Lig4 and Rad54 are required for repair of DNA double-strand breaks induced by P-element excision in *Drosophila*

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Dedicated to the memory of our colleague and friend Dr. Jan C.J. Eeken who died unexpectedly on the 24³ of May, 2002

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Site-specific double-strand breaks (DSBs) were generated in the white gene located on the X chromosome of Drosophila by excision of the \( w^{hd} \) P-element. To investigate the role of non-homologous end-joining (NHEJ) and homologous recombination (HR) in the repair of these breaks, the \( w^{hd} \) P-element was mobilized in flies carrying mutant alleles of either \( lig4 \) or \( rad54 \). The survival of both \( lig4 \) and \( rad54 \) deficient males was reduced to 25% in comparison to the wild type, indicating that both NHEJ and HR are involved in the repair P-induced gaps in males. Survival of \( lig4 \) deficient females was not affected at all, implying that HR using the homologous chromosome as a template, can partially compensate for the impaired NHEJ pathway. In \( rad54 \) mutant females survival was reduced to 70% after \( w^{hd} \) excision. PCR analysis indicated that the undamaged homologous chromosome may compensate for the potential loss of the broken chromosome in \( rad54 \) mutant females after excision. Molecular analysis of the repair junctions revealed microhomology (2-8 bp) dependent DSB-repair in most products. In the absence of Lig4, the 8-bp target site duplication is used more frequently for repair. Our data indicate the presence of efficient alternative end-joining mechanisms, which partly depend on the presence of microhomology but do not require Lig4.
INTRODUCTION

Double-strand breaks (DSBs) in DNA are induced by exogenous agents such as X-rays but can also arise during replication and other DNA metabolic processes. To restore the continuity of the genome and to prevent cytotoxicity and the formation of gross chromosomal aberrations, faithful repair is essential for each organism. In eukaryotes, DSBs are repaired via two main mechanisms, homologous recombination (HR) and non-homologous end-joining (NHEJ; reviewed by Pastink et al. 2001; van Gent et al. 2001). HR requires the presence of undamaged homologous DNA that can be used as a template for repair synthesis. In mammals, HR is restricted to late S and G2 phases of the cell cycle when the presence of the sister chromatid ensures accurate restoration of the genetic information. Copying information from the homologous chromosome may result in loss of heterozygosity and has deleterious consequences for the organism. Repair via NHEJ is based on direct ligation of the two broken DNA ends and does not require extensive processing of the DNA termini or the presence of extensive sequence homology on both sites of the break. However, NHEJ is frequently associated with the insertion or deletion of a few nucleotides at the site of the break (Pastink et al. 2001; van Gent et al. 2001).

HR is dependent on the RAD52 epistasis group proteins, which were first identified in Saccharomyces cerevisiae (reviewed in Paques and Haber, 1999; Symington, 2002). Homologous genes were also identified in higher eukaryotes including Drosophila (Pastink et al. 2001). One of the core components in HR is Rad54. Drosophila strains, which are deficient for rad54,
also known as okra, are hypersensitive to ionizing radiation and methyl methanesulfonate, implying that HR is essential for the repair of DSBs in flies (Kooistra et al. 1997). Recently, we have identified the ligase IV (lig4) gene in Drosophila, coding for one of the components required for NHEJ. In contrast to knock out mice, flies carrying lig4 null alleles are viable and display increased sensitivity to ionizing radiation during embryonic development (Gorski et al. 2003). To determine the relative contribution of NHEJ and HR to the repair of X-ray-induced DSBs in Drosophila, lig4; rad54 double mutants were generated. In the double-mutant flies, a synergistic increase in sensitivity to X-rays was seen in comparison with both single mutants, indicating that both DSB-repair pathways partially overlap. During the first hours of embryogenesis and at late stages of larval and pupal development, HR is the predominant repair pathway (Gorski et al. 2003).

P-elements in Drosophila are DNA transposons that are excised and inserted via a cut-and-paste mechanism that creates 8-bp target site duplications (TSD; see Figure 1A) upon insertion (O’Hare and Rubin, 1983; Kaufman and Rio, 1992). Full length P-elements have a size of 2.9 kb and are flanked by 31 bp inverted repeats (O’Hare and Rubin, 1983). The transposition requires an active transposase (P-element endonuclease) that generates two staggered DSBs containing 17-nucleotide 3’ overhangs within the 31-bp terminal inverted repeats (see Figure 1A) (Beall and Rio, 1997; Beall and Rio, 1998). Repair of P-induced double-strand gaps depends on host proteins and can occur via end-joining or through HR using the sister chromatid, the homologous chromosome, or an ectopically located homologous sequence as a template (reviewed by Lankenau, 1995).
Extensive genetic analysis showed only gene conversion events without crossing-over (Engels et al. 1990; Gloor et al. 1991; Johnson-Schlitz and Engels, 1993). On the basis of these results the synthesis-dependent strand-annealing (SDSA) model for homologous recombination was proposed (Nassif et al. 1994).

In premeiotic germline cells, the original P sequence is retained in most chromosomes, suggesting that sister chromatids are used efficiently as a template for repair synthesis (Johnson-Schlitz and Engels, 1993). In somatic cells, repair of P-induced gaps frequently results in imprecise excision of P-elements (Gloor et al. 2000; Rong and Golic, 2003). Imprecise excision of P-elements in both premeiotic germline cells and somatic cells often results in the retention of short P-element sequences at the site of the break in the DNA (O’Brochta et al. 1991; Takasu-Ishikawa et al. 1992; Staveley et al. 1995; Gloor et al. 2000). These sequences (also called footprints) are derived from P-element inverted repeats and can be divided into two classes. Double footprints are generated when P-element sequences are maintained on both termini and a single footprint is created when the P sequence of only one end is retained (Gloor et al. 2000). Differences in footprint distribution were observed upon excision of P-elements in premeiotic germline and somatic cells. Premeiotic germline cells typically show 15-18 bp double footprints (Takasu-Ishikawa et al. 1992; Staveley et al. 1995; Gloor et al. 2000), whereas in somatic cells usually single 4-7 bp footprints are retained (O’Brochta et al. 1991; Gloor et al. 2000).

To extend our previous studies on the survival of \textit{lig4} and \textit{rad54} single and double mutants after exposure to DNA damaging agents, we investigated
the role of *lig4* and *rad54* in P-element transposition. Survival studies after P-element excision indicate an important role for NHEJ and HR in repairing breaks generated during the process of transposition. Molecular analysis of the repair products provides evidence for a crucial role of alternative end-joining mechanisms in rejoining breaks after excision of P-element.
MATERIALS AND METHODS

Fly strains: Flies were grown on standard sugar-agar medium at 25°C.
The C(1)DX, y w f strain was obtained from the Drosophila Stock Center in Bloomington, Indiana. y^2 w^a spl and y w^{hd} spl strains were kindly provided by Carlos Flores and William Engels. Genetic symbols and nomenclature not described here are given in Lindsley and Zimm (1992) and FlyBase (http://flybase.bio.indiana.edu/). The lig4 deficient fly strain lig4^{57} has been described previously (Gorski et al. 2003). Briefly, the lig4^{57} mutant line carries a deletion of the X chromosome that uncovers nearly the entire lig4 gene, creating a lig4 null mutant. Okra^{A17-11} (abbreviated A17-11) is a null allele of rad54 and carries a CG-to-AT transition at the splice acceptor site of the second intron of the rad54 gene (located on the second chromosome) (Kooistra et al. 1997). The deficiency chromosome Df(2L)JS17 (abbreviated JS17) carries a multilocus deletion, which uncovers the rad54 gene.

In order to monitor directly the offspring and to suppress meiotic recombination, we used the first chromosome balancer Basc, also known as Muller 5 (abbreviated M5), the second chromosome balancer SM5 and the third chromosome balancer TM3 that carry the visible dominant marker mutations B, Cy and Ser, respectively (see Lindsley and Zimm, 1992).

P-element excision assay: To study the contribution of NHEJ to the repair of P-element-induced DSBs, the w^{hd} (w^{hd80617}) allele of white was used. w^{hd} flies have a bleached eye color (almost without pigment) due to the insertion of a 629 bp P transposable element in the sixth exon of the white gene (see Figure 1B; O'Hare and Rubin, 1983). The w^a (white-apricot) allele
contains a copia transposable element inserted in the second intron of the white gene, which results in an apricot-eyed phenotype (Bingham and Judd, 1981). This allele of white can be used in our crossing scheme as a template for recombination in females. By standard genetic crosses a recombinant first chromosome was generated that carries the lig4^57 mutation in combination with w^hd or w^a. The transposase required for the excision of w^hd is encoded by the P[ry^+ Δ2-3](99B) element (abbreviated Δ2-3) on the third chromosome (Robertson et al. 1988). To combine all the components required for P-element excision and subsequent repair, y w^hd lig4^57/y w^hd lig4^57 females were crossed to y^2 w^a lig4^57/Y; Sb P[ry^+ Δ2-3]/TM3, Ser males (see Figure 2). y w^hd lig4^57/Y; Sb P[ry^+ Δ2-3] males emerging from this cross were either directly used for PCR analysis or crossed individually to C(1)DX, y w f females. From this cross, y w^hd lig4^57 male progeny that did not carry the Δ2-3 element was recovered and analyzed by PCR. y w^hd lig4^57/y^2 w^a lig4^57; Sb P[ry^+ Δ2-3] females that were recovered from the original cross were either used for PCR analysis or mated individually to M5 males. In the next generation, y w^hd lig4^57 males that did not carry the Δ2-3 element were recovered and analyzed by PCR (Figure 2A). In the control crosses, y w^hd lig4^+/y w^hd lig4^+ females were crossed to y^2 w^a lig4^57/Y; Sb P[ry^+ Δ2-3]/TM3, Ser males and the offspring was analyzed as described above.

To investigate the role of Rad54 in the repair of P-element-induced gaps, we crossed y w^hd/y w^hd; JS17/SM5, Cy females to y^2 w^a/Y; A17-11/SM5, Cy; Sb P[ry^+ Δ2-3]/TM3, Ser males. In the next generation, the ratios of rad54 proficient and deficient male and female progeny were determined. According to Mendelian laws, y w^hd/Y; JS17/SM5, Cy; Sb P[ry^+ Δ2-3] and y w^hd/Y; A17-
11/SM5, Cy; Sb P[ry⁺ Δ2-3] rad54 heterozygous males should emerge in a 2:1 ratio with their y wʰd/Y; JS17/ A17-11; Sb P[ry⁺ Δ2-3] rad54 homozygous brothers. SM5, Cy/SM5, Cy flies are not recovered due to embryonic lethality. Both heterozygous and homozygous rad54 males as well as females that contain the Δ2-3 transposase should emerge from this cross in a 1:1 ratio with their siblings that do not contain Δ2-3. Both y wʰd/Y; JS17 (or A17-11)/SM5, Cy; Sb P[ry⁺ Δ2-3] and y wʰd/Y; JS17/A17-11; Sb P[ry⁺ Δ2-3] males as well as y wʰd/y² wᵃ; JS17 (or A17-11)/SM5, Cy; Sb P[ry⁺ Δ2-3] and y wʰd/y² wᵃ; JS17/A17-11; Sb P[ry⁺ Δ2-3] females were used for PCR analysis. In the control crosses, y wʰd/y wʰd females were crossed either to y² wᵃ; A17-11/SM5, Cy; Sb P[ry⁺ Δ2-3]/TM3 Ser or y² wᵃ; Sb P[ry⁺ Δ2-3]/TM3, Ser males and the offspring was analyzed as described above (see Figure 2B).

**DNA isolation and molecular analysis:** To perform PCR analysis, genomic DNA was isolated from adult flies. Single flies were homogenized in 50 µl of Buffer A (100 mM Tris-HCl pH 7.6, 100 mM EDTA, 100 mM NaCl and 0.5% SDS) and incubated for 15 min at 65°C. Next, 100 µl of Buffer B (1.5 M potassium acetate, 4.5 M LiCl) was added, the homogenate was thoroughly mixed and chilled on ice for 10 min. To remove the debris, the samples were centrifuged for 10 min at 15,000 rpm. The supernatant was retained and the DNA was precipitated in the presence of 0.6 volume of isopropanol.

PCR analysis of wʰd derivatives was carried out using primer p7 (5’-GGTTGTCGTACCTCTCATGG) and primer p9 (5’-GTGTTTTATGTACCAGAAACGAG) located upstream and downstream of the P-element insertion, respectively. After amplification of repair junctions in F₁ flies, the PCR products were gel-purified and cloned into the pGEM®-T
Easy Vector (Promega). 2-3 clones per fly were sequenced using either p7 or p9. In case of flies from the F2 generation, the PCR products were directly sequenced using the same set of primers. For each mating, 2-3 flies were selected for sequencing.

**Microinjections of Drosophila embryos:** 0-1 h wild type and lig4 deficient embryos were collected after crossing Berlin K females to Berlin K males and w, lig4\(^{57}/w\), lig4\(^{57}\) females to w, lig4\(^{57}/Y\) males. Embryos were injected with a mixture of 500 µg/ml of HindIII or HindIII-EcoRI linearized pUC18 plasmid DNA. Linear pUC18 DNA was gel purified after digestion of pKL59, a pUC18 derivative containing a 5 Kb HindIII fragment from *K. lactis* (van den Bosch *et al.* 2001). In this way, it is possible afterwards to discriminate between repair products and uncut plasmid molecules. 30 embryos per sample were incubated for 2 h at 25°C, frozen in liquid nitrogen, and low molecular weight DNA was isolated as described (Hagmann *et al.* 1998). DNA was dissolved in 10 µl of water and 1 µl of DNA was transfected into *E. coli* XL-1 Blue. Plasmid DNA was isolated from individual colonies and used for sequencing using plasmid specific primers.
**RESULTS**

Lig4 contributes significantly to the repair of DSBs created during **w\(^{hd}\) P-element excision in somatic cells:** One of the first steps in P-element excision in *Drosophila* involves the formation of two staggered DSBs with identical 17-nucleotide 3’-extensions (see Figure 1). To investigate the role of NHEJ in the repair of P-induced DSBs, the *lig4*\(^{57}\) mutation was introduced in flies containing all the elements required for P-element excision. As a test system we employed the *w*\(^{hd}\) allele of *white*, which carries a small P-element insertion of 629 bp in exon 6 (Figure 1A). To induce excision, *w*\(^{hd}\) was combined with the Δ2-3 element as a source of transposase. Reversion of *w*\(^{hd}\) to the wild type *white* sequence can occur in females by HR using the *w*\(^{a}\) allele on the homologous chromosome as a template for DNA synthesis. However, rejoining after exonucleolytic processing of the ends may also result in restoration of the wild type *white* sequence. Clonal expansion of correctly repaired cells in the eye imaginal discs leads to the formation of spots of wild type red tissue against an apricot background in the eyes of adult females or bleached eyes in adult males. When the sister chromatid is used as a template for recombination after excision, the *w*\(^{hd}\) sequence is restored. Finally, a fraction of the DSBs created upon P-element excision is repaired imprecisely, which prevents further cutting (Takasu-Ishikawa *et al.* 1992; Johnson-Schlitz and Engels, 1993; Staveley *et al.* 1995; Gloor *et al.* 2000; Rong and Golic, 2003).

To mobilize the *w*\(^{hd}\) P-element, *y* *w*\(^{hd}\) *lig4*\(^{57}\)/ *y* *w*\(^{hd}\) *lig4*\(^{57}\) females were mated to *y*\(^{2}\) *w*\(^{a}\) *lig4*\(^{57}\)/ *Y*; *Sb P[ry\(^{+}\) Δ2-3]/TM3, Ser* males. In the next
generation, \( y^\text{whd} \text{ lig}4^{57}/Y; \) Sb \( P[ry^* \Delta 2-3] \) (F1) males and \( w^\text{hd} \text{ lig}4^{57}/y^2 w^\alpha \text{ lig}4^{57}; \) Sb \( P[ry^* \Delta 2-3] \) (F1) females emerge theoretically in a 1:1 ratio with their siblings that do not carry the \( \Delta 2-3 \) transposase (see Figure 2A). If Lig4 is required for the repair of P-induced DSBs, the presence of the transposase will cause a decrease in this 1:1 ratio. In the \( lig4 \) proficient control, the ratios between flies without the \( \Delta 2-3 \) transposase and flies carrying the \( \Delta 2-3 \) element were 1.1 and 1.3 for males and females, respectively (Table 1). Apparently, the continuous presence of the transposase during development does not affect survival of DSB-repair proficient flies. P-element excision in a \( lig4 \) deficient background resulted in a 75% reduction in male survival. Among 1506 \( y^\text{whd} \text{ lig}4^{57}/Y; \text{TM3, Ser} \) males only 354 \( y^\text{whd} \text{ lig}4^{57}/Y; \) Sb \( P[ry^* \Delta 2-3] \) males were recovered (Table 1), which resulted in a survival ratio of 0.24. In males without transposase, no spots of wild type tissue were observed in the eyes of adult flies. Among 208 \( lig4 \) proficient males containing the \( \Delta 2-3 \) element, we recovered 34 flies that carried at least one spot in one of the eyes. In the absence of \( lig4 \), the spot frequency is increased 3.4-fold from 0.16 to 0.54 (Table 1).

In contrast to males, P-element excision did not result in a reduced survival of \( lig4 \) deficient females. The ratio of females with and without transposase was 1.1 and is similar to the survival ratio of 1.3 observed for repair proficient females (Table 1). These data suggest that gene conversion using the homologous chromosome can effectively compensate for the lack of Lig4. In agreement with this notion is the size of the spots observed in the eyes of mutant females after P-element excision. In all 1663 \( lig4 \) deficient females recovered, the eyes were almost completely (90-95%) covered with
spots of wild type red tissue. Individual spots were hardly discernible (see Figure 3). In lig4 proficient females, however, approximately 30-40% of each eye was covered with spots. Together, these observations indicate that Lig4 contributes significantly to the repair of DSBs after \( w^{hd} \) P-element excision in somatic cells in males, which contain only one X-chromosome. In females, however, gene conversion can partially compensate for the lack of Lig4 due to the availability of a homologous chromosome.

**Molecular analysis of repair junctions after \( w^{hd} \) P-element excision in lig4 deficient somatic cells:** To analyze the repair products after P-element excision in somatic cells, genomic DNA was isolated from single lig4 proficient \( y \ w^{hd} \) \( lig^{4+}/Y; \ Sb \ P[ry^+ \Delta 2-3] \) and deficient \( y \ w^{hd} \) \( lig^{457}/Y; \ Sb \ P[ry^+ \Delta 2-3] \) males as well as from single \( y \ w^{hd} \) \( lig^{4+}/y^2 \ w^a \ lig^{4+}; \ Sb \ P[ry^+ \Delta 2-3] \) and deficient \( y \ w^{hd} \) \( lig^{457}/y^2 \ w^a \ lig^{457}; \ Sb \ P[ry^+ \Delta 2-3] \) females. PCR primers p7 and p9 were used to amplify the region of the white gene containing the \( w^{hd} \) insertion site (see Figure 1). Amplification of the wild type white sequence resulted in DNA fragments of 505 bp. After insertion of the \( w^{hd} \) P-element a 1134 bp amplification fragment is produced. Analysis of DNA from females containing both the \( w^{hd} \) and \( w^a \) alleles showed that the P-element-containing 1134 bp fragment and the 505 bp fragment were amplified with the same efficiency (data not shown). Amplification of DNA from \( \Delta 2-3 \)-containing males and females revealed only DNA fragments of approximately 500 bp in size after fractionation on 2% agarose gels (results not shown). The absence of the 1134 bp fragment indicates efficient excision of the P-element during development. The size of the spots in the eyes of lig4 deficient females after P-element excision is in agreement with this notion. To study the repair
products after DSB-formation in more detail, amplified material obtained from single flies was cloned and sequenced (see Materials and Methods). From males of the lig4 proficient screen, 14 clones were analyzed. In 12 clones small footprints consisting of terminal inverted repeat sequences were present. In one of the clones analyzed, a double footprint was observed. The number of nucleotides retained ranged from 1 to 7 (Table 2). Additional nucleotides at the repair junction were observed in only 1 clone. In 9 of the junctions analyzed, short 2 to 4-nucleotide repeats present on both sides of the break could be identified. These small repeats or microhomologies were found mainly at the 3'-ends of the 17-nucleotide overhangs. Repair in 1 of the clones is based on a microhomology of 3 nucleotides within the 8 bp target site duplication (TSD). In a lig4 deficient background, footprints were observed in 4 out of 12 clones analyzed (Table 2). The size of the footprints varied between 6 and 30 bp. In 8 products, repair was based on the presence of microhomology. Interestingly, in 7 clones the 8-nucleotide GTCTGGCC TSD was used for microhomology-based repair (see Figure 1A). In these clones the entire P sequence and the TSD were deleted, leading to restoration of the wild type white sequence. In agreement with these data is the increase in the frequency of red spots observed in lig4 deficient males in comparison to the wild type.

Amplification of DNA isolated from single females after P-element excision also gave rise to PCR products of approximately 500 bp (results not shown). These products resulted from amplification of the w^a allele on one X-chromosome and the w^nd allele on the other X-chromosome. After P-element excision, the breaks can be repaired via end-joining (precise or imprecise) or
gene conversion using the \( w^a \) allele as a template. Precise end-joining products after excision of the \( w^{hd} \) element can be distinguished from products derived from the \( w^a \) allele on basis of a GGG to GTG change 125 nucleotides upstream of the \( w^{hd} \) integration site. Sequencing of 18 repair junctions from wild type females revealed 8 \( w^a \) alleles. In 2 out of 10 products recovered from the \( w^{hd} \) allele, DSBs were repaired using the TSD as a basis for microhomology-dependent end-joining. In 2 other clones, repair was based on a 2-nucleotide (CA) repeat. In the remaining clones analyzed, only footprints were seen, but no microhomologies (Table 3). Of 34 clones obtained from \( lig4 \) mutant females containing the \( \Delta2-3 \) P-element, 19 were derived from the \( w^a \) allele. Similar to \( lig4 \) deficient males, nearly half (7/15) of the remaining products were repaired via microhomology-based repair using the 8-nucleotide GTCTGGCC TSD. In 2 clones, additional nucleotides were observed at the repair junctions (Table 3). On basis of the increased size of spots in the eyes after P-element excision in \( lig4 \) deficient females, an increased frequency of \( w^a \) derived clones was expected. However, it cannot be excluded that the conversion tracts in some of the revertants do not include the polymorphism 125 nucleotides upstream of the \( w^{hd} \) integration site. Therefore, some of the clones showing loss of TSDs may be repaired via HR.

\textit{lig4} deficiency does not affect the type of excision products in premeiotic germline cells: Previously it was concluded that gene conversion using the sister chromatid is the predominant repair pathway in premeiotic germline cells (Engels \textit{et al.} 1990; Gloor \textit{et al.} 1991; Takasu-Ishikawa \textit{et al.} 1992; Johnson-Schlitz and Engels, 1993; Staveley \textit{et al.} 1995; Gloor \textit{et al.}}
A small fraction of the DSBs is, however, imprecisely repaired via end-joining. To investigate the contribution of Lig4 to the repair of P-induced gaps in the premeiotic germline cells, y w\textsuperscript{hd} lig4\textsuperscript{57}/Y; Sb P[ry\textsuperscript{*} Δ2-3] males and y w\textsuperscript{hd} lig4\textsuperscript{57}/y\textsuperscript{2} w\textsuperscript{a} lig4\textsuperscript{57}; Sb P[ry\textsuperscript{*} Δ2-3] females were crossed to C(1)DX, y w f females and M5 males, respectively (see Figure 2A). In the offspring of these crosses, y w\textsuperscript{hd} lig4\textsuperscript{57} F\textsubscript{2} males were recovered from both F\textsubscript{1} males (males from males) and F\textsubscript{1} females (males from females) and used for PCR and sequence analysis as described in Materials and Methods. In the control cross, y w\textsuperscript{hd} lig4\textsuperscript{*}/Y; Sb P[ry\textsuperscript{*} Δ2-3] males and y w\textsuperscript{hd} lig4\textsuperscript{*}/y\textsuperscript{2} w\textsuperscript{a} lig4\textsuperscript{57}; Sb P[ry\textsuperscript{*} Δ2-3] females were recovered and used for further crossings as described above.

Based on the eye-color phenotype, we detected 29 w\textsuperscript{*} revertants among 288 males from lig4 proficient females, resulting in a reversion frequency of 10%. 15 w\textsuperscript{*} revertants were identified among 284 males from lig4 deficient females (reversion frequency of 5%). (Reversion to the wild type sequence of white was confirmed by sequence analysis of 3 independent revertants from each group). In a similar study using wild type flies, Johnson-Schlitz and Engels (1993) obtained a reversion frequency of 14%. Molecular analysis revealed the presence of the 629 bp w\textsuperscript{hd} element in 95% (80/84) of the males from lig4 proficient females and 97% (85/88) of the males of lig4 deficient females analyzed (Table 4). For both classes of males from females, loss of the 629 bp P-element and retention of footprints, was seen in only 3 males. PCR analysis of males derived from wild type and mutant males revealed the presence of w\textsuperscript{hd} P-element in 68% and 71% of the flies analyzed, respectively (Table 4). Among the progeny of lig4 deficient males, 22 out of 90 showed excision of the P-element. In addition, 2 small and 2 large deletions
were observed. The small deletions result in the loss of *white* sequences on one or both sides of the *w*^{hd} P-element insertion, but are still positive after the PCR amplification using p7 and p9 primers. Large deletions were classified as those that lost either one or both sequences required for PCR amplification using the same set of primers. These deletions comprise only a minor class of all the repair products and were not further investigated. In F_{2} males from the experiment with repair proficient males, excision of the *w*^{hd} element was seen in 23 out of 85 flies. In 4 males deletions including flanking *white* sequences were identified (Table 4).

Sequence analysis of 23 repair products from the experiment with *lig4* proficient males showed footprints (18 to 33 bp) in all males analyzed (Table 5). In 9 males, the repair occurred via microhomology-mediated end-joining and 12 males contained additional nucleotides at the repair junctions (Table 5). Among 19 males derived from *lig4* deficient males that were analyzed, 9 were repaired using microhomology and 8 contained additional nucleotides. In the offspring of both *lig4* proficient and deficient F_{2} males, microhomology-dependent repair is based on short (2-3 bp) repeats present at the 5’-ends of the 17 bp overhangs. The most frequently observed repeats were AT and TA sequences (Table 5). These data indicate that inactivation of the *lig4* gene does not influence the type of products recovered after excision in premeiotic cells.

To study the footprint distribution, the size and frequency of each footprint was determined. No difference in footprint distribution was seen between males recovered from F_{1} *lig4* proficient and deficient males (Figure 5). The average footprint sizes were 28 bp and 29 bp for both classes,
respectively. As previously observed, footprints in repair proficient premeiotic cells were longer than in somatic cells (Gloor et al. 2000). The same result was seen in the absence of Lig4 (see Tables 2, 3 and 5).

**Rad54 plays a pivotal role in the repair of P-induced DSBs in somatic cells:** To investigate the role of HR in P-element transposition, the Df(2L)JS17 deficiency (abbreviated JS17) and the okra54^{A17-11} allele (abbreviated as A17-11) of rad54 were combined with the w^{hd} and Δ2-3 elements. To induce excision in a rad54 deficient background, y w^{hd}/ y w^{hd}; JS17/SM5, Cy females were mated to y^{2} w^{a}/ Y; A17-11/SM5, Cy; Sb P[y^{+} Δ2-3]/TM3, Ser males (see Figure 2B). According to Mendelian law, y w^{hd}/ Y; JS17/A17-11; Sb P[y^{+} Δ2-3] males and y w^{hd}/ y^{2} w^{a}; JS17/A17-11; Sb P[y^{+} Δ2-3] females emerge in 1:1 ratio with their rad54 siblings that do not carry the Δ2-3 transposase. If Rad54 is involved in the repair of P-induced gaps, the frequency of flies carrying the Δ2-3 transposase will decrease in comparison to those lacking Δ2-3. A drastic decrease in survival of rad54 deficient males and a moderate decrease in survival of rad54 deficient females were seen after the P-element excision. Among 128 y w^{hd}/ Y; JS17/A17-11; TM3, Ser males we recovered only 28 y w^{hd}/ Y; JS17/A17-11; Sb P[y^{+} Δ2-3] males, which results in survival ratio of 0.22 (28/128). In females, a survival ratio of 0.68 (132/196) was obtained by scoring 132 y w^{hd}/ y^{2} w^{a}; JS17/A17-11; Sb P[y^{+} Δ2-3] and 196 y w^{hd}/ y^{2} w^{a}; JS17/A17-11; TM3, Ser females (Table 6). In the same cross, heterozygous rad54-proficient males and females were obtained. Both y w^{hd}/ Y; A17-11 (or JS17)/SM5, Cy; Sb P[y^{+} Δ2-3] males and y w^{hd}/ y^{2} w^{a}; A17-11 (or JS17)/SM5, Cy; Sb P[y^{+} Δ2-3] females emerged in ratios of 0.97 (378/390) and 1.16 (403/346).
respectively, in comparison with their siblings lacking transposase (Table 6). Similar results were obtained in a control experiment in which homozygous wild type flies were used (data not shown). The frequency of spots of wild type tissue in the eyes was very low in both wild type and rad54 deficient males. In rad54 proficient females, spots were seen in all flies recovered (Table 6). However, in a rad54 deficient background, only 8 out of 132 recovered flies contained spots, indicating that Rad54 is important for allelic recombination.

To study the repair junctions in a rad54 deficient background following the P-element excision, single-fly genomic DNA was isolated from y w<sup>hd</sup>/Y; JS17/A17-11; Sb P[ry<sup>+</sup> ∆2-3] males and y w<sup>hd</sup>/ y<sup>2</sup> w<sup>a</sup>; JS17/A17-11; Sb P[ry<sup>+</sup> ∆2-3] females and subjected to PCR analysis and sequencing. Of 16 clones derived from rad54 mutant males, 6 had 1 to 11-bp insertions at the junctions and 8 were repaired using microhomology (Table 2). In 2 of these clones, the 8-bp target site duplication (TSD) was used for repair. The remaining clones were characterized by 2-4 bp homologies. Among the 16 junctions sequenced we observed 7 double footprints and 6 single footprints (Table 2). The size of the footprints varied between 1-4 bp and 20-33 bp. In wild type F<sub>1</sub> males the average footprint was 3 bp and large footprints were not observed. Among 36 clones obtained from rad54 deficient females, 27 carried the product of the w<sup>a</sup> allele. Among the remaining 9 clones, 3 were repaired using microhomology (2 using the TSD sequence) and 2 had additional nucleotides at the repair junctions.
DISCUSSION

Survival after $w^{hd}$ P-element excision is dependent on both NHEJ and HR: Much of our current knowledge of DSB-repair in eukaryotes is obtained from studies utilizing rare cutting endonucleases to generate site-specific DSBs in the genome. In *Drosophila*, P-element transposition has been widely employed as a system to generate site-specific chromosomal breaks (for a reviews see Lankenau, 1995; Gloor and Lankenau, 1998). In our study, excision of the 629 bp $w^{hd}$ P-element was used to induce two staggered DSBs with identical 17-nucleotide 3’ overhangs at the site of integration in exon 6 of the *white* gene. Repair of these breaks is achieved through NHEJ and HR, the two main DSB-repair pathways in eukaryotes (Pastink *et al.* 2001). Since the P-element-specific endonuclease is constitutively expressed during development, the P-element is a target to continuous excision. The formation of DSBs is prevented when the breaks are resealed incorrectly via end-joining or when in females the homologous chromosome, which does not contain the $w^{hd}$ P-element, is used as a template for repair. PCR analysis did not reveal the presence of the 629 bp $w^{hd}$ P-elements in somatic cells of flies containing the $\Delta2-3$ endonuclease. This observation indicates efficient formation of DSBs, resulting in a prevalence of cells in adult flies in which breakage is not possible anymore as a consequence of repair. In repair proficient flies, the presence of the $\Delta2-3$ element does not influence survival (see Table 2 and 6), implying efficient repair of DSBs. P-element excision in F$_1$ males deficient for *lig4* resulted in a 75% reduction in survival. In contrast, the survival of *lig4* deficient females
was not affected (see Table 1), indicating that allelic recombination can partially compensate for the absence of NHEJ in repair of P-induced gaps. All lig4 deficient females recovered had almost completely wild type colored eyes. In lig4 proficient females only 30-40% of each eye was covered with spots (see Figure 3). These observations provide direct evidence that repair by HR using the homologous chromosome as a template can effectively compensate for the lack of NHEJ in females in an error free manner. The drastic reduction in male survival indicates that NHEJ is an essential repair pathway in the absence of a homologous chromosome.

To determine the role of Lig4 in the repair of breaks after w<sup>hd</sup> excision in premeiotic germ cells, male progeny was analyzed by PCR. In males recovered after crossing wild type and lig4 mutant males the original P-element was found in about 70% of the flies, irrespective of the repair deficiency. In males obtained after crossing both wild type and lig4 mutant females over 95% of the flies still contain the w<sup>hd</sup> element. These findings suggest efficient repair through HR using the sister chromatid as a template as was previously suggested (Engels et al. 1990; Gloor et al. 1991; Johnson-Schlitz and Engels, 1993; Gloor et al. 2000). Nevertheless, we cannot exclude the possibility of reduced endonuclease activity during germ cell formation.

P-element excision in rad54 mutant males resulted in a 75% reduction in survival. This strong decrease in survival indicates an important role in males for recombination between sister chromatids in overcoming P-element-induced DSBs. In females, a reduction in survival of approximately 30% was observed. Remarkably, only 8 out of 132 females recovered contained spots of wild type tissue in the eyes. Apparently, after inactivation of rad54 the
homologous chromosome cannot be used anymore as a template for recombination. PCR analysis of females that were recovered showed the presence of the $w^a$ allele in 27 out of 36 clones studied. Possibly, breakage of the $w^{hd}$ chromosome leads to chromosome loss when repair is hampered. Loss of the $w^{hd}$ chromosome may be tolerated at later stages of development or compensated by duplication of the $w^a$ chromosome. Chromosome loss and duplication has also been observed in certain tumor cells and is one way to explain loss of heterozygosity (Cavenee et al. 1983; de Nooij-van Dalen et al. 1998).

In previous experiments, the role of Rad54 in P-element transposition was studied using a slightly different system. In these experiments the $w^{hd}$ element was combined with a $w^{alter}$ P-element on the second chromosome which can be used as an ectopic template for recombination. After excision of $w^{hd}$ hardly any flies were recovered (Kooistra et al. 1999). In the experiments described here, $rad54$ deficient flies that contained $\Delta 2-3$, emerged, albeit later than their siblings that did not contain the $\Delta 2-3$ transposase and appeared very weak. From males that were recovered, no offspring could be obtained. Females deficient for $rad54$ are sterile (Kooistra et al. 1997). The results obtained previously and the data presented here indicate that P-element transposition in the absence of HR has very severe effects on development and survival. Depending on the genetic background and/or variation in experimental conditions, delayed eclosion may be possible.

**Molecular analysis of repair junctions indicates efficient microhomology-mediated end-joining**: Sequence analysis of repair junctions revealed the presence of small 2-8 nucleotide repeats on both sides
of the break (see Table 2, 3 and 5). In premeiotic cells only 2-3 bp repeats were observed (Table 5). Whereas, in somatic cells from wild type and \textit{rad54} mutant flies the 8 bp target site duplication (TSD) was used occasionally for microhomology-dependent repair. In the absence of Lig4 the relative use of the TSD-repeat was increased. The presence of small sequence repeats on both sides of the break after \textit{w}^d excision strongly suggests repair through an alternative end-joining pathway, which is facilitated by the presence of microhomologies. This imprecise mechanism involves alignment of overhanging ends by pairing of one or a few nucleotides followed by trimming of the ends and/or gap filling and ligation to seal the break. The large footprints observed after excision in premeiotic cells can be explained by pairing of the terminal nucleotides, which are complementary (see Figure 1). In somatic cells both small and large footprints were observed, especially in the absence of Lig4 or Rad54. Possibly, in somatic cells the DNA ends are more vulnerable to exonucleolytic digestion.

Evidence for an alternative error-prone sub-pathway of end-joining that utilizes short stretches (1-7 bp) of homology on both sides of the break and that is independent of DNA-PK and XRCC4/Lig4 has been obtained from yeast as well as mammals (Feeney, 1992; Gerstein and Lieber, 1993; Moore and Haber, 1996; Boulton and Jackson, 1996a; Boulton and Jackson, 1996b; Bogue \textit{et al.} 1997; Kabotyanski \textit{et al.} 1998; Labhart, 1999; Feldmann \textit{et al.} 2000; Wang \textit{et al.} 2001a; Verkaik \textit{et al.} 2002; Yu and Gabriel, 2003; Ma \textit{et al.} 2003). This alternative pathway may also be independent of Ku80, although conflicting data have been reported (Izsvak \textit{et al.} 2004; Guirouilh-Barbat \textit{et al.} 2004). Recent investigations have demonstrated that \textit{in vitro} end-joining can
also occur via two distinct sub-pathways. One sub-pathway repairs DSBs with fast kinetics without excessive degradation of the DNA ends and relies on Ku, DNA-PKcs and Lig4 (Lobrich et al. 1995; DiBiase et al. 2000; Wang et al. 2001b, 2001c, 2003). The other sub-pathway is operating with 20-30-fold slower kinetics and is active mainly when one of the components of the DNA-PK complex or Lig4 is impaired (DiBiase et al. 2000; Wang et al. 2001c, 2003). Interestingly, this sub-pathway of NHEJ involves degradation of DNA termini (Lobrich et al. 1995; Wang et al. 2003). Similar studies by Udayankumar et al. (2003) also revealed the presence of two distinct sub-pathways. One sub-pathway relies on the Mre11-Rad50-Nbs1 complex and the other on a novel 200-kDa repair factor. In contrast to other observations, both sub-pathways converge on a common requirement for the XRCC4-Lig4 complex. Our results indicate that alternative end-joining in Drosophila does not require Lig4. To confirm this conclusion HindIII and EcoRI/HindIII linearized pUC18 plasmid DNA was injected into embryos isolated from wild type and lig457 mutant flies. After repair for 2 h, low molecular weight DNA was isolated and introduced into E. coli (see Materials and Methods). Sequence analysis of clones recovered showed an induction in the use of microhomology (predominantly 2-3 bp repeats) in mutant embryos injected with HindIII (25%) and EcoRI/HindIII (35%) in comparison to the wild type (6% and 19%, respectively). Restoration of the HindIII site did not differ between wild type and mutant embryos, indicating that Lig4 is not involved in this pathway or only plays a minor role.

The data presented here imply a pivotal role for both NHEJ and HR in the repair of P-element-induced DSBs in Drosophila. Defects in key
components of these pathways, Lig4 and Rad54, result in a reduced survival if a deficiency in repair cannot be effectively compensated. In flies proficient for DSB-repair, Ku80 and Lig4-dependent end-joining possibly results in restoration of the $w^{hd}$ allele. In agreement with this is the observation that the $w^{hd}$ element is occasionally inverted after excision in premeiotic germline cells (results not shown). Correct repair through NHEJ has also been observed in yeast after the introduction of linearized plasmid molecules containing complementary ends (Boulton and Jackson, 1996a; Boulton and Jackson, 1996b). The induction of DSBs during fly development is prevented after inaccurate repair or allelic recombination. As discussed by Jaklevic and Su (2004), loss of cells under repair-deficient conditions can be effectively compensated in a multicellular organism like Drosophila by proliferation of cells in which break formation is not possible anymore. This phenomenon may explain the recovery of flies that survive in the absence of NHEJ or HR.

ACKNOWLEDGEMENTS

We are grateful to M. Nivard and other members of the Drosophila group for stimulating discussions and moral support.
LITERATURE CITED


LEGENDS OF FIGURES

Figure 1. Structure and excision of the w<sup>hd</sup> P-element. (A) The w<sup>hd</sup> P-element is inserted into the 6<sup>th</sup> exon of the white locus on the X chromosome (O’Hare and Rubin, 1983). The analysis of the repair junctions following the mobilization of the w<sup>hd</sup> P-element was carried out using p7 and p9 primers (see Materials and Methods). (B) A DNA double-strand gap is generated after excision of the w<sup>hd</sup> P-element by the P-element-specific endonuclease. This endonuclease is encoded by the P[ry<sup>+</sup> A2-3](99B) P-element abbreviated here as A2-3. The endonucleolytic cleavage creates two staggered DSBs containing 17-bp 3’ overhangs within the 31-bp inverted repeats. TSD: target site duplication.

Figure 2. Schematic representation of the genetic crosses performed to mobilize the w<sup>hd</sup> P-element. (A) To study the contribution of Lig4 to the repair of w<sup>hd</sup> P-element-induced DSBs, y w<sup>hd</sup> lig4<sup>57</sup>/y w<sup>hd</sup> lig4<sup>57</sup> females were crossed to y<sup>2</sup> w<sup>a</sup> lig4<sup>57</sup>/Y; Sb P[ry<sup>+</sup> A2-3]/TM3, Ser males. In the next generation, both F<sub>1</sub> males and females containing A2-3 transposase can be recognized on basis of the Sb marker and were either directly analyzed by PCR and sequencing or mated to C(1)DX y w f females and M5 males, respectively. F<sub>2</sub> males emerging from these crosses were analyzed as described for F<sub>1</sub> progeny (see Materials and Methods). (B) The contribution of Rad54 to the repair of P-induced DSBs was studied by crossing y w<sup>hd</sup>/y w<sup>hd</sup>; JS17/SM5, Cy females to y<sup>2</sup> w<sup>a</sup>/Y; A17-11/SM5, Cy; Sb P[ry<sup>+</sup> A2-3]/TM3, Ser males. Both male and female F<sub>1</sub> progeny containing A2-3 transposase was used for PCR and sequence analysis. In the control cross (not shown), y w<sup>hd</sup>/y w<sup>hd</sup> females were crossed to y<sup>2</sup> w<sup>a</sup>; Sb P[ry<sup>+</sup> A2-3]/TM3, Ser males and the offspring was analyzed as described above.
Figure 3. Mosaic eye phenotypes of lig4 proficient (central picture) and lig4 mutant (right picture) females after the repair of w\textsuperscript{hel} P-element-induced DSBs. Use of the w\textsuperscript{a}-containing chromosome as a template for recombination leads to restoration of the wild type sequence of the white gene. Clonal outgrowth results in red (wild type) spots against an apricot (w\textsuperscript{a}) background. For comparison, the apricot eye of a w\textsuperscript{a}, lig4\textsuperscript{57} female not carrying the Δ2-3 transposase is shown on the left.
Figure 1

A

B

TSD

5'-site

GTCTGGCCC
CATGATGAAATAACATACGCTAGG
CAGACCAGTCTACTTTATTGTATTCACC

ATACAATGAATAGTACCAGACCAG

P seq

3'-site

GTCTGGCCC
CATGATGAAATAACATA

CCACCTTATGTTATTTCATCATGCTCTGGCCC

GTGGAATACAATAAAGTAGTACCAGACCAG

Δ2-3

Δ2-3

GTCTGGCCC
CATGATGAAATAACATA

ATACAATGAATAGTACCAGACCAG

5'-site

Δ2-3

5'-site
Figure 2

A

♀

PCR

w^m lig4^w

Δ2-3, Sb

TM3, Ser

♂

PCR

w^m lig4^w

Δ2-3, Sb

TM3, Ser

w^m lig4^w

♀

C(1)DX yw

♂

PCR

w^m lig4^w

M5

B

♀

w^m

JS17

SM5, Cy

♂

w^m

A17-11

Δ2-3, Sb

SM5, Cy

PCR

w^m

JS17

SM5, Cy

♂

w^m

A17-11

Δ2-3, Sb

SM5, Cy

PCR

w^m

JS17

SM5, Cy

♂

w^m

A17-11

Δ2-3, Sb

SM5, Cy

PCR

w^m

JS17

SM5, Cy

♂

w^m

A17-11

Δ2-3, Sb

SM5, Cy

PCR

w^m

JS17

SM5, Cy

♂

w^m

A17-11

Δ2-3, Sb

SM5, Cy

PCR

w^m

JS17

SM5, Cy

♂

w^m

A17-11

Δ2-3, Sb

SM5, Cy

PCR
Figure 3

\[ w^{mu} \text{ lig}^4 \text{ / he}; \text{ lig}^4 \text{ TM3, Ser} \]
\[ w^{mu} \text{ lig}^4 \text{ / he}; \text{ lig}^4 \text{ / Sb P(ry+ A2-3)} \]
\[ w^{mu} \text{ lig}^4 \text{ / he}; \text{ lig}^4 \text{ / Sb P(ry+ A2-3)} \]
Table 1. Survival and reversion frequency after \( w^{bd} \) P-element excision in \( lig4 \) proficient and deficient backgrounds

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<th>l4g deficient</th>
</tr>
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<tr>
<td></td>
<td>No. of flies</td>
<td>Ratio* Flies with spots</td>
<td>Spot frequency**</td>
</tr>
<tr>
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<td>-</td>
<td>195 0</td>
<td>1506 0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>208 1.1 34 0.16</td>
<td>354 0.24 192 0.54</td>
</tr>
<tr>
<td>Female</td>
<td>-</td>
<td>187 0</td>
<td>1509 0</td>
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<tr>
<td></td>
<td>+</td>
<td>237 1.3 237 1</td>
<td>1663 1.1 1663 1</td>
</tr>
</tbody>
</table>

* Ratio of flies with and without transposase. ** Flies containing spots in the eyes divided by the total number of flies recovered
**Table 2.** Analysis of repair junctions created after $w^{bd}$ P-element excision in male somatic cells (F1)

<table>
<thead>
<tr>
<th>Micro-homology</th>
<th>Footprint size (at 3')</th>
<th>Insertion</th>
<th>Micro-homology</th>
<th>Footprint size (at 3')</th>
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</tr>
</tbody>
</table>

* Repair junctions containing deletions of *white* sequences flanking the site of integration and which often include one or both target site duplication (TSD) sequences.
**Table 3.** Analysis of repair junctions created after \( w^{hd} \) P-element excision in female somatic cells (F1)

<table>
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<th>Micro-homology</th>
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<th>Footprint size (at 3’)</th>
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<td>31 (14)</td>
<td>acatg</td>
<td>AT</td>
<td>31 (17)</td>
<td>37 bp P seq</td>
<td></td>
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</tr>
<tr>
<td>31 (17)</td>
<td>37 bp P seq</td>
<td></td>
<td></td>
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<tr>
<td>28 (12)</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>33 (17)</td>
<td>g</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>30 (14)</td>
<td>tgtattatcattgtt</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>attataaca</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

| **lig4 deficient F1 females** | | | | | | | |
| **rad54 deficient F1 females** | | | | | | | |
| GTCTGGCC | 0 | No | GTCTGGCC | 0 | No | GTCTGGCC | 0 | No |

* Repair junctions containing deletions of white sequences flanking the site of integration and which often include one or both target site duplication (TSD) sequences. The \( w^d \) allele was recognized on the basis of a polymorphism (G to T) 125 nucleotides upstream of the \( w^{hd} \) integration site.
Table 4. PCR analysis of $w^{bd}$ P element transposition events in premeiotic germline cells

<table>
<thead>
<tr>
<th></th>
<th>males from females</th>
<th>males from males</th>
<th>PCR result</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wild type</td>
<td>lig4 deficient</td>
<td></td>
<td>wild type</td>
</tr>
<tr>
<td>$w^{bd}$</td>
<td>80 (95%)</td>
<td>85 (97%)</td>
<td>$w^{bd}$ retained</td>
<td>58 (68%)</td>
</tr>
<tr>
<td>lost (foot prints retained)</td>
<td>3</td>
<td>3</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Small deletions*</td>
<td>0</td>
<td>0</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Large deletions**</td>
<td>1</td>
<td>0</td>
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<td>0</td>
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Among 288 and 284 males recovered from wild type and lig4 deficient females, 29 and 15 $w^+$ revertants were identified, respectively. Of the remaining $w^-$ males 84 males derived from wild type females and 88 males derived from lig4 deficient females were used for further analysis. 85 and 90 males recovered from wild type and lig4 deficient males, respectively, were used for molecular analysis.

*Deletions including white sequences on one or both sides. **Deletions including one or both primer binding sites.
Table 5. Analysis of repair junctions created after \(w^{bd}\) P-element excision in male premeiotic germline cells

<table>
<thead>
<tr>
<th>Micro-homology</th>
<th>Footprint size (at 3’)</th>
<th>Insertion</th>
<th>Micro-homology</th>
<th>Footprint size (at 3’)</th>
<th>Insertion</th>
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<tr>
<td>19 (3)</td>
<td>tcaatttcaga</td>
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<td>28 (15)</td>
<td>No</td>
<td>33 (17)</td>
<td>No</td>
<td></td>
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</tr>
<tr>
<td>32 (16)</td>
<td>gttatataa</td>
<td>23 (16)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33 (17)</td>
<td>gtgtatgat</td>
<td>34 (17)</td>
<td>tgtatatatatata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AT</td>
<td>30 (16)</td>
<td>No</td>
<td>CAT</td>
<td>17 (4)</td>
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<td>33 (16)</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA</td>
<td>32 (17)</td>
<td>No</td>
<td>TA</td>
<td>32 (17)</td>
<td>No</td>
</tr>
<tr>
<td>29 (16)</td>
<td>No</td>
<td>AT</td>
<td>30 (16)</td>
<td>No</td>
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<tr>
<td>25 (17)</td>
<td>gatgactatgatatatatataatgaa</td>
<td>AT</td>
<td>30 (16)</td>
<td>No</td>
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</tr>
<tr>
<td>AT</td>
<td>30 (16)</td>
<td>No</td>
<td>TA</td>
<td>32 (17)</td>
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<tr>
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<td>AT</td>
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<td>cattaac</td>
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<tr>
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<td>No</td>
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<tr>
<td>AT</td>
<td>25 (11)</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>AT</td>
<td>30 (16)</td>
<td>No</td>
<td></td>
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**Table 6.** Survival and reversion frequency after \( w^{ld} \)-P-element excision in *rad54* proficient and deficient backgrounds

<table>
<thead>
<tr>
<th>Sex</th>
<th>Transposase</th>
<th>( rad54 ) proficient</th>
<th>( rad54 ) deficient</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. of flies</td>
<td>Ratio(^*)</td>
</tr>
<tr>
<td>Male</td>
<td>-</td>
<td>390</td>
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<tr>
<td></td>
<td>+</td>
<td>378</td>
<td>0.97</td>
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<tr>
<td>Female</td>
<td>-</td>
<td>346</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>403</td>
<td>1.16</td>
</tr>
</tbody>
</table>

\(^*\) Ratio of flies with and without transposase. \(^**\) Flies containing spots in the eyes divided by the total number of flies recovered.