The *Drosophila melanogaster* DNA *Ligase IV* Gene Plays a Crucial Role in the Repair of Radiation-Induced DNA Double-Strand Breaks and Acts Synergistically With *Rad54*

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ABSTRACT

DNA Ligase IV has a crucial role in double-strand break (DSB) repair through nonhomologous end joining (NHEJ). Most notably, its inactivation leads to embryonic lethality in mammals. To elucidate the role of DNA Ligase IV (Lig4) in DSB repair in a multicellular lower eukaryote, we generated viable *Lig4*-deficient Drosophila strains by *P*-element-mediated mutagenesis. Embryos and larvae of mutant lines are hypersensitive to ionizing radiation but hardly so to methyl methanesulfonate (MMS) or the crosslinking agent *cis*-diamminedichloroplatinum (cisDDP). To determine the relative contribution of NHEJ and homologous recombination (HR) in Drosophila, *Lig4; Rad54* double-mutant flies were generated. Survival studies demonstrated that both HR and NHEJ have a major role in DSB repair. The synergistic increase in sensitivity seen in the double mutant, in comparison with both single mutants, indicates that both pathways partially overlap. However, during the very first hours after fertilization NHEJ has a minor role in DSB repair after exposure to ionizing radiation. Throughout the first stages of embryogenesis of the fly, HR is the predominant pathway in DSB repair. At late stages of development NHEJ also becomes less important. The residual survival of double mutants after irradiation strongly suggests the existence of a third pathway for the repair of DSBs in Drosophila.

DNA double-strand breaks (DSBs) pose a serious threat to the stability of the genome. If left unrepaired, DSBs can cause cell death or contribute to the formation of gross chromosomal rearrangements such as translocations and deletions. A variety of damaging agents such as X rays and chemical compounds such as bleomycine can cause the formation of DSBs. Furthermore, DSBs arise as intermediates during V(D)J rearrangement in differentiating lymphocytes, meiotic recombination, and certain transposition events.

To counteract the deleterious effects of DSBs, two main repair pathways exist in eukaryotes: homologous recombination (HR) and nonhomologous end joining (NHEJ). HR requires the presence of an undamaged homologous DNA that can be used as a template. In this way, HR ensures accurate DSB repair. NHEJ is based on ligation of the two ends and does not require extensive sequence homology. Frequently, NHEJ is associated with insertion or deletion of a few nucleotides at the site of the break (for reviews see Pastink *et al.* 2001; van Gent *et al.* 2001). The relative contribution of both repair pathways depends on the organism, the phase of the cell cycle, the developmental stage, and, presumably, the structure of break formed. In lower eukaryotes, HR is the primary repair mechanism. In the yeast *Saccharomyces cerevisiae*, the contribution of the NHEJ to the repair of DSBs can be detected only when HR is impaired (Milne *et al.* 1996; Siede *et al.* 1996). In higher eukaryotes, both HR and NHEJ contribute to the repair of DSBs. HR is especially important during the S and G2 phases of the cell cycle when the sister chromatid can be used as a template and during early development (Takata *et al.* 1998; Essers *et al.* 2000; Richardson and Jasin 2000). NHEJ predominates in adult organisms and during the G1 and early S phases of the cell cycle. The NHEJ pathway was first studied in mammals using rodent cell mutants and involves a number of proteins including Ku70, Ku80, DNA-PKcs, Ligase IV and its associated protein XRCC4, and the Artemis protein. The current model of DSB repair by NHEJ assumes that a heterodimer of Ku70 and Ku80 binds to DNA ends and recruits DNA-PKcs to the site of the damage to form an active DNA-PK complex (Featherstone and Jackson 1999; Doherty and Jackson 2001; van Gent *et al.* 2001). Binding of Ku to the DNA ends is also required for recruitment of Ligase IV and XRCC4 to the site of the break, which results in stimulation of DNA end ligation (Critchlow *et al.* 1997; Grawunder

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et al. 1997; McElhinny *et al.* 2000). The newly identified Artemis protein binds to DNA-PKcs and has endo- and exonucleolitic activities required for processing of DNA ends (Moshous *et al.* 2001; Ma *et al.* 2002).

Mice deficient in one of the components of the DNA-PK complex display an increased sensitivity to ionizing radiation and a severe combined immunodeficiency phenotype due to defects in V(D)J recombination (Nussenzweig *et al.* 1996, 1997; Zhu *et al.* 1996; Gu *et al.* 1997). Mouse embryonic stem cells deficient in DNA-PKcs, however, are not hypersensitive to ionizing radiation (Gao *et al.* 1998a; Taccioli *et al.* 1998). Evidently, the repair of DSBs by NHEJ is independent of DNA-PKcs in these cells. In yeast and also in Drosophila and *Caenorhabditis elegans*, no obvious homolog of DNA-PKcs has been identified. The role of DNA-PKcs possibly could be restricted to cells that strongly rely on NHEJ for the repair of X-ray-induced DSBs. In contrast to the loss of DNA-PKcs or the Ku function, inactivation of *Ligase IV* or *XRCC4* in mice results in embryonic lethality as a consequence of massive apoptosis in the central nervous system (Barnes *et al.* 1998; Frank *et al.* 1998; Gao *et al.* 1998b; Grawunder *et al.* 1998a). Rescue of embryonic lethality is possible in a p53-, ATM-, or Ku-deficient background (Frank *et al.* 2000; Karanjawala *et al.* 2002). In addition to the repair of DSBs, studies in yeast and mammalian cells indicate that the Ku proteins and DNA-PKcs are also involved in maintenance of telomere length and normal chromosomal DNA end structure (Boulton and Jackson 1996a, 1998; Gravel *et al.* 1998; Bailey *et al.* 1999; Hsu *et al.* 1999; Chai *et al.* 2002).

Drosophila melanogaster has been used extensively to study the mutagenic effects of ionizing radiation (IR) and it represents an attractive system to study DSB repair in a multicellular organism (Pastink *et al.* 2001). Flies deficient in *Rad54* are highly sensitive to X rays and methyl methanesulfonate (MMS), implying that HR contributes significantly to the repair of DSBs in somatic cells (Kooistra *et al.* 1997). Inactivation of *Rad54* or *spindle-B*, one of the *Rad51* paralogs in Drosophila, leads to defects in meiosis (Ghabrial *et al.* 1998). Increased MMS sensitivity, as compared with wild-type strains, has not been observed for the *spindle-B* mutant.

To study the contribution of NHEJ to the repair of DSBs in flies and to investigate the role of DNA Ligase IV in a multicellular organism, we isolated the Drosophila DNA *Ligase IV* gene, *Lig4*, and examined its function by generating mutant strains. In contrast to mice, homozygous null flies are viable and show increased sensitivity to ionizing radiation. A strong synergistic effect for radiosensitivity was detected in *Lig4; Rad54* double-mutant flies.

MATERIALS AND METHODS

Drosophila DNA *Ligase IV* gene analysis: The Drosophila DNA *Ligase IV* gene (*Lig4*) was identified by screening the Berkeley *Drosophila* Genome Project database (http://www.fruitfly.org) and is located on the X chromosome at position 12A9-B1. A full-length *Lig4* cDNA clone (*RE37186*) was purchased from Research Genetics (Huntsville, AL).

Two-hybrid analysis: A 773-bp fragment encoding the C-terminal end of Drosophila DNA *Ligase IV* was amplified by PCR using the Expand High Fidelity PCR system (Roche, Indianapolis) and inserted as a *Sal*I-*Eco*RI restriction fragment into the single-copy two-hybrid vectors pPC97 and pPC86, carrying the GAL4 DNA-binding domain and the GAL4-activating domain, respectively (Chevray and Nathans 1992). In a similar fashion, a full-length cDNA fragment of 708 bp encoding the Drosophila XRCC4 protein was amplified by PCR and inserted into *Sal*I- and *Spe*I-digested pPC97 and pPC86. Two-hybrid studies were performed using the *S. cerevisiae* strain Y190 (Harper *et al.* 1993). Transfectants were selected for tryptophane and leucine prototrophy on YNB medium [0.76% yeast nitrogen base (Difco, Detroit), 2% glucose] containing 30 mg/liter adenine and 50 mm 3-aminotriazole. Protein-protein interactions were detected using a β-galactosidase colony filter assay.

Generation of *Lig4*-deficient flies: To generate *Lig4* mutant flies, the Drosophila *EP(X)0385* insertion line $CG12176^{EP(X)0385}$ (w^{1118} *P{w*$^{+mC}$ *EP}EP385*), abbreviated here as $Lig4^{EP385}$, was used. Sequence analysis showed that the site of integration of the EP element is 38 bp upstream of the ATG start codon of the *Lig4* gene and is located within the 5′-untranslated region (UTR) of the gene. To mobilize the EP element, we crossed w^{1118}, $Lig4^{EP385}$ females to *Sb P* [ry^+ *Δ2-3*]*/TM3* males. Males from this cross were subsequently crossed to *white* (*w*) females. On the basis of the eye color phenotype, four types of females could be distinguished among offspring of the last cross. Only those with an eye color darker than the original bleached eye color of the $Lig4^{EP385}$ line (putative insertion mutants) or those with white eyes (putative deletion mutants) were analyzed further. A PCR screen with the EP inverted-repeat primer PTR2 (5′-ACGGGACCACCTTATGTTATTTCATCATG-3′) and a *Lig4*-specific primer LHR2 (5′-GCGATGGCACTGATGTATCC-3′; nucleotides 2955–2975 of the genomic sequence) was used to identify insertion mutants (see Figure 1). The LGF4 forward primer (5′-TGCCGAGGCCTTGCACATCT-3′; nucleotides 364–344 upstream of the ATG start codon) and the LHR2 reverse primer were used to screen the putative deletion mutants. The following PCR conditions were used: 1 min 94°, 1 min 60°, 3 min 72° for 30 cycles.

To obtain *Lig4*-deficient flies, females containing a deletion in *Lig4* were crossed to *w* males. Individual males that could possibly carry the mutation in the *Lig4* gene were crossed to Muller 5 females and the female offspring were again screened for the presence of the deletion in the *Lig4* gene using the LGF4 and LHR2 primers. Next, *w Lig4*/Muller 5 females were crossed to Muller 5 males. In the following generation, *w Lig4* males were crossed to *w Lig4*/Muller 5 females to produce flies homozygous-deficient for *Lig4*. To determine the length of the deletions in the *Lig4* gene, PCR products were gel purified, cloned into pGEM-T Easy (Promega, Madison, WI), and sequenced. In total, 18 different *Lig4* deletion mutants were generated, of which the $Lig4^5$ and $Lig4^{57}$ lines were the subject of phenotypical analysis.

Treatment of Drosophila with DNA-damaging agents: In Drosophila, the sensitivity to DNA-damaging agents is dependent on the developmental stage and therefore embryos and larvae of different stages were used for treatment. *w, Lig4*-deficient females were crossed to *Lig4*-proficient Muller 5 males. After a 4-, 16-, 24-, or 28-hr period of egg laying, embryos and larvae of different developmental stages were treated directly or after further development with increasing doses of X rays. Fly cultures were grown at 25° and after 12–18 days the offspring were scored. In the untreated control, the ratio

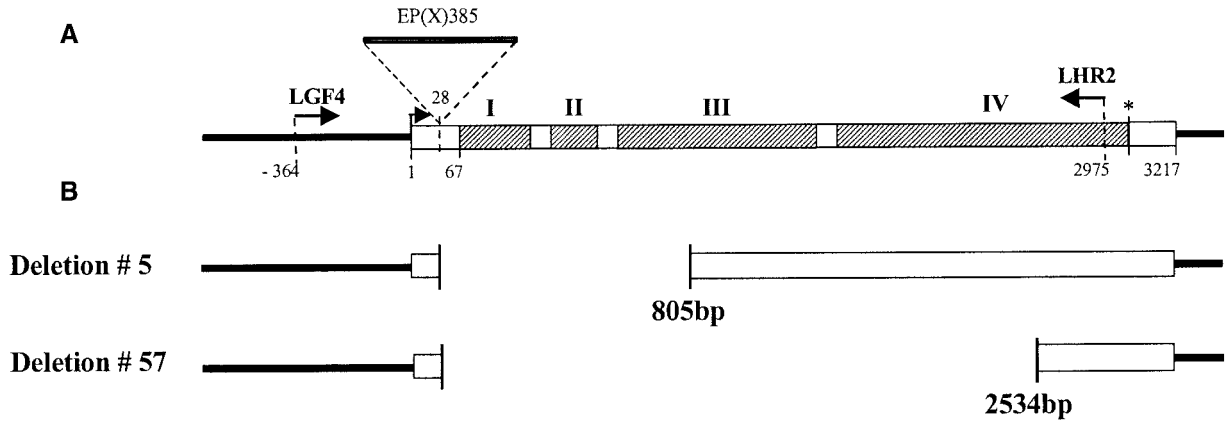

FIGURE 1.—Structure of the *Lig4* gene. (A) The intron-exon organization was determined after sequence analysis of a 3029-bp *Lig4* cDNA and comparison with the genomic sequence present in the database. The translated region of the *Lig4* gene is represented by hatched boxes, and introns and 5′- and 3′-UTRs are represented as open boxes. The ATG start codon is located at position 67. The stop codon, at position 3009, is indicated by an asterisk. To inactivate the *Lig4* gene, the *Lig4*^{EP385} line carrying an EP element inserted at position 28 was used. After mobilization of the EP element, the LGF4 and LHR2 primers were used to screen for the presence of deletions. (B) Two mutant lines generated and used for phenotypical analysis, *Lig4*^{5} and *Lig4*^{57}. The deletion in the *Lig4*^{5} mutant line extends up to nucleotide 805. *Lig4*^{57} uncovers nearly the entire *Lig4* gene extending up to nucleotide 2534, leaving only the last 475 nucleotides of the ORF.

of *Lig4*-deficient males to heterozygous females is theoretically 1.0. If the sensitivity to DNA-damaging agents is increased, this ratio will decrease with increasing dose.

To determine the effect of storage in the oocyte of maternal Lig4 protein and/or mRNA (maternal effect), *Lig4* heterozygous females were crossed to mutant males and the resulting progeny were tested for hypersensitivity to X rays in comparison to the reciprocal cross.

To investigate the contribution of HR to the repair of X-ray-induced DSBs in early developmental stages, we used the *okr*^{782} (originally called *okr*^{20682}) mutant allele of *Rad54*, named here *Rad54*^{782}. The *Rad54*^{782} allele carries a single-base-pair change, which results in a threonine-to-isoleucine change at position 660, which is located outside of the helicase domains but within a region conserved among *Rad54* homologs (K. MCKIM, personal communication). Unlike null alleles of *Rad54*, which result in female sterility, homozygous *Rad54*^{782} females are fertile. *JS17/cn Cy* males were crossed to *Rad54*^{782}/*Rad54*^{782} homozygous mutant females and in a parallel cross *Rad54*^{782}/*Rad54*^{782} males were crossed to *JS17/cn Cy* females. The deficiency chromosome *Df(2L)JS17*, referred to as *JS17*, uncovers the *Rad54* gene. The offspring were treated with increasing doses of X rays at different stages of development. In this generation, the expected ratio of *Rad54*^{782}/*Rad54*^{782} (*Cy*+) flies and *Rad54*$^{+}$/*Rad54*^{782} (*Cy*) flies is 1:1 according to Mendelian laws. The sensitivity of *Rad54*^{782}/*Rad54*^{782} mutant flies was calculated relative to *Rad54*$^{+}$/*Rad54*^{782} heterozygous flies.

To determine the relative contribution of NHEJ and HR to the repair of DSBs, *Lig4; Rad54* double-mutant flies were generated by crossing *Lig4*^{57}/*Lig4*^{57}; *Rad54*$^{A17\text{-}11}$/*cn Cy* females to *JS17/cn Cy* males. The *Rad54*$^{A17\text{-}11}$ mutation is a null allele of *Rad54* due to a GC-to-AT transition at the splice acceptor site of the second intron as has been previously described (KOOISTRA *et al.* 1997). In the untreated offspring, the expected ratio of *Lig4*^{57}; *Rad54*$^{A17\text{-}11}$/*Rad54*$^{A17\text{-}11}$ males, *Lig4*^{57}; *Rad54*$^{+}$/*Rad54*$^{A17\text{-}11}$ males, *Lig4*$^{+}$/*Lig*^{57}; *Rad54*$^{A17\text{-}11}$/*Rad54*$^{A17\text{-}11}$ females, and *Lig4*$^{+}$/*Lig*^{57}; *Rad54*$^{+}$/*Rad54*$^{A17\text{-}11}$ females is 1:2:1:2. Homozygous *cn Cy*/*cn Cy* flies were not recovered due to embryonic lethality. The sensitivity of single and double mutants was calculated relative to *Lig4*$^{+}$/*Lig*^{57}; *Rad54*$^{+}$/*Rad54*$^{A17\text{-}11}$ heterozygous females. *Lig4*; *Rad54* double mutants and *Lig4* and *Rad54* single mutants were treated at different developmental stages with increasing doses of X rays or 24–48 hr after egg laying with 0.2 ml/vial of 0.01, 0.02, 0.03, 0.06, or 0.08% MMS in phosphate buffer (pH 6) or with 0.2 ml/vial of 0.01, 0.05, 0.075, or 1.0 mM *cis*-diamminedichloroplatinum (cisDDP) in water.

X rays were generated with a SMART 225 machine at 200 kV, 4 mA, filter 1 mm Al at dose rates of ~1 Gy/min.

RESULTS

Sequence analysis of Drosophila DNA *Ligase IV* gene: The Drosophila DNA *Ligase IV* gene, *Lig4 (CG12176)*, was identified by searching the Drosophila Genome Database (http://www.fruitfly.org). The *Lig4* gene is located at position 12A9-B1 on the X chromosome. Sequencing of a *Lig4* cDNA plasmid clone (*RE37186*) revealed an insert of 3029 bp. Within this sequence an open reading frame (ORF) from position 67 to 3009 could be recognized. Comparison with the genomic sequence in the database confirmed the presence of the three predicted introns at positions 379–446, 612–670, and 1582–1643 in the *Lig4* gene (see Figure 1). Compared with the genomic sequence, base-pair substitutions were observed at positions 2091 (C to A) and 2093 (C to A), resulting in a leucine-to-isoleucine change. The sequence of the start codon AAAATGA matches the initiation consensus in Drosophila (C/A)AA(C/A)ATG very well (CAVENER 1987). A putative polyadenylation signal, AATAAA, is present starting at position 3176 in the 3′-UTR of the *Lig4* gene.

The predicted 918-amino-acid sequence of Lig4 protein is shown in Figure 2 aligned with human and yeast Lig4 proteins. The most extensive sequence homology is seen in the so-called "core" region conserved between eukaryotic DNA ligases, which includes the five motifs (I–V) that are conserved between ATP-dependent DNA

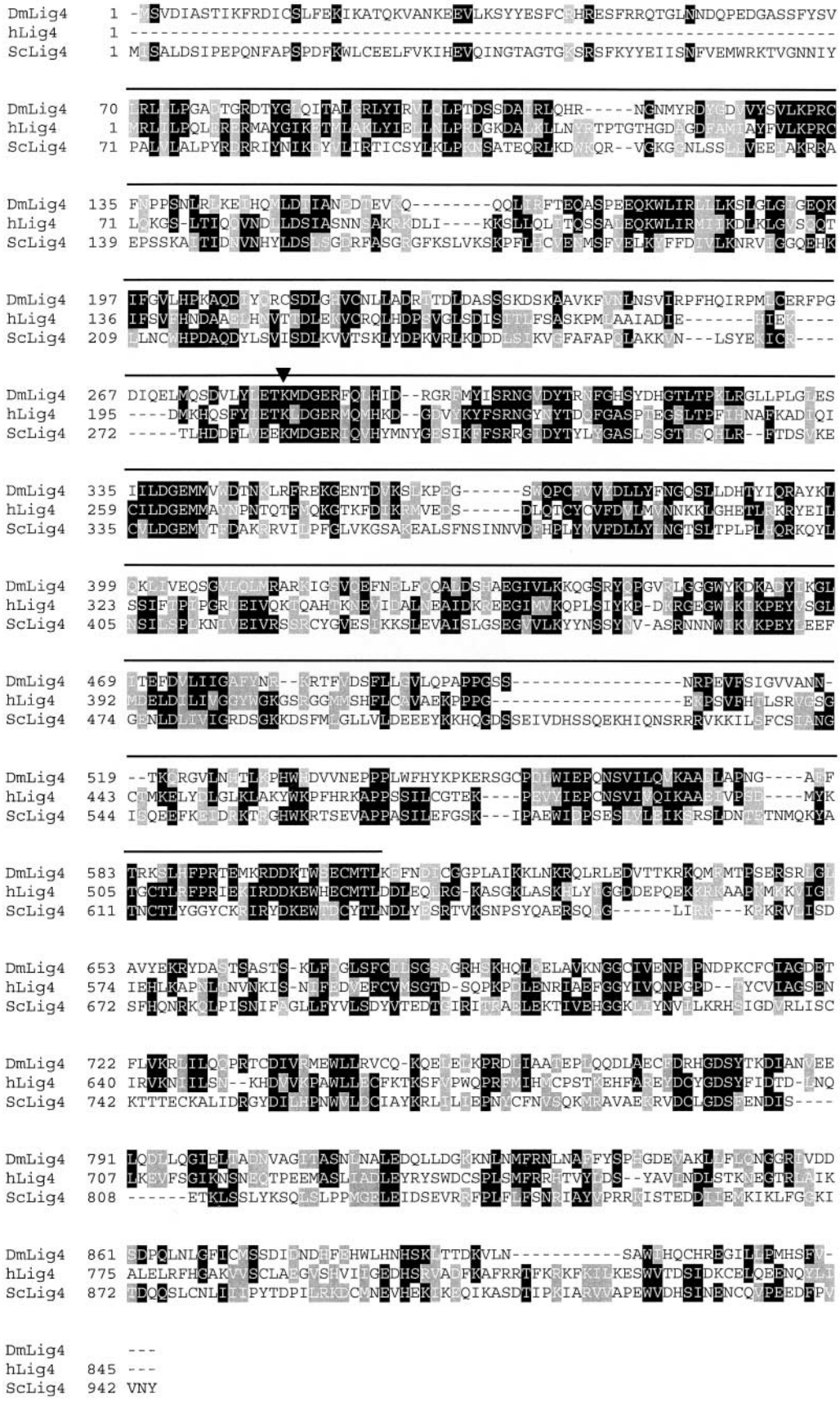

FIGURE 2.—Sequence alignment of Drosophila DNA Ligase IV (DmLig4), human Ligase IV (hLig4), and Ligase IV from *S. cerevisiae* (ScLig4). Protein sequences were aligned using the ClustalW algorithm. Identical and similar amino acid residues are indicated by solid backgrounds and shading, respectively, using the Boxshade program (CORPET 1988). The active site lysine residue at position 282 is indicated by an arrowhead and the core region conserved between eukaryotic DNA ligases is delineated by a thick line. Within this region the DmLig4 protein displays significant sequence homology with the human and the yeast Lig4 proteins (34 and 25% identity, respectively). The level of identity was determined using the Emboss local pairwise alignment algorithm (http://www.ebi.ac.uk/emboss/align/).

ligases and RNA capping enzymes and the conserved peptide that is found in all the ATP-dependent DNA ligases (Wei *et al.* 1995; reviewed in Tomkinson and Mackey 1998; Martin and MacNeill 2002). Within this region, DmLig4 shares 34% identity (53% similarity) and 25% identity (42% similarity) with hLig4 and ScLig4, respectively. The active site lysine is located at position 282 in the first motif.

At the C-terminal half of hLig4 and ScLig4, two BRCT (BRCA1 carboxy terminus) domains have been identified. In DmLig4, only one BRCT domain between residues 666 and 752 was recognized using the Prosite database (http://www.expasy.org/prosite/). The second BRCT domain, typically located at the C terminus, could not be identified. Although BRCT domains have been implicated in protein-protein interactions, human Lig4 binds to XRCC4 via a motif located between rather than within the BRCT domains (Grawunder *et al.* 1998b). On the other hand, data obtained by Herrmann *et al.* (1998) suggest that in the case of ScLig4 the second BRCT domain is required for binding of ScLig4 to Lif1 (yeast XRCC4). To study the interaction between Lig4 and the putative XRCC4 protein from Drosophila identified on the basis of homology by Sibanda *et al.* (2001), two-hybrid studies were carried out. A strong interaction was observed between the C-terminal part of the Lig4 protein fused to the GAL4 DNA-binding domain (bait vector) and the XRCC4 fused to the activation domain (prey vector) of the GAL4 transcription factor. The presence of a conserved BRCT domain at the C-terminal end of Lig4 is apparently not required for binding to XRCC4. This strong interaction clearly demonstrates the presence of a functional XRCC4 homolog in Drosophila. In the reciprocal two-hybrid experiment when the *Lig4* fragment was cloned in the prey vector and the XRCC4 cDNA in the bait vector, only a very weak interaction was seen. Unidirectional interactions have been observed previously using the yeast two-hybrid assay. For example, complex formation between Rad51 and Rad54 proteins can be detected in only one direction (Clever *et al.* 1997; Golub *et al.* 1997).

Generation of *Lig4*-deficient flies: To study the role of *Lig4* in the repair of DSBs, mutant flies were generated by *P*-element mutagenesis. The EP line *Lig4*^{EP385} contains a single *P* insertion in the 5′-UTR of the *Lig4* gene. To mobilize the EP element, *Lig4*^{EP385} females were crossed to *Sb P* [ry^+ $\Delta 2$-3]/*TM3* males. The male offspring were crossed to *white* females, and among the female offspring we selected newly induced insertion and deletion mutants on the basis of eye color phenotype (see materials and methods). Among ~800 females with an eye color darker than that of the original EP line, no EP insertions in the *Lig4* gene were found. Screening of ~200 white-eyed females and subsequent analysis of mutations resulted in the identification of 18 different *Lig4* deletion mutants. All the mutants were analyzed by sequencing. Since the LGF4 primer is located only 364 bp upstream of the original EP insertion site, the left side of each deletion maps relatively close to the original integration site. The right side of each deletion maps within the ORF of the *Lig4* gene. Two of these *Lig4* deletion mutants were used for the phenotypical analysis. The deletion in the *Lig4*^{5} mutant line extends until nucleotide 805 of the *Lig4* gene, completely deleting the first two exons. The *Lig4*^{57} mutant carries the largest deletion generated in our screen. It uncovers nearly the entire *Lig4* gene until nucleotide 2534, deleting three of four exons and leaving only 475 nucleotides of the ORF (Figure 1B).

Lig4-deficient mutants are viable and fertile as wild-type flies and do not show any signs of abnormal phenotype. *Lig4*-deficient males emerge in a nearly 1:1 ratio with their heterozygous sisters, indicating no measurable developmental retardations.

***Lig4* mutant flies are sensitive to ionizing radiation:** Homozygous *Lig4*-deficient females were crossed to (*Lig4*-proficient) Muller 5 males. In the F_1, the expected ratio of the *Lig4*-deficient males to heterozygous females is 1.0. If the *Lig4* deficiency results in increased sensitivity to DNA-damaging agents, this ratio will decrease with increasing dose given to the F_1 embryos and larvae. To determine the X-ray sensitivity of the original *Lig4*^{EP385} line as well as of the *Lig4*^{57} and *Lig4*^{5} mutant lines, 0- to 24-hr embryos, 24- to 48-hr larvae, and 48- to 72-hr-old larvae were exposed to a dose of 9 Gy (Figure 3A). The *Lig4*^{EP385}/*Lig4*^{EP385} line itself displayed a limited hypersensitivity to X rays at the embryonic stage. The ratio of males to females was 0.85 (53/62) compared to 1.08 (329/305) for the untreated control. The larval stages of the *Lig4*^{EP385}/*Lig4*^{EP385} line showed hardly any sensitivity to X rays in comparison to the heterozygous *Lig4*$^{+}$/*Lig4*^{EP385}. The ratios of males to females for 24- to 48-hr- and 48- to 72-hr-old larvae were 0.91 (300/330) and 1.02 (399/391), respectively. Apparently, the insertion of the EP element in the 5′-UTR of the *Lig4* gene does not severely interfere with the expression of the gene.

The two mutant lines generated in our screen, *Lig4*^{5} and *Lig4*^{57}, were hypersensitive to X rays (Figure 3A). For the nonirradiated controls, the ratios of males to females were 0.98 (538/550) and 0.89 (220/246) for the *Lig4*^{57} and *Lig4*^{5} mutant lines, respectively. *Lig4*^{57} and *Lig4*^{5} embryos that were 0–24 hr old showed a 4.1-fold [0.24 (82/343)] and a 4.7-fold [0.19 (14/74)] increase in sensitivity in comparison to the nonirradiated control, respectively. At later stages of development, the hypersensitivity of larvae to X rays gradually decreases. Larvae that were 24–48 and 48–72 hr old showed ratios of 0.68 (411/603) and 1.0 (665/660) for the *Lig4*^{57} mutant and 0.43 (119/279) and 0.76 (226/296) for the *Lig4*^{5} mutant. Both the *Lig4*^{57} and *Lig4*^{5} mutant lines were equally hypersensitive to X rays at the embryonic stage of development, indicating that both are null mutants.

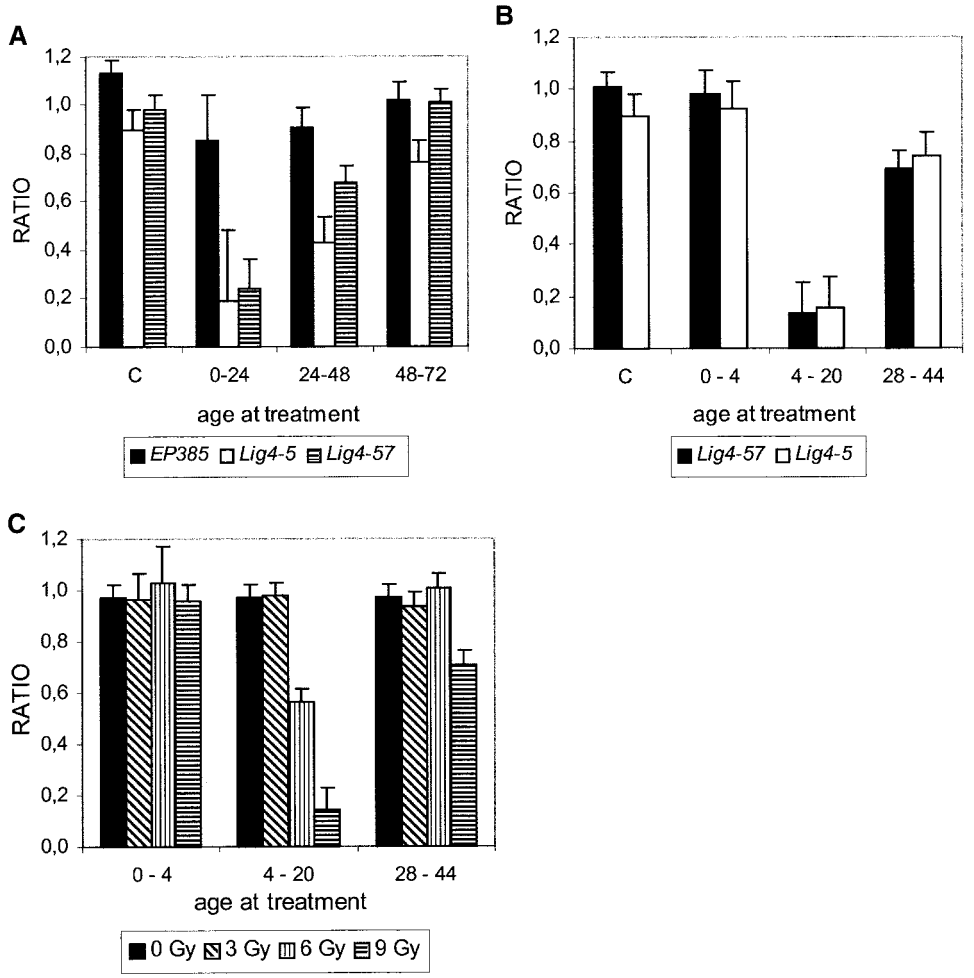

FIGURE 3.—Effect of X rays on *Lig4*-deficient mutants. On the *y*-axis the ratio of *Lig4*-deficient males to *Lig4*-proficient females is indicated. According to Mendelian laws, the expected ratio is 1.0. If the *Lig4* defect results in increased sensitivity to X rays, this ratio will drop. (A) $Lig4^{EP385}$ (EP-385) (solid bar), $Lig4^{57}$ (striped bar), and $Lig4^{5}$ (open bar) strains were exposed to a dose of 9 Gy at different stages of development (hours after egg laying). (B) Contribution of *Lig4* to the repair of X-ray-induced DSBs at embryonic and larval stages of development for $Lig4^{57}$ (solid bar) and $Lig4^{5}$ (open bar) after a dose of 9 Gy. (C) Dose-dependent decrease in recovery of $Lig4^{57}$ and $Lig4^{5}$ mutant lines (combined data). Nonirradiated controls are indicated as C or 0 Gy. Standard deviations are based on the total number of flies scored.

To investigate the contribution of NHEJ to the repair of DSBs at early stages of development, 0- to 4-hr- and 4- to 20-hr-old embryos and larvae and 28- to 44-hr-old larvae were exposed to a dose of 9 Gy (Figure 3B). Surprisingly, early embryos (0–4 hr) did not display an enhanced sensitivity to X rays. The ratios of males to females obtained for the $Lig4^{57}$ and $Lig4^{5}$ strains were 0.98 and 0.93, respectively. These values hardly deviated from those obtained from the untreated controls (1.01 and 0.9, respectively). However, in 4- to 20-hr-old embryos a strong increase in sensitivity to X rays was seen. In $Lig4^{57}$ and $Lig4^{5}$ mutant lines, a ratio of 0.14 (77/570) and 0.15 (77/500) was obtained for 4- to 20-hr-old embryos, resulting in a 7.2-fold (1.01/0.14) and a 6-fold (0.90/0.15) increase in sensitivity, respectively. In the case of 28- to 44-hr-old larvae, the hypersensitivity to X rays was less pronounced.

The sensitivity of the $Lig4^{57}$ and $Lig4^{5}$ mutant lines (data combined) to different doses of X rays was assessed at different stages of development (Figure 3C). At the most sensitive stage (4- to 20-hr embryos), the ratio of *Lig4*-deficient males to *Lig4*-proficient females was 0.56 after 6 Gy and 0.15 after a dose of 9 Gy. At the age of 28–44 hr, hypersensitivity was seen only after exposure to the highest dose. Again, 0- to 4-hr embryos did not exhibit an increased sensitivity to X rays.

Maternal effects contribute during the first 24 hr of development: In early Drosophila embryos, the repair of DNA damage is also dependent on maternal factors deposited in the egg. To determine the effect of storage in the oocyte of maternal Lig4 protein and/or mRNA, *Lig4* heterozygous females were crossed to mutant males. In the offspring, the ratio of mutant males and heterozygous females was determined and compared to the ratio of mutant males to heterozygous females obtained after crossing $Lig4^{57}$-deficient females to *Lig4*-proficient males (Figure 4). After treatment of 0- to 8-hr-old embryos and 8- to 24-hr embryos and larvae, the recovery of males originating from *Lig4*-deficient females was decreased in comparison with males obtained from *Lig4*-proficient females. Treatment of 0- to 8-hr-old embryos with a dose of 9 Gy resulted in a 2-fold difference in sensitivity (ratios 0.76/0.39) of males originating from $Lig4^{+}/Lig4^{57}$ females compared to those coming from $Lig4^{57}/Lig4^{57}$ females (Figure 4A). Treatment of 8- to 24-hr-old embryos and larvae with the

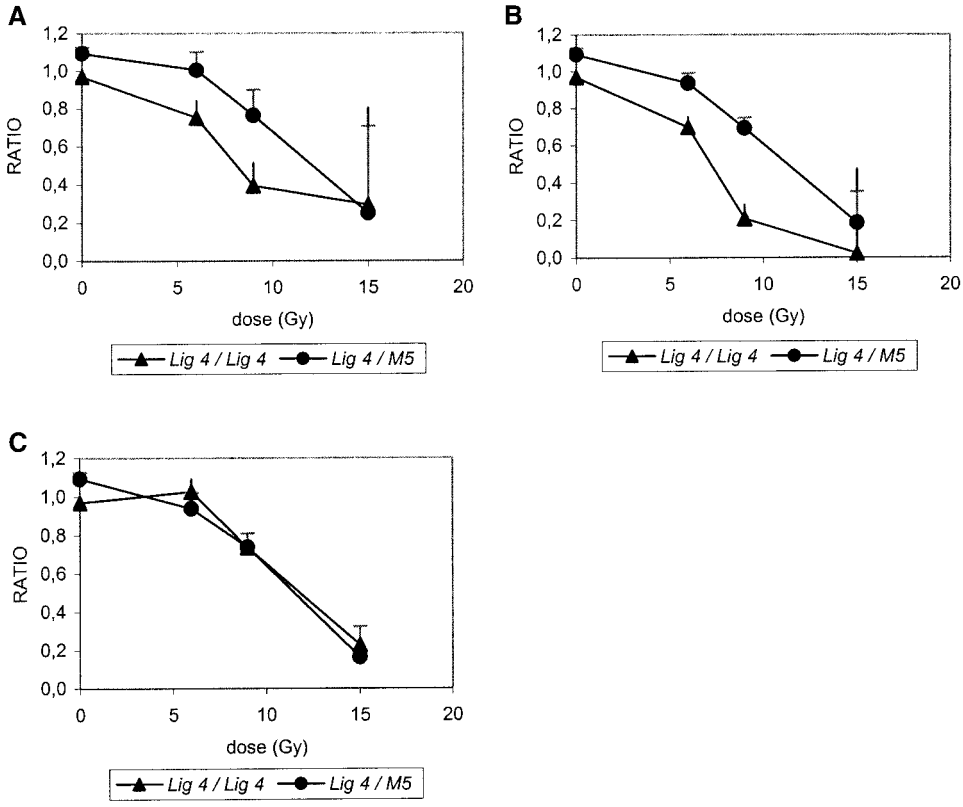

FIGURE 4.—Maternal contribution of Lig4 activity. Maternal effects were measured as the difference in recovery of *Lig4*-deficient males coming from *Lig4*-deficient females crossed to M5 males (▲) to those coming from heterozygous *Lig4* females crossed to *Lig4*-deficient males (●). Flies were irradiated with increasing doses of X rays (A) 0–8 hr, (B) 8–24 hr, and (C) 24–48 hr after egg laying. Standard deviations are based on the total number of flies scored.

same dose of X rays resulted in a 3.3-fold difference in sensitivity (0.69/0.21; Figure 4B). After 24–48 hr of larval development, no difference in sensitivity was seen among the offspring obtained from the two crosses (Figure 4C). Apparently, at this stage the maternally deposited Lig4 protein and/or mRNA is exhausted. At a dose of 9 Gy, the ratio dropped to 0.74 for both crosses, and at a dose of 15 Gy, dropped even further to 0.23 and 0.16 for males derived from *Lig4*$^{-}$/*Lig4*$^{-}$ and *Lig4*$^{+}$/*Lig4*$^{-}$ females, respectively.

HR repairs DSBs during very early embryonic stages of fly development: The data depicted in Figure 3, B and C, indicate that NHEJ hardly contributes to the repair of X-ray-induced DNA damage in very early embryos. To investigate the contribution of HR to the repair of X-ray-induced DSBs at these stages, we used the *Rad54*^{782} allele of *Rad54* (see MATERIALS AND METHODS). *Rad54*^{782}/*Rad54*^{782} females were crossed to *JS17/cn Cy* males and in a parallel control cross *JS17/cn Cy* females were mated to *Rad54*^{782}/*Rad54*^{782} males. The sensitivity of the *JS17/Rad54*^{782} (*Rad54*$^{-/-}$) mutant was calculated relative to *Rad54*^{782}/*cn Cy* (*Rad54*$^{+/-}$) heterozygous flies. Sensitivity of different developmental stages to X rays (0–8, 8–24, and 24–48 hr) was tested with increasing doses. In nonirradiated controls, the observed ratios were almost equal to the expected 1:1 ratio (Figure 5). In the first 8 hr of embryonic development, the ratio of *Cy*$^{+}$ (*Rad54*$^{-/-}$) to *Cy* (*Rad54*$^{+/-}$) flies in the offspring of *Rad54*^{782}/*Rad54*^{782} females decreased with increasing dose. In contrast, the ratio of *Cy*$^{+}$ to *Cy* flies in the offspring of heterozygous *JS17/cn Cy* females did not decrease with dose. At a dose of 9 Gy, nearly a fourfold difference in sensitivity was observed between the *Rad54*-deficient offspring from *Rad54*^{782}/*Rad54*^{782} females in comparison to the offspring from *JS17/cn Cy* females (Figure 5A). Among 8- to 24-hr-old embryos and larvae, no difference in sensitivity was observed between *Rad54*-deficient offspring from *Rad54*^{782}/*Rad54*^{782} females or *JS17/cn Cy* females (Figure 5B). Similar results were obtained for 24- to 48-hr-old larvae exposed to increasing doses of X rays (Figure 5C). The results suggest that after 8 hr of development the contribution of maternal Rad54 protein and/or mRNA is strongly reduced or exhausted. The significant increase in X-ray sensitivity seen in 24- to 48-hr larvae indicates also that at later stages of development HR plays an important role in the repair of X-ray-induced DSBs.

NHEJ and HR act synergistically in the repair of X-ray-induced DSBs: To determine the relative contribution of NHEJ and HR to the repair of DSBs, *Lig4; Rad54* double-mutant flies were generated by crossing *Lig4*^{57}/*Lig4*^{57}; *Rad54*$^{A17\text{-}11}$/*cn Cy* females to *Lig4*$^{+}$; *JS17/cn Cy* males. In this experiment only the *Lig4*^{57} strain was used, since the initial survival experiments did not show a difference between the *Lig4*^{57} and *Lig4*^{5} strains. The sensitivity of single and double mutants was calculated relative to *Lig4*$^{+/-}$; *Rad54*$^{+/-}$ heterozygous females (see MATERIALS AND METHODS). The embryos and larvae were treated with increasing doses of X rays at different developmental stages. After exposure of 0- to 24-hr-old

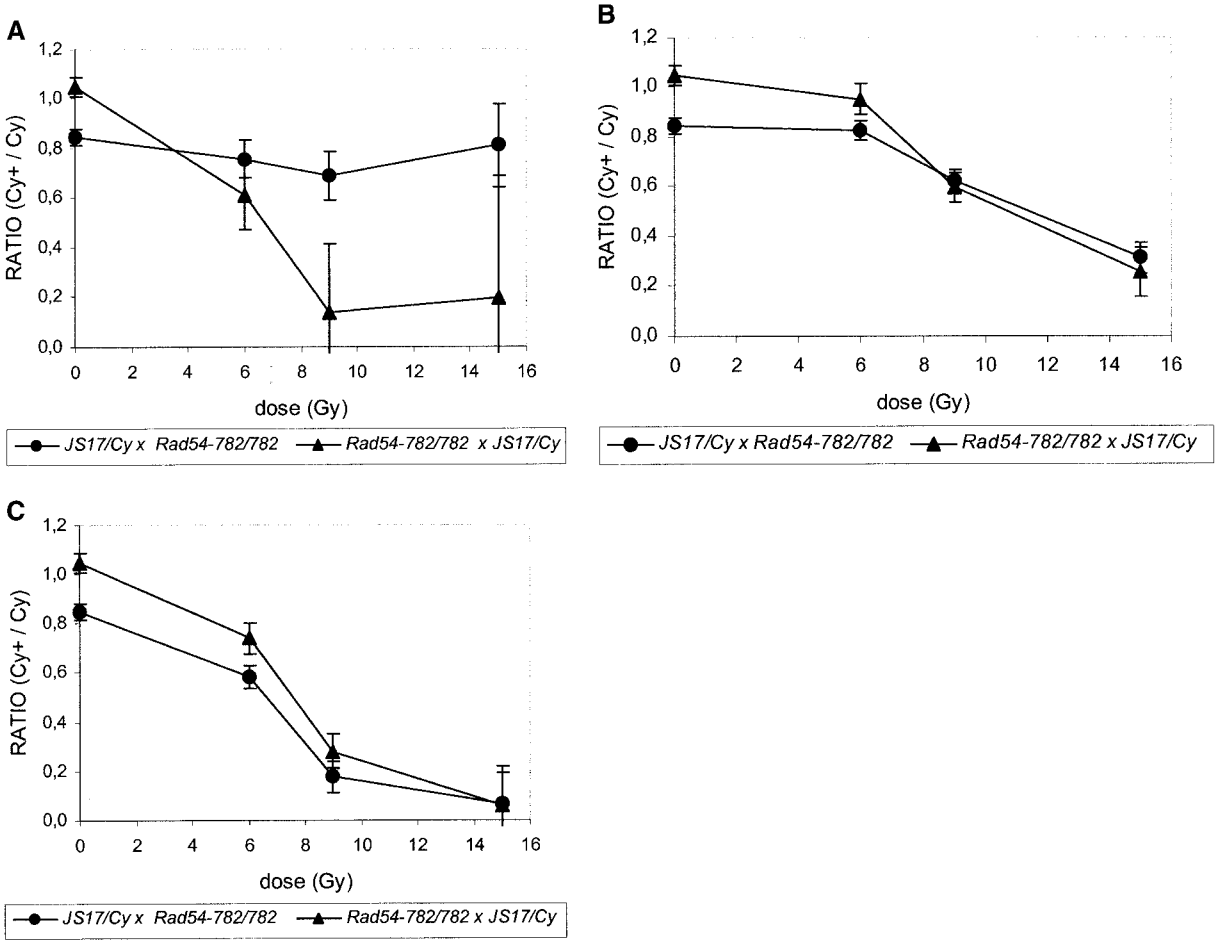

FIGURE 5.—Effect of X rays on *Rad54*-deficient mutants. The role of *Rad54* in the repair of X-ray-induced DSBs was determined using the $Rad54^{782}$ allele of *Rad54*. The sensitivity to X rays is given as the ratio of *Rad54*-deficient to *Rad54*-proficient flies. Maternal effects are seen as the difference in recovery of *Rad54*-deficient flies coming from $Rad54^{782}/Rad54^{782}$ females crossed to *JS17/cn Cy* males (▲) in comparison to those coming from *JS17/cn Cy* females crossed to $Rad54^{782}/Rad54^{782}$ males (●). Flies were exposed to X rays (A) 0–8 hr, (B) 8–24 hr, and (C) 24–48 hr after egg laying. Standard deviations are based on the total number of flies scored.

embryos and larvae to a dose of 3 Gy, a 3.2-fold and a 2.5-fold increase in hypersensitivity of the *Lig4*; *Rad54* double mutant was observed in comparison to the *Rad54* and *Lig4* single mutants, respectively (Figure 6A). At a dose of 6 Gy, *Lig4*; *Rad54* double-mutant flies displayed a 10-fold and a 4-fold increase in sensitivity in comparison to *Rad54* and *Lig4* single mutants, respectively. In 0- to 24-hr-old embryos and larvae, the difference in sensitivity observed between *Lig4* and *Rad54* single mutants can be partially ascribed to the maternal effect in the case of *Rad54*. Twenty-four hours later (24–48 hr), exposure to a dose of 3 Gy resulted in 12.5-fold and 11-fold increases in sensitivity of the *Lig4*; *Rad54* double mutant in comparison to *Rad54* and *Lig4* single mutants, respectively (Figure 6B). At higher doses, the toxic effect of the X rays becomes more severe and double-mutant as well as *Rad54* single-mutant flies were not recovered anymore. Treatment of 48- to 72-hr-old larvae with a dose of 3 Gy resulted in a 4-fold increase in sensitivity of the double mutant in comparison to both single mutants (Figure 6C). At a dose of 6 Gy and higher, both the *Lig4*; *Rad54* and *Rad54* larvae were killed. Only a relatively small increase in sensitivity is seen in *Lig4* larvae exposed to higher doses of X rays (Figure 6C). At 72–96 hr after egg laying, irradiation with a dose of 3 Gy did not result in an increased sensitivity of the single and double mutants. At higher doses, the *Rad54* single mutant and the *Lig4; Rad54* double mutant both showed the same drastic increase in sensitivity. The effects of increasing doses of X rays on the *Lig4* single mutant were much less severe (Figure 6D).

The results shown in Figure 6, C and D, indicate that at later stages of larval development the hypersensitivity of *Lig4* mutants is less pronounced. After a dose of 9 Gy, no *Lig4*; *Rad54* double-mutant flies could be recovered anymore. Exposure of *Lig4* mutant larvae of 48–72 and 72–96 hr resulted in a moderate increase in sensitivity at the higher doses applied. After a dose of 30 Gy, the

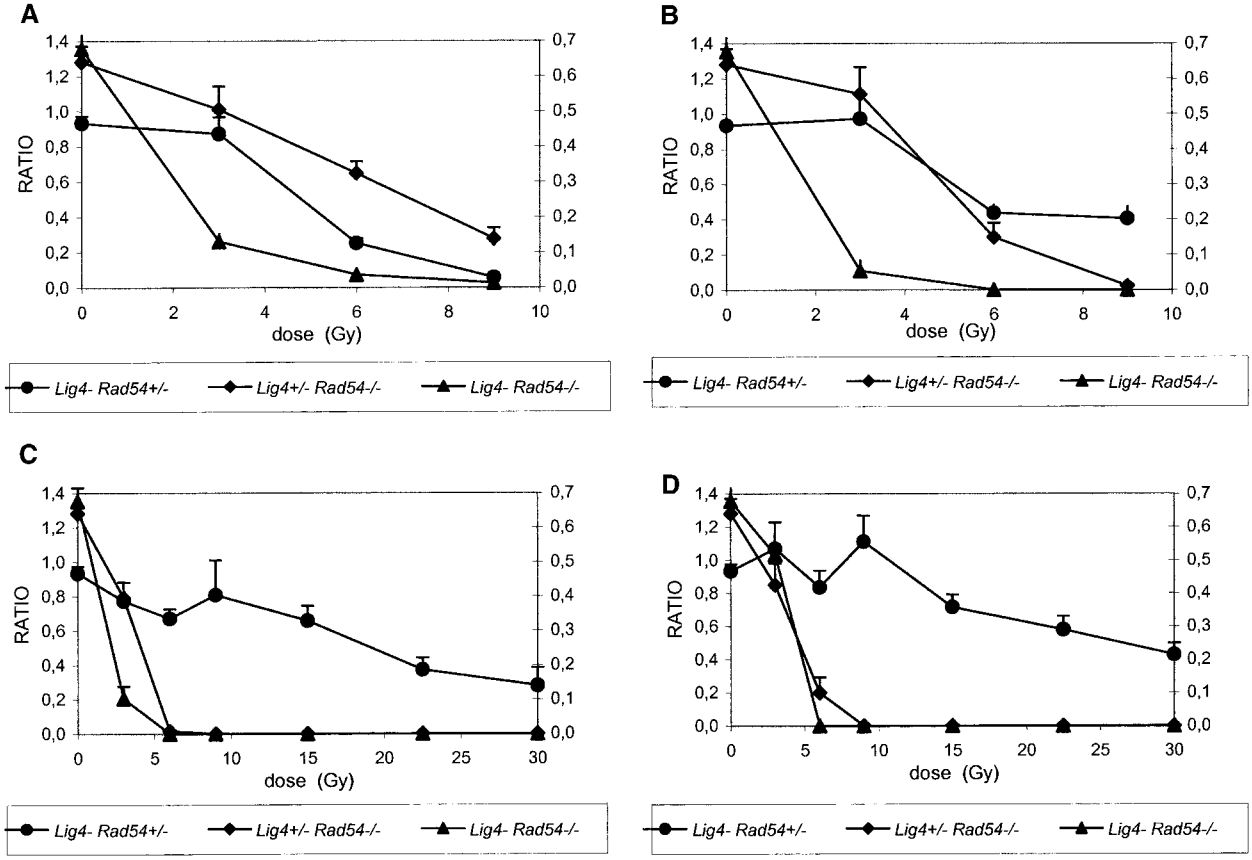

FIGURE 6.—Sensitivity of *Lig4; Rad54* double-mutant flies to X rays. Double-mutant flies were generated by crossing *Lig4*^{57}/*Lig4*^{57}*; Rad54*$^{A17\text{-}11}$*/cn Cy* females to *JS17/cn Cy* males. In the untreated offspring, the ratio of *Lig4; Rad54* double-mutant males, *Lig4* single-mutant males, *Rad54* single-mutant females, and *Lig4; Rad54* heterozygous females is expected to be 1:2:1:2 according to Mendelian laws. The ratio of single and double mutants was calculated relative to *Lig4; Rad54* heterozygous females. The ratios for the *Lig4* mutants are indicated on the left *y*-axis and the ratios for the *Rad54* mutants and the *Lig4; Rad54* double mutants are indicated on the right *y*-axis. The offspring were exposed to increasing doses of X rays (A) 0–24 hr, (B) 24–48 hr, (C) 48–72 hr, and (D) 72–96 hr after egg laying. (●) *Lig4*$^{-}$ *Rad54*$^{+/-}$, (◆) *Lig4*$^{+/-}$ *RAD54*$^{-/-}$, and (▲) *Lig4*$^{-}$ *Rad54*$^{-/-}$. Standard errors are based on fly count per vial.

ratio dropped to 0.24 and 0.37 for 48- to 72-hr and 72- to 96-hr larvae, respectively. These data indicate that after 48 hr of development, the role of NHEJ in the repair of X-ray-induced DSBs becomes less important than the role of HR.

***Lig4*-deficient flies are not sensitive to MMS and cisDDP:** As previously described, the *Rad54* mutant flies display a strong hypersensitivity to the alkylating agent MMS and to the crosslinking agent cisDDP (KOOISTRA *et al.* 1999). To determine if NHEJ is also involved in the repair of DSBs resulting indirectly as a consequence of exposure to these agents, *Lig4*^{57}*/Lig4*^{57}*; Rad54*$^{A17\text{-}11}$*/cn Cy* females were crossed to *JS17/cn Cy* males and the offspring were treated with MMS or cisDDP (see MATERIALS AND METHODS). Exposure of larvae at the age of 24–48 hr to increasing doses of cisDDP or MMS resulted in a nearly equal increase in sensitivity of the *Lig4*; *Rad54* double mutant and the *Rad54* single mutant (see Figure 7, A and B). Treatment of the *Lig4* single mutant with the same doses of cisDDP or MMS resulted in only an increased sensitivity at the highest doses used, suggesting no or a very minor role of NHEJ in the repair of crosslinks or alkyl lesions.

DISCUSSION

In higher eukyarotes, DNA Ligase IV is an essential protein used for the repair of DSBs via NHEJ. Inactivation of the DNA *Ligase IV* gene in mice results in embryonic lethality due to massive apoptosis in the central nervous system (BARNES *et al.* 1998; FRANK *et al.* 1998; GRAWUNDER *et al.* 1998a), which could be rescued in a p53- or ATM-deficient background or by deleting another member of the same repair pathway, namely Ku80 (FRANK *et al.* 2000; KARANJAWALA *et al.* 2002). In humans, partial defects in DNA *Ligase IV* result in developmental delay and immunodeficiency. Cells derived from patients suffering from this so-called LIG4 syndrome display increased sensitivity to ionizing radiation and impaired repair of DSBs (JEGGO and CONCANNON

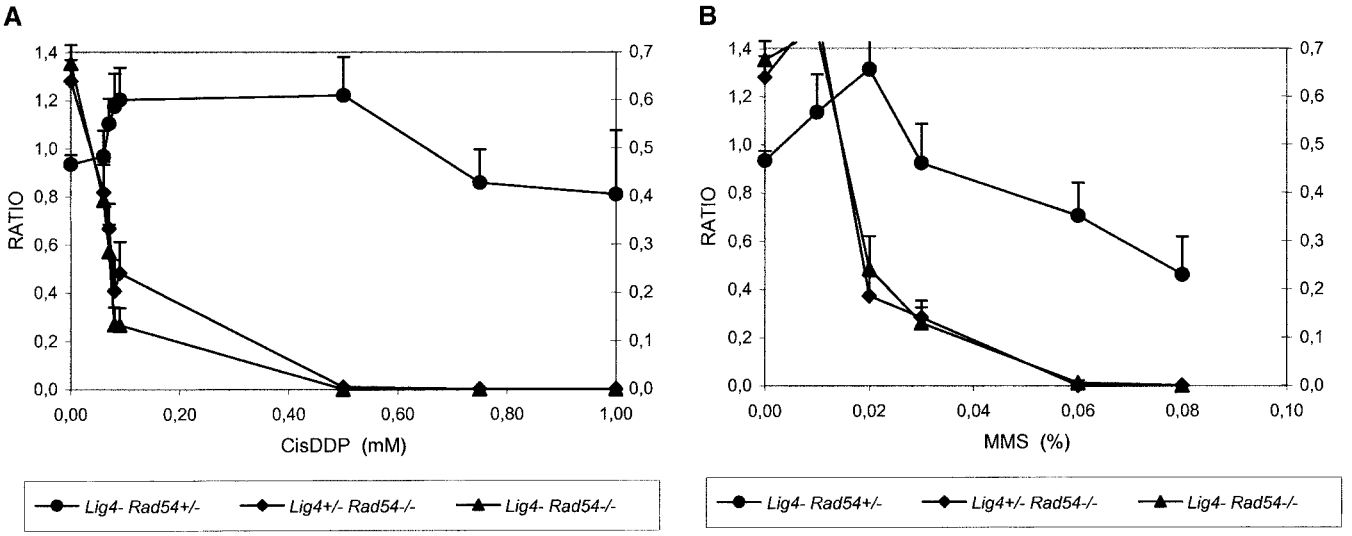

FIGURE 7.—Sensitivity of *Lig4; Rad54* double-mutant flies to cisDDP and MMS. Mutant flies were generated by crossing $Lig4^{57}$/$Lig4^{57}$; $Rad54^{A17\text{-}11}$/*cn Cy* females to $Lig4^{+}$; *JS17/cn Cy* males. The ratio of single and double mutants was calculated relative to *Lig4*; *Rad54* heterozygous females. The ratios for the *Lig4* mutants are indicated on the left *y*-axis and the ratios for the *Rad54* mutants and *Lig4; Rad54* double mutants are indicated on the right *y*-axis. After 24–48 hr of egg laying, larvae were exposed to increasing doses of cisDDP (A) or MMS (B). Standard errors are based on fly count per vial.

2001). To examine the effects of null mutations in the DNA *Ligase IV* gene at the organismal level, we employed *D. melanogaster* as a system. Sequence analysis of the Drosophila DNA *Ligase IV* gene, *Lig4*, revealed an ORF encoding a putative 918-amino-acid protein, which displays extensive sequence homology to other eukaryotic DNA Ligase IV proteins (TOMKINSON and MACKEY 1998). Within the so-called core region, DmLig4 shares 34 and 25% identity with hLig4 and ScLig4, respectively.

Lig4-deficient flies were generated by *P*-element-mediated mutagenesis (see MATERIALS AND METHODS). Two of the deletion mutant lines isolated, $Lig4^{5}$ and $Lig4^{57}$, were characterized in more detail. The $Lig4^{5}$ deletion extends until nucleotide 805 of the genomic sequence and the $Lig4^{57}$ deletion until nucleotide 2534 (see Figure 1). In contrast to *LIG4* mutant mice, flies deficient for *Lig4* are viable. Both males and females are fertile and show no obvious signs of defects or other abnormalities.

To investigate the role of *Lig4* in DNA repair, *Lig4*-proficient males were crossed to homozygous mutant females and the offspring exposed to DNA-damaging agents. The two mutant lines, $Lig4^{57}$ and $Lig4^{5}$, were equally hypersensitive to IR. The hypersensitivity was most severe after ~4 hr of embryonic development. Treatment of 4- to 20-hr-old embryos and larvae with a dose of 9 Gy resulted on average in a sevenfold increase in sensitivity. At later stages of development, the hypersensitivity of *Lig4*-deficient flies to IR is less severe.

Exposure of very young (0–4 hr) *Lig4*-deficient embryos to IR did not result in an increase in sensitivity (Figure 3B). Together these results imply that NHEJ contributes significantly to the repair of DSBs inflicted by IR but not during the first hours after fertilization. One possibility is that during early development DSBs are repaired through HR. By using the fertile $Rad54^{782}$ allele, we showed that HR is effective in the repair of DSBs in the first few hours of embryonic development (Figure 5).

To investigate the relative contribution of NHEJ and HR to the repair of DSBs in more detail, *Lig4; Rad54* double-mutant flies were generated by crossing $Lig4^{57}$/$Lig4^{57}$; $Rad54^{A17\text{-}11}$/*cn Cy* females to *JS17/cn Cy* males. Surprisingly, the *Lig4; Rad54* double-mutant flies were viable. When treated at early developmental stages (0–24 hr), the double-mutant flies were far more sensitive to IR than were either of the single mutants. These results indicate that in 0- to 24-hr-old embryos HR and NHEJ both contribute to the repair of IR-induced DSBs. Twenty-four hours later, when the maternal effect of *Rad54* wears off, a strong synergistic effect was observed in the double mutant. At higher doses, the toxic effect of IR becomes very severe and the double-mutant flies do not survive at all. Larvae that are 48–72 and 72–96 hr old rely predominantly on the HR for the repair of IR-induced DSBs, as shown by the relatively small increase in sensitivity seen for the *Lig4*-deficient flies. When treated with MMS or cisDDP, hardly any effect of *Lig4* deficiency was seen. Only at relatively high doses was a moderate increase in sensitivity observed (see Figure 7). Also, in yeast and humans NHEJ is not required for the repair of alkyl damage and crosslinks in DNA.

The survival data of double-mutant flies demonstrate that in Drosophila both NHEJ and HR contribute significantly to the repair of DSBs induced by ionizing radiation. The data also indicate that with the exception

of 0- to 4-hr embryos, both mechanisms can partially compensate for each other. At later stages of development (48–96 hr) the analysis of the double mutant suggests a less important role for NHEJ (see Figure 6, C and D). NHEJ and HR have been presented as competing pathways. Binding of Ku or Rad52 proteins to DNA ends at the site of the break would initiate DSB repair through NHEJ or HR, respectively (van Dyck *et al.* 1999). The result of such a competition is influenced by the relative amount of Ku70 and Rad52 (or by a functionally related protein in Drosophila, since a structural Rad52 homolog has not been identified), structure of the DSB, cell cycle phase, and stage of development (Hiom 1999). The pathway that is used has important consequences for the integrity of the genetic information of an organism. NHEJ is frequently associated with loss or gain of a few nucleotides. Correct restoration of the original sequence can occur via HR if the sister chromatid is used as a template. Using the homologous chromosome as a template could lead to loss of heterozygosity. Early embryonic development in Drosophila is a very rapid process. After fertilization the zygote nucleus undergoes nine divisions in a common cytoplasm to produce a multinucleate syncytium. After migration to the periphery of the egg, the nuclei undergo four more divisions before a cellular membrane is formed and somatic cells are produced. This process takes only 2.5 hr. To avoid accumulation of mutations during the rapid early divisions, which may have deleterious consequences at adult stages, it is beneficial to use HR as the principal mechanism in early development. Studies in mice also indicate that HR is especially important in early development (Essers *et al.* 2000). In contrast to mouse embryonic stem cells, a contribution of NHEJ cannot be detected in 0- to 4-hr-old Drosophila embryos, although we cannot exclude the possibility that defects in NHEJ can be fully compensated by HR in contrast to later stages. After the first 4 hr of embryonic development NHEJ does play an important role in the repair of DSBs. Between 4 and 20 hr of development *Lig4*-deficient flies are most sensitive to increasing doses of ionizing radiation (Figure 3, B and C). The hypersensitivity of *Lig4*-deficient larvae to IR is gradually reduced at later stages of development, indicating that the majority of the radiation-induced DSBs are repaired by HR and only a small fraction by NHEJ. It is difficult to speculate whether it is a competition between the repair pathways that causes those shifts or whether yet another repair system is active at later stages. Later in development the cell divisions definitely become much slower so it is not a matter of cell cycle stage and/or template availability, which would preferentially shift the repair toward HR. These observations differ from the data obtained from mouse studies. Mice deficient for *RAD54* are hypersensitive only at very early embryonic stages. In adult mice no hypersensitivity to ionizing radiation was seen in contrast to mice deficient in NHEJ (Essers *et al.* 1997, 2000).

The viability of the *Lig4; Rad54* mutant flies, as well as survival after low levels of X-ray irradiation, could be explained by evasion of checkpoint control and/or escape from checkpoint-triggered apoptosis at certain stages of the cell cycle or of development. Another possibility is that undamaged dividing cells in the imaginal discs can compensate for the loss of damaged and/or apoptotic cells. The viability of the double mutant after irradiation could also suggest the presence of another repair pathway that partially compensates for the impaired HR and NHEJ mechanisms. One possibility is single-strand annealing (SSA). This mechanism relies on the annealing of repeated sequences on both sides of the DNA break after the formation of 3′-single-strand tails (for review see Pastink *et al.* 2001). Evidence for the existence of SSA in Drosophila has been recently shown (Rong and Golic 2000; Preston *et al.* 2002). Another mechanism that possibly can overcome IR-induced DSBs in double-mutant flies is microhomology-dependent end joining (μEJ). Evidence for the existence of this pathway has been obtained from studies of mammalian cells mutated in one of the components required for NHEJ (Feeney 1992; Gerstein and Lieber 1993; Gottlich *et al.* 1998; Kabotyanski *et al.* 1998; Verkaik *et al.* 2002). Studies in yeast using engineered substrates transfected into *ku70/rad52* or *ku80/rad52* double mutants indicate the existence of a repair mechanism, which repairs DSBs on the basis of microhomology present on both sides of the break (Boulton and Jackson 1996a,b). Since small repeated sequences are frequently associated with the formation of chromosomal aberrations in mammalian cells, it will be of great interest to investigate the μEJ pathway in more detail in eukaryotic organisms, including Drosophila. The availability of *Lig4* and *Rad54* single and double mutants allows us to pursue further studies into the mechanisms of HR and NHEJ.

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