Synthetic Lethality of Drosophila in the Absence of the MUS81 Endonuclease and the DmBlm Helicase Is Associated With Elevated Apoptosis

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ABSTRACT

Mus81-Mms4 (Mus81-Eme1 in some species) is a heterodimeric DNA structure-specific endonuclease that has been implicated in meiotic recombination and processing of damaged replication forks in fungi. We generated and characterized mutations in *Drosophila melanogaster mus81* and *mms4*. Unlike the case in fungi, we did not find any role for MUS81-MMS4 in meiotic crossing over. A possible role for this endonuclease in repairing double-strand breaks that arise during DNA replication is suggested by the finding that *mus81* and *mms4* mutants are hypersensitive to camptothecin; however, these mutants are not hypersensitive to other agents that generate lesions that slow or block DNA replication. In fungi, *mus81*, *mms4*, and *eme1* mutations are synthetically lethal with mutations in genes encoding RecQ helicase homologs. Similarly, we found that mutations in Drosophila *mus81* and *mms4* are synthetically lethal with null mutations in *mus309*, which encodes the ortholog of the Bloom Syndrome helicase. Synthetic lethality is associated with high levels of apoptosis in proliferating tissues. Lethality and elevated apoptosis were partially suppressed by a mutation in *spn-A*, which encodes the ortholog of the strand invasion protein Rad51. These findings provide insights into the causes of synthetic lethality.

D NA repair and recombination processes involve formation of special DNA structures such as flaps, D-loops, and four-stranded Holliday junctions, which are processed by enzymes that recognize these specific structures. Mus81-Mms4 (or Mus81-Eme1) is one such enzyme. BODDY *et al.* (2001) reported that *Schizosaccharomyces pombe* Mus81-Eme1 displays endonuclease activity on Holliday junctions, but other studies have found that this enzyme and orthologs from yeast and mammalian cells have a higher affinity for other branched structures, such as 3′ flaps or substrates that mimic replication forks, and have the highest affinity for nicked Holliday junctions (KALIRAMAN *et al.* 2001; CONSTANTINOU *et al.* 2002; DOE *et al.* 2002; CICCIA *et al.* 2003; GAILLARD *et al.* 2003; OGRUNC and SANCAR 2003; OSMAN *et al.* 2003).

The *in vitro* substrate specificity of Mus81-Mms4 (or Mus81-Eme1) reflects the *in vivo* functions of this enzyme. In *S. pombe*, *mus81* and *eme1* mutations severely reduce the yield of viable meiotic spores (BODDY *et al.* 2001) and surviving spores display a near-complete absence of crossing over (OSMAN *et al.* 2003; SMITH *et al.* 2003; CROMIE *et al.* 2006). In *Saccharomyces cerevisiae*, *mus81* and *mms4* mutations eliminate the subset of meiotic crossovers that do not exhibit interference (DE

LOS SANTOS *et al.* 2003). These phenotypes presumably result from failure to cleave some recombination intermediate. Thus, Mus81 is required for a meiotic crossover pathway in *S. pombe* and *S. cerevisiae*, although both organisms also have Mus81-independent crossover pathways.

Yeast *mus81*, *mms4*, and *eme1* mutants are hypersensitive to agents that produce lesions that can block progression of replication forks, such as ultraviolet (UV) light and the alkylating agent methyl methanesulfonate (MMS) (BODDY *et al.* 2000; INTERTHAL and HEYER 2000). In *S. pombe*, *mus81* mutants are moderately sensitive to prolonged exposure to hydroxyurea (HU) (BODDY *et al.* 2000), an agent that causes replication fork stalling, although they are not highly sensitive to acute HU treatment (KAI *et al.* 2005). Yeast mutants are also hypersensitive to camptothecin (CPT), a topoisomerase inhibitor that generates double-strand breaks (DSBs) during replication (DOE *et al.* 2002; BASTIN-SHANOWER *et al.* 2003; KAI *et al.* 2005). These sensitivities suggest that Mus81 has a role in processing a DNA intermediate that arises during repair of replication forks or during replication restart.

An additional finding suggestive of a role for Mus81-Mms4 (or Mus81-Eme1) in replication fork repair comes from genetic interactions with RecQ helicases. In fungi, *mus81*, *mms4*, and *eme1* mutations are synthetically lethal with mutations in the RecQ helicase genes *SGS1* (*S. cerevisiae*) (MULLEN *et al.* 2001) and *rqh1* (*S. pombe*) (BODDY *et al.* 2000). RecQ helicases are thought to have

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important roles in repairing damaged, stalled, or blocked replication forks (Harmon and Kowalczykowski 1998; Courcelle and Hanawalt 1999; Doe *et al.* 2002; Liberi *et al.* 2005). One well-studied example is human BLM. Mutations in *BLM* cause Bloom Syndrome (BS), a rare hereditary disease characterized by short stature, severe sun sensitivity, and predisposition to a wide spectrum of cancers (reviewed in German 1993). A predominant feature of cells from BS patients is highly elevated levels of sister-chromatid exchange (Chaganti *et al.* 1974). These exchanges are believed to be the result of a change in processing of a DNA intermediate involved in replication fork metabolism. The synthetic lethality suggests that cleavage by Mus81-Mms4 is an alternative pathway for processing this intermediate. This interpretation is supported by the fact that the lethality of budding yeast *sgs1Δ mus81Δ* mutants is rescued in strains, such as *rad51* mutants, that are unable to exchange DNA strands for homologous recombination (Fabre *et al.* 2002; Bastin-Shanower *et al.* 2003).

Genetic studies of murine *Mus81* and *Eme1* have revealed some functional differences from fungal studies. *Mus81*$^{-/-}$ mice are fully fertile and show no defects in gametogenesis (McPherson *et al.* 2004; Dendouga *et al.* 2005), suggesting that this nuclease has little or no role in vertebrate meiotic recombination. In addition, the constellation of genotoxic agents to which *Mus81*$^{-/-}$ mouse cells are sensitive differs from that of *S. pombe* and *S. cerevisiae* mutants. While the fungal mutants are highly sensitive to MMS, UV, and CPT, the mouse mutants are only mildly sensitive to these agents, but are hypersensitive to agents that generate interstrand crosslinks (ICLs), such as cisplatin and mitomycin C (Abraham *et al.* 2003; McPherson *et al.* 2004; Dendouga *et al.* 2005; Hanada *et al.* 2006).

To better understand the roles of Mus81-Mms4 in metazoans, we generated mutations in the *mus81* and *mms4* genes of *Drosophila melanogaster*. We did not detect any defects in meiotic crossing over in these mutants. The mutants showed mild sensitivity to some DNA-damaging agents, but no differences from wild type for most agents tested. We also found that *mus81* and *mms4* mutations cause lethality when combined with null mutations in *mus309*, which encodes the Drosophila ortholog of the BLM helicase. This lethality was suppressed in triple mutants with *spn-A*, which encodes the Drosophila ortholog of the strand invasion protein Rad51. The *mus81* and *mms4* mutations were viable in combination with *mus309*^{N2}, a partial separation-of-function allele, providing novel insights into the cause of the lethality.

MATERIALS AND METHODS

Drosophila stocks: Flies were maintained on standard medium at 25°. Experiments with *mei-9* used the allele *mei-9*^{A2}, and experiments with *spn-A* used the *spn-A*^{093A} allele. Both are nonsense mutations that eliminate detectable protein expression (Staeva-Vieira *et al.* 2003; Yildiz *et al.* 2004). The *mei-41*^{29D} mutation deletes 975 bp of coding sequence and has a 1-bp insertion resulting in a frameshift; it is also genetically null (Laurencon *et al.* 2003). Several alleles of *mus309* were used; these are described in the text.

Mutations in *mus81* and *mms4*: A 3.9-kb fragment containing *mus81* and portions of the adjacent genes was cloned into pTV2. The 16-bp sequence between two *Pst*I sites in *mus81* was replaced with an *I–Sce*I recognition sequence. A 16-bp sequence containing an in-frame stop codon and an *Nhe*I site was inserted into a *Bgl*II site at codon 130. Ends-in targeting was conducted to generate integrations into the *mus81* locus, resulting in a tandem duplication in which one copy of *mus81* carried the insertion. Collapse of the tandem duplication, as in Rong and Golic (2000), produced the *mus81*^{Nhe} allele (Figure 1A).

To generate a mutation in *mms4*, we obtained the *P*-element line P{SUPor-P, *y*$^+$*w*$^+$}KG06402 from the Bloomington Drosophila Stock Center. We generated males that carried P{SUPor-P, *y*$^+$*w*$^+$}KG06402/*CyO*, H{w+, Δ2-3} to induce germline excision and crossed them to *y w*^{1118} females. *Cy*+ male progeny were screened for those that had lost the *y*+ and/or *w*+ markers on the *P* element. A total of 228 males, each from a different vial and therefore independent of one another, were crossed to generate progeny and then subjected to PCR to identify lines carrying deletions extending into *mms4*. We obtained three deletions: *mms4*^{ex1}, *mms4*^{ex2}, and *mms4*^{ex3}, which delete 839, 757, and 602 bp, respectively, of 958 coding base pairs in the gene (Figure 1B). We used *mms4*^{ex1} in the experiments described below.

Meiotic crossing over: Virgin females heterozygous for a series of markers on chromosome *3* (*cu sr e Pr ca*) were crossed to tester males (*cu sr e ca*) and progeny were scored for the five phenotypes. The female parents were *w*^{1118} (control), *y mus81*^{Nhe} *w*^{1118}, *w*^{1118} *mei-9*^{A2}, or *y mus81*^{Nhe} *w*^{1118} *mei-9*^{A2}. The white-eye phenotype of *w*^{1118} precluded scoring the claret-eye phenotype in males, so only female progeny were scored. Statistical comparisons were done for each pair of genotypes. For each interval, a χ^2 test was conducted to compare the number of progeny with a crossover in that interval *vs.* the number of progeny without a crossover.

Sensitivity to DNA-damaging agents: Crosses were designed to give a 1:1 ratio of mutant:control progeny. In most of the experiments, *mus81*^{Nhe} mutant males were crossed to *C(1)DX, y f/ Y* virgin females to generate *mus81*^{Nhe} (mutant) males and *C(1)DX, y f/ Y* (control) females. In some cases, *mus81*^{Nhe} males were crossed to *Df(1)AD11/FM7* or *y cv v f* virgin females, and female progeny, which were either *mus81*^{Nhe}/*Df(1)AD11* (hemizygous mutant) or *mus81*^{Nhe}/+ (control), were counted. For *mms4*, homozygous *mms4*^{ex1} virgin females were crossed to *Df(2R)E3363/CyO* males. Parents were allowed to mate and lay embryos for 3 days and then transferred to new vials and allowed to lay for 2 days before being removed. The first brood was left untreated. One day after removal of the parents from the second brood, 250 μl of a solution of the agent being tested was added to the food and ingested by feeding larvae. Adults were counted 9–17 days after removal of parents. To determine relative survival, the ratio of mutant:control was determined and then normalized to the mutant:control ratio found in untreated vials. All agents were dissolved in water except CPT. A stock solution of 5 mg/ml CPT in DMSO was made and this was diluted into 10% ethanol/2% Tween 20 in water. Mock treatment solution containing everything except CPT was added to the control brood. UV sensitivity assays were performed by irradiating third instar larvae in a Stratalinker (Stratagene, La Jolla, CA) as in Radford *et al.* (2005).

Synthetic lethality: Crosses were set up with parents homozygous for $mus81^{Nhe}$ or $mms4^{ex1}$ and heterozygous for *mus309* (*mus309* alleles were carried over a *TM3* balancer chromosome in the $mus81^{Nhe}$ background and *MKRS* in the $mms4^{ex1}$ background). The progeny were scored for survival to adulthood. Genotypes for experimental crosses are shown in Table 2. Control crosses were set up using females with the same genotype as those used in the experimental crosses and males that had wild-type *mus81* and were heterozygous for *mus309* (carried over the same balancer as used in the experimental crosses). Since *mus81* is on the *X* chromosome, the male progeny of the control cross were $mus81^{Nhe}$ mutants; therefore, only female progeny were used in calculations. Crosses to test for suppression of synthetic lethality by $spn\text{-}A^{093A}$ were identical except for the addition of $spn\text{-}A^{093A}$ to the $mus309^{N1}$ and $mus309^{D2}$ chromosomes.

Apoptosis measurements: Imaginal discs were dissected from wandering third instar larvae in Ringer's solution and fixed for 45 min in 4% formaldehyde and PBS with 0.1% Tween 20 (PBT). Discs were washed and blocked in PBT with 5% bovine serum albumin. Discs were incubated with 1:500 dilution of rabbit anti-human cleaved caspase-3 (Asp175) antibody (Cell Signaling Technology) in PBT overnight at 4°. Different lot numbers of the antibody stained different numbers of cells in wild-type discs; the experiments reported here were all done with the same lot number. Discs were incubated for 2 hr at room temperature with 1:1000 secondary goat anti-rabbit rhodamine-conjugated antibody (Molecular Probes, Eugene, OR) or secondary goat anti-rabbit fluorescein-conjugated antibody (Molecular Probes), stained with 10 μg/ml DAPI in PBT, and mounted with Flouromount-G (Southern Biotechnology Associates). Discs were visualized using TRIT-C and FIT-C filter of a Nikon Eclipse E800 fluorescent microscope. Quantification was performed by counting the number of antibody-stained cells in each of 5–15 wing discs of each genotype. Counts from each pair of genotypes were compared through an unpaired *t*-test with Welch's correction, using InStat (Graphpad) statistical software; two-tailed *P*-values are reported.

RESULTS

Mutations in *mus81* and *mms4*: The Drosophila ortholog of *mus81* maps to 1D2, near the tip of the *X* chromosome. We used the targeted mutagenesis method of RONG and GOLIC (2001) to introduce a mutation into *mus81* (see MATERIALS AND METHODS for details). A 16-bp fragment harboring a unique *Nhe*I site and an in-frame stop codon was inserted at a position predicted to terminate translation upstream of sequences encoding the conserved nuclease domain (Figure 1A); we refer to this allele as $mus81^{Nhe}$.

Since there is little sequence similarity between *S. pombe* Eme1 and *S. cerevisiae* Mms4, we used iterative BLAST searches to identify a Drosophila ortholog. This strategy pointed to *CG12936*, located in 47C1. After confirming that the product of this gene interacts with MUS81 in a yeast two-hybrid assay (J. LAROCQUE and J. SEKELSKY, unpublished data), we renamed the gene *mms4*. During the course of these studies, human *EME1* was identified (CICCIA *et al.* 2003; OGRUNC and SANCAR 2003). Eme1 proteins from sequenced mammalian genomes are predicted to be 570–590 residues and are
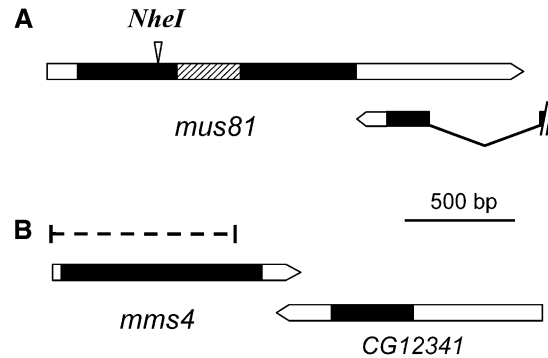

FIGURE 1.—Mutations in *mus81* and *mms4*. Schematics of (A) *mus81* and (B) *mms4* are shown. Protein-coding sequences are in solid. The region encoding the nuclease domain of MUS81 is stippled. The 16-bp insertion in $mus81^{Nhe}$ is indicated, and the 3'-end of the adjacent, overlapping gene is shown. In B, the region deleted in $mms4^{ex1}$ is denoted with a dashed bar. Both *mus81* and *mms4* lack introns. The 3'-UTR of each overlaps with the adjacent gene, which is transcribed in the opposite direction.

highly similar throughout. The predicted Drosophila MMS4 protein is only 309 residues; this protein shares sequence similarity (25% identity and 43% similarity) with the carboxy-terminal 300 residues of human Eme1. A full-length Drosophila *mms4* cDNA sequence has been reported (RE20777, GenBank accession no. AY071164), indicating that the size difference between human EME1 and Drosophila MMS4 is not due to an annotation error (there are no introns in the *mms4* gene, and there is another gene, *CG7637*, transcribed in the opposite direction, only 200 bp upstream of *mms4*). The N-terminal 200 residues of human Eme1 is conserved in other mammalian species but not in other vertebrates. *S. cerevisiae* Mms4 and *S. pombe* Eme1 are also longer at their amino termini, but conservation at the level of primary sequence is not evident. It is not known what function, if any, this N-terminal region has.

To generate mutations in *mms4*, we used the *P*-element insertion $P[SUPor\text{-}P]KG06402$, which is inserted 23 bp upstream of the ATG corresponding to the predicted start codon of *mms4*. No mutant phenotypes were observed in flies homozygous for $P[SUPor\text{-}P]KG06402$, so we conducted an excision screen to generate deletions (see MATERIALS AND METHODS for details). In the experiments described below, we used the deletion allele $mms4^{ex1}$, which removes 839 of 927 protein-coding base pairs (Figure 1B).

MUS81 is not required to generate meiotic crossovers: Yeast *mus81*, *mms4*, and *eme1* mutants have meiotic defects that include reductions in crossovers and spore viability (BODDY *et al.* 2001; DE LOS SANTOS *et al.* 2001; KALIRAMAN *et al.* 2001). In *S. pombe*, all meiotic crossovers require Mus81-Eme1 (OSMAN *et al.* 2003; SMITH *et al.* 2003), whereas in *S. cerevisiae* Mus81-Mms4 contributes to formation of the subset of crossovers that do not exhibit interference (DE LOS SANTOS *et al.* 2003). We found that

Genotype	Genetic distance (MU)					% of wild type	n
	cu–sr	*sr–e*	*e–Pr*	*Pr–ca*	Total		
w^{1118}	9.8	9.0	20.4	14.2	53.4	100	1463
$mus81^{Nhe}$	11.5	9.4	16.5	11.3	48.7	91	1967
$mei-9^{A2}$	1.5	0.7	2.4	1.9	6.5	12	538
$mus81^{Nhe}$ $mei-9^{A2}$	1.9	1.5	4.5	3.4	11.3	21	470

$mus81^{Nhe}$ flies have normal fertility and wild-type levels of meiotic nondisjunction of the X chromosome (data not shown). We measured meiotic crossing over along chromosome *3* (Table 1). Differences between *mus81* and wild type were significant in one of the four intervals, *e–Pr* ($P = 0.005$). Because this was the largest interval surveyed, spanning more than a third of the total genetic distance, this led to a slight decrease in the total map length of the region assayed; however, the map distances for both genotypes are within the range seen in different wild-type strains (KIDWELL 1977).

Previous work has shown that most crossovers in Drosophila require the nucleotide excision repair endonuclease MEI-9-ERCC1, in a complex with the novel protein MUS312 (BAKER and CARPENTER 1972; YILDIZ *et al.* 2002; RADFORD *et al.* 2005). Null mutations in *mei-9*, which encodes the catalytic subunit, eliminate 90% of crossovers (SEKELSKY *et al.* 1995). Interestingly, the nuclease domain in MEI-9 is related to that of MUS81 (ARAVIND *et al.* 1999). We hypothesized that MUS81-MMS4 might be involved in generating the residual crossovers observed in *mei-9* mutants. To test this hypothesis, we assayed meiotic crossing over in $mus81^{Nhe}$ $mei-9^{A2}$ double mutants (Table 1). Relative to wild-type or *mus81* single-mutant females, crossovers were significantly reduced in both *mei-9* single mutants and *mus81 mei-9* double mutants ($P < 0.0001$ for each interval). The increased frequency of crossovers in the double mutant *vs.* the *mei-9* single mutant is statistically significant over the entire interval ($P = 0.01$), but is not statistically significant in any single interval. We do not know whether the difference between *mei-9* single mutants and *mus81 mei-9* double mutants is biologically meaningful.

Sensitivity to DNA-damaging agents: Insight into functions of proteins involved in DNA metabolism can be gained by examining sensitivities of mutants to agents that damage DNA or interfere with DNA metabolism. Yeast *mus81*, *mms4*, and *eme1* mutants are hypersensitive to UV irradiation, MMS, HU, and CPT (BODDY *et al.* 2000; INTERTHAL and HEYER 2000; DOE *et al.* 2002; BASTIN-SHANOWER *et al.* 2003), whereas mouse $Mus81^{-/-}$ and $Eme1^{-/-}$ cells are hypersensitive to agents that cause ICLs (ABRAHAM *et al.* 2003; DENDOUGA *et al.* 2005;

HANADA *et al.* 2006). Although all these agents have multiple effects on cell metabolism, a commonality between them is that they have detrimental effects on replication forks: ICLs and damage induced by UV or MMS block DNA synthesis, HU treatment leads to stalled replication forks, and CPT causes replication-dependent DSBs (reviewed in FRIEDBERG *et al.* 1995). Hypersensitivities to these agents have been taken as evidence for the involvement of the Mus81-Mms4/Eme1 endonuclease in responding to replication fork problems.

We assayed $mus81^{Nhe}$ mutant larvae for sensitivity to UV, MMS, HU, CPT, ionizing radiation (IR), and nitrogen mustard. As a positive control, we conducted simultaneous assays of sensitivity on $mei-41^{29D}$ mutants. This gene encodes the Drosophila ortholog of ATR, which is required for the DNA-damage-dependent cell cycle checkpoint (HARI *et al.* 1995); *mei-41* mutants are hypersensitive to a wide range of DNA-damaging agents (BOYD *et al.* 1976; NGUYEN *et al.* 1979; MASON *et al.* 1981; BANGA *et al.* 1986). We did not detect hypersensitivity of $mus81^{Nhe}$ mutants to UV, MMS, or IR (data not shown). We did observe moderate hypersensitivity to CPT, which generates replication-dependent DSBs, and to the crosslinking agent nitrogen mustard (Figure 2). Surprisingly, $mus81^{Nhe}$ mutant larvae were less sensitive to HU than wild-type larvae (Figure 2). This was true for both hemizygous males ($mus81^{Nhe}/Y$) and hemizygous females ($mus81^{Nhe}/Df$), suggesting that the hyposensitivity to HU is due to mutation of *mus81*, rather than to some other mutation on the X chromosome; however, $mms4^{ex1}$ mutants were not hyposensitive (data not shown).

Mutations in *mus81* and *mms4* are synthetically lethal with null mutations in *mus309*: Mutations in *S. pombe mus81* are synthetically lethal with mutations in *rqh1*, which encodes a RecQ helicase (BODDY *et al.* 2000). Similarly, mutations in *S. cerevisiae MUS81* or *MMS4* are synthetically lethal with mutations in *SGS1* (MULLEN *et al.* 2001). There are three RecQ helicase genes in Drosophila: *RecQ4*, *RecQ5*, and *mus309* (SEKELSKY *et al.* 2000). Mutations in *mus309*, which encodes the Drosophila ortholog of the BLM helicase, DmBlm (KUSANO *et al.* 1999), result in hypersensitivity to MMS and IR, defects in DSB repair, maternal-effect embryonic lethality, and decreased meiotic recombination (BOYD *et al.* 1981; BEALL and RIO 1996; KUSANO *et al.* 2001; ADAMS *et al.* 2003; MIN *et al.* 2004; JOHNSON-SCHLITZ and ENGELS 2006; MCVEY *et al.* 2007, accompanying article in this issue).

We generated double mutants with $mus81^{Nhe}$ and various alleles of *mus309*. The $mus309^{D2}$ and $mus309^{D3}$ mutations were isolated in a screen for EMS-induced mutations causing hypersensitivity to MMS (BOYD *et al.* 1981); $mus309^{D2}$ is a nonsense mutation and is null, whereas $mus309^{D3}$ has a missense mutation that changes the glutamic acid residue in the DEAH motif to lysine, which should abolish helicase activity (KUSANO *et al.*
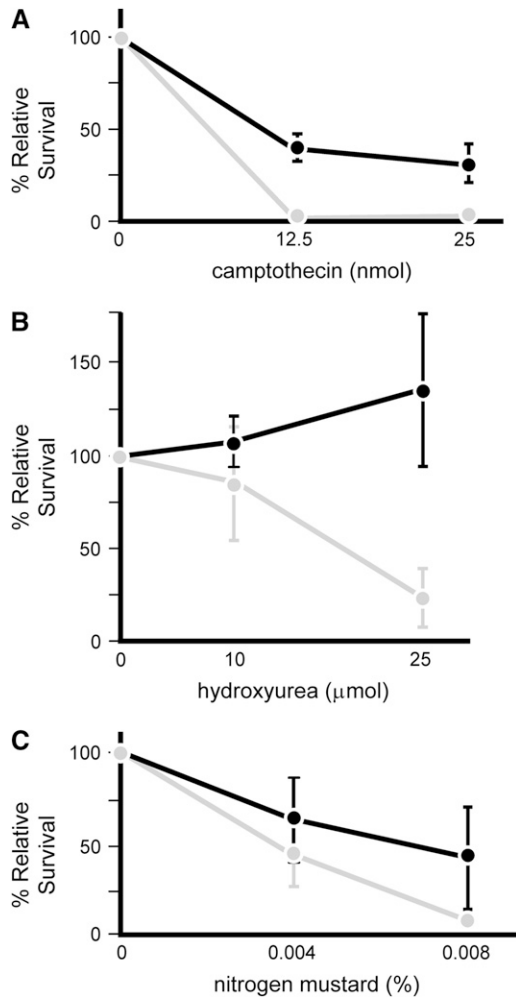
FIGURE 2.—(A–C) Sensitivity to DNA damage in *mus81*[Nhe]. Rates of survival to adulthood were calculated relative to control siblings in the same vial and normalized to ratios in untreated vials. Numbers on the *x*-axis represent the total quantity of agent added to the food (see MATERIALS AND METHODS), except for nitrogen mustard, which indicates the percentage (v/v) of agent in the 250 μl added to ∼8 ml of food. Solid lines, *mus81*[Nhe]; shaded lines, *mei-41*[29D] (positive control to verify activity of the agents). Relative survival for each point is the weighted mean from four to seven independent experiments, each including 7–10 independent vials. Error bars show standard deviation. For each point shown, there is a significant difference between survival of mutant and control siblings ($P < 0.05$), with the exception of *mus81*[Nhe] at the lower hydroxyurea dose ($P = 0.1221$).

2001). The *mus309*[N1] mutation is a deletion beginning 110 bp upstream of the coding region and extending for 2480 bp into the helicase domain; this allele is genetically null (MCVEY *et al.* 2007, accompanying article in this issue). Double mutants between *mus81*[Nhe] or *mms4*[ex1] and any of these null alleles of *mus309* were synthetically lethal (Table 2). We also tested for synthetic lethality between *mus81*[Nhe] and *mus309*[N2], a deletion that is predicted to truncate at least 566 residues from the amino terminus (MCVEY *et al.* 2007, accompanying article in this issue). The deletion does not extend

into the helicase domain, which begins at residue 736. This allele behaves like a null allele in a DSB repair assay, but is hypomorphic with respect to early embryonic function. Double mutants of the genotype *mus81*[Nhe]; *mus309*[N2]/*mus309*[N1] were viable (Table 2). In the cross reported in Table 2, survival was 83% relative to the control cross. In addition, these double-mutant adults are fertile. The *mus309*[N2] mutation therefore uncouples the role of DmBlm in DSB repair from the function required for survival in the absence of MUS81.

We examined the synthetic lethality between *mus81*[Nhe] and null alleles of *mus309* more carefully. Although we did not quantify lethality through all stages of development, we did observe many double-mutant pupae, often as pharate adults, indicating that double mutants can survive through embryonic and larval development. This observation is in agreement with a recent report by JOHNSON-SCHLITZ and ENGELS (2006), who found that lethality of *mus81; mus309* double mutants occurred primarily at the pupal stage. Pupal lethality often results from defects in cell proliferation (GATTI and BAKER 1989). This occurs when maternally deposited mRNA or protein is sufficient for embryonic development, but not for postembryonic proliferation of diploid tissues. Most larval growth is attributed to increases in cell size and ploidy rather than cell proliferation, so the mutant larvae grow and undergo pupariation; however, proliferation of diploid imaginal tissues and cells in the central nervous system is impaired, leading to the absence or reduction in size of the imaginal discs and the ventral ganglion. We dissected double-mutant larvae and did not detect any reductions in size of imaginal discs or the ventral ganglion.

Pupal lethality may also result from elevated cell death. We quantified the number of cells in wing imaginal discs that were undergoing apoptosis (Figure 3). There was no significant difference between wild-type and *mus81* single mutants ($P = 0.26$), but apoptosis was increased 3- to 4-fold in *mus309*[N1] single mutants ($P < 0.0001$ compared to wild type or *mus81*). Apoptosis was highly elevated in *mus81; mus309*[N1] double mutants, ∼15-fold over wild-type and 4-fold over *mus309*[N1] single mutants ($P < 0.0001$ for both comparisons). We did not detect any developmental delay in the double mutants, suggesting that the increased apoptosis is due to genome damage rather than to sampling of different developmental stages.

Synthetic lethality is suppressed by preventing homologous recombination: In *S. cerevisiae*, synthetic lethality between *sgs1* and *mus81* is suppressed by mutations in *RAD51* (FABRE *et al.* 2002; BASTIN-SHANOWER *et al.* 2003), which encodes a strand exchange protein essential for DSB repair through homologous recombination. One interpretation of this result is that Rad51-mediated strand invasion allows formation of a repair intermediate that must be processed by either the Sgs1 helicase or the Mus81-Mms4 endonuclease. In this model, the absence

TABLE 2

Synthetic lethality between *mus81^Nhe* and *mus309*

			Double mutant[c]		
Maternal genotype[a]	Paternal genotype[a]	Balanced[b]	Expected no.[d]	Observed no.	Survival (%)
mus81; mus309^D2/TM3	*mus81; mus309^D3/TM3*	1024	974	0	0
mus81; mus309^N1/TM3	*mus81; mus309^D3/TM3*	2489	2200	0	0
mus81; mus309^N1/TM3	*mus81; mus309^D2/TM3*	590	666	0	0
mus81; mus309^N1/TM3	*mus81; mus309^N2/TM3*	1924	1128	938	83
mms4; mus309^N1/TM3	*mms4; mus309^D2/TM3*	3984	1353	0	0
mus81; mus309^N1 spn-A/TM3	*mus81; mus309^D2 spn-A/TM3*	1710	1094	735	67

[a] Alleles used were *mus81^Nhe*, *mms4^ex1*, and *spn-A^093*. *mus309* alleles are indicated.

[b] Number of progeny carrying the *TM3* balancer and therefore heterozygous for the *mus309* chromosome.

[c] Number of double-mutant progeny (triple mutant, in the case of *mus81; mus309 spn-A*).

[d] The expected number is based on the number of balanced progeny, normalized to the ratio measured in the control crosses (identical except that the fathers did not carry a *mus81* or *mms4* mutation).

of Rad51 results in use of a different repair pathway that does not require Sgs1 or Mus81-Mms4. The Drosophila ortholog of Rad51 is encoded by *spn-A* (Staeva-Vieira *et al.* 2003). We found that mutation of *spn-A* partially suppressed the synthetic lethality between *mus81* and *mus309*, resulting in 67% of the mutants surviving to adulthood (Table 2).

We also quantified the effects of *spn-A* mutations on apoptosis (Figure 3). Apoptosis was elevated in *spn-A* single mutants relative to wild type ($P = 0.0003$), but not significantly different among *spn-A* single mutants, *mus309^N1 spn-A* double mutants, and *mus81; spn-A* double mutants ($P > 0.3$ for each comparison). Notably, apoptosis was lower in *spn-A* single mutants and *mus309^N1 spn-A* double mutants than in *mus309^N1* single mutants ($P = 0.0049$ and 0.0003, respectively), indicating that *spn-A* is epistatic to *mus309*. Consistent with this interpretation, we found that apoptosis was decreased in *mus81; mus309^N1 spn-A* triple mutants, relative to *mus81; mus309^N1* double mutants ($P = 0.0017$). However, the level in the triple mutants was still significantly higher than that in *spn-A* single mutants ($P < 0.0001$), suggesting that *spn-A* is only partially epistatic to *mus81* and

mus309. Interpretations of this finding are discussed below.

DISCUSSION

We generated mutations in Drosophila *mus81* and *mms4* and examined the effects of these mutations on processes in which fungal and mammalian Mus81, Eme1, and Mms4 have been implicated. In fungi, Mus81 is required to generate meiotic crossovers that do not display interference (Boddy *et al.* 2001; de los Santos *et al.* 2003; Argueso *et al.* 2004; Cromie *et al.* 2006). We observed a small decrease in crossing over in *mus81* mutants relative to the wild-type strain that we used as a control, but crossing over in the mutants was still within the range seen in different wild-type strains (Kidwell 1977). Our sample size was not large enough to rule out the possibility that Drosophila MUS81-MMS4 has a role in generating a subset of crossovers, such as those that do not exhibit interference. However, mathematical models of interference are consistent with a model in which all crossovers in Drosophila exhibit interference (Copenhaver *et al.* 2002).
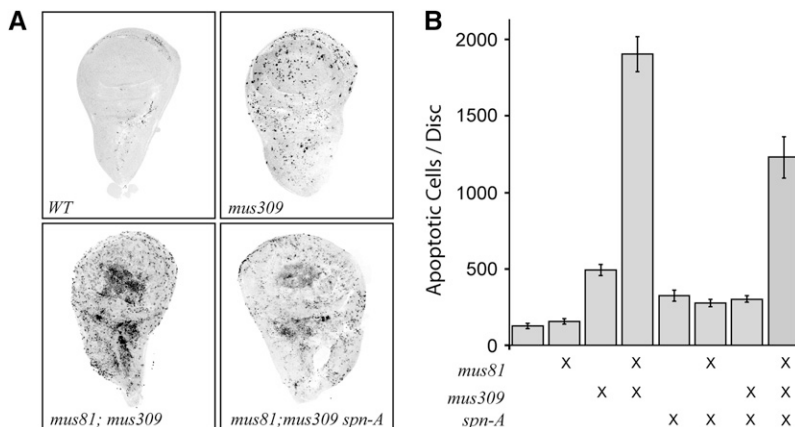

Figure 3.—Apoptosis in proliferating tissues. (A) Wing imaginal discs from mature third instar larvae, stained with an antibody that recognized cells undergoing apoptosis. Representative discs from four different genotypes are shown. The *mus309^N1* null allele was used in all cases. (B) Quantification of apoptosis in wing discs from larvae of various genotypes. Genotypes are given below the graph, with an "X" indicating a null mutation in the corresponding gene. Each bar represented the mean number of apoptotic cells ($n = 10, 5, 15, 12, 12, 11, 13$, and 8, left to right). Error bars are standard error of the mean.

Most crossovers in Drosophila require the MEI-9-ERCC1 endonuclease (Baker and Carpenter 1972; Sekelsky *et al.* 1995; Yildiz *et al.* 2004; Radford *et al.* 2005). Importantly, the residual crossing over that occurs in *mei-9* mutants was not eliminated by mutation of *mus81*. Our results do demonstrate, however, that MUS81-MMS4 does not have a major role in generating meiotic crossovers. This is similar to the case in other metazoans, such as mice and *Caenorhabditis elegans*, but differs from *S. cerevisiae* and *S. pombe*, where Mus81 is important in generating some or all meiotic crossovers. In other aspects of meiotic recombination, vertebrates appear to be more similar to *S. cerevisiae* than to Drosophila (McKim *et al.* 2002; Blanton and Sekelsky 2004). These similarities and differences reveal unexpected variability in this crucial process.

We measured the effect of various DNA-damaging agents on survival of *mus81* mutant larvae to adulthood. We detected hypersensitivity to some DNA-damaging agents that suggest a function in repairing damaged or stalled replication forks. Although these phenotypes were relatively mild, the spectrum of agents to which Drosophila *mus81* mutants are hypersensitive is more similar to that of mouse *MUS81* mutants than to that of *S. cerevisiae* and *S. pombe mus81* mutants. Nonetheless, each of these model organisms exhibits hypersensitivities that suggest a role in responding to damaged or stalled replication forks, and thus it appears that some important function is conserved, but that different response pathways are used to different extents in different species.

We found that *mus81* and *mms4* mutations are synthetically lethal with null mutations in *mus309*. Although we did not find any severe phenotypes in *mus81* single mutants, null mutants of *mus309* have several severe phenotypes, including strong hypersensitivity to MMS and IR, defects in DSB repair and meiotic recombination, and maternal-effect embryonic lethality (Boyd *et al.* 1981; Beall and Rio 1996; Kusano *et al.* 2001; Adams *et al.* 2003; McVey *et al.* 2007, accompanying article in this issue). Synthetic lethality cannot be caused by defects in meiotic recombination or, given the late stage at which the lethal phenotype manifests, to the essential early embryonic function of DmBlm. Synthetic lethality is also not due to the function of DmBlm in DSB repair, since the *mus309^{N2}* allele is not synthetically lethal with *mus81*, despite the fact that *mus309^{N2}* behaves like a null allele in DSB repair assays (McVey *et al.* 2007, accompanying article in this issue).

Insights into the etiology of synthetic lethality come from studies of the effects of *spn-A* on lethality and apoptosis. Similar to the case in fungi, we found that a mutation in *spn-A*, which encodes the Drosophila ortholog of the strand invasion protein Rad51, suppresses synthetic lethality. This result differs from a recent report by Johnson-Schlitz and Engels (2006), who were not able to suppress synthetic lethality with a *spn-A* mu-
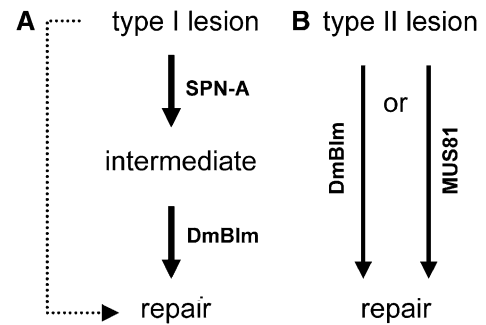

Figure 4.—Conceptual model for roles of SPN-A, DmBlm, and MUS81 in repair of spontaneous lesions. (A) Type I lesions are normally processed by SPN-A into an intermediate that is further processed by DmBlm. Our data do not reveal any evidence for a role of MUS81 in repairing this type of lesion. (B) Type II lesions are processed by either of two pathways, one requiring MUS81 and the other requiring DmBlm. Our data do not reveal a role for SPN-A in repairing this type of lesion.

tation. A likely explanation is that Johnson-Schlitz and Engels (2006) used *spn-A^1*, which is a hypomorphic allele (Gonzalez-Reyes *et al.* 1997), whereas we used the protein-null allele *spn-A^{093}* (Staeva-Vieira *et al.* 2003). Another difference is that the *mus81* deletion used by Johnson-Schlitz and Engels (2006) also removes the adjacent gene, *CG3703*. The function of this gene is unknown, but the protein it encodes is highly conserved (49 and 54% similarity to the human homolog, RUNDC1, over stretches of 379 and 204 residues, respectively) and contains a RUN domain characteristic of proteins involved in Ras-like GTPase signaling (Callebaut *et al.* 2001). In our experiments, suppression of synthetic lethality was incomplete (67% of the expected number of adults eclosed), suggesting that the *mus81; mus309^{N1} spn-A* triple-mutant genotype is near the threshold of viability. Additional mutations, such as deletion of *CG3703*, may decrease overall fitness so as to preclude survival of any individuals to adulthood.

We found that *mus309^{N1}* single mutants had elevated apoptosis. Apoptosis is also elevated in *spn-A* single mutants, but to a lesser degree. SPN-A is epistatic to DmBlm, since *mus309^{N1} spn-A* double mutants had the same amount of apoptosis as *spn-A* single mutants. We propose that there is some type of spontaneous lesion that is processed into a repair intermediate by SPN-A and then by DmBlm (type I lesions, Figure 4A). In the absence of SPN-A, there is a secondary pathway for repair of this type of lesion. This secondary pathway is not as efficient as the SPN-A-dependent pathway, or is error-prone, leading to some increased apoptosis in *spn-A* single mutants. Inability to process the SPN-A-dependent intermediate by DmBlm, however, results in more severe defects than using the SPN-A-independent pathway, so apoptosis is correspondingly higher in *mus309^{N1}* single mutants than in *spn-A* single mutants or *mus309^{N1} spn-A* double mutants.

In *mus81; mus309^{N1}* double mutants, apoptosis is dramatically elevated. This suggests that there is a second type of spontaneous lesion (type II) that can be repaired by either of two pathways, one requiring DmBlm and the other requiring MUS81 (Figure 4B). Synthetic lethality may be due to the sum of the apoptosis resulting from failure to repair type I and type II lesions. In the *mus81; mus309^{N1} spn-A* triple mutant, apoptosis is significantly reduced relative to the *mus81; mus309^{N1}* double mutant, but is still quite high. During proliferation of imaginal tissues, cell death caused by DNA damage is compensated for by increased proliferation, at least up to some threshold beyond which the tissue cannot regenerate (HAYNIE and BRYANT 1977; JAKLEVIC and SU 2004). The amount of apoptosis in the triple mutants may be near the threshold for viability, so that only a fraction of individuals can survive to adulthood. Alternatively, there may be tissue-specific effects on apoptosis, and the tissue that we examined (wing discs) may not be affected to the same extent as tissues that contribute to inviability (*e.g.*, central nervous system).

We speculate that type II lesions may be blocked replication forks, since both RecQ helicases and Mus81 have been implicated in processing blocked forks (reviewed in BRANZEI and FOIANI 2007; OSMAN and WHITBY 2007). Type I lesions may be DSBs associated with broken replication forks. Recombinational repair of these DSBs would require SPN-A and, subsequently, DmBlm. In *spn-A* mutants, repair could be accomplished through nonhomologous end joining. The *spn-A* mutation causes a similar reduction in apoptosis in *mus309^{N1}* single mutants and *mus81; mus309^{N1}* double mutants (34% *vs.* 35%, $P = 0.81$ by Fisher's exact test). The similar effect of loss of SPN-A on both genotypes can be explained if type II lesions that are not properly processed are converted into type I lesions; *i.e.*, in the absence of MUS81 and DmBlm, blocked replication forks break, generating DSBs.

The *mus309^{N2}* mutation is not synthetically lethal with the *mus81* mutation (Table 2). We hypothesize that the *mus309^{N2}* deletion destroys the ability of DmBlm to dissociate D-loops ("disruptase" activity), but does not eliminate the ability to promote Holliday junction branch migration. Thus, *mus309^{N2}* mutants are unable to repair type I lesions, consistent with the observed defects in DSB repair (MCVEY *et al.* 2007, accompanying article in this issue), but are competent to process type II lesions.

In conclusion, we found that Drosophila MUS81-MMS4 is not required to generate meiotic crossovers, but may have some functions in responding to lesions that arise during DNA replication. These functions are at least partially redundant with those of DmBlm, so that loss of both proteins is lethal. Lethality occurs in pupal stages, due to increased apoptosis. A subset of the damage leading to apoptosis is due to failure to process recombination intermediates generated by the SPN-A strand invasion protein.

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