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A Large-Scale Screen for Mutagen-Sensitive Loci in Drosophila

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ABSTRACT

In a screen for new DNA repair mutants, we tested 6275 Drosophila strains bearing homozygous mutagenized autosomes (obtained from C. Zuker) for hypersensitivity to methyl methanesulfonate (MMS) and nitrogen mustard (HN2). Testing of 2585 second-chromosome lines resulted in the recovery of 18 mutants, 8 of which were alleles of known genes. The remaining 10 second-chromosome mutants were solely sensitive to MMS and define 8 new mutagen-sensitive genes (*mus212*–*mus219*). Testing of 3690 third chromosomes led to the identification of 60 third-chromosome mutants, 44 of which were alleles of known genes. The remaining 16 mutants define 14 new mutagen-sensitive genes (*mus314*–*mus327*). We have initiated efforts to identify these genes at the molecular level and report here the first two identified. The HN2-sensitive *mus322* mutant defines the Drosophila ortholog of the yeast *snm1* gene, and the MMS- and HN2-sensitive *mus301* mutant defines the Drosophila ortholog of the human HEL308 gene. We have also identified a second-chromosome mutant, $mus215^{ZIII-2059}$, that uniformly reduces the frequency of meiotic recombination to <3% of that observed in wild type and thus defines a function required for both DNA repair and meiotic recombination. At least one allele of each new gene identified in this study is available at the Bloomington Stock Center.

THE ability of cells to reproduce their genome accurately requires both continuous monitoring of the integrity of the DNA complement and efficient repair of damage to the DNA. Coordination of these processes is required for proper completion of DNA replication and cell division, and it is crucial that cells be able to recognize damaged or incompletely replicated DNA to halt the cell cycle while damage is repaired and, most critically, to accurately repair that damage. In higher eukaryotes, impediments to repair can lead to high frequencies of mutation, cancer, and in some cases, cell or organismal death. DNA damage involves a variety of molecular lesions, including double-strand breaks (DSBs) of the DNA duplex, nicks in a single strand, creation of abasic sites, and a plethora of covalent chemical modifications. These modifications include covalent linkage of a variety of large and small adducts to the bases, as well as creation of intra- and interstrand crosslinks between bases. Damage can result both from external causes, including exposure to chemical mutagens and ionizing radiation, and from interaction with reactive species generated through normal cellular oxidative metabolism.

The repair of such disparate types of damage requires the action of a variety of qualitatively different DNA repair systems. To date, there is biochemical and genetic evidence from bacteria, yeast, and other higher eukaryotic systems for >130 distinct proteins involved in recognition and repair of DNA damage (Wood *et al.* 2001). Some of these proteins function quite specifically in repairing or removing damaged DNA (*e.g.*, photolyase), while others (*e.g.*, DNA ligases) play more general roles in cellular metabolism in addition to their specific functions in repair. There are five major categories of DNA repair, as reviewed in detail (Friedberg *et al.* 1995). These include damage reversal, in which the chemical alteration to the DNA molecule is reversed to restore the original sequence; base excision repair, in which damaged nucleotides are excised in a process that involves sequential base removal, endonucleolytic cleavage of the abasic site, and replacement of one or a few nucleotides; nucleotide excision repair, in which bases modified by bulky adducts are removed along with flanking bases by a pair of endonucleolytic cleavages releasing an oligonucleotide, followed by gap filling; mismatch repair, in which normal but mismatched base pairs trigger a strand-specific sequence of events, including endonucleolytic cleavage near the mismatch, exo-

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nucleolytic removal of sequences including the mismatched nucleotide, and polymerization to replace the sequences removed; and double-strand break repair, in which the broken ends of a duplex are rejoined by one of two methods: either direct ligation of the two ends after processing, which commonly results in the loss of sequence integrity at the junction, or repair by homologous recombination, in which the undamaged sequence present on the sister chromatid or homologous chromosome is used as a template for accurate repair of the break. Specific types of damage may require the action of more than one of these pathways. For example, genetic evidence from studies of interstrand crosslink repair mutants in *Saccharomyces cerevisiae* indicates that this process involves functions belonging to three separate repair pathways, as defined by genetic epistasis groups (Grossmann *et al.* 2001).

Genes that do not actually participate in the physical correction of damaged sequences may nonetheless be considered to be part of the DNA repair machinery of the cell. For example, error-prone DNA polymerases permit *trans*-lesion synthesis past damaged bases, allowing a cell to complete DNA replication despite the persistence of damage (Holmquist and Maher 2002). Likewise, "checkpoint" genes, which monitor genomic integrity and halt the progression of the cell cycle until damage is repaired by the mechanisms described above, are essential components of the cellular response to DNA-damaging agents (Weinert and Lydall 1993; Lehmann and Carr 1995).

Work on DNA repair in Drosophila began over 2 decades ago with the first screens for repair-deficient mutants (Boyd *et al.* 1976; Smith 1976). More than 30 Drosophila genes with mutagen hypersensitivity phenotypes have been identified in such screens to date. Genetic screens for mutagen-sensitive (*mus*) mutants have identified 8 DNA repair genes on the X chromosome (Boyd *et al.* 1976, 1981; Smith 1976; Nguyen *et al.* 1978; Yamamoto *et al.* 1990; Leonhardt and Boyd 1993), in addition to two mutagen-sensitive loci (*mei-9* and *mei-41*) that were first identified in screens for X-linked meiotic mutants (Baker and Carpenter 1972; Baker *et al.* 1976). Twenty-two DNA repair genes on the second and third chromosomes were likewise identified in screens for new *mus* mutants (Boyd *et al.* 1981; Snyder and Smith 1982; Henderson *et al.* 1987). The information currently available about these genes is summarized in Table 1.

Despite the success of these earlier screens, two lines of evidence suggested that there were many more such genes to be identified in Drosophila. First, numerous Drosophila proteins similar in amino acid sequence to known repair proteins from other species, but not associated with a specific mutagen-sensitive mutation, have been predicted through comparative analysis of the Drosophila genome sequence (Adams *et al.* 2000). Second, many of the genes for which mutants do exist remain defined by only a single allele, an observation that suggests that screens for mutagen-sensitive mutants have not yet reached saturation. For these reasons, we set out to exploit an important Drosophila resource, the collection of nearly 12,000 EMS-mutagenized lines created by Charles Zuker (E. J. Koundakjian and C. Zuker, personal communication) to carry out a screen for novel *mus* mutants.

We report here a screen of 6275 homozygous viable mutagenized lines (derived from the Zuker collection) for new mutants that were sensitive to methyl methanesulfonate (MMS) and/or nitrogen mustard (HN2). Of the 78 mutants recovered, 52 represent additional alleles of known genes. One of these, *mus301*, we have identified as the Drosophila ortholog of the human HEL308 gene. The remaining 26 new mutants define 22 new genes. Characterization of the 78 mutants is described below. One of these genes (*mus322*) has been characterized at the molecular level and shown to encode the Drosophila homolog of the interstrand crosslink repair gene *snm1* (Henriques and Moustacchi 1980; Dronkert *et al.* 2000). Another group has used the two *mus312* alleles generated in this screen to identify the *mus312* gene at the molecular level. In addition, a newly identified second-chromosome gene (*mus215*) has been shown to be required for normal levels of meiotic recombination, as well as for repairing DNA damage induced by MMS.

MATERIALS AND METHODS

Acquisition of stocks: The Zuker laboratory created a collection composed of 6000 second-chromosome and 6200 third-chromosome lines obtained following mutagenesis with 25 mm EMS (Niemeyer *et al.* 1996; Tsunoda *et al.* 1997; E. J. Koundakjian and C. Zuker, personal communication). The mutagenized autosomes were isolated over second- or third-chromosome balancers, and the stocks were provided and maintained in this state. The collection arrived in batches of 600 lines each week over a total of 7 months. All mutant lines were maintained at room temperature on a standard cornmeal-dextrose medium. The second-chromosome mutants were isolated on a c*n bw* chromosome and balanced over *CyO*, while the third-chromosome mutants were isolated on a *st* chromosome and received as stocks of genotype *bw/bw; st/TM6B*. Our screen compared the viability of mutagenized homozygotes to that of their mutagenized balancer heterozygote siblings. Mutant lines in which the frequency of homozygotes in the absence of treatment was <10% of the expected number were not tested further, since the presence of accumulated lethal or sublethal mutations interfered with the screen for mutagen sensitivity. The total number of discarded second-chromosome lines was 3415 (or 56.9%) of the 6000 lines. The total number of discarded third-chromosome lines was 2510 (or 40.2%) of the 6200 lines. The remaining 6275 mutant lines were tested in our screen.

At least one allele of each new gene identified in this study is available at the Bloomington Stock Center. The Hawley laboratory in Kansas City maintains all other alleles.

Screening for mutagen sensitivity: The screen consisted of three consecutive rounds of mutagen-sensitivity testing: In the first round, the 6275 lines from the second (2585) and third

TABLE 1

Summary of genes identified in earlier screens for *mus* mutants

Gene	Genetic location	CG no.	MMS	HN2	Previously reported EMS-induced alleles	*S. cerevisiae*/ mammalian ortholog	Reference for orthology
					Second chromosome		
mus201	2-34	CG32656	+	+	2	*Rad2/XPG*	Sekelsky *et al.* (2000)
mus202	2-		+	+	1		
mus203	2-		+	+	1		
mus204	2-		+	+	1		
mus205	2-54.9	CG1925	+	−	2	*Rev3/POLZ*	Eeken *et al.* (2001)
mus206	2-64.8		+	+	1		
mus207	2-		+	+	1		
mus208	2-89		+	±	2		
mus209	2-93.2	CG9193	+	−	10	*Pol30/PCNA*	Henderson *et al.* (1994)
mus210	2-69.1	CG8153	+	+	4	*Rad4/XPC*	Sekelsky *et al.* (2000)
mus211	2-47		+	+	2		
					Third chromosome		
mus301	3-23		+	+	5	None/*HEL308*	This study
mus302	3-45		+	+	6		
mus304	3-46	CG7347	+	+	6	None/*ATR-IP*	Cortez *et al.* (2001)
mus305	3-44		+	+	3		
mus306	3-56		+	−	1		
mus307	3-59		+	+	1		
mus308	3-51	CG6019	−	+	6	None/*POLQ*	Burtis and Harris (1997); Sharief *et al.* (1999)
mus309	3-	CG6920	+	+	3	*Sgs1/BLM*	Kusano *et al.* (2001)
mus310	3-47		+	−	1		
mus311	3-47		+	−	3		
mus312	3-18	CG8601	+	+	2	None/none	Yildiz *et al.* (2002)
					X chromosome		
mei-41	1-54.2	CG4252	+	+	48	*Mec1/ATR*	Hari *et al.* (1995); Laurencon *et al.* (2003)
mei-9	1-6.5	CG3967	+	+	16	*Rad1/XPF*	Sekelsky *et al.* (1995)
mus101	1-44.2	CG11156	+	+	10	*Dpb11/TopBP1*	Yamamoto *et al.* (2000); Makiniemi *et al.* (2001)
mus102	1-0.5		+	−	13		
mus105	1-13.7		+	−	7		
mus106	1-36-44		+	−	1		
mus108	1-10.8		+		1		
mus109	1-30.2		+	±	6		
mus111	1-26.9		+	+≅1			
mus112	1-32.8		+		3		

"+," mutagen sensitivity; "−," lack of sensitivity; "±," weak sensitivity (see materials and methods for details). Sensitivities indicated are based on information presented in the FlyBase database gene reports and references therein (http://flybase.bio.indiana.edu/).

(3690) chromosomes were tested with the appropriate concentrations of MMS and HN2 (see above). Young males and females from each line were transferred into a fresh vial and were allowed to lay eggs for 24 hr at room temperature. After 48 hr, these first vials were treated with MMS. At the end of the first 24 hr, the parents were transferred into a second vial and were allowed to lay eggs for an additional 24 hr. After 48 hr, these second vials were treated with HN2. At the end of 24 hr, the second-vial parents were transferred to a third vial and were allowed to lay eggs for 24 hr and then discarded. The third vials were the controls for round 1. All vials were scored on the fifteenth day after the beginning of egg laying. Lines were considered mutagen sensitive if either mutagen-treated vial had a homozygote/balancer heterozygote ratio that was <10% of the homozygote/balancer heterozygote ratio of the untreated control vials.

A total of 1558 balanced lines (681 from the second chromosome and 877 from the third chromosome) entered round 2 of the screen. For round 2, we followed a similar protocol as in round 1, except that all lines went through the screen as paired vials. The mutagen treatments also changed in this round, with lines receiving 0.05 and 0.025% MMS treatments and 0.003 and 0.005% HN2 treatments. During the first half of this round, it was determined that no significant difference in sensitivity occurred between the two concentrations of MMS or HN2; thus, the last half of the round 2 lines were treated with only 0.05% MMS or 0.005% HN2. A total of 507 lines (210 from the second chromosome and 297 from the third

chromosome) entered round 3 of the screen. To increase the number of progeny tested, round 3 lines were brooded in bottles. The transfer/treatment protocol was the same as round 1; the mutagen treatments remained at 0.04% MMS and 0.003% HN2 for both second- and third-chromosome lines.

Complementation testing: We isolated a total of 78 mutagen-sensitive lines: 18 on the second and 60 on the third chromosome. All 78 lines were complementation tested with the following extant mutagen-sensitive mutations: $mus201^{D1}$, $mus202^{A1}$, $mus204^{A1}$, $mus205^{A1}$, $mus206^{A1}$, $mus207^{D1}$, $mus209^{B1}$, $mus210^{B1}$, $mus211^{B1}$, $rad201^{1}$, grp^{1}, and okr^{WS} for the second-chromosome lines and $mus301^{D4}$, $mus302^{D5}$, $mus304^{D1}$, $mus304^{D3}$, $mus305^{D2}$, $mus306^{D1}$, $mus307^{D1}$, $mus308^{D16}$ (*mus308* was tested only with HN2-specific lines), $mus309^{D3}$, $mus310^{D1}$, $mus311^{D1}$, and $mus312^{D1}$ for the third-chromosome lines.

For complementation tests, 4–8 virgin heterozygotes of mutant line "A" were crossed to 5–10 male heterozygotes of mutant line "B." All crosses were performed at 25°. The virgins and males were placed in a fresh vial and maintained there for 48 hr. After 48 hr, flies were transferred to a second vial, and the first vial was treated with MMS. After 48 hr, flies were transferred from the second vial to a third vial, and the second vial was treated with HN2. The third vial served as the nonmutagenized control. All vials were scored on day 15. Mutants were arbitrarily defined as "failing to complement" if the ratio of [*trans*-heterozygote (*musA/musB*)]/[*balancer/mus*] in mutagen-treated vials fell between 0 and 30% of the control *trans*-heterozygote/balancer heterozygote ratios. Two mutants were likewise said to complement each other if the ratio of [*trans*-heterozygote (*musA/musB*)]/[*balancer/mus*] in mutagen-treated vials was at least 50% of the control homozygote/heterozygote ratios. Crosses were retested if they fell between 30 and 50%. In most cases, the profile of mutagen sensitivity observed for one of our newly isolated alleles matched that of other previously studied alleles of that gene.

Mutagen preparation: All mutagens were diluted to the appropriate concentration with double-deionized water. The MMS mutagen solution was made from a stock of 99.99% MMS. The HN2 mutagen solution was prepared from a stock of 10% HN2 in 0.1 N HCl. Vials (25 mm in diameter) were treated with 250 μl of mutagen solution, while bottles (64 mm in diameter) were treated with 1 ml of mutagen solution.

Measurement of meiotic nondisjunction: Selected homozygous mutagen-sensitive mutants were tested for chiasmate X nondisjunction, including $rad201^{ZII\text{-}0670}$, $mus201^{ZII\text{-}1953}$, $mus212^{ZII\text{-}1436}$, $mus212^{ZII\text{-}4368}$, $mus213^{ZII\text{-}6078}$, $mus215^{ZII\text{-}2059}$, $mus217^{ZII\text{-}5470}$, $mus218^{ZII\text{-}5841}$, $mus219^{ZII\text{-}5970}$, $mus302^{ZIII\text{-}1882}$, $mus302^{ZIII\text{-}6004}$, $mus305^{ZIII\text{-}1990}$, $mus305^{ZIII\text{-}2140}$, $mus305^{ZIII\text{-}5909}$, $mus312^{ZIII\text{-}1973}$, and $mus314^{ZIII\text{-}2504}$. One to three homozygous virgins were crossed to 6–10 XY,y^{+} *v f B; C(4), ci* ey^{R} males at room temperature. Crosses were scored and nondisjunction frequencies calculated as described (Hawley *et al.* 1992).

RESULTS

To begin the screen, it was necessary to determine the background sensitivity of the second- and third-chromosome parental flies to both MMS and HN2. For the third chromosome, we transferred the *bw/bw; st/st* siblings into fresh vials and allowed them to lay eggs for 24 hr at room temperature. The cleared vials were aged for an additional 24 hr to permit all embryos to hatch and then treated with varying concentrations of MMS (0.025–0.2%) or HN2 (0.003–0.02%). Parents transferred from the first vial were allowed to lay eggs in a second vial for an additional 24 hr and then removed. After an additional 24-hr period, the second vials were treated with water. Concentrations of MMS (0.04%) and HN2 (0.003%) were identified that permitted 50–80% survival when comparing the mutagenized *bw/bw; st/st* flies to unmutagenized *bw/bw; st/st* flies. These concentrations were used in subsequent studies. On the basis of similar studies using the second-chromosome parental line, the second-chromosome experimental lines were tested using the same dosages (0.04% MMS and 0.003% HN2).

The second chromosome: After discarding the lines bearing recessive lethal or semilethal mutations, 2585 second chromosomes were tested for mutagen sensitivity. Approximately 0.7% (18/2585) of these lines were shown to be sensitive to MMS. Sensitivity data for these lines are reported in Table 2. Although the frequency with which we recovered mutagen-sensitive mutants was approximately fivefold higher than that previously obtained from EMS-mutagenized second chromosomes (0.1%, or 5/4039; Snyder and Smith 1982), it should be noted that these authors do not report the fraction of the 4039 lines tested that were discarded due to the presence of recessive lethals or semilethal mutants. Moreover, Zuker and colleagues used a substantially higher dose of EMS (25 mM) than did Snyder and Smith (2.25 mM).

All 18 newly identified second-chromosome *mus* mutants were tested for the ability to complement known second-chromosome *mus* mutants $mus201^{D1}$, $mus202^{A1}$, $mus204^{A1}$, $mus205^{A1}$, $mus206^{A1}$, $mus207^{D1}$, $mus209^{B1}$, $mus210^{B1}$, $mus211^{B1}$, $rad201^{1}$, grp^{1}, and okr^{WS}. As shown in Table 3, of the 18 second-chromosome mutants, 8 are new alleles of existing mutants [*mus201* (1), *mus205* (4), *okra* (1), and *rad201* (2)]. As shown in Table 4, the remaining 10 mutants were complementation tested in all pairwise combinations and shown to define 8 new mutagen-sensitive genes, all of which were uniquely sensitive to MMS. Two of these genes, *mus212* and *mus213*, are defined by more than one new allele.

Both alleles of *mus212* ($mus212^{ZIII\text{-}1436}$ and $mus212^{ZIII\text{-}4368}$) are female sterile; females homozygous for either of the two mutants fail to lay eggs. When initially analyzed, $mus215^{ZII\text{-}2059}$ females, although fertile, displayed a severe defect in meiotic recombination. Females of the genotype *y cv v f car/+; $mus215^{ZII\text{-}2059}$* displayed both very high levels of X chromosome nondisjunction (20.9%, $N = 1331$) and levels of meiotic recombination that were <3% of those observed in wild type. Curiously, the observed decrease in recombination was not polar, as is observed for most recombination-defective mutants (Baker and Hall 1976), but rather uniformly distributed across the entire arm. This phenotype is exhibited only by loss-of-function mutants at the *mei-9* and *mus312* genes, both of which encode proteins thought to be involved in the process of resolving recombinational

TABLE 2

Newly identified *mus* mutants on the second chromosome

Line	0.04% MMS		0.003% HN2		Control		Sensitivity ratio		Final classification
	Homozygous	Heterozygous	Homozygous	Heterozygous	Homozygous	Heterozygous	MMS	HN2	
mus205$^{ZII\text{-}1713}$	1	153	51	83	53	149	0.018	1.727	MMS
mus205$^{ZII\text{-}2129}$	0	140	68	81	224	468	0.000	1.753	MMS
mus205$^{ZII\text{-}4981}$	0	228	136	296	229	484	0.000	0.971	MMS
mus205$^{ZII\text{-}5692}$	0	6	44	99	172	336	0.000	0.868	MMS
mus212$^{ZII\text{-}1436}$	1	190	54	196	26	139	0.028	1.470	MMS
mus212$^{ZII\text{-}4368}$	0	63	10	57	36	357	0.000	1.739	MMS
mus213$^{ZII\text{-}1520}$	0	83	18	214	96	356	0.000	0.312	MMS
mus213$^{ZII\text{-}6078}$	0	72	12	64	26	116	0.000	0.836	MMS
mus214$^{ZII\text{-}2010}$	1	97	56	97	44	276	0.064	3.620	MMS
mus215$^{ZII\text{-}2059}$	0	144	25	100	104	288	0.000	0.692	MMS
mus216$^{ZII\text{-}5359}$	0	11	39	125	160	440	0.000	0.858	MMS
mus217$^{ZII\text{-}5470}$	0	93	39	126	128	560	0.000	1.354	MMS
mus218$^{ZII\text{-}5841}$	0	58	33	24	108	188	0.000	2.394	MMS
mus219$^{ZII\text{-}5970}$	0	25	6	25	68	114	0.000	0.402	MMS
rad201$^{ZII\text{-}0670}$	0	509	25	366	72	305	0.000	0.289[a]	MMS and HN2
rad201$^{ZII\text{-}2512}$	0	132	3	102	224	420	0.000	0.055	MMS and HN2
okra$^{ZII\text{-}5130}$	0	100	0	99	54	127	0.000	0.000	MMS and HN2
mus201$^{ZII\text{-}1953}$	0	50	36	62	118	186	0.000	0.915[a]	MMS and HN2

[a] While these ratios do not meet the sensitivity cutoff of ≤0.1, other experiments (data not shown) and/or results from complementation tests did show sensitivity within the required range.

intermediates into crossover products (Yildiz *et al.* 2002). The observed defect in exchange resolution is consistent with a defect in the timing of synaptonemal complex dissolution observed in *mus215*$^{ZII\text{-}2059}$ oocytes by S. L. Page, C. M. Orme and H. K. Peters (personal communication). In the year since this analysis, the *mus215*$^{ZII\text{-}2059}$ stock has lost both the mutagen-sensitivity and meiotic chromosome segregation phenotypes, possibly as a consequence of the accumulation of genetic modifiers or perhaps due to loss by recombination, assuming that it maps to a region of the second chromosome not well balanced by the CyO balancer. Efforts to recover the original mutant phenotype are in progress in our laboratories.

The third chromosome: After discarding the lines bearing recessive lethal or semilethal mutations, 3690 third chromosomes were tested for mutagen sensitivity. Approximately 1.4% (53/3690) of these lines were shown to be sensitive to MMS. Sensitivity data for these lines are reported in Table 5. Again, the frequency with

TABLE 3

New alleles of known second-chromosome mutagen-sensitive genes

Gene	MMS	HN2	Previously reported EMS-induced alleles	Alleles reported here
mus201	+	+	2	1 (ZII-1953)
mus202	+	+	1	0
mus203	+	+	1	0
mus204	+	+	1	0
mus205	+	−	2	4 (ZII-1713, ZII-2129, ZII-4981, ZII-5692)
mus206	+	+	1	0
mus207	+	+	1	0
mus208	+	±	2	0
mus209	+	−	10	0
mus210	+	+	4	0
mus211	+	+	2	0
okra (okr)	+	−	9	1 (ZII-5130)
grapes (grp)	+	−	0	0
rad201	+	−	0	2 (ZII-0670, ZII-2512)

TABLE 4

Newly identified *mus* genes on the second chromosome

New genes	Zuker no.	MMS	HN2
mus212	ZII-1436	+	−
	ZII-4368	+	−
mus213	ZII-1520	+	−
	ZII-6078	+	−
mus214	ZII-2010	+	−
mus215	ZII-2059	+	−
mus216	ZII-5359	+	−
mus217	ZII-5470	+	−
mus218	ZII-5841	+	−
mus219	ZII-5970	+	−

which we recovered MMS-sensitive mutants was ~5-fold higher than the frequency (0.3%, 31/11,334) of MMS-sensitive mutants previously obtained (Boyd *et al.* 1981) from third chromosomes mutagenized at an ~10-fold lower dose (3 mm) of EMS.

All 60 newly identified third-chromosome *mus* mutants were tested for the ability to complement known third-chromosome *mus* mutants $mus301^{D4}$, $mus302^{D5}$, $mus304^{D1}$, $mus304^{D3}$, $mus305^{D2}$, $mus306^{D1}$, $mus307^{D1}$, $mus308^{D16}$, $mus\ 309^{D3}$, $mus310^{D1}$, $mus311^{D1}$, and $mus312^{D1}$. As shown in Table 6, 44 of the 60 third-chromosome mutants were alleles of existing mutants [*mus301* (8), *mus302* (5), *mus304* (2), *mus305* (24), *mus308* (3), and *mus312* (2)]. The identity of the two new alleles of *mus312* has been verified by sequence analysis (Yildiz *et al.* 2002). The reason that an inordinately large number of *mus305* alleles were recovered in our screen remains to be determined. This locus was not proven to be highly mutable in a previous screen for third-chromosome *mus* mutants (Boyd *et al.* 1981); thus, it seems unlikely that *mus305* is simply a very mutable gene, analogous to *mei-41*. An alternative hypothesis is that this allele preexisted at low frequency in the parental stock, prior to mutagenesis.

The remaining 16 mutants define 14 new mutagen-sensitive genes (*mus314–mus327*). Mutants in 7 of these genes were hypersensitive only to MMS (*mus314–mus320*), mutants in 3 genes (four total alleles) were hypersensitive only to HN2 (*mus321–mus323*), and mutants in 4 of these genes (five total alleles) displayed sensitivity to both MMS and HN2 (*mus324–mus327*). For the 16 novel third-chromosome mutants, *inter se* complementation tests were performed within phenotypic groups. Because it is possible that mutants in different phenotypic classes could be allelic, the number of unique new third-chromosome genes identified in this study may be an overestimate; indeed, one example of this was noted after completion of our studies.

All pairwise combinations of the seven MMS-sensitive mutants displayed full complementation, thus defining seven new genes. Among the five mutants that were sensitive to both MMS and HN2, one gene (*mus324*) was defined by two alleles. The remaining three mutants define novel genes. Finally, all three novel mutants that were solely sensitive to HN2 fully complemented each other in all pairwise combinations and also complemented an allele of the *mus308* gene. These three genes thus provide a substantial increase in the number of Drosophila genes whose products might function specifically in interstrand crosslink repair. As described below, one of these three genes was examined at the molecular level and shown to define the fly ortholog of the crosslink repair gene *snm1*, first characterized in *S. cerevisiae* (Henriques and Moustacchi 1980) and more recently in the mouse (Dronkert *et al.* 2000).

Identification of *snm1*: As noted above, our screen identified only seven lines that were uniquely hypersensitive to HN2. Of the seven new mutants, three were determined to be alleles of *mus308*, the only previously characterized Drosophila gene whose mutant phenotype included hypersensitivity only to agents capable of creating DNA interstrand crosslinks (Boyd *et al.* 1990), thus demonstrating the efficacy of the current screen in identifying this class of mutations. Hypersensitivity only to crosslinking reagents is a rare phenotype among known DNA repair genes; the only well-characterized example other than *mus308* is the *snm1* gene, first identified in *S. cerevisiae* by its sensitivity to nitrogen mustard and another crosslinking agent, psoralen (Henriques and Moustacchi 1980; Ruhland *et al.* 1981). Because this phenotype is so uncommon, and because the four novel crosslink hypersensitive mutations mapped to the same chromosome as did a predicted Drosophila gene (*CG10018*) highly similar to yeast *snm1*, it seemed likely that one or more of the four mutations might be mutant alleles of Drosophila *snm1*. Furthermore, the mutagen-sensitive phenotypes of two mutants, $mus322^{ZIII\text{-}4709}$ and $mus322^{ZIII\text{-}2589}$, were demonstrated by linkage mapping (data not shown) to reside in the chromosomal interval between thread and curled (72D1-86D4), consistent with the location of *CG10018* (83B1). We therefore determined the DNA sequence of all (ZIII-4709) or part (ZIII-2589) of the coding sequences of the *CG10018* gene from these two HN2 hypersensitive mutants, as well as the coding sequences from the *CG10018* gene of mutant strain ZIII-0708, which complements the mutagen sensitivity of ZIII-4709 and ZIII-2589 but was isolated on the same parental chromosome. The results of this analysis are presented in Figure 1.

Both ZIII-4709 and ZIII-2589 possess mutations not present in the ZIII-0708 sequence or in the sequence of the *CG10018* gene from GenBank (Figure 1A). The sequence of *CG10018* in $mus322^{ZIII\text{-}2589}$ flies revealed a missense mutation resulting in substitution of an evolutionarily conserved cysteine residue (Cys^{357} in the Drosophila sequence) by tyrosine, while the sequence in $mus322^{ZIII\text{-}4709}$ flies revealed a missense mutation resulting in substitution of an evolutionarily conserved

TABLE 5

Newly identified *mus* mutants on the third chromosome

Line	MMS 0.04%		HN2 0.003%		Control		Sensitivity ratio		Final classification
	Homozygous	Heterozygous	Homozygous	Heterozygous	Homozygous	Heterozygous	MMS	HN2	
mus302$^{\text{ZIII-1882}}$	0	83	6	23	32	93	0.000	0.758	MMS
mus302$^{\text{ZIII-2530}}$	0	80	24	124	45	90	0.000	0.387	MMS
mus302$^{\text{ZIII-5541}}$	0	60	78	159	82	128	0.000	0.766	MMS
mus305$^{\text{ZIII-1131}}$	0	220	48	103	66	186	0.000	1.313	MMS
mus305$^{\text{ZIII-1990}}$	6	297	43	82	115	196	0.034	0.894	MMS
mus305$^{\text{ZIII-2122}}$	1	238	36	71	88	272	0.013	1.567	MMS
mus305$^{\text{ZIII-2140}}$	7	244	40	188	30	225	0.243[a]	1.550	MMS
mus305$^{\text{ZIII-2150}}$	0	289	62	154	56	210	0.000	1.510	MMS
mus305$^{\text{ZIII-2339}}$	1	78	31	113	28	78	0.036	0.764	MMS
mus305$^{\text{ZIII-2739}}$	2	240	40	144	104	156	0.013	0.417	MMS
mus305$^{\text{ZIII-3064}}$	0	162	41	97	134	211	0.000	0.666	MMS
mus305$^{\text{ZIII-3068}}$	2	115	41	76	108	180	0.029	0.899	MMS
mus305$^{\text{ZIII-3077}}$	3	186	15	417	92	261	0.046	0.102	MMS
mus305$^{\text{ZIII-3135}}$	0	258	25	240	63	212	0.000	0.351	MMS
mus305$^{\text{ZIII-3162}}$	1	312	31	215	43	283	0.021	0.949	MMS
mus305$^{\text{ZIII-3355}}$	0	199	27	163	54	207	0.000	0.635	MMS
mus305$^{\text{ZIII-3983}}$	0	86	27	160	12	103	0.000	1.448	MMS
mus305$^{\text{ZIII-4861}}$	0	160	92	452	46	322	0.000	1.425	MMS
mus305$^{\text{ZIII-4863}}$	0	144	54	96	180	200	0.000	0.625	MMS
mus305$^{\text{ZIII-5443}}$	1	105	14	39	22	83	0.036	1.354	MMS
mus305$^{\text{ZIII-5588}}$	5	120	64	146	61	144	0.098	1.035	MMS
mus305$^{\text{ZIII-5675}}$	2	270	27	69	82	181	0.016	0.864	MMS
mus305$^{\text{ZIII-5711}}$	0	54	16	81	34	190	0.000	1.104	MMS
mus305$^{\text{ZIII-5751}}$	0	388	22	186	96	304	0.000	0.375	MMS
mus305$^{\text{ZIII-5909}}$	0	225	64	102	61	72	0.000	0.741	MMS
mus305$^{\text{ZIII-6104}}$	0	273	29	164	132	268	0.000	0.359	MMS
mus305$^{\text{ZIII-6140}}$	2	92	5	50	41	94	0.050	0.229	MMS
mus314$^{\text{ZIII-2504}}$	3	140	96	220	28	198	0.152[a]	3.086	MMS
mus315$^{\text{ZIII-2629}}$	1	194	20	140	24	258	0.055	1.536	MMS
mus316$^{\text{ZIII-2640}}$	0	206	10	208	36	270	0.000	0.361	MMS
mus317$^{\text{ZIII-4494}}$	2	178	9	145	37	352	0.107	0.590	MMS
mus318$^{\text{ZIII-4681}}$	0	141	22	170	18	246	0.000	1.769	MMS
mus319$^{\text{ZIII-5444}}$	2	168	26	146	62	138	0.026	0.396	MMS
mus320$^{\text{ZIII-6025}}$	0	348	3	39	11	171	0.000	1.196	MMS
mus308$^{\text{ZIII-0629}}$	22	95	0	128	30	108	0.834	0.000	HN2
mus308$^{\text{ZIII-2003}}$	72	128	0	186	40	132	1.856	0.000	HN2
mus308$^{\text{ZIII-3294}}$	104	176	0	178	124	229	1.091	0.000	HN2
mus321$^{\text{ZIII-0708}}$	33	107	0	81	41	93	0.700	0.000	HN2
mus322$^{\text{ZIII-4709}}$	156	220	0	240	128	254	1.407	0.000	HN2
mus322$^{\text{ZIII-2589}}$	48	105	1	88	88	212	1.101	0.027	HN2
mus323$^{\text{ZIII-2866}}$	58	160	0	186	62	110	0.643	0.000	HN2
mus301$^{\text{ZIII-2205}}$	0	405	0	309	27	213	0.000	0.000	MMS and HN2
mus301$^{\text{ZIII-2255}}$	0	238	0	82	44	276	0.000	0.000	MMS and HN2
mus301$^{\text{ZIII-3198}}$	0	231	3	176	38	134	0.000	0.060	MMS and HN2
mus301$^{\text{ZIII-3865}}$	0	237	22	384	110	265	0.000	0.138[a]	MMS and HN2
mus301$^{\text{ZIII-4875}}$	0	222	0	234	140	348	0.000	0.000	MMS and HN2
mus301$^{\text{ZIII-5121}}$	1	208	2	280	34	253	0.036	0.053	MMS and HN2
mus301$^{\text{ZIII-5813}}$	0	117	0	105	42	136	0.000	0.000	MMS and HN2
mus301$^{\text{ZIII-6030}}$	0	130	0	110	33	61	0.000	0.000	MMS and HN2
mus302$^{\text{ZIII-4933}}$	0	97	2	260	128	300	0.000	0.018	MMS and HN2
mus302$^{\text{ZIII-6004}}$	0	180	2	152	96	264	0.000	0.036	MMS and HN2
mus304$^{\text{ZIII-0610}}$	0	159	6	240	42	106	0.000	0.063	MMS and HN2
mus304$^{\text{ZIII-2785}}$	0	401	0	176	92	236	0.000	0.000	MMS and HN2
mus312$^{\text{ZIII-1973}}$	0	25	1	20	51	86	0.000	0.084	MMS and HN2
mus312$^{\text{ZIII-3997}}$	0	286	1	400	139	365	0.000	0.007	MMS and HN2
mus324$^{\text{ZIII-4325}}$	0	393	3	46	76	228	0.000	0.196[a]	MMS and HN2
mus324$^{\text{ZIII-5997}}$	0	324	0	222	42	208	0.000	0.000	MMS and HN2
mus325$^{\text{ZIII-4708}}$	2	132	0	153	30	188	0.095	0.000	MMS and HN2
mus326$^{\text{ZIII-4840}}$	4	134	3	276	88	240	0.081	0.030	MMS and HN2
mus327$^{\text{ZIII-5906}}$	1	124	0	133	112	240	0.017	0.000	MMS and HN2

[a] While these ratios do not meet the sensitivity cutoff of ≤ 0.1, other experiments (data not shown) and/or results from complementation tests did show sensitivity within the required range.

TABLE 6

New alleles of known third-chromosome mutagen-sensitive genes

Gene	MMS	HN2	Previously reported EMS-induced alleles	Alleles reported here
mus301	+	+	5	8 (ZIII-2205, ZIII-2255, ZIII-3198, ZIII-3865, ZIII-4875, ZIII-5121, ZIII-5813, ZIII-6030)
mus302	+	+	6	5 (ZIII-1882, ZIII-2530, ZIII-4933, ZIII-5541, ZIII-6004)
mus304	+	+	6	2 (ZIII-0610, ZIII-2785)
mus305	+	+	3	24 (ZIII-1131, ZIII-1990, ZIII-2122, ZIII-2140, ZIII-2150, ZIII-2339, ZIII-2739, ZIII-3064, ZIII-3068, ZIII-3077, ZIII-3135, ZIII-3162, ZIII-3355, ZIII-3983, ZIII-4861, ZIII-4863, ZIII-5443, ZIII-5588, ZIII-5675, ZIII-5711, ZIII-5751, ZIII-5909, ZIII-6104, ZIII-6140)
mus306	+	−	1	0
mus307	+	+	1	0
mus308	−	+	6	3 (ZIII-0629, ZIII-2003, ZIII-3294)
mus309	+	+	3	0
mus310	+	−	1	0
mus311	+	−	3	0
mus312	+	+	2	2 (ZIII-1973, ZIII-3997)

glycine residue (Gly[377]) by glutamic acid (Figure 1B). Drosophila SNM1 is a member of a large superfamily of proteins that include the metallo-β-lactamase fold domain (Aravind 1999). Within that superfamily, SNM1 is a member of the β-CASP subfamily, which is defined by additional conserved motifs and is composed primarily of proteins that are known or suspected to have nucleic acid substrates (Callebaut *et al.* 2002). Alignment of the Drosophila protein with other family members (Figure 1B) reveals that Cys[357] and Gly[377] each lie immediately adjacent to amino acids (histidine and aspartic acid shaded in Figure 1B) that are critical residues in the highly conserved motifs III and IV of the metallo-β-lactamase fold domain, involved in the coordination of zinc atoms and catalysis (Wang *et al.* 1999). Metallo-β-lactamase fold proteins are often involved in hydrolysis of ester linkages (Aravind 1999) and the Artemis protein, which, like SNM1, possesses both the metallo-β-lactamase fold and the β-CASP domains (Moshous *et al.* 2001; Callebaut *et al.* 2002), has been dem-

A

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14709    AGCTGTCCTGGAGCACTCATGTTCTTCTTCAAACTGAGCTCTGGCGAATGCATACTGCACACCGAGGACTTCCGAGCT
12589    AGCTATCCTGGAGCACTCATGTTCTTCTTCAAACTGAGCTCTGGCGAATGCATACTGCACACCGGGGACTTCCGAGCT
10708    AGCTGTCCTGGAGCACTCATGTTCTTCTTCAAACTGAGCTCTGGCGAATGCATACTGCACACCGGGGACTTCCGAGCT
wildtype AGCTGTCCTGGAGCACTCATGTTCTTCTTCAAACTGAGCTCTGGCGAATGCATACTGCACACCGGGGACTTCCGAGCT 115722
           CysProGlyAlaLeuMetPhePhePheLysLeuSerSerGlyGluCysIleLeuHisThrGlyAspPheArgAla
           Tyr (ZIII-2589)                                                 Glu (ZIII-4709)

AE003602.3
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B

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H.s.   VKVVLLDANHCPGAVMILFYLPNGT--------VILHTGDFRADPSMERS 825
M.m.   VKVVFVDANHCPGATMILFQLPNGA--------VILHTGDFRADPSMERS 808
A.t.   IKVTLIEANHCPGAALIHFRLLDGT--------CYLHTGDFRASKQMQTH 270
S.p.   ITVYVLDANHCPGSAMFVFETLQSNQT----RRVLHCGDFRASKDHVMH 279
S.c.   ISVVTLDANHCPGAIIMLFQEFLANSYDKPIRQILHTGDFRSNAKMIET 357
A.g.   VEIIALDANHCPGGIMFLFRLPNGS--------NVLHTGDFRASPEMEEY 268
D.m.   VQVTALEANHCPGALMFFFKLSSGE--------CILHTGDFRASADMESL 388
mut.             Y (ZIII-2589)                E (ZIII-4709)
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Figure 1.—Sequence analysis of *mus322* alleles. (A) Aligned partial nucleotide sequences of the *snm1* genes of Zuker strains ZIII-4709, ZIII-2589, and ZIII-0708 and the same sequence from the Drosophila genome (GenBank accession no. AE003602.3; 5′AG bases are from the first intron; coordinate of the 3′ base is indicated). The translation of this sequence is depicted immediately below. The single bases altered in ZIII-4709 and ZIII-2589 are shaded, and the consequences of these mutations on the amino acid sequence are indicated on the bottom line. (B) Aligned partial amino acid sequences of the SNM1 proteins from seven species are shown: *Homo sapiens* (AAH62582), *Mus musculus* (AAF64472), *Arabidopsis thaliana* (CAA66406), *Schizosaccharomyces pombe* (CAA93588), *S. cerevisiae* (CAA45405), *Anopheles gambiae* (EAA07248), and *Drosophila melanogaster* (AAF52011). Shaded amino acids indicate the highly conserved His and Asp residues. The identities of the mutant amino acid residues in alleles ZIII-4709 and ZIII-2589 are indicated on the bottom line, directly beneath the amino acid affected.

onstrated to possess $5' \rightarrow 3'$ exonuclease activity as well as endonucleolytic activity in complex with DNA-PK (Ma *et al.* 2002). No enzymatic activity has been reported for SNM1, although the possibility of a role involving cleavage of hairpin structures during crosslink repair has been the subject of recent speculation (Brendel *et al.* 2003). However, our results do indicate that the function of β-lactamase motifs III and IV is required for some aspect of interstrand crosslink repair, as has been demonstrated for motif II in *S. cerevisiae* (Li and Moses 2003). Although rescue of the *mus322* mutant phenotype by germline transformation has not yet been done, the identification of mutations affecting independent residues in highly conserved motifs in two alleles makes it highly probable that the crosslink sensitivity of *mus322* mutants is due to mutation of the Drosophila *snm1* gene.

The mus301/spnC gene corresponds to the Drosophila gene CG7972 and is the Drosophila ortholog of the human gene HEL308: The Drosophila gene *CG7972* encodes a polypeptide (CG7972-PA) very similar (29% identity) to the helicase domain of the interstrand crosslink repair gene *mus308*. The orthologous polypeptide encoded by the human gene HEL308 (41% identical to CG7972-PA) has been demonstrated to possess 3′-5′ DNA helicase and DNA-dependent ATPase activities *in vitro* (Marini and Wood 2002), but has not been linked to a mutant phenotype *in vivo*. A putative mutant allele of the Drosophila *CG7972* gene was created through the insertion of an element (KG09098) into the fourth intron of this gene in an ongoing screen for transposon insertions into the Drosophila genome (http://flypush.imgen.bcm.tmc.edu/pscreen/). Flies homozygous for this insertion allele (stock kindly provided by H. Bellen) were hypersensitive to the crosslinking agent cisplatin (average sensitivity ratio of 0.05 at 0.167 mm cisplatin), suggesting that this gene functions in DNA repair.

The discovery that an allele of CG7972 was mutagen sensitive led us to question whether it might correspond to any previously discovered *mus* genes not yet characterized at the molecular level. CG7972 maps to cytological location 66B8 (FlyBase 2003). Using the FlyBase Cytosearch tool, we examined this region of the genome for two lettered divisions to either side of this position. A total of 28 genes are predicted in this interval, including only one mutagen-sensitive gene, *mus301. mus301* was mapped to the 65F3-66B9 interval by its failure to complement Df(3L)pbl-X1 (Gonzalez-Reyes *et al.* 1997) and was shown to be allelic to the female-sterile mutation *spindle-C* (Ghabrial and Schupbach 1999), but has not been previously cloned. We therefore tested the possibility that *mus301* was an allele of *CG7972* using both genetic and molecular approaches.

The *P*-element-induced *CG7972* mutant allele was tested for its ability to complement the mutagen sensitivity of three alleles of *mus301*, using 0.08% MMS as a mutagen. Alleles tested included one previously isolated allele (*mus301*^{D5}; Boyd *et al.* 1981) and two of the eight new alleles of *mus301* identified in our screen: *mus301*$^{ZIII\text{-}2205}$ and *mus301*$^{ZIII\text{-}2255}$. In all three cases, the *P*-induced allele failed to complement (sensitivity ratio of 0.0 for each allele), suggesting that *CG7972* is the *mus301* gene. To confirm that the *P* element inserted in CG7972 was responsible for the mutant phenotype, we mobilized the element by the introduction of *P*-element transposase and identified revertants lacking the visible marker (*w*+) carried by this element. DNA sequencing of a PCR product generated using primers flanking the insertion site confirmed clean excision of the element. Complementation testing between the revertant and *mus301*$^{ZIII\text{-}2255}$ gave a sensitivity ratio of 0.83, confirming that the element was responsible for the mutagen-sensitive phenotype and suggesting strongly that *mus301* is indeed an allele of *CG7972*. Further supporting evidence was obtained by sequencing the entire open reading frame of two alleles of *CG7972*: *mus301*$^{ZIII\text{-}2255}$ and *mus301*$^{ZIII\text{-}3198}$. The sequence of the *mus301*$^{ZIII\text{-}2255}$ allele revealed a missense mutation, changing a GAC codon to TAC and resulting in the nonconservative substitution of tyrosine 455 of CG7972-PA by an aspartic acid residue. This residue is located in a region between the highly conserved motifs II and III of the superfamily II DNA and RNA helicases (Gorbalenya *et al.* 1989) and is in a region well conserved between the human, mouse, and Arabidopsis orthologs of HEL308 (Marini and Wood 2002). No missense mutations were identified in the open reading frame of allele *mus301*$^{ZIII\text{-}3198}$, suggesting that this allele may result from an alteration in regulatory sequences. Both alleles had in common two silent mutations relative to the published sequence (GenBank accession no. AE003735), presumably present in the parental chromosome. Although additional evidence for the identity of CG7972 and *mus301/spnC* might be provided by rescue of the mutant function by *P*-element transformation, the results presented strongly support this hypothesis.

The identification of the Drosophila ortholog of the human *HEL308* gene as *mus301/spn-C* provides the first evidence suggesting that the HEL308 protein may function in recombinational repair of DNA DSBs. Other members of the "spindle" class of female-sterile mutations in Drosophila have been identified as mutations in *okra*, the Drosophila ortholog of the *S. cerevisiae RAD54* gene (Ghabrial *et al.* 1998), and in three of the five known Drosophila *Rad51*-like genes: *spn-A* (Rad51; Staeva-Vieira *et al.* 2003), *spn-B* (XRCC3-like; Ghabrial *et al.* 1998), and *spn-D* (Rad51C-like; Abdu *et al.* 2003). The disruption of oogenesis in these mutants and in *spn-C* results from a failure in repair of meiotic DSBs, with subsequent activation of a meiotic recombination checkpoint (Ghabrial and Schupbach 1999; Abdu *et al.* 2003; Staeva-Vieira *et al.* 2003). In addition to their role in meiotic cells, the mutagen sensitivity of *spn-A*, *spn-C*, and (weakly) *spn-B* indicates that these gene products also play a role in the recovery of mitotic cells from elevated levels of DNA damage. The role in

recombination of the *RAD52* epistasis group of genes, which includes among it members *RAD54* and the *RAD51*-related genes, has been extensively characterized (reviewed by Symington 2002). Similar studies on the *HEL308*-related genes will be needed to determine the role of this helicase in recombinational repair.

DISCUSSION

Mutagen sensitivity and DNA repair pathways: There is a complex relationship between the specific hypersensitivity displayed by each *mus* mutant (MMS only, HN2 only, or both mutagens) and the repair pathway defined by that mutant.

The most abundant products of MMS treatment are 7-methyl guanine (7MeG) and 3-methyl adenine (3MeA) residues, which are nonbulky adducts classically repaired by the base excision repair (BER) pathway (Friedberg *et al.* 1995). However, genetic studies in *S. cerevisiae* have demonstrated that the nucleotide excision repair (NER) pathway plays an important role in competition with the BER pathway in the removal of the damaged bases 7MeG and 3MeA (Lee *et al.* 2002). Furthermore, these lesions are known to result in stalled replication forks, leading to the creation of DSBs that are characteristically found in dividing cells treated with MMS. Treatment with HN2 likewise leads to a complex array of DNA damage. The most frequent lesion is alkylation at the N7 position of guanine, creating a bulky helix-distorting adduct predominantly repaired by the NER system. However, as a bifunctional alkylating agent, HN2 is also capable of creating covalent crosslinks between either bases in close proximity on one strand or bases on opposite strands (Bauer and Povirk 1997). Creation of DSBs as a result of treatment with bifunctional crosslinking agents has been demonstrated in dividing *S. cerevisiae* (Magana-Schwencke *et al.* 1982) and mammalian cells (De Silva *et al.* 2000) and is probable but remains to be demonstrated in Drosophila. Given the complex array of damage resulting from exposure to MMS or HN2, we expect that our screen for mutagen-hypersensitive mutants will identify genes in a majority of the known repair pathways.

The most frequent class of mutants obtained in the screen reported here were those uniquely sensitive to MMS. The genes identified presumably encode functions not required for repair of types of damage either unique to HN2, such as interstrand crosslinks, or common to both mutagens, such as DSBs. The one known example found in this screen was new alleles of *mus205*, which encodes the catalytic subunit of DNA polymerase ζ. This polymerase is capable of *trans*-lesion synthesis across damaged bases on the template strand and is critical for survival of MMS-treated cells (damage tolerance). However, the lack of sensitivity to HN2 indicates that *mus205* is not essential for repair of the bulky adducts, interstrand crosslinks, and DSBs likely created by HN2 at the concentration tested. The *S. cerevisiae* homolog of *mus205*, *rev3*, is likewise not essential for DSB repair (Holbeck and Strathern 1997).

The second class of mutants identified was that sensitive to both mutagens. Although fewer of these were found in the screen reported here, this class is equal in abundance to the MMS-specific class among the entire set of Drosophila mutagen-sensitive mutants found to date. Examples of this class include components of the NER pathway, including *mus201* (XPG; Calleja *et al.* 2001) and *mus210* (XPC; Henning *et al.* 1994), as well as *mus309*, which participates in DSB repair through participation in the synthesis-dependent strand-annealing pathway (Adams *et al.* 2003).

An intriguing result of our screen is the identification of two new mutants, in addition to *mus308* and *snm1*, displaying specific hypersensitivity to HN2. Mapping and characterization of these genes is in progress, and preliminary results suggest the existence of at least two pathways as defined by epistatic interactions (L. Chang and K. C. Burtis, unpublished data). Although previous studies have indicated the importance of components of multiple pathways, including NER, recombinational repair, and damage tolerance, in repair of interstrand crosslinks, these pathways are also known to be involved in the repair of damage created by MMS (Friedberg *et al.* 1995). The existence of multiple HN2-specific mutants suggests the possibility of novel functions or pathways unique to the repair of interstrand crosslinks. Mutants lacking function of the *mus308* gene have been demonstrated to be hypersensitive to different chemical mutagens having in common only their ability to create interstrand crosslinks (Boyd *et al.* 1990), and to be insensitive to agents not creating crosslinks, indicating that it is the crosslink rather than the precise covalent modification that requires *mus308* function for repair. Similar studies using multiple crosslinking agents are in progress with the new mutants to confirm that they are likewise specific in their roles in crosslink repair. Hopefully, identification of the complete set of genes mutable to a crosslink-specific phenotype will lead to an understanding of how the various common repair pathways involved in crosslink repair coordinate with specific unique functions to repair this unusual type of damage. Understanding the function of these genes may also provide some insight into their normal physiological role. There is no evidence to date that interstrand crosslinks occur at significant levels under normal conditions in Drosophila; thus, it is unclear whether the evolutionarily selected function of these genes is actually to repair crosslinks or rather to function in the repair of another as-yet-unrecognized class of lesions. Given that there is only one extant mutant allele for two of the genes in this class, it is possible that more genes mutable to this phenotype remain to be identified. This is also the case for other repair pathways in Drosophila, as discussed below.

To what degree have this and previous screens saturated the autosomes for *mus* genes? Two alternative approaches were used to estimate the degree to which saturation has been achieved in the screens to date for autosomal genes that can be mutated to produce alleles that are both homozygous viable and mutagen sensitive. Both estimates—one derived by comparison with the number of known DNA repair functions identified to date in other organisms and the other derived from a statistical approach based on the number of alleles recovered for each complementation group—similarly conclude that mutations have been recovered in ~50% of mutable loci.

An analysis of the human genome suggests the presence of ~130 genes involved in DNA repair (Wood *et al.* 2001), a majority of which are evolutionarily conserved in other species. This may be an overestimate for Drosophila, given that there are genes on the human list for which no similar sequence appears in the Drosophila genome (*e.g.*, only one of seven Fanconi anemia genes, FANCD2, has a Drosophila homolog). Conversely, it is possible that there are repair functions unique to Drosophila. It is also important to note that it may not be possible to mutate some genes to alleles that are both homozygous viable and mutagen sensitive either because they are essential or because their products are functionally redundant. Still, making the assumptions that there are an equivalent number of repair genes in Drosophila and humans and that 80% of these genes are located on the second and third chromosomes, we might estimate that some 104 such genes are located on the second and third chromosomes. If this is indeed the case, then approximately half of those loci have been mutated in the screens performed thus far.

The traditional method of approximating the number of "hit-able" genes in Drosophila relies on the use of the Poisson distribution. This method assumes that all genes are mutable with an equal frequency and allows one to estimate the number of "un-hit" genes by the equation $P(0) = e^{-m}$ on the basis of the average number of alleles per mutable locus (m). Using this method, previous workers have estimated that the Drosophila genome might contain some 55–60 genes capable of mutating to mutagen sensitivity (Smith *et al.* 1980; Mason *et al.* 1981; Snyder and Smith 1982). Were that the case, the current number of loci identified might well represent a very large fraction of the possible mutational targets.

Unfortunately, the method in which the Poisson has classically been applied has two major problems. The first problem inherent in the application of the Poisson to estimate the number of target loci is that not all genes are equally mutable. The total collection of *mus* mutants in Drosophila and our own set of newly isolated mutants clearly contain a substantial number of hypermutable or "jackpot" loci. For example, while only 1 or 2 alleles were recovered for most loci, 8 alleles were recovered for *mus301* and 27 alleles were recovered for *mus305*. Inclusion of such loci would invalidate any estimate of saturation using a Poisson distribution, because it would artificially increase the mean number of alleles per locus. Although others have addressed this difficulty by simply excluding these types of outliers from the calculation of m, it is not clear where to draw the line when considering such loci as *mus205*, with six alleles.

The second difficulty in the use of the Poisson distribution lies in the method by which m is calculated. Traditionally, m has been determined simply by dividing the number of mutations recovered by the total number of loci they define. The problem is that an accurate estimation of the mean requires knowing the answer that one is trying to obtain, namely the number of unhit loci. When a screen is approaching saturation, the difference between total loci and loci for which mutant alleles have been recovered may become small enough that ignoring this difference will not greatly affect the result. However, when saturation is low or moderate, ignoring this difference will cause the degree of saturation to be drastically overestimated. These difficulties were anticipated by T. H. Morgan and H. J. Muller (Lefevre and Watkins 1986). If one assumes that among those alleles that are not hypermutable, mutability is relatively similar, it is still possible to estimate the degree of saturation by using the Poisson.

One effective method, first used by Cohen (1960), gives a maximum-likelihood estimate of m by simply taking the mean number of alleles per gene from the sample mean (alleles/identified locus) and then looking up m from a table. Again, this method requires the investigator to determine where to draw the line between what is an outlier and what is to be included in the calculation. We propose an alternative approach that does not require a subjective decision with respect to outliers. This method is derived from a comparison of the first few classes of the Poisson distribution itself: namely $P(0) = e^{-m}$, $P(1) = me^{-m}$, and $P(2) = m^2/2\ e^{-m}$ [where $P(X)$ is the proportion of loci with X alleles]. Dividing $P(1)$ by $P(2)$ gives you $2/m$. Note that although $P(1)$ and $P(2)$ are both proportions, and thus are dependent upon the size of all of the other classes, their ratio is independent of these classes. Thus, $P(1)/P(2) = N(1)/N(2)$, where $N(X)$ is the total number of loci recovered with X alleles, and m is easily calculated as:

$$2/m = N(1)/N(2) \quad \text{or} \quad m = 2\ N(2)/N(1).$$

As shown in Figure 2, the percentage of saturation follows directly from the Poisson distribution as $100(1 - e^{-m})$. We favor this method, because not only does it allow for calculation of m without assuming that the $P(0)$ class is small, but also it eliminates the need for the investigator to determine where the line should be drawn as to what is an outlier and what is not. Although we believe this approach to be preferable to a traditional use of the Poisson distribution, it is not without its prob-

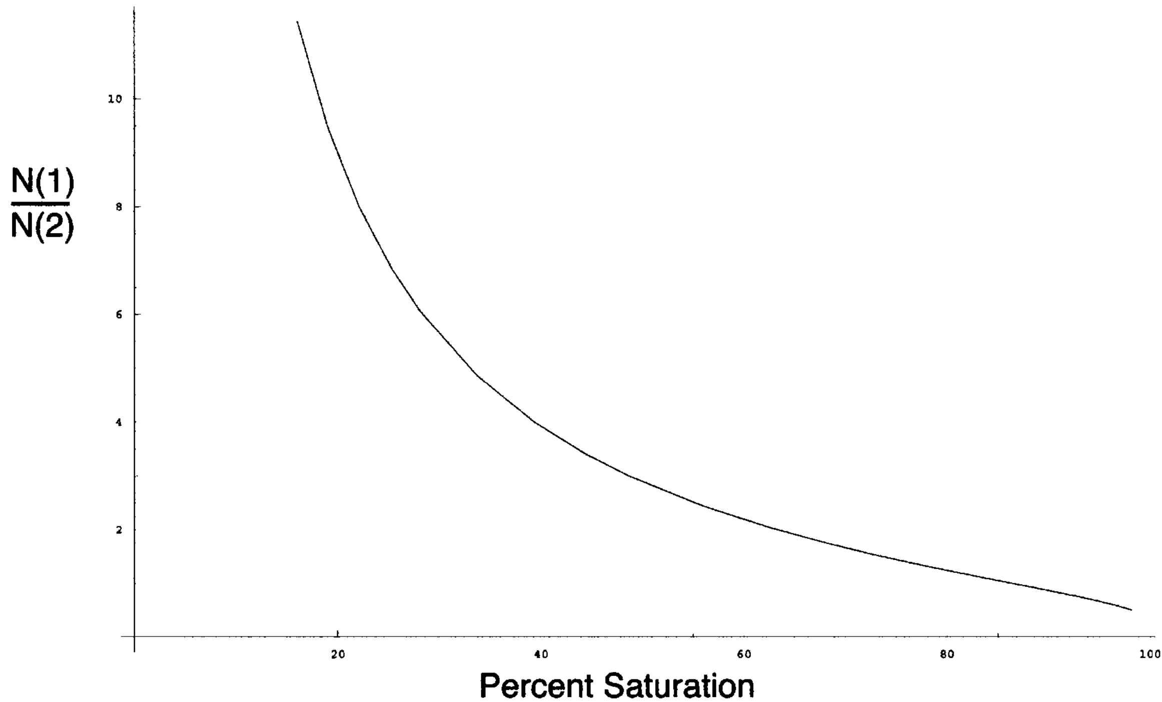

FIGURE 2.—A parametric plot of the ratio of $N(1)$ to $N(2)$ and percentage of saturation with respect to the parameter m. This plot is generated by further rearrangement of the previously outlined equations for each of the classes of the Poisson distribution.

lems: when either or both $N(1)$ and $N(2)$ are very small, the calculation of m is subject to large fluctuations as a result of sampling error.

The use of this method to estimate the degree of saturation for the second chromosome provides an answer that is pleasingly consistent with the estimate initially described above, which was based on genomic comparisons. For the second chromosome, 10 loci were defined by one allele and 6 loci were defined by two alleles. By using Figure 2, we can estimate that the second chromosome is 70% saturated and that the total number of *mus* loci on chromosome 2 may lie somewhere in the vicinity of 40. Application of the methods described by COHEN (1960) provides a similar result, an estimation of ~50% saturation. On the basis of these estimates, one would predict that there are ~35–50 genes on the second chromosome that can mutate to a *mus* phenotype, a number in reasonable agreement with the estimate of some 50 DNA repair genes obtained by genomic analysis.

TABLE 7

Newly identified *mus* genes on the third chromosome

New genes	Zuker no.	MMS	HN2
mus314	ZIII-2504	+	−
mus315	ZIII-2629	+	−
mus316	ZIII-2640	+	−
mus317	ZIII-4494	+	−
mus318	ZIII-4681	+	−
mus319	ZIII-5444	+	−
mus320	ZIII-6025	+	−
mus321	ZIII-0708	−	+
mus322	ZIII-4709	−	+
	ZIII-2589	−	+
mus323	ZIII-2866	−	+
mus324	ZIII-4325	+	+
	ZIII-5997	+	+
mus325	ZIII-4708	+	+
mus326	ZIII-4840	+	+
mus327	ZIII-5906	+	+

The analysis of the third chromosome is substantially more complicated for two reasons (Table 7). First, statistically, the estimation is made less useful because the number of genes defined by only two alleles is only 2. Given that the number of genes defined by a single allele (16) is large, it is very difficult to accurately assess the degree of saturation. The second problem lies in our suspicion that the excess of single-hit loci on chromosome 3 reflects a substantial number of genes that we would classify as *hypo*-mutable, either because only very rare changes produce a mutagen-sensitive phenotype or because that phenotype is exhibited only under certain treatment conditions or genetic backgrounds and not in others. We note, for example, that none of the new third-chromosome *mus* mutants reported here were allelic to the three previously characterized loci

that had been defined by single alleles (*mus306*, *mus307*, and *mus310*). In all cases where new alleles of extant genes were obtained, they fell in genes already defined by two or more alleles. Similarly, among the 14 new third-chromosome genes identified in our screen only 2 were defined by two or more alleles. We imagine that, had we continued our screens for third-chromosome *mus* mutants, the result would have been more alleles of already well-represented genes and a large population of new single-hit loci, rather than a further population of the class of existing loci defined by single mutants.

While we believe that the use of $N(1)$ and $N(2)$ to estimate saturation (when both are reasonably large) is an improvement over the classical method of estimation, because of its intuitive and practical simplicity, we anticipate that further progress in identifying repair-deficient loci in Drosophila will not be limited to screens such as the one described. Rather, we suspect that *mus* mutants will also be obtained by the use of reverse genetics to create mutants in genes defined by sequence homology or by the demonstration of a repair-deficient phenotype for mutants initially defined by other means. The *rad54* mutants created by Eeken and collaborators (Kooistra *et al.* 1997) serve as a successful example of mutants recovered using reverse genetics. An example of the demonstration of a repair-deficient phenotype for mutants initially defined by other means can be found in the analysis of the *haywire* gene. Initially defined by an inability to complement a tubulin mutation, the *haywire* gene encodes a Drosophila XPB homolog and displays a defect in the repair of UV-induced DNA damage (Mounkes *et al.* 1992; Mounkes and Fuller 1999; Merino *et al.* 2002). Furthermore, the observation that recombination-defective mutants that alter the processing of the initial DSB trigger the activation of a meiotic cell cycle checkpoint has resulted in the identification of a number of *rad51/dmc1* homologs in Drosophila (Ghabrial *et al.* 1998; Ghabrial and Schupbach 1999).

The continued acquisition of *mus* mutants by all of these means will further complete the genetic tool box for the analysis of DNA repair in Drosophila. As the remaining repair-deficient genes are identified and the biochemical functions of their protein products are elucidated, we will begin to address the variety of roles these proteins play not just in repair, but also in normal cellular processes, such as the control of meiotic and mitotic cell cycles, the coupling of replication to cell division induction, the facilitation of meiotic recombination, and the maintenance of active or inactive states of chromatin. The multiple roles played by some of the existing mutants in processes such as heterochromatic condensation and control of chorion gene amplification serve to remind us that the functions once thought of solely as "repair proteins" may indeed play much wider roles in the developmental process (Gatti *et al.* 1983; Yamamoto *et al.* 2000; Van Hatten *et al.* 2002).

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