IDENTIFICATION OF A SECOND LOCUS IN *DROSOPHILA MELANOGASTER* REQUIRED FOR EXCISION REPAIR

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ABSTRACT

The *mus(2)201* locus in Drosophila is defined by two mutant alleles that render homozygous larvae hypersensitive to mutagens. Both alleles confer strong *in vivo* somatic sensitivity to treatment by methyl methanesulfonate, nitrogen mustard and ultraviolet radiation but only weak hypersensitivity to X-irradiation. Unlike the excision-defective *mei-9* mutants identified in previous studies, the *mus(2)201* mutants do not affect female fertility and do not appear to influence recombination proficiency or chromosome segregation in female meiocytes.——Three independent biochemical assays reveal that cell cultures derived from embryos homozygous for the *mus(2)*^{D1} allele are devoid of detectable excision repair. 1. Such cells quantitatively retain pyrimidine dimers in their DNA for 24 hr following UV exposure. 2. No measurable unscheduled DNA synthesis is induced in mutant cultures by UV treatment. 3. Single-strand DNA breaks, which are associated with normal excision repair after treatment with either UV or N-acetoxy-N-acetyl-2-aminofluorene,* are much reduced in these cultures. Mutant cells possess a normal capacity for postreplication repair and the repair of single-strand breaks induced by X-rays.

EXCISION of DNA damage followed by resynthesis of the normal base sequence represents the predominant mode of DNA repair in a wide variety of prokaryotic and eukaryotic organisms (for review: HANAWALT *et al.* 1979). Genetic and biochemical studies with prokaryotes have outlined a complex excision mechanism involving a multiplicity of enzymatic steps (summarized in Table 1 of HANAWALT *et al.* 1979). Variation in the mechanism of incision in response to different lesions as well as partially overlapping redundancy in specific repair steps provide a rationale for the large number of genetic loci required for normal excision.

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* Abbreviations: AAAF—N-acetoxy-N-acetyl-2-aminofluorene; AAF—N-acetyl-2-aminofluorene; ara-C—cytosine-β-D-arabinofuranoside; EMS—ethyl methanesulfonate; HN2—nitrogen mustard; In(2L)Cy—In(2L)Cy, al^2 ast^3 b pr (*Cy* not present); In(2LR)CyO—In(2LR)O, dp^{lvI} Cy pr cn^2; MMS—methyl methanesulfonate; *mus*—refers to a mutation which confers mutagen sensitivity on larvae; UV—ultraviolet radiation—predominantly 254 nm.

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Studies designed to outline the molecular details of the excision mechanism in eukaryotes have focused on excision-defective mutants in yeast (*e.g.*, PRAKASH and PRAKASH 1979) and in humans (for review: PATERSON 1979). The identification of nine such loci in *Saccharomyces cerevisiae* (PRAKASH and PRAKASH, 1979) and seven in humans (BOOTSMA 1978) suggests that the excision process in eukaryotes will prove to be similarly complex.

Previous studies with *Drosophila melanogaster* have revealed the absence of detectable excision repair in the recombination-defective *mei-9* mutants (BOYD, GOLINO and SETLOW 1976; NGUYEN and BOYD 1977; HARRIS and BOYD 1980). In this report, we identify a locus on the second chromosome whose normal function is also essential for excision repair. Unlike the *mei-9* mutants, mutants at this locus do not exhibit extreme hypersensitivity to X-irradiation and do not influence meiocyte function. Summaries of this investigation have appeared (SNYDER and SMITH 1977; BOYD 1979; BOYD *et al.* 1980; SMITH, SNYDER and DUSENBERY 1980).

MATERIALS AND METHODS

Mutant Selection

The *mus(2)201* locus is defined by two mutant alleles that were recovered from experiments designed to isolate strains hypersensitive to MMS following EMS mutagenesis. Details of the isolation procedures leading to the recovery of these alleles will be reported elsewhere (BOYD *et al.*, *Genetics*, 1981; selection scheme B; HARDY, OREVI and MERRIAM, unpublished). Although the *mus(2)201*^{D1} chromosome is marked with *cn* and the *mus(2)201*^{A1} chromosome is marked with *bw*, stocks or cells homozygous for the *mus* chromosomes will be referred to as *mus201*^{D1} or *mus201*^{A1} throughout this report. A stock homozygous for *cn bw* is not hypersensitive to chemical mutagens and has been employed as the control strain for *in vivo* studies. Stocks homozygous for *w* or *cn* are both repair proficient and have been employed interchangeably as controls for *in vitro* repair studies. The *mus201*^{A1} allele has not yet been analyzed biochemically because the stock acquired a second site lethal mutation during the latter course of these studies.

Complementation Analysis

The recessive nature and allelic relationship of the *mus201* mutants was determined with tests based on larval survival after exposure to MMS. Ten virgin *In(2LR)CyO/mus* females were mated to either *In(2LR)CyO/mus* or Oregon-R wild type males in half-pint culture bottles. Parental flies were discarded after two days, and developing cultures were either treated with 1 ml 0.07% (v/v) MMS or left untreated. F_1 progeny were scored through day 17 of culture for the presence or absence of the *Cy* phenotype.

Genetic Localization

The absence of a morphological phenotype associated with *mus* mutations requires mapping procedures that depend upon isolation and establishment of recombinant chromosome stocks and subsequent testing for mutagen sensitivity. Because of the large size of the second chromosome, the location of the *mus 201* locus was determined with two experiments.

In the first experiment, the *mus 201* locus was localized to one arm of chromosome *II*. *In (2L)Cy, al*^{2} *ast*^{3} *b pr*, females were mated to *cn mus 201*^{D1} males and *In(2L)Cy, al*^{2} *ast*^{3} *b pr/cn mus 201*^{D1} heterozygous F_1 daughters were collected. These F_1 females were backcrossed to *cn mus 201*^{D1}, two two-day broods were established and one of these broods was treated with 1 ml of 0.07% MMS per bottle. F_2 progeny in the treated and replicate control cultures were scored for cinnabar or wild-type eye color. Because inversion heterozygosity reduces recombination throughout the left arm of chromosome *II*, the absence of cinnabar progeny in the treated cultures would indicate that the *mus 201* locus was either in the left arm or closely linked to cinnabar in the right arm.

In the second experiment designed to localize *mus 201* to a specific region, *al dp b pr* females were mated to *cn mus 201*^{D1} males. F_1 daughters were collected and backcrossed to *al dp b pr* males. F_2 recombinant males representing single crossover classes of the left arm were collected and mated individually to *In(2LR)CyO/l(2)91*^{DTS} females. From each F_2 cross, five males bearing the *In(2LR)CyO* chromosome heterozygous with either a single recombinant chromosome or the *al dp b pr* marker chromosome were selected and backcrossed individually to *In(2LR) CyO/l(2)91*^{DTS} females. This F_3 cross was raised at 29° to allow the automatic recovery and mating of a single genotype class of F_4 males and females, either *In(2LR)CyO/*recombinant chromosome or *In(2LR)CyO/al dp b pr*. The F_4 males and females were transferred to fresh vials and allowed to produce two replicate 24-hour broods. One brood was treated with 0.25 ml of 0.03% MMS. After 15 days, control and treated vials were scored for the presence of homozygous recombinant progeny and the location of the *mus 201* locus was determined from the pattern and frequency of MMS-sensitive recombinant chromosomes.

Mutagen Sensitivity

a. *MMS and HN2 Tests*: Ten pairs of *In(2LR)CyO/mus* flies were mated per half-pint culture bottle and allowed to lay eggs for two days. Parental flies were discarded and developing cultures were treated with MMS (1.0–6.0 mM) or HN2 (0.2–0.6 mM). Control cultures were left untreated. Eclosed F_1 adults were scored on days 14–16 of culture. Survival values are represented as a ratio of homozygous *mus* mutants to heterozygous *In(2LR)CyO/mus* controls. Recovery of homozygous *mus* mutants in untreated bottles is expected to be 50% of the heterozygous controls. The actual value obtained from untreated cultures was employed to normalize the data obtained from the treated sample.

b. *UV and X-ray tests*: *In(2LR)CyO/mus* cultures were established in half-pint culture bottles and parental flies were allowed to oviposit for two days. Mixtures of second and third instar larvae were collected from bottles on day 4 of culture by flotation in sucrose solution. Larvae were washed with water, apportioned in approximately equal aliquots to petri plates and irradiated with X-rays (500–2000 rads delivered at 60 R/min by a G.E. OX-250 industrial X-ray machine) or UV light (50–150 Jm^{-2} delivered in darkness by a G.E. G15T8 germicidal lamp). Irradiated larvae were returned to fresh media to complete development and the number and genotype of adults were determined on day 15 of culture.

Meiotic Analysis

a. *Reproductive capacity*: Virgin females and males, approximately 3–5 days old, were pair mated in vials for 24 hours and subsequently transferred to egg counting vials (regular media darkened with Welch's grape juice) for 15–24 hours. After the parents had been discarded, the number of eggs was determined and compared to the number of adults that eclosed by day 15 of culture.

b. *Chromosome nondisjunction*: The effect of the *mus201*^{D1} mutant on *X* and fourth chromosome nondisjunction in females was determined by mating *y*/+; *mus201*^{D1}/*mus201*^{D1}; *spa*^{pol}/*spa*^{pol} females in vials to Y^S $X{\cdot}Y^L$, *In(1)EN, v f B/O*; +/+; *C(4)RM, ci ey*^{R}*/O* males. Progeny were recorded from five two-day broods of individual pair matings and data were pooled for analysis.

c. *Recombination proficiency*: Females heterozygous for four recessive X-linked mutations (*y, cv, v, f*) and homozygous for *mus201*^{D1} were mated to males hemizygous for the X-linked mutations and homozygous for *mus201*+. Progeny resulting from five two-day broods of individual pair matings were recorded through day 15 of culture and data were pooled for analysis.

Unscheduled DNA Synthesis

Primary cell cultures derived from embryos (BOYD and SETLOW 1976; HARRIS and BOYD 1980) are prepared on glass coverslips in 35 mm tissue culture dishes. Twenty-four hours after the cultures have been established, one-third (0.5 ml) of the medium is removed from each dish and pooled separately for each mutant. Thymidine (methyl-^{3}H, 24 C/mM, Amersham Corp., Arlington Heights, Il.) is mixed with the pooled medium which is then returned to the plates for one hour. All medium is removed, pooled for individual mutants, and cleared of cells by centrifugation at 700 × *g* for 3 min. The adhering cells are irradiated with germicidal lamps (HARRIS and BOYD 1980) under 0.6 ml of PBS (DULBECCO and VOGT 1954). The PBS is replaced with 1.25 ml of cleared labeled medium and incubation is continued for three hours in the dark.

After incubation, the medium is replaced with 2 ml of PBS containing 10 μg/ml of unlabeled thymidine. Washing is repeated nine times at 10 min intervals with the exception that incubation in the fifth wash solution is extended to one hr at 25°. The cells are then fixed twice in 2 ml of methanol-acetic acid(3:1) for a total of 20 min. Coverslips are immersed in methanol for 5 min, air dried, and mounted on glass slides with Unimount (Brunswick Laboratories, St. Louis, Mo.). Feulgen staining is performed for over 1 hour following hydrolysis at 60° in 1N HCl for 12.5 min. The slides are placed in SO_2 water (0.05N HCl, 0.5% (w/v) $K_2S_2O_4$) for 5 min, rinsed in distilled water for 5 min, and dried.

Autoradiography is performed with nuclear track emulsion (NTB-2, Eastman Kodak Co., Rochester, N.Y.) which has been diluted 1:1 with 0.1% sodium dodecyl sulfate. Slides dipped at 45° are dried overnight in the dark and exposed at 4°. The emulsion is developed with one-half strength Kodak D-19 developer for 4.5 min at 15°. Silver grains are counted directly under oil over nuclei that are 8–12 μm in diameter after excluding cells in the S phase of the cell cycle. The primary cell cultures also contain clumps of cells whose nuclei are 2–5 μm in diameter. Since cells of this class are never in S phase during analysis and appear to possess greatly reduced levels of unscheduled DNA synthesis, they have been excluded from analysis.

Alkaline Elution Analysis

This assay, which measures single-strand molecular weights of DNA between 5×10^8 and 10^{10} daltons, is described in detail elsewhere (HARRIS and BOYD 1980). Briefly, cells containing DNA uniformly labeled with (^{14}C)thymidine are lysed on a teflon filter, and the released DNA is slowly eluted from the filter under denaturing conditions. The difference between the rate of elution of DNA from cells exposed to mutagen and the rate of elution of DNA from untreated cells is used to calculate termed "relative elution." This value is directly proportional to the frequency of experimentally-induced single-strand breaks in DNA.

Enzymatic Analysis of Pyrimidine Dimers

Our procedure for measuring pyrimidine dimers as UV-endonuclease-sensitive sites has been described (HARRIS and BOYD 1980). Briefly, UV-irradiated (^{3}H-labeled) and unirradiated (^{14}C-labeled) cells are mixed and their DNA is extracted and partially purified. This DNA is incubated with a crude endonuclease preparation from *Micrococcus luteus* which quantitatively nicks DNA in the vicinity of pyrimidine dimers. The resulting reduction in single-strand molecular weight of UV-irradiated DNA is quantified by sedimentation through alkaline sucrose gradients.

Analysis of DNA Synthesis and Postreplication Repair

Primary cultures are analyzed within twenty-four hours after the cells have been plated. Medium is removed and those cultures that are treated receive 10 Jm^{-2} of UV radiation. Fresh medium is added to the plates, which are then incubated for 30 min prior to receiving a 30 min pulse with 12 μC/ml [^{3}H] thymidine. The plates are then washed once with fresh medium and incubated in additional fresh medium for three hours. A control culture is labeled overnight with 0.5 μC/ml [^{14}C] thymidine and used as an untreated internal standard in all gradients. This culture is incubated with fresh medium for at least one hour prior to analysis. An aliquot of suspended cells labeled with ^{14}C is mixed with each sample of ^{3}H-labeled cells. DNA molecular weights are estimated after the cells have been lysed and subjected to alkaline sucrose gradient centrifugation. Molecular weight values are relative rather than absolute, because they have all been normalized to the internal ^{14}C standard (BOYD and SHAW, in press).

Similar manipulations were performed with isolated larval brain ganglia (BOYD and SHAW, in press). In the case of cultured organs, however, UV treatment was replaced by exposure to 8 μM AAAF for 15 min. The chase period was reduced to one hour and the tissue was homogenized prior to alkaline sucrose gradient sedimentations.

Analysis of Single Strand DNA Breaks

Primary cell cultures are labeled overnight with 0.5 μC/ml [^{14}C] thymidine in the case of control cultures and with 0.5 μC/ml [^{3}H] thymidine for mutant cultures. Cultures are incubated with fresh unlabeled medium for at least one hour prior to irradiation. The medium is removed from the plates before treatment with 10 kR of X-rays and fresh medium is added immediately thereafter. Molecular weight ratios are determined by centrifugation of alkaline sucrose gradients in a Sorvall TV865 vertical rotor (Boyd and Shaw, in press).

RESULTS

Complementation Analysis

The dominance relationship of both *mus* mutations with respect to MMS sensitivity was established in matings with an Oregon-R wild-type strain (Table 1). From MMS-treated cultures (crosses 1 and 2), contingency chi-square analysis of frequencies of recovery of *In(2LR)CyO/+* and *mus/+* adults indicates that both mutations are recessive at treatments up to 0.07% MMS. This conclusion permits complementation tests based on MMS sensitivity, and data establishing the allelic relationship of the *mus* mutants are also presented in Table 1. Crosses 3 and 4 establish that the *mus* mutations are fully viable in the absence of mutagen treatment but are highly sensitive to 0.07% MMS treatment. The same high MMS sensitivity observed for the non-Curly adults recovered from cross 5 establishes that the newly-induced *mus* mutants are allelic. Since these mutants represent the first MMS-sensitive strains identified in our laboratories on the second chromosome, they have been designated *mus(2)201*^{A1} and *mus(2) 201*^{D1} according to previously established convention (Smith 1976; Boyd *et al.* 1976).

Genetic Localization

Initial mapping studies using females heterozygous for a left arm inversion (Table 2A) indicated that the *mus 201* locus was tightly linked to *cn* and thus either was located within the left arm or in a proximal region of the right arm of chromosome *II*. Subsequent studies were conducted on the assumption that *mus 201* was in the left arm and used the recombinant marker stock *al dp b pr*. From data recorded in Table 2B, it was determined that the *mus 201* locus was located between *dp* and *b* at a map position of approximately 23.

TABLE 1

Complementation analysis of mus201 *allele*

Cross	Control			MMS-treated			Treated ratio
	Cy$^+$	Cy	Cy$^+$/Cy	Cy$^+$	Cy	Cy$^+$/Cy	Control ratio
Cy/201^{D1} × +	229	208	1.10	342	375	0.91	0.83
Cy/201^{A1} × +	411	367	1.12	159	166	0.96	0.86
Cy/201^{D1} × Cy/201^{D1}	272	511	0.52	10	514	0.02	0.04
Cy/201^{A1} × Cy/201^{A1}	328	612	0.54	6	419	0.01	0.03
Cy/201^{D1} × Cy/201^{A1}	105	250	0.42	1	218	0.005	0.01

TABLE 2

Mapping of the mus201 *locus*

A. Arm localization test

Genotype	Number of progeny Control culture	Treated culture
In(2L), al ast b pr/mus201^{D1} *cn*	202	308
mus201^{D1} *cn/mus201*^{D1} *cn*	194	10

B. Interval localization test

Genotype of recombinant chromosome	mus$^+$	mus	Total
al+ dp b pr	42	0	42
al+ dp+ b pr	4	10	14
al+ dp+ b+ pr	0	20	20

Mutagen Sensitivity

Since many of the known *mus* mutants confer meiotic defects (Baker *et al.* 1976a; Boyd *et al.* 1976; Smith 1976; Smith, Snyder and Dusenbery 1980), second chromosomal *mus* strains were initially maintained in heterozygous form with *In(2LR)CyO* and mutagen sensitivity tests utilized these stocks. Both *mus201* alleles were tested for sensitivity to MMS, UV, HN2 and X-rays (Figure 1). Since the *mus201*^{D1} allele is marked with *cn* and the *mus201*^{A1} allele is marked with *bw*, a *cn bw* strain was employed as a control for both stocks. In these experiments the dose ranges employed were such that survival of the *cn bw* control strain was not significantly reduced.

Both *mus201* alleles appear to be highly sensitive to MMS (Figure 1a) and UV (Figure 1B) but differ somewhat with respect to HN2 sensitivity (Figure 1c). The *mus201*^{A1} allele appears to be more sensitive to nitrogen mustard and X-rays than the *mus201*^{D1} allele. Neither allele, however, exhibits a particularly strong X-ray sensitivity. Contingency chi-square analysis indicates that, at the highest dose, both alleles show statistically demonstrable sensitivity. The weakness of this response, however, indicates that the *mus201* locus plays, at best, only a minor role in resistance to X-ray exposure.

Meiotic Analysis

Previous studies of X-linked mutagen-sensitive mutants have indicated that female infertility and associated meiotic abnormalities are often associated with the *mus* phenotype (Smith, Snyder and Dusenbery 1980). Investigations of the *mei-9* mutants have indicated that this gene function is required both for DNA repair of mutagen-induced damage in somatic cells and for normal chromosome disjunction and recombination in the germ line. Because of similarities between the *mei-9* and *mus201* loci established from studies of *in vivo* mutagen sensitivity and *in vitro* DNA repair (see below), the effect of the *mus201*^{D1} allele on meiotic chromosome behavior was analyzed.

a. *Reproductive capacity*: In initial studies, the reproductive capacity of *mus201* females and males was measured. In these experiments, the frequency

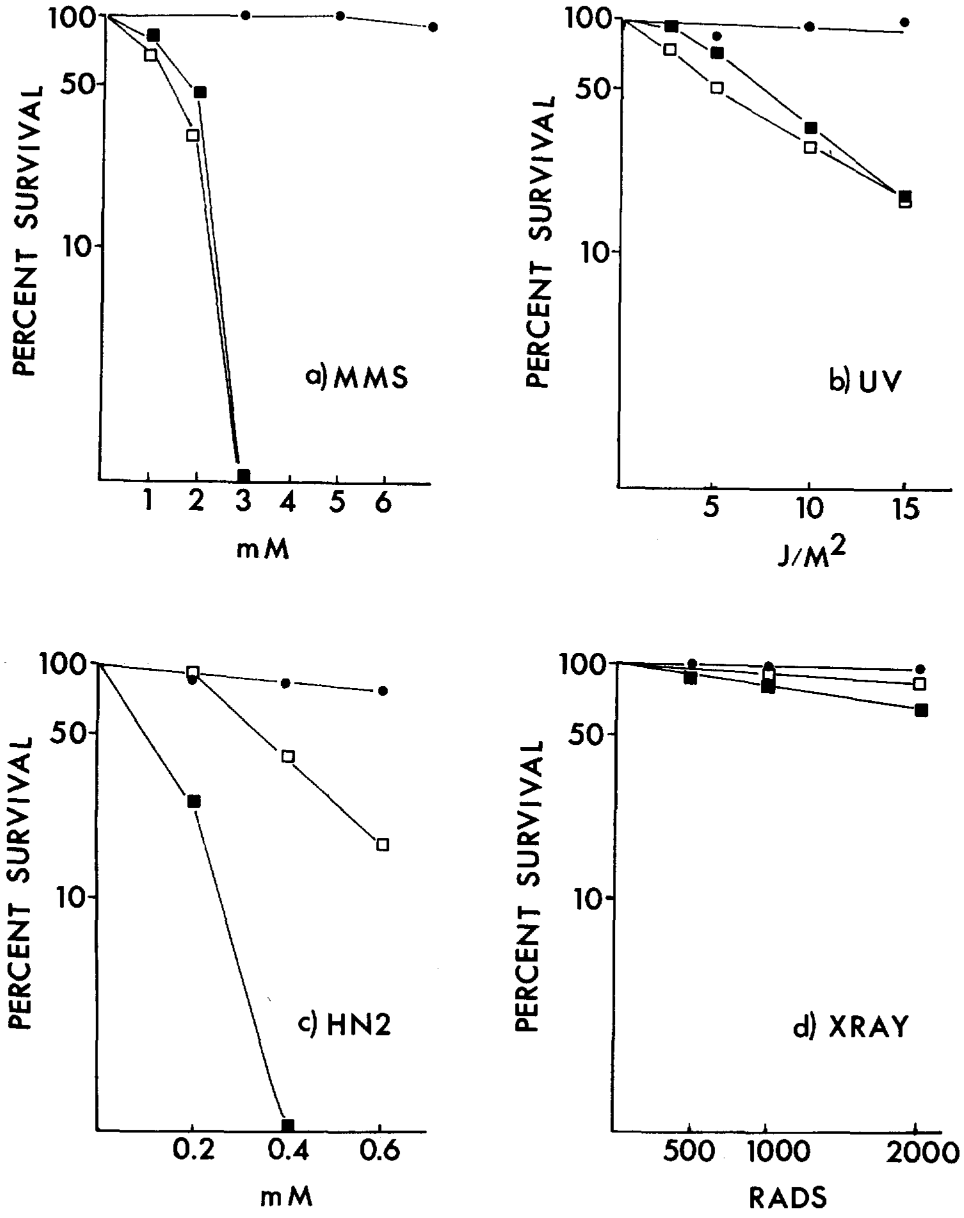

FIGURE 1.—Analysis of larval survival as a function of mutagen dose. The relative mutagen sensitivity of homozygous larvae was evaluated as described in MATERIALS AND METHODS. The average number of flies counted for any dose of a particular mutagen was 1241. No point is represented by fewer than 200 flies. Symbols: ● *cn bw*; ☐ *mus201*^{D1}; ■ *mus201*^{A1}. Mutagens: a. MMS, b. UV, c. HN2 and d. X-ray.

of eclosion derived from a number of comparative matings was measured relative to frequencies obtained with the *cn bw* stock that was utilized as a control strain. These data, presented in Table 3, suggest that *mus201* wild-type function is not necessary for either male or female fertility.

b. Meiotic nondisjunction: Studies contrasting the effect of *mus201*^{D1} with the *cn bw* control strain on female meiotic nondisjunction of the *X* and fourth chromosomes were performed. The frequencies of regular and exceptional F_1 progeny derived from the cross $+/y;201^{D1}$ *cn; spa*^{pol}/*spa*^{pol} × $Y^S{\cdot}X{\cdot}Y^L$, *In(1)EN, v f B/O; C(4)RM, ci ey*^{R}*/O* were recorded and the results are presented in Table 4. For comparative purposes, the effect of *mei-9*^{a} on female nondisjunction reported by BAKER and CARPENTER (1972) is also included in Table 4. Although the frequency of haplo-4 exceptional progeny was recorded, the numbers were excluded from the analyses presented in Table 4 because of the poor viability of such flies. The analysis of these data clearly indicates that the *mus201*^{D1} allele does not increase the rate of nondisjunction in female meiocytes above control levels. By comparison, the *mei-9*^{a} mutant causes a large increase in meiotic nondisjunction when compared to the control strain used in those experiments.

TABLE 3

Reproductive capacity of mus201 *strains*

Cross female × male	Number of eggs	Number of adults	Frequency of eclosion	Relative % eclosion
cn bw × *cn bw*	860	770	0.90	(100)
cn bw × *Oregon-R*	442	375	0.85	94
cn bw × *201*^{A1} *bw*	292	219	0.75	83
cn bw ×*201*^{D1} *cn*	418	359	0.86	96
201^{A1} *bw* × *201*^{A1} *bw*	346	307	0.89	99
201^{D1} *bw* × *201*^{D1} *cn*	914	712	0.78	87
201^{D1} *cn* × *Oregon-R*	417	341	0.82	91

TABLE 4

Analysis of nondisjunctional progeny

Progeny class	*cn bw*	*mus201*^{D1} *cn*	*mei-9*†
Regular	1990	2520	
X Exceptional	8	3	
4th Exceptional	1	0	
X-4th Exceptional	0	0	
Total	1999	2523	
* *X* Exceptional/10^3 ova	8.0	2.4	276.4
4th Exceptional/10^3 ova	0.5	0	188.5

* Since the identification of *X* exceptional progeny is 50% as efficient as the identification of regular and *4th* exceptions, the number of *X* exceptions has been doubled to allow a direct comparison of X and 4th exceptional rates.

† BAKER and CARPENTER (1972).

c. *Recombination proficiency*: The recombination proficiencies of *mus201*^{D1} and *cn bw* were compared by measuring the types and frequencies of recombinant offspring produced from females heterozygous for four recessive X-linked markers. Analysis of these data (summarized in Table 5) indicates that the *mus201*^{D1} allele produces a very slight decrease in recombination frequency and that this effect is considerably exaggerated by an unusually high rate of recombination in the *y-cv* interval of the *cn bw* control strain. By contrast, the *mei-9*^{a} mutant reduces the map intervals examined by BAKER and CARPENTER (1972) to 8% of the control values.

Unscheduled DNA Synthesis

Autoradiographic analysis of unscheduled DNA synthesis was performed in primary cell cultures prepared from embryos of homozygous stocks. Data presented in Figure 2 reveal the dose response of mutant and control cells to UV. In this figure the median labeling intensity for a given sample is found at the intercept of the curve with the 50% value on the ordinate. These data reveal an increase in unscheduled DNA synthesis between 0 and 40 Jm^{-2} in control cells. UV exposure does not, however, increase unscheduled DNA synthesis above background levels in cells homozygous for *mus201*^{D1}. Trivial explanations such as cell death or failure of thymidine uptake are unlikely in view of the fact that

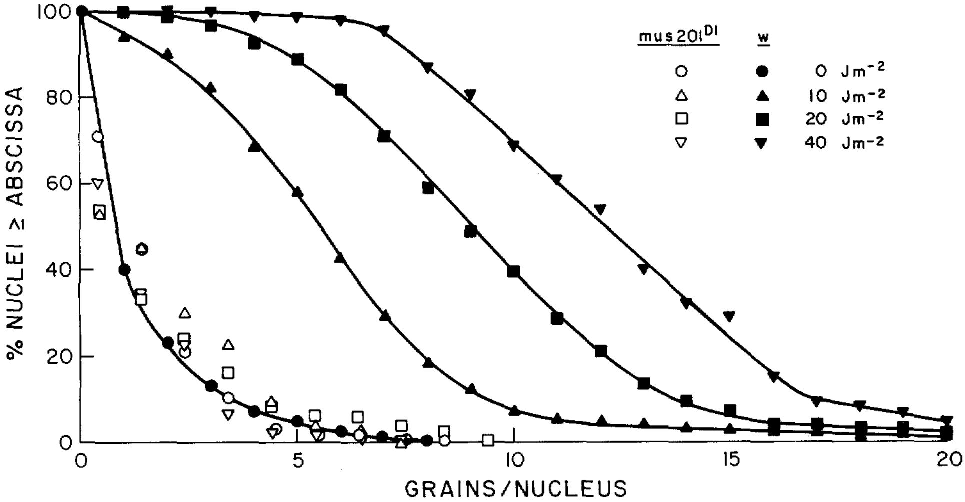

FIGURE 2.—Unscheduled DNA synthesis in primary cell cultures in *mus201*^{D1} and control (*w*) cells. Separate cultures were exposed to 0, 10, 20 or 40 Jm^{-2} UV. The curves derived from *w* cells are composites of 8, 6, 5 and 2 cultures analyzed, respectively, at these doses. One culture of mutant cells was employed at each of these radiation doses. Labeled thymidine was added at a concentration of 10 μC/ml. Autoradiographs were exposed for 12 days. Labeling intensity was analyzed in 50 cells of each culture. Data are tabulated to indicate the percentage of analyzed nuclei in a sample whose labeling intensity equals or exceeds each of the observed levels of labeling. Those values are plotted as a function of their respective intensity classes. Data obtained from mutant cultures has been normalized to the same background level as that exhibited by the control cultures.

TABLE 5

Analysis of recombination data

Strain	Total map distance	Total progeny scored	Map, individual regions *y-cv*	*cv-v*	*v-f*	Map, relative to control *y-cv*	*cv-v*	*v-f*	E_0	Tetrad distribution, frequency E_1	E_2	E_3
cn bw	60.8	3234	18.1	20.8	21.9	1.0	1.0	1.0	0.082	0.651	0.237	0.030
201^{D1} *cn*	50.1	2682	10.8	19.5	19.8	0.60	0.94	0.90	0.137	0.726	0.137	0

approximately normal numbers of heavily labeled cells were observed in these cultures (unpublished observations). In addition, BROWN and BOYD (1981) have demonstrated normal levels of thymidine incorporation in UV irradiated *mus201*^{D1} cultures.

Means derived from several such experiments are presented in Table 6. These data document a rapid rise in unscheduled DNA synthesis of control cells between 0 and 20 Jm^{-2}. Beyond 40 Jm^{-2} the repair response is more gradual. In contrast, UV exposure does not stimulate any detectable unscheduled synthesis in *mus201*^{D1} cells. The average error in the data derived from this mutant is about 4% of the incorporation observed in control cells at 40 Jm^{-2}.

Excision of Pyrimidine Dimers

The failure of *mus201*^{D1} cells to exhibit unscheduled DNA synthesis implies the existence of a metabolic block at or prior to the resynthesis step in excision repair. To pinpoint the lesion in this mutant more precisely, we have investigated earlier stages of the repair process by analyzing the fate of induced pyrimidine dimers. A sensitive enzymatic assay for such lesions has recently been refined by REYNOLDS (1978). In this assay DNA is isolated from cell cultures at various times after UV-irradiation. Alkaline sucrose gradients are then employed to detect single-strand breaks introduced at pyrimidine dimers by a UV-specific endonuclease. Application of this procedure to Drosophila cell cultures is presented in Figure 3. These data reveal no significant excision of pyrimidine dimers from DNA of *mus201*^{D1} cells during the day following irradiation. Control cultures (*w*), on the other hand, are seen to remove about 80% of the induced pyrimidine dimers from their DNA within this period.

Search for Strand Interruption

As opposed to chemical analyses for pyrimidine dimers, the enzymatic assay not only demonstrates the retention of pyrimidine dimers in DNA of mutant

TABLE 6

Unscheduled DNA synthesis in primary cell cultures

Strain	Ultraviolet dose	Grains/nucleus/day exposure—background (± SD)	Number of cultures analyzed
control (*w*)	10 Jm^{-2}	0.38 ± 0.07	8
	20 Jm^{-2}	0.62 ± 0.16	6
	40 Jm^{-2}	0.85 ± 0.19	4
	60 Jm^{-2}	0.96 ± 0.21	2
	80 Jm^{-2}	1.16 ± 0.05	2
mus201^{D1}	10 Jm^{-2}	0.00 ± 0.02	5
	20 Jm^{-2}	−0.02 ± 0.04	2
	40 Jm^{-2}	0.01 ± 0.03	5

The experimental protocol is described in MATERIALS AND METHODS. Total exposure times varied between 6 and 16 days. The concentration of the [^{3}H] thymidine employed varied between 5 and 15 μC/ml. Each value represents the mean of means obtained by evaluating the labeling intensity of 50 cells/culture. Within each experimental series the value obtained at 0 Jm^{-2} was subtracted from the results obtained after irradiation.

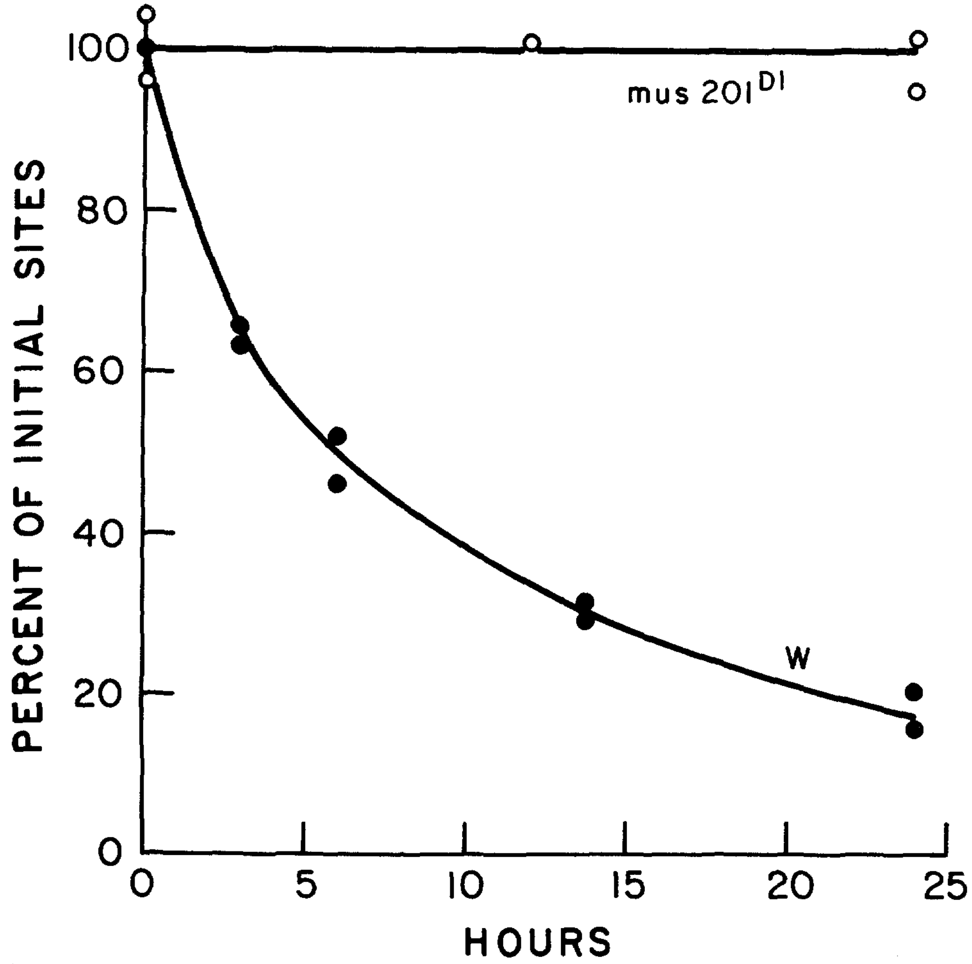

FIGURE 3.—Excision of pyrimidine dimers from DNA of UV-irradiated cells. Primary cell cultures were irradiated with 5 Jm^{-2} UV. Pyrimidine dimers were assayed enzymatically as a function of incubation time after irradiation. The initial sites referred to in the ordinate represent DNA sites that are susceptible to a UV-specific endonuclease preparation from *M. luteus*. Photorepair studies have previously identified such sites with pyrimidine dimers. The values obtained at 24 hr represent the typical excision capacity of 16 independently analyzed control cultures (range 10–35% remaining). Details of this assay are presented elsewhere (HARRIS and BOYD 1980).

cells, but it also reveals that such dimers are not associated with a significant fraction of single-strand DNA breaks (unpublished observations). This conclusion derives from the fact that the assay depends upon alkaline sucrose gradient centrifugation. If dimer-associated breaks had been present at high frequency in irradiated mutant cells, they would have been detected in control experiments in which the exogenous endonuclease was omitted. It is possible, however, that transient breaks are made and resealed in irradiated *mus201*^{D1} cells or that the level of such breaks is not detectable by alkaline sucrose gradient analysis. To pursue this question we turned to the alkaline elution procedure of KOHN *et al.* (1976) which provides a level of sensitivity for DNA breaks two orders of magnitude higher than that obtained with sedimentation analysis. This assay (HARRIS and BOYD 1980) generates a "relative elution" value that is directly proportional to the level of single-strand breaks in DNA ranging in size between 5×10^8 and 10^{10} daltons. Under our conditions a relative elution value of 0.1 corresponds to 2.12 experimentally-induced breaks per 10^{10} daltons of single-stranded DNA. Application of this assay to cultures of control and *mus201*^{D1} cells is presented in Table 7. In this series of experiments, cells were exposed to one of two mutagens and incubated in the presence or absence of DNA synthesis inhibitors. Values obtained in the absence of inhibitors reflect the mutagen-induced breaks which are present during the course of normal excision repair. The inhibitors serve to amplify the detection of such breaks by suppressing the resynthesis phase of excision repair. When the normal flow of repair is interrupted in this manner, gapped intermediates apparently accumulate. These phenomena are reflected in data obtained with control cultures following UV-irradiation (Table 7). Low, but detectable, levels of breaks are introduced by this treatment in the absence of inhibitors, and this break frequency is strongly enhanced in the presence of inhibitors. In *mus201*^{D1} cells, however, breaks are not detectable under either of these conditions. Mutant cells are thus apparently incapable of introducing interruptions into DNA single-strands following UV treatment.

This conclusion has been extended to cells treated with the mutagen AAAF, although the use of this compound entails an experimental complication. Even in the absence of inhibitors, this mutagen stimulates a high frequency of detectable DNA breaks. Previous analyses suggest that the majority of such breaks are chemically rather than enzymatically derived (HARRIS and BOYD 1980). We, therefore, infer that the level of breaks observed in *mus201*^{D1} cells is reduced

TABLE 7

Relative elution of DNA following mutagen treatment

	Treatment			
Cell origin	UV without inhibitors	UV with inhibitors	AAAF without inhibitors	AAF with inhibitors
control (*cn*)	0.091	0.417	0.346 ± 0.008	0.702 ± 0.021
control (*w*)	0.090 ± 0.031	0.386 ± 0.003	0.380 ± 0.010	0.636 ± 0.018
mus201^{D1}	0.0	0.005	0.254	0.282 ± 0.036

Relative elution values, which are directly proportional to single-strand interruptions in DNA, were determined as described elsewhere (HARRIS and BOYD 1980). By definition, untreated cultures have a relative elution of 0.0. Primary cell cultures were derived from homozygous stocks. Hydroxyurea (3mM) and ara-C (1mM) were added to one-half of the cultures one half-hour prior to mutagen treatment. After the cultures had been exposed to 5 Jm^{-2} UV or 10 μM AAAF, they were incubated for an additional one-half hour under the conditions existent prior to mutagen treatment. Single-strand DNA interruptions were monitored with the alkaline elution procedure. Where duplicate determinations were performed, one standard deviation is recorded. Data are corrected for minor effects of inhibitors alone.

relative to control cells, because it reflects only the chemically-mediated component of the break levels seen in control cells. This conclusion is substantiated by the observation that inhibitors enhance the levels of breaks observed in control cells, but they have no significant effect on the mutagen-induced break levels observed in *mus201*^{D1} cells. With the available technology it is thus not possible to demonstrate the existence of DNA interruptions related to repair in this mutant. It is, therefore, likely that this mutation blocks the initial incision step of excision repair, although we cannot at this time conclusively rule out the possibility of breakage followed by rapid religation.

Normal Features of DNA Repairs and Metabolism

In contrast to results obtained with several other mutagen-sensitive strains (BOYD *et al.* 1980), the data in Table 8A reveal no reduction in the capacity of *mus201*^{D1} cells to synthesize DNA. In the absence of any mutagen treatment, both primary cultures and brain ganglia derived from this mutant produce DNA equal in size to that of control cells after pulse-chase labeling. A slight reduction

TABLE 8

Normal features of DNA metabolism in mus201^{D1}

Treatment*	Molecular weight of *mus201*^{D1} DNA	Molecular weight of control DNA	Molecular weight ratio (mutant/control)
A. DNA synthetic capacity†			
1. Primary cultures			
UV, caffeine			
— —	163 ± 1.5(3)	163 ± 4.2(6)	1.01
+ —	139 ± 4.5(3)	147 ± 4.2(14)	0.95
+ +	131 ± 2.6(3)	140 ± 4.3(13)	0.94
2. Brain ganglia			
AAAF			
—	158 ± 0 (2)	160 ± 3.5(6)	0.99
+	124 ± 4.2(2)	132 ± 9.5(5)	0.94
B. Repair of X-ray-induced breaks in primary cultures‡			
Postirradiation incubation			
0 hour	63(1)	—	—
1 hour	110(1)	113(1)	0.97
3 hours	125(1)	126(1)	0.99

Details of these assays are found in BOYD and SETLOW (1976) and BOYD and SHAW (in press). Control cultures employed throughout these studies were derived from the repair-proficient *w* stock. In all cases weight average molecular weights ($\times 10^{-6}$) were determined by alkaline sucrose gradient centrifugation.

* + indicates treatment applied; — indicates treatment withheld.

† Primary cell cultures were treated with 10 Jm^{-2} of UV radiation, incubated in the presence of [^3H] thymidine for 30 min and in the absence of labeled precursor for an additional three hr prior to molecular weight analysis. Isolated brain ganglia were treated in an analogous fashion except that the UV treatment was replaced by 15 min exposure to AAAF, and the chase period was reduced to one hour. Molecular weight values have been normalized relative to an internal ^{14}C standard that was also present in each gradient. The number of independent determinations is indicated in parentheses.

‡ Single-strand DNA breaks introduced by 10 KR of x-rays were monitored by centrifugation as a function of time after irradiation. This radiation dose initially reduces the DNA of all cell cultures uniformly to a molecular weight of 63×10^6 daltons. In this experiment cellular DNA was uniformly labeled with either ^3H or ^{14}C which permits a comparison between control and mutant cells within the same gradient.

in this capacity is observed in mutant cells relative to controls after mutagen treatment. This small reduction is most readily explained by the demonstrated failure of *mus201*^{D1} cells to excise damage during this extended assay rather than to any significant reduction in postreplication repair capacity. Furthermore, caffeine is seen not to potentiate this reduction as it does in some postreplication repair deficient mutants (BOYD and SETLOW 1976). Finally, the data in part B of Table 8 reveal a normal capacity for the repair of single-strand breaks induced by X-rays in *mus201*^{D1} cells.

DISCUSSION

Mutagen Sensitivity: Hypersensitivity to physical and chemical mutagens has been utilized as a criterion for the isolation of mutant strains altered in DNA synthesis, recombination and repair. In Saccharomyces over 50 loci have been detected which influence sensitivity to UV, X-rays or MMS. Selection for yeast mutants specifically sensitive to MMS led to the identification of 28 complementation groups, 22 of which represent loci not previously detected by UV or X-ray studies (PRAKASH and PRAKASH 1977). In Drosophila, mutants at approximately 30 loci have been isolated on the basis of their hypersensitivity to the lethal effects of MMS (SMITH 1973, 1976; BOYD *et al.* 1976; SMITH, SNYDER and DUSENBERY 1980; BOYD *et al.*, 1981). Among both the yeast and Drosophila mutants, all possible combinations of cross-sensitivities to MMS, UV, and X-ray have been observed.

In the present report, we describe mutants at the *mus201* locus which exhibit strong *in vivo* hypersensitivity to UV, HN2, and MMS but not to X-rays. The spectrum of mutagen sensitivity exhibited by *mus201* larvae resembles that of cultures derived from classical forms of the human disorder, xeroderma pigmentosum (XP). Early studies with XP cell lines demonstrated hypersensitivity to UV and chemical agents such as 4-nitroquinoline-1-oxide and AAAF, which add bulky adducts to DNA (for review: CLEAVER and BOOTSMA 1975; ARLETT and LEHMAN 1978). Although initial studies did not reveal significant hypersensitivity to ionizing radiation or MMS, more recent work with cells from complementation group A has demonstrated enhanced sensitivity to MMS (THIELMANN and WITTE 1980).

Excision Deficiency: Studies with the *mei-9* mutants of Drosophila, which exhibit strong sensitivity to MMS, X-rays and UV, have demonstrated a deficiency in the excision of pyrimidine dimers (BOYD, GOLINO and SETLOW 1976; NGUYEN and BOYD 1977). Further studies have shown that the *mei-9* mutants interrupt the repair process at or before the incision step (HARRIS and BOYD 1980). In the present study, three independent biochemical assays reveal that cell cultures derived from embryos homozygous for the *mus201*^{D1} allele are devoid of detectable excision repair. Autoradiographic analysis failed to detect unscheduled DNA synthesis in mutant cells following UV irradiation, thereby identifying an excision deficiency at or before the resynthesis step of repair. Further studies utilizing a dimer-specific endonuclease demonstrate that mutant cells do not remove a significant proportion of UV induced pyrimidine dimers within 24 hours. Finally, the alkaline elution technique did not reveal any incision activity in mutant cells. Together, these experiments suggest that the *mus201* locus is required for the initial incision step of repair, although we cannot rigorously exclude the possibility that incision is followed by rapid religation. Thus, mutants at both the *mei-9* and *mus201* loci are defective in an early step of the excision process.

Biochemical studies of strains exhibiting UV hypersensitivity in yeast have identified mutants at nine loci which are defective in the excision of pyrimidine dimers (PRAKASH and PRAKASH 1979). Mutants of four of these loci are sensitive only to UV. Four additional loci are represented by mutants sensitive to UV and MMS, and mutants at the *rad 10* locus are sensitive to MMS and both forms of radiation. With respect to mutagen sensitivity and excision deficiency, the *mei-9* mutants in Drosophila are, therefore, analogous to the *rad 10* mutants of yeast. Similarly, the *mus201* mutants of Drosophila resemble the mutants *rad 1*, *rad 4*, *rad 14* and *mms 19*, in that all are hypersensitive to UV and MMS but not to X-rays. As in the case of *mei-9* and *mus201* mutants, present evidence suggests that the excision defects in yeast mutants occur at or prior to the incision stage (PRAKASH and PRAKASH 1979). Since a deficiency in the excision of UV damage can be associated with different combinations of mutagen sensitivity in both organisms, each probably possesses overlapping pathways of excision repair.

The excision deficiency observed for the *mus201*^{D1} mutant resembles mutants from five of the seven XP complementation groups (ZELLE and LOHMAN 1979). The corresponding loss of unscheduled DNA synthesis in *mus201* cells may even exceed the extent of the defect seen in the most severe cases of XP (ZELLE and LOHMAN 1979). Additional studies suggest that, as in the Drosophila and yeast mutants, XP complementation groups A, B, C and D are defective in the initial incision step of UV repair (FORNACE, KOHN and KANN 1976; DUNN and REGAN 1979). Furthermore, both *mus201* cells (Table 7) and cells from XP complementation groups A and C appear to be defective in the excision of damage produced by AAAF (AMACHER and LIEBERMAN 1977, REGAN and SETLOW 1974; MAHER *et al.* 1975). On the other hand, *mus201*^{D1} also resembles XP complementation group D in that both exhibit a partial reduction in AP endonuclease activity (KUHNLEIN *et al.* 1978; OSGOOD and BOYD, in press). The excision defects observed in selected mutants of these two organisms therefore appear very similar. However, in contrast to the highly excision-deficient mutants in man (LEHMAN *et al.* 1977), the corresponding Drosophila mutants do not exhibit strong defects in the capacity to synthesize high molecular weight DNA on a damaged template.

Relationship of Repair to Meiotic Recombination: Although mutagen sensitivity has been associated with recombination deficiency in a variety of eukaryotes (BAKER *et al.* 1976b), excision-defective strains generally do not exhibit defects in meiotic recombination. In yeast, for example, *rad* strains, which are both UV-sensitive and excision-deficient, do not affect sporulation whereas *rad* genes required for meiotic proficiency are most often associated with sensitivity to ionizing radiation (GAME *et al.* 1980). Among yeast mutants isolated on the basis of MMS sensitivity, however, more than half exhibit defects in sporulation. Furthermore, four of five mutants in that group that are sensitive to both X rays and UV are also defective in sporulation (PRAKASH and PRAKASH 1977).

Similarly, in Drosophila nearly half of the MMS-sensitive mutants display defects in meiotic functions (SMITH 1976; BOYD *et al.* 1976). The *mei-9* mutants are apparently unique in that they are defective in both meiotic recombination and in excision repair. In contrast, the *mus201*^{D1} mutant does not significantly affect male or female fertility, fourth or *X* chromosome nondisjunction or *X* chromosome recombination. Finally, studies of primary spermatocytes derived from XP patients have demonstrated normal levels of chiasma formation (HULTEN *et al.* 1974), suggesting that meiotic recombination is not affected by the XP lesion.

Conclusion: The *mus201* mutants described in the present report appear to be analogous to the excision-defective XP cell lines with respect to mutagen sensitivity, DNA repair and meiotic recombination. GOTH-GOLDSTEIN (1977) has demonstrated that XP-A cell lines are deficient in the removal of O^6-alkylguanine adducts induced by N-methyl-N-nitrosourea and N-ethyl-nitrosourea, and more recent studies have focused on possible enzymatic defects in XP cells associated with the repair of alkylation damage (KUHNLEIN *et al.* 1978; WITTE and THIELMANN 1979). Recent studies have identified additional mutagen-sensitive autosomal loci in Drosophila with partial defects in excision repair (BOYD and HARRIS 1981). Continued genetic and biochemical studies of the excision-deficient, alkylation-sensitive Drosophila mutants offers the potential for expanded knowledge of this fundamental process in animal cells.

The selection scheme employed to recover the *mus(2)201*^{D1} mutant was designed by M. M. GREEN. M. D. GOLINO isolated and performed the initial characterization of that mutant. We are indebted to D. L. LINDSLEY, R. HARDY and J. R. MERRIAM for providing us with their collection of stocks bearing mutagenized autosomes from which we derived the *mus(2)201*^{A1} mutant. K. E. S. SHAW documented the capacity of primary cells to repair single-strand breaks and to perform postreplication repair. C. HANCOCK assisted with the analysis of the UV sensitivity. The carcinogen AAAF was a generous gift from J. A. MILLER. We are grateful to J. J. BONNER for introducing us to the form of data presentation employed in Figure 2 and to R. L. DUSENBERY for her critical review of the manuscript. We would also like to thank our reviewers for suggesting a variety of valuable improvements in the manuscript. This investigation has been supported by grants from the Department of Energy (DE-AT03-79EV70210) and the Public Health Service (GM22221, GM25562, ES01101).

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