The *mus*309 mutation, defective in DNA double-strand break repair, increases the frequency of X-ray-induced somatic crossing over in *Drosophila melanogaster*, but the effect is not dose-rate dependent

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ABSTRACT

Effect of a 1000 R dose of hard X-rays, with two different dose-rates *viz*. 300 and 1000 R/min on somatic crossing over in the X chromosome of *Drosophila melanogaster* was studied in two different genotypes. Irradiation was given during the first-instar larval stage of the development. In the control crosses the flies carried wild-type autosomes, but in the experimental crosses the 3rd chromosomes carried a DNA double-strand break repair deficient *mus*309 mutant gene constitution. As expected, the frequency of X-ray-induced somatic crossing over increased in the mutant flies with both dose-rates of irradiation. As also expected, in the control flies irradiation given with the 300 R/min dose-rate caused more somatic crossovers than irradiation given with the 1000 R/min rate. However, rather unexpectedly, in the experimental flies there was no significant difference in the frequency of somatic crossing over between the two dose-rates of irradiation. The results can be explained by assuming that X-ray-induced somatic crossing over is a two-step event, and that the mechanism which repairs the lesion caused by the irradiation is controlled by the *mus*309 gene. In the control flies the repairing mechanism is capable to recover if the irradiation is given with a short term high dose-rate, but is not capable to recover if the irradiation is given with a long lasting low dose-rate. However, in the experimental mutant flies the repairing mechanism is only poorly recovered irrespective of the dose-rate.

Keywords: Ionizing Radiation; Mitosis; Mutagenic Sensitivity; RecQ Protein; X Chromosome

1. INTRODUCTION

1.1. General Introduction

DNA double-strand breaks (DSBs) are considered the most lethal form of DNA damage. They can result from either endogenous or exogenous sources. Naturally occurring DSBs are generated spontaneously during DNA synthesis when the replication fork encounters a damaged template, and during certain cellular processes. Exogenous factors which cause DSBs are for example ionizing radiation, UV light and radiomimetic drugs [1,2].

Failure to repair DSBs, or their misrepair, may result in cell death or chromosomal rearrangements, including deletions and translocations or genome instability in general. Two major pathways have evolved to repair DSBs and thereby suppress genomic instability. These are the non-homologous end-joining (NHEJ) pathway and homologous recombination (HR), also called homology-directed repair (HDR) pathway [1,2].

The HDR pathway is widely regarded as an accurate error-free form of repair, which requires the presence of a homologous template, such as a sister chromatid, and functions only after DNA replication [3-5]. The NHEJ pathway joins the two ends of a DSB through a process largely independent of homology. In its simplest form it entails straightforward ligation of DNA ends [1,2]. In contrast to HDR, NHEJ is active throughout the cell cycle [6], and it is also considered the major pathway for the repair of irradiation-induced DSBs at least in human cells [7]. Of these two major pathways, however, only the HDR pathway can lead to crossing over.

Two alternative pathways for the repair of the DSBs by homologous recombination are known. They are the synthesis-dependent strand annealing (SDSA) pathway

and the double-strand-break repair (DSBR) pathway [8]. The former pathway leads exclusively to non-crossover products and the latter to both crossover and non-crossover products [9,10].

1.2. The *mus*309 Locus of *Drosophila melanogaster* and Its Role in DSB Repair

The *mus*309 locus, also known e.g. as *DmBlm* and *Ku*70, on the right arm of chromosome three of *Drosophila melanogaster* has been identified as a mutagen sensitive locus [11]. It encodes, in a manner similar to its orthologues in other organisms, the mammalian *BLM* locus included, a RecQ helicase [12-15] and, accordingly, is involved in DSB repair [9,10,16]. Among other mutagens, *mus*309 mutants are, of course, sensitive to ionizing radiation as well [17]. In humans, gene defects in *BLM* cause Bloom's syndrome (BS), a rare, autosomal recessive disorder characterized for example by an increased incidence of many types of cancer [12].

It is known that in the female meiosis of *Drosophila melanogaster*, the product of the *mus*309 locus is involved in the SDSA pathway of the repair of the DSBs [18, 19]. More specifically, it is also known that in *mus*309 mutants the SDSA pathway is blocked, while the DSBR pathway remains functional [20]. Thus, the *mus*309 gene seems to control the choice made by the oocyte between the two alternative pathways of DSB repair. The same is also true for the *mus*309 orthologue, the *Sgs*1 locus, in meiocytes of yeast [21].

Two different models of mitotic DSB repair by homologous recombination which are not mutually exclusive have been proposed for *Drosophila melanogaster*. They are the "dissolution model" [22] and the "disruptase model" [23-25]. These models are based on studies of the effect of the *mus*309 gene on gap repair and on mitotic exchange in the germ line of the males, or on the interaction of *mus*309 with other genes being involved in DSB repair. Both models are modified versions of the DSBR model of meiotic crossing over presented by Szostak *et al.* [26], and the dissolution model was originally presented by Ira *et al.* [27] and Wu and Hickson [28].

The aim of the present study was to investigate the explanatory power of these two models of mitotic DSB repair by studying the effect of certain *mus*309 mutants, defective in DSB repair, on X-ray induced somatic crossing over.

The results support the hypothesis that X-ray-induced somatic crossing over is a two-step event both of the mechanisms of HDR pathway proposed being involved. The results, combined with the data of others, also suggest that the mechanisms for the repair of induced DSBs work differently in the somatic and germ line cells. Fur-

ther, it is proposed that in the wild-type flies the repairing mechanism is capable to recover if the irradiation is given with a short term high dose-rate, but is not capable to recover if the irradiation is given with a long lasting low dose-rate. In the *mus*309 mutant flies, however, the repairing mechanism is only poorly recovered irrespective of the dose-rate.

2. MATERIALS AND METHODS

2.1. Principle of the Investigation of Somatic Crossing Over in *Drosophila*
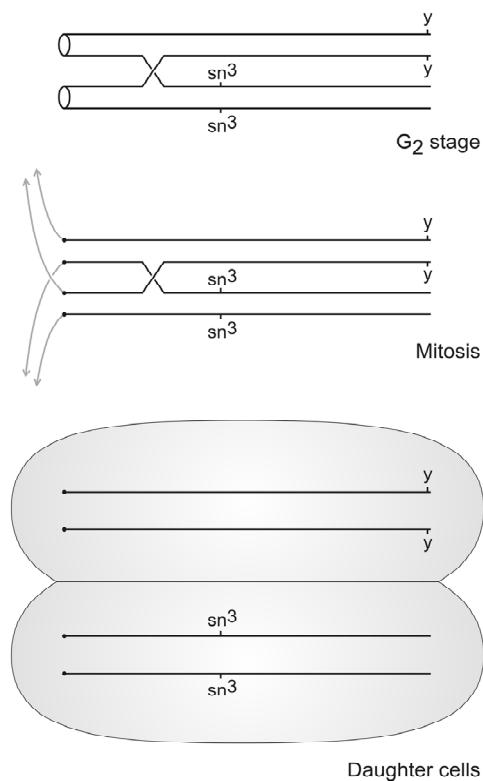
Curt Stern discovered in 1936 [29] that crossing over was not restricted to meiosis but also occurred during mitosis. Mitotic crossing over may occur in somatic cells, where its consequences may be visible as somatic mosaicism, because heterozygous recessive marker genes can become homozygous and give rise to mutant clones as shown in **Figure 1**. Irradiation will induce mitotic crossing over in the soma, leading to an elevation of the frequency of mosaicism for recessive markers [30]. This is now a widespread method for the generation of somatic mosaicism. For practical purposes, an X-ray dose of the order of 1000 R is used, usually for first-instar larvae [31].

Mitotic crossing over in somatic cells in the X chromosome most often occurs proximally to the *singed* locus (*sn*, 1 - 21.0) [32]. Provided that the homologous X chromosome is marked with the *yellow* marker (*y*, 1 - 0.0) and given a certain type of the segregation of chromatids in mitosis illustrated in **Figure 1** and called x-segregation by Stern [29], two cells homozygous for different marker genes are born. Regarding cuticular cells, this result of somatic crossing over could in principle be detected as a mutant twin spot on the otherwise wild-type cuticulum. This is so, because the cuticulum is a one-cell layer, and thus both results of somatic crossing over are visible. Always, as in the present study as well, however, more single spots than twin spots are found, and among the singles usually more *sn* than *y* [32]. The causes of these phenomena are obviously manifold [32-35], but they are irrelevant for the conclusions of this study.

2.2. Description of the *mus*309 Mutants Used

Two alleles of the *mus*309 locus were used. They were *mus*309^{D2} and *mus*309^{D3} both of which were coupled with a second site lethal gene and balanced with the *TM6* balancer marked with the *Tubby* (*Tb*, 3 - 90.6) marker in the stocks used.

Both the *mus*309 alleles used carry mutational changes that could potentially impair or abolish at least the helicase function of the MUS309 protein. In *mus*309^{D2}, there

Figure 1. Principle of the study of somatic crossing over in the X chromosome of *Drosophila melanogaster*. The X chromosomes are marked each with a different recessive marker gene, *yellow* (*y*) and *singed*-3 (*sn*^{3}) in this case. If somatic crossing over occurs between the proximal *sn*^{3} marker and the centromere during the G_2-stage of the cell cycle in a cell of the thoracic imaginal disc of a female larva, and if the centromeres orientate in the following mitosis in the way indicated, homozygous yellow and singed-3 daughter cells will be born. These will develop into yellow and singed-3 clones respectively, which can be detected as mutant spots on the otherwise wild-type cuticulum of the adult female individuals in which somatic crossing over occurred during the larval stage.

is a stop codon between the sequence motifs encoding the third and fourth helicase motif of the protein. *mus*309^{D3}, for its part, has a glutamic acid to lysine substitution in the conserved helicase II motif, in addition to another amino acid substitution close to the C terminus [35]. It has been demonstrated that the genotype *mus*309^{D2}/*mus*309^{D3} is semi-sterile [11,35,36].

2.3. Experimental Procedures

In the P-generation *y*; *mus*309^{D2}/*TM*6, *Tb* and *sn*^{3}; *mus*309^{D2}/*TM*6, *Tb* females were crossed with *y*/*Y*; *mus*309^{D3}/*TM*6, *Tb* and *sn*^{3}/*Y*; *mus*309^{D3}/*TM*6, *Tb* males respectively. (*y*, yellow 1 - 0.0; *sn*^{3}, singed 1 - 21.0;

*mus*309, mutagen-sensitive309 3 - 86E17). In the resulting F_1-generation *y*; *mus*309^{D2}/*mus*309^{D3} females were crossed with *sn*^{3}/*Y*; *mus*309^{D2}/*mus*309^{D3} males. These females and males were identified on the basis of their non-Tubby phenotype. The resulting F_2-generation was exposed to X-ray irradiation during the first-instar larval stage for the induction of somatic crossing over, or they were left without irradiation. After eclosion the F_2-females with the *y* +/+ *sn*^{3}; *mus*309^{D2}/*mus*309^{D3} genotype were investigated for the existence of *yellow* and *singed* macrochaeta on the otherwise wild-type cuticula of their mesonota. These are called experimental females, and the vast majority of all the F_2-females are of this genotype. (The rest constituted of the very few *mus*309^{D2} and *mus*309^{D3} homozygotes which were recombinants for the second site lethal genes). For a control, the respective irradiation and investigation procedure was made with the females of the *y* +/+ *sn*^{3}; +/+ genotype.

2.4. Details of the Irradiation Procedure

In the experimental F_1-generation crosses and in the respective control crosses the flies were allowed to mate in culture bottles for 2 - 3 days. After that the flies were transferred to Petri dishes containing standard *Drosophila* medium consisting of semolina, syrup, agar-agar and both dried and fresh yeast using very light ether narcosis, ten females and males to each dish. After a 24 h period of egg laying, the flies were discarded. After another 24 h interval the Petri dishes containing the developing larvae were irradiated with 1000 R of hard X-rays using a linear accelerator (Clinac 2100 C/D, Varian Medical Systems, CA) with a 6 MeV photon irradiation at the dose-rate of 3 Gy/min. Thus during the irradiation the larvae were in the mid first-instar stage of development. Two different sets were made: the distance of the focus to the Petri dishes was either 1 m or 77.5 cm the first giving the irradiation to the larvae with a slow dose-rate of 300 R/min and the second with a fast dose-rate of 1000 R/min. After irradiation, the medium cake containing the larvae was transferred from each Petri dish to a culture bottle containing fresh *Drosophila* medium, and the cultures were incubated in 25˚C.

After eclosion the females of the irradiated and non-irradiated progeny generations were carefully inspected under a dissecting microscope for mutant clones involving the mesonotal macrochaeta: yellow or singed single spots or yellow-singed twin spots, and notes were taken for the existence of the spots.

2.5. Statistical Methods

In the statistical analyses of the results Chi-square test, Fisher's exact test and logistic regression analysis [37] were used. All analyses were conducted with SAS statis-

tical software version 9.22 (SAS Institute Inc., Cary, North Carolina, USA

3. RESULTS

No mutant spots were observed either in control or experimental females if they were not exposed to irradiation (**Tables 1** and **2**). Thus it seems that the *mus*309 mutations investigated do not increase the frequency of spontaneous somatic crossing over. This is in contrast with the observation of Johnson-Schlitz and Engels [22] and McVey *et al*. [24] who found that in *mus*309 mutants the frequency of spontaneous mitotic crossing over in the germ line of the males was increased by several orders of magnitude.

On the other hand, the *mus*309 mutations investigated significantly increased the frequency of X-ray induced somatic crossing over with both 300 R/min and 1000 R/min dose-rate (**Tables 1** and **2**). The increase in the frequency of mutant spots in experimental *mus*309 females as compared to wild-type control females irradiated with the 300 R/min dose-rate was 1.80-fold (P = 0.0405; Fisher's exact test). The corresponding increase in females irradiated with the 1000 R/min dose-rate was 2.52-fold (P = 0.0160). The difference between the two factors of increase was significant (χ^2 = 3.95, d.f. = 1, P = 0.0468; multiple logistic regression model).

The distribution of the different types of spots was similar in the control and experimental females irrespective of the dose-rate. The significances of difference were as follows: 300 R/min set: χ^2 = 1.18, d.f. = 2, P = 0.5543; 1000 R/min set: χ^2 = 2.54, d.f. = 2, P = 0.2808. This observation indicates that the actual mechanism of X-ray induced somatic crossing over is the same in *mus*309 mutant and wild-type flies.

There was an overwhelming majority of the frequency of single spots over the frequency of twin spots, and of *singed* spots over *yellow* spots among the singles in every irradiation set. In addition to this the frequency of *yellow* single spots was never higher than the frequency of twin spots (**Tables 1** and **2**). Similar results have earlier been obtained by several authors [29,38-40].

However, the distribution of the different types of spots was similar with both dose-rates regardless of the genotype of the females. The significances of difference were as follows: control females: χ^2 = 2.58, d.f. = 2, P = 0.2753; experimental females: χ^2 = 0.1125, d.f. = 2, P = 0.9453. This latter observation indicates that the causes of the apparent non-reciprocality of somatic crossing over are not dependent of the dose-rate.

As appears from **Table 3**, in the control females irradiated with the 300 R/min dose-rate the frequency of mosaic mesonota was significantly higher than in females irradiated with the 1000 R/min dose-rate (P = 0.0477; Fisher's exact test). In the experimental females, however, the difference was not significant (P = 0.0702). Thus, the frequency of somatic crossing over in the wild-type flies was, as expected, dependent on the dose-rate of the irradiation. On the other hand, in the *mus*309 mutant flies the dose-rate effect was absent, or it was at most very weak, which has not been observed before.

4. DISCUSSION

4.1. General Discussion

It has been known since the pioneering work of Jutta Haendle [41-44] that the frequency of the X-ray induced somatic crossing over in *Drosophila melanogaster* increases with the increasing dose of the irradiation, and that irradiation given with hard X-rays and a slow dose-rate induces more exchanges than the same dose given

Table 1. Results from the control irradiation sets: distribution of different mosaic spots on the mesonota of the females of the genotype *y* +/+ *sn*^{3}; +/+ exposed to 1000 R of X-ray irradiation during the first-instar larval stage of development given with two different dos rates, 300 R and 1000 R/minute.

	Number of females investigated	Number of spots observed			
		twin spots	yellow spots	singed spots	Sum
Unirradiated control					
Number of spots	440	0	0	0	0
Number of spots/100 females		0	0	0	0
Irradiated with 1000 R, 300 R/min					
Number of spots	457	1	1	11	13
Number of spots/100 females		0.22	0.22	2.41	2.84
Irradiated with 1000 R, 1000 R/min					
Number of spots	519	1	1	5	7
Number of spots/100 females		0.19	0.19	0.96	1.35

Table 2. Results from the experimental irradiation sets: distribution of different mosaic spots on the mesonota of the females of the genotype $y +/+ sn^3$; $mus309^{D2}/mus309^{D3}$ exposed to 1000 R of X-ray irradiation during the first-instar larval stage of development given with two different dose rates, 300 R and 1000 R/minute.

	Number of females investigated	Number of spots observed			
		twin spots	yellow spots	singed spots	Sum
Unirradiated control					
Number of spots	440	0	0	0	0
Number of spots/100 females		0	0	0	0
Irradiated with 1000 R, 300 R/min					
Number of spots	430	4	3	15[a)]	22
Number of spots/100 females		0.93	0.70	3.49	5.12
Irradiated with 1000 R, 1000 R/min					
Number of spots	498	3	0	14	17
Number of spots/100 females		0.60	0	2.81	3.41

[a)]Two of the singed spots were in the same female.

Table 3. Comparison of the different irradiation sets: amount f mosaicism in the control ($y +/+ sn^3$; $+/+$) and experimental ($y +/+ sn^3$; $mus309^{D2}/mus309^{D3}$) females exposed to 1000 R of X-ray irradiation during the first-instar larval stage of development given with two different dose rates, 300 R and 1000 R/minute.

	Amount of mosaicism in the mesonota		
	Mosaic mesonota	Non-mosaic mesonota	Sum
Control females			
Dose rate 300 R/min	13 (2.84%)	444 (97.16%)	457 (100%)
Dose rate 1000 R/min	7 (1.35%)	512 (98.65%)	519 (100%)
Experimental females			
Dose rate 300 R/min	21 (4.81%)	409 (95.12%)	430 (100%)
Dose rate 1000 R/min	17 (3.41%)	481 (96.59%)	498 (100%)

with a fast dose-rate. With hard X-rays delivered at high dose-rates, the frequency of somatic crossing over increases nonlinearly with dose. (There is a marked shoulder at about 1000 R). With lower dose-rates or with softer X-rays the nonlinearity is less apparent. There is a very marked loss of efficiency in the induction of somatic crossing over with hard X-rays at dose-rates below 3000 R/min. These results were interpreted to mean that somatic crossing over induced with hard X-rays is a multihit event. One of these events is due to the soft component of irradiation and leads to induction of somatic crossing over by soft X-rays. This event is characterized by the fact that its recovery shows no time dependence. The other event is unique to hard X-rays and is distinguished by the fact that it is rapidly—within 30 - 150 seconds—repaired thus explaining the dose-rate effect of

hard X-rays [31,32].

4.2. The Difference between Somatic and Germ Line Cells

The result that no spontaneous somatic crossing over was observed either in the control or experimental females, as compared with the results of Johnson-Schlitz and Engels [22] and McVey *et al*. [24] that the frequency of mitotic crossing over in the germ line cells was greatly increased in the *mus*309 mutant males, suggests that the repairing mechanism controlled by the *mus*309 gene works differently in the male germ line and the female soma. This difference may be due to the rapid cell division rate of the spermatogonial cells as compared to the somatic cells in the imaginal discs. Based on the time table of the duration of different stages of spermatogenesis in *Drosophila melanogaster* [45] it can be calculated that the four synchronous spermatogonial mitotic cycles last ca. 4 h each. On the other hand, the doubling of the cell number in the wing imaginal disc is about 7.5 h [46]. The spermatogonial cells may be more sensitive to the lack of repair of the DSBs either because there are more DSBs in them or because the repairing mechanism has less time to be recovered.

4.3. Somatic Crossing Over Is More Frequent in *mus*309 Mutant Flies than in Wild-Type Flies

As could be expected, somatic crossing over is more frequent in the *mus*309 mutant flies, deficient in DSB repair, than in the wild-type flies. Contrarily with the expectations, however, X-ray irradiation given with the dose-rate of 300 R/min induced more somatic crossing

over than irradiation given with a dose-rate of 1000 R/min both in the wild-type and *mus*309 mutant flies. This result is a reflection of the dose-rate independence of somatic crossing over in the mutant flies, which will be discussed in the following section.

4.4. X-Ray Induced Somatic Crossing Over in the *mus*309 Mutants is Independent on the Dose-Rate

The most important result of this study was the unexpected observation that the dose-rate effect, found in the control flies, was absent or very weak in the *mus*309 mutant flies. In this respect the *mus*309 mutants are similar to the *c*(3)*G* homozygote flies, in which meiotic crossing over is completely suppressed (Haendle, quoted by Becker [32]). This suggests that both the *mus*309 and the *c*(3)*G* mutations affect the rapidly repaired breakage mechanism found by Haendle [41,42] (cf. [31,32]).

The results can be explained by assuming that X-ray-induced somatic crossing over is a two-step event, and that the mechanism which repairs the lesion caused by the irradiation is controlled by the *mus*309 gene. In the control flies the repairing mechanism is capable to recover if the irradiation is given with a short term high dose-rate, but is not capable to recover if the irradiation is given with a long lasting low dose-rate. However, in the experimental mutant flies the repairing mechanism is only poorly recovered irrespective of the dose-rate.

It is hypothesized that the two steps involved are the dissolvase and disruptase activities of the MUS309 protein, which are sub sequential [24]. One or the other of these activities is poorly recovered in the *mus*309 mutants, but the other one is normally functional. In the wild-type control flies both components act normally. These suggestions are supported by the fact that, based on the results concerning the distribution of mosaic spots, both genotypes studied have in principle the same repairing mechanism irrespective of the dose-rate; it is only the recovering time which differs between genotypes.

4.5. Suggestions for Further Studies

It remains to be studied in details what is the relation of the two steps of somatic crossing over observed in this study and characterized in terms of molecular genetics to the respective steps observed earlier by Jutta Haendle and characterized in terms of cellular and chromosomal genetics. It would be particularly interesting to know whether the rapidly repaired mechanism found by Haendle in actual fact is identical with the dissolvase or disruptase activity of the MUS309 protein. Specifically the dose-rate independence observed should, if possible, be studied in amorphic null mutants of the *mus*309 locus.

If the hypothesis of two steps presented is correct, no dose-rate dependence at all should be observed in such mutants. Unfortunately, however, the present author, being already retired, has no resources available any more to conduct these experiments.

5. ACKNOWLEDGEMENTS

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