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mus309 mutation, defective in DNA double-strand break repair, affects intergenic but not intragenic meiotic recombination in *Drosophila melanogaster*

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Summary

The effect was investigated of the hypomorphic DNA double-strand break repair, notably synthesis-dependent strand annealing, deficient mutation *mus309* on the third chromosome of *Drosophila melanogaster* on intergenic and intragenic meiotic recombination in the X chromosome. The results showed that the mutation significantly increases the frequency of intergenic crossing over in two of three gene intervals of the X chromosome studied. Interestingly the increase was most prevalent in the tip of the X chromosome where crossovers normally are least frequent per physical map unit length. In particular crossing over interference was also affected, indicating that the effect of the *mus309* mutation involves preconditions of crossing over but not the event of crossing over itself. On the other hand, the results also show that most probably the mutation does not have any effect on intragenic recombination, i.e. gene conversion. These results are fully consistent with the present molecular models of meiotic crossing over initiated by double-strand breaks of DNA followed by formation of a single-end-invasion intermediate, or D-loop, which is subsequently processed to generate either crossover or non-crossover products involving formation of a double Holliday junction. In particular the results suggest that the *mus309* gene is involved in resolution of the D-loop, thereby affecting the choice between double-strand-break repair (DSBR) and synthesis-dependent strand annealing (SDSA) pathways of meiotic recombination.

1. Introduction

(i) *Background*

Double-strand DNA break repair in *Drosophila melanogaster* usually occurs by homologous recombination (Engels *et al*., 1990). It is also well known that formation of double-strand DNA breaks is a necessary condition for crossing over in a variety of organisms (see e.g. Boyd *et al*., 1987 for a review). Present molecular models for meiotic crossing over and gene conversion suggest that crossing over is initiated by the formation of double-strand DNA breaks followed by formation of a heteroduplex, and rejoining of the ends born in the breakage. Accordingly, a structure called a Holliday junction is formed. Gene conversion, according to these models, is the consequence of mismatch repair in the region of the heteroduplex formation (see Olsen-Krogh & Symington, 2004 for a review). Thus, both double-strand DNA break formation and heteroduplex formation are necessary conditions of both crossing over and gene conversion. However, mismatch repair is, according to the present models, involved only in gene conversion but not in intergenic recombination, i.e. crossing over. On the other hand, resolution of Holliday junctions is only involved in crossing over.

mus309 is a well-characterized mutant on the third chromosome right arm (86F4) that is a member of a large family of mutagen-sensitive (*mus*) mutants in *D. melanogaster*. It is defective in synthesis-dependent strand annealing (SDSA), thus maintaining double-strand DNA breaks (Adams *et al*., 2003; Laurencon *et al*., 2004). Specifically Adams *et al*. (2003) suggested that a defect in *mus309* reduces the efficiency of the SDSA pathway during the course of homologous genetic recombination, and apparently at a stage after strand invasion in the formation of Holliday junctions. The *mus309* mutation is also known to be defective in double-strand DNA break repair after

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P-element excision (Beall & Rio, 1996; McVey *et al*., 2004) and notably also in meiotic checkpoint control in the oogenesis of *D. melanogaster* (Staeva-Vieira *et al*., 2003).

It is interesting to note that *mus309* is the *Drosophila* orthologue of the human *BLM* gene, mutations of which are responsible of Bloom's syndrome, which is characterized by a predisposition to a wide spectrum of cancers (German, 1993; Kusano *et al*., 2001; Min *et al*., 2004). Mutations of the human *BLM* gene, likewise its orthologue, *Sgs1*, in yeast, are also known to be defective in recombinational double-strand DNA break repair (Wu *et al*., 2001). In fact the *BLM* gene in man and its orthologues in other organisms encode a RecQ DNA helicase (Ellis *et al*., 1995; Karow *et al*., 1997; Mohaghegh *et al*., 2001). RecQ DNA helicase is known to take part in processing of DNA replication intermediates involved in, among other things, meiotic crossing over, mitotic sister chromatid recombination and, notably, double-strand DNA break repair in a variety of organisms from *Escherichia coli* to man (reviewed in Brabant *et al*., 2000; Heyer *et al*., 2003; Heyer, 2004).

Also it is known that the gene for RecQ helicase in *Escherichia coli* suppresses illegitimate recombination (Hanada *et al*., 1997; Harmon & Kowalczykowski, 1998; Yamagata *et al*., 1998). In conjunction with RecA and SSB proteins, RecQ helicase can initiate recombination events *in vitro* (Harmon & Kowalczykowski, 1998). The mutations of the orthologous gene in yeast, *Sgs1*, also increased the rate of recombination between DNA sequences that had 91% sequence homology but suppressed recombination between divergent DNA sequences (Myung *et al*., 2001).

It has also been demonstrated that RecQ helicases and DNA topoisomerases, which can break and rejoin DNA to alter its topology, act in concert to maintain genomic stability by preventing inappropriate recombination (reviewed by Wu & Hickson, 2001). Specifically it has been shown in budding yeast *Saccharomyces cerevisiae* that the helicase encoded by the *Sgs1* gene physically binds with DNA topoisomerase III (Gangloff *et al*., 1994; Fricke *et al*., 2001), and that this complex is important in, for example, regulation of genetic recombination in mitotic cells (Gangloff *et al*., 1994; Watt *et al*., 1995, 1996; Sinclair *et al*., 1997; Harmon *et al*., 1999). This type of interaction between RecQ helicase and DNA topoisomerase III has been conserved during evolution, and has been demonstrated for human mitotic cells also (Wu *et al*., 2000).

Most importantly the Bloom's syndrome helicase stimulates the activity of human topoisomerase IIIα to relax negatively supercoiled DNA (Wu & Hickson, 2002). By doing so, it suppresses mitotic crossing over via affecting the resolution of Holliday junctions by promoting branch migration (Karow *et al*., 2000; Wu & Hickson, 2003; Wu *et al*., 2005). Likewise it has been suggested that in yeast mitotic cells the helicase-topoisomerase, Sgs1–Top3, complex removes double Holliday junction intermediates from a crossover-producing repair pathway, thereby reducing crossovers (Ira *et al*., 2003).

Taking the foregoing into account it is interesting to study the effect of *mus309* on both intergenic and intragenic recombination in order to test, with formal *Drosophila* genetic methods, the models of meiotic recombination. The results of the present study are in full accordance with the models presented. They also shed new light on the question of the sequence and control of homologous recombination events in *Drosophila melanogaster*.

(ii) *Logic of the study of interference*

Sandler *et al*. (1968) have shown theoretically that a meiotic mutant that affects crossing over frequency without changing interference involves the event of crossing over itself, whereas a mutant that changes both crossing over frequency and interference affects some precondition of crossing over. The reasoning of Sandler *et al*. (1968) was as follows. Let a be the probability of the fulfilment of preconditions of crossing over in one region and only in that region in a three-point crossing over experiment. Let b be the probability of fulfilment of the same in another region and only in that region. Let d be the probability of the fulfilment of the preconditions in both regions at the same time, and x the probability of exchange, given the preconditions. Then the coefficient of coincidence, C, is

$$C = \frac{dx^2}{x(a+d)x(b+d)} = \frac{d}{(a+d)(b+d)}.$$

Since C is independent of x, if a mutant that acts on crossing over also affects interference, it must influence the preconditions of crossing over. If, however, interference remains unaltered, the target of the effect is the exchange itself.

2. Materials and methods

(i) *Experimental procedures*

Crossing over frequency and interference were studied in the X chromosome in the regions between cross-veinless (*cv*, 1–13·7), vermilion (*v*, 1–33·0) and forked (*f*, 1–56·7) markers. In the control flies cross, *cv v f*/+++ females were crossed to *cv v f*/*Y* males, and in the experimental cross, *cv v f*/+++; $mus309^{D2}$/$mus309^{D3}$ females were crossed to *cv v f*/*Y* males. Because the genotype $mus309^{D2}$/$mus309^{D3}$ is

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Table 1. *Effect of mus309^{D2}/mus309^{D3}, a DNA double-strand repair deficient gene on the third chromosome, on intergenic recombination in two regions of the X chromosome*

		Recombination frequency % ± SD	
Interval	Control	Effect of *mus309*	Significance of the difference
Region *cv v f*			
cv–v	18·51 ± 0·67 (630)	27·15 ± 0·91 (654)	$t = 8·284$; $P < 0·001$
v–f	23·13 ± 0·72 (787)	22·08 ± 0·85 (532)	$t = 0·942$; n.s.
Double crossovers	1·97 ± 0·24 (67)	4·28 ± 0·41 (103)	$t = 5·145$; $P < 0·001$
Total no. of flies	3403	2409	
Coefficient of coincidence	0·4599 ± 0·0505	0·7132 ± 0·0574	$t = 19·189$; $P < 0·001$
Region *y sc ec*			
y sc–ec	2·25 ± 0·17 (176)	5·60 ± 0·25 (491)	$t = 10·96$; $P < 0·001$
Total no. of flies	7810	8770	

Values in parentheses are the numbers of recombinant flies; $x + w$, $y + w$, and w respectively.

semilethal, the compound gives a hypomorphic condition of the function of the *mus309* gene (Janos Szabad, personal communication).

Crossing over was studied in the yellow (*y*, 1–0·0)–scute (*sc*, 1–0·0)–echinus (*ec*, 1–5·0) region of the X chromosome as well. In the control flies cross, y^2 sc^1 *ec*/+++ females were crossed to y^2 sc^1 *ec*/*Y* males, and in the experimental cross y^2 sc^1 *ec*/+++; *mus309^{D2}/mus309^{D3}* females were crossed to y^2 sc^1 *ec*/*Y* males.

Gene conversion, i.e. intragenic recombination, was studied in the white (*w*, 1–1·5) locus of the X chromosome. In the control flies cross y^2 sc^1 $w^a + ec$/+++ $w^e +$ females were crossed to y^2 sc^1 $w^a + ec$/*Y* males. The experimental cross was otherwise similar but the third chromosomes of the females carried the *mus309^{D2}/mus309^{D3}* mutant constitution. These crosses are selective for the gene conversion inside the white locus since recombinants were immediately observed as red-eyed flies.

The crosses were made as single female cultures, and progeny was raised at 25 °C on a standard *Drosophila* medium consisting of semolina, syrup, agar-agar, and both dried and fresh yeast.

(ii) *Measurement of interference*

The coefficient of coincidence, *C*, was calculated according to the following formula of Stevens (1936), which is a maximum likelihood equation:

$$\hat{c} = \frac{wn}{(w+x)(w+y)},$$

where w is the number of flies which were double crossovers, x and y are the numbers of flies which were single crossovers for *cv* and *v*, and *v* and *f*, respectively, and n is the total number of flies (data for w, x and y are given in Table 1).

The variance of *C* was calculated according to the following formula, also given by Stevens (1936):

$$V(\hat{c}) = \frac{c}{n}\left(\frac{1 - ca - cb - cab + 2c^2ab}{ab}\right),$$

where a and b are the recombination frequencies of *cv* and *v*, and *v* and *f*, respectively. This is also a maximum likelihood equation.

(iii) *Statistical methods*

In calculating the significances of difference the binomial *t*-test was employed.

3. Results

In the *cv v f* region, where intergenic crossing over was studied, the frequency of crossing over in the *cv–v* interval as well as the frequency of double crossing over was significantly increased due to the effect of *mus309* mutation (Table 1). However, crossing over frequency in the *v–f* interval was not affected. Notably, however, the crossing over interference was also significantly decreased (Table 1), indicating that some precondition of crossing over was affected due to the effect of *mus309* mutation.

In the *y sc ec* region the frequency of crossing over between *y–sc* and *ec* was more than doubled due to the effect of *mus309* mutation (Table 1). Thus, there is a gradient in the increase of crossing over frequency, the increase being most significant at the tip of the X chromosome, where crossing over is normally rare, and decreasing towards the centromere.

In the crosses planned to investigate the effect of *mus309* on gene conversion at the white locus, four red-eyed flies were observed in the control cross among $1·99 \times 10^5$ flies. Of these, 3 were recombinants for flanking markers also and 1 was not, the latter fly

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Table 2. *Effect of mus309^{D2}/mus309^{D3}, a DNA double-strand break repair deficient gene on the third chromosome, on frequency of intragenic recombination in the white locus in the cross* $y^2\ sc^1\ w^a + ec/+++\ w^e + \times y^2\ sc^1\ w^a + ec/Y$

	Total no. of flies	No. of red-eyed flies	Flanking marker phenotype
Control	$1{\cdot}99 \times 10^5$	4	$3++\ ec$ $1\ y\ sc\ ec$
Effect of *mus309*	$2{\cdot}01 \times 10^5$	3	$3++\ ec$

being a result of either gene conversion or mutation. In this connection it is worthwhile to note that usually most gene conversions in *Drosophila* are known not to be associated with recombination of the flanking markers (Finnerty, 1976).

In the experimental cross a total of 3 red-eyed flies were observed among virtually the same total number of progeny as in the control cross. All the 3 red-eyed flies were recombinants for the flanking markers also. Thus, contrary to its effect on intergenic recombination, the *mus309* mutation did not have any effect on intragenic recombination at the white locus, suggesting that the effect of *mus309* is different on intergenic and intragenic recombination. Though the material is rather small, it should be expected that the amount of red-eyed flies should approximately be doubled in the experimental cross. Namely, if the effect of *mus309* were the same on both intergenic and intragenic recombination, and since the *mus309* mutation more than doubled the frequency of intergenic recombination in the *y sc ec* region harbouring the white locus also, intragenic recombination should also be doubled. However, no increase in the frequency of red-eyed flies due to the *mus309* mutation was observed. The results concerning gene conversion are summarized in Table 2.

4. Discussion

In his seminal paper in 1964 Robin Holliday (Holliday, 1964) proposed a model for general recombination of which the two major postulated intermediates – heteroduplex DNA (hDNA) and the chiasma-like structure, now termed the Holliday junction (HJ) – have survived the test of time. Research primarily using the budding yeast *Saccharomyces cerevisiae* as an experimental organism has yielded a coherent molecular model for meiotic recombination (Szostak *et al*., 1983; Stahl, 1996; Hunter & Kleckner, 2001; Allers & Lichten, 2001).

According to this model, a double-stranded DNA break (DSB) is introduced in one of the four chromatids. Physical evidence suggests that the two ends are not equivalent and that only one end invades the unbroken homologue (Hunter & Kleckner, 2001; Allers & Lichten, 2001). The resulting single-end-invasion intermediate, or D-loop as it is also called, is processed to generate crossover (CO) or non-crossover (NCO) products involving a double Holliday junction (dHJ) intermediate. Mitotic recombination can be initiated by DSBs or gaps, and can proceed in a similar way to meiotic recombination, but was found to lead to significantly fewer COs than meiotic recombination (Paques & Haber, 1999).

This model, summarized by Heyer *et al*. (2003), has been well substantiated and documented in *S. cerevisiae*, but its confirmation in other organisms is not yet definitive. However, it is generally assumed that HJs, single or double, are the key intermediate for all COs. On the other hand, HJ resolution is still poorly understood in meiotic cells of eukaryotes (Symington, 2002). On the contrary, as suggested by studies involving Bloom's syndrome in man and mitotic crossing over in yeast, an increasing amount of evidence exists showing that in mitotic eukaryotic cells the RecQ helicase in conjunction with the DNA topoisomerase III resolves at least a proportion of Holliday junctions (see Section 1 for references).

It is commonly believed that there are at least two ways of processing of the single-end invasion intermediate, or D-loop, during the course of meiotic recombination, which can lead either to a crossover or to a non-crossover product. These models are the double-strand-break repair (DSBR) model and the synthesis-dependent strand-annealing (SDSA) model (summarized by Heyer *et al*., 2003 and Heyer, 2004). In *Drosophila melanogaster* DSBR occurs preferentially though the synthesis-dependent strand-annealing pathway (Kurkulos *et al*., 1994; Nassif *et al*., 1994).

In this connection, McVey *et al*. (2004) reported, basing their studies on molecular analysis, that DSBR in *D. melanogaster* in the absence of *mus309* was associated with frequent deletions into flanking sequences. Further, they observed that removing DmRad51, which mediates strand invasion, suppresses this deletion phenotype, suggesting that *mus309* acts behind the synthesis fork to unwind the nascent strand. McVey *et al*. (2004) logically suggested that in the SDSA pathway the nascent strand must first be dissociated from the template, resolving the D-loop, and proposed that *mus309* is involved in this dissociation step.

The results of the present study fully support the hypothesis of McVey *et al*. (2004) and in general the prevailing molecular models of the mechanism of meiotic crossing over. These models hypothesize that meiotic crossing over is initiated by formation of

double-strand DNA breaks followed by strand annealing and reunion of the ends caused by the breakage of DNA strands. The results show that the *mus309* mutation affects some precondition of crossing over, and that gene conversion remains unaltered in *mus309* mutants, indicating that *mus309* acts after heteroduplex formation.

It is already known that *mus309* affects synthesis-dependent strand annealing repair of DNA double-strand breaks (Adams *et al*., 2003; Laurencon *et al*., 2004). Thus, it is most likely that strand annealing in the SDSA pathway is precisely the 'precondition of crossing over' affected. Following the model of McVey *et al*. (2004), it is thus suggested that *mus309* acts after heteroduplex formation in the SDSA pathway, resolving the D-loop by dissociating the nascent strand from the template, and further that it does not have any effect in the DSBR pathway. This means that *mus309* acts as if it were controlling the choice between DSBR and SDSA pathways. In *mus309* mutants, where the SDSA pathway is defective, the oocytes preferentially enter the DSBR pathway.

Consequently, it is easy to understand that in *mus309* mutants the frequency of intergenic crossing over is increased, because in these mutants the SDSA pathway, leading exclusively to non-crossover products, is blocked, while the DSBR pathway, which leads both to crossover and non-crossover products, works. In other words, in *mus309* mutants an excess of crossover products is to be expected. Even though the material is small, this conclusion is supported by the observation that of the four gene conversions in the control cross one was not associated with recombination of the flanking markers, while all the three gene conversions in the experimental cross were associated with recombination of the flanking markers, i.e. crossing over. In the control females, meioses go via both the DSBR and the SDSA pathway, while in experimental females meioses go preferentially via the DSBR pathway. Gene conversion can occur in both pathways because heteroduplex formation precedes the choice between these pathways, but crossing over, i.e. Holliday junction resolution, can occur only in the DSBR pathway.

In this connection it is interesting to note that in the meiotic mutant *mei-9* of *D. melanogaster* the frequency of crossing over was dramatically decreased while gene conversions were found with normal frequency (McKim *et al*., 2002). Thus, in *mei-9* mutants, as in *mus309* mutants, the early steps of recombination, including the formation of heteroduplex DNA, appear unaffected because gene conversions were found with normal frequency. In contrast to *mus309*, however, in *mei-9* mutant females crossing over interference remained unaltered (Baker & Carpenter, 1972; Baker & Hall, 1976), indicating that *mei-9* affects crossing over *per se*. Thus, as Heyer *et al*. (2003) concluded, *mei-9* mutants show phenotypes predicted for the eukaryotic nuclear Holliday junction resolvase mutant.

Thus, it can be concluded that the steps of homologous meiotic recombination in *Drosophila melanogaster* females are most likely as follows: recombination is initiated by double-strand DNA breaks (DSB), followed by D-loop formation involving heteroduplex DNA. The D-loop enters either the DSBR pathway or the SDSA pathway for double-strand-break repair. In the DSBR pathway the D-loop forms a double Holliday junction, which can be resolved by resolvase encoded by *mei-9* to crossover and to non-crossover products. In the SDSA pathway the D-loop is resolved by the *mus309* gene product, which dissociates the nascent strand from the template.

McKim *et al*. (2002) proposed in general that in *D. melanogaster* the generation of crossovers involves the formation of an intermediate, possibly the Holliday junction, which is not formed in the non-crossover pathway. Further they suggested that all the precondition mutants alter the distribution of crossovers because they fail to produce the crossover-specific intermediate. For example, in precondition mutants low numbers of crossover intermediates may trigger a feedback response involving either the creation of additional DSBs or the conversion of more existing DSBs into crossovers. Such an effect could increase the frequency of crossovers in regions that normally experience few of these events, thus altering the overall distribution. This is precisely what was observed in the present study.

Using the same reasoning, McKim *et al*. (2002) also proposed that mutants which do not affect the formation of this intermediate, such as *mei-9*, do not alter the distribution of crossovers because they affect a step (resolution) after the crossover-specific intermediate has formed. Consequently, they proposed that the intermediate has a critical role in regulating the distribution and frequency of crossovers. The results of the present study are in full accordance with these proposals.

The results of the present study are also in full accordance with the results of the first study by Rockmill *et al*. (2003) involving the effect of the *Sgs1* gene, the yeast orthologue of *mus309*, on the frequency of meiotic recombination. Precisely as in the present study, it was observed that mutations or loss of *Sgs1* increased meiotic crossing over but gene conversion remained unaffected. Moreover, also in accordance with the present study, it was observed that the percentage of gene conversions associated with crossing over was increased in the mutant cells as compared to the wild-type control. The authors came to a similar conclusion as in the present study: They proposed that Sgs1 acts on recombination

intermediates that are not yet committed to whether a crossover or a non-crossover recombinant will be produced. Further, as in the present study, they suggested that Sgs1 favours the production of non-crossovers at the expense of crossovers.

Thus, it is almost certain that the effects of the yeast and fruit fly orthologues of the human Bloom's syndrome (BS) gene are identical. Because a hallmark of BS cells is increased crossing over (e.g. sister chromatid exchange), BS cells may be defective in a primary double-strand break repair pathway that does not generate crossovers, such as SDSA. Accordingly, there is now much evidence that this phenomenon is probably the explanation for the fact that BS cells are characterized by genomic instability.

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