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Research Article

Mus308 Processes Oxygen and Nitrogen Ethylation DNA Damage in Germ Cells of Drosophila

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The *D. melanogaster mus308* gene, highly conserved among higher eukaryotes, is implicated in the repair of cross-links and of O-ethylpyrimidine DNA damage, working in a DNA damage tolerance mechanism. However, despite its relevance, its possible role on the processing of different DNA ethylation damages is not clear. To obtain data on mutation frequency and on mutation spectra in *mus308* deficient (*mus308*$^{-}$) conditions, the ethylating agent diethyl sulfate (DES) was analysed in postmeiotic male germ cells. These data were compared with those corresponding to *mus308* efficient conditions. Our results indicate that Mus308 is necessary for the processing of oxygen and N-ethylation damage, for the survival of fertilized eggs depending on the level of induced DNA damage, and for an influence of the DNA damage neighbouring sequence. These results support the role of *mus308* in a tolerance mechanism linked to a translesion synthesis pathway and also to the alternative end-joinig system.

1. Introduction

Among the genes identified so far in *Drosophila melanogaster* that play a role in DNA damage repair, *mus308* presents some unique properties, because its cDNA sequence shows motifs characteristic of DNA helicase and DNA polymerases [1]. The putative product of this gene was indeed isolated as a new DNA polymerase, homologue to the *Escherichia coli* DNA polymerase I, carrying as well a DNA helicase domain at the N terminus region [2]. Orthologues of this gene have been found in *Caenorhabditis elegans* [1], *Arabidopsis thaliana* [3, 4], and mammals [3, 5–9]. In humans, three genes encoding proteins with sequence similarities to Mus308—one similar to Mus308 helicase, HEL308 [3], and two similar to the Mus308 polymerase, POLQ [5, 7] and POLN [6]—have been identified to date. POLQ, the most studied of these proteins, has also an ATPasehelicase domain at the N terminus and is able to perform DNA synthesis past an abasic site, following the A-rule [10]; however, there are contradictory results about its fidelity in a normal nondamaged template [10, 11].

The *mus308* gene is involved in the repair of cross-linking adducts [12, 13] and also of monofunctional damage [13], probably persistent and difficult to repair by other systems, such as the O-ethylpyrimidine damage induced by N-ethyl-N-nitrosourea (ENU) in postmeiotic male germ cells [14]. In addition, at least parts of ENU- and diethyl sulphate- (DES-) induced damages were repaired by Mus308 in female germ cells of Drosophila [15]. This protein works in a damage bypass mechanism [1, 13], which was originally related to homologous recombination, HR [14, 16]. Nevertheless, the isolation of the DNA polymerase encoded by this locus [2], its possible ability for DNA synthesis through abasic sites [10, 11], and the requirement of a functional Mus308 protein to prevent damage-induced DNA strand breaks in vivo in somatic cells of Drosophila [17], pointing to a translesion synthesis (TLS) mechanism as the activity of this protein [17]. In summary, along these years the

work of our laboratory have demonstrated that Mus308 works in the repair/processing of cross-links and oxygen ethylation damage [13–15, 17] whereas N-ethylation damage is apparently not substrate of this system, because no effect of methyl methanesulphonate (MMS) was detected either in germ cells [13] or in somatic ones [17]. Additionally, its mechanism of action is poorly understood, because it could be related to HR [14, 16] or to TSL [17]. Because of this, we have proposed that the *mus308* locus works in a bypass-mediated tolerance mechanism, BTM [15, 17].

Given the conservation of *mus308* among higher eukaryotic organisms, this locus is likely a part of a repair system relevant to DNA damage processing. Therefore, it would be important to elucidate what types of DNA damage, apart from cross-links and O-ethylpyrimidine adducts, are substrate of this system and to get information about which of the two possible mechanisms of action, HR or TLS, is actually involved in the damage bypass process.

To have more information about the role of Mus308 in the processing of DNA ethylation damage, we have studied here the effect of DES in postmeiotic male germ cells, analysing maternal repair and using the vermilion system [18]. This system combines the analysis of induced mutation frequencies, both at a single locus (*vermilion*, with a specific locus test) and at multiloci (700 loci in the X-chromosome, with the recessive lethal test), with the generation and analysis of mutation spectra [18]. Our data, together with other already published, indicate that Mus308 protein is involved in the processing of all types of oxygen ethylation damage, that it is also involved in the processing of nitrogen ethylation damage, that it prevents cell death at least when the amount of DNA damage is high, and that this protein could be working in a TLS mechanism as well as in an alternative end-joining system (alt-EJ).

2. Material and Methods

2.1. Chemicals. DES (CAS no. 64–67-5), obtained from Sigma Química (Spain), was dissolved in a solution of 3% ethanol-1% Tween-80 in 33.1 mM phosphate buffer (16.5 mM Na_2HPO_4, 16.6 mM KH_2PO_4, pH 6.8), containing 5% sucrose.

2.2. RL Test and Isolation of Vermilion Mutants. 1-2-days old *brown* (*bw*) males, in groups of 30 individuals, were placed in glass tubes, with eight layers of glass microfiber paper (Whatmann, GF/A) at the bottom, soaked with 0.9 ml of different DES concentrations. Negative controls were carried out treating males only with the solvent solution. After 3 hours treatment, males were mated to $In(1)sc^{S}1L\ sc^{8}R\ In(1)dl^{-}$ 49, *y*, $sc^{S1}sc^{8}$, *v*; *bw*; $mus308^{D2}$ (*I*, *v*; *bw*; *mus*308) virgin females (for marker descriptions see [19]). Protocols for fractionation the progeny in mature sperm and spermatids for the recessive lethal (RL) test and for isolation of F_1 and F_2 *vermilion* (*v*) mutants were described elsewhere [14].

At least five different experiments were carried out for each concentration and, since there were no differences among them, data were pooled. Statistical analysis of RL results was performed comparing mutant frequencies in treated flies with their respective negative controls, using the Fisher exact test.

The influence of *mus308* in the repair or processing of ethylation-induced damage was measured through the mutability index (MI) [20], and the statistical analysis of Aguirrezabalaga et al. [13] was carried out to determine whether MI values significantly differed from 1.

2.3. Molecular Analysis of Mutations. For each transmissible *vermilion* mutant, a homozygous strain was established to carry out the molecular analysis. All the isolated mutants were analysed.

The isolation of DNA and PCR amplifications were as described [21]. Mutant *vermilion* genes were cloned in the *M13mp19* vector or in a *pUC18* plasmid [22]. Sequencing reactions for the coding region were carried out using the dideoxy method, with a set of 10 internal primers. A fragment of about 1.8 Kb, localized upstream of the coding region, was analyzed as described before [21] in those mutants which did not show changes in the coding sequence. In order to exclude Taq polymerase-introduced errors, at least two plaques or colonies from independent PCR reactions were sequenced for each mutant.

Statistical analyses of differences between mutation spectra were carried out using the hypergeometric test for comparison of samples from mutational spectra [23, 24].

3. Results and Discussion

3.1. RL and Vermilion Mutation Frequencies. The RL and *vermilion* mutation frequencies, both spontaneous and induced by the different DES concentrations under *mus308* deficient (*mus308*$^{-}$) conditions, are presented in Table 1. Pooled data from mature sperm and spermatids are shown, because no differences between them were found in any case (not shown).

All chemically induced RL mutation frequencies are statistically higher than the spontaneous one, although their values decrease as DES concentration increases. Comparisons with the results previously obtained in *mus308* proficient (*mus*308^{+}) conditions [25] reveal two relevant differences (Table 1). First, the spontaneous RL frequency is statistically lower in *mus*308^{-} than in *mus*308^{+} conditions. Second, in *mus*308^{-} conditions, a decrease in RL frequencies is induced as DES concentration increases, whereas the opposite, that is an increase was detected in efficient repair conditions. Consequently, the value of the mutability index (MI) for 10 mM DES is statistically higher than 1 whereas for 15 mM and 25 mM the MIs are lower than 1 (Table 1).

To analyse the dose range between 10 and 15 mM DES, and to compare both repair conditions in the same experiment, a new and small experiment was carried out with 12 mM concentration. The obtained results confirmed that when the amount of DNA damage is low or moderate, hypermutability is obtained (4.8% and 8.2% RL mutation frequencies in *mus308*$^{+}$ and in *mus*308^{-}, respectively, with

Table 1: Recessive lethal (RL) and *v* mutation frequencies induced by DES on postmeiotic male germ cells of *D. melanogaster*, under *mus308* deficient (*mus*308$^-$) and efficient (*mus*308$^+$) conditions. Values of mutability index (MI) and their statistical signification are also presented.

			F_1 Analysis			F_2 Analysis				
Repair status	Treat.	Dose (mM)	Offspring	*v* mutants	Freq. ($\times 10^{-4}$)	Offspring[a]	*v* mutants	Freq. ($\times 10^{-4}$)	%RL	MI[b] (M*mus* 308$^-$/ M*mus* 308$^+$)
mus308$^-$	Control		24002	0	0	15698	0	0	0.17	
	DES	10	19582	0	0	12576	0	0	3.04***	1.55***
		15	154737	2	0.13	56093	11	1.96	2.91***	0.13***
		25	10092	0	0	6925	0	0	0.51***	0.01***
mus308$^+$	Control		8678	0	0	6766	0	0	0.28	
	DES[c]	10	54175	0	0	13090	2	1.53	2.13***	
		15	23209	4	1.72	9101	13	14.28	20.78***	
		25	15853	6	3.78	4703	5	10.63	29.22***	

[a]The F_2 offspring is the number of nonsterile treated X-chromosomes
[b]MI: mutation frequency induced in *mus*308$^-$/mutation frequency induced in *mus308*$^+$
[c]Data from Sierra et al. [25]. One experiment was carried out mating the treated males to *I,v;bw* females to check the validity of these previous data for comparisons.
$^*P < .05$; $^{**}P < .01$; $^{***}P < .001$.

Table 2: Molecular characterization of *v* mutants induced by DES in postmeiotic male germ cells, under *mus*308$^-$ conditions.

Mutant	Brood	Position	Mutation	Change	Sequence (5′–3′)[a]
D8-4	F_2-1B	92–556	464 bp deletion		
D8-5	F_2-1B	566	CG-TA	Leu-Phe	ACAG **C** TCCTG
D8-6	F_2-1B	6	TA-AT	Ser-Arg	TCAG **T** TCGC
		129	TA-CG	intron	tcag **t** tctg
		323	TA-CG	intron	tgag **t** aggt
		398	CG-AT	Gln-Lys	CAAG **C** AGAT
		416	GC-TA	Asp-Tyr	GTTC **G** ACTC
D8-7	F_2-1B	1167	GC-AT	Trp-STOP	AAGT **G** GAGA
D8-8	F_2-2C	648	CG-TA	Ser-Phe	GCAT **C** TGGT
D8-9	F_2-1A		No mutation		
D8-11	F_2-1C	−944	AT-TA		TATA **A** ATAT
		−243	TA-AT		TCAG **T** TATT
D8-12	F_2-1A	492	GC-AT	Arg-Gln	AACC **G** AGTG
D8-14	F_2-2A	494	GC-AT	Val-Met	CCGA **G** TGGT
D8-18	F_2-2A	1128	TA-CG	Leu-Pro	TTGC **T** CACC
		1168	GC-AT	Trp-STOP	AGTG **G** AGAT
D8-19	F_2-2A	596	AT-GC	Thr-Ala	GGAG **A** CCAT
D8-21	F_2-2A	974	CG-TA	Arg-STOP	GAAG **C** GACG
D8-23	F_2-2A	656	CG-TA	Gln-STOP	TTTT **C** AGTC
D8-26[b]	F_2-2A	promotor			
D8-27	F_2-1C	322	GC-AT	intron	gtga **g** tag
D8-29	F_2-2C	875	CG-TA	Gln-STOP	GTTT **C** AGGA
D8-30	F_1-2B	974	CG-TA	Arg-STOP	GAAG **C** GACG
D8-31	F_1-2C	−323	TA-AT		TCAG **T** TATT
		−945–935	10 bp deletion		

[a]Since for some mutation types the damaged base could not be identified, the sequence surrounding the detected change in the coding strand is presented. Intron sequences are shown in lower case letters, exon sequences in capitals.
[b]See text for details.

Table 3: Relative and absolute mutation frequencies (F_1 and F_2 values expressed as mutation frequencies $\times 10^{-5}$) of the different mutation types constituting the mutation spectra induced by DES in postmeiotic male germ cells, in *mus*308$^-$ and *mus*308$^+$ conditions.

Chemical	Mutation type	*mus308*$^-$			*mus308*$^{+(a)}$		
		Relative frequency	Absolute frequency ($\times 10^{-5}$)		Relative frequency	Absolute frequency ($\times 10^{-5}$)	
		%	F_1	F_2	%	F_1	F_2
DES	GC-AT	**47.8**	0.5	7.9	**73.3**	6.8	49.0
	AT-GC	**17.4**	—	5.3	**3.3**	—	3.3
	AT-TA	**17.4**	0.5	4.0	**10.0**	1.9	3.3
	AT-CG	—	—	—	**6.7**	1.0	3.3
	GC-TA	**8.7**	—	2.6	—	—	—
	Deletions	**8.7**	0.5	1.3	**6.7**	—	6.5

[(a)] Data of *mus308*$^+$ conditions are from Sierra et al. [25]. One experiment was carried out mating the treated males to *I,v;bw* females to check the validity of the previous data for comparisons.

a statistically significant MI of 1.7). It is noticeable that the very high rise in mutation frequency was detected between 10–12 mM and 15 mM DES in efficient repair conditions, but it is not unusual to find such a narrow window of increased activity in a chemical [26].

The obtained results demonstrate that Mus308 detects and processes DES-induced DNA damages. On one hand, low effectiveness DES doses, such as 10–12 mM (inducing low mutation frequencies), cause DNA damage, mostly oxygen alkylations [27], that seems to be processed through an error-free pathway, as pointed by the observed hypermutability. On the other hand, with high effectiveness DES doses (such as 15 and 25 mM), able to induce also considerable nitrogen alkylations [27], the obtained results indicate hypomutability; this fact, together with a decreased induced fertility, suggest that a functional Mus308 protein is necessary for the survival of the fertilized eggs.

The analysis of *vermilion* mutation frequencies (Table 1) show that, under *mus*308$^-$ conditions and considering all concentrations together, 2 mutants were isolated in F_1 (0.11×10^{-4} mutation frequency) while most *v* mutants, 11, were isolated among the F_2 offspring (1.46×10^{-4} mutation frequency). Other 5 *v* mutants were isolated from mass cultures, but they are not included in the mutation frequency estimations. Additionally, another mutant induced by DES was identified by genetic analysis as a translocation between the *X* and *Y* chromosomes that does not include the *v* locus. A comparison of these data with those obtained under *mus308* proficient conditions [25] reveals that the F_1 and F_2 induced mutation frequencies are much lower in *mus*308$^-$ than in *mus*308$^+$ conditions, indicating that the hypomutability observed with RL frequencies also extends to *v* mutation frequencies.

These results are in agreement with those previously obtained with ENU in the same cell type and under the same repair conditions. In that case, hypermutability was observed with a concentration that induced a moderate level of DNA damage (1 mM ENU), and similar results were found in RL and *vermilion* mutation frequencies analysis [14].

Results obtained here are consistent with a HR-mediated bypass of DNA damage if at least part of this damage induces cell mortality. However, a bypass tolerance system mediated by TLS could be also implicated in the processing of DES induced damaged. Thus, a DNA polymerase could process error-free some DNA damage, like oxygen alkylation, when the amount of DNA damage is low, but the processing of other types of induced DNA lesions, especially when they are present in high amounts (because other repair systems are saturated or inactive), like nitrogen alkylation, could be error-prone [28].

Moreover, there is another tolerance system, the alternative end-joining process (alt-EJ), independent of ligase 4 [29]. *mus308* was very recently discovered to be involved in this system [30], which processes DNA double strand breaks generated by replication blockage. Our results are compatible also with this system because nitrogen alkylations can be the source of DNA strand breaks [27].

3.2. Mutation Spectra. Details of DES-induced mutants are shown in Table 2. In D8-9 no mutation was found, and the same mutation was present in the independent mutants D8-21 and D8-30, as previously reported for other *vermilion* spectra [14, 18, 21, 31, 32]. In D8-26 no mutation was detected in the coding region nor in the proximal part of the promoter, but the distal part of the promoter could not be amplified, suggesting the presence of a mutation. Additionally, D8-6 presented five different mutations none of which was found in any other mutant. No mutants were isolated either from the 24002 F_1 and 15698 F_2 flies analysed in the concurrent control experiments or in the historical control; therefore, we consider that the observed mutations were induced by DES. The *v* mutation spectrum, constituted by the 23 obtained mutations, is summarized in Table 3 and includes two deletions (8.7%) and 21 base pair changes, distributed as follows: 11 GC-AT (47.8%) and 4 AT-GC (17.4%) transitions, and 4 AT-TA (17.4%) and 2 GC-TA (8.7%) transversions.

The pairing up of these mutations with the several adducts induced by DES indicates that: (i) the GC-AT and AT-GC transitions in the DES spectrum should be, respectively, the consequence of the O^6-ethylguanine and O^4-ethylthymine adducts [33–36], induced by this chemical [37–39]; (ii) AT-TA transversions are most probably due to N-ethylation [40], like the rest of transversions and the deletions [25, 27], because DES does not ethylate

O^2-thymine [37]. The two found deletions occur between direct repeats and, as the translocation, they can be indirectly generated from N-ethylation, as described before [27].

Comparison of the relative mutation frequencies of this *mus*308$^-$ spectrum with those previously obtained under *mus*308$^+$ conditions (Table 3) reveals clear differences ($P = .07$, with the hypergeometric test, and lower if the translocation is considered), including a strong decrease in the frequency of GC-AT transitions, and increases in the frequencies of AT-GC transitions, transversions and deletions under *mus308* $^-$ conditions. These results confirm that Mus308 is processing O^6-ethylguanine and O^4-ethylthymine, as indicated before [14], and reveal that this protein is also processing N-ethylation damage.

O^6-ethylguanine, like O^6-methylguanine, is a stable DNA lesion [37] that can mispair with T to produce GC-AT transitions as indicated but can also pair correctly with C [41] or can even block DNA polymerases [41, 42]. Therefore, O^6-ethylguanine can fit as a substrate of Mus308. O^4-ethylthymine is a DNA damage with a long half-life [37], difficult to repair in mammals [43–45]. Although it is not considered a lethal lesion [33, 36], it is able to block DNA replication in mammalian cells in a NER deficient background [46]. NER is apparently implicated in its repair in Drosophila [27, 32], although rather inefficiently, because AT-GC transitions are one of the most frequently ENU-induced damages in the repair-active premeiotic germ cells of this organism [21, 31]. Therefore, this adduct fits with the proposed requirements for the substrates of Mus308 [13, 14].

Since at least part of the N-ethylation damage can be persistent and can block DNA synthesis [37], its detection as substrate of Mus308, especially when the level of DNA damage is very high and repair is difficult, is not unexpected or strange. Additionally, it can be considered that N-ethylation is a source of DNA strand breaks [27], and this type of DNA damage is substrate of Mus308 in the alt-EJ system [30].

The sequence specificity of DES- induced mutations was studied determining the base pairs 5′ and 3′ of the damaged nucleotide (Table 2). The results of this analysis show that AT pairs are present at 5′ in 64% of GC-AT mutations in *mus*308$^-$ conditions whereas 64%–70% of this type of mutations is preceded by GC pairs at 5′ in *mus*308$^+$ conditions [25] and in NER deficient conditions [27], respectively. This means that the neighbouring sequences 5′ to O^6-ethylguanine change depending on the Mus308 status, which is in good accordance with the proposed polymerase function of Mus308, specially considering that no influence of surrounding sequences was found before for this chemical in this locus [25, 27], nor were expected for an S_N1/S_N2 alkylating agent [47].

In summary, the results presented in this paper demonstrate that Mus308 processes oxygen and nitrogen alkylations, and they support its role in a tolerance mechanism that is especially relevant in case of high DNA damage levels, because it prevents cell death. Additionally, these results suggest that this protein could act through a TLS pathway, because of (i) the detected neighbouring sequence influence, and (ii) its DNA polymerase activity. Finally, these results also agree with the Mus308 role in the alt-EJ system, for the processing of DNA damage-inducing strand breaks, which can be compatible with the TLS pathway [30].

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