# ARTÍCULO 3

## The *Drosophila melanogaster mus209$^{B1}$* mutant (PCNA) impairs genomic stability. Analysis by DNA fingerprinting

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# Spontaneous and bleomycin-induced genomic alterations in the progeny of *Drosophila* treated males depends on the *Msh2* status DNA fingerprinting analysis

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**Abstract**

Deficiency in DNA mismatch repair (MMR) confers instability of simple repeated sequences and increases susceptibility to cancer. Some of the *MMR* genes are also implicated in other repair and cellular processes related to DNA damage response. Supposedly, lack of their function can lead to a global genomic instability, besides microsatellite instability (MSI). To study the spontaneous and induced genomic instability in germ cells, related to the *Msh2* status, DNA alterations in the progeny of individual crosses of *Drosophila* deficient in one or two copies of the *Msh2* gene, were analysed by the arbitrarily primed polymerase chain reaction (AP-PCR). The results indicate that the progeny of homozygous parents for the normal *Msh2* allele (+/+) presents a significantly lower frequency of genomic alterations than those from heterozygous (+/−) or mutant homozygous (−/−) parents. In addition, the DNA damage transmitted to the progeny, after the adult parental males were exposed to bleomycin, indicates that whereas the induction of mutations related to MSI depends on the lack of the *Msh2* function, the induction of other mutational events may require at least one functional *Msh2* allele. Thus, the results obtained with heterozygous individuals may have special relevance for cancer development since they show that a disrupted *Msh2* allele is enough to generate genomic instability in germ cells, increasing the genomic damage in the progeny of heterozygous individuals. This effect is enhanced by mutagenic stress, such as occurs after bleomycin exposure.


*Keywords:* DNA mismatch repair; Genomic instability; *Drosophila melanogaster*; AP-PCR; Mutagenesis

## 1. Introduction

DNA mismatch repair (MMR) corrects DNA replication errors, being crucial for the maintenance of genomic stability. In humans, inheritance of one mutant allele in any of the *MMR* genes confers a high risk of developing hereditary nonpolyposis colorectal cancer (HNPCC), furthermore, the other allele is usually inactivated in these tumours by somatic mutation [1,2]. Complete MMR deficiency leads to a mutator phenotype mainly due to the accumulation of DNA replication errors. These errors usually manifest themselves as an increase in point mutations and as microsatellite instability (MSI) [3–6]. Initially, the origin of genomic

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instability in MMR-deficient cells was attributed to the failure in removing DNA mismatches arising from DNA replication; however, recent studies have also implicated several MMR components in other cellular processes. One example is the *Msh2* gene, which plays a central role in the MMR process as reflected by its predominant inactivation in HNPCC [7] and its role in MSI [8–10]. In addition, *Msh2* has been implicated in double-strand break repair [11], transcription-couple repair [12], meiosis [13] and DNA damage response [14–17]. These evidences suggest that, in addition to MSI and point mutations, other alterations related to the multifunctional properties of the MSH2 protein, might take place under *Msh2*-deficient conditions leading to a global genomic instability [16,17]. Furthermore, this genomic instability might depend on the number of alleles altered (homozygous versus heterozygous) and might be modulated by environmental factors.

An important question to cancer predisposition is to determine whether heterozygous individuals for mutations in *MMR* genes also manifest a higher degree of mutation frequency compared to normal individuals. Even a small increase in the mutation rate may be significant during the lifetime of an organism [18] and may be transmitted through the generations. On this matter, there is a lack of information in high eukaryote systems in vivo, and the experimental data obtained in *Msh2*-deficient murine and yeast cells are contradictory. Drotschmann et al. [9] by using a sensitive mutation detection system in yeast found an increased mutation rate in $Msh2^{+/-}$ cells. However, the result obtained with murine cells show no difference in mutation rates in normal $Msh2^{+/+}$ and heterozygous $Msh2^{+/-}$ cells [19–21].

The main purpose of the present study is to assess the implication of the *Msh2* gene in global genomic instability. Thus, the spontaneous and induced genomic alterations in a *Msh2* deficient background in vivo have been evaluated. To estimate the frequency of genetic alterations present in the parental germinal cells that are transmitted to the next generation, the *Drosophila spel1* mutant with a deleted *Msh2* gene, provides a good experimental approach, when analysing the genomic alterations in the offspring from selected crosses. Using this approach, we have recently demonstrated that the progeny of $Msh2^{-/-}$ shows a higher MSI compared to the progeny of normal $Msh2^{+/+}$ parents [10]. Here, we extend the analysis by including heterozygous $Msh2^{+/-}$ crosses and using the arbitrarily primed PCR (AP-PCR) technique that allows to analyse random sequences representative of the overall genome [22,23]. This PCR based DNA fingerprint method can detect quantitative and qualitative changes in DNA sequences [24] and it is useful to detect inherited DNA alterations [25–28]. In this study, crosses of individuals bearing either one or two null alleles of the *Msh2* gene, or two normal alleles of this gene have been established, and the spontaneous genomic damage in the germline, detectable in the progeny, have been analysed. In addition, exposure of the adult parental males to the radiomimetic compound bleomycin (BLM) allowed us to determine the induced DNA damage, related to the MMR status, transmitted to the progeny. Knowing that environmental factors are important determinants of human cancer and that their effects can be modulated by MMR function [29], an increased interest has arisen about the role of MMR in oxidative damage [21,30]. Since BLM produces reactive oxygen species [31], the study of the bleomycin-induced genomic damage in the heterozygous individuals is of special interest because they mimic the HNPCC syndrome.

## 2. Materials and methods

### 2.1. Drosophila stocks, culture and genetic crosses

The *Drosophila* stocks used were the following: (i) *Canton-S*(*CS*) strain, maintained in our laboratory and used as a wild-type strain; (ii) two chromosome II deficiency strains, *w;Df*(*2L*)*TE146*(*z*)*GW7 al dp bl*(*2*) *pwn cn/CyO* (abbreviated *Df*(*2L*)*GW7/CyO*) and *Df*(*2L*)$b80e^3$*/CyO*, used to create the *spel1* flies, which are *Msh2*-null mutants [32]. The deficiency strains were kindly provided by Carlos Flores (Laboratory of Genetics, University of Wisconsin, Madison). More details on mutations and specific markers are described in FlyBase [33]. The stocks were maintained in standard conditions at 25 °C and 60% of relative humidity.

After crossing the two deficiency strains, *Df*(*2L*) *GW7/CyO* and *Df*(*2L*)$b80e^3$*/CyO*, the offspring *spel1* and *Cy* were selected separately to establish two sets

of individual crosses: (1) *Msh2*$^{-/-}$ crosses, obtained by individually crossing *spel1* males and females and (2) *Msh2*$^{+/-}$ crosses, obtained by individually crossing *Cy* males and females. An additional set of crosses were established by individually crossing males and females of the *CS* strain (*Msh2*$^{+/+}$ crosses).

### 2.2. Bleomycin treatment procedure and sensitivity test

The bleomycin (BLM, CAS No. 11056-06-79) used in this study was supplied by Almirall Prodesfarma (Barcelona, Spain). Two to three day-old males, used to establish the individual crosses described above, were treated by feeding for 48 h with 10 μg/ml of BLM. The solution of 10 μg/ml of BLM was prepared in double-distilled water just before treatment, supplemented with sucrose (5%) to stimulate flies to ingest the product. Flies were transferred to special glass filter feeding units (1D3, Scott, Mainz, Germany) after 3–4 h of starvation. In order to set-up the *Msh2*$^{-/-}$, *Msh2*$^{+/-}$ and *Msh2*$^{+/+}$ crosses, treated males were mated individually with one virgin female of their corresponding genotype. They were allowed to lay eggs for 3 days to ensure that progeny comes from treated spermatozoa, and then the parents were removed and kept at −80 °C. Thereafter, the adult progeny was collected and analysed by AP-PCR together with the parental flies.

To test the sensitivity to BLM, early larvae *Df(2L)GW7/Df(2L)b80e*$^{3}$*(spel1)*, and early larvae from *Df(2L)GW7/CyO*, *Df(2L)b80e*$^{3}$*/CyO* and *CS* stocks were treated according to the following procedure: after mass crosses of the different stocks, 100 eggs were selected and transferred into vials with *Drosophila* Instant Medium (Carolina Biological Supply Co., Burlington, NC) rehydrated (v/v 1:1) with the desired solution. Surviving adults were scored to determine the survival frequency of *Msh2*$^{-/-}$ progeny (*Df(2L)GW7/Df(2L)b80e*$^{3}$ cross), *Msh2*$^{+/-}$ progeny (*Df(2L)GW7/CyO* or *Df(2L)b80e*$^{3}$*/CyO* crosses) and *Msh2*$^{+/+}$ progeny (*CS* cross). The number of surviving adults was corrected considering the ratio of lethal phenotypes expected in each cross. To compare the sensitivity of the different genotypes to BLM, the survival frequency of treatment experiments relative to control experiments was calculated.

### 2.3. Genomic DNA isolation

For each cross, the collected parental male and female and their corresponding offspring were used for individual genomic DNA extraction, following the method described by Roberts [34]. The obtained DNA was dissolved in 20–30 μl of double-distilled water.

### 2.4. AP-PCR amplification

A total of 25 μl of reaction was prepared using 1–2 μl of the DNA stock solution from individual flies (∼50 ng of DNA), 1 × PCR reaction buffer (50 mM KCl, 10 mM Tris–HCl pH 8.4, 0.01% gelatin), 4 mM $MgCl_2$, 125 μM of each dNTP, 3 μCi of [α-$^{33}$P] dATP, 1 U of Taq DNA polymerase and 0.25 μM of the arbitrarily primer. For each DNA sample, two DNA fingerprints were generated by performing two AP-PCR reactions. Fingerprint A was obtained using the arbitrarily primer WB (5′-GTTAGGGAGCCGATAAAGAG-3′) and fingerprint B was obtained using a combination of two arbitrary primers: D (5′-CGGAATTCCAAGACACATTG-3′) and F (5′-CGGAATTCCTAGGGCCGTTT-3′). The reactions were carried out in a Programmable Thermal Controller (PT-100 MJ Research, USA). A preincubation step for 3 min at 94 °C was followed by 5 cycles in low stringency conditions (94 °C 30 s, 40 °C 30 s and 72 °C 90 s) and 30 cycles in high stringency conditions (94 °C 15 s, 55 °C 15 s and 72 °C 1 min). Finally, 3 μl of the reaction was fractionated by electrophoresis on a 6% denaturing polyacrylamide gel (5 h 30 min-fingerprint A and 7 h-fingerprint B, at 90 W and 50 °C) and visualised by autoradiography.

### 2.5. Quantification of genomic damage by AP-PCR

Following our previous criteria [27], the fingerprinted bands considered for the assessment of genomic damage were those reproducible and clearly visualised in the autoradiography of each repeated experiment (see Figs. 1 and 2). The number of reproducible fingerprinted bands considered in this study was as follows: fingerprint A, 26 bands for the *Msh2*$^{+/+}$ individuals, 25 for *Msh2*$^{+/-}$ and 25 for *Msh2*$^{-/-}$; fingerprint B, 39 bands for the *Msh2*$^{+/+}$ individuals, 32 for *Msh2*$^{+/-}$ and 34 for *Msh2*$^{-/-}$.

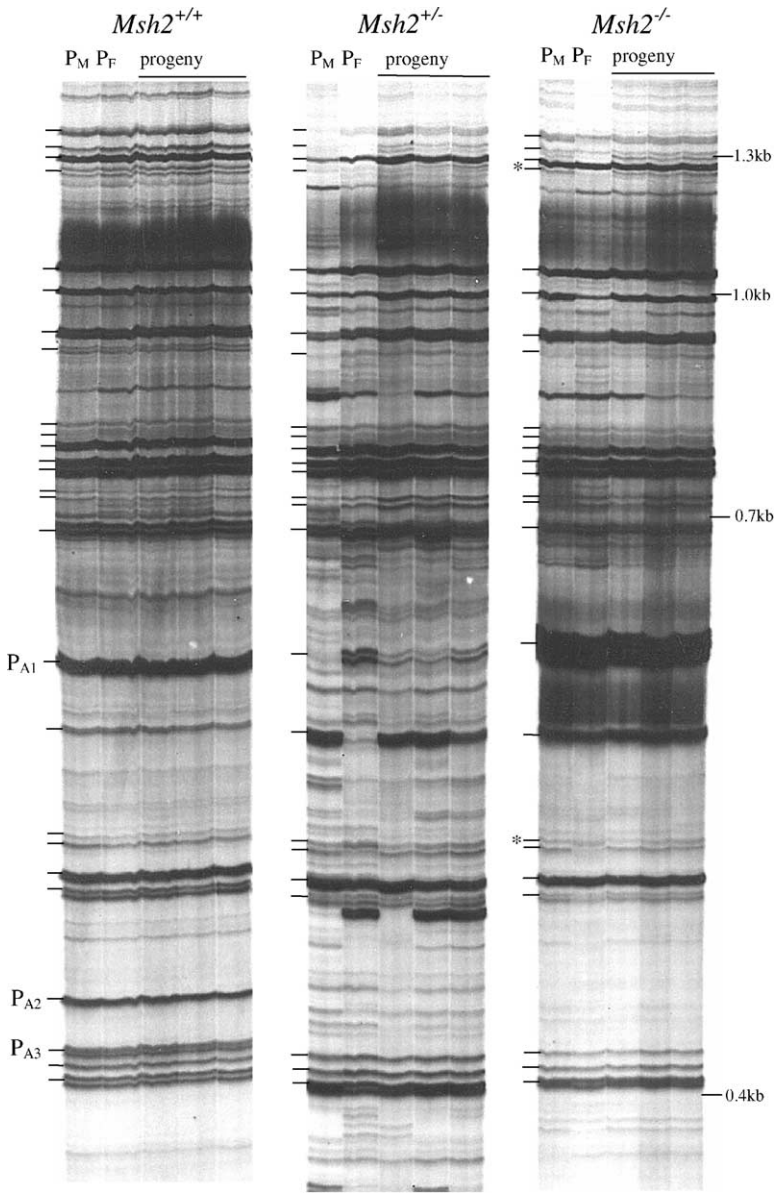


Fig. 1. DNA fingerprints by AP-PCR of parents and progeny of $Msh2^{+/+}$, $Msh2^{+/-}$ and $Msh2^{-/-}$ crosses, using the primer WB (fingerprint A). $P_M$, parental male; $P_F$, parental female. Lines on the left indicate the reproducible amplified bands used to quantify the genomic damage. $P_{A1–3}$ indicate the differences in reproducible bands between crosses of different genotypes. The polymorphic reproducible bands of individuals of the $Msh2^{-/-}$ genotype are marked with an asterisk (∗). The size of the bands is shown on the right.

To estimate the total genomic damage in the progeny, the number of altered bands in fingerprints A and B for each experiment was scored. The sum of all detected changes (loss of bands, new bands and band shifts) in the fingerprinted bands of the total progeny analysed in each experiment, was divided by the total number of analysed bands. This total number was calculated as follows: the number of reproducible amplified bands plus the number of new amplified bands for each primer, considering the latter as possible target

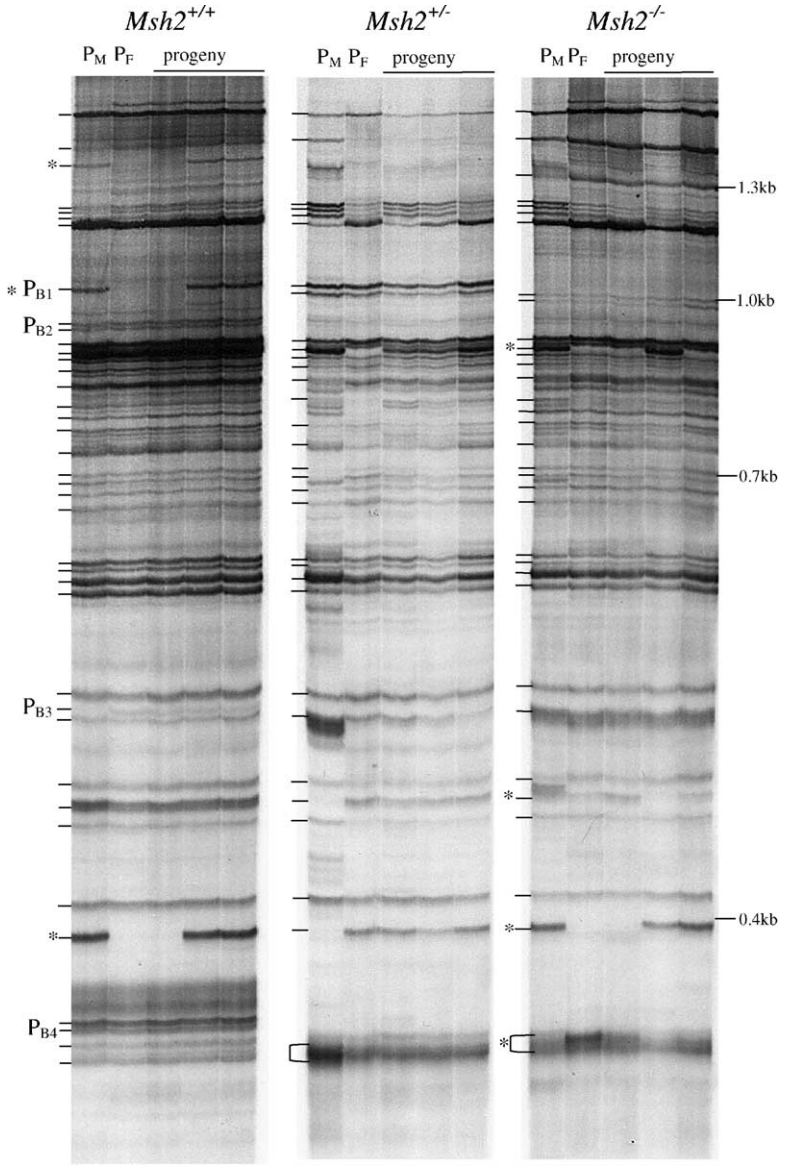


Fig. 2. DNA fingerprints by AP-PCR of parents and progeny of *Msh2*$^{+/+}$, *Msh2*$^{+/-}$ and *Msh2*$^{-/-}$ crosses, using the combination of primers D and F (fingerprint B). $P_M$, parental male; $P_F$, parental female. Lines on the left indicate the reproducible amplified bands used to quantify the genomic damage. $P_{B1–4}$ indicate the differences in reproducible bands between crosses of different genotypes. The polymorphic reproducible bands of individuals of the *Msh2*$^{+/+}$ or *Msh2*$^{-/-}$ genotype are marked with an asterisk (∗). The size of the bands is shown on the right.

sequences to amplify from fly genomic DNA of the different genotypes. Thus, the index corresponding to the total genomic damage (damage fraction, DF) was calculated. In addition, the band shift fraction (BSF) was also calculated by counting the number of mobility shifts of bands, separately. Statistical analysis was carried out by using the two-tailed Fisher's exact test.

## 3. Results

### 3.1. Genomic DNA fingerprints of $Msh2^{+/+}$, $Msh2^{+/-}$ and $Msh2^{-/-}$ individuals

Fingerprints obtained by AP-PCR are characteristic of the genome and the primer used, this property makes the technique useful for DNA typing of different organisms [35]. In Figs. 1 and 2, a representation of the DNA fingerprints obtained using two combinations of primers (fingerprint A and B, respectively) is shown. These fingerprints were obtained from parents and progeny of individual crosses of each studied genotype, $Msh2^{+/+}$, $Msh2^{+/-}$ and $Msh2^{-/-}$. As expected, the two combinations of primers gave different patterns of amplified sequences and, for each fingerprint, the reproducible amplified bands were selected to quantify the spontaneous and induced genomic alterations. The reproducible bands used in this analysis are indicated in Figs. 1 and 2. For each given primer, differences in some of the reproducible bands were found between crosses of different genotypes, due to the amplification of polymorphic sequences. Moreover, most of these genomic differences were found between the $Msh2^{+/+}$ and the $Msh2^{-/-}$ or the $Msh2^{+/-}$ individuals, showing the common origin of the two last genotypes (see Section 2); for example, $P_{A1–3}$ in Fig. 1 and $P_{B1–4}$ in Fig. 2. Some of the reproducible bands were also polymorphic for individuals of the same genotype, $Msh2^{+/+}$ or $Msh2^{-/-}$, these sequences are marked with an asterisk in Figs. 1 and 2. In the case of $Msh2^{+/-}$, differences between individuals are probably due to the deficient chromosome bearing the deleted *Msh2* allele *Df(2L)GW7* or $Df(2L)b80e^{3}$ (see Section 2).

### 3.2. Genomic alterations detected by AP-PCR in the offspring from crosses of individuals with one or two disrupted Msh2 alelles

To estimate the genomic instability inherent to the genotypes $Msh2^{+/+}$, $Msh2^{-/-}$ and $Msh2^{+/-}$ a comparative analysis of individual fingerprints of AP-PCR products, obtained from the matched progeny and parental flies of the control experiments, was carried out. The complete loss of bands, the new bands and the band shifts were computerised in the analysis, representing the total genomic damage in the parental germinal cells transmitted to the offspring. This assumption is based on the fact that most of the somatic alterations will be diluted by the normal DNA sequences and will not be detected in PCR amplifications. Moreover, when alterations occur in the first embrionary cell divisions, they will appear as changes of band intensity, therefore, this type of changes was ignored in our study because of their supposed somatic origin. An example of the genomic alterations detected in the AP-PCR fingerprints is shown in Fig. 3. The spontaneous fraction of damage in the germline (DF) was estimated as the frequency of genomic alterations, calculated as follows: the sum of altered bands in the AP-PCR fingerprints of the emerged individuals, divided by the sum of the total number of amplified bands (see Section 2).

As shown in Table 1, the DF in the germline detectable in the offspring of the control experiments differs between the three parental genotypes. The frequency of alterations in the progeny of deficient homozygous $Msh2^{-/-}$ parents, was 14-fold the frequency in the progeny of the normal homozygous $Msh2^{+/+}$ parents (DF, 0.057 and 0.004, respectively). The progeny of the heterozygous $Msh2^{+/-}$ parents also showed five-fold alterations more than the normal homozygous (DF, 0.021 and 0.004, respectively). Taking into account that, in the AP-PCR fingerprints, the mobility of bands are considered an indication of instability of simple repeated sequences [23,24,36], changes in band mobility (band shift fraction, BSF) were computerised separately, as an indication of microsatellite instability (MSI). In this case, it was also found that the progeny of the $Msh2^{-/-}$ crosses shows the highest frequency of this type of alteration, being 23-fold higher than in the $Msh2^{+/+}$ crosses ($P < 10^{-5}$). However, the BSF in the progeny of the heterozygous $Msh2^{+/-}$ crosses was not statistically different respect to the $Msh2^{+/+}$ crosses ($P = 0.233$). A graphic representation of the spontaneous DF and BSF in these three genotypes is displayed in Fig. 4. These results indicate that the total genomic instability depends on the number of altered copies of the *Msh2* gene, and that the BSF contributes more to the total genomic instability in the *Msh2*-null genotype than to that found in the heterozygous or normal *Msh2* geno-

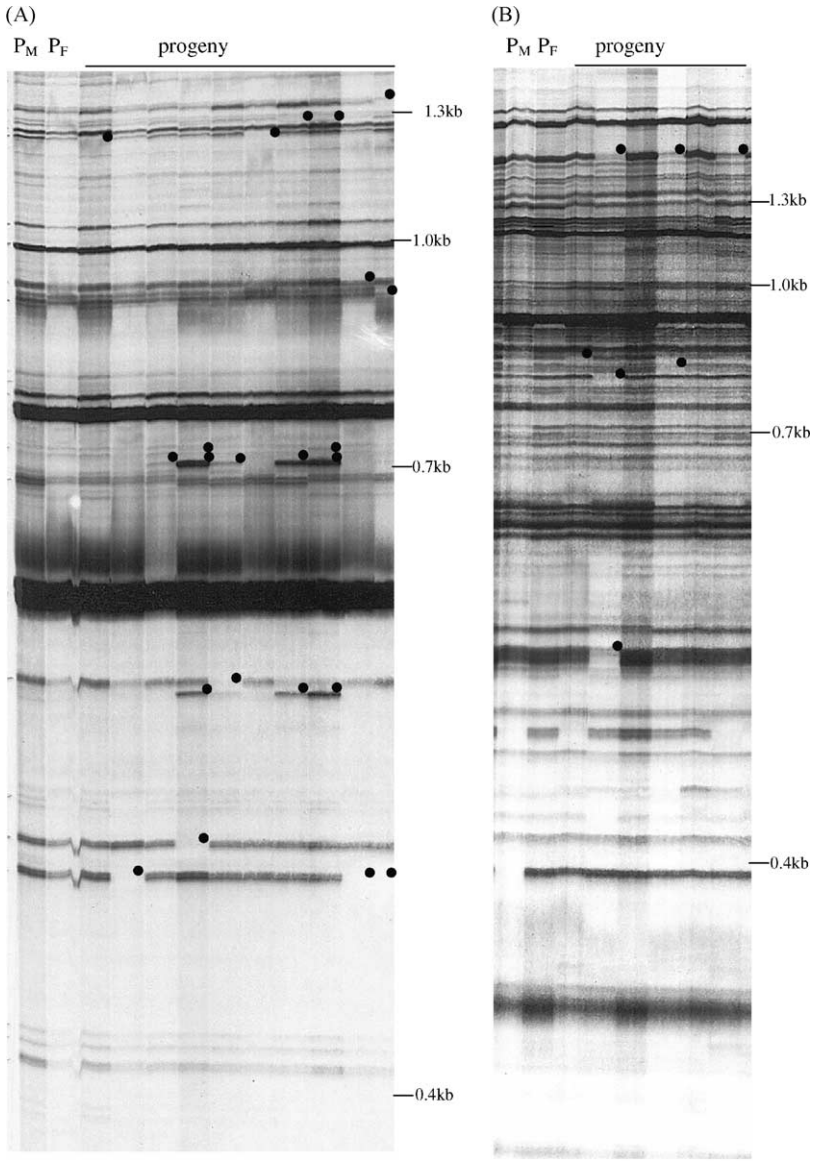


Fig. 3. Example of genomic alterations detected by AP-PCR fingerprints in the progeny of a *Msh2*$^{-/-}$ cross after exposure of the parental male to bleomycin. (A) Fingerprint A, (B) fingerprint B. Changes in the fingerprinted bands are indicated with a black dot (●). The size of the bands is shown on the right.

types. This fact was also put into manifest by the BSF/DF ratio shown in Table 1, being 8.05% for the *Msh2*$^{-/-}$ progeny, whereas a similar low BSF/DF ratio was found for the progeny of the *Msh2*$^{+/-}$ and the *Msh2*$^{+/+}$ crosses (4.13 and 5.55%, respectively).

### *3.3. Genomic damage induced by bleomycin in postmeiotic germ cells of Msh2 deficient and proficient males*

Bleomycin is considered a radiomimetic compound that induces DNA damage by liberation of oxygen

Table 1
Genomic damage detected by AP-PCR in the progeny from the *Msh2*$^{-/-}$, *Msh2*$^{+/-}$ and *Msh2*$^{+/+}$ crosses after exposure of parental males to bleomycin

| Crosses | Treatment | AP-PCR fingerprint[a] | No. of analysed individuals (lines) | No. of analysed bands | No. of altered bands | DF[b] | $P$[c] | No. of band shifts | BSF[d] | $P$[c] | Ratio of band shifts to altered bands (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Msh2*$^{-/-}$ | Control | A | 62 (7) | 2046 | 122 | 0.060 | | 5 | 0.0024 | | |
| | | B | 51 (6) | 2091 | 114 | 0.054 | | 14 | 0.0067 | | |
| | Total | | | **4137** | **236** | **0.057** | – | **19** | **0.0046** | – | 8.05 |
| | 10 μg/ml | A | 80 (7) | 2640 | 169 | 0.064 | | 25 | 0.0095 | | |
| | | B | 81 (7) | 3321 | 191 | 0.057 | | 36 | 0.0108 | | |
| | Total | | | **5961** | **360** | **0.060** | 0.520 | **61** | **0.0102** | **0.002** | 16.94 |
| *Msh2*$^{+/-}$ | Control | A | 97 (6) | 3201 | 96 | 0.030 | | 0 | – | | |
| | | B | 76 (5) | 2508 | 25 | 0.010 | | 5 | 0.0020 | | |
| | Total | | | **5709** | **121** | **0.021** | – | **5** | **0.0009** | – | 4.13 |
| | 10 μg/ml | A | 99 (7) | 3267 | 123 | 0.038 | | 8 | 0.0024 | | |
| | | B | 80 (6) | 2640 | 55 | 0.021 | | 11 | 0.0042 | | |
| | Total | | | **5917** | **178** | **0.030** | **0.003** | **19** | **0.0032** | **0.007** | 10.67 |
| *Msh2*$^{+/+}$ | Control | A | 69 (8) | 1932 | 13 | 0.007 | | 1 | 0.0005 | | |
| | | B | 54 (6) | 2322 | 5 | 0.002 | | 0 | – | | |
| | Total | | | **4254** | **18** | **0.004** | – | **1** | **0.0002** | – | 5.55 |
| | 10 μg/ml | A | 56 (8) | 1568 | 24 | 0.015 | | 3 | 0.0009 | | |
| | | B | 61 (8) | 2623 | 28 | 0.011 | | 0 | – | | |
| | Total | | | **4191** | **52** | **0.012** | **$3 \times 10^{-5}$** | **3** | **0.0007** | 0.369 | 5.77 |

[a] AP-PCR fingerprints obtained with the primer WB (A) and the combination of primers D and F (B) (see Section 2).
[b] DF: damage fraction calculated as the number of altered bands divided by the total number of analysed bands (see Section 2).
[c] Probability of the difference from the matched control (Fisher's exact test, two tailed; statistically significant values are in bold).
[d] BSF: band shift fraction calculated as the number of band shifts divided by the total number of analysed bands (see Section 2).

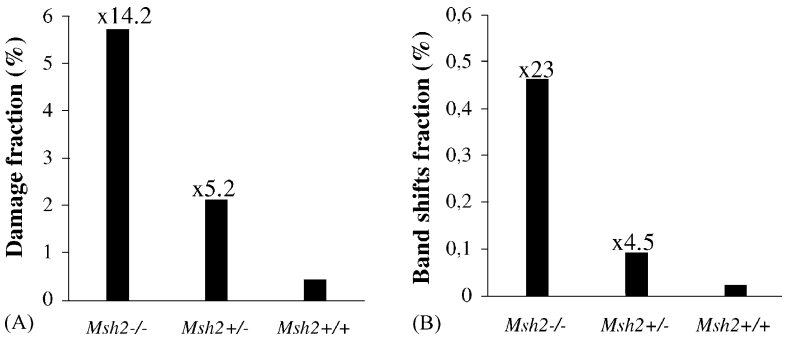


Fig. 4. Spontaneous genomic instability analysed by AP-PCR related to the *Msh2* condition. (A) Damage fraction corresponding to the frequency of the total genomic alterations detected in the fingerprinted bands; (B) Band shift fraction which represent the frequency of mobility of bands in the fingerprints. Numbers on top of bars indicate the times of the normal *Msh2* homozygous frequency in the homozygous deficient and the heterozygous.

radicals [31]. Recent studies using MMR deficient cells have shown that the mutagenic effect of oxidative stress depends on mismatch repair capability [21,30]. In addition, some reports indicate that oxidative damage produces MSI in human cells [37–39]. However, our recent in vivo results in *Drosophila*, under MMR deficient conditions, were not conclusive about the induction of MSI by BLM [10], which could be due to the sample size limitation of the in vivo studies when analysing MSI by locus specific PCR. Therefore, in the present study, we have extended the analysis using the multilocus AP-PCR technique which allows to analyse several random sequences in the overall genome and displays other types of sequence alterations besides the instability of simple repeated sequences [23,24,36]. We rationalised that, since BLM can induce a wide spectrum of mutagenic lesions including strand breaks and base damage [40,41], the AP-PCR analysis could reveal the global genomic alterations related to MMR capabilities, induced by BLM.

In Table 1, the genomic damage detected in the F1 generation after the exposure of the male germ cells (spermatozoa stage) to BLM is indicated. As it can be noticed, the frequency of alterations observed in the fingerprints A and B is similar, thus, thereafter, we only make reference to the sum of alterations observed in both fingerprints. Clearly, under our experimental conditions, BLM induces genomic damage detected by AP-PCR. As indicated above, the analysis was carried out by counting the total number of alterations observed in the progeny (DF) and, separately, computerising the number of band shifts (BSF). Interestingly, and as shown in Table 1, the type of damage induced by BLM depends on the parental *Msh2* genotype. Whereas BLM produced a significant increase of DF after treatment of normal homozygous and heterozygous *Msh2* males ($P < 0.0001$ and $<0.01$, respectively), no increases were observed in the damage fraction estimated from the progeny of the deficient homozygous *Msh2* males treated with BLM, respect to the control treatment (DF: 0.060 versus DF: 0.057, respectively). However, an opposite effect was detected when analysing the BSF under BLM treatment. Accordingly, a significant increase in the BSF was observed in the progeny of both deficient $Msh2^{-/-}$ and heterozygous $Msh2^{+/-}$ crosses with respect to the control ($P < 0.01$, in both crosses), but no differences were found between BLM treatment and control in the normal $Msh2^{+/+}$ crosses ($P = 0.369$). In Fig. 5, the effect of BLM in the genomic instability of germinal cells of $Msh2^{+/+}$, $Msh2^{+/-}$ and $Msh2^{-/-}$ individuals is represented. In this case, after BLM treatment, the number of band shifts respect to the total damage (BSF/DF ratio) increases in the $Msh2^{-/-}$ and $Msh2^{+/-}$ crosses; however, no change in the BSF/DF ratio was observed in the $Msh2^{+/+}$ crosses. This effect is not appreciated by looking at the DF, due to the fact that the BSF only represent the 4–8% of the total damage detected in the AP-PCR fingerprints obtained in our experiments (see Table 1). In summary, these results indicate that the induction of band shifts in the AP-PCR fingerprints

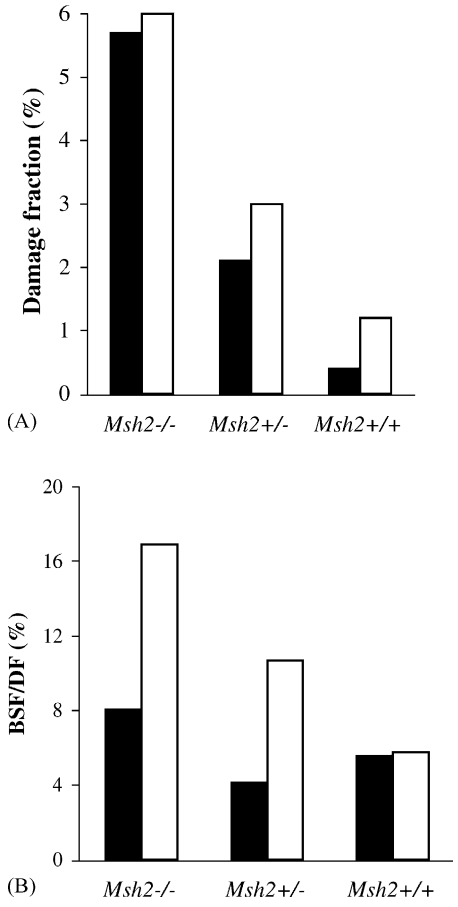


Fig. 5. Genomic damage induced by treatment of the germ cells with bleomycin, under different MMR conditions. (A) Frequency of genomic alterations detected in the progeny by AP-PCR (damage fraction); (B) The ratio of the number of band shifts alterations to the total number of alterations (BSF/DF), detected in the progeny by AP-PCR. Filled bars, 0 μg/ml; open bars, 10 μg/ml.

by BLM depends on the complete lack of function of the *Msh2* gene, whereas the induction of the other genomic alteration detected by AP-PCR (loss of bands and new bands) requires at least one functional *Msh2* allel; being the heterozygous condition where the induction of both types of alterations take place.

### 3.4. Mobility shifts of AP-PCR bands and microsatellite instability

Previous studies of genomic analysis by AP-PCR, consider that mobility of fingerprinted bands is an indication of instability related to simple repeated sequences [23,24,36] which is why the AP-PCR technique allowed to discover the microsatellite mutator phenotype for carcinogenesis [3].

Table 2
Comparison of spontaneous mobility shifts of bands found in AP-PCR analysis with spontaneous instability in microsatellite loci[a]

| Crosses | BSF[b] | Ratio[c] | MSI[d] | Ratio[c] |
|---|---|---|---|---|
| $Msh2^{+/+}$ | $2 \times 10^{-4}$ | – | $<2 \times 10^{-3}$ | – |
| $Msh2^{+/-}$ | $9 \times 10^{-4}$ | 4.5 | $9.5 \times 10^{-3}$ | 4.8 |
| $Msh2^{-/-}$ | $4.6 \times 10^{-3}$ | 23 | $4.8 \times 10^{-2}$ | 24 |

[a] Data provided from the analysis of the progeny.
[b] BSF: band shift fraction calculated as the total number of band shifts divided by the total number of analysed bands (see Section 2).
[c] Ratio to alterations in the progeny of $Msh2^{+/+}$ crosses.
[d] MSI: spontaneous microsatellite instability obtained after the analysis of five microsatellite loci. $Msh2^{+/+}$ and $Msh2^{-/-}$, data from [10]. $Msh2^{+/-}$, data from unpublished results.

In Table 2, the total changes due to band shifts (BSF) detected in the AP-PCR fingerprints of the progeny of the $Msh2^{+/+}$, $Msh2^{+/-}$ and $Msh2^{-/-}$ crosses in untreated experiments are shown. They are compared with our previous results of spontaneous microsatellite instability (MSI) in the progeny of $Msh2^{+/+}$ and $Msh2^{-/-}$ crosses [10], and unpublished results of MSI in $Msh2^{+/-}$ crosses. The ratio of the BSF in the offspring from parents bearing either one or two null alleles of the *Msh2* gene to the BSF in the offspring from normal homozygous ($Msh2^{+/+}$), was 4.5 and 23, respectively. A similar ratio was obtained for MSI analysis, where the progeny of $Msh2^{+/-}$ and $Msh2^{-/-}$ crosses presented a MSI frequency 4.8- and 24-fold higher, respectively, than the progeny from $Msh2^{+/+}$ individuals. The fact that, for all the crosses, the BSF was a range of magnitude lower than the frequency of MSI obtained by analysing five microsatellite loci, may be because, in our experiments, only a small number of the AP-PCR amplified sequences was susceptible to suffer this type of alteration.

### 3.5. Larvae survival after BLM treatment

Adult males, proficient and deficient on MMR, exposed to high doses of BLM (500 μg/ml) did not show any differential sensitivity (data not shown), indicating that cell division is a requirement for BLM

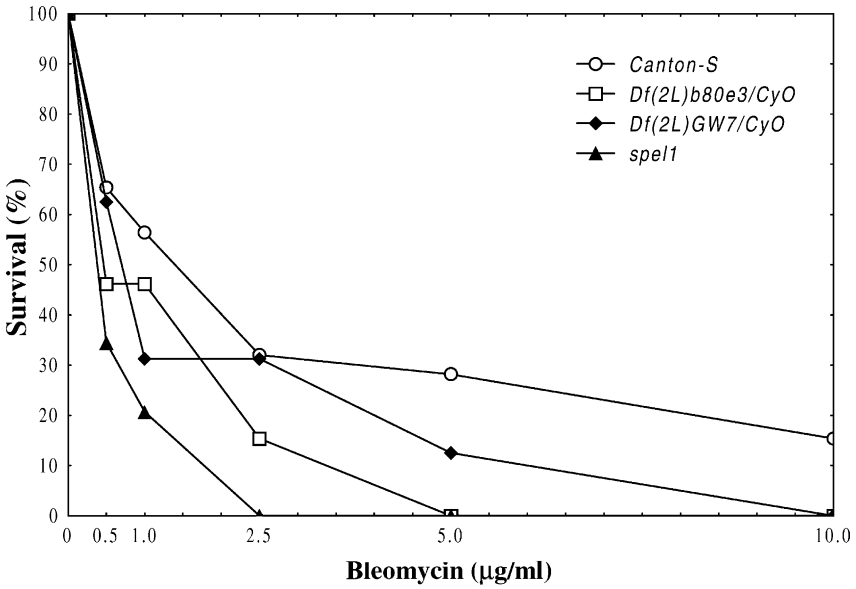


Fig. 6. Sensitivity of the different *Msh2* genotypes to bleomycin. Early larvae of *Canton-S* ($Msh2^{+/+}$), *Df(2L)GW7/CyO* ($Msh2^{+/-}$), $Df(2L)b80e^{3}/CyO(Msh2^{+/-})$ or $Df(2L)GW7/Df(2L)b80e^{3}$ ($Msh2^{-/-}$) crosses were exposed to different concentrations of bleomycin.

to induce cytotoxicity. Therefore, to determine the influence of MMR background on sensitivity to BLM, $Msh2^{+/+}$, $Msh2^{+/-}$ and $Msh2^{-/-}$ first instard larvae were chronically treated with BLM. As it can be appreciated in Fig. 6, $Msh2^{-/-}$ and $Msh2^{+/-}$ individuals show higher sensitivity than the $Msh2^{+/+}$, being the heterozygous $Msh2^{+/-}$ individuals less sensitive than the $Msh2^{-/-}$. Thus, in contrast to the alkylation tolerance of MMR deficient cells, in vivo BLM cytotoxicity depends on *Msh2* deficiency.

## 4. Discussion

The data obtained in this study indicate that the lack of function of the *Msh2* gene generates an overall genomic instability in germinal cells, reflected as genomic alterations in the progeny. This effect was also found in heterozygous individuals with a disrupted *Msh2* allele revealing the possible relevance of the global genomic instability found in heterozygous to tumorigenic process. Consistent with previous results [42], in our experiments the progeny of the $Msh2^{+/-}$ crosses did not show alterations related to the instability of simple repeated sequences; however, they did manifest a significant increase on the total genomic alterations respect to normal individuals ($P < 10^{-5}$). Thus, it seems that other functions of the MSH2 protein, different to MMR, are responsible for this global genomic stability. In addition, two functional *Msh2* copies are required to guarantee this stability. Therefore, and as suggested before, our results indicate that in heterozygous individuals, a limited function of the MSH2 protein could increase the risk of DNA alterations transmitted to the progeny [9,43]. In addition, in these individuals, the wild type *Msh2* allele would have a higher chance to mutate leading to a more severe genomic instability in somatic cells [9,44]. Thus, our in vivo results are in concordance with the mutator phenotype found in yeast cells containing only a disrupted *Msh2* allele [9].

The AP-PCR method has allowed us to study the implication of the *Msh2* gene in the genomic instability in the germline, through the fingerprint analysis of the genomic DNA of the progeny. This method has shown to be useful to detect DNA alterations in the human genome [3,24,45], as well as in experimental studies of transmitted DNA damage to the progeny of individuals exposed to genotoxic agents [25–28]. The genomic alterations detected in the AP-PCR fingerprints include loss of bands, new bands and band shifts. Band loss and gains could be caused by deleted

regions or modified primer annealing sites [10,24], whereas band shifts are related to simple repeated sequence instability [23,24,36]. Here, we show that the frequency of BSF, detected in the fingerprints, correlates with the frequency of MSI found by locus specific PCR (see Table 2). This indicates that the AP-PCR method can be used to estimate the instability of microsatellite related sequences, when the band shifts in the fingerprints are computerised separately.

In humans, there are evidences that environmental mutagens increase the mutation frequency and the risk of tumour development in the progeny of exposed parents [46–48]. These evidences are supported by experimental studies in mice [49]. In addition, mouse models for colon cancer have suggested that mutagen associated tumorigenesis is modulated by MMR function [29,49]. In the present study, we have found that the genomic damage induced by BLM in the *Drosophila* germline, and transmitted to the next generation, depends on the parental *Msh2* genotype. It is well documented that, under normal repair conditions, BLM induces a wide spectrum of mutagenic lesions, including DNA-strand breaks and base damage [40,41]. Interestingly, the progeny of the normal and heterozygous crosses for the *Msh2* gene present a significant increase in the total genomic damage, detected by AP-PCR, after exposure to BLM, but under complete lack of *Msh2* function no effect was detected. This result could be explained by germinal selection of the highly damaged spermatozoa of the treated $Msh2^{-/-}$ males. As shown in Table 1, the progeny of the $Msh2^{-/-}$ crosses present the highest level of spontaneous DNA damage that, together with the damage induced by BLM, could reach the tolerance limit. According to this assumption, in the $Msh2^{-/-}$ crosses an increase in sterility was observed after BLM treatment when compared to the control. This effect was not detected in heterozygous or wild type crosses (data not shown). In addition, the major sensitivity of $Msh2^{-/-}$ respect to $Msh2^{+/-}$ and $Msh2^{+/+}$ found in larvae, also agrees with this assumption (see Fig. 6). We can not discard that mutation-prone pathways, that require the Msh2 function, could process some of the lesions induced by BLM. In fact, recent studies have implicated the *Msh2* gene in double-strand break repair [11].

As indicated previously, a fraction of the damage detected by AP-PCR is related to microsatellite instability, i.e. BSF. Exposure of parental males to BLM produced a significant increase in the BSF in the progeny of individuals with one or two disrupted *Msh2* alleles, whereas no effect was detected in the progeny of normal individuals. A recent study indicates that oxidative stress induced by hydrogen peroxide generates MSI in MMR deficient human cells [37]. Therefore, the induction of BSF dependent on a defective MSH2 function found in our experiments, could be explained by the known effect of BLM producing oxygen radicals [30]. Furthermore, this result would confirm our previous observations about the possible effect of BLM in MSI under MMR deficient conditions [10].

In conclusion, our results indicate that the presence of one or two disrupted *Msh2* alleles produces genomic instability in germ cells leading to a heritable genomic damage, including MSI and other DNA alterations. In addition, the mutagenic stress produced by BLM is modulated by the *Msh2* genotype, being the heterozygous individuals susceptible to generate a wide spectrum of mutations after exposure to BLM. Hence, the use of an in vivo model system such as *Drosophila* could contribute to a better understanding of the implication of genetic and environmental factors in human cancer.

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# The *Drosophila melanogaster mus209*$^{B1}$ Mutant (PCNA) Impairs Genomic Stability. Analysis by DNA Fingerprinting

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## ABSTRACT

PCNA participates in multiple processes of DNA metabolism being essential for DNA replication and intervening in DNA repair. Temperature-sensitive PCNA mutants (*mus209*) of Drosophila are sensitive to mutagens, impair developmental processes and suppress positional-effect variegation. Here we show that the *mus209*$^{B1}$ allele has a mutator phenotype. Using the arbitrarily primed PCR fingerprinting technique (AP-PCR), we examined the accumulation of genetic alterations along the generations of *mus209*$^{B1}$ and *Canton-S* lines. In defective homozygous conditions, the *mus209*$^{B1}$ lines exhibited a high genomic instability that could be related to DNA synthesis defects as a cause of mutation. The analysis of changes in simple repeated sequences, either by AP-PCR or microsatellite loci, show that *mus209*$^{B1}$ also has a moderate effect increasing microsatellite instability, and we suggest that this effect might be due to a DNA polymerase processivity defects. The observed accumulation of genomic damage indicates that the *mus209*$^{B1}$ allele has different effects related to its role in DNA synthesis and repair.

## INTRODUCTION

Genomic stability is guaranted by the proper function of DNA metabolism. Hence, alterations in mechanisms as DNA replication, recombination, repair, or cell-cycle control suppose genetic changes with dramatic consequences to organisms. All these processes share some common factors; thus, a lack of function of shuch factors is expected to generate multiple alterations to the cells. On the other hand, these mutator phenotypes are characterized by increasing accumulation of DNA damage, a phenomenon well known as a putative cause of cancer (VOGELSTEIN *et al.* 1993). Proliferating cell nuclear antigen (PCNA) belongs to the group of genes involved in multiple processes of DNA metabolism for which a mutation suppressor effect has been reported (reviewed in WARBRICK 2000).

PCNA is a highly conserved protein that forms a homotrimeric complex that encircles double-strand DNA and slide along it, serving as the platform to bind diverse proteins (reviewed in KELMAN *et al.* 1998 and WARBRICK 2000). PCNA is essential for DNA replication and also participates in DNA repair. In DNA replication, PCNA is the processivity factor of DNA polymerases (KRISHNA *et al.* 1994; TSURIMOTO 1998) and stimulates the activity of FEN-1, involved in Okazaki-fragment maturation (LI *et al.* 1995; WU *et al*. 1996). It has also been reported that PCNA interacts with proteins involved in DNA repair (UMAR *et al*. 1996; GARY *et al.* 1997; GU *et al*. 1998; CLARK *et al*. 2000; FLORES-ROZAS *et al*., 2000) and cell cycle control proteins (WAGA *et al*. 1994; FLORES-ROZAS *et al*.1994; SMITH *et al*. 1994; WARBRICK *et al*. 1998). This wide capacity of PCNA to bind such diverse proteins suggests the important role of this factor in coordinating the cell cycle and DNA replication processes with DNA repair (WARBRICK *et al*. 1998; JONSSON *et al*. 1998).

In vivo studies show that mutations in the yeast PCNA (encoded by the *pol30* gene) cause sensitivity to genotoxic agents as UV and MMS (methyl methane sulfonate), have growth defects, and increase the level of spontaneous mutations (AYYAGARI *et al*. 1995; AMIN and HOLM 1996). Moreover, recent studies (CHEN *et al*. 1999; KOKOSKA *et al.* 1999) on the mutator phenotype of *pol30* mutants suggest that many of these mutants are simultaneously defective in multiple cellular processes, causing more that one mutagenic defect. Other studies have also implicated PCNA in DNA repair; thus, in vitro assays reveled that PCNA participates both in nucleotide and base excision repair (NICOLS and SANCAR 1992; SHIVJI *et al.* 1992; MATSUMOTO *et al*. 1994; FROSINA *et al*. 1996; KLUNGLAND and LINDAHL 1997), whilst in vivo genetic analysis demonstrated that PCNA is involved in mismatch repair (MMR) (JOHNSON *et al*. 1996; UMAR *et al*. 1996), postreplication repair (TORRES-RAMOS *et al*. 1996), and double-strand break (DSB) repair (HENDERSON and GLOVER 1998; HOLMES and HABER 1999).

Drosophila PCNA is encoded by the *mus209* gene (HENDERSON *et al.* 1994). The conditional lethality of temperature-sensitive (ts) *mus209* mutants, shows the essential role of this gene in cellular processes, and the genetic analysis of these mutants have been of great value to understand the role of PCNA in a high eukaryote organism. In addition to the role of Drosophila PCNA in essential cellular processes (HENDERSON *et al.* 1994; HENDERSON *et al.* 2000), *mus209* mutants are sensitive to MMS and γ-rays, indicating the participation of this gene in DNA repair (HENDERSON *et al.* 1994). Recent genetic studies with Drosophila PCNA mutants had reveled his involvement in DSB repair (HENDERSON AND GLOVER 1998).

Here we report the genomic damage acquired in the Drosophila *mus209*$^{B1}$ mutant, which is one of the best characterized PCNA alleles of Drosophila, having severe defects in ovary

development (HENDERSON 2000). This allele is a heat-sensitive lethal, in contrast to the majority of other alleles that, both in Drosophila and yeast, are cool-sensitive or unconditional lethals (AMIN and HOLM 1996; rewieved in HENDERSON *et al*. 1999).

In this study we have used a genomic DNA fingerprinting technique to measure the generalized genetic damage accumulated in *mus209*$^{B1}$ lines. Genomic fingerprints generated by arbitrarily primed polymerase chain reaction (AP-PCR) permit to detect DNA sequence variation in complex genomes (WELSH and MCCLELLAND 1990; WELSH and MCCLELLAND 1994). The technique is based on the amplification of random genomic sequences scattered in the genome by the use of PCR primers of arbitrary nucleotide sequence, and allows the detection of quantitative and qualitative changes in the genome. Previously, we have shown that this approach was useful to quantify the genomic damage in the Drosophila *mus201* mutant (LÓPEZ *et al*. 1999); as well as to detect tumor genomic alterations (PEINADO *et al*.1992).

Since it is well established that PCNA participate in multiple DNA metabolic processes, mutations in the *PCNA* gene could generate multiple genomic damage. Using the AP-PCR technique, we were able to display different types of genetic alterations accumulated in the *mus209* mutant. Thus, the analysis of DNA fingerprints and microsatellite loci reported here, shows that this allele has a mutator phenotype leading to general genomic instability and to a moderate increase of microsatellite instability. The observed genomic damage would indicate that the *mus209*$^{B1}$ allele is defective in DNA metabolism processes independently of mismatch repair.

## MATERIALS AND METHODS

**Drosophila *mus209$^{B1}$* strain and lines established:** The mutagen sensitive mutant *mus209$^{B1}$*: *b pr cn bw mus209$^{B1}$/Cy*, was kindly provided by DARYL S. HENDERSON (Department of Genetics, University of Cambridge, United Kingdom). For more details on the specific genetic markers see FLYBASE (1999). *mus209$^{B1}$* is a heat-sensitive lethal mutant that fails to complete development when it is cultured at 29º (HENDERSON et al. 1987). In addition, *mus209$^{B1}$/mus209$^{B1}$* females are unconditionally sterile, but males are fertile at permissive temperature, 22º ± 1º (HENDERSON et al. 1994).

Three sets of lines were established by selecting homozygous males *mus209$^{B1}$/mus209$^{B1}$*, heterozygous males *mus209$^{B1}$/Cy* or *Canton-S* individuals as parentals of each generation. For set (1), *mus209$^{B1}$/mus209$^{B1}$* males and *mus209$^{B1}$/Cy* females were selected and mated individually and 25 lines were constructed. From the offspring of these crosses, *mus209$^{B1}$/mus209$^{B1}$* sons and *mus209$^{B1}$/Cy* daughters were selected and mated to a ratio of two males with three females. To maintain these lines, the same selection procedure was followed every generation. For set (2), *mus209$^{B1}$/Cy* males and females were selected and mated individually to constitute 27 lines. From the offspring of each generation, *mus209$^{B1}$/Cy* sons and daughters were mated to a ratio of two males with three females, to maintain these lines. For set (3) *Canton-S* males and females were selected and mated individually to constitute 20 lines. The lines were maintained as in sets (1) and (2). We called these sets of lines *mus209$^{-/-}$*, *mus209$^{+/-}$* and *mus209$^{+/+}$*, respectively. The lines were maintained at 23º ±1º for six generations. From all the lines, the founders male and female, as well as the offspring from generation 3 (G3) and generation 6 (G6) of *mus209$^{-/-}$* and *mus209$^{+/+}$* lines, and offspring from G6 of *mus209$^{+/-}$* lines were collected and kept at –80º.

**Genomic DNA isolation:** Individual flies kept at –80º were used for genomic DNA extractions, following the method described by ROBERTS (1986) and dissolved in 20-30 µl of double-distilled water.

**AP-PCR amplification:** 25 µl of reaction was prepared using 1 µl of the DNA stock solution from individual flies (~ 50 ng of DNA) in 1 x PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 8.4, 0.01% gelatin), 4 mM $MgCl_2$, 125 µM of each dNTP, 3 µCi of [α-$^{33}$P]dATP, 1 U of *Taq* DNA polymerase and 0.25 µM of the arbitrary primer WB (5'-GTTAGGGAGCCGATAAAGAG-3'). The reactions were carried out in a Programmable Thermal Controller (PT-100 MJ Research, USA). A preincubation for 3 min at 94º was followed for five cycles in low stringency conditions (94º for 30 s, 40º for 30 s, and 72º for 90 s) and then for 30 cycles in high stringency conditions (94º for 15 s, 55º for 15 s, and 72º for 60 s). Finally, 3µl of reaction was fractionated by electrophoresis on a 6% denaturing polyacrylamide gel (6 h at 45 W) and visualized by autoradiography.

**Quantification of genomic damage by AP-PCR:** Following our previous criteria (LÓPEZ et al. 1999), the fingerprinted bands considered for the assessment of genomic damage were those reproducible and clearly visualized in the autoradiography of each repeated experiment. To estimate the total genetic alterations accumulated at the different times studied, G3 and G6 in *mus209*$^{-/-}$ and *mus209*$^{+/+}$ lines, and G6 in *mus209*$^{+/-}$ lines, the number of altered bands in the progeny of each line were scored. At each point of study, the sum of all detected changes (increases and decreases of band intensity, new bands and mobility shifts) in the fingerprinted bands of the total progeny analyzed was divided by the total number of reproducible amplified bands (28 or 27 fingerprinted bands with primer WB x nº of analyzed individuals, in *mus209*$^{B1}$ lines or *Canton-S* lines, respectively).

The indexes corresponding to the total accumulated damage (damage fraction, DF) and to the mobility shifts of bands (band shifts fraction, BSF) were calculated. The same procedure was used to estimate the accumulated damage per line or per individual, at the different times studied. Statistical analysis between the different lines and point of study was carried out using the Wilconxon test and the Fisher´s exact test, two-tailed.

**Microsatellite analysis:** The same individuals analyzed by AP-PCR were analyzed for microsatellite loci. PCR reactions were performed using 1 μl of the genomic DNA stock solution (~ 50 ng of DNA) in a 25 μl of reaction volume. PCR was carried out as described above, using 2.5 or 4.0 mM concentration of $MgCl_2$ and [$\alpha$-$^{33}$P]dATP or [$\alpha$-$^{33}$P]dCTP. The reaction took place for 30 high-stringency cycles using 55º to 58º, as the annealing temperature, depending on the primers. The primers used were described by SCHUG *et al*. (1998) and are summarized in Table 1, where the amplification conditions are also specified. PCR products were separated on 6% denaturing polyacrylamide gels (2h at 45W) and visualized by autoradiography. Fisher´s exact test, two-tailed, was used for statistical analysis.

## RESULTS

**Genomic fingerprints display genetic diversity in the *mus209$^{B1}$* strain:** As expected, AP-PCR DNA fingerprints of *mus209$^{B1}$* flies are different from those obtained of normal flies (*CS* strain), due to polymorphic differences between these strains (see Figure1). However, whereas we found no differences within *CS* stock after analysing 40 individuals (LÓPEZ et al., 1999), many of the fingerprint bands showed differences between *mus209$^{B1}$* flies. These findings reveled that genetic differences exist between individuals of the

*mus209*$^{B1}$ stock (Figure 1); specifically, analysing the fingerprints of 60 *mus209*$^{B1}$ individuals we observed that 14 out of the 28 reproducible bands were polymorphic in these flies.

Therefore, AP-PCR fingerprints give us an indication that there is an elevated rate of genetic variation in the *mus209*$^{B1}$ strain. Based on this observation, the aim of the current study was to analyze the accumulation of general genetic alterations in the *mus209*$^{B1}$ mutant over time, as consequence of endogenous mutation mechanisms associated to altered PCNA in this strain.

**Genetic alterations detected in *mus209*$^{B1}$ strain by AP-PCR:** To test the accumulated damage in the *mus209*$^{B1}$ mutant, two sets of *mus209*$^{B1}$ lines, called *mus209*$^{-/-}$ and *mus209*$^{+/-}$ lines were established, and compared to *Canton-S* lines used as control lines (*mus209*$^{+/+}$ lines). The *mus209*$^{B1}$ lines were obtained by selecting homozygous males *mus209*$^{B1}$*/mus209*$^{B1}$ and heterozygous males *mus209*$^{B1}$*/Cy* as parentals of each generation, respectively. In both type of lines, *mus209*$^{B1}$*/Cy* fertile females were used as parental (see MATERIAL AND METHODS). In addition to estimating the total genetic alterations arising in each genetic background, the present experimental design also allowed us to calculate the emerged mutations in the different lines (families), as well as to analyze the distribution of the genomic damage accumulated in the individuals of each set of lines.

At the different times evaluated (G3 and G6 for *mus209*$^{-/-}$ and *mus209*$^{+/+}$ lines and G6 for *mus209*$^{+/-}$ lines), genetic changes were quantified in each line by comparing the fingerprints of emerging flies with the fingerprints of the founders male and female (Figure 2). The most common type of changes found in all experiments was changes of band intensity and new bands (i.e. 94.8% and 93.3% of the total damage accumulated in *mus209*$^{-/-}$ lines at G3 and G6, respectively; 87.0% at G6 in *mus209*$^{+/-}$ lines; and 93.8% and 100% in

*mus209*$^{+/+}$). The remaining changes were due to mobility shifts of bands and, although these changes were computed to estimate the total damage, we also considered them separately, since this type of alteration is assumed to reflect instability of single repeated sequences (see below).

All the established mutant lines show accumulation of damage, and the average number of altered bands per individual was higher in *mus209*$^{-/-}$ lines than in *mus209*$^{+/-}$ lines, i.e. 2.90 ± 3.57 and 1.04 ± 1.51 at G6, respectively (Table 2), whilst in the control lines was 0.06 ± 0.24 at G6. The *mus209*$^{-/-}$ lines also show more alterations per individual at G6 than that at G3 (see Table 2), indicating that the genomic changes emerge progressively along the time. An example of fingerprints of individuals displaying high damage is shown in Figure 2.

It is important to point out that, when analyzing the AP-PCR fingerprints of the progeny of a line at each defined generation, we did not find more than two individuals showing the same type and number of alterations. This observation indicates that the progeny of any given line (family) have an independent origin; therefore, in our analysis we can discard the possibility of overestimating the number of alterations, when some of the siblings came from the same premeiotic event.

**Accumulated genomic damage in *mus209*$^{B1}$**: The total fraction of genetic alterations (DF), based on the number of altered bands found in the AP-PCR fingerprints was estimated. The total DF, at the different times studied, indicates that the *mus209*$^{B1}$ mutant is able to accumulate genetic alterations along the generations. As shown in Table 2, the DF estimated from all the *mus209*$^{-/-}$ lines at G6 is higher than at G3, although the difference is not statistically significant (G6: 0.102, G3: 0.080; $P = 0.195$). This increase in the DF can also be appreciated in each line at G6 and G3 (see Figure 3A). Seven of the ten *mus209*$^{-/-}$ lines

presented an increase of damage, and three of these lines (lines 9, 12, 32) show an increase of damage more that two-fold from G3 to G6. The slight reduction of damage from G3 to G6 in lines 5, 18 and 27, could be explained by negative selection of the highly damaged individuals arising over the generations in these lines. We also considered that this fact could be the cause of lack of statistical significance in the DF at G6 vs. G3 of the *mus209*$^{-/-}$ lines.

The analysis of *mus209*$^{+/-}$ lines shows that genetic damage was also accumulated when the lines were maintained in heterozygous conditions and compared with the *mus209*$^{+/+}$ lines (Figure 3B and 3C). However, the control *mus209*$^{+/+}$ lines showed a similar DF at G3 and G6, being an order of magnitud lower than in *mus209* mutant lines (see Table 2).

Therefore, these studies put into manifest that flies with altered *mus209* gene present genomic instability, being the damage accumulated dependent of the genetic background, being much lower in the *mus209*$^{+/-}$ lines than in the *mus209*$^{-/-}$ lines (see Figure 3). As indicated in Table 2, a significant difference in the DF was observed at G6 in the two sets of deficient lines respect to the control lines ($P<10^{-5}$ for both sets). A significant difference was also found at G6 between the two sets of deficient lines ($P<10^{-5}$).

**Distribution of genetic alterations in the analyzed individuals:** The distribution of the number of fingerprinted alterations in the analyzed individuals is represented in Figure 4. It is interesting to point out that the number of altered bands have a wider range in individuals of the *mus209*$^{-/-}$ lines than in individuals of the *mus209*$^{+/-}$ and *mus209*$^{+/+}$ lines, being the former at G6 where the individuals with the highest number of alterations were found; i.e.15% of individuals *mus209*$^{-/-}$ presented six or more alterations, with one individual showing 19 altered bands (data not shown). At G3, only 5.8% of individuals of this set presented six or more alterations, and a maximum of 10 alterations were found in two individuals. A maximum of seven alterations were found in one individual of the *mus209*$^{+/-}$ lines at G6,

where six alterations were also observed in three of these individuals (total 4.9%). However, the number of alteration found in the individuals of the *mus209*$^{+/+}$ lines was very low, with a maximum of two alterations per individual observed in 5% of individuals at G3 and 1% of individuals at G6.

We also want to point out that under the *mus209*$^{-/-}$ background the majority of individuals show at least one fingerprinted alteration, being the percentage of individuals altered similar for G3 and G6 (81.4% and 84.8%, respectively). Therefore, the difference between these two points of study resides in a major accumulation of genetic alterations for G6 individuals. In contrast, the amount of individuals showing alterations in the *mus209*$^{+/-}$ background was 54.3%; and the number of individuals with alterations decrease to 10% in the *mus209*$^{+/+}$ lines.

**Microsatellite instability in *mus209*$^{-/-}$ lines:** It has been suggested that PCNA contributes to general genomic stability; and, in addition, some of the yeast PCNA mutants also lead to microsatellite instability. To test whether the *mus209*$^{B1}$ mutation affects the stability of simple repetitive sequences we followed two different approaches. First, in the analysis of the AP-PCR, we quantified the bands that showed mobility shifts separately. It has been previously demonstrated that this type of qualitative change reflects microsatellite instability (PEINADO *et al*., 1992; IONOV *et al*., 1993). The other approach consisted in using the DNA of the same individuals analyzed by AP-PCR, to carry out PCR amplifications of five microsatellite loci.

In the DNA fingerprints, the fraction of band shifts (BSF) was estimated out of the total number of analyzed bands. In Table 2 we can appreciate that the BSF is similar in the homozygous and heterozygous *mus209*$^{B1}$ lines and significantly higher than the BSF in the *mus209*$^{+/+}$ lines. However, the rate of band shifts with respect to the total altered bands

reflects the contribution of the former type of alteration to the total damage. As shown in Table 2, 13.5% of the total alterations found in the *mus209*$^{+/-}$ lines at G6 correspond to mobility shifts; by contrast, this type of change represents only 6.8% and 6.6% of the total damage at G6, in the *mus209*$^{-/-}$ and *mus209*$^{+/+}$ lines, respectively.

As can be appreciated in Table 3, the results obtained analyzing the microsatellite sequences agree with those obtained by AP-PCR. No mutations were found in the *mus209*$^{+/+}$ lines, few mutations were found in the individuals with a *mus209*$^{-/-}$ background and the individuals with a *mus209*$^{+/-}$ background presented mutations in four out the five loci analyzed. Altogether these results suggest that the *mus209*$^{B1}$ mutant affects microsatellite stability with a major effect in heterozygous conditions.

## DISCUSSION

Drosophila PCNA mutants provide the opportunity to carry out complex genetic analyses to increase our understanding on the function of PCNA in a high eukaryote system. Previous studies of mutants of *Drosophila melanogaster*, carried out by HENDERSON *et al.* (1994; 1998) demonstrated the implication of PCNA in several cellular processes, with an essential role in development, in the maintenance of chromatin structure, and to overcome induced genetic damage. In the present work we ask if the Drosophila *mus209*$^{B1}$ mutant has a mutator phenotype that increases the level of spontaneous mutations. In fact a mutator suppressor effect has already been attributed to the yeast PCNA (reviewed in WARBRICK 2000).

Our first insight indicating that the Drosophila *mus209*$^{B1}$ mutant could have an intrinsic mutator phenotype came from the observation that individuals of this stock show a differential genomic fingerprint by AP-PCR, in comparison with a normal strain. In addition,

in the present study, using the AP-PCR technique in controlled experiments, we have shown that the *mus209*$^{B1}$ mutation leads to the accumulation of genomic damage along the generations, which prove genomic instability in the germ-line of these individuals. This observation provides the first experimental evidence of a PCNA mutant accumulating germ-line mutations. Since increases in the mutation rate were found in yeast PCNA mutants (MERRILL and HOLM 1998; CHEN *et al*. 1999), we assume that multiple genetic alterations might also arise in the somatic cells of Drosophila PCNA mutants. Taking into account that our studies on genomic instability with wild type Drosophila strain show no accumulation of genetic damage, under the same experimental conditions, the clear increase in genetic damage observed in *mus209*$^{B1}$, both in homozygous and heterozygous conditions, would indicate that the PCNA heterotrimers formed in the heterozygous individuals are important enough to deplete the normal function of PCNA.

The AP-PCR DNA fingerprinting technique can display genomic differences as quantitative or qualitative changes in the fingerprinted bands, and the origin of these changes lies in point mutations and rearrangements in the genome (PEINADO *et al*. 1992; IONOV *et al*. 1993; LÓPEZ *et al*. 1999). Taking into account that almost any kind of genomic change is potentially detected by AP-PCR, and that this technique allows the analysis of anonymous sequences in an unbysed manner, we chose this simple method to measure the accumulated genomic damage in a PCNA deficient background.

In both sets of *mus209*$^{B1}$ lines, a variety of changes in the fingerprinted bands (changes of band intensity, new bands and mobility shifts) were observed. Previous experimental evidences indicate that changes of band intensity and new bands could be originated by changes in the primer annealing sequences, and/or by more complex rearrangements (PEINADO *et al*. 1992; LÓPEZ *et al*. 1999); whereas changes in band mobility could be

originated by small changes in microsatellite sequences (IONOV *et al*. 1993). Furthermore, it must be pointed out that these changes might have an independent origin; and, thus, the genomic instability detected as quantitative changes by a PCR-based DNA fingerprinting technique, can be considered to be independent of the instability arising from DNA mismatch repair defects (BASIK *et al*., 1997; LÓPEZ *et al*. 1999). Therefore, the different types of alterations that we observed in the *mus209*$^{B1}$ lines could also be originated by different processes. Moreover, the fact that the protein encoded by *mus209*$^{B1}$ only has one aminoacid substitution (HENDERSON *et al*. 2000), indicates that this single change is enough to lead to multiple genetic alterations. Studies with yeast PCNA mutants have also shown more than one mutagenic defect, this would agree with the idea that multiple cellular processes could be affected in these mutants (CHEN *et al*. 1999; KOKOSKA *et al*. 1999).

The genomic instability, detected as new bands and quantitative changes by AP-PCR, represent the major type of alteration observed in our studies with the *mus209*$^{B1}$ mutant (93.2% and 86.5% of the total damage at G6 in *mus209*$^{+/-}$ and *mus209*$^{-/-}$ lines, respectively). This instability may be related to a DNA synthesis deficiency in the *mus209*$^{B1}$ mutant, because, as was previously suggested, the impair DSB-repair in Drosophila PCNA mutants might be due to a failure at the DNA synthesis step (HENDERSON and GLOVER 1998), which has already been demonstrated in yeast (HOLMES and HABER 1999). Since *mus209*$^{B1}$ flies are viable, at permissive temperature, a DNA synthesis deficiency must allow replication processes to be functional in this mutant. Another plausible explanation for this type of genomic instability can be the lack of stimulation of the FEN-1 activity. Mutations in the FEN-1 gene accumulate DSBs in yeast, increasing the number of deletions and duplications (TISHKOFF *et al*. 1997).

Microsatellite instability is a phenotype used as an indicator of defective DNA mismatch repair. However, the instability of simple repetitive sequences can also arise when components of the DNA replication machinery are altered; for example, DNA polymerase δ, FEN-1 and PCNA (KOSKOSKA *et al*. 1999 and herein references). Recent in vitro and in vivo studies indicate that PCNA is required for DNA mismatch repair (UMAR *et al*. 1996; GU *et al*. 1998; CLARK *et al*. 2000; FLORES-ROZAS *et al*. 2000) and, although the role of PCNA in this repair pathway is not completely understood, a model has been proposed with the implication of PCNA in strand discrimination (UMAR *et al*. 1996). All these evidences indicate that PCNA mutants could manifest microsatellite instability, either by impairing DNA mismatch repair or by an independent mechanism related to its role in genomic DNA replication.

In our studies, both AP-PCR and microsatellite analysis show that *mus209* is required to maintain the stability of simple repetitive DNA sequences, although the increase of mutations on these sequences is lower than for the other types of alterations detected (see Table 2). The alterations in microsatellite loci found in $mus209^{B1}$ indicate that these sequences are more unstable in this mutant than in wild type, where the average rate of changes in microsatellite sequences per generation has been reported to be about $6x10^{-6}$ (SCHUG *et al*. 1997; SCHÖLOTTERER *et al*. 1998). We also found that, under our experimental conditions, heterozygous background shows more alterations in microsatellite sequences than the homozygous mutants (BAIDA *et al.*, 2003). Taking together all our results, the moderate increase of microsatellite instability joined the high general genomic instability in $mus209^{B1}$, we can suggest that the $mus209^{B1}$ allele has a defect in DNA metabolism processes, independent of mismatch repair, leading to general genomic instability but allowing functional DNA replication. This genomic instability of $mus209^{B1}$ could be explained by two

non-exclusive pathways: mutant PCNA would confer deficiency in DNA polymerase processivity, and/or would make the repair DNA synthesis defective. Both interpretations would be in agreement with the implication of the *mus209* gene in DNA repair and in development processes (HENDERSON *et al*. 1994; HENDERSON and GLOVER 1998).

The instability of microsatellite sequences could be explained if the *mus209*$^{-/-}$ lines had a deficiency in DNA polymerase processivity, the effect of this alteration being less effective to produce microsatellite instability than a mutation in mismatch repair genes. Although we do not have an explanation for the higher microsatellite instability found in heterozygous, with respect to homozygous conditions in *mus209*$^{B1}$ lines, the contrary is true for the increase of general genomic instability. Since PCNA is a multifunctional factor, it is reasonable to think that a mutant PCNA homotrimer behaves differently depending on the processes where it is involved. Nevertheless, and regardless which is the specific mechanism through which the *mus209*$^{B1}$allele produces genomic instability, the data reported here indicates that the mutator phenotype shown by the PCNA mutants, would be particularly important in cancer susceptibility. In this context, different polymorphic variants of PCNA mutants might exist in humans and, those variants with a weak mutator effect and affecting the stability of simple repeated sequences, could allow the accumulation of genomic damage through generations.

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**FIGURES**

Figure 1

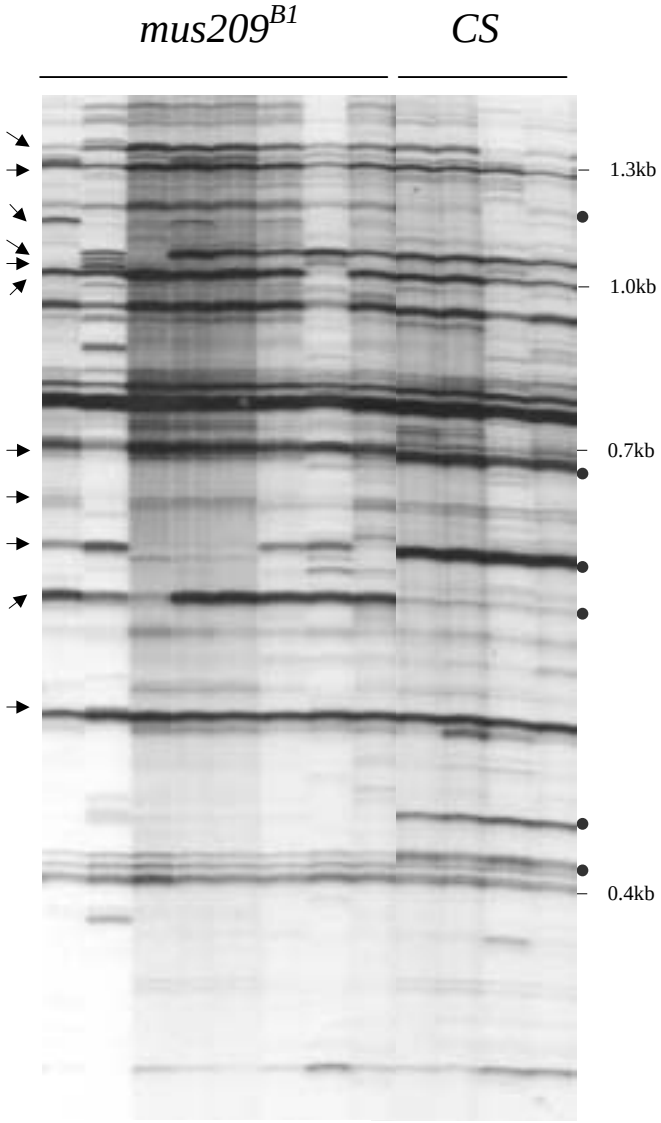
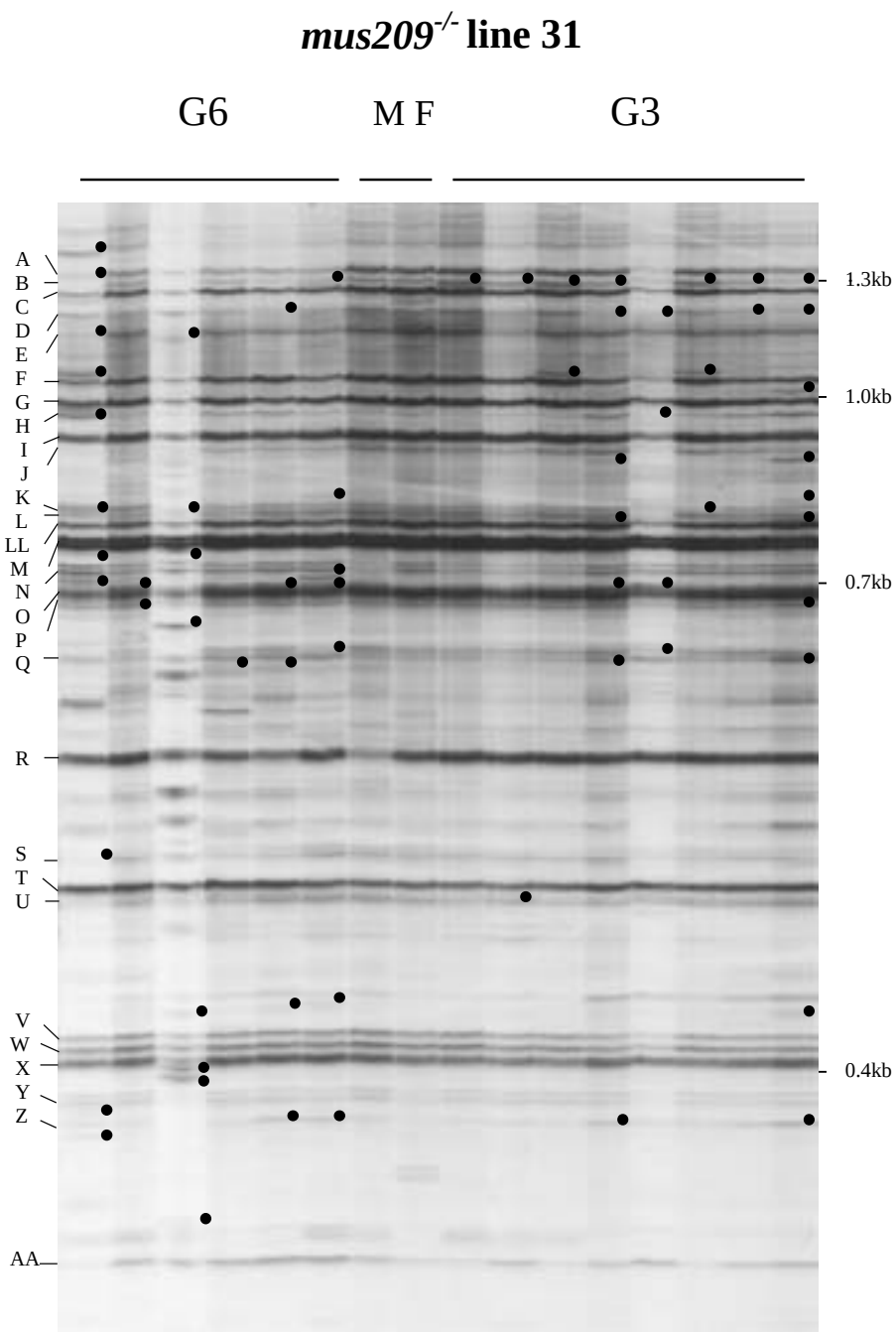


Figure 2


mus209-/- line 31
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Z
AA
1.3kb
1.0kb
0.7kb
0.4kb

Figure 3

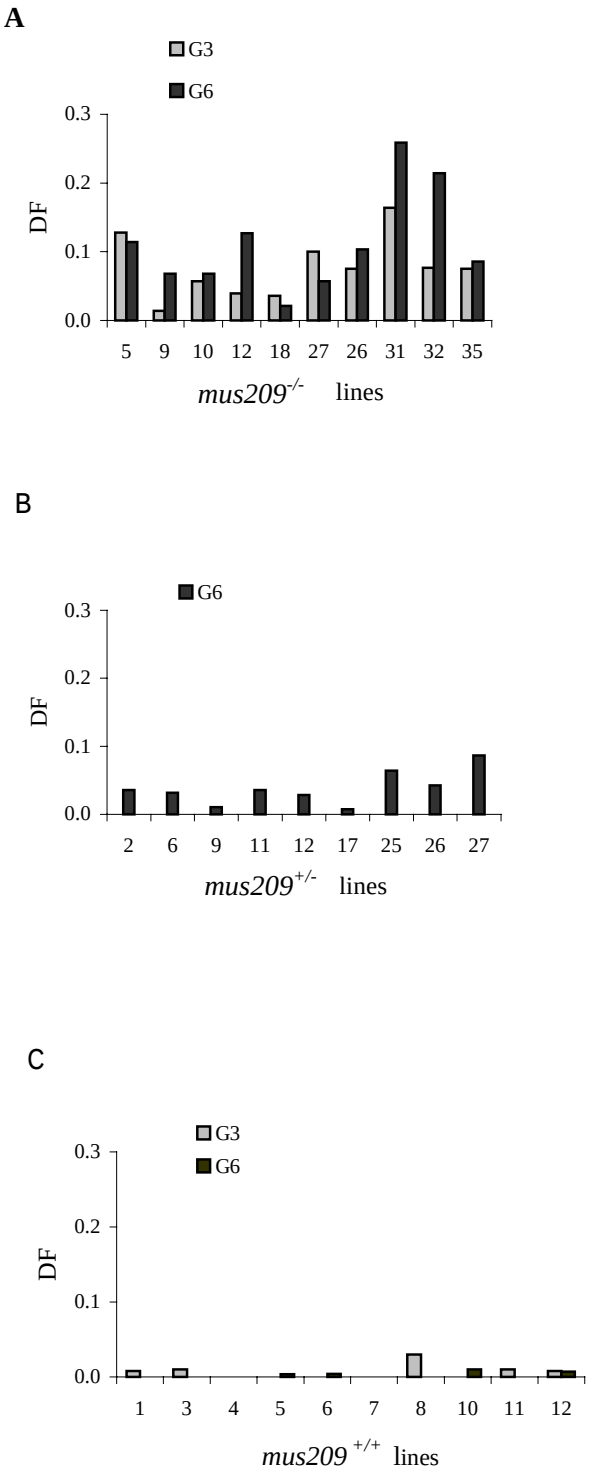
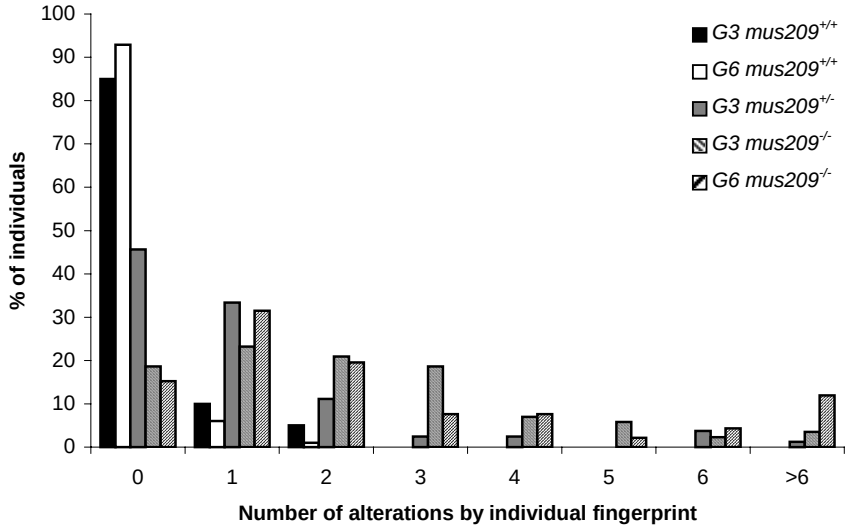

A
G3
G6
0.3
0.2
0.1
0.0
DF
5 9 10 12 18 27 26 31 32 35
mus209-/- lines
B
G6
0.3
0.2
0.1
0.0
DF
2 6 9 11 12 17 25 26 27
mus209+/- lines
C
G3
G6
0.3
0.2
0.1
0.0
DF
1 3 4 5 6 7 8 10 11 12
mus209 +/+ lines

Figure 4


100
90
80
70
60
50
40
30
20
10
0
% of individuals
0
1
2
3
4
5
6
>6
Number of alterations by individual fingerprint
G3 mus209+/+
G6 mus209+/+
G3 mus209+/-
G3 mus209-/-
G6 mus209-/-

## FIGURE LEGENDS

FIGURE 1.—AP-PCR fingerprints of genomic DNA of *mus209*$^{B1}$ and *CS* individuals. Polymorphic differences between *mus209*$^{B1}$ and *CS* strains are indicated with a black dot (•) on the right. Differences between *mus209*$^{B1}$ individuals are indicated with an arrow ( → ) the left. Sizes of the bands are shown on the right.

FIGURE 2.—Example of accumulation of genomic alterations at G6 and G3 of line 31 from *mus209*$^{-/-}$. AP-PCR fingerprints of genomic DNA of founders male (M) and female (F) and progeny at G3 and G6 are presented. Changes in the fingerprinted bands are indicated with a black dot (•). Sizes of the bands are shown on the right.

FIGURE 3. —Accumulated genomic damage in *mus209*$^{B1}$ lines. (A) Damage fraction (DF) obtained in *mus209*$^{-/-}$ lines, at G3 and G6. (B) DF obtained in *mus209*$^{+/-}$ lines at G6. (C) DF obtained in *mus209*$^{+/+}$ lines at G3 and G6.

FIGURE 4. —Distribution of genetic alterations found in individual fingerprints in *mus209*$^{-/-}$ lines at G6 and G3, in *mus209*$^{+/-}$ lines at G6 and in *mus209*$^{+/+}$ lines at G3 and G6.

# IV. DISCUSIÓN

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Tal como se ha mencionado en la introducción, durante estos últimos años se ha puesto especial interés en el estudio de los procesos implicados en el mantenimiento de la estabilidad del genoma, constituyendo una línea de investigación importante puesto que se encuentra relacionada con la susceptibilidad a padecer cáncer, así como, a desarrollar algunos síndromes genéticos.

Dentro de este contexto, nuestro interés general es conocer la implicación de la reparación del DNA en la estabilidad del genoma. Así, uno de los objetivos que nos hemos propuesto en este trabajo ha sido analizar, utilizando *D. melanogaster,* cuál es el papel de los genes *msh2* y *PCNA* en el mantenimiento de la estabilidad genómica. Para ello, hemos utilizado dos técnicas moleculares: la AP-PCR, que nos permite detectar daño general en el genoma, y el análisis de *loci* microsatélite por PCR, que nos permite detectar inestabilidad en puntos concretos del genoma. Además, hemos tratado de determinar, el papel del sistema de MMR en la modulación de la inducción de daño genómico producido por distintos compuestos químicos.

Así, antes de comenzar con estos estudios nuestro primer objetivo ha consistido en la puesta a punto de la técnica de AP-PCR en *Drosophila,* para validar su utilidad en el análisis del daño genómico en este organismo.

## 1. Detección de alteraciones genómicas en *Drosophila* mediante AP-PCR

Aunque la AP-PCR ha sido utilizada con éxito en distintos organismos para la detección de alteraciones en sus genomas (Kubota *et al.,* 1992; Peinado *et al.,* 1992; Ionov *et al.,* 1993; Kohno *et al.,* 1994; Kubota *et al.,* 1995; Shimada y Shima, 1998; Vasil´eva *et al.,* 2001), esta técnica no había sido usada anteriormente en *Drosophila*. Tal como ya se ha comentado, nos planteamos la utilización de esta técnica como base experimental para el

estudio de la inestabilidad genómica en *Drosophila*; por lo tanto, nuestro primer objetivo consistió en poner a punto la técnica de la AP-PCR en este organismo y, seguidamente, comprobar si el método nos permitía detectar de un modo eficaz daño en el DNA.

El primer paso consiste en seleccionar cebadores adecuados. En un principio, los cebadores utilizados en las reacciones de AP-PCR son elegidos arbitrariamente, con la condición de que generen un *fingerprint* reproducible del genoma, siendo suficiente un solo cebador para producirlo (Welsh y McClleland, 1990). Para poder seleccionar los cebadores que se utilizarían en nuestros experimentos se partió de una batería de cebadores que se usaron individualmente o en parejas en reacciones de AP-PCR preliminares, en las que se variaron las condiciones de amplificación (distintas temperaturas de apareamiento del cebador y de concentraciones de $Mg^{2+}$). Seis de estos cebadores proporcionaron un *fingerprint* reproducible de 20-30 bandas bien definidas (resultados no mostrados). Entre ellos, se seleccionó el cebador WB (ver Materiales y Métodos, **Anexo 1**) para llevar a cabo el primer trabajo de detección de daño genómico en *Drosophila* mediante el análisis de *fingerprints* obtenidos por AP-PCR (**Anexo 1**).

El análisis de daño genómico se llevó a cabo en un mutante deficiente en reparación, *mus201*, y en una cepa normal para la reparación, *Canton-S*. De esta forma podíamos determinar si el gen *mus201*, implicado en la reparación por escisión (NER) (Boyd *et al.,* 1987), tiene un papel la estabilidad del genoma. Además, ambas cepas se sometieron al tratamiento con 2-acetilaminofluoreno (2-AAF) con el fin de determinar si la técnica de AP-PCR nos permite detectar la ya conocida implicación del gen *mus201* en la reparación del daño inducido con este mutágeno (Heflich y Neft, 1994).

El estudio del daño genómico espontáneo e inducido por el tratamiento, relacionado con la distinta eficacia en la reparación del DNA de ambas cepas, se realizó en las células germinales, analizando para ello el daño transmitido a la descendencia. Así, se analizaron los *fingerprints* obtenidos a partir del DNA de moscas individuales de la descendencia de los cruces establecidos y se compararon con los *fingerprints* de los padres.

Los cruces y tratamientos se realizaron siguiendo el protocolo usado en los estudios de mutagénesis inducida en *Drosophila* (Vogel y Natarajan, 1995). En nuestro caso, se tratan

células postmeióticas de machos de la cepa *Canton-S* las cuales se cruzan con hembras *Canton-S* o *mus201* para determinar, así, la capacidad reparadora de las hembras.

Un análisis previo de los *fingerprints* obtenidos por AP-PCR de 40 individuos de cada una de ellas, *mus201* y *Canton-S* , mostró que entre los individuos de cada una de las cepas no había diferencias genómicas detectables en los patrones de bandas. Este hecho teniendo en cuenta nuestro sistema de análisis nos sugiere que el gen *mus201* no está directamente relacionado con la estabilidad genómica. Sin embargo, al comparar los *fingerprints* de ambas cepas detectamos diferencias en el patrón de bandas debido a la presencia de polimorfismos específicos de cada una de ellas (Figuras 1 y 2, **Anexo 1**). Estos polimorfismos son extremadamente útiles para caracterizar cada una de las cepas y para identificar cualquier error de cruzamiento, además, permiten identificar fácilmente la progenie de un cruce entre dos cepas cuando existe un polimorfismo de longitud (por ejemplo, banda I en la Figura 1, **Anexo 1**).

Para estimar el daño inducido por el 2-AAF en la línea germinal y no reparado tras la fecundación, se analizaron las alteraciones en las bandas de los *fingerprints* de la descendencia, contabilizando los incrementos y disminuciones de intensidad de bandas, cambios de movilidad, desaparición de bandas y la aparición de bandas nuevas. Así, se calculó la relación entre el número de bandas alteradas y el número total de bandas amplificadas por individuo, denominada PDF (***P****rogeny* ***D****amage* ***F****raction*), como una estima del daño genómico (Arribas *et al.,* 1997; Basik *et al.,* 1997). Nuestros resultados demuestran claramente que la inducción de daño por 2-AAF depende de la NER: el PDF en los cruzamientos de machos tratados con hembras *mus201* fue de un 21%, mientras que en los cruces con hembras *Canton-S* fue sólo del 1%. Estos resultados concuerdan con el hecho de que la mayoría de los aductos producidos por el 2-AAF son eliminados por la NER (Heflich y Neft, 1994).

Por otra parte en la puesta a punto de la técnica de AP-PCR en cualquier organismo es necesario comprobar que los cambios observados en los *fingerprints* se corresponden con alteraciones reales en el genoma. Para ello, se clonaron y secuenciaron dos de las bandas que presentaban cambios en la descendencia respecto a sus progenitores. A partir de la secuencia se diseñaron cebadores específicos internos que se utilizaron para la posterior amplificación específica por PCR de estas bandas utilizando DNA de individuos con y sin la

alteración. Esta amplificación específica muestra el mismo patrón que el obtenido por AP-PCR (Figura 5, **Anexo 1**), lo cual nos confirma que las alteraciones encontradas en los *fingerprints* se corresponden con alteraciones del genoma.

Tal como hemos indicado en la introducción (2.2.1), basándonos en estudios previos realizados con AP-PCR (Peinado *et al.,* 1992; Ionov *et al.,* 1993), las alteraciones de bandas se pueden clasificar en dos grupos: (I) cambios de intensidad en las bandas, desaparición de bandas y aparición de bandas nuevas, que pueden ser debidos a cambios en las secuencias de apareamiento del cebador o a reorganizaciones complejas del DNA, y (II) cambios de movilidad de las bandas, que pueden ser originados por pequeños cambios en secuencias microsatélite. En nuestros experimentos, de la fracción de daño total inducido por 2-AAF en *mus201*, el 93,3% representa alteraciones de tipo I y el 6,7% alteraciones de tipo II. La baja frecuencia de alteraciones de tipo II está correlacionada con la falta de inducción de inestabilidad de microsatélites por el 2-AAF en *mus201*, tal como comprobamos tras el análisis de 9 *loci* microsatélite. Se sabe que algunas de las lesiones inducidas por el 2-AAF son reconocidas por las proteínas de la MMR (Li *et al.,* 1996) y, por consiguiente, existe la posibilidad de producir inestabilidad de microsatélites en fondos genéticos deficientes en la MMR. En nuestro caso, tanto la cepa *mus201* como la *Canton-S* son normales para la MMR, por ello, la inducción de alteraciones de movilidad de bandas es baja.

Como conclusión, la técnica de AP-PCR es adecuada y suficientemente sensible para el análisis del daño genético en *D. melanogaster.* De manera sencilla se obtienen *fingerprints* reproducibles que nos permiten diferenciar cepas, así como cuantificar daño genómico y determinar la implicación de los sistemas de reparación del DNA. En esta tesis hemos aplicado esta técnica para establecer la implicación de los genes *msh2* (**Artículo 2**) y *PCNA* (**Artículo 3**) en la estabilidad del genoma de *Drosophila* .

# 2. Inestabilidad genómica espontánea en la línea germinal asociada a una deficiencia en *msh2*

El sistema de MMR es crucial para la corrección de los errores producidos durante la replicación. Como ya hemos indicado, en distintos organismos la alteración de alguno de los genes implicados en la MMR produce un incremento de la tasa de mutación espontánea, así como MSI (Ionov *et al.,* 1993; Bhattacharyya *et al.,* 1994; Umar *et al.,* 1994; Glaab *et al.,* 1997), siendo ambas, características de un fenotipo mutador.

Algunos de los genes implicados en la MMR también intervienen en otros procesos celulares, siendo el gen *MSH2* un buen ejemplo de ello. Su alteración provoca MSI (de Wind *et al.,* 1995; Drotschmann *et al.,* 1999) y está asociada al HNPCC (Peltomäki, 2001b). Además, este gen interviene en la DSBR (Sugawara *et al.,* 1997), en la TCR (Mellon *et al.,* 1996) y en la respuesta celular al daño en el DNA (Branch *et al.,* 1993; Fink *et al.,* 1996; Fritzell *et al.,* 1997; Yan *et al.,* 2001). Por lo tanto, las propiedades multifuncionales de la proteína MSH2 sugieren que una deficiencia o alteración de la misma puede provocar inestabilidad general en el genoma, aparte de MSI (Fritzell *et al.,* 1997; Yan *et al.,* 2001).

Para determinar el papel del gen *msh2* en la estabilidad genómica de *Drosophila* utilizamos el mutante *spel1*, que presenta una deleción de este gen (Flores y Engels, 1999), y la cepa *Canton-S* como control. Para dichos estudios, realizamos cruces individuales y analizamos el DNA tanto de los progenitores como de sus descendientes con el fin de poder determinar las alteraciones producidas en la línea germinal que son transmitidas a la descendencia. Utilizamos la técnica de AP-PCR para determinar la inestabilidad genómica general (**Artículo 2**), mientras que, paralelamente, también se determinó la MSI analizando un panel de 5 *loci* microsatélite (**Artículo 1**).

Al comparar los *fingerprints,* obtenidos por AP-PCR, de la descendencia con los de los progenitores de los distintos cruces, se observa que la falta de función del gen *msh2* genera inestabilidad genómica. Así, como se indica en la Tabla 3, la fracción de daño espontánea (DF, ***D****amage* ***F****raction*) obtenida en los cruces deficientes, $msh2^{-/-}$ y $msh2^{+/-}$, es significativamente superior a la de los cruces control $msh2^{+/+}$ ($P<10^{-5}$).

Teniendo en cuenta que en los *fingerprints* obtenidos por AP-PCR, los cambios de movilidad de las bandas representan inestabilidad en secuencias repetidas (Peinado *et al.,* 1992; Navarro y Jorcano, 1999), contabilizamos por separado este tipo de alteración como la fracción de bandas con cambios de movilidad (BSF, ***B**and **S**hifts **F**raction*). Observamos que la BSF, al igual que la DF, incrementa con el número de alelos *msh2* deficientes (ver Tabla 3), aunque este aumento, en el caso de la BSF, sólo es significativo respecto al control en el caso de los cruces con deficiencia en ambos alelos ($P<10^{-5}$).

Nuestros resultados sugieren que los individuos heterocigotos, aunque no presentan MSI, sí muestran una inestabilidad genómica elevada. Estos resultados están de acuerdo con los obtenidos en estudios con ratón y con cultivos celulares, que indican que una deficiencia de *MSH2* en heterocigosis no produce MSI (Reitmair *et al.,* 1997; Marra *et al.,* 2001) y en levaduras, en los que se demuestra que los individuos heterocigotos para deficiencia en *msh2* presentan un fenotipo mutador (Drotschmann *et al.,* 1999). Todo ello parece indicar que otras funciones de este gen, distintas a la de la reparación de apareamientos erróneos, están implicadas en la estabilidad general del genoma. Recientemente se ha descrito que el gen *msh2* se requiere tanto para la estabilidad de microsatélites como para el mantenimiento de la integridad del genoma en *Caenorhabditis elegans* (Degtyareva *et al.,* 2002).

Tabla 3. Inestabilidad genómica detectada por AP-PCR y análisis de microsatélites en la línea germinal de individuos con diferentes copias alteradas del alelo ***msh2***

| Cruce | AP-PCR | | | | | |
|---|---|---|---|---|---|---|
| | DF[a] | P[b] | BSF[c] | P[b] | MSI[d] | P[b] |
| *msh2*$^{+/+}$ | 0,004 | —— | 0,0002 | —— | $<2x10^{-3}$ | —— |
| *msh2*$^{+/-}$ | 0,021 | $<10^{-5}$ | 0,0009 | 0,233 | $9,7x10^{-3}$ [e] | <0,05 |
| *msh2*$^{-/-}$ | 0,057 | $<10^{-5}$ | 0,0046 | $<10^{-5}$ | $4,8x10^{-2}$ | $<10^{-5}$ |

[a]DF: número de bandas alteradas en los *fingerprints* respecto al total de bandas analizadas.

[b]Probabilidad de concordancia de los valores con respecto a los de *msh2*$^{+/+}$ (test exacto de Fisher, dos colas; valores significativos en negrita).

[c]BSF: número de bandas con cambios de movilidad en los *fingerprints* respecto al total de bandas analizadas.

[d]MSI: número de individuos con alteraciones en *loci* microsatélite respecto al total de individuos analizados.

[e]dato obtenido de Baida *et al.* (2003).

Los resultados que hemos obtenido del análisis directo de la inestabilidad de microsatélites en la línea germinal de los individuos deficientes para *msh2*, a diferencia de los anteriores, muestra la importancia que tiene el sistema de MMR en el mantenimiento de la estabilidad de este tipo de secuencias. Las frecuencias de MSI en heterocigotos y homocigotos deficientes son diferentes a las del control (Tabla 3), indicando que un solo alelo mutado de *msh2* es suficiente para producir MSI en las células germinales. Flores y Engels (1999) habían descrito previamente la aparición de MSI en la línea germinal de *Drosophila* en condiciones deficientes en *msh2* pero al cabo de 10-12 generaciones. Nosotros, analizando el mismo número de individuos por *locus* que el del experimento de Flores y Engels mostramos que una única generación es suficiente para detectar el efecto de la deficiencia de un alelo *msh2* en la estabilidad de los microsatélites en la línea germinal.

A partir del análisis de *loci* microsatélite calculamos la tasa de mutación espontánea en la línea germinal de este tipo de secuencias en el mutante *spel1* de *Drosophila* (*msh2*$^{-/-}$), siendo de $4x10^{-2}$ cambios por *locus* y generación. Esta frecuencia es muy superior a la descrita para individuos normales, $6x10^{-6}$ cambios por *locus* y generación (Schug *et al.,* 1997; Schlötterer *et al.,* 1998), sin embargo, es del mismo orden de magnitud que la tasa de mutación en secuencias microsatélite encontrada en levaduras, ratón y humanos bajo condiciones de deficiencia en MMR (Strand *et al.,* 1993; Kolodner, 1995; Sia *et al.,* 1997; Prolla *et al.,* 1998). Aunque estos estudios fueron realizados en células somáticas o cultivos celulares, en principio se esperaría que la MSI se produzca por mecanismos similares en células somáticas y germinales (Di Rienzo *et al.,* 1998; Sturzeneker *et al.,* 2000).

En conclusión, nuestros resultados indican que la falta de función del gen *msh2* produce inestabilidad genómica general, así como MSI, en la línea germinal, de tal forma que las alteraciones producidas por dicha inestabilidad son transmitidas a la descendencia. Además, la alteración en un solo alelo *msh2* es suficiente para manifestar tal fenotipo mutador. Este hecho puede ser de gran importancia en la susceptibilidad al cáncer ya que muchos de los casos de HNPCC son causados por la transmisión genética de un alelo mutante en uno de los genes que intervienen en la MMR.

# 3. Inestabilidad genómica inducida en la línea germinal del mutante ***spel1*** (***msh2***) de ***D. melanogaster***

Además de la predisposición genética a padecer cáncer, como en el caso ya mencionado de las familias con el síndrome de HNPCC (Kinzler y Vogelstein, 1996; Lynch *et al.,* 1996), existen factores de predisposición ambiental, muchos de los cuales son mutagénicos. De hecho, la mayoría de los cánceres humanos son de origen ambiental. Por otro lado, en humanos, la descendencia de individuos expuestos a ciertos mutágenos presenta altas frecuencias de mutación y un elevado riesgo de padecer cáncer (Dubrova *et al.,* 1996; Kastan, 1997; Roman *et al.,* 1999), lo cual también se observa experimentalmente en ratón (Nomura, 1982). Por lo tanto, cabe esperar que la deficiencia en la MMR y ciertos factores ambientales puedan interaccionar sinérgicamente para generar un estado de inestabilidad genómica con repercusiones en la carcinogénesis. De hecho, existen evidencias de que el sistema de MMR reconoce distintos tipos de daño (Feng *et al.,* 1991; Karran y Bignami, 1992; Li *et al.,* 1996) y se ha demostrado que células deficientes en MMR presentan hipersensibilidad al efecto mutagénico de ciertos compuestos (Kat *et al.,* 1993; De Wind *et al.,* 1995; Anthoney *et al.,* 1996; Fritzell *et al.,* 1997; Lin y Howell*.,* 1999). Todo esto sugiere que la MMR juega un papel importante en la detección de daño genómico promoviendo su eliminación o su reparación, e incluso desencadenando procesos de apoptosis si el daño es muy elevado (Hawn *et al.,* 1995; Nehmé *et al.,* 1997; Lin *et al.,* 2000).

En este contexto, hemos estudiado la contribución de diferentes mutágenos químicos en la MSI, bajo condiciones deficientes en MMR (**Artículo 1**). Este estudio se ha ampliado con el análisis por AP-PCR de las alteraciones en el genoma en el caso del tratamiento con bleomicina (**Artículo 2**).

En el primero de los artículos (**Artículo 1**) se estudió la MSI analizando cinco *loci* microsatélite por PCR en la descendencia de cruzamientos individuales de la cepa *spel1*, tras el tratamiento de los machos parentales y comparándola con los cruzamientos control sin tratamiento.

Aunque el tratamiento con bleomicina (BLM) produce un efecto marginal (ver Tabla 2, **Artículo 1**), podemos considerar que ni la BLM ni el peróxido de hidrógeno ($H_2O_2$), dos compuestos conocidos por producir radicales de oxígeno (ROS, ***R****eactive* ***O****xygen* ***S****pecies*), inducen mutaciones en microsatélites en condiciones deficientes en MMR. Estos resultados contrastan con la bibliografía creciente que muestra que el daño oxidativo produce MSI en bacterias y células humanas (Jackson y Loeb, 2000; Zienolddiny *et al.,* 2000a; 2000b); además, se ha visto que el estrés oxidativo tiene un efecto mutagénico en células humanas y de ratón deficientes en la MMR (De Weese *et al.,* 1998; Lin *et al.,* 2000). Sin embargo, debemos destacar que todos los trabajos mencionados se han realizado utilizando sistemas *in vitro* basados en métodos selectivos para el análisis de MSI, lo que permite detectar frecuencias de mutación muy bajas, mientras que los sistemas *in vivo*, como el que hemos usado, tienen una importante limitación en el tamaño de muestra cuando los efectos mutagénicos son bajos. Por lo tanto, para una correcta comparación de los distintos trabajos existentes se han de tener en cuenta las condiciones experimentales. Por otro lado, tampoco podemos descartar la posiblidad de que el estrés oxidativo produzca alteraciones en otros puntos del genoma, distintos de las secuencias microsatélite, en las moscas tratadas deficientes en la MMR, como ya se ha descrito en estudios con células humanas y de ratón deficientes en este sistema de reparación (De Weese *et al.,* 1998; Lin *et al.,* 2000).

El 2-AAF no induce MSI en un fondo genético normal en reparación, tal como hemos indicado anteriormente (**Anexo 1**), ni en condiciones deficientes para la MMR (**Artículo 1**) aunque, como en el caso de la BLM, observamos en este caso un aumento significativo en el número de alteraciones en uno de los microsatélites. Ya hemos mencionado que los aductos producidos por este compuesto son reparados por la NER (Heflich y Neft, 1994) y que las lesiones no reparadas pueden producir alteraciones en las secuencias repetidas (Bintz y Fuchs, 1990; Hiroaki *et al.,* 1993). Por lo tanto, la falta de efecto del 2-AAF en la MSI en nuestros experimentos puede ser debida a que el daño generado en individuos *spel1* es eliminado eficazmente por el sistema de NER, que no está alterado en esta cepa, y de esta forma las lesiones susceptibles a generar alteraciones en las secuencias repetidas simples son eliminadas.

Por último, el bromuro de etidio (EB) tampoco induce alteraciones en las secuencias microsatélite analizadas en la línea germinal del mutante *spel1*. El EB es un conocido

mutágeno que actúa como agente intercalante (Lecointe *et al.,* 1981) produciendo mutaciones de desplazamiento de lectura o *frameshifts* (Drake, 1970, 1976). Sin embargo, no se conoce su efecto en la MSI y su actividad genotóxica en *Drosophila* es poco conocida (López de Sepúlveda *et al.,* 1981; Xamena *et al.,* 1984).

Los resultados anteriores nos indican que la BLM induce un ligero efecto mutagénico en un fondo genético deficiente en MMR aunque no en un fondo normal. Estos resultados positivos pueden ser considerados como marginales y ser resultado de que el efecto genotóxico de la BLM podría estar relacionado con la deficiencia en *msh2*. Con la intención de profundizar en el papel papel de *msh2* en la inducción de alteraciones por la BLM, ampliamos nuestro estudio con el análisis de la inestabilidad general del genoma por AP-PCR (**Artículo 2**).

La BLM induce diversos tipos de lesiones en el DNA, desde roturas de cadena hasta daño en las bases nucleotídicas (Vig y Lewis, 1978; Traut, 1980) que al fijarse en forma de mutación pueden dar lugar a cambios genómicos detectables en los *fingerprints* obtenidos por AP-PCR. Nosotros encontramos que el daño inducido por la BLM en la línea germinal y que es transmitido a la descendencia depende del genotipo parental para *msh2*. La descendencia de cruces de individuos normales ($msh2^{+/+}$) y heterocigotos ($msh2^{+/-}$) muestra un aumento significativo del daño genómico total (DF, Figura 14A) después del tratamiento con BLM, comparado con la de los cruzamientos control sin tratamiento ($P<10^{-4}$ y $<10^{-2}$, respectivamente), mientras que en la de los cruzamientos homocigotos deficientes en *msh2* ($msh2^{-/-}$) no se aprecia el efecto del tratamiento con BLM (P=0,52). La aparente ausencia de daño inducido por la BLM en los homocigotos deficientes puede ser debida a un simple proceso de selección germinal de los espermatozoides con su material genético muy dañado. De hecho, la descendencia de los individuos $msh2^{-/-}$ presenta ya un nivel de daño espontáneo elevado y, posiblemente, tras el tratamiento con BLM, este nivel incrementa por encima del límite de tolerancia al daño en el DNA. Esta hipótesis se ve reforzada por la observación de un aumento de esterilidad en los machos $msh2^{-/-}$ tras el tratamiento con BLM que no se observa en los machos $msh2^{+/+}$ y $msh2^{+/-}$ (resultados no mostrados), así como, por la mayor mortalidad que presentan las larvas $msh2^{-/-}$ respecto a las larvas $msh2^{+/-}$ y $msh2^{+/+}$ tras el tratamiento con BLM (ver Figura 6, **Artículo 2**). Por otra parte, para explicar las diferencias halladas en la línea germinal de los machos $msh2^{+/+}$ y $msh2^{+/-}$ tratados con BLM y no hallada en la de los machos $msh2^{-/-}$, no podemos descartar

que intervengan procesos de reparación con tendencia a error, que requieran la función MSH2 en la reparación de algunas de las lesiones producidas por la BLM, que resultaría en un incremento de las alteraciones genómicas en los genotipos *msh2*$^{+/+}$ y *msh2*$^{+/-}$ pero no en *msh2*$^{-/-}$. De acuerdo con esta hipótesis, estudios recientes han implicado a *msh2* en la reparación de DSBs (Sugawara *et al.,* 1997).

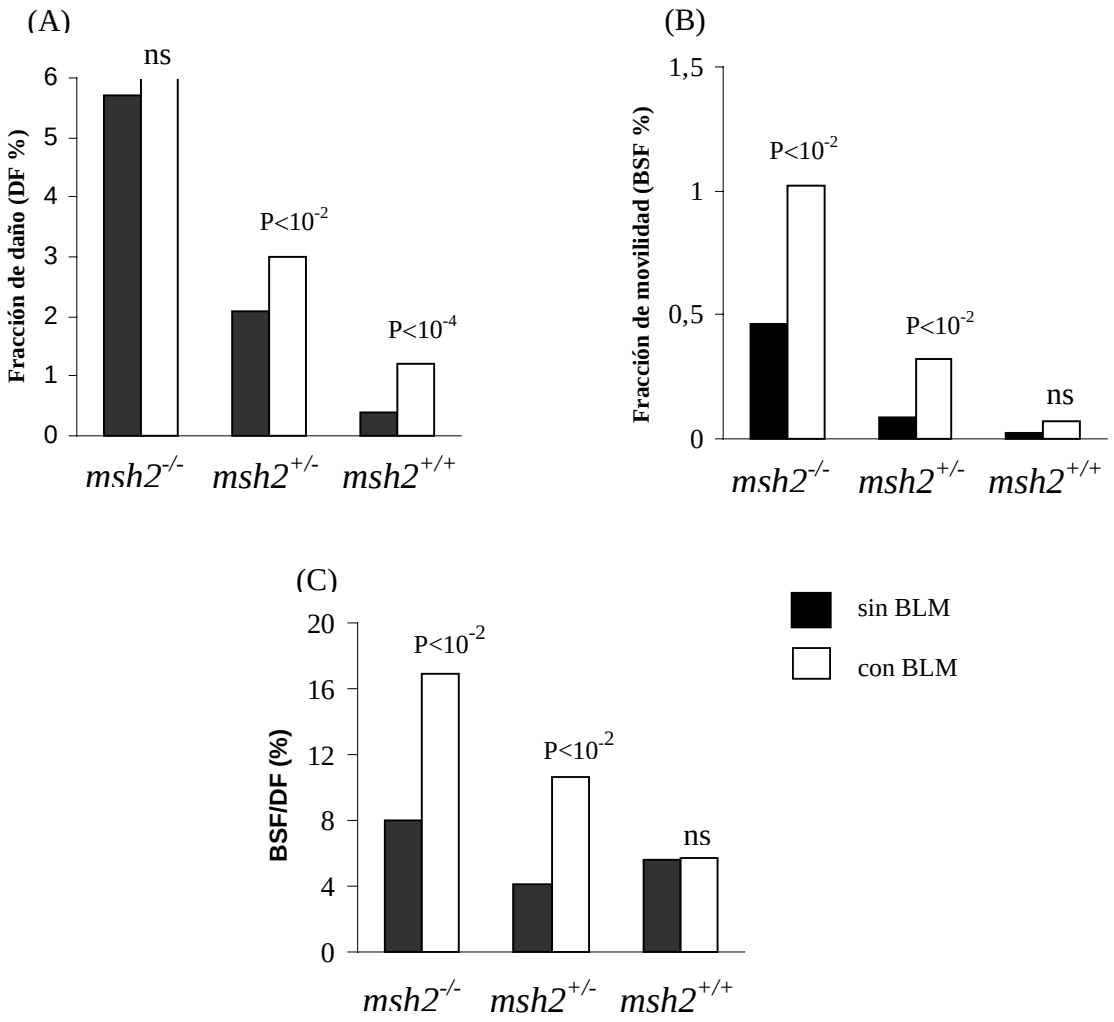


Figura 14. Inestabilidad genómica inducida por bleomicina (BLM) en la línea germinal y analizada por AP-PCR. (A) Fracción de daño total (DF %). (B) Fracción de alteraciones por cambios de movilidad (BSF %). (C) Relación entre la fracción de alteraciones por cambios de movilidad y la fracción de daño total. (P: significación respecto a las líneas sin tratamiento; test exacto de Fisher, dos colas; ns: diferencias estadísticamente no significativas.)

Al contabilizar los cambios de movilidad de las bandas en los *fingerprints* (BSF) observamos también que el efecto de la BLM depende del genotipo parental para *msh2*, pero en este caso, la BLM incrementa significativamente la BSF en la descendencia de los cruzamientos deficientes para este gen, $msh2^{-/-}$ y $msh2^{+/-}$ ($P<0,01$) aunque no se observa efecto en los cruzamientos de individuos normales ($P=0,37$) (Figura 14B). De esta forma, en la Figura 14C se puede observar que, tras el tratamiento con BLM, la proporción de alteraciones de cambios de movilidad de las bandas del *fingerprint* respecto al daño total (BSF/DF) incrementa en los cruzamientos deficientes para *msh2* pero se mantiene en los cruzamientos de individuos normales.

Como se ha indicado anteriormente, teniendo en cuenta que la BSF es un buen indicador directo de la MSI, podemos concluir que, en nuestras condiciones experimentales, la BLM induce o potencia la MSI en un fondo genético deficiente para *msh2.* Estos resultados indican que la MMR juega un papel importante en la MSI inducida por la BLM. Teniendo en cuenta que la BLM es un compuesto radiomimético y considerando que el estrés oxidativo produce MSI en células humanas deficientes en la MMR (Jackson *et al.,* 1998), la inducción de MSI puede ser resultado de las ROS producidas por la BLM (Lin *et al.,* 2000).

De este estudio cabe destacar que los individuos heterocigotos deficientes en *msh2*, además de manifestar un fenotipo mutador (ver apartado 2 de la Discusión), se muestran susceptibles a la inducción de daño genómico por la BLM. Esta observación es muy importante ya que nos indica que el riesgo de cáncer podría verse potenciado en los individuos con una predisposición genética a esta enfermedad en el caso de su exposición a ciertos factores ambientales. Por lo tanto, el uso del mutante *spel1* de *Drosophila* puede ser de importancia relevante en la identificación y estudio de factores ambientales implicados en procesos carcinogénicos relacionados con la inestabilidad genómica.

# 4. Inestabilidad genómica espontánea transmitida durante generaciones en la línea germinal asociada a una alteración en el factor de replicación PCNA

El PCNA es un factor implicado en múltiples procesos del metabolismo del DNA como la replicación, la reparación o el control del ciclo celular (Flores-Rozas *et al.,* 1994; Waga *et al.,* 1994; Umar *et al.,* 1996; Gary *et al.,* 1997; Tsurimoto, 1998), actuando como una plataforma capaz de interaccionar con proteínas muy diversas (Kelman y Hurwitz, 1998; Warbrick, 2000). Alteraciones en el gen que codifica para el factor PCNA de levaduras (*pol30*) producen un fenotipo mutador, sugiriendo el papel de este factor en la estabilidad genómica (Chen *et al.,* 1999; Kokoska *et al.,* 1999).

El mutante *mus209*$^{B1}$ de *Drosophila* nos permite estudiar *in vivo* el papel del gen *PCNA* en la estabilidad del genoma en la línea germinal, determinando así, si este factor garantiza la integridad del genoma a lo largo del tiempo. Para ello, estudiamos la acumulación de alteraciones a lo largo de varias generaciones utilizando la técnica de AP-PCR y el análisis de *loci* microsatélite (**Artículo 3**).

La primera indicación que tuvimos de que el gen *PCNA* podría jugar un papel importante en la estabilidad del genoma proviene de la comparación de los *fingerprints* obtenidos por AP-PCR de individuos de la cepa control *Canton-S* y de individuos mutantes *mus209*$^{B1}$. Mientras que entre los individuos *Canton-S* prácticamente no se observan diferencias en los *fingerprints*, entre los individuos *mus209*$^{B1}$ aparecen muchas bandas polimórficas, indicando una tasa alta de variación genética en esta cepa (ver **Artículo 3**, Figura 1).

Para estudiar la implicación del gen *PCNA* en la estabilidad genómica en la línea germinal a lo largo de las generaciones, se establecieron diferentes líneas con distinto número de alelos alterados para el gen *mus209*, así, se obtuvieron líneas homocigotas y heterocigotas para el alelo *mus209*$^{B1}$ y se usaron como líneas con los dos alelos salvajes los individuos *Canton-S*. Para la estima del daño genético acumulado se compararon los *fingerprints* obtenidos a partir de los individuos descendientes de cada línea, para las generaciones estudiadas, con la pareja fundadora de cada una de las líneas.

El análisis y comparación de las líneas *mus209*$^{-/-}$, en la tercera ($G_3$) y en la sexta generación ($G_6$), con las líneas *mus209*$^{+/+}$ nos muestra una acumulación progresiva del número de alteraciones genómicas a lo largo de las generaciones. Como se indica en la Tabla 4, la DF en las líneas *mus209*$^{-/-}$ aumenta en la $G_6$ repecto a la $G_3$, aunque este incremento no llega a ser significativo ($G_3$: 0,08, $G_6$: 0,102; P: 0,195); sin embargo, como era de esperar, en las líneas *mus209*$^{+/+}$ no se observa tal incremento del daño ($G_3$: 0,007, $G_6$: 0,003) (Tabla 4). Aunque el número de individuos *mus209*$^{-/-}$ que presenta alteraciones en $G_3$ es similar al de $G_6$ (81,4% y 84,8%, respectivamente), la media de las alteraciones por individuo en $G_6$ es superior a la de $G_3$ (2,90 y 2,23, respectivamente) (ver Tabla 2, **Artículo 3**). Además, el número de individuos con más de 6 alteraciones en $G_6$ es del 11,96%, mientras que en $G_3$ es sólo del 3,48% (ver Figura 4, **Artículo 3**)

Asimismo, como puede observarse en la Tabla 4, cabe indicar que el daño genómico acumulado a lo largo de las generaciones depende del genotipo *mus209*. Así, después de 6 generaciones de cruzamientos, el daño es progresivamente mayor cuanto mayor es el número de alelos *mus209* mutados, siendo los valores del daño de las líneas heterocigotas y homocigotas significativamente diferentes a los de las líneas normales ($P<10^{-5}$, ver figura 15A). Además, en la $G_6$ esta relación también se observa en el número de individuos que presenta un número elevado de alteraciones: en las líneas *mus209*$^{-/-}$ el 11,96% de los individuos presenta más de 6 alteraciones, en las líneas *mus209*$^{+/-}$ el 1,23% y en las líneas control no encontramos individuos con este número de alteraciones.

Con respecto al tipo de alteraciones observadas en los fingerprints, la mayoría de los cambios que se observan son cuantitativos y representan el 94,8% y el 87% de los cambios encontrados en $G_6$, en las líneas *mus209*$^{-/-}$ y *mus209*$^{+/-}$, respectivamente. Este tipo de alteraciones podría estar relacionado con la síntesis defectuosa del DNA durante la reparación, de hecho, el papel del factor PCNA en la síntesis reparativa ya ha sido sugerido anteriormente en *Drosophila* (Henderson y Glover, 1998) y en levaduras (Holmes y Haber, 1999). Sin embargo, otra posible causa podría ser la estimulación de la actividad de la proteína FEN-1 producida por el PCNA, ya que se ha observado que mutaciones en *fen-1* en levaduras producen una acumulación de deleciones y duplicaciones (Tishkoff *et al.,* 1997).

Tabla 4. Inestabilidad genómica acumulada a lo largo de las generaciones, detectada por AP-PCR y análisis de *loci* microsatélite, en distintas líneas de cruzamientos con mutación en ***mus209.***

| | AP-PCR | | | | | | |
|---|---|---|---|---|---|---|---|
| Líneas | DF[a] | P[b] | BSF[c] | P[b] | BSF/DF(%)[d] | MSI[e] | P[b] |
| *mus209*$^{+/+}$ | | | | | | | |
| G3 | 0,007 | —— | 0,0005 | —— | 7,14 | <0,002 | —— |
| G6 | 0,003 | —— | <0,0003 | —— | <10,00 | <0,002 | —— |
| *mus209*$^{+/-}$ | | | | | | | |
| G6 | 0,037 | **P<10$^{-5}$** | 0,0050 | **P<10$^{-3}$** | 13,51 | 0,0338 | **P<10$^{-4}$** |
| *mus209*$^{-/-}$ | | | | | | | |
| G3 | 0,080 | **P<10$^{-5}$** | 0,0040 | **P=0,01** | 5,00 | 0,0095 | P=0,22 |
| G6 | 0,102 | **P<10$^{-5}$** | 0,0070 | **P<10$^{-5}$** | 6,86 | 0,0073 | **P=0,12** |

[a]DF: número de bandas alteradas en los *fingerprints* respecto al total de bandas analizadas.

[b]Probabilidad de coincidencia de los valores con respecto a los de *mus209*$^{+/+}$ (test exacto de Fisher, dos colas; valores significativos en negrita).

[c]BSF: número de bandas en los *fingerprints* con cambios de movilidad respecto al total de bandas analizadas.

[d]BSF/DF: proporción BSF respecto a DF.

[e]MSI: número de individuos con alteraciones en *loci* microsatélite respecto al total de individuos analizados.

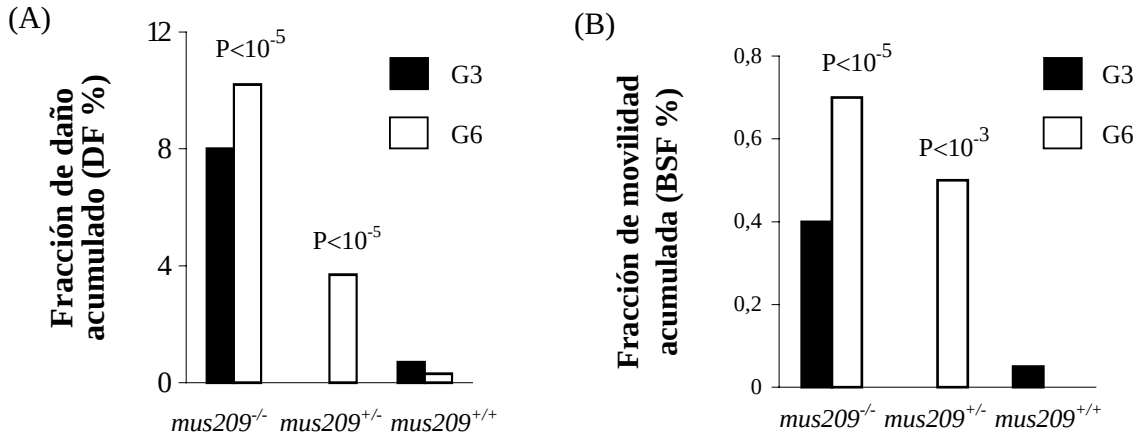


Figura 15. Inestabilidad genómica acumulada en la línea germinal de las líneas con diferente genotipo para *mus209,* analizada por AP-PCR . (A) Fracción de daño acumulado (DF %). (B) Fracción de alteraciones por cambios de movilidad acumulada (BSF %). (*P*: significación respecto las líneas *mus209*$^{+/+}$ en $G_6$; test exacto de Fisher, dos colas).

Aunque en menor grado, en los *fingerprints* analizados también se observan cambios de movilidad de bandas que fueron contabilizados de forma separada (BSF). En la Tabla 4 y en la Figura 15B se puede observar que este tipo de alteración aparece a lo largo de las generaciones dependiendo del genotipo *mus209*, siguiendo la misma tendencia que la acumulación de daño total en el genoma. Por lo tanto, las líneas *mus209*$^{-/-}$ son las que presentan mayor BSF, aunque tanto estas líneas como las líneas con un solo alelo mutado (*mus209*$^{+/-}$) muestran una acumulación de cambios de movilidad de las bandas del *fingerprint* significativamente superior a las de las líneas normales (*mus209*$^{+/+}$) ($P<10^{-5}$ y $P<10^{-3}$, respectivamente). Sin embargo, la relación entre los cambios de movilidad y el daño total (BSF/DF) es mayor en las líneas heterocigotas que en las líneas homocigotas para la mutación, indicando que este tipo de alteración contribuye en mayor proporción al daño total en las líneas heterocigotas. Esta observación se ve reforzada por los resultados obtenidos en el análisis de *loci* microsatélite, en el que también las líneas heterocigotas presentan una mayor MSI que las líneas homocigotas para la alteración en *mus209* y que las líneas normales.

Nuestros resultados apoyan la idea de que la proteína PCNA es un factor importante para el mantenimiento de la estabilidad del genoma *in vivo* en la línea germinal de *Drosophila*, garantizando su integridad a lo largo de las generaciones. Esta idea se ve reforzada por otras observaciones como la reciente descripción de un fenotipo mutador en mutantes para *PCNA* (*pol30*) en levaduras (Chen *et al.,* 1999; Kokoska *et al.,* 1999).

Dada la participación del PCNA en diferentes procesos celulares cabe pensar que mutaciones en el gen *PCNA* pueden afectar simultáneamente a múltiples procesos celulares, dando así lugar a distintos efectos mutagénicos. Además, de las alteraciones de tipo cuantitativo detectadas en los *fingerprints*, la MSI obtenida en este trabajo puede estar relacionada con la implicación del PCNA en la MMR (Umar *et al.,* 1996; Gu *et al.,* 1998; Clark *et al.,* 2000; Flores-Rozas *et al.,* 2000; Kleczkowska *et al.,* 2001; Lau *et al.,* 2002) o, de forma independiente, por el papel del PCNA en la replicación del DNA (Tsurimoto, 1998). En este momento no disponemos de una explicación convincente para la alta MSI encontrada en las líneas heterocigotas, pero cabe destacar la importancia que pueden tener estos resultados en la susceptibilidad al cáncer ya que en humanos pueden existir variantes polimórficas que provoquen la aparición de un fenotipo mutador débil, relacionándose con una mayor probabilidad de desarrollar algún tipo de cáncer, aunque

hasta ahora no se haya encontrado ninguna relación de este tipo con el *PCNA* (Ma *et al.,* 2000).

## 5. Inestabilidad genómica en individuos heterocigotos ***msh2***$^{+/-}$ y ***mus209***$^{+/-}$: haploinsuficiencia

El modelo de tumorigénesis basado en la inactivación de genes supresores de tumores propone que es necesaria la inactivación de los dos alelos del gen supresor para la formación del tumor (Knudson, 1971). Sin embargo, actualmente son cada vez más numerosos los estudios que muestran que la pérdida o inactivación de un solo alelo de estos genes puede ser suficiente para iniciar la tumorigénesis. Este efecto de dosis génica que contribuye al desarrollo tumoral se denomina **haploinsuficiencia**. Existen algunos ejemplos de haploinsuficiencia en humanos y ratón (Venkatachalam *et al.,* 1998; Song *et al.,* 1999) en los que los individuos portadores de una mutación en heterocigosis, que inactiva un solo alelo de un gen supresor de tumores, presentan una mayor incidencia de tumores, desarrollándose una parte de éstos sin que tenga lugar la mutación o pérdida del otro alelo normal. Además, mediante haploinsuficiencia también se puede modificar el riesgo de cáncer en el caso de exposición a mutágenos (Fero *et al.,* 1998) y en algunos casos en que los individuos son portadores de una mutación en heterocigosis en otro gen supresor (Smits *et al.,* 2000).

Los genes implicados en la reparación del DNA o en la segregación cromosómica se consideran un subtipo de genes supresores de tumores que actuan como guardianes del genoma. La haploinsuficiencia en estos genes puede dar lugar a una reparación o segregación deficiente, incrementando así la inestabilidad genómica, de tal forma, que pueden producirse mutaciones somáticas en otros genes supresores y oncogenes (revisado en Fodde y Smits, 2002). Así, cabe esperar que la haploinsuficiencia en estos genes vaya asociada a una mayor predisposición al cáncer como ocurre en los caso de *BLM* y *FEN-1* (Goss *et al.,* 2002; Kucherlapati *et al.,* 2002). Asimismo, la haploinsuficiencia de genes implicados en la respuesta celular al daño en el DNA, como *p53*, *ATM*, *BRCA2*, también parece estar relacionada con un alto riesgo de cáncer (Swift *et al.,* 1991; Venkatachalam *et al.,* 1998; Howlett *et al.,* 2002).

Aunque se sabe que las alteraciones en los genes que intervienen en el proceso de la MMR producen un alto riesgo de cáncer, las evidencias que se tienen hasta el momento parecen indicar que es necesaria la inactivación de ambos alelos de uno de estos genes para el desarrollo del tumor. Así, por ejemplo, en el síndrome de predisposición al cáncer HNPCC, la mutación germinal de uno de los genes de MMR, *MSH2* o *MLH1*, seguida de la mutación somática del otro alelo es la causa principal de la aparición de tumores (Lynch *et al.,* 1996). Asimismo, los estudios de inestabilidad genómica en fondos genéticos deficientes en la MMR se han realizado básicamente en modelos que contienen los dos alelos del gen alterados (Strand *et al.,* 1993; Umar *et al.,* 1994; Prolla *et al.,* 1998; Yao *et al.,* 1999). Los estudios realizados en condiciones heterocigotas son muy escasos y de resultados contradictorios. En todos ellos se han utilizado sistemas *in vitro* en levaduras y ratón (Reitmair *et al.,* 1997; De Weese *et al.,* 1998; Drotschmann *et al.,* 1999) y no existen en la bibliografía estudios de este tipo llevados a cabo *in vivo*. A pesar de ello, conocer la inestabilidad genómica en los individuos heterocigotos tiene un gran interés en la predisposición al cáncer, ya que la haploinsuficiencia en estos genes podría causar inestabilidad genómica, contribuyendo al inicio de la tumorigénesis en los individuos heterocigotos así como en su descendencia.

En nuestro trabajo con *Drosophila* hemos encontrado que uno de los principales genes que interviene en la MMR, el *msh2*, es importante en la estabilidad del genoma tanto a nivel de secuencias microsatélite como en el genoma en general. Además, el análisis de la inestabilidad genómica en individuos de *Drosophila* heterocigotos para la alteración en el gen *msh2* nos indica que la inactivación de un único alelo es suficiente para producir inestabilidad genómica general así como MSI en la línea germinal (ver **Artículo 2**). Aunque ello no representa en si una evidencia clara de haploinsuficiencia cabe considerar que estos mismos individuos (los heterocigotos $msh2^{+/-}$) muestran una mayor susceptibilidad a la inducción de daño genómico por agentes mutagénicos que los individuos normales para el gen *msh2.* En definitiva, nuestros resultados aportan la primera evidencia *in vivo* de la posible haploinsuficiencia de un gen que interviene en la MMR.

Otro aspecto importante con relación a la MSI asociada a ciertos tipos de cáncer es que algunos de estos tumores no presentan mutación en los genes de MMR (Bubb *et al.,* 1996; Senba *et al.,* 1998), por lo tanto surge la posibilidad de que otros genes implicados en la reparación del DNA y que juegan un papel importante en la MSI, podrían estar alterados en

estos casos. Así, recientemente se ha demostrado que la haploinsuficiencia en *FEN-1* acelera la progresión tumoral en ratón (Kucherlapati *et al.,* 2002).

Un caso semejante es el del gen gen que codifica el *PCNA*, que como ya hemos dicho es un factor clave en la replicación del DNA y está implicado en procesos de reparación del mismo (Umar *et al.,* 1996; Gary *et al.,* 1997; Tsurimoto, 1998). Nuestros resultados de inestabilidad genómica en mutantes de *Drosophila* para el *PCNA* nos indican la implicación de este factor en la estabilidad del genoma. Un aspecto importante de este estudio es que los individuos heterocigotos para la mutación en *PCNA* muestran inestabilidad genómica en la línea germinal, al igual que ocurre en los heterocigotos *msh2*$^{+/-}$, lo que podría indicar una posible haploinsuficiencia de este gen. Aunque no conocemos ningún trabajo que permita establecer una posible correlación entre alteraciones en el gen *PCNA* y cáncer en humanos, el papel que hemos encontrado del *PCNA* en la estabilidad genómica *in vivo* sugiere la posibilidad de la existencia de variantes alélicas que puedan afectar a la susceptibilidad al cáncer en humanos.

En resumen, podemos decir que la dosis en la actividad de los genes *msh2* y *PCNA* podría modificar la susceptibilidad al cáncer, produciendo un aumento de la inestabilidad genómica y/o alterando la respuesta al daño en el DNA.

# V. CONCLUSIONES

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1. La técnica de *fingerprinting*, AP-PCR, es útil para el análisis genómico en *Drosophila*. Permite diferenciar genéticamente distintas cepas de *Drosophila,* así como el análisis de mutaciones espontáneas e inducidas.

2. El análisis de los *fingerprints* obtenidos por AP-PCR permite estimar *in vivo* la inestabilidad genómica general en la línea germinal de *Drosophila*, asociada a deficiencias en reparación del DNA.

3. En *D. melanogaster*, la pérdida de función del gen *msh2* produce inestabilidad de microsatélites en la línea germinal, siendo la tasa de mutación de las secuencias microsatélite del mismo orden de magnitud que la encontrada en levaduras, ratón y humanos bajo condiciones de deficiencia en reparación de apareamientos erróneos.

4. La pérdida de función del gen *msh2*, tanto en homocigotos como en heterocigotos, produce inestabilidad genómica general en la línea germinal, detectada por AP-PCR, posiblemente debido a la implicación de la proteína MSH2 en otros procesos importantes del metabolismo del DNA.

5. El gen *msh2* interviene en la modulación del daño genético inducido, puesto que la deleción de uno o ambos aleos del gen genera inestabilidad genómica que se ve potenciada por la exposición a mutágenos, como es el caso de la bleomicina.

6. El factor de replicación PCNA juega un papel importante en la estabilidad general del genoma en la línea germinal de *D. melanogaster* debido a sus propiedades multifuncionales relacionadas con el metabolismo del DNA.

7. La mutación puntual en el gen *PCNA* de *D. melanogaster*, $mus209^{B1}$, produce inestabilidad genómica general dando lugar a la acumulación de alteraciones en el genoma a lo largo de las generaciones. El daño acumulado incrementa con el número de alelos *PCNA* mutados en las líneas de cruzamientos establecidas, aunque en las

líneas heterocigotas la inestabilidad de microsatélites contribuye en mayor proporción al daño genómico total que en las mutantes homocigotas.

8. Alteraciones en los genes *msh2* y *PCNA* de *Drosophila* en condiciones de heterocigosis muestran tanto inestabilidad genómica general como inestabilidad de microsatélites, indicando que un solo alelo funcional es insuficiente para garantizar la estabilidad del genoma. En el caso de humanos, esto podría provocar haploinsuficiencia y con sus consecuencias negativas en el desarrollo tumoral.

9. La técnica de AP-PCR permite cuantificar el daño general en el genoma. La fracción de dicho daño que se corresponde con la movilidad de las bandas obtenidas en los *fingerprints* muestra cierta correlación con el análisis de inestabilidad de microsatélites por PCR específica.


# VI. BIBLIOGRAFÍA

# VI. BIBLIOGRAFÍA

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# ANEXO 1

**TABLE 1**

**Primers of microsatellite loci**

| Locus | Repeat | Primer | Annealing Temperature | $MgCl_2$ |
|---|---|---|---|---|
| *Dmsgg3* | $(CAG)_{11}$ | TCCAGCAATCACAGCAAACT | 58º | 2.5 mM |
| | | TCTTTTGAAATTGCGGTTGA | | |
| *Mam* | $(CAG)_8$ | GGCGGCCTACCAGTTTTCCA | 58º | 2.5 mM |
| | | CCTGTTGCTCCCAGGTTTGC | | |
| *Dronanos* | $(AT)_{18}$ | CGCAAGTATTCATTTCAACACA | 55º | 4.0 mM |
| | | TGCTGGCGGTTGTTTCAT | | |
| *Droabdb* | $(AC)_{19}$ | TTCCAAGTCACACGGACGGG | 56º | 2.5 mM |
| | | GCACACCGACAACACAAG | | |
| *Dmtena* | $(AT)_{14}$ | CTCTTAGTGCGCAGGGATTC | 56º | 2.5 mM |
| | | GAGTCGCTCAATGGCAGGC | | |

**TABLE 2**

**Genomic damage detected by AP-PCR in *mus209*$^{B1}$ lines, at different generations**

| Type of lines | No. of analyzed lines | No. of analyzed individuals | No. of analyzed bands | No. of altered bands | Mean alterations per individual ± SD | DF[a] | No. of band shifts | BSF[e] | Ratio of band shifts to altered bands (%) |
|---|---|---|---|---|---|---|---|---|---|
| *mus209*$^{-/-}$ | | | | | | | | | |
| G3 | 10 | 86 | 2408 | 192 | 2.23±2.03 | 0.080[b] | 10 | 0.0040[f] | 5.01 |
| G6 | 10 | 92 | 2576 | 264 | 2.90±3.57 | 0.102[c] | 18 | 0.0070[g] | 6.83 |
| *mus209*$^{+/-}$ | | | | | | | | | |
| G6 | 9 | 81 | 2268 | 84 | 1.04±1.51 | 0.037[d] | 11 | 0.0050[g] | 13.51 |
| *mus209*$^{+/+}$ | | | | | | | | | |
| G3 | 10 | 80 | 2160 | 16 | 0.20±0.51 | 0.007 | 1 | 0.0005 | 6.60 |
| G6 | 10 | 99 | 2673 | 8 | 0.06±0.24 | 0.003 | 0 | 0.0000 | ——— |

[a] DF: damage fraction calculated as the total number of altered bands divided by the total number of analyzed bands.

[b] Statistically significant respect to *mus209*$^{+/+}$ at G3 ($P<10^{-5}$, Fisher´s exact test, two-tailed).

[c] Statistically significant respect to *mus209*$^{+/-}$ and *mus209*$^{+/+}$ lines at G6 ($P<10^{-5}$, Fisher´s exact test, two-tailed).

[d] Statistically significant respect to *mus209*$^{+/+}$ lines at G6 ($P<10^{-5}$, Fisher´s exact test, two-tailed).

[e] BSF: band shifts fraction, calculated as the total number of band shifts divided by the total number of analyzed bands.

[f] Statistically significant respect to *mus209*$^{+/+}$ at G3 ($P=0.01$, Fisher´s exact test, two-tailed).

[g] Statistically significant respect to *mus209*$^{+/+}$ at G6 ($P<10^{-5}$ at *mus209*$^{-/-}$ and $P<10^{-3}$ at *mus209*$^{+/-}$, Fisher´s exact test, two-tailed )

**TABLE 3**

**Instability in loci microsatellite**

| | *mus209*$^{-/-}$ lines | | | | *mus209*$^{+/-}$ lines | | *mus209*$^{+/+}$ lines | |
|---|---|---|---|---|---|---|---|---|
| | G3 | | G6 | | G6 | | G6 | |
| Locus | MSI[a] | % of changes | MSI[a] | % of changes | MSI[a] | % of changes | MSI[a] | % of changes |
| *DmtenA* $(AT)_{14}$ | 2/107 | 1.8 | 0/108 | 0.0 | 5/82 | 6.1[b] | 0/80 | 0.0 |
| *Droabdb* $(AC)_{19}$ | 0/107 | 0.0 | 4/110 | 3.6 | 3/85 | 3.5 | 0/100 | 0.0 |
| *Dmsgg3* $(CAG)_{11}$ | 0/104 | 0.0 | 0/106 | 0.0 | 3/75 | 4.0 | 0/89 | 0.0 |
| *Mam* $(CAG)_8$ | 0/96 | 0.0 | 0/108 | 0.0 | 0/83 | 0.0 | 0/89 | 0.0 |
| *Dronanos* $(AT)_{18}$ | 3/108 | 2.8 | 0/110 | 0.0 | 3/89 | 3.4 | 0/110 | 0.0 |

[a]MSI: total no. of changes/no. of analyzed individuals

[b]Significant difference against *mus209*$^{+/+}$ and *mus209*$^{-/-}$ lines at G6 ($P<0.05$, Fisher´s exact test, two-tailed)