Molecular study of the germinal reversions induced at the white-ivory locus in Drosophila melanogaster

S.Suárez, O.Cabrè, A.Velázquez, A.Creus, R.Marcos and N.Xamena

Grup de Mutagènesi, Departament de Genètica i de Microbiologia, Edifici Ca, Universitat Autònoma de Barcelona, 08193 Bellaterra (Cerdanyola del Vallès), Spain

1To whom correspondence should be addressed

The white-ivory somatic mutation test of Drosophila melanogaster is based on the reversion of the X-linked eye colour recessive mutation white-ivory to wild-type. Although the exact mechanism of white-ivory reversion is not quite understood, it has been suggested that such reversion, both in somatic and germ-line cells, could be due to the precise excision of the tandemly duplicated 2.96 kb DNA fragment characteristic of the white-ivory mutation. We have attempted to confirm this hypothesis analysing, at the molecular level, different germ-line revertants induced by chemical treatment with three well known alkylating agents: ethyl methanesulphonate, methyl methanesulphonate and N-nitroso-N-ethylurea. The molecular analysis of these germ-line revertants, using Southern blot hybridization and polymerase chain reaction techniques, shows that such reversions are associated with the deletion of the 2.96 kb tandemly duplicated DNA sequence of the white-ivory locus.

Introduction

The white-ivory somatic mutation test of Drosophila melanogaster, based on the reversion of the X-linked eye colour mutation white-ivory to wild-type, has been proposed as a sensitive assay to assess the genotoxic potential of chemicals (Green et al., 1986; Clements et al., 1990; Howe and Clements, 1990; Würgler and Küä, 1991; Xamena et al., 1991; Batiste-Alentorn et al., 1994). The white-ivory mutants revert to wild phenotype both in somatic and germinal cells. In somatic cells, the spontaneous frequency of reversion, measured as the proportion of flies with mosaicism, is 0.05–0.18% in adult females and 0.04–0.09% in adult males (Ryo et al., 1985; Green et al., 1986). The spontaneous frequency of germinal reversion for a strain bearing a single copy of the white-ivory allele is $5 \times 10^{-5}$ in homozygous females, and $5 \times 10^{-6}$ in hemizygous males and deletion-heterozygous females (Lewis, 1959; Bowman, 1965). Reversion can be significantly increased by X-ray and/or chemical treatment in germ-line cells (Lewis, 1959; Ryo et al., 1985; Howe and Clements, 1990) and also in somatic cells (Ryo et al., 1985; Green et al., 1986; Howe and Clements, 1990).

Karess and Rubin (1982) reported that the white-ivory mutants have a 2.96 kb internal tandem duplicated sequence of the white locus. This duplicated fragment comprises nucleotides –173 to +2795 of the white locus, showing a 6 bp duplicated sequence at the distal end of the duplication (O’Hare et al., 1984). This 2.96 kb internal duplicated sequence is responsible for the white-ivory phenotype, a pale yellow–pink eye colour that is darker in females than in males due to the absence of dosage compensation (Green, 1959). Furthermore, flies with an increased number of copies of the white-ivory allele have a progressively darker phenotype.

Although the exact mechanism of white-ivory reversion is not well understood, there are some references indicating that it could be due to the precise excision of the duplicated 2.96 kb DNA fragment (Karess and Rubin, 1982; Green et al., 1986).

To provide further information on the nature of the chemically-induced germ-line reversions of flies with the white-ivory allele, we present here the molecular analysis of several phenotypic revertants obtained after treatment of white-ivory larvae with three different alkylating agents.

Materials and methods

Chemicals

The reference mutagens used were: ethyl methanesulphonate (EMS; CAS No. 62-50-4), methyl methanesulphonate (MMS; CAS No. 66-27-3), and N-nitroso-N-ethylurea (ENU; CAS No. 759-73-9). They were supplied by Sigma Chemical Co. (St Louis, MO, USA). All compounds were dissolved in double-distilled water to the different concentrations used, just before the treatments.

Drosophila stocks

We used the following strains: (i) Canton-S (CS), a wild-type strain; (ii) C(1)DX, y females; (iii) white-one (w1), w1; (iv) white-ivory (w1), y w1. The w1 strain was purchased from Carolina Biological Supply Co. (Burlington, NC), while the other strains were obtained from the Umeå Drosophila Stock Center (Sweden). For a detailed description of the genetic markers and special chromosomes, see Lindley and Zimm (1992).

Treatment procedure

White-ivory larvae aged 24 or 48 h were washed from standard culture bottles, filtered and seeded in vials containing 9 ml of Drosophila Instant Medium (Carolina Biological Supply Co., Burlington, NC, USA) and 9 ml of the respective mutagen solution. The larvae remained in these vials until adults, and the emerged males were collected and crossed with C(1)DX, y f females, carrying attached X-chromosomes. The offspring was scored for the presence of males with wild red eye-colour which are the result of germinal reversion. Induced germinal revertants were maintained with C(1)DX, y f virgin females and were subsequently used for molecular analysis. All the crosses were carried out at 25 ± 1°C.

Quantitative measurement of red-eye pigments

To classify the induced revertants, we have used a quantitative measurement of red-eye pigments following the protocol described by Ashburner (1989). Twenty-five heads of 6 day old males were used and the relative absorbance was calculated as the ratio of the absorbance of the mutant (A455.11) with respect to the absorbance of wild-type (A455.c). corrected for the absorbance of white-l (A455.11) as follows:

\[
\text{Relative absorbance} = \frac{\text{A}455.11}{\text{A}455.c} - \frac{\text{A}455.11}{\text{A}455.11}
\]

Hybridization probe

The BgfII–BfII, HindIII–BamHI and SalI–SalI fragments of the white locus, between +6163 and +7476, +3171 and +1383, and +669 and –1530 positions respectively (see Figure 1), were used as probes in Southern blot experiments. These fragments were obtained from the plasmid pWP2 kindly provided by Dr W.J.Gerking, Department of Cell Biology, Basel University, Switzerland. The HindIII–BamHI fragment was cloned in pTZ18 (2.86 kb, Genescrite-Z; USB Corp., Cleveland, OH, USA), and BgfII–BfII and SalI–SalI fragments in pBluescript SK+ (2.96 kb, Stratagene Cloning Systems, La Jolla, CA, USA). To label the probes, the cloned fragments were first amplified by

© UK Environmental Mutagen Society/Oxford University Press 1996
polymerase chain reaction (PCR) using universal sequencing primers (M13/pUC sequencing primer 1, New England Biolabs Inc., Beverly, MA, USA; M13/pUC reverse sequencing primer 2, Promega Corp., Madison, WI, USA) and then labelled with digoxigenin-11-dUTP by random primed reaction using DIG DNA Labeling kit (Boehringer Mannheim, Germany) DNA analysis by Southern blot Genomic DNA extractions were carried out from 0.2 g of adult flies of CS, w, and the different revertant lines, as described by Pifóil et al. (1988), except that a phenol deproteinization step was added before deproteinization with chloroform. Genomic DNA (5 μg) was digested with HindIII and Sali according to the supplier’s instructions (Boehringer Mannheim, Germany), and the DNA fragments separated by electrophoresis on a 0.8% agarose gel Southern blotting on a positively charged nylon filter was carried out using a DIG Luminescent Detection Kit (Boehringer Mannheim, Germany) for detection of the amplified fragments was carried out on a 1% agarose gel electrophoresis.

Results and discussion
To obtain an adequate number of revertants to be analysed, we treated first-instar larvae of the w strain with three different well known strong mutagens, the alkylating agents EMS (0.25–2 mM), ENU (0.25–1 mM) and MMS (0.25–1 mM). The emerged males from these treatments were crossed with C(1)DX, y f virgin females, and their offspring was scored to detect males with wild red eye-colour in both eyes. Although some of these phenotypic revertants were sterile, we were able to establish 18 revertant lines with the fertile ones, maintaining them with C(1)DX, y f females (Table I). Eleven of these revertants (wR9 to wR16) were found in the same culture bottle, and a possible common premeiotic origin should be considered (cluster). All revertants were most likely induced by chemical treatment, since no germinal revertants were obtained between the 25 270 flies scored in the control crosses. These results indicate that the spontaneous frequency of reversion should be <3.96×10⁻⁵.

The molecular analysis of germinal revertants was carried out to establish whether, in our reversion experiments, the wild eye-colour phenotype was associated with the complete or partial loss of the 2.96 kb DNA duplicated segment of the white–ivory allele, accordingly with the previous observations of Karess and Rubin (1982).

Figure 1 shows a restriction map of the white locus of CS wild strain (from which the white–ivory mutant was originally isolated) and from the white–ivory mutant, indicating the probes used in this experiment (BglII–Bgll, HindIII–BanHlI and Sall–Sall). The first probe, BglII–Bgll, corresponds to a sequence of the regulatory region of the white locus, while the other two correspond to sequences from the structural region of this locus. The HindIII–BanHl probe was used to

**Fig. 1.** Restriction maps of a region of the white locus of the Canton-S wild type (a) and the w^I^ allele (b) strains. The two-headed arrows denote the 1.3 kb BglII–BglII, the 2.4 kb HindIII–BanHlI and the 0.8 kb Sall–Sall probes. Target points of HindIII and Sall restriction enzymes used in DNA digestions are indicated by vertical arrows. The thicker line indicates the 2.96 kb duplicated fragment in the w^I^ strain. Primers used in PCR experiments (wip1, wip2, wif3 and wif4) are denoted by horizontal arrows. (The maps are adapted from Karess and Rubin, 1982.)
Table I. Quantitative measurement of red-eye pigments of different mutants obtained after chemical treatment

<table>
<thead>
<tr>
<th>Strain or mutant</th>
<th>Compound and concentration (mM)</th>
<th>Possible cluster*</th>
<th>Absorbance at 485 nm ± SE</th>
<th>Relative absorbanceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>–</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>w^1</td>
<td>–</td>
<td></td>
<td>0.069 ± 0.066</td>
<td>0.070 ± 0.002</td>
</tr>
<tr>
<td>w^2</td>
<td>–</td>
<td></td>
<td>0.011 ± 0.024</td>
<td>0.002 ± 0.002</td>
</tr>
<tr>
<td>w^3</td>
<td>EMS 0.25</td>
<td>one</td>
<td>1.069 ± 0.070</td>
<td>0.977 ± 0.060</td>
</tr>
<tr>
<td>w^4</td>
<td>EMS 0.25</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^5</td>
<td>EMS 0.50</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^6</td>
<td>EMS 1.00</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^7</td>
<td>EMS 1.00</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^8</td>
<td>EMS 1.00</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^9</td>
<td>EMS 1.00</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^10</td>
<td>EMS 0.50</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^11</td>
<td>EMS 0.25</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^12</td>
<td>EMS 0.25</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^13</td>
<td>EMS 0.50</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^14</td>
<td>EMS 1.00</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^15</td>
<td>EMS 1.00</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^16</td>
<td>EMS 0.25</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^17</td>
<td>EMS 0.25</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
<tr>
<td>w^18</td>
<td>EMS 0.25</td>
<td>one</td>
<td>N.D.</td>
<td>–</td>
</tr>
</tbody>
</table>

*N.D.: non-determined value.

The relative absorbance was calculated as the ratio of the absorbance of the mutant (A_{485,w^1}) respect to the absorbance of wild-type (A_{485-CS}), corrected for the absorbance of white-1 (A_{485-w^1}) as follows:

\[ \frac{A_{485-CS}}{A_{485-w^1}} \]

The results obtained in our Southern blot experiments with different germ-line revertants agree with the previous indications from Karess and Rubin (1982) and Green et al. (1986), and they clearly show that in the 18 cases analysed, germinal reversion seems to be the result of a more or less precise excision of one of the two tandemly duplicated sequences of the white-ivory mutation. The results from Karess and Rubin (1982) indicated that four of five revertants, two of which arose from irradiated flies and two had spontaneous origin, showed a single copy of the 2.96 kb sequence as in the wild-type; the other revertant, X-ray induced, as well as one spontaneous partial revertant, showed a more complex pattern such as the introduction of a new DNA into the ivory duplication and the loss of some sequence from the same duplication. Moreover, it must be pointed out that the white-crimson, isolated as a partial phenotypic revertant of the white-ivory mutation, results from the insertion of a 10 kb DNA sequence into the white-ivory duplication (Collins and Rubin, 1982).

Our results over 18 revertants agree with the few cases described on complete germ-line white-ivory revertants. We
Our 18 revertants, as well as those analysed by Karess and Rubin (1982) and Green et al. (1986), show the loss of the white-ivory duplicated DNA fragment. Karess and Rubin (1982) also reported two cases with a more complex rearrangement, due to the introduction of new DNA into the site of the white-ivory mutation, and to the concomitant loss of some white-ivory DNA. Small deletions or any other type of rearrangements have not been observed in any of the cases. Although these observations could mean that the white-ivory system is highly selective and only allows to distinguish a narrow range of lesions, further investigations using a more sensitive method such as DNA sequencing are needed to confirm this assumption.

Another aspect to be considered is that, even though we clearly observed the loss of the 2.96 kb duplicated DNA segment, the question about which is the exact mechanism(s) involved in such loss induced by the action of mutagens remains open. Bowman (1965) in an early work proposed that a mechanism involving intrachromosomal recombination could explain the reversion of the white-ivory; however, some compounds that act as recombinagens in the Drosophila somatic wing test, such as methotrexate (Clements et al., 1990), strychnine (Würgler and Kägi, 1991), chromium (VI) oxide, potassium chromate and 2,4-dichlorophenoxyacetic acid (Graf and Würgler, 1996) are negative in the somatic white-ivory test. Therefore, the recombination mechanisms involved in both genetic systems are different, or it is possible that more than one mechanism is involved in the white-ivory reversion, as proposed by Howe and Clements (1990). Although these mechanisms have been proposed to explain the induced reversion in somatic cells, the same mechanisms could operate at germinal level; nevertheless, further work is needed to elucidate the mechanism(s) involved in such reversion and to know the molecular basis of the white-ivory somatic mutation assay. This knowledge is essential to decide the future use of the white-ivory system.

Acknowledgements

This research has been supported in part by the Spanish Ministry of Education and Science (Projects No. SAF92-0525 and SAF94-0697, CICYT) and by the Generalitat de Catalunya (CIRIT, GRQ93-2023).

References


Germinal reversions at the white-ivory locus in Drosophila melanogaster

Drosophila high molecular weight DNA to obtain genomic libraries. Nucleic Acid Res., 16, 2736.

Received on January 3, 1996; accepted on June 17, 1996