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

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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 Kägi, 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 5×10⁻⁵ in homozygous females, and 5×10⁻⁶ 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; Green et al., 1986; Howe and Clements, 1990) and also in somatic cells (Ryo et al., 1985; Green et al., 1986; Howe and Clements, 1990).

Kearss 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 (Kearss 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 f females; (iii) white-one (w¹), w¹; (iv) white-ivory (w¹), y y². 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 Lindsey 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 virgin 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 (A₄₅₀μm) with respect to the absorbance of wild-type (A₄₅₀μm), corrected for the absorbance of white-1 (A₄₅₀μm) as follows:

\[ \text{A₄₅₀μm - A₄₅₀μm / A₄₅₀μm} \]

Hybridization probe

The BglII–BglII, HindIII–BamHI and SalI–SalI fragments of the white locus, between +5163 and +7766, +3711 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, Genescribe-Z; USB Corp., Cleveland, OH, USA), and BglII–BglII 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

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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^E, and the different revertant lines, as described by Pittel 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) and then labelled with digoxigenin-11-dUTP by random primed reaction using DIG DNA Labeling kit (Boehringer Mannheim, Germany).

Results and discussion

To obtain an adequate number of revertants to be analysed, we treated first-instar larvae of the w^E 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 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 1). Eleven of these revertants (w^E to w^E16) 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–BglII, HindIII–BamHI and SalI–SalI). The first probe, BglII–BglII, 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–BamHI probe was used to detect the amplified fragments was carried out on a 1% agarose gel electrophoresis.

![Fig. 1. Restriction maps of a region of the white locus of the Canton-S wild type (a) and w^E (b) strains. The two-headed arrows denote the 1.3 kb BglII–BglII, the 2.4 kb HindIII–BamHI and the 0.8 kb SalI–SalI probes. Target points of HindIII and SalI restriction enzymes used in DNA digestions are indicated by vertical arrows. The thicker line indicates the 2.96 kb duplicated fragment in the w^E 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.)](image)
Germline reversions at the white-ivory locus in Drosophila melanogaster

Table 1. Quantitative measurement of red-eye pigments of different mutants obtained after chemical treatment

| Strain or mutant | Compound and concentration (mM) | Possible cluster* | Absorbance at 485 nm ± SE | Relative absorbance
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>-</td>
<td>-</td>
<td>1.09 ± 0.046</td>
<td>1.000</td>
</tr>
<tr>
<td>w</td>
<td>-</td>
<td>-</td>
<td>0.029 ± 0.002</td>
<td>0.050</td>
</tr>
<tr>
<td>wR1</td>
<td>EMS 0.25</td>
<td>one</td>
<td>0.011 ± 0.024</td>
<td>0.022</td>
</tr>
<tr>
<td>wR3</td>
<td>EMS 0.25</td>
<td>one</td>
<td>1.069 ± 0.070</td>
<td>0.977</td>
</tr>
<tr>
<td>wR23</td>
<td>EMS 0.50</td>
<td>one</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR24</td>
<td>EMS 1.00</td>
<td>one</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR5</td>
<td>MMS 0.50</td>
<td>one</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR1</td>
<td>ENU 0.25</td>
<td>one</td>
<td>1.096 ± 0.045</td>
<td>1.001</td>
</tr>
<tr>
<td>wR5</td>
<td>ENU 0.50</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR7</td>
<td>ENU 0.50</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR8</td>
<td>ENU 0.50</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR9</td>
<td>ENU 0.50</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR10</td>
<td>ENU 0.50</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR11</td>
<td>ENU 0.50</td>
<td>+</td>
<td>1.173 ± 0.080</td>
<td>1.071</td>
</tr>
<tr>
<td>wR12</td>
<td>ENU 0.50</td>
<td>+</td>
<td>1.289 ± 0.073</td>
<td>1.177</td>
</tr>
<tr>
<td>wR13</td>
<td>ENU 0.50</td>
<td>+</td>
<td>1.176 ± 0.010</td>
<td>1.074</td>
</tr>
<tr>
<td>wR14</td>
<td>ENU 0.50</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR15</td>
<td>ENU 0.50</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR16</td>
<td>ENU 0.50</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
</tr>
<tr>
<td>wR17</td>
<td>ENU 0.50</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
</tr>
</tbody>
</table>

*One = only one mutant was found in the culture; + = possible cluster (these mutants were found in the same culture bottle).

The relative absorbance was calculated as the ratio of the absorbance of the mutant (A<sub>485-w</sub>) respect to the absorbance of wild-type (A<sub>485-CS</sub>), corrected for the absorbance of white-1 (A<sub>485-w1</sub>) as follows:

(A<sub>485-w</sub> - A<sub>485-w1</sub>)/A<sub>485-CS</sub> - A<sub>485-w1</sub>.

N.D.: non-determined value.

We analysed the changes induced in the structural region of the white locus in the 18 induced germ-line revertants. Figure 2 presents the results of the Southern blot hybridization experiment with six revertants, compared with the CS and the w strains, using the probes from the structural region of the white-ivory locus. Hybridization of DNA digested with HindIII and Sall restriction enzymes with Sall-Sall probe shows a DNA fragment of 0.8 kb in all samples suggesting that no changes were produced in this region. On the other hand, when the HindIII-BamHI probe was used, the CS strain (lane 1) shows a DNA fragment of ~3.8 kb and the w strain (lane 2) a 6.8 kb fragment which correspond to their restriction maps (Figure 1). Revertants (lanes 3-8) show a DNA fragment of 3.8 kb like the CS strain, indicating the loss of the 2.96 kb DNA duplicated segment of the white-ivory allele. The remaining 11 revertants analysed showed the same pattern (data not shown).

In Figure 3 we can see the results of the Southern blot hybridization experiment using the BglII-BglII probe, after DNA digestion with HindIII and Sall enzymes, from the revertants previously indicated in Figure 2. In the lanes 1 and 2 (CS and w respectively), as well as in 4 and 8 (two of the six revertants analysed) we can observe a fragment of 5.7 kb, as expected from the white locus map (Levis et al., 1982) because the next HindIII target upstream is 5.7 kb apart, but in lanes 3, 5, 6 and 7, a longer fragment, of 6.8 kb, was observed. These results could be explained if, in these four revertants, one HindIII target was lost by a mutation. It must be indicated that three of the four revertants, w<sup>R11</sup>, w<sup>R12</sup> and w<sup>R13</sup> (lanes 5, 6 and 7), were found in the same culture bottle and therefore they could be considered a cluster, as we have previously discussed. In conclusion, at least two independent mutations affecting the HindIII target have been detected in the revertants analysed.

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, germlinal 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, 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.
Fig. 4. PCR amplification of the DNA sequences in the two boundaries of duplicated region of white-ivory mutation from CS, w<sup>1</sup> (lanes 3 and 4) and seven revertants (w<sup>1b</sup>, w<sup>1b2</sup>, w<sup>1b3</sup>, w<sup>1b4</sup>, w<sup>1b5</sup>, w<sup>1b6</sup> and w<sup>1b7</sup>; lanes 5–11 respectively). We used the HaeIII gX174 DNA restriction fragments of 1353, 1078, 872, 603 and 310 bp respectively, as molecular weight markers (lanes 1 and 13). Lanes 2 and 12 correspond to amplification reactions without DNA as a controls. (A) A fragment of 697 bp is amplified in all the samples using primers wip1 and wip2, which correspond to the upstream end of the duplicated fragment. (B) In the white-ivory sample (lane 4) a fragment of ~600 bp was amplified with primers wif3 and wip2, which were designed to amplify the junction sequence between the duplicated sequences, but no amplification was shown for CS and revertants as can be expected (C) With primers wif3 and wif4, the downstream end of the duplicated fragment was amplified (424 bp).

...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/are the exact mechanisms 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 germlinal 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.

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