

High genetic instability of heterochromatin after transposition of the LINE-like *I* factor in *Drosophila melanogaster*

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ABSTRACT In the present work, we have asked whether a group of 13 essential genes mapping to the heterochromatin of *Drosophila melanogaster* chromosome 2 are mutable following transposition of the *I* factor during I-R hybrid dysgenesis. We found that the frequency of lethal events mapping to chromosome 2 heterochromatin is surprisingly high, despite the low density of genetic functions identified in this region compared with euchromatin. Cytogenetic and molecular analyses indicated that the recovered mutations correspond either to insertions or to rearrangements. Moreover, chromosomes bearing specific heterochromatic lethal mutations were generated by recombination in the heterochromatin. Together, these data indicate that *I* factors transpose with high frequency into pericentric regions of chromosome 2 and may play a role in the evolution of constitutive heterochromatin.

Transposable elements (TEs) are repetitive mobile DNA sequences identified in a wide variety of organisms, ranging from bacteria to humans. These elements generally constitute 10–20% of the total eukaryotic genome (1). Besides their euchromatic location, TE-homologous sequences are frequently found in the heterochromatin of many species, including humans (2–7). In particular, recent studies have clearly established that in *Drosophila melanogaster* several clusters of retrotransposon-homologous sequences are present throughout the constitutive heterochromatin of all chromosomes (8).

TE accumulation represents one of the most intriguing aspects of the structure and organization of *Drosophila* heterochromatin. It has been suggested that heterochromatic regions may contain hotspots for TE insertions (9). Alternatively, the accumulation of TEs into heterochromatin might result from the combined effect of the genetic inertness and the inability of this part of the genome to undergo meiotic recombination (10); according to the latter hypothesis, transposition into constitutive heterochromatin would generally not give rise to mutational events with detrimental effects on fitness. However, *D. melanogaster* heterochromatin contains several single-copy and repetitive genetic loci (11) which *a priori* should not escape TE-mediated mutagenesis. Although it is well established that TEs are a major source of spontaneous euchromatic mutations in *D. melanogaster*, little is known about mutability of heterochromatic genes following transposition. Only a few studies have shown that normal or modified *P* elements, which mobilize through a DNA intermediate, are able to insert into the heterochromatin and can indeed cause insertional mutations (12–14). In addition, *R1* and *R2* retroelements insert at specific sites in the heterochromatic 28S rRNA genes of *Drosophila* (15), thus inactivating transcription of the rDNA unit. These elements belong to the evolutionarily widespread family of non-long terminal repeat

(non-LTR) elements which are mobilized by reverse transcription of an RNA intermediate. Representatives of this category also include mammalian *L1* sequences and both the *Het-A* elements and *I* factors of *Drosophila* (16–18). The *D. melanogaster I* factor belongs to the class of long interspersed nuclear elements (LINEs) and actively transposes in the germ line of the female progeny from crosses between reactive (R) females and inducer (I) males. This phenomenon, known as the I-R hybrid dysgenesis, gives rise to reduced fertility, increased frequency of euchromatic mutations, and X chromosome loss (19–21). Inducer strains contain 10–15 euchromatic dispersed copies of the 5.4-kb functional *I* factor, while several defective copies of *I* are embedded into the heterochromatin of both inducer and reactive strains (7, 20, 21). However, it remains uncertain whether the *I* factor can efficiently transpose in the heterochromatin.

To investigate LINE transposition in the heterochromatin and its mutagenic effect on heterochromatic genes, we have studied the mutability of a group of 13 vital loci mapping to the pericentric regions of *D. melanogaster* chromosome 2, following transposition of the *I* factor in the I-R hybrid dysgenesis. These genes have been extensively characterized at both the genetic and cytological levels and were found to behave like unique sequences (22–24). It has been shown that at least three of them, *light* (*lt*), *concertina* (*cta*), and *rolled* (*rl*), are indeed single-copy genes (11, 25). Our results show that the heterochromatic genes of chromosome 2 are highly mutable in I-R dysgenesis, and they provide evidence for a causal relationship between *I* factor transposition and heterochromatic mutability.

MATERIALS AND METHODS

***Drosophila* Stocks.** Genetic markers, mutations, and special chromosomes used in this work were described by Lindsley and Zimm (26). Genetic crosses were performed at 20°C ± 1°C. Reactive and inducer strains, which lack *P* elements, were provided by C. Vaury (Institut National de la Santé et de la Recherche Médicale, Clermont-Ferrand, France) and A. Pelisson (Centre National de la Recherche Scientifique, Gif-sur-Yvette, France).

Recovery of I-R Hybrid Dysgenesis-Induced Heterochromatic Lethal Mutations. Five dysgenic crosses involving the Charolles and *cn* reactive strains and the Canton-S, *Cy/Pm*, *M-5*, and *b lt rl cn* inducer strains were designed (Table 1). In these crosses, females from a reactive strain were crossed to males from an inducer strain; the F₁ dysgenic females were then mated to *CyO*, *S/Sco* males. F₂ males, heterozygous for the *CyO*, *S* balancer and its homolog transmitted from dysgenic

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Abbreviations: TE, transposable element; LTR, long terminal repeat; LINE, long interspersed nuclear element; EMS, ethyl methanesulfonate.

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Table 1. Heterochromatic lethals of chromosome 2 induced by I-R dysgenesis

Cross	Het.	Chr.	Lethals	Frequency	
1a	2R	1,564	11	7×10^{-3}	
1b	2R	1,003	0	0	
2a	2L	1,102	8	7.2×10^{-3}	
2b	2L	1,131	0	0	
3a	2R	1,204	5	4.1×10^{-3}	
3b	2R	896	0	0	
4a	2R	919	2	2.2×10^{-3}	
4b	2R	381	0	0	
5a	2R	1,024	2	1.9×10^{-3}	
5b	2R	967	1	1×10^{-3}	
6a	2L	909	2	2.2×10^{-3}	
6b	2L	712	0	0	
7a	2R	1,142	6	5.2×10^{-3}	
7b	2R	993	0	0	
8a	2L	746	1	1.3×10^{-3}	
8b	2L	520	0	0	
Exp. C.	2R	5,853	26	4.4×10^{-3}	$\chi^2 = 16.24$
	2R	4,240	1	2.3×10^{-4}	$P \leq 0.001$
Exp. C.	2L	2,757	11	4×10^{-3}	$\chi^2 = 11.17$
	2L	2,363	0	0	$P \leq 0.001$

Crosses 1a and 2a = Ch (R) \times C-S (I); 3a = Ch (R) \times M-5 (I); 4a = *cn* (R) \times *Cy/Pm* (I); 5a and 6a = *cn* (R) \times C-S (I); 7a and 8a = Ch (R) \times *b lt rl cn* (I), in which Ch = Charolles; C-S = Canton-S. The reciprocal control crosses are indicated with b. Het., the heterochromatic location of lethal mutations as judged by complementation with 2Lh or 2Rh deletions; Chr., number of chromosomes scored; Lethals, number of lethal chromosomes; Frequency, frequency of lethal chromosomes; Exp., total dysgenic crosses; C., total nondysgenic controls.

females, were individually mated to *Df(2R)M-S2¹⁰/Cy* or *Df(2L)Sd^{lt5}/Cy* females. *Df(2R)M-S2¹⁰* removes the bulk of the right arm heterochromatin of chromosome 2 (2Rh) with its vital genes, while *Df(2L)Sd^{lt5}* removes only the distal portion of the left arm heterochromatin of chromosome 2 (2Lh), a region which contains seven vital genes (24). The absence of *Cy⁺* progeny in the offspring of this cross indicates the induction of a lethal mutation on chromosome 2 which is uncovered by the heterochromatic deficiency. Chromosomes that failed to complement *Df(2L)Sd^{lt5}* were also tested over *Df(2L)C'*, which also lacks all the 2Lh genes (22). Chromosomes carrying lethal mutations mapping to heterochromatin were then tested for their ability to complement ethyl methanesulfonate (EMS)-induced alleles representing 13 genetic loci in 2Lh and 2Rh. For each complementation tests 150–200 progeny were counted. Among the chromosomes with 2Lh lethal alleles, *IR10* and *IR12* lines exhibited low viability and fertility and were subsequently lost. In the control crosses nondysgenic females were obtained by crossing inducer females to reactive males.

To compare the frequency of euchromatic vs. heterochromatic lethals simultaneously induced along chromosome 2, Charolles (R) females were crossed to Canton-S (I) males and the F₁ dysgenic females were then mated to *CyO*, *S/Sco* males. F₂ males, heterozygous for the *CyO*, *S* balancer and its homolog transmitted from dysgenic females, were individually crossed to *CyO*, *S/Sco* females to establish several lines. Homozygous lethal chromosomes were then tested for their ability to complement EMS-induced alleles representing 13 heterochromatic genes of chromosome 2.

Recovery of Heterochromatic Recombinants. Dysgenic females were recovered by crossing wild-type Charolles (R) females to *b lt rl cn/b lt rl cn* (I) males. In the control crosses *b lt rl cn/+ + + +* nondysgenic females were obtained by crossing *b lt rl cn/b lt rl cn* (I) females to wild-type Charolles

(R) males. The F₁ *b lt rl cn/+ + + +* dysgenic and nondysgenic females were then mated to *b lt rl cn/b lt rl cn* (I) males; the frequencies of euchromatic and heterochromatic recombinants in the F₂ offspring were then calculated. Recombinant lines were established by crossing single *b lt + +/b lt rl cn* and *b lt rl +/b lt rl cn* males to *Cy*, *cn/Sco* females, and *+ + rl cn/b lt rl cn* males to *Sco lt rl/Cy* females. The *b lt rl cn/+ + + +* females were also used to determine the frequency of heterochromatic lethals on chromosome 2 (Table 1: crosses 7a–7b and 8a–8b). The chromosomes with heterochromatic lethal alleles recovered in these experiments were backcrossed to *b lt rl cn* homozygous flies to establish whether they had been induced on chromosomes that had undergone heterochromatic recombination.

Nucleic Acid Manipulations, DNA Sequencing, PCR Amplification, and Cloning. DNA and RNA extraction, Southern and Northern hybridization assays, and recombinant DNA techniques were essentially performed according to standard procedures (27). The *I* factor insertion in the intron 2 of the *rl* gene was amplified by PCR (30 cycles; 94°C for 1 min, 55°C for 1 min, 72°C for 3 min) using 0.1 μ g of genomic DNA from the Canton-S, Charolles, and *rl^{IR1}* strains, and the following oligonucleotide primers: r13 (5'-GCGGATGACACGCTAACCA-3'), derived from exon 2 of the *rl* gene (M95124, nucleotides 415–432), and I5R (5'-TGCTGTTACATCCTCATCTG-3'), derived from the 5' end of the *I* factor (M14954, nucleotides 561–542). The 982-bp PCR product obtained from the *rl^{IR1}* strain was subcloned into the *EcoRV* site of the pBC KS (+) vector (Stratagene) and sequenced with the following primers: (i) T3; (ii) T7; (iii) I5R2 (5'-GGATTAGCGGTATTGTTG-3', M14954 nucleotides 260–243); (iv) rlint2 (5'-CAGGCATACCCCATGAGG-3'), and (v) B290 (5'-TCGAAAGAGTTGTGTGCA-3', M14954 nucleotides 392–375). The hybridization probes were a 1.4-kb cDNA *HindIII* restriction fragment containing the complete coding region of the *rl* gene (28), a 1.5-kb *SalI-HindIII* fragment containing the 5' end of the *I* factor (20), and a 530-bp *HindIII-DraI* fragment containing the 5' untranslated region and the first two exons of the *rl* cDNA (W. H. Biggs & K. Zavitz, personal communication).

RESULTS

Heterochromatic Mutations Induced by I-R Hybrid Dysgenesis. Our aim was to investigate whether activation of *I* factor transposition resulted in genetic instability of the heterochromatin. Five different dysgenic crosses were set up, and 37 lethal chromosomes 2 were recovered (Table 1). Of these, 26 of 5,853 chromosomes (4.4×10^{-3}) failed to complement *Df(2R)M-S2¹⁰*, thus mapping the lethal alleles to 2Rh, while the remaining 11, recovered from a total of 2,757 chromosomes (4×10^{-3}), failed to complement *Df(2L)Sd^{lt5}* and mapped to 2Lh. In nondysgenic controls, only one lethal mapping to 2Rh was recovered among 4,240 chromosomes, while no lethal mapping to 2Lh was recovered out of 2,363 chromosomes. Thus, I-R dysgenesis was highly effective in inducing mutations of chromosome 2 heterochromatic genes. The lethal chromosomes were further characterized in complementation assays with EMS-induced point mutations belonging to 13 heterochromatic complementation groups on chromosome 2 (22). The results of this analysis are summarized in Table 2. Twenty-two lethal mutations genetically behaved like point mutations. Interestingly, 8 chromosomes failed to complement more than one of the EMS-induced mutations and thus behaved as heterochromatic rearrangements. For example, *IR-1* failed to complement the four most proximal *41Ab*, *41Aa*, *rl*, and *41Ad* genes, while *IR-11* simultaneously lacked *40Fg* and *40Ff* loci, yet retained *40Fe*, which lies in between *40Fg* and *40Ff* (23), and thus likely corresponds to an inversion. In addition, *IR-45* behaved like a complex rearrangement, rather than a polar deletion. Finally, *IR-3*, *IR-4*, *IR-23*, *IR-24*, and *IR-30*, although

Table 2. Genetic analysis of heterochromatic mutations induced by the I-R dysgenesis

Cross	Lethals	Allelism test
1	<i>l(2Rh)IR1</i>	<i>41Ab; 41Aa; rl; 41Ad</i>
	<i>l(2Rh)IR2</i>	<i>41Ad</i>
	<i>l(2Rh)IR3</i>	Lethal with <i>M-S2¹⁰</i> and <i>M-S2⁸</i> ; vital with <i>e97</i>
	<i>l(2Rh)IR4</i>	
	<i>l(2Rh)IR5</i>	<i>41Ah</i>
	<i>l(2Rh)IR6</i>	<i>41Ah</i>
	<i>l(2Rh)IR7</i>	<i>41Aa; rl</i>
	<i>l(2Rh)IR8</i>	<i>41Ad</i>
	<i>l(2Rh)IR9</i>	<i>41Ad</i>
	<i>l(2Rh)IR17</i>	<i>41Ah</i>
	<i>l(2Rh)IR18</i>	<i>41Aa</i>
	<i>l(2Rh)IR37</i>	<i>rl</i>
	<i>l(2Rh)IR38</i>	<i>41Ad</i>
	<i>l(2Rh)IR39</i>	<i>41Ad</i>
	<i>l(2Lh)IR10</i>	Lethal with <i>Sd^{ht5}</i> and <i>C'</i>
	<i>l(2Lh)IR11</i>	<i>40Ff; 40Fg</i>
	<i>l(2Lh)IR12</i>	Semilethal with <i>Sd^{ht5}</i> and <i>C'</i>
	<i>l(2Lh)IR29</i>	<i>40Ff; 40Fe; 40Fg</i>
	<i>l(2Lh)IR31</i>	<i>40Fe</i>
	<i>l(2Lh)IR32</i>	<i>40Ff</i>
<i>l(2Lh)IR33</i>	<i>40Fe; 40Fg</i>	
<i>l(2Lh)IR34</i>	<i>40Fe</i>	
<i>l(2Lh)IR35</i>	<i>40Fe; 40Fg</i>	
<i>l(2Lh)IR36</i>	<i>40Ff; 40Fe; 40Fg; 40Fb</i>	
2	<i>l(2Rh)IR13</i>	<i>41Ae</i>
	<i>l(2Rh)IR14</i>	<i>41Ae</i>
3	<i>l(2Rh)IR21</i>	<i>41Ad</i>
	<i>l(2Rh)IR23</i>	Lethal with <i>M-S2¹⁰</i> and <i>M-S2⁸</i> ; vital with <i>e97</i>
	<i>l(2Rh)IR24</i>	
	<i>l(2Rh)IR27</i>	<i>rl</i>
<i>l(2Rh)IR30</i>	Lethal with <i>M-S2¹⁰</i> and <i>M-S2⁸</i>	
4	<i>l(2Rh)IR25</i>	<i>41Ae</i>
	<i>l(2Rh)IR26</i>	<i>41Ae</i>
	<i>l(2Lh)IR20</i>	<i>40Fg</i>
	<i>l(2Lh)IR28</i>	<i>40Fe; 40Fg</i>
5	<i>l(2Rh)IR40</i>	<i>41Ad</i>
	<i>l(2Rh)IR41</i>	<i>rl</i>
	<i>l(2Rh)IR42</i>	<i>41Ab; 41Aa; rl; 41Ad</i>
	<i>l(2Rh)IR43</i>	<i>41Ah</i>
	<i>l(2Lh)IR45</i>	Retains only <i>40Fc</i>
	<i>l(2Rh)IR46</i>	<i>41Ad</i>
	<i>l(2Rh)IR47</i>	<i>41Ad</i>

Crosses 1 = Ch × C-S; 2 = cn × Cy/Pm; 3 = Ch × M-5; 4 = cn × C-S; and 5 = Ch × *b lt rl cn*, in which Ch = Charolles and C-S = Canton-S. The following EMS-induced alleles representing 13 genetic loci mapping to the heterochromatin of chromosome 2 were used in complementation tests: *56-8*, *56-32*, *56-3*, *40-7*, *56-4*, *56-24*, and *40-5* for 2Lh and *45-10*, *31*, *64*, *45-17*, *34-25*, and *EMS 45-75* for 2Rh.

complementing EMS alleles of all six known 2Rh loci, failed to complement *Df(2Rh)M-S2⁸*, which removes the distal *41Ae* and *41Af* loci. The lethal mutations on these chromosomes were rescued by the *e97* duplication carrying the entire 2Rh (29). Thus, these chromosomes may carry mutations in genes mapping to the distal portion of 2Rh other than *41Ae* and *41Af*. We also performed an F₁ screen for the recovery of I-R dysgenesis-induced mutations in the *lt* and *rl* genes. Charolles (R) females were crossed to Canton-S (I) males, and their dysgenic daughters were subsequently mated to *b lt rl cn* homozygous males to select *lt* and *rl* phenotypes. Two lethal *light* (*lt^{IR1}* and *lt^{IR2}*) and two vital *rl* alleles (*rl^{IR1}* and *rl^{IR2}*) were recovered from 15,777 individuals, which corresponds to a frequency of 10⁻⁴ for both genes. No heterochromatic muta-

tion was recovered from 19,838 individuals scored in the reciprocal nondysgenic cross.

Euchromatic and Heterochromatic Lethals Induced by I-R Dysgenesis. We next performed dysgenic crosses designed to compare the frequency of euchromatic vs. heterochromatic lethals simultaneously induced along chromosome 2. A total of 45 homozygous lethal chromosomes were recovered from 830 chromosomes in I-R dysgenic crosses, while 6 lethal chromosomes were found over 539 chromosomes in nondysgenic controls; the difference in the lethal frequency between experimental and control groups is statistically significant ($\chi^2 = 15.73$; $P \leq 0.001$). Five lethal chromosomes from the dysgenic crosses (*IR-29*, *IR-36*, *IR-37*, *IR-38*, and *IR-39*) were found to carry heterochromatic lethals (Table 2). Among them, *IR-29* behaved like a deletion, while *IR-36* was likely to correspond to a multibreak rearrangement; the remaining 40 lethals complemented heterochromatic deficiencies of both 2R and 2L, and thus mapped to the euchromatin. None of the 6 lethals recovered from control crosses were heterochromatic; they may have been induced by a basal level of dysgenesis, and/or may correspond to preexisting lethals in Canton-S chromosome 2 that was not isogenized. To summarize, these experiments yielded euchromatic recessive lethals with a frequency close to 4.8×10^{-2} , and heterochromatic lethals with a frequency of 6×10^{-3} .

Cytological Analysis of I-R Dysgenesis-Induced Heterochromatic Lethals. To establish whether structural alterations had occurred in the heterochromatin of I-R-induced lethals, mitotic chromosomes of 39 lines were examined after Hoechst 33258 banding. The cytological data are summarized in the map in Fig. 1. The *IR-1*, *IR-7*, and *IR-42* lethals carry deletions affecting the 2R heterochromatin, in agreement with the genetic data (Table 2). The deletions occurring in both *IR-1* and *IR-42* spanned the region included between the proximal part of h41 and h44, while *IR-7* lacked a large part of h41. The *IR-2*, *IR-39*, *IR-40*, and *IR-47* mutations, which genetically belong to the *41Ad* complementation group only, were also found to be deletions: for example, both *IR-2* and *IR-40* span the region from h42 to h44. In contrast, no cytological abnormality was detected in *IR-11*, *IR-28*, *IR-29*, *IR-33*, *IR-35*, *IR-36*, and *IR-45*, which genetically behaved as rearrangements (see

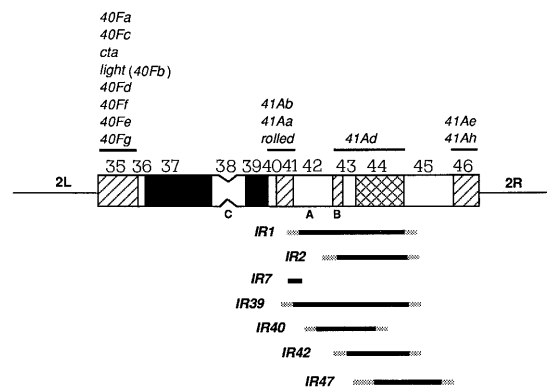


FIG. 1. Cytogenetic map of chromosome 2 heterochromatin, showing the extent of the I-R-induced deficiencies along the heterochromatin of the right arm. Thirteen cytologically distinct regions, designated h35 to h46, can be resolved in chromosome 2 heterochromatin (24). Filled areas represent the Hoechst 33258-bright regions; the shaded boxes represent regions of intermediate fluorescence, and the open boxes are regions of dull fluorescence. Only the heterochromatic regions are shown. Above the diagram are bars indicating the mapping of 13 vital heterochromatic genes. The genes in the left arm of chromosome 2 (2Lh) are indicated from top to bottom according to their left to right mapping along the chromosome. The extent of the I-R-induced deficiencies was determined cytogenetically as described in detail elsewhere (24, 30) and is represented by thick lines (below). 2L, left arm; 2R, right arm; C, centromeric region.

Table 2), or among the remaining lethals. In conclusion, genetic and cytological analyses show that almost 35% of all analyzed heterochromatic lethals were associated with chromosome rearrangements which mostly corresponded to large heterochromatic deletions. Interestingly, in an independent study about 30% of the I-R-induced euchromatic lethals of the *X* chromosome were also found to be associated with chromosomal rearrangements (31).

Heterochromatic Recombination Induced by I-R Dysgenesis. Although heterochromatin does not undergo meiotic recombination with an appreciable frequency, Hirazumi reported recombination between *lt* and Responder (*Rsp*) loci following I-R dysgenesis (32). We wished to investigate the possibility that heterochromatic recombination was involved in the generation of lethals. To that aim, we measured the frequency of both heterochromatic recombinants and heterochromatic lethals recovered from the same dysgenic cross involving the wild-type Charolles (R) strain and the *b lt rl cn* (I) strain (see *Materials and Methods* for details). The results of these experiments are shown in Tables 1, 3, and 4. The frequency of recombinants between the *b* and *lt* markers of 2L and between *rl* and *cn* in 2R does not increase in the dysgenic cross compared with nondysgenic control (Table 3). In contrast, the frequency of recombinants between *lt* and *rl* approaches 10^{-2} in the dysgenic experiment and is significantly higher, by nearly two orders of magnitude, than that observed in the control. Among a subset of 50 randomly selected recombinants between *lt* and *rl*, one chromosome, designated *R1(+ + rl cn)* had acquired a lethal allele of the heterochromatic *l(2Rh)41Ad* gene, which is located distally to *rl*. Therefore, the frequency of heterochromatic lethals within the group of recombinant chromosomes approached 2×10^{-2} (1/50) and was not significantly different from the 0.6×10^{-2} frequency that we found within the group of chromosomes that had not been selected for recombination (see crosses 7a and 7b in Table 1). When we determined the fraction of heterochromatic recombinants in the group of heterochromatic lethals, we found that two (*IR-40* and *IR-47*) of seven lethals (28%) were recombinants between the *rl* and *cn* markers (Table 4). In this case, the frequency of recombinants is significantly higher ($\chi^2 = 11.54$; $P \leq 0.001$) than that measured in a group of chromosomes that had not been selected for lethality (see Table 3). Both *IR-40* and *IR-47* were alleles of the *41Ad* gene. In addition, among a group of twenty *b lt rl +* recombinants, one (*R31*) was found to be a lethal allele of *41Ad*. It is intriguing that *IR-40*, *IR-47*, and *R31* had all independently accumulated a lethal allele of the same *l(2Rh)41Ad* gene, which maps to the h44 region of 2Rh (see Fig. 1) between *rl* and *cn*. Both the *IR-40* and *IR-47* lethals cytologically corresponded to deletions of 2Rh affecting regions h43–44–45 (Fig. 1), while *R31* showed no chromosomal alteration.

I Factor Insertion Within the *rl* Gene. To investigate the molecular nature of I-R dysgenesis-induced heterochromatic mutations, we initially carried out Southern blot hybridization experiments to analyze the *rl* region in chromosomes carrying the homozygous viable *rl^{IRI}* allele in comparison with wild-type parental chromosomes. The *rl^{IRI}* allele arose from dysgenic crosses between the Charolles (R) and Canton-S (I) strains. Consistent with the large size of the *rl* locus (W. H. Biggs III and K. Zavitz, personal communication), several genomic

Table 4. Recombinants between *rl* and *cn* within a group of chromosome 2 heterochromatic lethals

Lethals	Genotype	Allelism test
<i>l(2Rh)IRh40</i>	<i>b lt rl +</i>	<i>41Ad</i> (Df)
<i>l(2Rh)IRh41</i>	<i>b lt rl cn</i>	<i>rl</i>
<i>l(2Rh)IRh42</i>	<i>b lt rl cn</i>	<i>41Aa; rl; 41Ad</i> (Df)
<i>l(2Rh)IRh43</i>	<i>b lt rl cn</i>	<i>41Ah</i>
<i>l(2Rh)IRh46</i>	<i>b lt rl cn</i>	<i>41Ad</i>
<i>l(2Rh)IRh47</i>	<i>+ + + cn</i>	<i>41Ad</i> (Df)
<i>l(2Lh)IRh45</i>	<i>+ lt + +</i>	Retains only <i>40Fc</i>

IRh41, *IRh42*, *IRh43*, and *IRh42* are associated with the *b lt rl cn* chromosomes; *IRh40* and *IRh47* are associated with the *b lt rl +* and *+ + + cn* recombinant chromosomes, respectively; *IRh45* is a deletion which is likely to be induced on the Charolles wild-type chromosome. (Df) = cytologically detectable deletions.

fragments could be identified after digestion with *EcoRI*, which does not cut within the *I* factor. The only visible difference in the digestion pattern of the parental *rl⁺* Charolles compared with the mutant strain consisted in the altered mobility of one hybridizing band, which shifted from 10 kb to about 15 kb (Fig. 2A); this difference is consistent with what would be expected from the insertion of a full-length *I* factor. Reprobing the filter with a 1.5-kb probe containing the *I* factor 5' end (Fig. 2B) showed that the newly generated 15-kb band of *rl^{IRI}* indeed comigrated with *I* factor-homologous DNA sequences. Similar results were obtained after digestion with *PstI* and *XhoI* (data not shown). A more detailed mapping of the putative *I* factor insertion was obtained using a probe corresponding to a 500-bp *HindIII–DraI* fragment, containing exons 1 and 2 of the *rl* gene (W. H. Biggs III and K. Zavitz, personal communication). Only two bands, of about 3 kb and 3.5 kb respectively, hybridized with this probe in both Canton-S and Charolles *rl⁺* parental strains after *EcoRV* digestion (Fig. 2C). In the *rl^{IRI}* mutant, a novel 5-kb band could be seen, which replaced the parental 3.5-kb fragment. The observed difference in the size of these two bands corresponds to about 1.5 kb, in agreement with the presence of an *EcoRV* site at position 1570 in the *I* factor DNA. The *rl* exons 1 and 2 were found to be 350 and 140 bp long, respectively (W. H. Biggs III and K. Zavitz, personal communication), suggesting that the weaker signal intensity over the 3.5-kb parental band, which shifts to 5 kb in *rl^{IRI}*, might reflect the hybridization with exon 2 sequences. Together, these results suggest that *rl^{IRI}* corresponds to an *I* factor insertion closely linked to exon 2.

To learn more about the nature of the *rl^{IRI}* mutation, we used different pairs of oligonucleotide primers in PCR amplification reactions from genomic DNA of Charolles, Canton-S, and *rl^{IRI}* strains. Specific sets of primers were synthesized from different regions of *rl* exon 2 and from the *I* factor (see *Materials and Methods* for details), to amplify possible integration products of *I* within *rl* DNA, as suggested by the Southern analysis. Using the *rl3* and *I5R* combination of primers, directed respectively downstream of *rl* exon 2 and forward from the *I* factor 5' end, we could specifically amplify a 982-bp fragment from genomic DNA of *rl^{IRI}* homozygotes (Fig. 2D). Sequence analysis showed that the PCR fragment indeed contained 125 bp of *rl* exon 2, followed by a 394-bp region of unidentified homology, which likely corresponds to sequences of *rl* intron 2, and by 462 bp, representing the

Table 3. I-R dysgenesis-induced recombination between *lt* and *rl*

Cross	No. of parentals <i>b lt rl cn</i> + + + +	No. of recombinants			Frequency of recombinants		
		<i>b + + +</i> <i>+ lt rl cn</i>	<i>b lt rl +</i> <i>+ + + cn</i>	<i>b lt + +</i> <i>+ + rl cn</i>	<i>b</i> and <i>lt</i>	<i>rl</i> and <i>cn</i>	<i>lt</i> and <i>rl</i>
Exp.	6,298	338	126	75	4.9×10^{-2}	1.8×10^{-2}	1×10^{-2}
Control	8,037	457	138	2	5.3×10^{-2}	1.6×10^{-2}	2.3×10^{-4}

Exp. = dysgenic crosses; Control = nondysgenic controls.

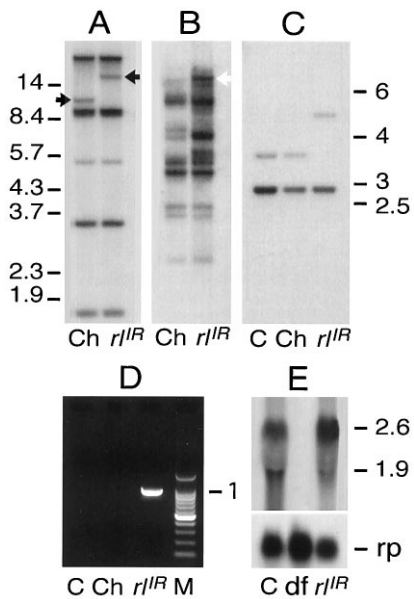


FIG. 2. (A) Southern hybridization of genomic DNA from the Charolles parental strain (Ch) carrying the rl^+ allele, and from rl^{IR} homozygotes (rl^{IR}), digested with *EcoRI* and probed with the 1.4-kb rl cDNA probe (30); black arrows indicate the bands whose mobility varies in the rl^{IR} mutant and in the Charolles strain. (B) Filters were stripped and reprobated with *I* sequences; the open arrow indicates *I*-homologous sequences in the dysgenic mutants that comigrate with *rl* homologous sequences. (C) Southern hybridization of genomic DNA from Canton-S (C) and Charolles (Ch) parental strains carrying the rl^+ allele, and from rl^{IR} homozygotes (rl^{IR}), digested with *EcoRV* and probed with the *HindIII*-*DraI* fragment. (D) PCR amplification reaction from genomic DNA of Canton-S (C), Charolles (Ch), and rl^{IR} strains. A 982-bp fragment was specifically amplified from genomic DNA of rl^{IR} homozygotes. M, molecular weight marker. (E) Northern blot assays of second-instar larvae total RNA from rl^+ , rl^{IR} , and *Df(2Rh)Rsp¹* homozygotes lacking the *rl* gene. The 1.4-kb rl cDNA probe reveals two major transcripts of 2.6 kb and 1.9 kb, both absent in larvae homozygous for *Df(2Rh)Rsp¹*. The same filter was reprobated with the rp49 ribosomal protein gene probe as a loading control.

terminal part of the *I* factor 5' end (GenBank accession no. Y11299). These results clearly show that the rl^{IR} allele is associated with a *de novo* insertion of the *I* factor into a *rl* intron, 400 bp downstream of exon 2 (Fig. 3).

Consistent with the findings that the *I* factor had been inserted in an intronic region of the *rl* gene, no detectable difference was observed in the size of mRNA transcripts homologous to the rl cDNA probe from the parental and rl^{IR} strains (Fig. 2E), although it remains possible that the *I* insertion caused abnormalities in the splicing and/or processing of the RNA transcripts.

DISCUSSION

In this work, we have investigated whether chromosome 2 heterochromatic genes of *D. melanogaster* are mutable after I-R hybrid dysgenesis, where transposition of the LINE-like *I*

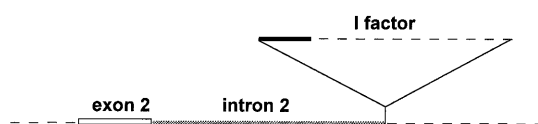


FIG. 3. Schematic representation of the *I* factor insertion in the rl gene. The white and shaded lines represent 125 bp of the rl exon 2, and 394 bp of the rl intron 2 respectively; the filled line represents the 5' end of the *I* factor. Dashed lines represent rl^{IR} genomic sequences and *I* factor sequences that were not included in the 982-bp PCR fragment. The *I* factor insert is not in scale.

factors is activated. An interesting outcome of our studies is the high overall frequency of lethal mutations mapping to chromosome 2 heterochromatin in dysgenic crosses, approaching 10^{-2} (Table 1). In particular, the *41Ad* gene is the most highly mutable heterochromatic gene of chromosome 2, and it exhibits an overall frequency of mutation (1.5×10^{-3}) which is one order of magnitude greater than that calculated for the most mutable euchromatic genes in I-R dysgenic crosses (17, 21). In addition, along chromosome 2, which carries at least 2,000 genes, euchromatic mutations were recovered with a frequency close to 5×10^{-2} ; in the same experiments, heterochromatic mutations were recovered with a frequency of about 0.6×10^{-2} ; thus, the euchromatin is only 8-fold more susceptible to mutate to recessive lethal than heterochromatin, despite its 150-fold enrichment in genetic functions. It follows that heterochromatic genes are about 20-fold more mutable than euchromatic genes in chromosome 2. About 35% of the analyzed heterochromatic lethals were associated with rearrangements which mostly correspond to large deletions spanning several megabases of DNA. The remaining lethals affecting individual loci may correspond to *I* factor insertions into the heterochromatic genes, as shown in the case of the rl^{IR} mutation (Figs. 2 and 3); in other cases, the occurrence of small lesions beyond the cytological level of resolution cannot be ruled out.

We have also found that I-R dysgenesis induces 1% recombination between the *lt* and *rl* genes in chromosome 2 heterochromatin, a value which is nearly two orders of magnitude higher than observed in nondysgenic controls. However, recombination between *rl* and *cn* is indeed involved in the specific generation of lethal alleles of the *41Ad* gene (Table 4). Interestingly, two of them (*IR40* and *IR47*) correspond to cytologically detectable deletions of 2Rh (Fig. 1) which might result from unequal exchanges. I-R dysgenesis was previously reported to induce both insertional mutations and chromosomal rearrangements in the *X* chromosome euchromatin (21, 31, 33). In most examined cases, *I* factor sequences were found to map at the breakpoints of the I-R-induced rearrangements, which were suggested to result either from recombination between transposing copies of *I* at the time of integration or, alternatively, from a target exchange between *I* factors. According to the latter model, the same element would join two nicks generated in nonhomologous chromosomal regions.

Non-LTR retrotransposons have been shown to integrate at specific heterochromatic sites in the *Drosophila* genome; for example, the *R1* and *R2* elements insert into specific sequences of the 28S rRNA genes (15). The *Het-A* and *TART* elements also specifically transpose with high frequency to broken chromosomal ends, which are regarded as being heterochromatic (18). The high frequency of mutations reported in the present study suggests that single-copy heterochromatic genes of chromosome 2 are a preferential target for *I* factor retrotransposition, which in turn would produce either insertional events or chromosomal rearrangements. This conclusion conflicts with the view that constitutive heterochromatin is mostly insensitive to the mutational effects caused by TEs (10).

Because TEs induce chromosome rearrangements in both natural and laboratory populations of *Drosophila*, they have been proposed to shape the genome structure (34). In addition, TEs may drive the concerted evolution of repetitive DNA sequences (35). It is tempting to speculate that the heterochromatic variability generated by both mutations and recombination events during I-R dysgenesis might have played a role in the evolution of *Drosophila* heterochromatin. Polymorphisms of constitutive heterochromatin are known to occur in natural populations of higher eukaryotes, including *Drosophila* and humans (36–38). Contraction or expansion of heterochromatic regions, such as those depicted in the *Rsp* locus (30, 39),

are thought to result from unequal exchanges between satellite DNA repeats. It is not unreasonable to suggest that these events could also be stimulated by I-R dysgenesis-induced heterochromatic recombination. Interestingly, *L1* elements were suggested to be involved in the generation of heterochromatic variability in human chromosomes (40). Further investigations on the interactions between LINEs and heterochromatic regions will be necessary to further clarify the impact of TEs upon heterochromatin evolution in eukaryotes. Finally, insertional mutagenesis with marked *I* factor might represent a powerful tool for molecular dissection of heterochromatin and cloning of heterochromatic genes.

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