Genetic Analysis of the Heterochromatin of Chromosome 3 in *Drosophila melanogaster*. II. Vital Loci Identified Through EMS Mutagenesis

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**ABSTRACT**

Chromosome 3 of *Drosophila melanogaster* contains the last major blocks of heterochromatin in this species to be genetically analyzed. Deficiencies of heterochromatin generated through the detachment of compound-3 chromosomes revealed the presence of vital loci in the heterochromatin of chromosome 3, but an extensive complementation analysis with various combinations of lethal and nonlethal detachment products gave no evidence of tandemly repeated vital genes in this region. These findings indicate that the heterochromatin of chromosome 3 is genetically similar to that of chromosome 2. A more thorough genetic analysis of the heterochromatic regions has been carried out using the chemical mutagen ethyl methanesulfonate (EMS). Seventy-five EMS-induced lethals allelic to loci uncovered by detachment-product deficiencies were recovered and tested for complementation. In total, 12 complementation groups were identified, ten in the heterochromatin to the left of the centromere and two to the right. All but two complementation groups in the left heterochromatic block could be identified as separate loci through deficiency mapping. The interallelic complementation observed between some EMS-induced lethals, as well as the recovery of a temperature-sensitive allele for each of the two loci, provided further evidence that single-copy, transcribed vital genes reside in the heterochromatin of chromosome 3. Cytological analysis of three detachment-product deficiencies provided evidence that at least some of the genes uncovered in this study are located in the most distal segments of the heterochromatin in both arms. This study provides a detailed genetic analysis of chromosome 3 heterochromatin and offers further information on the genetic nature and heterogeneity of Drosophila heterochromatin.

Genetic loci expressing vital functions have been identified within regions of the centromeric heterochromatin of chromosome 3 in *Drosophila melanogaster*. These loci were uncovered through the generation of deficiencies apparently restricted to the heterochromatic segment of the chromosome as a result of detaching compound-3 chromosomes in females (Marchant and Holm 1988). Although findings from the detachment study give no indication of repeated copies of vital loci, deficiency mapping alone provides insufficient evidence to conclude that the genes within chromosome 3 heterochromatin are single-copy loci. Consequently, it has been necessary to turn to a mutagen, ethyl methanesulfonate (EMS), that primarily induces point mutations and which had been most effective in producing single-gene mutations in the heterochromatic regions of chromosome 2 (Hilliker 1976).

The discovery of genes in heterochromatin contradicts earlier suggestions that heterochromatin is genetically inactive. Constitutive heterochromatin is greatly enriched in satellite DNA (Peacock et al. 1973) and this multiple-copy, short sequence DNA is almost certainly transcriptionally inert (Flamm, Walker and McCallum 1969; Southern 1970; Woodcock and Sibatani 1975; Bostock 1980). However, there is growing evidence that heterochromatin is not uniform in composition and exhibits a variety of molecular and genetic characteristics. For example, various organisms have disclosed exceptions to the general rule that heterochromatin is primarily composed of satellite DNA. Some blocks of heterochromatin have been reported to contain little or no satellite DNA (Hennig 1972; Arrighi et al. 1974; Cordeiro et al. 1975; Holmquist 1975; Wheeler et al. 1978; Ranganath, Schmidt and Hagele 1982). As well, some middle repetitive and unique sequences have been detected in blocks of heterochromatin that do contain mostly highly repeated satellite DNA (Comings and Mattoccia 1972). The heterochromatin of Peromyscus cells contains satellite DNA sequences covalently linked to nonrepetitive sequences (Kuo and Hsu 1978). Type I insertion sequences, originally discovered within the rDNA genes, have been found in *D. melanogaster* heterochromatin outside of the nucleolus organizer region (Peacock et al. 1973).
and one arm of the third chromosome is subdivided by a number of lightly stained bands. However, the chromosome and heterochromatin on the other arm chromosome, both arms of the second chromosome, genome reveals that the heterochromatin of the X 

ature-sensitive lethals were recovered. From the number of mutations recovered for each complementation groups, it would appear that all vital loci uncovered by deficiencies of chromosome-3 heterochromatin have been identified. Cytological examinations of three detachment products by S. PIMPINELLI (personal communications) have established relative positions for some of the genetic loci in the heterochromatin of chromosome 3.

MATERIALS AND METHODS

Inversion chromosomes and genetic markers used in this study are described in LINDSLEY and GRELL (1968). A strain isogenic for the third chromosome carrying the proximal genetic markers ri to the left and p^ to the right of the centromere was used for EMS mutagenesis. Induced lethal mutations were balanced over In (3LR) TM3, ri p^ sep Sb bx^w e'^Ser (hereafter referred to as TM3) which was maintained in stock heterozygous with the dominant mutation LyrA (Ly). Deficiencies used to identify and provide relative map positions of EMS-induced alleles of loci in 3L and 3R heterochromatin are described in the results section and diagrammatically represented in Figures 1 through 4 plus 6. Throughout this study flies were maintained on a standard Drosophila medium prepared from cornmeal, yeast, sucrose, dextrose and agar with propionic acid added as a mold inhibitor.

EMS mutagenesis: Newely closed males from the isogenic ri p^ stock were aged two days and then fed with 0.025 M EMS in 1% sucrose solution for 18 hours as described by LEWIS and BACHER (1968). After treatment, the males were allowed to recover for one day on standard medium and then mated to virgin TM3/Ly females. Male offspring heterozygous for the mutagenized ri p^ chromosome over Ly were collected and tested for lethals allelic to detachment-product deficiencies of the left and right arm. Each male was single pair mated at 29°C in shell vials to females carrying a detachment-product deficiency of the left arm balanced over TM3. After four days, the parents were removed, and the males were re-mated to females carrying a deletion of the right arm heterochromatin. In all, two different deficiencies of the left arm, Df(3L)1-16 and Df(3L)1-166, and two of the right arm, Df(3Ry)4-75 and Df(3Ry)5-65, were used. However, each mutagenized chromosome was tested against only one of the deficiencies for each of the arms. The offspring of each cross were examined for the presence or absence of ri p^/ri p^ and ri p^/TM3 individuals. Vials that contained no ri p^/ri p^ progeny were scored as putative lethal alleles of the detachment-product deficiency. The absence of ri p^/TM3 progeny indicated that a mutation had been induced that was allelic to one of the lethals carried on the TM3 chromosome.

All putative EMS-lethal alleles of deficiencies were re-tested at both 29°C and 29°C to confirm their lethality and to test for temperature-sensitivity. Each confirmed lethal was positioned relative to the deficiency complementation map by a series of lethality tests against small deficiencies of individual complementation regions. All EMS mutants that mapped to a particular complementation group were then complementation-tested against each other in all inter se combinations.

Putative EMS-induced lethal alleles of TM3 were also retained and tested for complementation in all inter se combinations. If the TM3 chromosome had accumulated recessive lethals in the centromeric region, the detachment products carrying a deficiency that included these loci would not have been recovered. To examine this possibility, and to identify the relative map positions of EMS-induced mu-
tations allelic to recessive lethals carried on the TM3 chromosome, crossover analyses were carried out as described in the previous paper (MARCHANT and HOLM 1988) using the two genetically marked third chromosomes st eg K1 and st in ri eg.

RESULTS

Isolating EMS-induced alleles of loci in chromosome 3 heterochromatin: EMS-induced alleles of loci in the proximal heterochromatic region of chromosome 3 were isolated by testing EMS-treated, ri p alleles against several different deficiencies generated through the detachment of compound-3 chromosomes (MARCHANT and HOLM 1988). Deficiencies 10-65 and 4-75 were used to screen for lethal alleles on the right arm; and deficiencies 1-166 and 1-16 were used for the left arm (see Figure 6 for a composite map of the region). The number of chromosomes treated and EMS-induced lethal alleles recovered is summarized in Table 1. The newly recovered EMS lethals were tested against a series of overlapping and nonoverlapping deficiencies of the left and right heterochromatic regions and positioned according to these complementation results. The EMS-lethals were then complementation-tested against all EMS lethals in and all available deficiencies for the same region. Each EMS-induced lethal allele has been assigned a code designating the deficiency with which it was identified, followed by the numerical order in which it was recovered (e.g., 10-65-2 is the second lethal recovered against the 10-65 deficiency chromosome).

Analysis of EMS-induced lethals on the right arm:
Two EMS-induced lethal alleles of l(3)Rh1 were recovered from 6301 ri p alleles tested against deficiency 10-65; and 13 lethals of l(3)Rh1 were recovered from 3342 chromosomes tested against the 4-75 chromosome. The two lethals recovered using the 10-65 chromosome failed to complement each other as well as ten additional deficiencies of the 10-65 region. The lethals recovered using deficiency 4-75 resulted in a more complex pattern that revealed a second locus on the right arm, as shown in Figure 1. Two of the lethals recovered using Df(3R)4-75, designated 4-75-12 and 4-75-17, failed to complement the two EMS lethals of l(3)Rh1 recovered using the Df(3R)10-65 chromosome. Ten of the remaining eleven complemented all EMS alleles of l(3)Rh1, as well as eight of the ten deficiencies assigned to the 10-65 class. These ten lethal alleles of 4-75 also failed to complement deficiency 5-53. This complementation pattern clearly defines a second gene in the right arm heterochromatin, designated l(3)Rh2. When tested against each other, the EMS lethals of l(3)Rh2 revealed a complementation pattern with two groups. Five lethals complemented neither group. The two complementation groups probably represent inter-

<table>
<thead>
<tr>
<th>Detachment product screened against</th>
<th>Deficiency region</th>
<th>No. of mutagenized chromosomes tested</th>
<th>No. of EMS lethals recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(3L)1-166</td>
<td>l(3)Lh1-4</td>
<td>7568</td>
<td>23</td>
</tr>
<tr>
<td>Df(3L)1-16</td>
<td>l(3)Lh4-6</td>
<td>2170</td>
<td>37</td>
</tr>
<tr>
<td>Df(3R)10-65</td>
<td>l(3)Rh1</td>
<td>6301</td>
<td>2</td>
</tr>
<tr>
<td>Df(3R)1-75</td>
<td>l(3)Rh1</td>
<td>3342</td>
<td>13</td>
</tr>
</tbody>
</table>

TABLE 1

Recovery of EMS-induced lethals uncovered by deficiencies associated with third chromosome detachment products

allelic complementation between lethal alleles of a single gene; otherwise, the five lethals that failed to complement both groups would be deficiencies, which seems unlikely. However, the last of the right arm EMS-induced lethals, 4-75-8 (in Figure 1), definitely appears to be a deficiency, as it fails to complement all other deficiencies and EMS-induced lethals of the right arm. These findings demonstrate the presence of at least two vital loci in the heterochromatin of 3R, the locus uncovered by Df(3R)10-65 and one distal to that. The deficiency analysis suggests that both of them lie close to the heterochromatic euchromatic junction.

Analysis of EMS-induced lethals on the left arm:
Lethal EMS-induced alleles of l(3)Lh1, l(3)Lh2 and l(3)Lh3 were recovered using Df(3L)1-166. These lethals produced relatively simple complementation patterns (Figure 2). All nine EMS-induced lethals of l(3)Lh1 failed to complement each other and all deficiencies that uncover this region. Curiously, only one of the nine EMS lethals exhibited the rotund-like phenotype observed for all of the most proximal deficiencies of the 3-9 class (MARCHANT and HOLM 1988). Three lethal alleles of l(3)Lh2 were recovered, all of which failed to complement each other and all deficiencies uncovering l(3)Lh2. Two alleles of l(3)Lh3 were recovered, and again they failed to complement each other and all deficiencies uncovering l(3)Lh3. Two additional EMS-induced lethals showed the properties of deficiencies. Lethal 1-166-12 failed to complement deficiencies for EMS-induced lethals of both l(3)Lh2 and l(3)Lh3. Similarly, 1-166-46 did not complement deficiencies and all EMS-induced lethal alleles of the three tightly linked proximal loci.

Complementation analysis of EMS-induced lethal alleles of l(3)Lh4 produced the most complex complementation pattern of this study. Since Df(3L)1-166 and Df(3L)1-16 overlap at this position, lethal alleles of l(3)Lh4 were recovered using both. A total of 30 alleles of l(3)Lh4 were recovered and tested in all inter se combinations as well as against all deficiencies of this region. The resulting complementation map shown in Figure 3 discloses that the 8A-80 deficiency, previously thought to define a single l(3)Lh4 locus, actually uncovers two separate genes. All 1-16 class
Figure 1.—Complementation pattern of deficiencies and EMS-induced lethals of loci in the heterochromatin of the right arm of chromosome 3. Detachment-product deficiencies are indicated by solid bars and EMS-induced mutations are designated by thin lines. The arrow points in the direction of the centromere.

deficiencies uncover both genes; but two of the four 1-166 class deficiencies, including 1-166 itself, only uncover the most proximal of the two genes, now designated l(3)Lh4A. The other two 1-166 class deficiencies, designated 6-21 and 9-37, uncovered both genes. The group 4A lethals in Figure 3 consisted of ten EMS-induced alleles, eight of which were recovered against Df(3L)1-166 and two against Df(3L)1-16. All ten lethals failed to complement each other.

All 20 EMS-induced lethals of group B (Figure 3)
were isolated using deficiency 1-16, and form a very complex complementation pattern. The results of inter se complementation tests between all twenty lethals are summarized in Table 2. All lethals fail to complement most of the other alleles in the group, but only two, 1-16-17 and 1-16-22, fail to complement at least one other allele. This complex pattern of complementing and noncomplementing alleles has been designated a single gene, \( l(3)Lh4B \).

Moving distally along the left arm, five EMS-induced lethal alleles of \( l(3)Lh5 \) were isolated using deficiency 1-16 (Figure 4). Four of these lethals failed to complement each other in all combinations, but the fifth lethal complemented the other four. All five lethals did not complement any of the deficiencies that uncovered the \( l(3)Lh5 \) region. This complementation pattern either may represent inter-allelic complementation or, alternatively, lethal 1-16-27 may be in a separate gene. The ten remaining EMS-induced lethals identified with the 1-16 deficiency, separated into three distinct complementation groups. Two of the lethals were alleles of \( l(3)Lh6 \); they failed to complement each other and all deficiencies of the \( l(3)Lh6 \) locus. The remaining eight lethals complemented deficiencies for all the previously identified complementation groups on the left arm. Further analysis against deficiencies belonging to the 1-16 and 9-52 classes, both of which uncover the \( l(3)Lh6 \) locus (Figure 4), defined two additional genetic loci, designated \( l(3)Lh7 \) and \( l(3)Lh8 \), that lie distal to \( l(3)Lh6 \). As demonstrated by the complementation map in Figure 4, four of the tested deficiencies from the 1-16 and 9-52 groups uncover both \( l(3)Lh7 \) and \( l(3)Lh8 \), whereas three of the deficiencies uncover only \( l(3)Lh7 \). Five of the EMS-induced lethals form an overlapping inter-allelic complementation pattern identifying the \( l(3)Lh7 \) locus. The three lethals of \( l(3)Lh8 \) are noncomplementing.

In summary, through complementation analysis and deficiency mapping of EMS-induced lethal alleles of proximal deficiencies generated through the detachment of compound autosomes, these studies have identified nine genetic loci, and possibly a tenth, in the heterochromatin of the left arm of chromosome 3, and two genes in the right arm heterochromatin.

Temperature-sensitive alleles: All EMS-treated
TABLE 2

Results of complementation analysis of 20 EMS-induced lethal alleles of i(3)Lh4B

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | |
chromosomes were recovered on the basis of their lethality over a detachment-product deficiency at 29°C. After retesting at 22°C, two of the lethals were found to be temperature-sensitive. The first, designated 1-166-5 was a temperature-sensitive allele of l(3)Lh4A, and has been included in the complementation map in Figure 3. This mutation was homozygous lethal at both the restrictive and the permissive temperatures, but survived over all deficiencies and all remaining EMS-induced lethal alleles of l(3)Lh4A at 22°C. The temperature-sensitive period of 1-166-5 was determined using shift-up and shift-down experiments. Progeny heterozygous for 1-166-5 over a deficiency of l(3)Lh4A had a very long temperature-sensitive period that began with the second larval instar stage and extended until late pupal stage.

The second temperature-sensitive mutation was designated 1-166-4. This mutation was homozygous viable at both 22°C and 29°C, and lethal over all deficiencies of l(3)Lh2 at 29°C but not 22°C. However, 1-166-4, which was not included in Figure 2, was only semi-lethal over the other EMS-induced alleles of l(3)Lh2 at the restrictive temperature. Shift-up and shift-down experiments determined that 1-166-4 had a sharply defined temperature-sensitive period early in the pupal stage.

As noted in MATERIALS AND METHODS, EMS-induced lethal alleles of TM3 were also recovered in this study and used to test for the presence of proximal lethals on the TM3 chromosome. A total of 24 lethal alleles of TM3 were recovered and complementation tested against each other in all possible combinations. The resulting complementation map included one group of eight lethals, one group of five, two groups of three, one group of two, and three groups with one lethal each. One lethal from each group with two or more alleles was chosen and mapped by the same recombination procedure used for the detachment-product deficiencies (MARCHANT and HOLM 1988). These lethal alleles of the TM3 chromosome mapped outside the proximal region. These results indicate that the TM3 chromosome used in this study did not harbor proximal lethals that would have precluded the unveiling of heterochromatic loci.

Cytological examination of detachment-products: Squashes of polytene and mitotic chromosomes carrying detachment-product deficiencies revealed no obvious duplications or deficiencies of third chromosome proximal regions. However, when S. PIMPINELLI (personal communication) examined three of the detachment-product deficiencies using the sophisticated fluorescence and N-banding techniques developed by GATTI and PIMPINELLI (1983), he found clear evidence of heterochromatic rearrangements. The results of his analysis are shown schematically in Figure 5. The top chromosome shows a standard Oregon-R wild-type third chromosome. The right arm heterochromatin carries a large, interstitial nonfluorescing block that appears as a dark-staining band in N-banding preparations. The left arm heterochromatin fluoresces brightly except for a narrow dark band indicated by a notch in the diagram.

Two detachment-product deficiencies of the left arm of chromosome three were examined. Detachment-product 2-85 was deficient for loci l(3)Lh1 through l(3)Lh7, and detachment-product 9-2 was deficient for genes l(3)Lh1 through l(3)Lh5 (Figure 4). Both the 9-2 and 2-85 chromosomes were generated from progenitor compounds that were free of duplications or deficiencies of vital loci. Detachment products carrying deficiencies of the left arm that are generated from such progenitor compounds must be formed by the joining of a centric fragment from C(3R) with an acentric fragment from C(3L). The centric fragment will carry one complete arm of the C(3R) progenitor chromosome, and in reverse orientation on the opposite side of the centromere, a duplication of proximal heterochromatin, from the other arm of the progenitor C(3R), that extends from the centromere to the radiation-induced breakpoint. The left acentric fragment will carry only a segment of the left heterochromatic block, starting with the left euchromatic-heterochromatic junction, and extending proximally towards the centromere.

For the 9-2 chromosome, the large fluorescence-dull band present on the left arm indicates that the duplication of right arm heterochromatin accounts for at least the proximal three-quarters of the heterochromatic block on the left arm. At most, only the small segment of heterochromatin distal to the fluo-
rescence-dull block on the left arm of 9-2 could possibly be normal left arm heterochromatin. Since 9-2 is deficient only for genes \( l(3)Lh1-5 \), the loci for \( l(3)Lh6 \) to \( l(3)Lh8 \) must remain in the 9-2 chromosome heterochromatin. Therefore, genes \( l(3)Lh6 \) to \( l(3)Lh8 \) must be located within the small segment of heterochromatin that accounts for about the distal one-eighth of the wild-type left arm heterochromatin block.

Two observations are of significance for explaining the cytological observations on the 2-85 detachment product. First, the 2-85 chromosome does not carry a duplication of the large right arm fluorescence-dull segment, so right arm heterochromatin can account for a maximum of about one-half of the heterochromatin on the left arm of \( Df(2L)2-85 \). Additionally, 2-85 is deficient for all loci except the most distal, \( l(3)Lh8 \). Combining these two observations, almost half of the heterochromatin on the left arm of \( Df(2L)2-85 \) cannot be accounted for either by distal left heterochromatin carried by the acentric detachment fragment, or by right arm heterochromatin. Therefore, this unaccounted heterochromatin must represent a duplication of nonvital proximal left heterochromatin present on the progenitor \( C(3R) \) chromosome, and carried by the centric detachment fragment. The cytological analysis of the 2-85 detachment product provides no further information about the distribution of genes within third chromosome heterochromatin.

The other chromosome examined cytologically is detachment-product 7-53, which carries a deficiency of the right arm heterochromatin. This chromosome had been generated from a set of progenitor compounds that carried no duplications or deficiencies on \( C(3L) \) but a duplication on \( C(3R) \) of left heterochromatin that included \( l(3)Lh1 \) through \( l(3)Lh6 \). From the analysis of possible breakpoints that could result in the formation of \( Df(3R)7-53 \), it seemed likely that this chromosome carried a large duplication of left heterochromatin on its right arm. The cytological analysis confirms this prediction by showing that at least the proximal two-thirds (and perhaps much more) of the heterochromatin on the right arm of \( Df(3R)7-53 \) is actually a duplication of left arm heterochromatin. This finding indicates that the locus \( l(3)Rh2 \), which is not deleted in \( Df(3R)7-53 \), must be present in the small amount of remaining distal right arm heterochromatin, in accord with its radiation mapping near the H-E junction.

Summarizing the results of this analysis, all three detachment-product deficiencies examined cytologically are missing almost all the heterochromatin normally found on the arm to which they had been assigned by the genetic analysis. However, in each case, the large deficiencies are accompanied by heterochromatin duplications of similar size from the opposite arm. The results also indicate that at least three genes in the left heterochromatin and one gene in the right heterochromatin are located very distally in their respective heterochromatic blocks.

**DISCUSSION**

EMS mutagenesis resulted in the recovery of 60 lethal alleles of loci uncovered by deficiencies of the proximal heterochromatin on the left arm of chromosome 3, and 15 lethal alleles of loci uncovered by deficiencies of the right arm. Complementation analysis against a set of deficiencies and of all *inter se* combinations of alleles within each of the vital regions uncovered by deficiencies, defined ten complementation groups on the left arm, of which nine were separated by deficiency break points. The EMS-induced lethals on the right arm separated into two complementing loci. Figure 6 contains a genetic map of the heterochromatic region of chromosome 3 showing the position of the complementation groups relative to each other and to representative deficiencies that establish all but one of the lethal loci as separate genetic sites.

Starting at the centromeric end of 3L, we find that the first three complementation groups defined by deficiencies each contain only one vital locus. The 30 EMS-induced lethals in region 4 belong to two complementation groups that are separated by deficiencies that uncover only one of the two loci. Deficiency region 5 also uncovers two complementation groups of EMS-induced lethals, of which one locus is represented by four alleles, the other by only one. Since these two complementing groups of alleles are not separated by deficiencies, we realize the distinct possibility of inter-allelic complementation. Nonetheless, for now we are considering each group as representing a separate gene in assigning genetic codes to the loci.

The sixth region uncovered by deficiencies contains three separate loci, each represented by two or more alleles and separated by the series of overlapping deficiencies that extend into this region. The distalmost two loci could not have been recognized from the detachment study as the compound autosomes involved did not carry deficiencies or duplications extending distal to complementation groups 5 and 6. Moreover, the preexisting deficiency for 3R heterochromatin, \( Df(3R)10-65 \), uncovered only the proximal locus, which precluded identifying the second complementation group on this arm prior to EMS mutagenesis. In Figure 6, in addition to identifying each genetic locus with an identifier that associates the gene with a heterochromatic region uncovered by a class of deficiencies, we have assigned each locus a descriptive code in keeping with the recommendations of LINDSEY and ZIMM (1986) for identifying genetic loci with their position on the cytological map (WRIGHT et al. 1981).
While we cannot definitely describe where, within the heterochromatin, the vital loci are situated, the three genetically deficient detachment products cytologically analyzed by S. Pimpinelli (personal communications) are missing almost all the heterochromatin normally found on the deficient arm. This loss is accompanied by duplications of heterochromatin from the opposite arm, an observation consistent with genetic findings for chromosome 2 (Hilliker 1981).

The cytological analysis places one and probably both genes of the right-arm heterochromatin very close to the euchromatic-heterochromatic junction, and at least three genes are identified with the distal 10–20% of the left-arm heterochromatin. However, the cytological results only demonstrate that genetic loci \( l(3)Lh1 \) to \( l(3)Lh5 \) definitely lie proximal to the most distal heterochromatic segment. The genetic map derived from the distribution frequency of detachment breakpoints (Marchant and Holm 1988) is in accord with the cytology, but should not be taken as evidence for the distribution of genes along the entire length of 3L heterochromatin. Radiation map distances are proportional to actual physical distances between genes if and only if radiation breakpoints are randomly distributed.

From the size of the deficiencies uncovering the right arm heterochromatin, and from the number of lethal alleles recovered from the EMS treatment, it seems reasonable to conclude that only two vital loci occupy heterochromatic regions to the right of the centromere. To the left of the centromere 60 alleles are distributed among ten loci, or nine if we consider as distinct loci only those separated by deficiencies. By applying the equation for a truncated Poisson distribution (Meneely and Herman 1979) to our data to estimate the number of genes with no alleles \( n_0 \), we find that it is unlikely that additional vital loci will be found. However, calculations based on the assumption of equally mutable loci, which is not the case in this study, will give underestimates of \( n_0 \) (LeFevre and Watkins 1986).

As with the second chromosome (Hilliker 1976), genes in the heterochromatin of chromosome three appear similar to single-copy genes found in the euchromatin. Inter-allelic complementation between the EMS-induced mutations was observed in three complementation groups and \( ts \) alleles were recovered for two loci. No evidence of repetitive genes was found from the testing of nonlethal detachment products over lethal deficiencies (Marchant and Holm 1988). All mutations were recessive lethals, with the only visible phenotype being the rotund-like displayed by rare surviving adults homozygous for deficiencies of the \( l(3)Lh1 \) group and for one of the nine EMS-induced alleles of this locus.

In contrast to the findings on chromosome 2, how-
ever, three of the EMS-induced lethals recovered in this study appear to be deletions, as they uncovered two or more genes separable by detachment-product deficiencies. Previous findings differ on the ability of EMS to induce deletions. While LIM and SNYDER (1974) and HILLIKER (1976) recovered only EMS mutations affecting one gene, others have reported EMS-induced deletions (WILLIAMSON 1970; BISHOP and LEE 1973; OLSON and LIM 1976). In two studies, it was found that EMS was more likely to induce deletions near or in heterochromatin than in euchromatin (BISHOP and LEE 1973; OLSON and LIM 1976). We tend to rule out the possibility of multiple hits as the only putative multiple events would have been in neighboring genes. However, we cannot dismiss the possibility of polar mutations as described by WRIGHT et al. (1981) for clustered events discovered in the 37BIO-37DI region of chromosome 2, nor the possibility of spontaneous deficiencies.

This analysis of chromosome 3 heterochromatin fails to detect any vital genes present in the heterochromatin of more than one chromosome in the D. melanogaster genome. Transcribed middle-repetitive DNA sequences are known to hybridize to the chromocenter of D. melanogaster polytene chromosomes, especially the more distal beta heterochromatin (SPRADLING, PENMAN and PARDE 1975; CARLSON and BRUTLAC 1978a; SPRADLING and RUBIN 1981), but none of these middle-repetitive sequences are confined strictly to the autosomes; as a rule they are found both on the Y chromosome and on one or more autosomes (LIFSCHYTZ and HAREVAN 1982). Therefore, it is possible that third chromosome heterochromatin carries vital genes other than those found in this study. Any such genes would be repetitive and present in the heterochromatin of one, or more, of the other chromosomes.

Vital loci are not uniformly distributed in chromosome-3 heterochromatin. More loci are found in the left arm and at least some of the genes are located very distally. LIFSCHYTZ (1978) has suggested that the euchromatic-heterochromatic junction (E-H junction) is a transitional zone, with more and more genes intermingling with the heterochromatic sequences as heterochromatin ends and euchromatin begins. This hypothesis is based on the discovery of alternating radiation “hot spots” and “cold spots” in the E-H junction region. Additional evidence for gene activity in the region of the E-H junction derives from molecular studies. The heterochromatic chromocenter of Drosophila polytene chromosomes consists of a dense inner core which does not incorporate [3H]uridine, surrounded by more diffuse regions near the E-H junction of each arm called beta heterochromatin which do incorporate [3H]uridine (LAHOTIA and JACOB 1974). Most mRNA that hybridizes to the chromocenter binds to beta heterochromatin (SPRADLING, PENMAN and PARDE 1975; RENKAWITZ 1978; GVOZDEV et al. 1980; YOUNG et al. 1983). A cloned DNA segment that codes for a 26,000 MW protein hybridizes to the E-H junction of the left arm of chromosome 3 of D. melanogaster (BIESSMAN et al. 1981). All this suggests that segments of “intercalary euchromatin” may be interspersed in the distal regions of heterochromatin. The genes in the distal region of chromosome 3 heterochromatin may be present in a segment of interspersed euchromatin in the transitional region between heterochromatin and euchromatin. An extensive cytological examination of a series of genetically defined proximal deficiencies as well as transposon tagging through P-element mutagenesis may offer a means of addressing this uncertainty.

Factors responsible for the restricted locations of vital genes in Drosophila heterochromatin are speculative. It has been proposed that the molecular structure of the surrounding heterochromatin provides an environment essential for the functioning of these loci (HILLIKER, APPELS and SCHALET 1980; PEACOCK and LOHE 1980). Perhaps only some regions of the heterochromatin have the molecular characteristics necessary for the presence of functioning genes. Fertility factors on the Y chromosome are specifically associated with segments of heterochromatin that are Hoechst-dull and N-banded (KENNISON 1981; GATTI and PIMPINELLI 1983). As well, within the X heterochromatin and on the heterochromatic Y chromosome, regions that interact with the abnormal oocyte maternal effect mutation are also Hoechst-dull (PIMPINELLI et al. 1985). The chromosome 2 and chromosome 3 heterochromatic blocks have five and three N-bands, respectively (GATTI and PIMPINELLI 1983). One of the genes defined by HILLIKER (1976) in the second chromosome heterochromatin is deleted by a deficiency of a single N-band (GATTI and PIMPINELLI 1983). Although the molecular nature of N-banded regions is unknown, it is possible that N-bands correspond to blocks of specific satellite sequences. The presence of the 1.705 g/cm³ satellite sequence has been specifically mentioned as the possible basis of N-banding (DENNIS and PEACOCK 1984). Again, a careful cytological analysis of a series of third chromosome deficiencies would be useful for determining whether heterochromatric sites of gene activity correspond to specific banding regions and/or specific satellite sequences.

The discovery of genes in heterochromatic segments raises important questions about how and when these genes are expressed. The condensed nature of heterochromatin does not seem compatible with transcription. However, since these regions are accessible for DNA replication, they may also be accessible for transcription. Another possibility is that heterochromatric genes are expressed early in development, before heterochromatric regions condense. The hetero-
chromatin of D. melanogaster does not condense until blastomere formation (Sonnenblick 1950); and the heterochromatin of Microtus agrestis is also relatively uncondensed early in development (Yunis and Yasmine 1971).

Lethal phases do not represent the stage at which a gene is expressed, but rather a critical period in development through which the organism is unable to pass successfully as a result of a mutant gene expressed earlier in development (Wright 1970). Nonetheless, some information about the timing of expression of genes in heterochromatin may be revealed by the temperature-sensitive periods (tsps) of temperature-sensitive (ts) mutations of heterochromatic genes. The one definite ts mutant of a heterochromatic gene recovered in this study (I-166-5) had a very prolonged tsp which began during the early second larval instar stage. Temperature sensitivity probably results from structural changes in heat-labile proteins (Jockusch 1966; Suzuki 1970), a finding which has two important implications for the results of this study. First, the recovery of a ts mutation of a heterochromatic gene provides further evidence that such genes code for proteins. Second, since a ts mutation must be transcribed prior to the tsp, if a heat-labile protein is responsible for the temperature sensitivity, at least one gene in chromosome 3 heterochromatin must be transcribed before the beginning of the second larval instar stage.

Pimpinelli et al. (1985) have recently put forward an intriguing suggestion about the region of D. melanogaster heterochromatin on the X and Y chromosomes that can rescue a series of maternal mutants including abnormal oocyte. Their experimental findings led them to conclude that heterochromatic regions may carry a duplicate set of the euchromatic maternal-effect genes. Based on the timing of rescue of the maternal-effect mutations, the heterochromatic gene set appears to be expressed early in development before nuclear migration and the general activation of zygotic gene action, while the euchromatic gene set is expressed after blastoderm formation. The authors suggest that one consequence of heterochromatic location is out-of-phase gene activity.

There is also evidence from a variety of organisms suggesting that genes in heterochromatin are expressed in the germ line rather than somatic tissues. During polytenization in several tissues of Drosophila larvae the heterochromatin is severely under-replicated, and a similar condition occurs in the polyploid somatic nuclei of adult tissues (Rudkin 1965; Gall and Atherton 1974; Spear 1977; Lakhotia 1984). In the insect Calliphora erythrocephala, satellite DNA is drastically under-replicated in polytene tissues of salivary glands and maphigian tubules, but is coordinately replicated with the non-satellite sequences during polytenization of nurse cell nuclei in the germ line (Dover 1980). And in irradiated cultured M. agrestis bone marrow cells, Cooper (1977) observed many stable rearrangements and deletions of the heterochromatin, but no such deletions have been observed in natural populations, suggesting that heterochromatic regions are essential for the germ line, but not required in at least some somatic cells.

Are there other implications of the presence of functioning genes in heterochromatin? The low gene density in heterochromatin supports the model that cytologically observed heterochromatin is usually a consequence of a high concentration of tandemly repeated satellite DNA sequences (Wollenzen, Baranti and Hearst 1977; Appels and Peacock 1978; Bostock 1980). Since all significant concentrations of satellite DNA are heterochromatic, and most but not all heterochromatic regions contain primarily satellite DNA sequences, satellite DNA seems to be a sufficient but not necessary condition for heterochromatin formation. Proteins that specifically bind to heterochromatic sequences (Hsieh and Brutlag 1979; Leviner and Varshavsky 1982; Strauss and Varshavsky 1984; James and Elgin 1986) may be responsible for its compact nature.

Heterochromatic genes may have profound consequences for the function and stability of heterochromatin. One of the problems in assigning a function to heterochromatin is that it is so variable, both within and between species. Variability is such a predominant feature of satellite DNA and of heterochromatin that several authors have proposed that the primary function of heterochromatin is to provide a rapid response to environmental changes (John and Miklos 1979; Bostock 1980; Miklos and Gill 1981). Rapid changes in the amount of heterochromatin may result in changes in the amount and distribution of crossing over, an altered nucleotype, new fertility barriers, and perhaps other effects.

At least one mechanism that seems to be involved in generating variations in heterochromatin content is unequal sister chromatid exchange (John and Miklos 1979; Kurnit 1979; Miklos and Gill 1981). Whatever the mechanisms, however, rapid expansion or contraction of heterochromatin could result in the deletion or duplication of genes present there (Miklos and Gill 1981). Therefore, the presence of functioning genes in heterochromatin may tend to restrict the variability of heterochromatin. The genes may function as anchors, impeding fluctuation in the region.

The amount of heterochromatic variation differs greatly between species. This difference in fluidity may reflect the presence or absence of heterochromatic genes. Heterochromatin in the human genome is very polymorphic on some chromosomes but not others (Craig-Holmes, Moore and Shaw 1975). Maize heterochromatin is highly conserved with no observed polymorphisms (Rhoaòes 1978); while rye
heterochromatin has extensive variations (Jones and Flavell 1982). In *D. melanogaster*, variations have been detected in X chromosome heterochromatin (Halfer 1981; Pimpinelli et al. 1985), which does not contain single-copy vital genes. However, no polymorphisms of autosomal heterochromatin, which does contain unique sequence vital genes, have been detected (Sved and Verlin 1980; Halfer 1981). In a *D. melanogaster* cell line examined for a seven year period, the loss or gain of sex chromosome heterochromatin, but not autosomal heterochromatin, was observed (Halfer 1978). The amount of intraspecific variation in heterochromatin may be a good indicator of the presence or absence of heterochromatic genes.

To summarize, the results of the genetic analysis of chromosome 3 heterochromatin in *D. melanogaster* provide further evidence that functioning genes are present in some heterochromatic regions, but not in others. At least some of the genes present in chromosome 3 heterochromatin are located in the most distal segments of heterochromatin on both arms. The next step in unlocking the mysteries of heterochromatin is to discern why genes are in some heterochromatic regions and not others; and to determine the costs and benefits of this arrangement for both the gene and the block of heterochromatin.

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