Cytogenetic Analysis of the Second Chromosome Heterochromatin of
Drosophila melanogaster

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ABSTRACT

This paper reports the cytogenetic characterization of the second chromosome heterochromatin of Drosophila melanogaster. High resolution cytological analysis of a sample of translocations, inversions, deficiencies and free duplications involving the pericentric regions of the second chromosome was achieved by applying sequential Hoechst 33258 and N-chromosome banding techniques to larval neuroblast prometaphase chromosomes. Heterochromatic rearrangements were employed in a series of complementation assays and the genetic elements previously reported to be within or near the second chromosome heterochromatin were thus precisely assigned to specific heterochromatic bands. The results of this analysis reveal a nonhomogeneous distribution of loci along the second chromosome heterochromatin. The l(2)41Aa, l(2)41Ah, rolled (l(2)41Ac) and l(2)41Ad loci are located within the proximal heterochromatin of 2R, while the nine remaining loci in the left arm and two (l(2)41Ae and l(2)41Ah) in the right arm map to h35 and to h46, respectively, the most distal heterochromatic regions. In addition, a common feature of these loci revealed by the cytogenetic analysis is that they map to specific heterochromatic blocks but do not correspond to the blocks themselves, suggesting that they are not as large as the Y fertility factors or the Rsp locus. Mutations of the proximal most heterochromatic loci, l(2)41Aa and rolled, were also examined for their phenotypic effects. Extensive cell death during imaginal disc development was observed in individuals hemizygous for either the EMS 31 and rolled mutations, leading to a pattern of phenotypic defects of adult structures.

THE Drosophila melanogaster heterochromatin can be subdivided into two broad distinct types (Heitz 1934): (1) the α-heterochromatin, which corresponds to a tiny compact mass located in the middle of the chromocenter and undergoes little if any replication during polytenization of the salivary gland chromosomes (Gall, Cohen and Polan 1971) and (2) the β-heterochromatin which appears as the diffusely banded material located at the junction between the well banded euchromatin and α-heterochromatin. The α-heterochromatin is almost exclusively comprised of large satellite DNA blocks (Appels and Peacock 1978), although the insertion of the copia transposon has been detected in a complex satellite DNA (Brutlag and Carlson 1978), while the β-heterochromatin is highly enriched in middle-repetitive DNAs (reviewed in Spradling and Rubin 1981; Nikolaos et al. 1988; Vaury, Bucheton and Pelisson 1989; Devlin, Bingham and Wakimoto 1990).

By combining Hoechst 33258 and N-banding techniques, the D. melanogaster mitotic heterochromatin can be subdivided into several regions having distinguishable cytological properties (Gatti and Pimpinelli 1983; Pimpinelli and Dimitri 1989). This characterization has led to the definition of a total of 63 bands in the heterochromatin of the D. melanogaster genome (S. Pimpinelli, S. Bonaccorsi, P. Dimitri and M. Gatti, manuscript in preparation).

Complementation analysis using rearrangements with cytologically determined heterochromatic breakpoints has yielded significant insight into the location and structural organization of the genetic elements mapping to the sex chromosome heterochromatin of D. melanogaster (Gatti and Pimpinelli 1983; Hardy et al. 1984; Pimpinelli et al. 1985; Bonaccorsi et al. 1988; reviewed in Pimpinelli et al. 1986). These elements are physically very large and consist of high and middle-repetitive DNAs. Two distinct types of organization are apparent. For example, the kl-5, kl-3 and kl-1 fertility factors on the Y chromosome, each correspond to a Y region which contains on the order of 4000 kb of DNA. These genes contain satellite DNA sequences (Bonaccorsi et al. 1990; S. Bonaccorsi and A. Lohe, personal communication), require structural integrity for function (Gatti and Pimpinelli 1983) and form giant loops (Bonaccorsi et al. 1988) actively transcribed in primary spermatocytes (Bonaccorsi et al. 1990). On the other hand, the bobbed (Ritossa 1976) and Suppressor of Stellate (Li-Vak 1984, 1990) loci are not inactivated by breakpoints within the loci but only by deletions and consist of an array of middle-repetitive DNA sequences whose number is critical for their function. Finally, the ABO factors (Pimpinelli et al. 1985) have multiple locations in the genome and may identify another kind of repeated genetic element.
The second chromosome heterochromatin, which accounts for about 20% of the entire heterochromatic content of the *D. melanogaster* karyotype, has been extensively studied in the last decade [reviewed in Pimpinelli et al. (1986) and Hilliker and Sharp (1988)]. Seventeen loci have been genetically mapped to this large chromosomal segment. These include Enhancer of *SD (E(SD))* (Brittnacher and Gannetzky 1984; Sharp, Hilliker and Holm 1985) and Responder (*Rsp*) (Gannetzky 1977; Sharp, Hilliker and Holm 1985), both belonging to the Segregation Distortion (*SD*) system. These genetic elements lie in the heterochromatin to the left (*2Lh*) and to the right (*2Rh*) of the centromere respectively. By applying banding techniques to characterize second chromosomes varying in the allelic *Rsp* status, we have shown that *Rsp* is a large locus that may be comprised of an array of AT-rich repeated DNA sequences (Pimpinelli and Dimitri 1989). A specific class of AT-rich repetitive DNA sequences correlated with the *Rsp* locus has been indeed isolated and characterized by Wu et al. (1988). In addition, an ABO element, a heterochromatic locus that interacts with the euchromatic abnormal oocyte (*abo*) mutation, resides in the *2Rh* (B. Gannetzky and J. Haemer in Sandler 1977; S. Pimpinelli and L. Fant, personal communication). At least 15 EMS-mutable loci have been identified, the concertina (*mat(2)ecta*) gene, a maternal effect gene mapping to the *2Lh* (Schupbach and Weischaus 1989) and 13 vital or semivital loci, 7 of which map to the left and 6 to the right of the centromere (Hilliker 1976).

The vital loci were identified using putative heterochromatic deficiencies generated by X-ray-induced interchanges between compound second chromosomes (Hilliker and Holm 1975). However, since neither the qualitative nor the quantitative heterochromatic content of these deficiencies were determined, the cytological mapping and the distribution of these loci along the mitotic heterochromatin of the second chromosome was uncertain.

To fill this gap I performed a genetic and cytological analysis of the elements which are known to be within or near the pericentric regions of the second chromosome. In particular, I asked the following questions: Are all loci genuinely heterochromatic? Are they homogeneously distributed along the heterochromatin? Are they similar to the *Y*-linked fertility factors or the *Rsp* locus in corresponding to large heterochromatin blocks? To answer these questions, chromosome rearrangements with presumed heterochromatic breakpoints were characterized cytologically by Hoechst- and N-banding techniques and genetically analyzed by complementation assays.

The results of this analysis show that the vital loci are not homogeneously distributed within the second chromosome heterochromatin. Only four of them, *l(2)41Aa, l(2)41Ab, rolled* and *l(2)41Ad*, are located within the very proximal heterochromatin on the right arm, while the remaining loci map to the most distal heterochromatic blocks.

The possible differences between these two groups of loci are discussed in the light of the recent findings on the organization of *D. melanogaster* heterochromatic genes.

**MATERIALS AND METHODS**

**Stocks:** Genetic markers, mutations and special chromosomes used in this work are described in Lindsley and Grell (1968) and Lindsley and Zimm (1986, 1987). Flies were grown on standard Drosophil medium. All genetic crosses were performed at 25 ± 1°. The nomenclature of the second chromosome heterochromatic loci was according to Lindsley and Zimm (1986).

**Complementation tests:** The heterochromatic rearrangements and EMS-induced lethal mutations employed for the genetic analysis were balanced over the *In(2R)JS1* chromosome which carries the dominant *Cy* mutation.

The genetic effects of cytologically characterized translocation breakpoints were assayed in a series of complementation tests with *Df(2Lc)Cv* and *Df(2Rhm)S210*, which are homozgyuous lethal deficiencies deleted for the *2L* and the *2R* proximal loci, respectively (Hilliker and Holm 1975; Hilliker 1976). In these experiments, *Df(2Lc)CV/SM1, Cy* and *Df(2Rhm)S210/SM1, Cy* females were crossed to *XY,y; T(1;2)/SM1, Cy* males. The genetic effects of cytologically characterized inversion breakpoints were assayed by crossing *Df(2Lc)CV/SM1, Cy* and *Df(2Rhm)S210/SM1, Cy* females to *In(2R)/SM1, Cy* males. The absence of *Cy* progeny among the offspring of these crosses indicates that a given breakpoint is lethal over the deficiency. For each complementation test, over 100–150 progeny were counted.

Different *2Rh* rearrangements were tested for their ability to complement the EMS-induced lethal alleles of the *2Rh* loci (see Table 1) by crossing EMS/*JS1* females to *R(2Rh)/SM1* males (where EMS, and *R(2Rh)*, indicate the EMS lethal mutation at the *2Rh* loci and the *2Rh* rearrangements, respectively). The absence of *Cy* progeny among the offspring from the cross indicate that the tested heterochromatic mutations fail to complement each other. Six EMS-induced lethal mutations isolated by Hilliker (1976) were used to define the six *2Rh* vital loci. For the *l(2)41Aa locus, EMS 31 was employed; for *l(2)41Ab*, EMS 45-10; for *l(2)41Ac*, EMS 64; for *l(2)41Ad*, EMS 45-17; for *l(2)41Ac*, EMS 34–25; and for *l(2)41Ad*, EMS 45–75. For each complementation test, over 150–200 progeny were counted.

**Generation of synthetic deficiencies:** Deficiencies of specific heterochromatic regions were generated either by recombination between inversions with different heterochromatic breakpoints or by the synthesis of segmental aneuploids as described by Lindsley et al. (1972).

The heterochromatic breakpoints of *In(2R)bw10*: and *In(2R)bwV*: fall between region h42 and h43 and between h44 and h45, respectively (see Results and Figure 7). In order to generate a chromosome deficient for region h43-h44, *In(2R)bwV*:/*bw*: males were crossed to *vg/vg* females. A crossover event within the *vg(2):57-L(2):72* region produces *In(2R)bwV*:/*bw*: female. These males were individually crossed to *SM1, Cy/SM1, Cy* virgin females to establish different *vg*:/*bw*: recombinant lines. Each recombinant chromosome was also cytologically examined to confirm that it was a heterochromatic defi-
ciency and was tested for its ability to complement each of six EMS lethal mutations which identify the 2Rh loci.

A deficiency of region h42-h43 was constructed by combining the elements of T(Y;2)A and T(Y;2)C, two fertile translocations whose 2Rh breakpoints were mapped to the h39-h40 and h43-h44 junctions, respectively (see Figure 7). XX, yu+;T(Y;2)A/SM1, Cy females were crossed to y; T(Y;2)C/Im(2R)bu 1042 mates to generate y/C 2Y/A 2Y′ 2SM1, Cy males. These males are fully fertile since the Y′ 2 element recovered from T(Y;2)A carries all the Y chromosome fertility factors. These males were individually mated to y/y; SM5, Cyn females to establish different lines. Each line was checked cytologically to verify the presence of the deficiency and was tested for its ability to complement each of six EMS lethal mutations which identify the 2Rh loci.

Cytology: Mitotic chromosome preparation, Hoechst staining and N-banding techniques were as described in Pimpinelli and Dimitri (1989).

Microphotography: Microphotographs were taken with a Kodak Pan-X film. To compare Hoechst- and N-banding staining sequentially performed on the same prometaphase chromosomes, microphotographs were printed both on normal paper (Ilfochrom) and on transparent Kodakith ortho film. Transparent prints were overlaid on normal ones and the different staining patterns exactly compared.

Selection of EMS 31 and rolled hemizygous larvae: EMS 31/SM3 and r1/SM5 females were crossed to Bf(2Rh)Rsp 31/SM5 males and the larval progeny was dissected in physiological solution. For each single individual, the neural ganglia were squashed in orcein to characterize the karyotype. While the imaginal discs were incubated in acridine orange in order to visualize cell death (Sprøj 1971; Russell 1974), SM5 is a submetacentric chromosome cytologically distinguishable from a metacentric one, therefore larvae whose brain cells did not carry SM5 were known to have imaginal discs hemizygous for EMS 31 or rolled. The EMS 31 hemizygous larvae were also selected for the presence of melanoic masses in their haemocoel, the mutant phenotype described by Hilliker (1976).

RESULTS

Cytological dissection of the second chromosome heterochromatin: Oregon-R and cn bw second chromosomes were processed by Hoechst 33258 staining and N-banding techniques, which are known to provide a fine longitudinal differentiation of constitutive heterochromatin (Holmquist 1975; Gatti, Pimpinelli and Santini 1976; Pimpinelli, Santini and Gatti 1976; Gatti and Pimpinelli 1983; Pimpinelli and Dimitri 1989). Chromosomes were sequentially stained, first with Hoechst and then with N-banding to increase the resolution of the cytological analysis. The Hoechst and N-banding patterns of the second chromosome heterochromatin are diagrammed in Figure 1. The nomenclature of the heterochromatic regions is according to Pimpinelli and Dimitri (1989). The diagrams were constructed after examining a large number of prometaphase figures and represent ideal chromosomes, in that the complete array of bands cannot always be resolved for each chromosome examined. The heterochromatin of the Oregon-R stock exhibits 12 differentially stained bands, while the cn bw heterochromatin shows an additional 2R band, that was designated h42B (Pim-

![Figure 1](image1.png)

**Figure 1.** - The cytological map of the second chromosome heterochromatin. (a) H33258-banding pattern of the cn bw second chromosome heterochromatin. (b) H33258-banding pattern of the Oregon-R second chromosome heterochromatin. (c) N-banding pattern of both cn bw and Oregon-R second chromosomes. The dark areas shown in (a) and (b) correspond to bright fluorescent regions; the cross-hatched areas to moderately fluorescent regions; the hatched ones to dull regions; the open areas to nonfluorescent regions. The dark areas shown in (c) correspond to the N-banding positive regions. 2L = left arm; 2R = right arm; C = centromere; the broken line indicates the secondary constriction of 2L.

Pimpinelli and Dimitri (1989). The Hoechst staining (0.5 µg/ml) revealed several regions with different degrees of fluorescence. For example, a specific bright spot is seen in 2L, corresponding to the h37 region which represents an unambiguous cytological marker. Since H-33258 is a general indicator of AT-rich chromosome regions (Comings 1975; Latt and Wohlfleib 1975), the brightness of h37 suggests that it contains AT-rich repetitive DNA sequences. The N-banding staining of the second chromosome heterochromatin revealed five heavily stained bands: h36, h38 which contains the centromere, h40, h43 and h45 (Figure 1c). Distally to h35, a secondary constriction (Kaufman 1934) is present which is associated with the histone gene cluster (S. Pimpinelli, personal communication).

Cytological analysis of 2h rearrangements: I characterized cytologically a series of putative heterochromatic rearrangements of the second chromosome. Each rearrangement was processed by Hoechst and N-banding and examined under the microscope after each step. Among the rearrangements examined are deficiencies derived from compound chromosomes (Figure 2), Rsp' deficiencies (Figure 3), SD-Roma' deficiencies (Figure 4), free duplications (Figure 5), inversions and translocations (Figure 6). The cytological characterization of these rearrangements is summarized by Figures 7 and 8.

Deficiencies: Proximal deficiencies of the second chromosome heterochromatin generated by the detachment of compound chromosomes after X-ray treatment represent useful tools for genetic analysis (Hilliker and Holm 1975; Hilliker 1976). However, the interpretation of the cytological pattern of these deficiencies is complicated since, due to the
FIGURE 2.—Sequential Hoechst and N-banding of Df(2L)C'. Hoechst (a) and N-banding (b) of Df(2L)C' and In(2LR)SM1. C, Cy promotaphase chromosomes. In the SM1 chromosome, which is Rsp insensitive and lacks region h39 (PIMPINELLI and DIMITRI 1989), regions h38 and h40 are fused. Df(2L)C', deleted for all defined 2Lh loci carries extra heterochromatin.

A well defined cytological pattern was obtained from the analysis of a group of Rsp' and SD Roma, 1t chromosomes. The Rsp' chromosomes are Rsp deficiencies, induced by X-rays on a Rsp' en bw chromosome; some of these deficiencies remove the vital loci of the 2R heterochromatin in addition to the Rsp locus (GANETZKY 1977). Hoechst and N-banding of four Rsp' chromosomes revealed an easily interpretable heterochromatic banding pattern showing that they are indeed deficiencies of the 2R heterochromatin (Figures 3 and 8).

Rsp'16 is deleted for almost the entire extent of 2Rh (Figure 3, a and b). It lacks the segment spanning from the distal part of region h38 to the proximal half of region h45. Rsp'11 carries a deficiency including regions h38-h42B; as a consequence, the h38 and h43 N-banded regions collapse in most nuclei, although some separation is seen in well elongated prometaphase chromosomes. Rsp'11 (Figure 3, c and d) carries a deficiency spanning from the right part of region h38 to region h42B. The remaining portion of region h38 is adjacent to h43. (e and f) Partial metaphase showing the Rsp'1 (left) and the SM1, C, Cy (right) chromosomes. Rsp'1 lacks a segment spanning from the right portion of region h38 to region h42A. Here, the remaining portion of h38 is well separated from h43. (g and h) The Rsp'16 chromosome lacks the h39 region. As a consequence, regions h38 and h40 are collapsed.

The analysis of the available 2Rh deficiencies was completed by the characterization of M-S210, M-S2' (MORGAN, SHULTZ and CURRY 1940) and r1106 chromosomes (Figure 7). The present cytological observation confirmed that M-S210 is a deficiency of the entire 2Rh (MORGAN, SHULTZ and CURRY 1940; HILLIKER and HOLM 1975). M-S2', which fails to complement mutations at the proximal euchromatic genes stw and ap (LINDSLEY and GRELL 1968), shows a
The h39 region of 2Rh corresponding to the Rspl locus (Pimpinelli and Dimitri 1989). e51 (Figure 5b), e61 (Figure 5c) and e57 (Figure 5d) share in common 2Lh, but differ for their 2R heterochromatic content. e57 is duplicated for the entire 2Rh. e61 carries a 2Rh segment spanning from region h38 to region h44; e51 is duplicated for regions h38-h41.

Translocations and inversions: I characterized 13 T(Y;2)′s induced by Lindsley et al. (1972), and three T(Y;2)s, T(Y;2)A, T(Y;2)B and T(Y;2)C, induced by Dobzhansky (see Lindsley and Grell 1968). I chose these translocations because their breakpoints on the salivary gland chromosomes are close to the chromosome center (regions 39-40 and 41-42), which suggests that they might be within the second chromosome heterochromatin. The T(2;3)E(da) was also selected by this criterion. Moreover, I analyzed a group of 2L, 2LR and 2R inversions whose genetic and cytological features suggest that their breakpoints involve the second chromosome heterochromatin. These rearrangements are variegated for either lt or bw and their breakpoints map close to the chromosome center (regions 39, 40 and 41 of the salivary gland chromosome map). In(2Lh)lt65, In(2Lh)lt628, In(2LhR)lt610, In(2LhR)lt616 (Brittnacher and Gantenzy 1989), In(2LhR)lt63 and In(2LhR)lt612 (Hesseler 1958) are all variegated for the lt gene, previously mapped to the pericentric region of 2L (Shultz 1936; Hesseler 1958). In(2LhR)bwV01 and In(2LhR)bwV02 are variegated for the euchromatic gene bw. Examples of cytological analysis of these rearrangements are shown in Figure 6. The breakpoint mapping is summarized in Figure 8. It should be noted that the 2L breakpoints of T(Y;2)B251 and T(Y;2)H54, which falls outside region h35 on mitotic chromosomes, are euchromatic since they map to region 39-DE of the salivary gland chromosomes (Siegel 1981). Among the remaining translocations, which map to region 40 on salivary gland chromosomes (Lindsley et al. 1972), one group

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**Figure 4.**—Examples of Hoechst and N-banding of SD-Roma-deficiencies. (a) The original SD-Roma chromosome from which the SD-Roma deficiencies were generated. In SD-Roma, which is Rspl insensitive and lacks region h39, regions h38 and h40 appear to be fused. (b) The H35258-banding pattern of the original SD-Roma (above) and SD-Roma (below) chromosomes. SD-Roma (right) lacks region h39, regions h38 and h40 appear to be fused. (c) The H35258- and N-banding patterns of the SD-Roma (right) and SD-Roma (left) chromosomes. Note that SD-Roma (right) lacks region h35 but still retains region h36. (d) A single SD-Roma chromosome lacking region h35. The cytological patterns of SD-Roma (right) and of SD-Roma (below) chromosomes.

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**Figure 5.**—Sequential Hoechst and N-banding of Dp(2;3). (a) e70, (b) e51, (c) e61 and (d) e57. Besides the 2Rh duplication, all mini-chromosomes carry a duplication of the 2Lh portion spanning from region h36 to the centromere.
exhibits 2L breakpoints cytologically similar to those of B251 and H54, while another group of translocations shows heterochromatic 2L breakpoints mapping proximally to region h35.

The 2L breakpoints of \( t^{G10}, t^{G16}, t^{G3} \) and \( t^{G28} \) occur proximally within region h35, since in all these rearrangements most of the region appears to be moved to a distal chromosomal location. The 2Lh breakpoints of \( l^{m3} \) and \( l^{t12} \) are located more proximally in region h37.

**Genetic analysis:** The following analyses were performed.

**\( T(Y;2h) \):** The genetic effects of cytologically characterized \( T(Y;2h) \) breakpoints were assayed in a series of complementation tests between these translocations and two lethal deficiencies, \( Df(2Lh)C' \) and \( Df(2Rh)M-S2' \), which uncover all the defined heterochromatic loci of 2L and 2R, respectively. This analysis showed that all breakpoints are fully viable over deficiency. Only the breakpoint of \( T(Y;2Rh)B \), falling at the h41-h42 junction, is lethal over \( Df(2Rh)M-S2' \). Further experiments showed that \( T(Y;2Rh)B \) is lethal when heterozygous with either EMS 31 and EMS 64 [which define the \( l(2)41Aa \) and rolled \( l(2)41Ac \) 2Rh loci, respectively] but is fully viable in combination with mutations at the \( l(2)41Ab, l(2)41Ad, l(2)41Ac \) and \( l(2)41Ah \) loci of the 2Rh (see Table 1). These findings suggest that \( l(2)41Aa \) and \( rl \) are adjacent genes in the 2Rh heterochromatin.

**\( l^t \) and \( bw^{10} \) inversions:** The effects of the cytologically determined inversion breakpoints were also assayed by complementation with \( C' \) and \( M-S2' \). This analysis showed that the breakpoints of \( In(2LRh)bw^{10} \) and \( In(2LRh)bw^{10} \) are viable over \( M-S2' \), while the \( l^t \) inversions fail to complement \( Df(2Lh)C' \). It should be noted that the breakpoints of \( l^{G10}, l^{G16}, l^{G3} \) and \( l^{G28} \) occur within region h35, while those of \( l^{m3} \) and \( l^{t12} \) are located more proximally in region h37 and exhibit a weaker \( lt \) variegated phenotype. Hence, these data suggest that the \( lt \) gene maps to the h35 region of 2Lh where the strongest \( lt \) variegator breakpoints are located. This suggestion is indeed supported by the analysis of the SD-Roma\(^{13} \) deficiencies (see below) and are in agreement with the results of the analysis of \( l^t \) rearrangements performed by WAKIMOTO and HEARN (1990).

**Mapping of 2Lh loci:** Nine loci have been assigned to the 2L heterochromatin by virtue of their failure to complement the \( Df(2Lh)C' \): The vital loci \( l(2)40Fa, l(2)40Fc, light \( (l(2)40Fb), l(2)40Fd, l(2)40Ff, l(2)40Fe \) and \( l(2)40Fg \) (HILLIKER 1976; using the LINDSLEY and ZIMM nomenclature) and the mat(2)cta gene (SCHUPBACH and WEISCHAUS 1989) whose relative position has been recently determined by WAKIMOTO.
TABLE 1

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Each deficiency was tested for its ability to complement the lethal mutations of 2Rh. EMS 31, EMS 45-10, EMS 64, EMS 45-17, EMS 34-25 and EMS 45-25 were used as representative alleles of the six loci of 2Rh. EMS/SJ1, Cy females were crossed to males bearing a specific heterochromatic rearrangement balanced over SM1. The expected ratio between Cy and Cy' progeny is 2:1.

* Malformed legs and irregular wing shape; many individuals died during the eclosion.

* Only the Cy and Cy' males progeny is included. In this case, the expected ratio between Cy and Cy' males is 1:1. ND = not determined.

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and Hearn (1990), the E(SD) locus (Brittnacher and Ganetzky 1984; Sharp, Hilliker and Holm 1985). The genetic analysis of the SD-Roma\textsuperscript{10} deficiencies performed by Brittnacher and Ganetzky (1984) showed that they remove other 2Rh loci in addition to l and E(SD). In particular, it is noteworthy that SD-Roma\textsuperscript{10} lacks all genetic loci of 2Lh, while SD-Roma\textsuperscript{13} retains only the most proximal locus associated to the 2Lh, l(2)40Fg, and SD-Roma\textsuperscript{18} carries both l(2)40Fg and the most distal locus associated to the 2Lh, l(2)40Fa. As shown in Figures 4 and 7, SD-Roma\textsuperscript{11}, SD-Roma\textsuperscript{13} and SD-Roma\textsuperscript{18} are deleted for region h35, but retain all remaining 2Lh regions. This indicates that l(2)40Fa, l(2)40Fc, eta, lt, l(2)40Fp, l(2)40Ff, l(2)40Fe, l(2)40Fg and E(SD) and all map to region h35 which is the most distal Hoechst 33258-dull region of 2L heterochromatin (Figure 9). These findings confirm the preliminary mapping of the l\textsuperscript{19} locus obtained from the cytogenetic analysis of the l\textsuperscript{19} inversion. Since Df(2Lh)SD-Roma\textsuperscript{10} retains the l(2)40Fa and l(2)40Fg genes, the most proximal and the most distal locus of the 2Lh respectively, that indicates that at least l(2)40Fg is proximally located within h35, while l(2)40Fa has to be distally located within h35 or at the euchromatin-heterochromatin junction.

Mapping of 2Rh loci: At least six vital loci l(2)41Ac, l(2)41Ab, rolled (l(2)41Ac), l(2)41Ad, l(2)41Ac and l(2)41Ah defined by EMS-induced lethal mutations were mapped using deficiencies to 2R heterochromatin (Hilliker 1976). Previous cytological analysis of Dp(2Rh;Y)\textsuperscript{s} (P. Dimitri, in Lyttle 1989) indicated that some of these complementation groups fall within the 2R heterochromatin. The extensive cytogenetic analysis of deficiencies and free duplications reported here permits the assignment of all six 2Rh loci to specific chromosome bands (Figure 9).

Each rearrangement was genetically assayed by complementation tests with the EMS-induced lethal mutations of 2Rh (Table 1). The genetic analysis of
order chromosome. Diagrams of the 2Lh (above) and 2Rh (below) show the extent of deficiency and duplication mapping results are consistent with each other.

An examination of Tables 1 and Figure 9 reveals that the free heterochromatic duplications had already been performed by BRITTNACHER and GANETZKY (1989). Hence it was necessary only to characterize these rearrangements for the presence of l(2)41Ah. The results of deficiency and duplication mapping are diagrammatically shown in Figure 9.

An analysis of the Table 1 and Figure 9 reveals that l(2)41Ab is not included in Df(2Rh)C2P A20. Thus l(2)41Ab maps proximally to region h40, very close to region h39 which corresponds to the Rsp locus. Since Df(2Rh)Rsp15 (Figure 3, g and h) and Dp(2hj)f70 (Figure 5a) genetically separate Rsp from l(2)41Ab (GANETZKY 1977; BRITTNACHER and GANETZKY 1989), it follows that l(2)41Ab might be located at the h39-h40 junction. Thus l(2)41Ab is the most proximal vital locus within 2R heterochromatin.

The l(2)41Aa and rolled(l(2)41Ac) loci are located within the h40-h41 segment which is limited proximally by the breakpoint of T(Y;2h)C used to construct the synthetic deficiency Df(2Rh)C2P A20 and distally by the distal end of Dp(2hj)f51 (l(2)41Ab+, l(2)41Aa+, rl+, l(2)41Aad-, l(2)41Ae+, l(2)41Ah+). An apparent difficulty in mapping the l(2)41Ab, l(2)41Aa and rolled loci is that the Rsp17 chromosome, which carries a deficiency spanning from the right half of region h38 to region h42A, fails to complement mutations at the rl locus but not at the l(2)41Ab and l(2)41Aa loci. One possible explanation for this apparent inconsistency is that the Rsp17 chromosome is a multiple hit rearrangement, rather than a simple polar deficiency. Hence it was necessary only to characterize these rearrangements for the presence of l(2)41Ab and l(2)41Aa, whose presence would be cytologically undetectable. Another possibility is that l(2)41Ab, l(2)41Aa and rl (l(2)41Ac) loci are not located in the same place on different chromosomes. However, the latter possibility seems unlikely given that, except for Rsp17, all other duplication and deficiency mapping results are consistent with each other.

Df(2Rh)b5mV32P_b5mV18 fails to complement only the EMS 45-17 lethal mutation at the l(2)41Ad locus, placing the l(2)41Ad locus within region h40-h44. In addition, Df(2Rh)Rsp131/EMS45-17 or Df(2Rh)b5mV32P_b5mV18/EMS45-17 hemizygous, die during eclosion and show a strong unextended phenotype (HILLIKER 1976) with malformed legs and unextended wings, while Df(2Rh)C2P A20/EMS45-17 hemizygotes exhibit a reduced viability (Table 1) and show an unextended phenotype weaker than that of Df(2Rh)Rsp131/EMS45-17. Thus, Df(2Rh)C2P A20, which is cytologically deleted for region h40-h43, behaves like a hypomorphic mutation at the l(2)41Ad locus. In principle, these findings might be explained by assuming that one or both of 2Rh breakpoints of T(Y;2h)C or T(Y;2h)A, used to generate Df(2Rh)C2P A20, induce a variegated position effect on the l(2)41Ad locus. However, this effect can be ruled out, since T(Y;2h)C/Df(2Rh)Rsp131/ or T(Y;2h)A/Df(2Rh)Rsp131 individuals are viable and exhibit a wild-type phenotype. An alternative possibility is that l(2)41Ad+ is repetitive in nature and that these repeats are partially deleted in Df(2Rh)C2P A20. If true, then a reduction in the number of repeats would partially impair the function of l(2)41Ad+, thereby resulting in a hypomorphic effect. A comparison of Df(2Rh)M-S210 and Dp(2hj)f57 with Df(2Rh)Rsp131 (see Figure 9) indicates that l(2)41Ae and l(2)41Ah map to the most distal regions of 2Rh. The cytological analysis of Df(2Rh)M-S24 confirms this conclusion and makes it possible to further limit the location of these loci. M-S24 fails to complement mutations at the l(2)41Ae and
$l(2)41Ah$ loci, strongly indicating that $l(2)41Ae$ and $l(2)41Ah$ map to the distal part of h46.

**Lethal phenotypes of mutations at the $l(2)41A$ and rolled loci:** The cytogenetic analysis presented above showed that two of the $2Rh$ ($l(2)41Ae$ and $l(2)41Ah$) and all of the $2Lh$ vital loci so far described map to the most distal block of heterochromatin, hence it cannot be determined here whether they are in heterochromatin or whether they lie at the very border between euchromatin and heterochromatin. However, the $l(2)41Ab$, $l(2)41Aa$, rolled and $l(2)41Ad$ loci are located within regions which are defined heterochromatic by standard cytological and molecular criteria. Therefore I have decided on concentrating on the functions of the proximal heterochromatic loci.

In a previous study, HILLIKER (1976) reported that EMS-31 hemizygous individuals survive until the third larval instar and develop large melanotic masses in the hemocoel. Furthermore, he found that individuals hemizygous for the $rl$ ipomorphic mutation are pupal lethals, while those hemizygous for the lethal alleles of $rl$ die as third instar larvae that completely lack imaginal discs. Here, I have found that 40% of the $rl$ hemizygotes die at the pharate adult stage. Interestingly, these individuals and rare eclosers show a strong $rl$ phenotype, with rough and reduced eyes, and frequently also exhibit missing structures or duplicated structures of the wings, legs and halteres (Figure 10). In order to find out whether the adult defects correlate with defects visible in larval stages, imaginal discs morphology of $rl/Df(2Rh)Rsp^I$ and $rl/Df(2Rh)Rsp^{31}$ late third larval larvae was examined. Imaginal discs of wing, legs and haltere were found to be malformed, reduced in size and with frequent deficiencies and duplications. Similar defects can be induced by X-ray irradiation (WADDINGTON 1942; POSTLETHWAIT and SCHNEIDERMAN 1973; POSTLETHWAIT 1975) or surgical bissection of discs (SHUBIGER 1971; BRYANT 1971). In addition, a group of mutations has also been identified which result in duplications of imaginal discs (for review see BRYANT 1978). A causal relationship between cell death in the developing imaginal discs and the occurrence of deficiencies and duplications has been previously demonstrated (FRISTROM 1968, 1969; RUSSELL 1974; ARKING 1975; SIMPSON and SCHNEIDERMAN 1975; CLARK and RUSSELL 1977; JAMES and BRYANT 1981). Therefore, I investigated whether cell death is also involved in producing the defects typically observed in $rl/Df(2Rh)Rsp^I$ or $rl/Df(2Rh)Rsp^{31}$ hemizygotes. In order to visualize cell death, imaginal wing discs were incubated in 1.6 × 10^{-6} M acridine orange (SPEIJ 1971; RUSSELL 1974). Using this technique small patches of naturally occurring cell death was detected in the imaginal wing discs of the $rl/cn bw$ control larvae (Figure 11a), while large patches of generally distributed cell death were apparent in the imaginal discs of $rl/Df(2Rh)Rsp^I$ and $rl/Df(2Rh)Rsp^{31}$ larvae of the same stage (Figure 11b).

I also examined the imaginal discs morphology of third instar larvae hemizygous for $EMS\ 31$, the late larval lethal mutation at the $l(2)41Aa$ locus. This analysis showed that some of the melanotic masses typically observed in $EMS\ 31$ mutants correspond to the degenerating thoracic discs. In addition, some mutant larvae exhibit abnormal imaginal wing discs, that are not melanotic but fail to undergo normal folding. Acridine orange incubation of $EMS\ 31$ hemizygous imaginal wing discs also revealed the presence of large patches of cell death (data not shown).

**DISCUSSION**

Much progress has recently been achieved on the cytogenetic properties of the genetic loci mapping to the $D.\ melanogaster$ heterochromatin (GATTI and PIMPINELLI 1983; HARDY et al. 1984; PIMPINELLI et al. 1985; BONACCORSI et al. 1988; PIMPINELLI and DIMITRI 1989; BONACCORSI et al. 1990). These study have concentrated largely on the sex chromosome heterochromatin and on elements cytogenetically very large. However, the localization and the distribution of the vital loci of the second chromosome presumed to be heterochromatic (HILLIKER 1976) and their relation-
ship to elements of the SD system were not clearly defined. In the present work, by combining cytological and complementation analyses, these elements have been mapped to specific heterochromatic regions.

The distribution of heterochromatic loci along the pericentric regions of the second chromosome: The results show that the genetic loci are non randomly distributed along the second chromosome heterochromatin. Only four of them, l(2)41Ab, l(2)41Aa, rolled and l(2)41Ad, are located within the most proximal regions of 2R heterochromatin (proximal loci). All remaining loci, which I will refer to as the distal loci, map to the distal heterochromatic blocks near the euchromatin-heterochromatin junction of both arms. Moreover, a common feature of the heterochromatic loci of the second chromosome revealed by the cytogenetic analysis presented here, is that they map to specific heterochromatic blocks but do not correspond to the blocks themselves, suggesting that indeed they are not as large as the Y chromosome fertility factors or Rsp.

Proximal loci: The cytogenetic studies reported here localize the l(2)41Ab, l(2)41Aa, rolled (l(2)41Ac) and l(2)41Ad loci in the most proximal regions h39-h40, h40-h41 and h43-h44, respectively, within the 2R heterochromatin (Figure 9). These regions contain blocks of α-heterochromatic highly repetitive satellite DNAs (AppeIs and Peacock 1978; A. Lohe and A. J. Hilliker, personal communication). The A-T rich middle-repetitive sequences related to Rsp (Wu et al. 1988) which has been shown to correspond to region h39 of the cn bw chromosome (Pimpinelli and Dimitri 1989) is also near these genes. In addition, a new class of middle repetitive DNA different from the Rsp sequences has been recently isolated mapping to h59 (R. CaiZzi, C. CagGeSe and S. Pimpinelli, personal communication). Thus, the heterochromatic segment extending from region h38 to region h46 contains both highly repetitive and middle repetitive DNA sequences. Furthermore, the presence of middle repetitive transposable elements within a complex satellite DNA of D. melanogaster (Brutlag and Carlsson 1978) suggests that blocks of highly repetitive DNAs are interrupted by other kind of sequences. It is currently unknown whether l(2)41Ab, l(2)41Aa, rolled and l(2)41Ad consist of unique coding sequences embedded in a high or middle-repetitive DNA environment or whether they are themselves comprised of repetitive sequences. However, the genetic behavior of D(2R)Czb A28 (see results), is compatible with the possibility that the l(2)41Ad locus, mapping to region h43-h44 which contains satellite DNAs (A. Lohe and A. J. Hilliker, unpublished results), is made up of repeated DNA sequences.

Possible functions of the l(2)41Aa and rl loci: The EMS 31 and rl hemizygous appear to exhibit a generalized syndrome disrupting development of larval and/or adult cuticular structures. The occurrence of extensive cell death in the imaginal discs appears to be causally related to the defects shown by the EMS 31 and rl hemizygous. The actual functions played by these loci during development are still unknown. However, the pattern of defects suggest that l(2)41Aa and rl represent a set of genes required for normal cell proliferation during imaginal disc development.

Distal loci: The analysis of the SD-Roma deficiencies indicates that l, E(SD) and the other 2h loci map to region h35, a Hoechst-dull band which corresponds to the most distal region of the 2L mitotic heterochromatin. The precise location of l and the remaining 2Lh loci along region h35 is currently undetermined. However, since Df(2Lh)SD-Rom 68 carries both l(2)40Fa and l(2)40Fg, the most proximal and the most distal locus of the 2Lh respectively, that indicates that at least l(2)40Fg is proximally located within h35 very close to h36, while l(2)40Fa has to be distally located within h35 or at the euchromatin-heterochromatin junction.

The l(2)41Ae and l(2)41Ah loci map to h46, a Hoechst-dull band which is the most distal region
of 2Rh. In particular, the genetic and cytological analysis of Df(2Rh)M-52 strongly suggests that these loci map to the distal part of region h46. Hilliker (1976) suggested that all heterochromatic vital loci of the second chromosome consist of unique DNA sequences occurring at a very low density relative to euchromatic regions. Devlin, Bingham and Wakimoto (1990) have shown in fact that the \( \text{lt} \) gene is made up of single copy exons and of a heterogeneous array of middle repetitive DNAs in the flanking and intronic regions. By \textit{in situ} hybridization experiments on salivary gland chromosomes \( \text{lt} \) has been mapped to the \( \beta \)-heterochromatin of 2L (Devlin et al. 1990; Wakimoto and Hearn 1990). Consistently, one can suggest that the remaining loci mapping to h35 are \( \beta \)-heterochromatic sequences with molecular organization that may be similar to that of \( \text{lt} \). The most distal loci of 2Rh which map to h46, \( k(2)41Ae \) and \( k(2)41Ah \), may also be \( \beta \)-heterochromatic sequences organized like the \( \text{lt} \) gene.

**On the genetic organization of regions h36, h37 and h38:** Based on the mapping results, it is worth noting that the 2Lh segment spanning from region h36 to region h38 appears to be devoid of genetic loci. This observation implies that nearly 70% of the 2Lh portion has no genetic function. However, it is possible that some heterochromatic loci mapping to this region are still undetected, despite the detailed genetic analysis performed by Hilliker and Holm (1975) and Hilliker (1976). Moreover region h36-h38 might also contain genes which are refractory to conventional genetic analyses and are revealed only in the presence of other specific genetic elements or mutations (Pimpinelli et al. 1986). For example, region h36-h38 may contain either genetic functions similar to \( \text{Rsp} \) or \( E(3D) \) which are dispensable for viability and fertility (Ganetzky 1977), or like the ABO elements which are repeated at several locations along the \( D. melanogaster \) heterochromatin (Pimpinelli et al. 1985). The generation of deficiencies removing regions h36, h37 and h38 will allow us to distinguish between these hypothesis.

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