THE GENETIC ANALYSIS OF THE HETEROCHROMATIN
OF CHROMOSOME 3 OF DROSOPHILA MELANOGASTER

by

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ABSTRACT

The heterochromatin of the third chromosome is the largest uncharacterized region of the *Drosophila melanogaster* genome, and the last major block of *D. melanogaster* heterochromatin to be thoroughly analyzed. In the present study, this region was genetically dissected by generating and analyzing a series of attached, detached and reattached third chromosomes. Separate detachment experiments were conducted for all twelve possible combinations of four newly synthesized sister-strand compound-3L's and three newly synthesized sister-strand compound-3R's. A total of 443 recessive lethal detachment products carrying putative heterochromatic deficiencies were tested for complementation in a several-stage complementation analysis. The results revealed the presence of seven separable vital regions in the heterochromatin of chromosome three. Attempts to reattach deficiency-carrying detachment products established that six of these vital regions are on the left arm, and only one is on the right arm. An analysis of the types and frequencies of detachment product deficiencies generated in each detachment experiment permitted the genetic characterization of the progenitor compounds. It was also possible to determine the proximal-distal orientation of the genes on each arm, and to identify possible breakpoints for each lethal detachment product produced. Seventy-five EMS-induced lethal alleles of detachment product deficiencies were also recovered and tested for complementation. Four additional genes in third chromosome heterochromatin were revealed, three on the left arm and one on the right arm. At least three of the EMS-induced lethals were small deficiencies. The inter-allelic complementation observed between some EMS-induced lethals, as well as the recovery of a temperature sensitive mutation of a heterochromatic gene, provided further evidence that there are single-copy, transcribed vital genes in third chromosome heterochromatin. Finally, a cytological analysis of three of the detachment product deficiencies by L. Sandler and S. Pimpinelli (personal
communication) provided evidence that at least some of the genes uncovered in this study are located in the most distal segments of third chromosome heterochromatin. The results suggest that the eleven vital genes discovered in the heterochromatin of the third chromosome are not randomly distributed between, nor within, the heterochromatic blocks of the left and right arms. This study provides further evidence that Drosophila heterochromatin is genetically heterogeneous, with vital genes being present in some heterochromatic segments but not others.
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INTRODUCTION

Since first described by the German investigator Emil Heitz (Heitz, 1928), heterochromatin has both puzzled and fascinated researchers in many fields of biology for over half a century. Although significant quantities of heterochromatin have been observed in the genomes of almost all higher organisms (Brown, 1966), no function has ever been clearly demonstrated for these chromosomal regions. Much remains unknown about the nature, composition, and properties of heterochromatin. Furthermore, the significance of what is known is often not readily apparent. Exceptions have been found to almost every rule or generalization about heterochromatin. To paraphrase Winston Churchill, heterochromatin appears to be "a riddle wrapped in a mystery inside an enigma".

Heterochromatin was originally defined by its differential staining pattern or "heteropycnosis" (Heitz, 1928). Unlike the other regions of the genome known as euchromatin, heterochromatin remains condensed and dark-staining throughout most of the cell cycle. However, there are problems with defining heterochromatin solely by the property of heteropycnosis. It is now known that there are several types of condensed, heteropycnotic chromatin. In some cases, such as the inactivated nucleated erythrocytes of birds, fish, and reptiles, the entire genome becomes permanently condensed. This type of condensed chromatin is not properly classified as heterochromatin, since it involves the general condensation of the entire genome rather than specific chromosomes or chromosomal regions.

Condensed chromatin that is true heterochromatin can be of two types - constitutive or facultative heterochromatin (Brown, 1966). Constitutive heterochromatin is present at identical positions on homologous chromosomes, and can
consist of an entire chromosome or a specific region of a chromosome. Facultative heterochromatin is formed by the condensation of only one of a pair of homologous chromosomes. A well-known example of facultative heterochromatin is the random inactivation and condensation of one of the X chromosomes in most female mammalian cells for the purposes of dosage compensation (Lyon, 1962). Since a given X chromosome is heterochromatic in some cells but a normal euchromatic X in others, facultative heterochromatin is considered to be a special condensed state of euchromatin. Constitutive heterochromatin, on the other hand, can be distinguished from both euchromatin and facultative heterochromatin by the C-banding technique which only stains constitutive heterochromatin (Pardue and Gall, 1970; Arrighi and Hsu, 1971).

A key discovery that clearly demonstrated that constitutive heterochromatin is chemically and structurally distinct from both euchromatin and facultative heterochromatin was the finding that constitutive heterochromatin is greatly enriched in highly repeated satellite DNA (Yasmineh and Yunis, 1969; Rae, 1970; Jones and Robertson, 1970; Pardue and Gall, 1970; Peacock et al., 1973; Gall and Atherton, 1974). Satellite DNA consists of very short sequences present in hundreds of thousands of copies per genome. Peacock et al. (1973; 1977a) have shown that the constitutive heterochromatin of Drosophila melanogaster and other organisms is almost entirely composed of long, homogeneous blocks of individual satellite DNA sequences tandemly arranged in a chromosome-specific order. The estimated amount of satellite DNA in each block of heterochromatin almost completely accounts for the total amount of DNA present (Peacock et al., 1977b), suggesting that there are few, if any, other types of DNA sequences within D. melanogaster heterochromatin. This view was supported by the discovery of junction DNA molecules that covalently link adjacent blocks of satellite DNA (Brutlag et al., 1977). While very few non-satellite sequences appear to be in heterochromatin, only trace amounts of satellite DNA are found outside of
heterochromatin (Peacock et al., 1977a, Cohen and Bowman, 1979).

The close correspondence between constitutive heterochromatin and satellite DNA has important implications for the possible function of heterochromatin. Satellite DNA is almost certainly genetically inert. Most satellite sequences would code for very odd proteins; and others contain such a high proportion of translational stop signals that it is very unlikely they could code for a protein (Southern, 1970; Bostock, 1980). More importantly, no RNA has been found to hybridize to satellite DNA (Flamm, Walker and McCallum, 1969; Woodcock and Sibati, 1975; Bostock, 1980). The only known example of satellite DNA transcription is the apparently accidental "run-on" transcription of some satellite DNA from newt lampbrush chromosomes (Baldwin and MacGregor, 1985; Varley, Macgregor and Erba, 1980).

The high concentration of satellite DNA found in constitutive heterochromatin is consistent with earlier evidence that heterochromatin is genetically inactive. Heitz (1929) was the first to suggest that the condensed nature of heterochromatin implies that these regions are of limited genetic importance. Drosophila geneticists discovered long ago a striking correlation between regions of the genome with little or no genetic activity and regions of heterochromatin. For example, Bridges (1916) observed that XO males in Drosophila melanogaster were phenotypically normal but sterile, and therefore concluded that the heterochromatic Y chromosome was devoid of any vital genes. Similarly, very few mutations were mapped to the X chromosomal and autosomal heterochromatin of this species (Muller and Painter, 1932; Kaufman, 1934). As well as having low mutation frequencies, heterochromatic regions are almost totally exempt from meiotic crossing-over (Muller and Painter, 1932). Baker (1954) later demonstrated that the absence of crossing-over is a general property of regions of constitutive heterochromatin.
The genetic inactivity of heterochromatin is further supported by the frequent polymorphic distribution of heterochromatin. In many species, the amount of heterochromatin on certain chromosomes varies considerably between individuals, with no apparent effect on phenotype or viability (e.g. Craig-Holmes, Moore and Shaw, 1975; Miklos and John, 1979; Kurnit, 1979; Gustafson, Lukasewski and Bennett, 1983; Maresca, Singer and Lee, 1984). In D. melanogaster, females with between one and four doses of the heterochromatic region of the X chromosome are phenotypically normal (Yamamoto and Miklos, 1977).

Biochemical studies have also suggested that heterochromatin is genetically inert. Hsu (1967) pulse-labelled mouse cells with tritiated uridine, and observed no radioactive grains over the heterochromatic regions. Sieger, Pera and Schwarzacher (1970) obtained similar results using Microtus agrestis. All this evidence led to the widely held belief that constitutive heterochromatin is genetically inert. However, it should be noted that none of the evidence excludes the possibility that heterochromatin possesses a low level of genetic activity.

Since constitutive heterochromatin exists in significant quantities in the genomes of almost all higher organisms and does not seem to code for appreciable amounts of protein or RNA, it is reasonable to expect that heterochromatin performs some other function. A number of functions have been proposed for heterochromatin, although there is no conclusive evidence to prove or disprove any of the functions that have been suggested.

Several researchers have speculated that the primary function of heterochromatin is to "protect" or "stabilize" centromeres, nucleolar organizer regions, and telomeres (Brown, 1966; Yunis and Yasmineh, 1971; Hsu, 1975). Although the exact mechanism by which heterochromatin would provide protection is usually not specified, it may be that heterochromatin acts as a passive spacer region that physically separates different regions of the chromosome.
Alternatively, heterochromatin may help to prevent specialized chromosomal regions, such as the tandemly arranged ribosomal RNA genes at nucleolar organizer regions, from being disrupted by meiotic crossing-over. Heterochromatin does have a non-random distribution in the genome and is usually found adjacent to centromeres, nucleolus organizers, and telomeres. However, there are exceptions, such as the large V chromosome in Drosophila nasutoides which is completely heterochromatic except for the centromeric region (Cordeiro et al., 1975). Also, there are a few higher organisms, such as the Norwegian rat, that do not have detectable amounts of heterochromatin and/or satellite DNA (Gosden et al., 1975; Timberlake, 1978; John and Miklos, 1979; Miklos, Willcocks and Baverstock, 1980; Sealy et al., 1981). If heterochromatin does function to protect or stabilize certain regions of the chromosome, then in at least some organisms such protection is not required.

A second function proposed for heterochromatin is that it initiates the synapsis of homologous chromosomes during meiosis (Goldring, Brutlag and Peacock, 1975; Peacock et al., 1977a; Beauchamp et al., 1979). The specific pattern of satellite DNA blocks in the heterochromatin of each chromosome could be the basis of recognition for proper homologue pairing. It has been shown that there are specific sequences, called "collochores", in the heterochromatin of the X and Y chromosomes of Drosophila melanogaster that serve as X-Y pairing sites in males (Cooper, 1964; Yamamoto and Miklos, 1977; Ault, Lin and Church, 1982; Appels and Hilliker, 1982). However, a variety of chromosomal manipulations in D. melanogaster have demonstrated that heterochromatin in general is not necessary for proper meiotic pairing (Yamamoto and Miklos, 1977; Yamamoto, 1979a; Hilliker, Holm and Appels, 1982). Further, cytological studies have detected no evidence that heterochromatin facilitates meiotic pairing (Maguire, 1972; John, 1976). Finally, the fact that some organisms contain no observable
amounts of heterochromatin is difficult to explain if heterochromatin is required for meiotic pairing.

A third proposed function for heterochromatin is the indirect control of cell size and the rate of cell growth and division (Bennett, 1972; Cavalier-Smith, 1978; Cavalier-Smith, 1980). Important characteristics such as cell and nuclear volume and the length of the cell cycle are indirectly controlled by the amount of chromatin in the nucleus, known as the "nucleotype". Intraspecific variations in heterochromatin content provides a possible means of altering the nucleotype and thus modifying important cell characteristics such as cell size and generation time. Variation in the amount of heterochromatin within a population may help a species adjust to a new environment. Although a nucleotypic function for heterochromatin is possible, there is no experimental evidence that heterochromatin performs such a function in nature.

At least two different roles have been suggested for heterochromatin in facilitating speciation. One possible role is that heterochromatic regions are preferentially involved in the initiation or fixation of chromosomal rearrangements which result in the formation of new species (Baimai, 1975; Hatch et al., 1976 a,b). According to this hypothesis, the more heterochromatin a species has, the more frequently it will be involved in speciation events. There is some supporting evidence that the amount of heterochromatin in a species correlates with the number of recognizable subspecies (Mazrimas and Hatch, 1972) and that there is a good correlation between high rates of speciation and chromosomal evolution (Bush et al, 1977). However, there is also some contradictory evidence. For example, a pocket gopher species group with a very small amount of heterochromatin has a very high frequency of chromosomal rearrangements (Patton and Sherwood, 1982).

A second way heterochromatin may facilitate speciation is by directly functioning as a sterility barrier (Corneo, 1978; Fry and Salser, 1977). If
substantial heterochromatic polymorphisms within a population disrupted meiosis, it may result in the effective genetic isolation of subgroups differing in amounts of heterochromatin, leading to sympatric speciation. There are many striking examples from a variety of organisms of closely related species whose only visible difference in chromosome structure is the loss or gain of blocks of heterochromatin (Pathak, Hsu and Arrighi, 1973; Holmquist, 1975; Rangonath, Schmidt and Hagele, 1982; Sen and Sharma, 1983). The pairing behaviour in the wheat-rye hybrid triticale shows that heterochromatin can disrupt meiotic pairing. A large block of heterochromatin on one of the rye chromosomes disrupts the pairing between the wheat and rye chromosomes in meiosis and results in chromosome bridges (Bennett, 1977; Bedrock, O'Dell and Flavell, 1980). However, there are also examples of subspecies with large differences in heterochromatin content that form viable and fertile hybrids (Blumenfeld, 1978 et al.; Patton and Sherwood, 1982).

The most substantiated of all the functions proposed for heterochromatin is that it modifies the amount and/or distribution of crossing-over. Although numerous studies have confirmed the quantitative and qualitative influence of heterochromatin on recombination (John, 1973; Miklos and Nankivell, 1976; Rhoades, 1978; Yamamoto, 1979b; John and King, 1982), it is not clear that heterochromatin evolved and exists in the genome for this purpose.

Although many functions have been suggested for constitutive heterochromatin, it is unlikely that the function(s) of heterochromatin will be clearly understood until some key questions about the nature of heterochromatin have been resolved. For example, is the function of satellite DNA equivalent to the function of heterochromatin? Are there DNA sequences other than satellite DNA in heterochromatin? If so, what role do these sequences have in determining the general properties and functions of heterochromatin? Do all DNA sequences within
a block of heterochromatin perform the same function? Does all constitutive heterochromatin have the same composition, properties and function? To quote Carlson and Brutlag (1978a), "the dearth of knowledge about the nonsatellite sequences present in heterochromatin clearly stands as a barrier to our understanding of the functions of heterochromatin".

There is growing evidence that heterochromatin is not uniform in composition and exhibits a variety of molecular and genetic characteristics. For example, there are 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 Drosophila melanogaster heterochromatin outside of the nucleolus organizer region (Peacock et al., 1981; Dawid et al., 1981; Appels and Hilliker, 1982; Hilliker and Appels, 1982; Cantu and Gay, 1984). Other moderately repetitive DNA sequences have been detected in significant quantities in Drosophila heterochromatin (Renkawitz, 1978; Mukherjee and Lakhotia, 1979; Leigh Brown and Ish-Horowicz, 1981; Lis, Ish-Horowicz and Pinchin, 1981; Spradling and Rubin, 1981). A moderately repeated DNA sequence that binds a 1.6 kilobase RNA isolated from embryos has been discovered adjacent to a cloned Drosophila melanogaster satellite sequence (Carlson and Brutlag, 1978b). It is now clear that non-satellite DNA sequences are present in constitutive heterochromatin, although in limited amounts. Until more is known about the role and genetic characteristics of these sequences it is premature to assume
that the properties and functions of satellite DNA automatically correspond to those of heterochromatin.

Most attempts to genetically characterize heterochromatin have used the fruitfly *Drosophila melanogaster*. This organism offers several important advantages for the study of constitutive heterochromatin. It has a low chromosome number (2n=8) and a relatively large heterochromatin content, comprising about 28% of the total genome (Peacock et al., 1973). The heterochromatin is present in a small number of easily observed large blocks which account for the proximal half of the acrocentric X chromosome, the entire Y chromosome, the proximal quarters of the large second and third metacentric autosomes, and most of the small fourth autosome. Other advantages of *D. melanogaster* include its short generation time, the ease with which it can be handled and raised, and the large number of mutations and chromosomal rearrangements that have been isolated and successfully mapped using polytene chromosomes. Furthermore, all the heterochromatin of *D. melanogaster* is constitutive and so there is no confusion between constitutive and facultative heterochromatin.

A number of genetic functions have been mapped to the heterochromatin of *Drosophila melanogaster* (reviewed by Hilliker, Appels and Schalet, 1980). Ritossa and Spiegelman (1965) determined that the tandemly repeated genes coding for rRNA are located at the nucleolus organizer regions present in both the X and Y heterochromatin. A later study showed that a partial deletion of the rRNA genes results in the *bobbed* (bb) phenotype (Ritossa, Atwood, and Spiegelman, 1966). Although the rRNA genes are present at large secondary constrictions in the heterochromatin, these genes retain many of the properties of heterochromatin even when separated from the surrounding blocks of heterochromatin (Hilliker and Appels, 1982).

Male fertility in *D. melanogaster* requires the presence of six distinct
fertility factors on the heterochromatic Y chromosome (Brousseau, 1960; Kennison, 1981; Hazelrigg, Forniti and Kaufman, 1982; Gatti and Pimpinelli, 1983). Each fertility factor is involved in a specific step in sperm development (Hardy, Tokoyasu and Lindsley, 1981) and seems to code for a different protein required for sperm development and fertility (Goldstein, Hardy and Lindsley, 1982). The recovery of EMS-induced (Williamson, 1970, 1972) and temperature-sensitive (Ayles et al., 1973) mutations of the male fertility factors suggest that they are single-copy, transcribed genes. However, on the basis of an exhaustive break-point analysis, Gatti and Pimpinelli (1983) have suggested that at least some of the Y fertility factors may have an enormous physical size, each containing up to several thousand kilobases of DNA. In addition to the six fertility factors there is another specific segment on the long arm of the Y chromosome that is required for normal sperm development. The deletion of this segment results in crystal formation in primary spermatocytes, but not sterility (Hardy et al., 1984; Livak, 1984).

Several other genetic properties have been mapped to specific regions of D. melanogaster heterochromatin, although it is not clear whether transcribed genes are involved. The chromosomal regions responsible for nucleolar dominance (Durica and Krider, 1978) and compensation response (Procunier and Tartof, 1978) are located in the X chromosome heterochromatin. A segment of the X heterochromatin also interacts with a family of recessive maternal mutations including abnormal oocyte (Sandler, 1970, 1972, 1977; Parry and Sandler, 1974; Pimpinelli et al., 1985). Extra doses of a specific segment of X heterochromatin can partially compensate for the maternal mutant defect in abnormal oocyte progeny. Two segments in the Y heterochromatin have a similar effect (Sandler, 1970; Pimpinelli et al., 1985). Finally, elements involved in the Segregation Distortion (SD) phenomenon have been mapped to the heterochromatin of chromosome two (Ganetsky, 1977; Brittnacher and Ganetsky, 1984; Sharp, Hilliker and Holm,
Thorough genetic dissections of the heterochromatin of the X (Shalet and Lefevre, 1973; Hilliker and Appels, 1982) and the Y (Kennison, 1981; Gatti and Pimpinelli, 1983) chromosomes have failed to detect any additional genetic functions in these regions. By comparison, very little was known about the genetic content of the autosomal heterochromatin of *D. melanogaster* until the recent development of an experimental procedure for selectively producing deficiencies of the proximal heterochromatin of the major autosomes.

The detachment of compound autosomes can be used to generate proximal deficiencies restricted to heterochromatin. A compound autosome is a chromosomal rearrangement which has two homologous autosomal arms attached to the same centromere. For example, a compound-3R, designated C(3R), has two right arms of chromosome 3 attached to a single centromere in a reversed metacentric configuration. To maintain diploidy, flies carrying a given compound autosome (such as a compound-3R) usually also carry the complementary compound autosome (in this case a compound-3L).

The origin and meiotic behaviour of compound autosomes has been examined in an extensive review by Holm (1976). Compound autosomes are formed by a translocation-like event, usually induced by radiation, between two breakpoints on opposite sides of the centromere of any two chromatids in a tetrad (Rasmussan, 1960; Leigh and Sobels, 1970; Holm, 1976). Rejoining between a centric and acentric fragment from different chromatids will result in the formation of a new compound autosome. This model for compound autosome formation predicts that both sister and non-sister strand attachments can occur. This prediction was confirmed by experiments showing that compound autosomes homozygous for a recessive visible mutation could be synthesized in heterozygous females (Leigh and Sobels, 1970; Holm, 1976). One sixth of all compounds synthesized from a stan-
standard pair of autosomes will be a sister-strand attachment between the two chroma­
ids of a given homologue.

Compound autosomes can be detached to form standard chromosomes by a simi­
lar two-hit, translocation-like event. Detachment occurs when flies carrying a
pair of complementary autosomes are irradiated and separate break points are
induced on both the left and right compound chromosomes. Rejoining of the
centric fragment from one compound chromosome with the acentric fragment from
the complementary compound will result in a reconstituted standard chromosome,
known as a detachment product.

Two important aspects of compound autosome synthesis and detachment make it
a useful procedure for screening for genetic loci in the proximal heterochro­
matin of autosomes. The first is that the formation of compound autosomes and
detachments seems to involve only breakpoints in heterochromatin (Hilliker and
Holm, 1975; Gibson, 1977). Secondly, the tranlocation-like event in both the
synthesis and detachment of compounds is asymmetrical with respect to the cen­
tromere. The breaks occur to one side of the centromere or the other, so that
rejoining can result in deficiencies and duplications of heterochromatic seg­
ments in the newly formed compound autosomes and detachment products. If a
detachment product carries a deficiency for a vital gene in heterochromatin, it
will be lethal when homozygous. For these reasons, the technique of compound
autosome formation and detachment offers a relatively easy procedure for
generating a series of proximal deficiencies of autosomal heterochromatin.

The compound detachment procedure was first applied to chromosome three of
D. melanogaster (Baldwin and Suzuki, 1971). Females carrying one of seven
different compound-3L chromosomes paired with the same compound-3R were
irradiated and mated to males carrying standard, balancer third chromosomes. A
total of 162 fertile progeny carrying reconstituted, standard third chromosomes
were recovered. Sixty-six of these detachment products behaved as recessive
lethals and were identified as putative deletions of vital heterochromatic loci. These chromosomes were tested for complementation in all inter se combinations. Thirteen of the lethals complemented all others, indicating that the lethality was a result of a random third-hit elsewhere on the chromosome. The remaining 52 lethals fell into 6 complementation groups, which formed a complementation pattern suggesting four distinct functional units. Mapping experiments demonstrated that the lethals of all four complementation units were between the most proximal markers known on the left and right arms of chromosome three. However, it was not possible to assign these lethals to heterochromatin with complete certainty.

Hilliker and Holm (1975) undertook a similar but more extensive study of the proximal heterochromatin of the second chromosome of D. melanogaster. Their analysis was facilitated by the existence of several known cytological and genetic markers present in the chromosome two centromeric region. A secondary constriction serves as a cytological landmark for the euchromatic-heterochromatic junction on the left arm, and the previously isolated deletion MS2-10 removes the entire block of heterochromatin on the right arm. As well, the recessive mutations rolled and light were believed to reside in second chromosome heterochromatin.

Hilliker and Holm (1975) recovered a total of 253 detachments from three compound-2 bearing strains. Of these, 122 behaved as recessive lethals and were tested for complementation in all possible inter se combinations. As well, the lethal detachment products were tested against MS2-10, rolled, light and some other known proximal deficiencies on chromosome two. The complementation results suggested that at least five vital genetic loci were present in the heterochromatin of chromosome two. Using the results of the complementation tests with MS2-10, Hilliker and Holm were able to assign three of the loci to the left arm.
heterochromatin and the other two to the right arm. Rolled and light were pseudo-dominant over deficiencies of the right and the left arm, respectively, confirming the heterochromatic location of these markers.

Cytological examination of polytene chromosomes carrying the proximal deficiencies failed to detect any extension of the deletions into the euchromatin. Further, complementation testing demonstrated that the detachment deficiencies were proximal to pre-existing deletions that extended from the euchromatin into the heterochromatin, proving that the deficiencies were restricted to heterochromatin.

Hilliker (1976) extended the analysis of second chromosome heterochromatin by collecting EMS-induced lethals which failed to complement the detachment product deficiencies. Complementation testing of the EMS-induced lethals identified at least thirteen genetic loci in chromosome two heterochromatin, seven on the left arm and six on the right arm. None of the EMS lethals behaved as a deficiency, and extensive inter-allelic complementation was observed. The genetic analysis of heterochromatin of chromosome two of D. melanogaster is the strongest evidence yet that single-copy, vital genes do reside in constitutive heterochromatin.

However, the euchromatin-like genes shown to be in the chromosome two heterochromatin do not seem to exist in the heterochromatin of the X or Y chromosome of the same organism (reviewed by Hilliker, Appels and Schalet, 1980). These findings raise the possibility that heterochromatin in D. melanogaster may be heterogeneous with respect to genetic activity. The heterochromatin of the sex chromosomes has a very different genetic composition than the heterochromatin of at least one of the major autosomes. It would be of significant interest to determine if the genetic content of chromosome three, the other major autosome, conforms to this dichotomous pattern. The results of Baldwin and Suzuki (1971) suggesting that chromosome three heterochromatin does contain
essential genes is strengthened by the finding that chromosome two detachment product deficiencies are restricted to heterochromatin (Hilliker and Holm, 1975). However, there is insufficient information about the number, distribution, repetitiveness and nature of genes in chromosome three heterochromatin to make a valid comparison between the heterochromatic gene content of the two major autosomes.

There is some evidence which hints that the genetic composition of chromosome two and chromosome three heterochromatin may differ. For example, satellite DNA accounts for close to 100% of the DNA in the heterochromatic blocks on the second, X and Y chromosomes; but a significant portion of chromosome three heterochromatin does not appear to be composed of these highly repeated sequences (Peacock et al., 1977b).

Banding studies also suggest differences between the heterochromatic blocks of chromosomes two and three. C-banding of the D. melanogaster genome reveals that the heterochromatin of the X chromosome, both arms of the second chromosome, and one arm of the third chromosome is subdivided by a number of lightly stained bands. However, the Y chromosome and heterochromatin on the other arm of chromosome three does not appear to be subdivided (Hsu, 1971). Pimpinelli, Gatti and De Marco (1975) also observed a heterogeneous response of Drosophila heterochromatin to treatment with 33258 Hoechst, an agent known to decondense mouse heterochromatin. In D. melanogaster, the heterochromatin of the right arm of chromosome three, both arms of the second chromosome, and part of the X chromosome was not influenced by this treatment. The left arm heterochromatin of chromosome three, the remainder of the X chromosome heterochromatin, and most of the Y chromosome was decondensed by Hoechst.

The differences in molecular structure and banding behaviour between chromosome two and three heterochromatin may reflect differences in gene con-
tent. An extensive genetic analysis of third chromosome heterochromatin is needed to determine if each heterochromatic block in *D. melanogaster* is genetically unique or if there are different classes (such as autosomal and sex heterochromatin) of genetically active Drosophila heterochromatin. No accurate generalization about the genetic activity of *Drosophila melanogaster* heterochromatin can be made until this last major uncharted block of heterochromatin in the genome of this species is analyzed.

A thorough genetic analysis of third chromosome heterochromatin is now feasible with improvements and extensions of the experimental procedures used by Baldwin and Suzuki (1971). The key difference is that the present study will utilize several different compound thirds for each arm, and all possible combinations of these will be detached. Furthermore, the detachment products from each pair of compound thirds will be analyzed separately. The advantage of this approach is that it allows the characterization of the progenitor compound autosomes used to generate each detachment.

A compound autosome can carry a duplication of a vital region of the opposite arm, a deficiency of a vital region on the same arm, or both or neither. (Figure 1) Each type of compound autosome will produce a different pattern of detachment products. There are many possible types of detachment products, and those with a deficiency can carry a polar, non-polar, centromere-spanning, or complex deletion. (Figure 2) The types and frequencies of deletions generated depends on the class of the progenitor compounds. For example, only polar deficiencies will be generated from compound autosomes carrying no duplications or deficiencies. Polar deficiencies can also be obtained from other classes of progenitor compounds. Non-polar deficiencies will only be recovered from progenitor compounds carrying a duplication or deficiency; and centromere-spanning deficiencies should only result from progenitors hemizygous for at least one heterochromatic locus.
Models showing the synthesis of the four possible types of compound autosomes. C(3L) chromosomes formed by sister-strand attachments are used as examples. Balancer chromosomes are shown as wavy lines, irradiation-induced breaks as solid arrows, 3L heterochromatin as cross-hatched blocks, and 3R heterochromatin as plain blocks. The symbols a, b, and c represent regions found in wild-type left heterochromatin; while d, e, and f designate regions in wild-type right heterochromatin.

TOP LEFT: Synthesis of a C(3L) chromosome carrying no proximal deficiencies or duplications of vital loci.

TOP RIGHT: Synthesis of a C(3L) chromosome carrying a proximal duplication of 3R heterochromatin.

BOTTOM LEFT: Synthesis of a C(3L) chromosome carrying a proximal deficiency of a 3L heterochromatic region.

BOTTOM RIGHT: Synthesis of a C(3L) chromosome carrying both a proximal 3R duplication and 3L deficiency.
FIGURE 2

Models showing the generation of four possible types of proximal deficiencies carried by detachment products. Irradiation-induced breaks are shown as solid arrows, 3L heterochromatin as cross-hatched blocks, and 3R heterochromatin as plain blocks. The symbols a, b, and c represent regions found in wild-type left heterochromatin; while d, e, and f designate regions in wild-type right heterochromatin.

TOP LEFT: Production of a polar deficiency of the left arm from progenitor compounds carrying no duplications or deficiencies of vital loci.

TOP RIGHT: Production of a non-polar deficiency of the left arm from progenitor compounds carrying a duplication of left arm heterochromatin.

BOTTOM LEFT: Production of a centromere-spanning deficiency from progenitor compounds carrying a deficiency of left arm heterochromatin.

BOTTOM RIGHT: Production of a more complex deficiency from progenitor compounds carrying duplications and deficiencies.
The frequency of lethal detachment products generated is also partially determined by the class of the progenitor compounds. For each detachment event, there is a reciprocal detachment which would be expected to occur at an equal frequency. Each time a compound autosome carrying a deficiency for a given region is detached, one of the two reciprocal detachment products will carry the deficiency. Therefore, a minimum of 50% of the detachment products from a deficiency-carrying compound will be deficient for the same region. If no deficiency is carried by the progenitor compounds, the proportion of detachment products deficient for a specific region cannot exceed 50%. The percentage of detachment products carrying any deficiency will also be partially dependent on whether the progenitor compounds are carrying deficiencies and/or duplications.

Additional information about the distribution of genes in the proximal heterochromatic region can be obtained from a detachment experiment if one knows the nature of the progenitor compounds. However, it is not possible to determine immediately to which of the four possible classes a particular compound autosome belongs. Any deficiency carried by a compound autosome is masked by the other arm of the same chromosome. It is assumed that progeny with compounds carrying a duplication are also phenotypically normal. A compound autosome can only be characterized by working backwards from the pattern of detachment products generated. Since the frequency and type of lethal detachments produced from a given compound autosome pair is a function of the interaction of the classes of both compounds in a pair, each compound autosome must be detached in several different combinations before it can be properly characterized. In the present study, these objectives are met by undertaking separate detachment experiments for all 12 possible combinations of three different C(3R) chromosomes and four different C(3L) chromosomes. It was necessary to analyze a relatively large number of detachment products to ensure a representative sample from each of the twelve detachment experiments.
Another improvement in the procedure used here was to detach only newly synthesized sister-strand compound thirds. Evidence suggests that compound chromosomes may accumulate spontaneous recessive lethals, especially near the centromere where homozygosis is infrequent (Parker, 1954). To avoid this problem, newly generated isogenic sister-strand attachments are used to ensure that the progenitor compounds are free of any contaminating lethal mutations.

One further refinement of the compound autosome synthesis and detachment procedure deserves mention here. Unlike chromosome two, there are no known deficiencies of chromosome three heterochromatin that can be used to determine on which side of the centromere newly discovered proximal genes reside. To overcome this disadvantage, an attempt was made to reattach newly synthesized detachment products carrying deficiencies. New sister-strand attachments will only be recovered from the chromosome arm not carrying the deficiency. Using this new approach, it was possible to assign each deficiency to either the right or left arm heterochromatin of chromosome three. Other improvements in the procedure are described later.

The procedural improvements described above have made possible an extensive genetic investigation of the proximal heterochromatin of chromosome three, extending the results of Baldwin and Suzuki (1971). The chromosome three heterochromatin is the largest uncharacterized region of the D. melanogaster genome, and the last major block of D. melanogaster heterochromatin to be thoroughly analyzed. The results of this study provide a detailed genetic map of chromosome three heterochromatin, and shed new light on the genetic nature and heterogeneity of Drosophila heterochromatin.
Drosophila Stocks

Several strains of *Drosophila melanogaster* with mutated or rearranged third chromosomes were used in this study. All new compound third chromosomes and detachment products that were generated carried the proximal recessive markers *radius incompleatus* on the left arm and *pink-peach* on the right arm. All detachment products were balanced over the In(3LR)TH3,Y ri p6 se bx34e e* chromosome (hereafter referred to as TH3), which suppresses crossing-over on chromosome three. Further information on all the mutations and rearranged chromosomes used in this study is given in Lindsley and Grell (1968).

Synthesis of New Compound-3 Chromosomes

Only newly synthesized isogenic sister-strand attachments were used for detachment. An isogenic *ri p6* strain was obtained by mating a single *ri p6/TH3* male to TH3/Ly females, and then mating siblings of the *ri p6/TH3* progeny to isolate a homozygous isogenic *ri p6* stock. Isogenic *ri p6* males were collected and mated to virgin TH3/Ly females. Approximately 1500 virgin F1 *ri p6/TH3* females were collected and treated with 2000 rads of gamma radiation from a *60*Co source. The irradiated females were mated in groups of 25 to *C(3L)VT1,se; C(3R)VK1,e* males in pint-sized bottles. The parents were cleared after ten days. New compounds generated in females heterozygous for TH3 could only be formed by the attachment of sister chromatids of the non-inverted *ri p6* homologue. The only surviving progeny carried a newly formed compound third, or were a rare nondisjunctional offspring. All *ri e* progeny carried a newly formed sister-strand C(3L) chromosome, while *se pp*
progeny carried a new sister-strand C(3R) chromosome. Separate stocks were established for each possible combination of newly synthesized compound thirds.

**Recovery of Detachment Products**

A separate detachment experiment was performed for each of twelve lines carrying a different combination of new C(3L) and C(3R) chromosomes. In each experiment, 2000 virgin compound-3 females were treated with 2200 rads of gamma radiation and mated in groups of fifty to TH3/Ly males in pint-sized bottles. The parents were cleared after 10 days, and offspring were collected at 18° for the next 20 days. Virgin females and males carrying detachment products recovered over TH3, and males carrying detachment products recovered over the Ly chromosome, were established in stock over TH3.

**Lethality Tests**

Each detachment product was tested for recessive lethality by mating males and females carrying the same detached ri p chromosome balanced over TH3. All lethality tests were carried out in duplicate in shell vials with two pairs of flies per vial. If the detachment carried a recessive lethal, no homozygous ri p progeny were produced. If the detachment product was homozygous viable, then ri p/TH3 heterozygotes and homozygous ri p flies were recovered in approximately a 2:1 ratio. All progeny in each vial were counted to ensure that any semi-lethals would be detected. Detachment products carrying recessive lethals were maintained in stock over TH3. Approximately 150 non-lethal detachment products were also saved.

**Complementation Tests**

To make possible the analysis of a large number of detachment products without requiring an astronomical number of crosses, complementation testing was done in several stages. First, the lethal detachment products from five of the
twelve detachment experiments were tested in all inter se combinations with all other lethal detachments from the same experiment. From these tests, a separate complementation map was produced for each of the five detachment experiments.

The second stage of the complementation analysis was to test between complementation groups of the five detachment experiments. Several detachment products were selected from every complementation group in each of the five experiments. These chromosomes were then pooled and tested in all inter se combinations, producing an accumulated complementation map for the five original experiments. Further complementation tests were carried out within complementation groups on the accumulated map wherever necessary to resolve any ambiguities.

The third stage of the complementation analysis was to test all lethal detachment products from the seven remaining experiments against a tester chromosome from each complementation group on the accumulated map. From the results of these crosses, all the detachment products of the seven remaining experiments were assigned to a complementation group on the overall map. Finally, some random complementation tests were conducted within each complementation group to ensure that none of the complementation groups could be further subdivided.

In each stage of the complementation analysis described above, all tests involved crossing a pair of males carrying one lethal balanced over TH3 to a pair of females carrying a second lethal over TH3. All crosses were carried out in shell vials at 25°C. Reciprocal crosses were performed for most complementation tests. Crosses between two non-complementing lethals produced only \( ri \, p^p/TH3 \) progeny. In crosses between complementing lethals, \(ripp/TH3 \) and \(ri \, p^p/ri \, p^p \) progeny were recovered in an approximate ratio of 2:1.
Recombination Mapping

The proximal position of the putative deletions carried by the detachment products was confirmed by a cross-over analysis. A tester detachment chromosome from each complementation class was tested in two recombination mapping experiments. The first experiment sought to determine if the deficiency on the tester chromosomes were to the left or right of eagle, a very proximal marker on the left arm of chromosome three. Females carrying a detachment product over the marker chromosome st eg Ki were mated to homozygous st in ri eg males. The F1 progeny was screened for crossovers between ri at map position 47.0 and eg at map position 47.3. One cross-over product, recovered in progeny with a radius incompletus, eagle, Kinked phenotype, included almost the entire left arm euchromatin of the original detachment chromosome. Progeny with a scarlet phenotype carried the reciprocal recombinant product which includes the entire right arm and the heterochromatin of the left arm of chromosome three. Both classes of recombinant products were recovered and tested for lethality over the original detachment product. If the recombinant chromosome recovered in the scarlet progeny fails to survive over the detachment product, then the deficiency carried by the detachment product is to the right of the ri-eg region. Conversely, if the recombinant product recovered in radius incompletus, eagle, Kinked progeny fails to survive, then the deficiency is in the left arm euchromatin.

A similar experiment was conducted for the right arm. Females carrying the detachment product over the marker chromosome eg Ki were mated to homozygous ri pp males. Cross-overs in the region between Kinked at map position 47.6 and pink-peach at map position 48.0 were recovered. Progeny with a Kinked, pink-peach phenotype carried a recombinant chromosome containing most of the right arm euchromatin whereas progeny with a radius incompletus phenotype carried the reciprocal recombinant product containing the entire left arm and the right arm heterochromatin. If the recombinant chromosome which includes the entire left
arm and the heterochromatin of the right arm is lethal over the original detachment product, then the detachment product deficiency is to the left of the Ki pp region. If the recombinant product with only the right arm euchromatin fails to survive over the complete detachment product, then the deficiency is distal to the Ki pp region on the right arm.

A putative detachment product deficiency can be assigned to the proximal region of chromosome three with certainty if it maps to the right of the ri eg region and to the left of the Ki pp region.

Reattachments

To determine whether proximal deficiencies were on the left or right arm of chromosome three, an attempt was made to synthesize new sister-strand reattachments of lethal detachment products from several complementation classes. Virgin females carrying the tester detachment product over TH3 were irradiated and mated to C(3L)VH3, st; C3R)SH19, + males. For each detachment product tested, 2500 females were treated with 2500 rads and mated, and in addition 1250 females were treated with 4000 rads and mated. Progeny with a radius incompletus phenotype carried a newly formed sister-strand C(3L); and those with a scarlet, pink-peach phenotype carried a new sister-strand C(3R). Only C(3L)'s should be recovered from detachment products with a right-arm deficiency, and vice versa.

EMS Mutagenesis

Newly eclosed males from the isogenic ri p° stock were aged two days and then fed with a 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 TH3/Ly females. Male progeny with a mutagenized ri p° chromosome recovered over Ly were collected and tested for allelism with detachment product deficiencies of the left and right
arm. Each male was single pair mated at 29° 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 re-mated to females carrying a deletion of the right arm heterochromatin. In all, two different deficiencies of the left arm and two of the right arm were used; although each mutagenized chromosome was only tested against one deficiency of each arm. The progeny of each cross was examined for the presence or absence of $ri\ p^p/ri\ p^p$ and $ri\ p^p/TH3$ progeny. Vials that contained no $ri\ p^p/ri\ p^p$ progeny were scored as putative lethal alleles of the detachment product deficiency. The absence of $ri\ p^p/TH3$ progeny indicated a lethal allele of TM3 had been produced.

All putative EMS-lethal alleles of deficiencies were re-tested at both 22° and 29° 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 maintained and tested for complementation in all inter se combinations. If the rearranged TM3 chromosome has accumulated any recessive lethals in the centromeric region, all detachment products carrying a deficiency that uncovered the TM3 lethal would not have been recovered. To test this possibility, several of the EMS-induced lethal alleles of TM3 were analyzed by the cross-over procedure described earlier to determine if any mapped to the proximal region of the third chromosome.
RESULTS

Synthesis of New Compound-3 Chromosomes

Six new sister-strand compound-3L and three sister-strand compound-3R chromosomes were recovered from approximately 1500 irradiated females. Each new compound third was assigned an alpha-numeric code according to the system described by Holm (1976). The three C(3R) chromosomes were designated as C(3R)VM1,pp to C(3R)VM3,pp; and the six C(3L) chromosomes were designated C(3L)VM1,ri to C(3L)VM6,ri. All three C(3R) chromosomes and the first four C(3L) chromosomes were used for the detachment experiments. Separate lines were established for all twelve possible combinations of these compound chromosomes.

Recovery of Detachment Products

A separate detachment experiment was undertaken for each of the twelve compound-3 combinations. In each experiment, 2000 virgin females carrying a pair of compound-3's were irradiated and mated to TM3/Ly males. Detachment products were recovered in the progeny over the TM3 and Ly chromosomes. The total number of detachment products recovered in each experiment is shown in Table I. Experiment 6 was repeated because of the low number of detachment products recovered.

It is interesting to note that the four detachment experiments involving C(3R)VM2 produced substantially fewer detachment products than any of the other experiments. To test whether this effect was a result of reduced fertility in stocks carrying the C(3R)VM2 chromosome, fecundity tests were carried out for each of the twelve compound lines. Two day old virgin females from each line were treated with 2200 rads and mated to males carrying a pair of compound thirds. Six bottles of 25 females and 10 males were mated for each compound line. The fecundity of stocks carrying the C(3R)VM2 chromosome was not significantly different from the other compound stocks.
TABLE I

The number of detachment products recovered from each detachment experiment.

<table>
<thead>
<tr>
<th>Expt. #</th>
<th>C(3L)</th>
<th>C(3R)</th>
<th># Females Irradiated</th>
<th>Detachments Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2000</td>
<td>218</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>2</td>
<td>2000</td>
<td>108</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1</td>
<td>2000</td>
<td>166</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>2000</td>
<td>201</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>2</td>
<td>2000</td>
<td>112</td>
</tr>
<tr>
<td>6A</td>
<td>3</td>
<td>2</td>
<td>2000</td>
<td>80</td>
</tr>
<tr>
<td>6B</td>
<td>3</td>
<td>2</td>
<td>2000</td>
<td>98</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>3</td>
<td>2000</td>
<td>178</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>1</td>
<td>2000</td>
<td>144</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>2</td>
<td>2000</td>
<td>93</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>3</td>
<td>2000</td>
<td>218</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>3</td>
<td>2000</td>
<td>163</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>3</td>
<td>2000</td>
<td>238</td>
</tr>
</tbody>
</table>
Another possible explanation is that the C(3R)VM2 chromosome carries a massive deficiency of proximal heterochromatin, and therefore presents a smaller target for gamma rays. The results reported later in this study do not substantiate this hypothesis. Nevertheless, the possibility that some intrinsic property of compound autosomes may influence their detachment frequencies is an interesting finding.

Detachment products recovered in males or females over TH3 and in males over Ly were maintained in stock balanced over TH3. Each detachment product was assigned a code number which identified the experiment and the detachment product. For example, 2-10 is the tenth detachment product recovered from experiment 2.

Lethality Tests

The results of lethality tests with the detachment products from each experiment are given in Table II. No significant semi-lethality or sterility was observed. It can be seen from the data that the percentage of lethal-carrying detachment products varied considerably from experiment to experiment. In three experiments, less than 35% of the detachment products carried lethals; while in five others over 60% of the detachment products were homozygous lethal. The other four experiments were within five percentage points of 50% lethals. These differences in lethality frequencies likely reflect different classes of progenitor compounds. At least 25 lethal detachment products from each experiment were maintained in stock and used in the complementation analysis. A total of approximately 150 non-lethal detachment products were also saved.

Complementation Analysis

A total of 443 lethal detachment products from the twelve separate experiments were tested for complementation following the several-step procedure described in the Materials and Methods section. First, a separate complement-
TABLE II

The results of lethality tests with detachment products from each detachment experiment. The "% Lethals" column lists the percentage of all detachment products in each experiment which carried recessive lethals.

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>C(3L)</th>
<th>C(3R)</th>
<th>LETHALS</th>
<th>NON-LETHALS</th>
<th>% LETHALS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>1</td>
<td>40</td>
<td>79</td>
<td>33%</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>2</td>
<td>53</td>
<td>31</td>
<td>63%</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>1</td>
<td>66</td>
<td>38</td>
<td>63%</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>38</td>
<td>116</td>
<td>25%</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>2</td>
<td>42</td>
<td>47</td>
<td>47%</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>2</td>
<td>42</td>
<td>16</td>
<td>72%</td>
</tr>
<tr>
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<td>62</td>
<td>31</td>
<td>67%</td>
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<tr>
<td>8</td>
<td>4</td>
<td>1</td>
<td>33</td>
<td>109</td>
<td>23%</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>2</td>
<td>39</td>
<td>32</td>
<td>55%</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>3</td>
<td>62</td>
<td>29</td>
<td>68%</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>3</td>
<td>50</td>
<td>51</td>
<td>50%</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>3</td>
<td>41</td>
<td>39</td>
<td>51%</td>
</tr>
</tbody>
</table>
ation map was produced for experiments 1, 2, 3, 4, and 10 by testing the lethal detachment products of each experiment in all inter se combinations. Testing between complementation groups from the different experiments produced an accumulated complementation map for the five experiments. Finally, an accumulated complementation map for all twelve experiments was produced by testing all the lethal detachment products from the remaining seven experiments against tester chromosomes from each complementation group in the five-experiment complementation map.

The accumulated complementation map of the lethal detachment products from all twelve experiments is shown in Figure 3. The complementation groups are indicated by solid bars, and those which are shown to overlap fail to complement. Each complementation group is designated by a representative detachment product, whose code number is given above the bar. The number under the bar represents the number of detachment products which fall into that particular complementation group.

As Figure 3 shows, there are 18 complementation groups, with as many as 146 and as few as 1 detachment product per group. This initial complementation map suggests that at least eight separate vital regions have been uncovered by the putative deficiencies on the detachment chromosomes. Small complementing deficiencies specific for seven of these regions have been recovered, and the presence of an eighth region between the 3-30 and 3-9 groups is implied by the complementation pattern. The two complementation groups shown as two solid blocks joined by a dotted line were interpreted as detachment products that carried double deficiencies, one on each side of the centromere. Such centromere-spanning double deficiencies can only be generated by progenitor compound chromosomes carrying a deficiency.

It should be noted that there is a certain degree of arbitrariness in the
Accumulated complementation map of putative deficiencies carried by detachment products from all twelve detachment experiments. Each bar represents a different complementation group. Bars that do not overlap represent complementing groups, and vice versa. The number above each bar designates the detachment product used to represent the complementation group. The number below the bar indicates the number of detachment products assigned to the complementation group. See text for further details.
way the complementation map is drawn in Figure 3. Other variations of this map are also consistent with the results. For example, a different position and polarity of some of the complementation groups is possible. These ambiguities will be resolved in later sections.

In addition to the detachment chromosomes shown on the complementation map, there were 44 chromosomes that behaved as random hits. These chromosomes complemented all others against which they were tested. Twenty of the putative random hit lethals from different experiments were selected and complementation tested in all inter se combinations. All crosses resulted in complementation, providing further evidence that these 44 chromosomes carry lethals elsewhere on the chromosome generated by random third-hits during the detachment event.

The only other detachment product included in the complementation analysis but not shown on the complementation map is lethal 11-64. This detachment chromosome is semi-lethal over all deficiencies of the region defined by lethal 3-30, and surviving heterozygotes have slightly outspread wings and only a partial posterior wing cross-vein. The 11-64 chromosome fully complements detachment products from all other complementation groups. Unfortunately, the 11-64 stock was lost, making further tests impossible.

Vials containing detachment products of the 3-9 complementation class also exhibited a phenotype. Rare homozygous adults would survive, but would die within 48 hours of eclosion. The surviving adults had a phenotype resembling rotund, with droopy wings and reduced sex combs in males. However, all detachment products of this class fully complemented rotund, which has been mapped to band 84D on the right arm of chromosome three (Duncan and Kaufman, 1975). The phenotype expressed by homozygotes of the 3-9 complementation group was designated rotund-like.

A number of non-lethal detachment products were also tested for complementation with some of the lethal complementation groups. If the heterochromatin of
Chromosome three contains tandemly repeated genes, it is possible that partial deletions will be homozygous viable. However, the partial deletions may be lethal over more severe deficiencies. To test this possibility, approximately 150 non-lethal detachment products were each crossed to lethals 3-126, 1-166, and 10-65, which collectively span the accumulated complementation map. However, all the crosses resulted in full complementation, and the non-lethal detachment product stocks were discarded. As well, to keep the number of stocks manageable, a maximum of ten lethal stocks continued to be maintained for each complementation group.

Recombination Mapping

The proximal position of the putative heterochromatic deficiencies was confirmed using the recombination mapping protocol described in the Materials and Methods section. Exchange events between tightly linked proximal markers on either the left or right arm were recovered from females heterozygous for a detachment product and a marker chromosome. For most detachment products, the lethal deficiency always remained with the recombinant product containing the centromeric fragment of the detachment chromosome. This was the case for detachment products 10-65, 10-42, 4-134, 10-39, 6B-29, 3-9, 6-61, and 4-75. These deficiencies, which include most of the complementation groups, are now confirmed to be located in the proximal region of chromosome three.

However, the lethals on detachment products 1-168 and 3-144 both mapped to the right arm euchromatin. The complementation group represented by deficiency 1-168 contains five lethal detachment products, all generated in detachment experiment 1. The deficiencies in this group complement all other complementation groups. It should be noted that the twelve compound stocks used for the detachment experiments were kept in stock for close to a year before being detached. Therefore, it seems likely that a spontaneous recessive lethal arose
in the right arm euchromatin and spread within a sub-population of the experiment 1 compound stock. Fifty percent of the detachment products generated from females bearing the lethal mutation would also carry the lethal. This explanation is consistent with the finding that spontaneous recessive lethals do accumulate on compound chromosomes (Parker, 1954).

A similar explanation is likely for lethal 3-144. The three other chromosomes that do not complement 3-144 are all from experiment 3. Two of these lethals, making up the 3-126 complementation group, also failed to complement a large group of deficiencies which were in the proximal region. The same recombination mapping procedure was carried out for detachment product 3-126, and it was found that this chromosome carried two separable lethals, one in the proximal region and one in the right euchromatin that did not complement 3-144. Therefore, it seems that a spontaneous lethal also arose in the right arm euchromatin of a sub-population of the compound line used in experiment three. Of the four detachment products recovered carrying this lethal, two also carried a proximal deficiency that resulted from the detachment event.

Four of the 44 detachment chromosomes that had been classified as random hits were also subjected to the same recombination mapping analysis. Three of the four lethals mapped outside of the proximal region, but the fourth, designated 10-33, mapped to the proximal region. This chromosome was tested against all detachment products still in stock from complementation groups which may possibly extend further than shown on the complementation map. Three of the ten stocks from the 10-39 complementation group and five of the ten stocks from the 4-134 group failed to complement 10-33. Therefore, 10-33 defines a new complementation group that was previously undetected because the tester chromosomes used to represent the 10-39 and 4-134 complementation groups did not extend out to include 10-33. The 10-39 and 4-134 groups were incorrectly classified as
single complementation groups, since they both contain some deficiencies that include the 10-33 region and some that do not. To determine if any other complementation groups had been overlooked in a similar way, ten other chromosomes classified as random hits were tested against all members of complementation groups that could possibly extend further than shown. However, no additional complementation groups were discovered and the results were consistent with the original classification of the ten lethals as random hits.

Reattachment of Detachment Products

The detachment product deficiencies were assigned to either the left or right arm heterochromatin by the results of attempts to reattach the detachment chromosomes. Females heterozygous for TM3 and a tester chromosome from various complementation groups were irradiated and mated to males carrying compound thirds. For each tester chromosome, 2500 females were treated with 2500 rads and 1250 females were treated with 4000 rads, and then mated. Except for rare non-disjunctional events, the only surviving progeny carried a newly synthesized sister-strand compound-3 chromosome. Since the new sister-strand attachments are homozygous for the appropriate chromosomal arm of the detachment chromosome, only new compound-3L's should be recovered from detachment products carrying a deficiency of the right arm, and vice versa. The results of the reattachment experiments are summarized in Table III. Only new C(3R)'s were generated from tester chromosomes representing the 3-9, 3-30, 1-16, 1-166 and 2-66 complementation groups. Therefore, these complementation groups and others that fail to complement them are presumed to represent deficiencies of the left arm heterochromatin. As expected, the 10-26 and 10-42 chromosomes failed to produce any new sister-strand attachments of the left or right arm, confirming that these detachment chromosomes carry double deficiencies.

The 10-65 chromosome reattachment experiment was repeated because of
TABLE III

Sister-strand compound thirds recovered from reattachment of selected detachment products.

<table>
<thead>
<tr>
<th>Detachment Product</th>
<th>Females Treated</th>
<th>Sister-Strand Compounds Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C(3L)'s</td>
</tr>
<tr>
<td>3-9</td>
<td>3750</td>
<td>0</td>
</tr>
<tr>
<td>3-30</td>
<td>3750</td>
<td>0</td>
</tr>
<tr>
<td>1-16</td>
<td>3750</td>
<td>0</td>
</tr>
<tr>
<td>1-166</td>
<td>3750</td>
<td>0</td>
</tr>
<tr>
<td>2-66</td>
<td>3750</td>
<td>0</td>
</tr>
<tr>
<td>10-26</td>
<td>3750</td>
<td>0</td>
</tr>
<tr>
<td>10-42</td>
<td>3750</td>
<td>0</td>
</tr>
<tr>
<td>10-65</td>
<td>3750</td>
<td>5</td>
</tr>
<tr>
<td>10-65 (repeat)</td>
<td>3750</td>
<td>4</td>
</tr>
<tr>
<td>1-168</td>
<td>3750</td>
<td>21</td>
</tr>
<tr>
<td>ri p&lt;sup&gt;+&lt;/sup&gt; (control)</td>
<td>3750</td>
<td>6</td>
</tr>
</tbody>
</table>
unusual results obtained in the first experiment. Five new sister-strand C(3L)'s and one new C(3R) were recovered. The recovery of compounds from both arms would suggest that the 10-65 chromosome does not carry a lethal on either arm. However, if this were the case, then new C(3L)'s and C(3R)'s would be expected to be recovered in equal numbers. Further, the 10-65 chromosome definitely does carry a recessive lethal. To resolve this anomaly, the 10-65 reattachment experiment was repeated, and this time only new C(3L)'s were recovered. It seems that the 10-65 chromosome does carry a lethal on the right arm; and that the single C(3R) recovered in the first experiment may have been caused by an experimental error, such as mislabelling or contamination of bottles used in the experiment.

The reattachment results with the 1-168 chromosome are also peculiar. As explained in the previous section, this chromosome carries a lethal outside the proximal region somewhere in the right euchromatin. The recovery of only C(3L)'s confirms that the lethal is on the right arm. However, the relatively large number of reattachments recovered is difficult to explain. It appears that this detachment chromosome differs significantly from the other detachment chromosomes tested in its ability to undergo further rearrangements. A similar finding was observed in the original detachment experiment, where one particular compound chromosome exhibited a significantly reduced propensity to be detached. These results suggest that third chromosomes that have previously been involved in attachment/detachment events with heterochromatic breakpoints differ widely in their competency to be involved in further such rearrangements.

A control reattachment experiment was conducted by reattaching the isogenic \( ri p^r \) chromosome while balanced over TM3. Since the \( ri p^r \) chromosome does not carry any recessive lethals, it should produce new sister-strand attachments of both the left and right arms. As predicted, both C(3L)'s and C(3R)'s were recovered in approximately equal numbers.
Further Analysis of Compound and Detachment Chromosomes

A revised complementation map that includes the results of the recombination mapping and reattachment experiments is shown in Figure 4. The revised map suggests the presence of six proximal genes on the left arm and one on the right arm. The right arm gene is designated L(3R)1, and the six genes on the left arm are numbered L(3L)1 to L(3L)6. Although the complementation map is shown with L(3L)1 proximal and L(3L)6 distal on the left arm, the results up to this point cannot distinguish between this possibility and the opposite orientation.

The orientation of the deficiencies on the left arm can be determined by genetically characterizing the progenitor compounds used in the detachment experiments, since deficiencies or duplications carried by the compounds will be polar (i.e. will include the most proximal gene and extend outwards). Individual complementation maps for each of the twelve detachment experiments were produced from the overall map, and then analyzed for clues about the genetic make-up of the progenitor compounds.

If a progenitor compound is carrying a deficiency, over 50% of all detachment products from that compound will carry a deficiency for the same specific region. An analysis of the data shows that over 50% of the detachment chromosomes produced in all four experiments involving C(3R)VM3 carried deficiencies of L(3R)1. Therefore, C(3R)VM3 also must have a deficiency of L(3R)1 on one arm. Similarly, two of three experiments using C(3L)VM3 produced over 50% lethals for a specific region on the left arm, indicating that C(3L)VM3 carries a deficiency of left arm heterochromatin. The third experiment involving this compound that did not produce over 50% lethals for any region must involve a C(3R) that carries a duplication that compensates for the deficiency carried on C(3L)VM3. The centromere-spanning deficiencies produced only in experiment 10, where C(3R)VM3 is paired with C(3L)VM3, are diagnostic of deficiency-carrying
Revised complementation map incorporating results of recombination mapping and reattachment experiments. The number above each bar designates the detachment product used to represent each complementation group; and the number below the bar indicates the number of detachment products assigned to each group. The number of detachment products in the 4-134, 1-16, 10-39, and 9-52 complementation groups is shown in brackets because they are estimates. Estimates were necessary because only some of the detachment products in these groups were available for complementation tests with the previously undetected 10-33 group.
compounds and confirms that at least one of these two compounds does carry a deficiency. None of the other progenitor compounds behaved as if they were carrying a deficiency.

Any detachment experiment that does not involve the two deficiency-carrying compounds and which results in non-polar detachment deficiencies must involve a progenitor compound carrying a duplication. However, it is not possible to identify non-polar deficiencies without first knowing the proximal-distal orientation of the deficiencies. To solve both unknowns simultaneously, a set of possible models of the progenitor compound chromosomes was constructed and analyzed to determine if it could account for all the detachment product classes generated in the twelve experiments. The only possible configuration that could explain all 443 detachment products had the orientation of the left arm shown in Figure 4 and the progenitor compounds described in Table IV. Using this set of progenitor compounds and orientation of the left arm, possible breakpoints for every detachment product were identified. No other models could come close to explaining the origin of all the detachment products.

Two ambiguities remained about the compound chromosome configurations described in Table IV. First, the duplication carried by C(3R)VH3 could extend to L(3L)5 or L(3L)6. Both possibilities are consistent with the data, and cannot be distinguished in any other way. The second ambiguity is that it is not known for certain whether any of the four C(3L) chromosomes carry duplications. Since there is only one complementation group on the right arm, it is not possible to identify non-polar deficiencies that would be diagnostic for duplications carried on C(3L)’s. This ambiguity was resolved by a reattachment experiment which tested the capability of progenitor compounds to rescue homozygous deficient sister-strand attachments. As described earlier, no C(3R) sister-strand attachments will be recovered from a detachment product carrying a deficiency on
**TABLE IV**

Duplications and deficiencies carried by progenitor compound-thirds.

<table>
<thead>
<tr>
<th>Progenitor Compound</th>
<th>Duplication</th>
<th>Deficiency</th>
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</thead>
<tbody>
<tr>
<td>C(3L)VM1</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>C(3L)VM2</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>C(3L)VM3</td>
<td>none</td>
<td>L(3L)1-5</td>
</tr>
<tr>
<td>C(3L)VM4</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>C(3R)VM1</td>
<td>L(3L)1-3</td>
<td>none</td>
</tr>
<tr>
<td>C(3R)VM2</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>C(3R)VM3</td>
<td>L(3L)1-6</td>
<td>L(3R)1</td>
</tr>
</tbody>
</table>
the right arm, and vice versa. However, when females carrying a detachment product with a deficiency on the right arm balanced over TM3 are irradiated and mated to males carrying a C(3L) compound with a duplication of the right arm, the duplication should be able to rescue homozygous deficient sister-strand C(3R)'s.

Each of the seven progenitor compounds used in the detachment experiments was tested for the presence of duplications by this method. The results of the reattachment experiments are shown in Table V. Each of the four C(3L) chromosomes was tested by mating 8000 irradiated females carrying the 10-65 right arm deficiency balanced over TM3 to males carrying the C(3L) chromosome being tested, paired with C(3R)SH19,+. The progeny was screened for the presence of newly synthesized compound-3R's. Since no sister-strand C(3R)'s were recovered, the four progenitor C(3L) chromosomes must not carry duplications of the region defined by the 10-65 deficiency.

The three C(3R) chromosomes used in the detachment experiments were tested in a similar manner. Females carrying the 3-9 left arm deficiency were treated with 4000 rads and mated to males carrying a progenitor C(3R) chromosome paired with C(3L)VH3, st. Newly synthesized sister-strand C(3L)'s were rescued by C(3R)VH1 and C(3R)VH3, but not C(3R)VH2. These results confirm that C(3R)VH1 and C(3R)VH3 are the only progenitor compounds carrying duplications.

Distribution of Putative Heterochromatic Loci

The breakpoints involved in the formation of the complete set of detachment products were not evenly distributed along the complementation map. Some regions of the map had a high frequency of breakpoints whereas in other regions breakpoints were rare. If the distance between two genes is assumed to be proportional to the number of breakpoints that occur in that region, it is possible to construct a distribution map of the genes in the third chromosome heterochromatin (Figure 5).
<table>
<thead>
<tr>
<th>Progenitor Compound</th>
<th>Females Treated</th>
<th>Predicted Duplications</th>
<th>Homozygous Deficient Compound-3's Rescued</th>
</tr>
</thead>
<tbody>
<tr>
<td>C(3L)VM1</td>
<td>8000</td>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td>C(3L)VM2</td>
<td>8000</td>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td>C(3L)VM3</td>
<td>8000</td>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td>C(3L)VM4</td>
<td>8000</td>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td>C(3R)VM1</td>
<td>8000</td>
<td>L(3L)1-3</td>
<td>-</td>
</tr>
<tr>
<td>C(3R)VM2</td>
<td>8000</td>
<td>none</td>
<td>-</td>
</tr>
<tr>
<td>C(3R)VM3</td>
<td>8000</td>
<td>L(3L)1-6</td>
<td>-</td>
</tr>
</tbody>
</table>
FIGURE 5

Distribution of genes in heterochromatin of chromosome three. Relative map distances are based on the number of radiation breakpoints induced in each region.
One difficulty in producing such a map was that in experiments involving a progenitor compound carrying a duplication or deficiency, there were often two or more possible sets of breakpoints for generating the same detachment product. Therefore, when calculating the map distances for either the left or right arm, experiments that involved compounds carrying a deficiency or duplication for the arm being examined were excluded from the analysis. As a result, the only experiments used in the calculations were those where the breakpoints could be assigned to a specific region with complete certainty.

For the right arm, four experiments involved compounds with a duplication or deficiency of the right arm and thus had to be excluded from the analysis. In the remaining eight experiments, only 22 out of a total of 649 lethal and non-lethal detachment products were deficient for the gene(s) defined by the 10-65 deficiency. If a breakpoint occurs on the right arm proximal to the 10-65 region, none of the resulting detachment products will carry a deficiency of the 10-65 region. If, on the other hand, the breakpoint occurs distal to the 10-65 region, then one-half of all detachment products will be deficient for this region. One of the two reciprocal detachment products that will result from such breakpoints will carry a duplication of the 10-65 region, and the reciprocal detachment product will carry a deficiency. Therefore, the actual frequency of breakpoints that occur distal to a particular gene is twice the frequency of deficiencies recovered for that gene. If it is assumed that detachment product breakpoints occur only in heterochromatin and are randomly distributed within the heterochromatic block, then the distance of the 10-65 gene relative to the centromere from the euchromatic-heterochromatic junction is calculated as follows:

\[
\frac{2 \times (\# \text{ of detachment products deficient for } 10-65)}{\text{Total } \# \text{ of detachment products}} \times 100\% = \frac{2 \times 22}{649} \times 100\% = 7\%
\]
Therefore, on the basis of breakpoint frequencies, the 10-65 gene is located at a position 7% of the distance from the euchromatic-heterochromatic junction to the centromere.

Similar calculations were made for each gene on the left arm. Only data from experiments 2, 5, and 9 were used, since these are the only experiments not involving compounds carrying duplications or deficiencies of the left arm. The relative positions of genes L(3L)1, L(3L)2, L(3L)3, L(3L)4, and L(3L)5 were 91%, 87%, 85%, 24% and 23% respectively of the distance from the left euchromatic-heterochromatic junction to the centromere. The map position of L(3L)6 was not calculated because of the extremely low number of detachment products tested for a deficiency of this gene. However, a very rough estimate would place it halfway between L(3L)5 and the left euchromatic-heterochromatic junction.

The map distances show in Figure 5 are proportional to the number of radiation breakpoints induced in each region. The map distances are proportional to the actual physical distances between genes if and only if radiation breakpoints are randomly distributed within the heterochromatic block.

Lethal Phases

The lethal phases of several of the detachment product deficiencies were determined using egg count experiments. Females carrying a deficiency over TH3 were mated to sibling males and then allowed to lay eggs on petri dishes containing fresh medium for three successive two-hour periods. The final petri dish in each series was examined and counted daily. All the deficiencies tested had larval lethal phases, either during the second or during the third instar stage.

EMS Induced Alleles

EMS-induced alleles of third chromosome proximal deficiencies were isolated by testing EMS-treated ri p chromosomes against several different deficiency-carrying detachment products. 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. The number of chromosomes treated and EMS-induced lethal alleles recovered is summarized in Table VI. The newly recovered EMS lethals were positioned relative to the accumulated deficiency complementation map by tests against deficiencies for each region of the map. The EMS-lethals were then complementation tested against all other EMS lethals and all deficiencies from the same region. Each EMS induced lethal is designated by the deficiency it was recovered against, followed by the numerical order in which it was recovered (e.g. 10-65-2 is the second lethal recovered against the 10-65 chromosome).

Analysis of EMS-Induced Lethals on the Right Arm

Two EMS-induced lethal alleles of L(3R)1 were recovered from 6301 ri p* chromosomes tested against 10-65; and 13 lethals of L(3R)1 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 and all ten deficiencies of the 10-65 region retained for testing. The lethals recovered using 4-75 resulted in a more complex pattern that revealed a second, and possibly a third, gene on the right arm, as shown in Figure 6. Two of the lethals recovered using 4-75, designated 4-75-12 and 4-75-17, failed to complement the two EMS lethals of L(3R)1 recovered using 10-65. Ten of the remaining eleven complemented all EMS alleles of L(3R)1, as well as eight of the ten deficiencies assigned to the 10-65 class. These ten lethal alleles of 4-75 did not complement deficiency 5-53 as well as 4-75 itself. This complementation pattern clearly defines a second gene in the right arm heterochromatin, designated L(3R)2. When tested against each other, the EMS lethals of L(3R)2 result in a complementation pattern with two groups. Five lethals fail to complement both groups. The two complementation groups probably represent inter-allelic complementation between lethal alleles of a single gene; but could represent complementation between
TABLE VI

Recovery of EMS-induced lethal alleles of deficiencies carried by third chromosome detachment products.

<table>
<thead>
<tr>
<th>Detachment Product Screened Against</th>
<th>Deficiency</th>
<th># Mutagenized Chromosomes Screened</th>
<th>EMS Lethals Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-166</td>
<td>L(3L)1-4</td>
<td>7368</td>
<td>23</td>
</tr>
<tr>
<td>1-16</td>
<td>L(3L)4-6</td>
<td>2170</td>
<td>37</td>
</tr>
<tr>
<td>10-65</td>
<td>L(3R)1</td>
<td>6301</td>
<td>2</td>
</tr>
<tr>
<td>4-75</td>
<td>L(3R)1</td>
<td>3342</td>
<td>13</td>
</tr>
</tbody>
</table>
FIGURE 6

Complementation pattern of deficiencies and EMS-induced lethals of proximal right arm. Deficiencies are indicated by solid bars and EMS-induced lethals are designated by thin lines. See text for further details.
alleles of two different genes. If the latter was the case, the five lethals that fail to complement both groups would be deficiencies. The 4-75-8 EMS-induced lethal definitely is a deficiency, as it fails to complement all other deficiencies and EMS-induced lethals of the right arm.

These results demonstrate the presence of at least one additional gene distal to L(3R)1 in the proximal region of the right arm of chromosome three.

Analysis of EMS-Induced Lethals on Left Arm

Lethal EMS-induced alleles of L(3L)1, L(3L)2 and L(3L)3 were recovered using the l-166 chromosome. These lethals produced relatively simple complementation patterns (Figure 7). All nine EMS-induced lethals of L(3L)1 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 with all deficiencies of the 3-9 class. Three lethal alleles of L(3L)2 were recovered, all of which failed to complement each other and all deficiencies uncovering L(3L)2. Two alleles of L(3L)3 were recovered, and again failed to complement each other and all deficiencies of L(3L)3. Two additional EMS-induced lethals behaved as deficiencies. Lethal l-166-12 failed to complement all deficiencies and EMS-induced lethals of both L(3L)2 and L(3L)3. Similarly, l-166-46 did not complement deficiencies and all EMS-induced lethal alleles of the three tightly linked genes L(3L)1, L(3L)2 and L(3L)3. The analysis of EMS-induced lethals failed to discover any additional genes in this region.

The complementation analysis of EMS-induced lethal alleles of L(3L)4 produced the most complex complementation pattern of this study. Since the l-166 and l-16 deficiencies overlap at this position, lethal alleles of L(3L)4 where recovered using both deficiencies. A total of 30 EMS-lethals of L(3L)4 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 8
FIGURE 7

Complementation pattern of deficiencies and EMS-induced lethals of genes L(3L)1, L(3L)2, and L(3L)3. Deficiencies are indicated by solid bars and EMS-induced lethals are designated by thin lines. See text for further details.
FIGURE 8

Complementation pattern of deficiencies and EMS-induced lethals of 8A-80 deficiency. Deficiencies are indicated by solid bars and EMS-induced lethals are designated by thin lines. All ten class A lethals fail to complement each other. The twenty class B lethals form a complex complementation pattern with considerable apparent inter-allelic complementation. See text for further details.
reveals that the 8A-80 deficiency, previously thought to define L(3L)4, actually uncovers two separate genes. All 1-16 class deficiencies uncover both genes; but two out of four 1-166 class deficiencies, including 1-166 itself, only uncover the most proximal of the two genes, now designated L(3L)4A. The other two 1-166 class deficiencies, designated 6-21 and 9-37, uncovered both genes. The group A lethals in Figure 8 consist of ten EMS-induced lethal alleles of L(3L)4A, eight of which were recovered against 1-166 and two against 1-16. All ten lethals failed to complement each other. The twenty EMS-induced lethals of group B in Figure 8 were all 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 VII. All of the lethals fail to complement most other lethals in the group, but only 1-16-17 and 1-16-22 don’t complement at least one other lethal. The complex complementation pattern suggests inter-allelic complementation of alleles of a single gene, designated L(3L)4B.

Moving distally along the left arm, five EMS-induced lethal alleles of L(3L)5 were isolated using deficiency 1-16. Four of these lethals failed to complement each other in all combinations. The fifth lethal complemented all of the other four. All five lethals did not complement any of the deficiencies which included the L(3L)5 region. This complementation pattern may represent either inter-allelic complementation or alternatively complementation between mutations of two separate genes. Two EMS-induced lethal alleles of L(3L)6 were also recovered using the 1-16 deficiency. These lethals failed to complement each other and all deficiencies of the L(3L)6 region.

Eight additional EMS-induced lethals of the 1-16 deficiency were recovered which complemented deficiencies for all the known complementation regions on the left arm. Further testing against deficiencies of the 1-16 and 9-52 classes revealed that these lethals defined two new genes, designated L(3L)7 and L(3L)8.
TABLE VII

Results of complementation analysis of twenty EMS-induced lethal alleles of L(3L)4B. Complementing crosses are indicated by a "+"; and non-complementating crosses are indicated by a "-".

<table>
<thead>
<tr>
<th>1-16-4</th>
<th>1-16-7</th>
<th>1-16-8</th>
<th>1-16-11</th>
<th>1-16-12</th>
<th>1-16-17</th>
<th>1-16-20</th>
<th>1-16-23</th>
<th>1-16-24</th>
<th>1-16-25</th>
<th>1-16-28</th>
<th>1-16-31</th>
<th>1-16-32</th>
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<th>1-16-43</th>
<th>1-16-44</th>
<th>1-16-45</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
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FIGURE 9

Complementation pattern of deficiencies and EMS-induced lethals of L(3L)5, L(3L)6, L(3L)7, and L(3L)8 regions. Deficiencies are indicated by solid bars and EMS-induced lethals are designated by thin lines. See text for further details.
that were distal to L(3L)6. The complementation results for the L(3L)5 to L(3L)8 regions are shown in Figure 9. Four of the deficiencies from the 1-16 and 9-52 classes uncover both L(3L)7 and L(3L)8, while three others uncover L(3L)7 but not L(3L)8. Five EMS-induced lethal alleles of L(3L)7 were recovered. These lethals form an overlapping complementation pattern that suggests inter-allelic complementation. The three lethals of L(3L)8 that were recovered all failed to complement each other.

To summarize these results, a total of at least nine putative genes were identified in the heterochromatin of the left arm, and at least two putative genes were detected in the right arm heterochromatin.

All EMS-treated chromosomes were originally recovered on the basis of their lethality with a detachment product deficiency at 29°. After re-testing at 22°, two of the lethals were classified as temperature-sensitive mutations. The first, designated 1-166-5, is a temperature-sensitive allele of L(3L)4A, and is included in the complementation map shown in Figure 8. This mutation is homozygous lethal at both the restrictive and permissive temperatures, but is lethal over all deficiencies and EMS-induced lethal alleles of L(3L)4A only at 29°. The temperature-sensitive period of the 1-166-5 mutation was determined using shift-up and shift-down experiments. Progeny carrying the 1-166-5 chromosome over a deficiency of L(3L)4A had a very long temperature-sensitive period that began at the start of the second larval instar stage and extended until late pupation.

The other temperature-sensitive mutation isolated is designated 1-166-4. This mutation is homozygous non-lethal at both 22° and 29°, and is lethal over all deficiencies of L(3L)2 at 29° but not 22°. However, 1-166-4 is only semi-lethal over the EMS-induced alleles of L(3L)2 at the restricted temperature. Shift-up and shift-down experiments determined that this mutation has a
sharply defined temperature-sensitive period early in the pupal stage. Because of the semi-lethality of 1-166-4 with other L(3L)2 EMS-induced lethals, it is not clear if this mutation is an allele of L(3L)2 or a mutation elsewhere on the chromosome which interacts with lethals of C(3L)2.

As explained in the Materials and Methods section, EMS-induced lethal alleles of TH3 were also recovered from the EMS screens and used to test for the presence of proximal lethals on the TH3 chromosome. A total of 24 lethal alleles of TH3 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 lethals, two groups of three, one group of two, and three groups with one lethal each. One lethal from each group with two or more members was chosen and recombination mapped by the same procedure used earlier for the detachment product deficiencies. All the lethal TH3 alleles mapped outside of the proximal region. These results indicate that the TH3 chromosome is likely not carrying a hidden proximal lethal.

Cytological Examination

Squashes of polytene and mitotic chromosomes carrying detachment product deficiencies did not reveal any obvious duplications or deficiencies of third chromosome proximal regions. However, Larry Sandler and Sergio Pimpinelli (personal communication) examined three of the detachment product deficiencies using the sophisticated fluorescence and N-banding techniques developed by Gatti and Pimpinelli (1983). The results of their analysis are shown schematically in Figure 10. The top chromosome shows a standard Oregon-R wild-type third chromosome. The right arm heterochromatin carries a large, interstitial non-fluorescing block that appears as a dark-staining band in N-banding preparations. The left arm heterochromatin is brightly fluorescing except for a narrow dark band indicated by a notch in the diagram.
Chromosome banding analysis of three detachment product deficiencies. Schematic representations of results of cytological examination by Sandler and Pimpinelli of Oregon-R chromosome and detachment products 2-85, 9-2, and 7-53 by fluorescence and N-banding techniques. Heterochromatic regions are represented by blocks, and the centromere by an open circle. Brightly fluorescing segments are represented by unshaded blocks; and fluorescence-dull, N-banded regions are indicated by solid blocks. The notch on the left arm represents a narrow fluorescence-dull gap. See text for further details.
3L  3R

- Bright fluorescence
- No fluorescence

Oregon-R

D.P. 2-85 (Def. L(3L)1-7)

D.P. 9-2 (Def. L(3L)1-5)

D.P. 7-53 (Def. L(3R)1)
Two detachment product deficiencies of the left arm of chromosome three were examined by Sandler and Pimpinelli. Detachment product 2-85 was deficient for genes L(3L)1-7, and detachment product 9-2 was deficient for genes L(3L)1-5. Both the 9-2 and 2-85 chromosomes were generated from progenitor compounds known to be carrying no 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 the right compound with an acentric fragment from the left compound. 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 heterochromatin block, starting with the left euchromatic-heterochromatic junction, and extending inwards 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 heterochromatin block on the left arm. At most, only the small segment of heterochromatin distal to the fluorescence-dull block on the left arm of 9-2 could possibly be normal left arm heterochromatin. Since 9-2 is only deficient for genes L(3L)1-5, the genes L(3L)6-8 must remain in the 9-2 chromosome heterochromatin. Therefore, genes L(3L)6-8 must be located within the small segment of heterochromatin that accounts for about the distal one-eighth of the wild type left-arm heterochromatic block.

Two observations are of significance for explaining the cytological analysis of 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 2-85. Combining these two observations, almost half of the heterochromatin on the left arm of 2-85 cannot be accounted for by either distal left heterochromatin carried by the acentric detachment fragment, or right-arm heterochromatin. Therefore, this unaccounted heterochromatin must represent a duplication of non-vital 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 additional evidence about the distribution of genes within third chromosome heterochromatin.

The other chromosome examined in the cytological analysis is detachment product 7-53, which carries a deficiency of the right arm heterochromatin. This chromosome was generated from a set of progenitor compounds carrying no duplications or deficiencies on the left compound and a large duplication of left heterochromatin that includes L(3L)1-6 on the right compound. From an analysis of the possible breakpoints that could result in the formation of 7-53, it is very likely that this chromosome carries a large duplication of wild-type left heterochromatin on its right arm. The cytological analysis confirms this prediction, showing that at least the proximal two-thirds (and perhaps much more) of the heterochromatin on the right arm of 7-53 is actually a duplication of left arm heterochromatin. This finding indicates that gene L(3R)2, which is not deleted in 7-53, must be present in the small amount of remaining distal right-arm heterochromatin.

Summarizing the results of the cytological analysis, all the detachment product deficiencies examined were 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 were partially or
completely compensated for by duplications of heterochromatin 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

In this study, a series of attached, detached, and re-attached third chromosomes were generated and used to dissect the heterochromatin of chromosome three of *Drosophila melanogaster*. The analysis of approximately 400 proximal deficiencies carried by detachment products, as well as 75 EMS-induced lethal alleles of the deficiencies, revealed the presence of at least eleven vital genes in the proximal region of chromosome three. Attempts to re-attach chromosomes carrying deficiencies of these genes established that nine of the genes were on the left arm and two on the right arm.

This analysis reinforces the usefulness of the compound autosome attachment and detachment procedure for analyzing the proximal regions of *Drosophila* autosomes. Using this basic technique, it was possible to generate a detailed genetic map of what was until now the largest uncharted region of the *D. melanogaster* genome. Several new modifications of the compound attachment and detachment procedure were used in the course of this study. First, reattachment of deficiency-carrying detachment products was used to determine which arm of the chromosome the deficiencies were on. Secondly, the ability of compound autosomes to rescue newly synthesized, homozygous deficient sister-strand attachments was used to test for the presence of duplications on the compound chromosomes. Finally, the use of many different combinations of compound chromosomes in detachment experiments permitted the genetic characterization of the progenitor compounds and therefore a detailed and useful breakpoint analysis of all the resulting detachment products.

As was the case with the second chromosome (Hilliker and Holm, 1975; Hilliker, 1976), the genes uncovered in the proximal region of chromosome three appeared similar to single-copy genes found in the euchromatin. Inter-allelic
complementation between EMS-induced mutants was observed in the larger complementation groups. No evidence of repetitive genes resulted from the testing of non-lethal detachment products over lethal detachment product deficiencies. All of the mutants were recessive lethals, with the only observed phenotype being the rotund-like phenotype displayed by rare surviving adults homozygous for deficiencies of the 3-9 complementation class.

However, unlike the Hilliker (1976) study, some of the EMS-induced lethals recovered in this study were clearly deletions, since they uncovered two or more genes separable by detachment product deficiencies. The EMS-induced deficiencies are probably quite small, as the genes they uncover appear to be tightly linked. Different findings have been reported about the ability of EMS to induce deletions. While Lim and Synder (1974) and Hilliker (1976) recovered EMS mutations affecting only one cistron, others have recovered EMS-induced deletions (Williamson, 1970; Bishop and Lee, 1973; Olson and Lim, 1976). In two of the studies where EMS-induced deletions were recovered, 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). It is not clear why EMS-induced deletions are found in some studies but not others.

There is convincing evidence that the genes uncovered by detachment product deficiencies, including those discovered in this study, are within heterochromatin. Gibson (1977) tested a large number of newly formed compound-2's for duplications of the complementary arm. Over half of the new C(2L)'s carried a duplication of the right heterochromatic marker r1, but never carried a duplication of the proximal right euchromatic gene stw. Further, detachment product deficiencies analyzed by Hilliker and Holm (1975) were genetically proximal to previously isolated deletions that extended from the euchromatin into the heterochromatin. Similarly, the deficiencies of the third chromosome recovered by Baldwin and Suzuki (1971) complemented all known proximal euchromatic markers on
the third chromosome. Finally, careful cytological examination of both polytene and mitotic chromosomes carrying detachment product deficiencies failed to detect any removal of euchromatin (Hilliker and Holm, 1975).

The results of the present study are also consistent with a heterochromatic location for vital genes uncovered by detachment product deficiencies. A cytological examination of three detachment products demonstrated that putative deficiencies genetically mapped to either the left or right arm correspond to deletions of most of the heterochromatic block of the appropriate arm. These findings verify that functioning genes do reside within the third chromosome heterochromatin of *D. melanogaster*.

The analysis of the three detachment product deficiencies examined both genetically and cytologically indicate that at least some of the genes in chromosome three heterochromatin are present in the most distal segments of centromeric heterochromatin on both arms. Another possible source of information about the distribution of genes in the third chromosome heterochromatin is the radiation map produced from the quantitative analysis of detachment product breakpoints (Figure 5). The results of the radiation breakpoint and cytological analyses are in agreement with positions for L(3R)2 in the distal right heterochromatin and L(3L)6-8 in the distal left heterochromatin. However, the cytological results do not indicate whether or not the proximal position of genes L(3L)1-3 shown in Figure 5 is accurate. The radiation map distances shown in Figure 5 are proportional to the actual physical distances between the genes if and only if it is assumed that radiation breakpoints are evenly distributed within the heterochromatic block.

There is some limited experimental evidence that radiation induced breakpoints are not evenly distributed within heterochromatin. Visual examinations of a series of radiation-induced rearrangements with heterochromatic breakpoints
detected a preponderance of breakpoints at or near the euchromatic-heterochromatic junction (Gatti, Tanzarella and Olivieri, 1974; Schubert and Rieger, 1976). Kennison (1981) also observed a striking non-random distribution of translocation breakpoints on the heterochromatic Y chromosome, with most if not all breakpoints within non-fluorescent segments, or near junctions between differentially staining regions.

If radiation breakpoints in heterochromatin are clustered near the euchromatic-heterochromatic junction, then the radiation breakpoint map in Figure 5 is skewed towards the centromere, and the real positions of the genes are more distal than shown. A more extensive cytological analysis is required to determine whether the map in Figure 5 is skewed, and if so, to what extent.

Two important conclusions about the distribution of genes in third chromosome heterochromatin have emerged from this study. First, the right arm heterochromatin of the third chromosome contains significantly fewer genes than does the left arm heterochromatin. Second, at least some of the genes present in the heterochromatin of chromosome-3 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 the heterochromatin ends and the euchromatin begins. This model is based on the discovery of alternating radiation "hot spots" and "cold spots" in the E-H junction region.

There is evidence that the E-H junction region is genetically active. The heterochromatic chromocentre of Drosophila polytene chromosomes consists of a dense inner core which does not incorporate $^3$H-uridine, surrounded by more diffuse regions near the E-H junctions of each arm called beta heterochromatin which do incorporate $^3$H-uridine (Lakhotia and Jacob, 1974). Most mRNA that hybridizes to the chromocentre bind to the beta heterochromatin (Spradling, Penman and Pardue, 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 three of D. melanogaster (Biessman et al., 1981). All this evidence suggests that there may be segments of "inter-
calary euchromatin" interspersed in the distal regions of blocks of heterochro-
matin. The genes known to reside in the distal region of chromosome-3 hetero-
chromatin may in fact be present in a segment of interspersed euchromatin in the
transitioning region between heterochromatin and euchromatin. An extensive
cytological examination of a large series of genetically defined proximal
deficiencies is necessary to further define the distribution of genes in third
chromosome heterochromatin.

The findings of this study confirm that the heterochromatin of D. melano-
gaster is genetically heterogeneous. Each major heterochromatic block in D.
melanogaster seems to have a novel gene content and distribution. Unique
sequence vital genes are found only in autosomal heterochromatin; and not the
heterochromatin of the sex chromosomes (reviewed by Hilliker, Appels and
Schalet, 1980). As well, significantly fewer genes are present in the right arm
heterochromatin of chromosome three than in the heterochromatin of the left arm
of chromosome three or either arms of chromosome two. Even within a specific
heterochromatin block, such as the heterochromatin of the left arm of chromosome
three, vital genes are not evenly distributed.

The factors responsible for the restricted locations of vital genes in
Drosophila heterochromatin are unknown. 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.

The 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, the 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 in the right-arm heterochromatin of chromosome-2 (Gatti and Pimpinelli, 1983). Although the molecular nature of N-banded regions is unknown, it is possible that N-bands correspond to blocks of a specific satellite sequence. 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 heterochromatic genes correspond to specific banding regions and/or specific satellite sequences. Although the molecular basis and significance of the genetic heterogeneity of Drosophila heterochromatin is uncertain, it is clear that genes are restricted to only some segments of the heterochromatin, and each chromosome in the Drosophila genome is unique with respect to gene content.

One limitation of the present analysis of chromosome three heterochromatin is that it would fail to detect any vital genes that are present in the heterochromatin of more than one chromosome in the D. melanogaster genome. It is known that a number of transcribed middle-repetitive DNA sequences hybridize to the chromocentre of D. melanogaster polytene chromosomes, especially the more distal beta heterochromatin (Spradling, Penman and Pardue, 1975; Carlson and Brutlag, 1978a; Spradling and Rubin, 1981). None of these middle-repetitive sequences are confined only to the autosomes; and as a rule they are found on both the Y chromosome and one or more autosomes (Lifschytz and Hareven, 1982). Therefore,
it is possible that there are vital genes in the third chromosome heterochromatin other than those found in this study. Such genes, if present, would be repetitive and dispersed to the heterochromatic block of one, or more, of the other chromosomes in the D. melanogaster genome.

Two other findings of this study are of interest but of unknown significance. The first is that attached or detached chromosomes can differ widely in their propensity to undergo further rearrangements. In the compound detachment experiments, all experiments involving C(3R)VM2 consistently generated significantly fewer detachment products than experiments involving the other compound autosomes. Similarly, the 3-126 detachment chromosome was several times more likely to undergo reattachment than the other detachment products tested.

The second observation of possible significance is that detachment products carrying a large deficiency also tended to carry a large duplication. All three detachment products analyzed by Sandler and Pimpinelli had relatively large deficiencies accompanied by large duplications. As a result, there was an approximately normal-sized heterochromatic block on both sides of the centromere of each detachment product. Hilliker and Holm (1975) observed a similar phenomenon with second chromosome heterochromatic deletions. Although their detachment products carried genetically large deficiencies, the mitotic chromosomes appeared normal, suggesting that the deletions were compensated by accompanying duplications. It is known that chromosomes with little or no heterochromatin on one arm can survive. The MS2-10 chromosome, which is not a detachment product, carries a cytologically visible deficiency of most if not all of the right arm heterochromatin (Hilliker and Holm, 1975). Therefore, there may be something intrinsic about the detachment process which generates offsetting duplications and deficiencies.

This study completes the genetic dissection of the major heterochromatic
blocks in Drosophila melanogaster, the only organism whose genome has been systematically analyzed for the presence of heterochromatic loci. However, there are indications that other organisms may also have genetically active heterochromatin. Khush, Rick and Robinson (1964) mapped a gene to the heterochromatin of the tomato genome, although it is possible that the gene is located at a small euchromatic gap in the heterochromatic block. The constitutive heterochromatin on the X chromosome of Microtus agrestis is very sensitive to DNase I, is not highly condensed at interphase, and appears to have newly synthesized RNA attached to it (Sperling et al., 1985). In the snake Elaphe radiak, segments of satellite-rich heterochromatin decondense at specific stages in development and are associated with transcription, as evidenced by $^3$H-uridine incorporation (Singh, Purdom and Jones, 1979).

The discovery of genes in at least some 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 heterochromatic genes are expressed early in development, before heterochromatic regions condense. The heterochromatin 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 Yasmineh, 1971).

If heterochromatic genes are expressed early in development, then mutants of these genes might be expected to have early lethal phases and temperature sensitive periods. The third chromosome heterochromatic deficiencies tested in this study had lethal phases during the second or third larval instar. Hilliker (1976) found that the EMS-induced lethal alleles of heterochromatic loci on the second chromosome had lethal phases in the late larval or pupal stages. While these results do not indicate that heterochromatic genes are expressed early in
development, they do not exclude such a possibility. Lethal phases do not represent the stage at which a gene is expressed, but rather a critical period in development during which the organism is unable to pass successfully as a result of a mutant gene expressed earlier in development (Wright, 1970).

More precise 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 only definite TS mutant of a heterochromatic gene recovered in this study (1-166-5) 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 do code for proteins. The second implication is that a TS mutation must be transcribed prior to the TSP if a heat-labile protein is responsible for the temperature sensitivity. Therefore, the results from the 1-166-5 TS mutant indicate that at least one gene in third chromosome heterochromatin must be transcribed before the beginning of the second larval instar stage. The recovery and analysis of more TS mutations of heterochromatic loci may help to answer the important question of when genes in heterochromatin are expressed.

There is evidence from a variety of organisms which suggests that genes present 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 many species of nematodes and copepods, satellite DNA-rich heterochromatic segments are specifically removed from
somatic cell chromosomes during the early cleavage stages (Moritz and Roth, 1976; Beerman, 1977; Streeck, Moritz and Beer, 1982). The germ line cells of all these organisms are diploid and retain a full complement of heterochromatin. In the insect Calliphora erythrocephala, satellite DNA is drastically under-replicated in polytene tissues of salivary glands and malphigian tubules, but is coordinately replicated with the non-satellite sequences during polytenization of nurse cell nuclei in the germ line (Dover, 1980). Cooper (1977) irradiated and observed cultured Microtus agrestis bone marrow cells, and found many stable rearrangements and deletions of the heterochromatin. However, no such deletions are observed in natural populations, suggesting that heterochromatic regions are essential for the germ line, but are not required in at least some somatic cells.

The condensation or under-replication of heterochromatin may be a mechanism for regulating the expression of genes in heterochromatin. 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 a heterochromatic location is an out-of-phase gene activity.

A second example of how heterochromatin may regulate the timing of gene activity comes from the snake Elaphe radiak (Singh, Purdom and Jones, 1979). In the growing oocytes of this species, the W chromosome satellite-rich heterochromatin decondenses completely and is associated with a period of $^3$H-uridine
incorporation, while the autosomal satellite-rich heterochromatin regions remain condensed. Later, the cycle is reversed and the W chromosome heterochromatin condenses while the autosomal heterochromatin decondenses. These examples suggest that one consequence of genes located in heterochromatin is that their expression may be restricted to certain periods in the development of an organism.

What are some of the other implications of the presence of functioning genes in heterochromatin? It is unlikely that the small number of genes in heterochromatin have a significant influence on the nature and properties of heterochromatin. In fact, 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 (Wollenzien, Barsanti 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 have been shown to specifically bind satellite DNA (Hsieh and Brutlag, 1979; Levinger and Varshavsky, 1982; Strauss and Varshavsky, 1984) may be responsible for the compact nature of heterochromatin.

Although heterochromatic genes are unlikely to have a significant effect on the nature and properties of heterochromatin, they may have a profound consequences for the function of heterochromatin. As discussed earlier, a number of functions have been proposed for heterochromatin, but none have been proven. 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 heterochromatin that several authors have now proposed that the primary function of hetero-
ochromatin is to provide organisms with a means to respond rapidly to environ­
mental 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 unknown effects. According to this hypothesis
heterochromatin provides a species with a dimension of flexibility unavailable
to organisms with only unique sequence DNA.

At least one mechanism that seems to be involved in generating variations
in heterochromatic content is unequal sister chromatid exchange (John and
Miklos, 1979; Kurnit, 1979; Miklos and Gill, 1981). Whatever the mechanism, the
rapid expansion or contraction of heterochromatin could result in the deletion
or duplication of genes present in heterochromatin (Miklos and Gill, 1981).
Therefore, the presence of functioning genes in heterochromatin may tend to
restrict the variability of heterochromatin. If, as suggested, the key property
of heterochromatin is its fluidity, then the presence of genes could interfere
with the proper functioning of heterochromatin. On the other hand, a species
which is well adapted to its environment may find it beneficial to have its
heterochromatin stabilized by the presences of heterochromatic genes. The genes
may function as anchors, impeding any fluctuations in heterochromatin.

The amount of intraspecific 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 poly­
morphic on some chromosomes but not others (Craig-Holmes et al., 1975). Maize
heterochromatin is highly conserved with no observed polymorphisms (Rhoades,
1978); while rye heterochromatin has extensive variations (Jones and Flavell,
1982). In Drosophila 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 Drosophila 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). These results re-affirm the heterogeneity of heterochromatin, as some blocks are more conserved and less fluid than other heterochromatic regions. The amount of intraspecific heterochromatin variation may be a good indicator of the presence or absence of heterochromatic genes.

Interspecific variations in heterochromatin between closely related species may help to reveal the significance of genes in heterochromatin. If it is important that genes are located in heterochromatin, then the heterochromatic position of the gene should be conserved between closely related species. On the other hand, if the gene's position is unimportant, it may not be conserved. A useful experiment would be to undertake a compound detachment study with the second or third autosome of D. simulans. D. simulans and D. melanogaster are sibling species, and their polytene and mitotic chromosome are almost identical. The blocks of heterochromatin in the two species are very similar in size and position, but there are drastic differences in the relative amounts of different satellite sequences present in the heterochromatin of the two species (Peacock et al., 1977b; Peacock and Lohe, 1980). The arrangement of sequence order within the heterochromatic blocks of the two species has been conserved, and so like the euchromatin, there have not been any significant rearrangements. However, there have been major saltatory changes in the relative amounts of particular DNA sequences. The increase of one sequence in D. simulans relative to D. melanogaster is compensated by a relative decrease in a different sequence, so that the total amount of satellite DNA in the two species remains equal.

It would be interesting to compare the gene content of, for example, the
third chromosome heterochromatin of both *D. simulans* and *D. melanogaster*. If the heterochromatic gene content is conserved between these two species despite the major changes in the surrounding satellite DNA, it would indicate that the heterochromatic position of these genes is significant. Conversely, if the heterochromatic genes were not conserved between the two closely related species, it would suggest that the location of heterochromatic genes is of little consequence.

To summarize, the results of the genetic analysis of chromosome three heterochromatin of *Drosophila melanogaster* provides 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 mystery of heterochromatin is to discern why genes are in some heterochromatic regions and not others; and what are the costs and benefits of this arrangement for both the gene and the block of heterochromatin.
LITERATURE CITED


