ANALYSIS OF CHROMOSOME *4* IN *DROSOPHILA MELANOGASTER*. II: ETHYL METHANESULFONATE INDUCED LETHALS[1,2]

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OUTSIDE the realm of the prokaryotes, it may appear unlikely that a complete inventory of the genetic material contained in the individual vehicles of hereditary transmission, the chromosomes, can be obtained. The chromosomes of higher plants and animals are either too large or the methods required for such an analysis are lacking. However, an approach to the problem is possible with the smallest autosome (number *4*) in *Drosophila melanogaster*. In this genetically best-known diploid organism appropriate methods are available and the size of chromosome *4* (0.2–0.3 μ at oogonial metaphase) suggests that the number of loci might be a relatively small, workable figure.

An attempt is being made to uncover all of the major loci on chromosome *4*, i.e., those loci capable of mutating to either a recessive lethal, semilethal, sterile, or visible state. In the first paper of this series (HOCHMAN, GLOOR and GREEN 1964), we reported that a study of some 50 spontaneous and X-ray-induced lethals had revealed a minimum of 22 vital loci on the fourth chromosome. Subsequently, two brief communications (HOCHMAN 1967a,b) noted that the use of chemical mutagens had substantially increased the number of lethal chromosomes recovered and raised the number of loci detected.

This paper contains an account of approximately 100 chromosome *4* mutations induced by chemical means (primarily recessive lethals which occurred following treatments with ethyl methanesulfonate). Analyses of the interactions of these mutations *inter se* and with the older spontaneous and radiation-induced factors have elevated the number of vital loci disclosed to 33 and strengthened our earlier estimate that the total number of essential loci on this microchromosome is less than 40. Evidence of the existence of the first complex locus on chromosome *4* will be presented here, as will an attempt to reconcile the disparity between our postulated number of microchromosomal genes and that derived from this autosome's DNA content as estimated from the work of others.

MATERIALS AND METHODS

Mutations of genes on chromosome *4* are detected by means of a crossing scheme spanning three generations. Normal males of the Oregon-R strain are treated with a chemical mutagen (in the manner specified below) and crossed *en masse* to virgin females of the cubitus interruptus–Dominant (ci^D)/eyeless–Dominant (ey^D) stock. F_1 males carrying a treated and a marked

[1] This paper is dedicated in friendship and respect to my teacher, Dr. CURT STERN.

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4($ci^D/+$ or $ey^D/+$) are individually mated in vials to ci^D/ey^D females. From each resulting vial, $ci^D/+$ virgin females and males are selected and crossed, and their progeny (generation 3) are examined. If no mutation was present on the treated fourth chromosome transmitted by his father to a given F_1 $ci^D/+$ or $ey^D/+$ male, the generation 3 progeny will contain $ci^D/+$ and $+/+$ individuals in approximately a 2:1 ratio. If an induced recessive lethal (or semilethal) mutation was transmitted, the generation 3 flies will lack (or have very few) non-ci^D flies. Recessive visible mutations will cause all (or some, if penetrance is less than 100%) of the generation 3 non-ci^D flies to appear phenotypically abnormal. The crossing scheme can be extended an additional generation to test for sterility mutations, temperature-sensitive mutations, etc. The methods employed to determine allelic relationships and location of the recovered mutations are described in HOCHMAN, GLOOR and GREEN (1964).

Three chemical mutagens were used. The majority of mutations occurred following treatment with ethyl methanesulfonate (EMS). In a 1965 series of experiments EMS was injected (0.05 M solution in saline), and in 1968 the flies were exposed to crumpled Kleenex tissues which had been saturated with 0.025 M EMS in sterile 1% sucrose as described by LEWIS and BACHER (1968). Experiments carried out in 1964 by a graduate student, Mr. THOMAS B. MCCUNE, utilized a quinacrine mustard now known as ICR-170 and a bifunctional alkylating agent [N,N-di-(2-chloroethyl)-amino-L-phenylalanine] called Melphalan, kindly supplied by Dr. M. FAHMY. In separate experiments, males were injected with either 0.05% ICR-170 in saline or 0.01 M Melphalan. Since Melphalan is relatively insoluble in water, it was first converted into a soluble sodium salt (dissolved in 0.1 N NaOH and then neutralized with 0.1 N HCl). The concentration was brought to the correct level by the addition of distilled water (MCCUNE 1965).

Most of the spontaneous and X-ray-induced mutations referred to in this paper were described in the first paper of this series. New spontaneous mutations are: 5 lethals (*1*^{e}, *2*^{h}, *16*, *20*, and *29*^{b}) recovered from a 1963 sample of adults inhabiting an orchard in Knoxville, Tennessee and 2 lethals (*1*^{f}, *10*^{a}) and 4 semilethals (*5*^{a}, *16*^{b}, *16*^{c}, *33*^{c}) which Dr. ALICE KENYON obtained from a laboratory population cage (KENYON 1967) and generously sent to me. Collections from natural populations in 4 different states from 1960–1963 yielded 21 lethals in 1721 microchromosomes tested, a frequency of 0.012.

Two new lethals of X-ray origin (*1*^{g}, *38*) were discovered in my laboratory in a small-scale 1964 experiment in which 235 fourth chromosomes were screened after parental males had been exposed to 4,000 r.

Additional testing of the lethal-bearing chromosomes induced by GLOOR and GREEN in 1957 (see HOCHMAN, GLOOR and GREEN 1964) has resulted in some corrections and reinterpretations. We have also decided to replace the nomenclature used earlier with one in which all lethals are symbolized by an italicized number, e.g., *l(4)1*, and all alleles found subsequently are given the same number and a letter superscript. Thus the lethals allelic to *1* described earlier (*27*, *28*, and *32*) become *1*^{a}, *1*^{b}, and *1*^{c}, respectively. This practice serves to release integers such as *27*, *28*, and *32* for use in naming newly detected loci and it also provides a means of computing rapidly the number of allelic mutations that have occurred at each locus. In a similar fashion, loci symbolized earlier by letters (such as the locus occupied by alleles *ST-4*, *SLC-1*, and *MW-1*) have been renamed. The locus is now designated *15* and the three alleles are *l(4)15*, *15*^{a}, and *15*^{b}, respectively.

Corrections of findings published in the first paper of this series are:

1. Lethal *30*, incorrectly called allelic to *ST-3*, is now symbolized *1*^{d}.
2. Lethal *16* turned out to be a translocation with the lethal not on the fourth chromosome. The number is now used to denote a valid chromosome *4* locus.
3. *l(4)ar*, while allelic to *spa*^{Cat} as reported, is not a member of locus *9* as are *l(4)9 (BU-1)* and *9*^{a} *(1-JFC)*. Instead, *l(4)ar* is now symbolized *16*^{a}.
4. Lethal *35* is not a lethal at the *ci* locus, but a *3;4* translocation giving a *ci* position effect (see DISCUSSION).
5. Lethal *3* was not lost. Indeed, it has proven to be a most useful and interesting mutant (see DISCUSSION).

Certain terms used in this paper may have different connotations to various investigators and hence require some clarification. The term "vital" or "essential" locus signifies a genetic unit of

function (the classical gene or the cistron) which may mutate to lethality. Geneticists including myself have sometimes called these "lethal" loci. I believe it is more appropriate to refer to the locus as vital, essential, or indispensable, and to speak only of the mutation as lethal. Several workers have reported the existence of nonessential loci in Drosophila which may be mutated or even deleted without causing the death of the homozygote, e.g., rosy (SCHALET, KERNAGHAN and CHOVNICK 1964) and white (LEFEVRE and WILKINS 1966). Moreover, there may be essential loci at which semilethal and visible, as well as lethal mutations occur.

The terms lethal and semilethal are used here to convey meanings somewhat different from those intended by some other investigators. Here, a lethal fourth chromosome means one which stays balanced over ci^D (or ey^D if necessary) and which typically never appears as a homozygous adult. The only exceptions are the extremely rare, weak, non-ci^D "escapers" which may be found principally in stocks of pupal lethals. From time to time a homozygote successfully traverses some critical period in metamorphosis, during which the lethal ordinarily acts, and thus escapes death. These escapers are invariably either sterile or so low in viability that they make no contribution to the next generation. The fourth chromosome semilethals that we deal with share that property of complete lethals of remaining in balanced stocks over ci^D or ey^D. While homozygous adults do occur at low frequencies, they are less fit than the ci^D (or ey^D) heterozygotes. They may exhibit phenotypic abnormalities (e.g., *33* and its alleles); one or both sexes may be sterile; or they may be ostensibly normal. The point to be stressed is that these semilethals are *not* like many of the viability-reducing mutations (having up to 50% of the fitness of wild-type flies) that one sees characterized as semilethals in certain papers dealing with population genetics. If one were to classify the latter in categories of fitness from 1–50% normal fitness, the chromosome *4* semilethals would all fall near the low end of the range. This is not to say that fourth chromosomes having homozygous fitnesses of about 50% normal do not exist. We simply have made no effort to identify and retain such chromosomes in our recent work.

There are 7 fourth chromosomes which are lethal when homozygous and also in heterozygous combination with lethals at two or more independent loci. For example, *lf(4)3* interacts lethally with lethals at loci *9*, *16*, *29*, *39*, and *40*. The dominant visible, recessive lethal, sparkling-Cataract, (spa^{cat}), does likewise and the *lf(4)3*/spa^{cat} genotype is also lethal. Without cytological proof (achieved through salivary gland chromosome inspection) that a deficiency is involved we hesitate to use the symbol *Df* for *3* or spa^{cat}. Since the probability that five lethal mutations occurred simultaneously is virtually nil, and the deficiency status is only putative, we make use of the term, lethal factor. The symbol *lf* is sort of a hybrid between that for lethal (*l*) and that for deficiency (*Df*). The *lf* designation is only a tentative one; further research may reveal a deficiency that has previously escaped detection, or that mutations such as *lf(4)3* involve regulatory genes, etc. The other *lf*'s are 2^c, $2^{bt\text{-}D}$, *38*, *40*, and ci^D.

Most, if not all, of the loci found on chromosome *4* are situated in the region delimited by a large proximal deficiency *Df(4)M*, the region marked off by a smaller terminal deficiency *Df(4)G*, or the area between these deficiencies. Figures 1A and B show, respectively, a moderately stretched pair of salivary fourth chromosomes (about 15 μ) and a greatly stretched single *4* (about 30 μ). Some of the major bands and the approximate extents of the two deficiencies, *M* and *G*, are indicated.

My preparations of giant chromosomes show, in accordance with those of BRIDGES (1935) and GRIFFEN and STONE (1940), about 50 bands in the right arm of *4*. SLIZYNSKI's (1944) report of over 100 bands in this limb cannot be substantiated by my examinations of microchromosomes stretched over 30 μ. This communication will employ a figure of 50 bands for *4R*. The small, thin left arm will be given but passing notice.

The photograph labeled C in Figure 1 displays the *Df(4)G* aberration as it appears in an unstretched polytene chromosome pair. One of the homologues can be observed to lack part of 102E and all of 102F. A distinct 2-banded cap (of *X*-chromosome origin) may be seen on the deficient *4*.

All of the visible microchromosomal mutants mentioned in this paper are named and symbolized. For detailed descriptions of their effects refer to LINDSLEY and GRELL (1968).

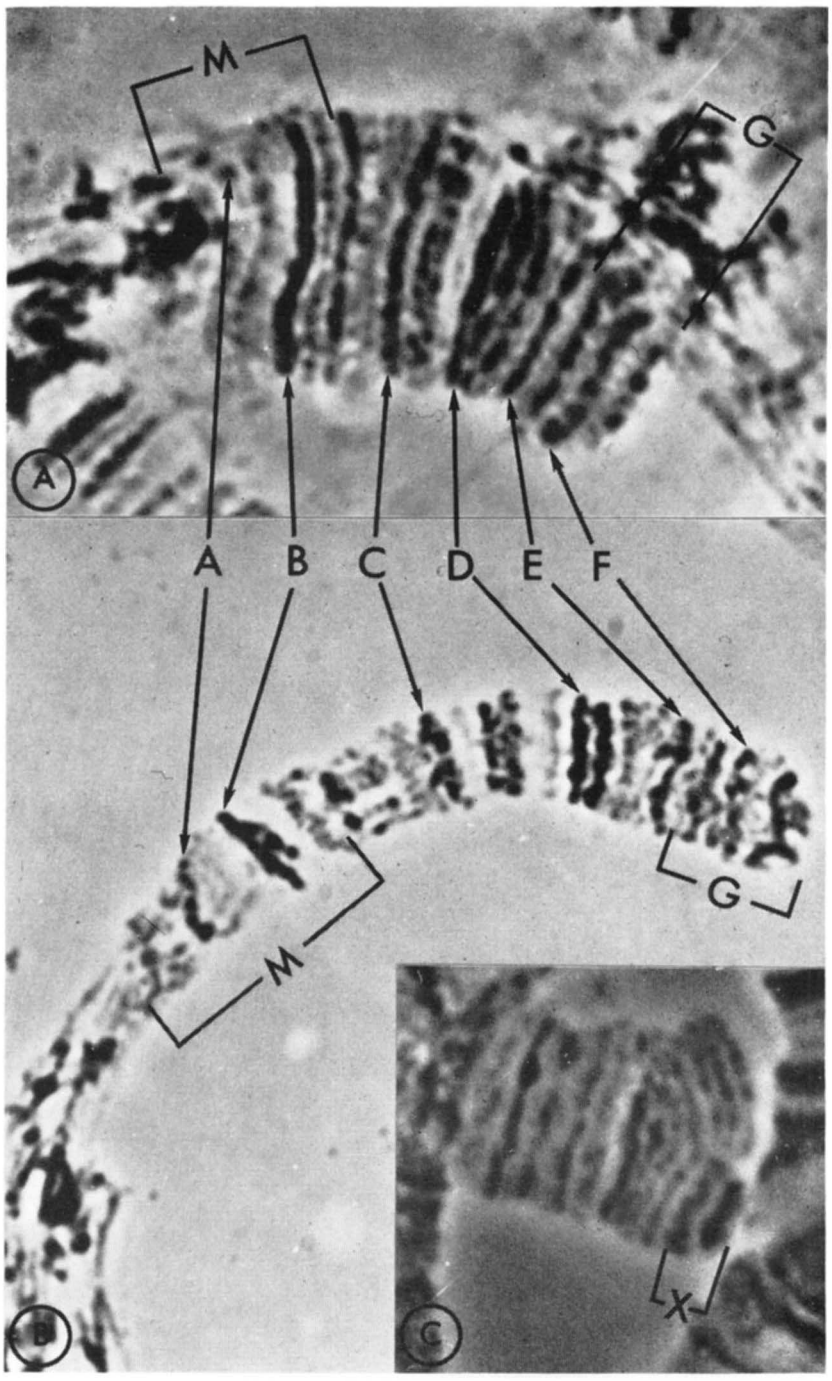

M
G
A
A
B
C
D
E
F
G
M
X
C

TABLE 1

Induced and spontaneous fourth chromosome mutations and aberrations

Source	Lethal chromosomes	Frequency	Point mutations		Deficiencies		Trans-locations	Lost	Recessive visibles
			Lethal	Semilethal†	*Df**	*lf*‡			
X rays	42	<0.01	30	0	4	3	4	1	3
EMS	72	0.04	70	11	0	1	1	2	3
ICR 170	10	0.04	10	1	0	0	0	0	0
Melphalan	3	<0.01	3	0	0	0	0	0	0
Spontaneous	26	0.01	25	4	0	0	0	1	0
Total	153	0.02	138	16	4	4	5	4	6

* Deficiency confirmed cytologically.
† No special effort made to detect semilethals.
‡ Deficiency suspected on genetic grounds only.

RESULTS

Table 1 lists the number of fourth chromosome mutations that have been induced by various treatments and the number extracted from natural or laboratory populations not knowingly exposed to any man-made mutagens. Since experiments involving abdominal injection of EMS (1965) and "feeding" EMS (1968) resulted in similar rates of lethal mutation (3.5% and 3.8%, respectively), the EMS data are pooled in Table 1. The 72 completely lethal chromosomes included two containing two point mutation lethals each (*28^a*, *37* and *2^x*, *36^a*), five bearing a lethal at one locus and a semilethal at a second locus (the lethals in question are *2^m*, *6^e*, *16^f*, *19^f*, and *23^c*) and one suspected deficiency lethal termed *lf(4)40*. There were an additional six semilethals (three at locus *33* and three unlocalized), two lethals that were lost before analysis, one translocation [*T(3;4)* with the lethal on chromosome *3*], and three recessive visible mutations of the abdomen rotatum locus (*ar^{65f}*, *ar^{65h}*, and *ar^{68i}*). A grand total of 88 mutations and aberrations were found on the 1978 chromosomes tested giving an overall frequency of detected genetic change of 4.4%.

Smaller-scale experiments involving ICR-170 produced 10 lethals and one semilethal in 266 chromosomes assayed, while those in which Melphalan was utilized yielded three lethals per 431 chromosomes examined. There were no double mutations, visibles, or aberrations in these two experimental series.

Nearly 6,000 chromosomes exposed to X-ray doses exceeding 3,000 r have had to be tested to uncover the 42 lethal ones listed in Table 1. Lethality of these chromosomes is due in 30 cases to presumed point mutations, in four cases to cytologically confirmed deficiencies (*Df(4)*'s *11*, *12*, *24*, and *G*), in three others

FIGURE 1.—Chromosome *4R* in larval salivary gland cells. The first doublet bands in each subdivision of 102 are indicated by A–F. Division 101 is poorly defined. *M* and *G* show the approximate extents of major deficiencies. A: a moderately stretched (about 15 μ) pair from an *M(4)^{63a}/ci^D* larva. B: a greatly stretched (about 30 μ) single *4*. C: an unstretched pair from a *Df(4)G/ci^D* larva. The lower homologue lacks the last three doublets and is capped by two bands of *X*-chromosome origin (*X*).

TABLE 2

Lethals and semilethals at 33 vital loci on chromosome 4

Region	Locus designation	Total alleles	Source				
			Spont.	X rays	EMS	ICR	Mel
	1	11	2	6	3	.	.
	13	2	.	1	1	.	.
	17	2	.	2	.	.	.
Df(4)M	*20*	2	1	.	1	.	.
	25	5	1	.	3	.	1
	26	2	.	.	1	1	.
	M	1	.	1	.	.	.
	9	4	2	.	2	.	.
	16	9	4	.	4	1	.
Df(4)G	*19*	8	.	.	2	4	2
	29	3	3	.	.	.	.
	39	1	.	.	1	.	.
	*40**	0	.	.	.	.	.
"bt^D"	*2*	29	3	6	17	3	.
	23	6	1	.	4	1	.
	4	12	2	3	6	1	.
	5	8	1	1	6	.	.
	6	6	1	3	2	.	.
	7	3	.	1	2	.	.
	8	3	.	3	.	.	.
	10	2	1	1	.	.	.
Between *M* & *G*,	*14*	6	1	2	3	.	.
proximal to *M*,	*15*	3	3	.	.	.	.
or in *4L*	*21*	2	1	.	1	.	.
	22	1	1	.	.	.	.
	27	1	.	.	1	.	.
	28	3	.	.	3	.	.
	30	1	.	.	1	.	.
	32	1	.	.	1	.	.
	33	4	1	.	3	.	.
	35	1	.	.	1	.	.
	36	2	.	.	2	.	.
	37	3	.	.	3	.	.
Total	33	147	29	30	74	11	3

* Locus *40* deduced from behavior of *lf(4)40*. See DISCUSSION.

to suspected deficiencies (*lf(4)3*, *lf(4)38*, and 2^c), and in four cases to translocations involving chromosome *4* and a lethal-bearing chromosome *2* or *3*. One lethal chromosome was lost and three recessive visibles (ar^{57d}, ar^{579}, and ci^{579}) were recovered.

Table 2 enumerates the 32 vital loci uncovered through analysis of 147 of the 154 lethal and semilethal mutations (seven semilethals are as yet unlocalized) and a 33rd locus deduced from the behavior of *lf(4)40*.

The 74 EMS-induced mutations exposed 26 loci, including nine not otherwise

revealed. The two quotients, 0.35 (26/74) and 0.12 (9/74), may be taken as measures of the usefulness of this mutagen in the search for microchromosomal loci. Comparable values for X rays are 0.43 (13/30) and 0.10 (3/30), and for spontaneous mutations the quotients are 0.59 (17/29) and 0.10 (3/29). The three main sources seem to be approximately equivalent except for the apparent tendency of spontaneous mutations to reveal more loci per mutation tested (17 per 29 is significantly higher than the 0.35 noted for EMS; $P<0.01$). On the other hand, when one takes into account the fact that the frequency of lethal mutations induced by EMS is four times greater than that induced by X rays and 2–4 × the frequency of lethal microchromosomes estimated to be in the wild populations sampled, it is obvious that EMS treatments provide the best opportunity for finding essential loci on chromosome *4*. Indeed, all but seven of the 33 vital loci detected thus far have one or more EMS-induced mutation and there is no theoretical reason why the base substitutions presumably provoked by EMS cannot convert any normal essential gene into a lethal mutant.

There were two EMS-treated chromosomes which were found to contain double lethals (2^{x}, 36^{a} and 28^{a}, *37*). This frequency of double mutation is 0.0010 (2 per 1,978) and the frequency expected is 0.0013 (which is the square of the frequency of induced lethals, 0.0354 (70 per 1,978)). An excess of double lethals induced by EMS [as reported by LIFSCHYTZ and FALK (1969)] was not noted here. The observed frequency of double mutation is increased if one includes the five chromosomes which carry a lethal and a semilethal (6^{e}, 23^{c}, 16^{f}, 19^{f}, and 2^{m}). However, since there was no special effort to identify and retain semilethals, I have no firm basis for an estimate of the combined lethal and semilethal mutation rate.

DISCUSSION

The results obtained have necessitated revisions in our delineation of the genetic content of the four "regions" of the microchromosome and these will be discussed first. A comparison of estimates of gene number based on the mutation work and on appraisals of DNA content in the autosome will follow.

The Df(4)M *region:* The fourth chromosome deficiency discovered by BRIDGES in 1925, now called *Df(4)M*, lacks approximately 1/4 of the bands seen in the normal right arm of the salivary gland chromosome *4* (see BRIDGES 1935; SLIZYNSKI 1944). There are still some uncertainties regarding the left and right breakpoints of the deletion. Our present best estimate is that 11–15 of the 50 bands are missing.

Seven distinct loci, which can be occupied by recessive lethal mutants, have been found in the region delimited by *Df(4)M*. These loci are designated *1*, *13*, *17*, *20*, *25*, *26*, and *M*. *M* is apparently a point mutation which produces the Minute phenotype in the heterozygous state ($M(4)^{57g}/+$). This Minute does not permit pseudodominant expression of recessive *ci* as do all other known chromosome *4* Minutes. None of the alleles at any of the seven vital loci interact with the three recessive visibles, grooveless (*gvl*), *ci*, or *ar*.

There are two dominant visible, recessive lethal mutants, Cell (Ce^{2}) and ci^{D},

which are placed in this region on the basis of their lethality in heterozygous combination with *Df(4)M*. The interactions of these two factors with one another and with recessive lethals at loci *13* and *17* are as follows: No ci^D/Ce^2 adults appear unless a third chromosome *4*, such as spa^{Cat}, is also present in the karyotype. Developmental tests (to be published elsewhere) reveal that whereas ci^D is an embryonic lethal and Ce^2 homozygotes die in either the embryonic or larval stages, the death of ci^D/Ce^2 individuals is delayed in most cases until the pupal period. This delayed lethality may be viewed as partial complementation, indicating Ce^2 and ci^D are not truly allelic in the functional sense.

Lethals at locus *13* kill larvae while those at locus *17* act in the embryonic and larval stages. $Ce^2/13$ and *17/13* individuals have normal viability but $ci^D/13$ animals die as embryos or larvae, $ci^D/17$ as larvae, and $Ce^2/17$ as embryos. These findings are interpreted conservatively as suggesting a minimum of two essential loci (*13* and *17*) within the Ce^2–ci^D "complex."

The sequence of loci within *Df(4)M* shown in Figure 2 is mainly arbitrary. *M*, *ci*, *13*, and *17* form a logical group that most likely excludes the other four vital loci, *1*, *20*, *25*, and *26*. One or more of these latter, however, may be located to the left of the most proximal member of the group. HOCHMAN (1965) and FAHMY (personal communication) agree that the small $M(4)^{63a}$ deletion involves bands 102A1,2 and perhaps 101F (see Figure 1A). This deletion is lethal with the point mutation *M* ($M(4)^{57g}$) and it uncovers recessive *ci*. Since band 102A1,2 is presumably closer to the left than to the right breakpoint of *Df(4)M*, the *M* and *ci* loci are tentatively placed in the proximal part of our map of the region. The dominant visible ci^D is put near *ci* as a consequence of the greater deviation from normal venation observed in ci^D/ci flies as compared to *ci/ci* or $ci^D/+$ individuals. The two mutations may actually be allelic although the fact that $M(4)^{63a}/ci^D$ is a viable fly whose abnormal venation is no more extreme than $ci^D/+$ argues for either pseudoallelism or nonallelism of ci^D and *ci*.

The diplo-*4* triploid crossing over data of STURTEVANT (1951) indicated that *gvl* was near and probably to the right of *ci*. Since *gvl* is not exposed by $M(4)^{63a}$ and does not interact with any other factor within *Df(4)M*, there is no compelling reason to place it between *M* and Ce^2. At this time, the sequence of *gvl*,

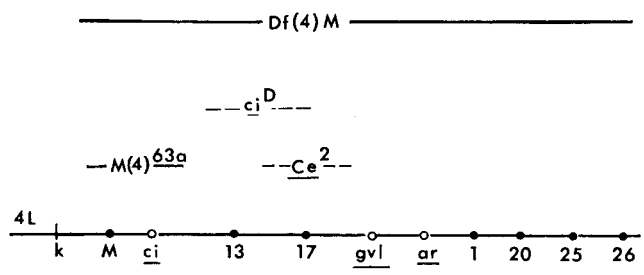


FIGURE 2.—Genetic factors within the region delimited by the proximal deficiency *Df(4)M*. See text for explanation of *M–17* sequence. No sequence intended for *gvl–26*. k = kinetochore, ● = vital loci, ○ = loci identified through recessive visibles. Dashed line indicates that extent of factor is based on complementation mapping.

ar, *1*, *20*, *25*, and *26* is unknown. Regardless of sequence, it seems likely that some or most of them are between 102A1,2 and 102B9,10, and distal to the *M-17* group.

In the first paper of this series a lethal named *35* (synonymous to lethal *18* recorded in LINDSLEY and GRELL 1968) was tentatively assigned to the *ci* locus because the *35/ci* heterozygote exhibited interruptions of vein *4*. Additional testing of this mutant has shown that it is actually a *3;4* translocation. It seems reasonable to assume that the mutant expression of the *35/ci* heterozygote is due to position effect (translocation of ci^+) and not to mutation or deletion of the ci^+ gene on the treated chromosome. Furthermore, the lethality is probably a result of a chromosome *3* lethal since a cross of $35/ci^D$ to an attached-*4* stock yielded viable haplo-*4* progeny. Consequently, we have concluded that the mutation initially designated *35* did not expose a microchromosomal locus.

The Df(4)G *region:* An X-ray-induced terminal deficiency of the right arm of chromosome *4* was discovered by GLOOR and GREEN in 1957. Examination of salivary gland chromosomes reveals that band 102E1,2 is the last major band remaining in the deleted chromosome (Figure 1C). Some 8–9 bands are missing and the deficient microchromosome is capped with a small piece of the *X* chromosome which includes the loci of y^+ and ac^+.

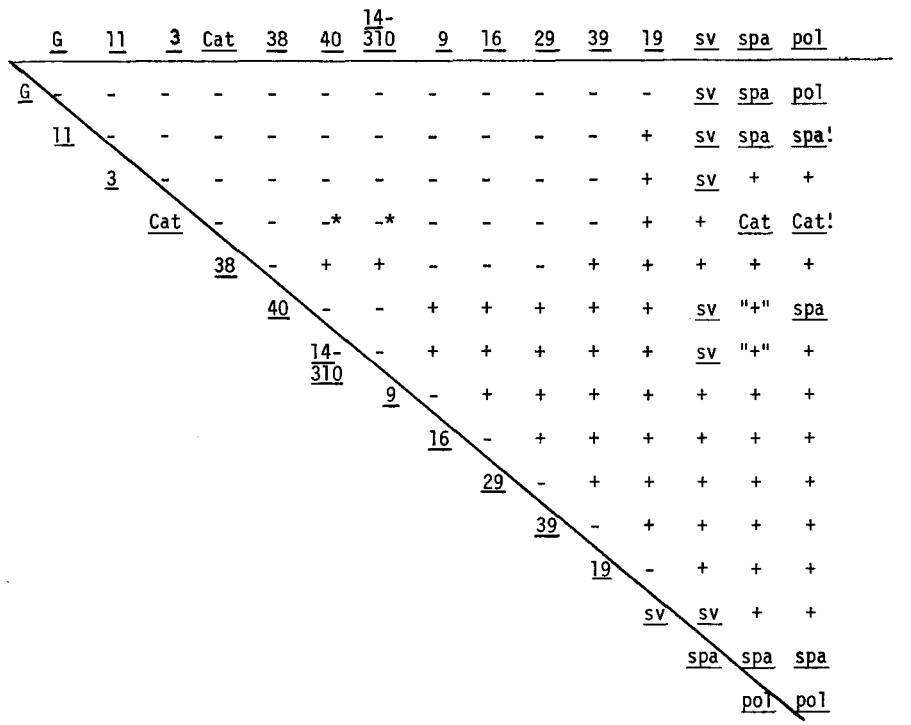
Six essential loci have now been found in this region, viz., loci *9*, *16*, *29*, *39*, *40*, and *19*. Two recessive visibles, shaven (*sv*) and sparkling (*spa*), are uncovered by *Df(4)G* and are therefore placed in the region. Interactions occurring between these genes and several verified and presumed deletions located within *Df(4)G* have permitted the construction of a complementation map which is "linear" for the most part and hence indicative of sequence. A matrix of crosses and resulting progeny is presented in Table 3.

Locus *19* is judged to be the most distal one on chromosome *4* since it is uncovered by *Df(4)G* but not by *Df(4)11*, and while the left breakpoints appear to be same for the two deficiencies, the distal tip of *4R* is present in the *Df(4)11* chromosome but missing in *Df(4)G*. The sequence for loci *9*, *16*, and *29* is unknown but, as they are the only ones interacting lethally with *lf(4)38*, it is assumed the three are neighbors. A lethal at locus *39* interacts with neither *lf(4)38* nor *lf(4)40*; it is placed between these factors although it could be to the left of *38*. The sixth vital locus, *40*, is deduced from the observations that *lf(4)40*, which was induced by EMS, and which permits pseudodominant expression of *sv* and *spa*, is lethal when homozygous or heterozygous with *Df(4)11*, spa^{Cat}, *lf(4)3*, and a recently discovered EMS-induced *lf* tentatively designated *14–310*. *lf(4)40* is fully viable over lethals from the other five essential loci within *Df(4)G*.

The complementation map in Figure 3 shows *sv* and *spa* as adjacent. It could be argued that *sv* is more proximal, i.e., to the left of *lf(4)38*. Either interpretation is subject to criticism. If the loci are distal and adjacent, spa^{Cat}, which apparently interacts with the mutants sparkling and poliert (spa^{pol}) but not with shaven, does not extend far enough distally to reach the *spa* locus. If one places *sv* to the left of *lf(4)38* and shifts *lf(4)3* (and *14–310*) to the left so that *sv* is

TABLE 3

Results of crosses involving factors located within Df(4)G



— = lethal prior to adult stage, —* = a few adults appear but they die shortly after eclosion, + = viable and wild type, "+" = wild type or very slight sparkling, *spa* = sparkling, *pol* = poliert, *Cat* = Cataract, *sv* = shaven, ! = extreme expression of mutant. The combinations *Cat/19* and *Cat/sv* should each be *Cat* rather than + (plus) as incorrectly shown in the body of Table 3.

overlapped, one has to assume that *lf(4)40* is a double mutation. I favor the first interpretation because one genetic change is more probable than two and because the interactions of Cataract and sparkling may not require an actual physical overlap of relevant chromosomal sections. A precedent for this type of interpretation is found in the case of ci^D and *ci*, where the effects and presumed location of $M(4)^{6sa}$ oppose the idea of structural allelism for the two interacting vein mutants.

The 2–23 region ("bt^D"): A dominant visible, recessive lethal induced on chromosome *4* with X rays by SCHULTZ in 1933 was named bent-Dominant (bt^D) because of phenotypic similarities to and interaction with recessive bent (*bt*), the first mutant found on *4*. The visible manifestations of bt^D are no longer perceived but the recessive lethality is still complete. Indeed, the bt^D chromosome is fully lethal both as a homozygote and when heterozygous with any of the alleles known at each of two independent loci designated *2* and *23*. BRIDGES

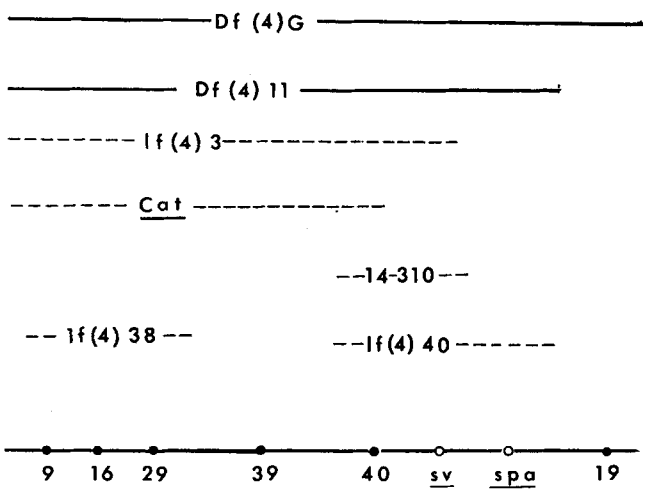


FIGURE 3.—Complementation map based on results presented in Table 3. *Df* = cytologically verified deficiency; *lf* = deficiency suspected on genetic grounds. See text for justification of sequence.

(1935) could discern no abnormality in the bt^D chromosome and so it might be thought that the chromosome carries two lethals, both perhaps dating back to the original X-ray exposure. However, one of the 1957 X-ray-induced lethals described in the first paper of this series, *29*, has also proven to be lethal in combination with all members of loci *2* and *23*. We have concluded that *29* or *l(4)2^c^* as it is now called (see LINDSLEY and GRELL 1968) and bt^D or $l(4)2^{bt^D}$ are actually deletions (too small to be resolved) or inactivations of two adjacent loci, *2* and *23*. Some additional salivary gland chromosome study will have to be done before the conjecture that we may be dealing with mutations (2^c, 2^{bt^D}) of a regulatory gene and two adjoining structural genes, *2* and *23*, can be elevated to the level of an hypothesis.

In any event, there are two aspects of locus *2* that deserve attention. Firstly, the 29 alleles of *2* comprise about 20% of all chromosome *4* lethals detected. Recovery of lethals at locus *2* was high from all mutation sources except the low-yielding Melphalan experiments. About 10% of spontaneous, 15% of X-ray-induced, 22% of EMS-induced, and 30% of ICR-170-induced lethals are alleles of *2*. It is interesting that four of the 13 X-ray-induced microchromosomal lethals studied by AMBELLAN and PROUT (1956) also were lethal with bt^D.

Secondly, there appears to be evidence for some kind of "intragenic" complementation at locus *2*. All 29 of the locus *2* mutations are completely lethal when homozygous or when heterozygous with 2^{bt^D} or 2^c. Twenty-four of the 29 are also fully lethal in all pairwise combinations. The remaining five (2^b, 2^f, 2^h, 2^k, and 2^t) fall into three "functional" units separable on the basis of partial complementation. Developmental tests (to be published elsewhere) show that 2^f, 2^h, and 2^t kill embryos, while 2^b and 2^k kill larvae. There are 10 different intercrosses possible with these five mutations and in seven of them, adults heterozygous for

TABLE 4

Results of crosses ($l_1/ci^D \times l_2/ci^D$) *involving the complementing alleles of locus 2*

Cross	Progeny		
	ci^D	non-*ci*^{D}	Percent non-*ci*^{D}
$2^f \times 2^h$	287	0	0
$2^f \times 2^t$	203	0	0
$2^h \times 2^t$	683	0	0
$2^b \times 2^k$	1,375	27	1.93**
$2^b \times 2^f$	366	105	22.29**
$2^b \times 2^h$	228	79	25.73**
$2^b \times 2^t$	730	110	13.10**
$2^k \times 2^f$	405	102	20.12**
$2^k \times 2^h$	431	85	16.47**
$2^k \times 2^t$	841	43	4.86**
$2^b \times 23$	133	64	32.49
$2^k \times 23$	332	154	31.69
$2^f \times 23$	69	51	42.50
$2^h \times 23$	138	60	30.30
$2^t \times 23$	426	225	34.56

** Chi-square tests give P $<$ .01 for (2 *ci*^{D} : 1 non-*ci*^{D}) ratio.

a pair of *2* "alleles" appear. As shown in Table 4, 2^f, 2^h, and 2^t act as alleles in that they permit no heterozygotes to reach the adult stage. However, a cross such as $l(4)2^b/ci^D \times l(4)2^k/ci^D$ yields about 2% non-*ci*^{D}, $2^b/2^k$ adults. Similar crosses involving either 2^b or 2^k as one parent and any member of the 2^f, 2^h, 2^t group as the other result in a greater proportion of non-*ci*^{D} adults. The conclusion that complementation is only partial is based on two facts: (i) the low frequency of non-*ci*^{D} adults (which is in all cases significantly below the $\simeq$ 33% + typical in crosses of nonallelic lethals, e.g., the *2* $\times$ *23* crosses presented in Table 4) and (ii) the phenotype of the non-*ci*^{D} imagoes (whose abdomens are clearly narrower than normal). Nevertheless, the surviving adults are fertile and a balanced stock, such as $2^b/2^f$, can be maintained.

The above facts of partial complementation and divergent developmental effects (2^b and 2^k act in the larval stage while all other *2* mutations affect embryos) suggest that *2* is a complex locus. The unusually high mutation rate recorded for this locus may indicate the presence of more mutable sites in the genetic unit termed locus *2* than in other microchromosomal loci. If locus *2* resembles other complex loci in Drosophila, it should be possible to separate by recombination the subunits we have designated 2^b, 2^f, and 2^k. However, because of the lack of fourth chromosome crossing over in diploids, detection of recombination within a suspected complex locus on the microchromosome will be much more difficult than a similar project involving loci on chromosomes *1*, *2*, or *3* of this species.

Six mutations have been found at locus *23*. Five of these are lethal in all homo-

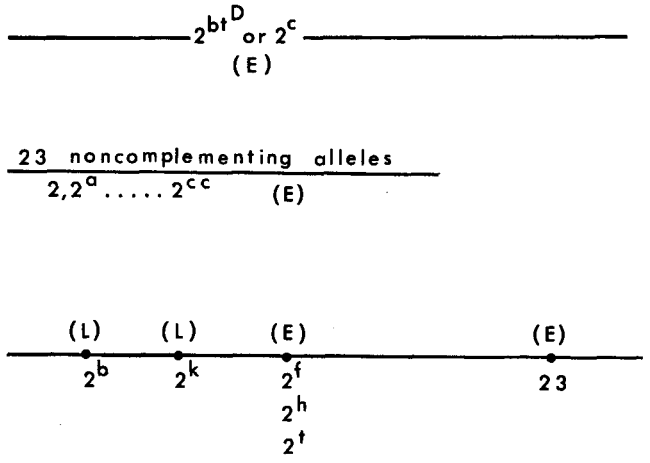


FIGURE 4.—Complementation map of *2–23* region. (E) and (L) indicate that death of lethal homozygotes occurs in embryonic or larval period, respectively.

zygous and heterozygous combinations with death occurring in the embryonic period. The sixth allele, 23^b, is probably a semilethal, and its presence in a chromosome carrying another nonallelic lethal, 6^e, has hindered its analysis. All alleles of *23* complement fully all alleles of locus *2* (excepting of course 2^{bt^D} and 2^c). The fact that *2* and *23* both function primarily in the embryonic stage may lend some support to the idea that the two are structural genes under coordinate control of one regulatory gene.

Figure 4 is a complementation map of mutations in the *2–23* region.

The remaining loci: Exactly half (20 of 40) of the loci detected on chromosome *4* do not interact with *Df(4)M*, *Df(4)G*, or 2^{bt^D}. Mutations at 17 of the 20 cause complete lethality (although in a few cases semilethal alleles occur). At one locus, *33*, four allelic mutations (3 EMS-induced and 1 spontaneous) have occurred. Homozygous adults having the reduced eye phenotype of eyeless appear regularly but at low frequency in stocks of these semilethals. The fitness of the homozygotes is so low that the stocks remain balanced ($33/ci^D$). Some of the individuals heterozygous for a *33* allele and ey^R show reduced eyes. For the present we shall regard the *33* locus as identical to *ey*. Finally, there are two loci which have been identified only by recessive visibles, *bt* and gouty (*gy*).

These loci, plus the two which are lethal with 2^{bt^D}, are considered unlocalized, but most of them doubtless reside in the area between the two major deficiencies, *M* and *G*. FUNG and STERN (1951) and STURTEVANT (1951) definitely located *bt* and *ey* in this area and it is logical to assume that most of the genes of *4R* should be in that portion of the arm which contains 0.52–0.62 of the bands in the salivary gland chromosome *4R*. At this time nothing definite can be stated regarding the sequence of the loci.

This discussion ignores the left arm of chromosome *4* because there is no evidence that it contains the type of major genes known to be in *4R*. The survival and fertility of *C(4)RM/0* flies (i.e., individuals having an attached-*4* condition

in which only the two right arms of *4* are presumably present) argue that some or all of *4L* is dispensable (see LINDSLEY and GRELL 1968). I have observed that *4L* has a diameter in salivary gland cells of less than one-half that of *4R*. (See also the drawings in GRIFFEN and STONE 1940 and in SLIZYNSKI 1944.) The drawings cited show about 20 bands in *4L* but I cannot confirm this. Part of the overall banding pattern seen in *4L* seems to me similar to that observed in the eyeless-Dominant duplication and I have hypothesized that *4L* is the source of this aberration (oral presentation at Austin Drosophila Conference, 1967).

If one compares (as in Table 5) the number of loci found within the two major deficiencies of the microchromosome (*M* and *G*) with estimates of the band content and relative length of these regions in salivary gland chromosomes, the lack of serious discordance between the relevant proportions conveys an impression that the loci detected are distributed more or less at random over the autosome. This impression cannot be confirmed until some additional mapping of the 22 unlocalized loci is accomplished. The discrepancies noted in the proportions of bands *versus* relative length are probably artifacts of the stretching procedures. Unless the squash pressure exerted during preparation of a slide is adequate, the fourth chromosome will remain attached to the chromocenter at both ends, severely hampering cytogenetic work. However, not all sections of the chromosome will necessarily be elongated equally during the stretching.

The number of vital loci to be expected on chromosome *4* was estimated as 34–38 in the first paper of this series (HOCHMAN, GLOOR and GREEN 1964). Judging from the frequency of repeat mutations (allelism) at the 33 loci discovered to date, there is no reason to increase the upper figure of the 1964 estimate. As seen in Table 2, there was only a single mutation at seven loci (*M*, *22*, *27*, *30*, *32*, *35*, *39*) and two mutations at seven other loci (*10*, *13*, *17*, *20*, *21*, *26*, *36*). If these 14 loci are taken as representative of all microchromosomal loci with low to average rates of mutation, the number of such loci that did not mutate (n_0) may be estimated from those which mutated once (n_1) and those

TABLE 5

The number of loci detected in the three regions of the right arm of chromosome 4 compared to estimates of length and band content of the regions in salivary gland chromosomes

	Regions			
	Df(4)M	*Df(4)G*	Between *M* & *G*	Total*
Number of loci	10	8	22	40
	(.25)	(.20)	(.55)	(1.0)
Number of bands	11–15	8–9	26–31	50
	(.22–.30)	(.16–.18)	(.52–.62)	(1.0)
Relative length†				
A	.27	.24	.49	1.0
B	.32	.19	.49	1.0

* The small region proximal to *Df(4)M* and the left arm are ignored.

† A or B = in moderately stretched or extremely stretched chromosomes shown in Figures 1A and B, respectively.

that did so twice (n_2) by employing a formula from the Poisson distribution as follows:

$$n_0 = \frac{(n_1)^2}{2 \times n_2}$$

In this case $n_0 = 3.5$ which, when added to the 33 loci already exposed, gives an estimate of 36–37 vital loci on the microchromosome. There may be some indispensable loci on *4* that mutate so infrequently that one cannot hope to uncover them regardless of the number of mutations induced and analyzed. As to the possibility that our screening methods overlook essential loci which can only mutate to dominant lethality (if indeed such loci exist), there is evidence from the survival of haplo-*4* flies that dominant lethals are scarce or absent on this autosome.

If there are but 40 or fewer essential loci on chromosome *4*, it may be difficult to detect the remaining ones. An evaluation of our success in finding loci from a chronological viewpoint strongly hints that we are approaching (have reached?) the economists' point of diminishing returns. The 1957 X-ray experiments revealed 12 loci. Of the naturally occurring lethals collected 1960–1963, only 10 of the 17 (0.6) exposed loci were new ones. The 1965 EMS treatments uncovered 23 loci of which only eight were new (0.35). Finally, in the 1968 EMS series only one of the seven loci revealed was a new one (0.14). Whether or not we continue to employ EMS in our search for microchromosomal loci will depend upon the results obtained in our current test series.

An expectation that 50 or fewer vital loci are located on the right arm of chromosome *4* comes from the finding [in close agreement with BRIDGES (1935, 1942)] that there are about 50 bands in the salivary gland chromosome *4R*. Actually some 33 lines can be discerned but the thicker ones are regarded as doublets, bringing the band total to 50. SHANNON, KAUFMAN and JUDD (1970) have reported that 113 lethal and semilethal recessive point mutations, which map between the *X* chromosome loci of zeste and white, belong to 12 functional units corresponding to the 12 salivary gland chromosome bands of the *z–w* region. If each band in a giant chromosome represents the site of an essential locus (or functional unit), JUDD and co-workers have saturated their section of interest and we are about two-thirds of the way to such saturation of chromosome *4*.

LIFSCHYTZ and FALK (1968, 1969) have been conducting a similar analysis of lethals confined to a small proximal section of the *X* chromosome said to comprise 5–10% of the euchromatic length of the salivary *X*. Tests of 35 X-ray-induced and 70 EMS-induced lethals have enabled these workers to construct a linear complementation map of 34 functional units within the section studied. Some points of resemblance between this work and mine are striking. The fourth chromosome's salivary band total of about 50 is about 5% of the *X* chromosome's total of 1,012. The lax length of the giant chromosomes averages 10 μ for chromosome *4*, and 144 μ for *X* (BRIDGES 1942). These figures suggest that the microchromosome and the part of *X* studied by LIFSCHYTZ and FALK may be quite comparable in size and/or number of genes. LIFSCHYTZ and FALK have tested 105 lethals (our X ray and EMS lethals total 100); they have detected 34 vital

loci (to our 33) and they have, as we do, evidence that most of the loci sought have been discovered.

LAIRD and MCCARTHY (1969) estimate that the amount of DNA in the haploid genome of *Drosophila melanogaster* is enough to account for 100,000 cistrons of 1,000 nucleotides each. If one assumes that chromosome *4* contains about 1% of the genetic material in this species (there are some 5,000 salivary gland chromosome bands of which 50 or so are microchromosomal) there should be approximately 10^6 nucleotides in each *4*. RUDKIN (1965) measured the DNA content of the *X* chromosome in *D. melanogaster* and found that the average amount per salivary band corresponds to 3×10^4 nucleotide pairs per haploid chromosome strand. A fourth chromosome with 50 average bands should therefore contain about 1.5×10^6 nucleotide pairs. Regardless of which of these two estimates is the more accurate, the presumed DNA content of the microchromosome far exceeds that expected for a chromosome with 50 or even 100 genes, if the average gene in Drosophila, like those in microorganisms, consists of 1,000–1,500 nucleotide pairs. One hundred genes averaging 1,500 nucleotide pairs would constitute a chromosome whose DNA content is still an order of magnitude below the estimates derived above!

Several possible explanations can be offered for the disparity in these estimates of microchromosomal gene number based on the mutation work, salivary band counts, (about 50), and on DNA measurements (about 1,000). The DNA calculations might be in error, although the basic agreement of the two independent appraisals (LAIRD and MCCARTHY 1969; RUDKIN 1965) is evidence of their validity. There could be considerable genetic redundancy in chromosome *4*. If certain loci (functional units) are present in two or more doses, the inactivation by a lethal mutation of one of them would be masked by the functioning of a replicate. Redundant loci would probably go undetected in the usual mutation experiments. Evidence for redundancy in the genomes of eukaryotes has been advanced by BRITTEN and KOHNE (1968). However, ROBERTSON, CHIPCHASE and MAN (1969) and LAIRD and MCCARTHY (1969) agree that the reiterated fraction of the genome in *D. melanogaster* is only about 10%.

Could the fourth chromosome be a carrier of a large number of genes whose mutant alleles serve merely to reduce viability so slightly that they escape detection by our screening methods? KENYON (1967) has compared chromosome *4* with chromosome *2* and she estimates that the maximum number of viability-affecting loci on *4* is 50 relative to an independent estimate of 400 on *2*. In my own tests of the viability of nonlethal microchromosomes, removed from wild populations and made homozygous, there is no indication that the proportion of semilethal plus deleterious chromosomes exceeds 10% (HOCHMAN 1962 and unpublished).

The possibility that most of the DNA in the microchromosome does not have a genetic function (i.e., in the coding of structural or regulatory genes) is rejected on the grounds that selection would have probably eliminated most of the waste by now. We are left finally with two alternatives which are not necessarily mutually exclusive. One is that there really are 1,000 or so genes on chromosome

4 and our method of detecting loci by mutation is inadequate. On the other hand, the functional units in Drosophila which we have referred to as genes may consist of 20 times the number of nucleotides typical of the cistrons of microorganisms. (The 3×10^4 nucleotide pairs estimated for the average band by RUDKIN is equivalent to 20 genes having a mean of 1.5×10^3 nucleotide pairs.) As RUDKIN (1965) states, ". . . these values (amount of DNA per average band) . . . are quite comparable to larger functional units such as the histidine 'operon' of P22 phage with 1.3×10^4 nucleotide pairs." Perhaps we have been scoring as allelic lethals any two mutational changes (each of which affects one of several critical points) in a long, integrated chain of DNA (located within a single chromomere and comprising, in a polytene chromosome, a single band) which normally controls one function indispensable for the development of the insect. Even though two mutations might impair widely separated portions of the long chain, the lack of complementation in *trans* heterozygotes leads to lethality and a conclusion that alleles are involved. The example of partial complementation noted at locus *2* above might be the exception that proves the rule.

Increased attention in my laboratory is currently being given to semilethal and sterility mutations and the possibility that electrophoretically separable protein variants are controlled by fourth chromosome genes. These approaches may disclose additional loci and/or demonstrate the range of mutational "versatility" at loci already recognized.

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SUMMARY

Allelism tests involving some 100 chemically induced mutations (primarily ethyl methanesulfonate induced lethals) and over 70 previously detected lethals and visibles of spontaneous or X-ray origin have revealed 33 loci capable of mutating to lethality on chromosome *4* in *Drosophila melanogaster*. In addition to these vital loci there are seven others which have been identified through recessive visibles only.—Ten of the 40 loci are situated in a proximal region of the autosome delimited by *Df(4)M* while eight others lie within the terminal deficiency, *Df(4)G*. The remaining loci, including two which interact lethally with the lethal factor on the bt^D chromosome, are unmapped but most are probably between *M* and *G*.—A high rate of mutation at locus *2*, plus the partial complementation and divergent developmental effects observed for five of its 29 alleles, suggest that it is the first complex locus to be recognized on the fourth chromosome.—The frequency of "repeats" encountered among the lethals confirms earlier estimates that there are no more than 40 essential loci on chromosome *4*. This figure is similar to what would be expected if each band in the polytene chromosome (about 50 for chromosome *4*) represented the site of a vital gene, but it is one or more orders of magnitude below that calculated from the amount of DNA estimated for this microchromosome. Attempts are made to explain or reconcile these disparate estimates.

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