GENETIC ANALYSIS OF CHROMOMERE 3D4 IN *DROSOPHILA MELANOGASTER*. II. REGULATORY SITES FOR THE DUNCE GENE[1]

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ABSTRACT

Chromomere 3D4 of the *X* chromosome of *D. melanogaster* contains two genes, dunce (*dnc*) and sperm amotile (*sam*). Mutations in *dnc* cause defects in memory formation and female fertility and reduce or eliminate the activity of a cAMP-specific phosphodiesterase designated form II. A fine structure map of this region has been constructed showing the locations of two *sam* mutations, five *dnc* mutations and a newly identified locus designated control of fertility (*cf*) that acts in *cis* to regulate the female sterility phenotype of *dnc*. The two *sam* mutations are separated by 0.02 ± 0.01 cM, the rightmost being located 0.08 ± 0.02 cM to the left of the null mutation dnc^{M11}. A cluster of null and form II-defective *dnc* mutations is located 0.04 ± 0.01 cM to the right of dnc^{M11}. The *cf* locus is 0.06 ± 0.02 cM to the right of this cluster. The location of the *dnc* and *cf* sites identify a region of approximately 0.10 cM that is required for proper expression of dnc^{+}. The dnc^{CK} mutation, associated with a reciprocal translocation between *3L* and the *X*, exhibits reduced form II activity and female sterility. This translocation breakpoint has been mapped to the left of the dnc^{+} gene and is near the breakpoint of $Df(1)N^{64j15}$ which also reduces expression of dnc^{+}. The effect of these independent chromosomal breaks on the dnc^{+} gene suggests the existence of a site to the left of dnc^{+} that is also required for proper expression of the gene.

DROSOPHILA has two major forms of phosphodiesterase that differ in molecular weight, substrate specificity, cation requirements and thermostability (KIGER and GOLANTY 1977, 1979; DAVIS and KIGER 1980). Form I, the cyclic nucleotide phosphodiesterase, hydrolyzes both cAMP and cGMP. This form of phosphodiesterase is activated by Ca^{2+} in the presence of calmodulin, a protein known to mediate the action of Ca^{2+} (KIGER and GOLANTY 1979; YAMANAKA and KELLY 1981; SOLTI *et al.* 1983; WALTER and KIGER 1984). Form II, the cAMP-specific phosphodiesterase, is not activated by calmodulin or Ca^{2+} (SOLTI *et al.* 1983; WALTER and KIGER 1984), has a high requirement for Mg^{2+} and is more heat labile than form I (KIGER and GOLANTY 1979).

KIGER and GOLANTY (1977) employed segmental aneuploidy in an attempt

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to locate the structural genes for these phosphodiesterases and located that for form II in chromomere 3D4. This region of the *X* chromosome is not required for viability, but it is essential for male fertility, normal female fertility and detectable form II phosphodiesterase activity (KIGER 1977; KIGER and GOLANTY 1979). Genetic analysis has revealed that chromomere 3D4 contains two genes: sperm amotile (*sam*), required for male fertility, and dunce (*dnc*), required for normal memory formation, female fertility and form II phosphodiesterase activity (BYERS, DAVIS and KIGER 1981; SALZ, DAVIS and KIGER 1982).

There are two lines of evidence suggesting that *dnc* is the structural gene for form II phosphodiesterase. First, there is a proportional relationship between the number of copies of chromomere 3D4 and form II activity (KIGER and GOLANTY 1979; SHOTWELL 1983). Second, two point mutations at the *dnc* locus, dnc^1 and dnc^2, exhibit reduced levels of form II activity associated with changes in the physical properties of the enzyme. The dnc^1 mutant possesses a thermolabile enzyme, and the dnc^2 mutant's enzyme has abnormal kinetics (KAUVAR 1982; DAVIS and KIGER 1981). As both of these properties persist after extensive purification of form II, it is probable that these alterations are due to changes in the primary structure of the enzyme (KAUVAR 1982).

The recombinational analysis presented here permits several *dnc* and *sam* mutations to be ordered in a fine structure map. During the course of this work the original dnc^1 strain was found to contain two closely linked mutations. Thermolability and reduction of form II enzyme activity appear to be associated with a mutation within a coding portion of the gene, whereas sterility is due to a mutation at a newly identified locus to the right of *dnc*, control of fertility (*cf*). This locus acts in *cis*, suggesting that it is a regulatory site required for normal expression of the dnc^+ gene. We propose that this site acts in conjunction with a functional *dnc* gene product to confer female fertility. The existence of two independent chromosomal breakpoints to the left of *dnc* that reduce the expression of the *dnc* gene suggests the possibility of additional regulatory sites to the left of the gene.

MATERIALS AND METHODS

Stocks: The deficiency and duplication chromosomes used have been described by KIGER and GOLANTY (1977). The deficiencies used in this study are $Df(1)N^{64i16}$ (3C3-3D4), $Df(1)N^{71i24-5}$ (3C4-3D4) and $Df(1)dm^{75e19}$ (3C12-3E4). The duplications used are $Dp(1;2)w^{+51b7}(dnc^+)$ (3C2-3D6), maintained in the second chromosome balancer *SM1;Cy* $Dp(1;2)w^{+51b7}$, and w^+Y (2D1-3D1). The right breakpoint for the w^+Y is that given by MCGINNIS, FARRELL and BECKENDORF (1980).

The isolation and characterization of mutations in the dunce and the sperm-amotile genes have been described by SALZ, DAVIS and KIGER (1982). The *dnc* mutations are balanced either with *C(1)DX;y w f* or *FM7* (MERRIAM and DUFFY 1972). Evidence for an additional locus adjacent to *dnc* is presented in this report. This locus is designated *cf* (control of fertility). The mutant allele, cf^1, is present in the original dnc^1 chromosome; the other chromosomes carry what we call the wild-type allele and designate cf^+. Whenever dnc^1 cf^1 and dnc^1 cf^+ are being compared, the stocks used are the parent stock, *y w* dnc^1 cf^1 *f* and *y* dnc^1 cf^+ *ec f*) (no. 74 from the cross between *y w* dnc^1 cf^1 *f* and *y* dnc^{M14} cf^+ *ec f*). The other *X* chromosomes used in this study are *y* dnc^{M14} *ec f*, *y w* dnc^{M14} *f*, *y* dnc^{M11} *ec*, *y w* dnc^{M11} *f*, *y w* dnc^{ML} f^{36a}, *y w* dnc^{GK} f^{36a}, *y w* dnc^2 *v f*, dnc^2 *ec f* and dnc^1 cf^1 *ec*.

GENERAL MAPPING STRATEGY

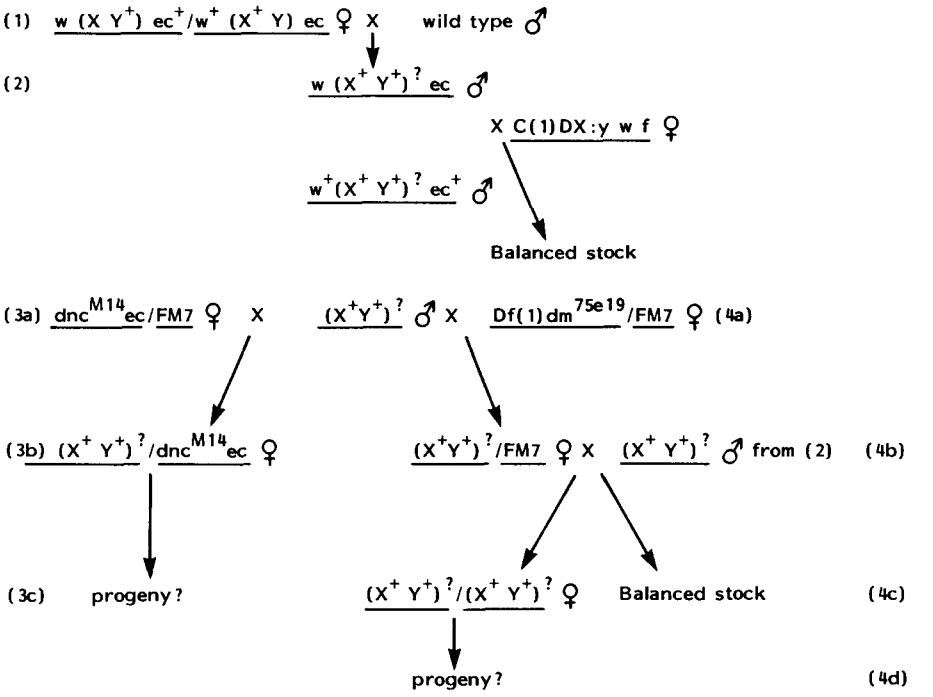

FIGURE 1.—Diagram of the general scheme used in constructing the fine structure map of chromomere 3D4. Details are given in the text.

Additional alleles of *sam* were sought by screening 2081 EMS-treated *X* chromosomes marked with *ec* for male-sterile mutations located between chromomeres 3D4 and 3D6. One new mutation, designated sam^2, was found, which, like sam^1, lacks motile sperm (SALZ, DAVIS and KIGER 1982). *y w* sam^1 f^{36a} and sam^2 *ec* are balanced either with *FM7* or with *C(1)DX:y w f;SM1:Cy Dp(1;2)*w^{+51b7}/+.

The wild-type stock used throughout is Canton-Special (Canton-S). Unless otherwise mentioned, LINDSLEY and GRELL (1968) have described the genetic markers and the balancers employed.

Genetic mapping: All crosses were carried out at 25° and will be described in RESULTS. Figure 1 is a diagram of the general strategy used in constructing the fine structure map of 3D4. Since the genes to be mapped are required either for male or for female fertility, only wild-type recombinants are recovered. The genes to be mapped (marked X and Y in the diagram) are coupled with either *w* or *ec*. Males recombinant for these flanking markers, produced in cross 1, are individually mated to attached-*X* females (cross 2). If these males are fertile (sam^+), a stock of the recombinant is established. When *dnc* alleles are mapped, each recombinant stock is then tested for the presence of *dnc* by backcrossing to dnc^{M14}/*FM7* females (cross 3a). If the nonbalanced females from this cross are sterile, then *dnc* is present. A stock producing homozygous females is then constructed by mating recombinant males from the attached-*X* stock to $Df(1)dm^{75e19}$/*FM7* virgins (cross 4a). In the next generation, balanced females are backcrossed to recombinant males and a balanced stock is established (cross 4b). If homozygous females from this stiock are fertile, then males from the attached-*X* stock are tested for form II enzyme activity. The map distances are reported in centimorgans (cM) ± the standard error as calculated by the method of STURTEVANT and BEADLE (1939).

In mapping the breakpoint of dnc^{CK}, *T(Y;3)R106* was used (LINDSLEY *et al.* 1972). *T(Y;3)R106* is

a translocation between the tip of the left arm of the third chromosome and the long arm of the *Y* chromosome. The breakpoint on the third chromosome is at 65D. The third chromosome with the small piece of the *Y* is marked with B^S, and the *Y* chromosome with the tip of *3L* is marked with y^+.

Fertility tests: The fertility of males was determined by mating two 1- to 5-day-old males with two to four virgin females. The vials were scored for the presence or absence of progeny on day 15. Three such matings were scored for each stock tested.

The fertility of females was determined by a two-step procedure. Initially, six females of each stock were tested, two to a vial with excess males. Each vial was scored for the presence or absence of progeny on day 15. If any vial contained ten or more progeny, ten females of that stock were retested individually. Single 0- to 5-day-old females (virgin or nonvirgin) were placed in a vial with two to three males and allowed to lay eggs for 6 days. The vials were then scored on day 17. Stocks often had to be retested in this way because *dnc* females, depending on the genetic background, occasionally produce progeny (SALZ, DAVIS and KIGER 1982). For a stock to be scored as fertile, the majority of females must each have 30 or more progeny. Testing ten females is sufficient to distinguish between stocks that are essentially sterile and those that are fertile. All tests were carried out at 25°.

Phosphodiesterase activity in whole-fly homogenates: Crude homogenates were prepared by homogenizing four male flies (3–5 days old) in 0.3 ml of buffer A (40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ and 2 mM 2-mercaptoethanol) at 0° followed by centrifugation for 5 min in the microfuge at 2°–5°. Aliquots (0.05 ml) of the supernatant were assayed for form II activity in a reaction mixture of 0.1 ml containing 20 μM [^{3}H]cAMP, 2 mM cold cGMP, in buffer A, incubated for 10 min at 30° (SHOTWELL 1983). The reaction was stopped by heating at 90° for 2 min. After cooling, 0.02 ml of carrier (5 mg/ml of 5′-AMP, cAMP, adenosine and inosine) was added to the reaction mixture. The 5′-AMP and nucleosides were separated from cAMP by a PEI chromatography separation similar to the one described by RANGAL-ALDAO, SCHWARTZ and RUBEN (1978). The reaction mixture, 0.005 ml, was spotted on a PEI cellulose strip and run in an ascending system in 50 mM KCl for 30 min. The spots were then viewed under a shortwave UV lamp; the 5′-AMP and nucleoside spots were cut from the strips and eluted with 1.0 ml of 100 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, for 30 min directly in the scintillation vials. Radioactivity was counted in 7 ml of scintillation cocktail (KIGER and GOLANTY 1979). Enzyme activity is calculated as pmoles of cyclic nucleotide hydrolyzed per minute per micrograms of protein. Protein determinations were done by the method of LOWRY *et al.* (1951). All activities reported are the means of the activities of three independent extracts of flies of the same genotype. We chose to average the activities of three independent extracts because the variation between different homogenates is larger than the variation inherent to the assay system.

Phosphodiesterase thermostability: Sixteen male flies (3–5 days old) were homogenized in 1.2 ml of buffer A on ice. The homogenate was then centrifuged for 5 min in a microfuge at 2°–5°. Aliquots were heated at 42° for various times and cooled immediately to 0°; 0.05 ml of each aliquot was then assayed as described before, incubating for 20 min at 21°. The reaction was stopped, and the reaction products were separated as described. The amount of activity remaining is expressed as the percent of initial activity in the untreated homogenate.

RESULTS

Form II phosphodiesterase activity of dnc *mutants*: The effect of different *dnc* mutations on the level of form II activity has previously been studied by estimating the amount of form II activity present after separation of form I and form II on sucrose gradients. However, the recovery of enzyme activities in such experiments is variable, making it difficult to quantify the precise amount of form II activity present in each mutant strain (DAVIS and KIGER 1981).

The amount of form II activity exhibited by the different *dnc* mutants is here measured directly in crude homogenates using an assay designed by SHO-

TABLE 1

Form II phosphodiesterase activities in males

Genotype	Activity (pmoles/min μg protein ± 2 SEM)	Wild type activity %
Canton-S	1.66 ± 0.16	100
dnc^1	0.85 ± 0.08	51
dnc^{CK}	0.58 ± 0.04	35
dnc^2	0.25 ± 0.03	15
dnc^{M14}	0.07 ± 0.01	4
dnc^{M11}	0.07 ± 0.01	4
dnc^{ML}	0.05 ± 0.03	3
$Df(1)N^{64i16}/w^+Y$	0.07 ± 0.02	4
$Basc/w^+Y$	1.58 ± 0.19	95

Males deficient for chromomere 3D4 and their control were generated by mating $Df(1)N^{64i16}/Basc;SM1:Cy\ Dp(1;2)w^{+51b7}/+$ females to $Df(1)w^{-67k30}/w^+Y$ males.

TWELL (1983). This assay takes advantage of the fact that cGMP is a competitive inhibitor of form I cAMP hydrolysis (DAVIS and KIGER 1980), permitting form II activity to be estimated by the amount of [^{3}H]cAMP hydrolysis in the presence of excess cold cGMP. The results are presented in Table 1. Three *dnc* alleles, dnc^1, dnc^2 and dnc^{CK}, exhibit, respectively, 51, 15 and 35% of wild-type activity. The alleles dnc^{ML}, dnc^{M11} and dnc^{M14} exhibit about 4% of wild-type activity, the same level exhibited by flies homozygous for a deletion of chromomere 3D4. This activity may be due to form I activity that is not completely inhibited by cGMP. Therefore, dnc^{ML}, dnc^{M11} and dnc^{M14} appear to be null alleles, supporting the previous conclusions of DAVIS and KIGER (1981).

Mapping the dnc^{CK} *breakpoint*: The *dnc* mutations, except for dnc^{CK}, are in cytologically normal *X* chromosomes. The dnc^{CK} mutation is associated with a reciprocal translocation between the tip of *3L* and the *X*. The cytological breakpoint on the *X* is just to the right of 3C and to the left of 4; the breakpoint on *3L* is at 63C (SALZ, DAVIS and KIGER 1982). The dnc^{CK} mutation exhibits residual form II activity, therefore, it is likely that the breakpoint is adjacent to the dnc^+ gene. The *X* chromosome breakpoint was mapped with respect to the *dnc* gene using the residual form II activity of dnc^{CK} as a marker. If the breakpoint is proximal to *dnc*, the *dnc* gene will have been translocated to the third chromosome. If this is the case, the residual form II activity of dnc^{CK} should map to the translocation third chromosome. A male hemizygous for dnc^{M14} and with one copy of the translocation third chromosome would be expected to have 35% of wild-type activity. On the other hand, if the *X* chromosome breakpoint is distal to the *dnc* gene, the *dnc* gene would remain on the *X* chromosome. A male hemizygous for dnc^{M14} and one copy of the translocation third chromosome would then be expected to have the same amount of activity as dnc^{M14} males.

The crosses to generate these males are diagrammed in Figure 2. Because the translocation third chromosome of dnc^{CK} is deficient for the tip of *3L*, males must also carry a *Y* chromosome duplication for the tip of *3L* in order

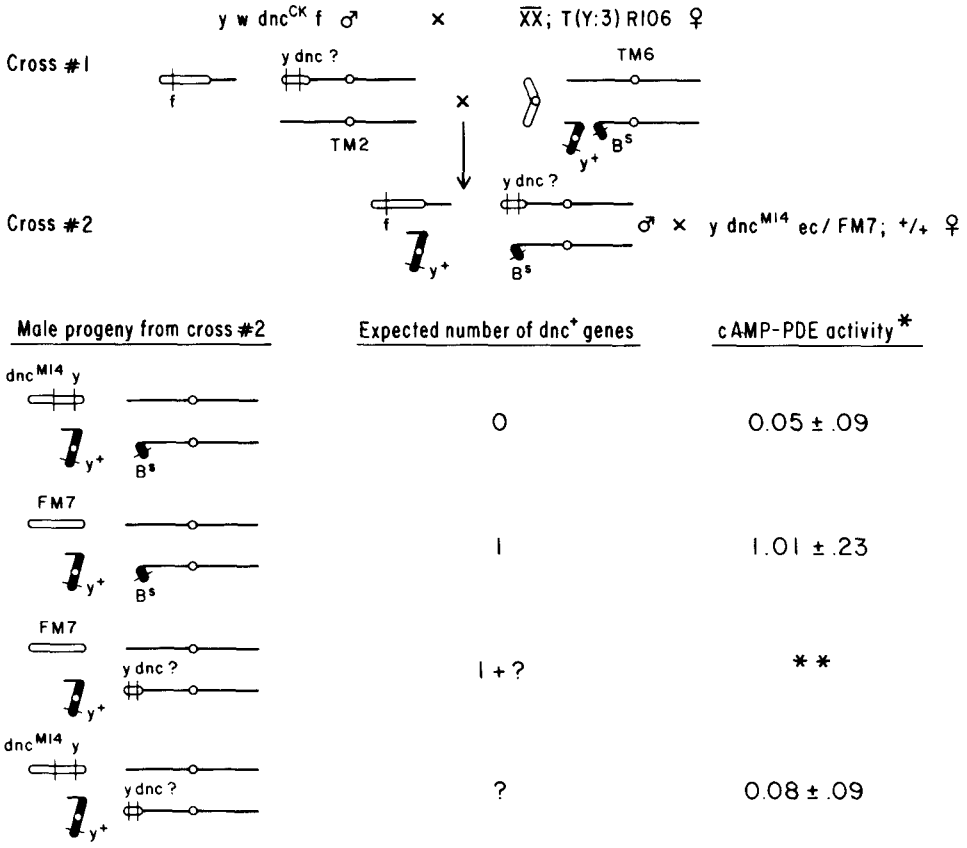

FIGURE 2.—A diagram of the scheme used to map the *X* chromosome breakpoint in dnc^{CK}. Details in text. *, The amount of activity is given in picomoles per minute per microgram of protein. **, These males are indistinguishable from *FM7;T(Y;3)R106* males. However, since they can only be generated from adjacent I segregation in the male they are rare.

to survive. Such a partial *Y* chromosome is derived from the *T(Y;3)R106* stock (cross 1). The control males, *FM7;T(Y;3)R106* with one copy of dnc^+, and dnc^{M14}*;T(Y;3)R106*, a null allele of *dnc*, are generated by alternate segregation in the male parent (cross 2). Males carrying both dnc^{M14} and the translocation third chromosome are generated by adjacent I segregation. An additional class of males is generated from adjacent I segregation that is indistinguishable from *FM7;T(Y;3)R106*. These males, hemizygous for *FM7* and with the translocation third chromosome, should be rare; it is likely that they were not assayed along with the control males.

The males carrying both dnc^{M14} and the translocation third chromosome of dnc^{CK} have the same activity as dnc^{M14}*;T(Y;3)R106* males (Figure 2). Thus, the translocation third chromosome does not carry dnc^+. The *X* chromosome breakpoint in dnc^{CK} is, therefore, distal to dnc^+.

Intragenic recombination between sam *mutations*: Since complementation of *X*-linked mutations in males is not possible, the newly isolated male-sterile mutation (tentatively designated sam^2) was mapped with respect to sam^1 (Table 2).

TABLE 2

Recombination in the region surrounding dnc

Maternal genotype	No. of males scored	No. collected		No. *sam*$^+$		No. *sam*$^+$ *dnc*$^+$		No. *dnc*$^+$	
		w ec	*w*$^+$ *ec*$^+$	*w ec*	*w*$^+$ *ec*$^+$	*w ec*	*w*$^+$ *ec*$^+$	*w ec*	*w*$^+$ *ec*$^+$
$\frac{w\ sam^1\ +}{+\ sam^2\ ec}$	30,853	744	485	0	3				
$\frac{w\ +\ dnc^{M11}\ +}{+\ sam^2\ +\ ec}$	18,407	433	313	131	115	7	0		
$\frac{+\ dnc^{M11}\ ec}{w\ +\ +}$	1,487	27	30					17	25

Male progeny recombinant in the white-echinus region, produced by females of the genotype *w sam*^{1} *ec*$^+$/*w*$^+$ *sam*^{2} *ec*, were individually mated to attached-*X* virgin females. Of the 1229 males tested, three were found to be fertile. The map distance between the two mutations is, therefore, 0.02 ± 0.01 cM, suggesting that the two male-sterile mutations are alleles of the same gene. The order, *w sam*^{1} *sam*^{2} *ec*, can be determined by the phenotypes of the *sam*$^+$ recombinants which are all *w*$^+$ *ec*$^+$.

Intergenic map distances between sam, dnc *and flanking markers*: The *dnc* gene is to the right of *sam*^{1} (SALZ, DAVIS and KIGER 1982). To establish the position of *sam*^{2}, it has been mapped with respect to *dnc*^{M11}, the leftmost allele of *dnc*. The cross and results are given in Table 2. Male progeny recombinant in the white-echinus region, produced by females of the genotype *w sam*$^+$ *dnc*^{M11} *ec*$^+$/*w*$^+$ *sam*^{2} *dnc*$^+$ *ec*, were individually crossed to attached-*X* females to establish stocks of *sam*$^+$ recombinants. Each of these stocks was then tested for the presence of *dnc*^{M11} by backcrossing recombinant males to *dnc*^{M14}/*FM7* females and testing the fertility of the non-*FM7* female progeny. The order *w sam*^{2} *dnc*^{M11} *ec* was determined by the phenotypes of the *sam*$^+$ *dnc*$^+$ recombinants: all were *w ec*. This confirms the gene order reported in SALZ, DAVIS and KIGER (1982), and it is further evidence that *sam*^{1} and *sam*^{2} are alleles of the same gene. Since *sam*^{2} is the rightmost allele of the *sam* gene and *dnc*^{M11} is the leftmost allele of the *dnc* gene, the maximum distance between the two genes is 0.08 ± 0.02 cM. From this same experiment the distance between *sam* and *w* can be calculated to be 2.3 ± 0.1 cM.

To complete the map of this region, *dnc*^{M11} was mapped with respect to *ec* (Table 2). Males recombinant in the white-echinus region, produced by females of the genotype *w dnc*^{M11} *ec*$^+$/*w*$^+$ *dnc*$^+$ *ec*, were individually crossed to attached-*X* females. Each stock was then tested for the presence of *dnc*^{M11} by backcrossing males to *dnc*^{M14}/*FM7* females and testing the fertility of the non-*FM7* females. The results show that *dnc*^{M11} is 1.5 ± 0.3 cM to the left of *ec*.

Intragenic recombination between dnc *mutations*: Intragenic recombinants were selected on the basis of female fertility and then tested for the restoration of normal form II enzyme activity. The strategy used to select intragenic recombinants is similar to that described in the preceding sections. Males recombinant in the white-echinus region, produced by females of genotype *w dnc*^{X} *ec*$^+$/*w*$^+$ *dnc*^{Y} *ec;SM1:Cy Dp(1;2)w*$^{+51b7}$/+, were mated individually to attached-*X* virgin

TABLE 3

Recombination that restores wild-type form II activity

Maternal genotype	No. of males scored	Classification of recombinants between *w* and *ec*: No. collected *w ec*	No. collected w^+ ec^+	No. fertile *w ec*	No. fertile w^+ ec^+	Summary of form II activity of each fertile recombinant[a]
$\frac{w\ dnc^{M14} +}{+\ dnc^{M11}\ ec}; \frac{Cy\ Dp(dnc^+)}{+}$	20,424	506	270	4	0	No. 35: 2.29 ± 0.10 No. 287: 2.31 ± 0.43 No. 756: 2.49 ± 0.61 No. 794: 2.45 ± 0.63
$\frac{w\ dnc^{2} +}{+\ dnc^{M11}\ ec}; \frac{Cy\ Dp(dnc^+)}{+}$	6,692	98	76	1	0	No. 214: 2.36 ± 0.15
$\frac{w\ dnc^{M14} +}{+\ dnc^{2}\ ec}; \frac{Cy\ Dp(dnc^+)}{+}$	18,485	506	298	0	6[b]	No. 445: 1.98 ± 0.36 No. 447: 1.89 ± 0.08 No. 448: 2.13 ± 0.39 No. 450: 2.44 ± 0.10 No. 452: 1.99 ± 0.28 No. 467: 1.92 ± 0.21
$\frac{w\ dnc^{ML} +}{+\ dnc^{M14}\ ec}; \frac{Cy\ Dp(dnc^+)}{+}$	20,905	290	297	1	0	
$\frac{w\ dnc^{ML} +}{+\ dnc^{2}\ ec}; \frac{Cy\ Dp(dnc^+)}{+}$	14,789	236	292	[d]		No. 195: 2.50 ± 0.26

Cy Dp is the abbreviation for *SM1:Cy Dp(1;2)w*$^{+51b7}$.

[a] The activity is expressed in pmoles/min/μg protein ± 2 SEM.

[b] Found as a cluster; therefore, they were generated by a premeiotic event (see text for details).

[c] Lost before enzyme level could be tested.

[d] A presumed mosaic (see text for details).

females. Each stock was then tested for female fertility. Those stocks that were fertile were then tested for form II enzyme activity. The fertile recombinant stocks fall into two classes: those that restore normal form II activity (Table 3) and those that do not (Table 4). Those recombinants that do not restore wild-type enzyme activity come from crosses that involve dnc^1.

The fertile recombinants from the females dnc^{M14}/dnc^{M11}, dnc^2/dnc^{M11} and dnc^{M14}/dnc^2 were found to have wild-type enzyme activity (Table 3). Four intragenic recombinants were found among the 20,424 progeny of dnc^{M11}/dnc^{M14} females. All of the recombinants are *w ec*, therefore, dnc^{M11} is 0.04 ± 0.01 cM to the left of dnc^{M14}. A single *w ec* recombinant was found from dnc^2/dnc^{M11} females, suggesting that dnc^2 is to the right of dnc^{M11}. The recovery of a single *w ec*, female-fertile strain produced from dnc^{ML}/dnc^{M14} females suggests that dnc^{ML} is to the right of dnc^{M14}. However, this strain was lost before its enzyme activity could be tested.

The "recombinants" from the dnc^2/dnc^{M14} females were found in a cluster, suggesting that a single premeiotic event was responsible for the restoration of dnc^+ activity. Therefore, it is unlikely that any of these were intragenic recombinants (see DISCUSSION).

TABLE 4

Recombination that does not restore wild-type form II activity

Maternal genotype	No. of males scored	Classification of recombinants between *w* and *ec*					
		No. collected		No. fertile		No. dnc^{+a}	
		w ec	w^+ ec^+	*w ec*	w^+ ec^+	*w ec*	w^+ ec^+
$\frac{w\ dnc^1\ +}{+\ dnc^{M14}\ ec}; \frac{Cy\ Dp(dnc^+)}{+}$	6,893	82	162	3	0	0	0
$\frac{w\ dnc^1\ +}{+\ dnc^{M11}\ ec}; \frac{Cy\ Dp(dnc^+)}{+}$	8,468	122	118	16	4[b]	0	0
$\frac{w\ dnc^1\ +}{+\ dnc^2\ ec}; \frac{Cy\ Dp(dnc^+)}{+}$	19,140	363	447	6	1[b]	0	0
$\frac{dnc^1\ ec}{Df(1)N^{71k24\text{-}5}}; \frac{Cy\ Dp(dnc^+)}{+}$	11,434		256		21		0

[a] Based on enzyme activity, all of the fertile recombinants have the same amount of activity as the dnc^1 parent.

[b] Generated by gene conversion (see text for details).

From females of genotype *y w* dnc^{ML} f^{36a}/dnc^2 *ec f* a single dnc^+ recombinant was recovered (Table 3). This stock was established by crossing a male of phenotype *y w ec* to an attached-X virgin female. In the following generations all of the males were found to be *y* w^+ dnc^+ *ec*. Evidently, the recombinant male was a mosaic for *w* and w^+ but did not possess a mosaic germ line. Possible origins of this mosaic will be considered in DISCUSSION.

Intragenic recombinants between dnc^1/dnc^{M14}, dnc^1/dnc^{M11} and dnc^1/dnc^2 were also selected on the basis of female fertility and then tested for form II activity (Table 4). Unexpectedly, these crosses produced female-fertile recombinants that exhibit form II activity comparable to that of the dnc^1 parent. The results in Table 5 show the activity of one of these recombinants (no. 74) from the dnc^1/dnc^{M14} cross. This stock is representative of all fertile recombinants generated between dnc^1 and the other mutations. To determine whether the dnc^1 level of enzyme activity exhibited by these recombinants is due to an unaltered dnc^1 allele, the thermostability profiles of a representative sample of these recombinants were compared with the profiles of wild-type and of the dnc^1 parent stock. Figure 3 shows the results obtained with one of these recombinants (no. 74) from the dnc^1/dnc^{M14} cross. The thermostability profile of this recombinant is similar to the profile of the dnc^1 parent. Therefore, the dnc^1 allele in no. 74 is unaltered.

To account for these unexpected observations, the existence of an additional locus, control of fertility (*cf*), has been postulated. The dnc^1 chromosome is postulated to carry a mutant allele cf^1, whereas the other *dnc* chromosomes carry the wild-type allele cf^+. Thus, the sterility of dnc^1 cf^1 females must be due to cf^1.

The majority of female-fertile recombinants recovered from females of the genotype *w* dnc^1 cf^1 ec^+/w^+ dnc^X cf^+ *ec;SM1:Cy* $Dp(1;2)w^{+51b7}/+$ are of the genotype *w* dnc^1 cf^+ *ec*. For these recombinants to contain the dnc^1 mutation, the

TABLE 5

Form II activity of dnc^1 cf^1 *and* dnc^1 cf$^+$ *genotypes*

	Males	Females
Canton-S	2.06 ± 0.43	2.30 ± 0.09
dnc^1 *cf*^{1}	0.85 ± 0.28	1.18 ± 0.20
dnc^1 *cf*$^+$ (No. 74)	0.93 ± 0.11	0.97 ± 0.06

Activities are expressed in pmoles/min/μg protein ± 2 SEM.

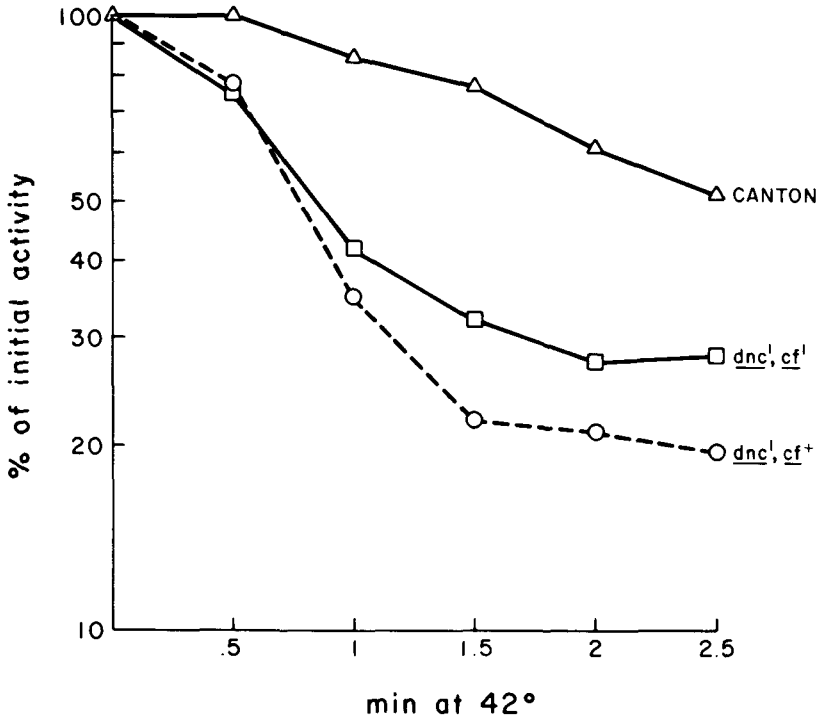

FIGURE 3.—Thermostability profiles of form II phosphodiesterase in crude homogenates of wild-type, *dnc*^{1} *cf*^{1} and *dnc*^{1} *cf*$^+$ males.

recombination event must have taken place between *dnc*^{1} and *cf*, placing *cf* to the right of *dnc*^{1}. The distance between *dnc*^{1} and *cf* can be estimated from the data in Table 4. Three *w dnc*^{1} *cf*$^+$ *ec* stocks were established from the 6893 progeny of *dnc*^{1}/*dnc*^{M14} females. The distance between *dnc*^{1} and *cf* from this cross is 0.09 ± 0.04 cM. Similarly, six *w dnc*^{1} *cf*$^+$ *ec* stocks were established from 19,140 progeny of *dnc*^{1}/*dnc*^{2} females giving a map distance of 0.06 ± 0.02 cM between *dnc*^{1} and *cf*. One *w*$^+$ *dnc*^{1} *cf*$^+$ *ec*$^+$ recombinant was also recovered from this cross. It was not included in the mapping data because it is most likely the result of a gene conversion event (see DISCUSSION). Four more potential gene conversion events were found among the progeny of *dnc*^{1}/*dnc*^{M11} females. These events will be considered further in the DISCUSSION. The frequency of gene conversion events in the progeny of *dnc*^{1}/*dnc*^{M11} females is

TABLE 6

Cis *action of* cf

Genotype	Mean no. of progeny/ female (± SEM)	
dnc^1 cf^1/dnc^1 cf^1	0	(n = 16)
dnc^{M14} cf^+/dnc^{M14} cf^+	0	(n = 10)
dnc^1 cf^+/dnc^1 cf^+	30.0 ± 6.5	(n = 9)
dnc^1 cf^1/dnc^{M14} cf^+	2.3 ± 1.0	(n = 10)
dnc^1 cf^+/dnc^1 cf^1	30.8 ± 6.5	(n = 10)

ten times higher than that found in the dnc^1/dnc^2 cross. Furthermore, six times as many female-fertile recombinants were recovered in the dnc^1/dnc^{M11} cross compared with the other two crosses. Because of the high frequency of conversion, the data from the dnc^1/dnc^{M11} cross have not been used to establish a distance between dnc^1 and *cf*.

The *cf* locus has also been mapped with respect to $Df(1)N^{71h24-5}$ (Table 4). Wild-type males (ec^+) produced by females of the genotype dnc^1 cf^1 *ec*/ $Df(1)N^{71h24-5}$;*SM1:Cy* $Dp(1;2)w^{+51b7}$/+ are the result of a recombination event between the deficiency breakpoint and *ec*. Stocks were established by individually mating ec^+ males to attached-*X* virgin females. Each stock was then tested for female fertility and form II enzyme activity. Twenty-one ec^+ recombinants were found to be female fertile and all of these exhibit form II activity indistinguishable from the dnc^1 parent (data not shown). The apparent map distance between the deficiency breakpoint and *cf* is 0.18 ± 0.04 cM and between the breakpoint and *ec* is 2.2 ± 0.14 cM (see DISCUSSION, however).

Interaction of cf *alleles with* dnc^1: The original dnc^1 strain, dnc^1 cf^1, does not complement the other *dnc* alleles with respect to fertility (SALZ, DAVIS and KIGER 1982). However, dnc^1 cf^+ recombinants were recovered because they did complement the sterility of dnc^{M14} cf^+. The nature of the interaction between *cf* and *dnc* can be deduced from the data in Table 6. It is noteworthy that, although dnc^1 cf^1/dnc^1 cf^+ females are fertile, dnc^1 cf^1/dnc^{M14} cf^+ females are sterile. Fertility is present only when dnc^1 is coupled with cf^+. The *cis*-acting nature of *cf* suggests that it is a regulatory locus required for the proper expression of the *dnc* gene.

DISCUSSION

Fine structure map: The mapping experiments described here permit *sam* and *dnc* mutations to be ordered in a fine structure map (Figure 4). In the preliminary mapping studies reported in the previous paper (SALZ, DAVIS and KIGER 1982), a significant reduction in recombination frequency over the white-echinus region was observed in crosses involving dnc^{M14}. The data presented here do not substantiate those observations. The recombination frequencies observed between *w* and *ec* in Table 2 are not significantly different from the standard recombination frequency of 4% (LINDSLEY and GRELL 1968), and the data in Tables 3 and 4 do not reveal any consistent reductions in the recom-

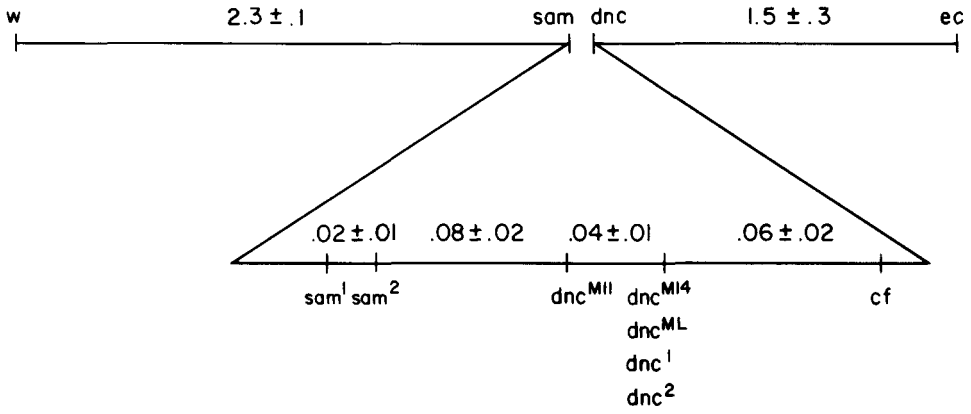

FIGURE 4.—Fine structure map of chromomere 3D4. Map distances are given in centimorgans.

bination frequency between *w* and *ec* associated with any *dnc* mutations. Nor do the data show any interchromosomal enhancement of recombination frequency by the *SM1* balancer (SCHULTZ and REDFIELD 1951).

Intragenic recombinants within the *dnc* locus, selected on the basis of restoration of female fertility, fall into two classes: those that exhibit restored normal form II activity and those that do not. Recombination events between null mutations that restore wild-type enzyme activity are clearly intragenic events. Four such events were observed among the 20,424 progeny of dnc^{M11}/dnc^{M14} females. These two null mutations are separated by 0.04 cM. No intragenic recombinants were found in 14,789 progeny of the cross between dnc^{ML} and dnc^{2} or in the 18,458 progeny of the cross between dnc^{2} and dnc^{M14}. Therefore, dnc^{2} must be less than 0.01 cM from dnc^{M14} and dnc^{ML}. A single fertile recombinant between dnc^{ML} and dnc^{M14} was recovered among 20,905 progeny. Since this recombinant was not tested for the restoration of form II activity, it is not possible to confirm it as a true intragenic recombinant; however, dnc^{ML} must be less than 0.01 cM from dnc^{M14}. In the cross between dnc^{2} and dnc^{1}, none of the recombinants exhibit restored wild-type enzyme activity. Consequently, dnc^{1} can be no more than 0.01 cM from dnc^{2}. This places dnc^{1} within the same cluster as dnc^{ML}, dnc^{M14} and dnc^{2}. On the other hand, dnc^{M11} is located 0.04 cM to the left of this cluster. A single recombinant among 6692 progeny was found in the cross between dnc^{M11} and dnc^{2}, confirming that dnc^{M11} is located to the left of dnc^{2} and, consequently, the other mutations in the cluster as well.

In the course of these experiments several unexpected classes of progeny were observed. The "recombinants" from the dnc^{2}/dnc^{M14} cross were found in a cluster, suggesting that a single premeiotic event was responsible for the restoration of dnc^{+} activity. This conclusion has been substantiated by R. L. DAVIS (personal communication) who has found that these recombinants each contain a 500-base pair insertion in or near the *dnc* gene.

In the cross between dnc^{2} and dnc^{ML} a single male was found that proved to be a mosaic for w^{+} and *w*. Unfortunately, the genotype of only the w^{+}-bearing chromosome is known because only *y* w^{+} dnc^{+} *ec* males were recovered

from the progeny of the mosaic male. To generate such a mosaic two rare events must have occurred. A single crossover event between the two *dnc* mutations accompanied by a spontaneous reversion at the white locus could have produced the *y* w^+ dnc^+ *ec* chromosome. On the other hand, mosaics can arise from the failure of heteroduplex DNA generated during recombination to be repaired (CARPENTER 1982; HILLIKER and CHOVNICK 1981). Thus, at the completion of meiosis the *X* chromosome may have contained heteroduplex DNA at the white gene. This mosaicism at the DNA level would be resolved after the first mitotic division, resulting in an embryo that is mosaic for *w* and w^+. To generate a chromosome that is dnc^+, the recombination event that generated the unrepaired heteroduplex DNA could have been accompanied by gene conversion or spontaneous reversion of *dnc* to dnc^+.

When mapping dnc^{M11}, dnc^{M14} and dnc^2 with respect to dnc^1, recombinants between *w* and *ec* were found to exhibit restored female fertility but not wild-type enzyme activity. Because these fertile stocks retain the thermolabile enzyme activity present in the parent dnc^1 stock we propose that these recombination events did not occur between the *dnc* alleles present in these crosses. We propose that the original dnc^1 strain is a double mutant. It has a mutation, within the *dnc* structural gene, associated with reduced and thermolabile enzyme activity, and it has another mutation, at *cf*, associated with female sterility. Based on the observation that the majority of dnc^1 cf^+ recombinants are *w ec*, *cf* must be to the right of dnc^1. However, five such recombinants exhibit the other flanking marker configuration w^+ dnc^1 cf^+ ec^+. CARPENTER (1982) has called such recombinants "reverse cross-overs."

Reverse crossovers are very rare. They have never been observed during the course of the extensive fine structure analysis of the rosy gene, except in the presence of *mei-9* (CARPENTER 1982). The *mei-9* mutant, defective in excision repair (BOYD, GOLINO and SETLOW 1976), increases conversion frequencies while decreasing the crossover frequency throughout the genome (BAKER and CARPENTER 1982; CARPENTER 1982). It is not inconceivable that there might be a meiotic mutation, similar to *mei-9*, in the *w* dnc^1 cf^1 stock. If such a mutation is assumed to be recessive (as all known meiotic mutations are), this mutation must also be in the dnc^{M14} *ec*, dnc^{M11} *ec* and dnc^2 *ec* stocks but not in the *w* dnc^{ML}, *w* dnc^{M14} or *w* dnc^2 stocks. Were it in the latter stocks as well, reverse crossovers should have been observed in other crosses, but none were observed in crosses that did not involve dnc^1. On the other hand, the average amount of recombination between *w* and *ec* is similar in those crosses that involve dnc^1 and those that do not (3.8 and 3.5%, respectively). Therefore, it is unlikely that a meiotic mutation is present in the dnc^1 stock.

The reverse crossovers could also be generated by two independent events: (1) a crossover event between *w* and dnc^1, resulting in a w^+ dnc^1 cf^1 ec^+ configuration, and (2) either a gene conversion event at *cf* or a spontaneous reversion of cf^1 to cf^+. Five reverse crossovers were found among the 727 w^+ ec^+ recombinants tested for fertility, giving a reversion or conversion frequency of 0.007. However, the *w* dnc^1 cf^1 stock appears to be stable, making it unlikely that cf^1 spontaneously reverted to cf^+. Therefore, it is likely that cf^1 converted to cf^+ and that cf^1 exhibits an unusually high frequency of conversion.

Gene conversion frequencies have been correlated with marker position within the gene; markers at either end of the gene have a higher conversion frequency than those in the middle of the gene (HILLIKER and CHOVNICK 1981). This polarity in gene conversion suggests that initiation of recombination occurs at specific sites. Therefore, the high gene conversion frequency at cf^1 might be interpreted as a high probability for initiation of recombination at this site. If this is the case, the distance calculated between *cf* and *dnc*, 0.06 ± 0.02 cM, could be an overestimate. The exceptionally high recombination frequency observed in the cross with $Df(1)N^{71h24-5}$ (Table 4) might also be explained in this way.

Regulation of dunce gene expression: The original dnc^1 strain is believed to contain two mutations, dnc^1, associated with reduced and thermolabile enzyme activity, and cf^1, associated with female sterility. Female fertility is restored when the wild-type allele, cf^+, is coupled to dnc^1, suggesting that the dnc^1 mutant allele provides enough enzyme activity for female fertility. The *cis*-acting nature of *cf* suggests that it is a regulatory locus. We do not suggest any mechanism for this regulation; we state only that the sequence of DNA identified by cf^+ is required for proper expression of the adjacent *dnc* locus.

The fertility defect of the dnc^1 cf^1 strain can be suppressed by *Su(fs)* (SALZ, DAVIS and KIGER 1982; SALZ 1983). *Su(fs)* (1-63.9), a dominant suppressor, restores the fertility of dnc^1 cf^1 females without changing either the level of activity or the temperature sensitivity of the form II enzyme. *Su(fs)* is allele specific and does not suppress the female sterility of other *dnc* mutations. This observation, and the observed fertility of dnc^1 cf^+ females, suggests that *Su(fs)* suppresses the effect of cf^1. Thus, the product of $Su(fs)^+$ may normally interact with the *cf* locus to effect proper expression of the dnc^+ gene. Mutations at either *Su(fs)* or *cf* do not detectably alter the form II levels in whole-fly homogenates. However, this does not preclude a tissue-specific change in enzyme activity. These observations suggest that both cf^+ and $Su(fs)^+$ may be involved in those aspects of dunce expression relevant to female fertility.

The dnc^{CK} chromosomal breakpoint affects both fertility and form II activity. The breakpoint has been mapped to the left of dnc^+. However, we have not yet mapped the dnc^{CK} breakpoint with respect to *sam*. The breakpoint of $Df(1)N^{64j15}$, to the left of *sam*, also reduces the level of form II activity and reduces the fertility of females (SALZ, DAVIS and KIGER 1982). Neither $Df(1)N^{64j15}$ nor dnc^{CK} affect male fertility (SALZ, DAVIS and KIGER 1982), however, a slight reduction in male fertility would not have been detected. Therefore, it is conceivable that both dnc^{CK} and $Df(1)N^{64j15}$ reduce the expression of *sam* as well as *dnc*.

Recently, DNA sequences within chromomere 3D4 have been cloned and dnc^2 tentatively located within a 10- to 12-kb segment (DAVIS and DAVIDSON 1984). The $Df(1)N^{64j15}$ deficiency breakpoint is located about 35 kb to the left of this region. However, there are two additional deletions and a small insertion in the $Df(1)N^{64j15}$ chromosome located closer to *dnc* (R. L. DAVIS, personal communication). It is not known which alteration is responsible for the reduction in *dnc* expression. The translocation breakpoint of dnc^{CK} is located within 5 kb of the $Df(1)N^{64j15}$ deficiency breakpoint (R. L. DAVIS, personal commu-

nication), confirming our genetic results. No additional DNA rearrangements have been noted. Curiously, in the Canton-S *X* chromosome, a middle repetitive sequence is found within a few kilobases of the $Df(1)N^{64j15}$ deficiency breakpoint; however, this 7.2-kb insertion appears to have no effect on *dnc* expression relative to other wild-type strains that do not have the insertion (DAVIS and DAVIDSON 1984).

A region of 0.04 cM, absolutely required for *dnc* expression, is identified by the null mutations dnc^{M11}, dnc^{M14} and dnc^{ML}. If one uses the conversion of 300 kb/cM found for the Notch region (ARTAVANIS-TSAKONAS, MUSKAVITCH and YEDVOBNICK 1983), *dnc* must be at least 12 kb in extent. Moreover, although the molecular analysis does not allow us to determine precisely which sequences distal to *dnc* are required for proper expression, the independent occurrence of two breakpoints to the left of *dnc* that affect its expression argues for the importance of this region in the proper expression of *dnc*. Furthermore, sequences (*cf*) 0.06 cM, or about 18 kb, to the right of *dnc* appear to be important in those aspects of *dnc* expression relevant to fertility. Thus, the *dnc* gene and its regulatory sequences may encompass more than 30 kb of DNA.

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