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Isolation of the *Drosophila melanogaster* Dunce Chromosomal Region and Recombinational Mapping of Dunce Sequences with Restriction Site Polymorphisms as Genetic Markers

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Using the method of chromosomal walking, we have isolated a contiguous region of the *Drosophila melanogaster* X chromosome which corresponds to salivary gland chromosome bands 3C12 to 3D4. This five-band region contains approximately 100 kilobases of DNA, including those sequences comprising dunce, a gene which functions in memory and cyclic nucleotide metabolism. Genome blots of DNA from flies carrying several different chromosomal aberrations with breakpoints in the region have been probed with the isolated clones to map the breakpoints on the cloned DNA and to delimit dunce sequences. This has localized dunce to a 50-kilobase region. In addition, we have searched this 50-kilobase region for restriction site polymorphisms between X chromosomes from different *Drosophila* strains by genome blotting experiments, and we have followed the segregation of detected polymorphisms and dunce alleles after meiotic recombination. The data map one dunce mutation between two polymorphisms located 10 to 12 kilobases apart.

The dunce (*dnc*) gene of *Drosophila melanogaster* is of special interest because it plays a role in cyclic nucleotide metabolism and a variety of behavioral processes. The *dnc* mutant flies execute poorly several different associative learning tasks, including those employing olfactory (9) and visual (11) cues, with positive (31) or negative (9) reinforcement. The associative learning deficit is manifest not only in flies but also in *dnc* mutant larvae (1). Operant conditioning is altered by lesions in this gene (3), as are the nonassociative learning responses of habituation and sensitization (10). In addition, *dnc* mutations disrupt one aspect of normal courtship behavior (13). Although normal learning by *dnc* mutants is not detected in some situations (11), the mutants learn normally but forget rapidly in others (8, 10, 31). Consequently, *dnc* flies are best classed as memory mutants.

The observation that the *dnc* mutation perturbs normal cyclic AMP metabolism (4) led to the suggestion that cyclic AMP is intimately involved in behavioral plasticity, a conclusion also reached from studies of learning and memory by *Aplysia* (15). More specifically, one of three normal forms of cyclic nucleotide phosphodiesterase expressed in normal *Drosophila* adults (6, 16; R. L. Davis and L. M. Kauvar, Adv. Cyclic Nucleotide Res., in press) is deficient in *dnc* mutants (4, 7). The current evidence suggests that *dnc* is the structural gene for this form of cyclic AMP phosphodiesterase (Davis and Kauvar, in press). Cyclic AMP levels are elevated in *dnc* mutants (4, 7), apparently due to the loss in one of the degradative enzymes.

We describe here the isolation of the chromosomal region which contains dnc^+, and also our experiments to map the gene on cloned DNA as the first step to probe the structure, regulation, evolution, and biological function of the gene. Chromosomal walking has been employed to isolate the dnc^+ chromosomal region, and principles of recombinational mapping advanced by Sturtevant (30) have been utilized to map *dnc* sequences on the isolated DNA.

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MATERIALS AND METHODS

Nucleic acid isolation. Bacteriophage λ DNA was isolated essentially as described by Maniatis et al. (21). Plasmid DNA was isolated by the methods of Ish-Horowicz and Burke (14).

Genome DNA was isolated from adult flies (12). Frozen flies were ground to a fine powder with a pestle in a mortar cooled on dry ice. The powder was suspended in ice-cold 0.35 M sucrose–0.050 M Tris-hydrochloride (pH 7.6)–0.025 M KCl–0.005 M magnesium acetate, and cell breakage was completed by Dounce homogenization. The homogenate was filtered once or twice through Nitex, and the filtrate was centrifuged at 4°C at 4,000 × *g* for 15 min. The nuclear pellet was suspended in homogenization buffer and recentrifuged. The nuclei were then suspended in 0.15 M NaCl–0.10 M EDTA–0.050 M Tris-hydrochloride (pH 8.0). Proteinase K was added to a concentration of 20 μg/ml, Sarkosyl was added to 2%, and the solution was incubated at 50°C for 2 h. Solid CsCl was added to $\rho = 1.7$, and the DNA was banded by centrifugation for 60 h at 20°C in a Ti50 rotor at 38,000 rpm. The genomic DNA was collected from the side of the tube as a viscous fraction and was dialyzed extensively against 0.010 M Tris-hydrochloride (pH 8.0)–0.010 M NaCl–0.001 M EDTA.

For rapid analysis of genome restriction sites, 10 adult flies were ground to a fine suspension in a ground-glass microhomogenizer in 0.4 ml of 0.05 M Tris-hydrochloride (pH 8.0)–0.010 M EDTA–0.5% sodium dodecyl sulfate. Proteinase K was added to a concentration of 1 mg/ml and was incubated at 37°C for 4 to 20 h. The solution was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) with incubation of the emulsion at 50°C for 15 min during the extractions. The aqueous phase was extracted with ether, and residual ether was removed under a stream of N_2. Sodium chloride was added to 0.3 M with 1 volume of isopropyl alcohol. After precipitation of the nucleic acids in the cold and centrifugation, the pellet was washed with 70% ethanol, dried under reduced pressure, and suspended in 20 μl of 0.010 M Tris-hydrochloride (pH 8.0)–0.001 M EDTA.

This procedure can be scaled up for larger numbers of flies. For restriction by six-hitters and blotting, we generally used DNA isolated from 10 flies per lane, although we have used DNA from as few as 2 flies. For restriction by four-hitters and blotting, 50 adult flies were used to isolate DNA for one gel lane.

Gel isolation of restriction fragments. Preparative restriction digests were fractionated on horizontal agarose gels and stained with ethidium bromide to visualize the DNA. A trough was cut adjacent to the fragment to be isolated, a strip of DE81 paper was inserted, and the fragment was electrophoresed into the paper; this electrophoresis was perpendicular to the direction used for initial fractionation. After all the DNA was electrophoresed into the paper, the paper was removed, trimmed, and washed extensively with 0.010 M Tris-hydrochloride (pH 8.0)–0.10 M NaCl–0.001 M EDTA. The DNA was eluted from the paper with six 100-μl washes with 0.010 M Tris-hydrochloride (pH 8.0)–1.0 M NaCl–0.001 M EDTA and was filtered through a plug of silanized glass wool. The filtrate was extracted twice with *n*-butanol and once with ether, and residual ether was removed with a stream of N_2. An equal volume of isopropyl alcohol was added to precipitate the DNA. After storage at −20°C, the DNA was recovered by centrifugation, and the pellet washed with 70% ethanol, dried, and suspended in 0.010 M Tris-hydrochloride (pH 8.0)–0.010 M NaCl–0.001 M EDTA. Recoveries ranged from 30 to 70%, depending on the fragment size. The DNA recovered was of high quality and could be used in all standard enzymatic reactions.

DNA labeling. Cloned DNA or isolated restriction fragments were labeled by nick translation as described by Mullins et al. (23).

Library screening and blot hybridizations. In general, eight genome equivalents from two different libraries were screened for chromosomal walking. Library screens and blot hybridizations were conducted by procedures described by Mullins et al. (23).

Subcloning. Restriction fragments from certain λ clones were subcloned into plasmid pBR322 or pUC8 by standard procedures.

Restriction digestions. Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories. The 24 restriction enzymes used which cut infrequently included *Acc*I, *Bcl*I, *Bam*HI, *Bgl*I, *Bgl*II, *Bst*NI, *Cla*I, *Eco*RI, *Hin*cII, *Hin*dIII, *Hpa*II, *Kpn*I, *Nae*I, *Nar*I, *Nru*I, *Pst*I, *Pvu*II, *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Sph*I, *Stu*I, and *Xho*I. Frequent cutters included *Alu*I, *Dde*I, *Hae*II, *Hae*III, *Hha*I, *Hin*fI, *Hph*I, *Mbo*I, *Mbo*II, *Msp*I, *Rsa*I, *Sau*96I, *Taq*I, and *Tha*I.

Fly strains and crosses. The fly strains utilized here have been described (7, 18), except for $Df(1)dm^{77h}$, which was induced by G. LeFevre in the Amherst wild type. The cytologies of chromosomal aberrations are those of G. LeFevre (personal communication).

To construct dunce region deficiencies, $C(1)DX, yf/w^{+}Y$ ♀ were crossed with $Df(1)N$; $SM1$, $CyDp(1;2)w^{+51b7}$ ♂, and the progeny males, $Df(1)N/w^{+}Y$, were selected. The $w^{+}Y$ chromosome covers the hemizygous lethality associated with notch (*N*) deficiency but does not extend to the *dnc* locus.

To generate recombinants near the *dnc* locus, males of the genotype $sc\ w^{bl}\ dnc^{2}$ were crossed to $Df(1)dm^{77h}/FM7$ ♀, and $sc\ w^{bl}\ dnc^{2}/Df(1)dm^{77h}$ progeny females further mated to produce recombinant X chromosomes recovered in their male progeny. Stocks were established with attached-X females. The marker scute (*sc*), although present in one parental chromosome, has been omitted in the text discussion since it played no role in the present analysis.

All 115 w^{+}-viable recombinant stocks analyzed were examined for female fertility by crossing males from each stock to $Df(1)dm^{75e19}/FM7$ females, selecting five non-*FM7* heterozygote progeny, and mating these to males. No intermediate fertility was observed; females from these tests produced very few or no progeny, or else they produced many progeny. These recombinants were then tested for the presence of the insertion element (see Fig. 6). The 20 recombinants which exhibited crossover to the right of this element were assayed for cyclic AMP phosphodiesterase activity by L. Kauvar (16). These recombinants were clearly dnc^{2} or dnc^{+} by the enzymatic assay.

RESULTS

Genetic organization of the chromosomal region containing dunce. Cytogenetic evidence indicates that *dnc* resides at chromomere 3D4 of the salivary gland X chromosome (Fig. 1). This assignment was made from the observation that the deficiency $Df(1)N^{64j15}$ does not completely remove the dnc^{+} function, whereas $Df(1)N^{64i16}$ and $Df(1)N^{71h24\text{-}5}$ do (7, 18, 26). Since the right breakpoint of $Df(1)N^{64j15}$ has been localized between chromomeres 3D3 and 3D4 and the right breakpoints of $Df(1)N^{64i16}$ and $Df(1)N^{71h24\text{-}5}$ have been localized between 3D4 and 3D5, the genetic analyses place *dnc* in chromomere 3D4. To the left of *dnc* but still within chromomere 3D4 is a gene named *sam*, whose normal function is required for sperm motility (26). The next known genetic function to the left of *sam* is *Sgs-4*, which resides in chromomere 3C11-12 and produces one of the larval glue polypeptides (19).

The breakpoints associated with the chromosomal aberrations $Df(1)dm^{75e19}$, $w^{+}Y$, and $Df(1)dm^{77h}$ are also of interest for the current analysis and are depicted in Fig. 1. The left breakpoint of $Df(1)dm^{75e19}$ and the right breakpoint of $w^{+}Y$ are both located to the left of *dnc*, as determined by genetic criteria (26). The chromosome $Df(1)dm^{77h}$ does not remove the dnc^{+} function; therefore, sequences removed by this deletion normally flank *dnc* on the right.

Isolation of the chromosomal region containing dunce^{+}. The molecular isolation of the *Sgs-4* locus (24) offered an entry point to the chromosomal region containing the *dnc* gene (Fig. 2). The clones λcDm1570 and λcDm1568 were isolated and characterized as part of *Sgs-4* studies (22, 24). In addition, those studies established the centromere-telomere orientation of the cloned region. Consequently, we isolated a 4.2-kilobase (kb) *Sal*I fragment from the centromere-proximal end of λcDm1568 as a probe to screen genome libraries, and we initiated a chromosomal walk in a centromeric direction.

Drosophila genome libraries from the strain Canton-S in bacteriophage λ vectors (5, 21) were screened with the 4.2-kb *Sal*I fragment and then successively, by using gel-isolated restriction fragments, from the centromere-proximal end of newly isolated clones as probes. At each step, we mapped the newly isolated clones with restriction enzymes. When necessary, we performed blotting experiments to determine the extend of overlap with characterized clones. In addition, qualitative genome blots were performed with each isolated fragment before screening the libraries to ensure that probe sequences were not repetitive in the genome. In this manner, we isolated a contiguous stretch of *Drosophila* genome DNA residing just proximal to the *Sgs-4* locus on the X chromosome. The restriction map of this region, deduced from

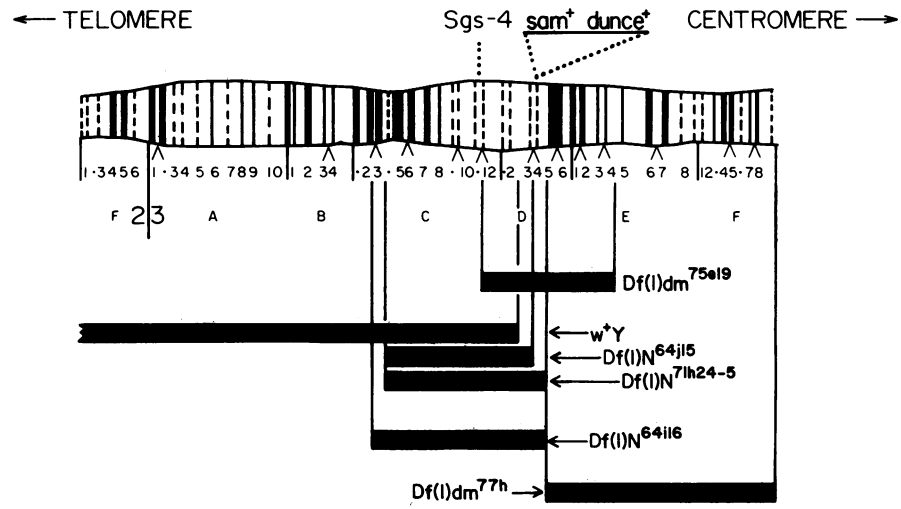

FIG. 1. Cytogenetic organization of the dunce chromosomal region (schematic illustration of a distal region of the X chromosome). The *Sgs-4* locus is in chromomere 3C11 or 12; *sam* and *dnc* both reside in 3D4. The cytological extents of several deficiency chromosomes and of w^+Y, an insertional translocation of X chromosomal region 2D1 through 3D2 into the Y chromosome, are shown below the chromosome. In this latter chromosome, the solid line segment indicates that portion of the X chromosome which is present in the translocation Y. Cytologies are those of G. LeFevre (personal communication).

analyzing the isolated clones, is depicted in Fig. 2. The extent of some of the clones recovered is also shown.

Mapping breakpoints of chromosomal aberrations. Since breakpoints associated with chromosomal aberrations perturb the restriction map at the site of the breakpoint, their location can be mapped by genome blotting experiments. If chromosomal aberrations have been defined genetically, they can be used to locate critical regions on cloned DNA. Several chromosomal aberrations breaking near *dnc* are depicted in Fig. 1. We expected to cross the following breakpoints during the walk in this order: the left breakpoint of *Df(1)dm*^{75e19}, the right breakpoint of w^+Y, the right breakpoint of *Df(1)N*^{64j15}, the right breakpoint of *Df(1)N*^{64i16} and *Df(1)N*$^{71h24-5}$, and the left breakpoint of *Df(1)dm*^{77h}, the last three being cytologically identical.

Since the *dnc* region can be completely deleted from the

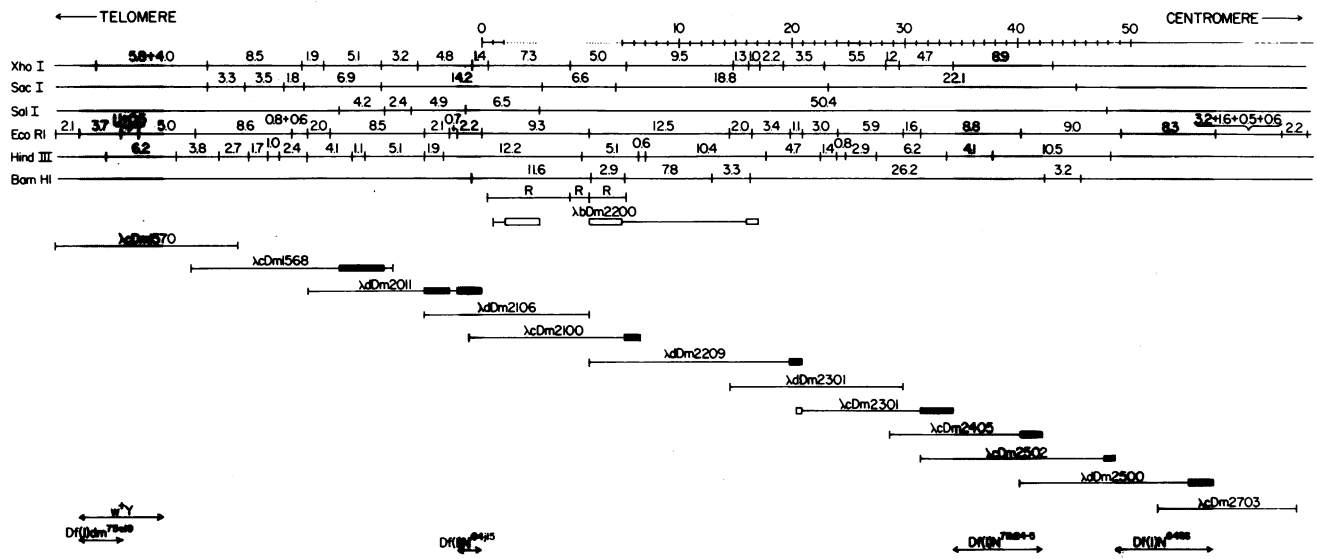

FIG. 2. Restriction map of the dunce chromosomal region (3C12 to 3D4 chromosomal region). Inserts of *Drosophila* genome DNA in λ vectors are shown as line segments below the map. Those isolated from the library prepared by shearing genome DNA and adding *Eco*RI linkers for cloning (21) are identified with a c in the nomenclature. Those from the library prepared from genome DNA partially digested with *Eco*RI (5) have been assigned a d. The blackened segments of certain clones shows the restriction fragments isolated to probe the libraries for each successive step. Open segments indicate uncertainties regarding the endpoints of some clones. The breakpoints of chromosomal aberrations in the region have been mapped (see Fig. 3) and are illustrated here. The widths of the broad shaded areas show our uncertainty from the mapping data. Certain restriction fragments in the region are known to contain repetitive sequences (R). Except for this one repetitive region, the cloned DNA to the right of the *Df(1)N*^{64j15} breakpoint is unique, established by genome blotting experiments. Above the restriction map is the arbitrary coordinate system set up for analyzing restriction site polymorphisms, expressed in kb of unique-sequence DNA from the right site of the 2.2-kb *Eco*RI fragment of λdDm2106. Note that the repetitive insertion sequence (7.3 kb) found in the Canton-S strain between coordinates 2 and 5 is not counted in the coordinate system.

fly without affecting viability (17), we have used a plus-minus (hybridization versus no hybridization) genome blot assay to determine whether cloned probe sequences are present in the DNA of flies genetically constructed to contain various extents of the *dnc* region. This method circumvents potential problems owing to restriction site polymorphism. McGinnis et al. (22) previously localized the left breakpoint of $Df(1)dm^{75e19}$ and the right breakpoint of w^+Y. Our data (not shown) map these breakpoints as shown in Fig. 2, confirming the localizations published previously.

The genome blots which establish the positions of the right breakpoints of $Df(1)N^{64j15}$, $Df(1)N^{64i16}$, and $Df(1)^{71h24\text{-}5}$ are shown in Fig. 3 and are summarized in Fig. 2. The 0.7-kb *Eco*RI fragment of λdDm2106 does not hybridize to any of the deficiency chromosomes, whereas a probe containing sequences just to the right (the 2.1- plus 2.2-kb *Eco*RI fragments) detects a 7.8-kb *Eco*RI fragment in the genome of flies $Df(1)N^{64j15}/w^+Y$ (Fig. 3A and 3B). Other unique-sequence probes to the left of the 2.2-kb *Eco*RI fragment but to the right of the w^+Y breakpoint have not detected homologous sequences in the $Df(1)N^{64j15}/w^+Y$ genome. This shows that the right breakpoint of $Df(1)N^{64j15}$ is within the 2.2-kb *Eco*RI fragment of λdDm2106 and that the 7.8-kb homologous fragment in the $Df(1)N^{64j15}/w^+Y$ genome is a fusion fragment.

Unique-sequence probes to the right of this region through sequences carried by λcDm2301 do not hybridize to the genomes of $Df(1)N^{64i16}/w^+Y$ and $Df(1)N^{71h24\text{-}5}/w^+Y$. The probe λcDm2405 does detect homologous sequences in the $Df(1)N^{71h24\text{-}5}$ chromosome (Fig. 3C), indicating that the right breakpoint of this deficiency is between the right ends of λcDm2301 and λcDm2405. The right breakpoint of $Df(1)N^{64i16}$ is between the right limits of λcDm2502 and λdDm2500, as shown by hybridization of $Df(1)N^{64i16}/w^+Y$ DNA to λdDm2500 but not to λcDm2502. We have searched for the left breakpoint of $Df(1)dm^{77h}$ but have not identified it within the interval of DNA which we have isolated.

Since the right breakpoint of $Df(1)N^{64j15}$ is to the left of *dnc* genetically and the deficiencies N^{64i16} and $N^{71h24\text{-}5}$ remove dnc^+ activity, these data suggest that the gene resides in the interval of ca. 50 kb between the breakpoints of $Df(1)N^{64j15}$ and $Df(1)N^{71h24\text{-}5}$. However, from these data we cannot exclude the possibilities that $Df(1)N^{64i16}$, $Df(1)N^{71h24\text{-}5}$, or both break within the gene and disrupt its function or that the gene resides to the right of these deficiencies and is structurally intact but inactive due to a position effect (28) of juxtaposed sequences on the gene.

Genomic representation of dunce chromosomal sequences. With one exception, the sequences of interest in the interval of ca. 50 kb are represented once per haploid genome. This was established by qualitative genome blots. We have probed Canton-S genome blots with a variety of cloned sequences spanning the 50-kb interval and find that the only genome restriction fragments which hybridize to the probes are those predicted by the map of cloned DNA (data not shown). Furthermore, the genome blots with *dnc*-deficiency DNA (Fig. 3) support this conclusion since certain clones show no hybridization to these genomes. Therefore, it is not possible that the *dnc* region is duplicated elsewhere in the genome.

The exception is a repetitive sequence encountered during the chromosomal walk which exists entirely within λcDm2100 (Fig. 2). This sequence is repeated between 30 and 50 times in the Canton-S genome, as indicated by probing genome blots with λcDm2100 or gel isolated restriction fragments (Fig. 2).

We probed an Oregon-R genome library (constructed by E. Meyerowitz) with the unique-sequence 1.3-kb *Xho-Eco*RI (synthetic) fragment and the 1.4-kb *Xho* fragment of λcDm2100, fragments which flank the repetitive sequence on the right and left, respectively. One positive identified with the 1.3-kb *Xho-Eco*RI fragment named λbDm2200 (Fig. 2) was characterized in detail. Restriction mapping and clone blotting experiments reveal that this clone spans the repetitive sequence region; however, genome blotting experiments show that the clone contains only unique sequences (data

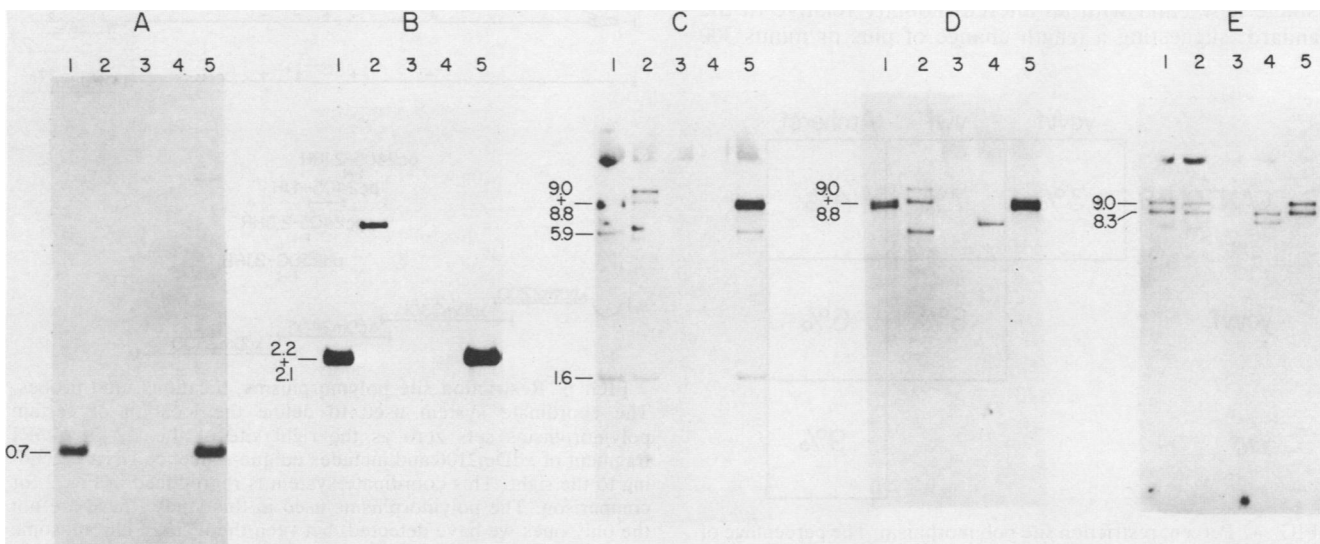

FIG. 3. Genome blots of DNA from dunce deficiencies. *Eco*RI digests of DNA from male flies of genotypes Canton-S (lanes 1 and 5), $Df(1)N^{64j15}/w^+Y$ (lanes 2), $Df(1)N^{64i16}/w^+Y$ (lanes 3), and $Df(1)N^{71h24\text{-}5}/w^+Y$ (lanes 4) were fractionated, blotted, and probed with the 0.7-kb *Eco*RI fragment of λdDm2106 (A) and the 2.2- plus 2.1-kb *Eco*RI fragments of λdDm2106 (B), λcDm2405 (C), λcDm2502 (D), and λdDm2500 (E). See the text for the genetic construction of the deficiency flies. The hybridizing restriction fragment in panel C, lane 4, is very faint in the original autoradiogram and did not reproduce well in photographs. This fragment comigrates with the smaller of the two bands observed in panel D, lane 4.

not shown). Probing genome blots of DNAs from different *Drosophila* strains with λbDm2200 and other unique-sequence restriction fragments in the vicinity disclose that Canton-S has a 7.3-kb insertion of DNA relative to other strains. It is likely that this is a transposable element since most of the middle repetitive DNA in *D. melanogaster* is nomadic (32).

In summary, these data indicate that chromomere 3D4, which is defined by the breakpoints of $Df(1)N^{64j15}$ and $Df(1)N^{71h24\text{-}5}$, is composed of approximately 50 kb of DNA and is unique, except for the presence of a repetitive insertion element in the Canton-S strain.

Restriction site polymorphisms as genetic markers. Chromosomal walking provides a useful method for isolating genome regions of interest. However, one major drawback is that there exists no general method of identifying the critical sequences on the hundreds of kilobase pairs of DNA one can isolate by this method. We have solved this problem in the case of *dnc* by mapping a *dnc* mutation by recombination on the cloned DNA, using restriction site polymorphisms identified and localized by prior survey as genetic markers.

This analysis is limited by the number of recombinants one can select and on the frequency of restriction site polymorphism. To determine whether polymorphism is frequent enough to make this approach feasible, we surveyed the restriction sites of several different X chromosomes across a ca. 40-kb stretch of chromomere 3D4. The X chromosomes utilized in this study were Canton-S, *y cv v f*, *y w f*, and Amherst, selected because they have served as parental chromosomes for the induction of *dnc* mutations or flanking markers (7). DNA was isolated from flies carrying these chromosomes, digested with 24 different restriction enzymes, most of which recognize hexanucleotide sequences, blotted after electrophoresis, and probed separately with λcDm2301, λcDm2405, and λdDm2500.

Most of the altered restriction patterns observed between different X chromosomes were of two types. Many were a change of one band to two smaller bands whose total length approximated that of the first. We interpret these as losses or gains of single restriction sites. A second major class showed a single new band with an altered mobility relative to the standard, suggesting a length change of plus or minus 300 base pairs. These could be small insertions or deletions, or they could be losses or gains of single restriction sites near one end of the standard fragment. We would not have detected on our blots fragments of <500 base pairs that would indicate the latter possibility. However, for the purpose of quantitating polymorphism, this class of alteration was counted as one change. In this manner, we tabulated the number of changes between the four chromosomes indicated.

	ycvvf	ywf	Amherst
CANTON-S	3%	7%	4%
ycvvf		8%	6%
ywf			9%

FIG. 4. Percent restriction site polymorphism. The percentage of restriction sites polymorphic in chromomere 3D4 between pairs of four different chromosomes is shown. Canton-S and Amherst are two standard wild-type strains. The chromosome *y cv v f* carries as visible markers yellow body color (*y*), crossveinless wings (*cv*), vermillion eyes (*v*), and forked bristles (*f*). The chromosome *y w f* carries *y*, *f*, and white eyes (*w*). All of these chromosomes are dnc^+.

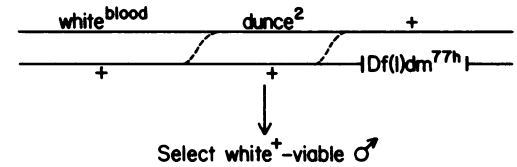

FIG. 5. Cross to map $dunce^2$. Females carrying $white^{blood}$ (w^{bl}) and $dunce^2$ (dnc^2) on one homolog and heterozygous with $Df(1)dm^{77h}$ were constructed and mated. Male progeny were scored and white$^+$-viable males mated to attached-X females.

Approximately 140 restriction sites were surveyed in the Canton-S chromosome. The *y cv v f* chromosome was closest to Canton-S, exhibiting about 3% divergence, and *y w f* was the most divergent, with about 7% of the sites different (Fig. 4). The Amherst/*y w f* comparison yielded a 9% polymorphic value. However, although most polymorphisms were scattered throughout the 40-kb interval, many of the *y w f* polymorphisms mapped to the area represented by the right side of λdDm2500. This suggests that the *y w f* chromosome may have a major sequence alteration in this vicinity, possibly the residence of a transposable element not found in the Canton-S chromosome. Consequently, we view the comparisons with *y w f* as overestimates of the amount of divergence due to random base change. A 3 to 6% estimate

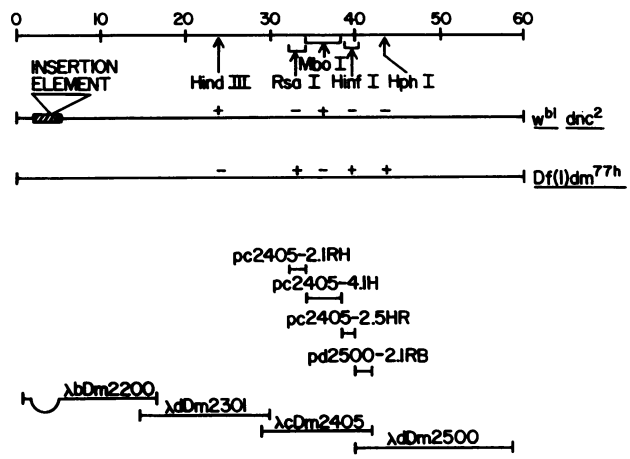

FIG. 6. Restriction site polymorphisms, locations, and probes. The coordinate system used to define the location of certain polymorphisms sets zero as the right site of the 2.2-kb *Eco*RI fragment of λdDm2106 and includes unique-sequence DNA extending to the right. This coordinate system is reproduced in Fig. 2 for comparison. The polymorphisms used in this study (these are not the only ones we have detected) between the $w^{bl}dnc^2$ chromosome and $Df(1)dm^{77h}$ include the insertion element between coordinates 2 and 5 detected by probing genome blots with λbDm2200, a *Hin*dIII polymorphism detected by λdDm2301, an *Rsa*I difference within the 2.1-kb subclone pc2405-2.1RH, an *Mbo*I difference within pc2405-4.1H, a *Hin*fI change in pc2405-2.5HR, and an *Hph*I polymorphism detected by pd2500-2.1RB.

of divergence between any two chromosomes is more conservative and realistic. This value is high enough to make efficient use of restriction site polymorphisms as genetic markers (see below).

Recombinational mapping of *dnc*^{2}. The female fly genotype that has produced the most informative recombinant progeny is shown in Fig. 5. One homolog carries the *dnc*^{2} allele, a mutation which alters the kinetic properties of cyclic AMP phosphodiesterase (7, 16) and which is therefore a good marker for the structural portion of the gene, and the left-side visible marker white^{blood} (w^{bl}). The other homolog is the *Df(1)dm*^{77h} chromosome.

From the heterozygous females depicted, 126 of 7,350 male progeny scored were white$^+$ (w^+). This gives a map distance between white and *Df(1)dm*^{77h} of 1.7 units, lower than the standard distance (20) of 3.1 between white and the diminutive (*dm*) locus, located in chromomere 3D5 or 3D6 (G. LeFevre, personal communication). This depressed value possibly reflects crossover suppression due to the deficiency. Most of the w^+ males were bred to establish a stock of each recombinant chromosome. DNA was then prepared from 10 male flies of each stock to analyze the spectrum of restriction sites carried by each recombinant chromosome.

Some of the polymorphisms detected in the survey described above have been employed as markers in recombination experiments. Others were sought, as the analysis pinpointed critical regions, by using additional enzymes which recognize tetranucleotide sequences and by using subclones of the *dnc*$^+$ region as probes. Those utilized here were detected and mapped by genome blotting experiments with DNA from Canton-S and Amherst flies, the parental stocks of the *dnc*^{2} and *Df(1)dm*^{77h} chromosomes, respectively. The w^{bl} marker was introduced into the *dnc*^{2} chromosome by recombination and potentially introduced restriction sites from a different genetic background, but the analysis presented below indicates that the crossover which produced this chromosome occurred to the left of the *dnc* region. Figure 6 presents the polymorphic sites employed and the probes utilized to detect these differences. An arbitrary coordinate system in kb has been assigned to this region, starting with the right-hand site of the 2.2-kb *Eco*RI fragment carried by λdDm2106, which is the approximate location of the *Df(1)N*^{64j15} right breakpoint (Fig. 2).

DNA from 115 white$^+$-viable recombinants was first analyzed for the presence or absence of the insertion element carried by the w^{bl}*dnc*^{2} chromosome at coordinate 2 to 5, by probing genome digests with λbDm2200. Twenty recombinants showed the *Df(1)dm*^{77h} restriction pattern (Fig. 7), indicating the absence of the insertion element. Thus, the crossovers which produced these recombinants occurred to the right of the insertion element. Phenotypic analysis of these indicates that both *dnc*$^+$ and *dnc*^{2} recombinants were recovered (Table 1). This reveals that the *dnc*^{2} lesion resides to the right of the insertion element.

These recombinants were then analyzed for the polymorphisms displayed in Fig. 6 by probing restriction digests with the appropriate cloned probes. We analyzed the 20 recombinants, first with respect to the *Hin*dIII polymorphism, then with respect to the *Hph*I configuration detected by pd2500-2.1RB followed by analysis of the *Mbo*I sites in pc2405-4.1H. Those recombinants displaying a crossover position in the *Hin*dIII-*Mbo*I interval were then analyzed for *Rsa*I sites; those in the *Mbo*I-*Hph*I region were examined for *Hin*fI restriction sites. Fig. 7 displays some of the data, and the data are summarized in Fig. 8 and Table 1. We have detected recombination in six different regions: to the left of the insertion element, between the insertion element and the *Hin*dIII polymorphism, between the *Hin*dIII and *Rsa*I polymorphisms, between the *Rsa*I and *Mbo*I polymorphisms, between the *Hin*fI and *Hph*I polymorphisms, and between the *Hph*I polymorphism and the left breakpoint of *Df(1)dm*^{77h}. Most importantly, we have recovered one

TABLE 1. Spectrum of polymorphisms in recombinant chromosomes

Recombinant[b]	Dunce phenotype[c]	Parentage of restriction sites[a]					
		*Hin*dIII at 24	*Rsa*I at 32–34	*Mbo*I at 34–38	*Hin*fI at 38–41	*Hph*I at 44	Crossover region[d]
6	m	*Df*	*dnc*^{2}	*dnc*^{2}		*dnc*^{2}	3
8	+	?		*Df*		*Df*	6
9	m	*Df*	*dnc*^{2}	*dnc*^{2}		*dnc*^{2}	3
10	m	*dnc*^{2}		*dnc*^{2}		*dnc*^{2}	2
11	m	*Df*	*Df*	*dnc*^{2}		*dnc*^{2}	4
12	+	*Df*		*Df*	*Df*	*dnc*^{2}	5
18	+	*Df*		*Df*		*Df*	6
20	+	*Df*		*Df*		*Df*	6
29	+	*Df*		*Df*		*Df*	6
33	+	*Df*		*Df*		*Df*	6
37	+	*Df*		*Df*		*Df*	6
40	m	*dnc*^{2}		*dnc*^{2}		*dnc*^{2}	2
46	m	*dnc*^{2}		*dnc*^{2}		*dnc*^{2}	2
48	+	*Df*		*Df*		*Df*	6
52	+	*Df*		*Df*		*Df*	6
53	+	*Df*		*Df*		*Df*	6
76	+	*Df*		*Df*	*Df*	*dnc*^{2}	5
128	m	?	*dnc*^{2}	*dnc*^{2}		*dnc*^{2}	2 or 3
131	+	*Df*		*Df*		*Df*	6
133	+	*Df*		*Df*		*Df*	6

[a] *dnc*^{2}, Parentage from the w^{bl}*dnc*^{2} chromosome; *Df*, parentage from *Df(1)dm*^{77h}.

[b] Twenty recombinants from w^{bl}*dnc*^{2}/*Df(1)dm*^{77h} females are identified by their assigned numbers. These have arisen from crossover to the right of the insertion element but to the left of the diminutive (*dm*) deficiency *77h* (Fig. 5).

[c] +, *dnc*$^+$; m, *dnc*^{2}.

[d] Numbers correspond to regions in Fig. 8.

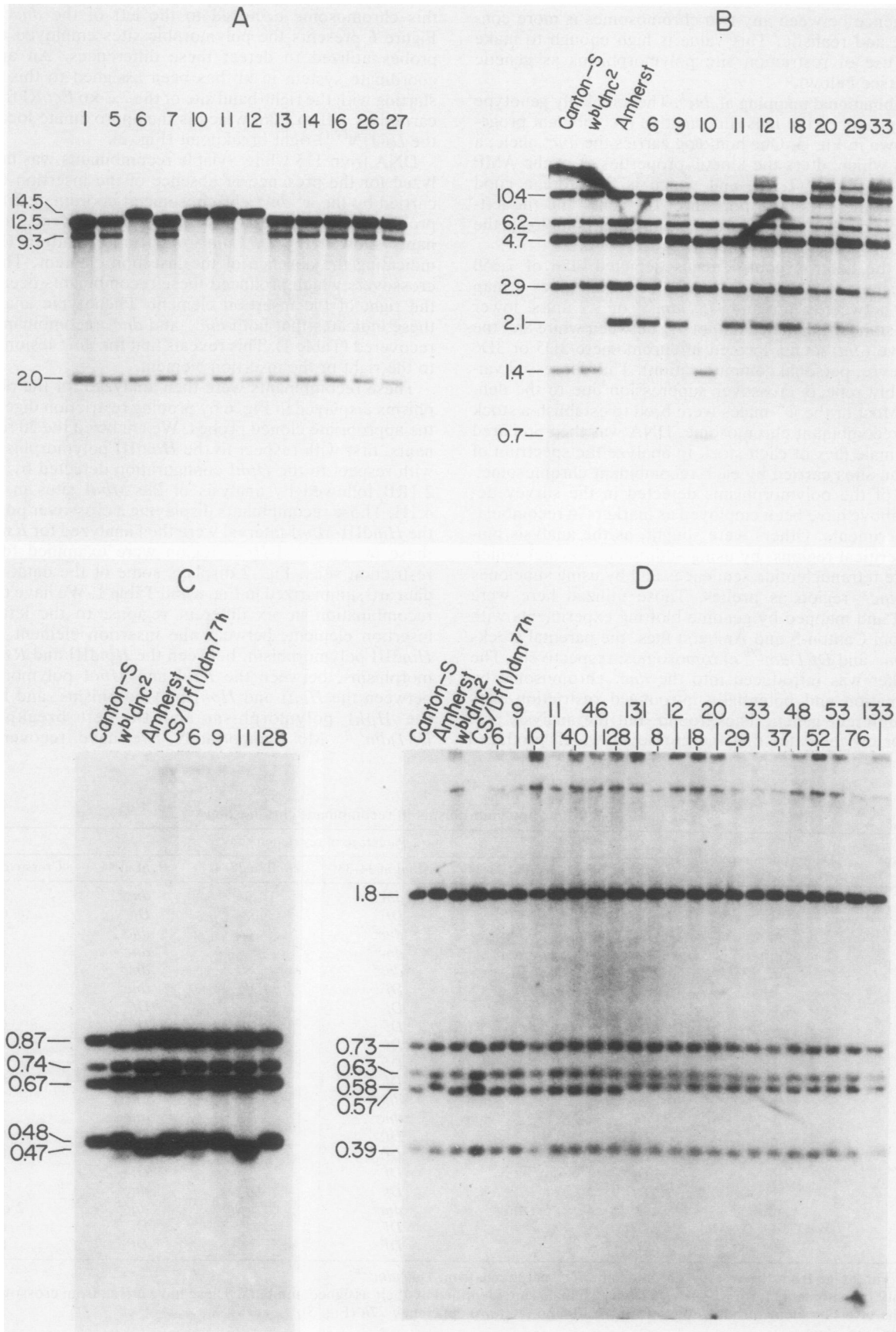

FIG. 7. A through D.

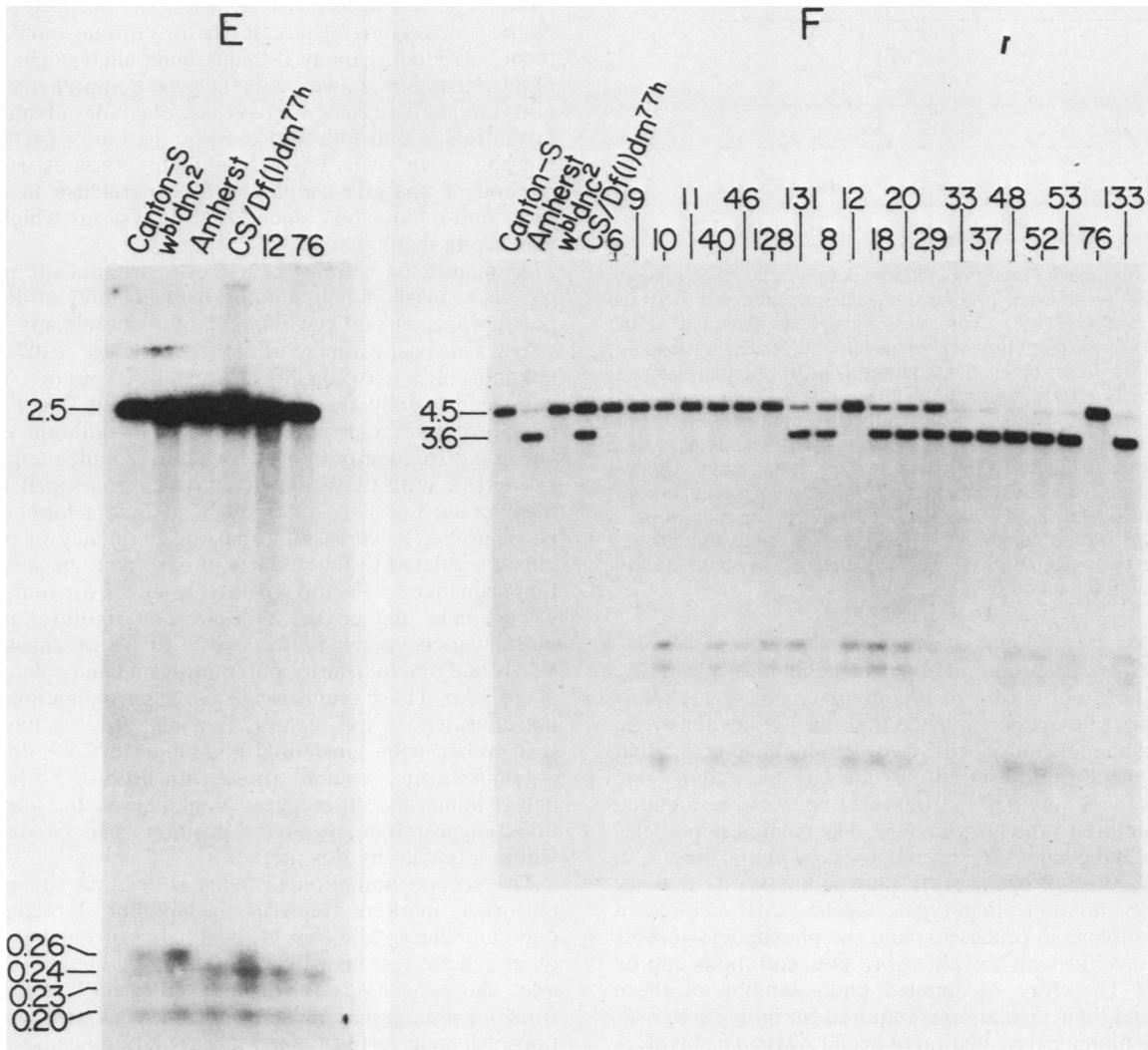

FIG. 7. Genome blots of recombinants. DNA prepared by a microprocedure (see text) was digested with restriction endonucleases, electrophoresed, blotted, and probed to detect the array of restriction sites in parental and recombinant chromosomes from the cross depicted in Fig. 5. (A) *Eco*RI digests probed with λbDm2200 to show the presence or absence of the insertion element (Fig. 6). This is 1 representative of 10 blots to probe the 115 white$^+$-viable recombinants. Canton-S and its derivative, $w^{bl}dnc^2$ (not shown), show a pattern identical to that of those recombinants with fragments of 12.5, 9.3, and 2.0 kb; Amherst and the derived chromosome $Df(1)dm^{77h}$ show the 14.5- and 2.0-kb bands (not shown). Recombinants 6, 10, 11, and 12 lack the insertion element found in $w^{bl}dnc^2$ and therefore must have arisen as a result of crossover to the right of the element. (B) *Hin*dIII digests of several recombinants and controls after probing with λdDm2301. The 1.4- and 0.7-kb *Hin*dIII fragments are diagnostic of Canton-S origin; the 2.1-kb fragment is diagnostic of the Amherst origin. Recombinant 10 exhibits the Canton-S pattern, and therefore, it exhibits crossover to the left of the *Hin*dIII polymorphism located at coordinate 24. (C) *Rsa*I digests probes with pc2405-2.1RH. Canton-S (CS) exhibits a 0.48-kb *Rsa*I fragment, and Amherst exhibits a 0.47-kb fragment. Note that the heterozygous control, Canton-S/$Df(1)dm^{77h}$, shows both. (D) *Mbo*I digests of recombinants and controls probed with pc2405-4.1H. The polymorphism here is between a 0.58-kb versus a 0.57-kb *Mbo*I band. (E) *Hin*fI digests of recombinants and controls probed with pc2405-2.5HR, showing the presence of an 0.26-kb band in Canton-S and its absence in Amherst. (F) *Hph*I digests. The probe is pd2500-2.1RB. The polymorphism has been mapped to a site comprising the right end of the 3.6-kb *Hph*I fragment observed in Amherst, which maps about 1 kb to the right of the right end of pd2500-2.1RB but is detected by this probe. The presence of the 3.6-kb band is diagnostic of the Amherst parentage. Partial digestion of an Amherst-derived chromosome results in the presence of a 4.5-kb *Hph*I band in addition to the 3.6-kb band.

recombinant (number 11) between the *Rsa*I and *Mbo*I polymorphisms which is phenotypically dnc^2, thus showing that the dnc^2 lesion is to the right of the *Rsa*I polymorphism. In addition, two dnc^+ recombinants (numbers 12 and 76) exhibited crossovers between the *Hin*fI and *Hph*I polymorphisms. Therefore, the dnc^2 lesion must reside to the left of the *Hph*I polymorphism. This maps dnc^2 to the 10- to 12-kb region defined physically on the left by the *Rsa*I polymorphism at coordinate 32 to 34 and the *Hph*I polymorphism at coordinate 44.

In summary, we have generated recombinant chromosomes produced by crossover near to, but on the left and right of, the dnc^2 mutation, and we have used restriction site polymorphisms between the two parental chromosomes as genetic markers to map this lesion. The data map dnc^2 to a 10- to 12-kb interval.

DISCUSSION

The *dnc* gene is intriguing in several respects. The variety of behavioral changes that occur when the gene undergoes

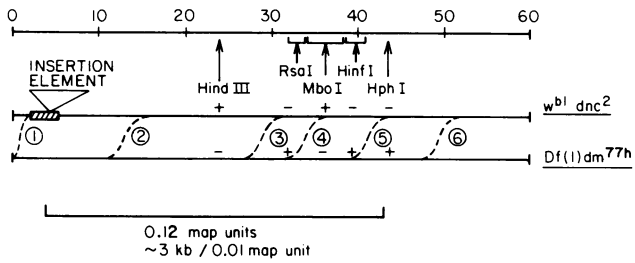

FIG. 8. Summary of recombinational data. A schematic diagram of the crossovers which produced the 20 recombinants between $w^{bl}dnc^2$ and $Df(1)dm^{77h}$. Crossovers have been detected at the numbered positions as follows: 1, to the left of the insertion element; 2, between the insertion element and the *Hin*dIII polymorphism; 3, between the *Hin*dIII and *Rsa*I polymorphisms; 4, between the *Rsa*I and *Mbo*I polymorphisms; 5, between the *Hin*fI and *Hph*I polymorphisms; 6, to the right of the *Hph*I polymorphism. The data for each recombinant are tabulated in Table 1. The genetic map distance between the insertion element and the *Hph*I polymorphism is shown and is converted to a DNA length equivalent. The value of ca. 3 kb/0.01 map unit is uncorrected for the reduced recombination observed in the cross (see text). This value compares favorably with that calculated for notch (2).

mutation show that it is involved in behavioral plasticity, although the exact nature of this involvement is not clear. Several lines of evidence indicate that dnc^+ codes for cyclic AMP phosphodiesterase, the most compelling being that dnc^1 affects the thermostability of the enzyme activity and dnc^2 alters the K_m, two properties one expects to be dictated by the structure of the enzyme (16). The current hypothesis is that the high cyclic AMP levels observed in *dnc* flies (4, 7) owing to the loss of cyclic AMP phosphodiesterase activity cause the behavioral phenotypes. Cyclic AMP mediates a variety of biological processes, and the phosphodiesterases form one level at which cyclic AMP concentrations can be controlled. Therefore, a detailed understanding of these enzymes and their regulation is required for in-depth knowledge of the biological regulation of cyclic AMP. The involvement of the gene in behavioral processes and in cyclic AMP metabolism have provided the impetus to initiate a study of the structure, regulation, function, and evolution of the gene.

Chromosomal walking was judged to be the method of choice for isolating the gene, and it has allowed the recovery of a large region of the X chromosome including dnc^+. Since genetic analysis of certain chromosomal aberrations had defined the gene to chromomere 3D4, we hoped early in this study that these aberrations would limit the gene to a manageable length of DNA sequence. The analyses of the aberrations delimit dnc^+ to about 50 kb, a region twice as large as anticipated from consideration of the average DNA content per chromomere. This required us to devise an additional method for localizing *dnc* sequences on the cloned DNA.

The problem was solved by mapping a *dnc* mutation by recombination, with restriction site polymorphisms as genetic markers. Others have effectively used restriction fragment polymorphisms as physical markers. Steinmetz et al. (29) analyzed two recombinants arising from crossover in the H-2D-Tla interval to map a polymorphic fragment homologous to a transplantation antigen pseudogene to the Qa-2,3 gene cluster. Polymorphic restriction fragments carrying H-2-related sequences have been mapped relative to other included markers in *t* chromosomes by recombination (27). Orkin et al. (25) used restriction site polymorphism in the β-globin gene region to characterize the chromosomal environment surrounding many β-thalassemia alleles. The analysis of the *dnc* region, however, is the most comprehensive study of its kind to date since we have searched for polymorphisms in the region and followed the segregation of certain polymorphisms in over 100 recombinant chromosomes. This method of analysis should be of general use in mapping important regions on cloned DNA in systems which can be genetically manipulated.

As mentioned before, the use of restriction site polymorphisms as markers is limited by the frequency of detectable polymorphisms and the number of recombinants one can select. Our best estimate of the frequency of restriction site polymorphism between any two strains is 3 to 6%. We have employed 24 enzymes which cut infrequently (most of which recognize hexanucleotide sites) and 14 different enzymes which cut frequently (most of which recognize tetranucleotide sites). With these, we detect in chromosomal DNA on the average 3 or 4 six-hitter sites and 25 four-hitter sites per kb of probe. If we assume that the frequency of polymorphism is related to the number of base pairs in the recognition sequence (~3% for six-hitters, so 2% for four-hitters), we estimate that on the average, one six-hitter polymorphism can be detected for every 10 kb of chromosomal DNA, and one four-hitter polymorphism can be detected for every 2 kb. These numbers are only approximations and do not consider several factors. For instance, we have noted that probes with substantial homology to RNA detect few polymorphisms, whereas those with little or no homology detect numerous differences. Nonetheless, the approximations suggest that crossover positions can be defined to within a few kb by this method.

The second limitation of using restriction site polymorphisms as markers concerns the number of recombinants one can select. The *dnc* locus is poor in this regard, and other genetic regions will undoubtedly be better suited than *dnc*. The easiest way for distinguishing dnc^+ recombinants from *dnc* mutant parental chromosomes is to test for restoration of female fertility since *dnc* mutations cause sterility. We have done this for the 126 w^+-viable recombinants. However, this phene is particularly sensitive to suppression effects incurred upon altering the genetic background (26; Davis and Kauvar, in press). Of the 126 w^+-viable recombinants we selected, 4 stocks were fertile yet showed crossover to the left of the insertion element and were clearly dnc^2 by the cyclic AMP phosphodiesterase assay. We conclude that these have an altered genetic background which suppresses the sterility phenotype. For the 20 recombinants to the right of the insertion element, phenotypic analysis has included both fertility tests and cyclic AMP phosphodiesterase assays.

Intragenic crossovers are potentially more useful than crossovers to the left or right of an allele for gene mapping. We have obtained a surprising result by analyzing polymorphisms in putative dnc^+ recombinants generated from females heterozygous for the alleles dnc^{M14} and dnc^2 (R. Davis, and H. Salz, unpublished data). The analyses of restriction site polymorphisms in the recombinants indicate that all of the sequence information within the 50-kb *dnc* gene region is derived from the dnc^{M14} chromosome. That is to say, we have failed to detect any evidence of recombination at the DNA level. However, the recombinants show a major sequence alteration of the *dnc* region, which is likely the result of an insertion element. The significance of this is not known and is under investigation.

Finally, we have examined the coding potential of the *dnc*$^+$ chromosomal region, and this is the subject of a forthcoming report (manuscript in preparation). We find that the region bounded by the *Rsa*I polymorphism on the left and the *Hph*I polymorphism on the right constitutes a single gene and that this gene produces multiple RNAs which are regulated in a complex fashion during development.

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