A DIPHENOL OXIDASE GENE IS PART OF A CLUSTER OF GENES INVOLVED IN CATECHOLAMINE METABOLISM AND SCLEROTIZATION IN DROSOPHILA. II. MOLECULAR LOCALIZATION OF THE *Dox-A2* CODING REGION

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ABSTRACT

Mutations at the *Dox-A2* (2-53.9) locus alter the A2 component of diphenol oxidase, an enzyme having an important role in cuticle formation. This locus is in the dopa decarboxylase, *Df(2L)TW130* region, which contains a cluster of at least 14 genes involved in catecholamine metabolism and the formation, sclerotization and melanization of cuticle in Drosophila. The region is subdivided by deficiencies, and localization of breakpoints in cloned DNA reveals a dense subcluster of six genes in the 23 kb proximal to *Ddc*. Five lethal loci distal to *Ddc* comprise a second such subcluster. The proximal breakpoints of deficiencies *Df(2L)hk18* and *Df(2L)OD15* define a 14.3- to 16.8-kb region containing *Dox-A2* and *l(2)37Bb*, and those of *Df(2L)OD15* and *Df(2L)TW203* define a 9.3- to 12.1-kb region containing *l(2)37Ba*, *l(2)37Bc* and *l(2)37Be*.—Southern blots show two of the *Dox-A2* mutations are small deletions (0.1 and 1.1 kb). The *Dox-A2* locus mRNA is 1.7 kb. cDNA clones indicate that the 3′ end is centromere proximal and that the coding region contains at least one small intron. The *Dox-A2* locus is within 3.4 to 4.4 kb of the *Df(2L)OD15* breakpoint, placing four of the vital loci within a maximum of 15.5 kb. The location of *Dox-A2* in a cluster of genes affecting cuticle formation is discussed.

THE major components of insect cuticle are cuticular proteins, chitin and catecholamines as crosslinking agents. In a holometabolous insect such as *Drosophila melanogaster*, there are several different cuticles formed during the lifetime of the animal. The synthesis of these cuticles requires the functioning of a large number of enzymes at specific times during development. One of the enzymes that plays a pivotal role both in catecholamine metabolism and the sclerotization and melanization of cuticle is phenol oxidase. The phenol oxidase enzyme system requires the participation of at least six proteins to go from the inactive proenzyme to the final activated forms (SEYBOLD, MELTZER and MITCHELL 1975). Due to the complexity of the phenol oxidase system, it is important to identify the individual genes coding for its components in order to be able to analyze their roles in cuticle formation. In the accompanying

paper (PENTZ, BLACK and WRIGHT 1986) we have presented data which show that mutations in the *Dox-A2* locus reduce diphenol oxidase activity by altering the A2 component of this enzyme.

The *Dox-A2* gene is located in the dopa decarboxylase (*Ddc*) region of the second chromosome, defined by the breakpoints of *Df(2L)TW130* (37B9-C1,2; 37D1-2, 8-12 bands). This chromosomal region contains a functional cluster of genes involved in cuticle formation and catecholamine metabolism. Of the 18 complementation groups in the region, 14 affect the formation, sclerotization or pigmentation of the cuticle in some way (WRIGHT *et al.* 1981, 1982), including at least three, *Ddc*, *Dox-A2* and *l(2)amd*, which affect catecholamine metabolism directly (WRIGHT, BEWLEY and SHERALD 1976; PENTZ, BLACK and WRIGHT 1986; B. C. BLACK and T. R. F. WRIGHT, unpublished results). Several genetic deficiencies subdivide the region, and GILBERT, HIRSH and WRIGHT (1984) localized some of these deficiency breakpoints in cloned DNA. They found a dense cluster of genes in the *Ddc* proximal area (six loci in a maximum of 23 kb). This is an exceptionally high gene density for Drosophila, particularly for genes which share little sequence homology. Most other clustered Drosophila transcription units contain repeated gene families with extensive homology (histones: LIFTON *et al.* 1978; chorion proteins: SPRADLING 1981; heat shock proteins: CORCES *et al.* 1980; VOELLMY *et al.* 1981; SIROTKIN and DAVIDSON 1982; AYME and TISSIÈRES 1985; cuticle proteins: SNYDER *et al.* 1982; SNYDER and DAVIDSON 1983; and salivary glue proteins: GARFINKEL, PRUITT and MEYEROWITZ 1983.)

Since the *Dox-A2* locus is one of the *Df(2L)TW130* region genes, the function of which in cuticle formation is known, we wished to locate it in the cloned DNA. This localization would show us its relation to the *Ddc* proximal gene cluster, would elucidate the arrangement of loci in the region around *Dox-A2* and would provide some information about the A2 protein coded for by *Dox-A2*. In this paper we report the localization of the *Dox-A2* coding region and four surrounding genes within the *Ddc* region DNA. The *Dox-A2* coding region contains at least one small intron, and the 3′ end of the 1.7-kb mRNA is centromere proximal. Mapping of genetically defined deficiency breakpoints showed that *Dox-A2* and the four surrounding vital genes are located in a region of only 28 kb of DNA. This cluster of five *Ddc* distal genes is located within 62 kb of the *Ddc* proximal gene subcluster.

MATERIALS AND METHODS

Drosophila stocks: All mutations and balancer chromosomes used are described in LINDSLEY and GRELL (1968) and WRIGHT *et al.* (1982), with the exception that *Df(2L)130* and *Df(2L)203* are now designated *Df(2L)TW130* and *Df(2L)TW203*, respectively. The diepoxybutane-induced deficiency, *Df(2L)OD15*, and its parental strain were given to us by D. CONTAMINE. The γ-ray-induced deficiency, *Df(2L)NST*, was given to us by P. GIBBS and J. L. MARSH. Flies were maintained on cornmeal, oats, agar, dextrose and yeast medium in half-pint milk bottles at 25°.

Materials: Restriction endonucleases and other enzymes were purchased from Bethesda Research Laboratories, New England Biolabs or Boerhinger-Mannheim. [α-^{32}P]-dCTP was purchased from Amersham or New England Nuclear.

λ-Drosophila hybrid phage stocks: The eight λ-Drosophila hybrids (λ1, λ5, λ6, λ7,

λ11, λ13, λ16, λ20) covering 100 kb of DNA in the *Df(2L)TW130* region were obtained from D. GILBERT and J. HIRSH (HIRSH and DAVIDSON 1981; GILBERT, HIRSH and WRIGHT 1984). Five λ-Drosophila hybrids (λRS21–λRS25) covering 38 kb distal to λ20 (the most distal clone from GILBERT's series) were obtained from R. STEWARD (STEWARD, MCNALLY and SCHEDL 1984). All clones except λRS25 were selected from the Canton-S DNA library cloned into the *Eco*RI site of the λ Charon 4 vector (MANIATIS *et al.* 1978); λRS25 contains Canton-S DNA in the *Bam*HI site of λ EMBL4.

Isolation of DNA: Phage particles were isolated and purified from *E. coli* KH802-infected cells according to the method of YAMAMOTO *et al.* (1970), as outlined in MANIATIS, FRITSCH and SAMBROOK (1982), except that the CsCl step gradient was omitted. DNA was released from phage particles by incubation in 100 μg/ml proteinase K and 0.1% SDS for 3–18 hr. After extraction with phenol, then chloroform, the DNA was precipitated with ethanol, washed in 70% ethanol and resuspended in TE (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, pH 8.0).

Drosophila DNA was extracted from whole flies (either fresh or stored at −70°) as described by GILBERT, HIRSH and WRIGHT (1984), with the exception that diethylpyrocarbonate was omitted.

Large-scale extraction of plasmid DNA was according to the alkaline lysis method of BIRNBOIM and DOLY (1979), as outlined by MANIATIS, FRITSCH and SAMBROOK (1982). Plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. Ethidium bromide was extracted with isopropanol saturated with TE and CsCl. After addition of 2 volumes of TE, plasmid DNA was precipitated with ethanol and was resuspended in TE.

Subcloning of genomic DNA fragments: Genomic Drosophila fragments for subcloning were obtained by digesting λ-Drosophila hybrids with the appropriate restriction enzyme(s), separating the fragments in agarose gels and collecting them onto Schleicher and Schuell NA-45 membrane. Restriction fragments were inserted into the plasmid pUC8 and were used to transform *E. coli* strain JM83, and insert-containing colonies were selected using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) color selection (MESSING, CREA and SEEBURG 1981).

Gel electrophoresis, DNA transfer and hybridization: Genomic Drosophila DNA was digested with restriction endonucleases and was electrophoresed in submerged horizontal 0.7, 1.0 or 1.2% agarose gels containing 1 μg/ml ethidium bromide in Tris-borate buffer (MANIATIS, FRITSCH and SAMBROOK 1982). Fragments were transferred to nitrocellulose filters in 20 × SSC as described by SOUTHERN (1975), with the addition of a 10-min treatment of the gel with 0.24 N HCl before the denaturation step. Each gel lane contained DNA from approximately ten flies. After transfer, filters were rinsed for 30 sec in 4 × SSC, air dried and then baked in a vacuum oven for 2–4 hr at 70°.

Dox-A2 hybridization probes were prepared from either whole λ-Drosophila hybrid clones or plasmid subclones by labeling with [α-^{32}P]dCTP by nick translation (RIGBY *et al.* 1977). Labeled probe was recovered by precipitation from 2 M ammonium acetate and was resuspended in TE. Specific activities ranged from 1 to 6 × 10^7 cpm per microgram of input DNA.

Filters were prehybridized for a minimum of 6 hr at 42° in (1) 50% formamide, 1.25 × Denhardt's solution, 3.75 × SSC, 62.5 mM sodium phosphate, pH 6.8, 1.25% glycine and 40 μg/ml denatured salmon sperm DNA or (2) 50% formamide, 1 × Denhardt's solution, 5 × SSC, 50 mM sodium phosphate, pH 6.8, and 250 μg/ml denatured salmon sperm DNA. Nick translated DNA (from 1 to 6 × 10^7 cpm) was hybridized to filter bound DNA in either of two solutions: (1) 50% formamide, 1.25 × Denhardt's solution, 3.75 × SSC, 40 mM sodium phosphate, pH 6.8, 6 μg/ml denatured salmon sperm DNA and 10% dextran sulphate or (2) prehybridization solution plus labeled probe. Hybridizations were incubated at 42° for a minimum of 18 hr. Filters were washed with agitation for four 30-min periods at 68° with 0.1 × SET (1 × SET = 150 mM NaCl, 20 mM Tris-Cl, pH 7.8, 1 mM EDTA), 0.1% SDS. In order to reuse

blots, they were kept moist at all times, and labeled hybridized probe was removed by boiling for 2 min per side in distilled water.

Screening cDNA libraries: Oregon-R cDNA libraries in λgt10 from crawling third instar larvae and 24 to 48-hr pupae were obtained from YEDVOBNICK (WHARTON *et al* 1985). A total of 200,000 plaques from each library was screened (MANIATIS, FRITSCH and SAMBROOK 1982) using a plasmid probe for the *Dox-A2* region.

Isolation of RNA and Northern blotting: Crawling third instar larvae were collected, weighed and stored at −70°. Larvae were ground to a powder in liquid N_2, suspended in 8 M guanidine hydrochloride (4 ml/g larvae) and extracted with an equal volume of phenol saturated with 10 mM Tris, pH 9.0. Debris was pelleted at room temperature; 2.5 volumes of 100% ethanol were added to the supernatant and RNA was precipitated at −70° and pelleted. The pellets were resuspended in TES (10 mM Tris, pH 9.0, 2 mM EDTA, 0.5% SDS) and were extracted sequentially with phenol and chloroform; the RNA was precipitated from sodium acetate and was pelleted. Samples were stored in distilled water at −70°. Embryo RNA (15–17 hr) was prepared by the method of GEITZ and HODGETTS (1985), and adult RNA (0–4 hr) was prepared according to PIROTTA, HADFIELD and PRETORIUS (1983).

RNA was denatured and electrophoresed on 1.6 or 1.8% agarose-formaldehyde gels (MANIATIS, FRITSCH and SAMBROOK 1982) at 30 mA, 4° for 4–6 hr, with buffer recirculation. Molecular weight standard lanes (*E. coli* rRNA, quail rRNA and Drosophila rRNA) were stained with ethidium bromide in 0.5 M ammonium acetate. The remainder of the gel was washed in distilled water (5 × 5 min), equilibrated in 20 × SSC and transferred to nitrocellulose membrane in 20 × SSC, or to GeneScreen*Plus* membrane in 10 × SSC. After transfer, the nitrocellulose filter was rinsed briefly in 4 × SSC and was baked immediately for 2 hr at 80° *in vacuo*: the GeneScreen*Plus* filter was rinsed in 2 × SSC, air dried and then baked as above. Nitrocellulose filters were prehybridized at 42° in DNA hybridization mix 2 (see above) for 18 hr and were then hybridized to 3–7 × 10^6 cpm of labeled probe for 24 hr in prehybridization solution plus 10% dextran sulphate. Washing was as for Southern blots. The GeneScreen*Plus* blots were hybridized in 50% formamide according to manufacturer's instructions and were washed (30 min per wash) once in 0.1 × SET, 1% SDS; once in 0.1 × SET, 0.5% SDS; and twice in 0.1 × SET at 60°.

RESULTS

Organization of genes in the region distal to *Ddc*: Three deficiency breakpoints genetically define two regions which contain the five *Ddc* distal lethal complementation groups, *l(2)37Ba*, *l(2)37Bb*, *l(2)37Bc*, *l(2)37Be* and *Dox-A2* (Figure 1). *Df(2L)hk18* deletes all five of the lethal complementation groups and the nonvital locus *hk*. The proximal breakpoint of this deficiency is located in the cloned DNA between coordinates −51.4 and −53.0 in GILBERT's clone λ20 (Figure 1) (GILBERT, HIRSH and WRIGHT 1984). The second deficiency, *Df(2L)OD15*, deletes *hook* (*hk*), *l(2)37Ba*, *l(2)37Bc* and *l(2)37Be*, but does not remove *l(2)37Bb* or *Dox-A2*. The third deficiency, *Df(2L)TW203*, removes only *hk*, the most distal gene of the *Df(2L)TW130* region, but does not delete any of the vital loci in question.

In order to locate the breakpoints of the deficiencies within the cloned DNA, restriction endonuclease digests of genomic DNA were probed with ^{32}P-labeled Drosophila DNA from clones distal to −53 (Figure 1). Since the deletions are homozygous lethal, genomic DNA was isolated from heterozygotes, and the restriction endonuclease fragment pattern was compared with that from homozygous parental DNA and DNA from the *CyO* balancer heterozygous with

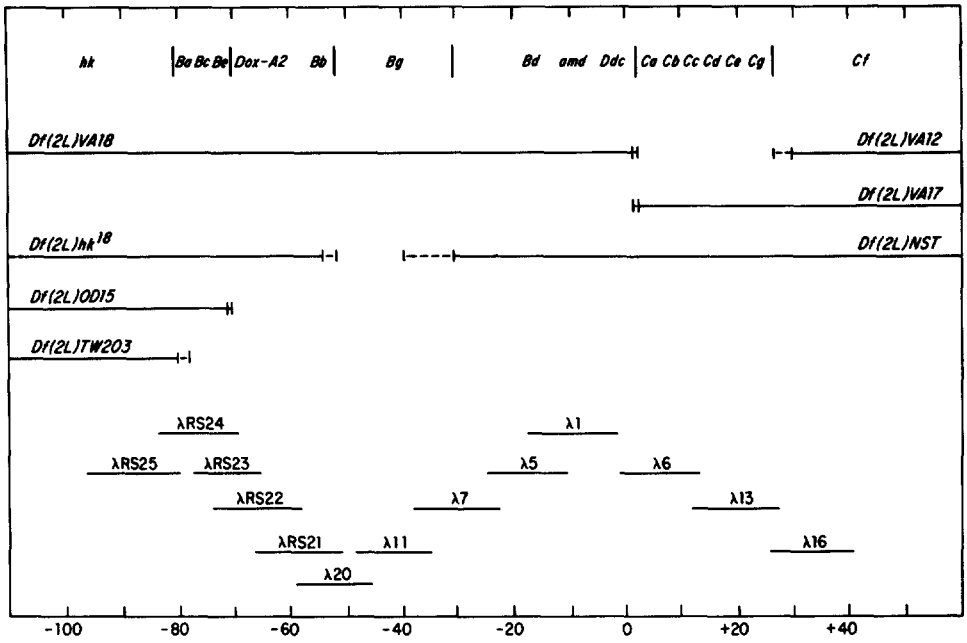


FIGURE 1.—Organization of lethal complementation groups in cloned Drosophila DNA of the *Df(2L)TW130* region. Coordinate 0 is assigned to the proximal end of λ1, and each coordinate unit corresponds to 1 kb of DNA. All centromere distal DNA is assigned a negative number and centromere proximal DNA a positive number. Lambda clone locations are as in GILBERT, HIRSH and WRIGHT (1984) and STEWARD, MCNALLY and SCHEDL (1984). The position of the 17 loci in the region is shown with respect to deletion breakpoints. Solid lines for the deletions represent the deleted region of DNA, and dashed lines represent the region of uncertainty in the endpoint.

Df(2L)TW130, which deletes all DNA of the region. The disappearance of fragments within the deletion is not expected due to the presence of the nondeficient, wild-type homologue. Therefore, a deletion endpoint is localized by using DNA probes which recognize new fragments representing the fusion of DNA flanking the deletion.

Genomic Southern blots probed with λRS22 and λRS23 reveal an extra *Eco*RI fragment in *Df(2L)OD15* DNA, but λRS21, λRS24 and λRS25 probes do not (not shown). Since the distal end of λRS21 and the proximal end of λRS24 are just 1.6 kb apart, this result indicates that the breakpoint of *Df(2L)OD15* must lie within this small region. Subclones which divide this region were constructed from λRS22 and used as probes. Figure 2A shows a genomic Southern blot probed with the 4.3-kb *Eco*RI fragment. This probe detects one extra fragment in each of the digests (*Pst*I, 1.85 kb; *Eco*RI, 1.35 kb; and *Eco*RI+*Pst*I, 1.25 kb). These results place the breakpoint of *Df(2L)OD15* within the 4.3-kb *Eco*RI fragment. Probing the same blot with the 6.5-kb *Eco*RI fragment (Figure 2B) shows that only in the *Pst*I digest is an extra band detected (1.85 kb). This locates the breakpoint of *Df(2L)OD15* distal to the *Eco*RI site at −67.3 and proximal to the *Pst*I site at −68.3. Thus, *Dox-A2* and *l(2)37Bb* are contained within 14.3 to 16.8 kb between coordinates

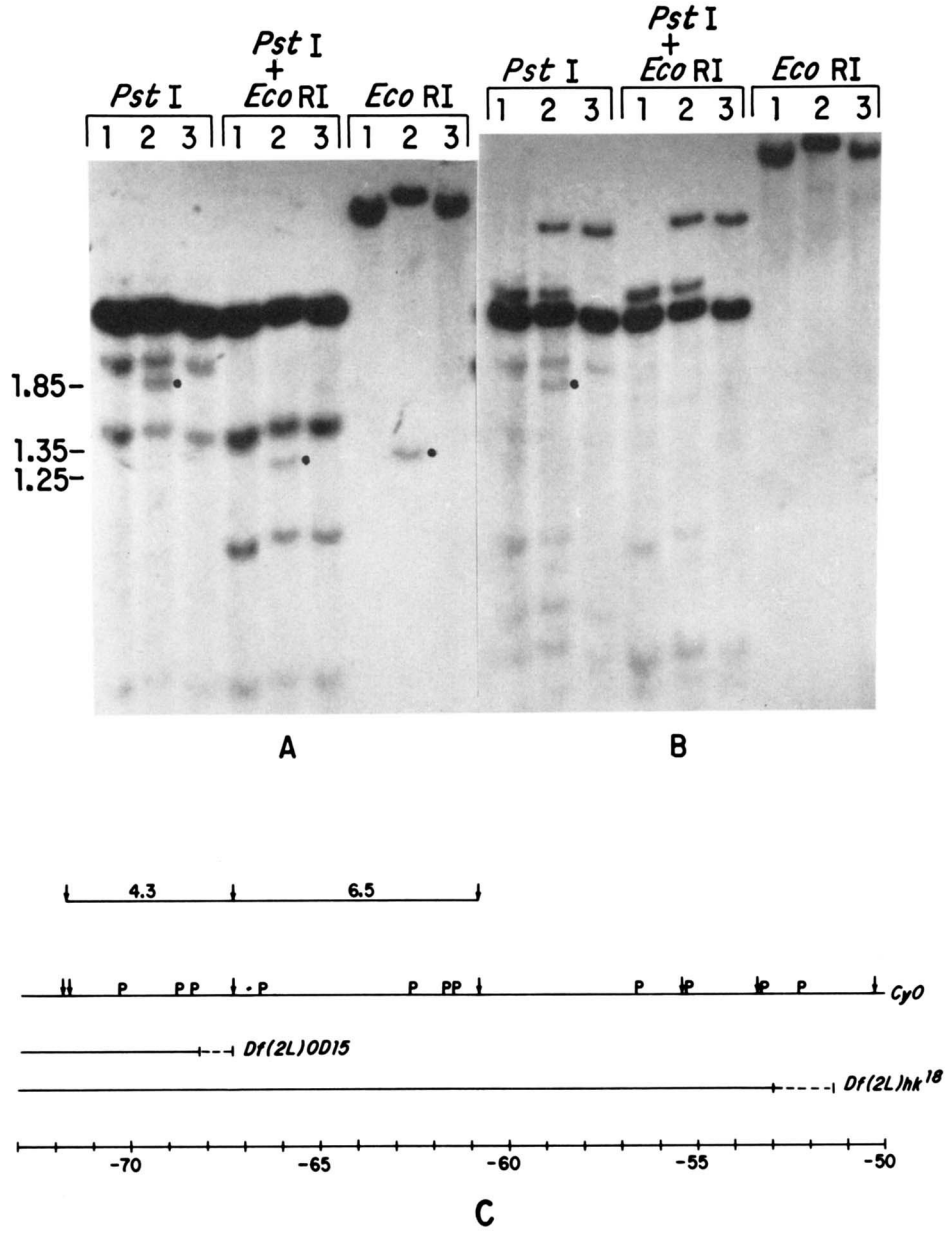


FIGURE 2.—Localization of proximal breakpoints of *Df(2L)OD15* and *Df(2L)hk18*. Genomic DNA blots of Drosophila DNA were probed with ^{32}P-labeled DNA fragments. The resultant pattern for the deficiency strain is compared with its parental strain and *CyO*. A, *Pst*I, *Eco*RI and *Pst*I+*Eco*RI digests of the deficiency heterozygote, *Df(2L)OD15/CyO* (2), its parent strain (1) and *Df(2L)TW130/CyO* (3) were probed with subclone 4.3. B, The same blot probed with subclone 6.5. Since *Df(2L)TW130* is a deletion removing all of the cloned DNA discussed, lanes (3) represent only the *CyO* chromosome. Dots next to gel bands indicate the extra fragment detected in the deficiency strain DNA. The sizes of these fragments in kilobases are shown. C, The location of the DNA probes and breakpoints with respect to the balancer (*CyO*) chromosomal DNA is diagramed. The restriction endonuclease map of *CyO* for the enzymes *Eco*RI (↓) and *Pst*I (P) is shown. The pattern for parental DNA is the same as that of *CyO* in this region. The solid horizontal line indicates deleted DNA, and the dashed line indicates uncertainty in the position of the breakpoints.

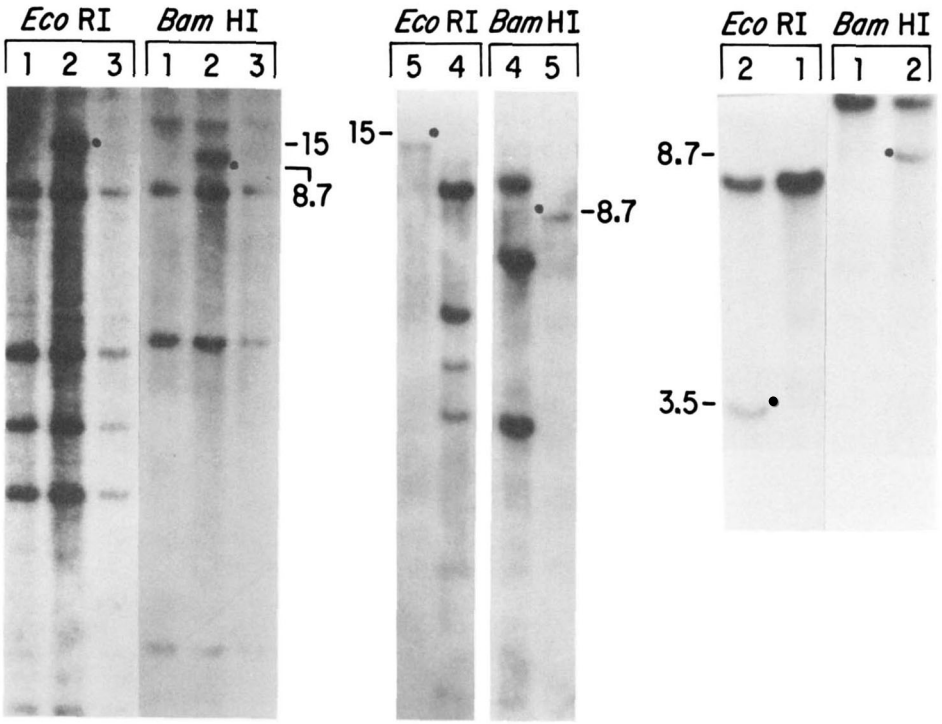
−51.4 and −68.3, as limited by the breakpoints of *Df(2L)OD15* and *Df(2L)hk18*.

Since *Df(2L)TW203* deletes only *hk* and none of the more proximal loci, genomic Southerns were probed first with λRS25. Figure 3A shows that, in both *Eco*RI and *Bam*HI digests, an extra fragment is detected in the deficiency DNA (2) (*Eco*RI, 15 kb; *Bam*HI, 8.7 kb). *Df(2L)TW130* deletes the entire region spanned by the cloned DNA, so DNA from heterozygotes of *Df(2L)TW203/Df(2L)TW130* probed with λRS25 will detect only those fragments of λRS25 present in *Df(2L)TW203*. Figure 3B shows that only one fragment is weakly detected in the *Eco*RI and *Bam*HI digests of *Df(2L)TW203/Df(2L)TW130* (5) DNA, and these correspond in size to the extra fragment detected in *Df(2L)TW203/CyO* (2). Therefore, *Df(2L)TW203* deletes all the DNA homologous to λRS25 except for the most proximal portions of the *Eco*RI and *Bam*HI fragments. In order to determine whether the breakpoint of *Df(2L)TW203* is in the λRS24 DNA, similar digests were probed with the 7.0-kb *Eco*RI subclone of λRS24. The results of this experiment (Figure 3C) show that this probe also detects an extra fragment in both *Bam*HI (8.7-kb) and *Eco*RI (3.5-kb) digests. Therefore, the breakpoint of *Df(2L)TW203* is near the *Eco*RI site at −78.8. [GILBERT (1984) confirms this localization in the region between −77.6 to −79.4.] Thus, the three lethal complementation groups *l(2)37Ba*, *l(2)37Bc* and *l(2)37Be* are located within a small region of 9.3 to 12.1 kb just proximal to *Df(2L)TW203*.

Location of *Dox-A2* in the cloned DNA: The three *Dox-A2* alleles were generated in a screen using a combination of EMS and γ-rays as mutagens (WRIGHT *et al.* 1982), making it likely that the mutant DNA would exhibit some detectable aberration. If this were the case, then probing mutant genomic DNA with DNA from the region delimited by the deficiency breakpoints might permit localization of the *Dox-A2* gene more precisely. Since these mutations are homozygous lethal, heterozygotes had to be examined and the fragment pattern compared both to the parental, *pr*, DNA and *CyO* DNA. A convenient *Pst*I site polymorphism between *CyO* and the *pr* parent (−65.8) permitted examination of some fragments separate from the *CyO* chromosome (Figure 4C).

Southern blots probed with λRS22 (−57 to −73.1) detect what appear to be deletions in *Dox-A2*^{1} and *Dox-A2*^{2} in the region of the *Pst*I polymorphism; *Dox-A2*^{3} DNA shows no difference from the parental pattern (not shown). Subclones from the area around −65.8 were used to probe mutant genomic DNA. Figure 4A shows a Southern blot probed with the 4.0-kb *Pst*I fragment that spans the polymorphic region. The *Pst*I digest of *Dox-A2*^{2} DNA (3) is missing the parental 3.0-kb *Pst*I fragment (1) and has, instead, a 1.9-kb fragment, indicating a deletion of 1.1 kb in the *Dox-A2*^{2} DNA. The *Pst*I digest of *Dox-A2*^{1} DNA (2) also shows a small difference: the expected 3.0-kb parental fragment is reduced in size to 2.9 kb, suggesting that *Dox-A2*^{1} is a deficiency of about 100 base pairs (bp). The deficiencies in *Dox-A2*^{1} and *Dox-A2*^{2} are placed between coordinates −62.8 and −65.8 by these *Pst*I digests.

The *Pvu*II digests (Figure 4A) of both *Dox-A2*^{1} (2) and *Dox-A2*^{2} (3) show no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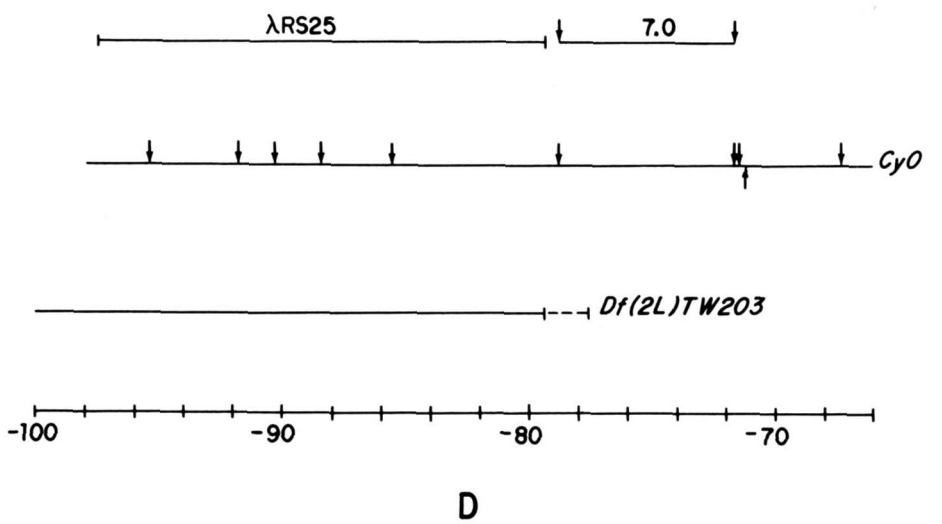
λRS25
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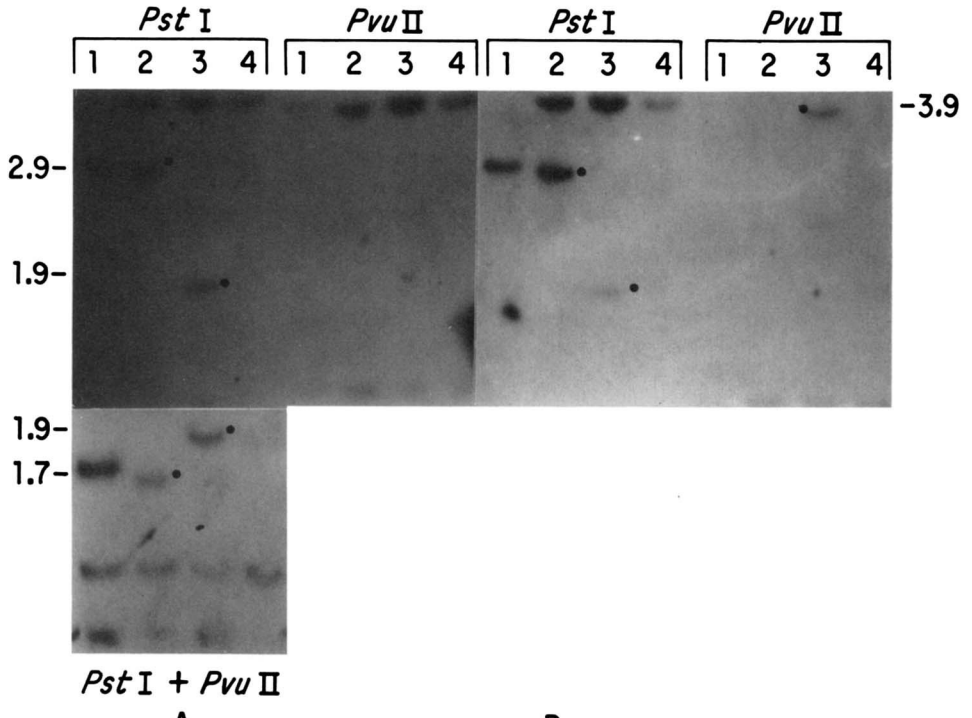
gross differences from the parental DNA (1). The small deletion in *Dox-A2*^{1} may be detected as a thickening of the 3.9-kb fragment. If the 1.1-kb deletion in *Dox-A2*^{2} removes the *Pvu*II site at −64.0, the resulting fragment would be the same size as the expected 3.9-kb parental fragment, which is the observed pattern. The *Pvu*II+*Pst*I double digests further localize the *Dox-A2* deletions (Figure 4A). In *Dox-A2*^{1} (2), the expected 1.8-kb fragment is missing and, instead, a 1.7-kb fragment appears, placing the *Dox-A2*^{1} deficiency between −64 and −65.8. The 1.9-kb *Pst*I+*Pvu*II fragment in *Dox-A2*^{2} (3) is larger than the parental fragment, confirming that the *Pvu*II site at −64.0 is deleted.

In Figure 4B, the same blot was probed with the 1.2-kb *Pvu*II fragment. This shows a difference in *Dox-A2*^{1} (2) only in the *Pst*I digest, as expected. In *Dox-A2*^{2} (3) differences are detected in both the *Pst*I and *Pvu*II digests. This result confirms the absence of the *Pvu*II site at −64.0 and suggests that the bulk of the 1.1 kb deleted in *Dox-A2*^{2} is in the region between −64.0 and −65.8, because this 1.2-kb *Pvu*II probe shares enough homology with the mutant DNA fragment to hybridize with it fairly strongly.

Selection of *Dox-A2* cDNA clones: Using the 4.0-kb *Pst*I fragment that spans the regions deleted in *Dox-A2*^{1} and *Dox-A2*^{2} as a probe, climbing third instar larval and 24- to 48-hr pupal cDNA libraries (WHARTON *et al.* 1985) were probed for sequences complementary to the *Dox-A2* region. A total of 2×10^5 plaques of each library was screened, and 61 larval and 19 pupal plaques were positive. These represent 0.03 and 0.01% of the total, respectively. A few of the most strongly hybridizing plaques were screened in a second round and resulted in the isolation of one pupal and five larval cDNAs. These clones are illustrated in Figure 5; they range in size from 0.68 kb to 1.6 kb. All five of the larval cDNAs and the one pupal cDNA have one end in common at approximately 100 bp proximal to the *Pvu*II site at −64.0. Since these cDNA libraries were constructed by priming the reverse transcript from the poly A end of the mRNA, this common end point of the cDNAs indicates that the 3′ end of the *Dox-A2* locus mRNA is near coordinate −64.0. The three larger cDNAs also contain the *Bam*HI site at −65.6 and extend about 50 bp beyond this site. In the genomic DNA, the *Pvu*II/*Bam*HI fragment measures 1.6 kb; however, in the cDNAs this region is shorter by 0.1 to 0.2 kb. (Although the cDNA libraries were constructed from the Oregon-R strain and the genomic

FIGURE 3.—Localization of proximal breakpoint of *Df(2L)TW203*. Genomic DNA blots of Drosophila DNA were probed with ^{32}P-labeled DNA fragments. A, *Eco*RI and *Bam*HI digests of the deficiency heterozygote *Df(2L)TW203/CyO* (2), its *al dp b Tft/CyO* parent strain (1) and *Df(2L)TW130/CyO* (3) probed with λRS25. B, *Eco*RI and *Bam*HI digests of the deficiency heterozygotes, *al dp b Tft/Df(2L)TW130* (4) and *Df(2L)TW203/Df(2L)TW130* (5) probed with λRS25. C, *Eco*RI and *Bam*HI digests of the deficiency heterozygote *Df(2L)TW203/CyO* (2) and its *al dp b Tft/CyO* parent strain (1) probed with subclone 7.0. Dots next to gel bands indicate the extra fragment detected in the deficiency strain DNA. The sizes of these fragments in kilobases are shown. D, The location of the DNA probes and breakpoints with respect to the balancer (*CyO*) chromosomal DNA is shown. The restriction endonuclease map of *CyO* is given for *Eco*RI (↓) and *Bam*HI (↑). The pattern for parental DNA is the same as that of *CyO* in this region. The solid horizontal line indicates deleted DNA, with the dashed line indicating the uncertainty in the position of the breakpoints.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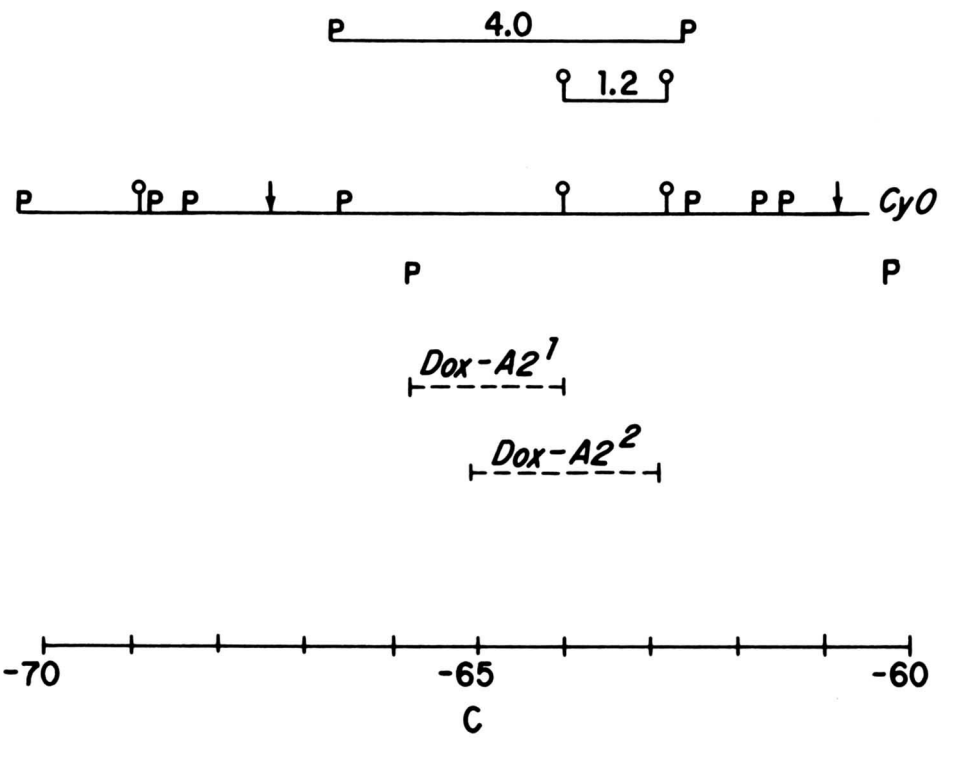
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P
P
P
P
CyO
P
P
Dox-A2 1
Dox-A2 2
-70
-65
-60
C

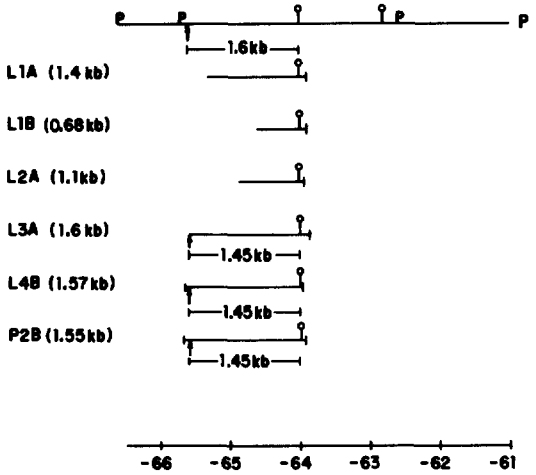

FIGURE 5.—*Dox-A2* cDNA clones. The cDNA clones were isolated from both crawling third instar larval (L) and 24- to 48-hr pupal (P) cDNA libraries (WHARTON *et al.* 1985). The restriction endonuclease map of the *pr* parent is shown for *Pst*I (P), *Pvu*II (⚲) and *Bam*HI (↑). The location of each cDNA clone is shown on the DNA map, and the size of the *Bam*HI/*Pvu*II fragment is indicated.

clones are from the Canton-S strain, the *Bam*HI/*Pvu*II fragment is the same size in genomic DNA from both strains.) Thus, there appears to be at least one 100–200-bp intron in the *Dox-A2* coding region located between −65.6 and −64.

***Dox-A2* locus mRNA:** cDNA copies of mRNA are not necessarily full-length copies of the message. To determine the full size of the *Dox-A2* locus mRNA, we analyzed RNA on Northern blots. Total cellular RNA was prepared from wild-type (*pr*) embryos (15–17 hr), larvae (crawling third instar) and adults (0–4 hr posteclosion) and *Dox-A2*^{2}/*CyO* larvae (crawling third instar). Northern blots probed with the 3.0-kb *Bam*HI-*Pst*I subclone (Figure 6A) show hybridization at one major band at 1.7 kb in embryos (E), larvae (L) and adults (A). The extent of hybridization is lower in embryos than it is in larvae and adults, as might be expected for the low level of phenol oxidase activity found during this stage. The largest molecular weight bands seen in the embryo and adult lanes are due to DNA in the samples, but the 5.0-kb band in embryos may be a precursor RNA. The same pattern of hybridization is found when the 1.6-kb *Bam*HI/*Pvu*II fragment, which contains sequences only internal to the *Dox-A2* coding region, is used as a probe (not shown). In Figure 6B, the blot of

FIGURE 4.—Localization of deletions in *Dox-A2* alleles. Genomic DNA blots of Drosophila DNA were probed with ^{32}P-labeled DNA fragments. A, *Pst*I, *Pvu*II and *Pst*I+*Pvu*II digests of mutant heterozygotes, *Dox-A2*^{1}/*CyO* (2) and *Dox-A2*^{2}/*CyO* (3), their *pr* parental strain (1) and *Df(2L)TW130/CyO* (4) probed with subclone 4.0. B, *Pst*I and *Pvu*II digests of mutant heterozygotes, *Dox-A2*^{1}/*CyO* (2) and *Dox-A2*^{2}/*CyO* (3), their *pr* parental strain (1) and *Df(2L)TW130/CyO* (4) probed with subclone 1.2. Dots next to gel bands indicate the deletion fragments and their sizes in kilobases are shown. C, The location of the DNA probes and the deletions with respect to the balancer (*CyO*) chromosomal DNA is shown. The restriction endonuclease map of *CyO* and the *pr* parent (P) is given for *Eco*RI (↓), *Pst*I (P) and *Pvu*II (⚲).

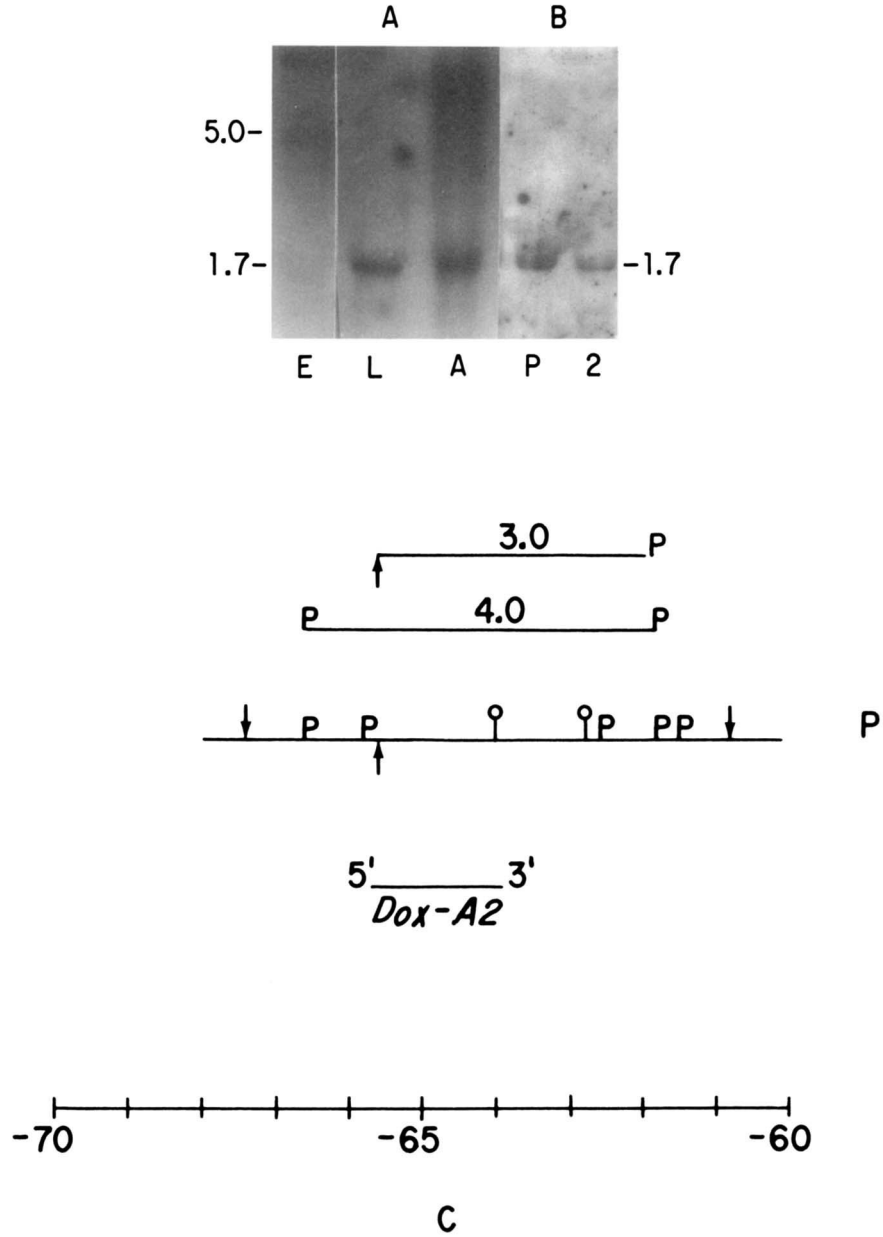

FIGURE 6.—*Dox-A2* locus RNA. A, Total cellular RNA from 15- to 17-hr embryos (E), crawling third instar larvae (L) and 0- to 4-hr adults (A) of the *pr* strain was isolated; 30 μg of each was electrophoresed in 1.6% agarose-formaldehyde gels, transferred to GeneScreen*Plus* and probed with the ^{32}P-labeled 3.0-kb *Bam*HI/*Pst*I subclone. B, Total cellular RNA from crawling third instar larvae of the *pr* parental strain (P) and *Dox-A2*^{2}/*CyO* (2) was isolated, and 50 μg of each was electrophoresed in a 1.8% agarose-formaldehyde gel, transferred to nitrocellulose and probed with the ^{32}P-labeled 4.0-kb *Pst*I subclone. Numbers indicate the size of the RNA bands in kilobases. C, The location of the DNA probes with respect to the parental (P) chromosomal DNA and the position of the *Dox-A2* coding region are indicated on the DNA. The restriction endonuclease map of the *pr* parent is shown for *Pst*I (P), *Bam*HI (↑), *Pvu*II (♀) and *Eco*RI (↓).

RNA from parental (P) and *Dox-A2*^{2}/*CyO* (2) larvae probed with the 4.0-kb *Pst*I subclone shows only one region of hybridization at the position of 1.7 kb in both the parent and mutant lanes.

DISCUSSION

GILBERT, HIRSH and WRIGHT (1984) have previously shown that *Ddc* and six lethal complementation groups in the proximal portion of the *Df(2L)TW130* region of the second chromosome of Drosophila are contained within a maximum of 31 kb. We have mapped the breakpoints of two distal deficiencies which further subdivide this *Df(2L)TW130* region. With this information we are able to localize five *Ddc* distal lethal complementation groups in the DNA. The deficiency breakpoints place three lethals, *l(2)37Ba*, *l(2)37Bc* and *l(2)37Be*, within a maximum of 12.1 kb and place two lethals, *l(2)37Bb* and *Dox-A2*, within a maximum of 16.8 kb. These five genes are thus contained in a region of no more than 30 kb. The localization of the 3′ end of the coding region for *Dox-A2* near −64 localizes the distal three genes and *Dox-A2* even closer together; four genes within a maximum of 15.5 kb. Our results and those of GILBERT, HIRSH and WRIGHT (1984) demonstrate that most of the loci in the *Df(2L)TW130* region are contained in two compact groups. Each of these represents a denser than usual clustering of genes for Drosophila, assuming an average of 15–30 kb of DNA per gene (based on an estimated 5,000–10,000 genes distributed among 1.6×10^8 bp of euchromatic DNA; RUDKIN 1961). In fact, all 15 of the vital genes so far localized at the molecular level in *Df(2L)TW130* are contained within 108 kb, which, even disregarding the subclustering, is a very dense arrangement.

Most other clustered Drosophila transcriptional units contain repeated gene families with extensive homology (histones: LIFTON *et al.* 1978; heat shock proteins: CORCES *et al.* 1980; VOELLMY *et al.* 1981; SIROTKIN and DAVIDSON 1982; AYME and TISSIÈRES 1985; chorion proteins: SPRADLING 1981; cuticle proteins: SNYDER *et al.* 1982; SNYDER and DAVIDSON 1983; salivary glue proteins: GARFINKEL, PRUITT and MEYEROWITZ 1983). Some of these clusters, however, have interspersed unrelated transcriptional units, the activities of which are not developmentally coordinated with the homologous genes. In addition, other regions of the genome have been described that have very high transcription unit densities, with no evidence of gene homology or coordinate regulation (BOSSY, HALL and SPIERER 1984; VINCENT *et al.* 1984). The region flanking *Ddc* differs from other gene clusters in that many of the loci appear to play a role in a common function, although most show no intergenic sequence homology and, hence, no close evolutionary relationship (GILBERT, HIRSH and WRIGHT 1984; E. S. PENTZ and T. R. F. WRIGHT, unpublished results).

The reason for the close proximity of the *Ddc* region genes may become apparent once their patterns of expression and functions are known. Each of the subclusters contains a gene that is known to be directly involved in catecholamine metabolism (*Ddc*, dopa decarboxylase; *Dox-A2*, diphenol oxidase). Examination of the few hemizygous pupae or pharate adults obtainable from

leaky alleles shows that all of these loci affect cuticle in some way; either the cuticle is incompletely formed or not properly sclerotized or pigmentation is incomplete or abnormally distributed (T. R. F. WRIGHT and E. Y. WRIGHT, unpublished results). It is known from *P* element-mediated reintegrations of fragments encoding *Ddc* and several other genes of the *Ddc* proximal subcluster (SCHOLNICK, MORGAN and HIRSH 1983; MARSH, GIBBS and TIMMONS 1985; J. KULLMAN and T. R. F. WRIGHT, unpublished results) that this entire region need not be intact for proper expression of the genes. Insight into the rationale for clustering should be available once the patterns of mRNA expression of these genes are known, but identification of the biochemical function of these loci will be required before it can be fully understood.

The *Dox-A2* locus may identify a structural locus for the A2 component of diphenol oxidase (PENTZ, BLACK and WRIGHT 1986). The location of the small deficiencies in *Dox-A2*^{1} and *Dox-A2*^{2} permitted unequivocal identification of the cDNA clones as coding for the *Dox-A2* gene. Our localization of the coding region for *Dox-A2* is the first demonstration of a DNA location for a probable structural component of the phenol oxidase enzyme system. Very little is known about the A2 component protein of phenol oxidase, beyond that it may be a lipoprotein (MITCHELL, WEBER and SCHAAR 1967). Our Northern analysis shows that *Dox-A2* locus mRNA is approximately 1.7-kb long, which can code for a protein of 68,000–70,000 daltons in molecular mass. The *speck* (*sp*) locus may also be a structural gene for A2 (WARNER, GRELL and JACOBSON 1975), so the A2 component may be a dimer of two nonidentical subunits coded for individually by *Dox-A2* and *sp*.

Analysis of cDNA clones shows that the coding region contains at least one intron of 100–200 bp in length. Such small introns (1 kb and less) seem to be typical of Drosophila enzymes and structural proteins (for example, alcohol dehydrogenase: GOLDBERG 1980; BENYAJATI *et al.* 1981; dopa decarboxylase: HIRSH and DAVIDSON 1981; actin: FRYBERG *et al.* 1981). The 3′ end of the mRNA is centromere proximal, as inferred from the common endpoints of all six cDNA clones. This indicates that the direction of transcription of the *Dox-A2* locus is opposite to that of *Ddc*, for which the 3′ end is centromere distal (HIRSH and DAVIDSON 1981; GILBERT, HIRSH and WRIGHT 1984).

The results of the Northern analysis show that mRNA for the *Dox-A2* locus is the same size at all stages of development examined. Furthermore, no difference is observed in the size of the *Dox-A2*^{2}/*CyO* RNA, which means that the hybridization is to *CyO* RNA and that no or very little RNA from *Dox-A2*^{2} (a 1.1kb-deletion, for which the mRNA would be expected to be abbreviated) is accumulated. The appearance of one major band of hybridization in all stages indicates that only one species of *Dox-A2* mRNA accumulates in the cell to any extent, although the 5.0-kb band in the embryo RNA may be a precursor RNA. The abundance of the RNA, as estimated from the intensity of hybridization, parallels the enzyme activity found at these stages (MITCHELL 1966; GEIGER and MITCHELL 1966; PENTZ, BLACK and WRIGHT 1986). When crawling third instar larval and 24- to 48-hr pupal cDNA libraries are screened for *Dox-A2* locus clones, the number of positive plaques found in the first

round of selection (0.03% of larval and 0.01% of pupal plaques) also reflects the differing levels of enzyme activity during these two stages of development. The correlation between the level of phenol oxidase enzyme activity and the stage-specific amounts of RNA detected in Northerns and the frequency of recovery of cDNA clones suggests that the stage-specific activity of diphenol oxidase may be under transcriptional control. Once other phenol oxidase component coding regions have been identified, it will be possible to analyze more fully the regulation of this complex enzyme system during development.

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