Molecular Analysis of Diepoxybutane-Induced Mutations at the *rosy* Locus of *Drosophila melanogaster*

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ABSTRACT

We have analyzed at the molecular level diepoxybutane-induced mutants determined to have lesions affecting expression of the *ry* locus. Of the 21 mutants analyzed here, genetic analysis suggested that five were putative deficiencies involving *ry* and adjacent lethal loci. However, molecular analysis confirmed that only two of these five putative deficiencies were in fact deletions detectable by the methods used in the analysis. The remaining 16 mutants were viable as homozygotes, suggesting that their lesions were confined to the *ry* locus. Seven of these 16 intragenic mutants were determined to be deletions of genetic material as evidenced by altered restriction patterns relative to the wild type patterns. Thus, nine of 21 (43%) diepoxybutane-induced mutants are due to deletions ranging in size from approximately 50 base pairs to more than 8 kilobase pairs. Most of the deletions (seven of nine or 78%) are intragenic and less than 250 base pairs in size; it seems that most, if not all, affect coding rather than regulatory sequences.

DIEPOXYBUTANE (DEB) is a potent chemical mutagen in a variety of biological systems (reviewed in EHRENBERG and HUSSAIN 1981). This difunctional alkylating agent is capable of producing alkali-labile sites in DNA and of forming inter- and intrastrand cross-links. Exactly how this compound acts and the precise end results of its premutagenic lesions are not clear, but there is evidence that this compound is mutagenic in *Drosophila melanogaster* (GRAF *et al.* 1984), is an efficient chromosome breaker (WATSON 1966; ZIMMERING 1983) and causes a high percentage of multilocus deletions in *Drosophila* (SHUKLA and AUERBACH 1980; OLSEN and GREEN 1982).

We chose to analyze DEB-induced mutations at the *rosy* (*ry*) locus of *D. melanogaster* on a molecular level since this type of analysis could allow us to classify intragenic mutations unambiguously as deletions. Recent studies by COTÉ *et al.* (1986) have demonstrated the utility of this system for the analysis of spontaneous and X-ray-induced mutations. A similar type of analysis has also been reported for X-ray-induced mutants at the *Adh* locus of Drosophila (KELLEY *et al.* 1985).

Forward mutations at the *ry* locus are easily detected by a phenotypic change in the eye color from the dull red wild type to a reddish brown. The *ry* locus and its gene product have been well characterized on a genetic, cytogenetic and biochemical level (CHOVNICK, GELBART and MCCARRON 1977; CHOVNICK *et al.* 1978) and the *ry* gene has recently been cloned and mapped to a specific polytene band (BENDER, SPIERER and HOGNESS 1983; SPIERER *et al.* 1983). Functional tests have shown that the entire *ry* locus is contained within an 8.1-kb *Sal*I restriction fragment (RUBIN and SPRADLING 1982). The *ry* transcriptional unit spans nearly the entire *Sal*I fragment (W. Bender, unpublished data) so one would expect to find mutations in most regions of the fragment.

MATERIALS AND METHODS

Materials: Diepoxybutane was purchased from Aldrich Chemical Company. Restriction endonucleases were purchased from Pharmacia P-L Biochemicals, Bethesda Research Laboratories and Boehringer-Mannheim. Proteinase K was obtained from Sigma and RNase A from Sigma or Pharmacia P-L Biochemicals. Nitrocellulose and DEAE membrane were from Schleicher and Scheull. Radiochemicals were obtained from New England Nuclear. Nick translation materials and agarose were from Bethesda Research Laboratories.

Mutagenesis and stock maintenance: Mutagenesis was done on the specifically characterized ry^{+11} allele which exhibits normal levels of enzyme activity and whose gene product has a fast mobility relative to other isoalleles (CHOVNICK, GELBART and MCCARRON 1977). Males of the genotype kar^2 ry^{+11} were collected within 6 hr of eclosion and aged with females for a 2-day period. After the 2-day period, the males were separated from the females, aged an additional 24 hr, starved for 3 hr and fed 5 mM DEB for 24 hr in a 5% aqueous sucrose solution by the method of AARON, NARDIN and LEE (1977). Treated males were mated individually for 2 days with two females, *y*; cn^{35}; ry^{41}. (For a description of symbols, see LINDSLEY and GRELL 1968.) In some experiments multiple broods were examined for the purpose of expanding the number of progeny. F_1 progeny were screened for the occurrence of the rosy phenotype. Such exceptional flies were mated with *Df(3R)* ry^{1608}/*MKRS* flies. *Df(3R)* ry^{1608} is a deletion of genetic material spanning the entire *ry* locus and adjacent loci and is maintained as a heterozygote over the third chromosome balancer, *Tp(3)MKRS, M(3)S34 kar* ry^2 *Sb*. F_2 progeny which exhibited

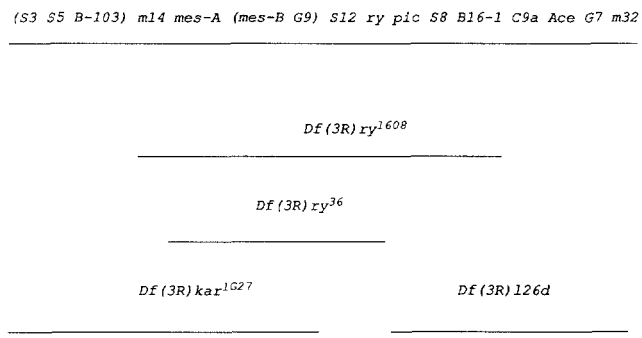

FIGURE 1.—Genetic map of polytene chromosome region 87DE. The map shows the relative order of the loci surrounding the *ry* locus and the extent of the deficiencies used in this analysis. [Adapted from HILLIKER *et al.* (1980).] The centromere is to the left of the *S3* locus.

the *kar ry* phenotype were inbred. If possible, the stocks were made homozygous; when not possible, the mutant chromosome was maintained as a heterozygote over the *MKRS* balancer chromosome.

DNA from the heterozygous DEB-induced mutants could not be easily analyzed as $ry^{DEB}/MKRS$ because the restriction fragments generated by ry^{2} on the balancer chromosome could not readily be distinguished from those fragments generated by the newly induced mutant allele. To circumvent this problem, females of the heterozygous DEB strains were mated with ry^{60} or ry^{506} males, strains with characterized intragenic deficiencies of the *ry* locus; ry^{60} involves a loss of approximately 1 kb of DNA and ry^{506} involves an approximately 3.5-kb deletion. DNA from the DEB/ry^{60} or DEB/ry^{506} flies was extracted and analyzed as described in the following sections.

Flies were grown at room temperature on standard cornmeal, agar, sugar and brewer's yeast medium containing Tegosept as a mold inhibitor and seeded with live baker's yeast.

Genetic analysis of heterozygous DEB-induced *rosy* mutants: $Df(3R)ry^{1608}/MKRS$ flies and other deletion mutants surrounding the *ry* locus were used in the genetic analysis of the heterozygous DEB-induced *ry* mutants. These deficiency strains are also maintained as heterozygotes over the *MKRS* chromosome and are characterized in HILLIKER *et al.* (1980, 1981) (Figure 1). More recently (SPIERER *et al.* 1983) the positions of the *ry* locus and the endpoints of the deficiencies used in this analysis have been more precisely defined. The *ry* locus is in polytene bands 87D12 and 13. The proximal breakpoint of $Df(3R)ry^{1608}$ is in 87D4-6 and the distal breakpoint in 87E1,2. The proximal breakpoint of $Df(3R)ry^{36}$ is in 87D7 and the distal breakpoint is 87D12. $Df(3R)kar^{1G27}$ is proximal to the *ry* locus since its distal breakpoint is in 87D8-10 and *Df(3R)l26d* is distal to the *ry* locus.

Appropriately aged virgin females of the heterozygous DEB-induced *ry* mutants were mated with males of these strains known to carry large deletions in the rosy microregion. F_1 progeny were screened to determine whether the various mutant stocks were viable in combination with the deletion strains.

Isolation of DNA from adult Drosophila: The method for isolation of genomic DNA from adult flies was a modification of a published procedure (BENDER, SPIERER and HOGNESS 1983). The modifications included treatment with RNase A (50 μg/ml, 37°, 15 min) followed by treatment with Proteinase K (150 μg/ml, 37°, 60 min) after removal of cellular debris. These enzymatic treatments were followed by three extractions with phenol and two extractions with chloroform:isoamyl alcohol (24:1). The DNA was recovered by ethanol precipitation.

Probes: The 8.1-kb *Sal*I and the 4.6-kb *Eco*RI fragments from the Canton S wild-type *ry* allele were collected in a chromosomal walk of the rosy region (BENDER, SPIERER and HOGNESS 1983) and subcloned into the plasmid vector pBR 322; both clones were obtained from W. BENDER and are referred to as pry8.1 and pry4.6 in this report. Additionally, it has recently been demonstrated that the *lS12* locus is contained entirely within pry8.1 in the region 5′ to the *ry* locus (Clark *et al.* 1986). Plasmid DNA was isolated from amplified bacterial cultures using an alkaline-SDS lysis procedure (ISH-HOROWICZ and BURKE 1981).

Restriction fragments of pry8.1 and pry4.6 were recovered from agarose gels (0.8–1.2%) using NA-45 DEAE membrane as the binding medium. This procedure was carried out according to the manufacturer's directions.

Plasmid DNA or DNA fragments were radiolabeled with [^{32}P]dCTP (600 Ci/mmol) using a nick translation kit according to the manufacturer's directions. Labeled DNA was separated from unincorporated nucleotides by gel filtration through Sephadex G-50-150 in 150 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 8). The typical yield was 25–80 × 10^6 cpm/μg DNA when plasmid DNA was nick translated and less than 3 × 10^6 cpm/μg DNA when a fragment was used in the reaction.

Restriction digests and blot hybridizations: Two to three micrograms of genomic DNA were digested to completion with a given restriction endonuclease(s) according to the manufacturer's directions. DNA was fractionated on 0.8–1.2% agarose gels using a Tris-borate buffer [0.089 M Tris, 0.089 M boric acid, 0.2 mM EDTA (pH8)]. pry8.1 was digested with various restriction endonucleases and used as molecular weight markers on the gels and resulting autoradiographs. Following electrophoresis, the gel was stained with ethidium bromide, illuminated with UV light and photographed using Polaroid type 665 film. Prior to blotting, the gel was soaked in a denaturing solution (1.5 M NaCl, 0.5 M NaOH) and then in a neutralizing solution [3 M NaCl, 0.5 M Tris (pH 7)]; each soaking was for one hour with gentle shaking. The DNA was transferred to nitrocellulose by standard capillary blotting techniques using 12 × SSC buffer (MANIATIS, FRITSCH and SAMBROOK 1982).

Prehybridizations were in 3 × SSC, 5 × Denhardt's, 0.1% SDS and 100 μg/ml sheared and sonicated calf thymus DNA, at 62° for 2–4 hr. Hybridizations were in 3 × SSC, 5 × Denhardt's, 0.1% SDS and 2–6 × 10^6 cpm of radiolabeled probe, at 62° for 15–20 hr. The filters were then washed four times for 30 min at 62° in 3 × SSC, 5 × Denhardt's and 0.1% SDS. Air-dried filters were exposed to Kodak XAR-5 film at −80° with a du Pont Cronex Lightning Plus intensifying screen. After the appropriate exposure time, the film was developed according to the manufacturer's directions.

RESULTS

Mutagenesis: Fifty-one DEB-induced *ry* mutants were recovered from an examination of approximately 189,000 F_1 test progeny (Table 1). In this report, these strains will be identified individually by isolation numbers, for example, 1-18 refers to a mutation isolated from vial number 18 of experiment 1. When strain numbers contain a lettered suffix after the experiment number, for example, 3A-118, it re-

TABLE 1

Isolation of DEB-induced *ry* mutants

F_1 examined	No. of mutants		Frequency of mutants
	Total	Transmitted	
189,263	51	23[a]	26×10^{-5}

[a] Twenty-one available for analysis.

fers to a specific brood in a multibrood mutagenesis experiment. When strain numbers contain a lettered suffix after the vial number, it indicates more than one mutant was recovered from the vial, for example, 3-20B refers to the second mutant recovered from vial 20 of experiment 3.

Of these 51 DEB-induced mutants, 23 involved germ-line mutations which were transmitted and 21 of these mutant strains were available for analysis in this research. A third strain, 1-81, was lost during this research but DNA had been extracted and was available for analysis. Of the 20 surviving strains, 15 are maintained as homozygotes and five (3-20A, 3-20B, 3A-118, 3B-65 and 5-97) are maintained as heterozygotes over the third chromosome balancer, *Tp(3)MKRS, M(3)S34, kar ry² Sb*.

Genetic analysis of DEB-induced mutants: The inability of five strains to survive as homozygotes suggested that the mutation affecting the *ry* locus might be a deletion extending from *ry* to a lethal locus on one or both sides of *ry*. As a preliminary screening, females of the heterozygous strains were mated with males of the *Df(3R)ry^{1608}/TP(3) MKRS, M(3)S34 kar ry² Sb* strain. Inviability over the *Df(3R)ry^{1608}* chromosome suggests a mutation in one of the lethal loci adjacent to the *ry* locus: *l(S12)* on the 5′ (centromeric) side or *pic* on the 3′ (telomeric) side. Three of the mutant chromosomes (3-20A, 3-20B and 3B-65) were viable over *Df(3R)ry^{1608}*, suggesting that their lethality was due to a second site mutation away from the *rosy* region.

Females of the two strains which were not viable over *Df(3R)ry^{1608}* were mated with males of the various strains which carry deficiencies in the *ry* region. The 3A-118 chromosome is not viable over *Df(3R)ry^{36}* or *Df(3R)l26d*, but is viable over *Df(3R)kar^{1G27}*. Assuming the mutation affecting *ry* is due to a single mutagenic event in the *ry* microregion, these data indicate a deletion spanning the entire *ry* locus and extending into both *l(S12)* and *pic*. The 5-97 chromosome is not viable over *Df(3R)ry^{36}* or *Df(3R)kar^{1G27}*, but is viable over *Df(3R)l26d*. Applying the same single mutagenic event assumption, these results indicate a substantial deletion of DNA 5′ to the *ry* locus and extending into the *ry* locus but probably not affecting *pic* on the 3′ side (summarized in Table 2).

The homozygous strains were not included in this analysis because their ability to survive as homozygotes

TABLE 2

Deficiency analysis of heterozygous strains

Mutant strain	Deficiency strains[a]			
	Df(3R)ry^{1608}	*Df(3R)ry^{36}*	*Df(3R)kar^{1G27}*	*Df(3R)l26d*
3-20A	+	NT	NT	NT
3-20B	+	NT	NT	NT
3B-65	+	NT	NT	NT
5-97	−	−	−	+
3A-118	−	−	+	−

[a] "+" indicates that the mutant is viable over the deficiency. "−" indicates that the mutant is not viable over the deficiency. "NT" indicates "not tested."

indicated that, if their mutations were due to deletions, the deletions did not extend beyond the *ry* locus to the lethal loci on either side.

Restriction endonuclease analysis: Twenty-one DEB-induced mutations at the *ry* locus were analyzed by comparing the restriction patterns of DNA from the mutant strains with the patterns observed in the DNA of wild type flies. It has been estimated that this method of analysis is sensitive enough to detect deletions of approximately 50 bp or larger (ZACHAR and BINGHAM, 1982) as well as smaller deletions or even single base substitutions which alter the recognition site of a restriction endonuclease used in the analysis.

pry8.1 was the primary probe used in the analysis of control and mutant DNA. Additional probes were used in the analysis of strain 5-97, and these probes are described in the discussion of strain 5-97.

In this analysis, DNA from the mutant strains was compared with *ry^{+11}* DNA, the paternal allele from which the mutants were derived and with *ry^{41}*, the mutant allele used in the initial genetic selection procedure. Digested DNA from *ry^{+11}* and *ry^{41}* flies were compared, and no differences were observed in the restriction fragments generated by *Pvu*II, *Bgl*II and *Sst*I (data not shown). These were the restriction endonucleases used initially to detect all of the mutations subsequently classified as deletions in this study. These data were used to eliminate the possibility that the same alteration observed in the mutant strains was present in either parental strain and also eliminated the need to use DNA from both parental strains as internal controls during this analysis.

Analysis of the homozygous strains: Of the 21 mutant strains examined in this analysis, 16 were maintained as homozygotes. Digestion with *Pvu*II (Figures 2, A and B, and 3, A and B) and *Sst*I (Figure 3C) and probing with pry8.1 gave normal (*i.e.*, wild type) restriction patterns for eight of the strains (1-12, 1-75, 1-81, 2-81, 4-65, 5-18, 6-74 and 6-138). Continued analyses indicated that these eight strains also had normal restriction patterns with *Bgl*II and *Nru*I digests (data not shown).

However, this preliminary analysis with *Pvu*II and

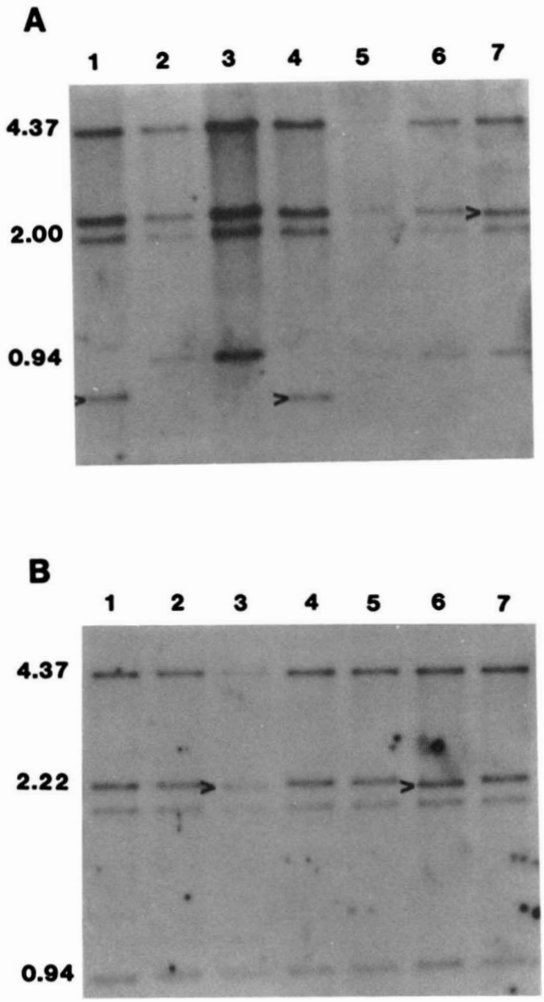

FIGURE 2.—Preliminary restriction analysis of homozygous strains. Digested DNA (2–3 μg) was applied to each lane, separated on 0.8% agarose gels, transferred to nitrocellulose and hybridized with pry8.1. The resulting autoradiographs are shown here. Arrowheads indicate altered restriction fragments. A, *Pvu*II digests of genomic DNA: lane (1) 2A-109, (2) ry^{+11}, (3) 3A-91, (4) 3B-38, (5) 6-138, (6) ry^{+11}, (7) 11B-115. B, *Pvu*II digests of genomic DNA: lane (1) 1-12, (2) ry^{+11}, (3) 1-18, (4) 1-75, (5) ry^{+11}, (6) 2-15, (7) 2-81.

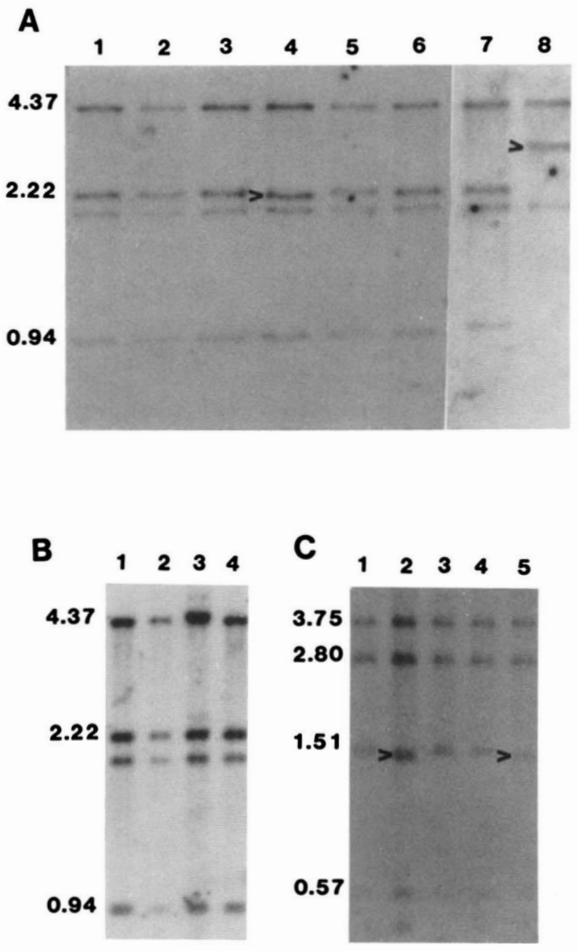

FIGURE 3.—Preliminary restriction analysis of homozygous strains. Digested DNA (2–3 μg) was applied to each lane, separated on 0.8% agarose gels, transferred to nitrocellulose and hybridized with pry8.1. The resulting autoradiographs are shown here. Arrowheads indicate altered restriction fragments. A, *Pvu*II digests of genomic DNA: lane (1) 4-65, (2) ry^{+11}, (3) 5-18, (4) 5-36, (5) ry^{+11}, (6) 6-74, (7) ry^{+11}, (8) 4-96. B, *Pvu*II digests of genomic DNA: lane (1) ry^{+11}, (2) 1-12, (3) 1-81, (4) ry^{+11}. C, *Sst*I digests of genomic DNA: lane (1) ry^{+11}, (2) 3A-91, (3) 6-138, (4) ry^{+11}, (5) 11B-115.

*Sst*I suggested that eight of the homozygous strains (1-18, 2-15, 2A-109, 3A-91, 3B-38, 4-96, 5-36 and 11B-115) involved deletions of genetic material. These strains were then subjected to further restriction analysis. The estimated size of the deletions is expressed as an average value since, in most cases, the size was derived from the data of multiple restriction analyses.

While 11B-115 was initially detected as a possible deletion in the 2.22-kb *Pvu*II fragment, 3A-91 presented the same restriction pattern as ry^{+11} (Figure 2A, lanes 3 and 7). Subsequent restriction analysis indicated both mutations were due to deletions in the 1.51-kb *Sst*I fragment (Figure 3C). Further analysis was accomplished with an *Sst*I/*Xho*I double digest (Figure 4A, lanes 4–6) and with an *Eco*RI/*Sst*I double digest (Figure 4B, lanes 4–6). Both *Xho*I and *Eco*RI cut the 1.51-kb *Sst*I fragment into two unequal fragments, the 3′ fragment being the smaller of the two (Figure 4C). While these 3′ fragments resulting from either double digest are evident in ry^{+11} DNA (lane 5 of Figure 4, A and B), they can not be detected in either mutant. This is not meant to imply that the fragments were deleted entirely in the mutant strains, but rather that the fragments generated by the double digests coincide with the small fragments from other parts of the *ry* locus and that the resulting doublets were not resolved. Taken together, these data indicate that both 11B-115 and 3A-91 are deletion mutants, the deletions being no greater than 50 bp and localized to the 3′ end of the 1.51-kb *Sst*I fragment.

Mutants 1-18, 2-15 and 5-36 were initially detected as possible deletions in the 2.22-kb *Pvu*II fragment (Figure 2B, lanes 3 and 6; Figure 3A, lane 4). Additional restriction analysis with *Bgl*II and *Sst*I (Figure

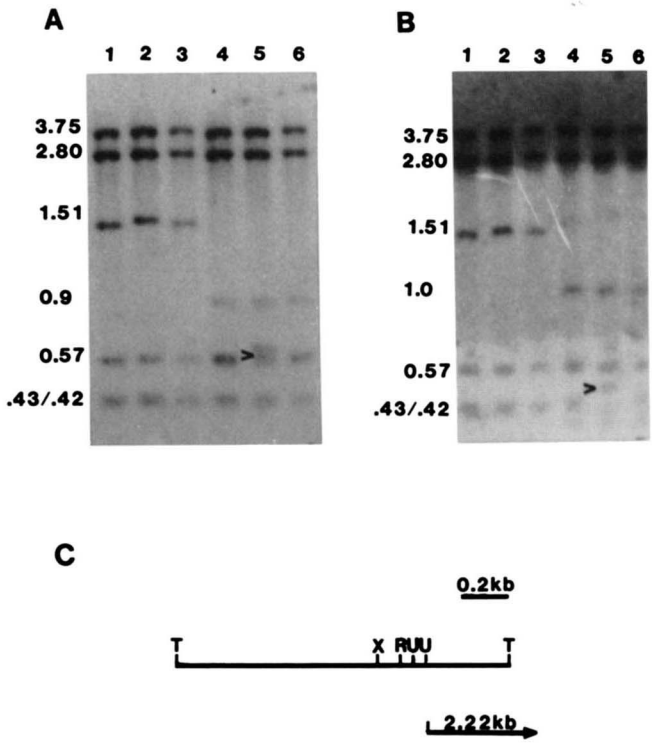

FIGURE 4.—Restriction analysis of 3A-91 and 11B-115. Digested DNA (2–3 μg) was applied to each lane, separated on 1.2% agarose gels, transferred to nitrocellulose and hybridized with pry8.1. The resulting autoradiographs are shown here. Arrowheads indicate the restriction fragments in ry^{+11} which were not detected in mutant strains. A, Comparison of *Sst*I and *Sst*I/*Xho*I digests of genomic DNA. Lanes (1) and (4) are 3A-91, lanes (2) and (5) are ry^{+11}, lanes (3) and (6) are 11B-115. DNA in lanes (1), (2), (3) are *Sst*I digests and in lanes (4), (5), (6) are *Sst*I/*Xho*I double digests. B, Comparison of *Sst*I and *Sst*I/*Eco*RI digests of genomic DNA. Lanes (1) and (4) are 3A-91, lanes (2) and (5) are ry^{+11}, lanes (3) and (6) are 11B-115. DNA in lanes (1), (2), (3) are *Sst*I digests and in lanes (4), (5), (6) are *Sst*I/*Eco*RI double digests. C, Restriction map of the 1.51-kb *Sst*I fragment showing the restriction sites pertinent to this analysis: (R) *Eco*RI, (T) *Sst*I, (U) *Pvu*II, (X) *Xho*I. The horizontal bar below the map indicates the 2.22-kb *Pvu*II fragment.

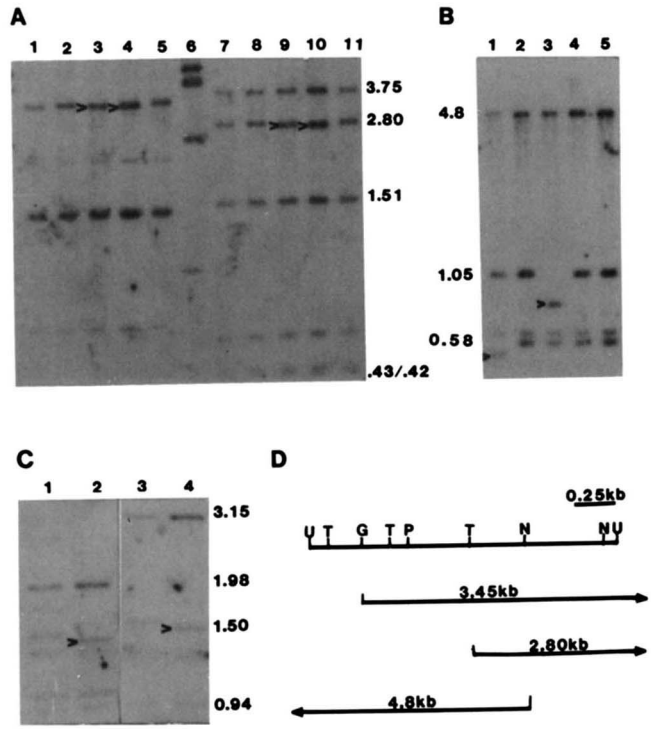

FIGURE 5.—Restriction analysis of 1-18, 2-15 and 5-36. Digested DNA (2–3 μg) was applied to each lane separated on 0.8% (A), 0.9% (B) and 1.0% (C) agarose gels, transferred to nitrocellulose and hybridized with pry8.1. The resulting autoradiographs are shown here. Arrowheads indicate altered restriction fragments. A, Comparison of *Bgl*II and *Sst*I digests of genomic DNA. Lanes (1) and (7) are 1-18; lanes (2), (5), (8) and (11) are ry^{+11}; lanes (3) and (9) are 2-15; lanes (4) and (10) are 5-36; lane (6) contains the size markers. DNA in lanes (1) through (5) was digested with *Bgl*II and in lanes (7) through (11) with *Sst*I. B, Comparison of *Nru*I digests of genomic DNA: lane (1) 2-15, (2) ry^{+11}, (3) 4-96, (4) 5-36, (5) ry^{+11}. C, Analysis of 5-36. Lanes (1) and (3) are ry^{+11} and lanes (2) and (4) are 5-36. DNA in lanes (1) and (2) are *Pst*I/*Pvu*II double digests and (3) and (4) are *Nru*I/*Pvu*II double digests. D, Restriction map of the 2.22-kb *Pvu*II fragment showing the restriction sites pertinent to this analysis: (G) *Bgl*II, (N) *Nru*I, (P) *Pst*I, (T) *Sst*I, (U) *Pvu*II. Horizontal bars below the map indicate the 3.45-kb *Bgl*II, 2.80-kb *Sst*I and 4.8-kb *Nru*I fragments.

5A) indicated deletions of 50–100 bp for 2-15 and 5-36 in the overlapping 3.45-kb *Bgl*II and 2.80-kb *Sst*I fragments (Figure 5D), but 1-18 presented a normal wild-type restriction pattern. Subsequent analysis with *Nru*I indicated a 50-bp deletion in the 0.58-kb *Nru*I fragment for 2-15 (Figure 5B, lane 1); however, 1-18 (data not shown) and 5-36 (Figure 5B, lane 4) presented normal patterns when digested with *Nru*I, suggesting that their alterations were in the 0.45-kb *Sst*I-*Nru*I fragment, contained in the 4.8-kb *Nru*I fragment (Figure 5D). Additional analysis of 5-36 with various double digests (Figure 5C) indicated a 75 ± 25-bp deletion localized to the 0.45-kb *Sst*I-*Nru*I fragment. Mutant 1-18 was analyzed in a similar manner (data not shown), but it again presented a normal restriction pattern suggesting that its alteration is not due to a detectable deletion and that the apparent restriction fragment polymorphism observed with the *Pvu*II digest (Figure 2B, lane 3) was due to anomalous migration of the DNA in the gel.

Mutant 4-96 was initially detected as a deletion mutant with a *Pvu*II digest showing that the 2.22-kb and 0.94-kb fragments characteristic of ry^{+11} were not present in the mutant DNA, but were replaced with a single larger fragment sized at approximately 3.0 kb (Figures 3A, lane 8 and 6A, lanes 3 and 4). This indicated a deletion of about 160 bp spanning the *Pvu*II site between the 2.22- and 0.94-kb fragments. The nearest restriction sites to this *Pvu*II site are *Nru*I on the 5′ side and *Bam*HI on the 3′ side; the *Nru*I-*Bam*HI fragment is approximately 0.3 kb (Figure 6B). Additional restriction analysis (Figure 5B, lane 3 and Figure 6) indicate that the deletion in 4-96 is 185 ± 25 bp and that the deletion does not affect the *Nru*I or *Bam*HI restriction sites adjacent to the altered *Pvu*II site.

Mutants 2A-109 and 3B-38 are the two strains with deletions initially localized to the 0.94-kb *Pvu*II frag-

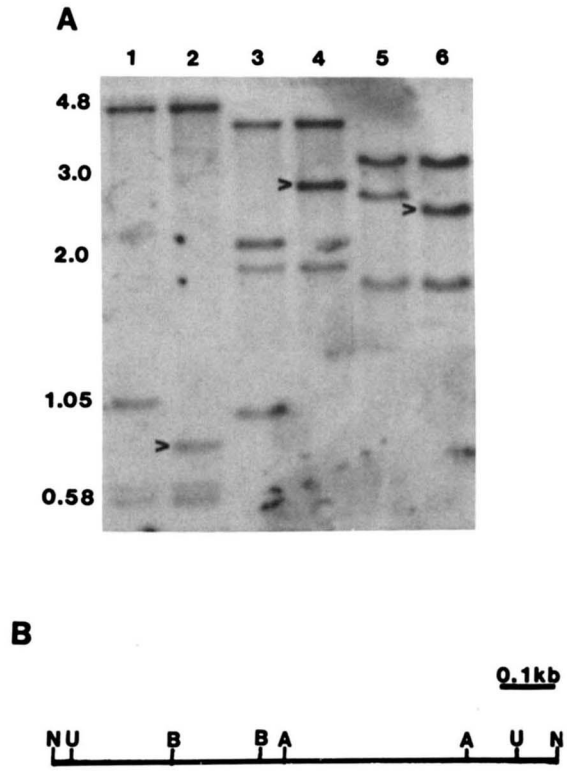

FIGURE 6.—Restriction analysis of 4-96. Digested DNA (2–3 μg) was applied to each lane, separated on a 1.0% agarose gel, transferred to nitrocellulose and hybridized with pry8.1. The resulting autoradiograph is shown here. Arrowheads indicate the altered restriction fragments. A, Analysis of 4-96. Lanes (1), (3), (5) are ry^{+11} and lanes (2), (4), (6) are 4-96. DNA in lanes (1) and (2) was digested with *Nru*I, in lanes (3) and (4) with *Pvu*II and lanes (5) and (6) are *Bam*HI/*Sal*I double digests. B, Restriction map of the 1.05-kb *Nru*I fragment showing the restriction sites pertinent to this analysis: (A) *Ava*I, (B) *Bam*HI, (N) *Nru*I, (U) *Pvu*II.

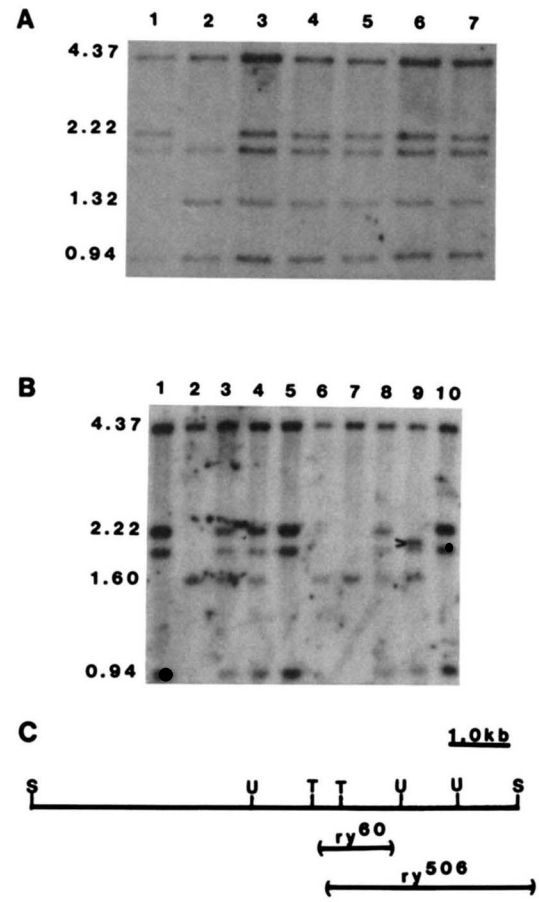

FIGURE 7.—Preliminary restriction analysis of heterozygous strains. Digested DNA (2–3 μg) was applied to each lane, separated on 0.8% agarose gels, transferred to nitrocellulose and hybridized with pry8.1. The resulting autoradiographs are shown here. A, *Pvu*II analysis of DEB/ry^{60} heterozygotes; lane (1) ry^{+11}, (2) ry^{60}, (3) ry^{+11}/ry^{60}, (4) 3-20A/ry^{60}, (5) 3-20B/ry^{60}, (6) ry^{+11}/ry^{60}, (7) 3B-65/ry^{60}. B, *Pvu*II analysis of DEB/ry^{506} heterozygotes: lane (1) ry^{+11}, (2) ry^{506}, (3) 3-20A/ry^{506}, (4) 3-20B/ry^{506}, (5) ry^{+11}, (6) 3A-118/ry^{506}, (7) ry^{506}, (8) 3B-65/ry^{506}, (9) 5-97/ry^{506}, (10) ry^{+11}. Arrowhead indicates the 2.1-kb fragment characteristic of 5-97. C, Restriction map of the *ry* locus showing the extent of the deletions of ry^{60} and ry^{506}: (S) *Sal*I, (T) *Sst*I, (U) *Pvu*II.

ment (Figure 2A, lanes 1 and 4). Subsequent restriction analysis (data not shown) identified the 2A-109 alteration as a deletion of 155 ± 25 bp in the 5′ region of the 0.94-kb *Pvu*II fragment and the 3B-38 alteration as a 200 ± 20-bp deletion in the 3′ region of this *Pvu*II fragment.

Analysis of the heterozygous strains: Initially, various restriction endonucleases were used to analyze the five DEB-induced mutant chromosomes carried as heterozygotes over the *Tp(3)MKRS, M(3)S34 kar* ry^2 *Sb* chromosome. This balancer chromosome carries the ry^2 allele, a mutation due to a B104 transposable element insertion within the 3′ end of the *ry* locus (COTÉ *et al.* 1986). It was not possible to discriminate between bands derived from the DEB-induced mutant allele and those generated by the ry^2 allele. To circumvent this problem, flies of the heterozygous strains were mated with ry^{60} or ry^{506} flies (both strains carry deletions as depicted in Figure 7C) and the DNA of F_1 progeny bearing the DEB/ry^{506} phenotype was analyzed.

Digestion with *Pvu*II gives normal restriction patterns for mutants 3-20A, 3-20B and 3B-65. When analyzed as heterozygotes over the ry^{60} deletion, these mutants show the five bands characteristic of ry^{+11} and ry^{60} (Figure 7A). When analyzed as heterozygotes over the ry^{506} deletion, all three mutants again show the normal restriction patterns for ry^{+11} and ry^{506} (Figure 7B).

*Sal*I/*Pvu*II double digests of 5-97/ry^{60} revealed a 2.1-kb fragment that is distinct from the bands observed in ry^{+11} or ry^{60} (Figure 8A). Digestion of 5-97/ry^{60} DNA and probing with pry4.6 (Figure 8G) also revealed the presence of this 2.1 kb fragment (Figure 8B), suggesting that the fragment is located within the 3′ region of the *ry* gene.

*Sst*I digests of ry^{+11} DNA typically generate six bands. Four of these bands are common to ry^{+11} and ry^{60}, but the 2.8- and 0.57-kb fragments found in ry^{+11}

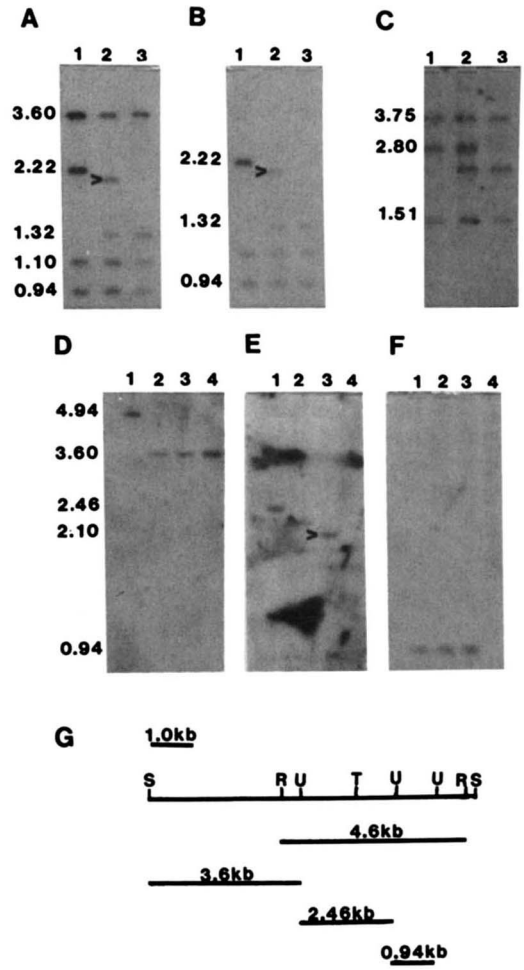

FIGURE 8.—Restriction analysis of 5-97. Digested DNA (2–3 μg) was applied to each lane, separated on 0.8% (C, D, E and F) and 1.0% (A and B) agarose gels, transferred to nitrocellulose and hybridized as indicated below. The resulting autoradiographs are shown here. Arrowheads indicate the 2.1-kb *Pvu*II fragment characteristic of 5-97. A, *Sal*I/*Pvu*II double digests of genomic DNA hybridized with pry8.1: lane (1) ry^{+11}, (2) 5-97/ry^{60}, (3) ry^{60}. B, *Sal*I/*Pvu*II double digests of genomic DNA hybridized with pry4.6: lane (1) ry^{+11}, (2) 5-97/ry^{60}, (3) ry^{60}. C, *Sst*I digests of genomic DNA hybridized with pry8.1: lane (1) ry^{+11}, (2) 5-97/ry^{60}, (3) ry^{60}. Panels D, E and F are *Pvu*II digests of pry8.1 and *Sal*I/*Pvu*II double digests of the same genomic DNA samples; they differ only in the probe used for hybridization. Lane (1) pry8.1, (2) ry^{+11}, (3) 5-97/ry^{506}, (4) ry^{506}. D, DNA probed with the 3.6-kb fragment. E, DNA probed with the 2.46-kb fragment. F, DNA probed with the 0.94-kb fragment. G, Restriction map of the *ry* locus showing the locations of the fragments used as probes in this analysis: (R) *Eco*RI, (S) *Sal*I, (T) *Sst*I, (U) *Pvu*II.

are replaced by a single 2.4-kb fragment in ry^{60} (Figure 8C). When 5-97/ry^{60} DNA was digested with *Sst*I (Figure 8C), the 2.8-kb band characteristic of ry^{+11} was intact in 5-97, again suggesting that the 3′ region of the *ry* locus is not affected by the mutation.

Further analysis of 5-97 was done using DNA from 5-97/ry^{506} heterozygotes. The parental allele and the deletion mutant (ry^{+11} and ry^{506}) have only the 5′ 4.37-kb fragment in common. When a *Pvu*II digest of 5-97/ry^{506} DNA was probed with pry8.1, the 2.1-kb fragment unique to 5-97 was observed in addition to the 2.00-kb and 0.94-kb fragments characteristic of ry^{+11} (Figure 7B, lane 9). To preclude the possibility that the 2.1-kb fragment was generated by an intragenic deletion in the 5′ region of the locus, 5-97/ry^{506} DNA was digested with *Sal*I and *Pvu*II and probed with *Pvu*II fragments of pry8.1 (Figure 8G). The 2.46-kb probe hybridizes to the 2.1-kb fragment (Figure 8E) but the 3.6-kb probe does not (Figure 8D). Likewise the 0.94-kb probe hybridizes only to the 0.94-kb fragments. When these results are considered in conjunction with the genetic analysis indicating that 5-97 is also deficient in the region 5′ to the *ry* locus, they suggest that the 5-97 mutation is due to a large deletion of DNA, including the 5′ region of the *ry* locus.

Various restriction endonuclease digests of mutant 3A-118/ry^{60} failed to show any detectable bands other than those derived from ry^{60} (data not shown). When a *Pvu*II digest of 3A-118/ry^{506} was analyzed, only the bands characteristic of ry^{506} were observed (Figure 7B, lane 6). These data indicate that the 3A-118 mutation is due to a deletion spanning the entire *ry* locus and this conclusion is consistent with the results of the genetic analysis.

DISCUSSION

The DEB-induced mutants analyzed here were all initially isolated over the intragenic ry^{41} mutation which is viable over the *Df(3R)*ry^{1608} chromosome, thus allowing for the recovery of deletions, both large and small. Two of the DEB mutant alleles (3A-118 and 5-97) are associated with large chromosomal aberrations. Although we have not rigorously demonstrated that *pic* is affected by a continuous deletion extending from *ry* to *pic*, the molecular analysis clearly indicates that the 3A-118 and 5-97 deletions extend to the *lS12* locus on the proximal side. The 3A-118 allele lacks DNA which hybridizes with pry8.1 and since this plasmid also contains the entire *lS12* locus, the 3A-118 deficiency must delete *lS12* as well as *ry*. The 5-97 allele retains approximately 4.1 kb of DNA which hybridizes with pry8.1 and further molecular analysis localized this 4.1-kb fragment to the 3′ end of the *ry* locus; thus, the 5-97 allele fails to hybridize with that portion of pry8.1 which contains the *lS12* locus. Thus, 3A-118 and 5-97 are intergenic mutations resulting from large continuous deletions of genetic material.

Seven of the DEB-induced mutations (2-15, 2A-109, 3A-91, 3B-38, 4-96, 5-36 and 11B-115) are due to small (*i.e.*, less than 250 bp) deletions within the *ry* locus. The remaining 12 strains carry intragenic alterations which may be either missense or nonsense mutations caused by base substitution, frameshift mutations or deletions too small to be detected by the

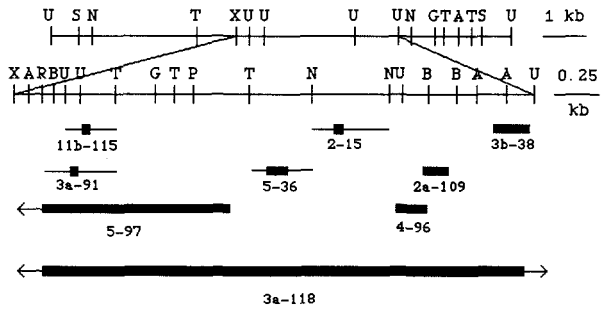

FIGURE 9.—Restriction map of *ry* locus including pertinent sites and location of DEB mutations. Sites for *Ava*I (A), *Bam*HI (B), *Bgl*II (G), *Nru*I (N), *Pst*I (P), *Eco*RI (R), *Sal*I (S), *Sst*I (T), *Pvu*II (U) and *Xho*I (X) are shown. A, Restriction map for the *ry* locus showing the *Pvu*II and *Sal*I sites, as well as other sites discussed in the paper. B, Enlarged map of the *Xho*I-*Pvu*II region in which the DEB-induced mutations were localized. C, Localization of the DEB mutations. Horizontal bars indicate approximate size of deletions; the thin lines extending from the ends of each deletion bar indicate the restriction fragment(s) to which the deletions were mapped. Arrowheads indicate the deletions extend beyond the limits of the map.

methods used in this analysis. Thus, 43% (9/21) of the DEB-induced mutations at the *ry* locus are caused by deletions ranging in size from approximately 50 bp to more than 8 kb.

The classification of only two of the 21 DEB-induced mutations as large intergenic deletions is in marked contrast to earlier reports indicating that at least one-third of DEB-induced mutations at the *y* and *w* loci are large, intergenic deletions (OLSEN and GREEN 1982) or that at least two-thirds of DEB-induced mutations at the *w*, *sn* and *m* loci are intergenic deletions large enough to include the locus of the visible mutation as well as a sex-linked lethal locus (SHUKLA and AUERBACH 1980). Both of these earlier reports are predicated upon the assumption that the observed mutations were due to a single mutagenic event, a continuous deletion extending to adjacent loci, and were not due to a cluster of point mutations in the adjacent loci. Since neither of the genetic tests used to detect the deletions were confirmed by cytogenetic or molecular analyses which would confirm that the mutations were due to deletions, the assumption of a single continuous deletion must be considered as not proven.

The demonstration that DEB causes translocations in Drosophila (WATSON 1966) indicates that DEB is capable of multiple hits during a single exposure to the mutagen. Such a multiple hit event could explain a possible clustering of point mutations leading to observations of a high frequency of deletions among DEB-induced mutations on the *X* chromosome and would explain the observed results for three of the DEB-induced mutations at the *ry* locus. The three heterozygous strains (3-20A, 3-20B and 3B-65) which are not viable as homozygotes but do not exhibit detectable deletions within the *ry* locus and which are viable over the $Df(3R)ry^{1608}$ chromosome are probably due to a point mutation (or very small deletion) within the *ry* locus and a second site mutation away from the *ry* region.

The seven intragenic DEB-induced deletions were mapped to the two internal *Pvu*II fragments (2.22 and 0.94 kb); the exception is 3A-91 which may be located within the 2.22-kb fragment or just 5′ to the fragment. This is consistent with the results of COTÉ *et al.* (1986) who localized 15 of 16 *ry* mutations identifiable by genomic blotting experiments to the two internal *Pvu*II fragments. Fine structure recombination analysis has defined the *ry* locus in terms of the genetic map positions of *ry* mutants and *ry* structural element variant alleles. ry^{23} and ry^{606} form the left boundary and ry^{2} and ry^{7} form the right boundary of the XDH structural element (GELBART, MCCARRON and CHOVNICK 1976). When this genetic map is "superimposed" on the restriction map of the *ry* locus, the genetic boundaries of the structural element coincide closely with the two internal *Pvu*II fragments (COTÉ *et al.* 1986).

Of the 21 DEB-induced mutations, the alterations of eight could be localized to a specific intragenic restriction fragment (Figure 9); 3A-118 is excluded from this discussion since it involves a total loss of the *ry* locus. These deletions are localized to the DNA regions that are reportedly within the defined boundaries of the XDH structural element. Thus, it would seem that the DEB-induced deletions cause the mutant phenotype by alteration of coding rather than regulatory sequences.

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LITERATURE CITED

AARON, C. S., H. E. NARDIN and W. R. LEE, 1977 Glass filter supports for treatment of adult *D. melanogaster* with chemical mutagens. Drosophila Inform. Serv. **52:** 166.

BENDER, W., P. SPIERER and D. S. HOGNESS, 1983 Chromosomal walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the bithorax complex in *Drosophila melanogaster*. J. Mol. Biol. **168:** 17–33.

CHOVNICK, A., W. GELBART and M. MCCARRON, 1977 Organization of the *rosy* locus in *Drosophila melanogaster*. Cell **11:** 1–10.

CHOVNICK, A., M. MCCARRON, A. HILLIKER, J. O'DONNELL, W. GELBART and S. CLARK, 1978 Gene organization in *Drosophila*. Cold Spring Harbor Symp. Quant. Biol., **42:** 1011–1021.

CLARK, S. H., M. MCCARRON, C. LOVE and A. CHOVNICK, 1986 On the identification of the *rosy* locus DNA in *Drosophila melanogaster*: intragenic recombination mapping of mutations associated with insertions and deletions. Genetics **112:** 755–767.

COTÉ, B., W. BENDER, D. CURTIS and A. CHOVNICK, 1986 Molecular mapping of the *rosy* locus in *Drosophila melanogaster*. Genetics **112:** 769–783.

EHRENBERG, L., and S. HUSSAIN, 1981 Genetic toxicity of some

important epoxides. Mutat. Res. **86:** 1–113.

GELBART, W., M. MCCARRON and A. CHOVNICK, 1976 Extension of the limits of the XDH structural element in *Drosophila melanogaster*. Genetics **84:** 211–232.

GRAF, U., F. E. WURGLER, A. J. KATZ, H. FREI, H. JUON, C. B. HALL and P. G. KALE, 1984 Somatic mutation and recombination test in *Drosophila melanogaster*. Environ. Mutagen. **6:** 153–188.

HILLIKER, A. J., S. H. CLARK, A. CHOVNICK and W. M. GELBART, 1980 Cytogenetic analysis of the chromosomal region immediately adjacent to the *rosy* locus in *Drosophila melanogaster*. Genetics **95:** 95–110.

HILLIKER, A. J., S. H. CLARK, W. M. GELBART and A. CHOVNICK, 1981 Cytogenetic analysis of the rosy microregion, polytene chromosome interval 87D2-4; 87E12-F1, of *D. melanogaster*. Drosophila Inform. Serv. **56:** 65–72.

ISH-HOROWICZ, D. and J. F. BURKE, 1981 Rapid and efficient cosmid cloning. Nucleic Acids Res. **9:** 2989–2998.

KELLEY, M. R., I. P. MIMS, C. M. FARNET, S. A. DICHARRY and W. R. LEE, 1985 Molecular analysis of X-ray-induced alcohol dehydrogenase (*Adh*) null mutations in *Drosophila melanogaster*. Genetics **109:** 365–377.

LINDSLEY, D. L. and E. H. GRELL, 1968 Genetic variations of *Drosophila melanogaster*. Carnegie Inst. Wash. Publ. 627.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

OLSEN, O.-A. and M. M. GREEN, 1982 The mutagenic effects of diepoxybutane in wild-type and mutagen-sensitive mutants of *Drosophila melanogaster*. Mutat. Res. **92:** 107–115.

RUBIN, G. M. and A. C. SPRADLING, 1982 Genetic transformation of *Drosophila* with transposable element vectors. Science **218:** 348–353.

SHUKLA, P. T. and C. AUERBACH, 1980 Genetic tests for the detection of chemically induced small deletions in *Drosophila* chromosomes. Mutat. Res. **72:** 231–243.

SPIERER, P., A. SPIERER, W. BENDER and D. S. HOGNESS, 1983 Molecular mapping of genetic and chromomeric units in *Drosophila melanogaster*. J. Mol. Biol. **168:** 35–50.

WATSON, W. A. F., 1966 Further evidence of an essential difference between the genetical effects of mono- and bifunctional alkylating agents. Mutat. Res. **3:** 452–455.

ZACHAR, A. and P. M. BINGHAM, 1982 Regulation of *white* locus expression: the structure of mutant alleles at the *white* locus of *Drosophila melanogaster*. Cell **30:** 529–541.

ZIMMERING, S., 1983 The *mei-9*^{a} test for chromosome loss in *Drosophila*: a review of assays of 21 chemicals for chromosome breakage. Environ. Mutagen. **5:** 907–921.

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