Mapping Point Mutations in the Drosophila *rosy* Locus Using Denaturing Gradient Gel Blots

Mark Gray,*,† April Charpentier,† Kathleen Walsh,† Pearl Wu† and Welcome Bender*

**Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and †Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, Tufts University School of Medicine, Boston, Massachusetts 02111*

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ABSTRACT

Mutations within the *rosy* locus of Drosophila were mapped using blots of genomic DNA fragments separated on denaturing gradient gels. DNA sequence differences between otherwise identical small *rosy* DNA fragments were detected among the mutants as mobility shifts on the blots. Mutations were mapped to within a few hundred base pairs of *rosy* sequence in 100 of 130 mutants tested—a 77% detection rate. The sequence changes in 43 *rosy* mutations are presented; all but six of these were single base changes. Thirty-four of 36 sequenced mutations induced by the alkylating agents *N*-ethyl-*N*-nitrosourea and ethyl methanesulfonate were transitions. All of the mutations mapped in the *rosy* transcription unit. Twenty-three of the 43 sequenced mutations change the predicted *rosy* gene polypeptide sequence; the remainder would interrupt protein translation (17), or disrupt mRNA processing (3).

MANY of the induced mutations used in experimental genetics are point mutations. For the purposes of this study, we define a point mutation as any DNA lesion not detectable by conventional blotting of DNA fragments electrophoresed in agarose gels. Typically, deletions or insertions of less than 50 bp are not detected by this method. Ethyl methanesulfonate (EMS) and *N*-ethyl-*N*-nitrosourea (ENU), the most commonly used chemical mutagens for Drosophila, appear to generate such point mutations (COTE *et al.* 1986). Also, point mutations are especially useful for studying mechanisms of gene expression, because they do not produce complex phenotypes. In contrast, chromosomal rearrangement mutations interrupt genes or move them to locations that change gene expression by position effect. Transposon insertion mutations are complicated even further by transcription originating within the transposon. In spite of their usefulness, only a small number of point mutations have been sequenced, because they are difficult to locate on a molecular map. Most of the molecular descriptions of point mutations have required the sequencing of cloned DNA from mutant and wild-type organisms. This approach can be impractical for characterizing many mutations in large genes, because of the effort required.

We have been studying mutations in the *rosy* locus of the fruit fly *Drosophila melanogaster* (CHOVNICK, GELBART and MCCARRON 1977). *rosy* mutant flies are deficient in the activity of xanthine dehydrogenase (XDH), an enzyme of the purine degradation pathway. XDH also affects, in an unknown way, the synthesis of the eye pigments (REAUME, CLARK and CHOVNICK 1988). Adult *rosy* flies have a brown-red eye color instead of the wild-type bright red. A genetic fine structure map of the *rosy* locus has been constructed using a variety of mutant alleles and polymorphic markers (CLARK, HILLIKER and CHOVNICK 1986).

Most of the *rosy* mutations that have been studied were induced by chemical mutagens. In our earlier study of 83 *rosy* mutants using Southern blots, 70 were indistinguishable from wild type in having no changes in the lengths of the expected *rosy* restriction fragments. These 70 mutations were probably point mutations (COTE *et al.* 1986). Only one of the 36 *rosy* mutations induced by the alkylating agent EMS had any mutant DNA lesion detectable on Southern blots.

Initially, we were interested in finding mutations that affect the regulation of *rosy* locus transcription. In our previous experiments, 61 ENU-induced *rosy* mutants that fit several criteria for having regulatory mutations were examined (LEE *et al.* 1987). The mutations were genetically mapped against markers within the *rosy* locus; DNA from six strains with mutations mapping near the 5′ end of the XDH gene was cloned and sequenced. All six mutations were found within the transcribed region of the gene, and either altered XDH polypeptide synthesis or mRNA processing.

To identify and sequence additional mutations in potential upstream regulatory regions, as well as other types of *rosy* mutations, it was necessary to use a method of intragenic mapping that was less laborious

than that used in our earlier studies. Denaturing gradient gel electrophoresis can be used to find mutations by detecting small differences in the melting behavior of short (200–700 bp) DNA fragments (FISCHER and LERMAN 1983). DNA fragments subjected to an increasingly denaturing physical environment partially melt. Rather than melting in a continuous zipper-like manner, most fragments melt step-wise, with discrete domains of the fragment becoming single-stranded in a very narrow range of denaturing conditions. When DNA fragments are electrophoresed in an acrylamide gel that contains an increasing gradient of denaturing solvents, their mobilities sharply decrease when they partially melt, because their shapes become more complex. The denaturants used are heat (a constant temperature of 60°) and a linear gradient of formamide (ranging from 0 to 40%) and urea (ranging from 0 to 7 M). The denaturant concentration, and position in the gel, at which a fragment partially melts (and thus nearly stops migrating) is dependent on the DNA sequence of the melted domain. This characteristic gradient concentration, and gel position, is often different even when only a single base in the sequence of the melted region is changed (FISCHER and LERMAN 1983). When a mixture of many different fragments is electrophoresed, each will stop at a sequence-dependent position in the denaturing gradient. Fragments containing an A + T-rich domain typically stop at relatively low denaturant concentrations (high on the gel); conversely, the more stable uniformly G + C-rich fragments usually run low in the gel.

In the experiments described below, *rosy* mutations were physically mapped to within several hundred bp of *rosy* sequence, by analyzing genomic DNA blots prepared from denaturing gradient gels. For some alleles, we cloned and sequenced only the small mutant portions of the gene, rather than the entire ~7.0-kb gene. The mutations sequenced include many left unresolved in our previous study, as well as mutations used in early genetic mapping experiments.

MATERIALS AND METHODS

Genetic strains: Virtually all *rosy* mutant strains tested in these experiments were isolated in mutagenesis experiments in the laboratory of ARTHUR CHOVNICK, with one exception, $ry^{L\cdot 19}$, a gift of E. B. LEWIS. The mutant alleles are numbered so that the first digit indicates the wild-type *rosy* locus background used to induce the mutation. For example, ry^{201} was induced on the ry^{+2} background, and ry^{606}, on the ry^{+6} background (CHOVNICK, GELBART and MCCARRON 1977). In all of these experiments, mutants were compared to their parental wild-type counterparts. Most of the mutations (102 of 135) were induced by ENU mutagenesis of the ry^{+5} wild-type strain; the remainder were induced by other mutagens, such as EMS, nitrogen mustard (HN2), triethylene melamine (TEM), diepoxybutane (DEB), and X-irradiation, as indicated below (MCCARRON and CHOVNICK 1981; LEE *et al.* 1987; LINDSLEY and ZIMM 1990).

Preparation of genomic DNA samples: Drosophila genomic DNA was prepared from adult flies using a rapid procedure (BENDER, SPIERER and HOGNESS 1983) and extracted once with phenol/chloroform (1:1) before precipitation in ethanol. Each electrophoresis sample contained 3 μg of genomic DNA digested in a total volume of 10 μl.

Denaturing gradient gel electrophoresis: Denaturing gradient gels were prepared as previously described, with only minor modifications (FISCHER and LERMAN 1983). The apparatus used for the preparation and electrophoresis of the denaturing gradient gels was designed by LEONARD LERMAN, and built from plans supplied by RICHARD MYERS (FISCHER and LERMAN 1979). All of the denaturing gradient gels were 6.5% acrylamide (37.5:1 acrylamide: bisacrylamide) in TAE buffer (40 mM tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 7.4). All gels were 0.6 mm thick and 13 cm long, with 20 wells, each 4 mm wide and 15 mm deep. Most of the gels had a linear gradient of denaturant of at least 50% (where 100% denaturant concentration is 7 M urea + 40% formamide; see FISCHER and LERMAN 1983). DNA samples were electrophoresed at 65–85 V, for 16–18 hr, at 60°. Afterward, the gels were stained in ethidium bromide (1 μg/ml) for 5 min and examined over a long-wave ultraviolet light source.

Preparation of DNA blots: The destained denaturing gradient gels were soaked in 0.5 M NaOH for 5 min, 0.5 M Tris, pH 8.0 for 5 min, and then in transfer buffer (20 mM Tris, 1 mM EDTA, pH 8.0) for at least 5 min. Next, each gel was placed in a stack with a nylon hybridization membrane (Nytran; Schleicher and Schuell) and the stack inserted into an electrophoretic transfer apparatus. DNA fragments were transferred by electrophoresis at 600 mA for 2 h. After electrotransfer, the DNA blots were rinsed briefly in 6 × SSPE (0.15 M NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA, pH 7.4), and baked under vacuum at 80° for 1–2 h.

Preparation of probes: Most of the blots were hybridized with single-stranded DNA probes. Single-strand recombinant pEMBL plasmids containing inserts up to 8.2 kb were prepared by standard methods (DENTE, CESARENI and CORTESE 1983). These DNAs were labeled by primer extension so that new strand synthesis occurs only in the vector portion of the molecule, leaving the insert region single-stranded (HU and MESSING 1982). Some DNA probes were labeled with ^{32}P by nick-translation of purified DNA fragments and recombinant plasmids (RIGBY *et al.* 1977). Other probes were ^{32}P-labeled DNA fragments prepared by primer extension after hybridization with random primers (FEINBERG and VOGELSTEIN 1983).

Hybridization of blots: The blots were soaked in 6 × SSPE for 5 min, and then prehybridized for at least 1 hr at 42° in a mixture of 50% formamide, 5 × SSPE, 1% SDS, 5 × Denhardt's solution (MANIATIS, FRITSCH and SAMBROOK 1982) and 100 μg/ml denatured sonicated salmon sperm DNA. Blots were hybridized at 42° for 16–20 hr in a mixture containing 10^6 cpm/ml ^{32}P-labeled DNA probe, 50% formamide, 5 × SSPE, 10% dextran sulfate (500,000 M_r), 1% SDS, 1 × Denhardt's solution, and 100 μg/ml denatured sonicated salmon sperm DNA. Blots were washed as follows: (a) 2 × SSPE + 1% SDS at 22° for 30 min, (b) 1 × SSPE + 1% SDS at 68° for 30 min, and (c) 0.1 × SSPE + 1% SDS at 68° for 30 min. Blots were dried briefly and exposed to X-ray film at −70° in the presence of an intensifying screen, for 2–20 hr.

Cloning of mutant genes: Most of the *rosy* mutant DNAs were isolated in recombinant plasmid clones using the fragment enrichment cloning strategy (NICHOLLS *et al.* 1985). By digesting the genomic DNA with enzymes cutting outside of the desired fragment, the complexity of the final plasmid

library was reduced approximately 10-fold. For *rosy* genes with mutations left of the *Pst*I site at +1.1-kb (see Figure 2), the 4.0-kb *Pst*I fragment (at −2.9 to +1.1) was cloned from genomic DNA. DNA was first digested with *Sal*I, *Kpn*I, *Apa*I, and *Hin*dIII, before digestion with *Pst*I. For genes with mutations to the right of the +1.1 *Pst*I site, the 3.0-kb *Pst*I/*Hin*dIII fragment (at +1.1 to +4.1) was cloned (Figure 2). Genomic DNA (20 μg) was first digested with *Xba*I, *Xho*I, *Sal*I, *Kpn*I, and *Apa*I, and then *Hin*dIII and *Pst*I, before fractionation by size. Usually, only ~2000 insert-containing colonies were screened to identify at least one with the desired *rosy* genomic fragment. The fragments were ligated with pEMBL8+ plasmid DNA (DENTE, CESARENI and CORTESE 1983), and the mixture used to transform JM101 or K802 host bacteria (HANAHAN 1983). Transformant colonies were screened for *rosy* DNA inserts by colony hybridization (GRUNSTEIN and HOGNESS 1975). Plasmid DNAs from positive colonies were prepared from 1-ml cultures (ISH-HOROWICZ and BURKE 1981). The clone's identity was rechecked by electrophoresis of the appropriate restriction fragments on a denaturing gradient gel.

Some of the *rosy* mutant DNAs were isolated in recombinant plasmid clones after DNA amplification by the polymerase chain reaction (PCR) strategy (SAIKI *et al.* 1988). A top strand oligonucleotide primer (5′-GAATTCCAGCCCTTGGATCC-3′; starting at nucleotide -1) was used together with a bottom strand primer (5′-CATTCCGTTCAGATCGGATCC-3′; at +2969), to amplify the intervening 2.9 kb of *rosy* DNA from ry^{8} genomic DNA. A different top strand oligonucleotide primer (5′-CGAGCTCAAGTCCTATTTCC-3′; at +1529) and a 3′ end bottom strand primer (5′-CTTCGAAACATACCTTGAGT-3′; at +4178) were used to amplify the downstream 2.6 kb portion of the *rosy* gene from the mutant strains ry^{1-19}, ry^{26}, and ry^{42}. PCR amplifications were done in a volume of 25 μl with 100 ng genomic DNA, 0.1 μM each primer, 200 μM deoxynucleotides, and 1 unit of *Taq* DNA polymerase. Denaturation for each of 30 cycles was at 94° for 1 min, annealing at 54° (62° for ry^{8}) for 1 min, and then synthesis at 72° for 1.5–2 min, using a Tempcycler model 50 (Coy Corp., Ann Arbor, Michigan). Amplified fragments were then cloned in the appropriate pEMBL plasmid vectors.

DNA sequencing: For most of the cloned mutant genes, small pEMBL subclones that placed the mutant DNA within 300 bp of the sequencing primer site were made. Single-strand DNA templates were prepared from the appropriate subclones and used in dideoxynucleotide chain termination sequencing reactions (SANGER, NICKLEN and COULSON 1977). For other cloned mutant genes, templates made from the entire pEMBL genomic clone were hybridized with complementary *rosy* oligonucleotide primers before the sequencing reactions.

Nondenaturing acrylamide gel electrophoresis: Genomic DNA samples prepared as described above were electrophoresed for 4–5 hr at 100 V in 4.5% acrylamide/TBE gels (MANIATIS, JEFFREY and VAN DE SANDE 1975). Blots were prepared and hybridized as described above.

RESULTS

Mapping *rosy* mutants using denaturing gradient gel blots

Selection of *rosy* mutants for study: Most of the 135 mutants selected were expected to be point mutants, because most were induced by mutagenic agents thought to give predominantly single base change substitutions, such as ENU and EMS. We were particularly interested in identifying mutations at sites in the gene that are signals for normal regulation of mRNA transcription and processing. Some mutants fit several biochemical and genetic criteria that suggested their mutations might affect transcription (MCCARRON and CHOVNICK 1981; LEE *et al.* 1987). Other mutants, such as those from earlier X-irradiation and EMS mutagenesis experiments, were included because of their frequent use as markers in recombination experiments. Because of our selection bias, the mutants included in this study may not represent a typical group (see DISCUSSION). Most (116/135) of the mutations were induced on the ry^{+5} wild-type background (CHOVNICK, GELBART and MCCARRON 1977). Others were induced on the ry^{+0}, ry^{+2}, ry^{+6}, and other well-characterized *rosy* wild-type strains (CHOVNICK, GELBART and MCCARRON 1977). In all cases, DNA from the mutant was compared to DNA from the appropriate parent strain.

Denaturing gradient gel blot strategy: Genomic DNA was prepared from *rosy* mutant adult flies, and analyzed by denaturing gradient gel electrophoresis, as follows. Genomic DNA samples (3 μg) were digested with at least three of the five restriction enzymes with 4 bp recognition sequences: *Alu*I, *Hae*III, *Hha*I, *Msp*I, or *Rsa*I. These enzymes cut *Drosophila* DNA frequently, producing many 200–700 bp DNA fragments. The digested DNA samples were electrophoresed in denaturing gradient gels with a wide range of denaturant concentration, such as 20–90% or 25–85% (see MATERIALS AND METHODS). After electrophoresis, most *rosy* DNA fragments reached a position in the gradient where their first melting domains denatured. The electrophoresed fragments were then transferred from the gel to nylon hybridization membranes, by electroblotting. The DNA blots were hybridized with radioactive *rosy* DNA probes and the *rosy* fragments visualized by autoradiography (Figure 1).

The order, from top to bottom, of the DNA fragments in denaturing gradient gels is not determined by molecular weight. Instead, the relative stabilities of the first melting domains in each fragment establish the fragment order in the gels. The molecular map positions of the *rosy* fragments detected on the denaturing gradient gel blots were identified by two methods. In the first, the blots were hybridized with probes prepared from short, cloned *rosy* fragments. In the second approach, cloned *rosy* fragments were electrophoresed in perpendicular denaturing gradient gels (FISCHER and LERMAN 1983). Electrophoresis of many fragments in these gels reveals simultaneously their relative molecular weights and melting order in a denaturing gradient.

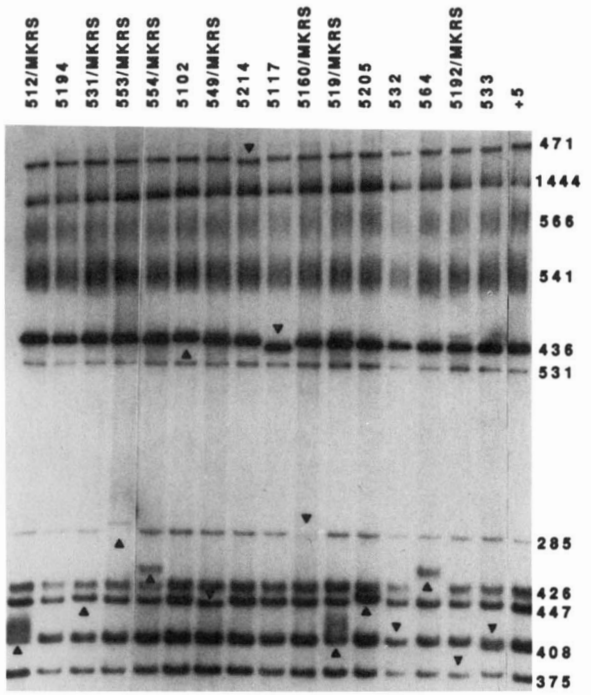

FIGURE 1.—Autoradiograph from a denaturing gradient gel blot of *rosy* mutant genomic DNA. This blot was prepared after electrophoresis of *Hae*III-digested *rosy* mutant DNA samples on a 30–80% denaturing gradient gel. The probe was prepared from the ry^{+5} 7.3-kb *Hin*dIII fragment (see Figure 2), and hybridized as described in MATERIALS AND METHODS; exposure time was 8 hr. The numbers at the top of each lane refer to the mutant DNA strain. Mutant fragments with altered melting behavior are marked with arrowheads. The identities of the fragments are marked on the right margin by their lengths in bp. Most of the mutant strains are homozygotes; some are heterozygous for the third chromosome balancer MKRS (LINDSLEY and ZIMM 1990). This chromosome has the ry^{2} allele, a mutation with a mobile element (B104) insertion which disrupts the 447-bp *Hae*III fragment.

Mapping of mutant fragments: Among the 135 mutant DNAs tested, 100 had at least one *rosy* restriction fragment with mutant (i.e., different from its wild-type parent) melting behavior; their map positions are shown in Figure 2. In many of the mutant strains, a mutant band was found in more than one of the four or five different restriction digests tested. In these mutants, all of the altered restriction fragments overlapped each other, suggesting that all of the melting behavior alterations found in one mutant gene were caused by the same base change. Five mutants had mobility shifts in fragments from all regions within and outside of the *rosy* gene, suggesting that they were induced on an unidentified chromosomal background; these mutants were not investigated further. For 30 of the mutants, fragment mobility shifts were not found among the 4–5 restriction enzymes tested. In total, 602 digests of genomic DNA from the 135 mutants were analyzed in these denaturing gradient gel blot experiments.

In order to assess the molecular weights of *rosy* fragments, all mutant genomic DNAs with at least one shifted fragment were analyzed using conventional blots prepared from nondenaturing acrylamide/TBE gels. Nylon blots were made from these gels and hybridized with radioactive *rosy* probes. Only four mutants had changes in the sizes of *rosy* fragments. Three of these, ry^{509}, ry^{539}, and ry^{5122}, had small deletions. The fourth, ry^{5207}, had a chromosomal rearrangement with a breakpoint in the first intron of the XDH transcription unit; this mutant was not investigated further. No alterations in the molecular weight of any *rosy* restriction fragments were found in the remaining mutants tested; these were expected to have point mutations. Excluding the five mutants on an unknown background, and the four rearrangement/deletion mutants, the rate of detection of point mutations, using denaturing gradient gel blots, was 76% (96/126).

Melting behavior changes in *rosy* mutants: Denaturing gradient gels with broad ranges of denaturant concentration were chosen in order to simultaneously compare fragments with very different stabilities. As a result, the mobility shifts of mutant fragments were often small–only 1 mm or less (see Table 1). The average magnitude of all of the mobility shifts was 2 mm. The shifts were always much greater (up to 10 mm) when mutant DNA fragments were electrophoresed in gels with the appropriate narrow range (30% or less) of denaturant concentration. Most of the DNA fragment shifts were changes in the position of a sharp band (*e.g.*, ry^{553}, ry^{5214}, ry^{5117}; Figure 1). In contrast to these, about one-third of the melting differences were changes in the position or the shape of diffuse, smeared bands (ry^{512}, ry^{564}, ry^{533}; Figure 1). Fragments with a high G+C content often produced diffuse bands, usually in the most denaturing region of the gradient. Most of the GC-rich *rosy* fragments never completely stopped during the course of electrophoresis. We suspect that the diffuse band phenotype was caused by the simultaneous melting of two or more melting domains in one DNA fragment. Each of the five restriction digests tested revealed mutant melting differences; mutant shifts were found in 26–36% of the DNA samples in each set of digests. Mutant fragments were found in two or more different restriction digests in 53 of the 100 mutant DNAs with an altered fragment.

Mutant fragments found by denaturing gradient gel blots were mapped throughout the *rosy* transcription unit; most were entirely within the large protein-coding second and third exons (Figure 2). In ry^{5331}, ry^{5102}, ry^{5117} and ry^{5324}, the mutant fragments include part of the small first exon. In ry^{5214}, all of the overlapping mutant DNA fragments map downstream from the XDH open reading frame. No mutant fragments mapped completely outside of transcribed DNA, or entirely within introns. However, examples

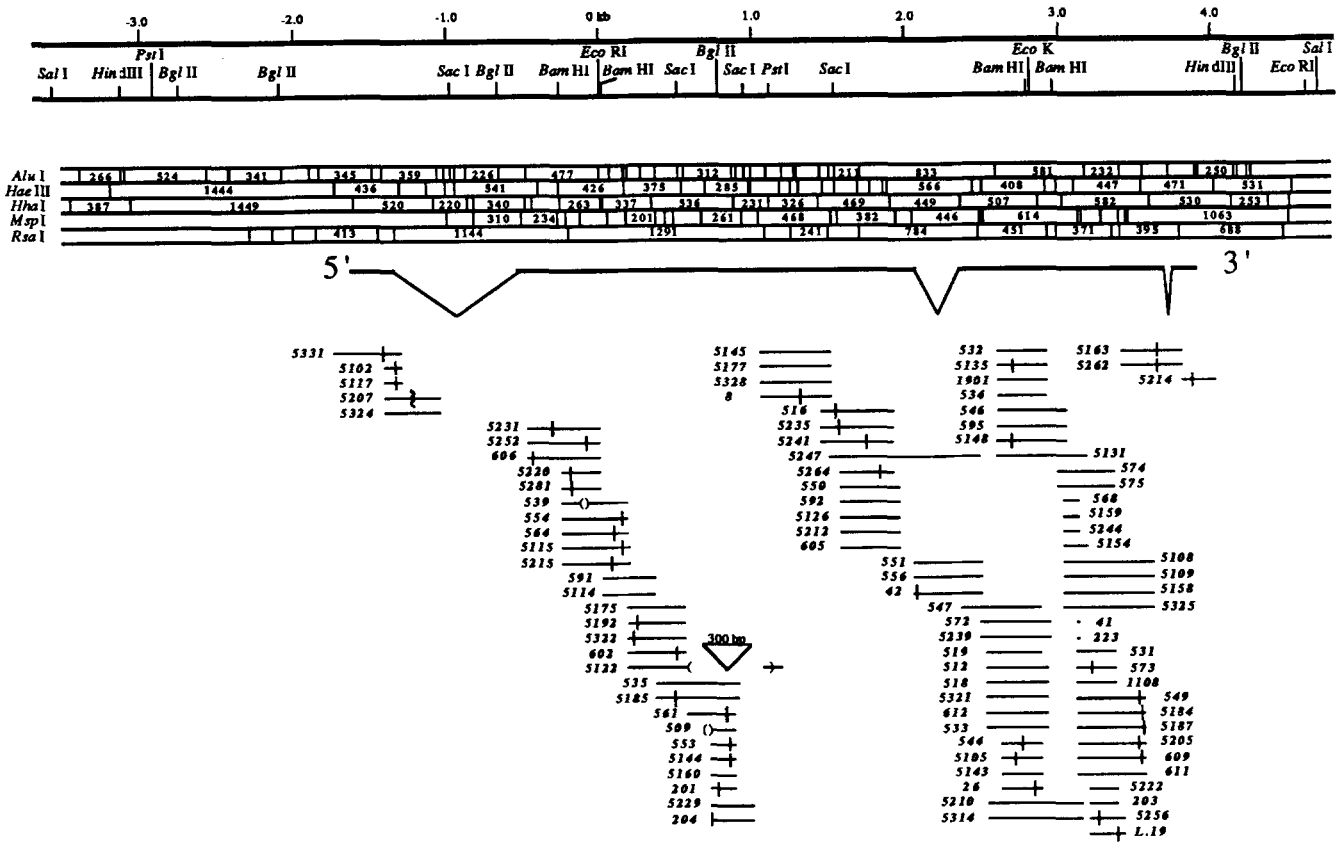

FIGURE 2.—Molecular map positions of *rosy* mutations found by denaturing gradient gel electrophoresis. At the top is a restriction map of the *rosy* locus. The bases are numbered as in previous studies (CURTIS *et al.* 1987; KEITH *et al.* 1987). The structure of the XDH mRNA is diagrammed below the restriction maps. The molecular positions of restriction fragments with altered melting behavior in *rosy* mutants is aligned with the restriction map, at the bottom. When a melting behavior shift was found in two or more restriction digests of the same mutant DNA, the position of the mutation was narrowed down to the region of overlap of the mutant fragments. The exact positions of sequenced mutations are indicated by the vertical slash marks. Four mutants had small deletions; these are indicated on the diagram at their respective map positions by lines with gaps indicating the lengths of the deletions (estimated by nondenaturing gel electrophoresis for ry^{539}). The position of the rearrangement mutation ry^{5207} is marked by the crooked line.

of mutations in these regions have been found and are described elsewhere (DUTTON and CHOVNICK 1988; CURTIS *et al.* 1989).

Some of the mutants analyzed with denaturing gradient gel blots were among the earliest mutations mapped genetically within the *rosy* gene (CHOVNICK 1966; CHOVNICK, GELBART and MCCARRON 1977). The genetic order of these mutations (ry^{606}, ry^{602}, ry^{204}, ry^{201}, ry^{8}, ry^{42}, ry^{26}, ry^{41} and $ry^{L.19}$) is consistent with the physical map order of the mutant fragments detected in genomic DNA.

DNA sequence changes in selected *rosy* mutations

Isolation of *rosy* mutant DNA: Genomic DNA from about one-half of the mapped *rosy* mutant genes was cloned and sequenced in the region of the restriction fragments with altered melting behavior (Figure 2). Since the molecular map positions of the mutations were localized to a few hundred base pairs, only the mutant portions of the genes were sequenced. In most of the experiments, *rosy* mutant DNA fragments were isolated in plasmid clones using the strategy of fragment enrichment (NICHOLLS *et al.* 1985). Some mutant DNA fragments were cloned in plasmids after PCR DNA amplification from genomic DNA (SAIKI *et al.* 1988).

To verify that each plasmid clone contained the correct mutant DNA, the clones were digested with the appropriate restriction enzyme(s), and electrophoresed in denaturing gradient gels similar to those used to detect the mutant fragment in genomic DNA. The melting behavior shifts of cloned fragments were identical to their genomic counterparts, with only one group of exceptions: all of the restriction fragments overlapping the +2.8-kb map position (Figure 2). Each of these fragments, when cloned in bacteria, stopped considerably higher (1–3 cm) in the denaturing gradient than the equivalent Drosophila genomic fragment. An *Eco*K recognition site (GCACGGAGGAGTT) is present in this region of the *rosy* gene, at nucleotide 2800 (Figure 2). We were unable to clone genomic *rosy* fragments (containing the *Eco*K site) in the bacterial strain JM101. This host strain has both

*Eco*K endonuclease and methylase activity (KESSLER, NEUMAIER and WOLF 1985). Genomic cloning in the host strain K802 was successful; this strain lacks the *Eco*K endonuclease, but retains methylase activity. Methylation (by host bacteria) most probably caused the large differences in the melting behavior of cloned fragments that include the *Eco*K site. This is consistent with reports of DNA fragment destabilization associated with adenine methylation (COLLINS and MYERS 1987).

Types of base changes and effect of mutagens: Forty-three different *rosy* mutations were sequenced (Table 1). All were within small restriction fragments with mutant melting behavior (Table 1; Figure 2). Most of the sequenced mutations (37 of 43) were single base changes; the remaining six changed more than one base pair. Five of these six, as well as a rearrangement and another small deletion (not sequenced), were induced by X-irradiation, HN2, and DEB. The ENU-induced mutant gene in ry^{5122} was missing ~700 bp of *rosy* sequence and had ~300 bp of DNA sequence from an unknown genomic source at the site of the deletion.

Most of the sequenced mutations were induced by ENU. All of the ENU-induced mutations, except ry^{5122}, were single base substitutions (Table 2). All four of the EMS-induced mutations were single base substitutions (Table 1). Twenty-seven had G to A, or C to T transitions; in seven, T was changed to C. In ry^{5192}, an A was replaced by a G. The only transversion mutation found was the C to A change in ry^{5215}.

Effect of mutations on *rosy* locus expression: All of the sequenced *rosy* mutations were in the XDH transcription unit. Approximately one-half (22 of 43) change the predicted amino acid sequence of the XDH polypeptide. Over half (12 of 22) of these missense mutations cause either the removal or insertion of a charged amino acid (Table 1). A serine residue would be removed in four mutants; 5 of the 22 missense mutants had substitutions involving a proline residue. Half of the missense mutants have a *rosy* null phenotype, and half have a partial-activity phenotype (Table 1). The remaining mutations apparently interrupt normal translation of the XDH mRNA, either by introducing in-frame nonsense codons or frameshifts, or interfering with post-transcriptional processing of the XDH mRNA (Table 1).

Ten mutants had single base changes resulting in a new translation termination codon. Seven nonsense mutations were amber codons (TAG), and three were opal codons (TGA). No ochre (TAA) nonsense mutations were found, probably because of the Drosophila codon bias (O'CONNELL and ROSBASH 1984) and the limited spectrum of ENU-induced base changes (Table 2). All but one of the nonsense mutants had a *rosy* null phenotype. The exception, ry^{5262}, has less than 5% of wild-type XDH activity (MARGARET MCCARRON, personal communication). This mutation creates an amber codon 23 codons upstream from the 3′ end of the XDH open reading frame, leaving intact most (1312 codons) of the coding sequence.

All but one of the seven sequenced deletion/insertion mutations placed frameshifts in the XDH open reading frame (Table 1). Frameshifts cause premature translation termination because of nonsense codons in the other two reading frames. All seven deletion/insertion mutants have a *rosy* null (eye color) phenotype. The ry^{42} mutation was exceptional because it complements with certain other *ry* null alleles, such as ry^{606} (GELBART, MCCARRON and CHOVNICK, 1976). This mutation removed seven amino acid residues, including a negatively-charged aspartic acid residue, and put in three new residues, including a negatively-charged glutamic acid residue, at the site of the deletion. The net loss of 9 bp keeps the *rosy* reading frame intact, without creating any in-frame termination codon.

Two single base change mutations, ry^{5102} and ry^{5117}, were substitutions of the first two bases in the first intron (Table 1). These mutations change the two most conserved bases in the 5′ splice donor consensus sequence G | $\underline{\text{GT}}\left(\frac{\text{A}}{\text{G}}\right)$AGT (MOUNT 1982). Normal splicing of the first and second exons of the XDH mRNA probably does not occur in these two mutants; both have a *rosy* null phenotype.

The mutation ry^{5331} places a new ATG methionine codon upstream from the normal XDH start codon; the entire XDH open reading frame is intact. The change found in the ry^{5331} mutation is identical to that in ry^{5182}, described in our previous study (LEE *et al.* 1987).

The mutation ry^{5214} is a T to C change 88 bp downstream from the wild-type XDH TAA stop codon, and 12 bp upstream from the mRNA polyadenylation site (KEITH *et al.* 1987). The mutation is in a sequence that could form a small hairpin structure at the 3′ end of the mRNA. A similar hairpin structure near the 3′ end of the mouse histone H3 pre-mRNA is bound *in vitro* by a non-snRNP factor when the 3′ end of the molecule is cut and polyadenylated (BIRNSTIEL, BUSSLINGER and STRUB 1985; MOWRY and STEITZ 1987). The ry^{5214} mutant has a partial activity phenotype; the efficiency of 3′ end XDH mRNA processing might be reduced in this mutant. No other *rosy* fragment melting behavior changes were found in ry^{5214} genomic DNA, and no additional mutant base differences were found in the DNA sequence of the 3.0-kb *Pst*I/*Hin*dIII fragment (Figure 2). We found no mutants with more than a single altered site, although such have been found and reported in our earlier study (LEE *et al.* 1987).

TABLE 1

Summary of DNA sequence changes in *rosy* mutations detected on denaturing gradient gel blots

Mutant	Mutagen	Altered fragment (mm shifted)	Sequence change	Outcome	Phenotype
5331, 5182[a]	ENU	*Hae*III 436 (1.5↑)	−1435: G → A	New ATG → frameshift	PA
5204[a] (1st)	ENU	*Hae*III 436 (2.0↓)	−1388: T → G		
(2nd)		*Hae*III 436 (2.0↓)	−1386: T → Δ	Frameshift	PA
5208[a]	ENU	*Hae*III 436 (1.0↑)	−1366: G → A	G/GT → A/GT (? splicing defect)	PA
5102	ENU	*Alu*I 359 (1.5↑)	−1365: G → A	GT → AT (no splicing)	Null
5117	ENU	*Alu*I 359 (3.0↓)	−1364: T → C	GT → GC (no splicing)	Null
523[a], *545*[a]	ENU	*Bgl*II-*Bam*HI 409 (1.0↑)	−551: G → A	AG → AA (no splicing)	Null
606	EMS	*Alu*I 477 (1.0↑)	−468: G → A	GGA → GAA: Gly → Glu^-	Null[d]
5231	ENU	*Msp*I 234 (1.0↑)	−328: G → A	GAG → AAG: Glu^- → Lys^+	PA
5198[a]	ENU	*Alu*I 477 (1.0↑)	−225: C → T	TCC → TTC: Ser → Phe	PA
5220	ENU	*Hae*III 426 (2.0↑)	−226: T → C	TCC → CCC: Ser → Pro	PA
5281	ENU	*Alu*I 477 (1.5↑)	−214: C → T	CTT → TTT: Leu → Phe	PA
538[a]	DEB	*Hae*III 426 (10.0↓)	−166 to −111: del56 bp	Frameshift	Null
5252	ENU	*Alu*I 477 (2.5↓)	−123: T → C	CTC → CCC: Leu → Pro	PA
5215	ENU	*Hae*III 426 (6.0↑)	43: C → A	CCG → CAG: Pro → Gln	PA
564	ENU	*Hae*III 426 (4.0↑)	75: C → T	CAG → TAG: Gln → term	Null
554	ENU	*Hae*III 426 (4.5↑)	109: G → A	TGG → TAG: Trp → term	Null
5115	ENU	*Hae*III 426 (3.0↑)	110: G → A	TGG → TGA: Trp → term	Null
5322	ENU	*Hae*III 375 (1.0↑)	180: G → A	GGC → AGC: Gly → Ser	PA
5192	ENU	*Hae*III 375 (1.0↓)	213: A → G	AAG → GAG: Lys^+ → Glu^-	PA[d]
406[b]	EMS	*Hae*III 375 (0.5↑)	451: G → A	GGA → GAA: Gly → Glu^-	Null[d]
5185	ENU	*Hha*I 536 (6.0↓)	466: G → A	GGC → GAC: Gly → Asp^-	PA
602	EMS	*Hae*III 375 (1.5↑)	478: C → T	TCC → TTC: Ser → Phe	Null[d]
5122	ENU	*Hae*III 375 absent	~500: del ~700 bp/ins ~300 bp	Frameshift	Null
509	HN2	*Hae*III 285 (17.0↑)	626–698: del 73 bp	Frameshift	Null
204	X-ray	*Hae*III 285 (16.0↑)	685: C → Δ	Frameshift	Null
201	X-ray	*Hae*III 285 (2.5↑)	737: ins. TT	Frameshift	Null
553, 5144	ENU	*Hha*I 536 (5.0↑)	816: C → T	CGA → TGA: Arg^+ → term	Null
561	ENU	*Hha*I 536 (6.0↓)	846: T → C	TGG → CGG: Trp → Arg^+	Null
8	X-ray	*Msp*I 468 (2.0↓)	1283–1299: del17 bp	Frameshift	Null
516	ENU	*Hha*I 469 (14.0↑)	1521: C → T	CAG → TAG: Gln → term	Null
5235	ENU	*Hha*I 469 (4.0↓)	1539: T → C	TCC → CCC: Ser → Pro	PA
5241	ENU	*Hha*I 469 (3.0↓)	1722: C → T	CAG → TAG: Gln → term	Null[c]
5264	ENU	*Hha*I 469 (4.0↓)	1807: G → A	GGA → GAA: Gly → Glu^-	Null[c]
42	X-ray	*Msp*I 446 (2.0↑)	2030–2045: del 16 bp/ins. 6 bp	Missense changes[f]	Null[d]
5148	ENU	*Msp*I 614 (2.0↑)	2573: C → T	CAG → TAG: Gln → term	Null
5105, 5135	ENU	*Rsa*I 451 (2.0↓)	2683: G → A	TGG → TGA: Trp → term	Null
544	ENU	*Hha*I 507 (2.5↑)	2721: G → A	GGA → GAA: Gly → Glu^-	Null
26	X-ray	*Hae*III 408 (9.0↑)	2804–5: GG → T	Frameshift	Null
41[r]	X-ray	*Rsa*I 371 (1.5↓)	3095–7: GGA → Δ	Gly codon deletion	Null
573	ENU	*Hae*III 447 (1.0↑)	3179: G → A	GGA → AGA: Gly → Arg^+	Null[d]
5256	ENU	*Hha*I 582 (1.0↑)	3221: C → T	CAG → TAG: Gln → term	Null[c]
531	DEB	*Rsa*I 371 (2.5↑)	3312: G → A	GGC → GAC: Gly → Asp^-	Null[d]
L.19	EMS	*Alu*I 232 (2.5↑)	3332: G → A	GAG → AAG: Glu^- → Lys^+	Null[d]
5205	ENU	*Hae*III 447 (1.0↓)	3486: G → A	GGT → GAT: Gly → Asp^-	PA[d]
549	ENU	*Hae*III 447 (1.5↓)	3498: T → C	CTC → CCC: Leu → Pro	Null
609	EMS	*Hae*III 447 (2.0↑)	3506: G → A	GGA → AGA: Gly → Arg^+	Null[d]
5184	ENU	*Hha*I 582 (1.5↑)	3513: C → T	TCT → TTT: Ser → Phe	Null[c]
5187	ENU	*Hha*I 582 (2.5↑)	3524: G → A	GCC → ACC: Ala → Thr	Null[c]
5262, 5163	ENU	*Rsa*I 395 (2.5↑)	3626: C → T	CAG → TAG: Gln → term	PA
5214	ENU	*Rsa*I 688 (1.5↓)	3850: T → C	ATGTTTT → ATGCTTT ? mRNA 3′ processing?	PA

PA refers to partial activity; the PA designation is based on the eye color of these mutants (GELBART, MCCARRON and CHOVNICK 1976; MARGARET MCCARRON, personal communication). The mutagens are: *N*-ethyl-*N*-nitrosourea (ENU), ethyl methanesulfonate (EMS), nitrogen mustard (HN2), diepoxybutane (DEB) and X-irradiation. The fragment with altered melting behavior listed is that with the largest shift found for the mutant; the magnitude (in mm) and the direction of the shift (↑ for upward and ↓ for downward) is indicated in parentheses. (As discussed in the text, other overlapping restriction fragments that include the mutation were often shifted as well). The positions in the gene are numbered as in Figure 2 (CURTIS *et al.* 1987; KEITH *et al.* 1987).

[a] Described in LEE *et al.* (1987).

[b] Described in CURTIS *et al.* (1989).

[c] Described in CURTIS and BENDER (1990).

[d] Complements with other *rosy* null alleles (GELBART, MCCARRON and CHOVNICK 1976; MARGARET MCCARRON, personal communication).

[r] Adult eye color is not the same as in most null mutants; some residual XDH activity might be present.

[f] In the ry^{42} mutant gene, 16 bp are removed and replaced by 7 new bp (5′-CCAAGAG-3′), for a net loss of 9 bp. This mutation removes the polypeptide sequence [Trp-Ser-Met-Asp^--Leu-Ser-Phe] and replaces it with [Cys-Gln-Glu^-].

TABLE 2

Types of ENU-induced single base change *rosy* mutations

Base change	Type	Examples[a]	Fraction of total[a] (%)
G → A	Transition	17	44.7
C → T	Transition	10	26.3
T → C	Transition	7	18.4
A → G	Transition	1	2.6
C → A	Transversion	1	2.6
T → G	Transversion	1	2.6
T → Δ	Deletion	1	2.6
All other transversions		0	0

[a] Includes the 34 ENU-induced mutations, and the seven ENU-induced mutations described in LEE *et al.* (1987).

DISCUSSION

Denaturing gradient blots for mapping mutations: Physical mapping of the *rosy* mutations with denaturing gradient gel blots is less laborious than traditional genetic mapping and genomic cloning. Since base differences must be in the first melting domain of a DNA fragment to be detectable in denaturing gradient gels (FISCHER and LERMAN 1983), we expected that mutations in G + C-rich sequence would be missed; G + C-rich sequence is usually found in the most stable melting domains. However, to our surprise, many of the mutations were mapped in the *rosy* DNA fragments with the highest G + C content. By using the different restriction enzymes, the melting domains of the fragments tested at any region of the gene were often rearranged, or "moved." In this way, mutations in higher melting domains of one fragment were detected by analyzing partially-overlapping lowest melting domains.

In general, G to A and C to T base changes caused fragments to shift upward in the gradient, and, conversely, A to G and T to C changes caused downward shifts. This is consistent with predictions of melting behavior changes made for all possible base changes in a mouse β-globin promoter DNA fragment (MYERS *et al.* 1985). In our experiments, exceptions to this rule were found only in fragments that produced smeared, diffuse bands in denaturing gradient gels (discussed above).

Success rate for detection of mutations: We have found 77% of the mutations in 130 *rosy* mutants tested (not including the five on an undetermined background). It cannot be determined from our results if any types of base changes were undetectable with denaturing gradient gel blots. In a computer simulation, 95% of all possible base substitutions in a 135-bp DNA fragment containing the mouse β-globin promoter would result in detection of a melting difference from the wild-type fragment, under ideal conditions of denaturing gradient gel electrophoresis (MYERS *et al.* 1985). The only substitutions that were predicted to be undetectable were some conservative transversions, although most (105/135) conservative transversions would be detectable. The magnitude of the fragment mobility shifts caused by conservative transversions was predicted to be much less than that of transitions and nonconservative transversions (MYERS *et al.* 1985). Nearly all types of base changes changed the melting behavior of mutant fragments made *in vitro* using chemical mutagens (MYERS, LERMAN and MANIATIS 1985). A portion of the 23% of *rosy* mutations not detected in our experiments could have been base changes, such as conservative transversions, that cause little or no melting behavior changes, as in the β-globin simulation. Almost all (95%) of the ENU-induced *rosy* mutations sequenced after detection of denaturing gradient blots were transitions. In a study of ENU-induced mutations at the *vermilion* locus, 79% were shown by DNA sequencing to be transitions, and 21% transversions (PASTINK *et al.* 1989). The *vermilion* mutant genes were sequenced directly, and may be a more representative sampling of the distribution of base changes caused by ENU. Thus, it seems likely that at least some of the 30 mutations not detected on denaturing gradient gel blots might have had transversions.

Some of the 30 mutations not detected were probably in the higher melting domains of the fragments tested, and the best restriction digest for analyzing these regions of *rosy* sequence have not yet been tested. For example, the splice acceptor site mutation ry^{545} (LEE *et al.* 1987) was not detected using the five restriction enzymes for the 135 new mutants. Most restriction fragments that include this site contain much of the A + T-rich first intron sequence; the mutation is at the edge of a very G + C-rich protein-coding sequence. We deliberately tested an alternative fragment (the *Bgl*II-*Bam*HI 409-bp fragment) that included a large portion of the G + C-rich sequence. On denaturing gradient gels, this fragment had distinctive melting behavior in genomic DNA of both ry^{545} and another mutant (ry^{523}) with the same base change (Table 1). Some of the other mutations might be detected in a similar manner, by analyzing additional genomic DNA digests, using different enzymes.

Double mutations: No *rosy* mutant genes were found with two or more non-overlapping mutant sites, suggesting that there were few, or perhaps no examples of multiple lesions in this group of mutant genes. Theoretically, the mutant fragment and base change that we found in some mutants could have had no effect on the *rosy* phenotype, and a second (important for the *rosy* phenotype) base change was present that escaped detection using the denaturing gradient gel blots. There are several reasons that we believe our mutations are the relevant ones. Some of the mutations were mapped genetically as single-site mutations. Much of the nonmutant (defined as such by denatur-

ing gradient gel electrophoresis) DNA in the mutant clones was used as wild-type control templates for sequencing new cloned mutant DNAs; no additional base changes were found. Almost all of our mutant base changes create in-frame nonsense codons, or nonconservative amino acid substitutions (Table 1). Double mutations separated by more than a few hundred base pairs have never been described in any ENU-induced mutant gene.

Regulatory mutations: All of the *rosy* mutations sequenced in this study either change the structure of the XDH polypeptide, cause premature termination of translation, or interfere with normal mRNA processing. No mutations were found in DNA upstream from the XDH gene. There are probably few point mutations in *rosy* regulatory sequences that would significantly alter the rate of XDH transcription by more than 50%. The sequences of promoters and enhancers identified in other genes are extremely variable and often repeated (SERFLING, JASIN and SCHAFFNER 1985). Most single base substitutions made *in vitro* in DNA upstream from the human β-globin gene have only subtle effects on the rate of transcription in a HeLa cell transient expression assay (MYERS, TILLY and MANIATIS 1986).

Effect of mutagen: The ENU and EMS results reported here are similar to what others have found for Drosophila. As discussed above, our data should not be regarded as fully representative of the spectrum of DNA lesions to be expected whenever ENU or EMS is used as the mutagen, because of the possible insensitivity of our detection scheme to conservative transversions and base changes in some regions of the gene. Also, the *rosy* mutants that were expected to have deletions and rearrangements were excluded from the study. ENU can certainly induce deletions and rearrangements, as in the case of ry^{5122} (described above), and the five ENU-induced large deletions extending beyond *rosy* into adjacent complementation groups (LEE *et al.* 1987). We have also found an ENU-induced 1.5-kb deletion in the *Ubx* gene (M. GRAY and W. BENDER, unpublished experiments). As discussed above, our ENU-induced *rosy* single base change mutations are almost exclusively (95%) transitions (Table 2), in contrast to the *vermilion* mutations, where only 79% were transitions (PASTINK *et al.* 1989). Twenty-seven of 39 previously reported ENU- and EMS-induced mutations in the *rosy*, *Adh*, *per*, *Ubx*, *Notch* and *vermilion* genes of Drosophila are G to A and C to T transitions (LEE *et al.* 1987; MARTIN *et al.* 1985; YU *et al.* 1987; WEINZIERL *et al.* 1987; KELLEY *et al.* 1987; PASTINK *et al.* 1989).

All five of our X-ray-induced *rosy* mutations change the total number of base pairs, either as small deletions, an insertion, or both. Similarly, five X-ray-induced *white* mutations had small (6–29 bp) deletions, with one (w^{17D2}) having an insertion of 10 bp of new sequence at the site of an 18-bp deletion (PASTINK *et al.* 1988). In contrast to the *white* mutations, our *rosy* deletions do not occur between 2- and 3-bp repeats (Table 1; KEITH *et al.* 1987). The inserted 7 bp of new DNA sequence at the site of the 16-bp deletion in ry^{42} is not repeated on either side of the deletion site, as in the X-ray-induced deletion/insertion w^{17D2} (PASTINK *et al.* 1988).

We have analyzed two mutations induced by diepoxybutane (DEB). One of these (ry^{539}) and one from our previous study (ry^{538}) were small deletions of less than 100-bp. The DEB-induced mutation ry^{531} is a single base substitution. Our DEB results are consistent with those of a previous study of 21 DEB-induced *rosy* mutations (REARDON *et al.* 1987), where 43% were deletions, ranging in size from 50 bp to 8 kb.

The 73-bp deletion mutation ry^{509} and the ry^{5207} rearrangement were induced by HN2. This mutagen, also known as mechlorethamine, is an alkylating agent that can also cross-link DNA (HOLLAENDER 1971). Our results suggest that the mutations may have arisen by DNA repair after a crosslinking event.

Point mutations and sequence polymorphisms: Point mutations can be used for a variety of biological investigations. They are helpful for studies of the biochemistry of genetic recombination and DNA repair. The physical mapping of point mutations causing a new and well-defined mutant phenotype can be used in identifying the relevant gene in chromosomal regions with many closely spaced genes. Missense mutations can help identify functional domains of proteins, by observing the biological consequences of amino acid substitutions; studies of this type are underway for Drosophila xanthine dehydrogenase, using *rosy* mutations (BRAY *et al.* 1990). Nonsense mutations can be used as substrates for the isolation of suppressor tRNA genes. Indeed, the *rosy* mutant eye color phenotype in the ry^{516} strain has been reverted to wild type when an active suppressor tRNA gene was introduced into the germline (DOERIG *et al.* 1988).

We have also used denaturing gradient gel blots to find DNA sequence polymorphisms, as well as mutant base changes. Laboratory and wild stocks of Drosophila have a high frequency of sequence polymorphisms. Between some pairs of strains, sequence differences can be found about every 100 bp, in the *rosy* and *Ultrabithorax* genes (LEE *et al.* 1987; CURTIS *et al.* 1989; BENDER, SPIERER and HOGNESS 1983). When two separate Drosophila genotypes are compared, many DNA fragments from all regions of the genome have different mobilities on denaturing gradient gels. When DNA from a heterozygous strain is electrophoresed on a denaturing gradient gel, sequence polymorphisms often cause the appearance of two bands for each fragment. Melting polymorphisms have been

used to map the lengths of gene conversion intervals, as well as mapping sites of crossovers (CURTIS *et al.* 1989; CURTIS and BENDER 1990).

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