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The Effect of Chromosomal Position on the Expression of the Drosophila Xanthine Dehydrogenase Gene

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Summary

Thirty-six isogenic D. melanogaster strains that differed only in the chromosmal location of a 7.2 or an 8.1 kb DNA segment containing the (autosomal) *rosy* gene were constructed by P-element-mediated gene transfer. Since the flies were homozygous for a *rosy*⁻ allele, *rosy* gene function in these indicated the influence of flanking sequences on gene expression. The tissue distribution of XDH activity in all the strains was normal. Each line exhibited a characteristic level of adult XDH-specific activity. The majority of these values were close to wild-type levels; however, the total variation in specific activity among the lines was nearly fivefold. Thus position effects influence expression of the *rosy* gene quantitatively but do not detectably alter tissue specificity. X-linked *rosy* insertions were expressed on average 1.6 times more activity in males than in females. Hence the gene acquires at least partial dosage compensation upon insertion into the X chromosome.

Introduction

In the eucaryotic genome, genes are subject both to controls that act locally, leaving adjacent genes unaffected, and to those that act over large chromosomal regions. Little is known about the DNA sequences required for either local or global gene programming. In particular, it is unclear whether the tissue-specific expression exhibited by many genes is a property of the genes themselves or derives from their presence in a larger chromosomal domain.

Studies of the effects of specific chromosome rearrangements have provided some information about the general features of genetic regulatory mechanisms. Chromosome rearrangement can alter or abolish the developmentally regulated expression of genes located great distances from the site of breakage. Dramatic examples of such "position effects" are often observed when euchromatic and heterochromatic genomic regions are juxtaposed in a rearranged chromosome (reviewed in Lewis, 1950; Eicher, 1970; Spofford, 1976). In contrast, extensive genetic and cytogenetic studies in Drosophila have demonstrated that a breakage and rejoining event involving two euchromatic regions has consequences that are almost always limited to a single gene near or at the breakpoint (reviewed in Lindsley and Grell, 1968; Lefevre, 1974). Thus if chromosomal domains are involved in developmental programming, these domains in Drosophila are generally no larger than single units of genetic function. These are estimated to be about 5–200 kb, based on the amount of DNA associated with single polytene chromosome bands.

The resolution of cytogenetic methods is insufficient to provide a detailed picture of the exact relatioship between a rearrangement breakpoint and the affected transcription unit. Two classes of rearrangement breakpoints are expected if sequences involved in developmental regulation extend beyond the transcribed region. Some breakpoints will simply disrupt the transcription unit itself, while others, located near but outside the transcription unit, will interfere with its regulation during development. Detailed molecular mapping of mutant chromosomes has provided several examples of this latter class of position effects (Fritsch, Lawn, and Maniatis, 1979; van der Ploeg et al., 1980; Klar et al., 1981; Nasmyth et al., 1981; Spradling and Mahowald, 1981). Further study of such effects will define the topography of sequences surrounding a gene that are involved in its developmentally regulated expression.

Gene transfer methods provide a powerful technique for determining the extent to which regulated expression of a gene depends on its normal chromosomal environment. An identical segment of DNA containing the gene of interest is introduced into the chromosomes of individuals of identical genetic background. Lines are then established that differ only in the site at which a single, intact copy of the gene has integrated. If all other sources of variability between the lines can be eliminated, then the ability of the gene to function in each line would reflect the influence of the surrounding chromosomal sequences. This paradigm has been used to examine the effect of flanking sequences on the expression of a retroviral provirus in mice (Jaenisch et al., 1981) and in rat HTC cells (Feinstein, Ross, and Yamamoto, 1982). However, it has not previously been applied to a specific chromosomal gene.

In Drosophila, transposable elements of the P family (Rubin, Kidwell, and Bingham, 1982; O'Hare and Rubin, 1983) can serve as efficient vectors for the introduction of defined segments of DNA into germ-line cells (Spradling and Rubin, 1982; Rubin and Spradling, 1982). We have used P-element-mediated transformation to construct a series of Drosophila strains that contain a single copy of the wild-type *rosy* gene at diverse sites in the genome. The *rosy* gene provides a sensitive assay for position effects since its product, xanthine dehydrogenase (XDH), is easily assayed and displays a distinctive pattern of expression during development. Examination of XDH production in 36 such lines revealed that tissue-specific *rosy* gene expression was not qualitatively influenced by chromosomal position. However, the quantitative level of enzyme activity produced was sometimes subject to position effect. In particular, *rosy* gene insertions on the X chromosome exhibit dosage compensation, a characteristic of normal X-linked genes in Drosophila.

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Results

Transposons containing a normal *rosy* gene are found at one or a few chromosomal sites in the wild-type offspring of *rosy* mutant flies injected as embryos with the cloned transposon DNA (Rubin and Spradling, 1982). XDH expression in flies containing *rosy* genes inserted by this method may be influenced by a variety of factors. These include the structure of the transposon containing XDH-coding sequences, the genetic background of the recipient strain, the number of sites of transposon insertion, the stability of the transposon during transposition and in subsequent generations, the stability and heritability of XDH expression from the transferred gene, and the local chromosomal environment into which the transposon has integrated. To determine the role of the last variable, the effect of genomic position on XDH regulation, it was necessary to control for the first five variables, each of which might influence *rosy* gene expression in the transformants.

Construction of Isogenic Lines Containing Single Transposon Insertions

Defective P elements containing either an 8.2 kb Sal I fragment or a 7.2 kb Hind III fragment of chromosomal DNA that includes the *rosy* gene were constructed and injected into embryos from a *rosy* mutant strain (ry^{42}) as described in Experimental Procedures. To be useful for studies of position effect, lines containing single transposon inserts had to be prepared from the initial population of transformants obtained. Since *rosy* gene expression is affected by several unlinked genes (see Finnerty, 1976), it was necessary to maintain a uniform genetic background in all the individual lines isolated. Consequently, all subsequent genetic crosses involved only the same *rosy* mutant strain, ry^{42}, that was used as a host for microinjection. Our strategy was to determine the number and location of the integration sites in each population of transformants. Then one or more sublines were derived containing only one of the inserts. The method used for this purpose is illustrated in Figure 1 and described in detail in Experimental Procedures.

By this approach, 36 isogenic lines were established that contained single transposon insertions. Of these lines 23 contained inserts of the chromosomal Sal I fragment and 13 contained the Hind III fragment. Figure 2 illustrates the chromosomal location of the *rosy* gene in each line (see also Table 1). Also shown are the locations of all the sites of transposon insertion detected in the chromosomes of the initial transformants.

The distribution of insertions is slightly nonradom with respect to the 100 cytogenetic chromosome subdivisions ($0.05 > p > 0.01$), but it is clearly very diverse. Only one insertion appeared to have occurred within heterochromatin (Figure 3). Either P elements do not frequently integrate within the approximately 30% of the genome represented in the polytene chromocenter or such insertions were not detected by in situ hybridization. The 36 sites selected for further study are a representative sample of the total population. No mutant phenotypes were visible in flies from any of the lines, but about 30% of the insertions appeared to be associated with recessive lethality. This is similar to previous estimates (25%–50%) of the frequency of lethality associated with unselected chromosome breaks induced by x-ray treatment (see Lefevre, 1974). Thus the distribution of transposon insertions is probably unbiased with respect to vital loci. Finally, the use of wild-type eye-color phenotype to select the initial transformant population probably did not result in the elimination of many nonfunctional sites. In all five cases in which at least three isogenic lines were established from lines containing two or three inserts, each of the 14 individual sites was shown to be active.

Stability, Expression, and Inheritance of Inserted DNA

Previous studies have indicated that exogenous DNA, following introduction into cells, may undergo rearrangement (Pellicer et al., 1980) and may not be transmitted or expressed in an orderly manner. Failure of some transformed mice to transmit introduced DNA according to Mendelian expectations has been reported (Palmiter, Chen, and Brinster, 1982). Reversible inactivation of genes introduced into mammalian cells by transformation has also been reported (Davidson, Adelstein, and Oxman, 1973; Davies, Fuhrer-Krusi, and Kucherlapati, 1982).

We therefore examined the isogenic lines for evidence of rearrangement, inactivation, or secondary transposition of introduced P elements, which could potentially interfere with the detection of position effects. Previous studies indicated that transposons introduced into germ-line chromosomes by transformation did not undergo detectable rearrangement (Spradling and Rubin, 1982; Rubin and Spradling, 1982). Southern blot analysis of five arbitrarily selected lines also failed to detect evidence of sequence alterations during insertion or during the etablishment of the lines (data not shown).

The possibility that the introduced transposons might undergo secondary transposition events was of greater concern. Because both complete and defective P elements were introduced into the host embryos, some recipients may have acquired a complete P element in their chromosomes. In such a strain, the *rosy* transposon would be expected to undergo further transposition or excision. During the isolation of the isogenic lines, there was no evidence of high rates of transposition.

To investigate the possibility of instability, even of low levels, the tests described in Figure 1D were carried out using eight lines containing X-linked insertions. Males from these lines were crossed to attached-X females that were also homozygous for a partial deletion of the normal *rosy* locus. In such lines, males will display wild-type eye color and females will have rosy eyes, as long as the transposon carrying the *rosy* gene remains X-linked. Movement of the transposon to an autosome would be detected because it

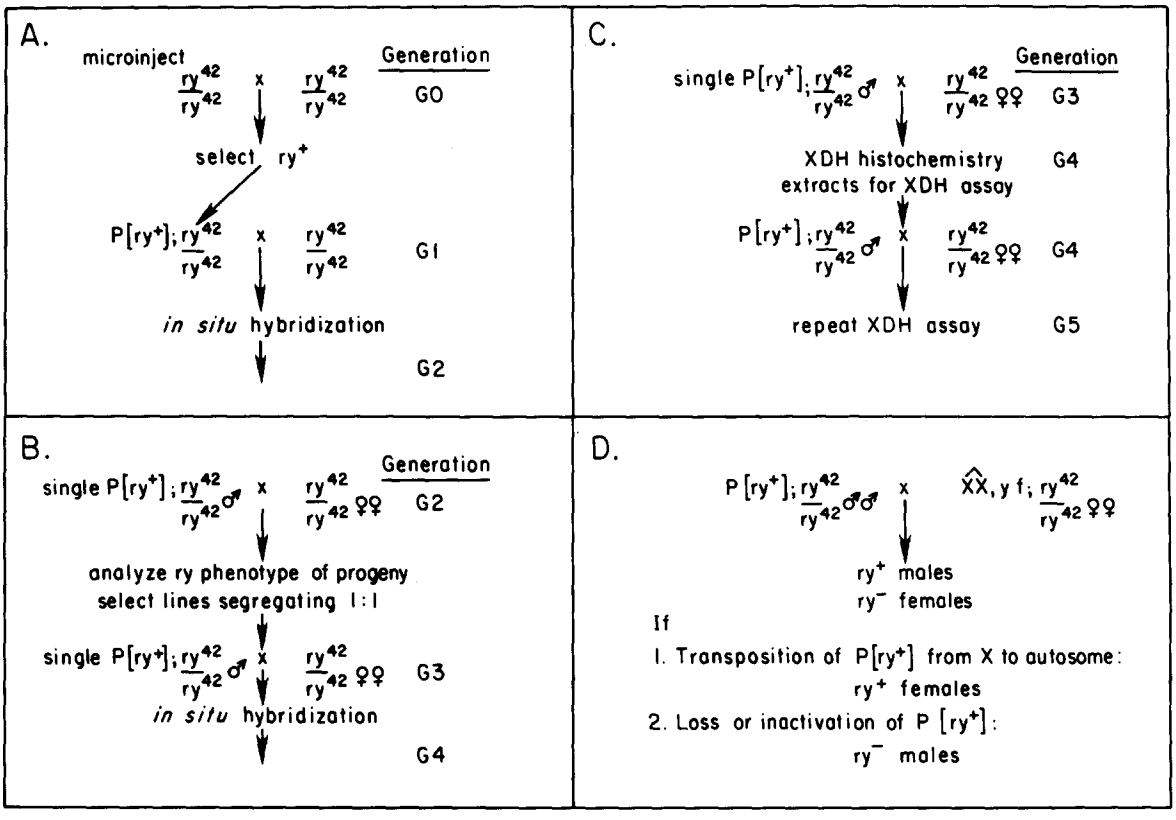

Figure 1. Protocol for Production of Stable, Isogenic Lines Containing Single Transposon Inserts

(A) Identification of chromosomal sites of insertion. Plasmid DNAs containing a *rosy* transposon and a complete P element were microinjected into (G0) host embryos of the ry^{42} strain. Surviving adults were mated to ry^{42} partners, and all the ry^+ G1 progeny derived each from individual G0 adult were maintained in a single culture. These G1 flies were again mated to ry^{42} partners, and the locations of the transposons (P[ry+]) were determined by in situ hybridization to larval salivary gland chromosomes.

(B) Isolation of lines containing single inserts. The segregation of wild-type and *rosy* mutant eye pigmentation was examined in isogenic lines derived by crossing individual wild-type G2 males to ry^{42} females. Lines known to contain closely linked sites of insertion were not used. Lines in which the eye-color phenotype showed 1:1 segregation were retained. The presence and location of a single transposon was verified by in situ hybridization to G4 larvae.

(C) Production of flies for histochemistry and enzyme activity assays. To obtain heterozygous flies for analyzing XDH expression, single G3 males from each isogenic line were crossed to ry^{42} females. Larval and adult tissues from the G4 generation were used for histochemical analysis and for the preparation of extracts. The tests were repeated in the G5 generation; in some cases they were also carried out in duplicate.

(D) Tests for stability and heritability. Cultures were established by crossing males from isogenic lines containing X-linked transposon insertions to attached-X females marked with *yellow* and *forked* (see Lindsley and Grell, 1968). Each generation, progeny were screened for the presence of exceptional ry^+ females or ry^- males.

would give rise to wild-type eye color in a female. Furthermore, if the *rosy* transposon is excised or inactivated by any mechanism not leading to lethality, it would result in a male with the mutant eye color. Among more than 12,000 individuals scored over four generations, no wild-type females resulting from such transposition events were observed, and only two $rosy^-$ males were detected. Hence the frequency of transposition or inactivation is not high enough to affect measurements of XDH gene function. The failure to observe secondary transposition suggested that complete P elements had not been retained in these lines. We were unable to detect any complete P elements by Southern blot hybridization in eight randomly selected lines (data not shown). Consequently, while complete P elements may have become integrated in a few of the transformed lines, as suggested by unusual inheritance patterns in some of the early crosses, they did not persist in later generations.

Tissue Distribution of XDH Activity in the Transformed Lines

During the life cycle of a wild-type fly, XDH activity shows both temporal modulation (Ursprung, 1961; Glassman and McLean, 1962; Munz, 1964; Sayles, Browder, and Williamson, 1973) and tissue-specific modulation (Hubby and Forrest, 1960; Ursprung and Hadorn, 1961; Munz, 1964). Activity is first detected shortly after the onset of gastrulation in the embryo and increases during the first half of embryonic development. XDH-specific activity varies two to three fold thereafter, being lower in pupae than in larvae or adults. Measurement of XDH activity in isolated tissues resulted in the identification of fat body and Malpighian

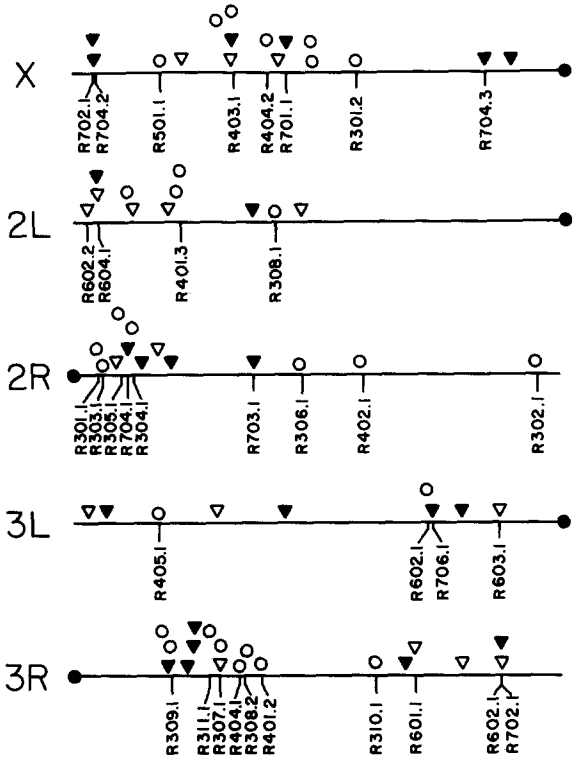

Figure 2. Chromosomal Sites of Transposon Insertion

All the sites of insertion of the indicated *rosy* transposons identified by in situ hybridization to G2 larvae are shown for each of the five major chromosome arms. The location of the single transposon present in each of the 36 isolines is indicated by the labels below the lines depicting each chromosome. (○) ry1 or ry3; (▽) ry10; (▼) ry11.

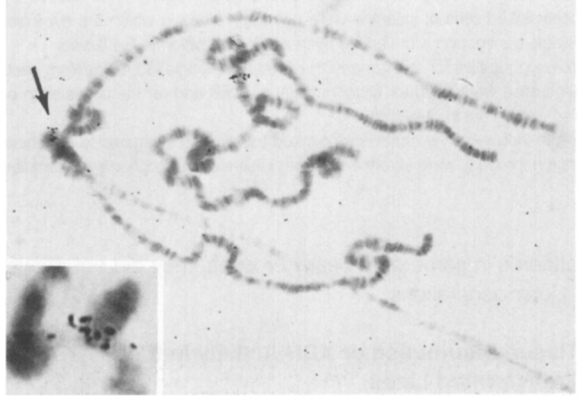

Figure 3. Location of the Insertion Site in Line R401.1 within the Heterochromatin of Chromosome 4

^{3}H-cRNA complementary to the chromosomal *rosy* gene was hybridized in situ to salivary gland polytene chromosomes from line R401.1. Hybridization was observed at the site of the normal *rosy* locus at 87D, as well as to *rosy* gene sequences present in the transposon (arrow). The inset shows that insertion has most likely occurred near the base of chromosome 4.

tubules as sites of enzyme in larvae. In adults, most of the activity is found in the hemolymph, with Malpighian tubules also containing significant levels. Only very low levels were found in testis. Tissue-specific patterns of XDH activity can

Table 1. XDH-Specific Activity in Adults from 36 ry^{+} Isogenic Lines

Strain	Type of Insert	Site of Insert	XDH-Specific Activity[a] Independent Assays	Mean
R301.1	Sal	42A	0.71, 0.53, 0.67	0.64
R301.2	ry1	12D	0.68, 0.64, 0.79	0.71
R302.1	Sal	60A	0.68, 0.74, 0.77	0.73
R303.1	Sal	42AB	0.38	0.38
R304.1	Sal	43C	0.58, 0.59	0.58
R305.1	Sal	42E	0.58, 0.63	0.60
R306.1	Sal	50B	0.51	0.51
R307.1	Sal	87A	0.48, 0.82	0.64
R308.1	Sal	29B	0.62, 0.64	0.63
R308.2	Sal	87F	0.68	0.68
R309.1	Sal	85A	0.44	0.44
R310.1	Sal	93AB	3.4, 3.4, 2.9, 3.7	3.3
R311.1	Sal	86D	0.56	0.56
R401.1	ry3	Chromocenter	0.36	0.36
R401.2	Sal	88E	0.58, 0.68	0.63
R401.3	Sal	25C	0.78, 0.82	0.80
R402.1	Sal	52F	0.86, 0.92	0.89
R403.1	Sal	7D	0.53, 0.67	0.60
R404.1	Sal	87F	0.55, 0.58	0.56
R404.2	Sal	9AB	0.27, 0.30	0.28
R405.1	Sal	64C	0.52, 0.56	0.54
R501.1	ry1	4D	0.55, 0.58, 0.70	0.61
R502.1	ry1	75CD	0.38	0.38
R601.1	ry10	95A	0.37, 0.37	0.37
R602.1	ry10	98C	0.38, 0.41, 0.38	0.39
R602.2	ry10	21D	0.68	0.68
R603.1	ry10	78BC	0.89, 0.81	0.85
R604.1	ry10	22A	0.60, 0.62	0.61
R701.1	ry11	9E	0.68	0.68
R702.1	ry11	1F	0.67	0.67
R703.1	ry11	48C	0.51, 0.44	0.47
R704.1	ry11	43	0.53	0.53
R704.2	ry11	1F	0.84	0.84
R704.3	ry11	18A	0.55, 0.71	0.63
R705.1	ry11	98C	0.31, 0.30	0.30
R706.1	ry11	75D	0.16, 0.22	0.19

XDH-specific activity was determined in extracts from adults, 3–7 days of age, of the indicated isogenic lines. Multiple experimental values represent assays of independent extracts, prepared in most cases from flies in successive generations (see Figure 1C and Experimental Procedures for details). Unusually high activity in line R301.1 is the result of a tandem insertion (Rubin and Spradling, submitted).

[a] Nanomoles product/min/mg protein at 25°C.

be conveniently assayed by histochemical staining (Carpenter, 1982).

Since other potential factors had been eliminated, any differences in XDH expression between the isogenic lines could be attributed to the influence of chromosomal sequences flanking each transposon. All 36 isogenic lines in Table 1 were screened to determine whether the pattern of tissue-specific expression of the *rosy* gene present on the transposon was distinguishable from that of wild type. Flies heterozygous for the transposon were selected from each strain (Figure 1C), and tissues were dissected and stained for XDH activity. XDH activity was present in the adult Malpighian tubules (Figure 4) and in the larval fat body cells of all the strains. As in the case of wild-type flies, little or no staining was seen in the adult testis. While

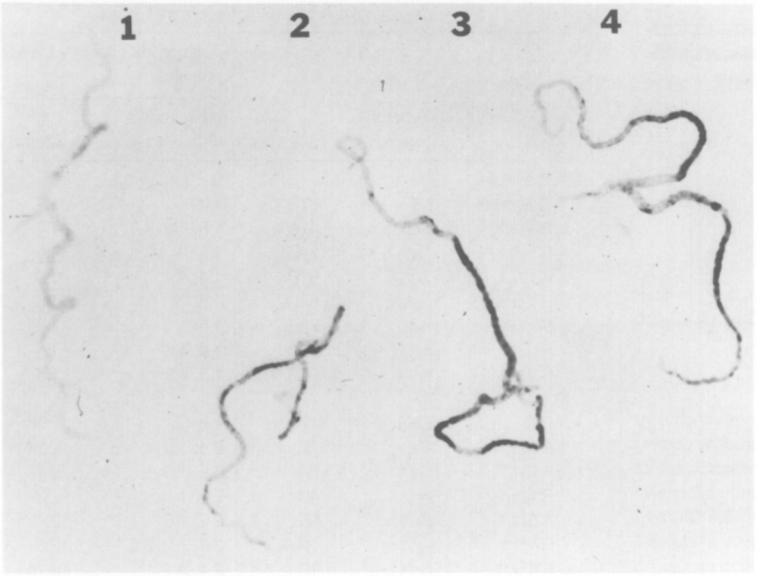

Figure 4. Histochemical Staining of Xanthine Dehydrogenase Activity

Tissues were dissected from wild type (Oregon R), ry^{42}, and each of the 36 transposon-bearing isogenic lines. Staining for XDH activity was carried out as described in Experimental Procedures. Shown are adult Malphigian tubes from host ry^{42} (1), wild type (Oregon R) (2), R301.1 (3), and R301.2 (4).

these studies detected no qualitative changes in the pattern of XDH expression as a result of chromosomal position, it was not possible to compare the levels of activity present in tissues of the different strains. Consequently, different methods were used for further examination of *rosy* gene expression in the transformed strains.

XDH-Specific Activity in the Transformed Lines

Since the XDH activity in an adult is the sum of activity in a variety of tissues, changes in either the tissue distribution or the level of *rosy* gene expression could alter total XDH-specific activity. Activity in adults increases following eclosion, but remains fairly constant after 2–3 days. Specific activity is higher in males than in females, presumably because of the high protein content of reproductively active females, which have numerous yolk-filled eggs in their ovaries. To control for these variables, all measurements of XDH-specific activity were made with equal numbers of male and female adults, 3 to 7 days of age. Extracts of such flies were prepared and assayed for XDH activity. Activity was normalized to the protein content of the homogenate. To demonstrate that the specific activity measured in these experiments was a heritable property of the strain in question, the measurements were repeated on flies from subsequent generations. The results of these measurements are given in Table 1.

The XDH activity of most lines was between 30% and 130% of the value for an Oregon R strain. The only exception was line R310.1, which had an XDH-specific activity four to five times that of wild type. Southern blot analysis (Rubin and Spradling, submitted) has shown that line R310.1 contains a transposon comprising four *rosy* genes that apparently derived from a tetramer of the injected plasmid. The XDH-specific activity per *rosy* gene in this strain is therefore similar to that of the other isogenic lines. The existence of R310.1 reinforces the expectation from previous studies (Grell, 1962) that XDH activity is proportional to the dosage of wild-type *rosy* genes, and therefore provides a direct assay for *rosy* gene activity.

The XDH-specific activity of each strain was highly reproducible in a subsequent generation and in duplicate assays (Table 1). The distribution of activities observed in the 36 lines is presented graphically in Figure 5. Comparison of the levels of XDH activity in different strains provides insight into some of the potential variables to be considered in evaluating position effects on *rosy* gene expression. For example, no correlation existed between XDH activity and the orientation of the Hind III fragment containing the *rosy* gene relative to the P element vector. (The orientation of the Sal I fragment in lines transformed with a mixture of ry1- and ry3-containing plasmids was not determined in most cases.) These observations suggest that the P element is not playing a simple role in the expression of the *rosy* gene contained on the transposon, for example by providing a strong promoter. They do not rule out the possibility that the P element plays some other role such as providing an enhancer-like activity.

Dosage Compensation of Transposons Inserted on the X Chromosome

In Drosophila, X-linked genes show equivalent activity in males and females despite the difference in gene copy number, a phenomenon called dosage compensation (reviewed in Lucchesi, 1977; Stewart and Merriam, 1980). It was therefore of interest to determine whether *rosy* genes inserted on the X chromosome came under such regulation. The XDH-specific activity in male and female flies from all eight lines containing X-linked *rosy* genes was measured. For comparison, identical mesurements were carried out on seven lines with autosomal insertions. Since reproductively active females such as those used in these measurements contain large amounts of yolk protein, their

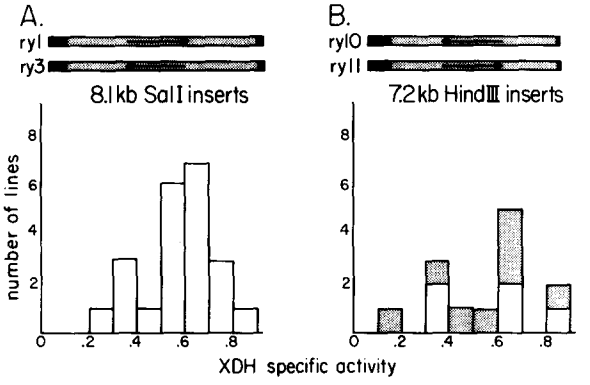

Figure 5. XDH-Specific Activity in Transformed Lines

The distribution of mean XDH-specific activities (nanomole isoxanthopterin/min/mg protein) from Table 1 among lines containing transposons bearing Sal I or Hind III is illustrated. Lines containing ry1 or ry3 inserts were not distinguished; however, those containing ry10 (open bars) are shown separately from those containing ry11 (stippled bars) in (B). The structure of these transposons is summarized at the top of the figure. P element sequences are shown in black; sequences from pDm2844S8.5 containing the chromosomal *rosy* gene are stippled. For comparison, a wild-type Oregon R line had an XDH-specific activity of 0.60. The width of the bars is approximately equal to the standard deviation of measurement within a line for lines with average activity.

Table 2. Dosage Compensation of Transformed *rosy* Genes

Strain	Type of Insert	Site	Ratio of XDH Activity Males/Females: Independent Measurements	Mean
Autosomal				
Oregon R		87D	1.7	1.7
R301.1	Sal	42A	1.8, 1.6	1.7
R308.1	Sal	29B	1.5	1.5
R310.1	Sal	93B	1.4, 1.1	1.3
R405.1	Sal	64C	1.8,1.4	1.6
R603.1	ry10	78BC	1.6	1.6
R703.1	ry11	22A	1.4, 1.7	1.6
R705.1	ry11	98C	1.5, 1.5	1.5
X				
R301.2	Sal	12D	2.8, 3.1	2.95
R403.1	Sal	7D	2.4, 1.9	2.2
R404.2	Sal	9A	3.2, 3.0	3.1
R501.1	ry1	4D	2.6, 1.9	2.3
R701.1	ry11	9E	2.2, 2.6	2.4
R702.1	ry11	1F	2.5, 2.5, 2.6	2.5
R704.2	ry11	1F	2.4, 3.1	2.75
R704.3	ry11	18A	2.5, 2.8	2.7

XDH-specific activity in extracts of 80 adult males or females, 3–7 days of age, was measured for each of the indicated isogenic lines. Both males and females contained a single copy of the *rosy* transposon. The ratio of male to female activity is given for seven lines containing autosomal insertions, eight lines containing X-linked insertions, and an Oregon R wild-type strain. Ratios measured more than once involved independent measurements of separate cultures of the line in question, which differed in the number of generations it had been maintained prior to testing.

XDH-specific activity is lower than that of males by an amount that depends on details of culture conditions, age, etc. These variables were controlled so that any differences between the X-linked and autosomal lines would reflect dosage compensation. The results of these measurements are shown in Table 2 and Figure 6.

The average ratio of the activity in males to that in females for the eight autosomal lines was 1.6. This was similar to a value of 1.7 in an Oregon R line containing active *rosy* genes at the normal autosomal location. In contrast to this, the activity ratio in the X-linked lines ranged from 1.9 to 3.2, with a mean of 2.6. Two lines, R404.2 and R301.2, showed nearly the twofold elevation expected for complete dosage compensation. In the other lines, a lower degree of elevation occurred, but all the lines showed some degree of dosage compensation.

Discussion

Are Large Chromosome Domains Required for Developmental Regulation?

A variety of studies suggest that DNA in eucaryotic chromosomes is organized into large domains encompassing tens to hundreds of kilobases (reviewed in Georgiev, Nedospasov, and Bakayov, 1978). The most obvious reflections of this organization are the detailed and highly reproducible banding patterns that can be elicited in metaphase chromosomes and are apparent in the polytene and lampbrush chromosomes from certain cells. Although the functional significance of these large regions is almost completely unknown, the concept of chromosomal domains provides a potential explanation for several perplexing observations. For example, although genes located on short DNA segments are sometimes transcribed in a normal manner when introduced into vertebrate cells, proper temporal and tissue-specific expression is absent. This result is observed whether the genes are introduced on extrachromosomal molecules or whether they have become integrated in a host chromosome. Even when the genes are introduced into a mouse and pass through repeated life cycles, they do not come under developmental controls.

Developmental regulation, as opposed to transcription per se, may require the presence of a gene in its normal chromosomal domain. According to this model, a transformed gene would acquire normal developmental controls only if large amounts of the sequences which normally flank it are included along with it at its now chromosomal location. Position effects should be common, since chromosome rearrangement breakpoints anywhere within a domain could potentially disrupt the function of the genes it includes. Results consistent with this view have come from experiments in which a defined DNA segment, i.e. a retrovirus provirus, has been inserted at a variety of locations in cells with similar genetic backgrounds. The ability of these viral sequences to be expressed depended on the site of insertion (Feinstein et al., 1982), and even the time of virus activation in an intact mouse varied with chromosomal position (Jaenisch et al., 1981).

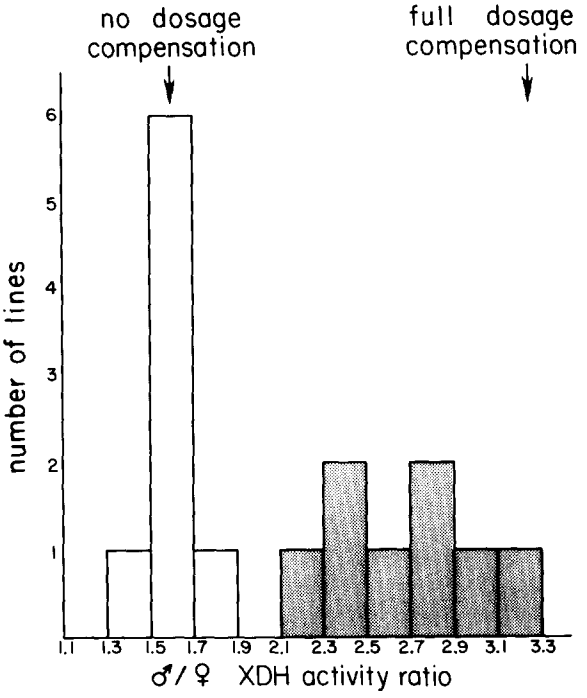

Figure 6. Dosage Compensation of X-Linked *rosy* Genes

The distribution of ratios of male to female XDH-specific activity is shown for eight lines containing a *rosy* gene on the X chromosome (stippled bars) and for seven lines containing an autosomal *rosy* gene (open bars). The mean for the autosomal lines is indicated as no dosage compensation (it is similar to the ratio obtained in a wild-type strain containing the *rosy* gene at its normal autosomal location). A value of twice the mean autosomal ratio is indicated as full dosage compensation. Data are from Table 2.

A 7.2 kb DNA Fragment Is Sufficient for Normal *rosy* Expression

The studies reported here represent a direct test of the sequence requirements for developmentally regulated expression of the *rosy* gene. At least 4.5 kb of DNA would be required to code for the 160 kilodalton XDH polypeptide (Chovnick et al., 1977). Therefore while the exact location of the *rosy* structural gene within the 7.2 kb Hind III fragment is unknown, at most 2–3 kb of flanking sequences could have been included in the transposons used. Despite the small amount of flanking DNA, flies containing a transposon expressed normal or nearly normal amounts of XDH. XDH activity was distributed among larval and adult tissues in a manner indistinguishable from that in wild-type flies. Clearly the *rosy* gene does not require a particular large chromosomal domain for apparently normal expression.

It is possible that the tissue distribution of XDH activity does not reflect only the distribution of XDH gene transcription or mRNA accumulation. The differential XDH activity among tissues observed in wild-type and transformant strains could result from posttranscriptional controls such as the tissue-specific availability of cofactors required for enzymatic activity. The fact that XDH activity is proportional to *rosy* gene dosage, however, argues that transcription is the actual limiting factor. No differences in tissue specificity were seen even in line R310.1, which has four times the XDH activity of the other isogenic lines.

Position Effects on *rosy* Expression

The absence of strong position effects on *rosy* expression not only indicates that a large region surrounding the normal gene is not necessary for its expression but also implies that other chromosomal domains capable of overriding the local controls on XDH expression are rare. Some quantitative variation in XDH activity was observed between the lines. The average specific activity of the lines transformed with the 8.2 kb Sal I fragment was 0.58 nanomole/min/mg, as compared with a value of 0.60 nanomole/min/mg per gene for a wild-type Oregon R strain. A majority of the isogenic lines had activities near 0.60, indicating that *rosy* gene function was unaffected by the local flanking chromosomal region. The standard deviation of activity measurements within individual lines averaged about 0.08 of the mean (Table 1). Consequently, lines differing from the expected "wild-type" value of 0.60 by more than two standard deviations—i.e., those with activities greater than 0.70 or less than 0.50—were considered to be likely examples of position effect. By this criterion, 20 of the 36 lines studied showed no local influence, 6 had elevated activity, and 10 showed reduced activity of the *rosy* gene.

Several mechanisms may underlie these position effects. The chromatin structure of the adjacent region could influence that of the inserted gene. Transcription from a promoter into which the transposon had inserted could interfere with XDH transcription; however, lines containing lethal insertions showed a frequency of position effects similar to that of lines containing nonlethal insertions. Whatever the cause, position effects were observed more frequently with the smaller transposons ry10 and ry11 (7 of 13 or 54%) than with the larger ry1 and ry3 transposons (9 of 23 or 39%). This suggests that the presence of 0.9 kb more flanking DNA in the Sal I fragment than in the Hind III fragment may have had a buffering effect.

Line R401.1 is a particularly interesting example of a position effect. This line exhibited one of the lowest specific activities of any of the ry1 or ry3 inserts. In situ hybridization showed that it contained an insertion very near or within the chromocenter of the polytene chromosomes, a region associated with reduced genetic activity and position effects on euchromatic genes (Lewis, 1950). Consistent with cytogenetic mapping of this insert to the fourth chromosome, genetic studies demonstrated that the wild-type *rosy* gene in R401.1 is linked to chromosome 4 (data not shown). Because of the small size of this chromosome it is difficult to determine if the site of insertion was truly within heterochromatin (see Figure 3); however, R401.1 would be a good candidate for further studies of position effects mediated by changes in chromatin structure.

Are the Sites of Transposon Insertion Unbiased?

The issue of whether the genomic sites containing inserted *rosy* genes in the lines studied here are representative of

the genome as a whole is of central importance. All available evidence suggests that DNA introduced by P-element-mediated transformation is integrated by the same mechanisms responsible for the transposition of intact P elements. P element insertions have been obtained in wide variety of genes (Simmons and Lim, 1980) but at differing frequencies. Analysis of the sequence of over 20 sites of P element insertion reinforces the view that these elements can integrate at a wide variety of genomic sites but have definite sequence preferences (O'Hare and Rubin, 1983). Three of four insertions in the *white* locus were found at identical sites (Rubin et al., 1982; O'Hare and Rubin, 1983). Consequently, the insertion sites observed with the *rosy* transposons used here probably represent a diverse but nonrandom subset of genomic sites. The wide diversity of location observed at the cytogenetic level is consistent with this expectation.

A further complication results from the use of *rosy* gene function itself to select and maintain lines containing transposons. This raises the possibility that only a limited subset of all possible P integration sites is actually represented in our studies. For several reasons, however, it is unlikely that the methods used to establish these lines have resulted in significant selection for genomic positions at which the *rosy* gene is highly functional. Wild-type eye pigmentation requires less than 1% of normal XDH activity (Chovnick et al., 1977) and behaves nonautonomously. The lowest activity of any functional *rosy* gene following transformation in these studies was about 30% of the wild-type activity in line R706.1. Thus if sites imposing greatly reduced activity on inserted genes exist, they must be relatively infrequent.

Although it is possible that some insertion sites eliminate activity altogether, we would have expected to detect inactive insertions in two ways. First, in lines containing multiple transposons, the activity of only one would be required. However, in the lines with multiple insertions tested, each individual transposon was capable of producing wild-type eye pigmentation. Second, if inactive insertions occur, they should segregate randomly in the crosses described in Figure 1 and occasionally end up in one of the isogenic lines. Inactive sites were not detected by in situ hybridization to chromosomes from 190 individual larvae from the 36 isogenic lines. However, it is still possible that genetically inactive insertions into a chromosomal region such as the underreplicated portion of the heterochromatin might have been missed.

The Mechanism of Dosage Compensation

Dosage compensation is accomplished in Drosophila by equalizing the activity of X-linked genes in males and females despite the differences in gene dosage. Measurements of phenotype (Muller, 1950; Smith and Lucchesi, 1969) or enzymatic activity (Seecof, Kaplan, and Futch, 1969) controlled by X-linked genes indicated that the activity of the single X chromosome in a male is equivalent to that of the two X chromosomes in a female. Spectrophotometric measurements revealed that differential replication of the X chromosome between the sexes cannot explain this compensation, at least in the case of the polytene salivary gland cells (Aronson, Rudkin, and Schultz, 1954). However, in males polytene X chromosomes appear to be larger and less compact than any of the other unpaired chromosomes (Dobzhansky, 1957), leading to the suggestion that genes contained on it are hyperactive, a view supported by rates of ^{3}H-uridine incorporation per unit length along these chromosomes (Mukherjee and Beermann, 1965). Models invoking either positive or negative regulators of gene activity have been proposed to explain this differential activity (reviewed in Stewart and Merriam, 1980), and genes that appear to be required for the establishment of dosage compensation in females (Cline, 1978) or males (Belote and Lucchesi, 1980) have been identified.

Both positive and negative regulatory models are compatible with regulatory products acting either at the site of each compensated X-linked gene or within larger domains of dosage compensation encompassing multiple genes. Previous studies failed to detect dosage compensation of autosomal genes translocated onto the X chromosome (Roehrdanz, Kitchens, and Lucchesi, 1977). Consistent with this lack of long-range influence, the differential compaction of the X and autosomal portions of such translocation chromosomes is maintained in male salivary gland cells (Muller and Kaplan, 1966). Only very large domains of compensation would have been detected in these studies, however, since the autosomal genes tested were distant from the site of the translocation breakpoint.

In contrast, the results reported here demonstrate that the mechanisms responsible for dosage compensation can spread to adjacent autosomal genes. In each of eight independent insertions of a 7 or an 8 kb autosomal DNA fragment into a site on the X chromosome, partial or complete dosage compensation of XDH-specific activity was observed. Furthermore, the increased ratio of XDH-specific activity in male to that in female extracts observed in the case of X-linked inserts (Table 2) was due to an increase in the absolute specific activity in males, rather than to decreased activity in females. The specific activities of the extracts from females were similar for autosomal and X-linked lines. Thus the dosage compensation of *rosy* genes we have observed occurs as a "position effect" exerted to different extents by different local X-chromosome sequences; it takes place by increasing the activity of the gene in the male X chromosome relative to that in the female X chromosome, or relative to the activity of that same gene at most autosomal locations. These observations suggest the existence of multiple sites located along the X chromosome which mediate a hyperactivation of gene activity in a local region.

Concluding Remarks

These studies have revealed that 2–3 kb of DNA flanking the *rosy* gene is sufficient to ensure normal gene expres-

sion at most chromosomal sites. If *rosy* proves to be unexceptional in this regard, and chromosomal context is often unimportant, what explains the failure of genes introduced into mammalian cells to come under normal controls, even after passage through the germ line as in the case of P element insertions? There are many possible explanations for the difference between gene transfer in mammals and in Drosophila. These include the mechanism by which DNA is introduced into host chromosomes, as well as possible modification of inserted DNA, for example by methylation. Transposable-element-independent integration may occur preferentially at sites that are not compatible with normal regulation; furthermore, such sites may be relatively frequent in the large genomes of mammals. Nonetheless, our results encourage the view that no fundamental barrier exists to the regulated expression of genes present on small segments of DNA.

For many purposes it would be useful to construct mutant genes in vitro and then test their ability to function by reintroducing each into the germ line by P-element-mediated gene transfer. In such a scheme, the properties of different constructs will have to be compared among lines where the chromosomal location of the inserted gene differs. In some cases it may be necessary to include an internal control gene within the transposon to monitor the potential activity of the particular site at which insertion has occurred. However, our results suggest that in many cases it may be possible to make direct conclusions based on the properties of transposons inserted at a few sites. Recent studies on the behavior of dopa decarboxylase genes (Scholnick et al., 1983) and alcohol dehydrogenase genes (Goldberg et al., 1983) introduced into flies by transformation suggest that this conclusion will apply to other genes in addition to *rosy*. Thus position effects do not appear to represent a serious obstacle to the analysis of gene function by in vitro mutagenesis and P-element-mediated transformation.

Experimental Procedures

Construction of Plasmids

The construction of plasmids containing the transposons ry1 and ry3 have been described previously (Rubin and Spradling, 1982). Plasmids containing the transposons ry10 and ry11 were synthesized as intermediates in the construction of a series of transformation vectors and will be described in detail elsewhere (Rubin and Spradling, submitted). Transposons ry1 and ry3 contain an 8.1 kb Sal I fragment from the wild-type *rosy* locus inserted at the Xho I site of the defective P element in plasmid p6.1 (Rubin et al., 1982). Transposons ry10 and ry11 contain a 7.2 kb Hind III fragment derived from within the 8.1 Sal I fragment, inserted at the rightmost Hind III site within the same defective P element. The Sal I and Hind III inserts therefore differ in the amount of DNA flanking the *rosy* transcription unit. The transposons in each pair differ only in the orientation of the chromosomal DNA relative to the P element.

Transformation

Microinjection of plasmid DNA containing a *rosy* transposon (300 μg/ml) and plasmid pπ25.1 (50 μg/ml) into 0–1.5 hr embryos of the ry^{42} strain was carried out essentially as described previously (Spradling and Rubin, 1982). Adults derived from injected embryos (G0 generation) were mated singly to ry^{42} partners, and phenotypically wild-type progeny in the G1 generation were selected. P element transposition under these conditions may occur premeiotically (Spradling and Rubin, 1982), hence the ry^+ progeny from a single G0 individual do not represent independent events. The exact number of independent transposition events occurring in the germ line of developing G0 flies is variable, and the relative frequency with which individual insertion events are represented in the G1 progeny will depend on the time of each insertion and the proliferation and lineage of the germ cells involved. Furthermore, transposons that are unable to express even low levels of XDH might escape detection since they would not alone result in production of wild-type eye color.

The significance of these variables was assessed by analyzing the chromosomal location of inserted *rosy* sequences in randomly selected G2 larvae by in situ hybridization (see Figure 1A). If, as expected, only a small number of independent insertion events had occurred, then larval chromosomes from the same line would often contain identical sites of transposon insertion. Examination of several larvae would reveal most of the sites present, although transposition events occurring late in development, or in a germ cell that contributed few gametes to the population sampled, would probably be missed. In contrast, if a large number of independent insertions occurred initially, only a few of which were capable of supporting *rosy* gene function, then the larvae should frequently contain insertion sites not found in other larvae from the same line, and some of these would represnt nonfunctional insertions segregating as unselected markers. Polytene chromosomes from four or six larvae of each line were hybridized in situ with sequences from the wild-type *rosy* gene (see Table 1). The larvae contained insertions that were present on average in 0.60 of the larvae analyzed. The insertions were located on all the chromosome arms and were labeled at an intensity consistent with a single insertion at each site (except in strain R310.1). These results argue that only a small number of independent insertion events not deleterious to the host cell occur following injection of DNA. Lines with multiple insertions sometimes contained more than one site within a single G2 larva, indicating that multiple insertions could occur within a single germ-line cell.

Construction of Isogenic Lines

Lines containing closely linked sites of transposon insertion were discarded, since single-insert lines could not easily be generated by the procedures used (see Figure 1B). Individual wild-type G2 males from each suitable transformed population were crossed to ry^{42} females to create a series of sublines, each of which should contain one or more of the transposon inserts segregating among the G2 progeny of each transformant. The eye-color phenotypes of the G3 descendants from these crosses were then scored. Over 50% of these sublines showed an approximately 1:1 segregation of the rosy and wild-type phenotype, indicating that only one unlinked, active *rosy* gene remained. (The actual segregation ratios were slightly skewed by the greater viability under standard culture conditions of ry^+ compared to ry^- individuals.) In some crosses the wild-type phenotype showed sex-linked inheritance; in all but one of these cases the localization studies had revealed the presence of a transposon on the X chromosome. The G3 progeny from other sublines showed 3:1 or greater segregation of wild-type eye color and thus contained two or more unlinked, active transposons. This pattern of segregation was observed in lines previously shown by in situ hybridization to contain two or more sites of transposon integration; furthermore, in all seven cases in which at least three isogenic lines were established from such a G2 population, at least one of them showed genetic evidence of multiple active *rosy* genes.

The segregation analysis indicated which sublines were likely to contain only a single insert. Single G3 males from such candidate isogenic lines were therefore mated to ry^{42} females (see Figure 1B), larvae from the G4 generation were analyzed by in situ hybridization, and the eye-color phenotypes of the G4 progeny were scored. As expected, in virtually all cases 1:1 segregation of the rosy phenotype was observed. Each isogenic line contained only a single site in its polytene chromosomes showing detectable hybridization to *rosy* DNA sequences in addition to the site of the normal *rosy* locus at 87D. Furthermore, the location of the transposon in the isogenic lines was almost always one of the sites expected from the initial cytological studies of the G2 generation. The failure to detect, among 190 larvae tested, any lines that contained multiple insertions but behaved genetically as single-site insertions strongly suggested that the *rosy* gene is able to function at most or all sites of transposon insertion. Furthermore, since each line contained a new site labeled at the expected intensity by

the probe, insertion of transposons into regions of DNA that do not replicate fully in polytene chromosomes is rare, or is not compatible with even low levels of *rosy* gene function.

Viability Tests

Stocks of the 36 isogenic lines were maintained by passaging five to ten flies with wild-type eye color onto fresh food every 2–3 weeks. After five to ten generations, the progeny of several individual ry^+ females from each stock were scored. The presence of wild-type females that produced exclusively wild-type offspring in an isogenic line was taken as evidence that the transposon insertion in that line was viable as a homozygote. Ten of the lines failed to yield such females and therefore appear to contain insertions associated with lowered viability or with lethality.

XDH Histochemistry

The protocol of Carpenter (1982) was followed except that staining was carried out on freshly dissected whole tissues. Following dissection of larval Malpighian tubules and fat body and of adult Malpighian tubules and testes, the tissues were washed in saline and then transferred to small chambers protected from light. Tissues were stained in 50 μl of 0.2 M Tris (pH 8.1), 400 μg/ml hypoxanthine, 1 mg/ml nitro blue tetrazolium, 300 μg/ml phenazine methosulfate, at room temperature for 30–60 min. In all cases tissues from wild-type and ry^{42} mutant strains were analyzed along with those containing *rosy* transposons. Differential staining of a tissue in a strain relative to the ry^{42} control was taken to indicate XDH activity if the staining was absent in controls in which hypoxanthine was omitted from the reaction mixture. The intensity of staining varied, particularly in the case of larval fat body cells. Under the conditions used, eggs stained nonspecifically and hence could not be used to assess XDH expression.

In Situ Hybridization

In situ hybridization to salivary gland chromosomes was carried out with ^{3}H-labeled cRNA probes complementary to the 8.2 kb Sal I fragment of pDm2844.S8.5 or subclones of the P element sequences in pπ25.1 (Spradling and Rubin, 1982).

Enzymatic Assay of XDH-Specific Activity

XDH assays were carried out essentially as described (Forrest, Glassman, and Mitchell, 1956; McCarron et al., 1979). Sixty male and sixty female adult flies (3–7 days of age) from each line were homogenized in 3 ml of 0.1 M Tris (pH 8.0) containing 2.7 mg/ml activated charcoal. Following centrifugation at 17,000 rpm for 30 min in a Sorvall SE12 rotor, the supernatant was removed, and 1 ml of 0.1 M Tris (pH 8.0) containing 8 mg/ml activated charcoal was added. The tubes were mixed thoroughly and centrifuged at 20,000 rpm for 30 min in the SE12 rotor. The supernatant was removed and filtered through a 0.45 μm HA (Millipore) filter to remove residual charcoal. Extracts were assayed within 6 hr of preparation, during which time no change in activity was detected. To determine enzyme activity the following components were mixed in a cuvette: 1.9 ml 0.1 M Tris (pH 8.0), 0.01 ml 10^{-3} M 2-amino-4-hydroxypteridine (Sigma), 0.02 ml 10^{-3} M methylene blue, 0.1 ml extract. Emission at 412 nm was determined with excitation at 336 nm at 25°C using a Aminco-Bowman spectrofluorometer. Under these conditions, reactions were linear for at least 20 min (except in the case of line R310.1) and were proportional to the amount of extract added. No activity was detected when extracts were prepared from the ry^{42} strain. Fluorescence emission was calibrated with quinine sulfate as standard. Activity was determined with reference to authentic isoxanthopterin, the product of the reaction catalyzed by XDH. Protein was determined by the method of Lowry et al. (1951).

In two experiments, 100 (or 120) male or female flies were homogenized in only 2 ml of buffer and processed as described. The absolute values of XDH-specific activity measured for strains in these experiments were systematically lower by about a factor of 2, suggesting that XDH activity is less efficiently extracted under these conditions. Since the ratios of male to female activity were indistinguishable from values measured previously, however, these values are included in Table 2. In only two cases, lines R404.2 and R702.1, absolute XDH-specific activity values from these experiments were included in Table 1, since these were the only measurements available. In these two cases, the values were corrected for the systematic error.

Nomenclature

We have used the symbol P[ry$^+$] to represent a defective P element transposon containing a functional *rosy* gene. In general, the brackets denote the limits of the transposable segment containing the indicated markers. For example, a strain containing a single X-linked insert between the markers *cv* and *v* in a ry^{42} background would be indicated:

$$cv\ P[ry^+]v;\ \frac{ry^{42}}{ry^{42}}$$

This notation succinctly summarizes the relevant genetic properties of the strain (including its expected behavior in a dysgenic cross).

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