THE UNDERLYING BASES OF GENE EXPRESSION DIFFERENCES IN STABLE TRANSFORMANTS OF THE ROSY LOCUS IN DROSOPHILA MELANOGASTER

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Manuscript received November 7, 1985
Revised copy accepted January 31, 1986

ABSTRACT

This report represents a continuation of our laboratory's effort to understand the major phenomena associated with P-M dysgenesis-mediated transformation in Drosophila. A group of stable transformants are characterized with respect to rosy gene expression. Stable, true-breeding, line-specific variants in gene expression are described. These are shown to be associated with single transposons present in each line, and the lines are free of functional P elements. The effects on expression are cis-acting, and there are no identifiable rosy DNA sequence lesions associated with these transposons. Evidence is presented that demonstrates that two features of the transformation experimental system are responsible for such variation. The first relates to the fact that the transposons insert at numerous genomic sites. Both heterochromatic and euchromatic position effects are characterized. The second relates to the fact that transformation involves dysgenic mobilization of a P-element transposon. This process is mutagenic, and such a mutation is characterized.

THE technique of P-M dysgenesis-mediated transformation in Drosophila (RUBIN and SPRADLING 1982) offers opportunity for new and exciting approaches in the experimental pursuit of several contemporary issues in Drosophila. In a previous report (DANIELS et al. 1985), we described the results of some of our early studies designed to understand the basis for transformant instability. The present report represents a continuation of our effort to understand the mechanisms underlying major phenomena associated with our transformants. In this paper, we consider the basis for gene expression differences seen in our stable transformants.

From our earliest efforts with transformation in Drosophila, it became apparent that gene expression, examined in several transformants, may exhibit stable, site-specific or line-specific differences. Other laboratories have made similar observations (SCHOLNICK, MORGAN and HIRSH 1983; SPRADLING and RUBIN 1983; GOLDBERG, POSAKONY and MANIATIS 1983; GEHRINC et al. 1984;
HAZELRIGG, LEVIS and RUBIN 1984; ZEHRING et al. 1984). A clear understanding of the nature of these true-breeding variants or mutations is essential for the design of future experiments.

Since the independent, stable transformants are located at different genomic sites, the observed differences in expression may be reflections of interaction with neighboring genomic sequences. Such effects, involving rearrangements that relocate euchromatic genes adjacent to or within heterochromatin, have been studied for many years, and are referred to as position effects (see review, LEWIS 1950; BAKER 1968; SPOFFORD 1976). In a recent report, LEVIS, HAZELRIGG and RUBIN (1985) have shown, in two instances, that white locus transposons, exhibiting white-variegated expression, are due to position effects. Whether these were heterochromatic position effects or true euchromatic position effects was never determined.

Well-defined position effects of euchromatic genes placed adjacent to foreign euchromatin are uncommon; the best-documented cases are those of Bar (STURTEVANT 1925; BRIDGES 1936; MULLER 1936; MULLER, PROKOFYEVA-BELGOVSKAYA and KOSSIKOV 1936), fa"ab (WELSHONS and KEPPI 1975; KEPPI and WELSHONS 1977; WELSHONS and WELSHONS 1985) and w"D2L (BINGHAM 1981; ZACHAR and BINGHAM 1982; LEVIS and RUBIN 1982). That euchromatic position effects may influence transformant gene expression is suggested by the instances of rosy locus transposons that exhibit sex-linked dosage compensation (SPRADLING and RUBIN 1983; DANIELS et al. 1985).

Another possible explanation for some of the site-specific differences in transposon expression is that of DNA sequence changes in the transformant brought about by the transposition process (P-M dysgenesis). In such cases, the site specificity does not relate to adjacent genomic sequences, but rather, reflects the fact that each transposition represents an independent opportunity for mutagenesis. This process is associated with a very high frequency of transposon DNA rearrangements varying greatly in size (DANIELS et al. 1985). Indeed, some of these sequence changes may be below the limits of resolution by restriction analysis (DANIELS et al. 1985; LEVIS, HAZELRIGG and RUBIN 1985).

THE EXPERIMENTAL SYSTEM

We have generated a number of rosy locus (ry:3-52.0) transformants over the past several years via P-element-mediated transformation (RUBIN and SPRADLING 1982). Our observations on site-specific variation of rosy locus expression in stable transformants confirm those of SPRADLING and RUBIN (1983). In the present instance, we are dealing with rosy locus transformants that exhibit normal eye color. They do exhibit strain-specific differences in expression at the level of xanthine dehydrogenase (XDH) activity ranging from apparent underproduction to apparent overproduction. Experiments are described that identify several causes of the differences in rosy locus expression exhibited by stable transformants. These are (1) Y-chromosome-modified heterochromatic position-effect; (2) non-Y-modified position effect, involving neighboring eu-
chromatic genomic sequences; and (3) a mutation in the coding region of a transformant recovered from a P-M dysgenesis-induced mobilization experiment.

MATERIALS AND METHODS

Strains employed: \( \text{ry}^{42} \) is an M strain, homozygous for an apparent point mutation (Côté et al. 1985).

\( \text{ry}^{506} \) is an M strain, homozygous for a 3.4-kb deletion that removes the 3' portion of XDH coding element (Côté et al. 1986).

\( C(1)DX, y; \text{ry}^{506} \) is a compound-X-bearing, M strain that is homozygous for \( \text{ry}^{506} \) (Daniels et al. 1985).

\([2216] = \text{sd} \text{ry}^{92216} \) is a \( \text{ry}^+ \) transposon in or near the scalloped (sd) locus in polytene region 13F. The transposon was formerly designated \( \text{sd}^{54} \) (Daniels et al. 1985). The transposon is maintained in a homozygous M strain of the constitution [2216]: \( \text{ry}^{506} \).

\([559] = \text{sd} \text{ry}^{92216-559} \) is a dysgenesis-induced derivative of [2216] maintained in homozygous strain [559]: \( \text{ry}^{506} \).

\( y \text{sn}; \text{bw}; \text{st} \) is an M strain, homozygous for the indicated recessive markers including the hypermutable \( \text{sn}^* \) allele. This strain was provided by William Engels.

\( \text{kar}^2 l(3)S12 \text{ry}^{111} / \text{MKRS} \) carries the third chromosome rosy region balancer MKRS = Tp(3)MKRS, M(3)S34 kar \( \text{ry}^0 \) sh and a \( \text{ry}^{111} \)-bearing chromosome. The \( \text{ry}^{111} \) allele is associated with normal levels of XDH (Chovnick et al. 1976).

\( Y^X Y^y / X; \text{kar}^2 l(3)S12 \text{ry}^{111} / \text{MKRS} \) carries the attached-XY chromosome \( Y^X Y^y = Y^X Y^y \).

\( \text{CyO}; TM2, \text{ry}^{2501} / T(2;3)\text{ap}^X \text{sa} \text{ry}^{2401} \) possesses the second chromosome balancer \( \text{CyO} = \text{IN}(2LR)O, \text{dp}^{191} \text{Cy pr cn} \), the third chromosome balancer TM2, \( \text{ry}^{2501} = \text{In}(3LR)\text{Ubx}^{136}, \text{ry}^{2501} \text{Ubx}^{130} \), and the chromosomes from T(2;3)ap\( ^X \), marked with \( \text{ry}^{2401} \). This strain was synthesized in our laboratory and has a rosy eye color phenotype. The \( \text{ry}^{2501} \) and \( \text{ry}^{2401} \) mutations were recovered from a gamma-ray mutagenesis.

\( \text{Canton-S} \) is a true M strain that is completely devoid of P-element sequences.

\( \text{Harwich} \) is a strong P strain containing >50 P-element copies per haploid genome (Bingham, Kidwell and Rubin 1982). This strain was provided by Margaret Kidwell.

Plasmids: \( \text{pry}8.1 \) consists of an 8.1-kb SalI \( \text{ry} \) locus fragment from Canton-S cloned into the SalI site of pBR322 (provided by Welcome Bender).

\( \text{pry}1 \) and \( \text{pry}3 \) contain the 8.1-kb \( \text{ry} \) locus fragment, derived from \( \text{pry}8.1 \), cloned into the Xhol site of the defective P element borne by the p6.1 plasmid (Rubin and Spradling 1982; Spradling and Rubin 1982). The \( \text{pry}1 \) and \( \text{pry}3 \) plasmids differ in the orientation of the \( \text{ry} \) DNA relative to the P element sequences.

\( \text{pr}25.1 \) contains an intact 2.9-kb P element with flanking DNA from polytene region 17C (Spradling and Rubin 1982; O'Har and Rubin 1983). A restriction map of this plasmid is shown in Figure 2. The \( \text{Xhol/SalI} \) and \( \text{SstI/SalI} \) fragments from the intact P element were used as probes in this study.

\( \text{pr}25.7 \) contains a functional P element that has a small segment deleted from one of its terminal repeats (Kares and Rubin 1984). This alteration renders the element incapable of transposition, but does not interfere with its ability to promote the transposition of other P elements with intact termini.

Southern blot analysis: The method for extraction of genomic DNA from adult flies is described in detail elsewhere (Daniels and Strausbaugh 1986). The procedures for restriction enzyme digestion, agarose gel electrophoresis, gel blotting, preparation of nick-translated probes and filter hybridization are described in Rushlow, Bender and Chovnick (1984).

In situ hybridization: Polytene chromosome spreads from larval salivary glands were prepared essentially by the methods described by Pardue and Gall (1975) as modified by Hyashi et al. (1978). \( ^3 \)H-labeled \( \text{pry}8.1 \) plasmid DNA was used as probe.
Polyacrylamide gel electrophoresis: Procedures are described by McCarron et al. (1979). The convention used to designate the electrophoretic mobility of XDH electrophoroms is given in the same reference.

Rocket immunoelectrophoresis: Methods are described by McCarron et al. (1979). Protein quantitation is directly related to area under the rocket curves (Laurell 1966; Weeke 1973).

Measurement of XDH activity: XDH activities were determined by the fluorometric assay described by McCarron et al. (1979).

Embryo injection: DNA was introduced into early syncytial embryos by the microinjection technique described by Spradling and Rubin (1982).

Malpighian tubule histochemistry: Late third-instar larvae were dissected in 0.2 M Tris, pH 8.5 (37°). Malpighian tubules were preincubated for approximately 4 min in 10 μl of buffer in a glass depression slide set in a dark 44° water bath. Following incubation, 200 μl of a stain solution [hypoxanthine (0.5 mg/ml), nitroblue tetrazolium (1.0 mg/ml), phenazine methosulfate (0.45 mg/ml) and nicotinamide adenine dinucleotide (1.0 mg/ml) in 0.2 M Tris, pH 8.5 (37°)] were added, and the organs were incubated for 10 min at 44°. The well was then filled with buffer and the organs were postincubated an additional 10 min at 44°. The staining fluid was carefully removed using a syringe, and 5% acetic acid was added to the organs before examination. It is important that tissues not be damaged during dissection. Also, culture conditions and the proper staging of larvae are critical for consistent results.

Genetic test for P factor activity: Approximately 20 males from the stock to be tested were mated en masse to a comparable number of y snw; bw; st, M strain females. Approximately 20-30 y snw F1 males were then crossed to C(I)DX, y f; ry506 females, and the resulting F2 males were screened for the presence of sn+ and sn- phenotypes.

RESULTS

Over the past 3 yr, we have analyzed 20 ry+ transformed lines that were generated in our laboratory by the P-element-mediated gene transfer technique described by Spradling and Rubin (1982). Germline transformants were obtained by injecting pry1 and/or pry3 plasmid DNA into homozygous ry506 embryos along with the pr25.1 plasmid, which contains a complete P element capable of providing transposase function. Of the 20 lines examined, six have continually exhibited signs of instability during propagation and upon outcrossing to laboratory strains. These unstable transformants will be considered in a separate report.

Stable ry+ transformants exhibit quantitative differences in XDH expression: The remaining 14 lines possess stably integrated ry+ transposons (13 autosomal, one sex-linked). These exhibit line-specific differences in XDH levels as measured by rocket immunoelectrophoretic determination of cross-reacting material (CRM) to an XDH antibody preparation. Each line is ranked in an ascending scale (1 to 4) relative to reference standards (Table 1). Eight of the 14 lines have a CRM profile approximating that of ry+, which is our reference standard of normal XDH expression. Of the remaining six lines, three produce greater and three produce lesser amounts of XDH than does ry+. In addition to the 20 transformed lines generated in this study, we have also examined several of the ry+ transformants described by Spradling and Rubin (1983), two of which are included in this report. The latter were generated with the same plasmid DNAs as were our transformants, but utilized ry42 homozygote embryos as recipients, in contrast to our use of ry506 homozygotes as recipients.
TABLE 1

CRM rankings for the 14 \( \text{ry}^+ \) stable transformants generated in our laboratory

<table>
<thead>
<tr>
<th>Rank no.</th>
<th>CRM range*</th>
<th>No. of transformants</th>
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<tbody>
<tr>
<td>1</td>
<td>( \text{ry}^{+10} ) or less</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>( \text{ry}^{+10} ) &lt; ( \text{ry}^{*5} )</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>( \text{ry}^{*5} )</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>( \text{ry}^{+5} ) &lt; ( \text{ry}^{*4} )</td>
<td>3</td>
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*See Figure 1 for CRM profiles of \( \text{ry}^{+10} \), \( \text{ry}^{*5} \) and \( \text{ry}^{*4} \).

FIGURE 1.—Rocket immunoelectropherograms illustrating the different XDH CRM levels of seven \( \text{ry}^+ \) transformants. Whole-fly extracts, containing equivalent amounts of protein, were run against anti-XDH serum. In A and B, wells 1–3 contain, in order, extracts from homozygous \( \text{ry}^{+10} \), \( \text{ry}^{*5} \) and \( \text{ry}^{*4} \) males. The \( \text{ry}^{+10} \) and \( \text{ry}^{*4} \) samples represent XDH control element variants associated with under- and over-production of XDH, respectively, whereas the \( \text{ry}^{*5} \) sample exhibits a normal level of XDH (Clark et al. 1984). A, Autosomal \( \text{ry}^+ \) transposons. Extracts from uniformly aged, homozygous males were applied to wells as follows: (4) \( \text{ry}^{+16} \); \( \text{ry}^{*306} \); (5) \( \text{ry}^{*62} \); [R401.1]; (6) \( \text{ry}^{*306} \) \( \text{ry}^{*72} \); (7) \( \text{ry}^{*241-8} \) \( \text{ry}^{*306} \) and (8) \( \text{ry}^{*201-4} \); \( \text{ry}^{*306} \). B, Sex-linked \( \text{ry}^+ \) insertions. Wells 4 and 5 contain extracts of hemizygous males from lines \( \text{ry}^{+27} \); \( \text{ry}^{*306} \) and [R403.1]; \( \text{ry}^{*62} \), respectively; wells 6 and 7 contain extracts from the corresponding homozygous females. The \( \text{ry}^{*306} \) and \( \text{ry}^{*62} \) mutant strains are CRM* (data not shown).

The range of \( \text{ry}^+ \) transformant line-specific variation in XDH expression is shown in Figure 1. The \([\text{ry}^{+14-16}]\) and \([\text{ry}^{+201-4}]\) strains exhibit, respectively, the lowest and highest XDH CRMs. Greater than half of the transformants display a CRM profile equivalent to the one produced by the \([\text{ry}^{*241-8}]\) and \([\text{R403.1}]\) strains, which have essentially normal amounts of XDH as defined by the \( \text{ry}^{*5} \) reference strain. Genetic and cytological localizations of the seven \( \text{ry}^+ \) transformants (Figure 1) are presented in Table 2.
Transposon expression and copy number: Differences in XDH expression of the transformant lines may relate to differences in transposon copy number (i.e., certain transformed lines may contain more than one functional ry+ transposon in some or all members of the population). There are two routes by which this may occur. The first is by the coincidental integration of two or more ry+ transposons into the same germline nucleus following embryo injection; the second is by the cotransformation of a nucleus with both a ry+ transposon and an intact P element from pr25.1. In the latter instance, the potential exists for subsequent transposition of the ry+ transposon. It has been our experience that cotransformed lines often display certain characteristic signs of instability (DANIELS et al. 1985). Although the ry+ transformants of Figure 1 manifest none of these features, it is nevertheless possible that some of these lines might contain P elements that are either sufficiently cryptic in their expression or present in such a small segment of the population that they have escaped detection by our test for P factor activity. Even low levels of transposase activity within a line might eventually result in the formation of subpopulations with varying numbers of ry+ transposons. We have eliminated this possibility, however, by the Southern blot analysis shown in Figure 2. None of
Control reconstruction experiment to determine the minimum number of P-element-bearing individuals that can be detected in populations of 125 flies by our Southern blotting procedure. DNA samples from populations containing defined proportions of P-element-bearing (Harwich = H) and P-element-free (Canton-S = CS) individuals were applied to lanes as follows: (1) 20 H and 105 CS, (2) 10 H and 115 CS, (3) 5 H and 120 CS, (4) 1 H and 124 CS and (5) 125 CS. As seen in lane 4, it is possible to detect a single P-element-bearing individual among 125 flies. When DNA containing complete P elements is digested with PvuII and probed with the XhoI-SalI fragment from p#25.1, each element produces an internally derived 0.9-kb fragment and a unique fragment of variable size that contains the right portion of the element and its flanking genomic DNA (see A). The variable fragment is characteristic of a particular element at a given location. C, Lanes 1 and 2 contain DNA from samples containing defined proportions of Harwich and Canton-S flies corresponding, respectively, to lanes 3 and 4 in B. Lanes 3–11 contain DNA samples, obtained from homozygous adults, as follows: (3) ryS6, (4) ry42, (5) ry46-1a; ryS66, (6) ryS66, (7) ry42, (8) ry42-1a; ryS66, (9) ry42-1a/ryS66, (10) ry42; [R401.1] and (11) [R403.1]; ry42. The single hybridization signal observed in each lane containing ry+ transformant DNA (lanes 5–11) corresponds to the rightmost PvuII fragment from the ry+ transposon, which shares sequences in common with the XhoI-SalI probe (see A and Figure 4A and B). The presence of a single signal confirms that there is only one ry+ transposon in the genome. Additionally, the absence of the 0.9-kb PvuII fragment (lanes 1 and 2), which appears in DNA containing the intact P element from p#25.1 (see A), confirms that these lines are devoid of functional P elements.
the transformants of Figure 1 show evidence of intact P elements within their populations. In addition, we have confirmed, both by Southern blot analysis (Figure 2C) and by in situ hybridization to polytene chromosomes (Table 2), that each of these lines contains only a single ry+ transposon. Therefore, the differences in XDH expression observed among this group are not due to differences in transposon copy number.

**XDH expression of the ry**+ **transposon is cis-acting:** Since the XDH holoenzyme is a homodimer, heterozygotes with fast and slow electromorphs produce three electrophoretically distinguishable enzyme forms: slow, intermediate and fast dimers. If monomers of each electrophoretic class are produced in equal numbers, then one would expect a 1:2:1 ratio of the respective dimer classes (i.e., 1SS:2SF:1FF). In contrast, if the electromorphs produce different numbers of monomers (i.e., as in cis-acting expression), then the pattern of XDH dimer classes should depart appropriately from the 1:2:1 ratio.

Figure 3A presents an XDH electropherogram demonstrating the electromorph diagnosis of a ry+ transposon (DNA from Canton-S) to be XDH^{1.02}. All transposons derived from pry1 and pry3 should express this electrophoretic character. Figure 3B presents an XDH electropherogram demonstrating the cis-acting expression associated with the ry+ transposon. Figure 3B, lane 5, presents the pattern of XDH dimers produced in flies carrying one dose of [ry^{+d+1a}] and a ry^{+15} allele. The very low productivity seen in CRM production of [ry^{+d+1a}] homozygotes (Table 2 and Figure 1) is paralleled in low production of the XDH^{1.02} monomers in contrast to the XDH^{0.90} product of ry^{+13}. Similarly, Figure 3B, lane 6, presents the pattern of XDH dimers produced in flies carrying one dose of [ry^{+201-4}] and a ry^{+15} allele. Here, we note that the high productivity seen in CRM production of [ry^{+201-4}] homozygotes (Table 2 and Figure 1) is seen, as well, in high production of the XDH^{1.02} monomers in contrast to the XDH^{0.90} product of ry^{+13}.

**Transposon expression and DNA sequence organization:** In a previous report (DANIELS et al. 1985), we have documented the effect of dysgenesis in the production of mutations, at high frequency, in an existing ry+ transposon. Most of the mutational events could be identified as deletions in the rosy locus DNA of the transposon. Indeed, several small deletions in noncoding regions were produced with tissue-specific, quantitative effects in XDH expression (M. McCARRON, C. LOVE and A. CHOVNICK, unpublished results). Consequently, we examined the rosy locus DNA sequence integrity of the transformants of Figure 1 by Southern blot analysis of genomic DNA (Figure 4). These experiments failed to find any indication of transposon perturbation in the sample of seven transposons examined. Although very small deletions might have been missed by this study, our past experience with this type of analysis (DANIELS et al. 1985) provides assurance that major reorganization of the transposon DNA cannot be the cause of the observed line differences in XDH expression.

**Transposon expression and vector effect:** Figure 4D presents a Southern blot of HindIII digested genomic DNA from homozygous transformants, the XDH levels of which are illustrated in Figure 1. Examination of the small restriction fragments of this blot permits determination of the orientation of
FIGURE 3.—Polyacrylamide gel electropherograms illustrating the cis-acting nature of XDH expression associated with ry+ transposons. A, Demonstration that a ry+ transposon derived from the 8.1-kb, Canton-S ry+ fragment (present in pry1 and pry3) encodes an XDH peptide with an electrophoretic mobility of 1.02. Lanes 2, 4, 6, 8 and 11 contain extracts from [ry++]. Control extracts were applied to wells as follows: (1) 0.90, (3) 0.94, (5) 0.97, (7) 1.00, (9) 1.02, (10) 1.03 and (12) 1.05. B, Electropherogram illustrating the relative amounts of slow, intermediate and fast dimers present in uniform, whole-fly extracts. Homozygous transformant (XDH1.02/XDH1.02) males were mated to ry+13/MKRS (XDH0.90/−) to produce F1 Sb* males that were used to provide the extracts for lanes 5 and 6. Control samples (lanes 1 through 4, 7 and 8) were obtained from males of the indicated homozygous strains or from heterozygous F1 males resulting from crosses of homozygous strains. Samples were applied to lanes as follows: (1) and (7) ry+13/ry−15 (0.90N/0.90N), (2) and (8) ry−11/ry+11 (1.02N/1.02N), (3) ry+13/ry+11 (0.90N/1.02N), (4) ry−13/ry−4 (0.90N/1.02H), (5) ry−-+13/−; ry508/ry+15 (1.02L/0.90N) and (6) ry508/ry+15 (1.02H/0.90N).

the rosy DNA in the transposon (pry1 or pry3), and enables us to examine the issue of vector specific effects on rosy expression (Figure 1). No vector specific effects are apparent in these data, nor have we noted such effect in other data.

Transposon expression and heterochromatic position effect: In Drosophila, the most extensively documented position effects are those involving the placement of euchromatic genes in or near heterochromatin (reviewed by Lewis 1950; Baker 1968; Spofford 1976). Recently, two such heterochromatic position effect variants have been described at the rosy locus by Rushlow and Chovnick (1984). An examination of the expression of rosy locus DNA transduced into heterochromatin constitutes, then, a reasonable starting point in the analysis of potential position effects on ry+ transposons. At present, there is only a single documented case of a ry+ transposon in or near heterochromatin. This transformant, [R401.1], was generated by Spradling and Rubin
FIGURE 4.—Restriction enzyme and gel blot analysis of whole-fly DNA from the seven ry⁺ transformants depicted in Figure 1. A and B, Restriction maps of the ry⁺ transposons borne by the pry1 (A) and pry3 (B) plasmids. These plasmids were constructed by inserting the 8.1-kb SalI ry locus fragment, obtained from pry8.1, into the XhoI site of the defective P element in plasmid p6.1 (Rubin and Spradling 1982; O'Hare and Rubin 1983; see also Figure 2). The resulting ry⁺ transposons are composed of two short P element segments (hatched boxes), 0.7 kb (left segment)
The CRM profile of [R401.1] relative to the other transformants in our study is shown in Figure 1A. One of the classic features of heterochromatic position-affected gene expression is that such expression is modified by the addition and subtraction of Y chromosome heterochromatin. Flies containing abnormal Y chromosome content (i.e., XO and XXY individuals) express, respectively, lower and higher amounts of gene product than do their XY and XX counterparts. By making the appropriate crosses, we have found that the XDH CRM levels of the R401.1 transposon are indeed altered by the addition and subtraction of Y heterochromatin (Figure 5A). Therefore, the interaction of this transposon with its genomic environment does constitute a true instance of heterochromatic position-affected transposon expression.

We next questioned whether the expression of other ry+ transposons might be affected by interaction with heterochromatin. Although all of the other ry+ transformants are located on the euchromatic chromosome arms, it is possible that some of these may reside in regions of intercalary heterochromatin and may exhibit a position-affected underexpression. To examine this possibility, we subjected two transformants with the most depressed XDH CRM levels, [ry+*1-1a] and [ry+*2-1] (Figure 1A) to the same analysis as described above for [R401.1]. In both cases (data shown only for [ry+*1-1a]) the addition and subtraction of Y chromosome heterochromatin has no effect on rosy locus expression.

and 0.4 kb (right segment) in length, that flank the 8.1-kb ry region DNA (thin bar). The pry1 and pry3 plasmids differ only in the orientation of the ry fragment relative to the P element sequences. The recognition sites for the three restriction enzymes used in this analysis are designated as: B = BglII, H = HindIII and P = PvuII. The open bars below each map represent the fragments observed when DNA containing the ry+ transposon is digested with HindIII and probed with pry8.1 DNA. The number above each bar indicates its length in kilobases. C, Restriction map of the ry region of chromosome 3, indicating the restriction enzyme recognition sites (see above) for three different alleles, ry+5, ry+6 and ry+4. The 8.1-kb ry fragment is depicted by the thin bar; thick bars represent adjacent sequences. The ry+6 deletion derives from a ry+5 allele, and the approximate location of the deleted segment is indicated below the map. A comparison of the 8.1-kb ry region DNA in ry+5, ry+6 and the ry+ transposons reveals two restriction site heterogeneities (indicated below the map): ry+5 lacks one of the BglII sites (boxed) that is present in ry+6 and the ry+ transposon; ry+5 and ry+4 contain a PvuII site that is not found in the ry+ transposon. The thin bars below the map represent the fragments observed when ry+5 and ry+6 DNAs are digested with HindIII and probed with pry8.1; numbers indicate lengths in kilobases. D, Southern blot of genomic DNA from homozygous adult flies digested with HindIII and the blot was probed with pry8.1 DNA. Samples were applied to lanes as follows: (1) ry+506, (2) [ry+*14-1a]; ry+506, (3) ry+506 [ry+*72-1], (4) [ry+*77-1]; ry+506, (5) [ry+*201-1]; ry+506, (6) [ry+*241-8]/ry+506, (7) ry+42, (8) [R403.1]; ry+42 and (9) ry+42; [R401.1]. In cases in which the ry+ transposon is sex-linked, DNA was obtained only from females (lanes 4 and 8); in all other instances, DNA was prepared from adults of both sexes. Transformants with pry1 transposons (see A) exhibit the hybridization pattern observed in lanes 2, 3, 4, 5 and 8; those with transposons of the pry3 orientation (see B) exhibit the pattern observed in lanes 6 and 9. Fragment sizes in kilobases are given on the left. No perturbations were noted in the HindIII fragments derived from the ry+ transposon in any of the seven ry+ transformants. We note that ry+ transformants in a ry+ background produce two indistinguishable 7.2-kb HindIII fragments. In such cases, any alteration in the fragment from the transposon would result in the appearance of an aberrant band. We have also examined the above DNA samples with two other restriction enzymes, BglII and PvuII. In all instances the fragments expected from an intact transposon (see A and B) were observed.
Figure 5.—The effects of heterochromatin content on the XDH levels of two transformed lines, R401.1 (A) and ry+4-1a (B). A and B, Rocket immunoelectropherograms. Extracts were obtained from uniformly aged adults and contain equivalent amounts of protein. Protein synthesis in ovaries contributes substantially to the total protein in extracts from females. Since XDH is not normally produced in ovaries, protein extracts from males will exhibit greater XDH levels than equivalent preparations from females. In this experiment, valid comparisons are limited to preparations from individuals of the same sex. Wells 1 and 2 contain control samples from ry+10/ry06 and ry+2/ry06 XY flies, respectively, and wells 5 and 6 contain samples from the corresponding XX individuals. (Refer to Figure 1 for a description of the ry+10 and ry+2 alleles.) Experimental samples from ry42/MKRS; [R401.1]/ or [ry+4-1a]/; ry06/MKRS flies were applied to wells as follows: (3) XO, (4) XY, (7) XXY and (8) XX. The XO and XXY flies were obtained by mating Y*X.YL/O MKRS males to homozygous ry+ females; XY and XX flies were obtained in the same fashion, except Y*; ry+11/MKRS flies were used in the P1 cross. A, Analysis of R401.1. Comparison of XO with XY males and XXY and XX females reveals that XDH CRM levels are dramatically influenced by the addition and subtraction of Y chromosome heterochromatin. B, Analysis of ry+4-1a. Alterations in Y chromosome heterochromatin content do not affect XDH levels.

Transposon expression and euchromatic position effect: We considered next the possibility that the XDH underexpression associated with [ry+4-1a] is due to a position effect not subject to Y chromosome modification. Such a position effect might relate to interaction with a neighboring euchromatic sequence; relocation to other genomic sites would create a new array of neighboring sequences for the ry+ transposon. Since [ry+4-1a] exhibits the lowest level of XDH expression of all transformants in the present study (Tables 1 and 2; Figure 1), relocation would, on this hypothesis, lead to the expectation of increased rosy locus expression in most new sites. Consequently, an experiment was carried out utilizing the protocol described in Figure 6 to promote and recover transpositions of [ry+4-1a]. From this experiment, one stable transposition was established. [ry+4-1a] resides at 57F on chromosome 2R (Figure 7A), and its transposition derivative, [ry+4-1a4], is located at 68A on chromosome 3L (Figure 7B). An examination of the XDH CRM of [ry+4-1a] reveals an almost twofold increase over that of the parental [ry+4-1a] line (Figure
FIGURE 6. — Diagram of the scheme employed to obtain stable transpositions of the \( \text{ry}^{+14-1A} \) transposon. Heterozygous embryos possessing a single \( \text{ry}^{+14-1A} \) chromosome were injected with \( \text{p} \times 25.7 \text{wc} \) plasmid DNA at a concentration of 250 \( \mu \text{g/ml} \). Injection survivors were outcrossed to a \( \text{ry}^{+} \) strain, and the appropriate progeny were screened for \( \text{ry}^{+} \) exceptional flies.

7C). We have also verified that the DNA structure of \( \text{ry}^{+14-1A} \) was not altered in the transposition process (Figure 7D), and we have confirmed that the new line carrying \( \text{ry}^{+14-1A} \) is stable and free of functional \( P \) elements (Figure 7E). These results demonstrate that the underexpression of \( \text{ry}^{+14-1A} \) is a position effect related to its specific position in polytene section 57F and that it is reversed by transposition to a new genomic site in polytene section 68A.

**Variegated and nonvariegated position effects:** In the case of \( \text{rosy} \) locus expression, eye color and total XDH activity of extracts of individuals are nonautonomous phenotypes. However, Rushlow, Bender and Chovnick (1984) were able to take advantage of the pleiotropic nature of \( \text{rosy} \) locus expression to examine enzyme distribution in the cells of the malpighian tubules where \( \text{rosy} \) locus expression is autonomous. They demonstrated the variegated nature of \( \text{rosy} \) locus expression in rearrangements that placed the \( \text{rosy} \) locus adjacent to heterochromatin. We examined XDH distribution in histochemical preparations of malpighian tubules from \( [R401.1] \) and \( [\text{ry}^{+14-1A}] \), as well as \( [\text{ry}^{+2216}] \), which is a transposon carrying the same wild-type \( \text{ry}^{+} \) allele. Only the tubules of \( [R401.1] \) individuals exhibit a variegated phenotype (Figure 8). Thus, only the \( Y \)-suppressed, heterochromatic position-affected expression appears to be variegated. In this context, we have also examined the
FIGURE 7.—Analysis of \([ry^{+1-a-4}]\). The \(ry^{+}\) transposon in this line is a derivative of \([ry^{+1-a}]\) and was obtained by the transposition scheme outlined in Figure 6. A and B, In situ hybridization of \(^{3}H\)-labeled \(p_r8.1\) DNA to salivary gland polytene chromosomes from homozygous larvae of \([ry^{+1-a}]\); \(ry^{506}\) (A) and \([ry^{+1-a-4}]\) \(ry^{506}\) (B). In both cases, two hybridization signals are evident, one at 87D, the location of the \(ro\) \(y\) locus on chromosome 3 (not shown), and the other at the site of insertion of the \(ry^{+}\) transposon. The \(ry^{+1-a}\) transposon is located at 57F (arrow in A) on chromosome arm 2R, and its transposition derivative, the \(ry^{+1-a-4}\) transposon, is inserted at 68A (arrow in B) on chromosome arm 3L. C, Rocket immunoelectropherogram. Extracts were obtained from homozygous, adult males and contain equivalent amounts of protein. Well 1 contains an undiluted control sample from \(ry^{+}\); wells 2 and 3 contain, respectively, dilutions with 50% and 25% of the extract in well 1. Wells 4 and 5 contain samples from \([ry^{+1-a}]\); \(ry^{506}\) and \([ry^{+1-a-4}]\) \(ry^{506}\), respectively. A nearly twofold increase in XDH CRM is observed in the transposition derivative. D, Verification that the \(ry^{+1-a-4}\) transposon DNA was unaltered by the transposition process. The autoradiogram shows two sets of three lanes. DNA samples are as follows: (1) \(ry^{506}\), (2) \([ry^{+1-a}]\); \(ry^{506}\) and (3) \([ry^{+1-a-4}]\) \(ry^{506}\). The left set of samples were digested with HindIII; the samples in the set on the right were digested with PvuII. The \(p_r8.1\) plasmid was used as probe. Arrows indicate the PvuII fragment that extends from the transposon's rightmost site into the flanking genomic DNA (see Figure 4A). This fragment is unique for each insertion. E, Confirmation that the \(ry^{+1-a-4}\) line is free of intact \(P\) elements. See Figure 2 for a description of this analysis. DNA samples are as follows: (1) 10 H and 115 CS, (2) 5 H and 120 CS, (3) \([ry^{+1-a}]\); \(ry^{506}\) and (4) \([ry^{+1-a-4}]\) \(ry^{506}\).
FIGURE 8.—Late third-instar larval malpighian tubules stained for XDH activity (see MATERIALS AND METHODS) of homozygotes $\text{ry}^{**}$ (A), $\text{ry}^{**/\text{lyz}}$; $\text{ry}^{\text{lyz}}$ (B), $\text{ry}^{\text{lyz}}$; $[\text{R401.1}]$ (C) and $\text{ry}^{\text{lyz}}$ (D). The variegated XDH expression of $[\text{R401.1}]$ (C) is compared to the uniform, but low, expression of $[\text{ry}^{**/\text{lyz}}]$ (B). The dark background due to overexposure of $\text{ry}^{\text{lyz}}$ (D) was necessary in order to visualize the unstained malpighian tubules associated with total absence of XDH activity.

Malpighian tubules of individuals with "low XDH" phenotypes associated with other variants of the ROSY locus, including the underproducer control element variant, $\text{ry}^{\text{lyz}}$, as well as various induced "leaky" site mutations. All exhibit low, but nonvariegated, XDH activity in malpighian tubules.

A dysgenesis-induced mutation that alters the structure of the XDH peptide encoded by the $\text{ry}^+$ transposon: Figure 3A demonstrates that the XDH encoded by the DNA of a normal ROSY locus transposon derived from either
Figure 9.—Comparative analysis of [2216] and [559]. A, Fluorometric assay of XDH activity in uniform extracts of adult males, aged 48 hr. Open circles represent sd [2216]; ry506/ry506. Closed circles represent sd [559]; ry506/ry506. B, XDH electropherogram of extracts of males, aged 48 hr, indicating the slower mobility associated with XDH from [559] (lane 2) compared to [2216] (lanes 1, 3 and 4). Both genotypes are homozygous for ry506. C, Gel blot of DNA prepared from adult males, digested with PvuII and probed with pry8.1. Samples are as follows: (1) ry506/ry506; (2) sd [2216]; ry506/ry506 and (3) sd [559]; ry506/ry506.

pry1 or pry3 should have a mobility of 1.02 on a scale developed in our earlier work (McCarron et al. 1979). All of the transposons described in Figure 1, including [ry+4-1a] (and its transposition derivative, [ry+4-1a4]), are characterized by this electromorph diagnosis. However, we have generated a rosy locus transposon that carries a dysgenesis-induced, apparent mutation associated with an altered XDH mobility.

In an earlier report (Daniels et al. 1985), experiments were described that characterized the nature of dysgenesis-induced perturbations of a ry+ transposon, [2216], located in polytene section 13F, in or near the scalloped (sd) locus (see Materials and Methods). One experiment involved the dysgenic perturbation of [2216] and the selective recovery of “leaky,” apparent XDH underproducer mutations by growth on standard Drosophila medium supplemented with an appropriate concentration of the irreversible XDH inhibitor, 4-hydroxypyrazolo-(3,4-d)pyrimidine (HPP or allopurinol). A number of such mutations of [2216] were recovered, and a detailed analysis will be reported elsewhere. However, one mutation, [ry+2216-559], bears on the present study, and consequently, is presented herein. This transposon, [559], remains located at polytene section 13F and, like [2216], exhibits an extreme sd wing phenotype (Daniels et al. 1985). These transposons are carried in strains homozygous for ry506 (Materials and Methods). The mutant [559] exhibits reduced levels of XDH compared to its parent, [2216], (Figure 9A) and is associated
with an XDH electrophoretic mobility change (Figure 9B), indicating a coding region mutation. Gel blot analysis, carried out on PvuII-, HindIII-, BglII- and SstI-digested DNA preparations from [559] and [2216] strains, revealed no restriction fragment differences. Figure 9C presents a gel blot illustrating the PvuII restriction analysis.

Since [2216], and other "leaky" mutant derivatives of it, retain the XDH\textsuperscript{1.02} mobility, one interpretation of the altered XDH mobility is that of a single base change mutation in the XDH coding region of the transposon. To preclude the unlikely possibility that the mutation involved some other sex-linked locus (e.g., maroon-like or cinnamon), mobility was examined in heterozygous females as well as in hemizygous males. It was identical for both (data not shown); therefore, the mobility change reflects a mutation in [559]. An alternative explanation would involve a small deletion, below the limits of our gel blot analysis, that retains translation and produces a functional peptide molecule. The latter interpretation would be consistent with our earlier observation that deletions of varying size are the most common result of P-M dysgenic perturbation of P-element transposons (Daniels et al. 1985).

DISCUSSION

One general class of experiments utilizing transformants in Drosophila involves in vitro mutagenesis and/or the production of hybrid gene constructs, followed by in vivo transformation to numerous possible chromosomal sites as P-element transposons. In such experiments, one hopes to relate the in vitro introduced lesion or hybrid construct to a specific or novel gene expression. Two qualifying factors in such experimental strategy are elaborated in this report. These relate to uncontrolled features of the experimental system that may give rise to true-breeding, stable variants or mutations even in the absence of preliminary in vitro gene perturbation.

The first relates to the fact that the transforming DNA inserts at numerous sites in the genome, and it provides an opportunity to observe position effects on gene expression. In the present report, we have demonstrated that some of the site- or strain-specific variation in rosy gene expression must result from influences of neighboring genomic sequences. In particular, we are able to identify classical, heterochromatic position effect variegation of rosy gene expression in a transposon, [R401.1], located in fourth chromosome heterochromatin. This position-affected gene expression is subject to modification by the presence or absence of Y chromosomes. Additionally, we describe a second category of position effect involving a rosy locus transformant, [\textsuperscript{ry+\textsuperscript{4}-1\textsuperscript{a}}], which is located in polytene segment 57F, a euchromatic segment of chromosome 2R. In this instance, stable underexpression of the rosy locus is not subject to Y chromosome modification. In the case of [\textsuperscript{ry+\textsuperscript{4}-1\textsuperscript{a}}], we demonstrate that the observed "underexpression" is relieved by transposition to another euchromatic genomic environment.

The second qualifying point relates to the fact that the transforming DNA inserts into the genome as an integral part of a P-element transposon mobilization event. In an earlier report (Daniels et al. 1985), we demonstrated that
dysgenesis-induced mobilization of a chromosomally integrated transposon is associated with a very high frequency of mutations (261 of 3071 transposon carrying chromosomes scored). On analysis, the exceptions were found to be largely associated with identifiable transposon DNA sequence rearrangements. Since both processes are similar events and share common requirements (responding transposons possess intact P-element "wings"; a source of "transposase" is present; the events take place in an M cytotype), we have assumed them to be comparable events in terms of the dysgenesis potential for mutagenesis. Moreover, the latter experiment (DANIELS et al. 1985) provides opportunity for quantitative assessment of this mutagenic potential on a scale not feasible in a transformation experiment. Most of the dysgenesis-induced transposon mutations involve large DNA sequence rearrangements resulting in total inactivation of the rosy locus. These are not a real problem for the transformation work, but the question of small lesions with subtle phenotypic effects is most pertinent. A second experiment, designed to examine this point was carried out utilizing allopurinol (HPP) to select for "leaky" mutations from the dysgenesis. In this latter study, 58,550 transposon carrying chromosomes were screened to yield a total of 836 sd ry- mutations on allopurinol. These included six "leaky" mutations that exhibit a ry+ eye color on standard medium, and are associated with XDH "underexpression." One of these, [559], is associated with slightly less than wild-type activity, exhibits an electrophoretic mobility change that is clearly associated with the rosy transposon and may be due to a single base change mutation in the XDH coding sequence. Still other "underexpression" variants recovered from the same experiment are associated with tissue-specific changes in rosy locus expression; these are associated with rearrangements in noncoding DNA of the rosy locus (M. MCCARRON, C. LOVE and A. CHOVNICK, unpublished results). These observations will be reported elsewhere. Fortunately, for the general class of experiments that involve in vitro perturbation followed by transformation, this class of mutations occurs with a low frequency, but one that is not to be totally ignored.

Considering both position effects and dysgenesis-induced mutations as qualifying factors in transformation studies involving in vitro perturbation, we note that both are readily obviated. Both factors involve unique events associated with individual transformants. Consequently, the production and analysis of several transformants for each perturbation should permit distinction of the underlying basis for the observed phenotypic response. Since position effects are quite common, and small DNA rearrangements do occur, interesting phenotypic effects found among transformants from in vitro DNA perturbation experiments may reflect multiple effect interactions. However, these too should be readily identified.

The rosy locus transformation system provides opportunity for elaborate observation and investigation of position-effect questions. The present report describes two very different position effects on rosy locus expression. Classical heterochromatic position effect is seen with [R401.I] that is (1) Y modified and (2) exhibits variegated expression in malpighian tubules with sectors of high and null activity, as is seen in traditional heterochromatic rearrangement
position effects on rosy locus expression (RUSHLOW, Bender and CHOVNICK 1984; RUSHLOW and CHOVNICK 1984). In contrast, the position-effect underexpression of \([ry^{+d-1a}}\) and \([ry^{+72-1a}}\) (the euchromatic transformants with the most depressed CRM levels) is not responsive to \(Y\) chromosome modification. Moreover, the malpighian tubules of \([ry^{+d-1a}}\) exhibit nonvariegated but low XDH activity levels. These observations imply that rosy DNA expression is influenced in a very different manner by heterochromatic and euchromatic sequences.

That these represent two very different kinds of position effects is supported by several other observations (S. CLARK and A. CHOVNICK, unpublished results). The gene, \(l(3)S12\), is located immediately proximal to the rosy locus and is included in the 8.1-kb \(SalI\) fragment of pry1 and pry3. Moreover, "leaky underexpression" of the \(l(3)S12\) gene is associated with a distinctive visible mutant phenotype, in contrast to the fully wild-type phenotype associated with one dose of \(l(3)S12^+\). Due to the proximity of these genes, coordinated observations have been made with respect to the responses of \(l(3)S12^+\) and \(ry^+\) to the same position effects in both transposon insertions, as well as in classical chromosome rearrangements. Thus, where rosy responds readily to both foreign heterochromatic and euchromatic DNA sequences, the \(l(3)S12\) locus, in contrast, is sensitive to euchromatization and totally insensitive to heterochromatin. Moreover, the euchromatic position effects on \(l(3)S12\) gene expression are also unresponsive to \(Y\) chromosome modification. These observations will be reported in detail elsewhere.

This investigation was supported by research grants GM-31853 and GM-09886 from the United States Public Health Service.

LITERATURE CITED


Communicating editor: A. SPRADLING