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

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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; GEHRING *et al.* 1984;

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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^{swb} (WELSHONS and KEPPY 1975; KEPPY and WELSHONS 1977; WELSHONS and WELSHONS 1985) and w^{DZL} (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: ry^{42} is an *M* strain, homozygous for an apparent point mutation (COTÉ *et al.* 1985).

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

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

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

[*559*] = *sd* [$ry^{ps2216\text{-}559}$] is a dysgenesis-induced derivative of [*2216*] maintained in homozygous strain [*559*]; ry^{506}.

y sn^w; *bw*; *st* is an *M* strain, homozygous for the indicated recessive markers including the hypermutable sn^w allele. This strain was provided by WILLIAM ENGELS.

kar^2 *l(3)S12* ry^{+11}/*MKRS* carries the third chromosome *rosy* region balancer *MKRS* = *Tp(3)MKRS, M(3)S34 kar* ry^2 *Sb* and a ry^{+11}-bearing chromosome. The ry^{+11} allele is associated with normal levels of XDH (CHOVNICK *et al.* 1976).

Y^SXY^L/X; kar^2 *l(3)S12* ry^{+11}/*MKRS* carries the attached-*XY* chromosome $Y^SX \cdot Y^L = Y^SX \cdot Y^L$, *In (EN), y v f B* $\sim y^+$.

CyO; TM2, $ry^{2301}/T(2;3)ap^{Xa}$ ry^{2401} possesses the second chromosome balancer *CyO* = $IN(2LR)O$, dp^{1V1} *Cy pr* cn^2, the third chromosome balancer *TM2,* ry^{2301} = $In(3LR)Ubx^{130}$, ry^{2301} Ubx^{130} e^s, and the chromosomes from $T(2;3)ap^{Xa}$, marked with ry^{2401}. This strain was synthesized in our laboratory and has a rosy eye color phenotype. The ry^{2301} and ry^{2401} mutations were recovered from a gamma-ray mutagenesis.

Canton-S is a true *M* strain that is completely devoid of *P*-element sequences.

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: pry8.1 consists of an 8.1-kb *Sal*I *ry* locus fragment from Canton-S cloned into the *Sal*I site of pBR322 (provided by WELCOME BENDER).

pry1 and pry3 contain the 8.1-kb *ry* locus fragment, derived from pry8.1, cloned into the *Xho*I site of the defective *P* element borne by the p6.1 plasmid (RUBIN and SPRADLING 1982; SPRADLING and RUBIN 1982). The pry1 and pry3 plasmids differ in the orientation of the *ry* DNA relative to the *P* element sequences.

pπ25.1 contains an intact 2.9-kb *P* element with flanking DNA from polytene region 17C (SPRADLING and RUBIN 1982; O'HARE and RUBIN 1983). A restriction map of this plasmid is shown in Figure 2. The *Xho*I/*Sal*I and *Sst*I/*Sal*I fragments from the intact *P* element were used as probes in this study.

pπ25.7wc contains a functional *P* element that has a small segment deleted from one of its terminal repeats (KARESS 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 pry8.1 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 electromorphs 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 sn*^{w}; *bw*; *st*, *M* strain females. Approximately 20–30 *y sn*^{w} F_1 males were then crossed to *C(1)DX*, *y f*; *ry*^{506} females, and the resulting F_2 males were screened for the presence of sn^{e} 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 *ry*^{506} embryos along with the pπ25.1 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*$^{+5}$, 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*$^{+5}$. 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 *ry*^{42} homozygote embryos as recipients, in contrast to our use of *ry*^{506} homozygotes as recipients.

TABLE 1

CRM rankings for the 14 ry^+ stable transformants generated in our laboratory

Rank no.	CRM range[a]	No. of transformants
1	ry^{+10} or less	1
2	$>ry^{+10}<ry^{+5}$	2
3	$\simeq ry^{+5}$	8
4	$>ry^{+5}<ry^{+4}$	3

[a] See Figure 1 for CRM profiles of ry^{+10}, ry^{+5} and ry^{+4}.

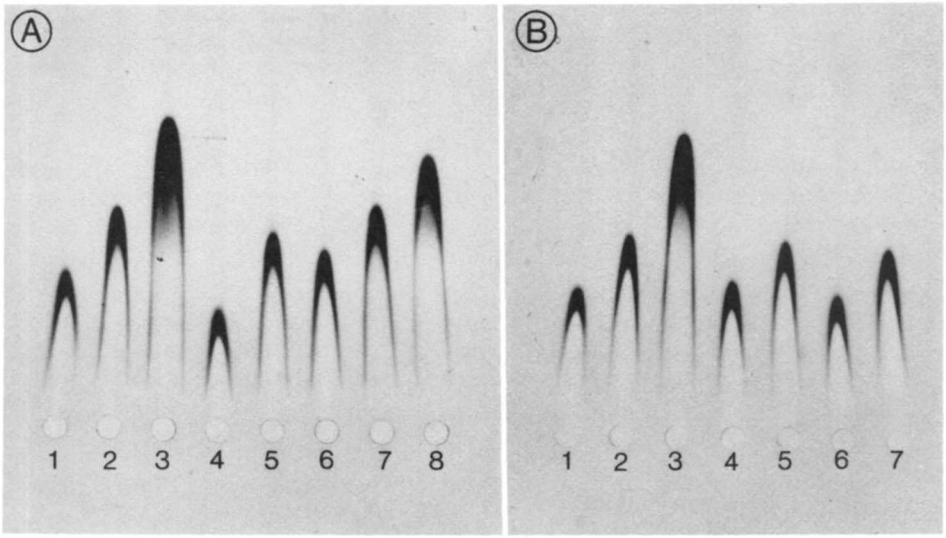


FIGURE 1.—Rocket immunoelectropherograms illustrating the different XDH CRM levels of seven ry^+ transformants. Whole-fly extracts, containing equivalent amounts of protein, were run against anti-XDH serum. In A and B, wells 1–3 contain, in order, extracts from homozygous ry^{+10}, ry^{+5} and ry^{+4} males. The ry^{+10} and ry^{+4} samples represent XDH control element variants associated with under- and over-production of XDH, respectively, whereas the ry^{+5} sample exhibits a normal level of XDH (CLARK *et al.* 1984). A, Autosomal ry^+ transposons. Extracts from uniformly aged, homozygous males were applied to wells as follows: (4) [$ry^{+i4\text{-}1a}$]; ry^{506}, (5) ry^{42}; [*R401.1*], (6) ry^{506} [$ry^{+72\text{-}1}$], (7) [$ry^{+241\text{-}8}$] ry^{506} and (8) [$ry^{+201\text{-}4}$]; ry^{506}. B, Sex-linked ry^+ insertions. Wells 4 and 5 contain extracts of hemizygous males from lines [$ry^{+77\text{-}1}$]; ry^{506} and [*R403.1*]; ry^{42}, respectively; wells 6 and 7 contain extracts from the corresponding homozygous females. The ry^{506} and ry^{42} mutant strains are CRM^- (data not shown).

The range of ry^+ transformant line-specific variation in XDH expression is shown in Figure 1. The [ry^{+i4-1a}] and [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 [$ry^{+241\text{-}8}$] and [*R403.1*] strains, which have essentially normal amounts of XDH as defined by the ry^{+5} reference strain. Genetic and cytological localizations of the seven ry^+ transformants (Figure 1) are presented in Table 2.

TABLE 2

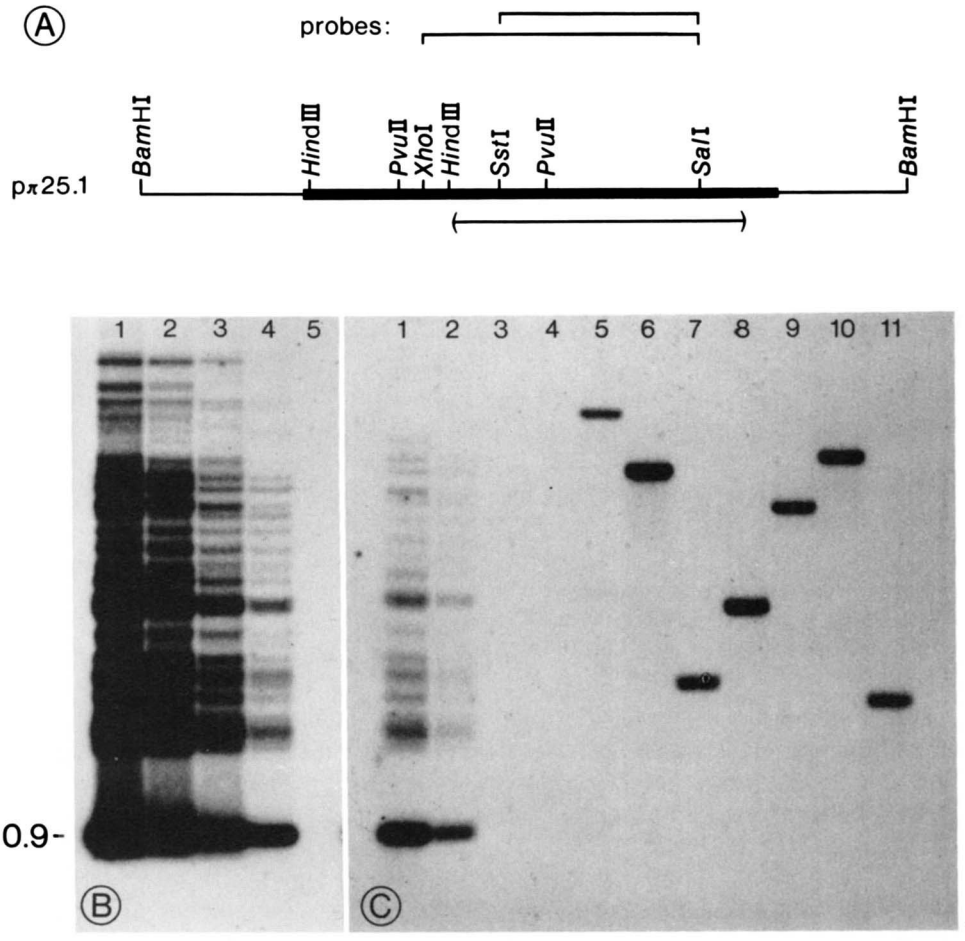
Genetic and cytological features of the seven ry^+ transformants shown in Figure 1

Strain	Linkage group	Cytological location	CRM rank[a]	Genotypic designation
ry^{+i4-1a}	2	57F	1	; [ry^{+i4-1a}]; ry^{506};
ry^{+72-1}	3	100D	2	; ; ry^{506} [ry^{+72-1}];
ry^{+i77-1}	1	16D	2	[ry^{+i77-1}]; ; ry^{506};
ry^{+201-4}	2	43EF	4	; [ry^{+201-4}]; ry^{506};
ry^{+241-8}	3	76F	3	; ; [ry^{+241-8}] ry^{506};
R401.1[b]	4	chromocenter	2	; ; ry^{42}; [*R401.1*]
R403.1[b]	1	7D	3	[*R403.1*]; ; ry^{42};

[a] See Table 1.
[b] SPRADLING and RUBIN (1983).

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 pπ25.1. 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

FIGURE 2.—The seven ry^+ transformed lines that exhibit differences in expression are devoid of intact *P* elements, and each contains only a single ry^+ transposon. A, Restriction map of the pπ25.1 plasmid. This plasmid contains a complete (functional) *P* element (thick bar) flanked by sequences from polytene region 17C (thin bars). The genomic fragment, obtained from a *D. melanogaster P* strain (π_2), was cloned into the *Bam*HI site of pBR322 (not depicted). For further details see SPRADLING and RUBIN (1982) and O'HARE and RUBIN (1983). The defective *P* element in the p6.1 plasmid, which was used as the vector in the construction of pry1 and pry3 (see Figures 4A and B), was derived from a complete *P* element by a single internal deletion, the location of which is indicated below the map. The injection mixture used in our transformation experiments contained pπ25.1 and pry1 and/or pry3 DNA. Transposition of the ry^+ transposon from the injected plasmid into the genome is catalyzed by a transposase molecule supplied by the functional *P* element borne by pπ25.1. When this *P* element integrates into the genome coincidentally with a ry^+ transposon, such cotransformants exhibit unstable ry^+ phenotypes (DANIELS *et al.* 1985), just as was shown for *white* locus transposons (HAZELRIGG, LEVIS and RUBIN 1984), and often manifest a protracted period of dysgenic potential. The *Xho*I-*Sal*I and *Sst*I-*Sal*I fragments, used as probes during the course of this study, are indicated above the map. B and C, Gel blots of DNA prepared by *en masse* extraction of 125 flies. DNA samples were digested with *Pvu*II and probed with the



*Xho*I-*Sal*I fragment from pπ25.1. B, 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 *Pvu*II and probed with the *Xho*I-*Sal*I fragment from pπ25.1, 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) ry^{506}, (4) ry^{42}, (5) [$ry^{+i4\text{-}1a}$]; ry^{506}, (6) ry^{506} [$ry^{+72\text{-}1}$], (7) [$ry^{+i77\text{-}1}$]; ry^{506}, (8) [$ry^{+201\text{-}4}$]; ry^{506}, (9) [$ry^{+241\text{-}8}$]/ry^{506}, (10) ry^{42}; [*R401.1*] and (11) [*R403.1*]; ry^{42}. The single hybridization signal observed in each lane containing ry^{+} transformant DNA (lanes 5–11) corresponds to the rightmost *Pvu*II fragment from the ry^{+} transposon, which shares sequences in common with the *Xho*I-*Sal*I 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 *Pvu*II fragment (lanes 1 and 2), which appears in DNA containing the intact *P* element from pπ25.1 (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 $[ry^{+i4-1a}]$ and a ry^{+13} allele. The very low productivity seen in CRM production of $[ry^{+i4-1a}]$ 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^{+13} 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 *Hin*dIII 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

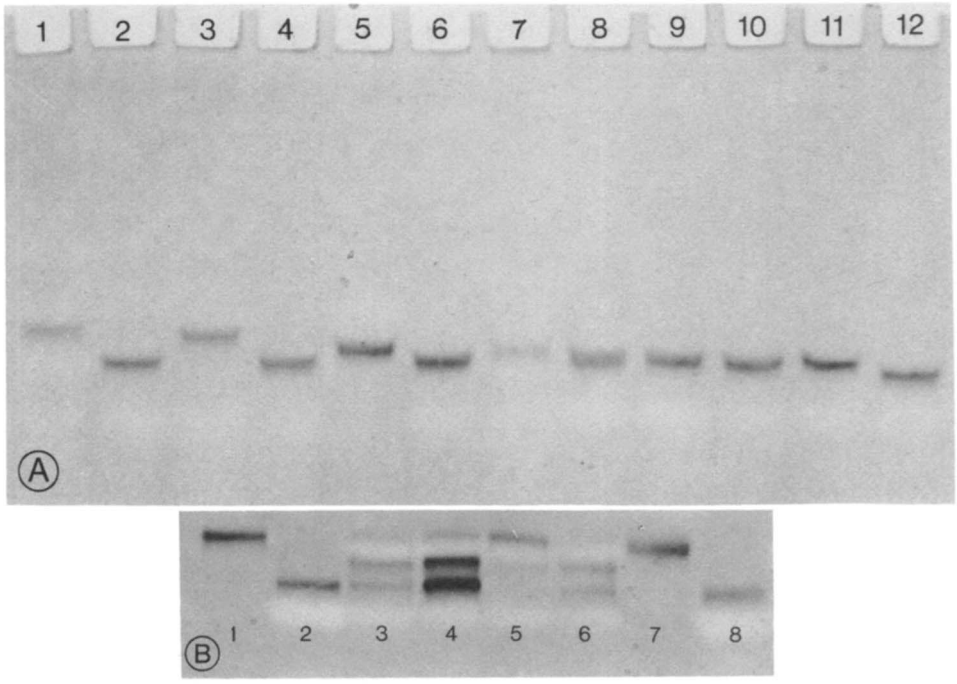


FIGURE 3.—Polyacrylamide gel electropherograms illustrating the *cis*-acting nature of XDH expression associated with ry^+ transposons. A, Demonstration that a ry^+ transposon derived from the 8.1-kb, Canton-S ry^+ fragment (present in pry1 and pry3) encodes an XDH peptide with an electrophoretic mobility of 1.02. Lanes 2, 4, 6, 8 and 11 contain extracts from [$ry^{+4\text{-}1}$]. 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 ($XDH^{1.02}/XDH^{1.02}$) males were mated to ry^{+13}/*MKRS* ($XDH^{0.90}/-$) to produce F_1 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 F_1 males resulting from crosses of homozygous strains. Samples were applied to lanes as follows: (1) and (7) ry^{+13}/ry^{+13} (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) [$ry^{+i4\text{-}1a}$]/−; ry^{506}/ry^{+13} (1.02L/0.90N) and (6) [$ry^{+201\text{-}4}$]/−; ry^{506}/ry^{+13} (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

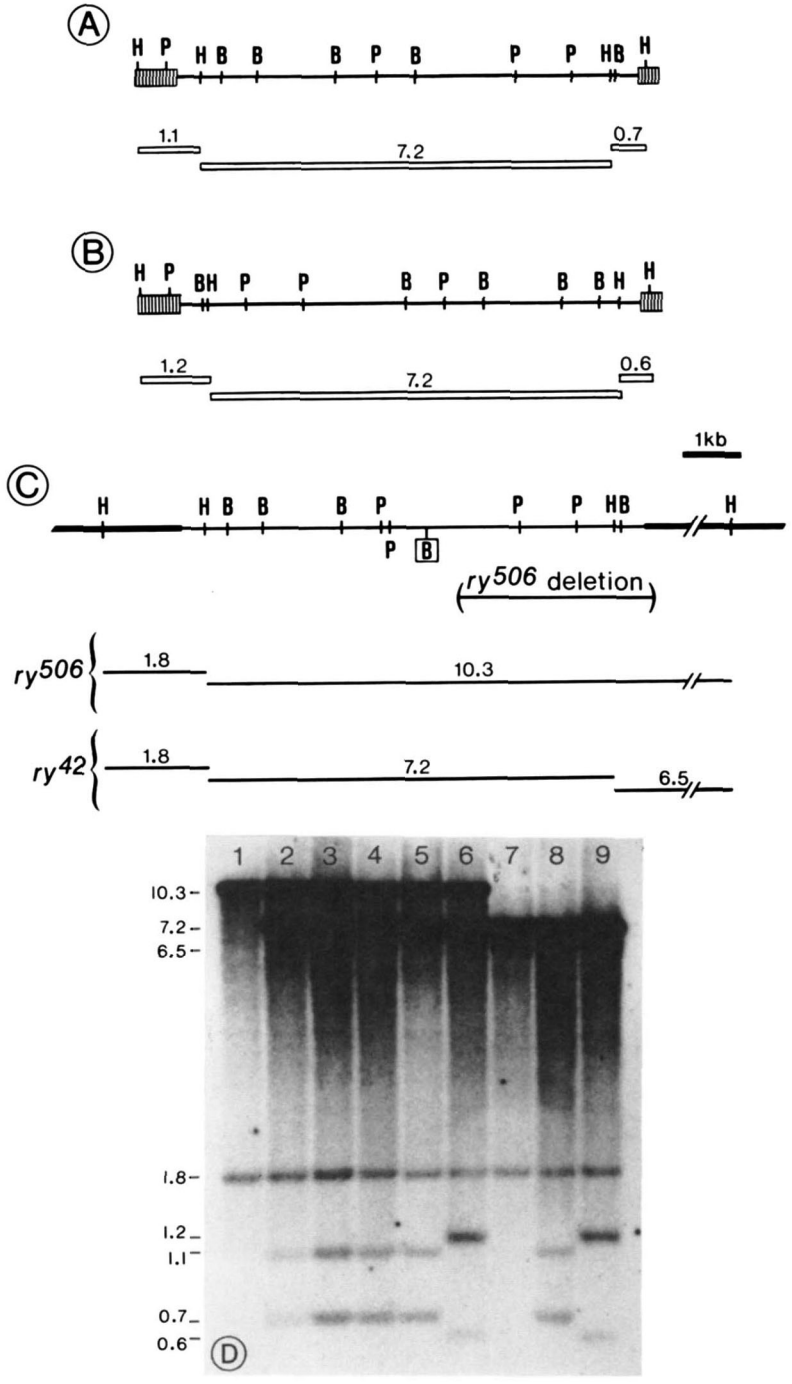


FIGURE 4.—Restriction enzyme and gel blot analysis of whole-fly DNA from the seven ry^+ transformants depicted in Figure 1. A and B, Restriction maps of the ry^+ transposons borne by the pry1 (A) and pry3 (B) plasmids. These plasmids were constructed by inserting the 8.1-kb *Sal*I *ry* locus fragment, obtained from pry8.1, into the *Xho*I 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)

(1983) and its ry^+ transposon was shown by *in situ* hybridization to reside in the chromocenter at the base of chromosome *4*. 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^{+i4-1a}] and [ry^{+72-1}] (Figure 1A) to the same analysis as described above for [*R401.1*]. In both cases (data shown only for [ry^{+i4-1a}]) the addition and subtraction of *Y* chromosome heterochromatin has no effect on *rosy* locus expres-

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 = *Bgl*II, H = *Hin*dIII and P = *Pvu*II. The open bars below each map represent the fragments observed when DNA containing the ry^+ transposon is digested with *Hin*dIII 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^{506} and ry^{42}. The 8.1-kb *ry* fragment is depicted by the thin bar; thick bars represent adjacent sequences. The ry^{506} 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^{42} and the ry^+ transposons reveals two restriction site heterogeneities (indicated below the map): ry^{+5} lacks one of the *Bgl*II sites (boxed) that is present in ry^{42} and the ry^+ transposon; ry^{+5} and ry^{42} contain a *Pvu*II site that is not found in the ry^+ transposon. The thin bars below the map represent the fragments observed when ry^{506} and ry^{42} DNAs are digested with *Hin*dIII and probed with pry8.1; numbers indicate lengths in kilobases. D, Southern blot of genomic DNA from homozygous adult flies digested with *Hin*dIII and the blot was probed with pry8.1 DNA. Samples were applied to lanes as follows: (1) ry^{506}, (2) [ry^{+i4-1a}]; ry^{506}, (3) ry^{506} [ry^{+72-1}], (4) [ry^{i77-1}]; ry^{506}, (5) [ry^{+201-4}]; 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 *Hin*dIII fragments derived from the ry^+ transposon in any of the seven ry^+ transformants. We note that ry^+ transformants in a ry^{42}, but not ry^{506}, background produce two indistinguishable 7.2-kb *Hin*dIII 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, *Bgl*II and *Pvu*II. In all instances the fragments expected from an intact transposon (see A and B) were observed.

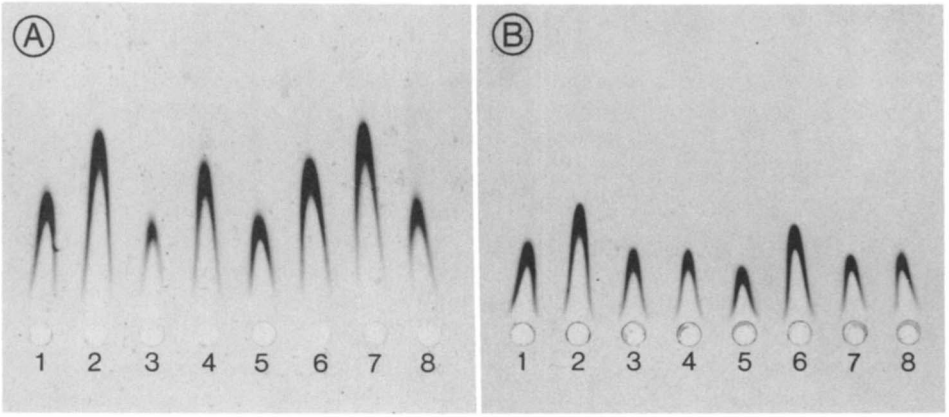


FIGURE 5.—The effects of heterochromatin content on the XDH levels of two transformed lines, *R401.1* (A) and $ry^{+i4\text{-}1a}$ (B). A and B, Rocket immunoelectropherograms. Extracts were obtained from uniformly aged adults and contain equivalent amounts of protein. Protein synthesis in ovaries contributes 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}/ry^{506} and ry^{+5}/ry^{506} *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^{+5} alleles.) Experimental samples from ry^{42}/*MKRS*; [*R401.1*]/ or $[ry^{+i4\text{-}1a}]/-$; ry^{506}/*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 $Y^{S}X{\cdot}Y^{L}/O$; ry^{+11}/*MKRS* males to homozygous ry^{42}; [*R401.1*] or $[ry^{+i4\text{-}1a}]$; ry^{506} females and selecting ry^{+} *Sb* progeny; *XY* and *XX* flies were obtained in the same fashion, except *XY*; ry^{+11}/*MKRS* flies were used in the P_1 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 $ry^{+i4\text{-}1a}$. Alterations in *Y* chromosome heterochromatin content do not affect XDH levels.

sion in the transposon (Figure 5B). Thus, it appears that the reduced expression of these transformants is not the result of interaction with heterochromatic sequences.

Transposon expression and euchromatic position effect: We considered next the possibility that the XDH underexpression associated with $[ry^{+i4-1a}]$ is due to a position effect not subject to *Y* chromosome modification. Such 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 $[ry^{+i4-1a}]$ 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 $[ry^{+i4-1a}]$. From this experiment, one stable transposition was established. $[ry^{+i4-1a}]$ resides at 57F on chromosome *2R* (Figure 7A), and its transposition derivative, $[ry^{+i4-1a\text{-}4}]$, is located at 68A on chromosome *3L* (Figure 7B). An examination of the XDH CRM of $[ry^{+i4-1a\text{-}4}]$ reveals an almost twofold increase over that of the parental $[ry^{i4-1a}]$ line (Figure

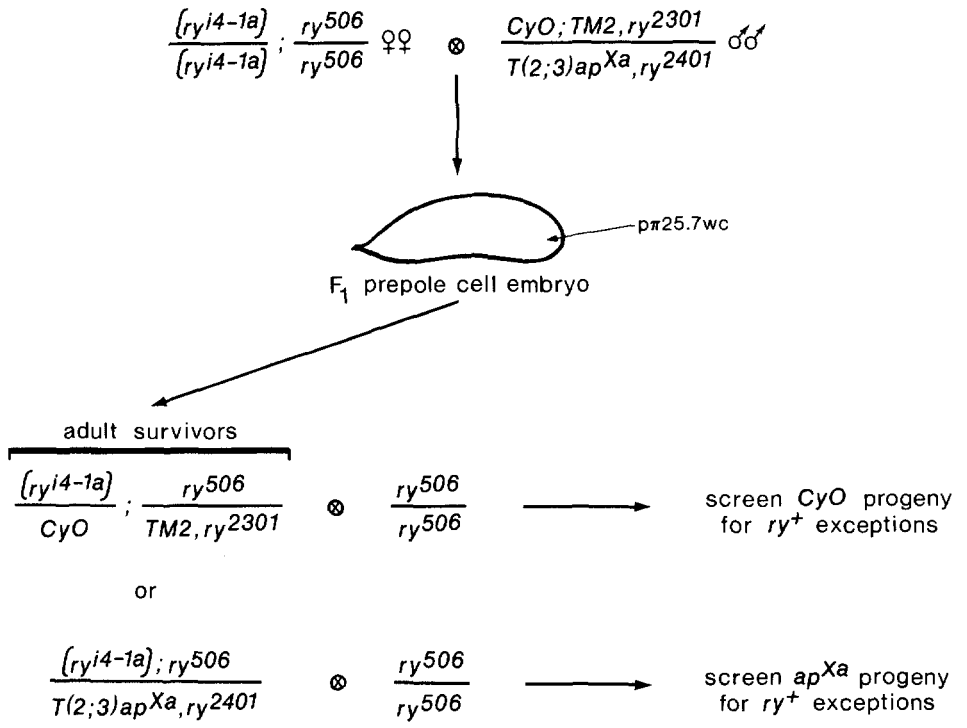


FIGURE 6.—Diagram of the scheme employed to obtain stable transpositions of the $ry^{+i4\text{-}1a}$ transposon. Heterozygous embryos possessing a single [$ry^{+i4\text{-}1a}$] chromosome were injected with pπ25.7wc plasmid DNA at a concentration of 250 μg/ml. Injection survivors were outcrossed to a ry^+ strain, and the appropriate progeny were screened for ry^+ exceptional flies.

7C). We have also verified that the DNA structure of [$ry^{+i4-1a\text{-}4}$] was not altered in the transposition process (Figure 7D), and we have confirmed that the new line carrying [$ry^{+i4-1a\text{-}4}$] is stable and free of functional *P* elements (Figure 7E). These results demonstrate that the underexpression of [ry^{+i4-1a}] 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 *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 *rosy* locus expression to examine enzyme distribution in the cells of the malpighian tubules where *rosy* locus expression is autonomous. They demonstrated the variegated nature of *rosy* locus expression in rearrangements that placed the *rosy* locus adjacent to heterochromatin. We examined XDH distribution in histochemical preparations of malpighian tubules from [*R401.1*] and [ry^{+i4-1a}], as well as [$ry^{+22\,16}$], which is a transposon carrying the same wild-type 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

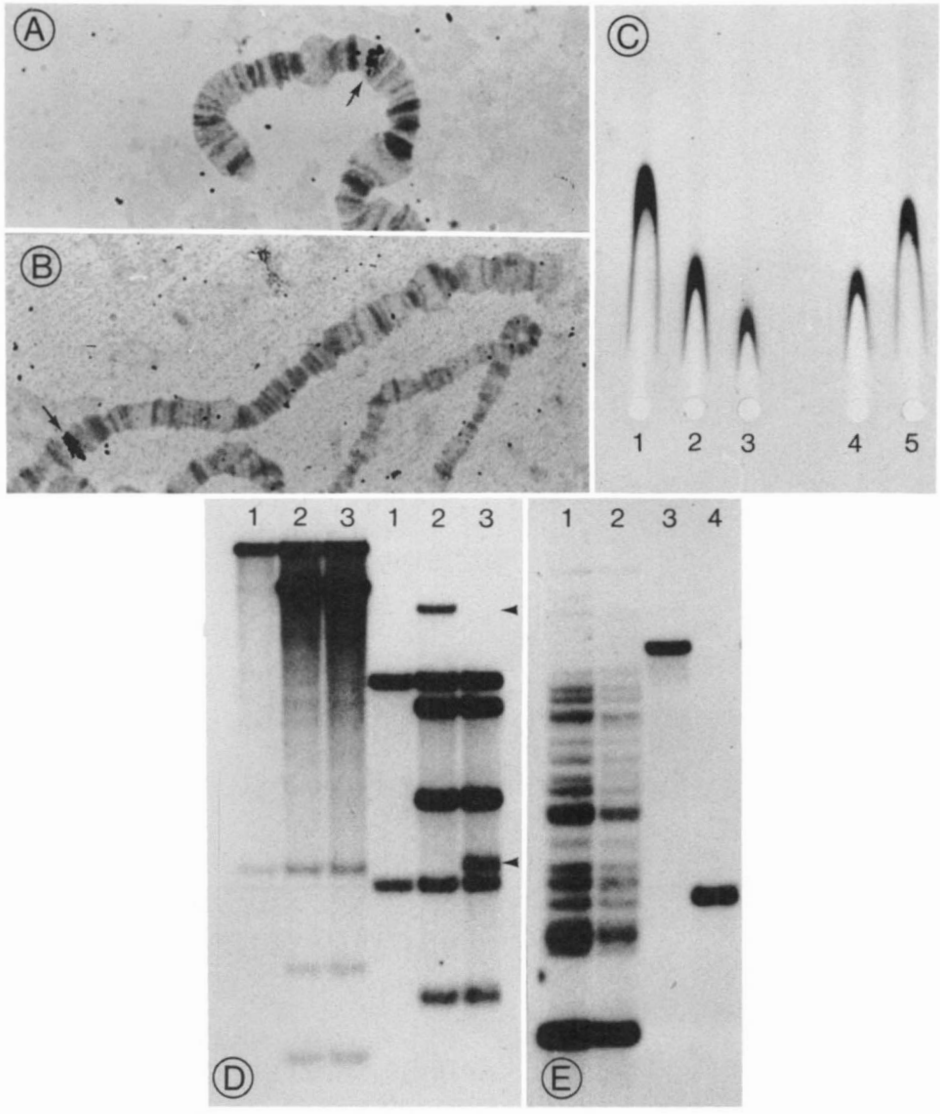


FIGURE 7.—Analysis of [$ry^{+i4\text{-}1a\text{-}4}$]. The ry^+ transposon in this line is a derivative of [$ry^{i4\text{-}1a}$] and was obtained by the transposition scheme outlined in Figure 6. A and B, *In situ* hybridization of ^{3}H-labeled pry8.1 DNA to salivary gland polytene chromosomes from homozygous larvae of [$ry^{+i4\text{-}1a}$]; ry^{506} (A) and [$ry^{+i4\text{-}1a\text{-}4}$] ry^{506} (B). In both cases, two hybridization signals are evident, one at 87D, the location of the *rosy* locus on chromosome *3* (not shown), and the other at the site of insertion of the ry^+ transposon. The $ry^{+i4\text{-}1a}$ transposon is located at 57F (arrow in A) on chromosome arm *2R*, and its transposition derivative, the $ry^{+i4\text{-}1a\text{-}4}$ 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^{+5}; wells 2 and 3 contain, respectively, dilutions with 50% and 25% of the extract in well 1. Wells 4 and 5 contain samples from [$ry^{+i4\text{-}1a}$]; ry^{506} and [$ry^{+i4\text{-}1a\text{-}4}$] ry^{506}, respectively. A nearly twofold increase in XDH CRM is observed in the transposition derivative. D, Verification that the $ry^{+i4\text{-}1a\text{-}4}$ 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) [$ry^{+i4\text{-}1a}$]; ry^{506} and (3) [$ry^{+i4\text{-}1a\text{-}4}$] ry^{506}. The left set of samples were digested with *Hin*dIII; the samples in the set on the right were digested with *Pvu*II. The pry8.1 plasmid was used as probe. Arrows indicate the *Pvu*II 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 $ry^{+i4\text{-}1a\text{-}4}$ 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) [$ry^{+i4\text{-}1a}$]; ry^{506} and (4) [$ry^{+i4\text{-}1a\text{-}4}$] ry^{506}.

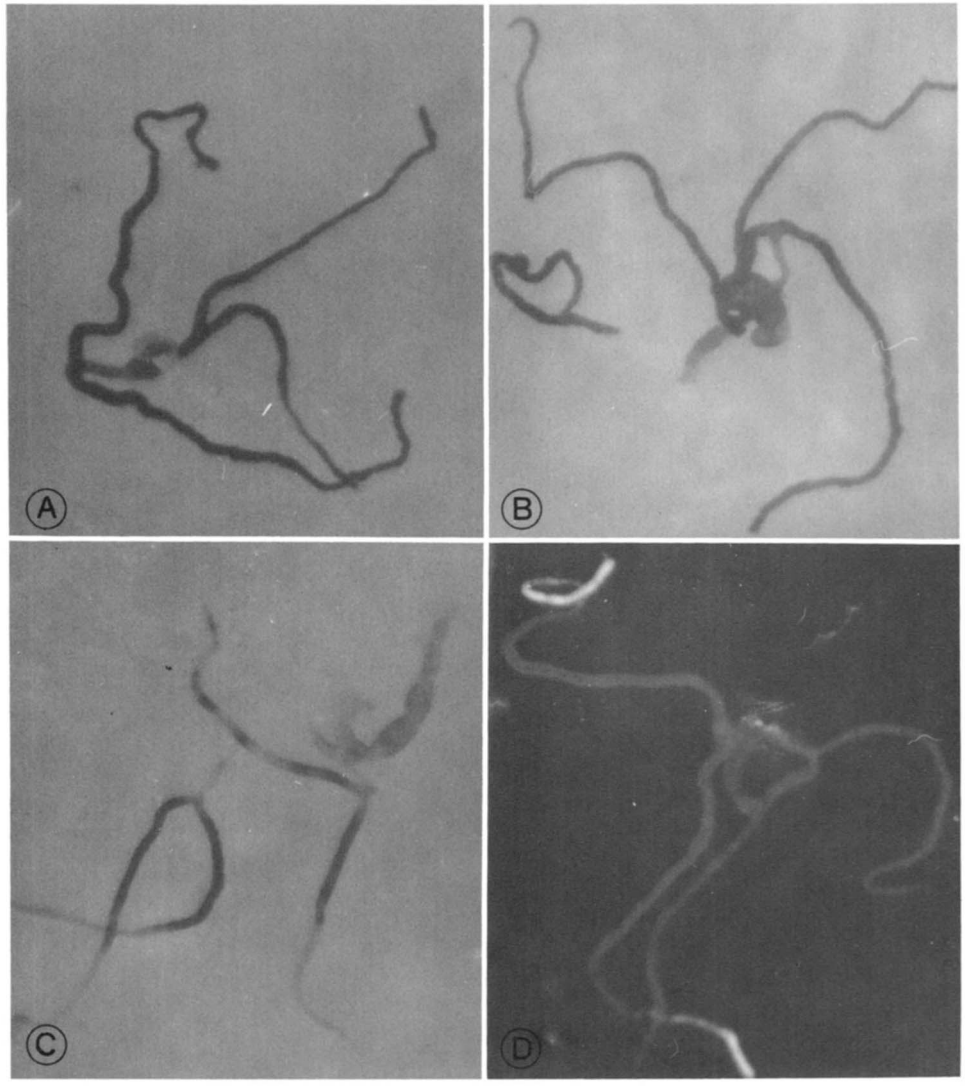


FIGURE 8.—Late third-instar larval malpighian tubules stained for XDH activity (see MATERIALS AND METHODS) of homozygotes ry^{+5} (A), $[ry^{+i4\text{-}1a}]$; ry^{506} (B), ry^{42}; *[R401.1]* (C) and ry^{506} (D). The variegated XDH expression of *[R401.1]* (C) is compared to the uniform, but low, expression of $[ry^{+i4\text{-}1a}]$ (B). The dark background due to overexposure of ry^{506} (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, ry^{+10}, 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 ry^{+} transposon: Figure 3A demonstrates that the XDH encoded by the DNA of a normal *rosy* locus transposon derived from either

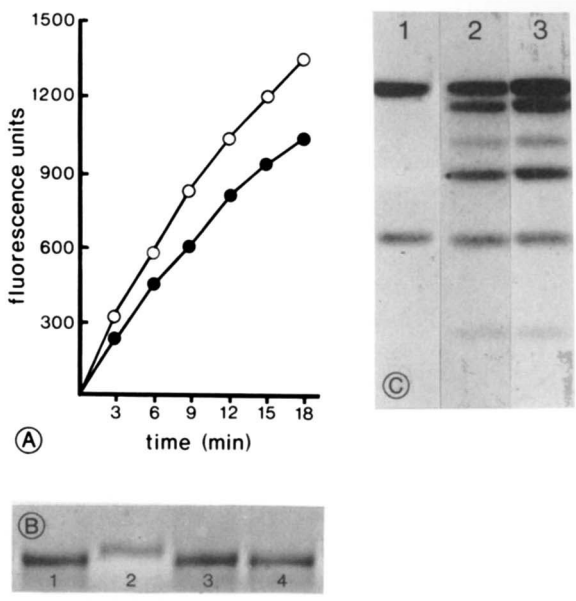


FIGURE 9.—Comparative analysis of [*2216*] and [*559*]. A, Fluorometric assay of XDH activity in uniform extracts of adult males, aged 48 hr. Open circles represent *sd* [*2216*]; ry^{506}/ry^{506}. Closed circles represent sd [*559*]; ry^{506}/ry^{506}. B, XDH electropherogram of extracts of males, 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 ry^{506}. C, Gel blot of DNA prepared from adult males, digested with *Pvu*II and probed with pry8.1. Samples are as follows: (1) ry^{506}/ry^{506}, (2) *sd* [*2216*]; ry^{506}/ry^{506} and (3) *sd* [*559*]; ry^{506}/ry^{506}.

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^{+i4-1a}] (and its transposition derivative, [$ry^{+i4-1a-4}$]), 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, [$ry^{ts2216-559}$], 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 ry^{506} (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 *Pvu*II-, *Hin*dIII-, *Bgl*II- and *Sst*I-digested DNA preparations from [*559*] and [*2216*] strains, revealed no restriction fragment differences. Figure 9C presents a gel blot illustrating the *Pvu*II restriction analysis.

Since [*2216*], and other "leaky" mutant derivatives of it, retain the $XDH^{1.02}$ 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, [ry^{+i4-1a}], 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 [ry^{+i4-1a}], 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 [*R401.1*] 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^{+i4-1a}] and [ry^{+72-1}] (the euchromatic transformants with the most depressed CRM levels) is not responsive to *Y* chromosome modification. Moreover, the malpighian tubules of [ry^{+i4-1a}] 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 *Sal*I 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 euchromatic position 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.

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