MOLECULAR AND CELLULAR BIOLOGY, Oct. 1985, p. 2567-2574
0270-7306/85/102567-08$02.00/0

Vol. 5, No. 10

P-Element-Induced Control Mutations at the *r* Gene of *Drosophila melanogaster*

STUART TSUBOTA,[1]* MICHAEL ASHBURNER,[2] AND PAUL SCHEDL[1]

Department of Biology, Princeton University, Princeton, New Jersey 08544,[1] and Department of Genetics, Cambridge University, Cambridge CB2 3EH, Great Britain[2]

Received 26 March 1985/Accepted 12 July 1985

The P-M hybrid dysgenesis system was used to produce five putative regulatory mutations at the rudimentary locus, *r*. All five mutations were the result of insertions at the 5′ end of the gene, upstream of the proposed start of transcription. All of the mutants displayed a leaky wing phenotype, and four of the mutants showed an uncoupling of the wing and female-sterility phenotypes, suggesting that they altered the normal spatial and temporal expression of the *r* gene. Four of the insertions were P elements. The fifth insertion, which was larger than an intact P element, consisted of a small P element connected to non-P-element DNA. Two of the mutants produced very little *r* transcript in adult females and were clustered 80 to 150 base pairs upstream of the start of transcription. The other three mutants had higher levels of *r* transcript in adult females and were clustered 440 to 500 base pairs upstream of the start of transcription. All of the data suggest that the insertions are in a 5′ noncoding region of the *r* gene involved in the control of its spatial and temporal expression.

The control of gene expression in higher eucaryotes, such as the fruit fly, *Drosophila melanogaster*, has been a major interest of both geneticists and developmental biologists, yet very little is known concerning the mechanisms involved in regulating gene expression. One of the major stumbling blocks in studying gene regulation in *D. melanogaster* has been the inability of geneticists to generate regulatory mutations. Selective screens have been available to recover regulatory mutations at the rosy (xanthane dehydrogenase), *adh* (alcohol dehydrogenase), and rudimentary (carbamylphosphate synthetase [CPSase]-aspartate transcarbamylase [ATCase]-dihydroorotase [DHOase]) loci; however, no regulatory mutations have been recovered in screens with conventional mutagens such as ethyl methanesulfonate (EMS) and X-rays. In fact the only putative control mutations at these loci have been isolated either from natural populations (6, 33) or as spontaneous mutations (35). Hence, it could be argued that control regions of *Drosophila* genes and perhaps genes of other eucaryotes may be refractory to many of the standard mutagens and that it would be worthwhile employing a somewhat different approach for the generation of regulatory mutations.

One such approach would be to use transposing elements as mutagens in schemes to isolate regulatory mutations. This possibility is suggested by the work of Barbara McClintock on controlling elements in maize (20). These elements are transposable elements that insert in and around a gene and alter its pattern of expression (4, 7). Transposable elements have been shown to be responsible for regulatory mutations in other organisms as well. These elements include the Ty elements in yeast cells (9, 27, 34, 36), avian leukosis viruses in both the 3′ and 5′ noncoding region of oncogenes (14, 22, 25), and insertions in the 5′ noncoding region of *Drosophila* genes (21, 31). To use transposing elements in schemes to isolate regulatory mutations, several requirements should be met. First, a transposing-element system that is capable of producing high mutation rates should be used. Second, these mutations should be induced in a gene in which a characteristic pattern of expression is seen during development and in which changes in the levels of expression can be easily detected.

The transposing-element system used in this study was the P-element system of P-M hybrid dysgenesis (2, 28). The P-M system consists basically of two strains of files, i.e., P strains, which for the most part are recent isolates from natural populations, and M strains, which are mainly established laboratory stocks. The P strains contain 30 to 50 members per genome of a heterogeneous family of sequences called P elements. The M strains, with rare exceptions, contain no P elements. When males from P strains are crossed to females of M strains, a syndrome known as hybrid dysgenesis occurs (8,16). This syndrome is characterized by temperature-dependent sterility, male recombination, and a high frequency of mutations and chromosomal breakage within the F1 progeny. The high mutation rate is caused by the mobilization and subsequent insertion of the P elements into genes (28). The largest P element is 2.9 kilobases (kb) long and codes for at least one *trans*-acting function which is necessary for transposition (24). Most P elements are smaller elements generated by internal deficiencies within the largest element. These defective P elements can transpose only in the presence of an intact P element. The mobility of P elements can be stabilized in a P strain or in an M strain if all the elements in the M strain are defective elements. Thus, the P-element family differs from other repeated-element families in that the mobility of its members can be experimentally controlled, and most importantly for the present study, conditions exist under which the mobility of the P elements is very high.

The gene chosen for the present study was the rudimentary gene, *r*, which codes for the first three enzymes in the pyrimidine biosynthesis pathway, i.e., CPSase, ATCase, and DHOase (3). All three activities are contained on a single polypeptide and have a characteristic developmental pattern of expression. The highest levels are observed in embryos and first-instar larvae. From the levels of these stages, the activities decrease during development and are at their lowest in pupae and adults. In addition, there are differences in the levels of activities between tissues. In third-instar larvae, which as a whole have low levels of *r* gene product,

* Corresponding author.

TABLE 1. Wing phenotype and complementation pattern

Mutation	Wing phenotype and value[a]	Complementation pattern
r^{hd1}	Moderate, 3	DHO^+ CPS^- ATC^+
r^{hd2}	Slight, 2	DHO^+ CPS^- ATC^+
r^{hd3}	Moderate, 3	DHO^+ CPS^- ATC^+
r^{hd4}	Moderate, 4	DHO^+ CPS^- ATC^-
r^{hd5}	Slight, 1–2	DHO^+ CPS^- ATC^+

[a] The numbering system is that of Green (13). A wild-type wing has a value of 1, and a severely truncated wing, characteristic of a deficiency, has a value of 5.

the imaginal disks have high levels (Tsubota, unpublished results). High levels are also seen in the ovaries and developing eggs of adult females (26; L. Ambrosio and P. Schedl, submitted for publication). These high levels result in the differences in *r* enzymatic activities between aged adult males and females. Null and hypomorphic mutations at the *r* locus result in a rescuable maternal-effect lethality and an oblique truncation of the wing. The lethality, seen as a female sterility, is caused by lack of *r* gene product in the embryo. To a certain extent, this lethality is rescuable by a wild-type gene introduced into the embryo by the sperm (Fig. 1B). The wing truncation is caused by a lack of *r* gene product in the wing during metamorphosis (10). The severity of the female sterility correlates very well with the severity of the wing phenotype. The wing phenotype is very useful, since the severity of truncation reflects the severity of the reduction in enzymatic activities. This fact was used to identify spontaneous overproducing mutations at the locus (35).

In the present study, a leaky allele, *Rev-r*$^{16-4}$, was used to increase the sensitivity of the mutation schemes. This allele facilitated the recovery of mutations which only slightly lower the expression of the *r* gene. As it turned out, this allele was very important, since four of the five mutations which were recovered would not have been detected if they had been induced in a wild-type allele.

MATERIALS AND METHODS

Stocks. See reference 17 for a further description of mutations. $r^{pyrI\text{-}16}$ is an EMS-induced mutation (11). *Rev-r*$^{16-4}$ was isolated as a revertant of a leaky *r* allele, $r^{pyrI\text{-}16}$ (35). It is a second-site mutation at the *r* locus resulting in a coordinate increase in the three enzymatic activities. *Rev-r*$^{16-1}$ is a spontaneous revertant of $r^{pyrI\text{-}16}$ (35). r^{56k} is an *r* allele which falls into the DHO^- complementation group (5). r^{29} *is an r* allele that falls into the ATC^- complementation group (5). $r^{pyrI\text{-}17}$ is an *r* allele which falls into the CPS^- complementation group (11). r^{3D} is an EMS-induced mutation in $r^{pyrI\text{-}16}$. r^{8B} is an EMS-induced mutation in $r^{pyrI\text{-}16}$. $r^{1\text{-}4}$ is an EMS-induced mutation in *Rev-r*$^{16-1}$. $r^{4\text{-}7}$ is an EMS-induced mutation in *Rev-r*$^{16-4}$. $r^{4\text{-}8}$ is an EMS-induced mutation in *Rev-r*$^{16-4}$. *C(1)RM* y^2 is a compound X chromosome. Male progeny from these females will inherit their X chromosome from their fathers. π2 is a wild-type strain isolated in Wisconsin. It is a P strain in the P-M hybrid dysgenesis system. Canton S is a wild-type strain. It is an M strain.

Drosophila media. Yeast-glucose medium contained 100 g of dried yeast, 100 g of glucose, 3 g of nipagin, 20 g of agar, and 1,000 ml of water. RNA medium contained yeast-glucose plus 1% (wt/vol) RNA. This medium increases the viability of *r* mutants but does not significantly affect their wing phenotypes. Unless indicated, all crosses were performed on yeast-glucose medium at 25°C.

Isolation of *r* mutations. To isolate mutations at the *r* locus which only slightly lowered the expression of the gene, an allele with a leaky structural gene mutation was used as the progenitor. The chosen allele was *Rev-r*$^{16-4}$. The majority of flies which are heterozygous for this allele and an *r* deficiency have normal wings. The remainder have disruptions of the marginal wing hairs, characteristic of very slight *r* mutations.

Hybrid dysgenesis shows a reciprocal cross-effect. High mutation rates are seen only when P males are mated to M females. Therefore, in the mutation schemes, π2 males were mated to *cv v Rev-r*$^{16-4}$ *f* females. The F1 females were crossed to wild-type males, and the F1 males were crossed to *C(1)RM* females. In both cases, the F2 males were scored for new *r* mutations. These final crosses were performed on RNA medium to increase the viability of any *r* males. These crosses are diagrammed in Fig. 1A.

Complementation. All hybrid dysgenesis-induced *r* mutations were crossed inter se and to the tester *r* alleles listed above which represent the three basic complementation groups of the *r* locus. The wings were scored for the rudimentary phenotype. Alleles were said to complement each other if the phenotype of the heterozygote was more wild type than that of either homozygote.

Sterility. Homozygous *r* females are conditionally sterile. If they are grown on media supplemented with pyrimidines, they are partially fertile. This sterility is actually a rescuable maternal-effect lethality. Progeny from homozygous *r* mothers die because of lack of pyrimidines, unless they are provided with sufficient pyrimidines either from the diet of the mother or by the introduction of an r^+ allele from the father. The rescue by an r^+ allele is diagrammed in Fig. 1B. This cross can be used to measure the fertility of a particular *r* mutant. The ratio of male progeny to total progeny is taken as a measure of the fertility of the mothers carrying the *r*

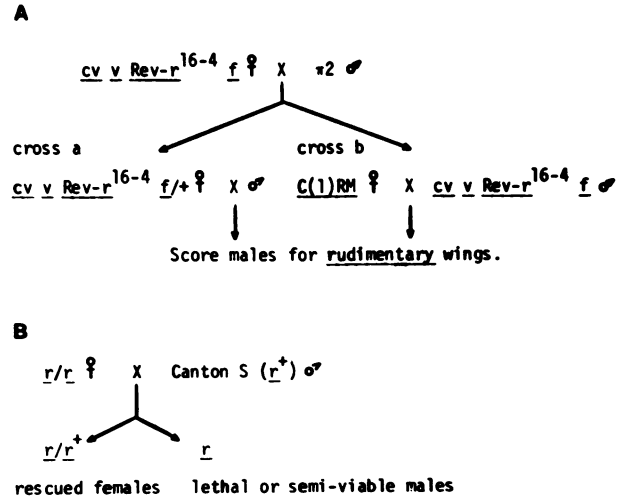

FIG. 1. (A) *r* gene mutation schemes. The crosses and the mutants employed in the screens are described in Materials and Methods and in Results. (B) Rescuability of maternal effect lethality. The cross used to score the fertility of each mutant is shown. The female progeny are rescued by the r^+ allele from their father. The viability of the male progeny depends on the activity of the maternal *r* allele during oogenesis and embryogenesis. A further description of this cross is in Materials and Methods and in Results.

gene. A value of 0 means total sterility, and a value of 0.5 means total fertility. The ratio of males to total progeny is more accurate than the total number of progeny produced, since there can be variability in the total number of progeny produced by females. Although the maternal-effect lethality is rescuable, in general the more extreme the *r* mutation, the lower the total number of progeny produced, so that the total number of progeny is also a measure of the fertility of the female. To measure the fertility of the various *r* mutants, 8 *r* females and 10 Canton S males were placed in a vial of food. Five tubes were set up for each mutant. The flies were transferred to new vials every 3 days for a total of 12 days of egg laying. The progeny were counted, and sexes were determined. The ratios of males to total progeny and the total number of progeny are listed in Table 2.

Southern blot analyses. DNAs from the various *Drosophila* stocks were digested with *Eco*RI, electrophoresed in 0.7% agarose gels, and transferred to nitrocellulose filters by the technique of Southern (32) with minor alterations. The clones used as probes in the analyses were previously isolated from the Canton S DNA library of Maniatis et al. (19). These clones have been mapped with respect to each other and the *r* mRNA (29, 30). The clones and the *r* mRNA are diagrammed in Fig. 2A. All of the clones except clone 313 were used in the present study. In addition, the nitrocellulose filters were probed with two plasmid clones, pBRH17 and pBRπ25.1. Plasmid pBRH17 is a clone of the 1.4-kb *Eco*RI fragment which contains the start of transcription of the *r* gene. The plasmid pBRπ25.1 contains an entire P element plus flanking DNA from cytological position 17C (24). pBRπ25.1 was kindly provided by Gerry Rubin.

Cloning of the mutations. Since all of the mutations were insertions within the same *Eco*RI fragment, the strategy for cloning them was to clone *Eco*RI fragments. DNA from each of the mutants was digested to completion with *Eco*RI and cloned into the *Eco*RI site of the vector lambda gt-lac. This procedure will clone genomically noncontiguous *Eco*RI fragments into the same vector. Recombinant clones were screened with a ^{32}P-labeled probe of pBRH17. DNA from the purified recombinant clones was cut with *Eco*RI and electrophoresed in a 0.7% agarose gel. The mutant *Eco*RI fragments were identified both by their size and homology to pBRH17. Each mutant fragment was isolated and cloned into the *Eco*RI site of pBR322. In the case of r^{hd4}, two *Eco*RI fragments were separately cloned, since this mutation contains an insertion with an *Eco*RI site.

TABLE 2. Fertility of homozygous *r* females

Mutation	Wing phenotype and value[a]	Total no. of progeny	Males/total progeny ratio[b]
$Rev\text{-}r^{16\text{-}4}$	1	5,595	0.488
$r^{pyrl\text{-}16}$	1	2,961	0.386
$r^{pyrl\text{-}17}$	1–2	405	0.085
r^{hd5}	1–2	0	
r^{hd2}	2	303	0.132
$r^{1\text{-}4}$	3	456	0.040
r^{hd1}	3	666	0.185
r^{hd3}	3	5	0.0
$r^{4\text{-}8}$	4	33	0.0
r^{hd4}	4	52	0.192
r^{3D}	5	0	
r^{8B}	5	4	0.0
r^{56K}	5	0	
$r^{4\text{-}7}$	5	3	0.0

[a] Wing phenotypes were determined as described in Table 1, footnote *a*.
[b] Total number of progeny and the male progeny/total progeny ratio were determined from the cross shown in Fig. 1B.

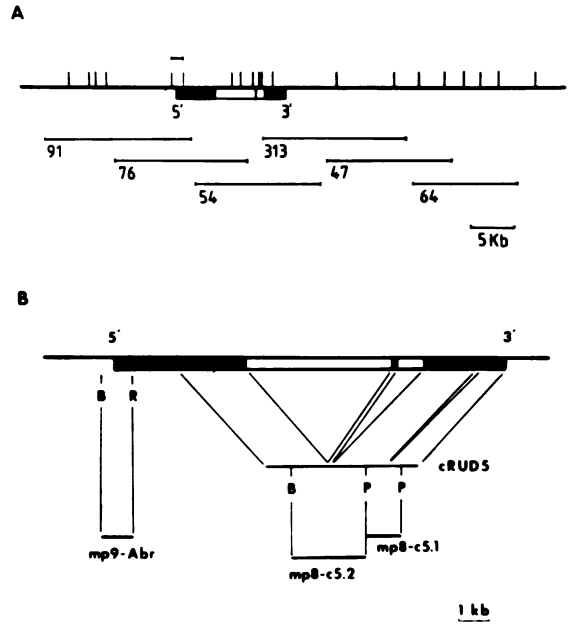

FIG. 2. (A) *r* region. The heavy horizontal line represents a region of 60 kb which includes the *r* gene. The vertical lines are *Eco*RI sites. The *r* transcription unit is drawn in. The black regions are exons, and the white regions are introns. The numbered horizontal lines are the lambda clones which define the region. The small horizontal line above the heavy line represents the 1.4-kb *Eco*RI fragment which is contained in pBRH17. The data are from references 29 and 30. (B) Probes for the *r* gene transcript. The three probes, mp9-Abr, mp8-c5.2, and mp8-c5.1, are clones into the vector M13. Their homology to the *r* transcript is shown. The clone cRUD5 is a cDNA clone. Its homology to the *r* gene is shown. The clones mp8-c5.2 and mp8-c5.1 are subclones of fragments from cRUD5. Abbreviations: B, *Bam*HI; P, *Pst*I; R, *Eco*RI.

Restriction mapping of the pBR clones. The clones were mapped by conventional restriction enzyme mapping techniques. Three enzymes, i.e., *Bam*HI, *Hin*dIII, and *Ava*II, were extremely useful. *Bam*HI was used to map the position of the insertions with respect to the *Bam*HI site within the 5′ *Eco*RI fragment. Since all P elements analyzed thus far have a *Hin*dIII site mapping 39 base pairs (bp) from one end, *Hin*dIII was used to map the position of the P insertion and its orientation within the 5′ *Eco*RI fragment. All P elements have an *Ava*II site within each terminal 31-bp inverted repeat. These sites are 22 bp from each end. *Ava*II was used to map the position of the P insertions and also to measure their size.

Northern blot analyses. Total RNA was isolated from 3- to 4-day-old adult females (23). The RNA was precipitated with 2.5 M LiCl and redissolved in 100 mM NaCl–1 mM EDTA–0.5% sodium dodecyl sulfate–10 mM Tris hydrochloride (pH 7.5). Samples were prepared and run on 0.7% (wt/vol) agarose–6% formaldehyde gels (12, 18). Approximately 20 μg of each sample was run. The RNA was transferred to nitrocellulose filters (32) and probed by a previously described method (30) with three single-stranded probes covering the rudimentary message. The probes were prepared from three M13 clones, i.e., mp9-Abr, mp8-c5.1, and mp8-c5.2 (Fig. 2B). The clone mp9-Abr is homologous to the first 0.7 kb of the message. The other two clones,

mp8-c5.1 and mp8-c5.2, are subclones of two restriction fragments from a cDNA clone, cRUD5 (30). They are homologous to 1.0 and 2.0 kb of the message, respectively.

The amount of rudimentary message was standardized to the amount of *adh* message. *adh* message was visualized with a nick-translated probe of pAdh-cDNA, a cDNA of *adh*. This clone was a gift from Cheeptip Benyajati. The amount of each message was measured with a Joyce, Loebl Automatic Recording Microdensitometer and a Zeiss MOP-3. To standardize the rudimentary message levels, the ratio of rudimentary message to *adh* message was calculated from the densitometric values.

RESULTS

Mutation schemes. To facilitate the recovery of mutations which only slightly lowered *r* gene expression, a leaky allele with a structural gene alteration was used as the progenitor. This allele, $Rev\text{-}r^{16\text{-}4}$, was isolated as an overproducing revertant of $r^{pyr1\text{-}16}$, which has 20% of the wild-type CPSase activity and normal levels of ATCase and DHOase activities (35). Although flies homozygous for $Rev\text{-}r^{16\text{-}4}$ have completely wild-type wings, they behave genetically as leaky mutants and have only 40% of the wild-type CPSase activity.

Five independent mutations out of 27,700 chromosomes were isolated. Two of 2,800 were isolated from females (Fig. 1A, cross A), and 3 in 24,900 were isolated from males (Fig. 1A, cross B). Only one mutation, r^{hd2}, arose as a cluster (in five males). The three mutations recovered from cross B occurred in the X chromosome of the M strain. The two mutations recovered from cross A most likely occurred in the X chromosome of the M strain, since they retained the closely flanking marker, forked, *f*. The recombination distance between *r* and *f* is 1.4 map units. Thus, all the mutations were induced in the allele, $Rev\text{-}r^{16\text{-}4}$.

Phenotypes and complementation mapping of the mutants. As was discussed in the introduction, the two rudimentary phenotypes, wing truncation and female sterility, result from the lack of *r* gene product in two different tissues and at two different stages in development. Mutations within the coding region of the *r* gene might be expected to result in a similar loss of gene activity in both the wing imaginal disks and the ovaries and thus exhibit a good correlation between the wing and sterility phenotypes. This indeed has been shown to be the case (5). On the other hand, mutations within a region of the gene concerned with the temporal and spatial distributions of the gene product might produce a differential expression of the *r* gene in the wing disks and the ovaries, resulting in the uncoupling of the wing and sterility phenotypes. Therefore, an examination of both phenotypes should offer some clues as to the nature of the mutations.

The wing phenotypes of the mutations and the complementation patterns are summarized in Table 1. Classified by their wing phenotypes, all of the mutations are hypomorphic. This fact suggests that the mutations are not in a polypeptide-coding region of the *r* gene if, as expected, they are insertion mutations. Two of the mutants, r^{hd2} and r^{hd5}, have very slight rudimentary wing phenotypes. The other three mutants, r^{hd1}, r^{hd3}, and r^{hd4}, have moderate rudimentary wings.

The second rudimentary phenotype, female sterility, was examined by crossing mutant females to Canton S males (Fig. 1B). The fertility was measured in two ways. First, the total number of progeny was measured. Second, from the surviving progeny, the ratio of males to total progeny was determined. This value measures the maternal contribution of functional *r* activity to the embryo and the activity of the maternal allele in the embryo, since the males get only the *r* allele of the mother, whereas the rescued females get an r^+ allele from the father. An examination of the wing phenotypes and the fertility of EMS-induced *r* mutants reveals a good correlation between the wing phenotype and the fertility of a given mutant (Table 2). Interestingly, some of the hybrid dysgenesis-induced mutants do not show this close correlation. The mutants r^{hd5} and r^{hd3} are more sterile than would be expected from their wing phenotypes. In particular, r^{hd5}, which has almost a wild-type wing phenotype, has the fertility of an extreme *r* mutant. The opposite result is seen with the mutants r^{hd1} and r^{hd2}. Both of these mutants have a higher male-to-total-progeny ratio than would be expected from their wing phenotypes. This uncoupling of the wing phenotype and female sterility suggests that the mutations differentially alter the amounts of functional *r* gene product in the ovaries and wing disks. This alteration could be explained by differential gene expression or differential stability of the product in the two different organs.

The mutant r^{hd4} has an interesting phenotype. According to its wing phenotype, it should be a female sterile. Indeed, r^{hd4} females produce very few progeny; however, a high proportion of these progeny are males. This fact suggests that just as the surviving females are being rescued by an r^+ allele from their fathers, the surviving males are also being rescued by r^+ activity. This rescue could occur if the r^{hd4} allele were being activated early enough in development to rescue the males. Thus the r^{hd4} mutation may be altering the regulation of the *r* structural gene by inactivating the gene in the ovaries, resulting in a small number of progeny, and activating the gene during embryogenesis, resulting in the rescue of some of the male progeny.

Both the hypomorphic nature of the wing phenotypes and the uncoupling of the wing and sterility phenotypes suggest that the hybrid dysgenesis-induced mutations result in modifications in the amount of *r* gene product in a tissue-specific and temporally specific manner. In agreement with this interpretation, the complementation analysis suggests that the level of active *r* gene product is reduced but not completely abolished. Although these mutants fail to complement each other, four of them will complement *r* alleles defective in ATCase or DHOase activity and thus are in the CPSase complementation group. The exceptional case, r^{hd4}, is the most severe of the five mutants; however, even this allele retains some *r* gene activity, as it will complement an *r* allele defective in DHOase activity. In view of these findings, it is important to note that the progenitor strain itself is defective in CPSase activity but compensates for this lesion by overproducing the multienzyme complex. Hence, the complementation pattern of the first four alleles can be explained by a reduction in the amount of an *r* gene protein defective in CPSase activity. The complementation pattern of r^{hd4} can be explained by postulating that a further reduction in the amount of this *r* gene protein results in a limiting amount of ATCase activity but not DHOase activity.

Analysis of the genomic sequence organization of the P-induced mutations. Since the phenotypic and complementation data suggest that the mutations alter the amount rather than the structure of the *r* gene product, it was of interest to see if the DNA sequence organization at the *r* gene corroborated these results. Moreover, since it had been shown that P-induced mutations are insertions (28), it was expected that the five mutations would be the result of insertions within noncoding regions of the *r* gene. To test this hypothesis, DNAs from the five mutations and the progenitor allele were digested with appropriate restriction enzymes, electropho-

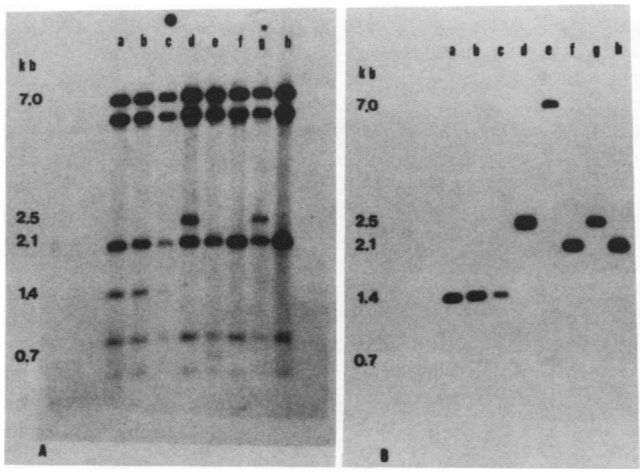

FIG. 3. Southern blots of the hybrid dysgenesis-induced *r* mutations. Genomic DNA was digested with *Eco*RI, electrophoresed in a 0.7% (wt/vol) agarose gel, and transferred to a nitrocellulose filter. The same filter was probed with ^{32}P-labeled phage 91 (A) and pBRH17 (B) (Fig. 2A). The genotypes that were probed are as follows: $r^{pyr1\text{-}16}$ (a), *Rev-*$r^{16\text{-}1}$ (b), *Rev-*$r^{16\text{-}4}$ (c), r^{hd1} (d), r^{hd4} (e), r^{hd2} (f), r^{hd5} (g), and r^{hd3} (h). The size of each appropriate fragment is on the left of each autoradiogram.

resed, and transferred to nitrocellulose filters. The filters were probed with a set of overlapping lambda clones which cover a 60-kb region, including the *r* gene (Fig. 2A; 29). This analysis revealed that the only difference between the five P-induced mutations and the progenitor allele throughout this 60-kb region is localized to a small 1.4-kb *Eco*RI fragment that contains the 5′ end of the *r* gene (Fig. 3). Figure 3A shows the arrangement of *Eco*RI fragments obtained when genomic digests were probed with the lambda phage recombinant 91, which contains this 5′ fragment. A comparison of the hybrid dysgenesis-induced mutations and the progenitor allele revealed that the 1.4-kb *Eco*RI fragment is missing in each of the mutations, whereas the other *Eco*RI fragments complementary to phage 91 are unaffected. These findings indicate that all of the mutations contain an insertion of foreign DNA in the 1.4-kb *Eco*RI fragment.

To confirm this conclusion, the same filter was probed with pBRH17, a product of the subcloning of the 5′ 1.4-kb *Eco*RI fragment into pBR322 (Fig. 3B). Four of the mutations, r^{hd1}, r^{hd2}, r^{hd3}, and r^{hd5}, can be interpreted as insertions of elements which do not contain *Eco*RI sites. The insertions of r^{hd1} and r^{hd5} are about 1.1 kb long, while those of r^{hd2} and r^{hd3} are about 700 bp in length. The fifth mutation, r^{hd4}, is probably an insertion which contains a single *Eco*RI site, as two new fragments are produced. Subsequent restriction mapping has confirmed this. This insertion is about 6.2 kb long.

Restriction mapping of the insertions. To further analyze the position of the insertions within the 5′ *Eco*RI fragment, the mutant 5′ *Eco*RI fragments were cloned into lambda gt-lac and then subcloned into pBR322. The subcloned fragments were then analyzed by restriction digests and by cross-hybridization with both the wild-type 5′ *Eco*RI fragment in pBRH17 and the full-length P element in pBRπ25.1. Several findings are of interest.

First, as was expected from the genomic analyses, all of the mutations are associated with insertions of DNA in the 1.4-kb *Eco*RI fragment (Fig. 4). Four of these insertions, r^{hd1}, r^{hd2}, r^{hd3}, and r^{hd5}, are small P elements. These elements retain at least a portion of the terminal 31-bp inverted repeats, judged by presence of the *Ava*II sites of the inverted

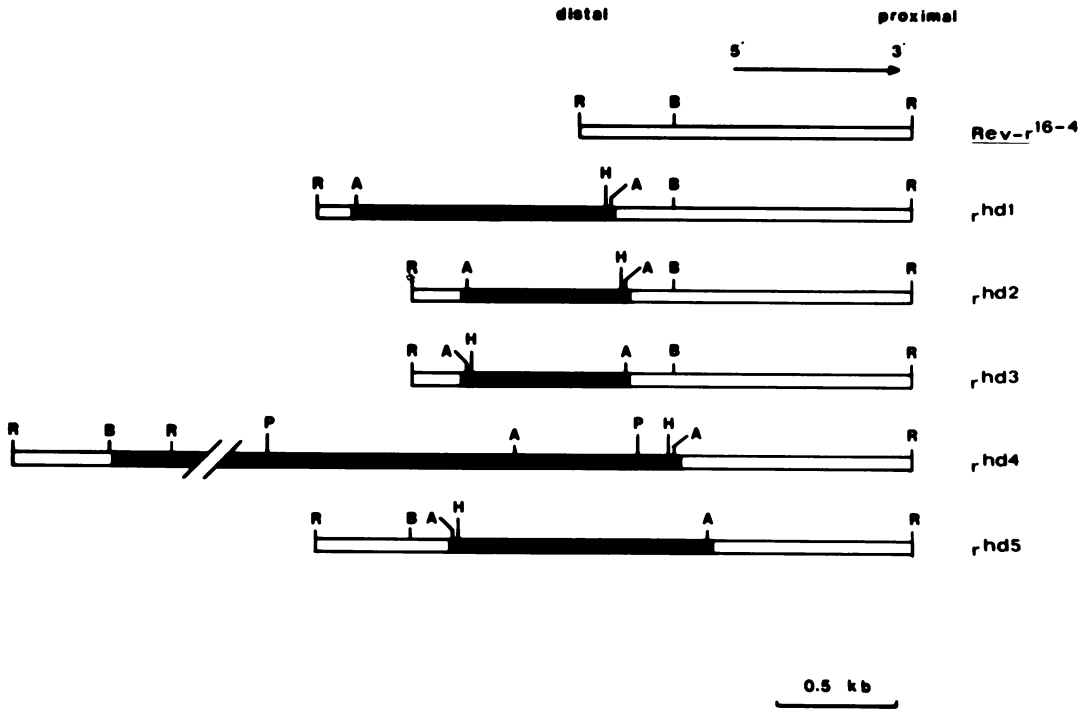

FIG. 4. Restriction map of *r* mutations. The inserted DNAs are indicated by the heavy black lines. The putative start of transcription and the orientation of the gene on the chromosome with respect to the centromere (distal and proximal) are indicated. Abbreviations: A, *Ava*II; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI.

TABLE 3. Gene message levels standardized to *adh* message levels[a]

Mutant	Relative message level
$r^{pyr1\text{-}16}$	1.00
$Rev\text{-}r^{16\text{-}4}$	3.03
r^{hd1}	1.99
r^{hd2}	0.88
r^{hd3}	0.74
r^{hd4}	0.33
r^{hd5}	0.29

[a] The relative amount of *r* gene message for each mutant was divided by the corresponding value for *adh* message. The quotients were then standardized to the value for $r^{pyr1\text{-}16}$, which was given the arbitrary value of 1.00.

repeats, and presumably are derived from the full-length 2.9-kb P element by internal deletions. Surprisingly, the fifth mutant, r^{hd4}, which is an insertion of 6.2 kb and hence more than twice the size of the full-length P element, also contains a small P element. This element is approximately 700 bp long and maps to the righthand edge of the insertion.The remaining DNA shows no homology to the P element and is presumably DNA from elsewhere in the genome. This possibility is currently being investigated.

Second, all of the insertions map upstream of the proposed start of transcription (Fig. 4). The 5′ end of the wild-type transcription unit is 200 to 300 bp to the right of the *Bam*HI site in the 1.4-kb *Eco*RI fragment (30; S. Tsubota, unpublished data; B. Zerges, personal communication). Three of the insertions, r^{hd1}, r^{hd2}, and r^{hd3}, map to the left of the *Bam*HI site and are therefore well outside the structural gene. The r^{hd1} insertion is located about 250 bp to the left of the *Bam*HI site or about 500 bp upstream of the start of transcription. Both the r^{hd2} and r^{hd3} insertions are about 700 bp long and map to about 190 bp to the left of the *Bam*HI site. Interestingly, the two P elements are in opposite orientation, which may account for the differences in the wing and female sterility phenotypes of the two mutants. The insertions of the two remaining mutants, r^{hd4} and r^{hd5}, are located about 100 and 170 bp to the right of the *Bam*HI site, respectively, and hence are closer to the start of transcription than are the other three insertions.

Transcripts in the P-induced mutations. The wing and fertility phenotypes and the positions of the mutations argue for the insertions being regulatory mutations upstream of the start of transcription. If this is indeed the case, one would expect that the amount of the transcript would be affected by these mutations. To test this possibility, total RNA from 3- to 4-day-old adult females was examined. Adult females were used because the expression in adult females should correlate well with the fertility of the flies. To correct for differences in the amount of RNA loaded onto the gel, the filter was probed first for *r* gene message and then for *adh* message. The amount of *r* gene message was standardized to the amount of *adh* message (Table 3). The northern blots of the *r* gene transcript are shown in Fig. 5. All of the stocks analyzed produce the wild-type-sized 7.3-kb transcript; however, the amounts in each stock vary (Fig. 5A). These results are consistent with the proposal that the mutations are all insertions upstream of the start of transcription. The first interesting comparison is between $r^{pyr1\text{-}16}$ and $Rev\text{-}r^{16\text{-}4}$ (Fig. 5A, lanes a and c). $Rev\text{-}r^{16\text{-}4}$ was isolated as a revertant of $r^{pyr1\text{-}16}$ and was proposed to be an overproducing mutation (35). Indeed, the transcript level in $Rev\text{-}r^{16\text{-}4}$ is threefold higher than the level in $r^{pyr1\text{-}16}$. The next comparison of interest is between the hybrid dysgenesis-induced mutants and their progenitor, $Rev\text{-}r^{16\text{-}4}$. Two of the mutants, r^{hd4} and r^{hd5}, have very low amounts of message and, as expected, are sterile. The other three mutants, r^{hd1}, r^{hd2}, and r^{hd3} produce higher amounts of the message. Of these mutants, r^{hd3} is sterile, a result that is not consistent with the considerable amount of transcript which is produced. However, some of the transcripts produced are clearly larger than the normal 7.3-kb message (Fig. 5A and B). Hence, the sterility of this mutant could be due to the production of aberrant *r* transcripts from new initiation sites. A quite different result is seen with r^{hd2}, which has a similarly sized P element inserted in the same position but in opposite orientation. This mutant produces a similar amount of *r* message, but the unusual transcripts are not present (Fig. 5A and B). This result is in agreement with the semifertility of r^{hd2}. (With regard to the production of aberrant transcripts, it may be significant that from the proposed direction of transcription of the P element [15], the P element in r^{hd3} could be transcribed in the same direction as the *r* gene.) Finally, r^{hd1} females have high levels of *r* message, i.e., almost twice as much as $r^{pyr1\text{-}16}$ females. Although r^{hd1} females are semifertile, they are not as fertile as would be expected if all the *r* message found in them was in the ovaries. This result suggests that in this mutant the *r* gene is being turned on in tissues other than those of the ovaries. This possibility is not too surprising, since the wing phenotype and fertility indicated that r^{hd1} affected the tissue distribution of the *r* gene product.

DISCUSSION

The purpose of the mutation schemes was to isolate regulatory mutations caused by the insertion of transposable

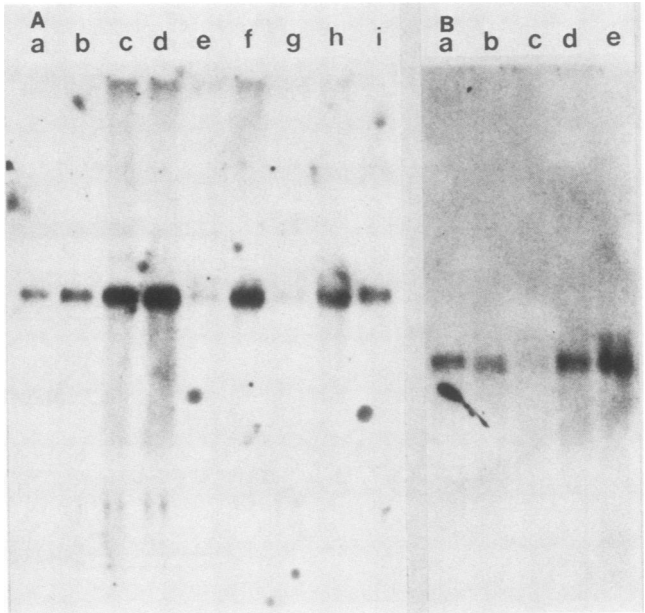

FIG. 5. Northern blots of *r* mutants. Total RNA from 3- to 4-day-old adult females was prepared, and 20 μg of each sample was electrophoresed in a 0.7% (wt/vol) agarose-formaldehyde gel and transferred to nitrocellulose filters. The filters were probed with three ^{32}P-labeled single-stranded probes (Fig. 2B). The genotypes tested are as follows: (A) $r^{pyr1\text{-}16}$ (a), $Rev\text{-}r^{16\text{-}1}$ (b), $Rev\text{-}r^{16\text{-}4}$ (c), r^{hd1} (d), r^{hd4} (e), r^{hd2} (f), r^{hd5} (g), r^{hd3} (h), and Canton S (i). (B) $Rev\text{-}r^{16\text{-}4}$ (a), r^{hd1} (b), r^{hd4} (c), r^{hd2} (d), and r^{hd3} (e).

elements. The mutations which were isolated have the properties one would expect of regulatory mutations. First, with respect to the wing phenotype, they are all hypomorphic mutations. Second, four of the mutations uncouple the wing phenotype and the maternal-effect lethality, which suggests alterations in the tissue distribution of the *r* gene product. Third, the complementation pattern of the mutations can be explained by a reduction in the amount of the *r* gene product. Fourth, the insertions are all clustered within a 400-bp region upstream of the start of transcription. Finally, the insertions affect the amount but, except in one case, not the size of the *r* gene transcript. Taken together, the data suggest that the insertions are upstream of the start of transcription, resulting in altered temporal and spatial patterns of regulation.

The transcript data reveal some interesting correlations between the positions of the insertions and the amount of transcript produced. Two of the mutants, r^{hd4} and r^{hd5}, have low levels of *r* transcript in adult females and are located 100 and 170 bp, respectively, to the left of the *Bam*HI site at the 5′ end of the gene. The remaining three mutants have nearly normal levels of *r* transcript and are clustered 190 to 250 bp to the left of the *Bam*HI site. These two clusters coincide with the position of two nuclease-hypersensitive regions at the 5′ end of the *r* gene. The first hypersensitive region maps from the *Bam*HI site to 200 bp to the right of the *Bam*HI site, and the second site maps 170 to 250 bp to the left of the *Bam*HI site (A. Udvardy and P. Schedl, unpublished results).

One plausible explanation for the data is as follows. The region upstream of the transcribed region of the *r* gene is involved in its normal spatial and temporal regulation. Structurally, this region can be divided into two nuclease-hypersensitive regions separated by a nonsensitive region. The nuclease-hypersensitive region closest to the start of transcription is involved primarily with the expression of the gene in the ovaries, and mutations in this region result in a lowering of transcription in the ovaries and, thus, female sterility. The second nuclease-hypersensitive region is not primarily involved with expression in the ovaries, and consequently, mutations in this region result in higher levels of *r* transcript in adult females and semifertility. Also, the data on r^{hd1} indicate that mutations in this region may result in alterations in *r* expression in other organs, e.g., a decrease in activity in imaginal disks and an increase in activity in certain adult tissues. Experiments such as in situ hybridization to the *r* transcript in tissue sections and in vitro site-specific mutagenesis can test the validity of this model.

The mutation schemes were very successful in isolating insertions in the 5′ noncoding region of the *r* gene. There are several reasons for the success of these schemes. First, P elements may preferentially insert in the 5′ noncoding regions of genes. None of the insertions recovered in this screen map within the structural gene, but instead are clustered within a 400-bp region at the 5′ end of the gene. This region is not a hot spot for insertion mutations in general, since none of the other known insertion mutations in the *r* gene are in this region. Presumably, this clustering of the P-element insertions could be explained at least in part by the partial sequence specificity of P insertions (24). However, other factors may be involved. The P-element insertions at the *r* gene suggest that the chromatin structure of this region determines susceptible sites of insertion, since all of the P elements inserted into nuclease-hypersensitive regions. In this regard, it will be of interest to determine if P-element insertions at other loci also map to nuclease-sensitive regions. Second, the use of the leaky allele, $Rev\text{-}r^{16\text{-}4}$, increased the sensitivity of the screens. This result is very important, since the P insertions did not drastically decrease the expression of the *r* gene in the wing imaginal disks. In fact, only r^{hd4} would have been isolated if a wild-type progenitor allele had been used, reducing the mutation frequency to 1 in 27,700. It may be that P elements insert in the 5′ noncoding regions of genes at relatively high frequencies and cause minor changes in the levels of transcription. These insertions will go undetected unless the screens are designed to detect leaky mutations.

The P insertions may offer a clue about why conventional mutagens have been ineffective in generating control mutations. The P insertions cause relatively minor decreases in *r* gene expression, yet the nature of the structural defects at the *r* gene is very drastic. The defects are all insertions ranging from 700 bp to 6.2 kb. If these drastic changes are having such minor effects, then one would not expect single-base-pair changes to have much effect in this region of the gene. This finding explains the ineffectiveness of EMS, a base substitution-inducing mutagen, in producing control mutations. In contrast, EMS is very effective in producing coding-region mutations. Irradiation can produce drastic changes such as chromosomal rearrangements. The ineffectiveness of irradiation in inducing control mutations is most likely due to the small target size of the control region, so that the majority of irradiation-induced mutations are large deficiencies which include the gene in question. This situation has been shown to be the case for gamma ray-induced mutations at the *adh* locus (1).

LITERATURE CITED

1. **Ashburner, M., C. S. Aaron, and S. Tsubota.** 1982. The genetics of a small autosomal region of *Drosophila melanogaster* containing the structural gene for alcohol dehydrogenase. V. Genetic characterization of X-ray induced *Adh* null alleles. Genetics **102**:421–435.
2. **Bingham, P. M., M. G. Kidwell, and G. M. Rubin.** 1982. The molecular basis of P-M hybrid dysgenesis: the role of the P element, a P strain-specific transposon family. Cell **29**:995–1004.
3. **Brothers, V., S. Tsubota, S. Germeraad, and J. Fristrom.** 1978. The rudimentary locus of *Drosophila melanogaster*: partial purification of a carbamyl phosphate synthetase-aspartate transcarbamylase-dihydroorotase complex. Biochem. Genet. **16**:321–332.
4. **Burr, B., and F. A. Burr.** 1982. *Ds* controlling elements of maize at the *Shrunken* locus are large and dissimilar insertions. Cell **29**:977–986.
5. **Carlson, P.** 1971. A genetic analysis of the *rudimentary* locus of *Drosophila melanogaster*. Genet. Res. **17**:53–81.
6. **Chovnick, A., W. Gelbart, M. McCarron, B. Osmond, E. Candido, and D. Bailie.** 1976. Organization of the rosy locus in *Drosophila melanogaster*: evidence for a control element adjacent to the xanthine dehydrogenase structural element. Genetics **84**:233–255.
7. **Doring, H.-P., M. Freeling, S. Hake, M. Johns, R. Kunze, A. Merckelbach, F. Salamini, and P. Starlinger.** 1984. A *Ds*-mutation of the *Adh1* gene in *Zea mays* L. Mol. Gen. Genet. **193**:199–204.
8. **Engels, W. R.** 1979. Hybrid dysgenesis in *Drosophila melanogaster*: rules of inheritance of female sterility. Genet. Res. **33**:219–236.
9. **Errede, B., T. S. Cardillo, F. Sherman, E. Dubois, J. Deschamps, and J. M. Waime.** 1980. Mating signals control expression of mutations resulting from insertion of a transposable repetitive element adjacent to diverse yeast genes. Cell **22**:427–436.
10. **Falk, D.** 1977. Genetic mosaics of the *rudimentary* locus of *Drosophila melanogaster*: a genetical investigation into the physiology of pyrimidine synthesis. Dev. Biol. **58**:134–147.

11. **Falk, D., and D. Nash.** 1974. Pyrimidine auxotrophy in *Drosophila*: normal-winged auxotrophic mutants and dominant auxotrophy at the *rudimentary* locus. Mol. Gen. Genet. **131**:339–349.
12. **Goldberg, D. A.** 1980. Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene. Proc. Natl. Acad. Sci. USA **77**:5794–5798.
13. **Green, M. M.** 1963. Interallelic complementation at the *rudimentary* wing locus in *Drosophila melanogaster*. Genetica **34**:242–252.
14. **Hayward, W., B. Neel, and S. Astrin.** 1981. Activation of cellular *onc* genes by promoter insertion in ALV-induced lymphoid leukosis. Nature (London) **290**:475–479.
15. **Karess, R., and G. Rubin.** 1984. Analysis of P transposable element functions in Drosophila. Cell **38**:135–146.
16. **Kidwell, M. G., J. F. Kidwell, and J. A. Sved.** 1977. Hybrid dysgenesis in *Drosophila melanogaster*: a syndrome of aberrant traits including mutation, sterility and male recombination. Genetics **86**:813–833.
17. **Lindsley, D., and E. Grell.** 1972. Genetic variations of *Drosophila melanogaster*. Carnegie Institution publications no. 627. Carnegie Institution of Washington, Washington, D.C.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, D. K. Sim, and A. Estradiatis.** 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell **15**:687–701.
20. **McClintock, B.** 1951. Chromosome organization and gene expression. Cold Spring Harbor Symp. Quant. Biol. **16**:13–47.
21. **McGinnis, W., A. W. Shermoen, and S. K. Beckendorf.** 1983. A transposable element inserted just 5′ to a Drosophila glue protein gene alters gene expression and chromatin structure. Cell **34**:75–84.
22. **Neel, B., W. Hayward, H. Robinson, J. Fang, and S. Astrin.** 1981. Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNA's: oncogenesis by promoter insertion. Cell **23**:323–334.
23. **O'Hare, K., R. Levis, and G. Rubin.** 1983. Transcription of the *white* locus in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **80**:6917–6921.
24. **O'Hare, K., and G. Rubin.** 1983. Structure of P transposable elements and their sites of insertion and excision. Cell **34**:25–35.
25. **Payne, G., J. Bishop, and H. Varmus.** 1982. Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. Nature (London) **295**:209–213.
26. **Rawls, J. M., Jr.** 1979. The enzymes for de novo pyrimidine biosynthesis in *Drosophila melanogaster*: their localization, properties and expression during development. Comp. Biochem. Physiol. **62B**:207–216.
27. **Roeder, G. S., and G. R. Fink.** 1980. DNA rearrangements associated with a transposable element in yeast. Cell **21**: 239–249.
28. **Rubin, G. M., M. G. Kidwell, and P. M. Bingham.** 1982. The molecular basis of P-M hybrid dysgenesis: the nature of induced mutations. Cell **29**:987–994.
29. **Segraves, W. A., C. Louis, P. Schedl, and B. Jarry.** 1983. Isolation of the *rudimentary* locus of *Drosophila melanogaster*. Mol. Gen. Genet. **189**:34–40.
30. **Segraves, W. A., C. Louis, S. Tsubota, P. Schedl, J. Rawls, and B. Jarry.** 1984. The *rudimentary* gene of *Drosophila melanogaster*. J. Mol. Biol. **175**:1–17.
31. **Snyder, M., D. Kimbrell, M. Hunkapiller, R. Hill, J. Fristrom, and N. Davidson.** 1982. A transposable element which splits the promoter region inactivates a *Drosophila* cuticle protein gene. Proc. Natl. Acad. Sci. USA **79**:7430–7434.
32. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
33. **Thompson, J., M. Ashburner, and R. Woodruff.** 1977. Presumptive control mutation for alcohol dehydrogenase in *Drosophila melanogaster*. Nature (London) **270**:363.
34. **Toh-e, A., Y. Kaneko, J. Akimaru, and Y. Oshima.** 1983. An insertion mutation associated with constitutive expression of repressible acid phosphatase in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **191**:339–346.
35. **Tsubota, S. I., and J. W. Fristrom.** 1981. Genetic and biochemical properties of revertants at the *rudimentary* locus in *Drosophila melanogaster*. Mol. Gen. Genet. **183**:270–276.
36. **Williamson, V. M., E. T. Young, and M. Ciriacy.** 1981. Transposable elements associated with constitutive expression of yeast alcohol dehydrogenase II. Cell **23**:605–614.