P Element-Mediated *in Vivo* Deletion Analysis of *white-apricot*: Deletions Between Direct Repeats Are Strongly Favored

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ABSTRACT

We have isolated and characterized deletions arising within a *P* transposon, *P[*hsw^a*]*, in the presence of *P* transposase. *P[hsw^a]* carries *white-apricot* (*w^a*) sequences, including a complete *copia* element, under the control of an *hsp70* promoter, and resembles the original *w^a* allele in eye color phenotype. In the presence of *P* transposase, *P[hsw^a]* shows a high overall rate (approximately 3%) of germline mutations that result in increased eye pigmentation. Of 234 derivatives of *P[hsw^a]* with greatly increased eye pigmentation, at least 205 carried deletions within *copia*. Of these, 201 were precise deletions between the directly repeated 276-nucleotide *copia* long terminal repeats (LTRs), and four were unique deletions. High rates of transposase-induced precise deletion were observed within another *P* transposon carrying unrelated 599 nucleotide repeats (yeast 2μ FLP; recombinase target sites) separated by 5.7 kb. Our observation that *P* element-mediated deletion formation occurs preferentially between direct repeats suggests general methods for controlling deletion formation.

P element-mediated transformation of Drosophila is an extremely useful technique for the study of recombinant genes *in vivo*. Although much insight into the mechanism of *P* element transposition has been gained, there is as yet no means of controlling where a *P* element will insert into the genome. Consequently, each independently derived transgene resides in a unique chromosomal context that affects its expression, and quantitative measurement of small differences in expression between transgenic alleles is problematic. The customary solution is to isolate several independent *P* element insertions for each allele and compare their expression. It has been repeatedly observed that all but a small number of transgenes (typically less than 5% of the total) show expression within twofold of each other (LAURIE-AHLBERG and STAM 1987; SPRADLING and RUBIN 1983). For most studies, these differences present no serious problems, and two or three independent insertions are sufficient. However, quantitative measurement of small differences in expression requires relatively more transformants. For example, LAURIE-AHLBERG and STAM (1987) recommend examination of no less than six transformant lines in order to be 80% confident of detecting a 50% difference in xanthine dehydrogenase expression. Clearly, the measurement of small differences in gene expression by *P* element-mediated transformation would be greatly simplified if position effects could be eliminated.

The ability to compare *P* transposons with identical flanking sequences would be extremely useful. Recently, GLOOR *et al.* (1991) and BANGA and BOYD (1992) have described strategies for gene replacement in Drosophila in which position effects are overcome by placing mutations in the context of the natural gene. While gene replacement does indeed eliminate the problem of position effect, these techniques require that one first obtain a *P* element insertion in the gene of interest. This requirement adds considerably to the time and manipulations required and is not always possible to fulfill. For example, we are interested in alterations at the *copia* insertion site of *w^a*. The only available *P* element insertion site within *white* (*w^{hd}*) is 2,030 nucleotides from the site of the *w^a copia* insertion. According to the estimates published by GLOOR *et al.* (1991) and NASSIF and ENGELS (1993), less than 8% of conversion events initiated by excision of that *P* element should include the *w^a* site. In fact, ENGELS *et al.* (1990) observed only two conversions to apricot among 358 reversions of the *w^{hd}* allele when the *w^a* allele was used as a donor for gene conversion. Presumably the vast majority of conversions were to wild type because the site of the *w^a copia* insertion was simply too far from the *w^{hd}* site for efficient co-conversion.

Another approach to the problem of position effects is the isolation of deletion derivatives from preexisting *P* element insertions. Such deletions arise at relatively high rates in response to *P* transposase, and this technique was proposed as a general strategy for analysis of gene structure by DANIELS *et al.* (1985), who examined deletions within the promoter of the *rosy* gene. The method was recently used by LAPIE *et al.* (1993) to

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examine the *Fat-body-protein-1* gene promoter. *P* element-mediated *in vivo* deletion analysis has also been used to examine the role of *cis* sequences in position-effect variegation at the *brown* locus (DREESEN *et al.* 1991).

In this report, we describe the application of *P* element-mediated *in vivo* deletion analysis to w^a. Interest in w^a arises from extragenic modifiers that alter its intermediate eye color phenotype, and the isolation of such modifiers appears to be an excellent way to identify genes involved in critical steps of RNA processing (X. PENG and S. M. MOUNT, submitted; ZACHAR *et al.* 1987). However, the sensitivity of the apricot phenotype to second site modifiers is due in part to a strong phenotypic response to quantitatively small alterations in *white* expression, making it desirable to reduce other influences on expression, such as position effects. Here we describe the successful isolation of a number of deletions within the *P[hsw^a]* allele, including several informative derivatives. However, in contrast to earlier studies, we also observed high rates of partial phenotypic reversion associated with a single reproducible event. The majority of the partial revertants isolated in our study are *P[hsw^{aRsLTR}]* alleles resulting from precise deletion between the 276-bp terminal repeats of the *copia* element in what appears to be a homology-dependent process. In order to investigate the possibility that high rates of direct-repeat recombination are a general property of *P* elements containing direct repeats, we looked for similar events involving unrelated 599 nucleotide repeats (FRTs) separated by 5.7 kb in another *P* transposon, and such events were indeed observed. Our observation that *P* element-mediated deletion formation occurs preferentially between direct repeats suggests general methods for generating useful derivatives of a parental *P* transposon in a common chromosomal context.

MATERIALS AND METHODS

Fly stocks (general): Alleles are generally described in LINDSLEY and ZIMM (1992) and the original sources of stocks used in this study are described in MOUNT *et al.* (1988) and KURKULOS (1991). w^{1118}; *P[>w^{hs}>]*67A and w^{1118}; *hsFLP Sco/S^2* CyO were provided by KENT GOLIC and are described in GOLIC (1991). *y* $w^{67c23(2)}$ was provided by VINCENZO PIRROTTA and is described in STELLER and PIROTTA (1985). *CyO/Sp*; *Sb*, *P[ry$^+$*, *Δ2-3]*(99B)/*TM6,Ubx* was obtained from WILLIAM ENGELS and is described in ROBERTSON *et al.* (1988).

Transformant nomenclature: Transformants are named as described in ASHBURNER (1989). Information in square brackets refers to sequences included within a *P* element, and information outside the brackets (sometimes omitted) indicates the particular insertional event. Thus, derivatives of the original *P[hsw^a]* allele described in this report that differ between the *P* termini but not in the flanking DNA are distinguished from it by superscripts within the brackets. For example, *P[hsw^{aR227}]*1 indicates a reversion of *P[hsw^a]*1, with 227 an arbitrary designation of the revertant line.

Generation of transgenic flies: The plasmid phswa was generated by substituting the *Xba*I-*Sac*I fragment from p3922a15 (MOUNT and RUBIN 1985) for the *Xba*I-*Sac*I fragment from CahswB4 (STELLER and PIROTTA 1985); it was used to transform *y* w^{67c23} flies using pπ25.7wc (KARESS and RUBIN 1984) as a transposase source and the hsw^a allele itself as a transformation marker.

Molecular biology: Standard polymerase chain reaction (PCR) amplification was conducted on genomic DNA (prepared as described below) for 30–35 cycles in the presence of 20 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 25 mM KCl, 50 mM dNTPs and 20 pmol of each oligonucleotide. Oligonucleotides were:

JW2, a 25-mer
(CGGAATTCAGCGACACATACCGGCG);
MK7, a 35-mer
(TGGAATTCAAATTGGTAATTGGACCCTTTATGTTG);
MK8, a 30-mer
(TATTTGAAGGGCACTATCGATATGAAATTG);
MG5, a 32-mer
(GCAGGGTCGTCTTTCCGGCACCGGAACTGCCC).

Pairs used in the initial screen of 234 lines were JW2 and MK7 ("primer pair 1") and MK7 and MK8 ("primer pair 2"). Relevant portions of *P[hsw^{aR197}]*, *P[hsw^{aR256A}]*, *P[hsw^{aR256C}]*, and 11 apparent *P[w^{aRsLTR}]* alleles, chosen at random, were amplified with appropriate primers, gel purified, and sequenced after subcloning. The allele *P[hsw^{aR227}]* was recognized as a negative for amplification with primer pair 1; in this case a 3.0% agarose gel was used to separate a novel 201-nucleotide PCR product obtained using primer pair 3 (JW2 and MG5) from the 195-nucleotide product derived from the w^{1118} allele, and this product was directly sequenced as described below.

Genomic DNA was prepared as described by BALLINGER and BENZER (1989). For Southern analysis, DNA was digested overnight at 37°. Digests were fractionated in agarose, transferred to nitrocellulose and probed with plasmid pm12.5 as described (MOUNT *et al.* 1988). Verification of flanking sequences was performed by digestion with *Eco*RV and probing with *P* sequences (pπ25.7wc). Derivatives of *P[>w^{hs}>]* were analyzed using the plasmid CarFRT [a *Hin*dIII-*Bam*HI FRT fragment cloned into Carnegie 1 (GOLIC and LINDQUIST 1989)], as the probe.

Direct sequencing of single stranded DNA was performed according to ALLARD *et al.* (1991). PCR amplification products were purified using Centricon-30 microconcentrators (Amicon), and single-stranded templates were generated using a single oligonucleotide in a Gene Machine II (USA/Scientific Plastics) thermal cycler. These products were again microconcentrated, and sequencing was performed using the other PCR oligonucleotide as primer and Sequenase (U.S. Biochemical Corp.) according to the manufacturer's protocol. Double-stranded sequencing of pUC19 subclones (HSAIO 1991) or single stranded sequencing of M13 mp19 subclones (SANGER *et al.* 1977; TABOR and RICHARDSON 1987), was used to obtain the sequence of PCR-generated restriction fragments.

Reversion of the *P[hsw^a]* element: *y* w^{67c23} *P[hsw^a]*1 females were mated to *CyO/Sp*; *Sb*, *P[ry$^+$,Δ2-3]*(99B)/TM6, *Ubx* males. Single *y* w^{67c23} *P[hsw^a]*1; *CyO/+*; *Sb*, *P[ry$^+$ (Δ2-3)]*(99B)/+ mosaic male G_1 progeny were crossed to w^{1118} f^5 virgin females and Sb^+ female G_2 progeny with eye color phenotype darker than that of *P[hsw^a]* were selected. G_2 females were mated individually to w^{1118} f^5 males, and *X* chromosomes from single red-eyed G_3 male progeny were isolated and maintained with *C(1)DX, y w f*.

***P* transposase-induced *FRT* recombination:** w^{1118}/Y; *ru P[>w^{hs}>]*(75A) *e* males were mated to w^{1118}; *CyO/Sp*; *Sb*, *P[ry$^+$*,

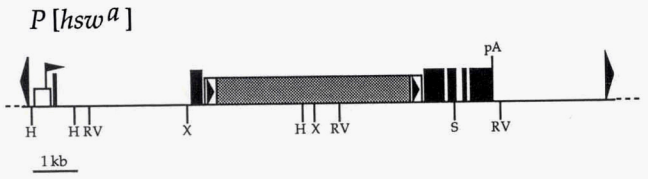


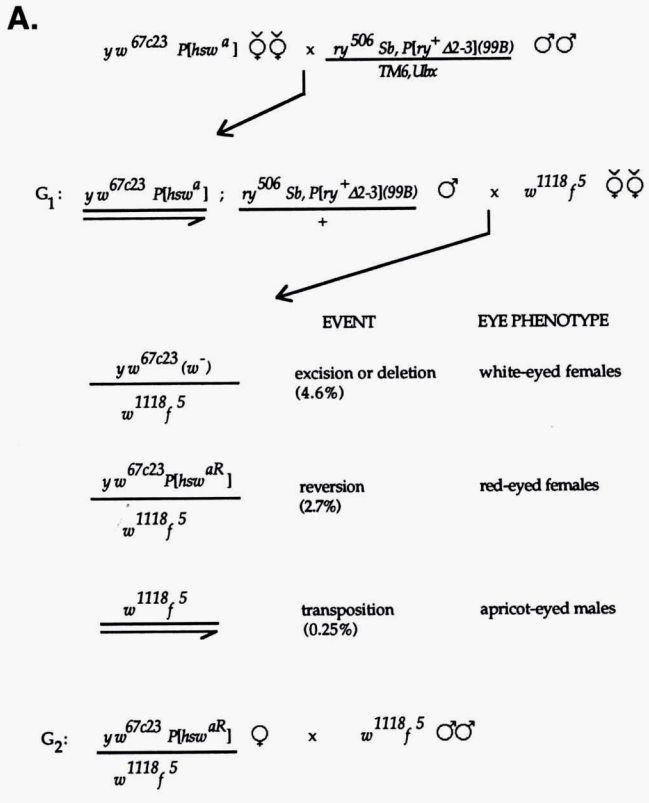
FIGURE 1.—Structure of the *P[hsw^a]* transposon. The structure of the phswa plasmid used for germline transformation to generate *P[hsw^a]* is shown. Sequences between the two *P* element termini (large black arrowheads) integrated into the *Drosophila* germline upon transformation. Exons from the *white* gene are indicated by solid black boxes. The start site for transcription from the *hsp70* promoter is indicated by the arrow, pA indicates the site of polyadenylation that forms the 3′ end of *white* sequences. The *copia* element is stippled, except for the LTRs, which are indicated by a white box with a small black arrowhead at each end. The *Xba*I (X) and *Sac*I (S) sites used to substitute sequences from w^a into the hsw^+ fusion gene are indicated, as are *Hin*dIII (H) and *Eco*RV (RV) sites.

Δ*2-3]*(99B)*/TM6,Ubx* females. 36 single mosaic male progeny of the genotype w^{1118}; *CyO* or *Sp*/+; *ru P[>w^{hs}>]*(75A) *e/Sb, P[ry$^+$, Δ2-3]* were mated to w^{1118}; *TM3/CxD* virgin females. One white-eyed Sb^+ male offspring from each cross was chosen at random for Southern analysis and subsequent genetic isolation of a *P[>w^{hs}*>]* derivative. As a positive control for homologous recombination between *FRT*s, w^{1118}/Y; *P[>w^{hs}>]*1A (67A) males were crossed to w^{1118}; *P[ry$^+$, hsFLP]*1A *Sco/CyO* females. The progeny of this cross were subjected to a 1 hr, 37° heat-shock approximately 48 hr after egg deposition in order to induce FLP activity. Genomic DNA was isolated from the mosaic male progeny of this cross and used for Southern analysis (see Figure 5B).

RESULTS

***P[hsw^a]* resembles the original *white-apricot* allele:** The application of *P* element-mediated deletion analysis to w^a required that we first generate a *P* element transgene that faithfully displayed all of the features that we wished to study. Using a previously described *hsp70-white* fusion (STELLER and PIROTTA 1985) as a starting point, sequences from *white-apricot* were substituted for the appropriate portions of *white* to create the *P[hsw^a]* element (see Figure 1). Following transformation, we observed that the *P[hsw^a]* allele conferred an apricot eye color similar to that of w^a flies (Figure 2B). This suggests that the *hsp70* promoter, without induction by heat shock, is comparable in strength to the *white* promoter in the relevant cells of the eye. Northern analysis of total RNA from *P[hsw^a]* flies shows that the pattern of RNAs generated from *P[hsw^a]* is very similar to that generated from w^a, and the response of the *P[hsw^a]* allele to *trans*-acting modifiers of w^a is identical to that of the original w^a allele (these results will be described in detail in a future manuscript [D. C. BRAATEN, M. KURKULOS and S. M. MOUNT, in preparation]). We concluded that the *P[hsw^a]* allele is a suitable surrogate for w^a in deletion analysis.

High rates of reversion of *P[hsw^a]* are observed in the presence of *P* transposase: In the course of other experiments, we noted that mosaic flies carrying both



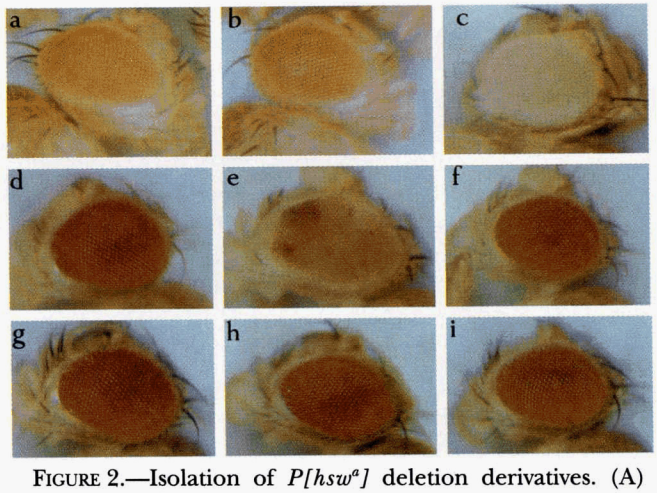


FIGURE 2.—Isolation of *P[hsw^a]* deletion derivatives. (A) The crossing scheme used to isolate deletion derivatives of the *P[hsw^a]*. Approximate rates are based on the experiments summarized in Table 1. (B) Phenotypes of selected alleles. **a**, A *white-apricot* male; **b**, a parental *P[hsw^a]* male. Genotype *y* w^{67c23}*P[hsw^a]*1; **c**, a *white* null male. Genotype w^{1118}; **d**, a wild-type *white* male. Genotype *y* w^{67c23}, *P[hsw$^+$]*; **e**, a G_1 mosaic male. Genotype *y* w^{67c23}*P[hsw^a]*1; *Sb P[ry$^+$, Δ2-3]99B/+*; **f**, a solo LTR deletion derivative. Genotype *y* w^{67c23}*P[hsw^{aRsLTR11A}]*1; **g**, the 256A deletion derivative. Genotype w^{1118}*P[hsw^{aR256A}]*1; **h**, the 227 (pseudo wild type) deletion derivative. Genotype w^{1118}*P[hsw^{aR227}]*1; **i**, the 197 deletion derivative. Genotype w^{67c23}*P[hsw^{aR197}]*1.

P[hsw^a] and the genomic transposase source *P[ry$^+$, Δ2-3]*(99B) (ROBERTSON *et al.* 1988) exhibited not only

TABLE 1

Rate of reversion and excision

Exp.	Source	Vials scored	Total no. of female progeny	Reddish female progeny: Vials	Reddish female progeny: Progeny
Reversion (red eye color)					
1	*P[hsw^a]1*	13	ND[a]	9[b]	26
2	*P[hsw^a]1*	48	1903	27 (56)[c]	51 (2.7)
				White female progeny: Vials	White female progeny: Progeny
Excision (white eye color)					
2	*P[hsw^a]1*	28	1003	13 (36)	46 (4.6)

[a] Not determined.

[b] A single *X* chromosome carrying a revertant allele was isolated from each of these nine vials, and each was shown by Southern and PCR analysis to be a *P[w^{aRsLTR}]* derivative (see text).

[c] Numbers in parentheses are percentages.

white patches in the eye, characteristic of clones resulting from somatic excisions of *P[hsw^a]*, but also unexpectedly dark red patches (see Figure 2Be for an example; every fly with this genotype shows a similar pattern). The color in these patches was clearly darker than would be expected from duplicative transposition, which suggested that *P* transposase was inducing phenotypic reversion of the *P[hsw^a]* allele in clones within the eyes of these dysgenic flies. In a pilot experiment to recover similar events in the germline (Table 1, experiment 1), we mated single *y w^{67c23} P[hsw^a];Δ2-3/+* mosaic males to *w^{1118}f^5* females. The progeny of 9 of 13 such crosses included flies with red rather than apricot eyes. A single line was established from each of the nine independent events. The phenotypes of these lines were indistinguishable from one another and very similar to that of a spontaneous partial revertant of *w^a* (*w^{aRsLTR}*) which retains a single LTR at the site of the *copia* insertion (CARBONARE and GEHRING 1985; ZACHAR *et al.* 1985; MOUNT *et al.* 1988). Southern analysis confirmed that all nine were identical to the *w^{aRsLTR}* allele with respect to *copia* sequences, and this was confirmed by PCR analysis of genomic DNA as described below (data not shown). All of the exceptional G_2 progeny (26/26) were female, and G_3 analysis showed *white* expression relatively closely linked to *forked*, indicating that reversion was probably not accompanied by transposition. This was confirmed by Southern analysis, which showed no *P* sequences with different flanking sequences in any of these lines (data not shown).

To obtain better estimates of the rate of reversion, this experiment was then repeated on a somewhat larger scale, with the results shown in Table 1 (experiment 2). The relatively high frequency of reversion was confirmed, with 27 of 48 vials containing some revertants, and 51 of 1903 transmitted chromosomes (2.7%) associated with reversion. In fact, the rate of mutation of *P[hsw^a]* to a darker phenotypic state is comparable to its rate of mutation to a null phenotypic state (4.6%; recorded in Table 1).

A screen for deletions within *copia* yielded primarily *P[w^{aRsLTR}]* derivatives: Previous studies of *w^{aRE714}*, an EMS-induced revertant of *w^a*, indicated the existence of a region required for *copia* polyadenylation at least 312 bases upstream of the site of polyadenylation in the 3′ LTR (KURKULOS *et al.* 1991). To isolate additional novel and informative deletions that would further define these or other regulatory sequences, we carried out a large-scale screen for revertants of *P[hsw^a]*. Following the experimental design shown in Figure 2A, we mated *y w^{67c23} P[hsw^a]1;CyO/+; Sb, P[ry$^+$,Δ2-3](99B)/+* males singly to *w^{1118}f^5* females. A range of revertant phenotypes was observed, from dark apricot or brown to wild type (robust red), although the majority exhibited a *w^{aRsLTR}* eye color phenotype (flat red). Of 342 vials with progeny, 245 contained revertant progeny, and recognizably distinct phenotypes allowed us to count a total of at least 362 independent events. One representative female from each recognizably distinct class was selected from each vial, and *X* chromosomes carrying revertant alleles were isolated from single non-mosaic males in the next generation.

We analyzed 234 of these lines further, using a PCR screen designed to identify *w^{aRsLTR}* revertants or deletions within the 3′ end of *copia* (Figure 3). One pair of oligonucleotides flanking the *copia* insertion site was used to identify *w^{aRsLTR}* revertants (primer pair 1 in Figure 3). Oligonucleotide JW2 hybridizes to the exon upstream of the *copia* insertion. Oligonucleotide MK7 hybridizes immediately adjacent to the *copia* insertion and has four nucleotides at its 3′ terminus complementary to *copia*, but not *white*, sequences. These four nucleotides prevent the amplification of *white* alleles that lack *copia* (including *w^{1118}* and *w^{67c23}*). PCR amplification with these two oligonucleotides yields a 404 bp product diagnostic of a *w^{aRsLTR}* derivative. A second pair of oligonucleotides (primer pair 2 in Figure 3) was used to identify any deletions affecting the 3′ portion of *copia*. Oligonucleotide MK8 hybridizes 120 bases upstream of the region deleted in *w^{aRE714}* and generates a 1067-nucleotide PCR product in combination with MK8 if the sequences between them have not been deleted. The vast majority of events analyzed (201 of 234, or 86%) gave the characteristic 404-bp *w^{aRsLTR}* amplification product with primer pair 1, and negative results with primer pair 2 (for an example, see Figure 3). All 201 putative *P[hsw^{aRsLTR}]* revertants exhibited the same flat-red eye color phenotype, as would be expected if they were identical at the molecular level, and the sequence of 11 such alleles, chosen at random, confirmed that they were indeed *P[hsw^{aRsLTR}]* revertants (data not shown). The second largest class of revertant (29, or 12%) gave negative results with primer pair 1, and 1.1-kb products with primer pair 2. These are the results that

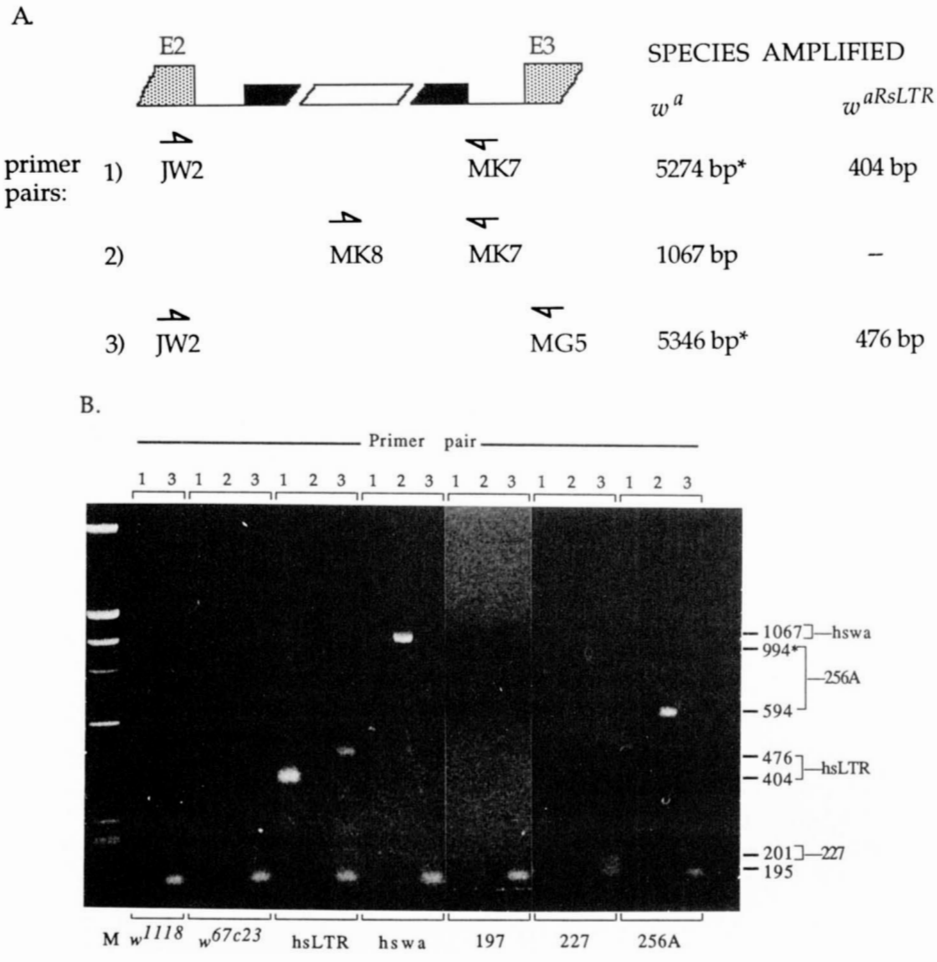
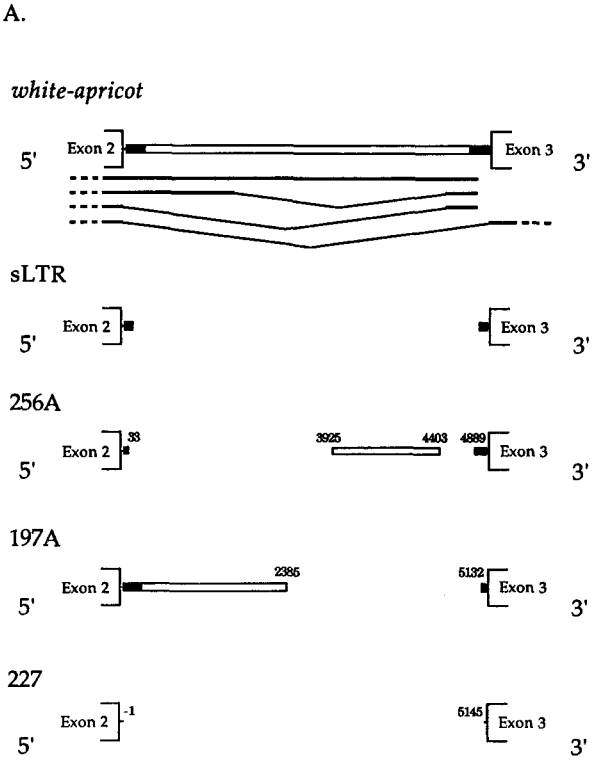


FIGURE 3.—**The PCR-based screen for deletions within *copia*.** (A) The positions of oligonucleotides used in the screen for deletion derivatives are shown beneath a schematic representation of the region of interest within the *P[hsw^a]* transgene. Primer pairs 1 and 2 were used in the initial screen of 234 lines, and primer pair 3 was used for follow-up confirmation. E2 and E3 (stippled) refer to exons 2 and 3 of the *white* gene, which flank the *copia* insertion. *Copia* sequences are represented by boxes that are thinner and either black (LTRs) or white (internal sequences). Products indicated with an asterisk (*) were not produced efficiently under our PCR conditions. (B) PCR analysis of genomic DNA from partial revertants of *P[hsw^a]*. The primer pairs used for each reaction are described in (A) and are indicated at the top of each lane. Genomic DNA templates are indicated below the gel: w^{1118}; w^{67c23}; hsLTR, *y* $w^{67c23}P[hsw^{aRsLTR11A}]1$; hswa, *y* $w^{67c23}P[hsw^a]$; 197, $w^{67c23}P[hsw^{aR197}]1$; 227, $w^{1118}P[hsw^{aR227}]1$; 256A, $w^{1118}P[hsw^{aR256A}]1$. Amplification products were resolved by electrophoresis in 1.6% agarose and TBE and stained with ethidium bromide. The 195-nucleotide product obtained using primer pair 3 results from the null *white* alleles w^{1118} or w^{67c23}, both of which are partial deletions that retain this portion of the *white* gene. The positions of the w^{aRsLTR} (hsLTR) 404- and 475-bp products, and the w^a (hswa) 1067-bp product are indicated to the right. Sizes of novel amplification products from the alleles 197, 227 and 256A are also shown. In the case of *P[hsw^{aR197}]*, neither the 2.5-kb amplification product expected with primer pair 1, nor the 2.6-kb amplification product expected with primer pair 3 were efficiently produced under our PCR conditions. The 994-bp amplification product from revertant *P[hsw^{aR256A}]* was visible on the original gel.

one obtains from the parental *P[hsw^a]* allele (the 5.3-kb amplification product expected from intact *P[hsw^a]* using primer pair 1 was only rarely seen under our PCR conditions). We concluded that alleles in this second class have no alteration within the 3′ portion of *copia*. Most of these revertants had lower levels of *white* expression than the w^{aRsLTR} alleles, but their phenotype was also more variable, ranging from dark apricot to nearly wild type. Southern analysis of two lines from this class with relatively dark pigmentation showed no changes within an interval spanning the *copia* insertion (*Hin*dIII-*Sal*I; data not shown). Either promoter rearrangement or local transposition of the entire *P[hsw^a]* element could account for these results. Although these two alleles showed no change, other derivatives in this class may be associated with the deletion of *copia* sequences upstream of the MK8 primer.

The structure of novel *P[hsw^a]* revertants; insertion/deletions and double deletions: Four revertants gave exceptional PCR products with one or both sets of oligonucleotides. All four, *P[hsw^{aR256A}]*, *P[hsw^{aR256C}]*, *P[hsw^{aR197A}]* and *P[hsw^{aR227}]* (referred to hereafter as 256A, 256C, 197 and 227) had eye color phenotypes distinguishable from w^{aRsLTR} alleles. 256A, 197 and 227 were more pigmented than w^{aRsLTR} alleles and indistinguishable from wild type (Figure 2B; the difference be-



B.

P[hswa^{R197}] 1 bp. repeat

Left	AGAAGCCATACTTAAAACATTTGAGAGTGTTTGGTGCAACTGTTTATGTGCATAT
Deletion	AGAAGCCATACTTAAAACATTTGAGATTATAAATTACAACATAAAGGGTCCAATT
Right	TTATTTATTAAGAAAGGAAATATAAATTATAAATTACAACATAAAGGGTCCAATT

P[hswa^{R227}] 2 bp. repeat

Left	CGGCTGATCTGTGTGAAATCTTAATAAAGTGTTGGAATATACTATTCAACCTACA
Deletion	CGGCTGATCTGTGTGAAATCTTAATAAACATAAAGGGTCCAATTACCAATTTGAA
Right	GAAAGGAAATATAAATTATAAATTACAACATAAAGGGTCCAATTACCAATTTGAA

P[hswa^{R256A}] (left deletion) 6 bp. repeat, 13 bp. insertion

Left	TATTCAACCTACAAAAATAACGTTAAACAACACTACTTTATATTTGATATGAATG
Deletion	TATTCAACCTACAAAAATAAAATAAATATGAATAAATAAATTATGAATTACTTAA
Right	ATTGTAATGCAGTTAGTACTCCTTTACCTAGTAAAATAAATTATGAATTACTTAA

P[hswa^{R256A}] (right deletion) 9/12 bp. repeat, 14 bp. insertion

Left	TATTAACATTAAACTAGAAAACCCCATTAAAATTTACGAAGACAATCAAGGCTGT
Deletion	TATTAACATTAAACTAGAAAACTAGAAAAACTAGAAAACCTACAAAAATAACGTT
Right	GTTATTTTTGAGGGGGCGTGTTGGAATATACTATTCAACCTACAAAAATAACGTT

P[hswa^{R256C}] 1 bp. repeat

Left	TTATAGACGTTATTTTTGAGGGGGCGTGTTGGAATATACTATTCAACCTACAAAA
Deletion	TTATAGACGTTATTTTTGAGGGGGCGTAAAACTCAAACATAAACTTGACTATTTA
Right	GTTGAAATAACTTAAAATATAAATCATAAAACTCAAACATAAACTTGACTATTTA

FIGURE 4.—The structure and deletion breakpoint sequences of *P[hsw^{aR}]* derivatives described in this study. (A) The structures in the vicinity of the second intron of *white-apricot* and four revertant alleles (sLTR, *P[hsw^{aRsLTR}]*; 256A *P[hsw^{aR256A}]*; 197A, *P[hsw^{aR197}]*; and 227, *P[hsw^{aR227}]*). Exons of the *white* gene are labeled, and the structures of the major RNA products of the *P[hsw^{a}]* allele are indicated schematically. *copia* sequences are indicated by a thin box; LTRs are shown in black. Numbers adjacent to the deletion endpoints refer to nucleotides within *copia* adjacent to the deletion (MOUNT and RUBIN 1985). Deletions are indicated by black spaces between boxes. For example, nucleotides 3925 through 4403 are retained in *P[hsw^{aR256A}]*, and this partial revertant carries two deletions, one removing nucleotides 34 through 3924, the other removing nucleotides 4404–4888. Because an identical *w^{aRsLTR}* product is generated by deletion between any two homologous points within the two 276-nucleotide LTRs, no numbers are listed. The LTRs extend from 1 to 276 and from 4871 to 5146. (B) The sequence across each of the novel deletion breakpoints depicted in (A) is shown, as is the sequence of a single deletion breakpoint from another allele, *P[hsw^{aR256C}]*. The one or two nucleotides present at both ends of the region deleted in *P[hsw^{aR197}]*, *P[hsw^{aR227}]* and *P[hsw^{aR256C}]* are underlined. In the case of the two deletions in *P[hsw^{aR256A}]*, the nucleotides repeated at each end of the deletion are underlined, and the inserted material is bracketed by a thin vertical line. The 15 nucleotide sequence AACCTACAAAAATAA, which flanks the two deletions in *P[hsw^{aR256A}]*, occurs once in each of the two LTRs in the wild type *copia* element (nucleotides 20–34 and 4889–4903).

tween these revertants and the *w^{aRsLTR}* alleles does not reproduce well). 256C, in contrast, had less pigmentation (not shown). Sequence analysis of PCR products, either directly or following cloning (see MATERIALS AND METHODS), provided the precise endpoints for each deletion indicated in Figure 4. 197A contains a single deletion that removes essentially the 3′ half of *copia* (nucleotides 2385–5132; MOUNT and RUBIN 1985). 256A is a double deletion, removing nucleotides 34–3924 and 4404–4888 of *copia*; each of these deletions is associated with a small insertion (discussed below). The remaining *copia* DNA in 256A is therefore a single LTR containing a 478 nucleotide insertion flanked by a 15 nucleotide duplication (see Figure 4B). 256C also contains two (or more) deletions within *copia*, only one of which (nucleotides 4871–5075) was sequenced. Unfortunately, this stock was lost after DNA had been isolated. Finally, 227 differs from a wild-type *white* allele by only 6 nucleotides, having deleted all but two nucleotides of *copia* and four nucleotides of the target site duplication. Southern analysis of 256A, 256C, 197 and 227 showed that these alterations were not accompanied by transposition (data not shown).

Recombination between 599-nucleotide FRT repeats: The high rate at which *w^{aRsLTR}* revertants were recovered in our screen, in particular the high proportion of all deletions that were of this type, led us to ask whether solo-LTR formation reflects a general tendency for deletions promoted by transposase to occur between directly repeated sequences. If this were the case, then one would expect that high rates of deletion could occur within other *P* elements that contain directly oriented repeats of a comparable length. To explore this possibility, we looked for deletions within *P[>w^{hs}>]*, a *P* transposon with two directly oriented copies of the 599-bp yeast 2μ FLP recombinase target (*FRT*; also abbreviated as ">") flanking a *white* minigene (GOLIC and LINDQUIST 1989). This *white* allele confers wild-type eye pigmen-

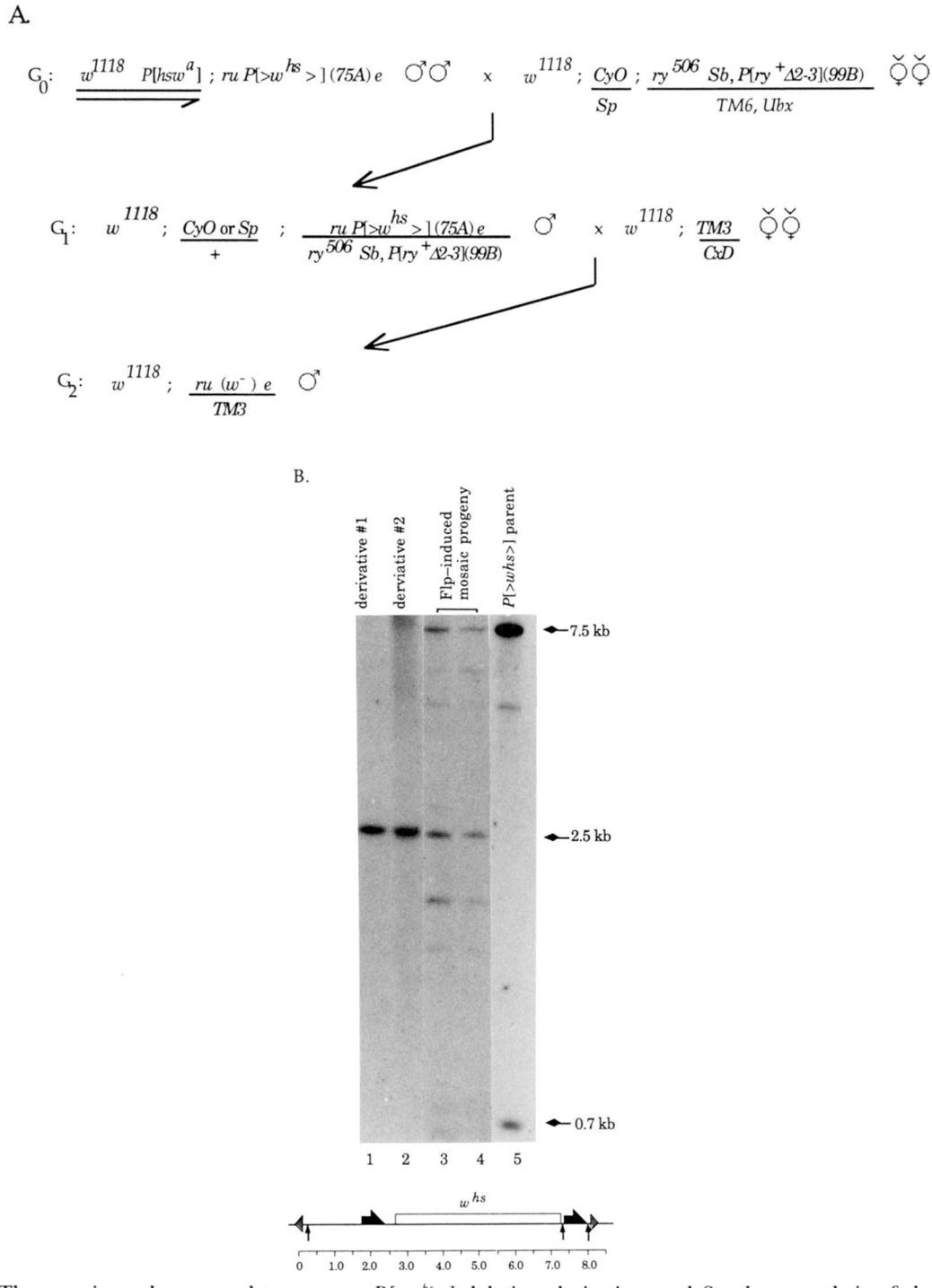


FIGURE 5.—The crossing scheme used to recover *P[>w^{hs}>]* deletion derivatives and Southern analysis of those derivatives. (A) Crossing scheme used to isolate *P[>w^{hs}>]* deletions. (B) Southern analysis of *P[>]* derivatives. Genomic DNA was digested with *Hin*dIII, and CarFRT was used as a probe. Note the comigration of 2.5-kb bands generated by *P* transposase mediated deletion (lanes 1 and 2) and FLP recombinase mediated deletion (lanes 3 and 4). Lanes 1 and 2 show DNA from two individual white-eyed G_3 males isolated as shown in (A). These were selected from among 36 candidates by an identical analysis on an earlier gel (not shown). Lanes 3 and 4 show DNA isolated from two single males made mosaic by heat-shock induction of the FLP recombinase as described in MATERIALS AND METHODS. Lane 5 shows DNA from flies carrying the parental construct. The 2.5-kb band is absent in the parental allele, but generated by either *P* transposase or FLP recombinase. The *FRT*-flanked w^{hs} gene (GOLIC and LINDQUIST 1989) is diagrammed beneath the autoradiogram. *Hin*dIII sites are indicated by vertical arrows. The *P* element terminal inverted repeats are indicated by shaded arrowheads. Coordinates indicate approximate distance in kilobase pairs from the leftmost *P* element end. Note the comigration of 0.7- and 7.5-kb bands in the mosaic flies and the parental allele.

tation, and site-specific recombination between the *FRT*s induced by FLP recombinase induces white-eyed derivatives with a single copy of the *FRT* (GOLIC and LINDQUIST 1989). To see if identical solo-*FRT* derivatives could be induced by *P* transposase in a reaction similar to that observed for *P[hsw^a]*, single males carrying both *P[>w^{hs}>]* and the Δ2-3 transposase source were generated and mated to the appropriate females (Figure 5A). An extremely high proportion of progeny inheriting the *P[>w^{hs}>]* chromosome were white-eyed (1, 250/1, 561), and every one of 36 *P[>w^{hs}>]*75A/*P[Δ2-3]*(99B) males yielded some white-eyed progeny. In contrast, control siblings lacking *P[Δ2-3]*(99B) yielded no white-eyed progeny. These white-eyed flies were either precise or imprecise deletion of the *w^{hs}* allele within the *P[>w^{hs}>]* element, and potentially represented solo-*FRT* derivatives of *P[>w^{hs}>]*. DNA was prepared from one white-eyed male offspring lacking *P[Δ2-3]*(99B) (as indicated by segregation of the *Sb* marker) from each mating, and examined by Southern analysis using an *FRT* DNA probe. Three of the 36 lines tested showed the 2.5-kb *Hin*dIII restriction fragment expected in the case of homologous recombination between the *FRT*s (two examples are shown in Figure 5B, lanes 1 and 2). Furthermore, this band comigrated exactly with that of a control solo-*FRT* derivative of *P[>w^{hs}>]* obtained from flies carrying both this target and a heat-inducible source of the yeast FLP recombinase (Figure 5B, lanes 3 and 4). All but one of the remaining 33 lines showed no signal, indicating that they retained no *FRT* sequences. These alleles probably result from excision of the entire *P[>w^{hs}>]* element, and the high rate at which they arise can be attributed to the presence of a homolog (see DISCUSSION). Thus, the solo-*FRT* derivatives expected in the case of homologous recombination were indeed generated, and at rates comparable to those at which the *P[hsw^{aRsLTR}]* derivatives arise.

DISCUSSION

One advantage of *w^a* as a system is that small differences in expression (less than twofold) result in phenotypic effects that are readily observable. While this is advantageous in the identification of extragenic modifiers with small but specific effects (X. PENG and S. M. MOUNT, submitted; RABINOW and BIRCHLER 1989; ZACHAR *et al.* 1987), it poses a problem for analysis of *cis*-acting sequences within *w^a*, because of variation in expression levels between insertion sites. Here, we have used the activity of *P* element transposase to isolate a number of deletions internal to the *P[hsw^a]* transposon. Despite the repeated isolation of a large number of identical events (discussed below), we have successfully generated a small collection of informative *w^a* derivatives in a chromosomal context identical to that of the original *P[hsw^a]* allele. These deletion derivatives have proven useful for studies of the molecular basis of *white-apricot* expression and its response to modifiers (KURKULOS 1993; D. C. BRAATEN, M. KURKULOS and S. M. MOUNT, in preparation).

Unique deletions are flanked by short repeats and can include short insertions that reiterate sequences immediately adjacent to the deletion: Previous descriptions of *P* element-mediated internal deletions (DANIELS and CHOVNICK 1993; DANIELS *et al.* 1985), carried out on target transposons lacking directly repeated sequences of significant length, did not identify preferred sites for deletion endpoints. Thus, it appears that internal deletions can occur at many sites. In this study, heterogeneous deletions would most likely result in white-eyed derivatives, and this class is indeed numerically comparable to the class of revertants (Table 1). We suspect that the four novel events whose sequences are presented in Figure 4 are typical of *P* element-mediated internal deletion breakpoints. Although a number of such breakpoints have been described previously, several points about these events are worth making.

Each of the two deletions in 256A is accompanied by insertion of DNA related to sequences at one end of the deletion. In the case of the left 256A deletion, the 13 inserted nucleotides (AATAAATATGAAT) are nearly identical to 14 nucleotides immediately to the right of the deletion (AATAAATTATGAAT). In the case of the right 256A deletion, the 14 inserted nucleotides (TAGAAAAACTAGAA) involve a repeat of eight nucleotides found to the left of the deletion breakpoint (AACTAGAA). Similar repetitious insertions of nucleotides immediately adjacent to the deletion are common in *P* element-mediated deletions, including both internal deletions and precise excisions (TAKASU-ISHIKAWA *et al.* 1992; JOHNSON-SCHLITZ and ENGELS 1993; reviewed by ENGELS 1989). This phenomenon was first described in the case of an internally deleted small *P* element (λπ51) by O'HARE and RUBIN (1983), who suggested a mechanism involving strand slippage and recopying of the same template by DNA polymerase. TAKASU-ISHIKAWA *et al.* (1992) favor a mechanism involving the nicking of hairpins formed by ligation of blunt ends. In the case of internal deletions, it is likely that repair synthesis initiated by excision of the entire *P* element precedes the generation of these inserted sequences. In any case, such structures probably result from repair synthesis rather than directly from the activities of *P* transposase. This idea is supported by the fact that similar structures have been observed at deletions induced by ionizing radiation. Examples include *exu^{DP3}* (T. CROWLEY and T. HAZELRIGG, personal communication) and *w^{sp4}* (DAVISON *et al.* 1985).

Recombination between long direct repeats is strongly favored: Perhaps our most striking observation is the high rate of precise deletions between the *copia* LTRs contained in *P[hsw^a]*. Flies carrying both *P[hsw^a]*

and the *P[ry*$^+$,*Δ2-3]*(99B) transposase source consistently displayed multiple red patches in each eye, indicative of high rates of reversion within the developing eye (Figure 2Be). That the majority of these reversion events are identical deletions between the *copia* LTRs is indicated by our observation of 201 independent but apparently identical events among 234 progeny with increased pigmentation. It is important to point out that the selection of these 234 examples from among 362 discernible events was not random. Preference was given to darker phenotypes (which would lead to an overcount of hsw^{aRsLTR} derivatives), and it is likely that in many cases multiple events went unrecognized because of identical phenotypes (which would lead to an undercount of hsw^{aRsLTR} derivatives). However, consideration of the overall rate of reversion (Table 1), and available data about what fraction of reversion events are likely to be w^{aRsLTR} derivatives, indicates that this specific molecular event occurs at a rate in excess of 2% in the germline of these males. We have unpublished data that these high rates of reversion depend on the presence of the LTRs. An allele that differs from *P[hsw*^{a}*]* by a deletion of the first 4,020 nucleotides of *copia* has a similar phenotype, but only a single LTR, and shows rates of reversion that are at least 10-fold lower (D. C. BRAATEN, unpublished observations). Most significantly, similarly high rates of deletion between direct repeats were observed in the case of a transposon with unrelated repeats (*FRT* sequences), implying that recombination between direct repeats within *P* elements is a general phenomenon.

It has been noted previously that many deletions within *P* elements occur preferentially at sites of short direct repeats [ENGELS (1989) and references therein]. Furthermore, it has been speculated that longer repeats might lead to higher rates of deletion, and this possibility was even suggested as a basis for the high rates of reversion of alleles involving duplicated *P* elements, *singed-weak* being the best known of these (ENGELS 1989). However, previous accounts of deletion formation at direct repeats within *P* elements have generally been anecdotal. For example, the reversion of another allele caused by an LTR-bearing element, *P[y*^{2}*]*, was reported, but rates were not determined (GEYER *et al.* 1990). Very recently, PÂQUES and WEGNEZ (1993) reported high rates (40%) of transposase-dependent precise deletions and amplifications of tandemly arranged 350-bp 5S ribosomal RNA genes located within a *P* element. Their study differs from ours in that the eight repeated 5S genes are tandemly arranged, while both *copia* LTRs and the *FRT* elements we examined are separated by unrelated sequence. It is possible that the greater number of repeats in their experiment is responsible for the higher rate of precise deletion. Whether or not that is the case, their study is further evidence that *P* element mediated direct-repeat deletions are a general phenomenon.

The data in Table 1 indicate that a significant fraction of all deletions within the 13-kb *P[hsw*^{a}*]* element are precise deletions between the two 276 nucleotide LTRs. Although it is possible that some deletions are without phenotypic consequence, the majority of deletions with randomly selected endpoints within the *P[hsw*^{a}*]* element (diagrammed in Figure 1) would be expected to remove either sequences required for *white* expression (resulting in loss of pigmentation) or *copia* sequences that interfere with white expression (resulting in darker pigmentation). Furthermore, although we cannot determine what fraction of white-eyed derivatives are deletions rather than excisions of *P[hsw*^{a}*]*, we think that the fraction of excisions is likely to be low because it has been observed that precise excisions of *P* transposons are rare in the absence of a homolog (ENGELS *et al.* 1990), and there was no homolog present in these experiments. This reasoning is supported by the observation that the rate of mutation to a white-eyed state is higher for autosomal *P[hsw*^{a}*]* insertions that do have a homolog (data not shown). In the case of the *FRT* recombination experiment, a homolog was present. This would account for the higher rate of white-eyed derivatives, which could result from precise deletions. In fact, an *FRT* probe detected no DNA fragments at all in 32 of the 33 *P[>w*^{hs}*>]* derivatives that were not solo-*FRT*s. Thus, the rate of formation of internal deletions involving the directly repeated *FRT* sequences, although difficult to estimate accurately from our data, appears comparable to the rate of formation of internal deletions involving directly repeated LTR sequences within *P[hsw*^{a}*]*.

Although the observation of rates of precise deletion in excess of 2% for two unrelated cases of direct repeats shows that direct-repeat deletion is a general phenomenon, there is evidence that the rate at which similar events occur may not always be so high. In the case of *P[y*^{2}*]*, mentioned above, rates were not determined, but appear to be less than those described here (GEYER *et al.* 1990).

Possible mechanisms for direct-repeat recombination; homology monitoring and single-stranded annealing: How is it possible that such a large fraction of deletions could be precise deletions between the relatively short 276-bp *copia* LTRs? We consider two possibilities.

One, which we refer to as homology-monitoring, is suggested by consideration of the model for repair of *P* element-induced gaps favored by GLOOR *et al.* (1991) for repair of double-strand gaps generated by *P* element excision. This model is based on one proposed by HASTINGS (1989), and a variant of this model, based on the work of FORMOSA and ALBERTS (1986), involves release of the growing strand behind a migrating replication bubble. In neither case is there resolution of Holliday junctions by endonuclease activity; instead, the two newly extended strands ultimately anneal. We consider

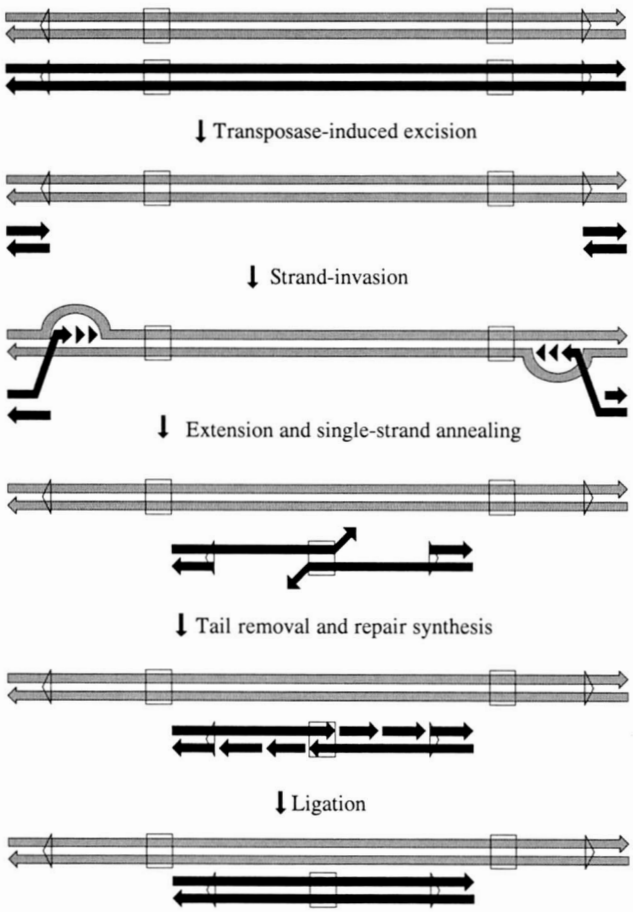


FIGURE 6.—A model for direct repeat-recombination based on single-stranded annealing [after GLOOR *et al.* (1991) and HABER (1992)]. Two sister chromatids are indicated. A double-strand break at *P* element inverted repeat termini (indicated by triangles) results from excision of the *P* element from the bottom duplex (black). Repair is initiated by strand invasion as described by GLOOR *et al* (1991), and the two extended 3′ termini anneal as described for single-stranded annealing in yeast (SUGAWARA and HABER 1992) and mammalian cells (LIN *et al.* 1990), resulting in a single copy of a direct repeat (indicated by the box) in the repaired duplex. This direct repeat could be either the *copia* LTR or the *FRT*. The model shown is single-stranded annealing. Homology monitoring would differ in that the two complexes would be associated (something that is difficult to depict in a drawing of this sort), and repair synthesis using the sister chromatid as template would terminate once common sequence was encountered, making removal of the 3′ "tails" of the newly synthesized DNA unnecessary.

the possibility that the two repair complexes remain associated with each other, and are compared by a proofreading function. Once common sequence is encountered, repair is terminated, and release of the newly synthesized DNA is accomplished by unwinding or topoisomerase activity. Normally, this would occur when a single complete copy of the intervening material has been synthesized, and repair would be error-free. However, in the case of direct repeats such as *copia* LTRs or *FRT*s, common sequence would be encountered prematurely, and sequences between direct repeats would be eliminated.

Another, similar, possibility is single-stranded annealing (HABER 1992). This mechanism has been proposed to explain deletions between repeated sequences that flank the site of a double-strand break, and involves the annealing of single-stranded DNA that has been generated from the double-strand break by exonuclease activity (SUGAWARA and HABER 1992). In the case of *P* element internal deletions (shown in Figure 6), the two single strands would be generated by repair synthesis using a copy of the parental *P* element present on the sister chromatid, rather than by exonuclease activity. However, the structure would be identical to that derived from exonuclease activity. Single-stranded annealing differs from homology monitoring in that the first involves synthesis past repeated sequences followed by annealing, while the latter involves termination of repair synthesis once common sequence is encountered. We note that the amplifications of tandemly arrayed 5S genes observed by PÂQUES and WEGNEZ are more consistent with single-stranded annealing rather than homology-monitoring.

Strategies for controlling deletion formation in transformation studies: The rates that we have observed here for recombination between direct repeats are high enough to be useful in the absence of selection (BALLINGER and BENZER 1989). Thus, engineering duplicated regions within *P* transposons prior to transformation should allow direct-repeat recombination to be used as a novel means for controlling deletion formation. Currently, *FRT*-mediated recombination is used for a variety of purposes, principally the creation of genetically distinct clones of cells during development (XU and RUBIN 1993; STRUHL and BASLER 1993; GOLIC 1991). Although minimal *FRT* sequences are quite short, they must be present in both the progenitor and product. In contrast, *P* transposase-mediated direct-repeat recombination has the advantage that it can be designed so that the final product has any sequence desired. Thus, it should be possible to place a repeat so that coding information is disrupted until direct-repeat recombination occurs. This technique should allow the study of effects of a protein in clones of cells during later stages of development despite unavoidable detrimental effects during earlier stages of development.

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