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Comparison of Targeted-Gene Replacement Frequencies in *Drosophila melanogaster* at the *forked* and *white* Loci

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P element-induced gene conversion has been previously used to modify the *white* gene of *Drosophila melanogaster* in a directed fashion. The applicability of this approach of gene targeting in *Drosophila melanogaster*, however, has not been analyzed quantitatively for other genes. We took advantage of the P element-induced *forked* allele, f^{hd}, which was used as a target, and we constructed a vector containing a modified *forked* fragment for converting f^{hd}. Conversion frequencies were analyzed for this locus as well as for an alternative *white* allele, w^{eh812}. Combination of both P element-induced mutant genes allowed the simultaneous analysis of conversion frequencies under identical genetic, developmental, and environmental conditions. This paper demonstrates that gene conversion through P element-induced gap repair can be applied with similar success rates at the *forked* locus and in the *white* gene. The average conversion frequency at *forked* was 0.29%, and that at *white* was 0.17%. These frequencies indicate that in vivo gene targeting in *Drosophila melanogaster* should be applicable for other genes in this species at manageable rates. We also confirmed the homolog dependence of reversions at the *forked* locus, indicating that P elements transpose via a cut-and-paste mechanism. In a different experiment, we attempted conversion with a modified *forked* allele containing the *su*(*Hw*) binding site. Despite an increased sample size, there were no conversion events with this template. One interpretation (under investigation) is that the binding of the *su*(*Hw*) product prevents double-strand break repair.

Gene targeting in eukaryotes through homologous recombination between exogenous DNA and its endogenous chromosomal homolog was first investigated with yeast cells (15, 31) and subsequently successfully tested with mammalian cells (1, 3, 21). In *Drosophila melanogaster*, P element-mediated germ line transformation (34, 37) was used to introduce modified DNA fragments to integrate at quasirandom sites into the genome. However, the altered chromosomal position of the modified gene often leads to unwanted position effects. Furthermore, many genes are too large to be incorporated in a fully functional P element and must be studied in situ.

More recently, directed modification of the *white* gene of *Drosophila melanogaster* has been carried out successfully (10). The technique depends on the presence of a P transposable element within or near the targeted gene. The mechanism involves a transposon-induced DNA double-strand break which subsequently is repaired by molecular processes of homologous recombination (for a review of P element-induced gap repair, see reference 22). The site of the double-strand break is precisely defined, and a template for repair can be designed in vitro. These studies suggested a new model for double-strand break repair, the synthesis-dependent strand annealing (SDSA) model (29), which may be of general relevance for homologous recombination in higher eukaryotes. According to the SDSA model, one or both ends of the double-strand break are assumed to locate template(s) and synthesize single-strand DNA. After enough such synthesis, the two broken ends, each a 3′ overhang, contain homologous single-stranded sequences. These then anneal and complete the synthesis. The result is double-strand break repair in which there is no opportunity for crossing over and in which the two templates can occasionally be used.

Most *Drosophila* work on gene replacement has been limited to the *white* locus. Although the technique has been applied to other loci, the data do not permit quantitative conclusions. The present study addresses the question of whether some of the insights into the double-strand break repair process gained from the *white* locus are applicable to other chromosomal sites. To evaluate the frequencies and to get a comparative estimate of the reliability, we performed P element-induced gap repair experiments at the *forked* locus. To aid in direct comparisons with the *white* locus, we used a new P element-induced *white* allele (w^{eh812}) and a P element-induced *forked* allele (f^{hd}) (17), which were combined onto the same chromosome and used as double targets in gene conversion experiments engaging an in vitro modified homologous template called P{w^+, *f*alter}.

Results from this study show that homologous recombination occurs at a reproducible rate and with similar frequencies at *forked* and at *white* loci. Conversion tract analyses are in agreement with the SDSA model (29). In a different experiment, we attempted to convert the *su*(*Hw*) binding sequence into the *forked* gene. Despite sevenfold increased sample sizes relative to experiments with templates lacking the *su*(*Hw*) binding site, which produced at least one conversion event, there was no conversion event with the template including the *su*(*Hw*) binding site. One interpretation (under investigation) is that the binding of the *su*(*Hw*) product not only interferes with enhancers and other chromatin proteins (4, 9), leading to chromatin condensation, but the binding of the *su*(*Hw*) product also prevents double-strand break repair.

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MATERIALS AND METHODS

***Drosophila* stocks.** Genetic symbols are defined in standard reference works (see reference 25 and the FlyBase database). The f^{hd} allele (17) was obtained

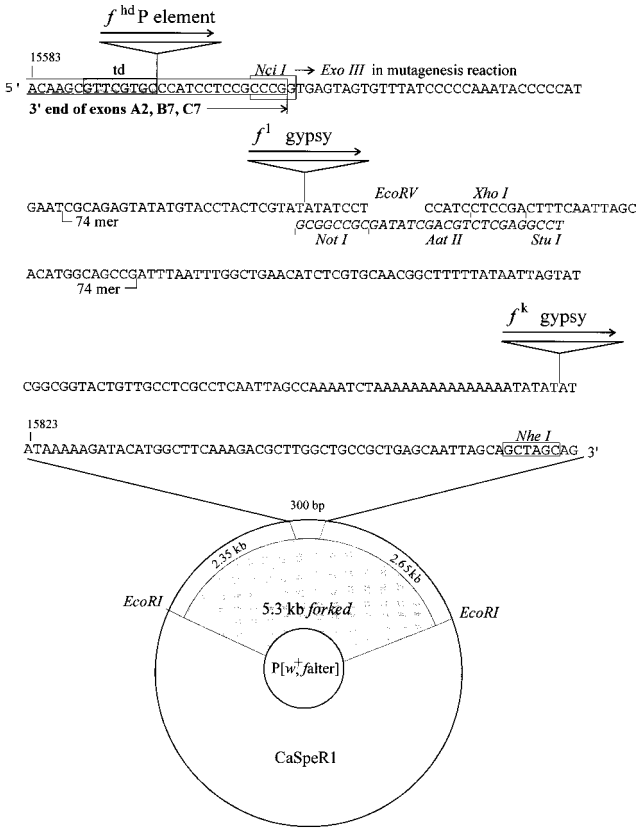


FIG. 1. Sequence structure of *forked* at the exon-intron boundary used in the P{w^+, *f*alter} construct. A 300-bp sequence fragment is shown representing the *forked* gene sequence from positions 15583 to 15883 as published (17). The insertion site of the P element in the f^{hd} allele is indicated, as is the 8-bp target site duplication (td). An *Nci*I restriction enzyme site used for Exonuclease III (Exo III) digestion in an in vitro mutagenesis reaction coincides with the intron exon boundary of the exons A2, B7, and C7 of *forked*. The insertion sites of the *gypsy* proviruses from the f^1 and f^k alleles are shown as well. The end points of a 74-mer oligonucleotide used in an in vitro mutagenesis experiment are indicated, and the altered sequence introduced into a 5.3-kb *Eco*RI *forked* fragment is shown in italic. The restriction sites *Not*I, *Eco*RV, *Aat*II, *Xho*I, and *Stu*I are unique to P{w^+, *f*alter} and can be used for cloning purposes. As shown in the lower part of the figure, the 5.3-kb *forked* fragment was cloned into the P element transformation vector CaSpeR1.

from D. Dorer and A. Christensen (Thomas Jefferson University, Philadelphia, Pa.). The TMSΔ2-3/TM2Δ2-3 balancer stock containing P{ry^+ Δ2-3}99B as a stable source of P transposase (33) was obtained from the Bloomington stock center. From here on P{ry^+ Δ2-3}99B is referred to as Δ2-3. The *B*, cs^{53}, and $w^{67c23(2)}$ stocks were obtained from the Bowling Green stock center. The other balancer stocks and the compound-X stock C(1)DX, *y w f/shi* were from the stock collection of W.R.E. Gap repair crosses were done at controlled temperatures at 18°C and at variable room temperatures averaging 23°C, as indicated in the text.

Construction of P{w^+, *f*alter} by in vitro mutagenesis. The structure and function of *forked* have been described by Hoover et al. (17) and Petersen et al. (32). A *forked* fragment spanning 5.3 kb from the *Eco*RI site at position 13163 to position 18476, to which an *Eco*RI linker (ATGAATTC) had been ligated (numbers are according to the *forked* sequence published in reference 17), was cloned into phage M13mp18/19, and single-stranded DNA was prepared and sequenced as described previously (23). In vitro mutagenesis of the f^1-*gypsy* insertion site was carried out with the aid of the oligonucleotide-directed in vitro mutagenesis system of Amersham, version 2.1 (38, 39). A 74-mer oligonucleotide containing novel restriction enzyme sites was synthesized by the trityl-on method. The sequence of the 74-mer primer is shown in Fig. 1. The same figure shows the *Nci*I site in which the DNA strand is not cut because of the incorporation of a thionucleotide. Only the non-thionucleotide-containing strand is nicked. Exonuclease III digestion was carried out at 37°C for 45 min, and repolymerization took place at 14°C for 5 h. A successfully mutagenized M13 clone was sequenced to confirm that no unwanted changes in the *forked* fragment sequence had taken place. The 5.3-kb *Eco*RI *forked* fragment subsequently was cloned into the *Eco*RI site of the pCaSpeR1 transformation vector (Fig. 1). The new construct was called P{w^+, *f*alter}, where w^+ indicates the mini-*white* gene included in the CaSpeR vector and *f*alter indicates the altered (mini) *forked* gene incorporated into the same vector.

Construction of P{w^+, *f*alter-*su*(*Hw*)bs} and transgenic lines. The *forked* fragment of the P{w^+, *f*alter} vector contains a multiple cloning site within the exons A2, B7, and C7 which is located at approximately the same location as the *gypsy* provirus insertion of the f^1 allele (Fig. 1). A 6.8-kb *Xho*I DNA fragment of the cloned *gypsy* provirus (27) was used to amplify the entire *su*(*Hw*) binding sequence by PCR. The amplified 409-bp sequence contained two 109-bp tandem repeats partially overlapping the entire array of 12 enhancer-like 12-bp repeats. This sequence interacts with the *su*(*Hw*) protein (36). Two oligonucleotides were used as PCR primers which hybridize to the ends of the *su*(*Hw*) binding region. The first oligonucleotide represents *gypsy* nucleotides 637 to 654 (27). It contains, in addition to the *gypsy* sequence, a *Not*I site at the 5′ end (5′-GTAGCGGC CGCGTTGTGTATCTGGCCACG-3′). The second oligonucleotide (5′-GTACTCGAGGCCGAGCACAATTGATCGG-3′) represents *gypsy* nucleotides 1045 to 1027 (27). It contains, in addition to the *gypsy* sequence, a *Xho*I site at its 5′ end. The 373-bp sequence flanked by these oligonucleotides was amplified by PCR with Hot Tub(Tm) DNA polymerase from Amersham. The amplification product was subcloned into the *Not*I and *Xho*I sites of the P{w^+, *f*alter} cloning site (Fig. 1). Subsequently the integrity of the fragment was confirmed by sequencing. The new construct was called P{w^+, *f*alter-*su*(*Hw*)bs}. Transformation of *y* w^{67c23} f^{36a} preblastoderm embryos with this construct was carried out as described below. The f^{36a} null phenotype is partially rescued in the transformants, giving rise to the expected f^1 phenotype.

P element transformation and in situ hybridization to polytene chromosomes. DNAs of the P{w^+, *f*alter} construct and of a P element with a defective inverted repeat used as a transposase source, P25.7wc (19), were injected into *y* w^{67c23} f^{36a} preblastoderm embryos as described previously (34, 37). The chromosomal insertions of P{w^+, *f*alter} were made homozygous, and their positions were mapped with the in situ hybridization protocol with biotinylated DNA probes as described previously (24).

Verification of precise excisions with PCR. Precise excisions of the f^{hd} P element obtained in crosses (see Fig. 3) in which f^{hd} was heterozygous over a wild-type forked allele were verified by PCR analysis. Sixteen flies phenotypically appearing to bear precise excisions were studied in detail. Lesions representing a multitude of three nucleotides and being larger than nine nucleotides (8-bp target site duplication plus one nucleotide) would have been recognized on the 4% agarose gel used in this experiment. In addition we digested five of the fragments with *Nla*III, which is an enzyme that makes a diagnostic cut for detecting the terminus of P elements. None of the fragments contained P sequences, confirming the assumption that fully reverted bristle phenotypes reliably represent precise excisions.

Phenotypic scanning of bristles and eye color. The phenotype of f^{hd} is that of a null mutant and is similar to that of f^{36a} (17). Reversion of the phenotype in order to identify conversion candidates was performed by scanning 32 macrochaetae: 4 humerals, 4 notopleurals, 4 supra-alars, 4 dorsocentrals, 4 postalars, 4 scutellars, 2 mesosternals, and 6 sternopleurals. The ocellars, postverticals, and orbitals of the head were not used for scoring, since the *B* eyes affected the phenotype of these bristles even in f^+ flies.

The w^{eh812} allele contains two mutations: a P element insertion in an exon and a 297-bp deletion in the 3′ transcribed untranslated region of the *white* gene. The *white* allele with only the P element insertion was called w^{eh}, and it has a white eye phenotype (18). The *white* allele with only the deletion was called w^{812}, and it expresses a brown eye phenotype. Therefore, the replacement of the P element insertion within the w^{eh812} allele for a wild-type *white* sequence would result in the w^{812} brown eye phenotype. Combination of the P{w^+, *f*alter} construct with w^{eh812} results in an orange eye phenotype. Revertants and convertants of the w^{eh812} allele were easily distinguished from the P{w^+, *f*alter}-derived orange eye color by their brown or wild-type eye colors. We did not try to distinguish phenotypically between the brown eye phenotype or wild-type eye color of a revertant but instead used PCR in order to distinguish between the two possibilities.

Combination of w^{eh812} and f^{hd} on the same X chromosome. The P{w^+, *f*alter} construct was injected into w^{67c23} *cs* f^{hd} *B* flies, and 12 independent chromosomal insertions were obtained. For the double target experiments, the w^{67c23} mutation had to be exchanged for the P element-induced w^{eh812} allele in order to combine this *white* allele with the *cs* f^{hd} *B* alleles. First, the w^{67c23} allele was exchanged for a Canton S [M] w^+ allele by crossing over, and subsequently the w^+ allele was exchanged by the w^{eh812} deletion and P element insertion allele. This was done for all lines carrying the P{w^+, *f*alter} construct.

DNA sequencing of *forked* revertants. DNA fragments were amplified from genomic DNA of *forked* revertants by PCR with two oligonucleotide primers (5′-TCAAGAGCATCAGCATCGAC-3′ and 5′-TTAAATCGGCTGCCATGT GC-3′) flanking the P element insertion site of f^{hd} and the mutagenized site within P{w^+, *f*alter}. The amplified product was cloned into a linearized plasmid with single overhangs of T incorporated by *Taq* DNA polymerase, which had been exposed to high concentrations of dTTP and *Taq* polymerase in order to add protruding T nucleotides to the ends (26). DNA was sequenced with double-stranded DNA and by the dideoxy-chain termination method (35).

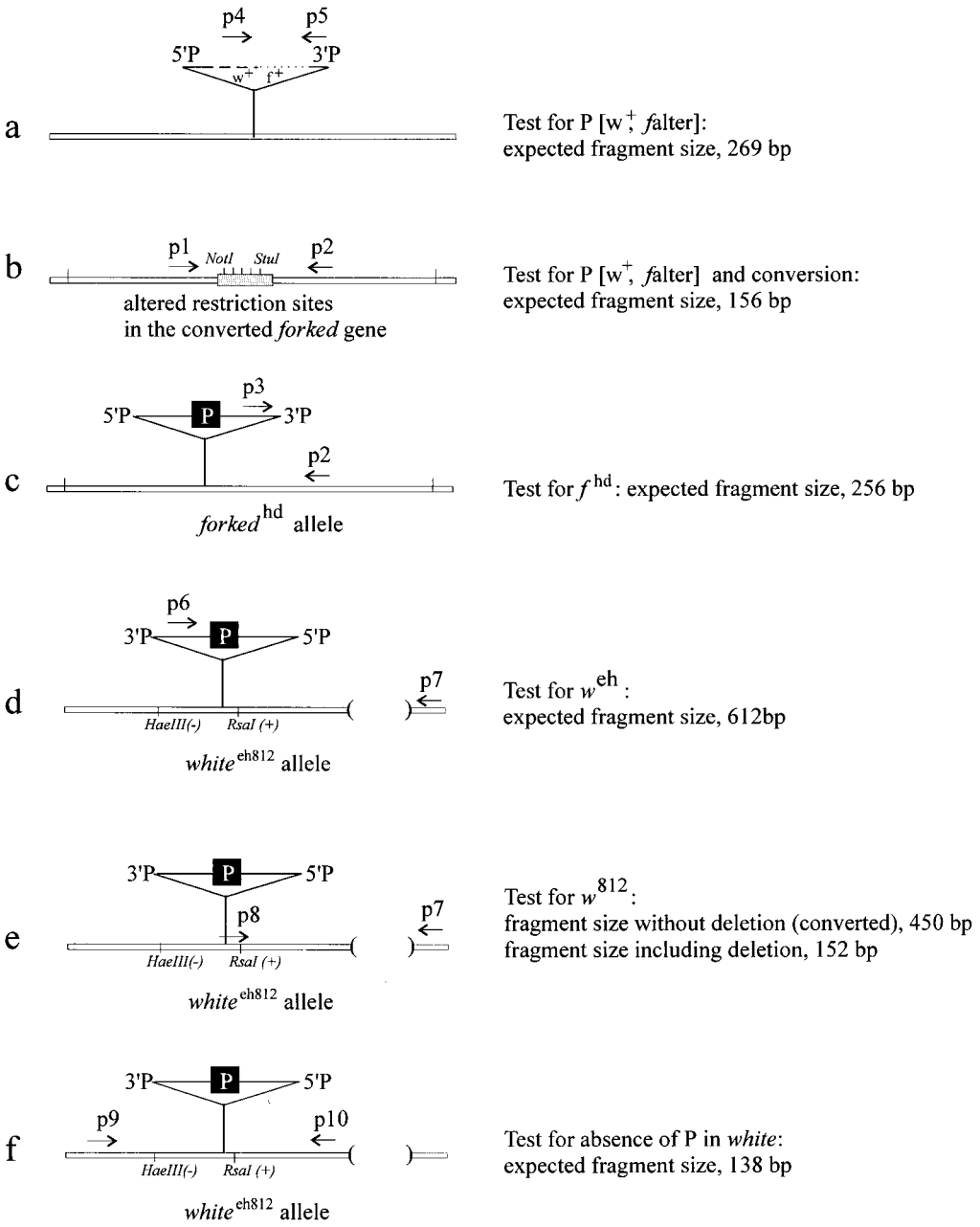


FIG. 2. Diagnostic PCR to analyze conversion events at *forked*^{hd} and *white*^{eh812}. (a through c) PCR experiments confirming true conversion events at *forked*. A true convertant was confirmed if reaction b produced a 156-bp PCR fragment which was digestible with *Not*I or any of the introduced restriction enzymes and when reactions a and c did not produce a product. If phenotypically *white*$^{-}$ and f^{+} conversion candidates gave the PCR and digestion products identifying the presence of P{w^{+}, *f*alter}, they were identified as false positives. In these cases, it was likely that P{w^{+}, *f*alter} had been modified. Partial revertants gave a variety of results. Reaction b gave a product of variable size, reaction c was either positive or negative, and reaction a did not produce any band. (d through f) PCR experiments identifying conversion at the w^{eh812} allele with the same P{w^{+}, *f*alter} template as for f^{hd}. w^{eh} is an allele in which a 629-bp P element was inserted just 23 nucleotides downstream relative to the w^{hd} P element insertion site. w^{812} is an allele in which a 297-bp deletion occurred 70 nucleotides downstream of the P element insertion of w^{eh}. w^{eh812} is an allele in which both mutations are combined. The restriction enzyme sites *Hae*III and *Rsa*I are in the opposite orientation in the *white* gene of the P{w^{+}, *f*alter} template; the deletion is not present in P{w^{+}, *f*alter}. The PCRs shown in this figure do not only allow the identification of true convertants but also allow analysis of the conversion tracts. (f) Test for the absence of the P element in the *white* gene. This reaction was only informative if P{w^{+}, *f*alter} had been removed by crossing out.

Molecular analysis to identify *forked* and *white* convertants. DNA for PCR was prepared from individual flies as previously described (11). Oligonucleotide priming sites for diagnostic PCR amplifications are shown for *forked* and *white* in Fig. 2a through f. Fig. 2a through f also show the expected PCR fragment sizes of converted or nonconverted revertants. In some of the reactions (Fig. 2a, c, and d), no PCR product was expected if the revertants were true convertants. In these cases, we used two additional PCR primers that recognized either a *micropia*-retrotransposon fragment (23) or a β_2-tubulin gene fragment to control for the successful PCR amplification. The following primer sequences were used: p1, 5′ TCAAGAGCATCAGCATCGAC3′; p2, 5′ TTAAATCGGCTGCCATGTGC3′; p3, 5′TCGCTGTCTCACTCAGACTC3′; p4, 5′ACCGATGGAAGCTGAGC GTG3′; p5, 5′CGTATGCTTGCAATAAGTGC3′; p6, 5′ CCTTAGCATGTC CGTGGGGT3′; p7, 5′ CGAAAGAGCAACTACGAAACG3′; p8, 5′ ACGT GGGTCTAGCCATTCTCATCGTGAGCTTCCGGGTACTCGCATATCT3′; p9, 5′ GGTCATCCTGGAGACGC3′; and p10, 5′ GTCGGCTACTCCTTG CGTCG3′.

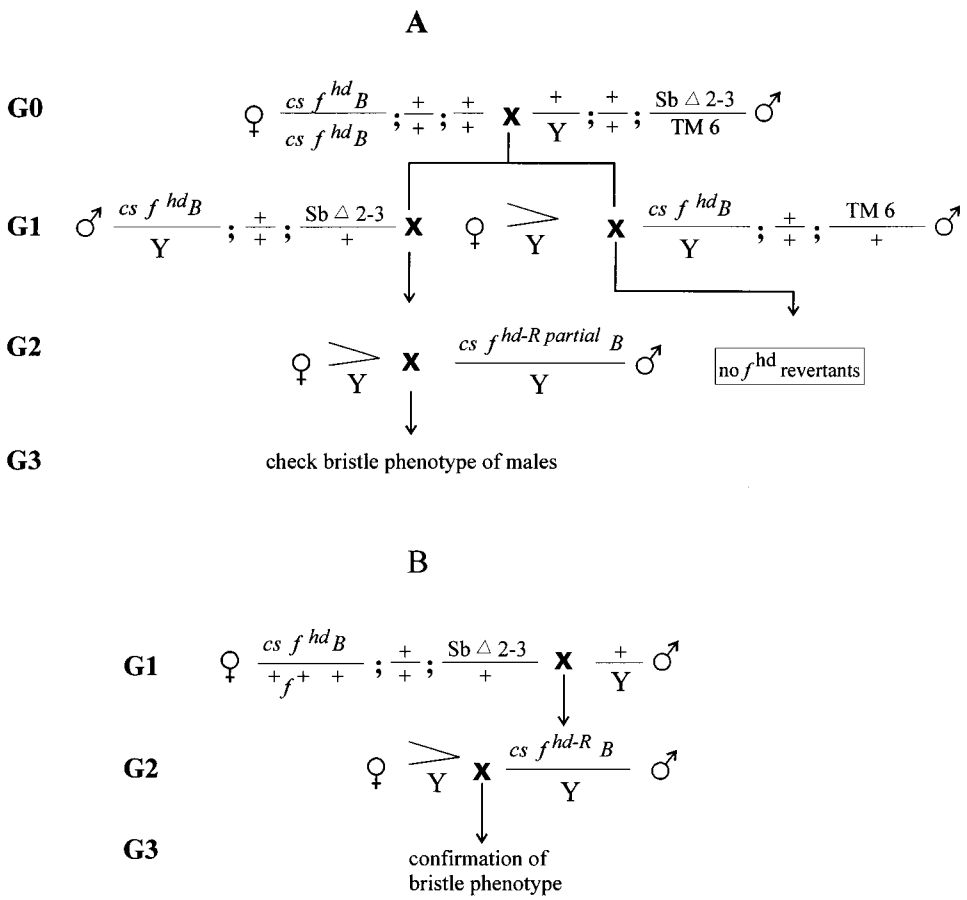


FIG. 3. Crosses to generate revertants of the f^{hd} allele in the male and female germ lines. (A) Cross-producing partial f^{hd} revertants. The hemizygous chromosome in G_1 (Y) is the Y chromosome. Note that except for the identical sister chromatid of the X chromosome, no other template for repair at f^{hd} is available. (B) Cross to produce complete revertants at f^{hd}. The f^{hd} chromosome was marked by the flanking marker alleles cs^{53} and *B*. These markers ensured a negligible crossing-over rate near the f^{+} allele and the f^{hd} allele of the two homologous X chromosomes. Male revertants were crossed to compound-X (indicated by >) females to pass the same X chromosome to the next generation, where numerous males can be analyzed phenotypically.

RESULTS

The insertion in f^{hd} is mobilized by P transposase. We used the two crosses shown in Fig. 3 to recover partial and complete revertants of f^{hd}. We measured the germ line reversion rate of f^{hd} by scoring for f^{+} phenotypes of the 32 thoracic macrochaetae. In contrast to w^{hd}, the *white* allele used in previous experiments (10), we observed that revertants of f^{hd} often encompassed partial revertants in which the severe bristle phenotype of f^{hd} had reverted to a less severe phenotype but not to the wild type. We also encountered somatic revertants, identified by mosaicism, in those cases in which the transposase source Δ2-3 was still present in the genome. Partial revertants were crossed with compound-X females to pass the reverted f^{hd} allele to the male offspring. At least 10 males of the G_3 progeny were then scored for the eventual appearance of a kinked bristle. If at least one kinked bristle occurred among the 320 bristles of 10 scored males, it was assumed to be a partial *forked* revertant. DNA sequencing of the PCR products spanning the P element insertion site confirmed our assumptions.

Four examples of partial and complete germ line revertants were selected for sequencing because they represented a wide range of phenotypes. The first was a complete excision of the P element, including one copy of the 8-bp target site duplication. There was no deviation from the wild type among 1,600 scored macrochaetae in 50 sons of the revertant male. The second was a partial revertant in which 25 nucleotides of the outer ends of the P element remained, as did both copies of the 8-bp target site duplication, resulting in an in-frame insertion of 33 bp. The bristle phenotype of this revertant was nearly wild type. However, among 22 scored flies, eight contained one or two bristles that were kinked once. The kinked bristles were randomly distributed on the various parts of the thorax. The third revertant also had a small portion of the P element outer ends remaining, but the number of P element bases was 29, thus resulting in a 37-bp frameshift mutation including the 8-bp duplication. This revertant presented a strong *forked* phenotype affecting all bristles. It was not, however, as severe as the f^{hd} phenotype. Finally, we examined a partial excision that left behind 322 nucleotides of the P element plus the 8-bp duplication, leaving an in-frame lesion. This line showed a mild *forked* phenotype, with every male out of 20 scored having at least one kinked macrochaeta. We conclude that even large in-frame insertions at this site can be tolerated without severe loss of *forked* function. This conclusion fits well with our present understanding of the function of *forked* proteins in bristle development (17, 32).

These results indicated to us that careful scoring of the bristle phenotype allows reliable judgment about the molecular events that take place at the reverted f^{hd} site.

f^{hd} reversion is homolog dependent. We measured the germ line reversion rate of f^{hd} when f^{hd} was hemizygous and compared this with the reversion rate in females when f^{hd} was heterozygous with a wild-type allele. Figure 3 shows the mating schemes for obtaining revertants. As a control, we scored 4,977 chromosomes of the G_2 males whose G_1 fathers lacked the Δ2-3 transposase source (Fig. 3A). As expected, no partial or complete f^{hd} revertants were obtained. In parallel, 73 independent crosses were carried out with G_1 males containing the transposase source. We obtained 6,023 sons, of which 544 (9.03%) reverted. These reversions, however, were all partial, as identified by a kinked bristle phenotype (compare previous section), and on the basis of PCR analysis, none of 26 lines with near wild-type bristles was due to precise excision of the f^{hd} P element.

Similar to observations at the w^{hd} allele (7), we found a dramatic increase in the rate of precise excisions when f^{hd} was heterozygous over a wild-type *forked* allele in females (Fig. 3B). As described in the previous section, fully reverted bristle phenotypes reliably represent precise excisions (see Materials and Methods). On the basis of this kind of phenotypic bristle analysis, the rate was 8.1% in 1,463 scored chromosomes compared with 0% complete revertants when f^{hd} was hemizygous. We were also able to estimate the number of partial *forked* revertants. Judging by the phenotype, 86 (5.9%) of the males scored possessed an intermediate *forked* bristle phenotype, representing partial revertants.

In an equivalent experiment, the reversion rate of w^{hd} was found to range between 5 and 18%, depending on the degree of homology of the template (7, 30). The frequency of 8.1% in the present experiment is at the lower range of the frequency variations (5 to 18%) found by Nassif and Engels (30). Since the *forked* homology between P{w^+, *f*alter} and f^{hd} was 100%, as far as we could tell from sequencing, this could be due to the fact that the w^{hd} experiment was carried out at 23°C, while the f^{hd} experiment was carried out at 18°C.

Also the larger size of the f^{hd} P element (2.6 kb) relative to the w^{hd} P element (629 bp) could have reduced the absolute rate of f^{hd} reversion. In the case of f^{hd}, the reduced temperature was necessary because several P elements left in the f^{hd} stock from the original mutagenesis caused a hybrid dysgenesis-like lethality of the G_1 flies in the presence of the transposase source P{ry^+ Δ2-3}99B (Fig. 3) (6). The lethal effects were reduced at 18°C, while the rate of complete and partial revertants was still respectable.

Our two experiments reveal that f^{hd} is a good candidate to be a target for P element-induced gap repair with an ectopic, marked template. The reversions at the *forked* locus seem to confirm the homolog dependence for reversions found for the w^{hd} allele (7), indicating that P elements jump by a cut-and-paste mechanism.

Construction of transformants carrying the template P{w^+, *f*alter}. The original idea was to introduce *gypsy* retrotransposon sequences into the same position of the *forked* gene as the *gypsy* transposon inserted within the f^1 allele (17) by P element-induced homologous recombination. Initially, we attempted to determine whether the *su*(*Hw*) binding site could be sufficient for the f^1 phenotype. We constructed a *forked* fragment, by in vitro mutagenesis, which contains a multiple cloning site at the position of the f^1-*gypsy* insertion. This fragment was cloned into the CaSpeR vector and could be used to introduce arbitrary DNA fragments and at the same time could be used to score for gene conversion events. The vector construct P{w^+, *f*alter} is shown in Fig. 1. The altered sequence introduces only 6 new nucleotides, but 23 nucleotides are exchanged, such that cutting sites of the restriction enzymes *Not*I, *Eco*RV, *Aat*II, *Xho*I, and *Stu*I are created. The altered sequence is located in an intron only 62 bp 3′ with respect to the exon in which the f^{hd} P element is located and 84 nucleotides downstream from the f^{hd} P element. The mutagenized fragment is 5.3 kb long. It starts at the *Eco*RI site located within the coding part of exon A1 according to the published nomenclature (17) and ends at the 3′ ends of the *forked* transcripts containing an *Eco*RI linker sequence. This fragment was cloned into the pCaSpeR vector and injected into w^{67c23} f^{36a} embryos. Twelve transformant lines were established, one of them containing two inserts of P{w^+, *f*alter}. Eleven of these lines were used in the gap repair experiment of this study. The cytological map positions were determined by in situ hybridization to polytene chromosomes.

The 5.3-kb *forked* fragment rescues the f^{36a} bristle null phenotype almost completely, leaving behind only a few thoracic bristles slightly kinked. (The same fragment does not rescue the f^{36a} phenotype of the marginal wing hairs [3a].) This result was not expected, since this fragment only encodes transcripts whose translational products were not believed to be sufficient for bristle development (17). Since we obtained an almost complete rescue of the thoracic macrochaetae in all transformant lines, this indicates that the introduced multiple cloning site in the intron of *forked* did not significantly interfere with the wild-type function of the *forked* gene. This finding was a prerequisite for phenotypically scoring *forked* convertants in which the P element-induced gap at f^{hd} was repaired with P{w^+, *f*alter} as an ectopic template.

Strategy for the gene conversion reaction at the *forked* locus. Figure 4 shows the cross used to replace the P element-induced f^{hd} allele with the in vitro-modified *f*alter template. G_2 males which had lost the P{w^+, *f*alter} template were expected to carry the *forked* null phenotype and to be *white* eyed. Candidates for *forked* conversion were identified phenotypically as *w cs* f^+ *B*. Each candidate then underwent a series of additional tests for confirmation as listed in Fig. 2a to c. First, a specific pair of primers was used to test for the presence of P{w^+, *f*alter} (Fig. 2a). Next, we used another set of primers spanning the f^{hd} P element integration site to determine whether the f^{hd} P element had been excised completely (Fig. 2b). Another test for the absence of the f^{hd} P element was performed with a primer binding to the 3′ end of the P element and a primer which binds downstream of the P insertion to the *forked* sequence (Fig. 2c). As a fourth means of identifying false revertants, G_2 males were crossed to compound-X females as in G_1. The G_3 offspring males were scored phenotypically for rarely appearing *forked* bristles which would indicate incomplete reversions. The successful conversion of the gap by the altered *forked* sequence of P{w^+, *f*alter} was further tested by restriction enzyme digests of the PCR product spanning the converted region (Fig. 2b). The restriction sites *Not*I, *Eco*RV, *Aat*II, *Xho*I, and *Stu*I are unique to P{w^+, *f*alter}. If these sites were found at the homologous sequence in the reverted f^{hd} allele, then gene conversion through the P element-induced gap repair in the sense described by Gloor et al. (10) had taken place.

Conversion at f^{hd}. We analyzed the conversion rates of f^{hd} with the ectopic template P{w^+, *f*alter} at 11 different autosomal sites. Figure 4 shows the crosses used to screen for conversion events. The results are summarized in Table 1. A total of 5,436 individual G_1 males were crossed to yield 69,416 sons that were scored for conversions. We found 105 of the crosses, or 1.93%, produced at least one convertant son. Many of these

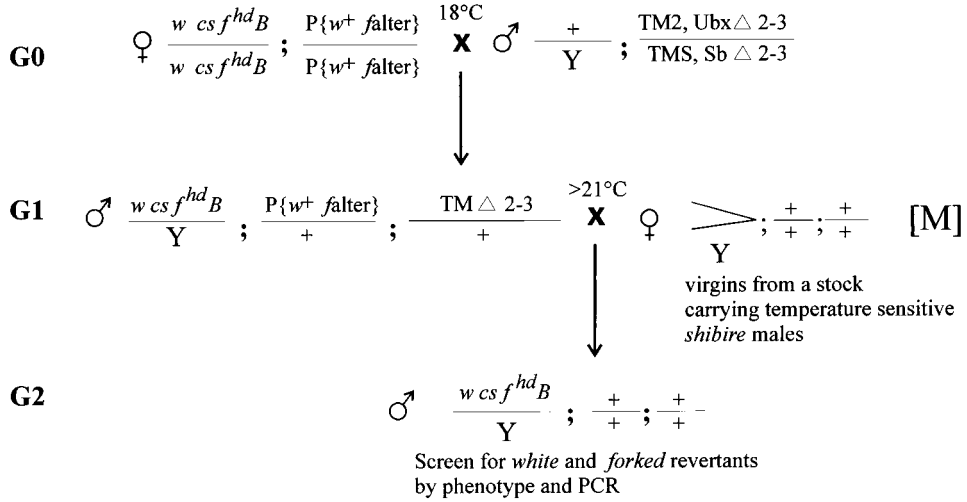


FIG. 4. Crosses to screen for conversion events at the P element-induced f^{hd} allele and at the P element-induced w^{eh812} allele. *w* represents the w^{67c23} allele in the case of the single-target experiment at f^{hd}. In the case of the double-target experiment, *w* symbolizes the allele w^{eh812}. Conversion occurs in the germ line of *w cs* f^{hd} *B*/Y G_1 males carrying the immobile Δ2-3 P element on chromosome 3 to provide transposase. The Δ2-3 P element is located on the two balancer chromosomes TM2 and TMS, which made it possible to safely remove this chromosome from the offspring; TMΔ2-3 was used to indicate either of these chromosomes. The P{w^+, *f*alter} template was present on the second chromosome in only two of the experimental lines constructed (shown in this figure), and it was located on the third chromosome in nine other lines used in this study (not shown here). Note that in the case of the third chromosome insertions, the TMΔ2-3 chromosome must be present in the scored G_2 progeny lacking P{w^+, *f*alter}. The presence of active transposase in G_2 was thought to reduce viability due to nonautonomous P elements also in the genome (6). This reduced the numbers of scoreable convertant events (see Table 2). Conversion at *white* could be scored in all G_2 sons, because wild-type *white* expression is easily distinguished from that produced by P{w^+, *f*alter} (see Table 2). However, only the sons not receiving the *f*alter chromosome could be scored for conversion at *forked*. These G_2 sons, therefore, always contained high levels of Δ2-derived transposase and suffered dysgenic effects, reducing their scorable numbers.

crosses yielded more than one convertant son, suggesting the events were premeiotic. The largest cluster included 21 convertants. The total number of convertants was 199, for a frequency of 0.29%.

This frequency may be compared to the 0.9% average conversion rate at w^{hd} from an ectopic *white* template (8, 10). Those studies revealed significant differences in the conversion rates among 18 different autosomal positions of the P{*w*alter} template. We used the present data to test whether the 11 different chromosomal positions gave equal conversion rates. For each of the 11 chromosomal sites, we used the numbers of crosses with and without conversion events to test for independence in a 2-by-11 contingency table. The results indicated significant variability between sites ($P < 0.01$).

w^{eh812} as a target for P{w^+, *f*alter}. While the average conversion rate at w^{hd} with P{*w*alter} as a template at 28 autosomal sites was 0.9% (8), the comparable conversion rate of f^{hd} was 0.29% (Table 1). To examine the basis for this threefold difference, we took advantage of the fact that the P{w^+, *f*alter} element contains templates for both the *white* and *forked* loci. Our strategy was to make double-strand breaks at both loci in the same individuals and thus measure the conversion rates simultaneously. This procedure was used to distinguish whether differences in conversion rate were intrinsic properties of the particular genes (*white* and *forked*) or whether both loci were under influences idiosyncratic of the used flystocks but possibly unrelated to the gap repair process itself. For example, such influences could be due to unknown background mutations present in the particular f^{hd} stock, or they could be due to the specific sequence structure of the P elements inserted within w^{eh812} and f^{hd}.

Figure 5 shows the experimental design. We used the f^{hd} allele to make breaks in *forked*, as in the previous experiment. For the *white* locus, we used an allele called w^{eh812}, described

TABLE 1. Conversion rates at *forked*

Template positions of P{w^+, *f*alter}	No. of single crosses	Total no. of males scored	No. of independent convertants	Rate of independent convertants (%)	No. of convertants, including clustering	Rate of convertants, including clustering (%)
3L74A	623	4,569	6	0.96	13	0.28
3L65 and 3L67A	857	5,804	19	2.2	27	0.47
3R90E-F	592	3,450	10	1.7	17	0.49
3L71-75	644	11,788	13	2.0	28	0.24
3R82	595	7,225	12	2.0	13	0.18
3L66E	344	2,413	1	0.29	1	0.04
3L66EF	608	7,464	9	1.48	15	0.20
3L74-77	210	919	2	0.95	3	0.33
3L79	376	12,123	18	4.79	58	0.48
2R59E	89	4,160	2	2.25	2	0.05
2R60B	498	9,501	13	2.61	23	0.24
Total	5,436	69,416	105	1.93 ± 1.37	199	0.29 ± 0.034

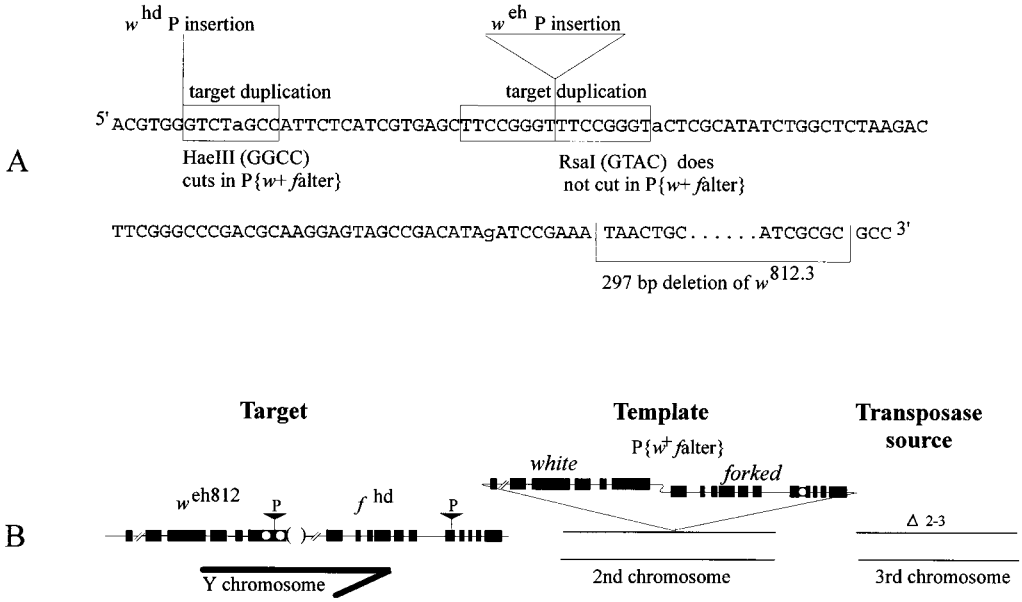


FIG. 5. Combined gap repair at the *white* and *forked* loci. (A) Sequence structure of w^{eh812} at the double-strand break site. (B) Three components necessary for the combined gap repair reaction at the *white* and *forked* loci. Solid blocks represent exons. The w^{eh812} and f^{hd} alleles are present on the same X chromosome and serve as targets for double-strand break repair after excision of the P element. A P element carrying an altered *forked* gene (alteration is shown as an open circle in P{w^+, *f*alter}) and a wild-type *white* gene serve as the ectopic template. The sequences differing from this gene in w^{eh812} are indicated as white dots (the *Hae*III and *Rsa*I sites), and the bracket indicates the 297-bp deletion of this allele. The stable transposase source P{ry^+ Δ2-3}99B is located on the third chromosome.

previously (18). We chose this allele rather than w^{hd}, which had been used in previous studies with *white*, because w^{eh812} carries a 297-bp deletion located approximately the same distance from the P element insertion as the alteration in *f*alter is from the insertion in f^{hd} (71 versus 84 bp [Fig. 5]). Nine different positions of the P{w^+, *f*alter} template in eight independent flystocks were used in this experiment (Table 2).

The results are listed in Table 2. The pooled conversion frequency at the w^{eh812} deletion was 0.17% in 100,144 scored chromosomes. In the same experiment at f^{hd}, the pooled frequency was 0.22% in 26,456 chromosomes. Note that fewer progeny were scored for *forked* reversion than for *white* reversion. This is because only those not receiving the P{*f*alter} chromosome could be scored for *forked*. The estimated conversion frequency was higher for *forked* than for *white* at five of the eight template positions, and higher for *white* in the other three, Thus, by the sign test, there was no significant difference between the two rates.

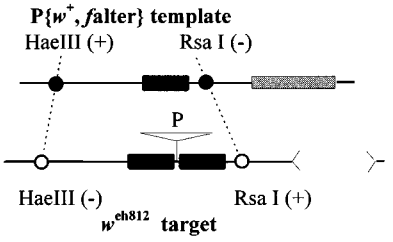
Similar to the case with *forked*, we tested the hypothesis that the same template construct at various different chromosomal locations gives equal conversion rates for *white*. Again, we found evidence for variability between template sites ($P = 0.013$).

Conversion tracts for w^{eh812} as a target and P{w^+,*f*alter} as a template. Note from Fig. 5, that the w^{eh812} allele differs from the template *white* gene by a *Hae*III site just to the left of the P element insertion, a *Rsa*I site just to the right, and the deletion further downstream (18). We made use of these dif-

TABLE 2. Conversion rates at *forked* and *white*

Template positions of P{w^+, *f*alter}	No. of single crosses for f^{hd} and w^{eh812}	No. of scored chromosomes for f^{hd} excluding P{f^+}[a]	No. of scored chromosomes for w^{eh812} including P{f^+}	No. of f^{hd} convertants		No. of w^{eh812} deletion convertants		Rate of f^{hd} convertants (%)		Rate of w^{eh812} deletion convertants (%)	
				id.[b]	cl.[c]	id.	cl.	id.	cl.	id.	cl.
3L74A	500	3,939	13,639	4	6	8	17	0.8	0.15	1.6	0.12
3L65 and 3L67A	411	1,811	14,784	2	3	18	35	0.48	0.16	4.4	0.24
3R90EF	250	1,504	8,943	5	9	5	7	2	0.6	2	0.08
3L71-75	250	1,864	8,632	2	3	8	13	0.8	0.16	3.2	0.15
3R82	300	3,980	12,119	6	6	14	26	2	0.15	4.7	0.21
3L66E	300	2,105	12,505	1	1	14	31	0.33	0.05	4.7	0.24
3L66EF	300	4,012	15,047	7	13	10	38	2.3	0.32	3.3	0.25
2R60B	331	7,241	14,475	11	18	3	3	3.3	0.25	0.91	0.02
Total	2,642	26,456	100,144	38	59	80	170	1.44 ± 1.11	0.22 ± 0.029	3.03 ± 2.16	0.17 ± 0.007

[a] P{f^+} represents P{w^+, *f*alter}
[b] id., number of convertants, including independent convertants only.
[c] cl., number of convertants, including clustering.



chromosomal position	3L65 and 3L67A		3L71-75		3R90E -F		3L74A		Total	
	id	cl	id	cl	id	cl	id	cl	id	cl
1)	6	8	6	11	3	5	7	16	22	40
2)	2	5	2	2	2	2	1	1	7	10
3)	1	1	0	0	1	3	0	0	2	4
4)	5	35	6	6	1	1	1	1	13	43
5)	2	1	0	0	0	0	0	0	1	2
6)	1	1	3	3	3	3	7	35	14	42
7)	0	0	0	0	0	0	0	0	0	0
8)	0	0	0	0	0	0	0	0	0	0
9) phenotypic revertants checked for single target site and loss of P by PCR	1	1	1	3	1	1	0	0	3	5
scored males	9,038		8,632		8,943		13,639		40,252	

abbreviations see Table 2

FIG. 6. Conversion tracts at w^{eh812} from the P{w^+, *f*alter} template. The data represent the clustered and independent conversion results of four different genomic template positions. The solid blocks represent the target site duplications. The solid and open circles represent the presence or absence of the *Hae*III and *Rsa*I sites as indicated. The gray block symbolizes the 297 bp deleted in w^{eh812}. This deletion is shown as a gap (< >). id, number of independent conversions; cl, number of convertants including clustering.

ferences as markers in order to examine the extent of the conversion tracts. The results (Fig. 6) are consistent with results seen previously in tracts generated from a different allele, w^{hd} (10, 18). The results are also consistent with the SDSA model mentioned above (29). The *Hae*III site was converted in 60% of the 141 tracts analyzed; The *Rsa*I site was converted in 68%, and the deleted sequence was restored in 36%. As expected, the sites closer to the P element insertion site were converted more frequently, and all tracts were continuous. In the 50 cases in which the deletion had been restored, the eye color was also fully wild type, whereas in the remaining 91 cases, the eye color was dark brown because of the deletion.

f^{hd} was not converted by a *forked* sequence including the *suppressor of Hairy-wing* binding site. The *suppressor of Hairy-wing* [*su*(*Hw*)] gene encodes a DNA-binding protein that binds to a specific sequence of the *gypsy* provirus (36 [for reviews, see references 4 and 5]). Using the vector P{w^+, *f*alter-*su*(*Hw*)bs} as an ectopic template, we attempted to introduce the *su*(*Hw*) binding site into the f^{hd} allele by P element-induced gap repair. The crossing scheme was analogous to that presented in Fig. 4, except that we used P{w^+, *f*alter-*su*(*Hw*)bs} instead of P{w^+, *f*alter} as a template. Because the P{w^+, *f*alter-*su*(*Hw*)bs} construct rescues the f^{36a} phenotype (only partially resembling the f^1 phenotype), we expected successful conversion events to exhibit this particular f^1-like phenotype. After P{w^+, *f*alter-*su*(*Hw*)bs} had been lost by segregation (G_2 in Fig. 4), we selected potential convertants that exhibited an f^1-like phenotype instead of a wild-type phenotype. Each candidate was tested by PCR (Fig. 2a to c) to ensure that the template P{w^+, *f*alter *su*(*Hw*)bs} had been lost by segregation. A total of 6,924 chromosomes were scored, and 110 conversion candidates were analyzed by PCR. Larger P element fragments were present in the majority of the candidates, but no true convertants were found. This result was in contrast to those of previous experiments (Tables 1 and 2), in which conversion events occurred readily with comparable sample sizes. One possibility,

as discussed below, is that the *suppressor of Hairy-wing* binding site acts to inhibit a sequence from serving as a gap repair template.

DISCUSSION

The present study addresses the question of whether some of the insights into the double-strand break repair process gained for the w^{hd} system are reproducible at other strictly defined double-strand breaks positioned at alternative chromosomal sites.

The transposable P element insertion allele w^{hd} has been mainly used to introduce DNA double-strand breaks at this locus in order to study specific aspects of the repair of this break with an in vitro-designed template, P{*w*alter} (10, 22). With various derivatives of P{*w*alter}, several important questions were addressed, such as how the efficiency of DNA double-strand break repair can be stressed by alternative template positions or how deleted and inserted nonhomologous sequences within the template could be converted into the target, and which effects the different properties of sequences flanking the break impose on the repair process. A number of insights were gained. (i) Analysis of conversion tracts suggested a new model for the molecular processes of double-strand break repair, the SDSA model (29). (ii) Large nonhomologous insertions of 8 kb could be introduced at the target site (18, 29). (iii) Large deletions could be introduced equally well (18). (iv) DNA repair was more efficient if the template and target possessed identical sequences flanking the initial break (18). (v) Ectopic templates present in the same chromosome in *cis* are preferred by a factor of 5 over templates present in *trans* (8). (vi) To find the break, a small window of about 100 bp of identical sequence seems to be necessary for the successful homology search of the template (30). Recently it was also shown that plasmid-borne sequences which were not integrated into a chromosome could be targeted to a double-strand break (20).

Including the results of this paper, the in vivo gene targeting by P element-induced gap repair has been successful at eight different positions in the *Drosophila* genome so far: at two sites in the *white* gene (reference 10 and this paper), at the *singed* locus (7), at two sites in the *bithorax* complex (16, 28), at a site at 50C (32a), at the *su*(*f*) locus (40), and at the *forked* gene (this paper). The exchange of one P element for another P element was explained by P element-induced double-strand-break repair mechanisms; however, the process could involve multiple step mechanisms in which the gap repair reaction is only one part of several recombinatorial steps (12, 14).

Conversion frequency. Examination of tens of thousands of chromosomes carrying the w^{hd} target and with P{*w*alter}-derived ectopic templates revealed an average conversion rate of 0.9% (including clustered events) for 28 autosomal sites (8, 10, 29). Our data on conversions at *forked* reveal a frequency of 0.29% (including clustering) averaged from 12 template positions. Frequencies were suspected to be lower compared with those of w^{hd}, because crosses were conducted at a lower temperature (18 versus 23°C) in order to avoid the pupal lethality associated with the presence of several nonautonomous P elements in the f^{hd} stock. Differences in the size of the mobile P element and the genetic background might also contribute.

Our experiments with w^{eh812} and f^{hd} as combined targets utilizing P{w^+, *f*alter} templates indicate that w^{eh812} and f^{hd} are converted with very similar frequencies (0.17 and 0.22%, respectively [Table 2]) in an identical genetic context and where developmental temperatures and other environmental variables are also identical. The w^{eh812} allele differs from w^{hd} in having its P element insertion orientation reversed and offset by 23 nucleotide sites (Fig. 5). In addition, w^{eh812} carries a 297-bp deletion downstream of the insertion site. There is some evidence that the P element insertion in w^{eh812} is at least equal to the w^{hd} insertion in its mobilization frequency. In an independent experiment (18a) with w^{eh}, which is identical to w^{eh812} except that it lacks the downstream deletion, conversion from a template on the homologous chromosome was found to occur at a high frequency. The measured rates were 6.3% (n = 1,161) and 21.4% (n = 5,271), depending on the degree of homology in the template allele. These frequencies are comparable to the analogous results with w^{hd} (10). Therefore, this observation combined with the present results indicating rough equivalence between w^{eh812} and f^{hd} with the same ectopic autosomal templates can be used to argue that all three P element insertions, w^{hd}, w^{eh}, and f^{hd}, undergo conversion events at similar frequencies.

No convertants were detected with the *su*(*Hw*) binding region within the template. We attempted to convert the DNA sequence which interacts with the chromatin insulator protein, *su*(*Hw*), into the f^{hd} allele. Almost 7,000 chromosomes were scored by selection of males expressing the f^{1}-like phenotype, a phenotype expected in true convertants containing the incorporated *su*(*Hw*) binding sequence. One hundred-ten conversion candidates showing the f^{1}-like phenotype were analyzed by PCR, but no conversion events were detected. In order to explain this result, one has to assume that either true conversion events did not occur with the P{w^+, *f*alter-*su*(*Hw*)bs} template or that they may have occurred but that we failed to pick them up.

The first assumption seems unlikely, because sample sizes of 1,000 scored chromosomes with the P{w^+, *f*alter} template resulted in at least two conversion events (Tables 1 and 2). Nevertheless, we cannot exclude this possibility.

The second explanation assumes that scoring the f^{1} phenotype has led to a bias, failing to isolate true convertants of this template. This could be true, but then we would have to explain why the *su*(*Hw*) binding sequence built into the P{w^+, *f*alter} vector results in a rescue of the f^{hd} and f^{36a} null mutants but would not render the converted f^{hd} allele into the same phenotype.

While the two explanations cannot be ruled out from the data presented, our failure to convert sequences that bind the *su*(*Hw*) protein into the gap at the *forked* locus could be alternatively explained by direct interference of the *su*(*Hw*) protein or its associated modifying protein [*mod*(*mdg4*)] (9) with the gap repair processing proteins. The *su*(*Hw*) gene is expressed in most tissues (13), which would explain its possible interaction with gap repair in the germ line.

Our results also may explain unsuccessful attempts by others to convert *gypsy* sequences into the *yellow* and *white* genes by P element-induced gap repair (9a). Since the *su*(*Hw*) protein is known to interfere with enhancer-driven promoter activity (5) and because of its involvement in position effect variegation (9), it is possible that our results reflect an incompatibility between the presence of the *su*(*Hw*) binding site and the repairosome involved in the gap repair reaction. Since the *su*(*Hw*) and the *mod*(*mdg4*) proteins are part of a chromatin insulator complex (2, 4), our results point toward a possible interaction of these chromatin insulators and DNA double-strand break repair.

While our attempt to convert the *su*(*Hw*) binding site into the *forked* gap failed, we may draw some useful conclusions from this effect. The system described here could be useful to identify sequences to which DNA binding proteins bind which may suppress or enhance gap repair frequencies by interacting

with the gap repair-performing proteins. Thus, questions about the involvement of chromatin during double-strand break repair could be addressed by this approach.

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ADDENDUM IN PROOF

A ninth locus which had been successfully targeted by P element-induced conversion was reported while this paper was in press. C. Merli, D. Bergstrom, J. Cygan, and R. Blackman replaced the endogenous *out at first* (*oaf*) promoter with a promoter capable of interacting with the *decapentaplegic* (*dpp*) enhancers (reported at the 37th Annual Drosophila Research Conference, San Diego, Calif., 27 April to 1 May 1996; Genes Dev., in press).

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