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DNA homology requirements for mitotic gap repair in *Drosophila*

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ABSTRACT **We used *P* transposable-element mobilization to study the repair of double-strand DNA breaks in *Drosophila melanogaster* premeiotic germ cells. Distribution of conversion tracts was found to be largely unaffected by changes in the length of sequence homology between the broken ends and the template, suggesting that only a short match is required. However, the frequency of repair was highly sensitive to single-base mismatches within the homologous region, ranging from 19% reversion when there were no mismatches to 5% when 15 mismatches were present over a 3455-bp span.**

Efficient recombination between two stretches of DNA requires that the two sequences locate each other in the genome and form the stable recombinational intermediate complex. Some degree of homology between the two interacting sequences is needed for efficient recombination by most pathways (1).

The questions of how long the stretch of homology must be and how closely the sequences must match within this stretch have been approached with various experimental systems in bacteria (2–5), yeast (6–8), and mammals (9–15). The general conclusion from these studies is that the recombination mechanisms are much more sensitive to degree of homology than would be predicted purely on the basis of DNA-duplex stability. In *Escherichia coli*, for example, a single-base mismatch in 53 bp resulted in a 4-fold reduction in recombination (4), and a 40-fold reduction was seen when the mismatch proportion was increased to 10% (3). The minimum length of homology required has been estimated at 20–50 bp for *E. coli* (2–4) and 63–89 bp for *Saccharomyces cerevisiae* (7). For mammalian cells, estimates range from 163 to 300 bp (9, 13), but some studies indicate efficient recombination with as little as 20 bp (9, 10). One suggestion (2, 5) is that the length and stringency of homology requirements reflect an evolutionary adaptation to prevent recombination between unrelated sites and are, thus, expected to depend on size and complexity of the genome.

In this report we make use of the recently developed method of transposable-element-induced gap repair to address the question of homology requirements for recombinational DNA repair. *P* elements are DNA transposons that have been used for a variety of techniques in *Drosophila* (16). Recent data (17–19) led to a model in which *P* element excision leaves behind a double-stranded DNA break. According to this interpretation, such breaks are often extended into various-sized gaps through exonuclease activity. Repair occurs when the ends of the gap invade a homologous DNA sequence from which to copy the missing genetic information. The result is unidirectional transfer of information from the template sequence to the excision site. The template can be the sister strand or an ectopic site located elsewhere in the genome or extrachromosomally (17, 18, 20).

The system allows us to examine the length and stringency of homology requirements in *Drosophila melanogaster*. A further advantage of this approach is that recombination occurs between sequences on different chromosomes, thus requiring a homology search that covers the entire genome. In contrast, note that in all the previous studies cited above the two interacting sequences were either closely linked within a few kbp (6–9, 13, 14) or else one or both of the sequences was present on a multicopy plasmid (2, 3, 4, 8, 11, 12, 14). Thus, the effective concentration of the searched-for sequence was much higher than in the system used here, and the search for homology was correspondingly less demanding. We find that a short stretch of homology is still sufficient for a high frequency of recombinational repair, even when there is only one copy of the sequence per chromosome. However, the frequency of successful repair is highly sensitive to single-base mismatches within this stretch.

METHODS

***Drosophila* Strains and Crosses.** All crosses were set up in standard cornmeal/molasses/agar medium at ≈22°C. The stocks have been described (17, 18); see Lindsley and Zimm (21) for genetic symbols. All females with mobilized *P* elements were mated individually, so that independent events could be distinguished. In previous experiments (D. Johnson-Schlitz and W.E., unpublished work) we observed up to a 2-fold increase in reversion frequency among the progeny of parents that had aged 15–20 days after eclosion. For all experiments reported here we used only young female parents and scored their progeny on a consistent schedule (Fig. 1) to avoid age-related variability.

Mapping Conversion Tracts. The restriction-site alterations in the white gene used as markers for determining conversion tracts have been described (18), as has our method for determining them. Briefly, we extracted DNA from single *Drosophila* males (23), amplified parts of the white gene by the PCR, and then cut with restriction enzymes corresponding to the marker site changes.

EXPERIMENTS AND RESULTS

Gap-Repair Strategy. Fig. 1 shows the general procedure used to replace a *P* element-bearing allele with its homolog. We used a *P* element insertion allele of the X chromosome-linked white locus known as $w^{hd80k17}$ (abbreviated w^{hd}) as our target for repair. This allele carries a small, nonautonomous *P* element inserted into one of the exons, resulting in a recessive bleached-white eye color. The closely linked markers yellow and split were used to identify chromosomes. Transposase was provided by an immobile *P* element on another chromosome (22).

The template was any of a series of revertants of w^{hd}, generically designated $w^{hd\text{-}R}$, in which the *P* element and some of its flanking sequences were replaced by sequences from an *in vitro* modified white gene (18). Each $w^{hd\text{-}R}$ allele carries up to 12 single-base substitutions that served as restriction-site markers without preventing white gene ex-

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Abbreviation: w^{hd}, $w^{hd80k17}$.

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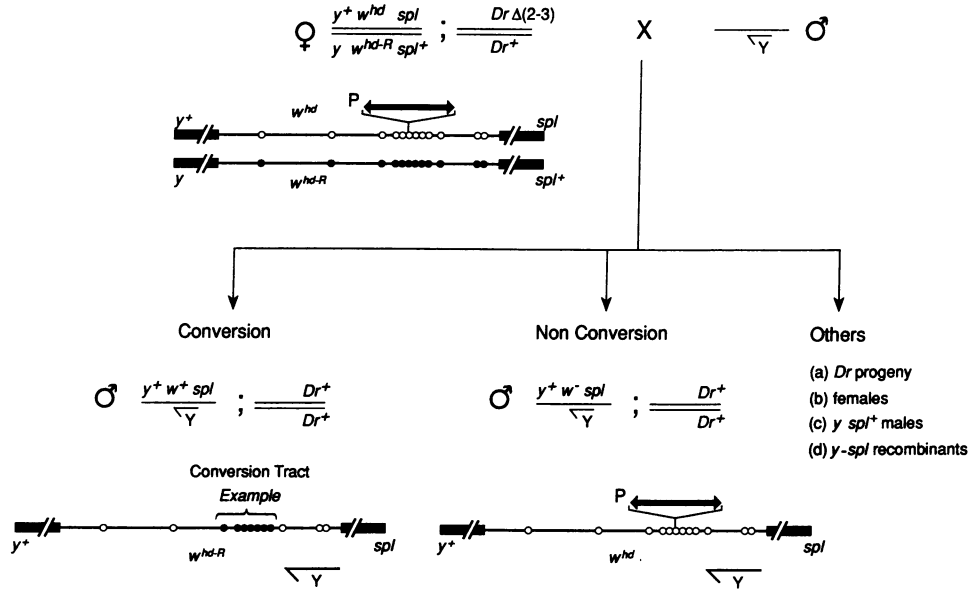

FIG. 1. Genotypes used for generating conversions at the w^{hd} gene. A diagram of the white locus is shown under each genotype. Marker sites are indicated by ○ for wild-type site and ● for the alteration introduced into *P*[walter], as described (18). Females are heterozygous for w^{hd} and one of the $w^{hd\text{-}R}$ alleles and also for flanking markers yellow (*y*) and split (*spl*), which are closely linked on the X chromosome (21). These females also have the *P*[Δ2-3](99B) transposase source (22) on a third chromosome marked with Drop (*Dr*). Each female was mated individually 2–6 days after eclosion to produce the first brood and transferred 5 days later for a second brood. Progeny were scored until 23–25 days after each brood was initiated. Up to three y^+ *spl*; Dr^+ sons with wild-type eye color, indicating potential $w^{hd\text{-}R}$ revertants, were saved from each cross and mated to compound-X females to establish lines, but only one per cross was used in this analysis to ensure independent events. In the pictured example, the template is a $w^{hd\text{-}R}$ allele in which all 12 marker sites of *P*[walter] had been copied in. This allele was derived from a conversion event in a previous experiment (18). The newly derived $w^{hd\text{-}R}$ allele has a conversion tract that includes seven of the marker sites.

pression and up to three other known sites of naturally occurring differences from w^{hd}. Repair events were identified as phenotypic reversions of white among sons with the same flanking markers as the parental w^{hd} chromosome.

Length of Homology. To assess the length of homology required for repair, we determined the conversion tract distribution obtained with a $w^{hd\text{-}R}$ allele as the template and compared it to analogous data from previous work (18) in which the same 12 markers were present ectopically on a transposon, *P*[walter]. With *P*[walter] as the template, the length of flanking homology varies depending on how much gap widening has occurred before strand invasion, but this length can never exceed 2456 bp on the left side of the break or 999 bp on the right (Fig. 2). If the gap expands to include the extreme left or right marker site, then the homology is <550 bp or 115 bp, respectively. However, with $w^{hd\text{-}R}$ as template, the flanking homology extends to the ends of the chromosome. Therefore, the homology is effectively infinite on both sides of the break, regardless of the extend of gap widening. We analyzed 81 independent reversions in which the template was a $w^{hd\text{-}R}$ allele carrying all 12 marker sites (Fig. 3). Each revertant had at least one of the marker sites copied into the newly reverted white gene. The conversion tracts and their distribution were very similar to the previous data from the *P*[walter] template (Fig. 4). If the model of double-strand gap enlargement is correct, this similarity implies that the minimum length of homology needed for efficient recombination is less than the 115 bp that separates the most distal marker site from the homology boundary of *P*[walter]. Other possibilities are considered below (see *Discussion*).

Gap-Enlargement Model. Note that for the rightmost two marker sites the $w^{hd\text{-}R}$ template had somewhat higher conversion rates than *P*[walter]. This difference is expected if we assume that gap repair fails when the gap extends beyond the boundary of homology in *P*[walter], and such progeny are not

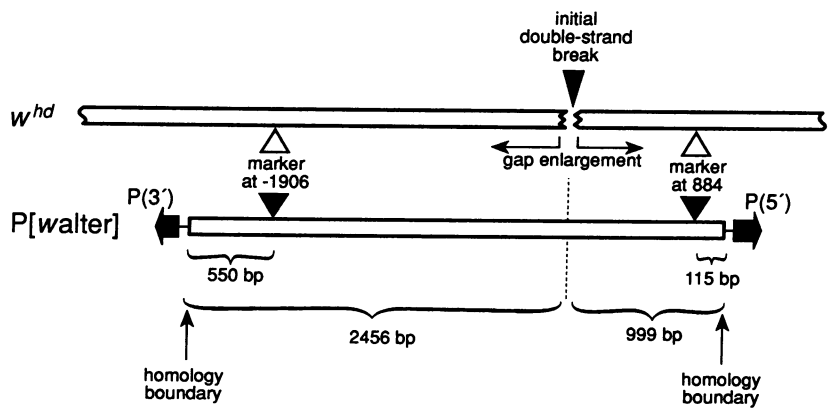

FIG. 2. Comparison of the white sequences in the w^{hd} allele and *P*[walter]. Open bars represent sequences derived from the white gene and surrounding sequences. Only the two marker sites most distal from the initial breakpoint are shown.

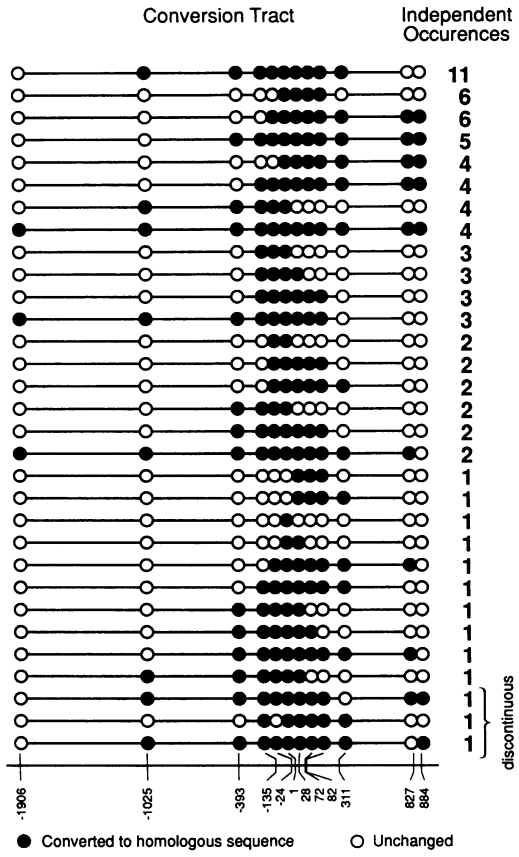

FIG. 3. Conversion tracts. Each of 81 y^+ *spl*; Dr^+ males carrying new $w^{hd\text{-}R}$ alleles obtained, as shown in Fig. 1, was analyzed as described (18) to determine the conversion tract. Only one such son per female was used to ensure that all events were independent. Note that the last three conversion tracts are discontinuous, which is approximately the same frequency of discontinuous tracts that has been seen with an ectopic template (18).

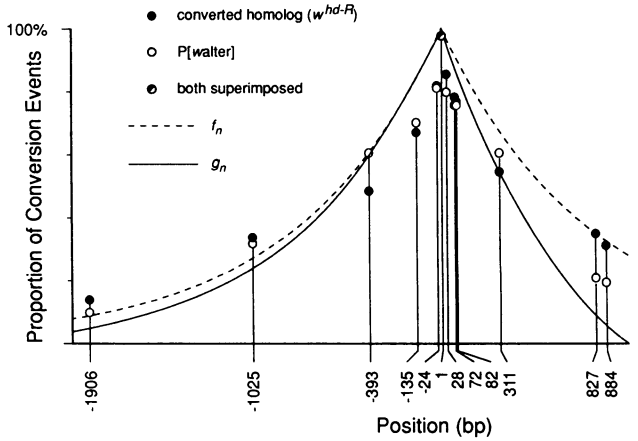

FIG. 4. Distribution of conversion tracts. Comparison of the distribution of conversion frequencies when the template is the homologous $w^{hd\text{-}R}$ allele (Fig. 3), as opposed to previous data (18) using an ectopic template, *P*[*w*alter], with limited flanking homology. Only 73 conversion tracts from the *P*[*w*alter] template were plotted, as opposed to the 111 plotted in figure 7 of ref. 18. The difference reflects the exclusion of 21 aberrant tracts and 17 conversions derived from *P*[*w*alter](34C), which was found to carry a deletion of the right end of the white gene (18). Similarly, only the 78 continuous tracts from Fig. 3 were used. The theoretical curve $f_n = x^n$ is the same as that reported (18), and $g_n = (x^n - x^H)/(1 - x^H)$, which is the conditional probability that a gap covers point n, given that it does not extend beyond point H, the boundary of the homologous sequence. The values of H were 2456 on the left side and 999 on the right side, based on the sequence of *P*[*w*alter]. We used maximum likelihood (18, 24) to estimate the value of x at 0.99873 ± 0.000096 based on f_n for the 78 tracts from Fig. 2 and g_n for the 73 tracts from *P*[*w*alter].

recovered. We can easily compute the probability of such events based on a model of exonuclease action in which each new nucleotide is cleaved independently with constant probability x. Thus, the gap-widening process is terminated at a given nucleotide site with probability $1 - x$. The expected conversion-tract curve for the $w^{hd\text{-}R}$ template is then determined by the waiting-time distribution described (18), and the probability that the endpoint of a tract lies between nucleotides j and k is $(x^j - x^k)$, where j and k are counted from the site of the original breakpoint. The curve for *P*[*w*alter] is similar, except that the probability must be made conditional on the gap not being widened beyond the homology boundary (Fig. 2). The conditional probability is $(x^j - x^k)/(1 - x^H)$, where H is the nucleotide position of the homology boundary, either 2456 bp or 999 bp, depending on whether points j and k lie to the left or right of the breakpoint. An implicit assumption of these models is that the minimum length of homology needed for efficient repair is small relative to the distance between the most distal marker and the homology boundary, 115 bp. If this assumption is invalid, the observed frequencies for the more distal markers in the *P*[*w*alter] series will tend to be lower than the predicted curve, g_n in Fig. 4. The results (Fig. 4) show that these models can account for the observed differences in conversion rates for the distal marker sites. Moreover, conversion rates for the distal markers in the *P*[*w*alter] series actually exceed the predictions (g_n), thus reinforcing the above conclusion that the search window is <115 bp.

The Effect of Mismatches. The same kind of crosses (Fig. 1) were used to determine the effect of single-base mismatches on the frequency of repair. We measured the reversion rates for a series of $w^{hd\text{-}R}$ templates, each with a different subset of the 12 marker sites plus 3 other variable sites that were not used as markers, spread over a 3455-bp span. Reversion frequencies were estimated as the proportion of y^+ *spl*; Dr^+ sons with wild-type eyes. The results (Figs. 5 and 6) showed that the reversion frequency decreased linearly from ≈19%, when none of the sites was heterozygous, down to 5%, when all of them were. The negative correlation (Kendall's τ) between number of heterozygous sites and reversion rate was significant at $P < 0.002$. We conclude that the DNA gap-repair mechanism in *Drosophila* exhibits a high degree of sensitivity to single-base mismatches.

Such high sensitivity could also explain an apparent dearth of very short conversion tracts in our data, as indicated in Fig. 4, where most of the central points lie below the theoretical curves. The shortest gaps will have the highest concentration of mismatches near the edge of the gap due to the placement of our marker sites (Fig. 5). These gaps will, therefore, be less likely to undergo successful repair and, thus, be underrepresented in our sample.

Lack of Associated Crossing Over. Previous work indicated that gap repair at the w^{hd} gene is associated with very little, if any, increased crossing over of outside markers (17), and the present data concur. We observed an average of 3.27% crossing over between the yellow and split loci in the experiments described above (Fig. 1), which was slightly greater than the transposase-free control (2.61%, N = 20,286), but the difference was not significant. The correlation between the crossover frequency and reversion rates was actually less than zero but was also not significant. The results are consistent with the previous conclusion (18) that strand exchange does not occur in most gap-repair events. However, the data cannot rule out the possibility that there is a small amount of crossing over in order to explain the enhanced male recombination seen at *P* element-containing intervals (27, 28).

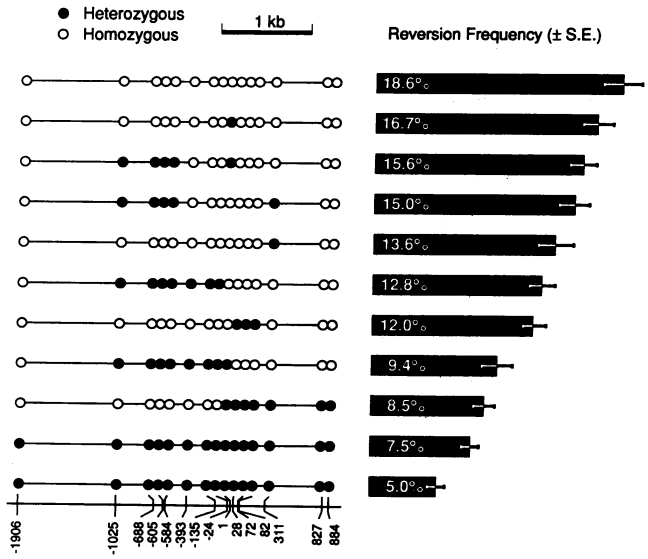

FIG. 5. Mismatches of each $w^{hd\text{-}R}$ allele used and corresponding reversion rates. Data were derived from the crosses described in Fig. 1, except that the $w^{hd\text{-}R}$ allele in the female parent was one of the 10 indicated alleles. Note that the actual spacing between some of the marker sites is less than that of their symbols, ○ and ●, which were separated for clarity here and in Fig. 1. The $w^{hd\text{-}R}$ allele with no substitutions was obtained as a precise excision in a w^{hd} male in the absence of any homolog or ectopic template (17), and the others were obtained by copying in parts of the *P*[*w*alter] transposon (18). Two of the $w^{hd\text{-}R}$ alleles have discontinuous conversion tracts, which were probably formed in two steps (18). The two values for the allele with all 15 sites converted came from two independent experiments, which were the first and last of the 11 tests done. Reversion frequencies were estimated as the proportion of y^+ *spl*;*Dr*.$^+$ sons with wild-type eyes, and the SE values were determined by a method that is not biased by clustering due to premeiotic events (25). The reversion rate of the $w^{hd\text{-}R}$ allele with no substitutions was obtained as part of another experiment (D. Johnson-Schlitz and W.E., unpublished work). The numbers of individually mated females per genotype were 132, 210, 202, 147, 132, 148, 136, 124, 207, 231, and 199 from top to bottom, and the total numbers of y^+ *spl*;*Dr*$^+$ progeny were 1743, 2288, 2215, 3534, 1768, 2986, 2789, 1815, 2153, 4631, and 2848, respectively. In addition to the 12 marker sites described (18), there were three other differences between w^{hd} and some of the $w^{hd\text{-}R}$ alleles, which are the result of natural polymorphisms. The substitutions were ATT(w^{hd}) → TTC, T(w^{hd}) → A, and C(w^{hd}) → T, at positions −1447 to −1445, −1424, and −1341 in the coordinates of O'Hare *et al.* (26), corresponding to sites −584, −605, and −688 above. These sites were determined for each $w^{hd\text{-}R}$ allele by DNA sequencing or by assuming continuity of conversion tracts. Other heterozygosities could exist in unsequenced parts of the w^{hd} allele, but these are mostly exonic regions where variability is less likely.

DISCUSSION

Implications for Gene Replacement. The technical application of *P* element-induced gap repair as a method for gene replacement in *Drosophila* has been demonstrated (18, 20), and an analogous system has been used in nematodes (29). The present results suggest that the constructs used for such procedures require only a few hundred base pairs of homologous sequence on either side of the target, but that such sequences should match the genomic DNA as closely as possible.

DNA Repair and Recombination in *Drosophila*. It is possible that the present observations are specific to *P* element-induced breaks and do not apply to other types of recombination and repair of DNA damage. However, given the small size of *P* elements, <3 kb, they seem unlikely to encode extensive recombination functions in addition to the transposase and regulation functions already identified (16). Therefore, we suggest that the characteristics of DNA repair

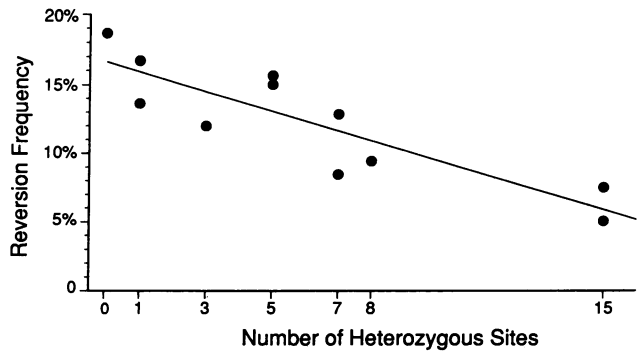

FIG. 6. Effect of heterozygosity on reversion frequency. Plotted points correspond to the $w^{hd\text{-}R}$ alleles in Fig. 5, and the line was fitted by least squares. In addition to the nonparametric correlation test mentioned in the text, we also performed an exact permutation test with the product–moment correlation coefficient (24). We computed the correlation for each of the 11! = 39,916,800 permutations of the data and found only 10,256 permutations with a correlation less (i.e., more negative) than or equal to the one seen. The *P* value is, therefore, 0.00026.

reported here actually reflect the processes that normally occur in *Drosophila* mitotic cells.

In contrast to the present data, Hilliker *et al.* (30) have argued that heterozygosities have no detectable effect on meiotic crossing over and gene conversion in *Drosophila*. This apparent discrepancy might reflect a true difference between recombination in meiosis versus mitotic processes. Most or all of the events we observed occurred in mitotic cells before meiosis, as indicated by clusters of revertants in the progeny of individual flies (17). Moreover, previous work has shown that homolog-dependent reversion of w^{hd} occurs somatically as well as in the germ line (17). Another indication that the process differs from meiotic recombination is the lack of a substantial enhancement of crossing over associated with w^{hd} reversion. However, it is still possible that some of the early steps, such as the search for homology and strand invasion, are shared between gap repair in mitotic cells and meiotic recombination. The effect might be too subtle to detect with the necessarily small sample sizes possible in studies of meiotic recombination.

Length of the Homology Search Window. Our data place an upper limit for the search window at 999 bp, the distance from the initial breakpoint to nearest-homology boundary. We further suggest that the window is actually <115 bp. This argument relies on our interpretation that the distribution of conversion tracts reflects differential gap widening before the search for homology and repair. This interpretation agrees well with the fit to the models of gap enlargement shown in Fig. 4.

However, several other interpretations should be considered. (*i*) It is possible that the conversion tracts we observed were from mismatch repair of heteroduplex DNA rather than from gap widening, thus allowing for longer search windows. Such repair would have to be strand-biased (31) to explain the preponderance of continuous tracts we observe. This interpretation does not provide an explanation of the reduced conversion rate for the central marker sites (Fig. 4), whereas gap widening can account for the effect, as discussed above. In addition, it is not clear how mismatch repair alone can result in transfer of insertions and deletions, as has been observed for w^{hd} (D. Johnson-Schlitz, N.N., G. Gloor, and W.E., unpublished work). (*ii*) The homology search could occur before the gap widening. This possibility seems less likely if we assume that the broken ends remain in a protein–DNA complex during the homology search and repair steps. (*iii*) The homology search might occur with only one end of the break used as a search window and the other end being

carried along to the repair site. In that case, the window would still have to be no more than 550 bp, the distance from the leftmost marker to the homology boundary, to account for cases in which all 12 of the marker sites lie within the conversion tract.

The Search for Homology. A diploid *Drosophila* cell contains 370 million bp including heterochromatin and many dispersed families of moderately repeated sequences (32). Our data suggest that the ends of a *P* element-induced chromosome break can locate a single homologous template anywhere in the genome by using only a short stretch of closely matching sequence as a search window. Moreover, this search is so efficient that up to several percent of progeny from our crosses carried conversion events at the white locus. It seems unlikely that such a powerful search mechanism would rely on random collisions between homologous DNA sequences.

This conclusion is strengthened by the observations discussed above for bacteria, yeast, and mammals, and corroborated here for *Drosophila*, that the search mechanism is hypersensitive to a small proportion of mismatches. We observed a 3-fold decrease in the conversion rate associated with a slight reduction of homology from 100% to 99.6%. In fact, the sensitivity might be even greater because many mismatches in our templates (Fig. 5) lie close to the *P* insertion point and might, thus, be removed by gap widening before the homology search.

Several lines of evidence suggest that this search occurs in two steps: the selection of a DNA molecule to scan and the scanning itself. Kinetic studies of DNA pairing mediated by the RecA protein of *E. coli* (5, 33, 34) indicate that the reaction is close to first order in DNA concentration, supporting the conclusion that the search is "processive" rather than based on random collisions, which would require second-order kinetics. Furthermore, increasing the size of the target sequence by adding heterologous DNA favored the reaction, suggesting that the search is more efficient for longer DNA molecules. Selection of the molecule to scan appears to occur preferentially when the target homology is embedded in a longer region of relatively weak homology (8) or when it is linked to the site of chromosome breakage (35).

In our data, the finding that the absolute frequency of repair and conversion is greater when the template is on the homolog rather than an ectopic site suggests that the homolog is favored in the selection step. There is also preliminary evidence for an enhanced conversion rate when the template is linked to the point of chromosome breakage, as in the case of *P*[*w*alter](6F) in figure 3 of ref. 18 and similar data for other X chromosome-linked sites (D. Johnson-Schlitz, C. Preston, and W.E., unpublished work). This observation suggests that the scanning step can occur over stretches of several million base pairs. Such efficient scanning combined with the short search window and the high sequence precision seems to reflect a remarkable mechanism for finding homology in a large and complex genome.

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