*Proc. Natl. Acad. Sci. USA*
Vol. 89, pp. 1735–1739, March 1992
Genetics

# Oligonucleotide-directed site-specific mutagenesis in *Drosophila melanogaster*

(*P*-element transposition/double-strand break/mutation/DNA repair/recombinant DNA)

SATNAM S. BANGA* AND JAMES B. BOYD

Department of Genetics, University of California, Davis, CA 95616

*Communicated by R. W. Allard, October 11, 1991 (received for review July 18, 1991)*

ABSTRACT **An efficient technique has been developed for performing *in vivo* site-directed mutagenesis in *Drosophila melanogaster*. This procedure involves directed repair of *P*-element-induced DNA lesions after injection of a modified DNA sequence into early embryos. An oligonucleotide of 50 base pairs, whose sequence spans the *P*-element insertion site, mediates base replacement in the endogenous gene. Restriction mapping, DNA sequencing, and polymerase chain reaction analysis demonstrate that base substitutions present in an injected oligonucleotide are incorporated into genomic sequences flanking a *P* insertion site in the white gene. This analysis suggests that progeny bearing directed mutations are recovered with a frequency of about $0.5 \times 10^{-3}$. Because *Drosophila* remains a premier organism for the analysis of eukaryotic gene regulation, this system should find strong application in that analysis as well as in the analysis of DNA recombination, conversion, repair, and mutagenesis.**

Understanding of a genetic function frequently requires mutational analysis of the gene in question. Although chemical and physical mutagens can be used to obtain mutations, these agents act randomly. In selected organisms gene replacement after *in vitro* mutagenesis of the target gene can also provide valuable mutations (1, 2). Gene targeting is not currently possible in *Drosophila*, however, where transformation is generally mediated by *P* transposable elements that integrate randomly (3). *P*-element transformation also suffers from the limitations that expression of the integrated gene can be affected by its ectopic chromosomal location and that large genes cannot be transformed.

To circumvent these problems we have developed an efficient procedure for performing *in vivo* site-directed mutagenesis in this organism. The following three observations suggested that it might be possible to direct the repair of a *P*-element-induced DNA lesion by injecting embryos with exogenous DNA sequences that span the *P*-element insertion site: (*i*) *P*-element transposition can occur in early embryogenesis (3, 4). (*ii*) *P*-element transposition is postulated to generate a double-strand DNA break that is repaired with the aid of homologous sequences (5, 6). (*iii*) In *Escherichia coli*, oligonucleotides have been shown to direct the repair of double-strand breaks present in plasmid DNA (7). To test our hypothesis that *P*-element-induced breaks can undergo oligonucleotide-directed repair, we employed the X chromosomal white mutation $w^{hd80k17}$. This mutation was generated by insertion of a nonautonomous *P* element into the 3′ coding region of the white gene (8, 9). To repair the *P*-element-induced break in this gene, wild-type oligonucleotide sequences spanning the *P* insertion were provided during transposition in early embryogenesis. To distinguish between $w^+$ revertants obtained by precise excision of the *P* element and $w^+$ revertants obtained through oligonucleotide-directed repair, oligonucleotides carrying silent base changes were injected. These changes alter the base sequence but not the amino acid sequence of the white gene. Chromosomal repair mediated by such an oligonucleotide should produce phenotypically wild-type progeny carrying the silent base substitutions present in the injected sequence. Here we demonstrate that an oligomer of 50 base pairs (bp) can mediate base replacement in the vicinity of a *P* element in the white gene.

## MATERIALS AND METHODS

***Drosophila* Stocks and Culture Conditions.** The $P[ry^+\ \Delta 2\text{–}3](99B)$ stock (abbreviated as Δ2–3) was used as a stable source of transposase (10) to mobilize the nonautonomous *P* element in the $y\ w^{hd80k17}\ f$ stock as shown in Fig. 1. These crosses were made at 18°C and all other crosses were made at 25°C. Flies were reared on standard *Drosophila* medium. Descriptions of chromosomes or mutations not presented here can be found in Lindsley and Grell (11).

**Oligonucleotide Synthesis.** Oligonucleotides were synthesized with a Cyclone Plus DNA synthesizer (MilliGen/Biosearch, Novata, CA) and were purified by Oligo-Pak columns according to the manufacturer's specifications (MilliGen/Biosearch). Oligonucleotides were either resuspended in injection buffer (4) for injecting embryos or in water for DNA amplification. To synthesize a double-stranded oligonucleotide, two complementary strands were mixed in equal amounts, incubated at 90°C for 2 min, and then allowed to anneal at room temperature for 2 h.

**Embryo Injection.** Synthetic oligonucleotides were injected into embryos prior to germ-cell formation as described (3, 4).

**DNA Amplification.** Genomic DNA used in polymerase chain reaction (PCR) amplification was isolated from single flies in 50 μl of squishing buffer as described (5). PCRs were performed in 20- or 50-μl volumes for 30 cycles with an annealing temperature of 64–68°C for 30 sec, extension at 72°C for 1 min, and denaturation at 92°C for 45 sec. For 50-μl reaction volumes, 5 μl of squishing buffer containing genomic DNA, 5 μl of 10× *Taq* buffer (Promega), 50 pmol of each primer, all four dNTPs (each at 10 mM), and 3–4 units of *Taq* polymerase (Promega) were used. For 20-μl reaction volumes, 2 μl of squishing buffer containing genomic DNA, all four dNTPs (each at 4 mM), and 2 μl of 10× *Taq* buffer were used in addition to 50 pmol of each primer and 3–4 units of *Taq* polymerase.

The presence of a directed base-pair change in the genomic DNA was detected by amplifying genomic DNA with one of the primers that carries the respective base modification at the 3′ end as described (12) (see Fig. 5 *A* and *B*).

**Restriction Endonuclease Mapping.** To determine the presence or absence of *Hae* III or *Sau*3AI sites, PCR-amplified

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*To whom reprint requests should be sent at present address: Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, 185 South Orange Avenue, Newark, NJ 07103-2714.

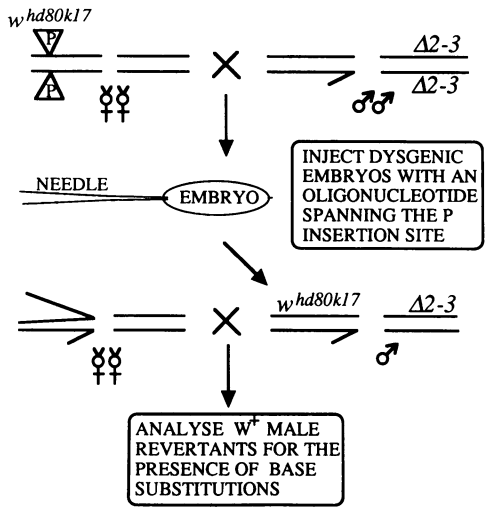


FIG. 1. Mating scheme for recovering $w^+$ revertants after oligonucleotide injection. The *P* element present in the white gene mutation $w^{hd80k17}$ was mobilized in embryos by the modified *P* element Δ2–3. Dysgenic embryos of genotype $w^{hd80k17}$; +/Δ2–3 were injected at 18°C either with single-stranded or double-stranded oligonucleotides at concentrations of 7 mg/ml and 3 mg/ml, respectively. After hatching, larvae were raised at 25°C. Males injected with the double-stranded oligonucleotide were mated individually to four or five compound double X chromosome females. Males injected with the single-stranded oligonucleotide were mated two at a time to four or five compound double X chromosome females. In the latter case the number of fertile males given in the text may be an overestimate because one member of a pair could have been sterile.

products were cleaved with either *Hae* III or *Sau*3AI according to the manufacturer's specifications (Promega). Electrophoretic fractionation was conducted in 12% polyacrylamide gels.

**DNA Sequencing.** Dideoxyribonucleotide sequencing (13) was performed on single-stranded amplified DNA to determine the sequence of the oligonucleotide-directed revertant (O-dR1) around the *P* insertion site using the Sequenase kit (United States Biochemical). The gel-purified 223-bp fragment (indicated in Fig. 3) was asymmetrically amplified (14) using the primer 5′-GTCGGCTACTCCTTGCGTCG-3′. Amplified product was then sequenced by employing the internal primer 5′-GCACATCGTCGAACACCACG-3′. Sequencing products were analyzed in 7% polyacrylamide gel.

## RESULTS

**Experimental System.** To test the possibility that a *P*-element-induced break can be repaired by using an oligonucleotide, embryos of genotype $w^{hd80k17}$; Δ2–3 were injected with both single-stranded and double-stranded oligonucleotides as shown in Fig. 1. Nucleotide sequences of both oligomers including the base modifications are shown in Fig. 2 *C* and *D*. Injected males were crossed individually or in pairs to compound double X chromosome females, and the progeny were scored for the presence of $w^+$ revertants. The DNA of these revertants was examined by restriction enzyme analysis, by sequence analysis, or by PCR analysis to identify oligonucleotide-directed $w^+$ revertants.

**Analysis of $w^+$ Revertants Obtained by Injecting a Single-Stranded Oligomer.** Three $w^+$ revertants (recovered from a culture with two males) were produced among 923 progeny of 24 males that had been injected with the single-stranded oligonucleotide. Restriction enzyme analysis of a 223-bp PCR-amplified DNA fragment (spanning the *P* insertion site) from each of these three revertants revealed that one possesses the restriction pattern carried by the injected oligonucleotide (Fig. 3*B*) and two exhibit the wild-type restriction pattern (Fig. 3*A*). Data from the oligonucleotide-directed revertant and one wild-type individual are depicted in Fig. 3*C*. The revertant O-dR1 (oligonucleotide-directed) carries base modifications present in the single-stranded injected oligonucleotide, because the most internal *Hae* III site, which is present in the wild-type sequence, has been eliminated, and a new *Sau*3AI site has been created, which is absent from wild-type. The sequences presented in Fig. 2*C* reveal that both substitutions are required to generate the *Sau*3AI site. The spontaneous occurrence of both mutational events in the

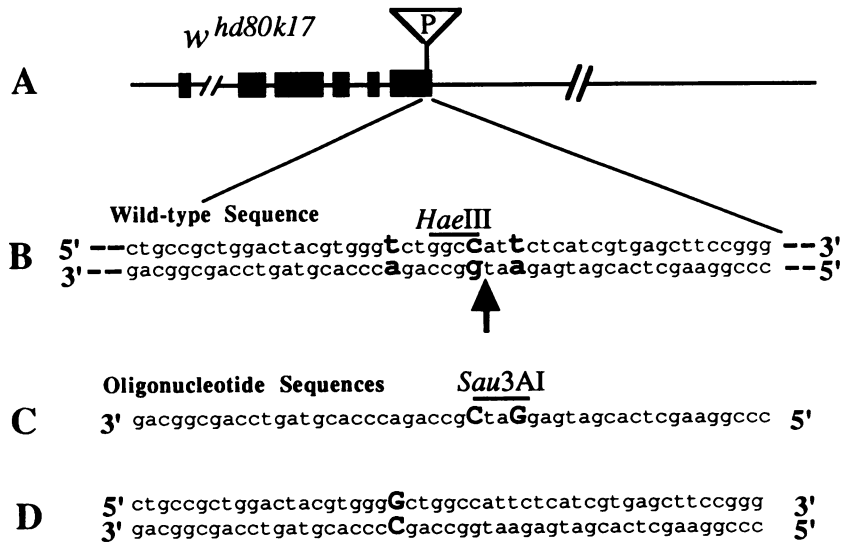


FIG. 2. Partial sequence of the white gene and the corresponding synthetic oligonucleotides. (*A*) Black boxes represent exons of the white gene located on the X chromosome. The position of the *P* element in the $w^{hd80k17}$ mutant is indicated by a triangle. (*B*) The 50 bp of the wild-type sequence of the white gene encompassing the *P* insertion site. The *P* insertion site is indicated by an arrow. The bases identified by boldface type have been modified in the synthetic oligonucleotides shown in *C* and *D*. Broken lines indicate continuity of the white gene sequence. The horizontal line above the sequence indicates a *Hae* III site (ggcc). (*C*) Sequence of the 50-bp single-stranded synthetic oligonucleotide indicating base alterations (boldface uppercase type) that abolish the *Hae* III restriction site and create a new *Sau*3AI restriction site (GatC, 5′ → 3′, indicated by a horizontal line). These substitutions do not alter the corresponding amino acids. (*D*) Sequence of the 50-bp double-stranded synthetic oligonucleotide indicating a base substitution (boldface uppercase type). This alteration also fails to affect the corresponding amino acid.

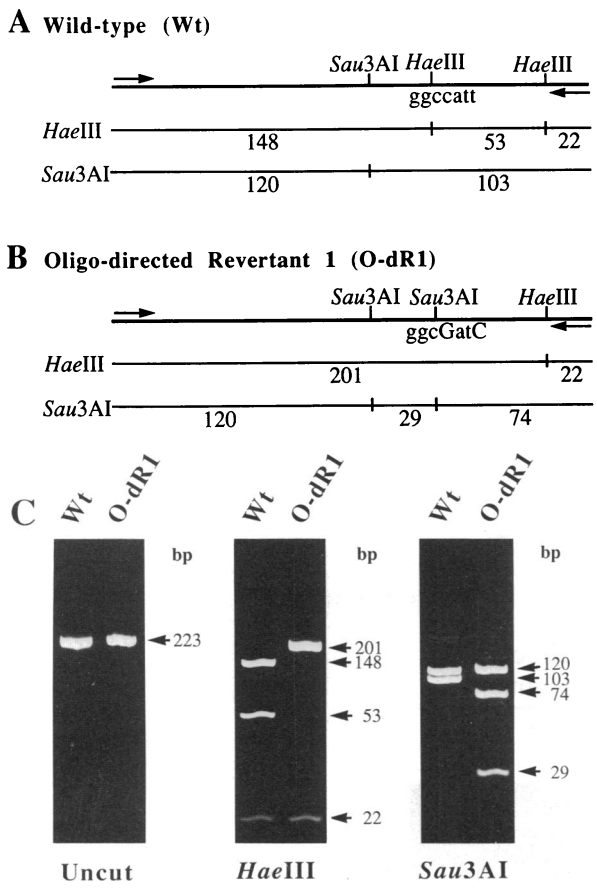


FIG. 3. Identification of oligonucleotide-directed revertants by restriction mapping. Restriction maps of the PCR-amplified 223-bp genomic fragment of the wild-type white gene (*A*) and the oligonucleotide-directed revertant (*B*) are shown. Arrows indicate primers (5′-GTCTGCTGATTAACCAATGG-3′ and 5′-GTCGGCTAC-TCCTTGCGTCG-3′) used for amplification. Partial sequence given below the most internal *Hae* III site has been altered by making the changes indicated by uppercase letters in *B*. The base modification c to G eliminates the most internal *Hae* III site whereas an additional base modification of t to C creates a new *Sau*3AI site (GatC). Restriction fragments generated by the restriction endonucleases *Hae* III and *Sau*3AI are shown below the restriction map. (*C*) PCR-amplified genomic fragments of wild-type (Wt) and the oligonucleotide-directed revertant (O-dR1) were digested with either *Hae* III or *Sau*3AI or remained undigested. Various restriction fragments were visualized by ethidium bromide staining after electrophoretic fractionation in a 12% polyacrylamide gel.

absence of the oligonucleotide is expected to be exceedingly rare. Direct confirmation of the presence of directed base modifications was provided by sequencing the PCR-amplified genomic DNA from the oligonucleotide-directed revertant O-dR1 (Fig. 4).

**Analysis of $w^+$ Revertants Obtained by Injecting a Double-Stranded Oligomer.** Embryos undergoing *P* transposition were also employed to determine whether a double-stranded oligonucleotide can be utilized in the repair of *P*-element-induced lesions. Thirty fertile injected males produced 13 $w^+$ males among 2600 male progeny. Because at least four revertants were obtained from different injected males, this collection of revertants represents at least four mutational events. A 100-bp genomic fragment encompassing the *P* insertion site was amplified by PCR. Flies from a cluster of 10 revertants derived from a single male exhibit a directed base alteration as evidenced by PCR analysis (Fig. 5 *A* and *B*). Data from three members of that cluster (O-dR20-1, O-dR20-3, and O-dR20-7) are shown in Fig. 5*C* along with

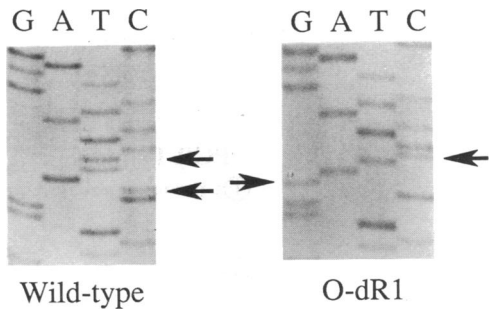


FIG. 4. Sequence analysis of the 3′ region of the white gene spanning the *P* insertion site in wild type and the O-dR1 revertant. Altered bases are indicated by arrows.

results from the three other independent wild-type revertants (Wt7-1, Wt13-1, and Wt15-1). Amplification of the genomic fragment from the wild-type revertants was observed with the wild-type primers 1 and 3, but little or no amplification of that fragment was observed when primer 1 was replaced by mutant primer 2. Since primer 2 carries the same base substitution at the 3′ end that was present in the injected oligonucleotide, this result indicates that these revertants do not carry the directed alteration (see Fig. 5*C*). On the other hand, amplification of the same genomic fragment in the oligonucleotide-directed revertants (O-dR20-1, O-dR20-3, and O-dR20-7) by primers 2 and 3 and not by primers 1 and 3 indicates that these three revertants carry the base modification present in the injected oligonucleotide. Similar analyses involving seven other members of that cluster also reveal incorporation of the substituted base (data not shown). It should be pointed out here that since the double-stranded oligonucleotide was generated by mixing equal amounts of two complementary strands, it is possible that residual single-stranded oligonucleotide may have been involved in the repair event.

## DISCUSSION

Since *P* elements preferentially insert into the 5′ regulatory regions of structural genes, the expanding collection of *P* inserts already represents a rich opportunity for application of this approach to the analysis of gene regulation. Because conversion tracts in *Drosophila* have been demonstrated to extend >1000 bases (15), this range provides the potential for extensive mutational analysis of 5′ regulatory sequences. It is therefore likely that this approach can be extended to the use of cloned DNA constructs that can be readily mutated *in vitro*. The current alternative of cloning, *in vitro* mutagenesis and *P*-element transformation, is considerably more laborious and can introduce a number of artifacts arising from the construct itself and from the relocation of the sequences in the genome. The system described here eliminates the need for exogenous gene sequences and retains the genomic position of the gene under study. The possibility of employing oligonucleotides for site-directed mutagenesis in *Drosophila* also provides an approach for investigating the mechanisms of mutagenesis, recombination, conversion, and DNA repair in this model organism. Since our understanding of these processes at the molecular level remains rudimentary in eukaryotes, this approach should also contribute to our understanding of these processes.

Overall, about 4% of the fertile males injected with single- or double-stranded oligomers produced progeny that carry directed alterations (2 out of 54 tested males). The frequency of a directed change among their progeny is about $0.5 \times 10^{-3}$ (11 oligonucleotide-directed revertants out of 3523 progeny scored). A single individual present at that frequency in a

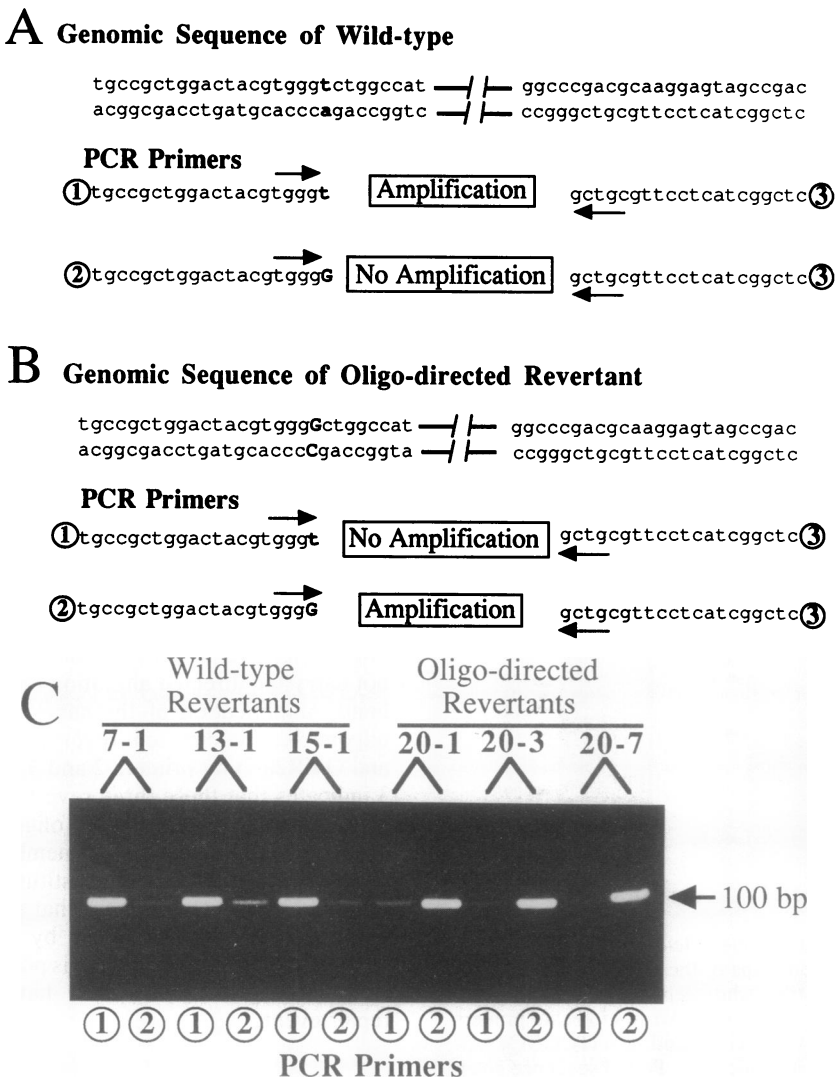


FIG. 5. Identification of oligonucleotide-directed revertants by PCR analysis. The sequence of the 100-bp PCR-amplified fragment of the white gene spanning the *P* insertion site in wild type (*A*) and the oligonucleotide-directed revertant (*B*) is shown. Uppercase type indicates substituted bases. The combinations of primers used in the PCR to detect oligonucleotide-directed revertants are given below the sequence. Primers 1 and 3 are wild type in sequence whereas primer 2 ends with the same base substitution present in the injected oligonucleotide. Arrows indicate the 5′–3′ orientation of the primers. A combination of primers 1 and 3 will amplify the indicated genomic fragment from wild-type DNA whereas primers 2 and 3 will catalyze amplification only if the substituted base is present in the genome. (*C*) The 100-bp genomic fragment was amplified in parallel reactions with each revertant using combinations of primers 1 and 3 or primers 2 and 3 as shown in *A* and *B*. The common primer 3 is not shown. PCR products were visualized by ethidium bromide staining after fractionation in a 12% polyacrylamide gel. Data for three wild type (Wt7-1, Wt13-1, and Wt15-1) and three oligonucleotide-directed revertants (O-dR20-1, O-dR20-3, and O-dR20-7) are shown. The other seven members of the genetic cluster (20 series) have also incorporated the oligonucleotide-directed base substitution by this analysis (data not shown).

population can readily be detected by PCR amplification using one of the primers that carries the directed substitution at the 3′ end (12). This approach is, therefore, available for the recovery of mutations whose phenotypes are not as readily detected as the white mutation employed here.

One requirement of this approach is that a chromosomal lesion must be generated in the vicinity of the site targeted for mutagenesis. Attempts to mutagenize the white gene by injecting single-stranded oligonucleotides into embryos in the absence of a DNA lesion have thus far proven unsuccessful (S. W. Hills and J. A. Kiger, Jr., personal communication). The need for a directed lesion in this system is being met by the increasing availability of *P*-element insertions throughout the *Drosophila* genome. In addition, application of PCR technology now permits recovery of *P*-element insertions in much of the currently cloned portion of the *Drosophila* genome (16–18). Coinjection of the *P* transposase and oligonucleotides could further enhance the efficiency of this approach (19). Alternatively, transposable elements such as *hobo* (20) that employ transposition mechanisms similar to that of the *P* element could also be used to create the requisite chromosomal lesions.

A second requirement of this technique is the absence of endogenous wild-type homologous sequences. This situation can be achieved for X chromosome-linked genes by injecting hemizygous male embryos. For autosomal genes, embryos heterozygous for a deficiency uncovering the locus of interest can be injected. Alternatively, hemizygosity may not be a necessary condition because the vast excess of the injected oligonucleotide sequence present may out compete the endogenous template. An alternative approach to site-directed mutagenesis involving the introduction of altered sequences by *P* transformation has been suggested (5). That method, however, would be considerably more laborious than the approach described here.

Several mechanisms have been considered that might be responsible for the directed repair of chromosomal breaks by

oligonucleotides (7). (*i*) It is possible that the oligonucleotide serves as a template for the repair of double-strand breaks by a gene conversion mechanism. (*ii*) Information present in the oligonucleotide may be incorporated into chromosomal DNA by template switching of DNA polymerase (21). (*iii*) It is possible that information present in the oligonucleotide is physically incorporated into chromosomal DNA by homologous recombination. The system described here may be exploited to distinguish between these alternatives.

**Note Added in Proof.** While this manuscript was under review, an alternate approach of gene replacement in *Drosophila melanogaster* was published (22).

We thank J. A. Kiger, Jr., M. M. Green, R. S. Hawley, and D. S. Henderson for comments on the manuscript, and Angela Rosenstein for technical assistance. We also thank W. R. Engels for fly stocks and M. Cadle, S. Reinl, and J. Presley for synthesizing oligonucleotides. S.S.B. is indebted to his parents (Ram S. Banga and Gian K. Banga) and family (Satwant K. Banga, Inderpreet S. Banga, and Jasjit S. Banga) for moral and financial support. This work was supported by the Department of Energy (Grant EV70210).

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