A Targeted Gene Knockout in Drosophila

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ABSTRACT

We previously described a method for targeted homologous recombination at the *yellow* gene of *Drosophila melanogaster*. Because only a single gene was targeted, further work was required to show whether the method could be extended to become generally useful for gene modification in Drosophila. We have now used this method to produce a knockout of the autosomal *pugilist* gene by homologous recombination between the endogenous locus and a 2.5-kb DNA fragment. This was accomplished solely by tracking the altered genetic linkage of an arbitrary marker gene as the targeting DNA moved from chromosome *X* or *2* to chromosome *3*. The results indicate that this method of homologous recombination is likely to be generally useful for Drosophila gene targeting.

TO carry out gene targeting in Drosophila (Rong and Golic 2000) a donor construct carrying DNA from the gene to be targeted is randomly inserted into the genome by *P*-element-mediated transformation. Then, a site-specific recombinase (FLP) and a site-specific endonuclease (I-*Sce*I) are used to generate, *in vivo*, an extrachromosomal DNA molecule that carries a double-strand break (DSB) within the gene of interest. The presence of this DSB stimulates homologous recombination between the excised donor and the homologous chromosomal target locus (Figure 1). Our initial experiments were based on recombination events that rescued a mutant allele of the Drosophila *yellow* gene. Homologous recombination between the donor and target locus was frequent, occurring approximately once in every 500 female gametes. We recovered several classes of recombinant at the target locus that included allelic substitutions and integration of donor DNA. The promise of this technique is that, starting with only a DNA sequence, animals with specific mutations can be generated and their phenotypes studied to deduce the normal function of the mutated gene. It is therefore important to know whether this method can be generally applied for targeted gene modification in Drosophila.

One issue of concern is whether genes that are not located near telomeres (as is *yellow*) can be targeted. Engels (2000) raised the possibility that the targeted recombination that we observed relied on a type of DNA repair termed break-induced replication (BIR; Malkova *et al.* 1996). He proposed that a single one-ended homologous exchange occurred between the chromosomal target locus and the excised and cut donor molecule, leaving the recombinant chromosome with a truncated terminus. To be recovered as a viable product this chromosome would be repaired by BIR, wherein the broken terminus invades the homolog, prompting unscheduled replication to the end of the chromosome (Figure 2). Since the *yellow* gene that we targeted lies only ~110 kb from the *X* chromosome telomere, it is not unreasonable to imagine that a chromosome break at this location could be repaired by replication to the end of the chromosome. Additionally, *yellow* targeting was much more efficient in the female germline (with two *X* chromosomes) than the male germline (with one *X*), and Engels' BIR model, wherein repair of a one-ended recombination event relies on replication templated from a homolog, provides an explanation for this difference. As a practical matter, the most significant implication of Engels' model is that if targeting must involve BIR, then it is likely that only genes situated near telomeres can be successfully targeted because of the requirement for continuous replication to the end of the chromosome.

A second question is whether methods can be developed to produce targeted mutation and whether this can be done without relying on knowledge of the phenotype produced by mutation in the target locus (Anonymous 2000). Homologous recombination reactions similar to that which we used at *yellow* have been modified to generate targeted mutations in *Escherichia coli*, in yeast, and in mice (Shortle *et al.* 1982; Shen and Huang 1986; Thomas and Capecchi 1987). It is likely that similar methodology can be used in Drosophila. The work presented here demonstrates the use of one such method in Drosophila for targeted gene modification.

MATERIALS AND METHODS

DNA constructs: A 2.5-kb *Nru*I-*Pst*I fragment of the *pugilist* (*pug*) genomic region was cloned into pBS(Not), which is a derivative of pBluescript II KS (+) from Stratagene. In

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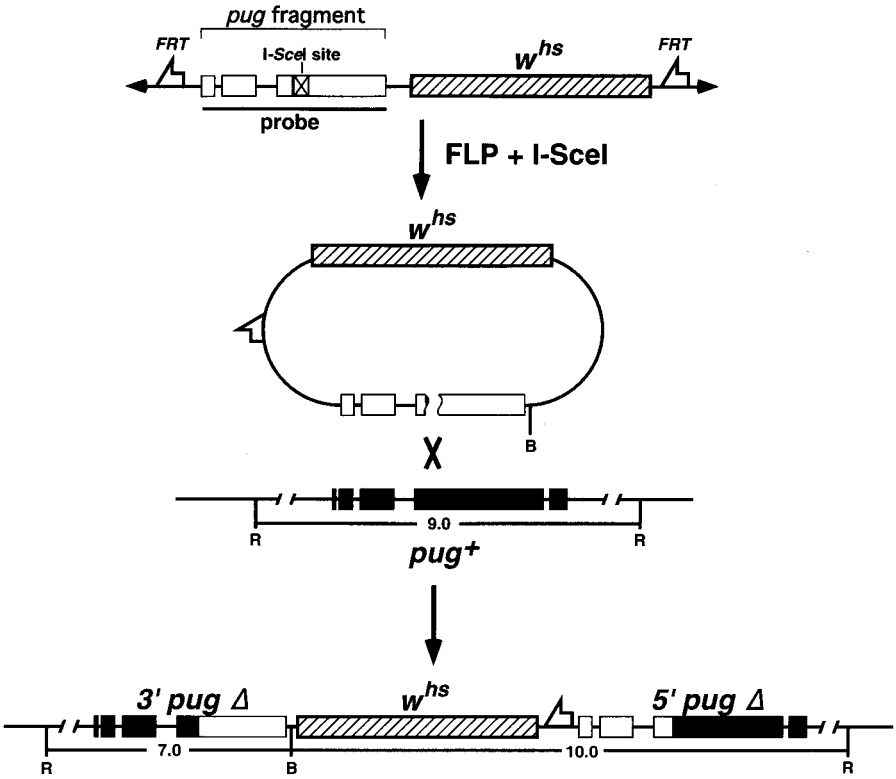

FIGURE 1.—The targeting scheme. The donor construct is diagrammed at the top. FLP-mediated excision and I-SceI-mediated cutting produce the extrachromosomal targeting molecule shown. A 2.5-kb internal fragment of the *pug* gene was used to construct the P[>w^{hs}·*MTH*Δ>] donor. It lacks 5′ regulatory sequences and the first, part of the fourth, and the fifth exons. This targeting molecule is expected to recombine with the endogenous *pug* locus, as shown, to produce a tandem duplication. Arrowheads at the donor site represent *P*-element ends. The direction of *pug* transcription is from left to right. The restriction sites and the location of the probe used in Southern blot analysis are shown with sizes indicated in kilobases. R, *Eco*RI; B, *Bam*HI.

pBS(Not), the *Apa*I site in the multiple cloning site was changed to a *Not*I site by site-directed mutagenesis. An I-*Sce*I recognition site was synthesized as two oligos: Apa-I-plus, 5′-gctagggataacagggtaatggcc; and Apa-I-minus, 5′-attaccctgttatcc ctagcggcc. They were allowed to anneal and were cloned into the unique *Apa*I site in the 2.5-kb *pug* fragment. The same *pug* fragment, now flanked by *Not*I sites, was cloned into the *Not*I site in the *P*-element vector pP[>w^{hs}·*N*>], which is a modified form of pP[>w^{hs}>] (GOLIC and LINDQUIST 1989), to produce the donor construct pP[>w^{hs}·*MTH*Δ>] for *pug* targeting. This construct was transformed into flies by standard *P*-element transformation.

Molecular analyses of targeting events: Southern blot analyses were performed as described (RONG and GOLIC 1998). To verify the restoration of the *Apa*I site in the targeted *pug* allele, primers proxH3 (RONG and GOLIC 1998) and pugseq1.rev (5′-ttcagacgctccaacactga-3′) were used. PCR reactions were done using genomic DNA from flies homozygous for the targeted *pug* alleles. PCR products were digested with *Apa*I and run on a gel along with uncut controls.

Fly stocks and crosses: Description of stocks not provided here can be found at http://flybase.bio.indiana.edu. The deficiency that deletes the pug^{+} region is in the stock *Df(3R)cu/ TM6B, Tb,* provided by the Bloomington Stock Center. The stock *v;* pug^{Dru18} (RONG and GOLIC 1998) was used to provide the *pug* null allele. For targeting we used insertions of the P[>w^{hs}·*MTH*Δ>] donor on either chromosome *X* or *2*. We used either a chromosome *2* or *3* that carries both the *70FLP* and *70I-SceI* transgenes. Crosses generated flies with a single copy donor element and *70FLP* and *70I-SceI*. All flies carried either the w^{1} or the w^{1118} null alleles on their *X* chromosomes. For most screening crosses, the heat-shocked flies were mated to y^{1} w^{1}*; Sb/ TM6, Ubx* flies, and the w^{hs} gene was mapped with respect to chromosome *3* in all progeny with pigmented eyes. In a small number of cases the heat-shocked flies were mated to y^{1} w^{1} *70FLP*3F; *Sb/ TM6, Ubx* and the offspring were screened for a white^{+} nonmosaic phenotype, which could indicate that the w^{hs} gene was no longer flanked by FLP recombination targets (FRTs), as expected for a targeting event. Positives were retested for FLP-mediated white mosaicism and mapped with respect to chromosome *3*. The reported numbers are corrected for the fact that in some crosses only half the tested flies had the appropriate genotype.

RESULTS

Insertional, or ends-in, targeting of an internal gene fragment could conceivably generate a targeted gene knockout. The expected product would be a tandem partial duplication of the target gene, with both copies defective because they each lack a portion of the gene (as shown in Figure 1). We tested this targeting scheme by using it to generate a null mutation in the *pug* gene, which encodes a homolog of the trifunctional form of the enzyme methylenetetrahydrofolate dehydrogenase. Null mutations in this gene produce a recessive eye color defect (RONG and GOLIC 1998). The gene is located at 86C on the right arm of chromosome *3*, ~20 Mbp from the nearest telomere. A 2.5-kb internal fragment of *pug* was engineered to carry a recognition site for the I-*Sce*I endonuclease. This *pug* fragment was then placed into a modified form of the *P*-element vector P[>w^{hs}>] (GOLIC and LINDQUIST 1989) to produce the donor construct for *pug* targeting (Figure 1).

The w^{hs} gene of this vector is a hypomorphic allele of the *white* eye color gene and serves as a w^{+} marker for transformation and for the recovery of targeting events. The *pug* gene fragment and w^{hs} are flanked by direct repeats of the *FRT*. When FLP mediates recombination

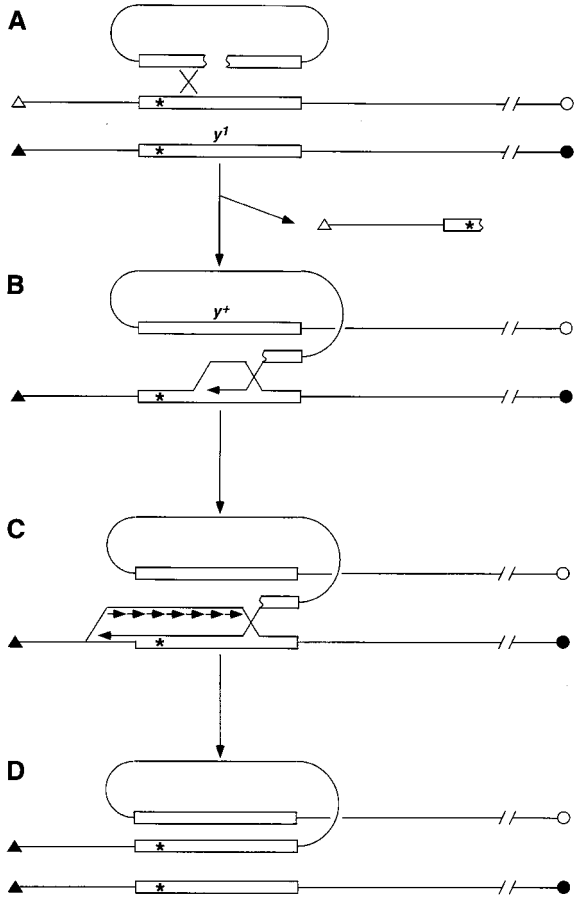

FIGURE 2.—Break-induced replication model for *yellow* targeting (adapted from ENGELS 2000). This model proposes that in a y^1 female, the extrachromosomal y^+ donor pairs with a chromosomal y^1 allele, and DNA to the left of the break recombines with the chromosome (A). This gives rise to a truncated *X* chromosome missing all DNA distal to *yellow*. The reciprocal product is an acentric fragment, which is lost as a result of exonucleolytic degradation or failure to segregate when the cell divides. It is then proposed that the broken end of the centric *X* chromosome invades the homolog at *y*, initiating DNA replication toward the telomere (B and C). This produces two intact *X*'s, one of which carries a targeted *y* allele. The resulting cell should be homogyzous for all the alleles distal to *y*, exemplified by both *X* telomeres having the same shade (D). The *yellow* gene is represented as a rectangle, the rest of the chromosome as a single line. Telomeres are represented by triangles; centromeres are indicated as circles. The asterisk indicates the site of the point mutation in y^1. Left-facing arrows indicate leading-strand DNA synthesis. Right-facing arrows indicate lagging-strand synthesis.

between these *FRT*s, both the w^{hs} gene and the partial *pug* gene are excised from the chromosome on a circular DNA molecule. Subsequent I-SceI cutting generates the recombinogenic *pug* targeting molecule. Homologous recombination between this molecule and the resident *pug* locus is expected to carry the w^{hs} gene into *pug* (Figure 1). If the donor is located on a chromosome other than chromosome *3*, a targeting event can be recognized by the altered genetic linkage of w^{hs} that will

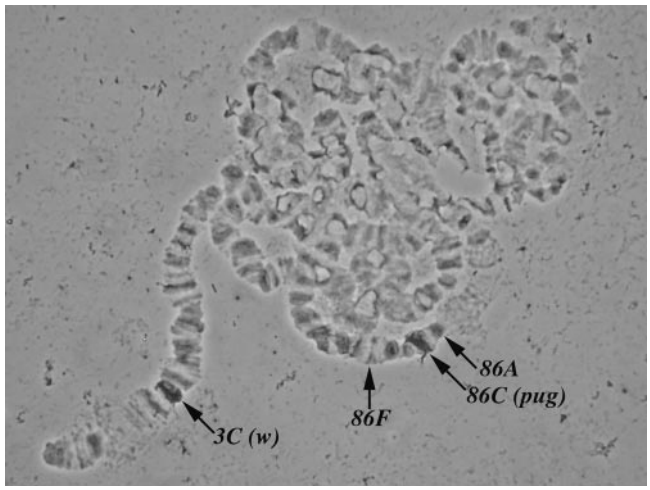

FIGURE 3.—Cytological mapping of targeted insertion. Chromosomes from flies homozygous for a targeted *pug* allele were probed with labeled *white* gene DNA (random-primed pP[>w^{hs}>] DNA). Two signals were detected: one at the 3C locus, which is the endogenous *white* locus, and the other at 86C, which is the targeted *pug* locus. Chromosome landmarks are indicated.

result. The modified *pug* allele produced by targeted insertion of this donor molecule is expected to be mutant.

To carry out targeting we produced flies that had the transgenic *pug* donor construct, on either the *X* chromosome or chromosome *2*, and the heat-inducible *70FLP* and *70I-SceI* transgenes. These flies were heat shocked as described (RONG and GOLIC 2000) and test-crossed to flies carrying the w^1 mutation. The vast majority of progeny were white eyed, owing to FLP-mediated excision and loss of the *FRT*-flanked w^{hs} gene (GOLIC and LINDQUIST 1989). In most cases, when offspring with pigmented eyes were observed the w^{hs} gene was mapped to detect its mobilization to chromosome *3*. A small number of crosses were screened with an alternative technique that is explained in the DISCUSSION.

A total of 455 vials (generating 100–150 progeny per vial) were set up to screen for mobilization of w^{hs} in the female germline: we recovered three independent cases of w^{hs} mobilization to chromosome *3*. Chromosome *in situ* hybridization was used to locate the w^{hs} gene on chromosome *3*. In two cases, w^{hs} was located at 86C, the normal location of *pug* (Figure 3). In the third case w^{hs} was located in cytological region 97 and was not characterized further.

We also tested for targeting in the male germline. We recovered no *pug* targeting events from 308 vials, although we did recover one example of nontargeted mobilization (to chromosome *2*).

Southern blot analysis was performed on one of the targeted alleles to confirm its molecular structure. DNA from homozygous flies was digested with restriction enzymes *Eco*RI and *Bam*HI and hybridized with a probe

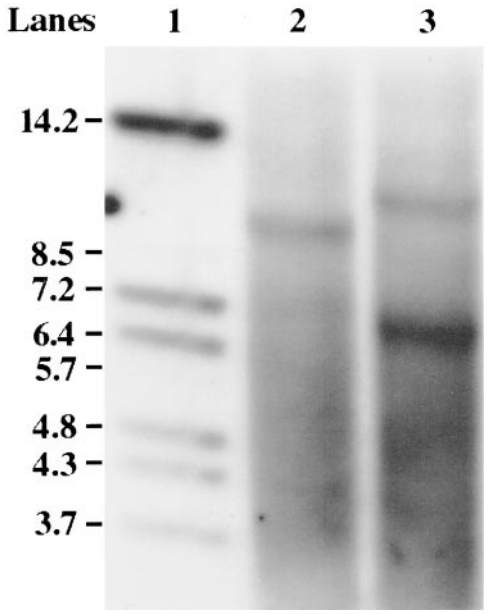

FIGURE 4.—Southern blot analysis of a *pug* targeting event. Genomic DNA was digested with *Eco*RI and *Bam*HI and blotted to a membrane. The membrane was hybridized with a 2.5-kb *pug* probe (Figure 1). Lane 1, molecular markers with indicated sizes; lane 2, pug^{+} control showing the endogenous 9-kb band; lane 3, DNA from flies homozygous for the targeted *pug* allele showing, as predicted, the 7-kb and the 10-kb fragments.

made from the 2.5-kb *pug* fragment (Figure 1). As expected, the wild-type 9-kb band was converted into two bands of 7 and 10 kb (Figure 4). Hybridization with a *white* probe detected only the 10-kb band and the endogenous *white* bands (not shown).

To assess the phenotype of the two *pug* alleles produced by targeting we generated the following three genotypes: (1) flies homozygous for the targeted alleles; (2) flies that carried the targeted alleles over a *pug* deficiency; and (3) flies that carried the targeted alleles heterozygous with a previously isolated *pug* null allele (RONG and GOLIC 1998). For both targeting events, all three genotypes displayed the *pug*-null phenotype of slightly reduced pigmentation in the center of the eye (not shown). By this assay, the alleles generated by targeted integration of the donor are functional nulls.

Further analysis suggested that the *pug* targeting events arose by precise homologous recombination. The I-SceI cut site in the donor was inserted at a unique *Apa*I site in the 2.5-kb *pug* fragment, destroying the *Apa*I site in the process. The DSB repair model for recombination (SZOSTAK *et al.* 1983) predicts that the DSB generated by I-*Sce*I will be enlarged to a gap by exonuclease activity. During integration, this gap will be repaired by DNA synthesis that uses the wild-type target locus as a template. It was therefore expected that the *Apa*I site would be restored in the targeting process, leading to an *Apa*I site in each of the two *pug* copies. This was confirmed by Southern blot analysis of *Apa*I-digested genomic DNA, or by *Apa*I digestion of PCR-amplified DNA fragments (not shown). In summary, cytological, phenotypic, and molecular data support the conclusion that these represent cases of targeted modification of the *pug* locus.

DISCUSSION

The general application of targeted mutagenesis: The most important aspect of our results is the demonstration that a gene far removed from the nearest telomere can be successfully targeted. Since the *pug* gene is very much closer to centric heterochromatin than it is to a telomere it seems likely that most euchromatic genes will be susceptible to targeted modification by this technique.

These experiments also show that nontargeted insertions, although they do occur, are not so frequent as to be a significant nuisance. Here, the ratio of targeted to nontargeted recombinants was 2:1 in females (or 1:1 overall). In the previous *yellow* targeting experiments, targeted recombinants also outnumbered nontargeted events (RONG and GOLIC 2000). Nontargeted events that arise on chromosomes other than the target chromosome can be easily detected by mapping the w^{hs} marker gene. Southern blot analysis can be used to detect and discard nontargeted events that are linked to the target chromosome and to confirm the expected structure of targeting events.

Our results address the concerns of whether this method can be used for targeted mutagenesis. We generated a directed mutation without relying on prior knowledge of the mutant phenotype. Instead, we recovered targeting events by following the segregation of an easily visible genetic marker. In addition, our results demonstrate the production of a functional null allele, even though the gene is partially duplicated. The right-hand copy is almost certainly a null owing to the absence of upstream regulatory elements and the first exon of the *pug* gene. The phenotypic studies suggest that the left-hand copy is also a functional null, although it is possible that the phenotypic assay may not be sensitive to a small amount of *pug* activity. Even so, it is not unreasonable to imagine that, by strategically placing point mutations in the donor, the two copies would assuredly be nulls. Therefore, we believe that this targeting method provides a general tool for directed mutagenesis of the Drosophila genome.

The mechanism of targeted homologous recombination: The recovery of targeted alleles at *pug* does not rule out the possibility that some of the targeting events we previously recovered at *yellow* did arise by homologous recombination and BIR. The BIR model for *yellow* targeting provided an explanation for the observed female:male difference in targeting efficiency. It predicts that there should be no difference between the sexes

in the efficiency of targeting an autosomal gene. In the experiments reported here, the *pug* targeting events recovered from females do outnumber those recovered from males, but the number of events is too few to reasonably conclude that this difference is significant, and thus cannot be used to address the BIR model.

Targeting of *yellow* was substantially more efficient (1 in 4 vials for the female germline; Rong and Golic 2000) than targeting of *pug* (1 in 228 vials). This could be taken as support of the hypothesis that the majority of *yellow* targeting events relied on the mechanism of BIR. According to this view, *pug* targeting is less efficient because of the much greater gene-telomere distance involved and the correspondingly lower probability of a replication fork sucessfully proceeding to the end of the chromosome. On the other hand, it seems at least equally likely that the explanation for this disparity owes to the different amounts of donor:target homology in the two experiments—8 kb in the *yellow* experiments *vs.* 2.5 kb in the *pug* targeting experiments reported here. In other targeting systems, the extent of donor:target homology does influence the frequency of homologous recombination (Shen and Huang 1986; Deng and Capecchi 1992; Dray and Gloor 1997). Further experiments (Engels 2000) are required to determine whether BIR is part of the mechanism of Drosophila gene targeting.

Targeting methodology: Altered genetic linkage was used to detect *pug* targeting events in most of the work reported here. The essence of this screen is the detection of marker gene movement from one chromosome to another. This works well but is time consuming since it requires mapping of the donor *P* element and construction of special strains so that the donor chromosome or its homolog is dominantly marked.

To speed the process of gene targeting we have begun to use an alternative method to detect targeted homologous recombination events (Figure 5). The w^{hs} marker has an easily scored cell-autonomous phenotype. The infrequent progeny of targeting crosses that exhibit the w^+ phenotype arise as a result of the donor element escaping FLP-mediated excision or by mobilization of the donor to the target site (or a nontargeted site). The original donor is distinguished from cases of mobilization by the fact that, in the former case, the w^{hs} marker gene is flanked by *FRT*s, but in the latter it is not (see Figure 1). FLP will cause eye-color mosaicism when w^{hs} is flanked by *FRT*s but not when there is only a single *FRT* adjacent to w^{hs}. This difference can be employed to aid in the detection of gene targeting events. Flies that carry the donor insertion, *70FLP*, and *70I-SceI* are heat shocked and crossed to flies that carry *70FLP*. The progeny of this targeting cross are heat shocked during the first few days of development to induce FLP synthesis. The mosaic or nonmosaic phenotype of the infrequent w^+ flies tells whether they carry the original donor element or a targeted insertion. Molecular characterization by Southern blotting can then be used to confirm that the recovered events resulted from homologous recombination at the target locus. One of the two *pug* targeting events was recovered using this method.

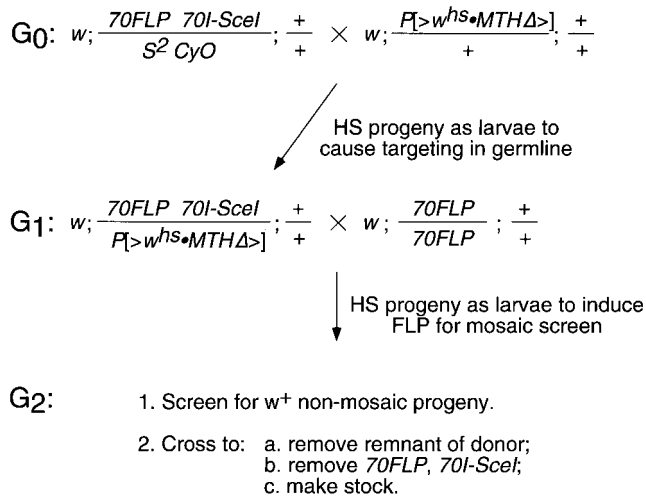

Figure 5.—A rapid targeting scheme. In this example a donor element on chromosome *2* is used for targeting *pug* on chromosome *3*. If the donor parent in G_0 is heterozygous for that insertion (as indicated here) then only half of the progeny will carry the donor element. This may often be the case if the donor element has not yet been mapped. Many of the G_1 flies that receive the donor element will be white-eyed owing to the high rate of somatic excision and loss, but these are still suitable for use in the targeting cross. S^2CyO is a chromosome *2* balancer. HS, heat shock.

This alternative procedure significantly simplifies and speeds the process of gene targeting by allowing targeting to be carried out without knowing the location of the donor element. The process can be made even easier by using a *70FLP* line that has strong constitutive expression of FLP. We have generated such a line (unpublished results). This eliminates the need for heat-shocking the progeny of the targeting cross for the mosaic/nonmosaic test.

The crosses to obtain targeting can be carried out contemporaneously with crosses to map the donor element. The purpose of mapping is to provide the information needed to remove, by crossing, the remnant donor *P* element after targeting has been achieved.

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