METHODS: A Companion to Methods in Enzymology 14, 000–000 (1998)
Article No. ME980591

# Use of FLP/FRT System to Study *Drosophila* Development

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Marked clones in mosaic animals have been used extensively to answer developmental questions in *Drosophila*. Recently, the use of the FLP/FRT system has allowed for the high-frequency production of mosaic animals for 95% of *Drosophila* genes. Cell markers have been engineered in this system to label mutant clones in both developing tissues and adult cuticle. Strains carrying these genetically marked FLP/FRT chromosomes have greatly enhanced our ability to study gene function in both germline and somatic *Drosophila* cells.
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In studying the developmental biology of different organisms, several fundamental questions recur. What cells in the early embryo give rise to certain adult structures and does a given structure develop independent of a cell lineage? In a particular organ or tissue, how are progenitor cells specified in correct numbers, and how do these founder cells become compartmentalized during development? At the level of molecular genetics, the requirement of a gene's function in neighboring cells reveals its role in cell interaction mechanisms. Is a given gene required for multiple developmental stages? If so, how can its function be studied in a later developmental process? And ultimately, how can we identify new genes involved in a given developmental process?

Historically *Drosophila* mosaics, composed of cells of different genotypes, have been useful in addressing these developmental questions. Various methods have been employed to generate mosaics: chromosome loss induced by unstable chromosomes or mutations, cell or nuclear transplantation, local gene inactivation/activation, and mitotic recombination induced by ionizing radiation (1–6). However, serious limitations in all these methods render mosaic analysis impractical for studying many genes and developmental processes. Critical limitations include the low frequencies of mosaicism and the limited availability of cell markers for labeling clones in developing tissues.

Ionizing irradiation by X rays is the most frequently used method for inducing mitotic recombination. X-ray irradiation produces rare chromosomal breaks, which can lead to the exchange of homologous chromosome arms. When sister chromatids segregate at mitosis, a cell acquires a homozygous region distal to the point of recombination. Mutant clones are recognized only if they comprise cells homozygous for a genetic marker closely linked to the mutation of interest. Mitotic recombination events take place at low frequencies when induced by X rays. The low frequency of mitotic recombination impedes analysis of clones in internal tissues, which require tedious dissections. On the other hand, increasing the ionizing radiation to raise the recombination frequency causes excessive cell death. These shortcomings greatly limit the use of genetic mosaics for biological studies.

Use of the yeast site-specific recombination FLP/FRT system to induce mitotic clones in *Drosophila* (Fig. 1A) together with the generation of novel cell markers has helped overcome most of the above-described problems [see reviews (7–10)]. The construc-

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tion of *Drosophila* chromosomes integrating FLP/FRT elements and cell markers (9) has made mosaic analysis a routine and easy procedure. To date, more than 200 papers have been published using these chromosomes. Additionally, many studies have reported using the germline clone system (11, 12) and the FLP-out technique (Fig. 1B) (7, 13). Here, we discuss the applications of these techniques, with a focus on common questions raised by users, and review recent technical improvements.

## 1. USE OF FLP/FRT SYSTEM AND CELL MARKERS TO PERFORM MOSAIC ANALYSIS FOR EXISTING MUTATIONS

A general scheme for inducing mitotic clones using the site-specific recombinase FLP, its target FRT sites, and cell markers to label mutant cells in *Drosophila* is illustrated in Fig. 1A. When marked with cell-autonomous markers, clones can be readily identified, allowing analysis of these cells in both developing and adult tissues. A genetic scheme to recombine a mutation of interest onto an FRT-carrying chromosome arm for mosaic analysis is diagrammed in Fig. 2. Information regarding the use of specific FLP/FRT chromosomes and cell markers is described in the following paragraphs [also see review (10)].

### A. FLP Strains

To control the induction of mitotic recombination events, the most commonly used FLP constructs are expressed under the control of the heat-shock promoter. This allows for high levels of recombinase to be expressed at specific developmental stages. The original FLP construct (*P[ry$^+$;hs-FLP1]*) generated by Golic and Lindquist (7) has since been jumped onto different chromosomes (Table 1). A second FLP construct, hsFLP122 or hsp70-FLP1 from Struhl and Basler (13), contains a modified coding sequence for FLP and produces mitotic recombination at frequencies severalfold higher than that of the original

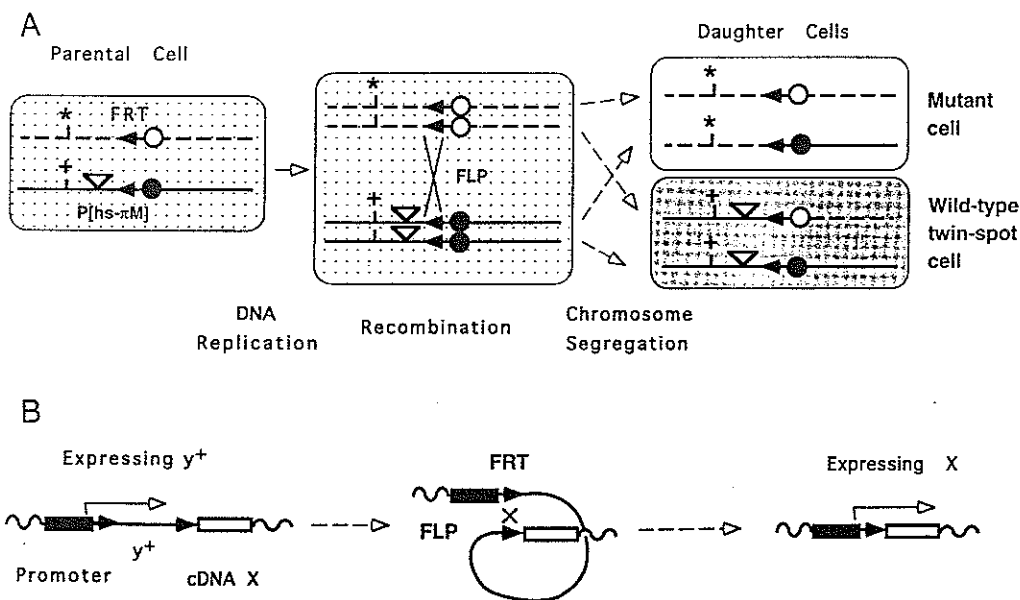


**FIG. 1.** Generating and labeling mutant clones using FLP/FRT and cell markers. (A) In a heterozygous parental cell, FLP induces mitotic recombination between FRT sites (solid arrows) on homologous chromosome arms. Segregation of recombinant chromosomes at mitosis produces two daughter cells: a mutant cell bearing two copies of the mutant allele (*) and a wild-type twin-spot cell containing only the wild-type form of the gene (+). The cell marker *P[hs-πM]* (∇) cosegregates with the wild-type gene, labeling the mutant cell by its absence. Subsequent cell divisions result in clones from each of these original daughter cells. (B) The FLP-out technique has been used to activate a gene in genetically marked cells. In the parental cell, a constitutive promoter drives the expression of the marker gene $y^+$. The $y^+$ gene is flanked by two FRT elements with the same orientation. On induction, FLP mediates mitotic recomination between these FRT sites, excising the intervening $y^+$ gene. Consequently, the constitutive promoter drives expression of a downstream gene, *X*, in $y^-$ cells.

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hsFLP1 lines. In addition, hsFLP122 exhibits a lower background of mitotic recombination in uninduced flies compared with the original hsFLP1 line.

In practice, the X-linked FLP insertions are used in combination with autosome FRT sites, whereas the autosome FLP insertions are used with FRT-carrying X chromosomes (Tables 1, 3, 4, and 5). Strains carrying both FLP and FRT are generally stable. Many of these strains have been cultured in our laboratory without problems for 5 years at 18°C. However, if a problem such as a drop in frequency of mitotic recombination does develop with a particular strain, then the FLP- and FRT-carrying chromosomes should be reintroduced into the same strain. We caution users to test FLP/FRT strains before commencing clonal analysis of mutations of interest. It is a good idea to test two independent FRT insertions, which makes it easier to distinguish potential

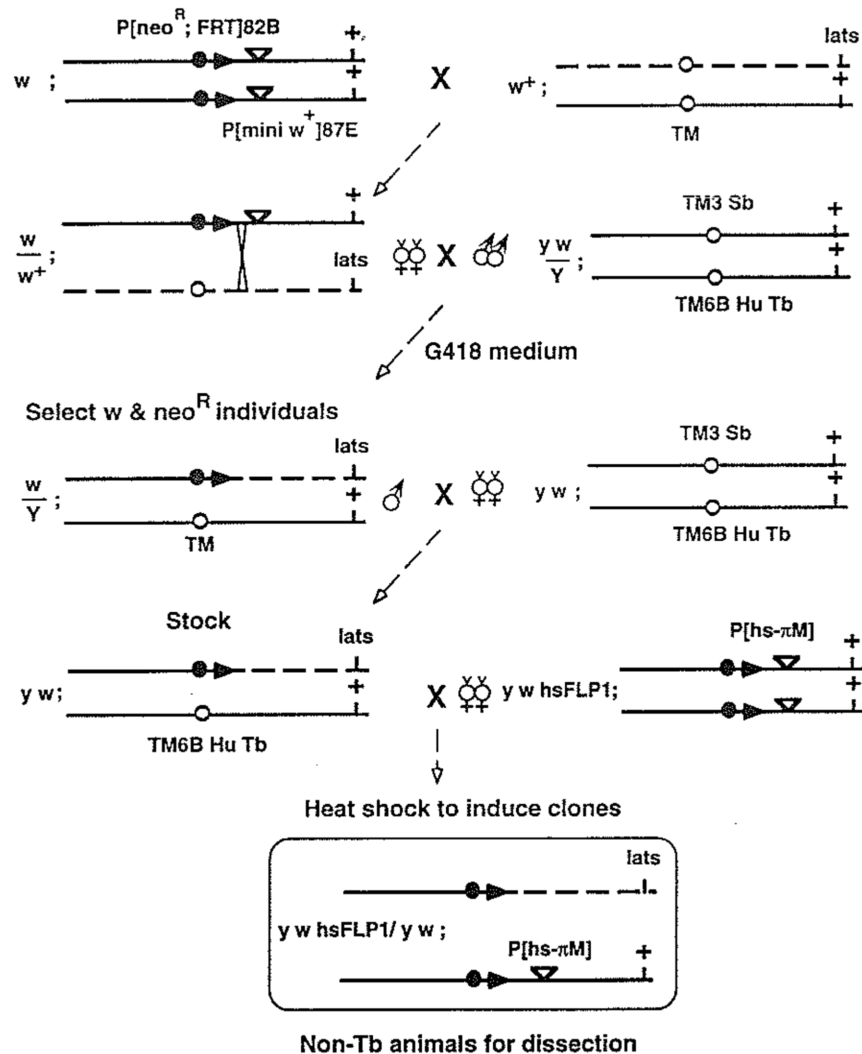


**FIG. 2.** Placing a mutation on an FRT chromosome and producing mutant clones. To recombine a mutation (*lats*) onto an FRT-carrying arm (solid arrow), *lats/TM6B* flies are mated to *w;FRT82B P[mini w+;hsπM]87E* animals. Non-TM heterozygous female progeny are crossed to balancer males on G418 medium to select for neomycin-resistant, white-eyed individuals which are likely carrying the recombinant chromosome (*w;P[neo, FRT]82Blats/TM*). Candidates are individually mated to balancer flies to establish individual lines. These lines are checked for the existence of the *lats* mutation by complementation tests. To produce *lats* mutant clones, *yw;FRT82Blats/TM6B* males from the established stock are crossed to *yw hsFLP1;FRT82B P[hsπM]* virgins, and progeny are heat-shock treated to induce the expression of FLP recombinase (see text for details). Non-*Tb* animals are dissected for analyzing *lats* clones in developing tissues.

problems caused by either a particular FLP/FRT strain or an improper method of inducion.

There are two caveats to the present FLP/FRT system for inducing mitotic clones. First, induction of clones by FLP/FRT-mediated mitotic recombination is restricted to dividing cells in early developmental stages. Second, the heat-shock promoter is not active in early embryo divisions or in germline cells during oogenesis. Other promoter-driven FLP constructs have been made [such as UAS/FLP (Xu, unpublished)], but not extensively tested. Since neither the heat-shock nor UAS promoter expresses well in germline cells during oogenesis, a FLP construct driven by a strong maternal promoter will be particularly useful for inducing mutant clones in ovariole germline and young embryos.

### B. FRT Strains

FRT sites have been introduced onto each major chromosome arm. Their insertions, chromosomal locations, abbreviations, and ability to mediate mitotic recombination are listed in Table 2.

There are three types of FRT-carrying chromosomes. The FRT sites in the FLP-out construct generated by Struhl and colleagues, in general, do not support frequent recombination between homologous chromosomes. This may be because they contain shortened versions of the FRT target sequence (13).

The original $P[>w^+>]$ construct was made by Golic and Lindquist (7) and mobilized into new locations (Table 2) (12). The insertions of this construct are indicated by either $P[>w^+>]$ or $P[mini\ w^+; FRT]$, followed by a stock number in superscript (Table 2). To simplify the written genotypes for strains carrying FLP/FRT constructs, these insertions are abbreviated as FRT followed by a superscript number (Tables 2–4; $FRT^{101}$, $FRT^{9\text{-}2}$, $FRT^{2R\text{-}G13}$, and $FRT^{3L\text{-}2A}$). This FRT element is marked with the *mini* $w^+$ marker. The *mini* $w^+$ gene in this construct is flanked by two FRT sites; thus, induction of FLP/FRT-mediated recombination results in loss of the $w^+$ marker. Moreover, the association of the $w^+$ marker with the FRT element makes it unsuitable for analyzing adult clones, which are marked with $w^-$ mutations.

The third construct, $P[ry^+;hs\text{-}neo;FRT]$, was designed to mediate mitotic recombination between homologous chromosomes (Table 2) (9). These sites are abbreviated as FRT followed by their chromosomal locations (e.g., FRT40A). To use the FLP/FRT system to generate homozygous mutant clones, the mutation of interest must first be recombined onto a FRT-containing chromosome arm. The $P[ry^+;hs\text{-}neo;FRT]$ element is dominantly marked with a neomycin gene (*hs-neo*) (14) to allow antibiotic selection for FRT-containing chromosomes (Fig. 2). Flies carrying the *hs-neo* element can be selected on G418-containing medium without heat-shock treatment, provided they are cultured at 25°C. The selection medium can be made by adding G418 solution to premade vials with a few holes made in the standard fly medium [0.2 to 0.3 ml of 25 mg/ml Geneticin (GIBCO, Grand Island, NY) in 10 ml of medium]. G418 medium can be stored at 4–18°C for at least 2 weeks. It should be pointed out that G418 solution does not soak evenly into large volumes of premade medium in bottles. Since selection for the FRT chromosome is dependent on the leaky expression of the neomycin gene (without heat shock), it is often desirable to work out the appropriate concentration of G418 to use. In such tests, two pairs of $ry^-$ and

**TABLE 1**

FLP Chromosomes

| Chromosome | Strain | References[a] |
|---|---|---|
| X | *y w hsFLP1;Adv/CyO* | (7, 9; Xu, T., *et al.*, unpublished) |
| | *y w hsFLP1;TM3, Sb/TM6B, Hu* | (7, 9; Xu, T., *et al.*, unpublished) |
| | *y hsFLP1;Bc;* $kar^2\ ry^{506}$ | (7; Heitzler, P., unpublished) |
| | *y w hsFLP122* | (13) |
| | *y w hsFLP122;TM3,* $ry^{RK}Sb$*/TM6B, Hu* | (13; Ito, N., *et al.*, unpublished) |
| | *y w* $hsFLP^{12}$*;Sco/CyO* | (7, 12) |
| | *y w* $hsFLP^{22}$*;CxD/TM3, Sb* | (7, 12) |
| 2 | *y;hsFLP38 Bc/CyO;Ki* $kar^2\ ry^{506}$ *Tb* | (7; Heitzler, P., unpublished) |
| | *pr pwn hsFLP38/CyO;Ki* $kar^2\ ry^{506}$ | (7; Heitzler, P., unpublished) |
| 3 | *hsFLP3, MKRS/TM6B* | (7; Jan, Y. N., *et al.*, unpublished) |

*P[ry⁺;hs-neo;FRT]*-containing flies should be mated in the same G418 vial, which best mimics the selection conditions in recombination experiments. Testing wild-type flies alone on G418 medium often results in escapers. However, in actual recombination experiments such wild-type escapers are competed out by their FRT-carrying siblings. For strains that grow poorly on G418, or when it is desirable to select on medium with high levels of G418, it is helpful to heat-shock vials once or several times during early larval stages.

To select for recombinant chromosomes that carry both the FRT element and the mutation of interest, one should use an FRT chromosome that also contains a distally located adult marker (Fig. 2, Table 3). Selection for neomycin resistance (FRT positive) and loss of the adult marker (gain of the mutant chromosome region) facilitate the identification of the desired recombinant chromosomes. For this purpose, a set of chromosomes that contain both *P[ry⁺;hs-neo;FRT]* and *P[mini-w⁺]* elements are listed in Table 3. It should be pointed out that any FRT chromosome with a distally located visible marker would be suitable for the recombination experiment. Furthermore, one may wish to choose an FRT chromosome with a visible marker that is positioned similarly or proximally to the mutation of interest.

Below is a discussion of the FRT insertions available for each chromosome arm. Two *P[ry⁺;hs-neo;FRT]* insertions located on the X chromosome produce high mitotic recombination frequencies and confer good neomycin resistance: FRT18A and FRT19A (Table 2) (9). FRT18A, however, is used more often because it was the first isolated on the X chromosome. Besides FRT18A and FRT19A, another *P[ry⁺;hs-neo;FRT]* insertion, FRT 19F, mediates mitotic recombination at a lower frequency and is located closer to the centromere. Two *P[mini w⁺;FRT]* insertions are also located on the X chromosome [Table 2; $FRT^{101}$ at 14A-B and $FRT^{9\text{-}2}$ at 18E-F] (12). They are linked with the $ovo^D$ mutation, which can be used to produce germline clones for maternal-null embryos (also see below). Since the $ovo^D$ mutation is linked with a *P[mini w⁺;FRT]* element, a mutation of interest has to be recombined onto the same *P[mini w⁺;FRT]*-carrying arm for inducing maternal-null clones. It would be desirable to place $ovo^D$ on the FRT19A arm so that any X-linked mutation only needs to be recombined onto the FRT19A chromosome to analyze clonal phenotypes in all tissues.

**TABLE 2**

FRT Elements

| Chromosome | Insertion | Code | Frequency of recombination | References |
|---|---|---|---|---|
| X | *P[mini w⁺;FRT]14A-B* | $FRT^{101}$ | High | (7, 11, 12) |
| | *P[ry⁺, hs-neo;FRT]11A* | FRT11A | ND[a] | (9) |
| | *P[mini w⁺;FRT]18E-F* | $FRT^{9-2}$ | High | (7, 11, 12) |
| | *P[ry⁺, hs-neo;FRT]18A* | FRT18A | High | (9) |
| | *P[ry⁺, hs-neo;FRT]19A* | FRT19A | High | (9) |
| | *P[ry⁺, hs-neo;FRT]19F* | FRT19F | Low | (9) |
| 2L | *P[ry⁺, hs-neo;FRT]29D* | FRT29D | ND | (9) |
| | *P[ry⁺, hs-neo;FRT]34B* | FRT34B | ND | (9) |
| | *P[ry⁺, hs-neo;FRT]40A* | FRT40A | High | (9) |
| 2R | *P[mini w⁺;FRT]42B* | $FRT^{2R-G13}$ | High | (7, 11, 12) |
| | *P[ry⁺, hs-neo;FRT]42B* | FRT42B | Low | (9) |
| | *P[ry⁺, hs-neo;FRT]42C* | FRT42C | Low | (9) |
| | *P[ry⁺, hs-neo;FRT]42D* | FRT42D | Medium | (9) |
| | *P[ry⁺, hs-neo;FRT]43D* | FRT43D | High | (9) |
| | *P[ry⁺, hs-neo;FRT]50B* | FRT50B | ND | (9) |
| 3L | *P[ry⁺, hs-neo;FRT]69A* | FRT69A | ND | (9) |
| | *P[ry⁺, hs-neo;FRT]72D* | FRT72D | High | (9) |
| | *P[mini w⁺;FRT]79D-F* | $FRT^{3L-2A}$ | High | (7, 11, 12) |
| | *P[ry⁺, hs-neo;FRT]80B* | FRT80B | Medium | (9) |
| 3R | *P[ry⁺, hs-neo;FRT]82B* | FRT82B | High | (9) |
| | *P[ry⁺, hs-neo;FRT]89B* | FRT89B | ND | (9) |
| | *P[ry⁺, hs-neo;FRT]93D* | FRT93D | ND | (9) |

[a] Not determined.

For chromosome arm 2L, FRT40A is the best to use; it mediates a very high frequency of mitotic recombination and works very well for G418 selection. All of the available cell markers including $ovo^D$ have been placed on this FRT40A chromosome arm (Tables 3–6).

FRT43D on 2R is the choice for high-frequency recombination and G418 selection. Another *P[ry⁺;hs-neo;FRT]* insertion, FRT42D has a lower mitotic recombination frequency. Two more FRTs, FRT42C and FRT42B, have much lower mitotic recombination frequencies. In addition, $ovo^D$ has been recombined onto a $P[mini\ w^+;FRT]42B$-containing chromosome for generating germline clones (Tables 2 and 6).

Initially, the $P[ry^+;hs\text{-}neo;FRT]$-carrying chromosome for 3L contained two inserts: FRT73D and FRT80B. FRT73D was originally missed as it did not give a strong *in situ* hybridization signal (10). The FRT80B strains that also contain the $P[mini\text{-}w^+\ hs\pi M]75C$ cell marker have only the FRT80B insert, as the FRT73D was recombined off with the addition of *hs-πM*. However, the other FRT80B strains carrying two FRT insertions have low mitotic recombination frequencies (15). With the removal of FRT73D, the new FRT80B strains now have a high frequency of mitotic recombination (N. Ito, personal communication). One should confirm that received FRT80B strains contain only the FRT80B element (Tables 3–5).

For 3R, FRT82B has a high mitotic recombination frequency and good G418 selection. All cell markers including $ovo^D$ have been placed on the FRT82B chromosome arm (Tables 3–6).

In addition to these proximally located FRT insertions, a set of FRT elements located at the middle of chromosome arms have been collected. These are listed in Table 2 and may be useful when it is necessary to make homozygous only half of the chromosome arm.

### C. Analyzing Clones in Adult External Tissues

#### *i. Adult Cuticle*

A number of cell markers exist to identify clones in the adult cuticle. The *yellow* (*y*) gene is widely used as *y* mutant bristles are easily identified in a $y^+$ background. The $P[y^+]$ insertions on autosome arms have been placed onto FRT-carrying arms, allowing clones mutant for X-linked and autosomal genes to be marked with the $y^-$ marker. In additiont to $y^-$, *f* and *sn3* on the X chromosome are also good markers and label macro- and microchaete.

To identify individual cells of a clone, P. Heiztler (personal communication) placed a combination of adult cuticular markers onto the FRT-carrying arms. These markers allow single-cell resolution of clones and label a variety of cells including bristles, trichomes (epidermis), and naked cuticle (neuron) (Table 4). *Dp(3;Y;1)*, a duplication for the $mwh^+$ gene, has been introduced onto the FRT19A arm in a $mwh^-$ background to label trichomes mutant for X-linked genes. *f* is used in combination with *mwh* to label bristles. The $ck^{CH52}$ and *pwn* markers have been recombined onto FRT40A and FRT42D chromosome arms to distinguish both bristles and trichome cells mutant for genes on 2L and 2R, respectively. For chromosome arm 3L, *mwh* and *trc* mark trichomes, and *jv* marks bristles. In addition to *Sb*, which labels bristles, the *Dp(2;3)P32* ($pwn^+$) has been introduced onto the FRT82B arm in a *pwn* background, thus identifying cells mutant for genes on 3R.

**TABLE 3**

Strains for Introducing Mutations onto FRT Arms

| Chromosome | Strain | Reference |
|---|---|---|
| X | *w P[mini-w+ hsπF]17B FRT18A* | (9) |
| | *y w P[mini-w+ hsπM]5A, 10D FRT19A* | (9) |
| | $f^{36a}$ *FRT19A;mwh* $kar^2$ $ry^{506}$ | Heitzler, P., unpublished |
| 2L | $w;P[mini\text{-}w^+\ hs\pi M]36F$ *FRT40A* | (9) |
| | $y;P[y^+\ ry^+]25F\ ck^{CH52}$ *FRT40A/CyO;* $kar^2$ $ry^{506}$ | Heitzler, P., unpublished |
| 2R | $w;FRT42D\ P[mini\text{-}w^+,\ hs\pi M]45F$ | (9) |
| | $y;FRT42D\ pwn\ P[y^+,\ ry^+]44B/CyO;kar^2\ ry^{506}$ | Heitzler, P., unpublished |
| | $w;FRT43D\ P[mini\text{-}w^+,\ hs\pi M]45F$ | (9) |
| 3L | $y\ w;P[mini\text{-}w^+\ hs\pi M]75C$ *FRT80B* | (9) |
| | *yy;mwh (FRT73D?) FRT80B* $kar^2$ $ry^{506}$ | Heitzler, P., unpublished |
| 3R | $w;FRT82B\ P[mini\text{-}w^+\ hs\pi M]87E$ | (9) |

An example of using these chromosomes to generate and mark adult clones is given below. For this example, *mwh* and *f* are used to mark adult clones mutant for a X-linked mutation (*sgg*) (see Fig. 2 and Table 4). First, to generate an FRT-mutant chromosome cross *sgg w/FM7c;+/TM* virgins to *f*$^{36a}$ *FRT19A;mwh kar*$^{2}$ *ry*$^{506}$ males. Cross *sgg w/f*$^{36a}$ *FRT19A/TM;mwh kar*$^{2}$ *ry*$^{506}$ virgin progeny to *FM7c;mwh kar*$^{2}$ *ry*$^{506}$*/TM* males on G418 medium. Select *sgg w f*$^{36a}$ *FRT19A/FM7c;mwh kar*$^{2}$ *ry*$^{506}$ virgins ($w^{a}$, neoR, and non-TM) to mate with *FM7c;mwh kar*$^{2}$ *ry*$^{506}$ males to establish a stock. Check for the existence of the *sgg* mutation in stocks by complementation tests. To generate clones, cross *Dp(3;Y;1)M2 y FRT19A/FM7;emc*$^{FX119}$ *mwh kar*$^{2}$ *ry*$^{506}$ virgins to *y;hsFLP38 Bc/CyO;Ki kar*$^{2}$ *ry*$^{506}$ males. Male progeny from this cross with the genotype *Dp(3;Y;1)M2 y FRT19A;hsFLP38 Bc/+;emc*$^{FX119}$ *mwh kar*$^{2}$ *ry*$^{506}$ */Ki kar*$^{2}$ *ry*$^{506}$ are mated to *sgg w f*$^{36a}$ *FRT19A/FM7c;mwh kar*$^{2}$ *ry*$^{506}$ females. Heat-shock larval progeny at late first instar to early second instar stages for 1 h at 38°C, and score *f* and *mwh* cuticle clones in $y^{+}$ $B^{+}$ *Bc* $Ki^{+}$ female adults. The wild-type twin-spot clones are marked with $y^{-}$.

*ii. Eye*

The *white* (*w*) *gene* is used to analyze clones in the adult eye. The *FRT-w*$^{+}$*F* strains contain the full-length *w* gene and are able to produce pigment at wild-type levels. It should be reemphasized that when viewed under the dissecting scope, mutant clones lacking the endogenous $w^{+}$ gene or a $P[w^{+}]$ insertion appear as black patches in the eye. Only $w^{-}$ clones large in size or located at the eye periphery appear white. To analyze clones at a single-cell level, animal heads must be embedded in Durcapan resin for sectioning (10, 16). The $P[w^{+}]$ insertions recombined onto FRT chromosomes are good for identifying the genotypes of individual cells in plastic sections (Table 4).

Sometimes mutant cells do not survive or divide to produce visible clones in mosaic adults. Thus, one has to rely on the existence of wild-type twin-spot clones to determine whether clones have been induced in prospective adults. The *mini* $w^{+}$ gene can be used to better visualize twin-spot clones under a dissection scope. In this case, the *mini* $w^{+}$ gene produces lower levels of pigmentation, and twin-spot clones that contain two copies of *mini* $w^{+}$ appear

**TABLE 4**

Strains for Adult Cuticular Clones

| Chromosome | Strain | References |
|---|---|---|
| X | *FRT18A;hsFLP3, MKRS/TM6B* | (9) |
| | *FRT19A;hsFLP3 MKRS/TM6B* | (9; Xu, T., *et al.*, unpublished) |
| | *y w FRT19A* | (9; Xu, T., *et al.*, unpublished) |
| | *w sn*$^{3}$ *FRT19A* | (9; Xu, T., *et al.*, unpublished) |
| | *f*$^{36a}$ *FRT19A;mwh kar*$^{2}$ *ry*$^{506}$ | Heitzler, P., unpublished |
| | *Dp(3;Y;1)M2 y FRT19A/FM7;emc*$^{FX119}$ *mwh kar*$^{2}$ *ry*$^{506}$ | Heitzler, P., unpublished |
| | *Dp(3;Y;1)M2 y M(1)o*$^{Sp}$ *FRT19A/FM7;kar*$^{2}$ *ry*$^{506}$ | Heitzler, P., unpublished |
| 2L | *y w hsFLP1;P[y*$^{+}$ *ry*$^{+}$*]25F P[w*$^{+}$ *ry*$^{+}$*]30C FRT40A* | (9; Xu, T., *et al.*, unpublished) |
| | *y;P[y*$^{+}$ *ry*$^{+}$*]25F ck*$^{CH52}$ *FRT40A /CyO;kar*$^{2}$ *ry*$^{506}$ | Heitzler, P., unpublished |
| 2R | *y w hsFLP1;FRT42D P[y*$^{+}$*, ry*$^{+}$*]44B P[w*$^{+}$*, ry*$^{+}$*]47A/CyO* | (9; Xu, T., *et al.*, unpublished) |
| | *y;FRT42D pwn P[y*$^{+}$*, ry*$^{+}$*]44B /CyO;kar*$^{2}$ *ry*$^{506}$ | Heitzler, P., unpublished |
| | *y w;FRT42D P[mini-w*$^{+}$*, hs*$\pi$*M]45F M(2)S7/CyO;kar*$^{2}$ *ry*$^{506}$ | Heitzler, P., unpublished |
| | *y w hsFLP1;FRT43D P[w*$^{+}$*, ry*$^{+}$*]47A* | (9; Xu, T., *et al.*, unpublished) |
| | *y w hsFLP1;FRT43D P[y*$^{+}$*, ry*$^{+}$*]44B* | (9; Xu, T., *et al.*, unpublished) |
| 3L | *w hsFLP122;P[w*$^{+}$*]70C FRT80B* | Xu, T., *et al.*, unpublished; Ito, N., *et al.*, unpublished |
| | *y w hsFLP122;P[ry*$^{+}$ *y*$^{+}$*]66E P[w*$^{+}$*]70C FRT80B* | Xu, T., *et al.*, unpublished; Ito, N., *et al.*, unpublished |
| | *y;mwh (FRT73D?) FRT80B kar*$^{2}$ *ry*$^{506}$ | Heitzler, P., unpublished |
| | *y;trc FRT80B kar*$^{2}$ *ry*$^{506}$*/TM6C ry*$^{CB}$ *Sb Tb* | Heitzler, P., unpublished |
| | *y w;jv P[ry*$^{+}$ *y*$^{+}$*]66E P[mini-w*$^{+}$ *hs*$\pi$*M]75C FRT80B kar*$^{2}$ *ry*$^{506}$*/TM3 ry*$^{RK}$ *Sb* | Heitzler, P., unpublished |
| | *y w;M(3)i*$^{55}$ *P[mini-w*$^{+}$ *hs*$\pi$*M]75C FRT80B kar*$^{2}$ *ry*$^{506}$*/TM3 ry*$^{RK}$ *Sb* | Heitzler, P., unpublished |
| 3R | *y w hsFLP1;FRT82B P[w*$^{+}$*;ry*$^{+}$*]90E P[y*$^{+}$ *ry*$^{+}$*]96E* | (9; Xu, T., *et al.*, unpublished) |
| | *y w hsFLP1;FRT82B P[mini-w*$^{+}$ *hs*$\pi$*M]87E Sb*$^{63b}$ *P[y*$^{+}$ *ry*$^{+}$*]96E* | (9) |
| | *FRT82B kar*$^{2}$ *ry*$^{506}$ | Heitzler, P., unpublished |
| | *pr pwn;FRT82B kar*$^{2}$ *ry*$^{506}$ *bx*$^{34e}$ *Dp(2;3)P32/FRT82B kar*$^{2}$ *ry*$^{506}$ | Heitzler, P., unpublished |

darker than the heterozygous background. For example, one could find a lack of $y^-$ mutant bristles when analyzing the effect of a mutation on bristle development, in prospective mosaic adults. This could either be caused by a lack of mitotic recombination or reflect the true effect of the mutation of interest. The existence of darkly pigmented twin-spot clones in the eyes of the same animals indicates that mutant clones were produced and failed to survive or proliferate to form clones in adults.

An example of using $P[w^+]$ to mark adult eye clones (*lats*) is given below (see Fig. 2 and Table 5). First, to generate an FRT-mutant chromosome cross *lats/TM* males to *w;FRT82B P*[*mini-*$w^+$ *hsπM*]87E virgins. Cross *w*/$w^+$;*lats/FRT82B P*[*mini-w+ hsπM*]-*87E* virgin progeny to *y w;TM3/TM6B* males on G418 medium. Select $w^-$;*FRT82B lats /TM* individual males ($w^-$ and neoR) and cross to *y w;TM3/TM6B* virgins to establish stocks. Check for the existence of the *lats* mutation in stocks by complementation tests. To generate clones, cross *y w hsFLP1;FRT82B P*[$w^+$;$ry^+$]*90E P*[$y^+$ $ry^+$]*96E* virgins to *y w;FRT82B lats/TM6B* males. Heat-shock larval progeny at late first instar to early second instar stages for 1 h at 38°C and score $w^-$ eye clones in non-Tb adults.

### D. Internal and Developmental Tissues

The ability to label cells in developing tissues enables the roles of multifunctional genes to be more readily dissected throughout development. Genes involved in multiple processes often display multiple phenotypes; thus, it could be misleading to make any conclusions from their adult clonal phenotypes alone.

Clones of mutant cells can be detected by antibody staining for an associated cell marker. The πM and NM cell markers were generated to label cells in developing tissues and have been recombined onto all FRT chromosome arms. They are fusions of the MYC epitope with either P-transposase or Notch sequences (9). πM localizes to the nucleus and NM to the Golgi and plasma membrane. The choice of cell markers depends on the tissue type, the involvement of other markers, and any expected phenotype of the mutant cells. For further flexibility, πF, which expresses the FLAG epitope, was constructed (9). Another cell marker for developing tissues is CD2, a gene encoding a transmembrane protein from the rat immunoglobulin superfamily (17). This marker has been used in labeling both homozygous mutant cells and FLP-out clones (13) (see below). *lacZ* enhancer–trap lines and promoter–*lacZ* fusions can also serve as good cell markers (*arm–lacZ*) (18, 19). In addition, many of the enhancer–*lacZ* lines are excellent markers for cell identity and can be used in double-labeling experiments to reveal the developmental properties of mutant cells. Use of a ubiquitously expressed *lacZ* gene to label mutant clones hinders such applications.

**TABLE 5**

Strains for Clones in Developing and Internal Tissues

| Chromosome | Strain | References |
|---|---|---|
| X | *w P[mini-w+ hsπM]5A, 10D FRT18A;hsFLP3, MKRS/TM6B* | (9) |
| | *w P[mini-w+ hsNM]8A FRT18A* | (9) |
| | *w P[mini-w+ hsπF]17B FRT18A* | (9) |
| | *y w P[mini-w+ hsπM]5A, 10D FRT19A* | Heitzler, P., unpublished |
| | *y w P[mini-w+ hsπM]5A, 10D* $M(1)o^{Sp}$ *FRT19A /FM7* | Heitzler, P., unpublished |
| 2L | *w hsFLP1;P[mini-*$w^+$ *hsπM]21C, 36F FRT40A* | (9) |
| | *w hsFLP1;P[mini-*$w^+$ *hsNM]31E FRT40A* | (9) |
| 2R | *w hsFLP1;FRT42D P[mini-*$w^+$*, hsπM]45F/CyO* | (9) |
| | *y w;FRT42D P[mini-*$w^+$*, hsπM]45F M(2)S7/CyO;*$kar^2$ $ry^{506}$ | Heitzler, P., unpublished |
| | *y w hsFLP1;FRT42D P[mini-*$w^+$*, hsNM]46F* | (9) |
| | *w hsFLP1;FRT43D P[mini-*$w^+$*, hsπM]45F, 47F* | (9) |
| | *y w hsFLP1;FRT43D P[mini-*$w^+$*, hsNM]46F* | (9) |
| 3L | *y w hsFLP122;P[mini-*$w^+$ *hsπM]75C FRT80B* | (9; Ito, N., unpublished) |
| | *y w hsFLP1;P[mini-*$w^+$ *hsNM]67B (FRT73D?) FRT80B* | (9) |
| | *y w; jv P[*$ry^+$ $y^+$*]66E P[mini-*$w^+$ *hsπM]75C FRT80B* $kar^2$ $ry^{506}$*/TM3* $ry^{RK}$ *Sb* | Heitzler, P., unpublished |
| | *y w;*$M(3)i^{55}$ *P[mini-*$w^+$ *hsπM]75C FRT80B* $kar^2$ $ry^{506}$*/TM3* $ry^{RK}$ *Sb* | Heitzler, P., unpublished |
| 3R | *w hsFLP1;FRT82B P[mini-*$w^+$ *hsπM]87E,97E* | (9) |
| | *y w hsFLP1;FRT82B P[mini-*$w^+$ *hsNM]88C* | (9) |

Most of the cell markers are expressed under the control of the *hsp70* promoter. As these markers have short half-lives, the heat pulse used to generate mitotic clones does not result in sustained high levels of marker expression. On the other hand, tissues must be dissected, fixed, and stained within a limited period following cell marker induction by heat shock (see below). The staining density of the marked cell reveals its genotype. Cells containing zero, one, or two copies of the cell marker stain with different intensities. The staining intensities can thus be used to predict the genotypes of the marker gene and its associated mutant allele. Cells staining twice as intensely as heterozygous cells are the twin-spot clones of the nonstaining cells. This property of twin-spot clones is very useful in detecting any growth defects a gene may confer.

Many questions have frequently been asked regarding use of the hs-πM and hs-NM markers. The standard staining protocol is described below, followed by a detailed discussion of variables that may hinder its reliability:

Set up appropriate crosses and culture at 25°C (Fig. 2). To induce clones in imaginal disks, heat-shock late first instar larvae for 60 min in a 38°C water bath. After several days, pick wandering third instar larvae to a new vial and heat-shock again for 60 to 90 min to induce the expression of the πM or NM cell markers. Return vial to 25°C for 60 min prior to dissecting. After dissecting imaginal disks in cold 0.1 M phosphate buffer, fix for 40 min on ice in PLP (2% paraformaldehyde, 0.01 M $Na_3H_2IO_6$, 0.075 M lysine, 0.037 M sodium phosphate, pH 7.2). Wash tissues on ice by transferring into fresh PSN (0.1 M phosphate, pH 7.2, 1% goat serum, 0.1% Saponin) four times for 10 min to remove any fixing agent. Tissues are then incubated overnight at 4°C with a 1:50 to 1:200 dilution (1 μg/ml) of anti-MYC [MAb MYC 1-9E10.2 (20); American Type Culture Collection, Rockville, MD), or a 1:1 dilution for 1-9E10.2 supernant, in PSN. Wash four times with PSN, and incubate with secondary antibodies for 2 to 4 h at room temperature or overnight at 4°C (1:200 dilution of goat anti-mouse, Jackson Laboratories). Rewash tissues in PSN four times, and mount in 90% glycerol, 1× PBS, 0.5% *n*-propyl gallate for fluorescence staining.

It is necessary to culture flies at 25°C, as larvae grown at 18°C often die on heat shock. When heat-shocking larvae, push the cotton stop down so that the water level is higher than the wandering space for the larvae to ensure proper heat shock. For inducing clones, 38°C is more effective than 37°C. However, temperatures higher than 40°C kill the animals. It is essential to induce FLP expression when cells in the tissues of interest are dividing. For example, cells in the eye imaginal disk do not proliferate during the first 6 h after hatching. Induction of FLP expression during that period will not produce any mitotic clones in the eye. Induction of FLP immediately following this 6-h period results in large-sized clones at low frequencies. The eye disks of early third instar larvae contain many more dividing cells, and inititiating mitotic recombination at this time produces small clones at high frequencies. It is important to design your heat-shock regime according to the proliferation property of the tissue of interest.

When inducing cell marker expression by heat shock, the preceding recovery period is also critical. For the πM and πF markers, an incubation of 60 min at 25°C is important for the proteins to be translated and localized to the nucleus. However, if too much time is taken before fixation the protein may degrade and the signal will be weak.

The anti-MYC antibody is very sensitive to fixing solution. More washes are always helpful. By transferring tissues into fresh buffer, only a minimal amount of solution will contaminate a new wash. The choice of antibodies is obviously also of importance. Although the anti-MYC antibody is derived from the same hybridoma, different subclones often give variable qualities of antibodies. For secondary antibodies, those with multiple absorptions do not appear to work well. We have best experience with goat anti-mouse secondary antibodies from Jackson Laboratories (115-095-146 minus rabbit, 115-095-100 minus rat, 115-085-146 minus rabbit, and 115-085-100 minus rat).

### E. Use of *Minute* in Mosaic Analysis

Historically, the *Minute* (*M*) mutations have been used to increase the size of mutant clones. Homozygous $M^+/M^+$ clones sustain a growth advantage over their $M/M^+$ neighboring cells. García-Bellido and Dapena (2) took advantage of the *M* mutation by generating large somatic clones, which enabled them to provide the first evidence for the compartmentalization of cells during development.

*Minute* compensated for the low frequencies and small sizes of clones induced by X-ray irradiation. With the FLP/FRT system, however, high clone frequencies are now possible, and large-sized clones can be produced by inducing FLP expression at earlier

developmental stages. Although several *Minute* mutations have been recombined onto FRT chromosome arms (Tables 4 and 5; P. Heitzler, unpublished), we recommend initially examining clones in the absence of the *M* mutation. Additionally, recent data show that the *M* phenotype is complex as there is more cell proliferation and cell death in $M/M^+$ cells in comparison to $M^+/M^+$ cells (B. Edger, personal communication). Thus, *M* may interfere with the analysis of the clonal mutant phenotype of a gene, especially in the case of proliferation defective genes.

### F. Use of $ovo^D$ in Generating Maternal Null Germline Clones

The maternal contribution of both protein and RNA is essential for embryonic development. However, maternal products often interfere with analysis of the zygotic function of a gene. Phenotypes of homozygous mutant embryos are often masked by the wild-type products deposited by the heterozygous mothers. In fact, screens for zygotic embryonic lethals have revealed morphological defects in approximately 240 loci among the 5500 zygotic embryonic lethal genes (21). In these cases, it is desirable to eliminate the maternal contribution. One solution is to produce embryos from homozygous mutant germline cells in heterozygous females.

Dominant female-sterile (DFS) mutations have been used to select for germline clones (22–24). Females heterozygous for DFS mutations will not produce any eggs. However, if mitotic recombination occurs in germline cells, then resulting clones of cells lacking DFS mutations will produce eggs (25). Perrimon and colleagues combined FLP/FRT with the DFS technique and placed the $Ovo^{D1}$ mutation onto each FRT chromosome arm (Table 6) (11, 24, 26). These lines made it possible to produce maternal-null embryos for genes throughout the genome. One should be aware that the FRT elements in several of the $ovo^D$ chromosome arms (X, 2R, 3L; Table 6) are *P[mini* $w^+$*;FRT]* insertions. To use *FRT-*$ovo^D$ chromosomes, the mutation of interest must be recombined onto the same FRT elements.

The highest frequencies of germline clones are produced when FLP is induced in third instar larvae (26). In addition to larvae, clones can also be generated in adults. As ovary cells divide once every 24 to 48 h, induced flies must be sufficiently aged to allow clones to grow for producing maternal null eggs. To initiate mitotic recombination in ovariole germline cells, transfer adult females into new vials and incubate in a 38°C water bath for 60 min. Alternatively, to induce germline clones in larvae, females are allowed to lay eggs for 1 day. Progeny from these females are heat-shocked twice for 2 h over a period of several days until they reach late second to third instar stages. Larvae and adults should come from well-fed crosses.

**TABLE 6**

Strains for Generating Germline Clones

| | |
|---|---|
| X | $C(1)DX, y\ f/w\ ovo^{D1}\ v^{24}\ FRT^{101}/Y;hsFLP38$ |
| | $C(1)DX, y\ f/ovo^{D2}\ v^{24}\ FRT^{9\text{-}2}/Y;hsFLP38$ |
| 2L | $P[mini\ w^+;ovo^{D1}]^{2L\text{-}13X13}\ FRT40A/S\ Sp\ Ms(2)M\ bw^D/CyO$ |
| 2R | $FRT^{2R\text{-}G13}\ P[mini\ w^+;ovo^{D1}]^{2R\text{-}32X9}/S\ Sp\ Ms(2)M\ bw^D/CyO$ |
| 3L | $w;P[mini\ w^+;ovo^{D1}]^{3L\text{-}2X48}\ FRT^{3L\text{-}2A}/ru\ h\ st\ \beta Tub85D^D\ ss\ e^S/TM3, Sb$ |
| 3R | $w;FRT82B\ P[mini\ w^+;ovo^{D1}]^{3R\text{-}C13a31n9}/ru\ h\ st\ \beta Tub85D^D\ ss\ e^S/TM3, Sb$ |

## 2. USE OF THE FLP-OUT TECHNIQUE TO ACTIVATE GENES

FLP was first described to mediate recombination between FRT sites on the same chromosome in *Drosophila* (7). In this case, induction of mitotic recombination by FLP results in loss, or FLPing-out, of a marker gene flanked by two FRT sites. Struhl and Basler (13) used this property to constitutively express *wingless* (*wg*) in genetically marked clones (Fig. 1B) (13). In their construct, *wg* is transcriptionally inactive as it is separated from a constitutive promoter by a marker gene ($Actin5C{>}y^+{>}wg$). On induction, FLP mediates recombination between FRT sites flanking the marker gene, excising the intervening marker gene ($y^+$ or a cell marker). Consequently, *wg* under the constitutive Act5C promoter in clones of $y^-$ cells, *tubulin* $\alpha$, a stronger promoter than Act5C, has been used in a similiar manner (27). In addition, this approach has been useful to express a cell marker, *lacZ,* allowing positively labeled cells and their lineages to be traced during development.

## 3. USE OF THE FLP/FRT STRAINS TO PERFORM GENETIC SCREENS

A major application for the genetically marked FLP/FRT strains has been in performing $F_1$ genetic

screens. A detailed description of $F_1$ mosaic screens is reviewed in Xu and Harrison (10). Traditional genetic screens required three generations of crosses to establish individual lines for identifying potential mutants. In addition, many genes are required for proper development in multiple stages, and homozygous mutants for these genes are often embryonic lethal. Thus, the requirement for these genes in later developmental processes was often not disclosed in screens examining homozygous mutants. Both these limitations are circumvented by screening for mutations in mosaic animals. First, mutations are identified in one generation. Second, mosaic animals carrying small patches of mutant cells are viable, and the phenotypes of mutant clones are readily identified. These special features make $F_1$ screens efficient and effective in identifying mutations affecting many biological processes. Such screens have already identified important genes that escaped detection in traditional screens (18, 21). In addition, one can use these strains for performing modifier screens or genetic interaction screens in $F_1$ mosaic animals.

X-ray irradiation is preferred over chemicals mutagens for $F_1$ screens. X rays usually induce changes in both DNA strands and all the cells of the resulting $F_1$ progeny will carry the identical mutagenized chromosomes. In contrast, EMS often generates mutations in only one DNA strand, creating $F_1$ progeny mosaic for the mutagenized chromosome. Consequently, not all mutant-bearing individuals produce $F_2$ offspring with the mutant chromosomes [reviewed in (6)]. Ideally, it is desirable to use P elements as mutagens in $F_1$ mosaic screens. The *P[ry+;hs-neo;FRT]* construct which carries two marker genes, was designed to allow for "crippling" of the P-element ends by genetic selection. Future efforts to immobilize the established *P[ry+;hs-neo;FRT]* insertions will be worthwhile for performing $F_1$ mosaic screens with P-element mutagens.

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