Use of a Yeast Site-Specific Recombinase to Produce Female Germline Chimeras in Drosophila

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ABSTRACT

We describe an efficient method for generating female germline mosaics by inducing site-specific homologous mitotic recombination with a yeast recombinase (*FLP*) which is driven by a heat shock promoter. These germline mosaics are produced in flies heterozygous for the agametic, germline-dependent, dominant female sterile (*DFS*) mutation ovo^{D1}, where only flies possessing germline clones are able to lay eggs. This method, the "FLP-DFS" technique, is very efficient because more than 90% of females with germline clones can be recovered. We show that this heat-inducible, site-specific mitotic recombination system does not affect viability and that the germline clones recovered are physiologically the same as those created by X-ray induced mitotic recombination. We describe the parameters of *FLP*-recombinase induced germline mitotic recombination and the use of the "FLP-DFS" technique to analyze the maternal effect of *X*-linked zygotic lethal mutations.

THE production of germline chimeras is invaluable for analyzing the tissue specificity (germline *vs.* somatic) of recessive female sterile mutations (WIESCHAUS, AUDIT and MASSON 1981; PERRIMON and GANS 1983), as well as detecting the maternal effect of recessive zygotic lethal mutations (PERRIMON, ENGSTROM and MAHOWALD 1984, 1989). Two techniques have been commonly used to generate these mosaics. The first one is pole cell transplantation in which mutant pole cells are injected into otherwise wild-type female embryos (ILLMENSEE 1973). The second one is the "dominant female sterile" technique which utilizes germline-dependent dominant female sterile (*DFS*) mutations (WIESCHAUS 1980; PERRIMON and GANS 1983; PERRIMON 1984). The "DFS" technique is technically easier than pole cell transplantation [see Perrimon and Gans (1983) for discussion].

The *X*-linked *DFS* mutation ovo^{D1}, or *Fs(1)K1237* (BUSSON *et al.* 1983; PERRIMON 1984), has been used extensively to analyze the tissue specificity of recessive *X*-linked female sterile mutations (PERRIMON and GANS 1983; PERRIMON *et al.* 1986) as well as the maternal effect of *X*-linked zygotic lethal mutations (PERRIMON, ENGSTROM and MAHOWALD 1984, 1989). Females heterozygous for ovo^{D1} do not lay eggs and develop only atrophic ovaries containing no vitellogenic eggs. Previously, X-ray irradiation was used to recover germline mosaics in individuals heterozygous for ovo^{D1}. A mitotic exhange occurring in the female germ cells results in recombinant daughter cells which have eliminated ovo^{D1} and thus can produce eggs. To generate germline chimeras of an *X*-linked mutation (*m*) using the "DFS" technique, individuals *trans*-heterozygous for both the *DFS* and *m* mutations are treated with X-rays and *m/m* homozygous germline clones are recovered.

One technical problem of the "DFS" technique using ovo^{D1} is the low frequency of mosaic females recovered following X-ray irradiation (PERRIMON 1984). To increase the frequency of germline mosaics in females heterozygous for ovo^{D1}, we have taken advantage of the properties of the yeast "FLP-FRT" site-specific recombination system. Recently, the yeast *FLP*-recombinase and its recombination targets (*FRTs*) from the 2 μm plasmid of *Saccharomyces cerevisiae* (see review by COX 1988) were successfully transferred into the Drosophila genome (GOLIC and LINDQUIST 1989). The heat-inducible *FLP*-recombinase gene, under the control of an *hsp70* promoter, recognizes and promotes recombination specifically at the level of the *FRT* sequences. GOLIC and LINDQUIST (1989) demonstrated that a mini-*white* gene flanked with *FRT* elements can be excised resulting in the production of mosaic eyes. In addition, GOLIC (1991) has recently shown that *FLP*-recombinase can catalyze site-specific recombination between homologous chromosomes in both somatic and male germline tissues. In this paper, we report the combination of the "FLP-FRT" recombination system with the "DFS" technique to develop the "FLP-DFS" technique which allows the recovery of more than 90% of mosaic females. We have applied this technique to the analysis of the maternal effect phenotypes of a large number of newly induced *X*-linked lethal mutations and discuss its extension to the autosomes.

MATERIALS AND METHODS

Flies were raised on standard Drosophila media at 25° unless indicated. Descriptions of balancers and mutations

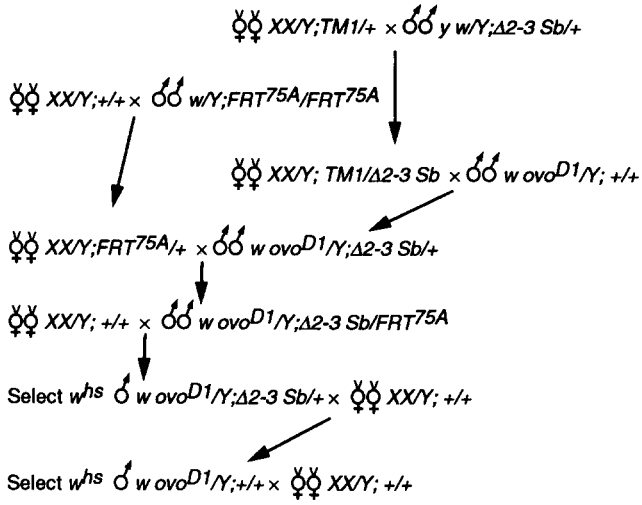

FIGURE 1.—Genetic crosses to recover *X*-linked *FRT* insertions. Only the *X* and third chromosomes are depicted. Four *X*-linked *FRT* insertions were recovered using this scheme (see MATERIALS AND METHODS). Description of the chromosomes: *XX/Y* is the attached-*X* chromosome: *C(1)DX, y f/Y*. FRT^{75A} is the third chromosome *FRT* insertion marked with the mini-*white* (w^{hs}) gene (see MATERIALS AND METHODS). *TM1* is a third chromosome balancer (LINDSLEY and GRELL 1968). It carries the dominant marker *Moire*.

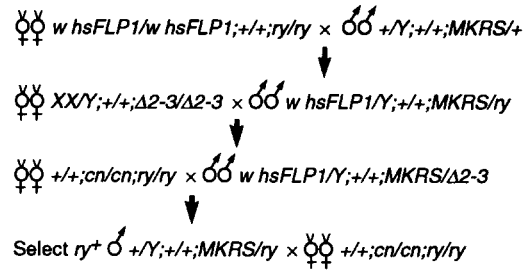

FIGURE 2.—Genetic crosses to recover autosomal *FLP*-recombinase insertions. The *X*, second and third chromosomes are depicted. Due to the design of this experiment, all second chromosome *FLP* insertions are on a wild-type chromosome and all third chromosome *FLP* insertions are on the *MKRS* chromosome. A total of 62 autosomal independent *FLP* insertions were recovered out of 120 crosses of 3 males of genotypes *w FLP1/Y;+/+;MKRS/Δ2-3* crossed with 5 *+/+;cn/cn;ry/ry* females. Description of the chromosomes: *ry* is ry^{506}; *FLP1* is the *hs-FLP1* described by GOLIC (1991). It carries the ry^{+} gene. *MKRS* is a third chromosome balancer described in LINDSLEY and ZIMM (1990). It carries the dominant marker *Stubble* (*Sb*) and the recessive marker *ry*.

that are not described in the text can be found in LINDSLEY and GRELL (1968) and LINDSLEY and ZIMM (1985, 1986, 1987, 1990).

Transposase stocks: The "jumpstarter" strain we utilized is *Δ2-3 (P[ry⁺;Δ2-3] 99B)* that carries a defective *P* element on the third chromosome at 99B which constitutively expresses high levels of transposase but cannot itself transpose (ROBERTSON *et al.* 1988). The two transposase stocks used in this study were obtained from the Bowling Green Stock Center. These are: ry^{506} *P[ry⁺ Δ2-3]* and *CyO/Sp;*ry^{506} *Sb P[ry⁺;Δ2-3]/TM6, Ubx.*

Dominant female sterile stock: The *X*-linked dominant female sterile mutation ovo^{D1} (BUSSON *et al.* 1983) is maintained as an attached-*X* stock: *C(1)DX, y f/Y* females crossed to ovo^{D1} v^{24}/Y males. The *white* (*w*) mutation on the ovo^{D1} chromosome was generated following mutagenesis of the original ovo^{D1} v^{24} chromosome by ethyl methanesulfonate (EMS) (R. COYNE, personal communication). The stock is also kept with *C(1)DX, y f/Y* females.

***FRT* stocks:** The *FRT* element, *P[>*w^{hs}*>]*, constructed by GOLIC and LINDQUIST (1989) contains a mini-*w* gene (w^{hs}) marker flanked by two direct repeats of *FRT* sequences in the *P* element vector. To generate *X* chromosomes that carry the *FRT* element proximal to the ovo^{D1} mutation, we destabilized an *FRT* element (FRT^{75A}) located on the third chromosome (GOLIC and LINDQUIST 1989). The scheme used for the recovery of the *X*-linked *FRT* insertions onto the *w* ovo^{D1} chromosome is shown in Figure 1. Since the *FRT* element carries the w^{hs} marker, we used the *w* ovo^{D1} chromosome in this scheme. We recovered ten independent *FRT* insertions of which four were *X*-linked (FRT^{101}, FRT^{106}, FRT^{107} and FRT^{109}). These insertions were recovered at a very low frequency because they were isolated after examination of about 14,000 progeny.

FLP* stock:** The *FLP* element, *P[ry⁺ hsFLP]*, constructed by GOLIC and LINDQUIST (1989) carries an *hsp70-FLP* fusion gene and ry^{+} as a selectable marker in the *P* element vector. To generate various *FLP* strains, we destabilized a *FLP* element located on the *X* chromosome (*hsFLP1*; GOLIC and LINDQUIST 1989) and isolated insertions on the autosomes (see scheme in Figure 2). Sixty-two independent *FLP* insertions were recovered. In this study we utilized six of these: five are inserted on the *MKRS* balancer chromosome: FLP^{39}, FLP^{42}, FLP^{48}, FLP^{90} and FLP^{99}; and one, FLP^{38}, is inserted on the second chromosome. *FLP* insertions on the third chromosome are kept as: *w* ovo^{D1} FRT^{101}*/Y; MKRS, FLP/nkd* crossed with *C(1)DX, y f/Y; MKRS, FLP/nkd* females. ***naked (***nkd***) is a third chromosome embryonic lethal mutation (JURGENS *et al.* 1984). The second chromosome FLP^{38} insertion is kept as *w* ovo^{D1} FRT^{101}*/Y;*FLP^{38}*/*FLP^{38} crossed with *C(1)DX, y f/Y;*FLP^{38}*/*FLP^{38} females.

Recombinant chromosomes: The following recombinants were constructed to test the efficiency of the site-specific recombination technique: *FM7/l(1)zw3*^{M11} *w* FRT^{101}; *FM7/l(1)csw*^{VA199} *w* FRT^{101}; *FM7/l(1)ph*^{107} *w* FRT^{101} and *FM7/fs(1)K10 w* FRT^{101}.

X-ray-induced mitotic recombination: Progeny were irradiated at a constant dose of 1000 rad (Torrex 120D X-ray machine; 100 kV, 5 mA, 3-mm aluminum filter) as described in PERRIMON, ENGSTROM and MAHOWALD (1984). The efficiency of X-ray mitotic recombination in the ovo^{D1} background is around 5–8% when 1000 rad are delivered at the end of the first instar stage (36 ± 6 hr).

Heat shock treatment: Heat shocks were performed at 37° using either a water bath or an air flow incubator. Staging was determined as hours following egg deposition.

Embryonic development and lethal phase determination: Eggs were scored as unfertilized if no embryonic development was detectable following dechorionation in 50% bleach. Larval cuticles were prepared in Hoyers' mountant as described by VAN DER MEER (1977). The cuticles were examined using bright field or dark field illumination. Lethal phases were determined as previously described (PERRIMON, ENGSTROM and MAHOWALD 1989).

Detection of germline clones: Germline clones in females heterozygous for ovo^{D1} were identified by two methods. When accurate identification of the number of mosaics was required, females were dissected four days following eclosion. Since the ovo^{D1} mutation perturbs early oogenesis (BUSSON *et al.* 1983; PERRIMON 1984; OLIVER, PERRIMON and MAHOWALD 1987), a germline clone is identified by the presence of vitellogenic egg chambers. Alternatively, females with germline mosaics were detected by their egg laying ability.

To identify females possessing *fs(1)K10* homozygous germline clones, single females mated with one *Oregon-R P2* (*Ore-R*) male were transferred to plastic tubes and the num-

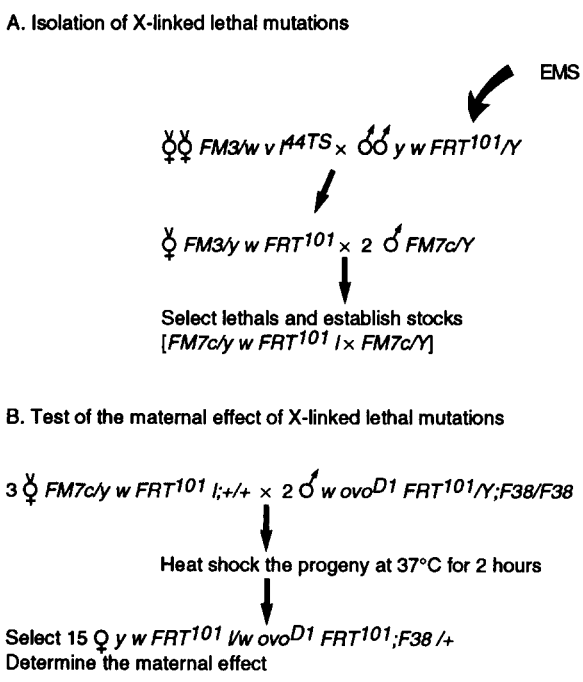

FIGURE 3.—Mutagenesis scheme and method used to analyze the maternal effect of X-linked lethal mutations. A total of 871 X-linked lethal (*l*) mutations were recovered from 2950 successful single pair matings. See MATERIALS AND METHODS for details.

ber of both wild-type and K10 eggs scored daily (WIESCHAUS and SZABAD 1979).

***In situ* hybridization to polytene chromosomes:** *In situ* hybridization to polytene chromosomes was carried out as described by GALL and PARDUE (1971). The probe for *in situ* hybridizations was nick translated with biotinylated dUTP (LANGER-SAFER, LEVINE and WARD 1982), and detected using a Detek-1-HRP kit from ENZO Diagnostic Inc. The probe used was the p*P[>whs>]* plasmid containing the *FRT*s, the mini-*w* (w^{hs}) gene and the *P* element terminal inverted repeat sequences (GOLIC and LINDQUIST 1989).

Mutagenesis: The method used to isolate *X*-linked lethal mutations is shown in Figure 3A. Two- to three-day-old males of genotype *y w* FRT^{101}/*Y* were fed a 20 mM solution of EMS (Sigma) in 1% sucrose for 18 hr (LEWIS and BACHER 1968). Batches of 10 mutagenized males were crossed at 25° to 15 virgin females heterozygous for the lethal-bearing balancer *FM3* and the temperature sensitive lethal chromosome wvl^{44TS} (KOMITOPOULOU *et al.* 1983). Flies were transferred daily for a period of 4 days and the F_1 progeny shifted to 29° to ensure lethality of the wvl^{44TS}/*Y* males. Single pair matings between one *FM3/y w* FRT^{101} virgin female and two *FM7c/Y* males were established and their progeny examined for the absence of *y w* FRT^{101}/*Y* males. Lines lacking *y w* FRT^{101}/*Y* males were subsequently balanced.

To analyze the maternal effect phenotypes of a zygotic lethal mutation (Figure 3B), three *FM7/y w lethal* FRT^{101} virgin females were crossed with two *w ovoD1* FRT^{101}/*Y; F38/F38* males. The progeny were heat shocked for 2 hours at 37° during larval stages and 15 females, of genotypes *y w lethal* FRT^{101}/*w ovoD1* FRT^{101}*; F38/+*, examined for the presence of germline clones. These females were mated with five *Ore-R* males. If the lethal mutation analyzed does not affect germ cell viability, at least 10 mosaic females are recovered under these conditions. The analysis of 10 females with germline clones is sufficient to determine the maternal expression of a lethal mutation (PERRIMON, ENGSTROM and MAHOWALD 1984, 1989).

RESULTS

Design of the "FLP-DFS" technique: Figure 4 shows the method used to produce flies carrying germline clones using the "FLP-DFS" technique. Since this technique was used to detect the maternal effect of recessive *X*-linked zygotic lethal mutations, a lethal mutation (*l*) is drawn in this figure. Although not described in this paper the "FLP-DFS" technique can also be used to generate germline clones to test the tissue specificity of recessive female sterile mutations as well. Chromosomes were constructed such that germline mosaics are recovered as the result of a chromosomal exchange at the site of the *FRT* elements. This exchange is strictly dependent on the presence of the *FRT* sequences and of the *FLP*-recombinase activity (see below and Table 1).

Building the chromosomes: The "FLP-DFS" technique (Figure 4) required the construction of various chromosomes. First an *FRT* element needed to be introduced proximal to the ovo^{D1} mutation. Since no *FRT* insertions were available on the *X* chromosome, we generated such insertions by destabilizing an *FRT* insertion on the third chromosome using the *Δ2-3* transposase (Figure 1, MATERIALS AND METHODS). Among the four different *X* chromosome *FRT* insertions recovered on the *w* ovo^{D1} chromosome, FRT^{101} was determined by *in situ* hybridization to map to position 14A-B of the salivary gland polytene chromosomes (data not shown). This insertion is thus proximal to ovo^{D1}, since the *ovo* locus maps to position 4E of the salivary gland polytene chromosomes (BUSSON *et al.* 1983; OLIVER, PERRIMON and MAHOWALD 1988), and was subsequently used to test the efficiency of the site-specific recombination system in inducing germline mosaics.

Second the homologous chromosome devoid of the ovo^{D1} mutation needed to be recovered. The *FRT* chromosome without ovo^{D1} is referred to as + FRT^{101} This chromosome was recovered as the result of an X-ray-induced germline mitotic exchange that occured between ovo^{D1} and the FRT^{101} element. *y w/y w* virgin females were mated to *w* ovo^{D1} FRT^{101}/*Y* males and their progeny irradiated at the end of the first instar larval stage. Females of genotype *y w/w* ovo^{D1} FRT^{101} carrying germline clones were recovered and crossed individually with *y w/Y* males. If a crossover occurs proximally to the FRT^{101} insertion, only *y w* progeny are expected. However, if a crossover occurs between ovo^{D1} and FRT^{101} both *w* and w^{hs} progeny are obtained. Among 110 germline clone-bearing females examined, we recovered eight such distal crossovers. Stocks homozygous for the + FRT^{101} chromosome were subsequently established.

***FLP*-recombinase can induce mitotic recombination in the female germline:** We tested whether the *FLP*-recombinase can promote chromosomal site-specific exchange to generate germline mosaics. Progeny

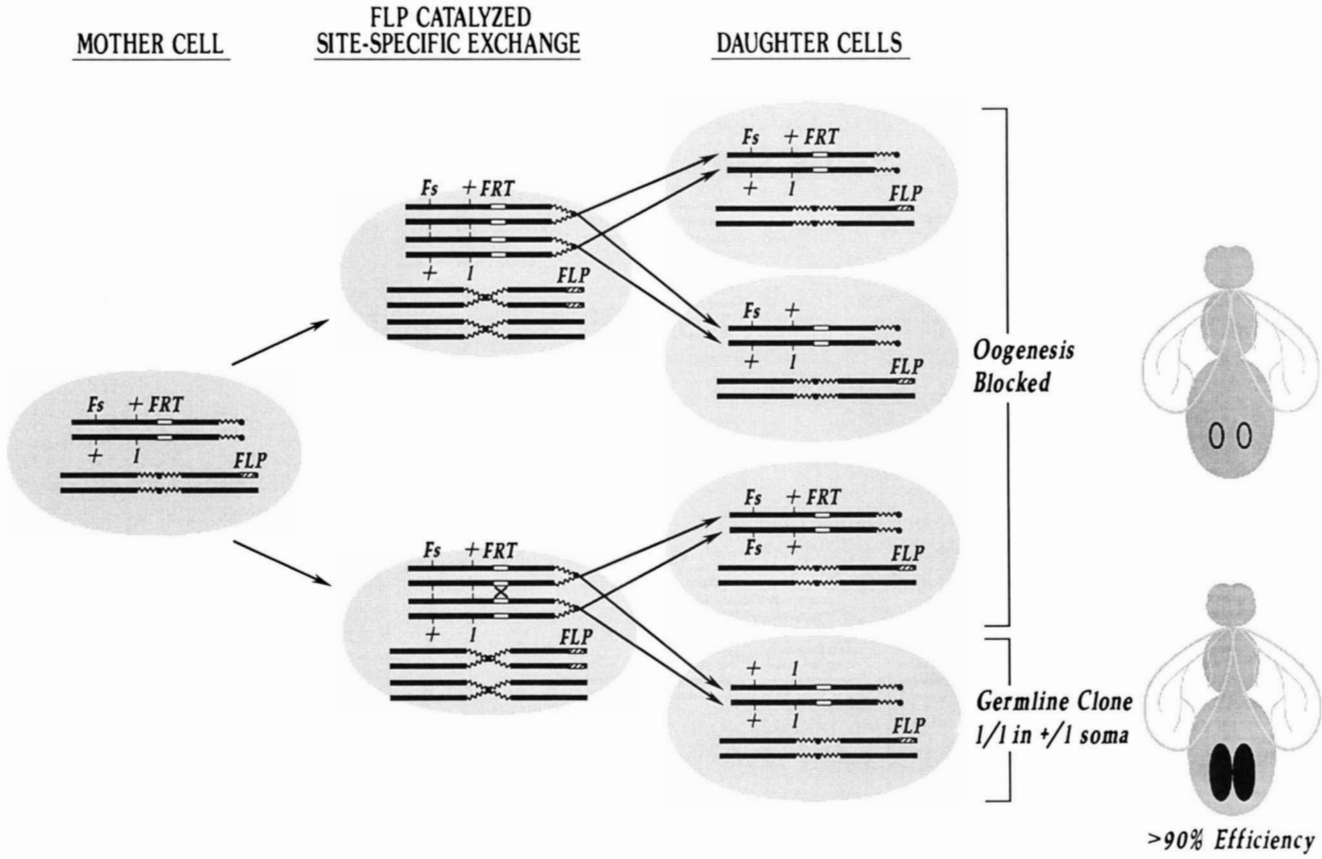

FIGURE 4.—The "FLP-DFS" technique. *FLP*-recombinase induced site-specific exchange: A chromosomal exchange that occurs in the euchromatin of a fly of genotype *Fs + FRT/+ l FRT;FLP/+* is shown. In this scheme, the *FRT* insertion is located proximally to both *Fs* and *l* on the *X* chromosome. The autosomally located *hsp70-FLP* can provide sufficient recombinase activity following heat induction to catalyze site-specific chromosomal exchange at the position of the *FRT* sequences. *FLP*-catalysed recombination can result in the recovery of greater than 90% of females with *l/l* germline clones (see text). Nomenclature: Atrophic ovaries are shown as empty ovals and developed ovaries as black ovals. *FLP*-recombinase target sequences (*FRT*) are depicted as blank boxes and *FLP* as stippled boxes. Dominant female sterile (*Fs*), recessive zygotic lethal mutation (*l*), *hsp70-FLP* (*FLP*).

TABLE 1

***FLP*-recombinase can induce female germline mosaics**

Genotype	*N* tested	*N* fertile
FM7/D1 101;+/+	170	1[a]
FM7/D1 101;FLP[42]/+	182	0
+101/D1 101;+/+	202	3 (1.5%)
+101/D1 101;FLP[42]/+	131	61 (47%)

Progeny from the cross *FM7/+ FRT[101]; +/+* with *ovo[D1] FRT[101]/Y; MKRS, FLP[42]/+* were heat shocked for 2 hr at 37° at 48–72 hr of development. Flies were subsequently grown at 25°. Females of four different genotypes were examined for the presence of germline clones. The number of females tested (*N*) does not reflect the ratio at which each genotypic class was recovered. In this experiment, the percentage of spontaneous mitotic exchange is 1.5%, a value comparable to those obtained in previous studies (PERRIMON and GANS 1983; BUSSON *et al.* 1983; PERRIMON 1984).

[a] Nondisjunction: the genotype of this female was *FM7/+ FRT[101]*. Nomenclature: *D1* is *ovo[D1]* and *101* is *FRT[101]*.

from females *FM7/+ FRT[101]* mated with males *ovo[D1] FRT[101]/Y; MKRS, FLP[42]/+* were aged 48–72 hr following oviposition and heat shocked for 2 hr at 37°. Female progeny of various genotypes were individually analyzed for the presence of germline clones (Table 1). Germline clones were recovered only in flies homozygous for the *FRT[101]* insertion. In the case of females without *FLP*-recombinase, 3 clones among 202 flies were recovered (1.5%) while 61 clones among 131 flies (47%) were recovered from females with *FLP*-recombinase following the heat treatment. The 3 clones obtained in the flies without *FLP*-recombinase are the result of spontaneous mitotic exchange. These experiments demonstrate that *FLP*-recombinase can promote exchange at the position of the *FRT* elements in the female germline and thus is an efficient system to generate germline mosaics. The high frequency of recombination occurs only in females with *FRT* elements on homologous chromosomes and in the presence of *FLP*-recombinase. We did not detect any effect on survival rates since all classes of females were recovered at the expected ratios (data not shown).

Embryos derived from germline clones induced with *FLP*-recombinase have the same phenotype as those derived from X-ray-induced mitotic recombination: We tested whether the phenotypes of embryos derived from germline clones induced by site-specific mitotic exchange were similar to those derived from

X-ray-induced germline clones. We examined the maternal effect phenotypes of mutations in the three essential genes, *l(1)zeste-white 3* (*l(1)zw3*) (PERRIMON and SMOUSE 1989), *l(1)pole hole* (*l(1)ph*) (AMBROSIO, MAHOWALD and PERRIMON 1989) and *l(1)corkscrew* (*l(1)csw*) (PERRIMON, ENGSTROM and MAHOWALD 1985), which have been previously shown to be associated with specific maternal effect lethal phenotypes. Mutations from each locus were recombined with + FRT^{101} (see MATERIALS AND METHODS) and the embryos derived from females of genotype ovo^{D1} FRT^{101}/ *lethal* FRT^{101};*MKRS*, FLP^{42}/+ were analyzed. First we examined the frequency of unfertilized or abnormal eggs obtained from *FLP*-induced germline clones. As shown in Table 2, the frequency of unfertilized eggs in the case of *l(1)csw* and *l(1)ph* is similar to that of the *y w* control. A decrease in the fertilization rate is observed for *l(1)zw3*; however, this is due to the mutation tested rather than the *FLP*-induced germline clone procedure (PERRIMON and SMOUSE 1989). Second, we examined the phenotypes of embryos derived from *FLP*-induced germline clones. Examination of the embryonic phenotypes of the fertilized eggs demonstrated that they are indistinguishable from those derived from X-ray induced germline clones (Table 2). From these experiments we conclude that the *FLP*-induced germline clones are the same as those derived from X-ray induction.

Efficiency of different *FLP*-recombinases and length of heat shock: To optimize the frequency of *FLP*-induced germline clones using ovo^{D1}, we tested whether the length of heat shock would affect the frequency of germline mosaics recovered. Progeny from + FRT^{101}/+ FRT^{101} females crossed with ovo^{D1} FRT^{101}/*Y;MKRS, FLP*/+ males were heat shocked during larval stages at 60 ± 10 hr of development and the resulting female progeny, of genotype ovo^{D1} FRT^{101}/+ FRT^{101};*MKRS FLP*/+ were examined for the presence of germline clones. The results are shown in Table 3. Larvae were subjected to elevated temperatures for a range of 0–120 min and four different *FLP* insertions on the *MKRS* chromosome were tested. Although some fluctuations were observed (Table 3) in the frequency of mosaic females recovered using the various *FLP*-recombinase insertions, a 2-hr period of heat shock at this developmental stage is necessary to recover 75–85% mosaic females (Table 3). The non-heat-shocked controls show only either 0 or 3% mosaic females. Clones obtained in the control could result from the leakiness of the *hsp70* promoter which may provide some low level of *FLP*-recombinase activity. Alternatively, these germline mitotic recombination events could be the result of spontaneous mitotic exchange.

In conclusion, the length of the heat shock affects the frequency of germline clone induction. A heat shock of at least 120 min during larval stages is required to recover about 80% mosaic females.

TABLE 2

***FLP*-recombinase induced germline clones and embryonic phenotypes**

Genotype	N	N_{unh}	N_{unf}	$\frac{N_{unf}}{N}$ (%)	$\frac{N_{unh}-N_{unf}}{N-N_{unf}}$ (%)
+/+	786	121	99	12.6	3.2
+*101/D1 101*;FLP^{42}/+	289	36	27	9.3	3.4
VA199 101/D1 101;FLP^{42}/+	175	175	19	10.9	100
107 101/D1 101;FLP^{42}/+	200	200	25	12.5	100
M11 101/D1 101;FLP^{42}/+	281	281	80	28.5	100

The following mutations were examined: $l(1)corkscrew^{VA199}$ (*VA199*), $l(1)pole\text{-}hole^{107}$ (*107*) *and* $l(1)zeste\text{-}white\ 3^{M11}$ (*M11*). The number of unhatched eggs is indicated. All the embryos derived from *FLP*-recombinase induced *VA199* germline clones had a *corkscrew* phenotype in which embryos have a U-shaped or twisted phenotype (PERRIMON, ENGSTROM and MAHOWALD 1985). Embryos derived from *107* germline clones fell into two classes. If only maternal activity is missing, embryos complete embryogenesis and show deletions of both anterior and posterior structures. However, if both maternal and zygotic $l(1)ph^{+}$ activity is absent, embryos degenerate at germ band elongation (AMBROSIO, MAHOWALD and PERRIMON 1989). We scored 157 embryos derived from *FLP*-recombinase induced *107* germline clones; 72 showed deletions of both anterior and posterior structures and 85 showed poor cuticular differentiation. All embryos derived from *FLP*-recombinase induced *M11* germline clones die during embryogenesis and exhibit a segment polarity phenotype similar to embryos mutant for the segment polarity gene *naked* (PERRIMON and SMOUSE 1989). These embryos lack all denticle belts which are derived from the anterior most part of each segment.

Females with clones were derived from heat-shocked progeny (2 hr at 48–72 hr of development following oviposition) of crosses between *FM7/lethal* FRT^{101};+/+ with ovo^{D1} FRT^{101}/*Y;MKRS*, FLP^{42}/ +. The ratio of unhatched eggs derived from two different controls are also shown.

Controls: Control 1, eggs derived from females homozygous for the + chromosome (in this case + refers to an *X* chromosome that carries both *yellow* and *white*). Control 2, eggs derived from + FRT^{101} homozygous *FLP*-recombinase induced germline clones.

Nomenclature: See Table 1. N is the total of eggs examined. N_{unh} is the number of unhatched eggs and N_{unf} the number of unfertilized eggs.

Test of different *FLP*-recombinases and the developmental stage of heat shock: To determine the optimal time during zygotic development at which the heat treatment should be delivered, the frequency of mosaic females recovered following heat shock was determined at various developmental times. We tested three different *FLP*-recombinase insertions in this experiment (FLP^{38}, FLP^{42} and FLP^{99}). The results, shown in Figure 5, indicate that a similar developmental profile of clone induction is generated for all three *FLP*-recombinase insertions tested. The frequency of mosaic females increases gradually during embryonic and larval stages, and reaches a plateau during pupation. For FLP^{38} and FLP^{99}, approximately 40–50% of females with germline clones are recovered when the heat shock is performed during embryogenesis. If the heat shock is administered after pupal stages, a plateau of 90–100% of mosaic females is reached. Interestingly, the *FLP*-recombinase insertion FLP^{42} shows a similar developmental profile; however, clones are recovered at a lower frequency. Heat

TABLE 3

Level of *FLP*-recombinase activity and the frequency of mosaic females

Genotype	Heat shock (min)						
	0	30	45	60	75	90	120
+ *101/D1 101;FLP^{39}*/+	0/47 (0%)	5/82 (6%)	2/67 (3%)	34/125 (27%)	27/86 (31%)	3/26 (12%)	74/106 (70%)
+ *101/D1 101;FLP^{48}*/+	0/68 (0%)	0/15 (0%)	1/35 (4%)	4/21 (20%)	11/40 (27%)	3/4 (75%)	31/40 (78%)
+ *101/D1 101;FLP^{90}*/+	2/66 (3%)	3/80 (4%)	20/70 (29%)	14/56 (25%)	5/22 (23%)	26/34 (47%)	35/40 (87%)
+ *101/D1 101;FLP^{99}*/+	0/35 (0%)	3/99 (3%)	5/65 (8%)	33/63 (52%)	9/26 (35%)	24/80 (30%)	32/38 (84%)

Females derived from heat-shocked progeny of crosses between + *FRT^{101}*/+ *FRT^{101}*;+/+ with *ovo^{D1} FRT^{101}/Y;MKRS, FLP*/+ were examined for the presence of germline clones. Results are presented as the number of females with clones divided by the total number of females analyzed. Heat shock was performed at 60 ± 10 hr of development in an air flow incubator. The four different *FLP*-recombinases (see MATERIALS AND METHODS). *FLP^{39}*, *FLP^{48}*, *FLP^{90}*, *FLP^{99}*, which are inserted on the *MKRS* balancer chromosome were tested.

Nomenclature: See Table 1.

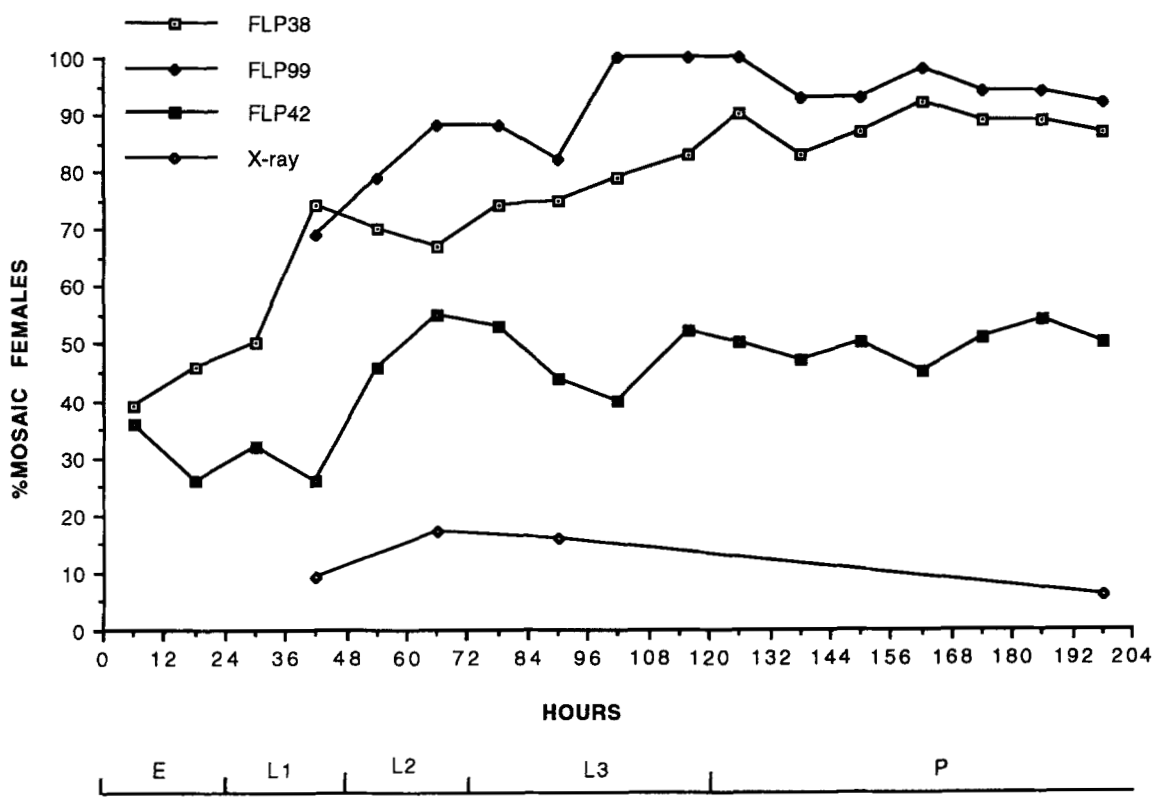

FIGURE 5.—Developmental profile. Three different *FLP*-recombinases *FLP^{38}*, *FLP^{42}* and *FLP^{99}* were tested. Progeny derived from the three crosses: (1) females + *FRT^{101}*/+ *FRT^{101}*;+/+ × males *ovo^{D1} FRT^{101}/Y;FLP^{38}/FLP^{38}*; (2) females + *FRT^{101}*/+ *FRT^{101}*;+/+ × males *ovo^{D1} FRT^{101}/Y;MKRS, FLP^{99}*/+; and (3) females + *FRT^{101}*/+ *FRT^{101}*;+/+ × males *ovo^{D1} FRT^{101}/Y;MKRS, FLP^{42}*/+, were heat shocked for 2 hr at 37° at various developmental stages. In this experiment flies emerged at around 228 hr. Females of genotype + *FRT^{101}/ovo^{D1} FRT^{101};FLP*/+ were examined for the presence of germline clones. The percentage of mosaic flies is calculated as the number of mosaic females/total number of flies analyzed × 100. The average number of flies examined at each developmental time point is 119 for *FLP^{38}*, 113 for *FLP^{42}* and 45 for *FLP^{99}*. Also indicated in this graph is the frequency of germline clones recovered from X-ray-induced mitotic recombination (data from PERRIMON 1984). Nomenclature: embryonic (E), larval (L1, L2, L3) and pupal (P) stages.

shock during embryonic stages results in 25–35% of females bearing germline clones, while an heat shock during pupal stages produces approximately 50% of mosaic females.

We conclude that various *FLP*-recombinases insertion lines have a similar developmental profile of mitotic recombination activity, although they can show different levels of recombinase activity. *FLP^{38}* and *FLP^{99}* are more efficient recombinases than *FLP^{42}*. To conveniently generate 70–90% germline clones with the "FLP-DFS" technique, we recommend a heat treatment of 2 hr at 37°, 1 day before pupation

using either FLP^{38} or FLP^{99} recombinases.

To compare the relative efficiency of the "FLP-DFS" technique *vs.* the "DFS" technique, we have indicated in Figure 5 the frequency of X-ray-induced female mosaics recovered in a similar genetic background (data from PERRIMON 1984). It is evident from this comparison that *FLP*-recombinase can produce, at least by one order of magnitude, a higher frequency of mosaic females than X-ray irradiation.

Clone size: How many independent germline clones can be induced using *FLP*-recombinase in one individual female? To determine such numbers, we dissected $+\ FRT^{101}/ovo^{D1}\ FRT^{101};FLP/+$ females following heat shock at various developmental stages and examined their ovaries for the presence of germline clones (Table 4). Since a mosaic ovary is easily identified by the presence of vitellogenic oocytes, the total number of ovaries with clones could be obtained. To estimate the number of individual clones present in these mosaic ovaries, we used the Poisson distribution (Table 4). Two different *FLP*-recombinase insertions were used in this study. Using FLP^{M42}-recombinase, each mosaic ovary is found on average to contain less than 1.3 clones when the heat shock is performed at any time throughout development (Table 4). In no cases did more than 26% of the mosaic ovaries contain more than 2 clones (calculated from Table 4 as: $f(2) + \ldots f(n)/f(1) + \ldots f(n) \times 100$). However, using FLP^{38}, a more efficient recombinase (Figure 5), more clones are recovered. When the heat shock is performed after pupation, 1.5–2 clones per mosaic ovaries can be induced. When the heat shock is performed after 36 hr of development, more than 30% of mosaic ovaries contain 2 clones (calculated from Table 4 as: $f(2) + \ldots f(n)/f(1) + \ldots f(n) \times 100$), and after 120 hr more than 50% of mosaic ovaries contain 2 clones and more than 20% of mosaic ovaries contain 3 clones (calculated as: $f(3) + \ldots f(n)/f(1) + \ldots f(n) \times 100$).

Since the ovo^{D1} mutation affects the development of germ cells (PERRIMON 1984; OLIVER, PERRIMON and MAHOWALD 1987) the numbers of *FLP*-recombinase germline clones calculated in the previous analysis is probably an underestimation of the actual number of clones that can be induced in a wild-type background. To estimate the number of independent germline clones that can be induced in a physiological background closer to wild type, we used the recessive maternal effect mutation *fs(1)K10* which has been previously used in a clonal analysis of the female germline (WIESCHAUS and SZABAD 1979). Eggs derived from oocytes homozygous for *fs(1)K10* are easily identified among a population of wild-type eggs because their dorsal appendages are fused ventrally. The results of this clonal analysis using FLP^{M42}-recombinase is shown in Table 5 and Figure 6. On average each mosaic female contains 2.84 clones when the heat shock is delivered at the first instar larval stage and 6.2 clones when heat shocked at the pupal stage (see legend of Figure 6 for calculations). Such an increase in the number of K10 clones was expected since the number of germ cells increases at least three times between these two developmental stages (WIESCHAUS and SZABAD 1979).

Screen: To test the efficiency of the "FLP-DFS" technique to analyze the maternal effect of zygotic lethal mutations we conducted a germline clone analysis of 871 newly induced *X*-linked lethals. The isolation of the lethals and the method we used to detect their maternal effects is described in Figure 3 and MATERIAL AND METHODS. Maternal expression was detected for 59.4% of the mutations (Table 6). No clones were recovered from 39.1% of them and 8% showed defects in oogenesis leading to the production of abnormal eggs. 4.3% were associated with a strict maternal effect and 8% with a paternally rescuable maternal effect. These results are similar to those previously described by PERRIMON, ENGSTROM and MAHOWALD (1989) who used X-ray-induced mitotic recombination to analyze the maternal expression of a random set of *X*-linked lethal mutations.

DISCUSSION

We have used a yeast *FLP*-recombinase to produce germline clones in the background of the *X*-linked dominant female sterile mutation, ovo^{D1}. More than 90% of females bearing germline clones can be easily recovered depending upon the *FLP*-recombinase insertion utilized, and the length and stage of heat shock. This technique is at least 10 times more efficient than the use of X-rays to induce germline clones (see Figure 5).

To demonstrate that *FLP*-induced mitotic exchange does not generate unexpected defects in germline and embryonic development, we showed that embryos derived from *FLP*-recombinase induced germline clones of three previously known maternal effect loci are indistinguishable from embryos derived from X-ray-induced germline clones. The high frequency of recovery of mosaic females and the production of physiologically normal germline clones indicates that the "FLP-DFS" technique is the best tool to create female germline mosaics.

Unlike X-irradiation (HAYNIE and BRYANT 1977), use of *FLP*-recombinase has the significant advantage of not causing cell death. Additionally, inducing germline clones with *FLP*-recombinase reduces the frequency of distal crossovers since the site of mitotic exchange between homologous chromosomes is fixed (by the position of the *FRT* sequences) and the only crossovers that can occur between the *FRT* element and the *DFS* are derived from spontaneous mitotic exchange which occur at a very low frequency (see Table 1). These experiments demonstrate that the use of *FLP*-recombinase to generate germline mosaics is

TABLE 4

Germline clonal analysis of ovo^{D1}

Age of HS (hr)	N	%MF	%MO	f(0)	f(1)	f(2)	f(3)	f(4)	f(5)	#CL
FLP^{M42}										
0–12	36	36	24	0.76	0.21	0.03	0	0	0	1.13
12–24	78	26	18	0.82	0.16	0.02	0	0	0	1.11
24–36	25	32	20	0.80	0.18	0.02	0	0	0	1.10
36–48	125	26	15	0.85	0.14	0.01	0	0	0	1.07
48–60	69	46	31	0.69	0.26	0.05	0	0	0	1.22
60–72	24	55	44	0.56	0.32	0.09	0.02	0	0	1.30
72–84	167	53	39	0.61	0.30	0.07	0.01	0	0	1.24
84–96	158	44	33	0.67	0.27	0.05	0.01	0	0	1.21
96–108	130	40	27	0.73	0.23	0.04	0	0	0	1.15
108–120	131	52	36	0.64	0.29	0.06	0.01	0	0	1.22
120–132	104	50	39	0.61	0.30	0.07	0.01	0	0	1.24
132–144	137	47	38	0.62	0.29	0.07	0.01	0	0	1.24
144–156	188	45	32	0.68	0.26	0.05	0	0	0	1.22
156–168	158	51	40	0.60	0.30	0.08	0.01	0	0	1.26
168–180	127	54	44	0.56	0.32	0.09	0.02	0	0	1.30
180–192	133	50	43	0.57	0.32	0.09	0.02	0	0	1.30
192–204	141	56	43	0.57	0.32	0.09	0.02	0	0	1.30
FLP^{F38}										
0–12	78	39	22	0.78	0.19	0.02	0	0	0	1.10
12–24	54	46	26	0.74	0.22	0.03	0	0	0	1.12
24–36	102	50	30	0.70	0.25	0.05	0	0	0	1.17
36–48	66	74	51	0.49	0.35	0.12	0.03	0	0	1.36
48–60	74	70	51	0.49	0.35	0.12	0.03	0	0	1.36
60–72	66	67	45	0.55	0.33	0.10	0.02	0	0	1.31
72–84	124	74	57	0.43	0.36	0.15	0.04	0	0	1.42
84–96	174	75	51	0.49	0.35	0.13	0.03	0	0	1.37
96–108	126	79	60	0.40	0.37	0.17	0.05	0.01	0	1.50
108–120	179	83	65	0.35	0.37	0.19	0.07	0.02	0	1.60
120–132	152	90	75	0.25	0.35	0.24	0.11	0.04	0.01	1.83
132–144	175	83	72	0.28	0.36	0.23	0.10	0.03	0	1.72
144–156	183	87	77	0.23	0.34	0.25	0.12	0.04	0.01	1.86
156–168	124	92	74	0.26	0.35	0.23	0.10	0.03	0	1.73
168–180	187	89	77	0.23	0.34	0.25	0.12	0.04	0.01	1.86
180–192	76	89	83	0.17	0.30	0.27	0.16	0.07	0.03	2.11
192–204	93	87	72	0.28	0.36	0.23	0.10	0.03	0	1.72

+ FRT^{101}/ovo^{D1} FRT^{101};FLP/+ female progeny (from + FRT^{101}/+ FRT^{101};+/+ females crossed with ovo^{D1} FRT^{101}/Y;FLP/FLP males) were dissected 4 days following eclosion and examined for the presence of developed ovaries. Two *FLP*-recombinase insertions were analyzed: FLP^{38} and FLP^{M42}. Heat shock (HS) was delivered for 2 hr at 37° at various time periods following egg deposition.

N is the total number of females examined. %MF represents the percentage of mosaic females. %MO corresponds to the percentage of mosaic ovaries. %MO = [number of ovaries with clones/total number of ovaries examined] × 100. Because females have two ovaries, a mosaic female can possess one or two developed ovaries. #CL is the average number of clones present in a single ovary. #CL = $f(1) + 2f(2) + \ldots + nf(n)/f(1) + f(2) + \ldots + f(n)$.

The Poisson distribution (FELLER 1968) was used to calculate the occurrence of *n* clones per ovary. The frequency of ovaries with no clones = $f(0)$ = [number of ovaries without clones/number of ovaries examined] × 100) = e^{-u}. The frequency of ovaries with *n* clones = $f(n) = u^n e^{-u}/n!$. Values have not been corrected for the spontaneous clones that occur at a very low frequency in these experiments (about 1%, see Table 1).

significantly more efficient than the use of X-rays.

The developmental profile of *FLP*-recombinase activity (Figure 5) shows that the frequency of mosaic females increases gradually during larval stages to reach a plateau at pupation. The developmental pattern of *FLP*-induced mosaics (Figure 5) is reminiscent of the growth of the female germline (WIESCHAUS and SZABAD 1979). Germ cell divisions follow a logarithmic curve during larval stages. One day following pupariation, a constant number of germline cells is established and maintained by stem cell divisions. The similarity between these two developmental profiles suggests that the frequency of mosaic females reflects the number of germline cells available for *FLP* to act upon. More germline cells provide more targets for *FLP*-recombinase and hence a higher frequency of mosaic females.

We conducted two different clonal analyses to calculate the number of germline clones that are induced using *FLP*-recombinase. The first one was aimed to estimate the number of independent clones induced in females heterozygous for ovo^{D1}. Our analysis (Table 4) demonstrates that depending upon the *FLP*-recombinase insertion used, a mosaic ovo^{D1}/+ ovary is derived from 1 to 3 independent clones (see RESULTS). The aim of the second analysis was to determine the

TABLE 5

Germline clonal analysis of *fs(1)K10*

Heat shock	*N* females	Total eggs	*N* mosaics	Total eggs	K10 eggs	$\frac{N\ K_{10}\ eggs\ (\%)}{N\ mosaic\ total}$
L1	121	25,893	98	21,390	1,012	4.73
P	1,016	207,961	681	155,026	8,827	5.69

Progeny from the cross: *FM7/fs(1)K10 w FRT^{101}* females crossed with + *FRT^{101}/Y*; *MKRS, FLP^{42}/+* males, were heat shocked for 2 hr at 37° and the F_1 female progeny *fs(1)K10 w FRT^{101}/+FRT^{101}/Y*; *MKRS, FLP^{42}/+* crossed with *Ore-R* males were scored for the presence of K10 eggs. Two developmental stages were analyzed: first instar larval stage (L1: 24–48 hr after oviposition) and ths pupal stage (P: white pupae to white pupae + 24 hr). In this study, only the flies which lay the minimum number of eggs necessary to detect 95% of mosaics are reported. This number is 25 for first instar larval stage and 55 for the pupal stage. Pupae were staged visually following selection of the white pupae.

As in the previous experiment (Table 4), values were not corrected for the spontaneous K10 clones which occur at a very low frequency (data not shown, see also WIESCHAUS and SZABAD 1979).

number of independent clones that can be induced using *FLP*-recombinase in a physiologically wild-type background. Results from this experiment (Table 5, Figure 6) demonstrate that 2–3 clones per mosaic female are recovered when the heat shock is delivered at the first instar larval stage and an average of 6 clones are induced when the heat shock is delivered at the pupal stage. These results are consistent with a previous analysis that utilized X-ray induced mitotic recombination (PERRIMON 1984). Previously, PERRIMON (1984) found that the frequency of X-ray-induced germline clones at the first instar stage is similar for *ovo^{D1}* and *fs(1)K10*; however, when clones were induced later in development the clone frequency in *ovo^{D1}/+* females decreased in comparison to those found in *fs(1)K10/+* females. Such differences were hypothesized to reflect the detrimental effect of *ovo^{D1}* mutation on germ cells development (see also OLIVER, PERRIMON and MAHOWALD 1987).

It should be noted that at the pupal stage 33% of females do not possess any K10 clones (Table 5). This observation is puzzling since the average number of clones per mosaic female is 6. It indicates that the probability of induction of germline recombination is not random from female to female. The reason for this is unclear and may be due to differences in the microenvironment of the larvae during heat shock. Alternatively, it may reflect synchronization of germ cell divisions. Since the action of *FLP*-recombinase is most likely cell cycle dependent, only germ cells at a specific stage in the cell cycle might be able to undergo *FLP*-induced mitotic recombination (see also GOLIC and LINDQUIST 1989).

The "DFS" technique has been used to identify previously unknown genes involved in embryonic patterning. PERRIMON, ENGSTROM and MAHOWALD (1989), using *ovo^{D1}* and X-irradiation to generate germline mosaics, conducted an extensive screen, near saturation, for all *X*-linked zygotic lethal mutations

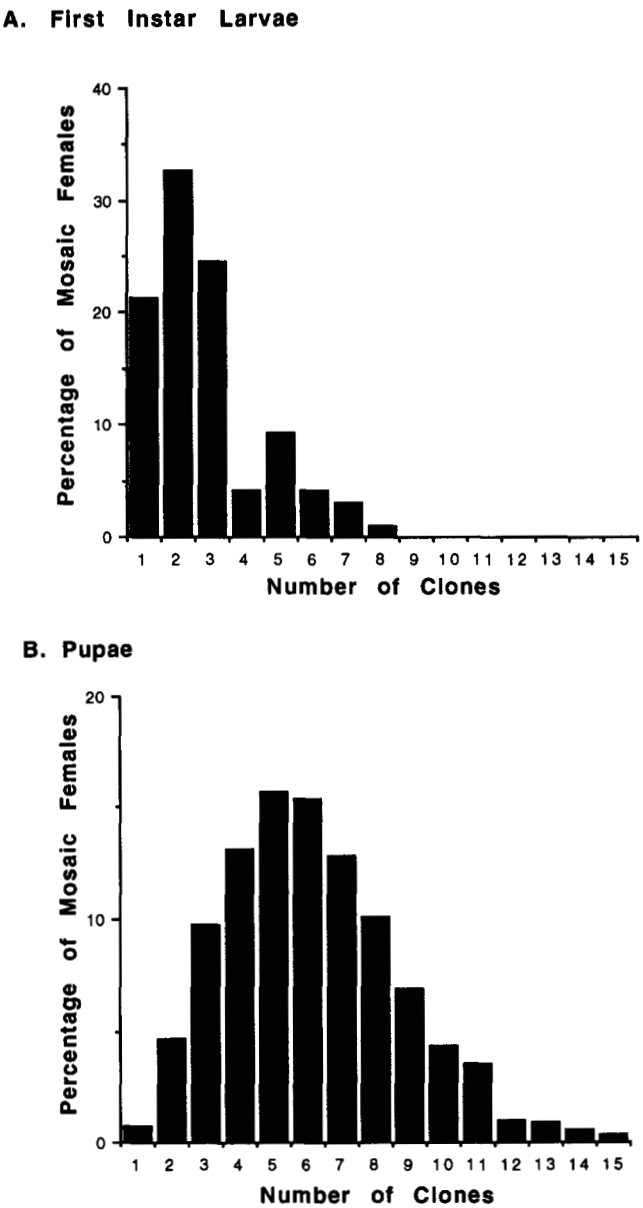

FIGURE 6.—Number of K10 clones. The percentage of mosaic females carrying *n* clones is shown for two developmental stages: (A) first instar larval stage and (B) pupal stage (see Table 5 for details). Calculations of the number of clones present in mosaic females is based upon the data from WIESCHAUS and SZABAD (1979). At the first instar larval stage, WIESCHAUS and SZABAD estimated that each female possesses an average of 30 germ cells. If a female carries a single germline clone induced during the first instar larval stage, the fraction of K10 eggs laid by this female should be 1/60 (since once cell division has to occur to generate an homozygous *fs(1)K10* cell). The total number of clones (n) in a single female was calculated as: [*N* K10 eggs/*N* total eggs] × 60. At the pupal stage, WIESCHAUS and SZABAD estimated that 109 germ cells were present. Unlike larval germ cells, the number of pupal germ cells does not increase but remains constant due to a stem cell mode of division. The total number of clones (*n*) in a single female was calculated as: [*N* K10 eggs/*N* total eggs] × 109. The average number of clones per mosaic female can be directly calculated from the numbers shown in Table 5. The average number of clones is 0.0473 × 60 = 2.84 clones when the heat shock is delivered at the first instar larval stage and 0.0569 × 109 = 6.2 when pupae are heat shocked. These calculations assume that clone sizes per female do not vary greatly from female to female.

TABLE 6

Germline clone phenotypes of newly induced X-linked lethal mutations

	Germline clone phenotypes				
N	L	AO	ME	MER	NME
Experimental numbers					
871	290	59	32	60	430
Corrected numbers					
742	290	59	32	60	301
	(39.1%)	(8%)	(4.3%)	(8%)	(40.6%)

N is the total of zygotic lethal mutations tested in germline clone mosaics. The phenotypes observed from homozygous germline clones of zygotic lethal mutations have been classified into five groups. Group 1: Lethal (L), no mosaics are recovered. Group 2: Abnormal oogenesis (AO). This group includes those lethals that lay abnormal eggs; *i.e.*, collapsed, abnormal shape. Group 3: Maternal effect (ME). Group 4: Rescuable maternal effect (MER). Group 5: No maternal effect (NME). A detailed description of this classification can be found in PERRIMON, ENGSTROM and MAHOWALD (1989).

Since the zygotic lethal mutations were isolated at random on the *X* chromosome (Figure 3), approximately 30% of them should be localized between the *FRT* insertion (at position 14A-B on the polytene chromosomes) and the centromere. Germline clone analysis of these mutations would be classified into the NME group because a mitotic exchange occurring at the level of the *FRT* element does not lead to homozygosity of the lethal. We have corrected the numbers to take this into account.

with specific maternal effect phenotypes. This analysis was labor intensive due to (1) the low frequency of mosaic females recovered and (2) the recovery of crossovers induced between ovo^{D1} and the lethal mutation analyzed. The use of *FLP*-recombinase to induce germline clone production has solved these problems. First, the number of females needed to be analyzed to determine the maternal effect of a zygotic lethal mutation, using X-ray-induced mitotic recombination, required the screening of at least 300 females of the appropriate genotype in order to isolate enough mosaics (around 10). The "FLP-DFS" technique reduces considerably the amount of work involved since only 15 females need to be analyzed to recover the same number of mosaics. Second, because the mitotic exchange is localized at the position of the *FRT* element, the only crossovers that may occur between the lethal and the *DFS* are of spontaneous origin. These events occur at a very low frequency (around 1%) and can be easily identified by the analysis of independent batches of mosaic females.

Due to the efficiency of the "FLP-DFS" technique, we can envision the feasibility of conducting screens to identify autosomal zygotic lethal mutations with specific maternal effect phenotypes. A prerequisite for such autosomal screens is the availability of *FRT* elements located at the base of each chromosomal arm, and of autosomal germline-dependent dominant female sterile mutations. Some autosomal *DFS* mutations which might be appropriate for the production of germline mosaics have been reported (YARGER and KING 1971; ERDELYI and SZABAD 1989; SZABAD *et al.* 1989). Alternatively, transposons that carry ovo^{D1} might become available since the *ovo* gene has recently been cloned (MEVEL-NINIO *et al.* 1991).

The germline clone analysis of *fs(1)K10* (Figure 6, Table 5) demonstrates that when using FLP^{M42}-recombinase approximately 4% of the total number of eggs are derived from homozygous germline clones. Possibly, this frequency could be increased by using a more effective *FLP*-recombinase or multiple copies of various *FLP*-recombinase insertions. To identify eggs derived from homozygous germline clones, one could take advantage of *P[lacZ]* strains that express β-galactosidase during oogenesis and early embryogenesis. In this scheme, a lethal mutation, located distally to the *FRT* element, is crossed with a strain that carries both the *P[lacZ]* insertion, located distally to the same *FRT* element, and the *FLP*-recombinase gene. Following an heat shock treatment, a mitotic exchange occurring at the level of the *FRT* element will render the lethal (*l*) mutation homozygous and eliminate the *P[lacZ]* insertion. Eggs derived from *l/l* germline clones can be identified by lack of β-galactosidase activity. A number of enhancer trap strains have been recovered that should be suitable for these purposes (FASANO and KERRIDGE 1988; GROSSNIKLAUS *et al.* 1989; N. PERRIMON, unpublished). Although appropriate for the study of a small number of mutations, the use of such a technique would be very tedious and not as advantageous as the use of *DFS* mutations to conduct large screens to detect the maternal effect of zygotic lethal mutations.

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