Use of a Yeast Site-Specific Recombinase to Generate Embryonic Mosaics in *Drosophila*

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ABSTRACT An efficient method for generating embryonic mosaics using a yeast site-specific recombinase (*FLP*), under the control of a heat shock promoter, is described. *FLP*-recombinase can promote mitotic exchange between homologous chromosomes that contain *FRT* (*FLP* Recombination Target) sequences. To demonstrate the efficiency of *FLP*-recombinase to generate embryonic mosaics, clones of the recessive and cell autonomous mutation *armadillo* (*arm*), detected by their ability to differentiate ectopic denticles in the naked cuticle of each abdominal segment, have been induced. We have analyzed the parameters of *FLP*-recombinase induced embryonic mitotic recombination and have demonstrated that clones can be efficiently induced during the postblastoderm mitotic divisions. We discuss applications of this technique for the analyses of the roles of various mutations during embryonic patterning.
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Key words: FLP-recombinase, FRT, embryonic mosaics

INTRODUCTION

The ability to generate mosaics is a powerful technique for studying the mechanisms underlying pattern formation, as well as for analyzing the roles of specific genes in patterning. In *Drosophila,* mosaic analyses have been used extensively to determine the effects and cellular autonomy of mutations on adult patterning. The most commonly used technique to generate adult somatic mosaics is mitotic recombination, which can be induced following X-ray treatment at various developmental stages. A number of cell autonomous recessive markers [Lawrence *et al.,* 1986] are available to detect mutant clones in the adult cuticle (e.g., *forked, multiple wing hairs*) and the eye (e.g., *white*). In addition, a number of histochemical markers have been used to detect clones of homozygous cells in internal structures [e.g., *aldehyde oxidase;* Janning, 1972; *acetylcholinesterase;* Ferrus and Kankel, 1981; *succino-dehydrogenase;* Lawrence, 1981]. Other methods used to generate adult somatic mosaics include nuclear transplantation, the use of an unstable *ring-X* chromosome

(the gynandromorph technique), and the use of mutations that cause somatic chromosome elimination [e.g., *paternal loss* and *mitotic loss inducer;* Hall *et al.,* 1976].

Contrary to adult mosaics, the analyses of the effects and cell autonomy of mutations affecting embryonic patterning have been poorly analyzed. The reasons for this include the inefficient means of generating large numbers of mosaic animals, as well as the paucity of embryonic markers. A rather laborious method for generating embryonic mosaics consists of the transplantation of cells or nuclei. For example, labeling *Drosophila* cells by injection of markers such as horseradish peroxidase and subsequent transplantation into developing embryos [Technau, 1986], has been used to test the autonomy of several neurogenic genes [Technau and Campos-Ortega, 1987].

Genetically, two methods, gynandromorphs and X-ray induced mitotic recombination, have been used to produce embryonic mosaics. Gergen and Wieschaus [1986] generated mutant patches in embryos using the unstable *Ring-X* chromosome. To identify these mutant territories, the cell autonomous mutation *shaven baby* (*svb*), which decreases the number and size of larval denticles, was used. This technique allowed the determination of the cellular autonomy of a number of embryonic lethal mutations [Gergen and Wieschaus, 1986]. There are three problems associated with the use of *Ring-X* chromosomes: first, this technique is limited to studies of X-linked mutations; second, the clones generated are large; and third, the time of clone induction cannot be controlled. An alternative method to generate and recognize small mutant patches in an otherwise heterozygous animal makes use of the cell autonomous segment polarity mutation *armadillo* (*arm*). *arm* embryos exhibit a segment polarity phenotype in which all naked cuticle of the larvae is deleted and replaced by denticles. Wieschaus and Riggleman [1987] demonstrated that clones of *arm* cells, generated by X-ray

induced mitotic recombination, can develop ectopic patches of denticles in part of the naked region of every segment [see also Klingensmith et al., 1989]. To analyze if the mutations Notch [N; Hoppe and Greenspan, 1986] and polyhomeotic [phm; Smouse and Perrimon, 1990] which perturb the differentiation of the ventral epidermis, are cell autonomous, doubly mutant clones of arm N and arm phm were induced in heterozygous animals. Two problems arose from the use of this technique: first, X-ray treatment of embryos generates a large number of non-specific defects that make the identification of some clones difficult; and second, clones of homozygous cells are recovered at a very low frequency.

Recently, an efficient way to generate mosaics using the site-specific recombinase, FLP, was developed and used successfully to induce clones in the imaginal discs and germ cells [Golic, 1991; Chou and Perrimon, 1992]. FLP-recombinase promotes mitotic exchange between homologous chromosomes that contain FRT sequences. Unlike X-ray induced mitotic recombination [Haynie and Bryant, 1977], the use of FLP-recombinase is not associated with cell death. Furthermore, since it is heat inducible, the timing of clone induction is tightly regulated. We have tested whether FLP-recombinase could be used to induce embryonic mosaics in animals heterozygous for the cell autonomous, segment polarity gene arm. We report the parameters of this clonal analysis and describe applications of this method to study embryonic patterning.

MATERIALS AND METHODS
Strains

We used the X-linked FRT $(P[>w^{hs}>])$ insertion [Golic and Lindquist 1989], FRT^{101}, located at cytological position 14A-B on the X-chromosome [Chou and Perrimon 1992]. We used the FLP insertion $[P[ry^{+}hsFLP]$; Golic and Lindquist, 1989], FLP^{38}, that is located on the second chromosome [Chou and Perrimon, 1992]. In this study we used a single FLP insertion (FLP^{38}) which has been previously shown to be extremely efficient [Chou and Perrimon, 1992].

The recombinant chromosome y arm^{XK22} FRT^{101} was constructed to test the efficiency of the site-specific recombination technique. arm^{XK22} behaves genetically as a null arm allele [Peifer and Wieschaus, 1990]. This stock is maintained using the FM7c balancer. Embryos of the genotype arm^{XK22} $FRT^{101}/$ + FRT^{101}; +/FLP^{38} were derived from crosses of FM7c/arm^{XK22} FRT^{101}; +/+ females with + FRT^{101}/Y; FLP^{38}/FLP^{38} males.

Flies were raised on standard Drosophila media at 25°C. Descriptions of balancers and mutations that are not described in the text can be found in Lindsley and Zimm [1992].

Egg Collection and Heat Shock Treatment

Eggs were collected on petri dishes containing an agar-molasses medium supplemented with dry yeast. Females were mated for at least 24 hours prior to the egg collections and were allowed to lay eggs in a quiet environment to optimize the synchronization of the eggs deposited. The first collections were discarded.

Heat shock treatments of the synchronized embryos were performed at 37°C in a circulating waterbath. The petri dishes were covered with parafilm and floated on the water. Following the heat shock, the plates were immediately transferred into a 25°C incubator.

Cuticle Examination and Recording of the Arm Clones

To detect the presence of arm clones, embryos were allowed to fully develop (approximately 24 hr at 25°C) and their cuticles prepared in Hoyers' mountant as described by van der Meer [1977]. The positions and numbers of denticles of all arm clones recovered were recorded on schematic drawings of a ventral cuticle of a first instar larvae. The cuticles were examined using bright field or dark field illumination. arm clones were recognized as ectopic denticles in the naked regions of the seven (A1 through A7) abdominal segmental intervals.

RESULTS AND DISCUSSION
FLP-Recombinase Can Promote Mitotic Exchange During Embryonic Development

Previously, Wieschaus and Riggleman [1987] showed that homozygous clones of null alleles of armadillo (arm), generated by X-ray induced mitotic recombination in heterozygous embryos, led to the ectopic occurrence of denticles in the naked region of the larval cuticle. The method we used to test the efficiency of FLP-recombinase to induce embryonic mosaics, using arm as a cuticular marker, is shown in Figure 1. Embryos of genotype arm $FRT^{101}/$ + FRT^{101};$FLP^{38}/$ + were heat shocked at 37°C at various times during embryonic development and examined for the occurrence of arm clones.

Results shown in Table 1 indicate that FLP-recombinase can promote mitotic exchange during embryonic development. While arm clones are found in heat treated embryos that carry one copy of FLP-recombinase, no arm clones are recovered in the heat shocked animals without FLP-recombinase. The percentage of mosaics increases with the length of the heat shock. Following a 30 min heat shock, a small number (12.5%) of mosaic larvae are recovered; however the number of mosaics increases to 100% following a 120 min heat shock. Although the number of clones recovered increases with heat shock, the percentage of segmentation defects due to the heat treatment also increases, especially when a heat shock of 180 min is administered (Table 1). Because the presence of these defects makes the identification of arm clones difficult, we decided to use a 120 min heat shock to generate arm clones in all the following experiments. Such condi-
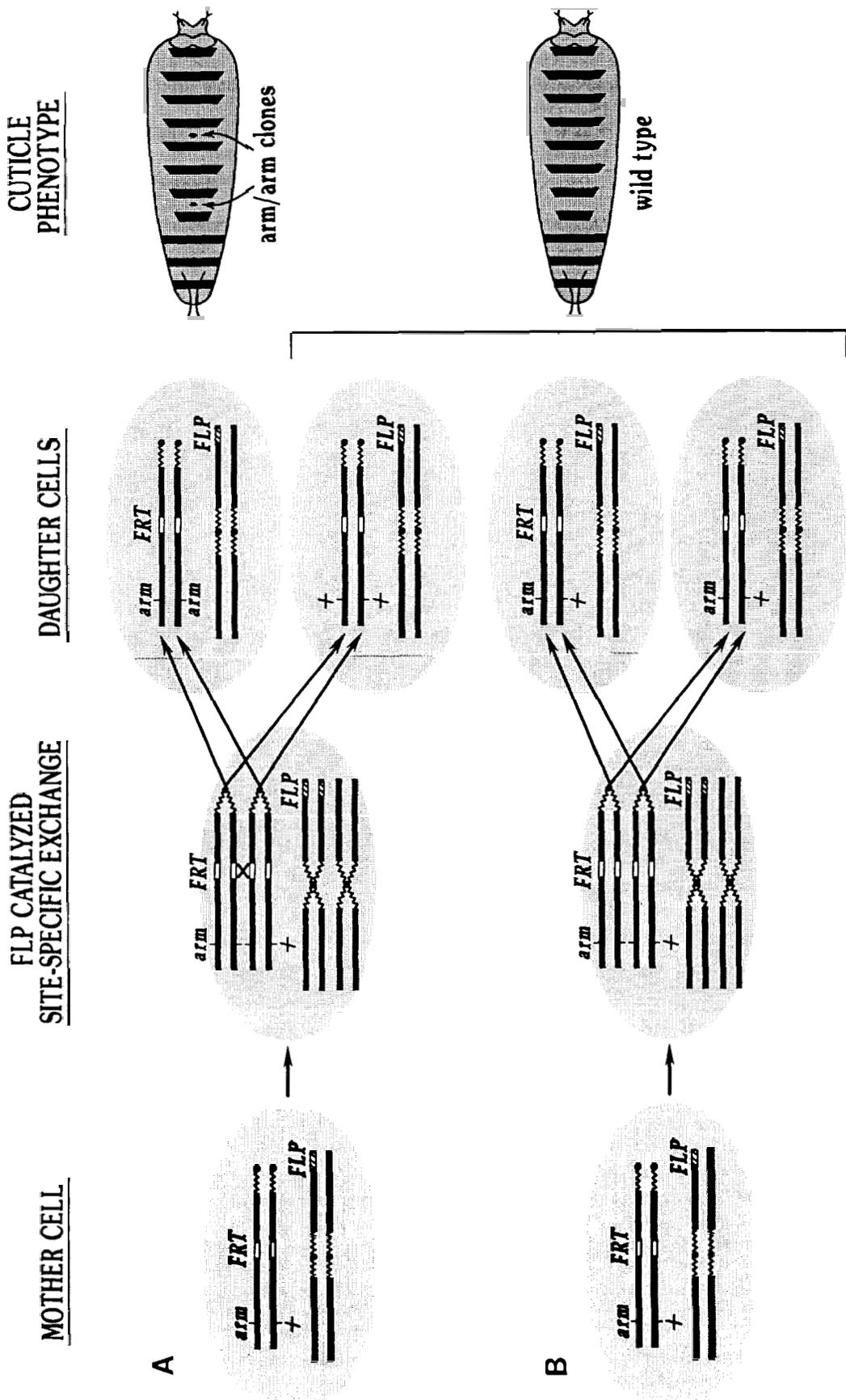
CUTICLE
PHENOTYPE

arm/arm clones

wild type

FLP CATALYZED
SITE-SPECIFIC EXCHANGE

MOTHER CELL

DAUGHTER CELLS

arm FRT

FLP

arm FRT

arm

FLP

+

+

FLP

arm FRT

FLP

+

arm

FLP

+

A

B

Fig. 1. *FLP*-recombinase induced site-specific exchange. **A:** *FLP*-recombinase induced mitotic recombination exchange occurring on the X-chromosome of a cell of genotype *arm FRT^{101}/FRT^{101}; FLP^{38}/+* is shown. Following heat induction, the *hsp70-FLP^{38}* provides the recombinase activity necessary to catalyze the chromosomal exchange at the level of the *FRT* sequences. *FLP* catalyzed recombination results in the recovery of a cell homozygous for *arm*. **B** shows a cell that does not undergo *FLP* catalyzed recombination, resulting in two daughter cells of the same genotype as the mother cell. Nomenclature: *armadillo (arm)* clones are shown as patches of denticles in the naked region of the segment. *FLP*-recombinase target sequences (*FRT*) are depicted as blank boxes and *FLP*-recombinase as stippled boxes. The *FRT* is proximal to *arm* on the X chromosome and the *hsp70-FLP^{38}* is located on the second chromosome.

TABLE 1. Efficiency of Clone Induction as a Function of the Length of Time of the Heat Shock

Length of heat shock (min)	N			%	
	Total	Exp	Mosaics	Mosaics	Defects
Experiment					
30	169	56	7	12.5	1.2
60	294	98	78	80	0.7
120	192	64	72	100*	1.6
180	112	37	39	100*	11.6
Control					
120	134	45	0	0	4.5

*0–6-hour-old embryos, derived from crosses of $FM7c/arm^{XK22} FRT^{101}$; $+/+$ females with FRT^{101}/Y; FLP^{38}/FLP^{38} males, were heat shocked at 37°C for designated times (length of heat shock is shown in minutes). The embryos were allowed to develop until cuticle formation (approximately 24 hr) and their cuticles were prepared for examination. As a control, embryos derived from crosses of $FM7c/arm^{XK22} FRT^{101}$ females with FRT^{101}/Y males were heat shocked at 37°C for 120 min In each experiment, approximately one quarter of the embryos recovered were of the arm mutant phenotype, consistent with the expected fraction to be of the arm/Y; $FLP^{38}/+$ genotype (data not shown). The calculated percentage of mosaic larvae when the heat shock is performed for 120 min and 180 min is higher than 100% due to the method of determining the number of heterozygous animals. These numbers have been corrected to 100% (*). Nomenclature: N total is the number of larvae examined which do not show the arm phenotype. Since there is no independent method of determining the number of $arm^{XK22} FRT^{101}/+$; $+/FLP^{38}$ from their siblings ($FM7c/Y$; $+/FLP^{38}$ and $FM7c FRT^{101}$; $+/FLP^{38}$), the expected number of larvae of genotype $arm^{XK22} FRT^{101}/FRT^{101}$; $FLP^{38}/+$ is calculated as: N exp = N total/3. N mosaics represents the number of larvae with arm somatic clones. The percentage of $arm^{XK22} FRT^{101}/FRT^{101}$; $FLP^{38}/+$ larvae with clones (% mosaics) and the percentage of non-arm embryos with segmentation defects (% defects) due to the heat shock treatment is indicated. The length of heat shock is indicated in minutes (min).

tions lead to the recovery of 100% of mosaicism among the animals of the appropriate genotype. In addition, these parameters generate a minimal percentage of heat-induced segmentation defects.

Somatic clone induction using FLP-recombinase is more efficient than following X-ray treatment. For example, Wieschaus and Riggleman [1987] found, using X-ray induced mitotic recombination, that 49% of the animals with the appropriate genotype are mosaics, with an average of 1 to 2 clones per animal. Using FLP-recombinase at a similar developmental stage we are able to induce mosaics in 100% of the animals with the appropriate genotype, with an average of 4.8 clones per animal. In addition, defects due to the heat treatment are less deleterious to the embryo than X-ray irradiation since, in our experiments, a heat shock treatment of 120 min only induces a minimal percentage of segmentation defects (Table 1).

Clone Size

Cells from the ventral epidermis undergo an average of two to three mitotic divisions following the cellular

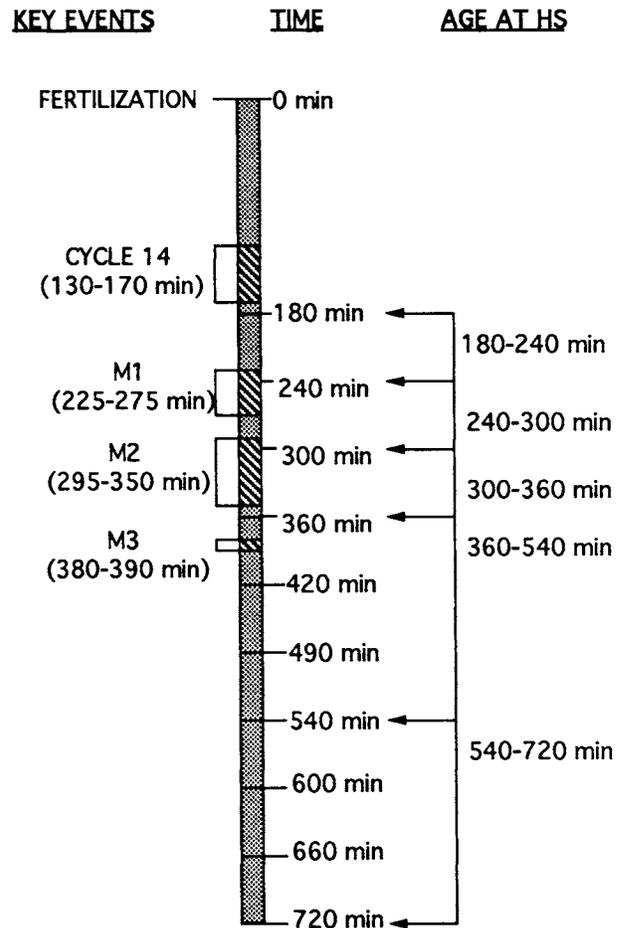Fig 2. Relationship between the age of the embryo at heat shock and the post-blastoderm mitotic events. The chronology of the mitotic events in the early embryo from fertilization to 720 min is depicted. The formation of the cellular blastoderm at 170 min is followed by three post blastoderm mitosis, M1, M2, and M3, that begin at 225 min, 295 min, and 380 min, respectively [Campos-Ortega and Hartenstein, 1985; Foe 1989]. The hatched boxes represent the onset and duration of each mitosis. The times of the embryo collections performed (age at heat shock) are shown in brackets (see also Table 2). Based on the estimation that a single arm epidermal cell gives rise to an average of 2.6 denticles, we expected the average size of arm clones induced during M2 to be 5.2 denticles (clone size of 2 cells) and 10.4 denticles (clone size of 4 cells) for clones induced during M1.

blastoderm stage (Fig. 2) [Campos-Ortega and Hartenstein, 1985]. Although most epidermal cells undergo two mitotic divisions, only a minor fraction is believed to undergo a third division. To determine whether FLP-recombinase can promote mitotic exchange during all postblastoderm mitoses, a detailed analysis of the various clone sizes was conducted (Fig. 2, Table 2).

The clone size is estimated as the number of denticles in a single clone. To determine the average number of denticles secreted by a single arm cell, clones were induced around the last mitotic division at 380 min post-fertilization (M3, Fig. 2). When embryos at ages 360–

TABLE 2. Parameters of *arm* Clonal Analysis*

LEC (min)	Age at HS (min)	N			%		A	
		Total	Exp	Mosaics	Mosaics	Defects	Clones	Denticles
60	180–240	183	61	58	95	4.2	4.2 ± 3	4.5 ± 2.9
60	240–300	218	73	56	77	2.7	4.8 ± 2.7	3.7 ± 2.7
60	300–360	245	82	41	50	3	3.1 ± 1.9	3.9 ± 1.8
180	360–540	203	54	12	17	1.2	1.3 ± .5	2.6 ± .8
180	540–720	173	58	0	0	0	0	0

*Eggs of various ages, derived from the cross of *FM7c/arm*^{XK22} *FRT*^{101}; +/+ females with *FRT*^{101}/*Y*; *FLP*^{38}/*FLP*^{38} males, were heat shocked at 37°C for 120 min. LEC is the length of egg collection in minutes. Age at HS (heat shock) represents the age of the embryos at the time of heat treatment. The average number of *arm* clones per animal (A clones) is calculated as the total number of *arm* clones divided by the total number of larvae with clones (N mosaics). The average number of denticles per clone (A denticles) is the total number of denticles divided by the total number of *arm* clones. The standard deviation is shown for both averages. Details on the total number of *arm* clones as well as the total number of denticles per age groups is shown in Figure 3. See Table 1 for additional nomenclature.

540 min are heat shocked for 120 min (Table 2), the size of the clones ranged from 1.8 to 3.4 denticles with an average of 2.6. The average of 2.6 denticles secreted by a single *arm* cell is in accordance with the results of Wieschaus and Riggleman [1987] who estimated that an epidermal cell gives rise to 3 denticles on average. It is of interest to note that when embryos at ages 540–720 min are heat shocked, no clones are recovered. This is expected since most mitotic division in the ventral epidermis are completed by 390 min.

Three earlier time points were analyzed in order to assess whether *FLP*-recombinase can promote mitotic recombination during the first two postblastoderm divisions (Table 2, Fig. 2). When the heat shock is delivered to embryos at ages 300–360 min, the sizes of the clones ranged from 2.1 to 5.7 denticles with an average of 3.9 denticles; when the heat shock was performed on embryos at ages 240–300 min, the sizes of the clones ranged from 1 to 6.4 denticles with an average of 3.7 denticles; and when the embryos are heat treated at ages 180–240 min, the sizes of the clones ranged from 1.6 to 7.4 with an average of 4.5 denticles. The distribution of the clone sizes is shown in Figure 3.

Clone Frequency

To determine whether the age of the embryos at the time of the heat shock treatment affects the number of clones per animal, the frequency of *arm* clones induced in heat shocked *arm FRT*^{101}/+ *FRT*^{101}; *FLP*^{38}/+ embryos collected at various embryonic stages was determined (Fig. 2). The efficiency of mosaic induction increases when the embryos are heat shocked at younger ages. Few clones are recovered when embryos are heat shocked for 120 min at ages 360 to 540 min (17% of the embryos with the appropriate genotype are mosaics with an average of 1.3 clones per animal), as well as at ages 300–360 min (50% of the embryos of the appropriate genotype are mosaics with an average of 3.1 clones per animal). However, more clones are recovered in embryos heat shocked at ages 240–300 min (77% of the embryos with the appropriate genotype are mosaics

with an average of 4.8 clones per animal) and at ages 180–240 min (95% of the embryos with the appropriate genotype are mosaics with an average of 4.2 clones per animal).

Spatial Distribution of Arm Clones

We were concerned that we may be under-estimating clone sizes due to our inability to detect the full extent of *arm* clones. The size of large *arm* clones which overlap with the segmental denticle band may be under-estimated since wild-type and *arm* denticles are morphologically similar. Additionally, the size of *arm* clones that span the most posterior third region of the naked cuticle may be under-estimated since *arm* clones are not found in this domain due to either cell death or cell transformation [Wieschaus and Riggleman, 1987; Klingensmith et al., 1989].

We examined the distribution of small clones (1 to 4 denticles) induced in embryos heat shocked at ages 180–240 min and 240–300 min (Fig. 4). We reasoned that if these clones represented large clones whose sizes have been under-estimated based upon their position within the segmental unit, they should be preferentially localized either near the denticle belts or near the most posterior region of the naked cuticle of the segment. Results of this analysis, shown in Figure 4, demonstrate that the small clones are not preferentially distributed in the areas that may affect our ability to determine the full size of the *arm* clones. 90% of the clones are in the central part of the naked region (between 20 and 80% in relative distance from the anterior and posterior denticle belts). Examples of the position of some of these clones are shown in Figure 5.

The Mode of Action of *FLP*-Recombinase

Our mosaic analyses demonstrate that the efficiency of mosaic induction increases when the embryos are heat shocked at younger stages. As expected, since only a minor fraction of epidermal cells undergo a third postblastoderm division [Campos-Ortega and Hartenstein, 1985], we recovered more clones in animals un-
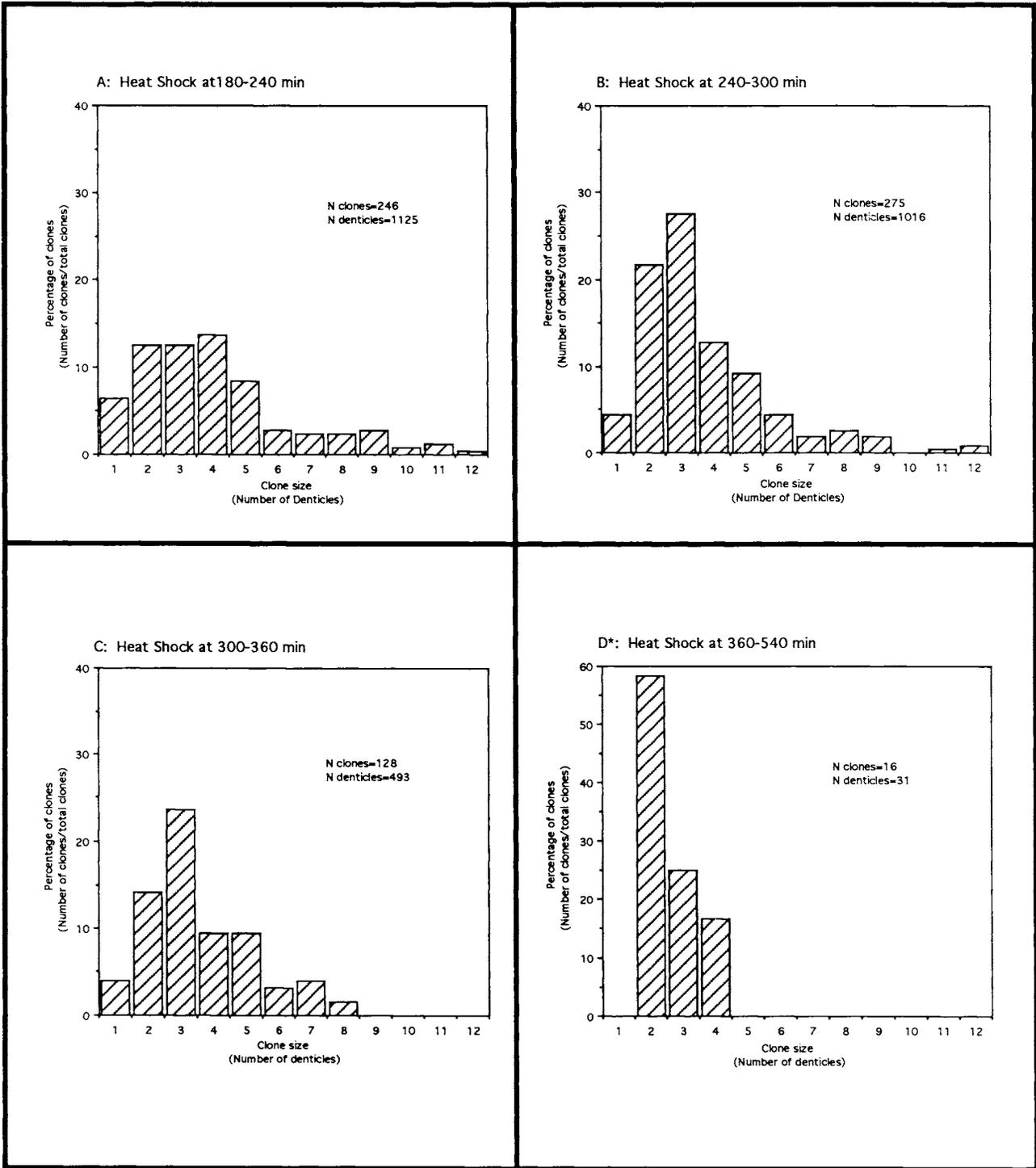
Fig. 3. Distribution of *arm* clones. The percentage of clones (number of clones with a specific clone size/total number of clones) with various clone sizes, estimated by their number of denticles, is depicted at four heat shock time points. N clones is the total number of clones recovered and N denticles is the total number of denticles recovered. These numbers were used to calculate the values of A clones and A denticles shown in Table 2.

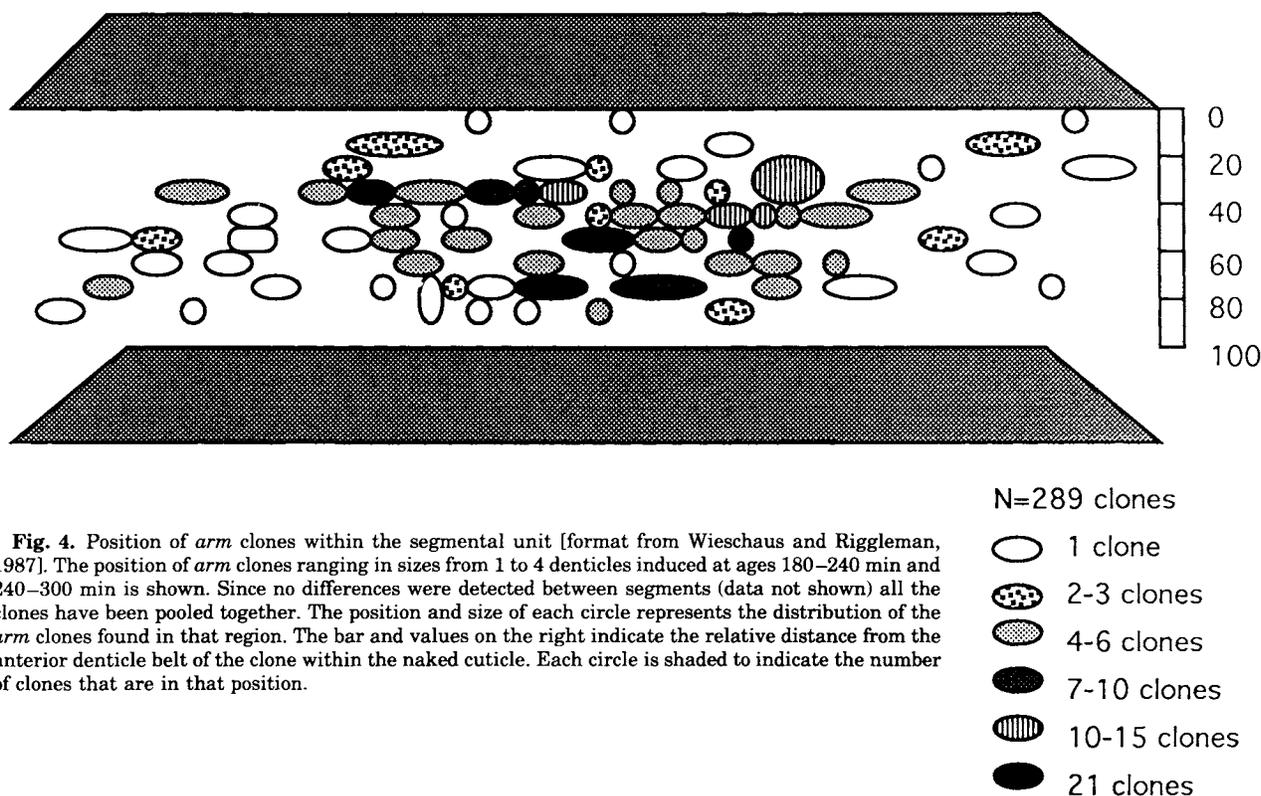*Graph D has a greater Y-axis range than the other graphs.

Fig. 4. Position of *arm* clones within the segmental unit [format from Wieschaus and Riggleman, 1987]. The position of *arm* clones ranging in sizes from 1 to 4 denticles induced at ages 180–240 min and 240–300 min is shown. Since no differences were detected between segments (data not shown) all the clones have been pooled together. The position and size of each circle represents the distribution of the *arm* clones found in that region. The bar and values on the right indicate the relative distance from the anterior denticle belt of the clone within the naked cuticle. Each circle is shaded to indicate the number of clones that are in that position.

N=289 clones

◯ 1 clone

⬮ 2-3 clones

⬯ 4-6 clones

⬤ 7-10 clones

⬛ 10-15 clones

⬤ 21 clones

dergoing M1 and M2 than during M3. In addition, we found that more *arm* clones are recovered when the heat shock is performed during M1 than during M2. This observation is unexpected because more cells divide during M2 than M1 [Campos-Ortega and Hartenstein, 1985]. This result suggests that *FLP*-recombinase, induced under our heat shock conditions, can exert its effect through more than one postblastoderm mitosis. In this case the size of a clone will represent the additive effect of clones induced during multiple mitoses. Heat shocking embryos at ages 180–240 min and 240–300 min may lead to the recovery of clones induced not only in M1 but also M2 and M3. Similarly, heat shocks performed in embryos at ages 300–360 min may represent the additive effect of clones induced during M2 and M3.

Estimation of the Total Number of Mitotic Clones

The *arm* marker we utilized to identify the somatic clones only allows us to detect clones in a small fraction of the ventral larval epidermis. The abdominal segments are composed of approximately 90 epidermal precursor cells, half of which secrete the denticle belts [Campos-Ortega and Hartenstein, 1985; Bejsovec and Martinez-Arias, 1991]. Since *arm* clones cannot be detected in the most anterior half of the segmental domain [approximately 6 cells in length; Bejsovec and Martinez-Arias, 1991] which is covered with denticles

and the most posterior one third of the naked region [approximately 2 cells in length; Wieschaus and Riggleman, 1987; Klingensmith *et al.*, 1989], we estimate that there are only 210 epidermal cells (1/3 × 90 × 7) per embryo where *arm* clones can be found. In this calculation, 7 represents the abdominal regions of the ventral epidermis where *arm* clones were scored in our analysis (see Materials and Methods). The average number of clones per animal out of these 210 scorable cells is approximately 4.5 or 2.1% (Table 2). Extrapolation to the 6,000 cells present at the cellular blastoderm stage indicates that in the order of 126 cells (2.1% of 6,000) per blastoderm may undergo a site-specific mitotic exchange.

Extension of the Technique

As described in the Introduction, the use of mosaics to determine the effects and cell autonomy of mutations affecting embryonic patterning has been poorly investigated. This is due to limitations in the efficiency of generating mosaics, and the paucity of embryonic markers available. In this paper we have shown that *FLP*-recombinase can be used to solve the first problem. The second problem, however, still needs work. The detection of clones using *arm* is restricted to a small region of the cuticle and is not appropriate to detect clones of homozygous cells in internal tissues. One method to identify clones in all embryonic cells is to use markers that ubiquitously stain every cell in the
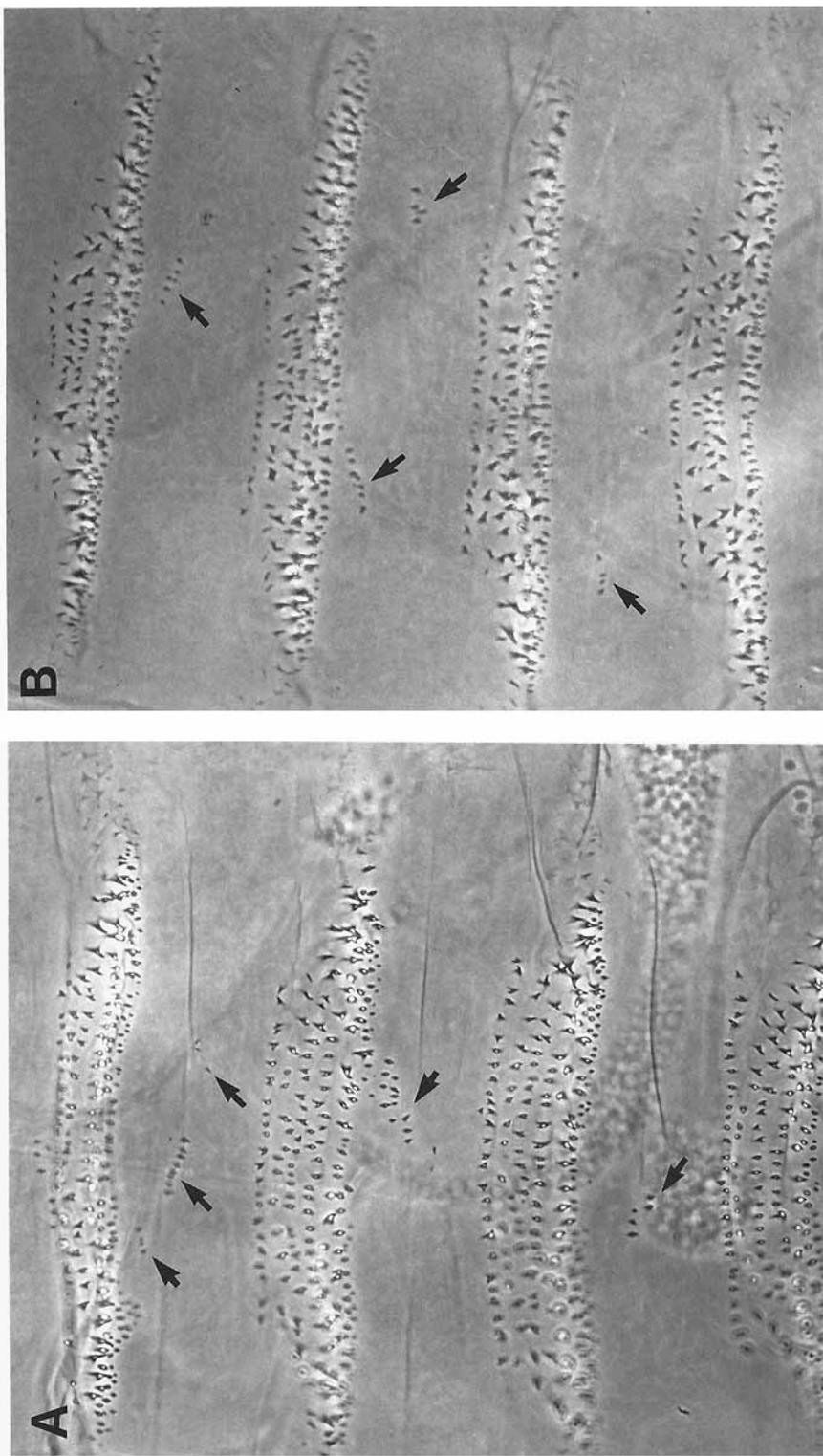
Fig. 5. Example of *arm* clones. The cuticle of the embryo shown in **A** possesses at least 5 individual *arm* clones in a three segment interval and the embryo in **B** exhibits at least 4 clones. Clones are indicated by arrows.

embryo. For example, to detect homozygous somatic clones, one could take advantage of the *Sex-lethal* gene (*Sxl*). *Sxl* protein is ubiquitously expressed in female embryos but is not expressed in either male or *diplo-X* embryos carrying a null *Sxl* mutation (*Sxl^0*) [Bopp et al., 1991]. In this scheme, a lethal mutation (*l*), located distally to *Sxl^0* and the *FRT* element, is crossed with a strain that carries the same *FRT* element and an autosomal *FLP*-recombinase gene. Following an appropriate heat shock treatment, a mitotic exchange occurring at the level of the *FRT* element in these *l Sxl^0 FRT/FRT*; +/*FLP* animals, will yield cells homozygous for both *l* and *Sxl^0* mutations. *l/l* somatic clones can thus be identified by loss of *Sxl* protein expression. An alternative method is to use enhancer trap strains, *P[lacZ]*, that ubiquitously express β-galactosidase. In this scheme, flies that carry a *l* mutation located distally to the *FRT*, are crossed with flies that carry a *P[lacZ]* inserted distally to the *FRT*. *l/l* somatic clones, induced following a heat shock treatment in *l FRT/ P[lacZ] FRT*; +/*FLP* animals, can then be identified by loss of β-galactosidase staining.

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