Simple and efficient generation of marked clones in *Drosophila*

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Background: Cell lineage analysis and mosaic analysis of mutations are important techniques that are used to study the development of many organisms. Unfortunately, the methods employed for such analyses are usually inefficient, technically demanding or labor intensive. In *Drosophila*, the most common methodology used for the generation of mosaic animals is mitotic recombination, which is induced by X-rays. Although this technique is simple, it has the undesirable characteristics of a low efficiency and a high rate of cell death. Furthermore, although a large number of marker systems has been employed to detect mitotic recombinants, none allows easy identification of clones for all cell types.

Results: A system is described here that allows a highly efficient generation of clones with the concomitant expression of an easily detectable cellular marker. This method can be applied to cell lineage and mosaic analysis in *Drosophila*. The site-specific yeast FLP recombinase, under the control of a heat shock-inducible promoter, efficiently catalyses mitotic recombination specifically at the site of a FLP recombination target (FRT). In this system, recombination fuses the α-tubulin promoter to the *lacZ* gene, allowing transcription of the marker. Recombinant cells and their progeny can, therefore, be detected by standard assays for β-galactosidase. Of particular importance is the fact that only the cells of interest stain, thus allowing their simple detection in any tissue.

Conclusions: We demonstrate that, by intermolecular recombination, we can use FLP recombinase to generate marked clones efficiently in embryonic, larval and adult tissues. This simple and efficient technique is well suited to cell-lineage analysis and can be easily extended to the generation and detection of mutant clones in mosaic animals.

Current Biology 1993, **3**:424–433

Background

The ability to trace cellular lineages is critical to the study of many developmental phenomena. Analysis of cell lineages allows us to determine the number of cells that give rise to a given structure, the rates at which particular cell populations grow, and the origin of the various cell types that form a tissue in the mature animal. Unlike in *Caenorhabditis elegans*, where the origin and destiny of all cells can be easily visualized [1], the cellular lineages of most animals cannot be readily analysed. Many techniques have therefore been developed to make lineage analysis feasible in other organisms [2,3]. A straightforward approach that is used in many animals is the direct injection of high molecular weight dyes or histochemical markers into single cells of the embryo [4–6]. Another method that has been used is the generation of chimeric animals, such as chick–quail hybrids [7]. An elegant technique currently used in some vertebrates involves injection of replication-defective, histochemically-detectable retroviral vectors into specific regions of the developing animal [8,9]. These approaches have led to significant discoveries, such as observations indicating that the specific fates of most vertebrate neural precursors are undetermined before the last mitotic division [10–12] and that neural precursors are able to migrate over large distances [13].

In *Drosophila*, lineage analysis has been accomplished primarily through the generation of mosaic animals by genetic techniques. Cell lineage has been most extensively studied in the embryonic and adult epidermis, where a number of useful visible markers can be used to detect clones [3,14]. Animals mosaic for these markers have been generated by a variety of techniques, including nuclear transplantations and genetically-induced loss of chromosomes [3,15,16]. However, the technique most often used to induce mosaics in *Drosophila* is X-ray mitotic recombination. To generate recombination, an animal heterozygous for a recessive marker is exposed to X rays during development. X-irradiation induces chromosome breaks, which are sometimes repaired in such a way as to exchange arms between homologous chromosomes. As a consequence, one of the daughter cells arising from the recombinant mother will be homozygous for the marker, thus resulting in patches of mutant tissue in a wild-type animal. It is by using such techniques that *Drosophila* fate maps were generated and the 'compartment hypothesis' was formulated [17–19].

There are two major problems with the use of X-irradiation for clonal induction. The typical dose of X-rays used in such experiments gives a low rate of clone production and a high rate (40–60%) of cell lethality [20]. Recently, these problems have been overcome by using two different strategies that exploit the site-specific yeast recombinase, FLP. The first strategy uses mitotic exchange in much the same manner as X ray-induced recombination [21–24]. Two homologous

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chromosomes carry a FLP recombination target site (FRT) at the same chromosomal position, with a mutation on one homologue. FLP recombinase is then expressed under heat shock control and catalyzes the exchange of the chromosome arms specifically at the FRTs, generating a patch of mutant tissue in an otherwise wild-type animal. A second strategy that could also be used for lineage analysis involves intramolecular recombination rather than chromosome exchange [25]. In the 'flp-out' technique, a constitutive promoter is fused to the *lac Z* gene that contains at the 5′ end a transcriptional stop sequence flanked by FRTs. Expression of FLP recombinase under heat shock control results in the specific excision of the transcriptional stop sequence by recombination occuring between the FRTs. This results in heritable activation of *lac Z* in the recombinant cell lineage. These two FLP-mediated systems avoid the drawbacks associated with X-rays. The efficiency of FLP recombination is at least an order of magnitude greater than X-rays and cell lethality is avoided because recombinase is expressed by heat shock, which does not affect cell viability.

By extension of the mitotic exchange technique, it is possible to create mosaic animals in which only some cells are mutant for a gene of interest. Such an animal can be used to study the autonomy of gene function, thus providing clues to the mechanism by which a gene acts. For example, *engrailed* mutant cells exhibit cell autonomy [26], whereas *wingless* mutant cells are non-autonomous [27]. This is consistent with the molecular nature of engrailed protein as a transcription factor and wingless protein as a secreted signalling molecule [28]. Additionally, mosaics can be useful in analysing the tissue-specific effects of zygotic lethal mutations as it is possible to generate animals in which some tissues are homozygous for the lethal mutation. Mosaics allow the analysis of the function of a gene product beyond the stage in development when a whole animal of that genotype would die. In this manner, it has been possible to examine the roles of many essential proteins in later developmental processes. For example, this methodology uncovered the maternal role of the *Drosophila raf* kinase in the establishment of embryonic terminal structures [29].

Although the cuticle markers described above have been useful in the analysis of epidermis, they cannot be employed for other tissues. Enzymatic markers, such as aldehyde oxidase, succinate dehydrogenase and β-galactosidase, have been more generally used [30–32]. In conjunction with mitotic exchange, enzymatic markers have been used to create and mark mosaics in the internal tissues of the fly. However, additional complexity is introduced because the marker is heterozygous in the animal before mitotic recombination. The result is that the entire animal, except for the clone, stains positively for the marker. Consequently, the cells of interest are unstained within a background of heterozygous cells that do stain. Although this is of relatively minor consequence in analysis of unicellular layers, it has made analysis of internal tissues very difficult [31].

Our goal was to develop a technique that uses efficient mitotic exchange to label clones heritably with an easily detectable marker, in a background that does not express the reporter. In such a system, there would be no staining for the marker in heterozygous cells; therefore clones would be detected by the generation of staining rather than by the loss of it, thus facilitating analysis of both internal and external tissues. The technique should be applicable to both cell-lineage analysis and mosaic analysis of mutations. We describe such a system that uses highly efficient, site-specific mitotic exchange catalysed by the FLP recombinase to reactivate the expression of the *lacZ* gene by placing it downstream of the α-tubulin promoter. We demonstrate that this system can be applied to cell lineage analysis and describe how it can be extended to the mosaic analysis of most mutations.

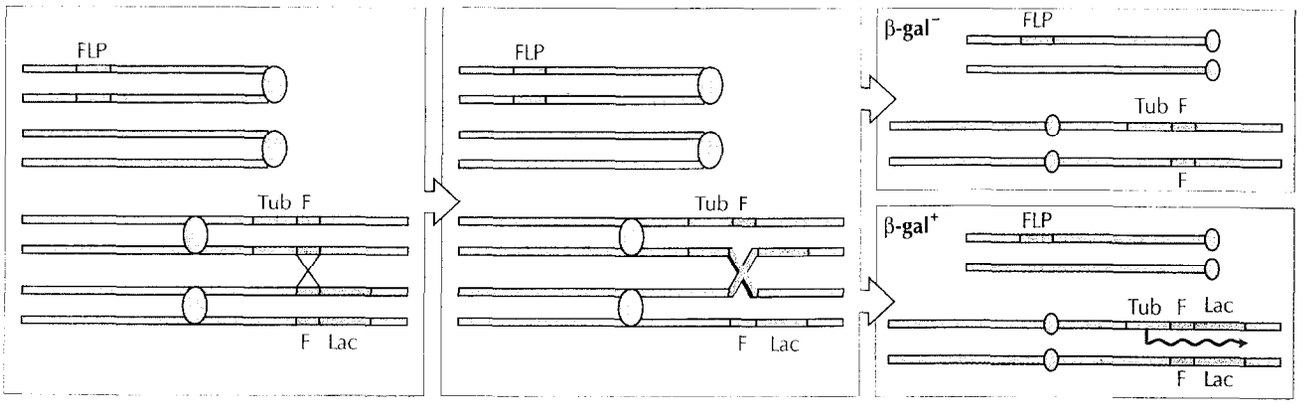

Fig. 1. Summary of chromosome segregation associated with FLP-mediated mitotic exchange at 37°C. Chromosomes of mother cell (left) are shown after DNA synthesis, directly preceding cell division. Chromosomes of daughter cells (right) are shown with the normal diploid content following mitosis. β-galactosidase expression is shown in blue. Tub, α-tubulin promoter; F, FLP recombination target (FRT); Lac, *lacZ*-coding region; FLP, site-specific FLP recombinase gene.

Results

Experimental design

A method is described here that overcomes many of the limitations and difficulties of previous techniques that have been employed in cell lineage analysis and mosaic analysis of mutations. Simultaneously, clones are induced and cells are heritably marked. In this procedure, summarized in Figure 1, the *lacZ* gene is activated to produce β-galactosidase by its juxtaposition with the promoter region for the *Drosophila* α-tubulin gene as a consequence of FLP-mediated recombination. The α-tubulin promoter was chosen for its strong expression in a wide range of cell types (KH O'Donnell, C-T Chen and PC Wensink, unpublished data). β-galactosidase is an excellent reporter because it is stable, present in a low abundance in the background in flies, and easy to detect using either histochemical reactions or commercially available antibodies that are raised to the protein. In addition, the *lacZ* gene was tagged with a sequence encoding the SV40 nuclear localization signal in order to restrict the reporter to the nucleus, thus facilitating the identification of stained cells.

To fuse the promoter to the reporter efficiently, mitotic recombination mediated by the yeast site-specific recombinase, FLP, is employed. Previous work has shown that FLP can induce recombination with much greater frequency and viability than X-irradiation [21–23,33]. The promoter region flanked on the 3′ side by a FLP target site (FRT) is introduced on one chromosome, whereas the *lacZ* gene flanked by an FRT at the 5′ end is introduced on the homologous chromosome. The hsp70-driven FLP recombinase is then expressed following heat shock and catalyses recombination between the two homologous chromosomes at the FRT. This brings the α-tubulin promoter upstream of the *lacZ* gene and results in the expression of β-galactosidase in all cells derived from that recombinant.

To ensure efficient recombination and avoid the generation of large chromosomal duplications or deletions, it is necessary to place the promoter and reporter gene constructs in exactly the same chromosomal position. This allows maximal chromosomal pairing around the FRT, which is a prerequisite for efficient mitotic recombination. Unfortunately, site-directed homologous DNA insertion is technically difficult in *Drosophila* [34]. To circumvent this problem, it was necessary to make, and insert, a transposon construct that contains both the promoter and reporter elements, separated by an FRT. Following chromosomal integration, portions of the transposon were deleted *in vivo* to produce one chromosome with only the promoter/FRT and another with only the FRT/*lacZ*. These deletions were made using P element transposase, by introducing the original integrated chromosome into the *Δ2-3* strain [35] to generate excisions of the transposon. P element excision is often imprecise and can result in the removal of only part of the transposon. In the original plasmid, the promoter/ FRT/ reporter cassette was flanked by the *yellow* gene on one side and the *white* gene on the other (Fig. 2a). These markers allow the detection of potentially useful imprecise excision events simply by selecting the resulting progeny that lack either *white* or *yellow* gene products, but not both (Fig. 2b).

The complete construct described above, pCXY21, was introduced into flies mutant for *yellow* and *white*. Nine independent y^+ w^+ transformant lines were recovered and stained for β-galactosidase activity. A single line, X15, was chosen for its consistently strong staining and used in the experiments described below. Examination revealed that staining begins at gastrulation and becomes more intense throughout embryogenesis. All tissues examined, except nervous system tissue, stained strongly and uniformly (data not shown).

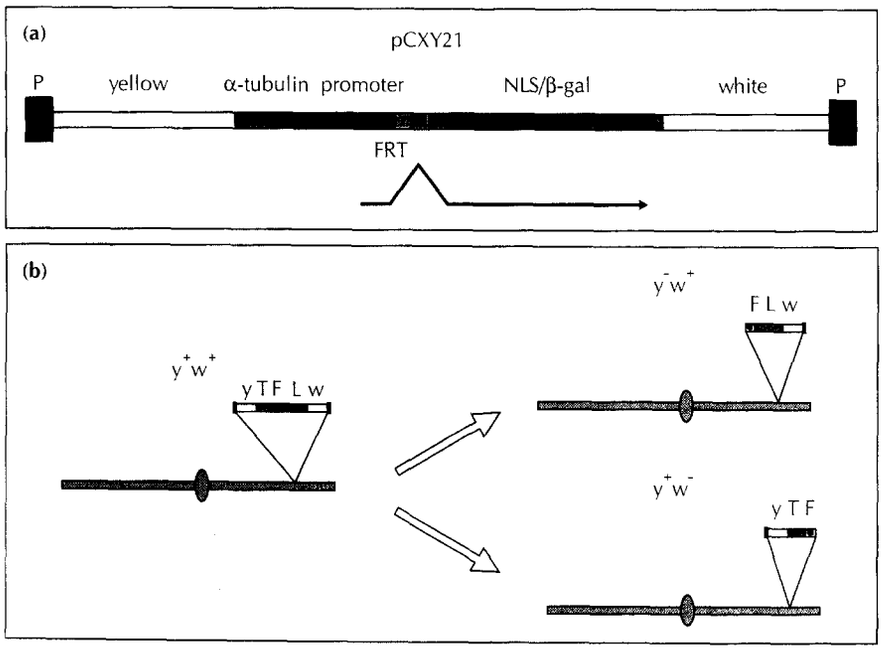

Fig. 2. Generation of chromosomes. **(a)** Shows the general structure of the pCXY21 P element plasmid. The resulting fusion transcript, including the FRT-containing intron, is shown below. (See Materials and methods for a detailed description of this construction.) **(b)** Shows the imprecise excision events that generate the two classes of chromosomes necessary for the recovery of marked clones using the strategy described in Figure 1. The parental X15 insertion chromosome, which carries both the *yellow* and *white* genes, is drawn to the left and two types of imprecise excision (y^+ w^- and y^- w^+) are represented on the right. T, α-tubulin promoter; F, FRT; L, lacZ-coding region; y, *yellow* gene; w, *white* gene.

Similar staining was detected in larval epidermis, salivary glands, gut and gonadal tissues. Non-uniform staining was seen in the larval brain and imaginal discs. Only ovarian tissue has been examined in adults, and both follicle and germ cells exhibit strong staining throughout the ovary. Localization of this insertion revealed that the transposon is at polytene map position 60B near the tip of the right arm of the second chromosome.

To generate partial deletions of the P element, males were generated that carried both the X15 chromosome and the $\Delta 2\text{-}3$ *Sb* chromosome. 200 such males were mated individually to 3 $y^- w^-$ virgin females each. 36 independent $y^+ w^-$ or $y^- w^+$ progeny were recovered. Stocks were made and tested for β-galactosidase activity and for presence of the FRT sequence. Ideally, a line should have no detectable reporter activity but must retain the FRT sequence for subsequent recombination. Embryos from each of the excision lines were tested for marker expression using anti-β-galactosidase antibodies. There were 29 $y^- w^+$ lines, 27 of which did not stain for β-galactosidase activity. Genomic DNA from each line that did not stain was then subjected to PCR using a primer within the FRT site. Only two of the 27 lines also contained the FRT. One of these (X15-29) was used in the experiments described below. Only 7 $y^+ w^-$ lines were recovered, none of which stained for β-galactosidase. Two of these 7 lines, X15-4a and X15-33, retained the FRT and both were used as described below. To generate animals in which the promoter/reporter is reconstituted by recombination, a source of heat shock-inducible FLP recombinase was introduced into animals that carried complementary, partially deleted transposons on each of its second chromosomes (that is, $y^+ w^-/y^- w^+$ flies). Animals of this genotype are referred to as 'recombination competent'.

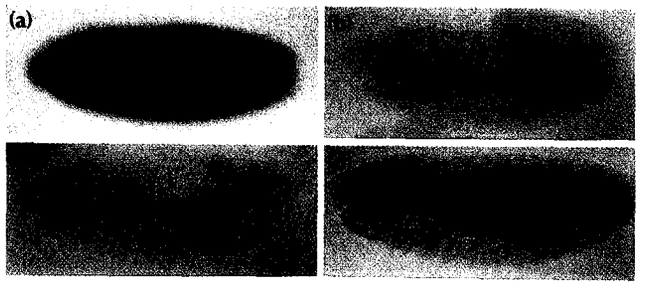

Fig. 3. Embryonic clones. FLP recombinase was expressed using heat shock at 3–4 hours after egg deposition in recombination competent embryos. Animals were aged and β-galactosidase was detected using antibodies conjugated to horseradish peroxidase. Stained clones appear brown. Animals are oriented with anterior to the left and dorsal to the top. **(a)** A stage 14 embryo [36] carrying the parental X15 chromosome before imprecise excisions were made. The animal stains strongly throughout, except for the nervous system. **(b)** Recombination competent stage 12 embryo heat shocked for 20 minutes. One two-cell clone and one four-cell clone are visible in the plane of focus at the anterior of the embryo. **(c)** and **(d)** Recombination competent stage 14 embryos heat shocked for 60 minutes: the embryo in (c) shows several clones from one to four cells in size; the embryo in (d) shows more than 100 clones of varying size.

Generation of clones in the embryo

The nuclei of the *Drosophila* embryo undergo 14 cycles of rapid synchronous division during the first three hours (at 25°C) after fertilization. At cycle 14, membranes form between the nuclei, and the blastoderm is cellularized. It is at the time of cellularization that most of the early zygotic gene expression begins. Subsequent to cycle 14, three slower, asynchronous post-blastoderm cell divisions occur in most of the embryonic tissues. These mitoses are completed approximately 3.5 hours after blastoderm cellularization [36,37]. Accordingly, recombination competent embryos were heat shocked to induce the expression of the FLP recombinase immediately following cellularization. Embryos were aged to 3–4 hours, then heat shocked at 37°C for 20, 40 or 60 minutes, and allowed to recover from heat shock for approximately 16 hours at 16°C. This protocol allowed sufficient time for the embryos to progress through all three of the post-blastoderm divisions, thus resulting in the largest possible clone size. These embryos were subsequently fixed and immunostained for the presence of β-galactosidase.

As can be seen in Figure 3, FLP-mediated recombination can be induced in embryos with as little as 20 minutes heat shock; shorter heat shocks were not done. Although there was large variability in the number of clones induced per embryo for a given heat shock duration, it was observed that longer heat shocks produced more clones per embryo. Many embryos heat shocked for 20 minutes produced only one or two clones (Fig. 3b); however, other embryos contained more than 40 clones. The same wide range of clone production was seen in the embryos heat shocked for 60 minutes. In that experiment, there were as few as two and as many as several hundred clones (Fig. 3d) per embryo.

The size of the induced clones was also variable within a single embryo. Because the FLP recombinase was expressed between the time of the fourteenth synchronous nuclear division and the first post-blastoderm division, it would be expected that clones induced at that time would have undergone three divisions. As the first of the divisions results in only one daughter that expresses β-galactosidase, the expected clone size would be four cells. In this experiment, clones of one to eight β-galactosidase-expressing cells can be detected within any given embryo, presumably indicating that not all recombination events are occurring at the time when FLP expression is induced. This is similar to results seen elsewhere [23] suggesting that the FLP recombinase is relatively stable in the embryo and persists throughout later post-blastoderm divisions to catalyse recombination beyond the time of heat shock. Persistence of FLP recombinase would explain the presence of the one and two cell clones seen in the experimental embryos.

The eight-cell clones observed may arise because some of the embryos in a collection were slightly lagging

developmentally and were therefore heat shocked before nuclear cycle 14. This hypothesis is supported by previous observations that expression from the heat-shock promoter can be detected before cellularization [38]. Alternatively, these clones may be representative of a small number of embryonic cells that normally divide more than the usual three times after cellularization [37]. Finally, it is possible that FLP-mediated recombination may be occurring before DNA replication in the cell cycle. If this has occurred, then both daughter cells would express β-galactosidase, thus giving rise to an eight-cell clone after three post-blastoderm divisions. This last alternative seems unlikely because embryos heat shocked after the third post-blastoderm division did not produce detectable β-galactosidase-expressing cells (data not shown), suggesting that perhaps diploid embryonic cells cannot undergo FLP-mediated recombination.

Generation of clones in ovaries

To establish the utility of this technique further, clonal induction was examined in ovaries. Adult females have two ovaries that consist of approximately 20 ovarioles each (for review, see [39]). An ovariole consists of a chain of developing egg chambers with the germarium at the apical tip, a structure that contains two or three germline stem cells [40] and approximately 500 somatically-derived pre-follicle cells. Germ cells are derived from a small population of cells that is set aside during embryogenesis and divides throughout larval development. In the adult, a germline stem cell divides once to give rise to another stem cell and a cystoblast. This cystoblast then divides incompletely four times to generate a cyst of 16 cells, one of which will become the oocyte while 15 will become nurse cells; the later will produce many of the molecules necessary for the developing oocyte. The cyst is associated with 60–80 of the pre-follicle cells and leaves the germarium. These follicle cells then divide until approximately 1100 follicle cells cover the egg chamber. As the oocyte begins to increase in volume, most of the follicle cells migrate posteriorly to lie over the oocyte and subsequently begin yolk uptake, secrete the extra-embryonic membranes and finally degenerate. Specialized sub-populations of follicle cells are designated to generate structures necessary for fertilization and respiration.

To generate clonal patches in ovarian cells, recombination-competent animals were heat shocked for varying times during first and third larval instars, or as adults (Figures 4, 5 and 6, respectively). Ovaries were then dissected from adults, fixed and stained for β-galactosidase activity. After staining, ovarioles were teased apart and mounted for examination. At all stages, this procedure resulted in the production of clones in both follicle cells and nurse cells. The relative proportions of

Table 1. Frequency of ovarian clone induction.

(a) Numbers of ovarioles

Stage at heat shock	Total	Stained	% Stained	Follicle cell clones	Germ cell clones
First larval instar	240	96	40	92	5
Third larval instar	172	47	27	9	40

(b) Numbers of egg chambers per staining ovariole

Stage at heat shock	Average % egg chambers with follicle cell clones	Average % egg chambers with germ cell clones
First larval instar	76	33
Third larval instar	32	28

(a) Summary of the frequency of ovarioles that possess marked clones. Clones can be found in both follicle cells and germ cells. The efficiency of clonal induction was determined as the proportion of ovarioles stained relative to the total number of ovarioles examined. Although the efficiencies of mosaic production are consistently quite high (40% at the first instar and 27% at the third instar), the target cell population for recombination is changed dramatically with the time of induction. This difference is reflected in the number of clones in follicle versus germ cells at the two induction times. There is approximately a 20-fold preference for follicle cell clones at the first instar induction and a 4.4-fold preference for germline clones at the third instar inductions, thus these tissues undergo an 80-fold change in relative suitability to undergo FLP recombination. **(b)** Summary of the clonal induction seen within single ovarioles. Among ovarioles with follicle cell clones from the first instar inductions, a large proportion (76%) of the egg chambers on an individual ovariole contained clones. Among the ovarioles with follicle cell clones induced at the third instar, an average of 32% of the egg chambers on a given ovariole contained β-galactosidase staining cells. Consistent with expectations, this seems to indicate that the recombination event has occurred much later in pre-follicle cell development than the first instar clones and reflects the proliferation of the pre-follicle cells that occurs between the first and the third larval instar. The percentage of cysts on a given ovariole that contain germ cell clones is approximately the same for either the first or the third instar inductions (33% and 28%, respectively). This result is expected because the germline-derived components of each egg chamber arise from stem cell divisions. Clonal induction during larval stages would occur while the stem cell population is still being produced. In the resulting adults, the nature of stem cell division ensures that the number of marked stem cells remains constant, and so, therefore, should the rate of production of marked eggs. Production of ovarioles, in which approximately one-third of the egg chambers are marked, fits well with the data that each ovariole contains 2 or 3 functioning stem cells [40].

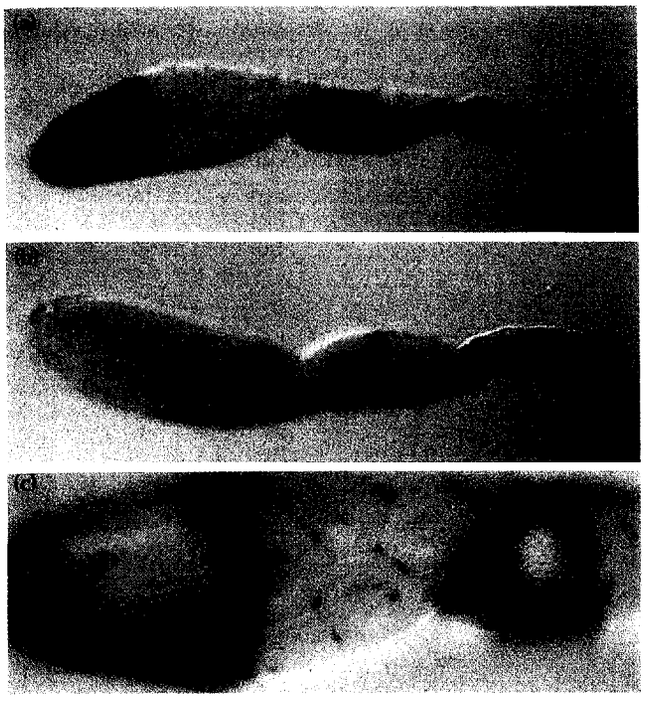

Fig. 4. Ovarian clones induced at first larval instar. FLP recombinase was expressed using heat shock in recombination competent animals at the first larval instar. Ovaries were dissected from adults and stained for β-galactosidase activity that appears blue. **(a)** An ovariole in which all follicle cells stain for β-galactosidase. **(b)** An ovariole in which a subset of follicle cells in each egg chamber are derived from β-galactosidase-positive clones. **(c)** A higher magnification of another ovariole that shows clonal patches in each egg chamber.

each, as well as the size and frequency of clones, were dependent on the developmental stage of the animal during heat shock (Table 1).

The overall rate of clonal induction, as assayed by staining in adult ovaries, was quite high. Individual ovarioles were scored as units, as the egg chambers of each ovariole are derived from a common pool of stem cells in the germarium. A single 1 hour heat shock at the first larval instar stage produced clones in 40% of the ovarioles examined, and at the third larval instar stage resulted in 27% stained ovarioles (for a summary of clones, see Table 1). As each female contains approximately 20–25 ovarioles per ovary, virtually all animals competent for FLP recombination carried clones. In animals heat shocked at first instar, the resultant clones were primarily found in follicle cells, whereas most ovarian clones from animals heat shocked at third instar were in the germline. When compared, there is an 80-fold relative difference in the target cell preference for recombination between the two developmental stages. As pairing of homologous chromosomes is necessary for recombination, it may be that germ cells are less active mitotically at first instar. Alternatively, perhaps the hsp70 promoter-driven recombinase is not expressed at high levels in the germline at the first larval instar.

As oogenesis is an ongoing process, it is also possible to generate clones in adults. Recombination competent females were heat shocked as adults and then dissected, fixed and stained for β-galactosidase activity 1–10 days after heat shock. Within 1–2 days of heat shock, clones of one or two cells were recovered in follicle cells. 3–4 days after heat shock, clones of two to four cells were recovered in follicle cells, whereas one- or two-cell clones were found in the germline (Fig. 6). Clones found at later times post-heat shock are larger, as would be expected. In general, the recovered clone sizes fit well with the estimated division time of 24–48 hours for follicle and germ cells.

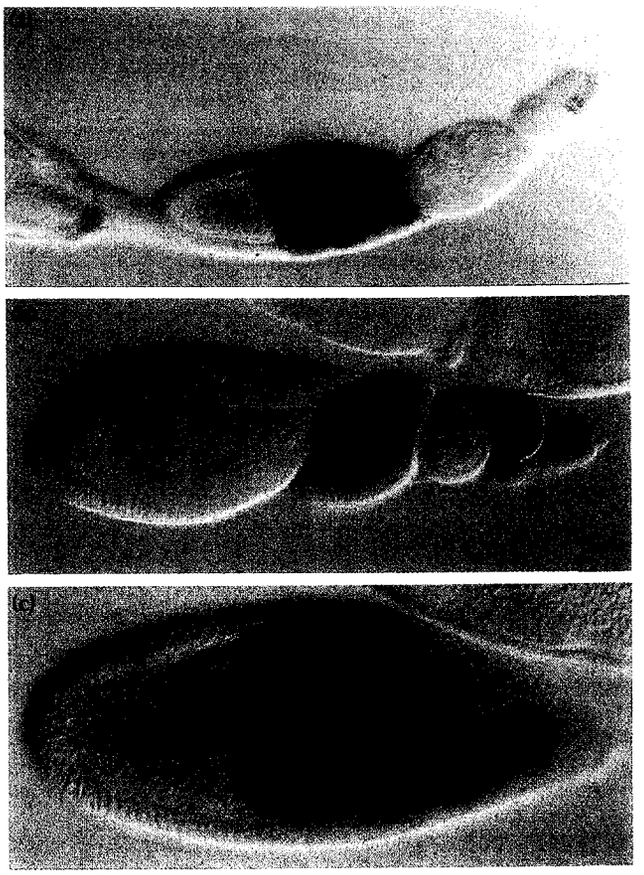

Fig. 5. Ovarian clones induced at the third larval instar. Clones were induced by heat shock of recombination competent third instar larvae and stained as in Figure 4. **(a)** An ovariole with germ cell clones in two egg chambers and in the germarium. **(b)** Staining of germ cell clones in 3 egg chambers, as well as staining in the germarium. **(c)** A higher magnification of an egg chamber in which the 16 germ cells are all clonally-derived from a β-galactosidase-positive recombinant.

Discussion

Using the site-specificity of the yeast FLP recombinase, it has been possible to develop a system that couples highly efficient mitotic exchange with a simultaneous activation of a cell autonomous marker. The α-tubulin promoter, adjacent to and 5′ of an FRT element, is placed on one chromosome, whereas the β-galactosidase coding region, adjacent to and 3′ of an FRT, is placed in the same position on the homologous

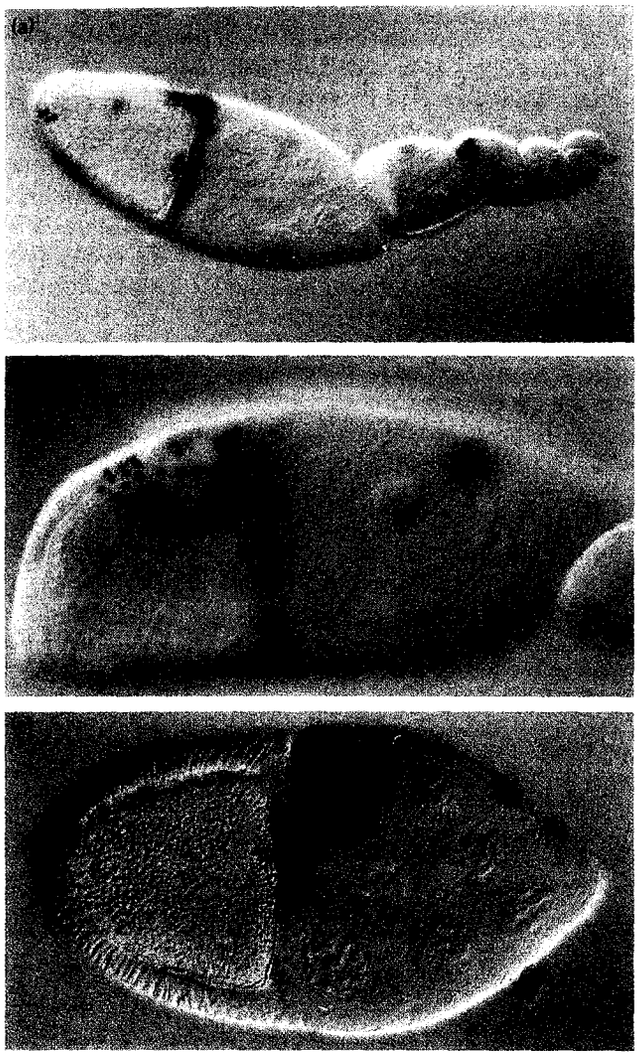

Fig. 6. Ovarian clones induced in adults. Clones were induced by heat shock of recombination competent adults. 3–4 days after heat shock, animals were stained as previously described. **(a)** An ovariole with both follicle cell and germ cell clones. Several follicle cell clones that contain two and four cells, can be seen in the oldest chamber, whereas clones of single germ cells can be seen in two of the youngest egg chambers. **(b)** A higher magnification of a chamber showing a single germ cell clone as well as a follicle cell clone or clones. **(c)** A higher magnification of a chamber with clones among the border cell population of follicle cells and a single germ cell clone.

chromosome. A source of FLP recombinase is introduced on another chromosome and is expressed during development by heat shock-induction. FLP catalyses mitotic recombination between the paired FRTs and thus places the β-galactosidase coding region downstream of the α-tubulin promoter on the same chromosome. This fusion results in the heritable expression of β-galactosidase in the recombinant cell lineage.

The system described here has many advantages over presently used techniques. First, FLP-mediated recombination is inherently 10–100 times more efficient than X-ray-induced recombination. Second, induction of the recombinase is accomplished by heat shock, a much milder treatment than X-irradiation or microinjection. Third, the detection method is based on a positive method of cell marking, thus making it possible to analyse clones in any tissue without tedious sectioning. With these advantages, this methodology can be applied to mosaic analyses in any tissue and with virtually any mutation.

Although this system will be of general use, some of the potential weaknesses should also be noted. The major drawback to this, or any FLP-mediated approach, is that the precise timing of mitotic recombination is not known. When using X-rays, chromosome breaks occur at the time of irradiation and recombination occurs before the next cell division. In contrast, the FLP recombinase is relatively stable in flies and this results in recombination well after the time of heat shock. Persistence of FLP is of particular significance in the embryo, where cell divisions occur very rapidly. The problem may be overcome by the construction of a labile FLP recombinase, perhaps by adding a protein degradation signal (PEST sequence) to the recombinase [41], thereby allowing much more precise timing of clonal induction.

Another drawback at present is that the *lacZ* gene under control of the α-tubulin promoter is not uniformly expressed in all tissues, thus restricting analysis to the tissues where detectable levels of β-galactosidase are produced. Although this is not necessarily undesirable, a universal system in which the marker is ubiquitously expressed could be designed by the replacement of the α-tubulin promoter with a generally expressed promoter (perhaps from ubiquitin or RNA polymerase subunits). Such a construct could be used as described here, but could be employed for lineage analysis in all tissues at all developmental stages.

As described, this technique, used for the generation of marked cell lineages, should be widely useful for the analysis of patterns of cell division, migration and differentiation. It could also be applied to the mosaic analysis of *Drosophila* mutations. By recombining the mutation of interest onto the chromosome that bears the distal portion of the marker cassette, it will be possible to produce mutant clones that express β-galactosidase. For general use, this will require the construction of stocks with complementary halves of the recombination targets near the base of each of the five major chromosome arms, but such an effort should be worthwhile. The same factors that make this technique so powerful for cell-lineage analysis also apply to the generation and analysis of mosaics; the efficiency of the generation of clones would be very high and the identification of mutant tissue would be easy, even in internal tissues.

The classes of clones that have been seen in these preliminary studies suggest a range of questions that may be addressed using this technique. The facile generation of marked clones in follicle cells should allow a detailed analysis of follicle cell lineage. Induction of clones in various sub-populations of follicle cells provides a mechanism for the analysis of

mutations involved in the specific functions of these cell types. Also, the ability to make and mark germline clones within a single cyst may provide a novel genetic method for functionally distinguishing the oocyte from the nurse cells. That capability would make it possible to examine more precisely the function of gene products implicated in oogenesis and would provide a tool for studying the role of the oocyte nucleus during oogenesis.

The technology described here may also be applicable to other organisms. For example, it has already been shown that FLP recombinase can function in mammalian cells [42]. The development of a technique that involves complementary cassettes in mice should be straightforward as it is possible to target constructs to specific chromosomal sites by homologous recombination. Specific targeting to the same genomic insertion site of two separate cassettes, one with the promoter/FRT and the other with FRT/marker, would eliminate the need for the type of excision scheme described here for flies. The development of this system in mice may be potentially useful for the mosaic analysis of mutations.

Conclusions

It is clear that the FLP recombinase can be used to reconstitute a promoter/marker cassette by intermolecular recombination. This recombination occurs with a high frequency and without causing any harm to the animal. The technique can therefore be employed as an excellent means to perform cell lineage analysis. Additionally, the technique could be extended to generate and mark mutant cells for mosaic analysis. Specific expression of the marker in only mutant cells will make it easy to analyse the effects of mutations on any tissue throughout development. We expect such a technique to be of general interest and utility to the *Drosophila* community and may also prove applicable for other organisms.

Materials and methods

Plasmid construction

The P element plasmid was constructed using the Tα1-lacZ plasmid (KH O'Donnell, C-T Chen and PC Wensink, unpublished data). This construct contains the *lacZ* protein-coding region fused to 2 kb of the promoter of *Drosophila* α1-tubulin gene, including the 5′ leader, intron, and coding region for the first 21 amino acids of α-tubulin. Tα1-lacZ was cut with *Bam*H I to linearize at the α-tubulin/*lacZ* junction. A double-stranded fragment encoding the SV40 nuclear localization signal was ligated into the *Bam*H I site to generate the plasmid Tα1-NLS. This double-stranded fragment was generated by annealing oligonucleotides NLS-1 and NLS-2 (sequence GATCCGCGGGTGCCCAAGAAGAAGCGCAAGGTC and GATCGACCTTGCGCTTCTTCTTGGGCACCCGCG, respectively). The resulting plasmid was sequenced using the dideoxy-termination method [43] to confirm the insertion of the fragment in the sense orientation. Tα1-NLS was linearized within the tubulin intron using *Xba* I, blunt-ended, and then ligated with the blunted 45bp *EcoR*I to *Bam*H I FRT-containing fragment of pJFS36 [44]. The entire 6.8 kb *Xho* I fragment containing the tubulin-FRT-β-gal fusion was blunted and ligated into the blunted *Bam*H I site of the CaSpeR 4 P element vector [45]. A 5 kb intronless *Sal* I fragment containing the *yellow* gene [46] was then ligated into the *Xho* I site of the P element construct. The resulting plasmid, pCXY 21, was then introduced into flies of the genotype *y w; Δ2-3, Sb/TM6* [35] by germline transformation [47]. Nine independent insertion lines were generated. Insertions on the X chromosome were detected by examining segregation of the y^+ and w^+ markers. Autosomal insertions were mapped by standard genetic techniques using the stocks *w; CyO/Sco;* +/+ and *w;* +/+*; TM3, Sb/CxD.*

In situ hybridization to polytene chromosomes

In situ hybridizations to polytene chromosomes were performed by standard techniques [48] using biotinylated probes prepared by random priming [49]. The Vectastain Elite HRP kit from Vector Labs and diaminobenzidine from Polysciences were used to detect hybridization.

Generation of excision lines

One of the transformant lines, *X15*, was subjected to P element mobilization by the introduction of *Δ2-3* transposase. *y w/y w; X15[*y^+ w^+*]/X15[*y^+ w^+*]* females were mated to *y w; Δ2-3, Sb/TM6* males, generating dysgenic *y w; X15[*y^+ w^+*]/+; Δ2-3, Sb/+* males. 200 dysgenic males were individually mated to three *y w/y w* females. Progeny were examined for individuals that were either y^+ w^- or y^- w^+. In vials containing more than one fly of either phenotype, only one representative of each was selected for further analysis. 36 independent excisions were recovered. Of these, 29 were y^- w^+ and 7 were y^+ w^-.

PCR analysis of excisions

Two pairs of PCR [50] primers were used to analyze excision lines for the presence of FRT sequences. The pairs used were FRT-f (GAAGTTCCTATACTTTCTAGA) with BW1(CCTCTCCCACTTCAGTTA) and FRT-r(GAAGTTCCTATTCTCTAGAAA) with NLS-2 (sequence above). These pairs amplify DNA from the FRT to the α-tubulin promoter and from the FRT to the nuclear localization signal of *lacZ*, respectively. Genomic DNA from excision lines was subjected to 30 cycles of amplification consisting of 1 minute at 94°C, 1 minute at 57°C and two minutes at 72°C. Products were analyzed by agarose gel electrophoresis.

X-gal staining

Ovaries were stained for β-galactosidase activity using standard procedures. Briefly, ovaries were fixed for 5 to 10 minutes in 2.5% glutaraldehyde in PBT and then stained in X-gal staining solution [51] for 15 to 60 minutes at 37° until color developed.

Antibody staining

Embryos were stained using standard antibody staining procedures. Embryos were dechorionated in 50% bleach, washed and fixed in 3.7% formaldehyde in PBT with heptane for 15 minutes. Vitelline membranes were removed with methanol/heptane. Embryos were incubated with a mouse monoclonal anti-β-galactosidase antibody from Boehringer Mannheim Biochemical, followed by biotinylated horse anti-mouse antibodies from Vector Labs which was then detected using the Vectastain Elite ABC horseradish peroxidase kit from Vector Labs with diaminobenzidine (DAB).

Heat shock induction of FLP recombinase

Heat shock was used to induce the expression of FLP recombinase to generate mitotic clones. Two heat shock-recombinase insertion chromosomes were used, FLP1 [33] on the first chromosome and FLP99 [22] on the third chromosome. Two com-

binations of the y^+w/yw^+ strains were used and two different recombinase lines were used, for a total of four possible genetic combinations. As the results obtained with each of the combinations were quite similar, no distinction between these genotypes was made. All heat shocks were done in plastic vials using a circulating water bath at 37°C. Embryos and larvae were heated for 30, 60 or 120 minutes and adults for one to four times for 30 minutes each on the same day.

Acknowledgements. We would like to thank Drs P. Wensink, P. Geyer, and E. Wilder for sharing their reagents with us and Drs E. Wilder and R. Finkelstein for their advice from earlier efforts to design similar techniques. We would also like to thank Drs E. Siegfried and J. Duffy for their critical reading and comments on the manuscript. D.A.H. was supported by the Damon Runyon-Walter Winchell Cancer Research Fund fellowship No.DRG 1154. This work was supported by Howard Hughes Medical Institute.

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Received: **24 May 1993;** revised: **11 June 1993.**
Accepted: **11 June 1993.**