Requirement of *flex* (*f*emale *le*thal on *X*) in the development of the female germ line of *Drosophila melanogaster*

ANANYA BHATTACHARYA[1*], S. SUDHA[1], SWATHI BALAKRISHNA[1] and H. SHARAT CHANDRA[1,2]

[1]*Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560 012, India*
[2]*Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore 560 064, India*

Abstract

Drosophila melanogaster females homozygous for *flex*, an X-linked recessive mutation, do not survive. Hemizygous males are unaffected. Homozygous embryos appear to lack SXL, the product of the *Sex-lethal* (*Sxl*) gene, apparently as a result of disruption of *Sxl* splicing. It is known that both *Sxl* and its somatic splicing regulators [*snf* and *fl*(2)*d*] also function in the development of the female germ line. For this reason, we investigated the role of *flex* in the germ line by generating *flex/flex* clones in *flex*/+ females. Females carrying such clones in their germ lines do not lay eggs whereas females carrying $flex^+$ eggs lay viable eggs. Additionally, DAPI staining of ovarioles showed that diploid germ cells that are homozygous mutant for *flex* do not complete oogenesis. These results indicate that the $flex^+$ gene product may be required for the development of the female germ line.

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Introduction

In *Drosophila melanogaster*, the process of sex determination is initiated by a cell-autonomous mechanism which functions transiently in somatic cells of the early embryo (Sanchez and Nöthiger 1983; Cline 1984). This involves measurement of the ratio of the number of X chromosomes (X) to the number of sets of autosomes (A). The X : A ratio activates a special embryonic promoter, *Pe*, of *Sxl* (Keyes *et al.* 1992). At this stage, the initiation of *Sxl* transcription is limited to females; later in embryogenesis, transcription shifts to a maintenance promoter, *Pm*, which is active throughout development in both sexes. SXL, produced from Sxl_{Pe} mRNA, initiates an autoregulatory feedback loop that directs the productive splicing of transcripts expressed from Sxl_{Pm} (Bell *et al.* 1991; Bopp *et al.* 1991). *Sxl* controls three subordinate pathways that are necessary for the viability and fertility of the fly: the processes leading to dosage compensation, somatic sexual development and germ line sex determination (for reviews see Baker and Belote 1983; Steinmann-Zwicky *et al.* 1990; Belote 1992; Steinmann-Zwicky 1992a, b; Kuroda *et al.* 1993; Baker *et al.* 1994; Gorman and Baker 1994).

Regulation of sex determination in the germ line is substantially different from that in the soma. Germ line sex determination is regulated by cell-autonomous as well as cell-nonautonomous factors (reviewed by Pauli and Mahowald 1990; Steinmann-Zwicky 1992a, b; Burtis 1993). Female germ cells require the activity of *Sxl* to proceed normally through oogenic differentiation (Schupbach 1985; Nöthiger *et al.* 1989; Steinmann-Zwicky *et al.* 1989). Studies by Bopp *et al.* (1993) have shown that *Sxl* is required for some aspect of germ line development other than establishment and maintenance of sexual identity. The ultimate effect of X : A signals in the germ cells appears to be the same as in the soma: removal of the male-specific exon from *Sxl* pre-mRNA (Bopp *et al.* 1993; Oliver *et al.* 1993). Many of the known components of the X : A signalling elements do

* For correspondence. Present address: Waksman Institute, Rutgers University, 190 Frelinghuysen Road, Piscataway, NJ 08854-8020, USA. E-mail: bhatta@waksman.rutgers.edu.

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not seem to play any role in the germ line (Schupbach 1985; Granadino *et al.* 1993; Steinmann-Zwicky 1993). In the sexual differentiation pathway, genes downstream of *Sxl*—*tra*, *tra2* and *dsx*—are not required in female germ cells but rather must function in the surrounding somatic tissues (Marsh and Wieschaus 1978; Schupbach 1982). The *tra*→*dsx* signalling pathway may direct differentiation of the female germ line by feminizing the expression of some as yet unknown genes. *tra2* is thought to act as a cofactor for some other sex-specific gene which finally activates the somatic signalling pathway (Horabin *et al.* 1995). The products of otu^+, ovo^+, snf^+, fu^+ and $fl(2)d^+$ are required for the female-specific splicing of Sxl^+ pre-mRNA and thus for specifying female differentiation of the germ line (Oliver *et al.* 1988, 1990, 1993; Steinmann-Zwicky 1988; Granadino *et al.* 1992; Pauli *et al.* 1993).

During a search for additional X-linked genes affecting sex determination, a female-lethal mutation was isolated in our laboratory from an EMS mutagenesis screen (Anand 1993). Females homozygous for this mutation, named *flex*, do not survive; heterozygous females and hemizygous males are viable and fertile. In contrast to most of the genes affecting sex determination, *flex* does not exhibit female lethality in transheterozygous combination with loss-of-function alleles of *Sxl, sisA, sisB, da, snf* and *fl(2)d* (A. Anand and H. S. Chandra 1993 Abstract, Proceedings of the XVII International Congress of Genetics, Birmingham, UK).

Three new alleles of *flex* were subsequently isolated: $flex^2$, a mutation induced by EMS, and $flex^3$ and $flex^4$, induced by gamma irradiation (Bhattacharya *et al.* 1999). Investigations on the status of *Sxl* regulation in *flex*/*flex* embryos showed that SXL is not present in such embryos. Also, studies of *Sxl* transcription suggested that *Sxl* splicing could be disrupted in homozygous embryos (Bhattacharya *et al.* 1999). Most splicing regulators of *Sxl* also function in the female germ line (Oliver *et al.* 1988; Granadino *et al.* 1992). It was considered necessary to generate germ line chimaeras to find out whether the $flex^+$ gene product is essential for the development of the female germ line and whether diploid germ cells homozygous mutant for *flex* have a recognizable phenotype. Here we report on the generation of *flex*/*flex* mitotic clones in *flex*/+ females with the help of the FLP–FRT recombination system (Chou and Perrimon 1992). Our results showed that the females with *flex*/*flex* clones lay very few eggs. Also, DAPI staining of dissected ovarioles from these females indicated that oogenesis does not proceed to completion when *flex*/*flex* clones were generated, suggesting that the $flex^+$ gene product is apparently required for the development of the female germ line.

Materials and methods

Flies were maintained on standard cornflour–yeast–sugar–agar medium at 25°C unless otherwise stated.

Stocks used: $y\,w\ FRT^{9-2}/y\,w\ FRT^{9-2}$ (Chou and Perrimon 1992) contains two direct repeats of *FRT* sequences cloned in a P element vector with $white^+$ as selectable marker and *nptII* gene conferring resistance to the antibiotic geneticin. C(1)DX $y\,f\ ovo^{D2}\ v^{24}\ FRT^{9-2}$/Y; FLP^{38}/FLP^{38} (Chou and Perrimon 1992) contains, along with the *FRT* sites, a dominant female-sterile mutation (ovo^{D2}). The FLP-recombinase is also cloned in a P element vector under a hsp70 promoter with $rosy^+$ as a selectable marker. Both these stocks were gifts from Prof. Norbert Perrimon. Among the *flex* alleles, $flex^2$ and $flex^3$ were used in this set of experiments. The description of balancers can be obtained from Flybase.

Generation of germ line clones: The two *flex* alleles, $flex^2$ and $flex^3$, were recombined onto the chromosome bearing *FRT* at 18E. Females carrying the recombinant chromosome (*flex FRT*) were crossed to males with ovo^{D2} *FRT*. The progeny of such crosses were given heat shock at appropriate stages of development to induce the synthesis of FLP-recombinase. Recombination occurs between the *FRT* sites leading to the formation of mitotic clones (*flex*/*flex*) in the developing germ line. The presence of ovo^{D2} marks the products of recombination. For example, $ovo^{D2}/+$ is the nonrecombinant genotype, and ovo^{D2}/ovo^{D2} and *flex*/*flex* are the recombinant classes. To check the effects of such clones in the female germ line, each of these females was crossed to wild-type males and their egg-laying ability monitored.

DAPI staining of ovaries: (after Warn *et al.* 1985) The ovaries of each genotypic class of females (3–5 days old) were dissected in cold 1 × EBR (130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 10 mM HEPES, pH 6.9) and transferred to an Eppendorf tube containing 1 × EBR on ice. EBR was removed and 100 µl of devitellizing buffer [1 vol buffer B (whose composition is given below), 1 vol formaldehyde 36%, 4 vol H_2O] and 600 µl of heptane were added. The mix was shaken vigorously to saturate the buffer with heptane and then gently for 10 minutes at room temperature. This solution was removed and the ovaries were rinsed with 1 × PBS. The composition of buffer B was 100 mM KH_2PO_4/K_2HPO_4 (pH 6.8), 450 mM KCl, 150 mM NaCl, 20 mM $MgCl_2$. Tissues were incubated for 5 minutes at room temperature in 1 µg/ml DAPI solution in PBS. They were then rinsed with PBS and mounted in 50 µl glycerol : PBS (50% glycerol). The ovaries were placed on a slide, and individual egg chambers were dissected and examined in the UV channel of a fluorescence microscope.

Results

Generation of flex/flex clones in the germ line

The effect on oogenesis of a clone of *flex*/*flex* cells in the germ line of a *flex*/+ female was investigated (for details see Materials and methods). $flex^2$ was recombined onto a

Table 1. Clonal analysis of *flex*^{2} in the germ line.

Genotype of females	Stage of heat shock	Per cent egg-laying efficiency*
w f FRT/*w f FRT* (I)	48–72 hours 72–96 hours	 60 (150/250)
w f flex^2 *FRT*/*ovo*^{D2} *FRT* (II)	48–72 hours 72–96 hours	12 (8/66) 6 (4/66)
y cho cv v f flex^2 *FRT*/*ovo*^{D2} *FRT* (III)	48–72 hours 72–96 hours	3 (2/63) 0 (0/45)
w f FRT/*w f FRT* & *w f flex*^{2} *FRT*/*ovo*^{D2} *FRT* (IV)	Nil	0 (0/85)

* Expressed as the ratio of the number of females laying viable eggs to the total number of females tested. The detailed description of each of the crosses is given below.
(I) *w f FRT*/*w f FRT* females (which served as positive controls) were crossed to *ovo*^{D2} *FRT*/*Y*; *FLP*^{38}/*FLP*^{38} males and the progeny were given heat shock at the two stages indicated. The F_1 females were crossed to wild-type males and the egg-laying ability of such females was tested.
(II) *w f flex*^{2} *FRT*/FM7 females were crossed to *ovo*^{D2} *FRT*/*Y*; *FLP*^{38}/*FLP*^{38} males and the progeny were given heat shock as described in the table. Among the F_1 females, the non-Balancer females were collected and their egg-laying ability was monitored as before.
(III) To monitor effect of genetic background *y cho cv v f flex*^{2} *FRT*/FM7 females were used instead of *w f flex*^{2} *FRT*. The crosses are identical to those described in row II.
(IV) As negative controls in this analysis, both *w f flex*^{2} *FRT*/FM7 females and *w f FRT*/*w f FRT* females were used but no heat shock was administered. The crosses were the same as those described earlier.

chromosome carrying the *FRT* insertion at 18E and such recombinant females were crossed to males bearing *ovo*^{D2} *FRT* and FLP-recombinase. The progeny were subjected to heat shock at 37°C for two hours at the middle of the second instar (48–72 hours after oviposition) and late third instar (72–96 hours after oviposition). As positive control, *w f FRT* flies which are *flex*$^+$ were also subjected to heat shock to test for the activity of the FLP-recombinase. As negative control, all females (with and without *flex*^{2}) were tested for their egg-laying ability but no heat shock was administered. The results of one such experiment are summarized in table 1.

The frequency of mitotic recombination is around 60% in the positive control (*flex*$^+$ *FRT*), which agrees well with published data for *FLP*^{38} (Chou and Perrimon 1992). Flies that received no heat shock (i.e. *flex*$^+$ *FRT* and *flex*^{2} *FRT*, which served as negative controls) did not lay any eggs. These data show that there is no leakiness associated with the recombinase and that the clones formed in the heat-shocked flies are entirely products of recombination. It was observed that *flex*^{2} *FRT*/*ovo*^{D2} *FRT* females carrying the *flex*/*flex* clones, upon heat shock, lay very few eggs in comparison to *flex*$^+$ *FRT*/*ovo*^{D2} *FRT* females. To examine if there are allele-specific variations in this phenotype, a similar analysis was carried out with *flex*^{3} instead of *flex*^{2}. The egg-laying of *flex*^{3} *FRT* flies was monitored following heat shock conditions similar to those indicated in table 1. The results are summarized in table 2.

Hence, the observation that females in which presumptive *flex*/*flex* clones had been induced lay few or no eggs suggests the possibility that such chimaeric germ lines do not support the formation of viable eggs. To verify the formation of mitotic clones and to see the effect of *flex*/*flex* clones on the development of the female germ line, ovarioles from such females were dissected and stained with DAPI.

Staining of ovaries with DAPI

The ability of germ cells homozygous for *flex* to progress through oogenesis and their capacity to form functional gametes was explored. We had observed that SXL is not present in the somatic tissues of embryos homozygous for *flex* owing to a disruption in the splicing regulation of *Sxl* (Bhattacharya *et al.* 1999). Therefore an attempt was made

Table 2. Clonal analysis of *flex*^{3} in the female germ line.

Genotype of females	Stage of heat shock	Per cent egg-laying efficiency*
w f FRT/*w f FRT* (I)	48–72 hours 72–96 hours	53.2 (83/156)
w f flex^3 *FRT*/*ovo*^{D2} *FRT* (II)	48–72 hours 72–96 hours	10.86 (5/46) 8.37 (3/37)
w f FRT/*w f FRT* & *w f flex*^{3} *FRT*/*ovo*^{D2} *FRT* (III)	Nil	0 (0/40)

* Expressed as the ratio of the number of females laying viable eggs to the total number of females tested.
Experiments in rows I–III are identical to those described in table 1. Here, instead of *flex*^{2}, *flex*^{3} have been used to analyse whether any allele-specific variations exist.

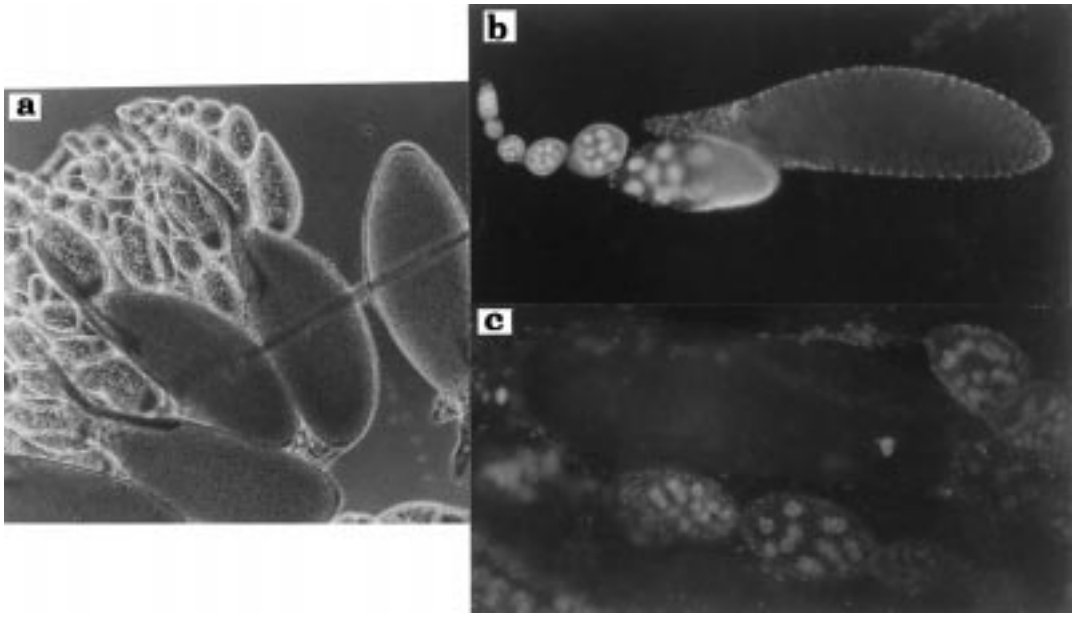

Figure 1. Dissected egg chambers stained with DAPI: (a) bright field image of a dissected ovary from a wild-type female; (b) typical egg chamber from a wild-type female after staining with DAPI; (c) ovary from an ovo^{D2}/Bal female showing arrest of oogenesis at stage 6.

to investigate whether *flex*/*flex* germ cells mimic the behaviour of diploid germ cells homozygous for loss-of-function mutations of *Sxl*. As in the previous experiment, $flex^2$ was recombined onto a chromosome carrying the *FRT* insertion at 18E and such recombinant females were crossed to males bearing ovo^{D2} *FRT* and FLP-recombinase. The progeny were subjected to heat shock at 37°C for two hours according to the protocol given in the previous experiment. Ovaries from *flex FRT*/ovo^{D2} *FRT* and $flex^+$ *FRT*/ovo^{D2} *FRT* females (with and without heat shock) were dissected and stained with DAPI.

Figure 1a shows the bright field image of a dissected ovary from a wild-type female and 1b a typical egg chamber after staining with DAPI. An ovary from an ovo^{D2}/Balancer female showing arrest of oogenesis at stage 6 is shown in figure 1c. It can be seen that *w f* $flex^+$ *FRT*/ovo^{D2} *FRT* females which did not receive any heat shock have rudimentary ovaries (figure 2a) compared to ovaries from wild-type females (figure 1). In almost all the ovarioles examined, oogenesis had been arrested at around stage 6. Upon receiving heat shock at 72–96 hours after oviposition, the morphology of the ovarioles changed drastically, and the progress of germ cells following oogenesis could be observed (figure 2b). Oocytes in different stages of development as well as mature eggs could be observed. It will be recalled from the previous experiment that such females are capable of laying viable eggs (tables 1 and 2). The results of DAPI staining of ovaries from these $flex^+$ *FRT* females are thus consistent with the results of the egg-laying experiments.

Similar experiments were carried out with *flex FRT*/ovo^{D2} *FRT* females. The results of staining ovarioles from these females with DAPI are shown in figures 3 and 4. As described in table 1, both *w f* $flex^2$*FRT*/ovo^{D2} *FRT* and *y cho cv v f* $flex^2$ *FRT*/ovo^{D2} *FRT* flies were used to see the effect of genetic background. We observed that flies that received no heat shock had rudimentary ovaries in which oogenesis had been arrested around stage 6 (figures 3a and 4a) as in $flex^+$ *FRT*/ovo^{D2} *FRT* females without heat shock. Upon heat shock (48–72 hours and 72–96 hours) oogenesis appeared to proceed normally in some ovarioles and immature oocytes could be seen (figures 3b and 4b). But neither mature oocytes nor eggs were observed in any of these ovaries. Similar results were obtained when ovaries from $flex^3$ *FRT*/ovo^{D2} *FRT* females were dissected and their nuclei stained with DAPI (data not shown). It will be recalled that in comparison with $flex^+$ *FRT*/ovo^{D2} *FRT* females the egg-laying ability of these females is drastically reduced (tables 1 and 2). Taken together, these observations indicate that *flex*/*flex* clones generated in the germ line of *flex*/+ females do not give rise to normal eggs. On the other hand, wild-type ($flex^+$) clones generated under identical conditions supported the development of viable eggs. Hence, the $flex^+$ gene product is probably necessary for the formation of a viable germ line in females.

Discussion

Germ line chimaeras are invaluable for analysing the tissue specificity (germ line vs somatic) of recessive female-sterile mutations (Wieschaus *et al.* 1981; Perrimon and Gans 1983) as well as for detecting the maternal effect of recessive zygotic lethals (Perrimon *et al.* 1984, 1989). Pole cell transplantation and utilization of a dominant female-sterile

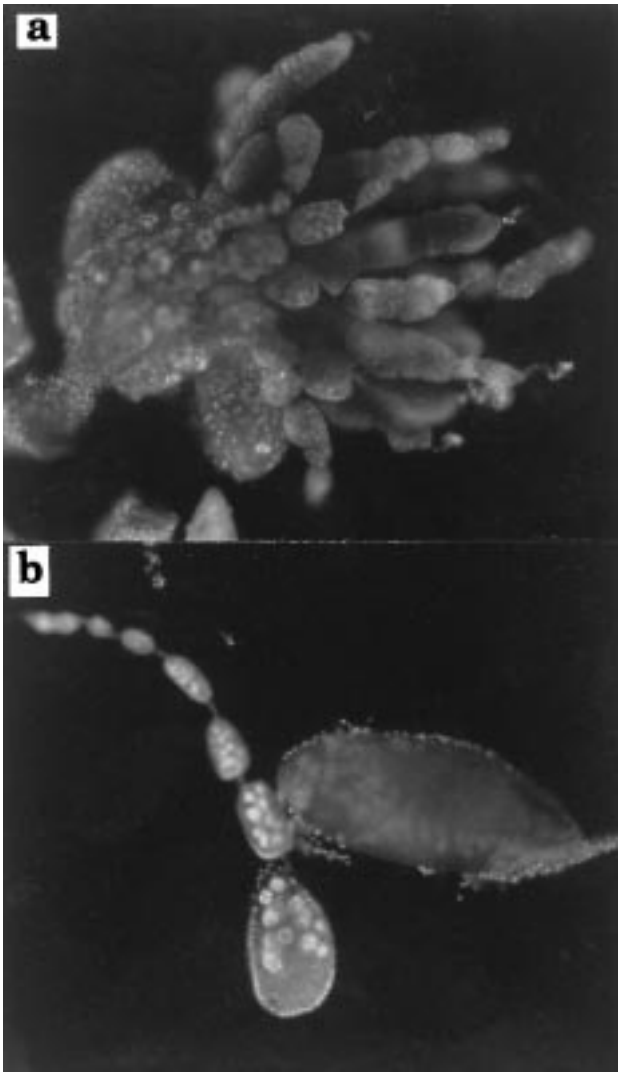

Figure 2. Dissected egg chambers stained with DAPI. Ovaries from *w f flex*$^{+}$ *FRT*/*ovo*^{D2} *FRT* females were dissected and stained with DAPI: (a) ovary from a female which was not subjected to heat shock; (b) typical egg chamber from a female which had received two hours of heat shock at third instar. Oogenesis proceeded normally in such flies.

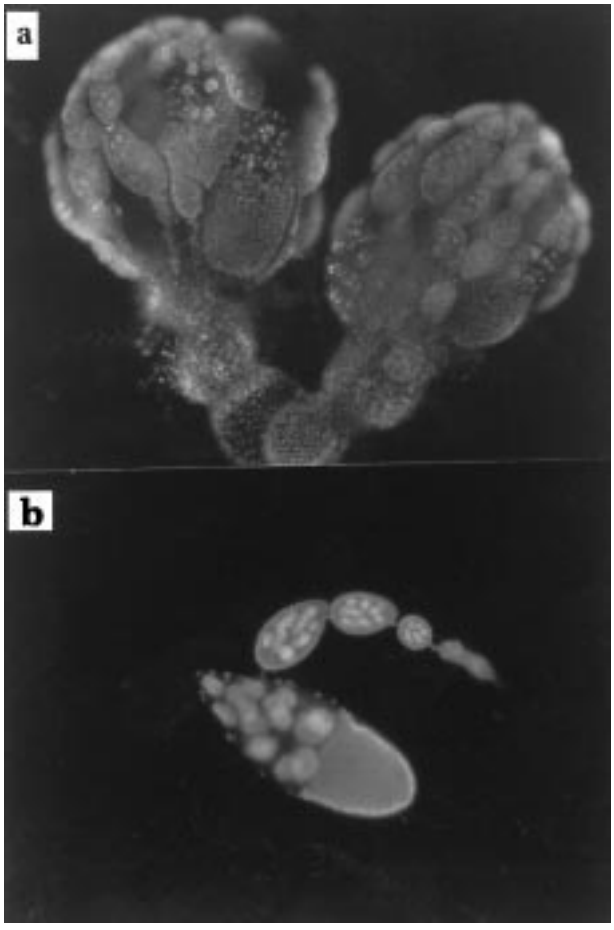

Figure 3. Dissected egg chambers stained with DAPI. Ovaries from *w f flex*^{2} *FRT*/*ovo*^{D2} *FRT* females dissected and stained with DAPI: (a) ovary from a female which received no heat shock; (b) a typical egg chamber from a female which, as a third instar larva, had received two hours of heat shock; oogenesis is initiated but neither mature oocytes nor eggs were seen.

mutation (DFS) have previously been employed to address such questions (Illmensee 1973; Perrimon and Gans 1983; Perrimon 1984). A modification of the latter method was made by Chou and Perrimon (1992) in which mitotic exchange was induced with the help of the FLP–FRT system in female germ cells.

flex is a zygotic female-lethal mutation (Anand 1993). Experiments aimed at investigating the status of *Sxl* regulation showed that SXL is absent in *flex*/*flex* embryos. The absence of SXL is most likely the result of a defect in the female-specific splicing of *Sxl* (Bhattacharya *et al.* 1999). This raises the possibility that *flex* could be a regulator of *Sxl* splicing or it could be regulating the function of some other gene which directly affects the splicing of *Sxl*. Since most of the genes involved in the maintenance of *Sxl* activity (*snf* and *fl*(*2*)*d*) also function in the germ line of the female and they display a characteristic phenotype, it was necessary to find out whether *flex*$^{+}$ gene product is essential for the development of the female germ line, and whether diploid germ cells homozygous for *flex* have a recognizable phenotype.

In the present investigation, the approach of Chou and Perrimon (1992) was used to generate *flex*/*flex* germ line clones in *flex*/+ females. It was observed that females bearing such clones lay few eggs compared to females carrying *flex*$^{+}$ clones generated under similar heat shock conditions (tables 1 and 2). This could be because the *flex*$^{+}$ gene product is necessary for the successful completion of the development of the female germ line. This effect appears to be independent of the genetic background.

To verify the formation of these mitotic clones in the germ line and to see the progress of such germ cells through oogenesis, ovarioles from each of these classes of females (described in table 1) were stained with DAPI. Our experiments showed that *flex*/*flex* clones are indeed generated in

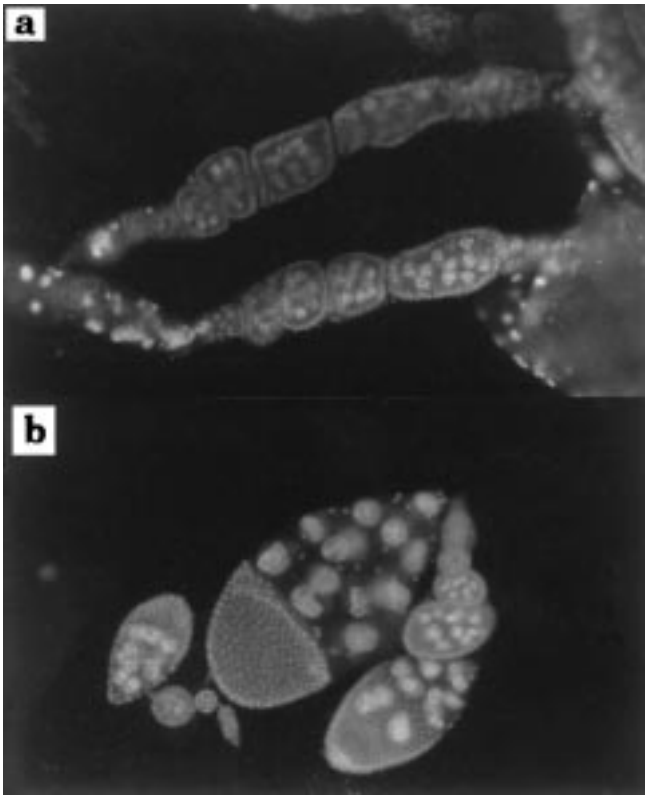

Figure 4. DAPI staining of dissected egg chambers. Ovaries from females of the genotype *y cho cv v f flex*^{2} *FRT*/*ovo*^{D2} *FRT* were dissected and stained with DAPI: (a) ovary from a female which did not receive heat shock; (b) typical egg chamber from a female which had received two hours of heat shock as a third instar larva. Here also, oogenesis does not proceed to completion and no mature eggs were seen.

the germ line of the females following heat shock. Although oogenesis appeared to proceed normally in some of these ovarioles, neither mature oocytes nor eggs could be seen. In such ovarioles oogenesis does not proceed beyond stage 10.

We were also interested in the question whether *flex*/*flex* germ cells mimic the behaviour of diploid germ cells homozygous for loss-of-function mutations of *Sxl*. Ovaries of females in which the germ cells are *Sxl*$^{-}$ are filled with large numbers of small cells that are not morphologically female. They appear either undifferentiated or similar to primary spermatocytes (Schupbach 1985; Oliver *et al.* 1988, 1990; Steinmann-Zwicky *et al.* 1989). *otu*, *ovo*, *fu* and *snf* are required for the female-specific splicing of *Sxl*$^{+}$ pre-mRNA in the female germ line. Diploid germ cells mutant for *otu*, *ovo* or *snf* form tumorous structures which are suppressed by the constitutive expression of *Sxl*$^{+}$ (Steinmann-Zwicky 1988; Oliver *et al.* 1990; Salz 1992; Pauli *et al*. 1993). Females transheterozygous for *snf* and *Sxl* mutations show sexual transformations, ovarian tumours, and greatly reduced viability. These results led to the proposition that *snf* is needed to activate or maintain *Sxl* expression (Oliver *et al.* 1988). Additionally, pole cell transplantation experiments have shown that the phenotype of *fl(2)d* in diploid germ cells resembles that exhibited by *Sxl*^{f4} and *Sxl*^{f5}, suggesting the likely involvement of *fl(2)d* in *Sxl* splicing in the female germ line (Granadino *et al.* 1992).

When ovaries from *flex FRT*/*ovo*^{D2} *FRT* and *flex*$^{+}$ *FRT*/*ovo*^{D2} *FRT* females were dissected and their nuclei stained with DAPI, it was observed that the phenotype of the presumptive *flex*/*flex* clones was different from that exhibited by *Sxl*/*Sxl* clones. Our results show that *flex* affects the development of the female germ line, but *flex*/*flex* clones neither exhibit an early-arrest phenotype nor form tumorous structures (figures 3b, 4b). In presumptive *flex*/*flex* clones, the initiation of oogenesis could be observed but neither mature oocytes nor eggs were seen. These results suggest that the requirement of *flex*$^{+}$ is probably not at the stage of establishment of sexual identity in the germ line but at later stages of differentiation. As mentioned before, *flex* appears to be involved in the splicing of *Sxl* in the somatic tissues (Bhattacharya *et al.* 1999). Thus *flex* may have different roles in the somatic and germ line tissues. Since we do not yet have a female-viable but sterile allele of *flex*, experiments aimed at deciphering the epistatic relationship between *flex* and other genes involved in germ line sex determination (*ovo*, *otu*, *snf* and others) could not be done. We cannot comment at this stage whether the effect of *flex* on the development of the female germ line is autonomous, since these clones were generated in females which were also heterozygous for *ovo*^{D2}. Hence, it is necessary to generate *flex* homozygous clones in females which do not have any other dominant female-sterile mutation to answer the question whether *flex* acts autonomously in the germ line.

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