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Expression of the *Sex-lethal* gene is controlled at multiple levels during *Drosophila* oogenesis

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SUMMARY

In addition to controlling somatic sexual development in *Drosophila melanogaster*, the *Sex-lethal* (*Sxl*) gene is required for proper differentiation of female germ cells. To investigate its role in germ-line development, we have examined the expression of *Sxl* in wild-type ovaries and ovaries that are defective in early steps of germ cell differentiation. As in the soma, the basic mechanism for on/off regulation of *Sxl* relies on sex-specific processing of its transcripts in germ cells. One class of female-sterile mutations, which includes *fs(1)1621* and the tumorous-ovary-producing allele of the *ovarian tumor* gene, *otu[1]*, is defective in the splicing process. These mutants have germ lines with high amounts of *Sxl* RNA spliced in the male mode and a severe reduction of protein levels in the germ cells. Another class of female-sterile mutations produces a phenotype similar to that seen in *fs(1)1621* and *otu[1]* but appears to express normal levels of Sxl protein in the germ cells. However, this second class does not show the changes in protein distribution normally observed in wild-type germ cells. In the wild-type germarium, the non-differentiated germ cells show a strong cytoplasmic accumulation of Sxl protein followed, as the germ cells differentiate, by a dramatic reduction and redistribution of the protein into nuclear foci. Interestingly, two female-sterile alleles of *Sxl*, *Sxl^{f4}* and *Sxl ^{f5}* belong to the second class, which shows persistent cytoplasmic accumulation of Sxl protein. These *Sxl* female-sterile mutants encode an altered protein indicating that *Sxl* regulates processes that eventually lead to the changes in Sxl protein distribution. Lastly, we demonstrate that during the final stages of oogenesis several mechanisms must operate to prevent the progeny from inheriting Sxl protein. Conceivably, this regulation safeguards the inadvertent activation of the *Sxl* autoregulatory feedback loop in the male zygote.

Key words: *Sex-lethal*, germ line, sex determination, splicing regulation

INTRODUCTION

Somatic sexual development in *Drosophila* has provided an excellent system to study the mechanisms that are responsible for initiation and maintenance of developmental decisions. The initial choice of sexual identity in somatic cells is made early in embryogenesis in response to the primary sex-determining signal, the ratio of X chromosomes to autosomes (for reviews see Hodgkin, 1990; Steinmann-Zwicky et al., 1990). In normal diploid flies, the female developmental pathway is selected when two X chromosomes are present while the male pathway is chosen when there is only a single X chromosome. The target for the X:A signalling system is the binary switch gene, *Sex-lethal* (*Sxl*); it is turned **on** in chromosomally female cells, while it remains **off** in chromosomally male cells. The initial choice of *Sxl* activity is controlled at the level of transcription. A special early *Sxl* promoter is transiently activated in female embryos, while this promoter is not turned on in male embryos (Keyes et al., 1992).

Once the appropriate pathway has been selected the X:A signalling system shuts down. The protein produced from the early RNAs then mediates the transition from the initiation to the maintenance phase of regulation where the activity of *Sxl* is controlled by alternate splicing. In females, *Sxl* is maintained in the on state by an autoregulatory feedback loop (Bell et al., 1991). In this feedback loop, female Sxl proteins promote their own expression by directing the splicing machinery to skip the 3rd exon of transcripts (see Fig. 1A) from the constitutive *Sxl* late promoter which is active in both sexes. This generates mature *Sxl* mRNAs, which encode female protein species that show sequence similarity to a family of RNA-binding proteins (Bell et al., 1988). In males, the X:A ratio does not activate *Sxl*. The *Sxl* autoregulatory feedback loop is not set in motion and the male determined state is maintained by default. In the default state, the splicing machinery incorporates exon 3 into mature *Sxl* mRNAs. This male-specific exon contains in-frame stop codons, which truncate the open reading frame

leading to the production of small, presumably non-functional, peptides.

Sxl sits at the top of several gene cascades that control different aspects of somatic sexual development. Of these, the sexual differentiation pathway mediated through the gene *doublesex* (*dsx*) is the best understood. In females, *Sxl* turns on *transformer* (*tra*) by specifying the female-specific splicing of *tra* transcripts (Nagoshi et al., 1988; Sosnowski et al, 1989; Inoue et al., 1990). The tra protein produced from these female mRNAs together with tra2 (*transformer2*) protein, which is expressed in both sexes, then directs the female-specific splicing of *dsx* transcripts (Nagoshi and Baker, 1990; Hedley and Maniatis, 1991; Hoshijima et al., 1991). In males, where Sxl protein is absent, *tra* is not turned on and *dsx* is spliced in the male mode. The other known cascade that *Sxl* controls is the dosage compensation pathway (Cline, 1984; Gorman et al., 1993). Dosage compensation in males is achieved by hypertranscription of the single X chromosome (Lucchesi and Manning, 1987). This system is turned off in females by *Sxl*. Although the immediate targets have not been identified, *Sxl* may also control this pathway by splicing regulation.

Apart from its somatic functions, *Sxl* is also required for proper development of the female germ line (reviewed in Pauli and Mahowald, 1990; Steinmann-Zwicky, 1992a,b). While much less is known about its role in oogenesis, both the regulation and function(s) of *Sxl* in the germ line seem to differ in important ways from the soma. First, the early *Sxl* promoter is activated in the female soma but not in the progenitors cells of the germ line, the pole cells (Keyes et al., 1992). Additionally, mutations in components of the X:A signalling system that disrupt activation of *Sxl* in the soma have no apparent effect on female germ-line development (Cline, 1983, 1986; Schüpbach, 1985; Steinmann-Zwicky, 1993). Second, in contrast to the soma in which *Sxl* regulation is cell autonomous, it appears that activation of *Sxl* in the germ line not only depends on the chromosomal constitution of the germ cells, but also on inductive signals from the soma (Steinmann-Zwicky et al., 1989; Nöthiger et al., 1989). Finally, the downstream targets of *Sxl* in the germ line are likely to be different from those in the soma. Neither *tra*, *tra2* nor *dsx* are required in the germ line (Marsh and Wieschaus, 1978; Schüpbach, 1982). Furthermore, *Sxl* may not have a role in germ-line dosage compensation. Whereas somatic diplo-X cells deprived of *Sxl* activity die because they fail to turn off the X-chromosome hyperactivation system, the lack of *Sxl* activity in the germ line has no apparent effect on germ cell viability (Schüpbach, 1985).

Transplantation of female pole cells carrying null mutations of *Sxl* into a wild-type female host has provided insights into the possible germ-line function of *Sxl* (Schüpbach, 1985; Steinmann-Zwicky et al., 1989). These germ cells do not enter oogenic differentiation in the adult gonad. Instead, they continue to proliferate forming large cysts that contain many small, undifferentiated germ cells that have a spermatocyte-like appearance. A similar phenotype is exhibited by a special class of recessive female-sterile *Sxl* alleles (Perrimon et al., 1986; Salz et al., 1987). Though the somatic activity of *Sxl* in these alleles appears to be essentially wild type, there is some defect in a germ-line function(s) and mutant females produce ovaries consisting almost entirely of small undifferentiated cells. Interestingly, *Sxl* is not the only gene which, when mutated, exhibits this defect in oogenesis. The tumorous ovary group of female-sterile loci displays a similar phenotype (King and Mohler, 1975), and it is possible that the functions of these genes in germ-line development are related to that of *Sxl*. To learn more about the mechanisms that control *Sxl* activity in oogenesis, we have examined the expression and distribution of *Sxl* RNA and protein in wild-type and various tumorous ovaries.

MATERIAL AND METHODS

Fly strains

Flies were maintained on standard yeast/cornmeal medium and kept at 25°C if not otherwise indicated. The following loci are described by Lindsley and Zimm (1992): *fs(1)1621=fs(1)A1621*, otu^1, otu^3, fu^1, fu^{33}, $ovo^{Drv23}=ovo^{D1rs1}$, Sxl^{f4}, Sxl^{f5}. The *bamΔ86* deletion was generated by imprecise excision of the original P-element mutant line and deletes the entire coding sequence except for 6 residues at the carboxy-terminal end (Dennis McKearin, personal communication). The $otu^{PΔ3}$ mutation deletes about 450 bp upstream of the cDNA start site and displays a tumorous phenotype when homozygous (Steinhauer and Kalfayan, 1992). The *Df(1)fu* stock was obtained from the Bowling Green stock center.

PCR analysis of ovarian RNA and sequencing of mutant alleles

Total RNA was prepared from dissected ovaries or ovary-depleted carcasses. For reverse transcription, we used an internal primer hybridizing to exon 10 of the late transcript (Samuels et al., 1991) following the procedure as described in Frohman et al. (1988). Approximately 4% of the cDNA mixture was then amplified by PCR using a 5′ primer located in exon 2 and a 3′ primer in exon 7. Each amplification employed one cycle of 95°C for 3 minutes, 62°C for 2 minutes, 72°C for 40 minutes followed by 26 repeats of the cycle 95°C for 45 seconds, 58°C for 2 minutes, 72°C for 1.5 minutes. Detection of products was done by Southern analysis with either a female-specific probe derived from cDNA cF1 (Bell et al., 1988) or male exon sequences. Sequences: exon 10 primer 5′ GAGCAGCAACATTGCCCAGGCG 3′; exon 7 primer 5′ CGTGTCCAGCTGATCGTC 3′; exon 2 primer 5′ GTGGTTATCCCCCATATGGC 3′

cDNAs for sequencing were generated by reverse transcription with an exon 10 primer followed by PCR amplification with kinased primers from exons 2 and 10. PCR products were blunt-ended with Klenow and digested with *Cla*I (which cuts once in the cDNA). The final products were cloned into the *Eco*RV and *Cla*I sites of the Bluescript vector. Dideoxy sequencing was performed by the method of Sanger et al. (1977). Exon 10 reverse transcription primer 5′ GTGGAAGTTGATGCAGCCGCTG 3′. Other primers were the same as above.

Analysis of genomic DNA of the *Sxl* female-sterile mutants exploited the mismatch scanning system of Montandon et al. (1989) which detects point mutations other than AT→TA transversions. The method was modified as described by Klein and Meyer (1993). For the analysis of *Sxl*, nineteen pairs of PCR primers were synthesized and used to amplify overlapping 1.5 to 2.0 kb regions that together covered the entire 23 kb transcription unit. Once a mismatch was detected and its general position identified, the change in sequence was determined by producing single-

stranded template DNA by asymmetric PCR (McCabe, 1990) and sequencing by the method of Sanger et al. (1977).

Generation of polyclonal anti-Sxl antibodies

Production and purification of the Sxl/lacZ fusion protein is described in Bopp et al. (1991). This antigen was emulsified in equal volume of Freund's complete adjuvant and injected in multiple subcutaneous sites of two female rabbits (NZW). After 3 boosts with the Sxl/lacZ fusion protein, rabbits were killed. Sera were purified over an affinity-adsorbent column (Boehringer) coupled to Sxl protein purified from bacteria expressing the complete coding sequence of cDNA cF1 (Mark Samuels, manuscript in preparation). Coupling reaction was performed in PBS, 0.1% SDS for 6 hours at room temperature. Prior to passing sera, the column was washed for 90 minutes in 0.35 M triethanolamin-HCl pH 7.8 and thoroughly rinsed with PBS. Bound antibodies were eluted with 0.5 M NaCl/0.2 M glycine-HCl pH 2.8.

Immunoblot analysis

Ovaries were dissected in PBS and frozen on dry ice. While thawing, they were homogenized in 2× loading buffer. The samples were boiled for 5 minutes and loaded on SDS-PAGE. After separation, protein was electrotransferred to nitrocellulose paper in Tris-glycine-methanol. Blots were blocked in 5% low-fat dry milk powder in TBS, 0.05% Tween-20 (TBST). Anti-Sxl antibody was applied as a 1:10 dilution of supernatant of hybridoma line mSXL104 or a 1:500 dilution of affinity-purified anti-Sxl polyclonal serum. anti-BicD monoclonal antibodies were applied as a 1:30 dilution of hybridoma supernatant from line 4C2 (Suter and Steward, 1991). For detection of antibody-antigen complexes, we used the horseradish peroxidase-enhanced chemiluminescence system according to the manufacturer's directions (ELC: Amersham).

For immunoprecipitation, we dissected the gonads of 25 flies and homogenized the tissue in RIPA buffer in the presence of protease inhibitors (see Suter and Steward, 1991). The sonicated extract was precleared with unloaded Protein A-Sepharose beads (Pharmacia) and then incubated in a 1:50 dilution of affinity-purified polyclonal serum for 1 hour and 20 minutes at 4°C. Protein A-Sepharose beads were added and incubated for 2 hours in the cold. Immunoprecipitated material was then washed several times in RIPA buffer prior loading on SDS-PAGE. Sxl protein was probed with monoclonal antibody mSXL104 and the bound rabbit Ig detected with a secondary anti-rabbit antibody coupled to alkaline phosphatase (Promega).

Immunocytochemistry

Mutant and wild-type ovaries were dissected from third instar larvae and 5- to 7-day-old females and fixed for 20 minutes at room temperature in 4% paraformaldehyde (Polysciences EM grade) in phosphate-buffered saline (PBS). The fixative was removed by rinses with PBSTT (PBS, 0.1% Triton-X 100, 0.05% Tween 80) and ovaries were then blocked and permeabilized in 1 mg/ml bovine serum albumin (BSA) in PBSTT for 5-6 hours at room temperature. The first antibody was applied as a 1:10 dilution of supernatant from the hybridoma line mSXL18 (Bopp et al., 1991) in PBSTT containing 0.1 mg/ml BSA, incubated overnight at 4°C and 1 hour at room temperature. After several rinses and washes in PBST, ovaries were treated with biotinylated anti-mouse (3 μg/ml of IgG (H+L); Vector Laboratories) for 1 hour at room temperature. After several washes, ovaries were incubated with FITC or Cy3-labeled Streptavidin (2 μg/ml, Jackson Laboratories) for 45 minutes at room temperature. The samples were counterstained with the nuclear dye propidium iodide (5 μg/ml in mounting solution) when FITC-labeling was applied to detect Sxl protein or, in the case of Cy3 labeling, they were incubated in 1 μM YO-PRO (Molecular Probes) for 10 minutes and subsequently washed in PBS. The stained samples were mounted in 75% glycerine and analyzed by laser scanning confocal microscopy (Krypton-Argon Laser, Biorad MRC 600).

Whole-mount in situ hybridization

Whole-mount in situ hybridizations were performed with PCR-

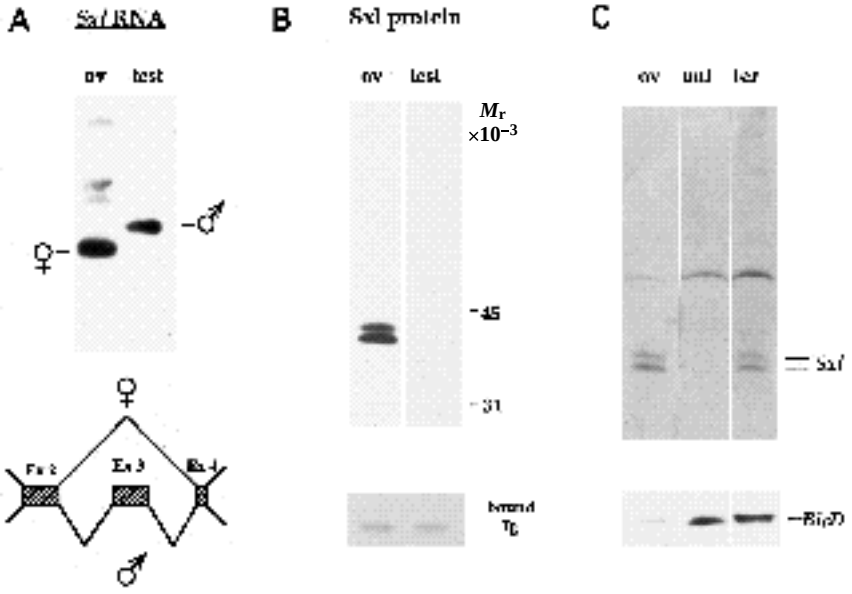

Fig. 1. Analysis of *Sxl* RNA and protein in adult gonads. (A) PCR-amplified splice products in wild-type ovaries (ov) and testes (test) detected with ^{32}P-labeled *Sxl* cDNA probe. The sizes of the expected female- and male-specific fragments are indicated. Minor upper bands in ovaries derive from incomplete processing. (B) Sxl protein was immunoprecipitated from ovarian extracts (ov) and testis extracts (test) with an affinity-purified anti-Sxl polyclonal antibody raised in rabbits (see Material and Methods) and detected with the monoclonal antibody mSXL104 (Bopp et al., 1991) on western blot. Rabbit Ig bound to sepharose beads was detected with an anti-rabbit secondary antibody. (C) Immunoblot containing total protein extracts from dissected ovaries (ov), unfertilized eggs (unf) collected over 6 hours and fertilized eggs (fer) collected over the same period of time. Sxl protein was detected with the anti-Sxl polyclonal antibody. The 55×10^3 M_r band, which appears in all lanes, is due to cross-reactivity of the polyclonal serum. Protein-products of the *Bic-D* gene were detected with a monoclonal antibody (Suter and Steward, 1991) to control for the total amount of protein loaded.

generated single-stranded probes as described by Clifford and Schüpbach (1992). Sense and antisense probes for female-specific Sxl RNA were derived from cDNA MS3 (Samuels et al., 1992). The antisense male exon probe was generated from the genomic clone 619 (G. Calhoun, unpublished result) using an internal primer at the 3′ end of the male exon. The male exon probe contains an additional 293 bp of flanking intronic sequence. The hybridization procedure was basically according to Tautz and Pfeifle (1989) incorporating the modifications for ovary staining as suggested in Suter and Steward (1991). Ovaries were fixed in 200 μl of 4% paraformaldehyde in PBS (PP), 20 μl of dimethyl sulfoxide, 600 μl of heptane for 20 minutes at room temperature. After proteinase K treatment (10 minutes in 50 μg/ml), the ovaries were washed and refixed in PP for 15 minutes. Prehybridization and hybridization were performed at 45°C in 40 μl hybridization buffer containing 50 μg of boiled digoxigenin-labeled probe. For detection we used the alkaline-phosphatase conjugated anti-digoxigenin antibody (1:2000, Genius Kit) and visualized the bound complexes by incubating the tissue with NBT and X-phosphate at 37°C for 1 hour. After stopping the staining reaction with rinses of PBS, the samples were mounted in Aquamount and examined under a Zeiss Axioplan microscope using Nomarski optics.

RESULTS

Post-transcriptional control of *Sxl* in the germ line

In the soma, *Sxl* is transcribed in both sexes and expression of *Sxl* gene products is regulated by sex-specific splicing of the nascent RNA (Bell et al., 1988; Bopp et al., 1991). Since the gene is also transcribed in the germ cells of both sexes, we tested whether the same mechanism of post-transcriptional regulation operates in the germ line. Primers complementary to sequences in exons 2 and 7 (Samuels et al., 1991) were used to amplify cDNAs from RNA purified from hand-dissected female and male adult gonads (Fig. 1A). In ovaries, we observed fragments of the size expected for the female-specific form of *Sxl* mRNAs in which exon 2 is

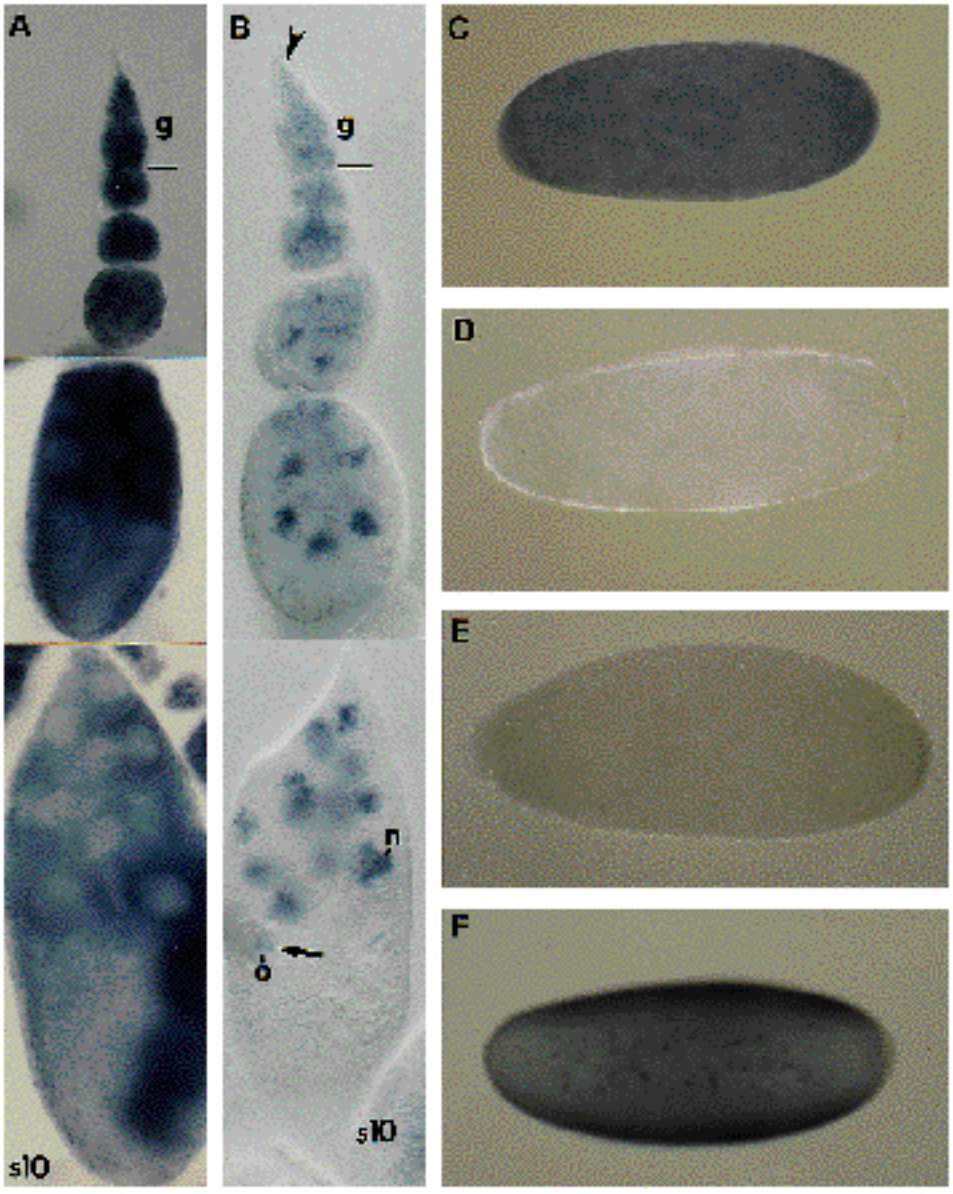

Fig. 2. Distribution of *Sxl* RNA in ovaries and early embryos (A-D) and Sxl protein in early embryos (E,F). (A) Whole-mount ovaries were probed with an anti-sense DNA derived from the female-specific cDNA, cF1 (Bell et al., 1988). While we detect large amounts of *Sxl* transcripts in germ cells of all stages with the antisense probe, little or no hybridization is observed with the sense cDNA probe (not shown). (B) Whole-mount ovaries were probed with an anti-sense DNA generated from the male exon. Arrowhead in B points to the anteriormost germ cells in the germarium and the arrow marks the position of the oocyte nucleus in an early stage 10 egg chamber (g, germarium; n, nurse cell nucleus; o, oocyte nucleus; s10, stage 10). (C,D) Cleavage stage embryos were probed with either the female-specific DNA or the male exon, respectively. (E,F) Embryos were treated with monoclonal antibodies against female-specific proteins of *Sxl* as described in Bopp et al. (1991). The embryo in E was arrested at the cleavage stage, while embryo in F was arrested at nuclear division 14 during cellularization.

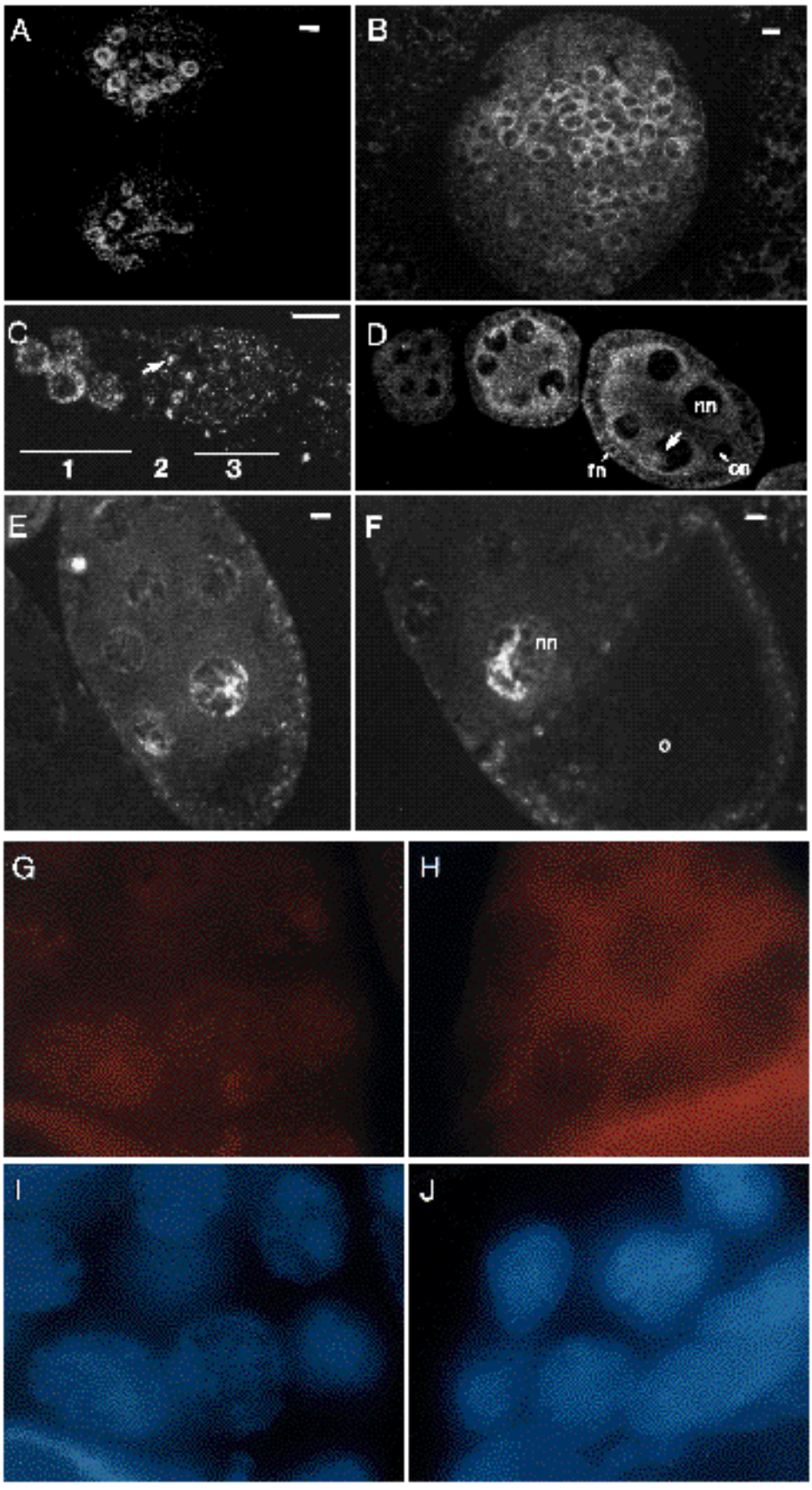

Fig. 3. Distribution of Sxl protein in female gonads. Whole mount ovaries were treated with anti-Sxl monoclonal antibody mSXL18 and analysed by confocal microscopy (A-F) or by whole-mount immunofluorescence (G-J). (A) Two optical sections through different planes in the female gonad of an early third instar larva. (B) Confocal image of a late third instar ovary showing apical (top) to basal (bottom) polarity. (C) Distribution of Sxl protein in the three regions of the germarium (arrow points to a focus of concentrated protein in region 2). (D) Optical section through early previtellogenic stages (arrow indicates localization of Sxl protein in the nurse cell nucleus). (E) Confocal image of a stage 8 egg chamber. (F) Section through an early stage 10 egg chamber. (G) Sxl protein in late stage 10 nurse cells and corresponding Hoechst staining (I). (H) Sxl protein in stage 11 nurse cells and corresponding Hoechst staining (J). (nc, nurse cell nucleus; on, oocyte nucleus; fn, follicle cell nucleus; o, oocyte). Bar, 10 μm.

joined directly to exon 4. In contrast, these female-specific spliced products were not detected in testes. Instead, the amplified fragments in testes were the size expected for the male-specific splice products where the translation-terminating exon 3 is included in the *Sxl* mRNA. These findings predict that full-length Sxl proteins should be expressed in ovaries but not in testes. Immunoprecipitation of Sxl protein from a large number of hand-dissected gonads with an affinity-purified polyclonal antiserum (see Material and methods) confirmed this expectation. As shown in Fig. 1B, the two major full-length forms of Sxl protein that are observed in the female soma are present in ovarian extracts but not in testis extracts. We conclude that sex-specific processing of nascent *Sxl* RNA is responsible for maintaining the appropriate state of *Sxl* activity not only in the soma but also in the germ line.

Distribution of *Sxl* RNA in adult ovaries and early embryos

To investigate the transcription of *Sxl* in the female germ line, we hybridized single stranded probes from a female-specific cDNA, cF1 (Bell et al, 1988), to whole-mount ovaries isolated from wild-type flies. *Drosophila* ovaries are composed of 16-18 ovarioles, which contain multiple egg chambers at different stages of differentiation (reviewed by King, 1970; Mahowald and Kambysellis, 1980). The anteriormost structure of the ovariole is the germarium where the stem cells and their progeny undergo a series of divisions to form a cluster of 16 interconnected cells, the egg chamber. One of the cells in this chamber becomes the oocyte, while the remaining 15 become nurse cells. As shown in Fig. 2A, a substantial amount of *Sxl* RNA is present in all cells of the germarium including the stem cells. The egg chamber matures as it proceeds down the ovariole, and this process has been divided into 14 stages. In the early stages (previtellogenesis), the nurse cells undergo multiple rounds of endomitotic replication and grow in size. During these stages, high levels of *Sxl* RNA accumulate in the cytoplasm of nurse cells while lower levels are detected in the somatically derived follicle cells that surround each chamber. At the onset of yolk deposition (vitellogenesis), stage 8 of oogenesis, the nurse cells begin transporting a wide range of factors into the maturing egg. *Sxl* mRNA appears to be included in this process as considerable levels of RNA are detected in the growing oocyte (see stage 10 egg chamber in Fig. 2A). At the end of vitellogenesis, stage 11, the nurse cells dump their remaining contents into the oocyte and then degenerate. The *Sxl* RNA deposited in the oocyte appears to be stable as it persists after fertilization through the early cleavage stages in embryogenesis (Fig. 2C).

We also hybridized whole-mount ovaries with a single stranded probe that only detects male exon sequences. In contrast to the hybridization pattern observed with the female cDNA probe, no cytoplasmic staining was evident even after prolonged incubation. However, we did observe stained dots in the nuclei of both the germ cells and follicle cells (see Fig. 2B). Since we failed to detect male-spliced *Sxl* RNAs in wild-type ovaries (Fig. 1A), it is likely that the nuclear signal is due to hybridization to incompletely processed nascent transcripts. These nuclear dots are present in the anteriormost cells of the germarium beneath the terminal filament indicating that *Sxl* is transcriptionally active in the stem cells (arrowhead in Fig. 2B). With progression through the pre- and vitellogenic stages, the number of dots in nurse cell nuclei increases correlating with the increase in haploid genome equivalents that occurs during polyploidization. Nuclear dots are also seen in the oocyte nucleus at stages 9 and 10 indicating that the germinal vesicle transcribes *Sxl* at these stages (arrow in Fig. 2B). From stage 11 on, nuclear staining in the nurse cells and the oocyte begins to fade and disappears completely by stage 12 (not shown).

Distribution of Sxl protein during oogenesis

The distribution of Sxl protein in the germ line is considerably more complex than that of the mRNA. The female gonads of early third instar larvae contain mostly large proliferative germ cells, oogonia, that have not yet undergone differentiation. By the late third instar, these large germ cells are confined to the middle region of the gonad with smaller somatic cells of mesodermal origin extending from the apical and basal poles (Lasko and Ashburner, 1990). In both the early (Fig. 3A) and late third instar gonads (Fig. 3B), high levels of Sxl protein are found in the oogonial cells, while much lower levels are detected in the somatic mesodermal cells. Surprisingly, most of the Sxl protein appears to accumulate in the cytoplasm, not in the nucleus. This distribution is in marked contrast to the predominantly nuclear localization seen in somatic cells (Bopp et al., 1991).

The unusual cytoplasmic localization of Sxl protein is also seen in the large cells in the anteriormost region of the adult germarium (Figs 3C, 4B). As interpreted from EM sectioning studies by Koch and King (1966) (Fig. 4A), this part of the germarium contains two or three stem cells, one or two cystoblasts (daughters of the stem cells), and one or two 2-cell cysts (cystoblasts that have undergone one division with incomplete cytokinesis). Approximately the same number of cells, 5-9, display the strong cytoplasmic accumulation of Sxl protein. In the posterior half of region 1, which contains the proliferating cystocytes that form 4-cell, 8-cell and 16-cell cysts, there is an abrupt transition in the staining pattern (Figs 3C, 4B). The level of cytoplasmic Sxl protein appears to drop precipitously and only a low level of diffuse staining is observed in the small cystocytes found in this part of the germarium. By the time the mesodermal cells start to invade centripetally and surround the newly formed 16-cell cluster in region 2 of the germarium, a second change in localization occurs. The protein now becomes concentrated in foci within the nuclei of the cystocytes. The nuclear localization of these stained foci was confirmed by double staining with a nuclear dye (Fig. 4B). In region 3 of the germarium, these nuclear foci become less conspicuous and, instead, we observe low levels of diffuse staining. To demonstrate that these changes in localization are specific to Sxl protein, we examined the distribution of the *Drosophila* antigen that is recognized by a monoclonal antibody directed against the rat B″ U2 snRNP protein (Habets et al., 1989). In contrast to Sxl protein, the *Drosophila* antigen recognized by this monoclonal antibody appears exclusively nuclear in all cells of the germarium (Fig. 4C).

The amount of Sxl protein increases again and most of it

appears to be localized to the cytoplasm of the nurse cells during the previtellogenic stages of oogenesis. While there seems to be some Sxl protein in the growing oocyte (Fig. 3D), the level appears lower than in the nurse cell cytoplasm. Sxl protein is also evident inside the polyploid nurse cell nuclei at these stages and appears to be located close to the nuclear envelope (arrow in Fig. 3D). Also, we often observe strong staining in the cytoplasm of the somatically derived follicle cells that surround the egg chamber (Fig. 3D). During the vitellogenic stages, much of the Sxl protein appears to accumulate in the nurse cell nuclei, particularly in the posteriormost nurse cells which are closest to the oocyte (Fig. 3E,F). There is a low level of cytoplasmic staining in the nurse cells at these stages; however, little or no protein is evident in the growing oocyte (Fig. 3E,F). By stage 10 of oogenesis, staining of Sxl protein coincides largely with Hoechst-stained structures in the nurse cell nuclei (Fig. 3G,I).

An abrupt change in localization is again observed in chambers at stage 11 and older. Sxl protein becomes excluded from the nurse cell nuclei and instead accumulates in the nurse cell cytoplasm (Fig. 3H,J). In follicle cells, the protein predominantly localizes to the nucleus forming a number of foci of concentrated protein (Fig. 3E,F). After the nurse cells start to degenerate at stage 11/12, the staining in the follicle cell nuclei is progressively confined to one or two brightly fluorescent spots per nucleus (not shown).

Maternal contribution of *Sxl*

Throughout most of oogenesis, Sxl protein is localized predominantly in nurse cells while there appears to be little or no protein in the maturing oocyte. This appears to be true even near the end of oogenesis when the nurse cells dump their contents into the oocyte. We tested whether Sxl protein is deposited and stably stored in the mature oocyte by immunoblot analysis. Protein extracts from ovaries, unfertilized eggs and fertilized eggs of wild-type flies were probed with a polyclonal anti-Sxl antiserum. As shown in Fig. 1C, Sxl proteins are abundant in extracts from ovaries but not detectable in extracts from unfertilized eggs. An equivalent amount of protein extract from fertilized eggs shows considerable levels of Sxl protein which is derived from zygotic expression of the gene (Bopp et al., 1991).

The absence of Sxl protein in unfertilized eggs despite the presence of large quantities of maternal *Sxl* mRNAs indicates that these mRNAs are not translated prior to fertilization. The transcripts persist for 1-2 hours after fertilization (Salz et al., 1989) and appear to be evenly distributed in early cleavage embryos (Fig. 2C). Even though these maternal RNAs are exclusively processed in the female mode (no hybridization to male-specific exon sequences was observed, Fig. 2D), they do not appear to be translated as no protein is detected in early cleavage stage embryos of both sexes (Fig. 2E). Sxl protein is first detected when *Sxl* is zygotically activated at the blastoderm stage (Keyes et al., 1992), as shown in Fig. 2F.

Abnormal Sxl expression in tumorous ovary mutants

Genes in the tumorous ovary group of female-sterile loci appear to function in early germ-line differentiation. Mutations in these loci disrupt the formation of the 16-cell egg chamber and generate, instead, cysts that contain large numbers of small undifferentiated cells. Since a similar phenotype is exhibited by the female-sterile alleles of *Sxl*, it was of interest to determine if any of the genes in the tumorous ovary group influenced either the expression or localization of *Sxl* gene products in the female germ cells. Our analysis suggests that the tumorous ovary genes can be divided into two classes on the basis of their effects on *Sxl*. Mutations in the first class block *Sxl* expression, while in the second class both the expression and the localization of *Sxl* protein is perturbed.

Mutations that block Sxl expression

Defects in the germ-line expression of Sxl protein are observed in tumorous ovary alleles of two female-sterile loci, *fs(1)1621*(also named *liz* in Steinmann-Zwicky, 1988 or *sans-fille* in Oliver et al., 1988) and *ovarian tumor* (*otu*; King and Storto, 1988). As can be seen for *fs(1)1621* in Fig. 5A,B abnormalities in *Sxl* expression are already evident in immunostained gonads from female third instar larvae. In gonads from animals heterozygous for the *fs(1)1621* mutation (Fig. 5B), the expression and distribution of Sxl protein resembles that observed in wild type (compare Fig. 3B). There is a high level of cytoplasmic Sxl protein in the oogonial cells, while the somatic cells at the poles display a weak and diffuse staining pattern. In contrast, gonads from homozygous mutant animals show that Sxl protein expression in the oogonial cells is severely reduced, and only uniformly weak and diffuse staining is observed (Fig. 5A). The expression of protein is also affected in gonads dissected from adult females. The germ cells populating the mutant gonad have little or no Sxl protein, while apparently normal levels are expressed in the surrounding somatic follicle cells (Fig. 5C,D). Occasionally, we observe single or small clusters of germ cells displaying high levels of cytoplasmic protein like that seen in wild-type oogonial cells (arrow in Fig. 5C). Consistent with these immunostaining results, protein extracts prepared from mutant *fs(1)1621* ovaries show levels of Sxl protein which are comparable to that found in the agametic ovaries of ovo^{-} mutant animals (Fig. 6C).

Like in *fs(1)1621,* expression of Sxl protein is severely reduced in the germ cells from females homozygous for otu^{1}. The whole-mount staining of otu^{1} ovaries is shown in Fig. 5E, and the Western blot is shown in Fig. 6C. We also analyzed the expression of Sxl protein in two other *otu* alleles, $otu^{P\Delta 3}$ and otu^{3} (Steinhauer and Kalfayan, 1992). The tumorous ovaries of $otu^{P\Delta 3}$ homozygous females were similar to otu^{1} and have little or no Sxl protein in the germ cells. A different result was obtained with otu^{3}. Unlike either otu^{1} or $otu^{P\Delta 3}$, the tumorous cysts in ovaries of otu^{3} homozygous females have readily detectable levels of Sxl protein (not shown). However, the distribution of Sxl protein in otu^{3} appears to be abnormal. As is observed for the tumorous ovary mutants described in the next section, small undifferentiated germ cells that populate the otu^{3} mutant ovaries have predominantly cytoplasmic Sxl protein.

The severe reduction in Sxl protein expression in *fs(1)1621* and otu^{1} mutant ovaries is likely to be due to a

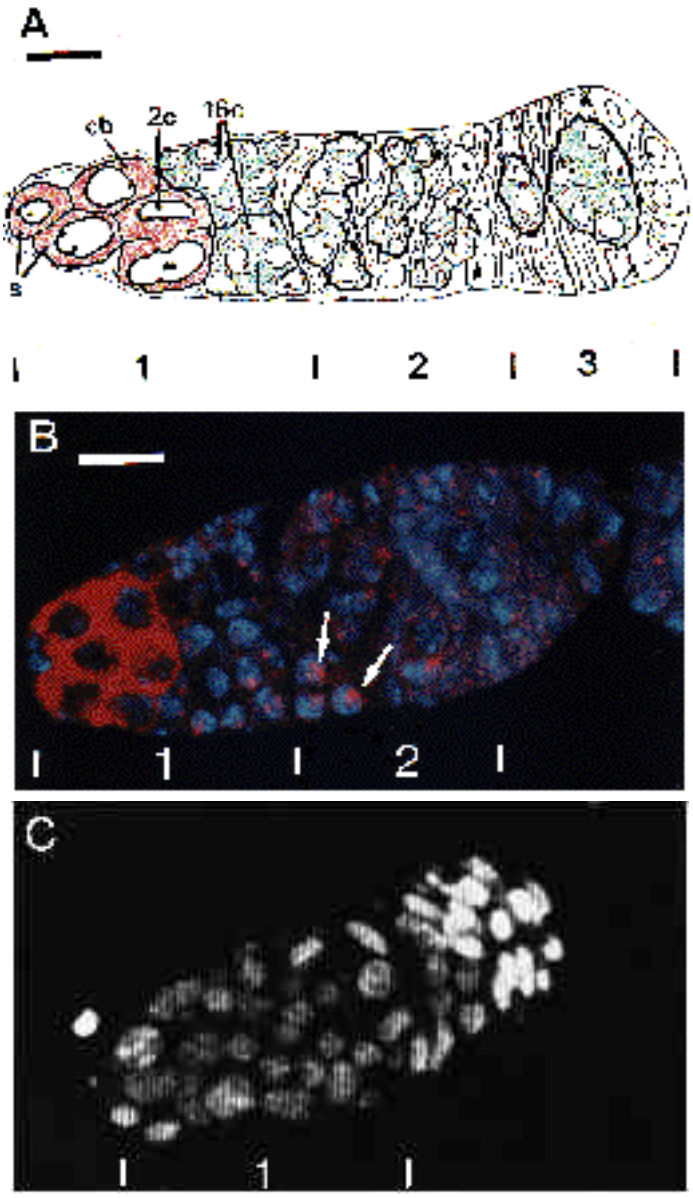

Fig. 4. Distribution of Sxl protein in the germarium. (A) Drawing of an EM section through the germarium (taken from Koch and King, 1966). 16-cell cysts (16c) are colored in green, while earlier stages are labeled red (s, stem cell; cb, cystoblast; 2c, 2-cell stage). (B) Double labeling for Sxl protein (red) and a nuclear dye, propidium iodide (blue). Arrows point at foci of concentrated protein in cystocyte nuclei. (C) Germarium stained with a monoclonal antibody against the rat B″ snRNP. The anterior tips of the germaria are pointing to the left and the extensions of region 1, 2 and 3 are marked with vertical bars. Bar, 10 μm.

failure to initiate and/or maintain the female splicing mode properly. To test this possibility, we examined the splicing pattern of *Sxl* transcripts in ovaries mutant for *fs(1)1621* by reverse transcription and PCR amplification. In contrast to wild-type ovaries, substantial levels of male-spliced *Sxl* RNA were detected (Fig. 6A,B). This result is consistent with the idea that regulation of *Sxl* splicing is impaired in germ cells mutant for *fs(1)1621*. That *fs(1)1621* affects the autoregulatory feedback loop of *Sxl* is also suggested by analysis of *Sxl* RNA processing in the somatic tissue of homozygous mutant females. As can be seen in Fig. 6B, there is a small but clearly detectable level of male-spliced RNA which is never observed in the soma of wild-type females. Even though this mutation by itself has no apparent phenotypic defect in the soma, splicing regulation of *Sxl* is not as efficient as in the wild-type condition. We also examined the splicing regulation of *Sxl* in the otu^1 background. As was the case for *fs(1)1621*, we observe substantial levels of male-spliced RNA in the otu^1 ovaries (Fig. 6A).

Mutations that alter Sxl protein distribution

Members of this class are the female-sterile loci *bag-of-marbles* (*bam*, McKearin and Spradling, 1990) and *fused* (*fu*; King, 1959). Unlike either *fs(1)1621* or otu^1, high levels of Sxl protein are present in tumorous ovaries mutant for these loci (Fig. 7A,B). The Sxl proteins from ovaries of females homozygous for a null allele of the *bam* gene, *bamΔ86* (Dennis McKearin, personal communication), comigrate with the two major protein variants seen in wild-type ovaries (Fig. 6C). However, immunolocalization in the mutant ovaries indicates that the distribution of Sxl protein is abnormal. In wild-type ovaries, high levels of cytoplasmic staining are confined to cells in the anterior part of the germarium, while there is a marked reduction in the level of cytoplasmic staining in the cells immediately posterior. Following this reduction in the levels of cytoplasmic staining, there is a redistribution of protein into the nuclei forming foci (see Fig. 4B). In both the *bam* and *fu* ovaries, this transition in the level and distribution of Sxl protein does not seem to occur and most if not all the small germ cells that populate the tumorous cysts show high levels of cytoplasmic Sxl protein.

We also examined the processing of *Sxl* mRNAs in *bam* and *fu* ovaries by reverse transcription and PCR amplification. As can be seen in Fig. 6A, in *fu* mutant ovaries *Sxl* transcripts are exclusively processed in the female mode indicating that splicing regulation of *Sxl* is not affected. A small amount of male-spliced RNA can be detected in *bam* mutant ovaries; however, Sxl protein levels appear normal in *bam* (Fig. 6C).

Sxl protein distribution in a temperature-sensitive fused mutant

To learn more about the role of the *fused* gene in the subcellular localization of Sxl protein, we stained ovaries from females homozygous for a temperature-sensitive allele of *fused*, fu^{33}, with an anti-Sxl antibody at different times after a shift from the permissive (18°C) to the restrictive temperature (29°C). At the permissive temperature, oogenesis in fu^{33} females appears normal, and the subcellular distribution of Sxl protein in the germarium and in egg chambers at different stages resembles that observed in wild-type ovaries. No differences in the Sxl staining pattern or in oogenesis were evident when the fu^{33} flies were shifted to the restrictive temperature for one day. After 3 days at the non-permissive temperature, there appears to be a slight increase in the number of cells in the germarium that

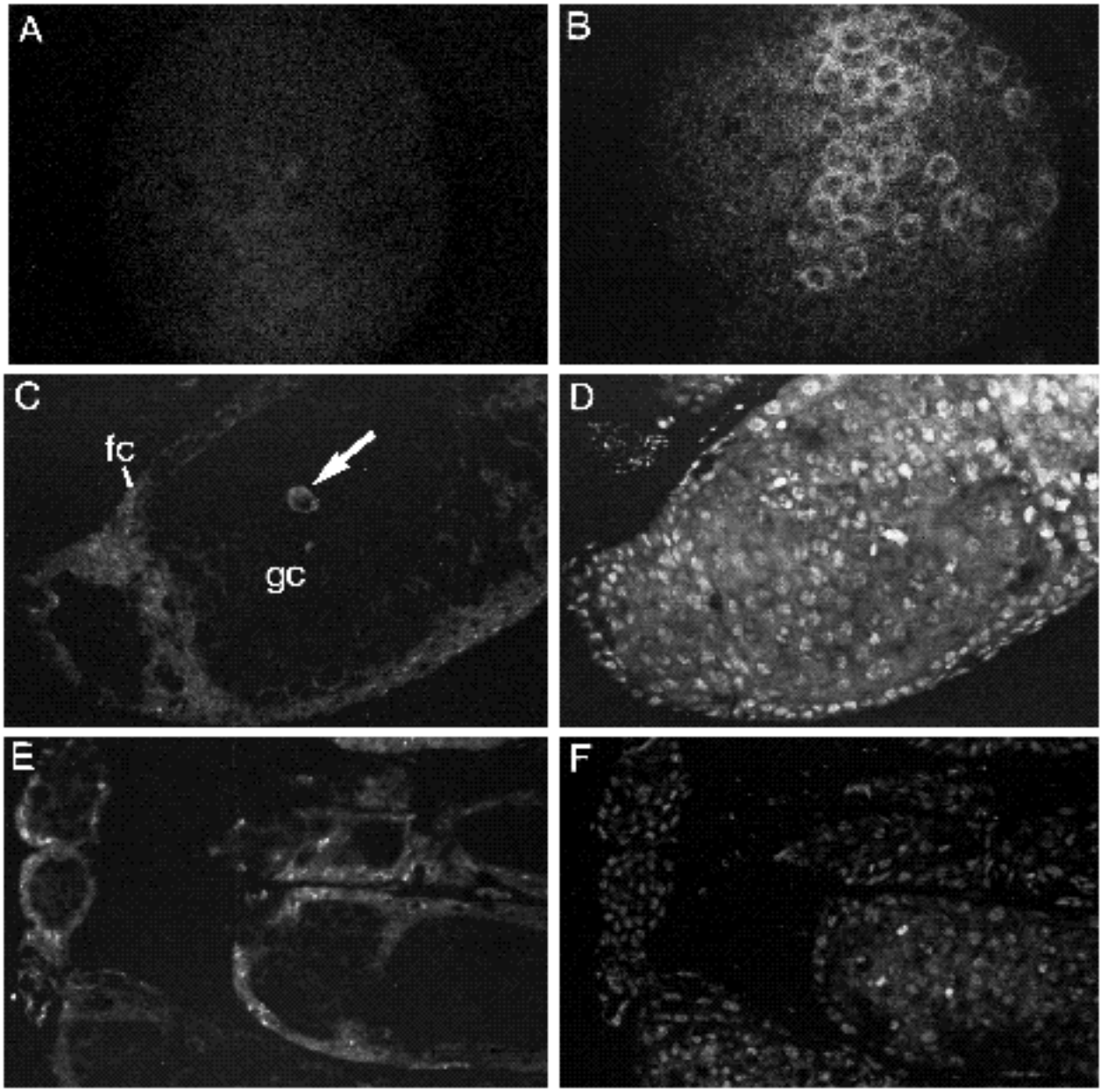

Fig. 5. Analysis of Sxl protein distribution in germ cells mutant for *fs(1)1621* or *otu*^{1} using confocal microscopy. (A) Optical section through an ovary from a late third instar larva homozygous for *fs(1)1621*. (B) Ovary of the same stage from a heterozygous animal (*fs(1)1621*/Binsc). (C) Sxl protein in the adult gonad of a female homozygous for *fs(1)1621*. Arrow points at a single germ cell that expresses high levels of Sxl protein (fc, follicle cell; gc, germ cell) and nuclear staining of the same section (D). (E) Sxl protein in the adult gonad of a female homozygous for *otu*^{1} and corresponding nuclear staining (F).

contain high levels of cytoplasmic Sxl protein (Fig. 7C). Some of these cells appear smaller in size than the cells normally observed (arrows in Fig. 7C). However, the egg chambers posterior to the germarium show normal Sxl protein distribution and morphology. After 5 days at 29°C, aberrations in the early stages of oogenesis become clearly apparent. First, the number of cells in the anterior region of the germarium that exhibit high levels of cytoplasmic Sxl protein increases (see Fig. 7D). Second, the posterior region of the germarium becomes filled with a number of small cells which also exhibit cytoplasmic staining. In some instances, we observe structures posterior to these enlarged germaria which resemble cysts of tumorous ovaries. These aberrations appear to be confined to the early stages of oogenesis, while cysts and egg chambers that have proceeded farther in the oogenic pathway seem to be unaffected by the temperature shift. At these later stages, the distribution of Sxl protein is similar to that in wild-type flies, as is the morphology of the developing egg chambers. Our analysis suggests that this temperature-sensitive allele of *fu* affects only early steps in oogenic differentiation during cystocyte proliferation.

Redistribution of Sxl protein does not occur in female-sterile mutations of *Sxl*

Two recessive *Sxl* mutants, Sxl^{f4} and Sxl^{f5}, have been identified that have early defects in oogenesis similar to those observed in the tumorous ovary loci described above (Perrimon et al., 1986; Salz et al., 1987). Neither of these female-sterile alleles exhibits any obvious phenotypic effects in the soma, and both complement complete null mutations for viability and somatic differentiation (Salz et al., 1987). It is thus likely that these alleles either are misregulated in the germ line or encode proteins that are defective in germ-line function(s). To distinguish between these alternatives, we examined the splicing pattern of *Sxl* transcripts and the expression of Sxl protein in females mutant for these alleles.

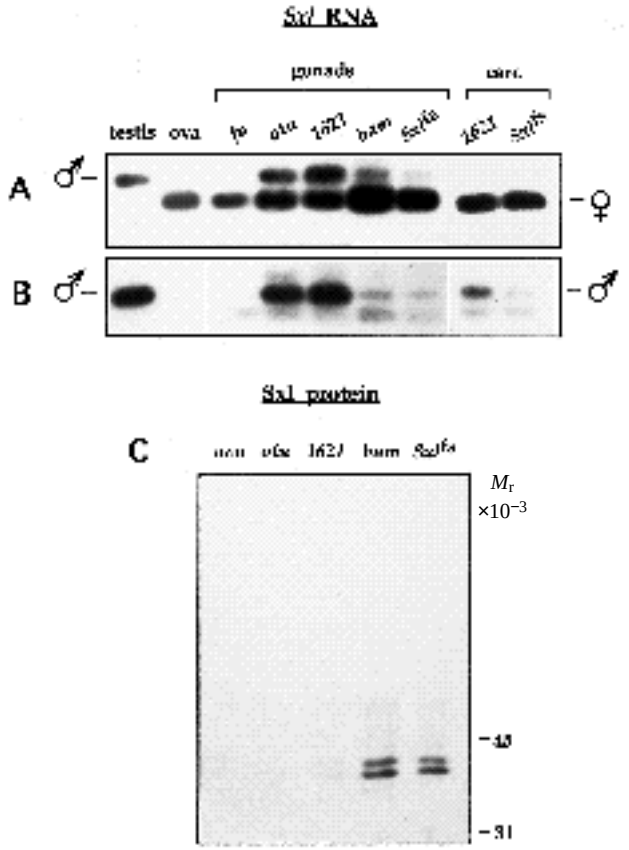

Fig. 6. Processing of *Sxl* RNA and expression of Sxl protein in tumorous ovaries. PCR-amplified splice products in ovarian (ova) or testes extracts were resolved by electrophoresis and detected by either a female *Sxl* cDNA probe (A) or a probe-specific for the male exon (B). Mutant flies were analysed by amplifying *Sxl* RNA from dissected gonads (gonads) or carcasses depleted of gonads (carc). Exposure times for gonads and carcass blots are different so the band intensities do not reflect relative amounts. The amount of male-spliced RNA in the carcasses is low and difficult to observe with the common female probe but reproducibly detected with the male exon probe. Genotypes of the mutant flies are the following: *fu=fu*^{1}*/Df(1)fu*; *otu=otu*^{1}*/otu*^{1}; *1621=fs(1)1621/fs(1)1621*; *bam=bamΔ86/bamΔ86*; $Sxl^{fs}=Sxl^{f4}/Sxl^{f4}$. The expected sizes of female- and male-specific fragments are indicated. (C) Immunoblot of Sxl protein in mutant gonads. The amount of protein equal to two ovaries were loaded in each lane and Sxl protein was detected with the monoclonal antibody mSXL104. Control for somatic contribution of Sxl protein is shown in the *ovo* lane containing ovarian extracts from ovo^{Drv23}/ovo^{Drv23} females. This amorphic allele of *ovo* produces gonads that are depleted of germ cells (Oliver et al., 1987).

Sxl splicing in the female steriles

We find that splicing regulation of *Sxl* in both the germ line and the soma is not completely normal in these mutants. As shown in Fig. 6A,B, a very small but detectable level of male-spliced *Sxl* RNA is present in ovaries as well as the soma of females mutant for Sxl^{f4}. A small amount of male-spliced RNA is also observed in the Sxl^{f5} mutant ovaries (not shown). In spite of this defect, it seems unlikely that a failure of these mutants to establish properly or maintain the autoregulatory feedback loop is responsible for their phenotype. Although male-spliced RNA is detected in both mutants, most of the *Sxl* transcripts are processed in the female mode and immunostaining of mutant gonads reveals high levels of Sxl protein in the germ cells of both Sxl^{f4} (Fig. 8) and Sxl^{f5} (not shown). Moreover, as shown in Fig. 9, both of the major Sxl protein variants are detected in extracts from homozygous mutant gonads.

Sxl protein distribution in female steriles

While autoregulation does not seem to be significantly impaired in these two female-sterile alleles, there are abnormalities in the distribution of Sxl protein in the mutant germ cells. In wild-type ovaries, we observe an abrupt transition in the distribution and level of Sxl protein in region 1 of the germarium (see Fig. 4B). As shown in Fig. 8A, this transition does not occur in ovaries from Sxl^{f4} mutant females. Most, if not all, the germ cells in the germarium show high levels of cytoplasmic Sxl protein, while the characteristic nuclear foci that normally appear in region 2 of wild-type germaria are not observed. Moreover, this high level of cytoplasmic protein persists even in older cysts (Fig. 8B, C, D). The tumorous ovaries of Sxl^{f5} mutant females display the same alteration in protein distribution (not shown).

The female-sterile alleles of Sxl express an altered gene product

Since the Sxl proteins expressed in the mutants exhibit normal electrophoretic migration (Fig. 9), we suspected that the mutations arise from minor alterations in the protein structure. To identify these changes, we examined the protein-coding sequence of *Sxl* mRNAs from homozygous mutants. cDNAs covering the bulk of the open reading frame were generated by reverse transcription and PCR amplification and sequenced. In both mutants, we detected a single base change that alters the predicted Sxl protein sequence. These alterations appear to be the only changes in the entire *Sxl* gene. When genomic DNA sequences of Sxl^{f4} and Sxl^{f5} were compared by the heteroduplex mismatch detection system of Montandon et al. (1989) only one region of mismatch was detected. When sequenced, the mismatch was found to be derived from the same changes observed in the cDNAs.

As shown in Fig. 9, the mutations in both alleles are

located in the carboxy-terminal part of the protein, well downstream from the second Sxl RRM (RNA Recognition Motif; compare sequences in Bell et al., 1988 and Samuels et al., 1991). This is an unusual region of the protein in that it contains a stretch of 7 proline residues interrupted by a single glutamine. In Sxl^{f4} the CCA codon for the proline residue at position 318 is changed to TCA so that it encodes a serine residue. The alteration in Sxl^{f5} is located

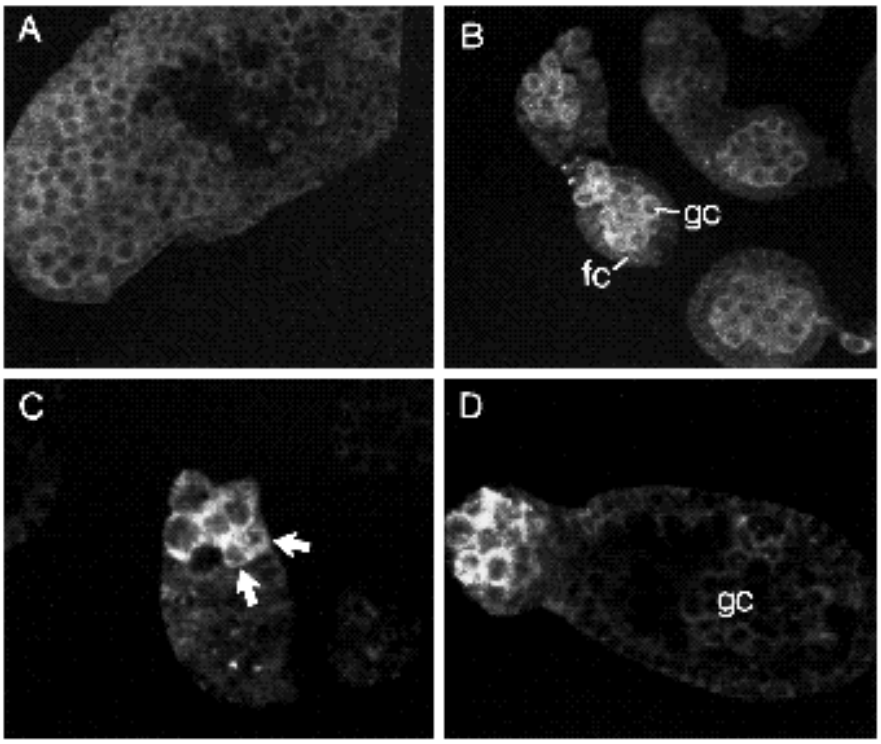

Fig. 7. Distribution of Sxl protein in *bam* and *fu* mutant ovaries. (A) Optical section through the apical part of an ovariole from homozygous *bamΔ86* females. (B) Optical section through tumorous cysts in females transheterozygous for $fu^1/Df(1)fu$. (fc, follicle cell; gc, germ cell). (C,D) Confocal images of germaria homozygous mutant for a temperature-sensitive allele of *fu*, fu^{33}, maintained at the restrictive temperature (29°C) for 1 day (C) or 5 days (D). Arrows in C show persistent staining in small proliferating cystocytes (gc, germ cell). Bar, 10 μm.

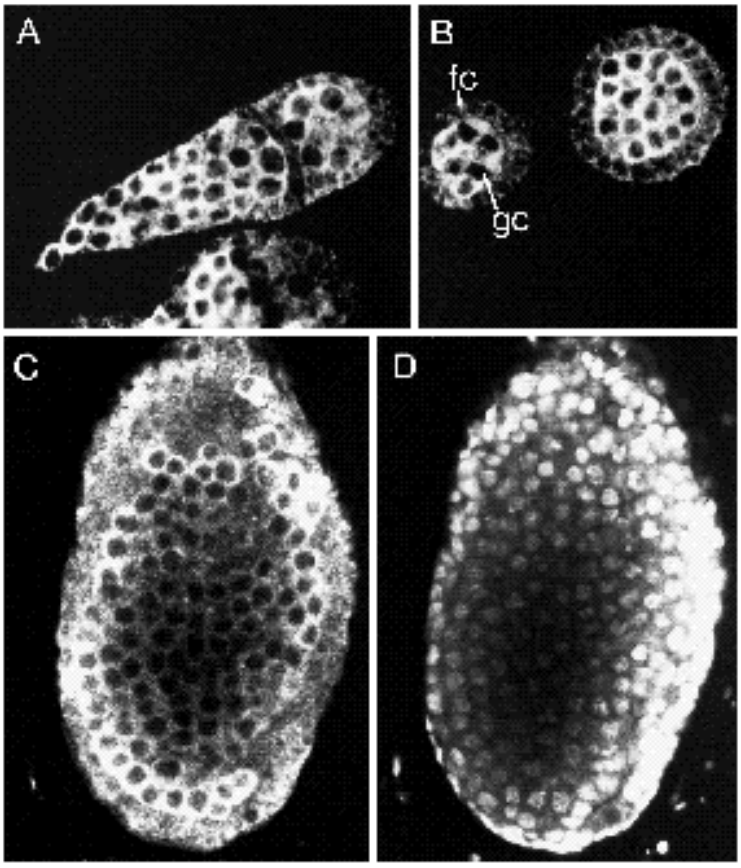

Fig. 8. Distribution of Sxl protein in ovaries homozygous for the female-sterile *Sxl* mutation, Sxl^{f4}. Three different optical sections of one mutant ovariole are shown in A-C. (A) The anteriormost end of the ovariole. (B) Two tumorous cysts located posterior to section shown in (A). (C) A large tumorous cyst at the posterior end. (D) Propidium iodide nuclear staining of the cyst shown in C. (fc, follicle cell; gc, germ cell).

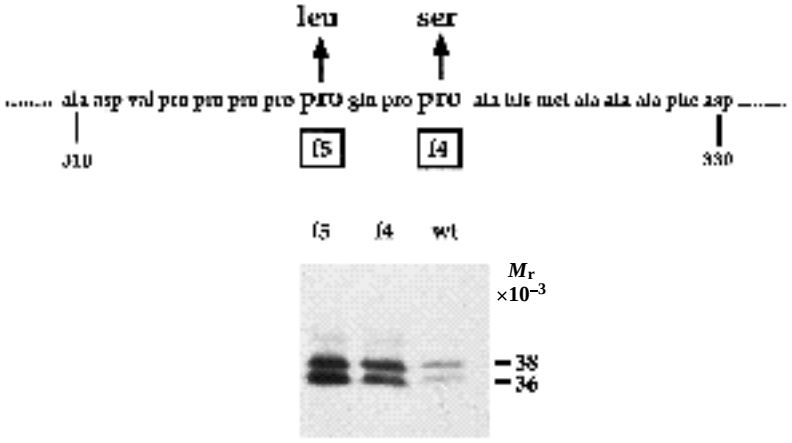

Fig. 9. Sequence analysis of female-sterile mutations of *Sxl*. The Sxl^{f4} and Sxl^{f5} alleles express altered gene-products with a single non-conservative change close to the carboxy-terminal end of the protein (for complete sequence see Bell et al., 1988). Immunoblot analysis of mutant and wild-type ovaries demonstrate that the mutant alleles generate two protein variants which co-migrate with the major antigens observed in wild type (f4=Sxl^{f4}/Sxl^{f4}; f5=Sxl^{f5}/Sxl^{f5}; wt=*Oregon R* wild type).

only 3 amino acids upstream, and also affects a proline residue. In this case, the CCA codon for the proline at position 315 is changed to CTA so that it encodes leucine. It is unlikely that either of these changes derive from naturally occurring polymorphisms as they have not been found in any of the cDNAs derived from wild type or genomic sequences which have been analyzed (Bell et al., 1988, Samuels et al., 1991; Keyes et al., 1992). Additionally, since both mutants were derived from the same parental chromosome, the unique change observed in each mutant would indicate that the chromosome was originally wild type at that position.

Localization of the mutant protein in the soma and heterozygous germ line

The changes in Sxl^{f4} and Sxl^{f5} proteins are only 3 residues apart and both alter a proline residue. When the two alleles are combined, they fail to complement each other for germline function and give rise to the same pattern of Sxl protein distribution as either mutant alone. These observations raise the possibility that the amino acid substitutions cause a defect in a localization domain, influencing the ability of the protein to redistribute and, thus, resulting in aberrant germ-line development. To address this possibility, we first tested whether the mutant Sxl^{f4} protein is localized normally in somatic cells. A collection of embryos, in which half the female animals are expected to be homozygous for Sxl^{f4} and the other half heterozygous, were stained with Sxl antibodies. Sxl protein was found to be predominantly nuclear in the somatic cells of all the embryos examined and there were no conspicuous differences in protein distribution amongst the embryos (not shown). Hence, the Sxl^{f4} mutation does not appear to affect the subcellular localization of Sxl protein in somatic cells. In a second experiment, we examined the distribution of Sxl protein in germ cells from females heterozygous for the Sxl^{f4} mutation. If the change in the Sxl^{f4} mutation inactivates a domain required for redistribution, the protein that is expressed by the Sxl^{f4} allele might be expected to persist at high levels in the cytoplasm of differentiating cystocytes even though the development of these cells is proceeding normally. Contrary to this expectation, the protein distribution in the heterozygote appears normal and is indistinguishable from wild type (as in Fig. 4B).

DISCUSSION

Female germ cells require the activity of the *Sxl* gene to proceed normally through oogenic differentiation (Schüpbach, 1985; Steinmann-Zwicky et al., 1989; Nöthiger et al., 1989). In the studies reported here, we have examined how *Sxl* activity is controlled in wild-type germ cells and in germ cells defective in early steps of oogenic differentiation. Like the soma, it appears that regulation by alternate splicing is the basic mechanism for controlling *Sxl* activity in the germ line. However, the complex and dynamic patterns of Sxl protein expression and distribution during oogenesis suggest that the function of the gene may be controlled at several levels in the germ line.

Autoregulation in the germ line

Our analysis of *Sxl* transcripts in wild-type ovaries and testes indicates that the on/off regulation of *Sxl* in the germ line is based on the alternate splicing of the translation-terminating exon, exon 3. In addition, though our evidence is indirect, it seems likely that the mechanism of regulation in the germ line is an autoregulatory feedback loop in which female Sxl protein directs the female processing of *Sxl* transcripts. This view is supported by our finding that the female-sterile alleles of *Sxl* have low levels of male-spliced RNA both in the soma - where the case for autoregulation is well documented - and in the germ line. Since the only mutation in these flies is in *Sxl*, the presence of male RNA would argue that *Sxl* must regulate its own expression in the germ line. As discussed below, studies on the female-sterile locus *fs(1)1621* would also suggest that the *Sxl* autoregulatory feedback loop operates in the germ line.

We have shown that *fs(1)1621* is defective in the splicing regulation of *Sxl* transcripts and high levels of male RNAs accumulate in the female germ line. Significantly, the *Sxl* splicing defect in the *fs(1)1621* mutant is not confined to the germ line; we also observe male-processed RNAs in the soma of adult females (~5 % of the *Sxl* RNA). Since clones of somatic cells showing male differentiation are not observed in homozygous *fs(1)1621* females, it seems unlikely that these male-spliced *Sxl* RNAs are from cells in which *Sxl* was never activated by the X:A signalling system during early embryogenesis. Instead, these male-spliced RNAs are probably derived from a large number of cells in

which autoregulation is marginally impaired or from cells in which autoregulation failed at some point late in development. In either case, this would mean that *fs(1)1621* must function in somatic *Sxl* autoregulation. The idea that *fs(1)1621* plays a role in autoregulation in the soma would account for a number of other observations. First, it would explain why *trans*-heterozygotes of *fs(1)1621* and a *Sxl* null allele show reduced female viability and masculinization of somatic tissue (Oliver et al., 1988; Steinmann-Zwicky, 1988). Consistent with this genetic interaction, we have found that the suboptimal splicing regulation observed in *fs(1)1621* females becomes exacerbated when only one copy of *Sxl* is present (J. I. H., unpublished results). Second, defects in somatic autoregulation would explain why males carrying the constitutive Sxl^{M1} allele can be rescued by *fs(1)1621* (Steinmann-Zwicky, 1988).

Since the germ line defect in *fs(1)1621* females is rescued to full fertility by a constitutive *Sxl* allele, Sxl^{M1}, (Steinmann-Zwicky, 1988; Salz, 1992) it would appear that the oogenesis defect of the *fs(1)1621* mutation is due to the misregulation of *Sxl* in the germ line. Given the role of *fs(1)1621* in the somatic expresssion of *Sxl*, this misregulation can be expected to lie in autoregulation. It should be noted that autoregulation is much more severely impaired in the germ line of *fs(1)1621* females than it is in the soma. This difference could be due to the fact that in the early stages of germ line development (eg., the non-differentiated larval oogonial cells) much of the *Sxl* protein is located in the cytoplasm rather than the nucleus. This predominantly cytoplasmic localization might make autoregulation (or perhaps the transition from the initiation to the maintenance mode) especially sensitive to a reduction in *fs(1)1621* activity.

In contrast to *fs(1)1621*, which affects both the germ line and soma (and functions in both sexes), *otu* seems to be required only in the female germ line (Steinhauer and Kalfayan, 1992). The otu protein is cytoplasmic and believed to control the cytoskeletal organization in female germ cells (King and Storto, 1988; Steinhauer and Kalfayan, 1992). Thus, it would seem unlikely that otu protein is directly involved in the splicing regulation of *Sxl*. Rather, we suspect that *otu* may be involved in the initiation of the *Sxl* autoregulatory feedback loop, perhaps, playing a role in the subcellular trafficking of components involved in signalling sexual identity. Whatever the precise function of *otu* in the female germ line, several observations indicate a requirement for *otu* beyond its involvement in *Sxl* regulation. First, unlike *fs(1)1621*, the constitutive Sxl^{M1} mutation does not rescue the tumorous ovaries of otu^{1} to fertility. While Sxl^{M1} improves the otu^{1} tumorous phenotype so that differentiating egg chambers are formed, these egg chambers are abnormal in their development (Daniel Pauli, personal communication) indicating that processes in addition to *Sxl* regulation require wild-type *otu* activity. Second, the otu^{1} allele is a hypomorphic mutation while loss-of-function alleles of *otu* produce adult gonads that have few if any functional stem cells (King and Storto, 1988). This phenotype is more severe than that observed for germ cells lacking *Sxl* activity (Schüpbach, 1985). The lack of functional stem cells in the absence of *otu* may reflect a very early defect in germ-line sex determination as has been suggested for *ovo* (Oliver et al., 1987). Third, in another *otu* allele, otu^{3}, relatively high levels of Sxl protein can be detected in germ cells in spite of the fact that this mutant exhibits a tumorous ovary phenotype which is quite similar to that of otu^{1}. Presumably, in this *otu* background where expression of *Sxl* is not severely perturbed, the tumorous phenotype arises from a defect in some other process that *otu* regulates.

How the *Sxl* autoregulatory feedback loop is set in motion in the germ line remains to be determined. *Sxl* is not activated in the progenitors of the germ line, the pole cells, at the time when somatic cells begin to express the gene (Bopp et al., 1991; Keyes et al., 1992) and deletion mutants suggest that the early somatic promoter is distinct from the promoter used to activate *Sxl* in germ cells (Salz et al., 1987). *Sxl* must be activated between pole cell migration and the early third instar larval stage, presumably, by a mechanism that responds to both the autonomous chromosomal constitution and a signal from the soma (Steinmann-Zwicky et al., 1989; Nöthiger et al., 1989). It is intriguing that both *ovo* and *otu* are located on the X chromosome. Perhaps, these genes contribute to or are part of the X:A counting system that germ cells utilize.

Distribution of Sxl protein in the early stages of oogenesis

In somatic sex determination, Sxl protein appears to function primarily, if not exclusively, as a splicing regulator controlling the processing of its transcripts and those of downstream genes. Accordingly, the protein is localized predominantly in the nucleus where it appears to interact with nascent transcripts (Bopp et al., 1991; D. B., unpublished results). In contrast to the soma, Sxl protein displays a complex distribution pattern in the germ line, and there are quite extensive changes in its subcellular localization during the course of oogenesis. Perhaps the most dramatic alterations in the adult gonad occur in the germarium. At the very tip of the germarium, we observe very high levels of cytoplasmic Sxl protein. The cytoplasmic protein appears to be restricted to the anterior part of region 1, and there is a precipitous drop in level of cytoplasmic staining in the posterior part of region 1. This transition occurs either just before or during the second round of cystocyte division suggesting that a stage-specific signal induces a process that mediates the apparent disappearance of cytoplasmic Sxl protein. Though we suspect that the bulk of the cytoplasmic Sxl protein is degraded during this transition, we can not exclude the possibility that the protein is relocalized in a manner that makes it inaccessible to our antibodies. In the newly formed 16-cell cysts, the remaining and/or newly synthesized protein becomes concentrated in nuclear foci, in marked contrast to the predominantly cytoplasmic localization in the stem cell and its immediate progeny.

It is possible that the cytoplasmic localization of Sxl protein may be directly relevant to the functioning of the *Sxl* gene during the initial stages of oogenesis. It is during this phase of oogenesis that the differentiation pathway leading from the stem cell to a 16-cell cyst is initiated. In the absence of normal *Sxl* activity, this differentiation pathway seems to be disrupted and tumorous ovaries consisting of small undifferentiated cells are formed. It would not be unreasonable to

suppose that the protein exerts some regulatory function in the cytoplasm controlling, perhaps, the translation or stability of cytoplasmic RNAs.

The tumorous ovary phenotype is also observed in the two female-sterile *Sxl* alleles, Sxl^{f4} and Sxl^{f5}. Both alleles express apparently normal levels of cytoplasmic protein in early stages. However, the normal alterations in the level and distribution of Sxl protein during cystocyte proliferation does not occur and all germ cells in the gonad continue to produce high levels of cytoplasmic protein. Two possibilities could explain the failure of the mutant Sxl proteins to undergo the normal transitions in level and distribution. First, the mutations might affect a protein domain that is critical for the degradation/redistribution process. However, in heterozygous females, this defect is rescued by the presence of wild-type protein. Such a rescue would require the wild-type protein to facilitate the degradation/redistribution of the mutant protein. The second, and more likely possibility, is that these two mutants fail to execute properly some early germ-line function that is dependent on the carboxyl domain of the protein. The differentiation pathway of germ cells is thus altered, perhaps blocked at a point prior to the transition from the 2-cell to the 4-cell cyst, making them incompetent to downregulate protein levels. While the precise nature of this germ-line function is unknown, it is reasonable to speculate, based on the RNA-binding properties of Sxl protein, that this activity involves the interaction of Sxl protein with some RNA species. In this regard it is worth noting that while the mutations in the two alleles map outside the *Sxl* RNA-binding domains, autoregulation in the soma of these female-sterile alleles is not completely normal.

Cytoplasmic staining also persists in the germ line of females mutant in two other loci, *bam* and *fu*. Both *bam* and *fu* mutants have a tumorous ovary phenotype much like the female-sterile mutations in *Sxl*. *bam* is thought to control the early cystocyte divisions (McKearin and Spradling, 1991). Since we observe cytoplasmic staining in all germ cells mutant for *bam*, it would appear that this mutation arrests cystocyte proliferation at a point prior to the stage when Sxl protein is downregulated. *fu* encodes a serine/threonine kinase and in embryogenesis it is thought to function in the segment polarity pathway by mediating cell-cell communication (Preat et al., 1990). If *fu* has an analogous role in early oogenesis, then the differentiation pathway that leads to the degradation of Sxl protein in region 1 of the germarium would depend on a cell-cell signalling process. Since we have been unable to detect any phosphorylated forms of Sxl protein (D. B., unpublished results), *Sxl* is unlikely to be the direct target for *fu* phosphorylation in such a signalling pathway.

In principle, this signalling system could involve communication between the interconnected daughter cells of the cystoblast. Alternatively, the signalling system might mediate cell-cell communication between somatic and germ cells. In this respect, it is of interest that high levels of cytoplasmic Sxl protein have been observed in the germ cells of *tra2/tra2* ^{ts2} pseudomales raised at 25°C (D. B., unpublished results). Genetic studies have shown that *tra2* is required in the soma but not the germ line for normal oogenesis so the persistence of cytoplasmic Sxl protein in the germ cells of testes from *tra2* ^{ts} pseudomales is likely to be caused by some failure in communication between the masculinized somatic cells and the female germ cells.

Sxl protein at later stages of oogenesis

In the period between the initial formation of the 16-cell cyst in the germarium until near the end of oogenesis at stage 11, we detect significant levels of nuclear Sxl protein. Although there are no mutations known to disrupt *Sxl* function specifically during these stages of oogenesis, the nuclear localization of the Sxl protein suggests that it may be involved, as in the soma, in regulating RNA splicing. We would anticipate that there are targets for *Sxl* regulation in addition to the autoregulatory feedback loop. What these targets are is not clear. However, given the fact that Sxl protein is localized predominantly in the nurse cells rather than the oocyte, it is possible that it functions in some aspect of nurse cell determination or differentiation. The distribution of Sxl protein contrasts with that of gene products involved in oocyte determination or differentiation, which show preferential accumulation in the developing oocyte (Suter and Steward, 1991; Lantz et al., 1992).

In the chicken but not the egg

In addition to the possible function of *Sxl* in some aspect of nurse cell development, there are other reasons why it may be important to exclude Sxl protein from the oocyte - the presence of significant levels of functional Sxl protein in the mature egg could potentially activate the *Sxl* gene in the early embryo independently of the X:A signalling system. Since the inappropriate activation of *Sxl* in male embryos would cause a son-less phenotype, it seems likely that there are specific mechanisms that ensure that Sxl protein is not stably deposited in the mature oocyte. Between stages 1 and 11, when high levels of Sxl protein are present in the nurse cells, the transport of Sxl protein from nurse cells to the oocyte appears to be prevented. The Sxl protein may lack an appropriate oocyte targeting signal; alternatively, it may be specifically retained in the nurse cells. At stage 11, when the nurse cells begin to dump much of their contents into the oocyte, Sxl protein first disappears from the nurse cell nuclei and thereafter from the cytoplasm. The specific turnover of Sxl protein during this phase of oogenesis suggests that there may be a mechanism that targets it for degradation.

While Sxl protein is largely excluded from the oocyte, substantial levels of *Sxl* mRNA accumulate in the oocyte and even persist in the zygote for 1-2 hours. The *Sxl* mRNA in the egg does not appear to be translated either before or after fertilization. Possibly, the maternal *Sxl* mRNA lacks some signal that is normally required for translation in the egg. Alternatively, the RNA may contain signals that block translation and instead mark it for degradation shortly after the egg is fertilized. Depleting the embryo of any maternal contribution of Sxl protein allows a new cycle to begin where the choice of sexual identity depends solely on the chromosomal sex of the zygote, and not on the mother.

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