virilizer regulates Sex-lethal in the germline of Drosophila melanogaster

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SUMMARY

In Drosophila, the gene Sex-lethal (Sxl) is required for female development. It controls sexual differentiation in the soma, dosage compensation and oogenesis. The continuous production of SXL proteins in XX animals is maintained by autoregulation and depends on virilizer (vir). This gene is required in somatic cells for the female-specific splicing of Sxl primary transcripts and for an unknown vital process in both sexes. In the soma, clones of XX cells lacking Sxl or vir are sexually transformed and form male structures; in the female germline, XX cells mutant for Sxl are sterile. Certain alleles of fl(2)kd and vir cause female-specific lethality and a sexual transformation of somatic cell clones into male tissue. Both effects result from a male-specific expression of Sxl in XX animals. In addition, all three genes have a vital function in both sexes.

We now studied the role of vir in the germline by generating germline chimeras. We found that XX germ cells mutant for vir, in contrast to cells mutant for Sxl, perform oogenesis. We show that the early production of SXL in undifferentiated germ cells is independent of vir while, later in oogenesis, expression of Sxl becomes dependent on vir. We conclude that the early SXL proteins are dispensable for this process.

However, vir must be active in the female germline to allow normal embryonic development because maternal products of vir are required for the early post-transcriptional regulation of Sxl in XX embryos and for a vital process in embryos of both sexes.

Key words: Drosophila, Sex determination, Oogenesis, Autoregulation, Maternal effect, Sxl, virilizer (vir)

INTRODUCTION

Sex-lethal (Sxl) is the key gene for sex determination in Drosophila melanogaster. It controls three different pathways: somatic sex determination, dosage compensation, which equalizes the amount of X-chromosomal transcripts in males and females, and oogenesis. The primary signal for sex determination in the soma is the ratio of X chromosomes to autosomes (X:A) (Bridges, 1921, 1925; reviewed by Cline, 1993). If this ratio is 1.0 (XX:AA), Sxl is transcriptionally activated. As a consequence, the sex-determining genes transformer (tra) and doublesex (dsx) are female-specifically expressed, which leads to female somatic differentiation. If the X:A ratio is 0.5 (X:AA), Sxl and tra remain inactive, and the bifunctional locus dsx is expressed in the male mode, which results in male somatic differentiation (for reviews see Belote, 1989, 1992; Steinmann-Zwicky et al., 1990; Cronmiller and Salz, 1994; Cline and Meyer, 1996). In the absence of SXL protein, the dosage compensation gene male-specific lethal-2 (msl-2) expresses a functional product and promotes, together with the products of the other male-specific lethal genes (msl-1, msl-3, mle) and males-absent on the first (mof), the hypertranscription of the single X chromosome in males (Belote and Lucchesi, 1980; Kelley et al., 1995; Zhou et al., 1995; Hilfiker et al., 1997; for reviews see Kuroda et al., 1993; Baker et al., 1994; Gorman and Baker, 1994). Mutations in Sxl or upstream of it or in the msl genes lead to sex-specific lethality because of disturbance of dosage compensation. Mutations downstream of Sxl in the somatic sex determination pathway cause sexual transformation.

The differential expression of Sxl in XX embryos is achieved in two steps. The first level of control is transcriptional. Products of numerator and denominator genes, which form the X:A signal regulate an establishment promoter, P_E. In XX embryos, this promoter is activated at blastoderm stage and produces an early SXL protein. Later, Sxl is regulated by alternative splicing. Around early gastrulation, transcription from P_E is switched off and a maintenance promoter, P_M, becomes active in both sexes. The early SXL protein, present only in females, promotes the female-specific splicing of its own pre-mRNA, thus starting an autoregulatory loop. In males, which have no preexisting SXL protein, the Sxl mRNA includes an exon (#3) with several in-frame stop codons that terminate translation prematurely (Bell et al., 1988; Bopp et al., 1991; Keyes et al., 1992; Wang and Bell, 1994). Three additional genes are known to be involved in the female-specific splicing of Sxl pre-mRNA: sans-fille (snf) (Salz, 1992; Albrecht and Salz, 1993), female-lethal-2-d (fl(2)kd) (Granadino et al., 1990, 1992) and virilizer (vir) (Hilfiker and Nöthiger, 1991; Hilfiker et al., 1995). Females mutant for snf¹⁶²¹ are sterile. Certain alleles of fl(2)kd and vir cause female-specific lethality and a sexual transformation of somatic cell clones into male tissue. Both effects result from a male-specific expression of Sxl in XX animals. In addition, all three genes have a vital function in both sexes.

Regulation and function of Sxl in the germline are less well understood. Females mutant for Sxl¹⁴, a mutation that
particularly disrupts the germline function, and females with transplanted pole cells mutant for the null allele Sxl\(null\), develop ovaries with tumorous cysts due to an excessive proliferation of the germ cells and to a partial transformation into spermatocytes (Schüpbach, 1985; Steinmann-Zwicky et al., 1989; Bopp et al., 1993). The genes snf, fl(2)\(d\), ovo and ovarian tumor (ota) are required for proper expression of Sxl in XX germ cells (Salz, 1992; Granadino et al., 1992; Oliver et al., 1993; Pauli et al., 1993). Mutations in any of these genes cause tumorous ovaries. This shows that Sxl is necessary for the differentiation of female germ cells. The initial expression of Sxl is probably regulated by a cell-autonomous signal, the germline X:A ratio, which is measured by elements different from those used to determine the X:A ratio in the soma and by an inductive signal that is provided by somatic cells (Nöthiger et al., 1989; Steinmann-Zwicky et al., 1989; Steinmann-Zwicky, 1993; Granadino et al., 1993; Horabin et al., 1995; reviewed by Steinmann-Zwicky, 1992). It was recently shown that, in parallel to the soma, Sxl in the germline is also maintained by autoregulation (Hager and Cline, 1997). In our paper, we analyzed this second step of regulation and asked whether Sxl in germ cells depends on vir, which is involved in the autoregulation of Sxl in somatic cells.

**MATERIALS AND METHODS**

**General techniques**

Unless noted otherwise, all crosses were done at 25°C. Flies were reared on standard food (corn meal, sugar, yeast, agar, Nipagin). For genetic symbols, see Lindsley and Zimm (1992).

**vir alleles**

\(\text{vir}^{\text{tsi}}\) is a temperature-sensitive allele that transforms XX animals into intersexes at 29°C; \(\text{vir}^{\text{tsf}}\) causes female-specific lethality. Both alleles have no effect on XY animals. \(\text{vir}^{\text{ps}}\) is lethal for females and semilethal for males. \(\text{vir}^{\text{s}}\) and \(\text{vir}^{\text{ds}}\) are lethal for both sexes (Hilfiker and Nöthiger, 1991; Hilfiker et al., 1995). \(\text{Df}(2R)\text{vir}^{\text{130}}\) is a deficiency that uncovers \(\text{Frd}, \text{twi}\) and \(\text{vir}\).

**Clones in the germline**

Cell clones homozygous for \(\text{vir}\) were generated by the FLP-DFS technique (Chou and Perrimon, 1992, 1996; Chou et al., 1993; Xu and Rubin, 1993). Females of the genotype \(\text{w}; \text{hs}-\text{neo}; \text{FRT}^{\text{42}}\text{D}, \text{P}[\text{ry}^{+}; \text{FLP}]^{\text{38}}\) were crossed to \(\text{y}; \text{w}\) \(\text{FL}122.16\); \(\text{P}[\text{ry}^{+}; \text{hs}-\text{neo}; \text{FRT}^{\text{42}}\text{D}, \text{P}[\text{ry}^{+}; \text{FLP}]^{\text{38}}\). To induce mitotic recombination, their offspring was heatshocked for 30 minutes at 37°C between 24 and 32 hours after oviposition. \(\text{f}^\omega \text{w}\); \(\text{FLP}, \text{FRT}^{\text{42}}\text{D}, \text{P}[\text{ry}^{+}; \text{FRT}]^{\text{101}}\text{Av} \text{oov}^{\text{D1}}, \text{y}^{\text{24}}\text{P}[\text{min}^{+}\text{w}^{+}; \text{FRT}]^{\text{101}}, \text{P}[\text{ry}^{+}; \text{FLP}]^{38}\) were crossed to \(\text{y}\); \(\text{w}\) \(\text{CM}1\); \(\text{P}[\text{min}^{+}\text{w}^{+}; \text{FRT}]^{\text{101}}, \text{P}[\text{ry}^{+}; \text{FLP}]^{38}\). They were heatshocked for 1 hour at 37°C between 24 and 32 hours after oviposition.

**Staining procedures**

Embryos of different stages derived from homozygous \(\text{vir}\) germ cells were stained with anti-SXL antibody as described by Bopp et al. (1991). The paternal X chromosome was marked with \(\text{Df}(\text{X})\text{F1}^{+}; \text{lacZ}\) (Zeng et al., 1994) to distinguish XX and XY embryos. Adult ovaries with clones of stem cells homozygous for \(\text{vir}\) and \(\text{Sxl}\) were stained with anti-SXL antibody and DAPI (Bopp et al., 1993).

**Examination of moribund embryos**

Females with germ cells mutant for \(\text{vir}^{\text{s}}\) and \(\text{vir}^{\text{ds}}\) were crossed to males carrying the following enhancer trap constructs: \(\text{Sxl}_{\text{F1}}^{+}\); \(\text{lacZ}\) (Keyes et al., 1992), \(\text{hh}^{+}; \text{lacZ}, \text{en}^{+}; \text{lacZ}, \text{fz}^{+}; \text{lacZ}, \text{hh}^{+}; \text{lacZ}\). Their embryos were stained with X-Gal and DAPI according to standard protocols.

**Transplantation of larval gonads**

Gonads of third instar larvae of \(\text{XY}; \text{cn} \text{vir}^{\text{ps}}\) \(\text{bw}\); \(\text{Df}(2R)\text{vir}^{\text{130}}\) and \(\text{XY}; \text{cn} \text{vir}^{\text{s}}\) \(\text{bw}\); \(\text{Df}(2R)\text{vir}^{\text{130}}\) males were transplanted (Ursprung, 1967) into adult females and cultured in their abdomen for 6 days. Afterwards, the gonads were analysed by phase-contrast microscopy.

**Transplantation of pole cells**

Females homozygous for \(\text{osk}^{\text{301}}\) were crossed to Oregon R wild-type males. Due to a maternal effect of \(\text{osk}\), the resulting embryos lack endogenous pole cells and served as hosts for transplanted pole cells (Lehmann and Nüsslein-Volhard, 1986). The donor embryos were obtained from a cross of \(\text{y sn}^{\text{r}}\); \(\text{vir}^{\text{s}}\) \(\text{bw}\); \(\text{T}(2;3)\text{Xa}\) females with \(\text{B}^{\text{B}1}\text{Y}; \text{Df}(2R)\text{vir}^{\text{130}}\text{SM5}\) males. For technical details, see Van Deusen (1976) and Steinmann-Zwicky et al. (1989).

**RESULTS**

**XX germ cells homozygous for \(\text{vir}\) are able to complete oogenesis**

In XX animals, somatic cell clones that become homozygous mutant for \(\text{vir}\) switch to the male pathway (Hilfiker et al., 1995). We now tried to determine the sexual fate of XX; \(\text{vir}\) germ cells. The FLP-DFS technique was used to generate clones homozygous for \(\text{vir}^{\text{s}}\), \(\text{vir}^{\text{ps}}\), \(\text{vir}^{\text{ds}}\) and \(\text{vir}^{\text{ds}}\) in heterozygous \(\text{vir}\)/\(\text{ovo}^{\text{D}}\) females (see Materials and Methods). These females are sterile due to \(\text{ovo}^{\text{D}}\) unless they lose this dominant female-sterile mutation by mitotic recombination and the resulting stem cells homozygous for \(\text{vir}\) are able to form eggs. Germ cells mutant for any of the four tested \(\text{vir}\) alleles were able to complete oogenesis (summarized in Table 1). Females with germ cells mutant for the female-specific lethal allele \(\text{vir}^{\text{ps}}\) gave rise to male and female offspring in a 1:1 ratio when mated to \(\text{vir}^{\text{ps}}\) males, and to purely male offspring when mated to homozygous \(\text{vir}^{\text{ps}}\) males. Females with germ cells mutant for the stronger \(\text{vir}^{\text{ds}}\) allele produced no daughters, even when the father contributed a \(\text{vir}^{\text{ds}}\) allele. But even of the male offspring, only a few per cent survived to adulthood. In contrast to these two alleles, females with germ cells mutant for the strong alleles \(\text{vir}^{\text{ps}}\) and \(\text{vir}^{\text{ds}}\) formed eggs, but no larvae hatched (except very few escapers with \(\text{vir}^{\text{ps}}\)). In addition, the fecundity was also affected: the number of females laying eggs as well as the number of eggs produced per female were significantly reduced for \(\text{vir}^{\text{ps}}\) (18 eggs per female in 3 days, \(n=48\)) and even more so for \(\text{vir}^{\text{ps}}\) (9 eggs per female in 3 days, \(n=48\)), compared to 57 eggs for \(\text{vir}^{\text{ps}}\) (\(n=52\)).

All these phenotypes were rescued if the clones were made in females that carried a transgene containing a genomic 10 kb fragment with the \(\text{vir}^{\text{ps}}\) gene (M. Niessen, unpublished). This shows that the defects are due to \(\text{vir}\) and not to any other unknown mutations that become homozygous after mitotic recombination. Such mutations would not be rescued by the \(\text{vir}^{\text{ps}}\) transgene.

Thus, XX germ cells mutant for \(\text{vir}\), in contrast to somatic cells, are not sexually transformed. The embryonic defects that occur despite a paternal \(\text{vir}^{\text{ps}}\) gene point to a maternal effect and the reduced number of eggs to a vital function in female germ cells.
Table 1. Sex and viability of offspring derived from females with vir/vir germ cells

<table>
<thead>
<tr>
<th>Father</th>
<th>Mother</th>
</tr>
</thead>
<tbody>
<tr>
<td>vir⁺/vir⁺</td>
<td>vir²/vir² vir⁴/vir⁴ vir¹/vir¹ vir⁴/vir⁴</td>
</tr>
<tr>
<td>c♀, ᵃ♀(1)</td>
<td>c♀, ᵃ♀(6), ᵃ♂(5)</td>
</tr>
<tr>
<td></td>
<td>SxlP active</td>
</tr>
<tr>
<td></td>
<td>SxlP active</td>
</tr>
<tr>
<td></td>
<td>SxlP inactive</td>
</tr>
<tr>
<td>vir²/vir²</td>
<td>c♂(2), ᵃ♀(3)</td>
</tr>
<tr>
<td></td>
<td>SxlP active</td>
</tr>
<tr>
<td></td>
<td>Sxl mosaic</td>
</tr>
<tr>
<td>vir¹/vir¹ts</td>
<td>c♂(2), ᵃ♀(4)</td>
</tr>
<tr>
<td></td>
<td>n.d.</td>
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<td></td>
<td>n.d.</td>
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<td></td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Full maternal genotype: w/y w FLP; FRT vir bw/FRT ovoD.

n.d. = not determined.

(1) Normal viability (equal numbers of males and females).

(2) Apparently normal viability.

(3) Daughters die because of zygotic effect (acting on top of maternal effect).

(4) Daughters die because of a combined maternal/zygotic effect.

(5) Daughters die because of maternal effect.

(6) Number of surviving sons derived from vir⁶ germ cells is reduced to a few percent of all eggs deposited (data not shown).

(7) Very rare escapers.

(8) Unspecific effect (not related to sex).

Maternal vir product is required for the autoregulation of Sxl

Since earlier results had shown that vir is required for female-specific expression of Sxl (Hilfiker et al., 1995), we looked at the expression of Sxl in embryos derived from females with germ cells mutant for vir. To monitor the early transcriptional expression of Sxl, we introduced a SxlP::lacZ construct. In addition, the embryos were stained with anti-SXL antibody. To distinguish the two sexes, XX embryos were marked with Dfd(F1)::lacZ transmitted by the paternal X chromosome. In homozygous vir² embryos from vir²/vir² germ cells, the establishment promoter of Sxl was activated at blastoderm and SXL protein was uniformly distributed. At the beginning of gastrulation, the expression of SXL became mosaic and finally disappeared (Fig. 1B-D).

All vir alleles are truly recessive. vir²/vir¹ts females are viable when they derive from heterozygous vir² female germ cells (Hilfiker et al., 1995), but did not survive when they derived from homozygous vir² germ cells (Table 1). Females with germ cells mutant for the stronger allele vir⁶ never produced any daughters, even when the father contributed a vir⁺ allele. Similar to vir²/vir² embryos, vir⁶/vir⁺ embryos correctly initiated transcription of Sxl, but failed to maintain expression (Fig. 1E,F). Thus, maternal vir⁺ product is necessary for the autoregulation of Sxl during early development of XX embryos.

Maternal vir product has a vital function for embryogenesis

Germ cells mutant for the strong alleles vir² and vir⁴ were able to form eggs, but no larvae hatched except for very few escapers with vir³. Even with vir⁶, the viability of XY embryos was strongly reduced and XX animals were completely inviable. The genotype of the father had no influence on survival. The eggs were fertilized as shown by the presence of dividing nuclei in developing embryos. The developmental potential of heterozygous vir² embryos is variable: some were arrested already at blastoderm stage, others died as differentiated embryos or rarely as larvae. To determine the lethal period and to identify possible specific defects, several enhancer trap lines were introduced via the father (see Materials and Methods). In vir²/+ embryos, the establishment promoter of Sxl was activated and the segmentation genes hunchback (hb), fushi tarazu (ftz), engrailed (en) and hedgehog (hh) were correctly expressed. This suggests that the known maternal components necessary for formation of the anterior-posterior axis are normally distributed. vir⁴ embryos are more strongly affected. Already at blastoderm stage, reporter genes like hb and Sxl were not expressed. vir⁶/+ embryos showed a general disturbance in morphology with an irregular distribution of nuclei.

SxlM partially rescues the germline defects

We tested whether constitutive alleles of Sxl can rescue any of the three germline defects of vir, namely the reduced egg production of the females themselves, the maternal effect on the autoregulation of Sxl in XX embryos, and the maternal effect on the viability of XX and XY embryos.

We used two constitutive mutations of Sxl, SxlM¹ and SxlM⁴ (Cline, 1978; Maine et al., 1985). SxlM¹ is not fully constitutive and still depends to some extent on the regulation by vir, snf and fl(2)d. SxlM¹ males die, but survive if they are also mutant for vir² (Hilfiker et al., 1995), snf¹⁶21 (Steinmann-Zwicky, 1988; Salz, 1992) or fl(2)d¹ (Granadino et al., 1992). On the other hand, vir², snf¹⁶21 and fl(2)d¹ females are rescued by SxlM¹. SxlM⁴ is fully constitutive and males mutant for SxlM⁴ cannot be rescued by mutations in vir, snf or fl(2)d.

Experimental females with SxlM⁴; vir/vir germ cells were tested for the three germline defects. Presence of SxlM⁴ neither increased production of eggs in females with germ cells mutant
were themselves homozygous for cells. If XX embryos, derived from homozygous independent of female germ cells and a vital maternal effect that are both despite the presence of SXL protein, develop as strongly reported previously that XX animals with these genotypes, were sexually transformed (Table 2, lines I, II). It has been maternal, depending on Sxl females. Thus, the autoregulation of germ cells. This rescue must be zygotic since has no Sxl Sxl M4; vir 6 /vir + et al., 1993). maternal effect on zygotic activity of (Cline, 1978; Bopp Sxl Sxl M1; vir 2f /vir 2f has a vital function in vir 6 /vir 2f derived from these eggs. Thus, vir is able to rescue the daughters of females with vir 6 and vir + can be introduced either by the female or by the male can be introduced either by the female or by the male for vir 2 and vir 4 nor did it rescue the lethality of embryos derived from these eggs. Thus, vir has a vital function in female germ cells and a vital maternal effect that are both independent of Sxl (Table 2, line III).

Sxl M4 was able to rescue the daughters of females with vir 6 germ cells. This rescue must be zygotic since Sxl has no maternal effect on zygotic activity of Sxl (Cline, 1978; Bopp Sxl M1; vir 2f /vir 2f is necessary for the differentiation of female germ cells. A specific allele, Sxl 4 , disrupts only the germline activity of Sxl. Sxl 4 females are viable, but sterile due to an excessive proliferation of undifferentiated germ cells (Bopp et al., 1993). Since the ovarian phenotypes of Sxl 4 and vir are different, and since Sxl M4 rescues the defects in female embryos derived from vir germ cells only zygotically, Sxl function is expected to be independent of vir in germ cells. To test this assumption, ovaries with vir clones were stained with anti-SXL antibody to monitor the expression of Sxl. In wild-type females, SXL protein is present in nurse cells and follicle cells, but not in the oocyte (Fig. 2A,B).

SXL protein was present in clones of all vir alleles at the tip of the gerarium, but the amount in nurse cells decreased already at stage two. In later stages, SXL was nearly or totally absent. Despite the reduction of SXL protein, no tumorous cysts were observed as they occur in Sxl 4 ovaries (Fig. 2C-E). Thus, it is possible that SXL is only needed for the first steps of oogenesis in the gerarium.

### Table 2. Effects of Sxl M on vir/vir germ cells and their offspring

<table>
<thead>
<tr>
<th>Father</th>
<th>Sxl M4; vir 2f/vir 2f</th>
<th>Sxl M4; vir 2f/vir 6</th>
<th>Sxl M4; vir 2f/vir 2f</th>
<th>Sxl M4; vir 2f/vir 6</th>
<th>Sxl M4; vir 2f/vir 2f</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+/Y; vir 2f/vir 2f</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>Sxl M4/Y; vir 2f/vir 2f +/Y; vir 2f/vir 2f</td>
</tr>
<tr>
<td></td>
<td>85♂</td>
<td></td>
<td></td>
<td></td>
<td>815♂</td>
</tr>
<tr>
<td></td>
<td>Sxl M4/+; vir 2f/vir 2f</td>
<td></td>
<td></td>
<td></td>
<td>Sxl M4/+; vir 2f/vir 2f</td>
</tr>
<tr>
<td></td>
<td>55♀(2)</td>
<td></td>
<td></td>
<td></td>
<td>4♀(2)(4)</td>
</tr>
<tr>
<td>II</td>
<td>n.d.</td>
<td>+/Y; vir 2f/vir 2f</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+/Y; vir 2f/Cyo</td>
<td></td>
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<td></td>
<td></td>
<td>55♂</td>
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<tr>
<td></td>
<td></td>
<td>Sxl M4/+; vir 2f/vir 2f</td>
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<td></td>
<td>15♀(2)</td>
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<td></td>
<td>Sxl M4/+; vir 2f/Cyo</td>
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<tr>
<td></td>
<td></td>
<td>19♀(3)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>III</td>
<td>n.d.</td>
<td>n.d.</td>
<td>†</td>
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<td>n.d.</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Father</th>
<th>Sxl M1; vir 2f/vir 2f</th>
<th>Sxl M1; vir 2f/vir 6</th>
<th>Sxl M1; vir 2f/vir 2f</th>
<th>Sxl M1; vir 2f/vir 6</th>
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<tbody>
<tr>
<td>IV</td>
<td>+/Y; vir 2f/+</td>
<td>+/Y; vir 2f/+</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td></td>
<td>114♂</td>
<td>47♂</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>snf Sxl M1; vir 4(5)</td>
<td>snf Sxl M1; vir 4(5)</td>
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<tr>
<td></td>
<td>74♀(1)</td>
<td>10♀(3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>+/Y; vir 2f/vir 2f</td>
<td>+/Y; vir 2f/vir 2f</td>
<td>†</td>
<td>†</td>
</tr>
<tr>
<td></td>
<td>440♂, XX †</td>
<td>39♂, XX †</td>
<td></td>
<td></td>
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</tbody>
</table>

Full maternal genotype: a w Sxl M/y w FLP; FRT vir bw/FRT ovo D; b y w FLP/+; FRT vir bw/FRT ovo D.

n.d. = not determined.

(1) Also viable without Sxl M.
(2) Rescued by Sxl M, but sexually transformed by vir.
(3) Rescued by Sxl M, female phenotype due to vir +.
(4) Escapers.
(5) Sxl used to introduce Sxl M1 from father.
Sxl is required for the early steps of oogenesis

To test if Sxl and vir mutations really disrupt different processes in oogenesis, we generated germline clones in Sxl<sup>fl1</sup>/ovo<sup>D</sup> and Sxl<sup>fl4</sup>/ovo<sup>D</sup> females at the same stage as the vir clones. In contrast to vir, these females never laid any eggs. Sxl<sup>fl4</sup> clones showed the typical phenotype with tumor cysts, which we never observed in vir clones. These results suggest that Sxl is necessary for the early phase of differentiation of germ cells and that vir is involved in the regulation of Sxl only later when oogenesis is already initiated and can be completed without Sxl.

Male germ cells are not affected by vir

The gene vir has a female-specific function in the soma and the germline. In addition, it plays a role in an important vital process in somatic cells of both sexes. As a last point, we examined a possible role in the male germline. By transplanting whole larval gonads of the lethal genotypes XY; vir<sup>2f</sup>/Df(2R)vir130 (5 cases) and XY; vir<sup>2f</sup>/Df(2R)vir130 (12 cases), we could show that sperm was formed after culturing the gonads in the abdomen of adult females. We also transplanted XY; vir<sup>2f</sup>/Df(2R)vir130 pole cells into agametic XY embryos. Three fertile males with integrated pole cells of the desired genotype were recovered (Table 3). These results suggest that vir is not required for spermatogenesis.

**DISCUSSION**

Besides its requirement for the female-specific splicing of Sxl and tra and a yet unknown vital process in the soma of both sexes, we found that vir has several functions in the female germline: maternal product of vir is required for the regulation of Sxl in daughters and for the viability of both sexes. In addition, vir is necessary in the female germline for a normal rate of egg production and for the maintenance of late Sxl activity. The early production of SXL, however, is independent of vir and is sufficient to initiate and execute oogenesis. Finally, vir is dispensable for spermatogenesis.

**Table 3. XY germ cells mutant for a lethal vir allele perform spermatogenesis when transplanted into a male host**

<table>
<thead>
<tr>
<th>Genotype of integrated germ cells*</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>T(2;3)Xa/SM5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Df(2R)vir130/T(2;3)Xa</td>
<td>0</td>
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<td>vir3/SM5</td>
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<tr>
<td>vir3/Df(2R)vir130</td>
<td>1</td>
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</table>

*Adult flies were tested for fertility with cn vir<sup>ts</sup> bw/cn vir<sup>ts</sup> bw females, and the genotype of the integrated germ cells was determined from the phenotype of the progeny. Of 113 adult male hosts, only 7 were fertile.

**Functions of vir in oogenesis**

In contrast to somatic cells, XX germ cells do not need vir for their female identity. Oogenesis can proceed normally in vir mutant germ cells. However, germ cells mutant for strong alleles which affect the vital somatic function form much smaller clones. Either some of these mutant germ cells die, or they divide and differentiate more slowly. Since this defect cannot be rescued by a constitutive allele of Sxl, the affected function must be independent of Sxl. This conclusion is further supported by vir<sup>2l</sup>, which reduces expression of Sxl in germ cells but allows egg production at a normal rate. The vital functions of vir, both in somatic and female germ cells, are unknown. It is possible, however, that vir is involved in the same process in both cell types.

**Maternal functions of vir**

daughterless (da), hermaphrodite (her) and snf are known to have a maternal effect on the viability of daughters. XX embryos from da or her mutant mothers fail to initiate Sxl (Cline, 1980, 1993; Albrecht and Salz, 1993; Pultz and Baker, 1995).

Our work has now identified vir as a further component that is maternally required for the regulation of Sxl in XX embryos. Unlike da and her, but similar to snf, it acts at the level of post-transcriptional control. The establishment promoter of Sxl is activated both in vir<sup>fl1</sup>/+ and vir<sup>2f</sup>/vir<sup>2l</sup> embryos derived from vir mutant mothers, but Sxl expression is not maintained. The lethality of vir<sup>fl1</sup>/+ embryos indicates that maternal product of vir is responsible for the autoregulation of Sxl during early development. In addition, maternal vir function is also required for viability of offspring of both sexes as shown by strong alleles disrupting the vital function. For such alleles, neither the autoregulation of Sxl nor the vital process can be rescued by a paternal vir<sup>+</sup> allele. The reason may be that the zygotic gene is either not yet active or not yet sufficiently active.

**Regulation and function of Sxl and vir in the female germline**

Most genes of the somatic sex determination cascade are dispensable within germ cells (Marsh and Wieschaus, 1978; Schüpbach, 1982; Granadino et al., 1993; Steinmann-Zwicky, 1993, 1994a; Horabin et al., 1995). Sxl, although necessary for oogenesis, does not have a master regulatory function for sex determination in the germline, as indicated by the following observations. (1) Expression of Sxl in germ cells is first detected in 16- to 20-hour-old embryos (Horabin et al., 1995). A male-specifically expressed gene, however, is already observed in germ cells of 10-hour-old embryos, suggesting that some aspect of sexual development must have been determined prior to expression of Sxl (Staab et al., 1996). (2) Sxl<sup>fl4</sup>/Sxl<sup>fl1</sup> and
Sxl f1/Sxl f1 larvae have female gonads by the criteria of size and morphology; only as adults do the germ cells form abnormal multicellular cysts. Sxl does not seem to control the sex-specific differentiation of germ cells in larvae, but is required later for oogenesis (Steinmann-Zwicky, 1994b). (3) XY germ cells containing Sxl M1 or Sxl M4 are not feminized, but instead form fertile sperm (Steinmann-Zwicky, 1993).

The expression of Sxl in the germline depends on inductive signals from the gonadal soma and on an autonomous signal given by the germline X:A ratio, which is measured by elements different from those used to determine the somatic X:A ratio (Granadino et al., 1993; Steinmann-Zwicky, 1993). Thus, initiation of Sxl is different in soma and germline. We now showed that, in a second phase, expression of Sxl in differentiating cysts becomes dependent on vir. This parallels the regulation of Sxl in somatic cells. In contrast to the soma, however, the consequences of misexpression of Sxl are different: germ cells are not sexually transformed.

The different germline phenotypes of Sxl and vir mutations may have trivial reasons. A perdurance effect of VIR protein made in heterozygous stem cells prior to mitotic recombination could maintain the production of SXL protein in vir clones. However, the tested females continued to lay eggs even two weeks after the induction of clones and after several rounds of cell divisions. By this time, VIR protein should have been diluted or eliminated and should no longer be able to regulate Sxl. A second possibility would be that the first 24 hours of development in heterozygous condition before the induction of clones determine the female fate of a germ cell irreversibly. In all previous experiments with ovaries mutant for Sxl M4 and with transplanted pole cells mutant for Sxl f1, the germ cells were homozygous mutant from the beginning (Schüpbach, 1985; Bopp et al., 1993). But even when we induced Sxl clones under the same conditions as the vir clones, the females remained sterile as in earlier experiments showing that Sxl is necessary after the induction of the clones. Therefore, the phenotypic differences of Sxl and vir mutants are caused by different requirements for the two genes.

Based on these results, we suggest the following model (Fig. 3). The initiation mechanism of Sxl in the germline is unknown. Early SXL protein, however, is necessary in gonial cells at the tip of the germarium in the adult ovary for female development. Later in oogenesis, the germ cells may become independent of the primary signals by the soma and the X:A ratio and maintain SXL production by autoregulation (Hager and Cline, 1997).

This later post-transcriptional regulation depends on vir. Late SXL protein, however, is no longer necessary for the female differentiation of germ cells, once these cells have embarked on the oogenic pathway. It is not yet clear what other functions SXL has in later stages of oogenesis. SXL protein is redistributed during oogenesis (Bopp et al., 1993). In stem cells and early cystoblasts in the germarium, SXL is predominantly cytoplasmic. During the mitotic divisions, the level of cytoplasmic SXL drops drastically. In the cluster of 16 cells, the protein becomes concentrated in the nuclei of the cystocytes. This transition of SXL protein may reflect the early and late functions of Sxl. We conclude that SXL is required for a short period during the transition from stem cells to cystoblasts. Lack of SXL during this phase results in tumorous cysts.

snf and fl(2) d are two other genes involved in the autoregulation of Sxl in the soma. Salz et al. (1992) and Granadino et al. (1992) showed that these two genes are also required for oogenesis. Mutant females produce no eggs, but instead form multicellular cysts. We can only speculate why mutations in snf, fl(2) d and vir have different consequences in the germline. They may still all affect the autoregulation of Sxl, as they do in somatic cells, but at different times. It is possible that the production of early SXL protein depends on a post-transcriptional regulation that requires snf and fl(2) d, whereas vir may only act later in oogenesis.

The regulation of Sxl in the germline is certainly more complex than in the soma, and the germline function of this gene is also less clear. A comparative analysis of the regulation and function of Sxl in soma and germline is therefore worthwhile and may contribute to our understanding of how a gene operates in different cell types.

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REFERENCES


