The gene virilizer is required for female-specific splicing controlled by Sxl, the master gene for sexual development in Drosophila

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SUMMARY

The gene virilizer (vir) is needed for dosage compensation and sex determination in females and for an unknown vital function in both sexes. In genetic mosaics, XX somatic cells mutant for vir differentiate male structures. One allele, vir2f, is lethal for XX, but not for XY animals. This female-specific lethality can be rescued by constitutive expression of Sxl or by mutations in msl (male-specific lethal) genes. Rescued animals develop as strongly masculinized intersexes or pseudomales. They have male-specifically spliced mRNA of tra, and when rescued by msl, also of Sxl. Our data indicate that vir is a positive regulator of female-specific splicing of Sxl and of tra pre-mRNA.

Key words: sex-specific splicing, genetic mosaics, msl genes, transformer, Drosophila, virilizer

INTRODUCTION

The primary signal for sex determination in Drosophila melanogaster is the ratio of X chromosomes to sets of autosomes (X:A). This signal acts on the sex-specific differentiation genes via a small number of sex-determining genes, organized in a hierarchical fashion and regulated by alternative splicing. The X:A signal is read by the key gene Sex-lethal (Sxl) which controls three distinct pathways: (i) sex determination of the soma, (ii) sex determination of the germ line and (iii) dosage compensation, a process that equalizes the amounts of X-chromosomal transcripts in males and females (for reviews see Steinmann-Zwicky et al., 1990; Belote, 1992; Steinmann-Zwicky, 1992a,b; Kuroda et al., 1993; Gorman and Baker, 1994; Baker et al., 1994).

With an X:A ratio of 1.0, the Sxl gene is activated at the blastoderm stage (Sánchez and Nöthiger, 1983; Gergen, 1987; Erickson and Cline, 1991) and produces a functional protein (Keyes et al., 1992). As a consequence, female-specific products of the sex-determining gene transformer (tra) are made. This in turn causes female-specific expression of doublesex (dsx) which now results in female somatic sexual differentiation (for reviews see Baker, 1989; Belote, 1989, 1992; Steinmann-Zwicky et al., 1990; Cline, 1993). In addition, Sxl activity is required to achieve a female-specific mode of dosage compensation which is characterized by a low transcription rate of X-chromosomal genes. Disturbances in dosage compensation lead to sex-specific lethality (reviewed by Lucchesi and Manning, 1987; Kuroda et al., 1993; Gorman and Baker, 1994; Baker et al., 1994). Specifically, XX animals that lack Sxl function overexpress their X-linked genes (Lucchesi and Skripsky, 1981; Gergen, 1987) and die during embryogenesis (Cline, 1978).

With an X:A ratio of 0.5, the Sxl gene remains functionally inactive. As a consequence, no active product of tra is made. This causes the bifunctional locus dsx to be expressed in the male mode which dictates male development (Burtis and Baker, 1989). A male-specific high rate of X-chromosomal transcription is achieved in the absence of a functional Sxl product and by the activity of a set of male-specific lethal genes, collectively called msl genes (mle, msl-1, msl-2, msl-3). Disturbance of dosage compensation, brought about by loss of function of any one of the msl genes, or by gain of function of Sxl (SxlM), leads to male-specific lethality (Fukunaga et al., 1975; Tanaka et al., 1976; Belote, 1983; Bachiller and Sánchez, 1989; Cline, 1978; for review see Kuroda et al., 1993; Gorman and Baker, 1994; Baker et al., 1994).

The differential expression of Sxl is achieved in two steps. An early promoter, P e , is activated in diplo-X but not in haplo-X individuals around the blastoderm stage as a consequence of a two-fold difference in the number of X-linked numerator genes (Parkhurst et al., 1990; Keyes et al., 1992). This leads to the early female-specific production of SXL protein. Later, around germband extension, transcription from P e is switched off, and a late promoter, P m , becomes active in both sexes. SXL protein, present only in female embryos (Bopp et al., 1991), now initiates an autoregulatory loop by promoting female-specific splicing of the Sxl pre-mRNA (Bell et al., 1991). In males, the Sxl mRNA includes an exon (#3) with a stop codon that terminates translation prematurely (Bell et al., 1988; Bopp et al., 1991). Two genes necessary for female-specific splicing of Sxl pre-mRNA have
so far been identified, fl(2)id and snf (Granadino et al., 1992; Albrecht and Salz, 1993).

We have previously described a newly discovered gene involved in sex determination, called virilizer (vir) which acts upstream of tra in the sex-determining hierarchy (Hilfiker and Nöthiger, 1991). A temperature-sensitive mutation, vir\(^{ts}\), transforms XX animals into intersexual flies of a type similar to intersexes produced by certain alleles of dsx. The present paper describes a genetic and developmental analysis of vir. Our results show that the vir locus is complex, harboring female-specific functions that are required for sex determination and dosage compensation, as well as functions that are zygotically required for viability in both sexes. Our genetic and molecular analyses suggest that vir is required for female-specific splicing of Sxl and of tra pre-mRNA.

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 Lindsey and Zimm (1992). Flies to be analyzed under a compound microscope were macerated in hot 10% NaOH and mounted in Faure’s solution.

EMS mutagenesis

The gene vir was mapped to the tip of the second chromosome between Frd (2-103.1) and bw (104.5) at 2-103.9 (Hilfiker and Nöthiger, 1991). Homozygous bw males were treated with EMS according to Lewis and Bacher (1968) and crossed to L2/CyO.513 females. Resulting \(L^2/bw\) males were individually crossed to females of genotype Df(2R)vir130/CyO.513 or Df(2R)vir130/F(2;3)Xa to check for sexual phenotype or lethality of the Df(2R)vir130/bw animals. The tester chromosome Df(2R)vir130 carries a deficiency which uncovers Frd, twi (Simpson, 1983) and vir. Of 2453 such crosses, some 85% produced offspring. Balanced stocks were established from 22 matings in which the mutagenized chromosome was lethal over the deficiency. The 22 detected lethals were retested at 29°C in trans with the vir\(^{ts}\) mutation for changes in sexual phenotype. Six of them produced intersexual XX animals, indicating a new mutation in the vir gene. None of these six chromosomes showed any cytologically visible rearrangements. These mutations, collectively called vir\(^{EMS}\), were named vir\(^{2}\), vir\(^{3}\), vir\(^{4}\), vir\(^{5}\) and vir\(^{6}\).

The phenotype of XX; vir\(^{2}\)/vir\(^{EMS}\) intersexes, raised at 29°C, is very similar to intersexes caused by dsx mutations (Hildreth, 1965; Nöthiger et al., 1987; see also Fig. 1 in Hilfiker and Nöthiger 1991). XX; vir\(^{2}\)/vir\(^{EMS}\) animals, raised at 25°C or below, developed into phenotypically wild-type females, which, however, were mostly sterile. Males of genotype X/Y; vir\(^{2}\)/vir\(^{EMS}\) were morphologically not affected and were mostly fertile. The mutant vir\(^{EMS}\) bw chromosomes were recombined with a cn chromosome and tested for complementation by crossing y sn\(^{1}\); cn vir\(^{EMS}\) bw/SM5 females to cn vir\(^{EMS}\) bw/SM5 males at 18°C, 25°C and 29°C. XX animals had a wild-type dark body color and normal bristles (y sn\(^{1}\) y sn\(^{+}\) sn\(^{+}\)) whereas XY animals showed a yellow body color and singed bristles (y sn\(^{1}\)).

Lethal period of vir\(^{EMS}\)

The lethal period was determined by counting, at various times of development, the offspring of the cross y w y sn\(^{1}\); Df(2R)vir130/bw females to vir\(^{EMS}\) bw/bw males. Eggs, collected for 2 hours, were counted and placed on Petri dishes filled with agar and covered with a suspension of live yeast. Hatching male and female larvae were later separated and then recounted every 24 hours. XX larvae have dark mouth hooks (y y\(^{+}\)) whereas those of XY larvae are yellow (y/Y).

Clonal analysis of cuticular structures

Cell clones homozygous for either vir\(^{2}\), vir\(^{3}\) or vir\(^{6}\) were generated by X-ray-induced mitotic recombination. Females of genotype y; M(2)S7 Df(2R)B80, y\(^{+}\)/SM5 were crossed to y sn\(^{1}\); vir\(^{EMS}\) bw/SM5 males, and, as a control, to y sn\(^{1}\); bw/bw males. Mitotic recombination will produce a cell marked with y, homozygous for vir and having lost M(2)S7. Loss of this Minute will confer a relative growth advantage onto the cell so that large clones result. Eggs were collected for 2 hours, and larvae were irradiated with 10Gy (Philips MG 160, 2 mm Al filter, 25 cm distance, 150 kV, 14 mA, 2 minutes) at 24±1 hours, 48±1 hours, 72±1 hours, and 96±1 hours after oviposition. After eclosion, phenotypically wild-type male and female flies were selected, mounted under coverslips and screened with a compound microscope for the occurrence of yellow clones in the foreleg, the terminalia and the abdominal segments.

Transplantation of vir imaginal discs

Because XX animals mutant for vir never reach adulthood, but may in some allelic combinations survive to the third larval instar or even early pupal stage, we transplanted imaginal discs of such larvae into normal host larvae of genotypes cn or cn bw or cn; ry. Females y sn\(^{1}\); cn vir\(^{6}\) bw/SM5 were crossed to either cn vir\(^{2}\) bw or cn vir\(^{6}\) bw/SM5 males, and late third instar larvae showing dark mouthhooks (y y\(^{+}\)) and white Malphigian tubules (cn bw/cn bw) were selected. The single genital disc, the two foreleg discs and the two eye discs of an individual donor larva were transplanted (Ursprung, 1967). The eye discs served to verify homozygosity for cn bw which confirmed the desired mutant vir genotype. Eecosed host flies were dissected and their implants mounted in Faure’s solution for microscopical analysis.

Interactions with genes involved in dosage compensation

The following stocks were established to test possible interactions between vir and genes involved in dosage compensation, and the desired genotypes (see Results) were obtained by performing the appropriate crosses:

**Sxl\(^{2}\) stocks:**

- y; Y; Y; md1; F(2;3)M; CyO: cn vir\(^{4}\) bw/SM5
- Males of this stock were crossed to females of genotype cn vir\(^{EMS}\) bw/SM5 whereby vir\(^{EMS}\) stands for any of the six new alleles described in this paper.

**Sxl\(^{2}\) stocks:**

- B\(^{y}\); y cn Sxl\(^{2}\); vir\(^{2}\) bw/SM5; cn Sxl\(^{2}\)/FM6; cn vir\(^{2}\) bw/CyO
- Sxl\(^{2}\) F1#19:

- w\(^{1118}\); TM3, Ser P[w\(^{+}\), SxlcF1#19]/+

**msl\(^{2}\) stocks:**

- B\(^{y}\); msl-1\(^{2}\) vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-1\(^{2}\) vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
- B\(^{y}\); msl-2\(^{3}\) cn vir\(^{2}\) bw/SM5
**Results**

Complementation analysis reveals female-specific and non sex-specific vital functions of *vir*

In an EMS-screen, we produced six new alleles of *vir*, namely *vir*2f, *vir*3, *vir*4, *vir*5, *vir*6 and *vir*7, which we will collectively call *vir*EMS in this paper (see Materials and Methods).

The new alleles were tested in all combinations. XX animals, homozygous or trans-heterozygous for any of the *vir*EMS alleles, died at 18°C, 25°C and 29°C. For XY animals, the situation is more complex although most of the alleles are also lethal for males. Normal male viability and fertility, however, were observed for *vir*2f, either when homozygous or trans-heterozygous with any of the other *vir*EMS alleles. This identifies *vir*2f as a female-specific lethal allele. The other alleles, when homozygous, produced either no, or very few, male escapers, except for *vir*6. A few heteroallelic combinations showed complementation around 10%. Compared to their *vir*EMS/+ brothers, surviving males that were homozygous or trans-heterozygous for any of the *vir* alleles, except for *vir*2f, emerged late and were very weak.

The lethal period of *vir* mutant animals was determined by counting eggs and larvae at various times of development. The tested animals were trans-heterozygous for *vir*EMS over the deficiency *Df(2R)vir130* (see Materials and Methods). No lethality during embryogenesis was detected in XX or XY animals. In all cultures with an expected frequency of 25% of trans-heterozygous animals, approximately a quarter of the larvae died during larval or pupal development, except XY animals with the male *vir*2f which showed no noticeable lethality (data not shown).

**XX cells mutant for *vir*EMS differentiate male structures**

All *vir* alleles in trans with *vir*1ts transform XX animals into intersexes at 29°C. To study the sex-transforming effect of lethal *vir* alleles, we produced mosaic animals by mitotic recombination and by transplantation of imaginal discs (see Materials and Methods). Clones of homozygous *vir* cells were found in all body parts, irrespectively of the *vir* allele used and of the time of irradiation. For XY animals, the average sizes and frequencies of clones were roughly the same in *vir* and control clones, indicating no significantly reduced viability of the homozygous *vir* cells. In XX animals, a slight reduction in the size of the clones was apparent for *vir*2f and *vir*6 in the forelegs, but not in the tergites and sternites (data not shown). The important point of the clonal analysis is that homozygous *vir* clones, when they occurred in the sexually dimorphic regions of XX animals, differentiated male structures (Fig. 1A-C). In XX animals, all clones produced normal male structures. The results confirm our earlier conclusion that *vir* function is required for female sexual differentiation (Hilfiker and Nöthiger, 1991). Since sexual transformation occurred even when the clones were generated towards the end of the last larval instar (96 hours after oviposition), we conclude that *vir* is required throughout development, as is the case for *Sxl*, *tra* and *tra2* (Baker and Ridge, 1980; Epper and Nöthiger, 1982; Sánchez and Nöthiger, 1983).

Imaginal discs of genotypes *XX; *vir*2f/vir*6 and *XX; *vir*6/vir*7 were transplanted into normal *vir*+ hosts. We recovered the adult derivatives of 28 foreleg, of 18 genital and of 36 eye discs. Discs of genotype *vir*2f/vir*6 differentiated well-developed adult structures, but structures homozygous for *vir*6 were often poorly developed. The foreleg and the genital discs formed male structures (Fig. 1D-F).

**viral interacts with *Sxl* and with the msl genes**

An interaction between *vir*EMS and *Sxl*1 was tested in *XX; *vir*1ts/vir*EMS animals that were heterozygous *Sxl1/+* (for crosses see Materials and Methods). Whereas *Sxl*/Sxl*; *vir*EMS/*vir*1ts, or *Sxl1/*Sxl*1; *vir*EMS/*vir*1ts are viable, no doubly heterozygous females of genotype *Sxl1/*Sxl*1; *vir*EMS/*vir*1ts were found for any of the six *vir*EMS alleles; with only one dose of *Sxl*, even homozygous *vir*1ts animals died (Table 1).

| Table 1. Viable combinations of *vir* alleles cause lethality of XX animals that are heterozygous for *Sxl*1 |
|---------------------------------|-------------------------------------------------|-------------------------------------------------|
| *vi* EMS*/v*1ts                  | +/Y                                             |
| +/Y animals                      | +/Y animals                                     |
| *vi* EMS*/v*1ts                  | +/Y                                             |
| |                                   |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| *Sxl1/+* animals                 | 52     | 2.08  |       |       |       |       |       |       |       |       |       |       |       |       |
| *vi* EMS*/v*1ts                  | 87     | 1.18  |       |       |       |       |       |       |       |       |       |       |       |       |
| +/Y animals                      | 75     | 1.49  |       |       |       |       |       |       |       |       |       |       |       |       |
| *vi* EMS*/v*1ts                  | 69     | 0.81  |       |       |       |       |       |       |       |       |       |       |       |       |
| +/Y animals                      | 99     | 1.00  |       |       |       |       |       |       |       |       |       |       |       |       |
| *vi* EMS*/v*1ts                  | 83     | 0.86  |       |       |       |       |       |       |       |       |       |       |       |       |
| +/Y animals                      | 47     | 0.95  |       |       |       |       |       |       |       |       |       |       |       |       |

All crosses were done at 25°C.

1. *vir*= regulates *Sxl* expression.
In genetic mosaics, *vir* mutant cells differentiate male structures. The figure shows clones generated at 48 hours after oviposition (A–C), and structures formed by transplanted imaginal discs (D–F). (A) A large clone in the female genital region differentiated into external male genitalia. (B) A clone in the basitarsus produced 5 sex comb bristles. (C) A clone in the female dorsal anal plate produced part of a male anal plate (mAP). Altogether, we found 14 clones with sex combs, two with male genitalia and one with male analia in XX animals heterozygous for the alleles *vir*2, *vir*3 or *vir*6. Transplanted foreleg discs (D) and genital discs (E,F) formed male structures; D,E = X/X; *vir*2/*vir*5, F = X/X; *vir*6/*vir*6. Symbols: Female structures: T8, eighth tergite; dAP, dorsal anal plate; vAP, ventral anal plate; VP, vaginal plate. Male structures: AP, anal plate; CL, clasper; GA, genital arch; LP, lateral plate; PA, penis apparatus; SC, sex comb. Bars = 100 µm.
alleles and also carried Sxl1, Sxl4 or SxlcF1#19, a transgene with a constitutively expressed female-specific cDNA of Sxl (Bell et al., 1991). Only genotypes containing vir2 were rescued (Table 2). The rescued XX animals were strongly masculinized and in some cases transformed to pseudomales. The results obtained with Sxl4 were essentially the same as those with Sxl1, except that the rescue was higher and the surviving animals were less masculinized than those carrying Sxl1 (data not shown). Our findings indicate that vir2 is defective in functions required for dosage compensation and sex determination, and that the defect in dosage compensation, and to a lesser degree in sex determination, can be corrected by constitutive expression of Sxl.

Inappropriate expression of female-specific functions of Sxl is lethal for XY animals (Cline, 1978; Bell et al., 1991). Interestingly, the lethal effect of Sxl1 and SxlcF1#19, but not of Sxl4, on XY males can be rescued by vir2/virEMS (Table 2). The rescued XY animals were fertile males.

XY animals mutant for any of the male-specific lethal (msl) genes die during the third larval instar (Fukunaga et al., 1975; Tanaka et al., 1976; Belote and Lucchesi, 1980a,b; Belote, 1983; Bachiller and Sánchez, 1989), as do XX animals mutant for virEMS. XX animals mutant for the female-specific lethal allele vir2, but not for any other virEMS allele, can be rescued by male-specific lethal mutations in msl genes (Table 3). The percentage of rescued animals was variable and low (between 0.4 and 40%), and all rescued XX animals were transformed into pseudomales.

Mutations in vir interfere with the female-specific processing of the transcripts of Sxl and tra

Female sexual differentiation requires female-specific splicing of tra pre-mRNA. Genetic data had placed vir upstream of tra in the sex-determining pathway (Hilfiker and Nöthiger, 1991). We would thus expect that rescued XX; vir2/msl pseudomales express the male pattern of tra mRNA. As Fig. 2A shows, these animals (lanes 3 and 4) have tra transcripts identical to wild-type males (lane 2) and lack female-specific tra mRNA (lane 1). The molecular data thus confirm that vir acts upstream of tra and, directly or indirectly, controls the mode of expression of this gene.

The genetic interactions with Sxl described above suggest that vir is a positive regulator of Sxl. We therefore analyzed the sex-specific processing of Sxl transcripts in rescued XX; vir2/msl pseudomales (Fig. 2B). Such flies contain only male-

**Table 3. Mutations in male-specific lethal genes (msl) partially rescue XX animals from the female-specific lethal effect of the allele vir2**

<table>
<thead>
<tr>
<th>Allele</th>
<th>vir2/vir2</th>
<th>vir2/vir2</th>
<th>vir2/vir2</th>
<th>vir2/vir2</th>
<th>vir2/vir2</th>
<th>vir2/vir2</th>
<th>vir2/vir2</th>
<th>vir2/vir2</th>
</tr>
</thead>
<tbody>
<tr>
<td>msl-1/mle</td>
<td>7 (777)</td>
<td>10 (1387)</td>
<td>10 (1618)</td>
<td>10 (1618)</td>
<td>10 (1618)</td>
<td>5 (255)</td>
<td>5 (255)</td>
<td>5 (255)</td>
</tr>
<tr>
<td>msl-2/mle</td>
<td>0.4 (1139)</td>
<td>0.4 (1139)</td>
<td>0.4 (1139)</td>
<td>0.4 (1139)</td>
<td>0.4 (1139)</td>
<td>1 (1391)</td>
<td>1 (1391)</td>
<td>1 (1391)</td>
</tr>
<tr>
<td>mle3/mle</td>
<td>0 (219)</td>
<td>0 (219)</td>
<td>0 (219)</td>
<td>0 (219)</td>
<td>0 (219)</td>
<td>1 (148)</td>
<td>1 (148)</td>
<td>1 (148)</td>
</tr>
<tr>
<td>mle2/mle</td>
<td>2 (566)</td>
<td>2 (566)</td>
<td>2 (566)</td>
<td>2 (566)</td>
<td>2 (566)</td>
<td>1 (1391)</td>
<td>1 (1391)</td>
<td>1 (1391)</td>
</tr>
</tbody>
</table>

*Relative viability in % of the expected number of males, whereby the number of heterozygous sisters (n) serves as reference. The rescued XX animals are transformed into pseudomales.*
specifically spliced transcripts of Sxl (compare lanes 3 and 4 with lane 2). These results indicate that vir is required for the female-specific splicing of Sxl transcripts. Consistent with this conclusion, only male-specific transcripts of Sxl and tra were found in X/X; vir pseudomales, namely X/X; msl-2 vir 2f/msl-1 mle vir 5 (3); and X/X; msl-2 vir 2f/msl-2 vir 7 (4); yeast tRNA (5). A female-specific and a non sex-specific band of ~290 and ~465 nucleotides, respectively are expected and observed. Note that the DNA marker shown in the right panel runs slightly (2-4%) faster than RNA molecules of the same length. (B) RT-PCR. 1 μg of RNA from animals of different genotypes was reverse transcribed and amplified using oligonucleotide primers complementary to sequences in exon 2 and 4 of Sxl. The generated PCR products were separated on an agarose gel stained with ethidium bromide. The structure of the Sxl transcripts and the origin of primers are shown below (see also Materials and Methods). The sizes of the expected PCR products are 343 base pairs for the male-specific transcript and 153 base pairs for the female-specific transcript. For genotypes of animals whose PCR products are shown in panel 1 to 4 see Fig. 2A. Panel 5 shows a negative control (yeast tRNA).

**DISCUSSION**

Our results identify vir as a gene that harbours functions for viability in both sexes, and for female mode of dosage compensation and sex determination. The main experimental evidence for this statement is summarized in Fig. 3. Our current view of the position of vir in the sex determination hierarchy and of its role in the various developmental processes is shown in Fig. 4.

**vir regulates splicing of Sxl and tra in sex determination**

All the tested alleles that cause lethality to males and females are also deficient for the female-specific functions of dosage compensation and sex determination. These pathways are controlled by Sxl, and vir could thus be a regulator of Sxl.

The role of Sxl is well established in the pathway of somatic sex determination. Its protein, besides regulating the splicing of its own pre-mRNA (autoregulation), promotes the female-specific splicing of the tra pre-mRNA (transregulation) by blocking the 3' splice site that is used in males (Belote et al., 1989; Sosnowski et al., 1989; Inoue et al., 1990; Horabin and Schedl, 1993).

The most obvious and best understood aspect of vir is its role in sex determination, as demonstrated by XX; vir mutant cells forming male structures in genetic mosaics (Fig. 1). We have previously shown (Hilfiker and Nöthiger, 1991) that the temperature-sensitive intersexual phenotype of XX; vir 50/vir 1ts animals is rescued by the construct hs-tra-female which constitutively expresses the female mode of tra (McKeown et al., 1988). This indicates that vir acts upstream of tra. Our new findings confirm the high hierarchical position of vir in the cascade of sex-determining genes and suggest that it plays a role in the regulation of Sxl and tra.

Our genetic arguments receive support from molecular
analyses which show that surviving XX; \textit{vir}^{2f}/\textit{mle} pseudomales produce mRNAs of \textit{Sxl} and \textit{tra} that are male-specifically spliced (Fig. 2). The male-specific products of \textit{tra} could be the consequence of the male-specific expression of \textit{Sxl}. The next experiments, however, indicate that \textit{vir} is also directly engaged in the splicing of \textit{tra} transcripts.

Since \textit{Sxl}^{M/+}; \textit{vir}^{2f} animals are efficiently rescued from the female lethal effect of \textit{vir}^{2f}, but remain sexually transformed pseudomales or strongly masculinized intersexes (Table 2), we conclude that the sex-transforming effect of \textit{vir} mutations is not exerted via \textit{Sxl} alone. When the female-specific function of \textit{Sxl} is constitutively provided in XX; \textit{vir}^{2f} animals by \textit{Sxl}^{M} or \textit{SxlcF1#19}, the female-specific function of \textit{tra} is still not guaranteed, but in addition appears to require an active product of \textit{vir}. To test this conclusion we subjected the rescued animals to Western analysis. As Fig. 5 shows, substantial levels of SXL protein are present in strong intersexes (lane 2) and pseudomales (lane 5). Even animals with normal levels of SXL, such as the intersexes in lane 2 (compare with females in lane 3) still splice the pre-mRNA of their \textit{tra} gene largely in the male mode, as indicated by their strongly masculinized appearance. These intersexes are not mosaics of

<table>
<thead>
<tr>
<th>Function</th>
<th>\textit{vir}^{1f}</th>
<th>\textit{vir}^{M}</th>
<th>\textit{vir}^{EMS}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole flies</td>
<td></td>
<td>XX</td>
<td>XY</td>
</tr>
<tr>
<td>Males</td>
<td>25° f</td>
<td>f</td>
<td>f</td>
</tr>
<tr>
<td>Females</td>
<td>25° s</td>
<td>s, t</td>
<td>f</td>
</tr>
<tr>
<td>Mosaics clones and transplanted imaginal discs</td>
<td>n.d.</td>
<td>n.d.</td>
<td>f</td>
</tr>
</tbody>
</table>

Fig. 3. Summary of results:
\( \delta = \text{male} \)  \( \varphi = \text{female} \)  \( \varphi = \text{intersex} \)
\( \psi \delta = \text{pseudomale} \)  \( f = \text{fertile} \)  \( s = \text{sterile} \)
\( \dagger = \text{lethal} \)  n.d. = not determined.

Fig. 4. Current view of the role of \textit{vir} in somatic cells. \textit{vir} is required for female-specific splicing of \textit{Sxl} (thick arrow) and also of \textit{tra} and presumably (?) of \textit{msl-2} (thin arrows). Furthermore, \textit{vir} provides a vital function in both sexes (thick arrow). Female-specific expression of \textit{Sxl} is initiated at the transcriptional level by an X:A ratio of 1, and is later maintained by splicing regulation: SXL protein catalyzes the female-specific splicing of its own pre-mRNA (autoregulatory function for maintenance of \textit{Sxl}-activity) and that of \textit{tra} and probably of \textit{msl-2} (transregulatory function). It is this second level of \textit{Sxl} regulation by splicing where \textit{vir} and also \textit{snf} and \textit{fl}(2)d are involved.

Fig. 5. Expression of SXL in \textit{vir} mutant flies rescued by \textit{Sxl}^{M}, and of control flies (Western blots). The arrows indicate the two major isoforms of SXL. \( \delta = \text{male} \)  \( \varphi = \text{female} \)  \( \varphi = \text{intersex} \)
\( \psi \delta = \text{pseudomale} \)
male and female cells, but display an intermediate sexual phenotype at the cellular level characteristic of flies expressing only low levels of female-specific products of \( \text{tra} \) (Butler et al., 1986). The results thus support our conclusion that \( \text{vir} \) is needed not only for female-specific expression of \( \text{Sxl} \), but also of \( \text{tra} \). This may also be the case, although to a lesser extent, for \( \text{fl}(2) d \) since XX animals mutant for \( \text{fl}(2) d \) are rescued by \( \text{Sxl}^{\text{M1}} \) and are sexually transformed into intersexes at 29°C (Granadino et al., 1992).

The Western data (Fig. 5) furthermore suggest that presence of \( \text{SXL} \) protein still requires \( \text{vir} \) for the splicing of its own pre-mRNA: the pseudomales in lane 5 have substantially less \( \text{SXL} \) protein than the females in lane 6 which suggests that the transcripts produced by the \( \text{Sxl}^* \) allele in these genotypes are efficiently spliced only in the presence of \( \text{vir}^* \). This difference is not apparent between lanes 2 and 3, most likely because \( \text{Sxl}^{\text{M4}} \) is the much stronger constitutive allele than \( \text{Sxl}^{\text{M1}} \) (Bernstein et al., 1995). This interpretation is supported by the observation that \( \text{Sxl}^{\text{M1}}/\text{Y} \) males mutant for \( \text{vir}^{2f} \) survive (lane 4) whereas \( \text{Sxl}^{\text{M4}}/\text{Y} \) do not.

Based on our results, \( \text{vir} \) belongs to the class of genes that are involved in the regulation of \( \text{Sxl} \). Recessive mutations in the genes \( \text{fl}(2) d \) (Granadino et al., 1990) and \( \text{snf} \) (Albrecht and Salz, 1993) were shown to prevent female-specific splicing of \( \text{Sxl} \) pre-mRNA in XX animals; and, like \( \text{vir}^{2f} \), the female-specific mutant effects of both genes are rescued by \( \text{Sxl}^{\text{M}} \) mutations (Steinmann-Zwicky, 1988; Salz, 1992; Granadino et al., 1992).

**The role of \( \text{vir} \) in dosage compensation**

The sex-specific lethality of XX animals mutant for \( \text{vir}^{2f} \) by itself was suggestive of a role of \( \text{vir} \) in dosage compensation. Interactions between \( \text{vir}^{2f} \) and mutations in genes known to play a role in dosage compensation, such as \( \text{Sxl} \) (Cline, 1978; Lucchesi and Skripsky, 1981; Gergen, 1987; Bernstein and Cline, 1994) and the male-specific lethal genes (msl genes) (Belote and Lucchesi, 1980a,b; Belote, 1983) further support the proposed role of \( \text{vir} \).

As shown in Table 1, XX animals with otherwise viable combinations of \( \text{vir} \) alleles, e.g. \( \text{vir}^{109}/\text{vir}^{2f} \), die when they have only a single dose of \( \text{Sxl}^* \). However, animals with two X chromosomes and mutant for \( \text{vir}^{2f} \) are rescued by constitutive expression of \( \text{Sxl} \) (Table 2). These results indicate that \( \text{vir} \) mutations achieve their female-specific lethal effect via \( \text{Sxl} \), which explains why males are not affected by these same allelic combinations.

Animals with two X chromosomes and mutant for \( \text{vir}^{2f} \) are also rescued, although with low frequency, by loss of function of msl genes (Table 3). In view of the sex-transforming effect of mutations in \( \text{vir} \), and since mutations in msl genes do not interfere with sexual differentiation, it was not surprising that the rescued animals were transformed into sterile pseudomales. We conclude that \( \text{vir}^{2f} \) allows the inappropriate activity of the msl genes in XX zygotes, and that the affected animals then die as a result of overexpression of X-linked genes. Elimination of any one of the msl genes lowers X-chromosomal transcription towards a level that is lethal for XY animals, but is appropriate for a single X in XX animals (Belote and Lucchesi, 1980a,b; Breen and Lucchesi, 1986). The fact, however, that the rescue of \( XX; \text{vir}^{2f} \) animals by mutations in msl genes is weak, is a warning that the regulatory network of dosage compensation is more complex. Bernstein and Cline (1994) have recently shown that \( \text{Sxl} \) controls an early female-specific vital function that is not dependent on msl gene activity.

Our interpretation is supported by the recent finding that the MLE and MSL-1 proteins bind to the polytene X-chromosomes in salivary glands and Malpighian tubules of XX animals mutant for \( \text{vir}^{2f} \) or \( \text{vir}^{109} \); furthermore, these animals have the male-specifically acetylated form H4Ac16 of histone H4 (Hilfiker et al., 1994). Binding of the MLE and MSL-1 proteins to the X-chromosome and acetylation of histone H4 at lysin 16 normally occur only in males and are strongly, although not absolutely, correlated with hypertranscription of their single X-chromosome (Kuroda et al., 1991; Palmer et al., 1993; Bone et al., 1994; Hilfiker et al., 1994).

**How could \( \text{vir} \) participate in the regulation of the msl genes?**

Recent studies have shown that, of all the msl genes [mle; Kuroda et al. (1991); msl-1; Palmer et al. (1993, 1994); msl-3; Gorman et al. (1995)], only msl-2 is differentially expressed due to sex-specific splicing: Only males are capable of producing a functional protein; in females, the productive splice seems to be prevented by \( \text{Sxl} \), as proposed by Zhou et al. (1995) and Kelley et al. (1995). Thus, it appears that the \( \text{Sxl} \) protein acts again, as in the regulation of its own mRNA and of that of \( \text{tra} \), by blocking a splice site in the \( \text{msl-2} \) transcripts. Since the rescue of \( XX; \text{vir}^{2f} \) animals by \( \text{Sxl}^{\text{M}} \) is not complete (Table 2) and since the \( \text{Sxl}^{\text{M1}}/\text{Y} \) males in lane 4 of Fig. 5 survive despite the presence of \( \text{SXL} \), we conclude that the \( \text{SXL} \) product, as for the regulation of \( \text{tra} \), requires \( \text{vir} \) function to efficiently prevent the male-specific processing of \( \text{msl-2} \) transcripts.

**\( \text{vir} \) acts at two levels in the sex determination hierarchy**

It was unexpected that \( XX; \text{vir}^{2f} \) animals rescued by \( \text{Sxl}^{\text{M}} \) were transformed into pseudomales or strongly masculinized intersexes. A simple model in which \( \text{vir} \) acts above \( \text{Sxl} \) predicts that the rescued animals, due to the female-determining effect of \( \text{Sxl} \) (Cline, 1979), would be females; alternatively, if \( \text{vir} \) acted below \( \text{Sxl} \), there would be no rescue of the lethality. The actual results are best interpreted by a model in which \( \text{vir} \) acts at both levels, upstream and downstream of \( \text{Sxl} \), but ostensibly with differential effectiveness (Fig. 4). Its function appears to be absolutely required for female-specific splicing of \( \text{Sxl} \) transcripts, but seems less important for the regulation of \( \text{tra} \), and even less for \( \text{msl-2} \) which we assume to be the other target. We infer this from our observation that the function provided by \( \text{Sxl}^{\text{M1}} \) or \( \text{Sxl}^{\text{M4}} \) in \( XX; \text{vir}^{2f} \) animals is largely, but not completely, sufficient to prevent the inappropriate activity of the msl genes, but insufficient to make enough female-specific products of \( \text{tra} \) necessary for female sexual development.

**\( \text{vir} \) is active in males**

We have seen that \( \text{vir}^{2f} \) rescues XY males from the male-specific lethal effect of \( \text{Sxl}^{\text{M1}} \) and partially of \( \text{SxICF1#19} \) (Table 2), but not of \( \text{Sxl}^{\text{M4}} \). These results can be understood by recalling that \( \text{Sxl}^{\text{M1}} \), in contrast to \( \text{Sxl}^{\text{M4}} \), is not unconditionally constitutive (Cline, 1979; Bernstein et al., 1995), and XY animals mutant for \( \text{Sxl}^{\text{M1}} \) and \( \text{snf}^{1621} \) (Steinmann-Zwicky, 1988; Salz, 1992) or \( \text{fl}(2) d \) (Granadino et al., 1992) can survive as males some of which are even fertile. As our results
show, surviving males of genotype Sxl\textsuperscript{M1/Y}; vir\textsuperscript{2f} do produce some SXL protein (Fig. 5, lane 4), but not enough to make vir function dispensable. This implies that Sxl\textsuperscript{M1}, besides the functions of snf\textsuperscript{+} and fl(2)d\textsuperscript{+}, also requires vir\textsuperscript{+} to become fully functional in XY animals, and hence that an active product of vir must also be present in males.

If vir is active in males, as our results imply, then it is unlikely to play a discriminatory role in sex determination. Rather, it may be comparable to da and tra2, two genes whose products are present in both sexes, but serve sex-determining functions only in females (Cronmiller and Cline, 1987; Amrein et al., 1988).

**vir is an essential gene**

Most alleles of vir are zygotic lethals. Since our first screen, we have generated many more vir alleles, and still only two, vir\textsuperscript{Is} and vir\textsuperscript{2f}, are truly female-specific. This indicates that most lesions induced in vir affect a general non sex-specific function which, however, is dispensable in somatic cells of genetic mosaics: homozygous mutant cells survive in clones and in transplanted imaginal discs (Fig. 1).

Female-specific alleles and regular lethal alleles are also known for snf and fl(2)d, two genes that are, like vir, involved in the female-specific splicing of Sxl (Oliver et al., 1988; Steinmann-Zwicky, 1988; Granadino et al., 1990; 1992; Salz, 1992; Albrecht and Salz, 1993; Flickinger and Salz, 1994). Whereas putative null alleles of vir and fl(2)d are viable in cell clones, snf\textsuperscript{null} is required for normal cell proliferation (Helen Salz, personal communication). The gene snf codes for the Drosophila homolog of the mammalian U1A snRNP, a general component of the splicing machinery (Flickinger and Salz, 1994). The molecular structures of vir and fl(2)d are not yet known. The vital function of vir, fl(2)d and snf, however, cannot depend on Sxl as this gene is completely dispensable in males (Cline, 1988), and constitutive expression of Sxl does not rescue animals mutant for those alleles that abolish this vital function.

If, as our results suggest, vir is involved in splicing of the pre-mRNAs of Sxl and tra, and perhaps msl-2, it could also participate in the splicing of pre-mRNAs of vital genes. Mutations in snf (Flickinger and Salz, 1994), in fl(2)d (Granadino et al., 1990; 1992), and in vir, can cause lethality to both sexes. However, since clones of vir and fl(2)d, in contrast to those of snf\textsuperscript{null}, are viable in males and females, neither vir nor fl(2)d can encode a general and absolutely necessary factor for cell growth and cell viability. We do not know whether vir is directly involved in the splicing of tra and msl-2 transcripts, or whether it somehow activates the SXL protein. The answer to this question must await the molecular analysis of vir.

In summary, vir emerges as a gene that functions in several developmental pathways: sex determination, dosage compensation and vital processes. As depicted in Fig. 4, it seems to achieve this by regulating Sxl and tra, and presumably msl-2, as well as yet unknown target genes required for vital functions in both sexes. The common molecular mechanism by which vir performs these various functions may be its involvement in the process of splicing whereby the splicing of Sxl, and to a lesser extent of tra and msl-2, appears particularly sensitive to malfunction of vir.

We are grateful to many colleagues in the laboratory for critical and fruitful discussions and to Pia Meier-Gerschwiezer and Margrit Schmet for technical assistance. We want to thank Drs. Daniel Bopp, Monica Steinmann-Zwicky and Mariana Wolffner for constructive comments on the manuscript, and Prof. Dr. John Lucchesi for some of the msl stocks and for his generous hospitality during the stay of R. Nöhöger at Emory University in Atlanta. Our work was supported by the Swiss National Science Foundation, the ‘Roche Research Foundation’, the ‘Georges und Antoine Claraz-Schenkung’, the ‘Karl Hescheler-Stiftung’, the ‘Julius Klaus-Stiftung’ and the ‘Stiftung für wissenschaftliche Forschung an der Universität Zürich’.

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