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Abstract

Sex-lethal (Sxl) is the master switch gene for somatic sex determination in Drosophila melanogaster. In XX animals, Sxl becomes activated and imposes female development; in X(Y) animals, Sxl remains inactive and male development ensues. A switch gene for sex determination, called F, has also been identified in the housefly, Musca domestica. An active F dictates female development, while male development ensues when F is inactive. To test if the switch functions of Sxl and F are founded on a common molecular basis, we isolated the homologous Sxl gene in the housefly. Though highly conserved in sequence, Musca-Sxl is not sex-specifically regulated: the same transcripts and protein isoforms are expressed in both male and female animals throughout development. Musca-Sxl is apparently not controlled by the primary sex-determining signal and, thus, is unlikely to correspond to the F gene. Ectopic expression of Musca-SXL protein in Drosophila does not exert any noticeable effects on the known target genes of endogenous Sxl. Instead, forced overexpression of the transgene eventually results in lethality of both XY and XX animals and in developmental abnormalities in some escaper XY animals. Similar results were obtained with the Sxl homologue of Ceratitis capitata (Saccone, G., Peluso, I., Artiaco, D., Giodano, E., Bopp, D. and Polito, L. C. (1998) Development 125, 1495-1500) suggesting that, in these non-drosophilid species, Sxl performs a function different from that in sex determination.
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SUMMARY

Sex-lethal (Sxl) is the master switch gene for somatic sex determination in Drosophila melanogaster. In XX animals, Sxl becomes activated and imposes female development; in X(Y) animals, Sxl remains inactive and male development ensues. A switch gene for sex determination, called F, has also been identified in the housefly, Musca domestica. An active F dictates female development, while male development ensues when F is inactive. To test if the switch functions of Sxl and F are founded on a common molecular basis, we isolated the homologous Sxl gene in the housefly. Though highly conserved in sequence, Musca-Sxl is not sex-specifically regulated: the same transcripts and protein isoforms are expressed in both male and female animals throughout development. Musca-Sxl is apparently not controlled by the primary sex-determining signal and, thus, is unlikely to correspond to the F gene. Ectopic expression of Musca-SXL protein in Drosophila does not exert any noticeable effects on the known target genes of endogenous Sxl. Instead, forced overexpression of the transgene eventually results in lethality of both XY and XX animals and in developmental abnormalities in some escaper XY animals. Similar results were obtained with the Sxl homologue of Ceratitis capitata (Sacccone, G., Peluso, I., Artiaco, D., Giodano, E., Bopp, D. and Polito, L. C. (1998) Development 125, 1495-1500) suggesting that, in these non-drosophilid species, Sxl performs a function different from that in sex determination.

Key words: Sex determination, Evolution, Insect, Sex-lethal, Drosophila, Musca domestica

INTRODUCTION

In Drosophila melanogaster, the gene Sex-lethal (Sxl) acts as the master switch gene at the top of the sex determination cascade. It interprets the primary sex-determining signal and then transduces it to different subordinate pathways in the cascade (Cronmiller and Salz, 1994; Cline and Meyer, 1996). When Sxl is active, its products instruct the somatic cells to establish and maintain a female identity and, in addition, to set the transcriptional activity of X-chromosomal genes into the low mode typical and necessary for animals with two X chromosomes (Lucchesi, 1996). When Sxl is inactive, the alternative pathway of male differentiation and a high level of transcription of X-linked genes ensues. The gene is first regulated by a chromosomal signal that is constituted by the number of X chromosomes relative to sets of autosomes, the X:A ratio (Cline, 1993; Parkhurst and Meneeley, 1994). Sxl is turned ON in 2X:2A cells by a high dose of products of X-linked numerator genes, while Sxl remains OFF in X:2A cells where the dose of X-linked numerator products is below a threshold for activation. The target of this ON/OFF regulation is a dose-sensitive establishment promoter (Pe) of Sxl, which becomes transiently active only in 2X:2A cells at the cellular blastoderm stage (Keyes et al., 1992; Estes et al., 1995). Around gastrulation, Sxl becomes transcribed from a more distally located maintenance promoter (Pm) in both sexes. In the absence of active Sxl proteins, as in males, Sxl mRNA retains a translation-terminating exon (exon 3 in Fig. 1C), which prematurely truncates the open reading frame and prevents the generation of active full-length protein. Hence, the gene remains functionally OFF. In female cells, the presence of early SXL protein, translated from transcripts of Pe, achieves the exclusion of this exon from the mature transcript. The resulting mRNAs encode active full-length products which, in turn, sustain the productive splicing mode. This autoregulatory mechanism guarantees a continuous production of functional SXL proteins in females (Bell et al., 1988, 1991). Functional SXL is necessary to control two downstream genes: it activates transformer (tra) (Sosnowski et al., 1989; Inoue et al., 1990) and represses male-specific lethal-2 (msl-2) (Bashaw and Baker 1995; Kelley et al. 1995, 1997; Zhou et al., 1995). The TRA protein is necessary for female development and must be absent for male development; the MSL-2 protein is necessary for a high rate of transcription of X-chromosomal genes and must be absent for the female-specific low rate. SXL protein contains two RNA Recognition Motifs (RRM1 and RRM2 in
Fig. 1A), which have been shown to interact directly and sequence-specifically with nascent RNA of its target genes (Valcărcel et al., 1993; Sakashita and Sakamoto, 1994, 1996; Samuels et al., 1994; Wang and Bell, 1994).

In *Musca domestica*, the primary signal is given by a dominant male determiner (*M*; Perje, 1948). When *M* is present in the zygote, it imposes male development by preventing the activity of the switch gene *F*. When *M* is absent, *F* becomes active and directs female development (Dubendorfer et al., 1992). *F* could be the direct target of the primary sex-determining signal and thus would functionally correspond to *Sxl*. Parallels between *Sxl* and *F* are seen in the existence of a dominant mutation in *F*, *FD* (McDonald et al., 1978), and a recessive mutation, *F*<sup>man</sup> (Schmidt et al., 1997). These behave like dominant gain-of-function or recessive loss-of-function alleles of *Sxl* in *Drosophila*; regardless of the primary signal, the dominant mutations enforce the female mode of development, the recessive mutations the male mode. It is thus conceivable that these analogous functions are executed by the same regulatory molecules. We decided to test this assumption by searching for homologous *Sxl* sequences in the housefly genome. We report the isolation and characterisation of a gene in *Musca* that is the structural homologue of *Sxl* in *Drosophila*.

**MATERIALS AND METHODS**

**Procedure for sexing houseflies**

For isolation of cDNA, we used a wild-type strain with XX/XY sex determination. For RNA and protein analysis, unisexual progeny was produced as follows. (1) Purely male progeny was obtained by crossing *M*<sup>III</sup>/M<sup>III</sup> males (from an *M* strain described in Hilfiker-Kleiner et al., 1993) to XX wild-type females. All offspring receive the male-determining *M* factor and develop into fertile males. (2) Purely female progeny was obtained by crossing NoM males (derived from an Ag strain with maternal sex determination; Vanossi Este and Rovati, 1982) to XX wild-type females. In the absence of *M*, all embryos develop into fertile females. Stocks were cultured as described in Hilfiker-Kleiner et al. (1993).

**Isolation and analysis of *Sxl* homologous sequences in *M. domestica***

**Amplification with degenerated primers**

A codon bias of *M. domestica* was established by a statistical analysis of sequences derived from formerly isolated genes, *pgd*, *sod* and *Zw* (D. H.-K., unpublished results), Krüppel and hunchback (Sommer and Tautz, 1991). The bias was used to design degenerated PCR primers flanking the RRM of *D. melanogaster*. 1 µg of cDNA prepared from adult male and female houseflies was amplified in 100 µl with 50 µM of primers:

5′ : ATI GTI AA(C/T) TA(T/C) TTG CCI CA pos. 502
3′ : TG TGC CTC (T/C) (T/C) (T/C) (T/C) (T/C) (T/C) common pos. 918

The following conditions were chosen for 33 cycles: denaturation at 94°C for 1 minute, annealing at 42°C for 1 minute and extension at 72°C for 2 minutes. The amplified fragment was isolated and subcloned following routine DNA procedures.

**cDNA library screen**

Using the ZAP-cDNA synthesis kit from Stratagene, we constructed a *Musca* cDNA library with 5 µg of poly(A)<sup>+</sup> RNA isolated from ovaries of adult wild-type flies (XX). cDNAs were ligated into the Uni-ZAP<sup>TM</sup> XR vector and the packaged recombinant phages were transfected into XL1-blue MRF<sup>+</sup> cells (according to manufacturer’s directions). For the screen, we used an unamplified library containing 2×10<sup>6</sup> plated phages and probed it with the P<sup>32</sup>-labelled amplification fragment. Screening conditions, routine nucleic acid analysis and sequence analysis were done essentially as described in Sambrook et al. (1989).

**RT-PCR analysis**

1 µg of poly (A)<sup>+</sup> primed with oligo(dT) was reverse transcribed by Superscript RNaseH (Gibco BRL). 1/50 of the reversed transcribed material was used per PCR reaction for a specific pair of primers. Cycling conditions: denaturation at 92°C for 50 seconds, annealing at 55°C for 50 seconds and elongation at 72°C 70 seconds. The following oligonucleotides were used as primers in PCR reactions:

DB2 5′ CT TTC TGT GTT GTT ACC TAA common pos. 158
DB3 3′ AAA CGC TCT TAG TTT GCC TTC MdSxl2-specific pos. 1175
DB4 3′ CGA TGA TGT GAA ATC TGT GTC common pos. 310
DB5 3′ ATT TAC ACT AGA GTG TGG CTG MdSxc1-specific pos. 959

**Immunoblot analysis**

Purely male and purely female progeny of different stages were obtained from crosses as described above. The same amount of male and female material was collected in microfuge tubes and frozen in liquid N<sub>2</sub>. While thawing, material was homogenised in 2× SDS loading buffer. Samples were then boiled for 5 minutes to denature proteins and insoluble material was removed by centrifugation. An equal volume of the supernatant per lane was separated on 12% SDS-PAGE. After electrophoresis, 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 1:200 dilution of affinity-purified anti-SXL polyclonal serum (Bopp et al., 1993). Washes were performed in TBST. For detection of antibody-antigen complexes, we used an alkaline-phosphatase-conjugated secondary antibody (Promega) according to the manufacturer’s directions.

**Immunocytochemistry**

Embryos of wild-type houseflies were collected and washed in water. Subsequent handling and antibody incubations were done following the protocol as described by Bopp et al. (1991). For tissue staining, material was dissected from males and females in PBS 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 PBST (PBS, 0.1% Triton-X 100) and tissues were then blocked and permeabilized in 1 mg/ml bovine serum albumin (BSA) in PBST overnight at 4°C. For detection of SXL, the first antibody was applied as a 1:10 dilution of supernatant from the hybridoma line mSXL18 combined with a 1:500 dilution of ascites fluid from the same line in PBST containing 0.1 mg/ml BSA for 1 hour at room temperature. For MSL-2 detection, the first antibody was applied as a 1:100 dilution of a mouse monoclonal line (A. Hilfiker and J. Lucchesi, personal communication). After several rinses and washes in PBST, samples were treated with 15 µg/ml of Lissamine-Rhodamine-conjugated anti-mouse IgG H+L (Jackson Immuno-Research-Laboratories) for 1.5 hour at room temperature. After several washes, samples were counterstained with 50 µg/ml of DAPI, a nuclear dye. For better results, the material was postfixed in 15 minutes in 4% paraformaldehyde in PBS. After final washes in PBS, the stained samples were mounted in 80% glycerine containing 2.5% of DAPKO. Preparations were visualised by laser scanning confocal microscopy (Molecular Dynamics) and analysed on a Silicon Graphic workstation.

**Construction of MdSxl2 Drosophila transformants**

A 2.4 kb EcoRI fragment of MdSxl2 lacking 354 bp of trailer sequence was introduced into the unique EcoRI site of the pkB 256 transformation vector. This vector, a derivative of Carnegie 4, contains hsp70 promoter sequences upstream and tubulin trailer sequences.
including transcriptional stop sites downstream of the insertion site. The construct carries inverted repeats of P-elements for integration and a mini-white gene as selectable marker. *Drosophila w1118;1188* host embryos were injected with 417 μg/ml of construct DNA and 83 μg/ml of helper plasmid supplying P-transposase.

For heat-shock experiments, we inserted tubes containing freshly laid eggs into a heat-cycling device and programmed it for pulses of 30 minutes at 37°C in intervals of 8 hours when grown at 25°C. Flies were counted and inspected after eclosion. For salivary gland staining, early third instar larvae containing two doses of the transgene were exposed to 37°C for 30 minutes and left 1 day at 25°C before dissection.

RESULTS

A gene homologous to *Sxl* is present in the housefly

With a set of degenerate primers flanking the conserved RRM of *Sxl* in *D. melanogaster* (see Materials and Methods), a fragment of 407 bp was amplified from cDNAs prepared from adult houseflies. The isolated fragment revealed 75% homology at the nucleotide level and hybridised to a single band on a genomic Southern blot of *Musca*, indicating that this sequence is unique in the genome of the housefly (Fig. 1B). With this fragment as a probe, we isolated two cDNAs, MdSxl1 (1.2 kb) and MdSxl2 (2.8 kb), from an ovarian library prepared from adult flies (Fig. 1A). Sequences of MdSxl1 and MdSxl2 (GenBank accession numbers AF025689 and AF025690) are identical until nucleotide position 1099 where an alternative donor site is used in MdSxl1. The MdSxl1 sequence downstream of this position corresponds to the DmMS16 splice variant in *Drosophila*, whereas the MdSxl2 sequence downstream of position 1099 corresponds to that of *Drosophila* DmMS3 (Samuels et al., 1991). Overall homology at the amino acid level is 84% between MdSxl1 and DmMS16 and 83% between MdSxl2 and DmMS3 (Fig. 2). No homology could be detected in the untranslated sequences. In the amino terminal region upstream of RRM1, several blocks of well-conserved peptide stretches are interspersed with diverged sequences rich in gly, ser and asn. Several gaps had to be introduced into the *Musca* sequence to allow the best possible alignment of this part of the protein. The similarity in the first 124 amino acids is 77%. The highest degree of conservation (95%) is found in the region encompassing the two RRM, with only 8 non-conservative changes in 160 residues. This remarkably high degree of conservation extends another 18 residues downstream of RRM2 and is then followed by a poorly conserved region located around a proline-rich domain in *Drosophila Sxl*. The alternative carboxy ends again display a significant degree of similarity to the corresponding sequences in *Drosophila*: 52% for MdSxl2/DmMS3 and 85% for MdSxl1/DmMS16 (Fig. 2). **Sxl of Musca does not express sex-specific gene products**

We next investigated if *Sxl* of *Musca*, like the *Drosophila* gene, expresses sex-specific gene products. Using a polyclonal antibody against *Drosophila* SXL protein, two prominent classes of antigens, 36 and 38 kDa, are detected throughout *Musca* development (Fig. 3A). In contrast to *Drosophila*, however, the same protein isoforms are present at all stages in both male and female extracts. No sex-specific differences in mobility were detected suggesting that *Musca* *Sxl* produces full-length proteins in both sexes. The higher amount of SXL protein in extracts of adult females can be attributed to accumulation of SXL protein in ovaries, since extracts of adult males and of ovariectomized females contain similar amounts of SXL protein. Consistent with these results, northern blot analysis revealed no size differences in the *Sxl* transcripts of males and females (data not shown). We performed PCR experiments on cDNA templates prepared from poly(A)+ RNA of adult males and females to resolve small differences in RNA size that may have escaped detection in northern blot analysis. Primers derived from the leader sequence of MdSxl2 and trailer sequences of MdSxl1 and MdSxl2, respectively.

![Fig. 1.](image-url)

(A) Schematic diagram of *Sxl* cDNAs from *Musca*. The original amplified 407 bp fragment is depicted as a bar flanked by two half arrows which represent the primers. The broken vertical line marks the position of an alternative donor site. *MdSxl1* is incomplete at its 5’ end starting 10 residues downstream of the putative translation start signal. (B) Genomic Southern probed with the 407 bp fragment. DNA was isolated from adult males of the genotype *bw b ge M* (lanes 1 and 3) and from females of the genotype *bw b ge pw* (lanes 2 and 4), digested with *EcoRI* (lanes 1 and 2) or with *HaeIII* (lanes 3 and 4). (C) Schematic representation of two differentially processed *Sxl* transcripts of *D. melanogaster*. The carboxyl end of DmMS3 corresponds to that of MdSxl2 (stippled box), while that of DmMS16 is homologous to MdSxl1 (hatched box). The position of the translation terminating male exon (3), which is not included in DmMS3 and DmMS16, is indicated. No homology to a second large ORF present in the trailer of DmMS16 (marked as a grey box; see Samuels et al., 1991) is found in the *Musca* transcripts. Sequences of MdSxl1 and MdSxl2 have GenBank accession numbers AF025689 and AF025690.
amplified fragments of the same size in males and females (Fig. 3B). Also, amplification with primer pairs that correspond to sequences flanking the insertion site of the male exon in *Drosophila* yielded fragments of the same size in males and females. This analysis confirmed that the ORFs of both splice variants are indeed uninterrupted in both sexes.

Polyclonal as well as monoclonal antibodies against *Drosophila* SXL protein recognise nuclear antigens in *Musca* tissues. These antigens first appear in blastoderm embryos after onset of cellularisation (Fig. 4A). They are expressed in all somatic cells, but not in the pole cells, the progenitor cells of the germline (arrows in Fig. 4B). The detected antigens are predominantly localised to the nucleus where they display dots of accumulation in an otherwise diffusely stained nucleoplasmic background (Fig. 4C,F,G). Like in *Drosophila*, these antigens show an unusual association with condensed chromatin in mitotically active cells (Fig. 4C). Consistent with our immunoblot analysis, we find ubiquitous expression of these antigens in both sexes throughout development. The expression pattern in *Musca* tissue, as detected by our antibodies, corresponds to that of *Sxl* in *Drosophila* females. This and the fact that the same antibodies recognise ectopically expressed *Musca* SXL protein in *Drosophila* males (see Fig. 5) suggest that the cross-reacting antigens in *Musca* are products of its *Sxl* gene.

**Overexpression of Musca SXL protein in D. melanogaster causes lethality in XX and XY animals, but has no feminising effect**

Given the remarkable correspondence in sequence, it is

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**Fig. 2.** Comparison of the SXL proteins of *D. melanogaster* and *M. domestica*. A BESTFIT comparison (GCG tools package) was performed. DmSXL, sequence of *D. melanogaster*; MdSXL, *M. domestica*. The two RNA-binding motifs (RRM1 and RRM2) are boxed. Residues that are identical do not appear in the *Musca* sequence. Horizontal bars represent gaps introduced into the sequence to allow a best fit. (A) Alignment of the first 303 Musca residues which are identical in MdSxlc1 and MdSxlc2, and the corresponding part of the *Drosophila* protein. (B,C) Alignment of the carboxyl sequences of DmMS16 with that of MdSxlc1 (B) and of DmMS3 with that of MdSxlc2 (C).
conceivable that Musca SXL protein is functionally equivalent to that of Drosophila when expressed in the same molecular environment. To test this, we introduced a transgene containing MdSxlc2 sequences under the control of the heat-shock promoter hsp70 into the germline of D. melanogaster. With one copy of the transgene, viability and morphology at 25°C were the same as in non-transgenic siblings (Table 1). When animals carried two copies of the transgene, viability of both males and females was reduced by about 50% compared to siblings with one copy of the transgene; no sign of feminization was detected in surviving XY animals, which were fertile males. To increase MdSXLc2 expression, we exposed the transgenic flies to multiple heat pulses throughout their life cycle. With one copy of the construct, males of lines C2.1 and C2.3 show a significantly decreased viability when compared to their non-transgenic brothers (Table 1). Females with one copy of the construct were not affected by heat treatment. In animals with two copies, however, the lethal effects were more

Table 1. Relative viability of Drosophila flies carrying the MdSxlc2 construct was measured for flies growing at constant temperature of 25°C, or with heat pulses given for 30 minutes every 8 hours (HS) throughout development

<table>
<thead>
<tr>
<th>Line</th>
<th>Chromosome</th>
<th>One copy/no copy</th>
<th>Two copies/one copy</th>
<th>One copy/no copy</th>
<th>Two copies/one copy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25°C HS</td>
<td>25°C HS</td>
<td>25°C HS</td>
<td>25°C HS</td>
</tr>
<tr>
<td>C2.1</td>
<td>X</td>
<td>1.02 (294)*</td>
<td>0.63 (178)*</td>
<td>0.98 (220)*</td>
<td>1.05 (164)*</td>
</tr>
<tr>
<td>C2.2</td>
<td>2</td>
<td>1.30 (136)‡</td>
<td>1.10 (132)‡</td>
<td>1.23 (187)‡</td>
<td>1.05 (162)‡</td>
</tr>
<tr>
<td>C2.3</td>
<td>2</td>
<td>1.03 (200)‡</td>
<td>0.68 (123)‡</td>
<td>2.24 (305)‡</td>
<td>1.25 (142)‡</td>
</tr>
<tr>
<td>C2.4</td>
<td>3</td>
<td>1.30 (239)‡</td>
<td>1.60 (116)‡</td>
<td>1.70 (219)‡</td>
<td>2.30 (139)‡</td>
</tr>
<tr>
<td>C2.6</td>
<td>3</td>
<td>0.90 (42)‡</td>
<td>0.82 (49)‡</td>
<td>1.0 (38)†</td>
<td>0.71 (58)†</td>
</tr>
</tbody>
</table>

The total number of animals is indicated in parentheses.

* w1118 C2/w1118 × w1118/BY.
† w1118 C2/Binsc × w1118 C2.1/BY.
‡ w1118/BY: C2/CyO or TM3 × w1118/b1118 +/+.
§ w1118 BY: C2/CyO or TM3 × w1118/b1118; C2/CyO or TM3.

Fig. 4. Distribution of SXL protein in M. domestica. Mixed populations of Musca and D. melanogaster embryos were stained with monoclonal antibodies of line mSXL18 (Bopp et al., 1991). (A–C) Late blastoderm embryo with high levels of nuclear staining in somatic cells (sc), but not in the pole cells (pc). (C) Higher magnification: white arrowheads point to punctuated distribution of SXL in interphase nuclei. In the mitotically active region (demarcated by two black arrows), SXL protein remains associated with condensed chromatin. (D) Gastrulating embryo and (E) embryo 8-9 hours after fertilisation with fully extended germband. Staining is detected in all Musca embryos, while 50% of the added Drosophila embryos (Dm) remained unstained. Staining of salivary glands in a male larva (F) and in a female larva (higher magnification in G). In both sexes, salivary gland nuclei (sn) display foci of SXL protein (arrows).
dramatic: in line C2.2, we found only 4% males and 16% females compared to siblings with one insert of the construct and, in line C2.1, only one out of 292 females survived (Table 1).

In XY animals of Drosophila, expression of Drosophila SXL MS3 protein under the same promoter leads to strong feminization already at 25°C: many XY zygotes die due to a female setting of dosage compensation, and rare survivors appear morphologically as females as a consequence of female-specific expression of tra (Bell et al., 1991). These effects are in part directly induced by SXL protein and in part by activating the female mode of splicing of the endogenous Sxl in XY animals. To test for a feminising activity of Musca SXL, we examined the effects of the transgene on the three known target genes of Sxl.

Sxl
In transgenic males, anti-SXL antibody detects a 36 kDa antigen that is absent in extracts of non-transgenic control males (Fig. 5A). This protein isoform becomes greatly enriched in transgenic males after heat treatment and is present in males with or without an endogenous Sxl gene (Fig. 5B). Expression of the endogenous Sxl gene in XY males would result in two prominent protein isoforms of 36 and 38 kDa in size. As only one 36 kDa band appears in transgenic males, we conclude that Musca SXL is not able to impose the female mode of splicing on the endogenous Sxl gene.

msl-2
The lethality that is observed in males expressing MdSXLc2 protein could be caused by repressing translation of msl-2, a gene essential for the male mode of X-linked hypertranscription. Despite the presence of large amounts of Musca SXL protein in salivary gland cells of transgenic XY larvae, we find that MSL-2 is expressed and properly distributed to the X chromosome (Fig. 6).

tra
Ectopic expression of tra transforms XY animals into morphological females (McKeeown et al., 1988). Examination of XY animals expressing MdSXLc2 did not reveal any clear evidence for female transformation. In cases where males of lines C2.2 and C2.4 had been exposed to heat shocks, we occasionally found a reduced number of sex comb bristles, 3 or 4 instead of 12, but no female bristles. In other transgenic males, the genitalia were not fully rotated. The lack of any female structures argues against significant expression of TRA
induced by MdSXLc2. Furthermore, no yolk proteins, a typical product of tra activation, were detected in protein extracts of escaper males (data not shown).

**DISCUSSION**

**Sxl is conserved in Diptera, but regulation and function have diverged**

We describe the isolation and characterisation of a unique Musca gene that is highly homologous to Sxl in Drosophila. A well-conserved Sxl gene has also been found in other Diptera, such as Chrysomya rufifacies (Müller-Holtkamp, 1995), Megaselia scalaris (Sievert et al., 1997) and Ceratitis capitata (Saccone et al., 1998). Consistent with their closer phylogenetic relationship, Sxl products of M. domestica share a higher degree of structural similarity to those of C. rufifacies (95%) than to those of D. melanogaster (83%), or to those of D. virilis (82%) (Bopp et al., 1996). By sequence comparison, Sxl of C. capitata is as distantly related to that of Musca and Chrysomya (77-79%) as to that of Drosophila (73-75%) (Saccone et al., 1998). These findings reflect their phylogenetic relationship remarkably well and suggest that these genes have evolved from a common ancestor that must have existed at least 150 million years ago.

In D. melanogaster, Sxl is differentially regulated by the primary sex-determining signal to control sexual differentiation of the developing zygote. The presence of sex-specific Sxl transcripts in D. subobscura (Penalva et al., 1996) and D. virilis (Bopp et al., 1996) demonstrated that the same mechanism of sex-specific splicing is operational in these Drosophila species. In contrast, the Sxl genes of C. rufifacies (Müller-Holtkamp, 1995), Megaselia scalaris (Sievert et al., 1997), C. capitata (Saccone et al., 1998) and M. domestica (this work) produce identical transcripts in females and males. The regulatory mechanism by which a translation-terminating exon is inserted into the open reading frame of male transcripts is absent in these non-drosophilid species. This regulation may have evolved more recently in the genus Drosophila to provide a molecular basis for the control of sexual development. Our transgenic studies and those performed by Saccone et al. (1998) also show that, despite a high conservation in sequence, the biochemical property of the protein has diverged. Neither Musca nor Ceratitis SXL protein is capable of effectively mimicking the functions of the endogenous protein in Drosophila. Since the RNA-binding domains are almost identical and, hence, are likely to have preserved the same binding specificity, the cause for this functional disparity probably resides in sequences outside of the RRM. For instance, the amino-terminal region of Drosophila SXL has been implicated in cooperative binding of SXL to its target sequences on nascent RNA (Wang and Bell, 1994; Sakashita and Sakamoto, 1996). As this region is less well-conserved between Drosophila and Musca, the Musca SXL protein could fail to interact efficiently with other protein components necessary for stabilising its binding to target RNAs in Drosophila.

**What is the role of Sxl in Musca?**

The absence of sex-specific Sxl products in Musca argues against a correspondence of Sxl and the sex-determining gene F. Accordingly, we find no detectable changes in the expression pattern of Sxl in houseflies that carry a dominant gain-of-function allele of F, F<sup>1493</sup> (McDonald et al., 1978), or a loss-of-function allele, F<sup>man</sup> (Schmidt et al., 1997) (data not shown). Dismissing Sxl as a candidate for F raises the question whether F may have structural correspondence to any of the other sex-determining genes in Drosophila. It remains possible that F corresponds to tra, the next ON/OFF switch downstream of Sxl in the cascade. In Drosophila, tra appears to control all aspects of somatic sexual development, except dosage compensation. In Musca, the X and Y chromosomes are interchangeable, i.e. genetically equivalent, except for the male determiner on the Y (Rubini et al., 1972). Thus, there is no obvious need for a mechanism that compensates for different doses of X-linked genes in XX and XY houseflies. A tra-like gene would therefore suffice to act as the first gene in the sex determination cascade in Musca to govern sexual development. It is an interesting thought that Sxl may have been recruited in the genus Drosophila to coordinate the controls of dosage compensation and of sexual differentiation.

Different from Drosophila, Musca Sxl could be engaged in processes that are not related to sex determination. It is intriguing that most of the transcriptional regulators of Sxl in Drosophila also participate in controlling the neural pathway (Erickson and Cline, 1991; Parkhurst and Meneeley, 1994). Thus, Sxl and its regulators may have originally functioned as a regulatory cassette in neurogenesis. It is possible that this function of Sxl has been preserved in M. domestica. A role for Sxl in neurogenesis finds support in the observation that male embryos of D. virilis express a male-specific SXL protein that specifically accumulates in the developing central nervous system (Bopp et al., 1996).

A function of Sxl common to both Musca and Drosophila may reside in the germline. In Drosophila, the role of Sxl in the germline appears to be fundamentally different from that in the soma: rather than having a decisive sex-determining function, the germline activity of Sxl is needed to control some early steps in oogenic differentiation (Horabin et al., 1995). As the mode of oogenesis is meroistic-polytrophic in both Musca and Drosophila, the mechanisms of differentiation and, in particular, those early processes in which Sxl participates may well be conserved between the two species. As in Drosophila, SXL protein is already expressed in early gonial cells of the adult ovary of the housefly (D. B., unpublished results). Furthermore, ectopic expression of Musca SXL protein in Drosophila females that are sterile due to a germline-specific mutation in the endogenous Sxl gene can partially restore fertility (D. B., unpublished results). Thus, in contrast to the somatic function, Musca protein seems to be able to substitute the oogenic function in Drosophila. These preliminary data suggest that Sxl shares a well-conserved function in germline development of these dipteran insects. Support for this idea comes from studies in Megaselia scalaris, a phorid fly, where Sxl is expressed exclusively in the germline, but not in the soma of adult males and females (Sievert et al., 1997).

A conclusive answer as to the role of Sxl in Musca development has to await functional studies in the housefly. Such studies will not only reveal some of the principles by which sex-determining systems evolve, but also help to understand the evolutionary mechanisms that recruit regulatory molecules for different pathways.
REFERENCES


