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Dosage compensation in *Drosophila*: the X-chromosomal binding of MSL-1 and MLE is dependent on *Sxl* activity

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In *Drosophila*, dosage compensation, i.e. the equalization of levels of X-linked gene products in the two sexes, is achieved by the hypertranscription of most X-linked genes in males relative to females. The products of at least four genes, collectively termed male-specific lethal (*msl*) genes, are required for this process and, at least in the case of three of them, mediate this function through an association with the X chromosome in males. We have studied some of the parameters that affect the association of the *msl-1* gene product and found that its presence is dependent on the wild-type function of the other three genes, leading to the conclusion that these gene products contribute to the formation of a multi-subunit complex. Furthermore, the X-chromosomal association of the *msl-1* and *mle* gene products is negatively correlated with the level of function of the master regulatory gene *Sxl* and can assume either a mosaic or a uniform distribution in the tissues of mutant XX individuals. Surprisingly, we also found that the association of these two *msl* gene products with the two X chromosomes in females of certain mutant genotypes does not result in the hypertranscription of X-linked genes or in any apparent reduction in viability.

Key words: hypertranscription/male-specific lethal

Introduction

Although male *Drosophila* have a single X and females have two X chromosomes in their somatic cells, the products of most X-linked genes are present in equivalent amounts in the two sexes. This equalization was first noted by Stern (1929) and termed dosage compensation by Muller (1932, 1950); it is achieved by an enhancement of the transcriptional rate in males relative to females (Mukherjee and Beermann, 1965) and it depends on the activity of at least four autosomal genes: *male-specific lethal-1* (*msl-1*), *male-specific lethal-2* (*msl-2*), *maleless* (*mle*) and *maleless-on-the-third* (*mle3*, also known as *msl-3*) (Belote and Lucchesi, 1980; Uchida *et al.*, 1981; Lucchesi *et al.*, 1982); these genes are collectively referred to as *msl* genes. Loss-of-function mutations in any one of these genes are lethal to males because they result in a relative insufficiency in the level of X-linked gene products. Such mutations have no effect on the viability of females, leading to the conclusion that the products of the *msl* genes are not required in this sex. Male-specific transcriptional enhancement of X-linked genes also depends on the absence of an active product from the master regulatory gene, *Sex lethal* (*Sxl*). This conclusion is based principally on the observation that a constitutive allele of *Sxl* causes male-specific lethality (Cline, 1978) and that partial loss-of-function alleles of *Sxl* lead to an abnormally high rate of transcription of both X chromosomes in mutant larvae (Lucchesi and Skripsky, 1981).

The *mle* gene has been cloned and found to encode a polypeptide that contains several short motifs characteristic of a superfamily of DNA and RNA helicases. The *mle* polypeptide (MLE) is associated with hundreds of sites along the X chromosome in males, but not in females (Kuroda *et al.*, 1991). This association is dependent on the absence of *Sxl* function and requires the wild-type activity of the other *msl* genes (Gorman *et al.*, 1993). Similar results have been obtained with the *msl-3* gene product (M.Gorman and B.Baker, personal communication).

The *msl-1* gene encodes a novel polypeptide containing an acidic terminus characteristic of factors involved in transcription and chromatin modeling. As in the case of MLE, the *msl-1* polypeptide (MSL-1) is associated specifically with numerous sites along the length of the X chromosome in males (Palmer *et al.*, 1993). In the present paper, we report the results of a study by cyto-immunofluorescence of some of the parameters governing this sex-specific chromosomal association. Our results strongly support the hypothesis that the four *msl* gene products interact to form a multiprotein complex. Furthermore, our results indicate that the presence of an appropriate level of functional *Sxl* gene product (SXL) is necessary to prevent the association of MSL-1 with the X chromosomes in females, as is the case for MLE (Gorman *et al.*, 1993), and MSL-3 (M.Gorman and B.Baker, personal communication). Surprisingly, we have discovered that MSL-1 and MLE are bound to the two X chromosomes of females homozygous for vir^{1ts}, an allele of the *virilizer* gene (Hilfiker and Nöthiger, 1991), without resulting in the hyperactivity of X-linked genes and the concomitant reduction in the viability of these mutant females.

Results

MSL-1 fails to bind to the X chromosome in the absence of the other msl gene products

As previously reported (Palmer *et al.*, 1993), MSL-1 is present along the X chromosome and, in a reduced manner,

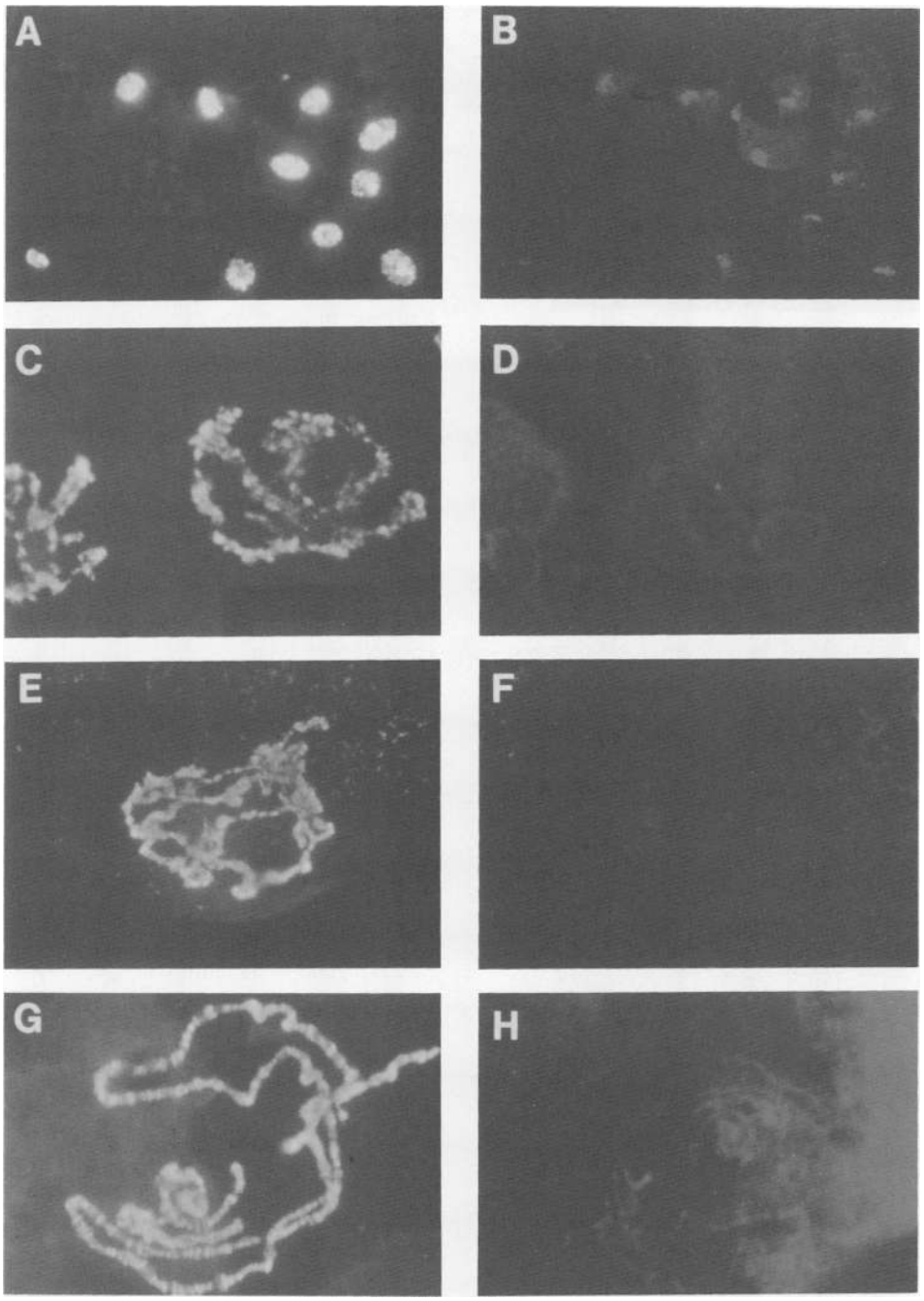

Fig. 1. Anti-MSL-1 staining of salivary gland nuclei from wild-type male and mutant male larvae. Chromosomes are stained with Hoechst 33258 (A, C, E and G) or with an anti-MSL-1 antibody (B, D, F and H) and photographed under oil immersion with a 100 × objective; (**A** and **B**) male-specific, anti-MSL-1 staining is evident in all nuclei from very young second instar wild-type male larvae; (**C** and **D**) nucleus from a *cn msl-2*^{Y9} bw homozygous male larva; (**E** and **F**) nucleus from a *msl-3 red* homozygous male larva; (**G** and **H**) nucleus from a *cn mle*^{Y38} *bw* homozygous male larva. There is no evidence of MSL-1 antibody binding to the X chromosome in any of these nuclei.

at a very few autosomal sites in wild-type male larvae but not in female larvae. To determine what effect the other *msl* genes may have on this binding, we generated male larvae homozygous for loss-of-function mutations of *mle*, *msl-2* and *msl-3*. Because such larvae are moribund, they almost invariably exhibit poorly developed salivary glands with small and ill-defined polytenic chromosomes. In light of this fact, it was necessary for us to establish that MSL-1 could be detected in such nuclei, if it were present. To this end, we stained nuclei of comparable size and morphology obtained from wild-type males. The results presented in Figure 1A and B demonstrate that the presence of MSL-1 can be clearly detected in nuclei with a low level of polytenization where the X chromosome cannot be distinguished from the other chromosomes in the complement. In fact, we have been able to extend this observation to the diploid nuclei of imaginal disks (data not shown). MSL-1 does not appear to be associated with the X chromosome in the nuclei of mutant male larvae homozygous for the loss-of-function alleles *mle*^{Y38}, *msl-*

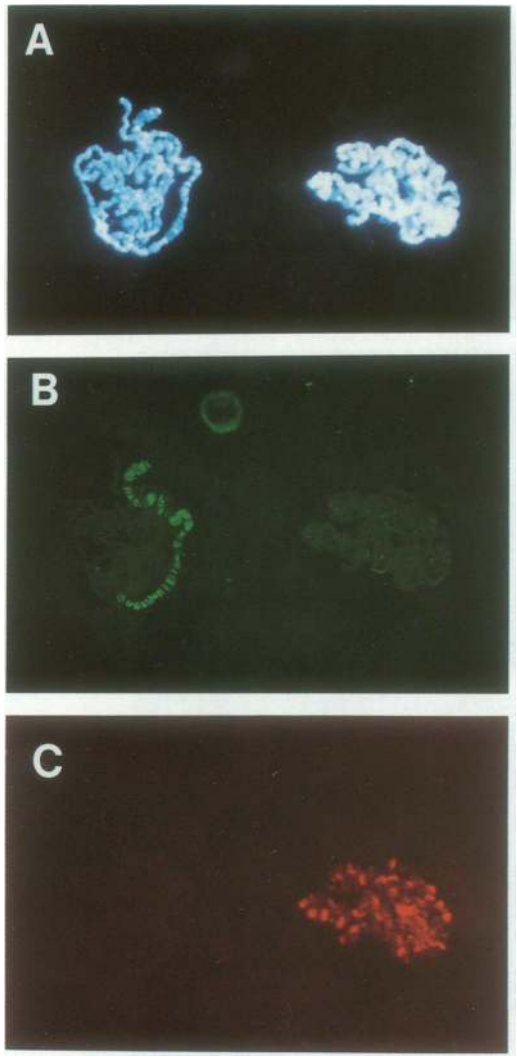

Fig. 2. Anti-MSL-1 and anti-SXL staining of salivary gland nuclei from a *y* Sxl^{fhv1} *mal/cm* Sxl^{f1} *ct* larva. Chromosomes are stained with Hoechst 33258 (**A**), with MSL-1 antibody (**B**) and with SXL antibody (**C**) and photographed under oil immersion with a 100 × objective. The nuclei that stain with one antiserum do not stain with the other.

2^{y9} or *msl-3* (Figure 1C–H). These results support the conclusion that the wild-type products of these genes are necessary for the binding of MSL-1 to the X chromosome in males.

Reduced Sxl gene function is required for MSL-1 binding

Since the occurrence of dosage compensation is known to be controlled by the presence or absence of the functional product of the regulatory gene *Sxl*, the role that this product may play in the binding of MSL-1 to the X chromosome was investigated. Because loss-of-function alleles of *Sxl* are lethal to females during embryonic development, a heteroallelic combination, Sxl^{fhv1}/Sxl^{f1}, was used to generate individuals with reduced *Sxl* function which could, nevertheless, develop to the third instar and yield salivary glands amenable to cytological investigation (Lucchesi and Skripsky, 1981). In addition, we collected larvae homozygous for the Sxl^{f2} allele that allows the recovery of some escaper adult females. When polytene chromosome spreads from such larvae were treated with MSL-1 antiserum, the majority showed a positive reaction indicating the presence of MSL-1 at numerous sites along the paired X-chromosome elements (103 positive nuclei from a total of 114 nuclei examined at random in 12 glands of Sxl^{fhv1}/Sxl^{f1} larvae; 59 positive nuclei from a total of 110 nuclei examined at random in eight glands of Sxl^{f2}/Sxl^{f2} larvae). A positive nucleus from a larva with the heteroallelic combination is shown in Figure 2A and B).

To determine if the nuclei that exhibit MSL-1 binding differ from those that do not with respect to the level of *Sxl* gene product, we exposed salivary gland preparations to two primary antisera, simultaneously: the rabbit anti-MSL-1 serum described above and a mouse monoclonal antiserum generated against SXL (line 18, Bopp *et al.*, 1991). When polytene chromosomes of wild-type females are exposed to SXL antiserum, the presence of the antigen is revealed at a large number of sites on all chromosomes (D.Bopp, personal communication). Using anti-rabbit and anti-mouse secondary antisera labeled with different fluorochromes we determined that a perfect negative correlation existed between the presence of SXL and the binding of MSL-1 in all of the nuclei that we observed (Figure 2C). These results are comparable with those obtained by Gorman *et al.* (1993) with respect to MLE.

Mutations that cause late female-specific larval lethality allow binding of MSL-1 and MLE in XX individuals

We wished to extend our investigation to mutant alleles of genes whose products appear to be required for proper Sxl^{+} function. To this end, we examined XX larvae carrying mutations of the *virilizer* (*vir*) gene (Hilfiker and Nöthiger, 1991) or mutations of the *female lethal 2d* [*fl(2)d*] gene (Granadino *et al.*, 1990). In the case of *virilizer*, we generated larvae homozygous for a female-specific lethal allele (vir^{2f}) or heteroallelic for this allele and a non-sex-specific lethal allele (vir^{3}). In the case of *fl(2)d*, we generated larvae homozygous for the $fl(2)d^{1}$ allele, which is a temperature-sensitive mutation, lethal in females and semi-lethal in males at 29°C and semi-lethal in females at 18°C (Granadino *et al.*, 1991). We also generated larvae heteroallelic for $fl(2)d^{1}$ and $fl(2)d^{2}$, a non-conditional lethal allele in both sexes; the heteroallelic combination is a temperature-sensitive lethal genotype: females die at 29°C and have greatly reduced viability at 18°C (Granadino *et al.*, 1992). We reared mutant larvae at the intermediate temperature of 25°C and we observed that MSL-1 and MLE are associated with numerous sites along the paired X chromosomes in all of the nuclei examined (Figure 3A and B). The absence of mosaicism in these mutant larvae is in contrast with the results obtained with the *Sxl* mutant alleles reported above.

MSL-1 and MLE bind to the two X chromosomes in mutant vir^{1ts} *individuals without apparent hyperactivation of X-linked genes or loss of viability*

The conditional vir^{1ts} mutant allele allows normal female development at rearing temperatures of ≤25°C, while at 29°C XX individuals are transformed into intersexes (Hilfiker and Nöthiger, 1991). We wished to determine if the binding of MSL-1 and MLE was correlated with

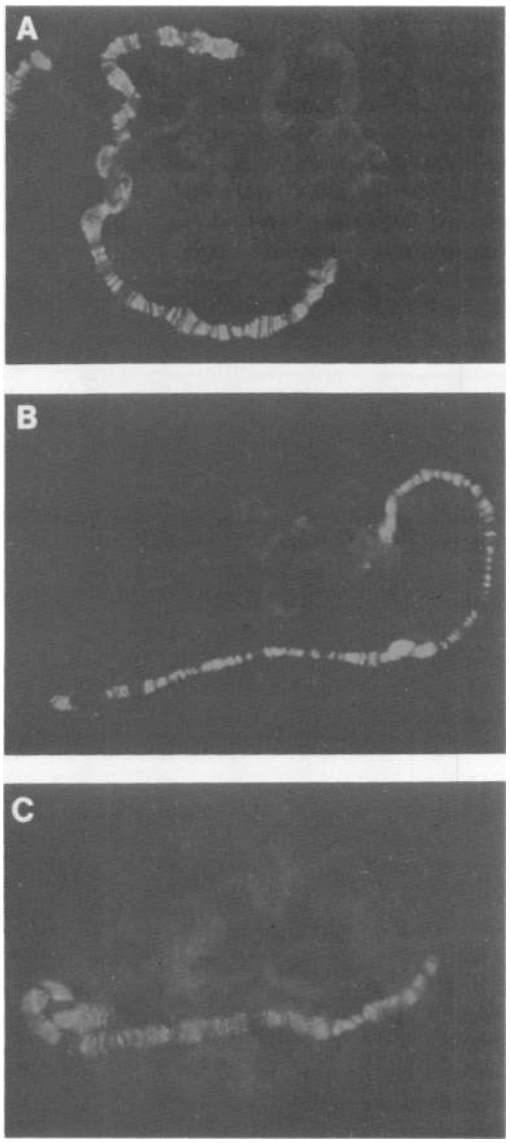

Fig. 3. Anti-MSL-1 staining of salivary gland nuclei from mutant XX larvae. Chromosomes are stained with MSL-1 antibody and photographed under oil immersion with a 100 × objective; (**A**) nucleus from a *y w; cn vir^{2f} bw* homozygous larva; (**B**) nucleus from a *y w; cn fl(2)d^{1} bw* homozygous larva; (**C**) nucleus from a *cn vir^{1ts} bw* homozygous larva raised at 18°C. Staining of the paired X chromosomes can be clearly seen in all three mutant larvae.

sexual transformation, i.e. if it was inversely correlated with an increase in rearing temperature. To our surprise, we observed staining of the X chromosomes with the MSL-1 and MLE antisera in all salivary gland nuclei, at all temperatures (Figure 3C). Close examination revealed that the pattern of antiserum binding is the same as in the case of *fl(2)d* and *vir^{2f}* mutant females and of wild-type males. We stained Malpighian tubules with anti-MSL-1 serum and confirmed that the binding of *msl* gene products to the X chromosome occurs in other larval tissues with polytenic nuclei (data not shown). The association of these *msl* gene products with the X chromosomes suggests the occurrence of hypertranscription of X-linked genes which, in females, is expected to result in decreased viability. To assess the viability of *XX;vir^{1ts}/vir^{1ts}* females reared at 25°C, we collected first instar larvae from the homozygous

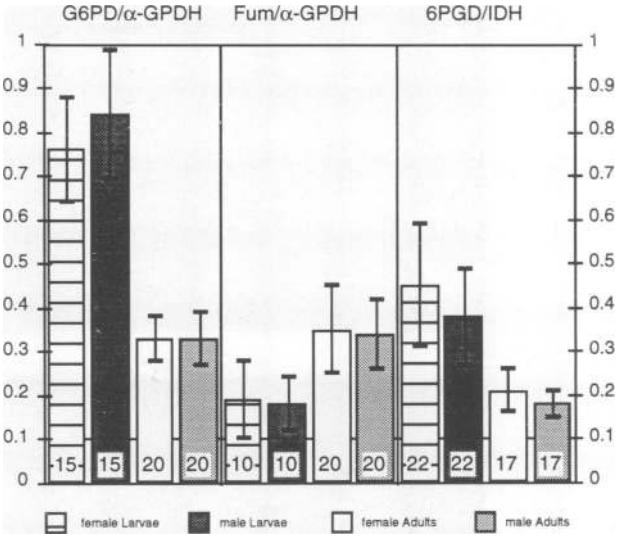

Fig. 4. Enzyme activities in mutant *vir^{1ts}* female larvae and adults. Glucose-6-phosphate dehydrogenase (G6PD), α-glycerophosphate dehydrogenase (α-GPDH), fumarase (Fum), 6-phosphogluconate dehydrogenase (6PGD) and isocitrate dehydrogenase (IDH) were measured in crude extracts of *cn vir^{1ts} bw* homozygous females; males of the same genotype were used as controls. The data reported in each panel represents the rate of activity for a particular X-linked enzyme normalized to the rate of activity of an autosomal enzyme in the same extract; the number of separate extracts assayed are given at the base of each column and the standard deviations from the mean are indicated.

stock and followed their development to adulthood. A total of 250 larvae produced 100 adult males and 91 adult females. These results suggest that the mutation has little effect on female viability. Since the imaginal disks contribute an overwhelming proportion of structures to the adult, we stained wing, leg and eye-antennal disks of *XX;vir^{1ts}/vir^{1ts}* larvae. While male larvae exhibited clear evidence of binding of the MSL-1 antiserum in all of their disk nuclei, we found no evidence of this binding in female larvae (data not shown). Finally, we measured several X-linked (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and fumarase) and autosomal (α-glycerophosphate dehydrogenase and isocitrate dehydrogenase) enzyme activities in crude extracts of mutant larvae and adults (Figure 4). With the exception of a slightly elevated 6-phosphogluconate dehydrogenase level in mutant female larvae there appears to be no increase in the relative level of X-linked enzyme activities in XX individuals due to the presence of the *vir^{1ts}* mutation.

The binding of SXL to multiple sites along the chromosomes of wild-type females is absent in vir^{1ts} mutant females

As mentioned above, SXL is found associated with numerous sites on the polytene chromosomes of wild-type females. Such an association is not detectable in the nuclei of *XX;vir^{1ts}/vir^{1ts}* female larvae (Figure 5A–D). Yet, a sufficient level of functional SXL product must be present since these larvae develop into normal, fertile females. We confirmed that this is the case by Western blot analysis, using a polyclonal antiserum that recognizes SXL polypeptides. The results, presented in Figure 6, show the presence of the two major female-specific SXL

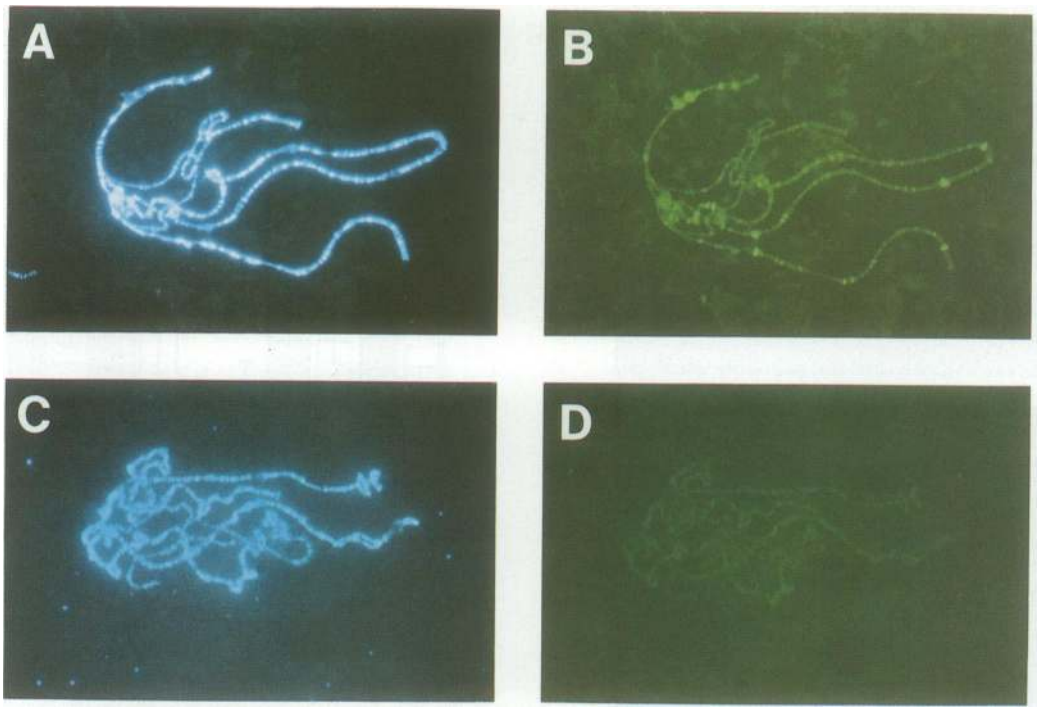

Fig. 5. Anti-SXL staining of salivary gland nuclei from wild-type Samarkand (**A** and **B**) and *cn* vir^{1ts} *bw* mutant (**C** and **D**) XX larvae. Chromosomes are stained with Hoechst 33258 (A and C) and with SXL antibody (B and D) and photographed under oil immersion with a 100 × objective. The association of the SXL antiserum with numerous sites on the chromosomes of the wild-type female larva is absent in the mutant larva.

products (Bopp *et al.*, 1993) in vir^{1ts} mutant females at levels that correspond to those of wild-type females.

To investigate further whether the binding of the *msl* gene products to the X chromosomes of vir^{1ts} mutant females is the result of the effect of the mutation on the function rather than on the level of SXL in these individuals, we used *SxlcF1*, a transduced Sxl^{+} cDNA sequence that is under the control of a heat shock promoter. In line #19, this construct is constitutively expressed even in the absence of heat shock (Bell *et al.*, 1991). We reared larvae of the genotype *XX;*vir^{1ts}*/*vir^{1ts}*;SxlcF1#19/+* at 25°C and exposed their salivary gland nuclei to MSL-1 and SXL antisera. Although we observed clear evidence of SXL antiserum binding throughout the genome of these larvae, MSL-1 was still associated with their X chromosomes (Figure 7A–C). We also introduced the constitutive allele Sxl^{M1} in vir^{1ts} females. In this case, as well, we observed MSL-1 antiserum binding to the X chromosomes of mutant larvae. Surprisingly, the SXL antiserum binding pattern was missing (Figure 7D–G).

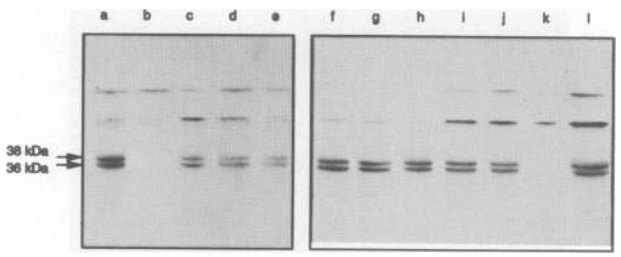

Fig. 6. Western blot analysis of SXl protein in wild-type and mutant larvae and adults. Blots of total protein extracts were treated with the anti-SXL polyclonal antibodies (Bopp *et al.*, 1993). Lane a: wild-type (Samarkand) adult females; b: wild-type males; c: *y* Sxl^{f2593}*/y cm* Sxl^{fPb} adult females; d: *y* Sxl^{f2593}*/y* Sxl^{fLS} *oc v f* adult intersexes; e: *y* Sxl^{f2593}*/y* Sxl^{f2593} adult pseudomales; f: *XX;cn* vir^{1ts} *bw/cn* vir^{1ts} *bw* third instar larvae raised at 29°C; g: *XX;cn* vir^{1ts} *bw/cn* vir^{1ts} *bw* third instar larvae raised at 25°C; h: wild-type third instar female larvae; i: *XX;cn* vir^{1ts} *bw/cn* vir^{1ts} *bw* adult intersexes raised at 29°C; j: *XX;cn* vir^{1ts} *bw/cn* vir^{1ts} *bw* adult females raised at 25°C; k: wild-type males; l: wild-type females. The two major SXL proteins are present in all XX individuals analyzed; the bands that are present in all lanes are due to cross-reactivity of the polyclonal antiserum and serve as a control for the total amount of protein loaded in each lane.

The isoform of histone 4 acetylated at lysine 16 (H4Ac16) is found on the X chromosomes of vir^{1ts} *mutant females*

Using a specific polyclonal antiserum, Turner *et al.* (1992) have shown that H4Ac16 is preferentially associated with the X chromosome in the salivary gland nuclei of male but not of female larvae. Having established that at least two *msl* gene products bind to multiple sites along the X chromosomes in *XX;*vir^{1ts}*/*vir^{1ts} larvae, we wished to determine if the histone isoform characteristic of X-chromosome chromatin in males would also be present in these mutant females. We exposed the polytene chromosomes of mutant larvae raised at 25°C to H4Ac16 antiserum and observed clear, positive staining along the paired X chromosomes (Figure 8A and B). To determine the relationship between the binding of the *msl* gene products and the presence of H4Ac16, we introduced the loss-of-function allele *msl-*1^{Y222} or mle^{Y38} in vir^{1ts} homozygous female larvae and exposed their salivary gland chromosomes to H4Ac16 antiserum. We found no evidence of the presence of this isoform in these mutant larvae (data not shown).

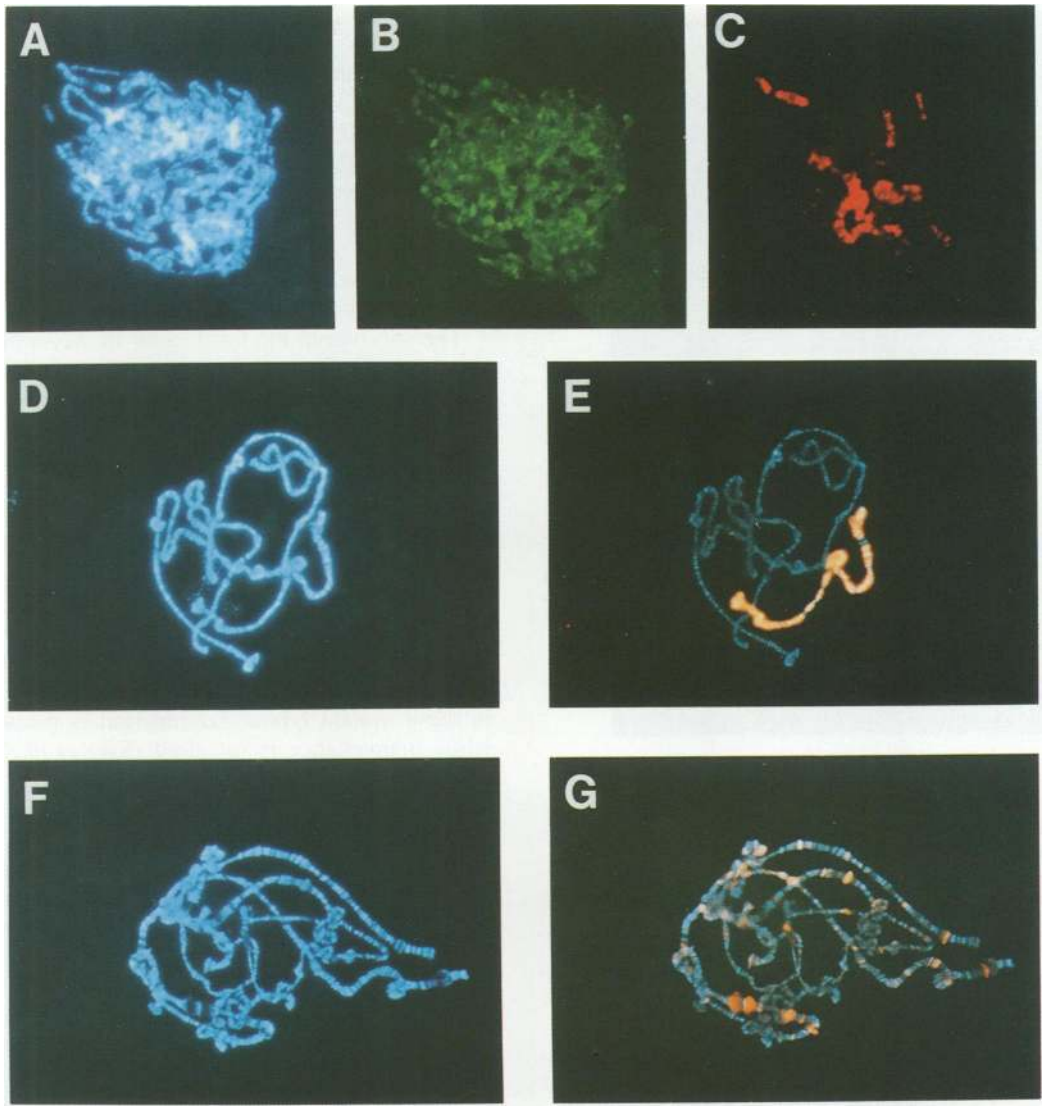

Fig. 7. Anti-MSL-1 and anti-SXL staining of vir^{1ts} homozygous mutant females carrying the constitutive alleles of *Sxl*. (**A**–**C**) Nucleus from a *XX; cn* vir^{1ts} *bw/cn* vir^{1ts} *bw;TM3, Ser SxlcF1#19/+* larva stained with Hoechst 33258 (**A**) and with mouse anti-SXL followed by goat fluorescein-labeled anti-mouse serum (**B**) or with rabbit anti-MSL-1 followed by Texas Red-labeled goat anti-rabbit serum (**C**); although the chromosomes are poorly spread because of the presence of the TM3 balancer, binding of SXL and MSL-1 antisera can be seen. (**D** and **E**) Nucleus from a *y cm* Sxl^{M1}*/+;cn* vir^{1ts} *bw/cn* vir^{1ts} *bw* larva stained with Hoechst and with mouse anti-SXL and mouse anti-MSL-1 followed by cyanine-labeled goat anti-mouse serum; only the binding of MSL-1 antibodies to the paired X chromosomes is evident. (**F** and **G**) Nucleus from a control larva of genotype *y cm* Sxl^{M1}*/+;cn* vir^{1ts} *bw/T(2;3)ap*^{Xa}, *Xa* stained with Hoechst and with mouse anti-SXL and mouse anti-MSL-1 followed by cyanine-labeled goat anti-mouse serum; in this nucleus, due to the presence of a wild-type allele of *vir*, the pattern of SXL staining is evident and MSL-1 staining is absent. All nuclei were photographed with a 40 × objective.

Certain Sxl mutations that affect maintenance but not initiation of SXL synthesis give the same results as vir^{1ts}

We raised Sxl^{f2593}/Sxl^{f2593} larvae at 25°C and stained their polytene chromosomes for the presence of MSL-1 and MLE. We also generated Sxl^{f2593}/Sxl^{fPb} and Sxl^{f2593}/Sxl^{fLS} larvae and stained their chromosomes for the presence of MSL-1. As in the case of vir^{1ts} mutant larvae, all nuclei examined exhibited the presence of these *msl* gene products on the paired X chromosomes (data not shown). Staining with the SXL monoclonal antiserum failed to reveal any SXL associated with the chromosomes in Sxl^{f2593}/Sxl^{f2593} and Sxl^{f2593}/Sxl^{fPb} larvae. Western blot analysis of extracts from adults of all three of the above genotypes show the presence of the female-specific *Sxl* gene products (Figure 6).

Discussion

Our results indicate that the preferential association of MSL-1 with the X chromosome in males depends on the presence of the wild-type products of the other three *msl* genes. The same relationship holds for MLE (Gorman *et al.*, 1993) and for MSL-3 (M.Gorman and B.Baker, personal communication). These observations lead to the important conclusion that the *msl* gene products must interact as a precondition to X-chromosome binding in males. This conclusion is supported by the cytological

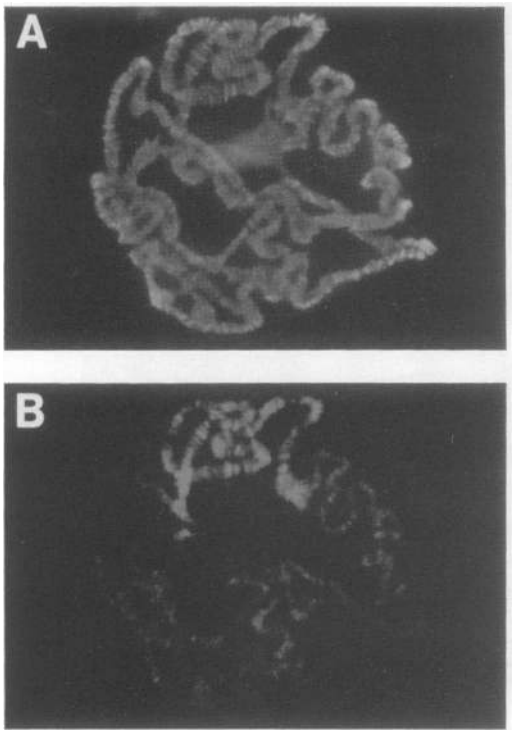

Fig. 8. Anti-H4Ac16 staining of vir^{1ts} homozygous mutant females. Chromosomes of a *XX;cn* vir^{1ts} *bw/cn* vir^{1ts} *bw* larva raised at 25°C are stained with Hoechst 33258 (**A**) and with anti-H4Ac16 (**B**). Photographed under oil immersion with a 100 × objective. Histone 4 acetylated at lysine 16 is preferentially associated with the paired X chromosomes.

co-localization of MSL-1 and MLE observed by Bone *et al.* (1994).

As predicted by Cline (1978), the absence of *Sxl* function (or a significant reduction of this function) is deleterious to XX individuals because of the inappropriate hypertranscription of the two X chromosomes (Lucchesi and Skripsky, 1981). In accordance with this conclusion we have observed that MSL-1 binds to the two X chromosomes in ~90% of the nuclei of Sxl^{fhv1}/Sxl^{f1} individuals and in ~55% of the nuclei of Sxl^{f2}/Sxl^{f2} individuals. The binding of MSL-1 to the two X chromosomes in a particular nucleus is correlated with the absence of a detectable level of SXL functional product. Such a correlation was clearly established by Gorman *et al.* (1993): all nuclei whose chromosomes stained with the SXL antiserum were negative for anti-MLE staining of the X chromosomes and vice versa. We have observed the same correlation with respect to the binding of MSL-1.

Chromosomal female (XX) individuals homozygous for vir^{2f} or heteroallelic for this allele and vir^{3} die during larval development; these individuals can be shown by analysis of genetic mosaics to be highly masculinized. Furthermore, $XX;vir^{2f}/vir^{2f}$ individuals can be rescued by introducing a constitutive *Sxl* mutation in their genome (A.Hilfiker and R.Nöthiger, personal communication). These as well as other studies with vir^{1ts} (Hilfiker and Nöthiger, 1991) indicate that the *vir* gene product is necessary for the proper function of SXL in females. Binding of MSL-1 to the X chromosomes in all nuclei of XX mutant larvae is concordant with the presumed diminished female-specific *Sxl* function in these larvae.

The wild-type *fl(2)d* gene appears to be another gene involved in the generation of functional, female-specific SXL product since $XX;fl(2)d^{1}/fl(2)d^{1}$ and $XX;fl(2)d^{1}/fl(2)d^{2}$ mutant females exhibit male-specific *Sxl* transcripts (Granadino *et al.*, 1990) and their temperature-sensitive lethality can be rescued by a constitutive allele of *Sxl* (Granadino *et al.*, 1992). Larvae of these genotypes, reared at the intermediate temperature of 25°C, were found to exhibit anti-MSL-1 staining of the two X chromosomes in accord with their reduced level of *Sxl* function.

The survival to the third instar of XX individuals with impaired *Sxl* function can be achieved if a mixture of mutant and wild-type cells are present or if the SXL product or its function decline gradually, in all cells, as development proceeds. In our experiments, inferences with respect to *Sxl* function are based on the binding of MSL-1 and MLE to the X chromosomes. In the case of Sxl^{fhv1}/Sxl^{f1}, the mosaicism is due to a failure in initiation of *Sxl* activity in some but not all cells, whereas in Sxl^{f2}/Sxl^{f2} larvae initiation occurs in all cells but maintenance fails (Bernstein and Cline, 1994) in a subset of these cells. In the case of combinations of Sxl^{fLS}, Sxl^{fPb} with the Sxl^{f2593} allele, the absence of mosaicism is caused by the fact that in these mutant larvae *Sxl* function is properly initiated while maintenance is impaired (Salz *et al.*, 1987) in all cells. By analogy, and consistent with the genetic data mentioned above, vir^{2f} and *fl(2)d* interfere with the maintenance of *Sxl* function, as well.

In all of the cases discussed so far, the binding of MSL-1 or MLE to the X chromosomes in XX individuals is concomitant with a reduction of SXL function and a decrease in viability. It was surprising, therefore, to observe binding of these two *msl* gene products to the X chromosomes of salivary glands and Malpighian tubules of $XX;vir^{1ts}/vir^{1ts}$ larvae reared at 25°C, a permissive temperature that allows them to develop into fertile adult females. Given the abundance of tissues with polytenic nuclei relative to diploid tissues, the apparent lack of hypertranscription of X-linked genes in these mutant larvae was unexpected. This result could be explained if the male X-specific isoform of histone 4 (H4Ac16) were a required intermediate in this process and if it were absent in these larvae. We established that H4Ac16 is, in fact, present on the X chromosomes of vir^{1ts} mutant larvae, thereby negating this possible explanation. These experiments, nevertheless, led us to an important ancillary conclusion. When MSL-1 and MLE are prevented from binding to the X chromosomes in $XX;vir^{1ts}/vir^{1ts}$ larvae by making them homozygous for an *msl-1* or an *mle* loss-of-function allele, H4Ac16 is also absent from these chromosomes. This observation suggests that the presence of H4Ac16 on X chromosomes is a consequence rather than a precondition to the binding of the *msl* gene products.

The absence of MSL-1 staining in imaginal disks may explain the normal viability of adult $XX;vir^{1ts}/vir^{1ts}$ females and it is possible that the apparent lack of hypertranscription in mutant vir^{1ts} larvae could have a trivial explanation. Since our cytological techniques do not permit us to quantify the amount of complex bound to the X chromosomes, this amount could be less than in the case of other mutant females, leading to some low level of hyperactivation of X-linked genes that we are unable to

measure with our enzymological methods and that is compatible with high viability.

Although *XX;vir^{1ts}/vir^{1ts}* individuals develop into normal females at permissive temperatures, their level of *Sxl* function must be barely above the threshold needed for normal female development. This contention is supported by the observation that removal of one of the two functional doses of the *tra* gene in these individuals leads to their differentiation into intersexes (Hilfiker and Nöthiger, 1991) and removal of one of the two functional doses of the *Sxl* gene leads to lethality (A.Hilfiker and R.Nöthiger, personal communication). Furthermore, even though the major SXL protein bands are present in Western blots of *XX;vir^{1ts}/vir^{1ts}* individuals, the SXL antiserum fails to bind to their polytene chromosomes. It should be noted that the SXL protein must be present in these nuclei because only XX individuals that can splice *tra* transcripts in the female mode—an SXL-dependent process—develop into phenotypic females as *XX;vir^{1ts}/vir^{1ts}* individuals do.

We attempted to increase *Sxl* activity in these individuals by introducing the constitutive allele *Sxl^{M1}*; the presence of this allele failed to restore the dispersed anti-SXL binding pattern and did not eliminate the anti-MSL-1 staining of the paired X chromosomes. These results suggest that the staining pattern in *XX;vir^{1ts}/vir^{1ts}* individuals is not correlated simply with a reduced level of female-specific splicing of the *Sxl* primary transcript since this level is augmented by the presence of the *Sxl^{M1}* allele. We also introduced the *SxlcF1#19* fusion gene in *XX;vir^{1ts}/vir^{1ts}* individuals and observed that it restores SXL chromosomal binding, yet without interfering in any noticeable manner with the binding of MSL-1. This fusion gene encodes one particular SXL protein which is capable of splicing the primary transcripts of the endogenous *Sxl* gene in males, initiating the autoregulatory production of SXL and resulting in lethality. Nevertheless, the *SxlcF1#19* protein product is insufficient to rescue XX embryos homozygous for loss-of-function alleles such as *Sxl^{f1}* (Bell *et al.*, 1991). In *XX;vir^{1ts}/vir^{1ts}* individuals, although *SxlcF1#19* raises the level of SXL gene product sufficiently to allow SXL binding it does not prevent the association of MSL-1 with the X chromosomes. We can conclude that, in a *vir^{1ts}* background, XX individuals lack a sufficient level of a particular SXL product that normally prevents MSL-1 and MLE from binding to the X chromosomes. Alternatively, the level of this particular SXL product may be adequate but its function may be impaired by the absence of wild-type *vir* products. To distinguish between these alternatives will require a detailed comparison of the transcripts and of the protein inventories generated by the two constitutive *Sxl* alleles used in the present study with those of the wild-type allele, in the presence or absence of the *vir^{1ts}* mutation.

Materials and methods

Antibody preparation

A 965 bp *Bam*HI fragment from cDNA *msl*-1(ZV)6 encoding a polypeptide of 321 amino acids (Palmer *et al.*, 1993) was subcloned into pGEMEX-2 (Promega). The glutathione-S-transferase−MSL-1 fusion protein was purified on a glutathione column according to the manufacturer's specifications (Promega) and used to immunize a rabbit from which pre-immune serum had been collected. Another antiserum against a polypeptide corresponding to the entire open reading frame was generated in mice and was used to stain the nuclei described in Figure 7E and G.

Culture conditions

Drosophila stocks were maintained and crosses were performed on standard cornmeal-molasses-yeast agar medium containing propionic acid and methylparaben as mold inhibitors and seeded with active dry yeast. For the collection of larvae, ~20–30 pairs of parents (depending on the fecundity of the females involved) were placed in a culture bottle and were transferred daily to fresh bottles. The cultures were overlaid with yeast paste and reared at 25°C unless noted otherwise. The *msl*, *vir* and *fl(2)d* mutant alleles are described in the text. All other mutant alleles and the special chromosomes listed in the text or in the figure legends are described in Lindsley and Zimm (1992). Depending on the particular cross, mutant larvae were recognized by the absence of the *Bc* dominant marker or the presence of red Malpighian tubules, yellow versus black mouth hooks, or by the cytological identification of a balancer chromosome in the polytene nuclei preparations.

Cytoimmunofluorescence

Salivary glands were dissected in 0.7% NaCl and fixed in PBS, 0.1% Triton X-100, 3.7% formaldehyde for 20 s, then in 50% acetic acid, 3.7% formaldehyde for 3 min according to the method of Kuroda *et al.* (1991). The glands were squashed and the slides were immersed in liquid nitrogen and allowed to accumulate on dry ice. Blocking of the chromosomes, incubation with the primary antiserum (diluted 1:500) and with Texas Red-conjugated goat anti-rabbit secondary antiserum (diluted 1:200; Jackson ImmunoResearch Laboratories, Inc.), staining of the chromosomes with 0.01 mg/ml Hoechst 33258 and mounting in 80% glycerol, 2% *n*-propyl gallate were performed according to Kuroda *et al.* (1991). When the anti-SXL monoclonal antiserum was used (diluted 1:5), the secondary antiserum was goat anti-mouse conjugated with fluorescein, Texas Red or cyanine. The chromosomes were examined with a Zeiss Axioplan Universal microscope equipped for incident light fluorescence and photographed with an MC100 camera. Positive controls were included in every series of slides to confirm that the absence of staining in certain genotypes was a valid experimental result.

Western blotting

Preparation of the extracts, SDS−PAGE and transfer of the proteins to nitrocellulose were performed according to Bopp *et al.* (1991). The efficiency of transfer was monitored by staining the gels with Coomassie Blue and the nitrocellulose blots with Ponceau S Red. Following blocking, the blots were treated with anti-SXL polyclonal serum (diluted 1:500) followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antiserum.

Enzyme assays

Samples of hemisected adult flies, aged for 3 days, or freshly collected larvae were weighed and homogenized in 0.1 M Tris, 1.0 mM EDTA, 7.0 mM β-mercaptoethanol buffer (pH 8.5) at a concentration of 10 mg live weight/ml. The reaction mixture for α-glycerophosphate dehydrogenase was 4.5 mM NAD^+, 30 mM α-glycerophosphate in 0.1 M glycine-NaOH (pH 9.5) buffer; for isocitrate dehydrogenase, 1.4 mM $NADP^+$, 6.0 mM isocitrate, 20.0 mM $MgCl_2$ in 0.1 M Tris (pH 8.5) buffer; for glucose-6-phosphate dehydrogenase, 0.6 mM $NADP^+$, 6.6 mM glucose-6-phosphate, 10.0 mM $MgCl_2$ in 0.2 M Tris (pH 8.5) buffer; for 6-phosphogluconate dehydrogenase, 1.4 mM $NADP^+$, 2.2 mM 6-phosphogluconate, 20 mM $MgCl_2$ in 0.2 M Tris (pH 7.5) buffer; for fumarase, 50.0 mM sodium malate in 0.1 M $NaPO_4$ (pH 7.8) buffer. Enzyme activities were monitored as the rate of increase in absorbance at 340 nm for all enzymes except fumarase which was monitored at 250 nm.

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