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Evidence that MSL-mediated dosage compensation in *Drosophila* begins at blastoderm

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SUMMARY

In *Drosophila* equalization of the amounts of gene products produced by X-linked genes in the two sexes is achieved by hypertranscription of the single male X chromosome. This process, dosage compensation, is controlled by a set of *male-specific lethal (msl)* genes, that appear to act at the level of chromatin structure. The properties of the MSL proteins have been extensively studied in the polytene salivary gland chromosomes where they bind to the same set of sites along the male X chromosome in a co-dependent manner. Here we report experiments that show that the MSL proteins first associate with the male X chromosome as early as blastoderm stage, slightly earlier than the histone H4 isoform acetylated at lysine 16 is detected on the X chromosome. MSL binding to the male X chromosome is observed in all somatic tissues of embryos and larvae. Binding of the MSLs to the X chromosome is also interdependent in male embryos and prevented in female embryos by the expression of *Sex-lethal (Sxl)*. A delayed onset of binding of the MSLs in male progeny of homozygous mutant *msl-1* or *mle* mothers coupled with the previous finding that such males have an earlier lethal phase supports the idea that *msl*-mediated dosage compensation begins early in embryogenesis. Other results show that the maleless (MLE) protein on embryo and larval chromosomes differs in its reactivity with antibodies; the functional significance of this finding remains to be explored.

Key words: dosage compensation, *Drosophila*, *male-specific lethal*, X chromosome

INTRODUCTION

Organisms with a different number of X chromosomes in males and females have to equalize the level of transcripts produced by X-linked genes. This process, termed dosage compensation, was first described in *D. melanogaster* (Muller, 1932), where dosage compensation is achieved by hypertranscription of the single male X chromosome, so that it produces as many transcripts as the two female X chromosomes (Mukherjee and Beermann, 1965). Hypertranscription is correlated with an altered structure of the male X chromosome, which, in the light microscope, has a more diffuse appearance than other chromosomes and is as wide as the two female X chromosomes (Offermann, 1936; Dobzhansky, 1957). Four genes are known that regulate dosage compensation: *maleless* (*mle*), *male-specific lethal-1, -2* and *-3 (msl-1, msl-2, msl-3*, respectively; Fukunaga et al., 1975; Belote and Lucchesi, 1980a; Belote and Lucchesi, 1980b; Uchida et al., 1981; Lucchesi et al., 1982). Mutations in these loci, which are collectively referred to as the *male-specific lethals (msls)*, reduce the transcription rate of X-linked genes in males to about 60% of wild-type levels (Belote and Lucchesi, 1980a), leading to lethality at late larval/early pupal stages. In *msl* mutant males there is also a change in the structure of the X chromosome, which now has the same appearance as other chromosomes (Belote and Lucchesi, 1980a; Lucchesi et al., 1982; Okuno et al., 1984; Gorman et al., 1993). Mutations in the *msl* loci do not have any obvious effects in females.

Antibodies against the MSL proteins have revealed a striking, common feature of all four proteins. Immunostaining of polytene chromosomes from third instar larval salivary glands shows binding of these proteins to hundreds of sites along the male X chromosome and a small number of sites on the autosomes, whereas essentially no binding is observed on chromosomes from female larvae (Kuroda et al., 1991; Palmer et al., 1993; Gorman et al., 1993; Bashaw and Baker, 1995; Gorman et al., 1995; Kelley et al., 1995; Zhou et al., 1995). The sites at which these four proteins are bound to chromosomes are identical, with the exception that MLE is found at a few dozen sites where the other MSL proteins are not bound. Strikingly, binding of each of the MSL proteins is dependent on the presence of all four proteins, suggesting that the MSL proteins may be associated in a heteromeric protein complex (Gorman et al., 1993; Hilfiker et al., 1994; Palmer et al., 1994; Bashaw and Baker, 1995). The first biochemical support for this hypothesis is provided by the finding that the MSL-1 and MSL-2 proteins can be co-immunoprecipitated from protein extracts of male larvae (Kelley et al., 1995).

An important clue as to the function of the *msls* came from the finding that histone H4 acetylated at the lysine 16 residue

(H4Ac16) is also associated with hundreds of sites along the male's X chromosome (Turner et al., 1992). H4Ac16 is additionally associated with 30-40 autosomal sites in both sexes, and a small number of X chromosome sites in females (Turner et al., 1992). The enrichment of H4Ac16 on the male's X is dependent on *msl* function, and the positions on the male X chromosome where H4Ac16 is detected coincide largely with MSL binding sites (Bone et al., 1994). Although far from conclusive, the simplest interpretation of these data point to a role of the MSLs in changing chromatin structure to bring about dosage compensation (reviewed by Baker et al., 1994).

The recent molecular characterization of the *msl-2* gene (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995) provided substantial insight into the molecular mechanism by which dosage compensation is restricted to males. Although it was previously shown that *Sex-lethal* (*Sxl*) prevents binding of the MSL proteins in females (Gorman et al., 1993; Hilfiker et al., 1994; Palmer et al., 1994), the molecular nature of this regulation was unclear. Analysis of the *msl-2* gene showed that *msl-2* mRNA has the same ORF in males and females, but is translated only in males. Sequences in both its 3′ and 5′ untranslated regions (UTRs) have segments that match consensus sequences for SXL binding sites (as defined by Samuels et al., 1994) suggesting that *msl-2* may be a direct target of *Sxl* regulation which prevents the translation of the *msl-2* mRNA in females (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995). Strikingly, transgenes lacking either the 3′, or 3′ and 5′, *msl-2* UTRs ectopically express MSL-2 protein in females and this leads to binding of all four MSL proteins to the female X chromosomes (Bashaw and Baker, 1995; Kelley et al., 1995). These findings suggest that *msl-2* is the only sex-specifically regulated member of the *msls*.

Here we focus on two conceptually distinct, but technically closely intertwined aspects of dosage compensation. First, while it is clear that dosage compensation occurs from the early embryo through adult stages, and in diploid as well as polytene cells, most studies of dosage compensation have been done at the organismal level (by analyzing the levels of proteins or RNAs) and thus there are only a few specific tissues (e.g. larval salivary gland cells, the pigment cells of the eye, and imaginal discs) in which dosage compensation has been documented to occur (for review see Lucchesi and Manning, 1987). Moreover, essentially all of the work that has been reported on the characterization of the properties of MSL proteins has been carried out in polytene salivary gland cells. While the properties of the MSL proteins in salivary gland cells may be representative of other cell types the only data on other cell types are the findings that in third instar larval brains the MSL-1, MLE and H4Ac16 proteins are co-localized on the X chromosome in males during cell division (Lavender et al., 1994).

Second, there is substantial evidence that there are two distinct processes of dosage compensation in *Drosophila*. Dosage compensation mediated by the *msls* occurs (at least) during the larval, pupal and adult periods (for reviews see Baker et al., 1994; Lucchesi and Manning, 1987). However, in the early embryo dosage compensation has only been assayed at two genes (Gergen, 1987; Polito et al., 1990) and for the one of those at which the regulation of dosage compensation was examined it was found to be under the control of *Sxl*, but independent of the *msl* genes (Gergen, 1987; Bernstein and Cline, 1994). The findings of an *msl*-independent dosage compensation process (referred to herein as 'early dosage compensation') are all based on assaying the phenotypic effect of mutations in the *runt* gene, which functions during an approximately one-hour time period at blastoderm (Gergen, 1987). There is currently little data on the relative domains of early dosage compensation and *msl*-mediated dosage compensation. They could be temporally sequential processes, or function simultaneously in distinct tissues or on distinct sets of genes (Bernstein and Cline, 1994; Kelley et al., 1995).

In order to better understand the temporal domains of early, *msl*-independent, dosage compensation and *msl*-mediated dosage compensation, we have undertaken studies to analyze at what time during embryonic development the *msls* start to function to regulate dosage compensation. We show that the MSL proteins accumulate in a subregion of male nuclei beginning at late blastoderm stage. We demonstrate that this nuclear compartment is the X chromosome by showing that MSL staining exactly coincides with the in situ signal from an X-specific DNA probe. The X chromosome binding of the MSLs is observed throughout embryonic and larval development in both diploid and polytene tissues. H4Ac16 colocalizes with the MSLs in embryos, although it is first detected at slightly later stages of embryogenesis. Binding of the MSLs is interdependent in diploid cells and is prevented in female embryonic cells by *Sxl*. We observe a delay in the onset of binding of the MSLs in male embryos derived from mothers homozygous mutant for *mle* and *msl-1*. This delay in MSL binding coupled with the observation that mutant male progeny of mutant mothers have a slightly earlier lethal phase (Belote and Lucchesi, 1980b), suggests that the X chromosome binding of the MSL in early embryogenesis reflects the actual occurrence of MSL mediated dosage compensation at this time.

MATERIALS AND METHODS

Drosophila stocks

Descriptions of mutants and rearrangements can be found in Lindsley and Zimm (1992) and Gorman et al. (1995). The 'blue' balancer chromosomes used were a CyO-derivative expressing β-galactosidase driven by the actin promoter (from C. S. Goodman, UC Berkeley) and a TM3-derivative expressing β-galactosidase driven by the Ubx promoter (Irvine et al., 1991).

Antibody production

Antibodies against the N-terminal portion of the MLE protein were raised against a MLE-β-galactosidase fusion protein. To generate the fusion protein a 1150 bp *Eco*RI/*Xho*I restriction fragment (aa 1-378) from the *mle25* cDNA (Kuroda et al., 1991) was subcloned into the pWR 590 vector (Guo et al., 1984). Generation and purification of the antibodies followed the protocol of Gorman et al. (1995). The generation of antibodies against MSL-1, MSL-2, MSL-3, the C-terminal part of the MLE protein and H4Ac16 is described by Bashaw and Baker (1995), Gorman et al. (1995), Kuroda et al. (1991) and Turner et al. (1992).

Embryo staining

Immunostaining of whole-mount embryos was based on the method described by MacDonald and Struhl (1988). Embryonic cells were fixed and immunostained essentially as described by Franke et al. (1995). For stainings with the anti-H4Ac16 antibody the protocol was modified by adding 50 mM sodium butyrate to the fixation solution

to inhibit histone deacetylases.

For either whole-mount or embryonic cell stainings, antibodies against the MSL proteins and the H4Ac16 were used at a 1:100 dilution, antibodies against SXL were used at a 1:50 dilution. Secondary anti-rat, anti-mouse and anti-rabbit antibodies (Jackson ImmunoResearch) conjugated to either Horseradish peroxidase, alkaline phosphatase, Fluoresceine or Cy-3 were all used at a dilution of 1:200.

In situ hybridization and immunostaining

Salivary glands from male third instar larvae were dissected in PBS/0.1% Triton X-100 and fixed in lactic acid/water/acetic acid (1:2:3) for 3-5 minutes. The glands were then squashed onto poly-L-lysine treated slides and the coverslips were flipped off after freezing the slides in liquid nitrogen. The slides were then incubated in PBS for 5 minutes and the chromosomes postfixed in PBS/1% Triton X-100/3.7% formaldehyde for 20 minutes at room temperature. The slides were then washed in PBS for 15 minutes, dehydrated in 95% ethanol for 5 minutes and air dried.

Embryos were collected from apple juice plates, dechorionated by hand and then placed on a poly-L-lysine treated slide in a drop of lactid acid/water/acetic acid (1:2:3). The embryos were covered with a coverslip and dissociated by lightly tapping on the coverslip with a pen. After fixation for 2-4 minutes the embryonic cells were squashed onto the slide. Removal of the coverslip, washes and postfixation were done in the same way as for the polytene chromosomes.

The chromosome-specific biotinylated DNA probe (200 ng in 3× SSC/50% formamide) was obtained by microdissection of the specific chromosome arms from salivary gland polytene chromosomes, PCR amplification of the DNA and end-labeling of the PCR fragments with biotinylated nucleotides. The exact protocol for the generation of the chromosome-specific probes will be published elsewhere. The probe was placed on the slides and covered with a coverslip which was then sealed with rubber cement. Chromosomes and hybridization probe were denatured by heating the slides to 95°C for 3 minutes.

The slides were then incubated in a moist chamber at 37°C for 12-18 hours. After removal of the coverslips, slides were washed in 2× SSC/50% formamide at 37°C for 2× 20 minutes, in 2× SSC/50% formamide with PBS/0.1% NP40 (3:1) at 37°C for 20 minutes, in 2× SSC/50% formamide with PBS/0.1% NP40 (1:1) at 37°C for 20 minutes, in 2× SSC/50% formamide with PBS/0.1% NP40 (1:3) at 37°C for 20 minutes and finally placed in PBS/0.1% NP-40 at room temperature.

Chromosomes/embryonic cells were then incubated with a rat anti-MSL-1 antibody (1:50 dilution) in PBS/0.1% NP40/5% dry milk at 4°C overnight. Slides were washed in PBS/0.1% NP40 3× 5 minutes and then incubated with a secondary Cy-3-conjugated anti-rat antibody and fluoresceine-conjugated streptavidin. The DNA was counterstained with Hoechst dye (0.01 μg/ml in PBS) for 15-30 seconds and then mounted in 90% glycerol/2% propyl-galleate under a coverslip. The preparations were analyzed under epifluorescence optics using a Zeiss axiophot microscope. Pictures were taken with a Photometrics CCD camera using IPLab Spectrum software. Colors were added with the Adobe Photoshop 3.0 software.

RESULTS

MSL proteins and histone H4Ac16 accumulate in a subnuclear compartment beginning at late blastoderm

The characteristics of the binding of the MSL and H4Ac16 proteins to chromosomes has thus far only been examined in polytene chromosomes from third instar salivary glands. In order to determine (1) whether X chromosome binding of these proteins is also observed in other tissues in males, (2) whether they are regulated in the same way in other tissues, as well as (3) the earliest developmental stage at which the MSL proteins and the H4Ac16 are associated with the male X chromosome, we stained various embryonic and larval stages with antibodies against MSL-1, MSL-2, MSL-3, MLE and H4Ac16. To determine the sex of embryos and young larvae they were

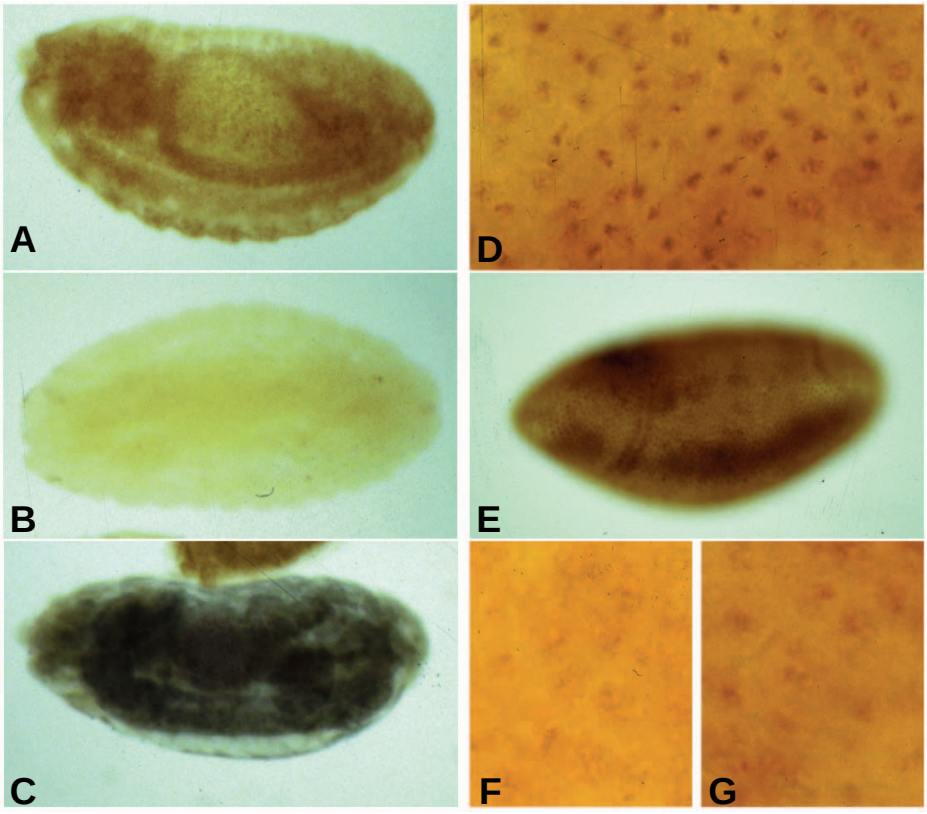

Fig. 1. Anti-MSL-3 and anti-SXL (A,C) immunostaining of wild-type embryos. (A) A stage 16 male embryo displaying the subnuclear localization of the MSL-3 protein in all somatic cells. A close-up (D) shows the concentration of the MSL-3 protein in about 20-30% of the nucleus. The sex of the embryo is confirmed by absence of staining with a female-specific anti-SXL antibody. Female embryos do not show any localized staining in the nuclei (B) and are identified by the detection of SXL protein (C). (E,G) A stage 6 male embryo, in which MSL-3 protein is localized in a subnuclear compartment in all cells in contrast to the pattern in the blastoderm stage (F), where only a subset of cells shows the male-specific MSL-3 pattern.

simultaneously stained with an anti-SXL antibody that stains only females (Bopp et al., 1991).

The anti-MSL-1, -2, -3 and MLE antibody stainings revealed that in male embryos these proteins accumulate in all nuclei in a compartment encompassing about 20-30% of the nucleus, suggesting that in other cell types they may also be specifically associating with the X chromosome as they are in salivary gland cells. One exception is the pole cells, which during blastoderm- and gastrula-stages, when they are still visible in the whole-mount preparations, do not show any male-specific subnuclear localization of the MSL proteins. We further examined all three larval stages to ascertain whether the MSL proteins showed the same subnuclear localization pattern as seen in embryos. In all cases a similar subnuclear localization pattern of MSL staining was observed and we never observed a significant group of cells/tissue where such staining was absent. As these observations are made on preparations that were obtained by squashing slightly dissected whole larvae onto slides, they should represent all cells/tissues of the larvae. Although we cannot exclude there being some tissues in which the MSLs do not mediate dosage compensation, these results indicate that if there are any such tissues they must be very limited.

As a major focus of this study was on the analysis of the early stages in which the MSLs first associate with the male's X chromosome, we concentrated on the analysis of staining patterns in embryonic stages. As shown in Fig. 1A,D, staining with anti-MSL-3 antibodies reveals that the MSL-3 protein is concentrated in just one part of the nucleus in all somatic cells of male embryos. Female embryos stained only very weakly with the antibodies against MSL-3 (Fig. 1B,C). A similar subnuclear staining pattern in male embryos is seen with antibodies against MSL-1 and -2, whereas female embryos stain only weakly, or not at all, with these antibodies (data not shown). These observations are consistent with those from third instar larval stages, which showed that MSL-1 and -3 are expressed at a reduced level in females and that the MSL-2 protein is not expressed at all in females (Bashaw and Baker, 1995; Gorman et al., 1995; Kelley et al., 1995; Palmer et al., 1994; Zhou et al., 1995). Staining with antibodies against the N-terminal part of the MLE protein, which is expressed at the same level in male and

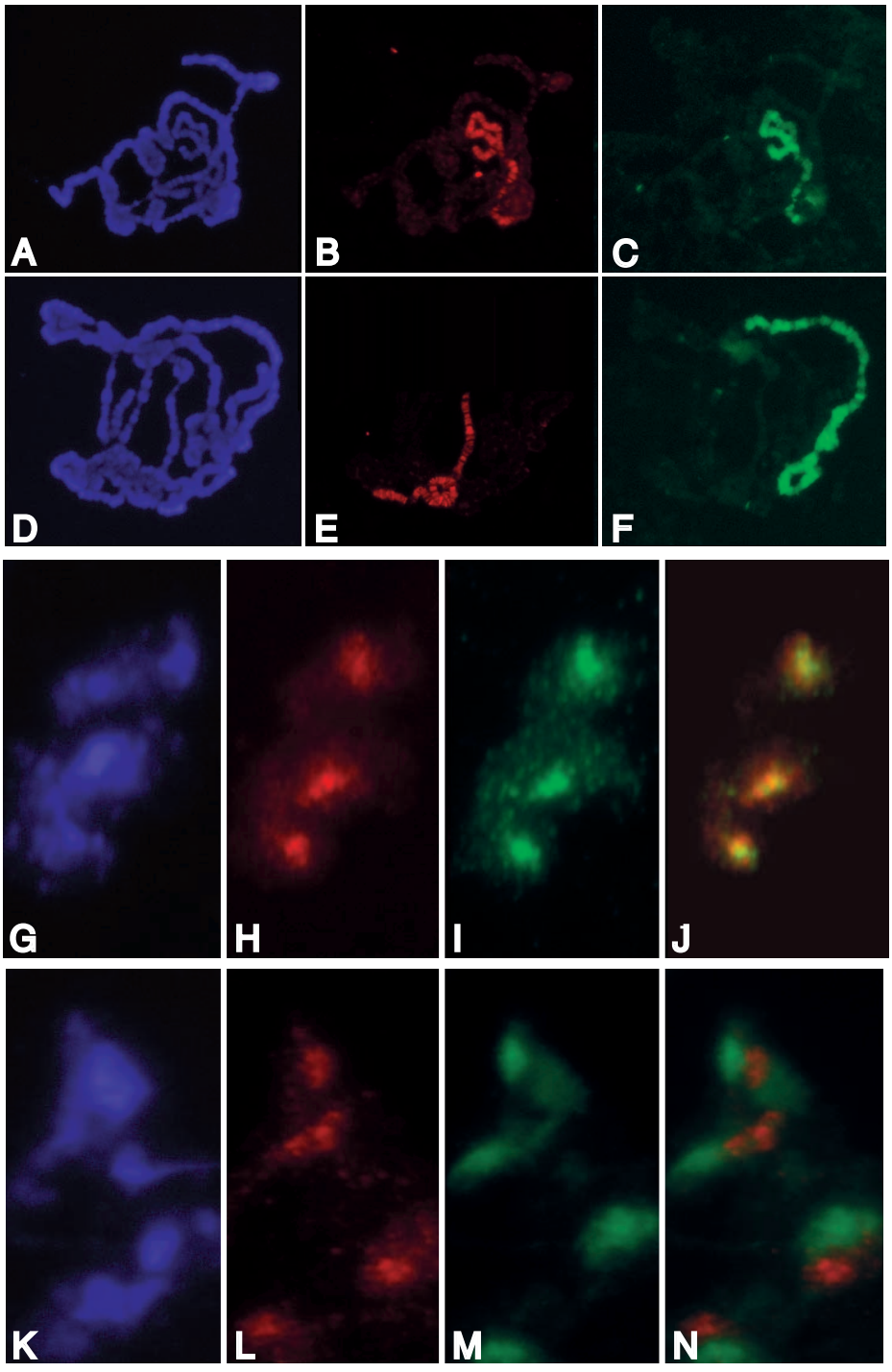

Fig. 2. Immunofluorescence staining with anti-MSL-1 antibodies in conjunction with fluorescence in situ hybridization with X- and 2R-chromosome-specific DNA probes on salivary gland polytene chromosomes (A-F) and diploid embryonic cells (G-N). In both tissues the MSL-1 signal and the signal from the X-specific probe completely overlap, whereas the MSL-1 signal and the 2R-specific signal light up different compartments of the nuclei. Staining was done with Hoechst DNA stain (A,D,G,K), anti-MSL-1 antibodies (B,E,H,L) and DNA probes from the X (C,I) and 2R (F,M) chromosomes. Stainings in embryonic cells are superimposed to visualize the overlap (J) or non-overlap (N) of MSL-1 signal and in situ signal.

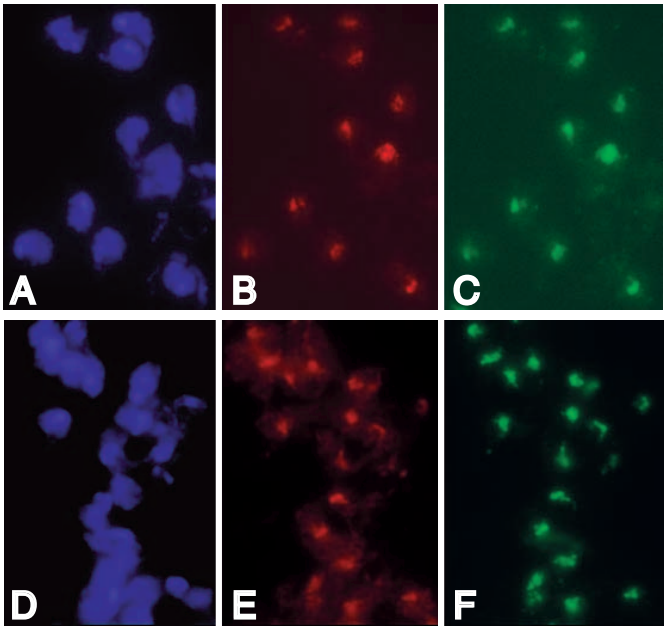

Fig. 3. Double immunofluorescence staining of embryonic cells with anti-MSL-2 (B), anti-H4Ac16 (E) and anti-MSL-1 (C,F) antibodies. The signals in B,C and E,F overlap. Nuclei are visualized with Hoechst DNA stain (A,D).

female third instar larvae (Kuroda et al., 1991), does give a positive result in both male and female embryos, but the subnuclear staining pattern as described above for the other MSL proteins is only detected in the male embryos (not shown). H4Ac16 is also found in a similar subnuclear staining pattern in male embryos (Fig. 3E).

The MSLs and H4Ac16 bind to the X chromosome in embryos

To establish that the subnuclear localization of the MSL proteins and H4Ac16 seen in male embryonic cells was due to their association with the X chromosome we simultaneously determined the locations within embryonic nuclei of the X chromosome (by fluorescent in situ hybridization (FISH) with DNA probes covering the entire length of the X) and the MSL-1 protein (by immunostaining with anti-MSL-1 antibodies). Fig. 2A-C,G-J shows that the MSL-1 staining completely overlaps with the X-specific chromosome 'paint' in both polytenic larval and diploid embryonic cells. A control experiment that similarly determined the locations of chromosome 2R and the MSL-1 protein revealed that they are in different compartments of nuclei (Fig. 2D-F,K-N). These results establish that the MSL-1 protein is associated with the X chromosome in male embryonic cells.

Because immunostaining in conjunction with FISH did not work with all of the antibodies against the MSLs and H4Ac16,

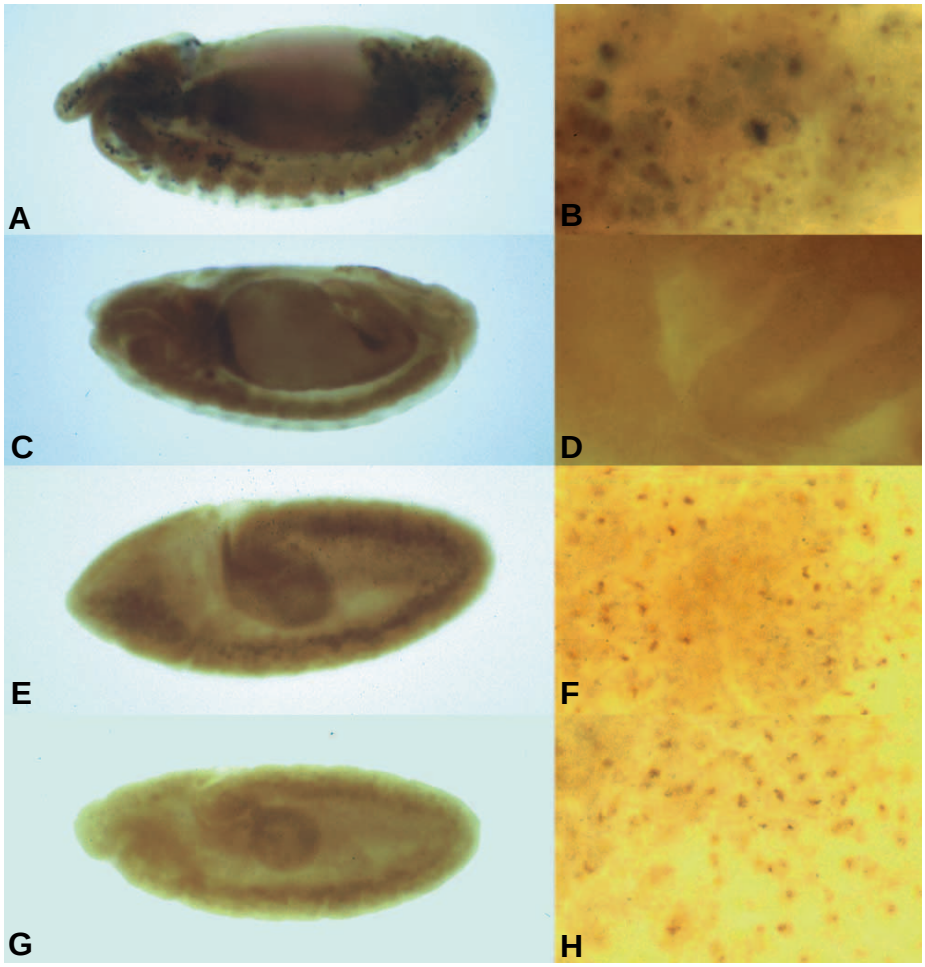

Fig. 4. Triple immunostaining of male embryos with antibodies against MSL-3, β-galactosidase and SXL. (A,B) *msl-1* heterozygous mutant embryo (identified by the actin-β-gal pattern) showing binding of the MSL-3 protein to the X chromosome. (C,D) *msl-1* homozygous mutant embryo showing no association of MSL-3 protein with the X chromosome. (E,F) *msl-1* homozygous mutant embryo (stage 9) derived from an *msl-1* heterozygous mutant mother. The MSL-3 protein is associated with the X chromosome. (G,H) *mle* homozygous mutant embryo (stage 10) derived from an *mle* heterozygous mutant mother. The MSL-3 protein is associated with the X chromosome.

due to the loss of antigen on the chromosomes during the procedure, we carried out double immunostainings of embryonic cells in order to analyze whether MSL-1 protein localization is coincident with the localization of the other MSLs and the H4Ac16. As shown in Fig. 3A-C, the MSL-1 and MSL-2 protein are detected in exactly the same location in the nucleus. The same result was obtained with antibodies against H4Ac16 and MSL-1 (Fig. 3D-F). Similar experiments with MSL-3 and MLE (data not shown) establish that all the MSL proteins and the H4Ac16 are localized on the X chromosome in male embryonic nuclei.

The binding of MSL proteins is interdependent

Binding of each of the MSLs and H4Ac16 to the polytene X chromosome in salivary glands requires the presence of all four MSL proteins. This observation has led to the suggestion that the MSL proteins form a complex that binds to the X chromosome and all the MSLs must be present to get binding (Bashaw and Baker, 1995; Bone et al., 1994; Gorman et al., 1993; Hilfiker et al., 1994; Palmer et al., 1994). In order to analyze whether this is also true in the diploid embryonic tissues we constructed stocks where *msl* mutations were balanced by chromosomes that express β-galactosidase under the control of promoters that are active during embryogenesis. Simultaneous staining with anti-MSL, anti- β-gal and anti-SXL antibodies allowed us to identify the male heterozygous and homozygous *msl* mutant embryos and analyze the MSL staining pattern. Shown in Fig. 4C,D is the MSL-3 staining pattern in a *msl-1* homozygous mutant male embryo. No association of the MSL-3 protein with the X chromosome is detected, whereas such staining is clearly visible in the heterozygous *msl-1* embryo identified by the β-gal expression (Fig. 4A,B). In none of the male embryos homozygous mutant for *msl-2* or *msl-3* could we detect any association of the other MSL proteins or H4Ac16 with the X chromosome throughout embryogenesis. Embryos homozygous mutant for *mle* and *msl-1* show a different staining pattern in early embryonic stages. *msl-1* homozygous mutant embryos show association of the MSL proteins with the X chromosome up to stage 9 (Fig. 4E,F), while in *mle* homozygous mutant embryos such an association is seen up to stage 10 (Fig. 4G,H). The *mle* and *msl-1* mutants used in this experiment do not produce any protein (Kuroda et al., 1991; Gorman et al., 1995), therefore we attribute the staining to the presence of maternal *mle* and *msl-1* products in these embryos. These data support the idea that the MSLs have to form a protein complex in order to bind to the male X chromosome and that this complex is already formed in the early stages of embryogenesis.

SXL prevents MSL binding in female embryos

In female larval salivary gland cells the absence of MSL protein binding to the X chromosomes is due to the function of SXL. This was demonstrated using female larvae that are heteroallelic for the viable partial loss-of-function allele Sxl^{fhv1} and the null allele Sxl^{f1} and develop as mosaics with respect to SXL expression (Gorman et al., 1993). In these mosaics, cells that express SXL do not show binding of the MSLs to the X chromosomes, whereas in cells that do not express SXL the MSLs are associated with the female X chromosomes (Gorman et al., 1993; Hilfiker et al., 1994; Palmer et al., 1994; Bone et al., 1994; Bashaw and Baker, 1995; Kelley et al., 1995). We wanted to investigate whether the same inverse correlation between SXL expression and MSL binding to the X chromosomes is already evident at embryonic stages. We therefore squashed single embryos from the cross $Sxl^{fhv1}/Sxl^{fhv1} \times Sxl^{f1}/Y$ onto a coverslip and performed double immunostaining using anti-SXL and anti-MSL-1 antibodies. In about 50% of the embryos we could detect mosaic SXL expression, which we assume are the female Sxl^{f1}/Sxl^{fhv1} progeny. Fig. 5 shows that the binding of the MSLs is prevented in the SXL-expressing cells, whereas in cells that do not show any SXL expression, the MSLs are associated with the X chromosomes. This demonstrates that in female embryonic cells binding of the MSLs to the X chromosomes is prevented by the expression of SXL, as it is at later stages.

The initiation of MSL protein association with the X chromosome in embryos

The earliest stage at which the MSLs are detected in the subnuclear localization pattern characteristic of male embryos is in late blastoderm/early gastrula stages. Fig. 1E,G shows an early gastrula stage embryo were the male-specific MSL-3 staining is observed in all cells in the pattern that is maintained throughout embryogenesis and all three larval stages. A close-up picture from a blastoderm stage embryo (Fig. 1F) shows MSL-3-specific staining on the X chromosome is detected in only a subset of cells. This subnuclear localization is not observed in earlier stages, where the MSLs are rather homogeneously distributed in the nucleus. This indicates that the MSLs are beginning to accumulate on the X chromosome at this time. None of the MSLs could be detected in the male-specific pattern with others being homogeneously distributed in the nucleus. These observations suggest that either the MSLs become localized to the X chromosome simultaneously, or if there is an order to their association with the X, they are all binding within a very short period of time, such that the order cannot be resolved by our experiments. Staining with H4Ac16 antibodies in these early stages of embryogenesis did not allow us to establish the exact time at which this protein is detected on the male's X due to high background staining in these early stages. To get convincing staining with those antibodies the embryos had to be squashed to reduce the thickness of the preparation, therefore prohibiting the exact determination of the developmental stage. We could nevertheless detect cells showing MSL localization to the X chromosome without colocalized detection of H4Ac16. As our one-hour egg collections

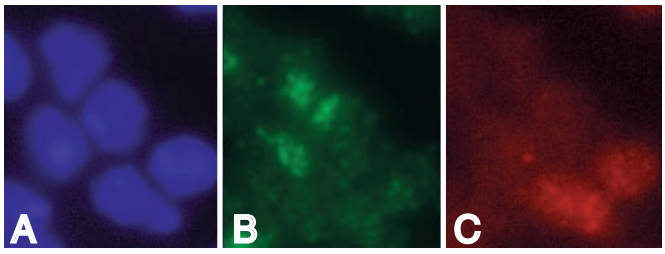

Fig. 5. Double immunostaining of embryonic cells from single embryos from the cross $Sxl^{fhv1}/Sxl^{fhv1} \times Sxl^{f1}/Y$. Shown are cells from an embryo which most likely is of the genotype Sxl^{fhv1}/Sxl^{f1}. Staining with anti-MSL-1 (B) and anti-SXL (C) antibodies shows the inverse correlation between MSL-1 X chromosome binding and SXL expression.

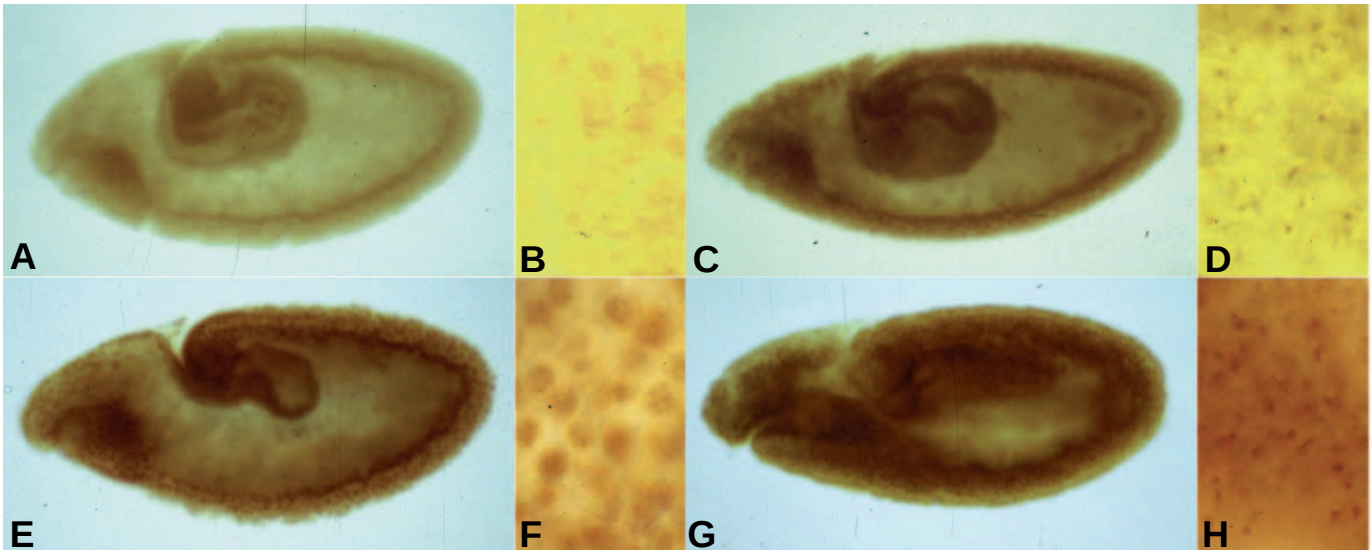

Fig. 6. Immunostaining of male embryos with anti-MSL-3 antibodies derived from mothers homozygous mutant for *msl-1* (A-D) and *mle* (E-H). Binding of the MSL-3 protein is first detected in stage 10 (C,D), but not in earlier stages (A,B), if the mothers are mutant for *msl-1*. In male progeny from *mle* mutant mothers binding is observed in stage 11 (G,H), but not in earlier embryonic stages (E,F).

contained embryos between stage 5 and 8, we conclude that the delay in detection of H4Ac16 compared to the time when the MSLs are first detected in their specific staining pattern does not exceed 60 minutes.

The onset of X chromosome binding is changed in embryos derived from mothers homozygous for *mle* or *msl-1* mutations

The preceding results establish that the association of the MSL proteins with the male's X chromosome begins during the early stages of embryogenesis. However, it is unclear whether this binding also means the MSL proteins are functioning at this stage to bring about hypertranscription of the male's X chromosome. To address this question we asked whether the maternal effects of mutations in the *msl* genes, which are seen as slight shifts of the lethal phase towards earlier larval stages (Belote and Lucchesi, 1980b), are correlated with changes in the MSL binding pattern during embryogenesis.

We stained embryos derived from homozygous *msl* mutant mothers with antibodies against the four MSL proteins. In embryos derived from wild-type or *msl* heterozygous mutant mothers we first detect the MSL-3 protein on the male's X chromosome at stage 5, which corresponds to the late blastoderm stage. As shown in Fig. 6 we see a clear shift of the timepoint when the MSLs are first detected on the male X chromosome in embryos derived from homozygous *mle* and *msl-1* mutant mothers. Embryos from homozygous *msl-1* mutant mothers show X chromosome binding starting at stage 10 of embryogenesis with no, or very few, cells showing X chromosome binding of the MSL-3 protein in stage 9 embryos (Fig. 6A-D). Embryos from *mle* homozygous mothers exhibit X chromosome staining with anti-MSL-3 antibodies in stage 11 with almost no X chromosome staining in stage 10 embryos (Fig. 6E-H). Embryos derived from homozygous *msl-2* or *msl-3* mutant mothers do not show any differences when compared to embryos from wild-type mothers (not shown). We obtained the same results with antibodies against MLE, MSL-1 and MSL-2. These data correlate with the reported maternal effects of *msl* mutations (Belote and Lucchesi, 1980b). Such maternal effects are most obvious in progeny from *mle* and *msl-1* homozygous mothers and are not detected in the progeney of *msl-2* or *msl-3* homozygous mothers (Belote and Lucchesi, 1908b; Uchida et al., 1981). This correlation suggests that the lack of MSL binding to the X in male embryos between stage 5 and stage 10 is in fact responsible for the earlier lethal phase of these progeny. This is supported by the fact that embryos homozygous mutant for *mle* and *msl-1* derived from heterozygous mutant mothers show staining up to stage 9-10 of embryogenesis (Fig. 4E,F). These data suggest that *msl*-mediated dosage compensation is occurring during embryogenesis.

Antibodies against different parts of the MLE protein show a different staining pattern in embryonic, but not in larval cells

As described above we detect all four MSL proteins bound to

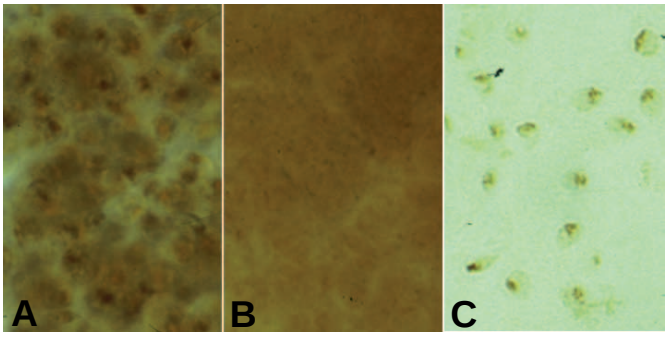

Fig. 7. Immunostaining of cells from male embryos (A,B) and male first instar larvae. Anti-MSL-1 clearly shows association of the protein with the X chromosome (A), as seen for all other MSL proteins. Antibodies against the C-terminal 132 amino acids of the MLE protein fail to detect the protein in embryos (B), but clearly stain cells from first instar larvae (C).

the X chromosome in male embryos. The results with respect to MLE's distribution were obtained using anti-MLE antibodies against the N-terminal 382 amino acids of MLE. However, when we use a serum directed against the C-terminal 132 amino acids of the MLE protein, we observe a different staining pattern. The latter antibodies show a uniform staining in embryonic cells in both whole-mount preparations and in squashed cells without any subnuclear localization of MLE being observed. We attribute the homogeneous staining to the background staining obtained with this particular antiserum and we conclude from our results that this antibody does not, in embryonic stages, recognize the MLE protein, which is associated with the X chromosome based on the results with the antibodies against the N-terminal portion of MLE. By staining first instar larval cells with the C-terminal antibody we could clearly detect the association of the MLE protein with the male X chromosome without any background (Fig. 7A-C). The staining, in larval tissues, with the antibody against the C-terminal portion of the MLE protein lets us conclude that the failure of this antibody to recognize the MLE protein in embryos is not due to technical difficulties, but rather is due to the fact that either (a) in embryonic cells the only MLE protein present lacks the C-terminal epitope recognized by the antibodies, (b) that this epitope is in some way modified in embryonic stages, or (c) that this part of the protein is inaccessible to the antibodies in embryos. So far we have not been able to detect different forms of the MLE protein in western blots of embryonic extracts using both the N- and C-terminal antibodies and thus at this point we are not able to explain these potentially significant differences in staining.

DISCUSSION

Although dosage compensation in *Drosophila* was discovered over 60 years ago relatively little is known about dosage compensation from a developmental standpoint. The recent molecular characterizations of the properties of the MSL proteins in the polytene cells of larval salivary glands have provided a set of criteria that we have used to address, at the cellular level, whether these proteins are functioning analogously in other cell types and developmental stages to mediate dosage compensation. In particular, in larval salivary gland cells antibodies to the MSL proteins have been used to show that (1) all four of the MSL proteins bind specifically to the X chromosome in male, but not female, cells; (2) these proteins bind to identical sets of sites along the male's salivary X chromosome; and (3) the binding of each MSL protein to these sites is dependent on the presence of the other three MSL proteins (reviewed by Baker et al., 1994). In addition, the H4Ac16 isoform has also been shown to be highly concentrated on the male X chromosome and its presence there depends on the functioning of the MSL proteins (Turner et al., 1992; Bone et al., 1994). Determinations of whether the MSL proteins and H4Ac16 display these properties in other cell types and at other developmental stages has allowed us to delimit the temporal and spatial domains of *msl*-mediated dosage compensation. Our experiments also provide some insights into the relationship between the process of early, *msl*-independent dosage compensation and *msl*-mediated dosage compensation. While our results were being written up a paper appeared that also addressed some of these questions (Rastelli et al., 1995).

The chromosomal association of the MSL proteins in non-salivary gland cells

Our results show that in males beginning at about the end of blastoderm and continuing throughout the larval period all four of the MSL proteins appear to be expressed ubiquitously in somatic cells. Strikingly, in all somatic tissues (both diploid and polytene) of males and at all postblastoderm stages, MSL staining is restricted to a subregion of the nucleus. We never observed a significant group of somatic cells/tissue where such staining was absent. All four MSL proteins, as well as H4Ac16, colocalize to the same subnuclear region in diploid cells; similar observations are reported by Rastelli et al. (1995) for MSL-1, MSL-2, MLE and H4Ac16 in embryos. We have further demonstrated using FISH with probes prepared form microdissected chromosomes that the subregion of the nucleus where the MSLs and H4Ac16 are localized in diploid cells corresponds to the location of the X chromosome.

In male pole cells, which give rise to the germline, we did not observe any subnuclear localization of the MSL proteins during blastoderm and gastrula stages, when pole cells are still visible in the whole-mount preparations. This is consistent with the previous finding that, while there is a requirement for *mle* in the male germline, germline cells homozygous for *msl-1*, or *msl-2* mutations produce functional sperm (Bachiller and Sanchez, 1986). Thus if there is dosage compensation in the male germline it must be by a mechanism that is, at least in part, distinct from that which functions in somatic cells. The unique requirement for *mle* in the male germline may not reflect a role in dosage compensation. Perhaps related to this is the fact that in salivary gland cells there are some sites where MLE, but not the other three MSL proteins, bind to chromosomes. Thus MLE may have a function(s) outside of its role in *msl*-mediated dosage compensation.

The binding of the MSLs and H4Ac16 requires all four MSL proteins

Binding of the MSLs and H4Ac16 to the male's X chromosome in salivary glands requires the presence of all four MSL proteins, suggesting that the MSL proteins have to form a heteromeric protein complex in order to bind to the X (Gorman et al., 1993; Bashaw and Baker, 1995; Gorman et al., 1995; Kelley et al., 1995; Zhou et al., 1995). Our experiments show that in embryos the binding of the MSLs and H4Ac16 to the X chromosome is also interdependent from the beginning of the time when we can detect their association with the X during late blastoderm/early gastrulation; the absence of any one of the MSL proteins abolishes the binding of all the MSls and H4Ac16 in embryonic cells. A dependence of MSL-1 and MLE binding on the zygotic genotype at *msl-2* has also been reported by Rastelli et al. (1995).

Taken together the above observations suggest that *msl*-mediated dosage compensation is ubiquitous at postblastoderm stages in the soma and occurs by the same mechanism in all somatic cell types.

On the initiation of *msl*-mediated dosage compensation

These findings are significant with respect to how dosage com-

pensation is achieved in the early embryo and the relative domains of early dosage compensation and MSL mediated dosage compensation. Transcription of a few genes, including *sisterless-a* (*sis-a*) and *sisterless-b* (*sis-b*) (Erickson and Cline, 1993), is detected beginning as early as cell cycles 8-9 (approx. 1 hour postfertilization) although general transcription does not begin until about cell cycle(s) 10-14 (Anderson and Lengyel, 1979, 1980; Weir and Kornberg, 1985). As *sis-a* and *sis-b* are X-linked, and function as dosage-dependent numerator elements in sex determination in these preblastoderm stages, it would appear that dosage compensation is not operational at this stage (Cline, 1988); the levels of early *sis-a* and *sis-b* transcripts peak at nuclear cycle 12 suggesting that dosage compensation remains absent at least until this point (Erickson and Cline, 1993). Differences in the amount of SIS-B protein can be detected in syncitial blastoderm stages, whereas it is detected at equal levels, and thus is dosage compensated later in development where it functions in nervous system development (Deshpande et al., 1995). At nuclear cycle 12 (mid-stage 4) the first transcripts from the (early) establishment promoter of *Sxl* (Sxl-P_e) are detected and this marks the point when the process of early dosage compensation could begin, as it is controlled by the product of the Sxl-P_e (Bernstein and Cline, 1994). Strongly supporting the idea that early dosage compensation does begin at this time is the finding that the expression of *runt*, the X-linked gene whose expression has been used to define the process of early dosage compensation as Sxl-P_e dependent and *msl* independent, begins at nuclear cycle 12 and is required for about an hour (until mid-stage 5) (Gergen, 1987; Bernstein and Cline, 1994). We detect the start of MSL binding at mid-stage 5 and MSL binding to the X chromosome is complete by the end of stage 5. The *Sxl* maintenance promoter becomes active around nuclear cycle 14 (Barbash and Cline, 1995) therefore we cannot distinguish whether the early or late Sxl products are functioning to make *msl*-mediated dosage compensation male-specific by preventing the expression of *msl-2* in females (Bashaw and Baker, 1995; Kelley et al., 1995; Zhou et al., 1995) at these early stages. Assuming that the binding of the MSL proteins to the X chromosome by early gastrula is indicative that *msl*-mediated dosage compensation has begun by that time (a point we consider below), this leaves a time window of approximately an hour between when general transcription has begun and when *msl*-mediated dosage compensation could be functional.

The temporal patterns of gene expression just outlined are compatible with a view that early dosage compensation is a process that has evolved to cope with a need for dosage compensation prior to the time when the MSL based system becomes functional; i.e. that these two systems operate sequentially. However, from our current state of knowledge there is nothing that requires that these two processes be sequential. Indeed, it has been suggested that they may operate on distinct sets of targets (Bernstein and Cline, 1994; Kelley et al., 1995).

Although we do not have a definitive answer to the question of when the *msls* start to function to regulate dosage compensation, two of our observations bear on this question. Our results from staining embryos that are derived from homozygous *msl* mutant mothers suggest that the *msls* are functional in the early embryo. Specifically, we found that male embryos derived from homozygous mutant *msl-1* or *mle* mothers, show a delayed onset of MSL binding to the X chromosome (from stage 5 to stages 10-11). This means that the homozygous mutant sons of these mothers show no MSL binding to the X chromosome throughout embryogenesis in contrast to homozygous mutant sons of the heterozygous mothers. These show binding in early stages of embryogenesis due to the perdurance of the maternal gene product. This could explain the slightly earlier lethal phase in homozygous mutant sons of homozygous mutant *msl-1* or *mle* mothers compared to homozygous mutant sons of heterozygous mutant *msl-1* or *mle* mothers (Belote and Lucchesi, 1980b) suggesting that the delay in MSL binding seen in progeny of homozygous mutant mothers is biologically significant. If this absence of binding of the MSL proteins over a short period of time is the only difference in those progeny derived from homozygous mutant *msl-1* and *mle* mothers, it would indicate that *msl*-mediated dosage compensation is already important at this time and, if missing, affects the development of larvae by not generating enough product(s) of X-linked genes important for larval development.

One other result that may be relevant to whether *msl* mediated dosage compensation is occurring during embryogenesis, is the finding that the MLE protein appears to be either modified, or is in a different context on the X chromosome, in embryonic cells compared to later stages. In particular we find that at embryonic stages our antibodies against the C-terminal part of the protein do not recognize the MLE protein, which antibodies against the N-terminal part of the protein show is present on the X chromosome at these times. Moreover, in first instar larvae the MLE protein is detected by the antibodies against both the C-terminal and N-terminal parts of the MLE protein, suggesting that a change in either a posttranslational modification of the MLE protein or in its X-chromosomal environment happens at this time. Whether this finding is indicative of a functional difference is currently unclear.

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