Drosophila Male-Specific Lethal 2 Protein Controls Sex-Specific Expression of the *roX* Genes

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ABSTRACT

The MSL complex of Drosophila upregulates transcription of the male X chromosome, equalizing male and female X-linked gene expression. Five male-specific lethal proteins and at least one of the two noncoding *roX* RNAs are essential for this process. The *roX* RNAs are required for the localization of MSL complexes to the X chromosome. Although the mechanisms directing targeting remain speculative, the ratio of MSL protein to *roX* RNA influences localization of the complex. We examine the transcriptional regulation of the *roX* genes and show that MSL2 controls male-specific *roX* expression in the absence of any other MSL protein. We propose that this mechanism maintains a stable MSL/*roX* ratio that is favorable for localization of the complex to the X chromosome.

A fundamental aspect of development is the establishment of complex patterns of gene expression. Specific regulatory mechanisms operate at the level of individual genes, groups of genes, or over an entire chromosome, to control gene expression. The process of dosage compensation presents an example of how transcription of an entire chromosome is globally regulated. Acting on many different genes, dosage compensation functions on top of the local control mechanisms that operate on individual genes. The male-specific lethal (MSL) complex of *Drosophila melanogaster* upregulates transcription of most of the genes on the male X chromosome, equalizing male and female X-linked gene expression. The complex consists of at least five MSL proteins, MSL1, MSL2, MSL3, MLE (maleless), and MOF (males absent on first), and two noncoding RNAs (*roX1* and *roX2*). Each MSL protein is required for male viability. MSL1 is a novel acidic protein, MSL2 is a RING finger protein, MLE is a DExH RNA/DNA helicase, and MSL3 and MOF are chromodomain proteins with RNA-binding activity in vitro (reviewed in Meller and Kuroda 2002). The MSL complex is thought to be responsible for targeting MOF, a histone acetyltransferase, to the male X chromosome, where it acetylates histone H4 on lysine 16 (H4Ac16), a chromatin modification associated with increased transcription (Hilfiker et al. 1997; Akhtar and Becker 2000; Smith et al. 2000). Gene-specific regulation has not been described for the MSL complex. In addition, no function outside of their role in the intact dosage compensation complex has been attributed to any of the MSL proteins.

One of the most intriguing aspects of fruit fly dosage compensation is the role the noncoding *roX* RNAs play. Despite the lack of significant sequence similarity, the two *roX* RNAs are redundant male-specific lethal genes (Meller and Rattner 2002). Their participation in male dosage compensation is dual. As integral components of the ribonucleoprotein complex, the presence of either *roX1* or *roX2* RNA is essential for targeting of MSL complexes to the X chromosome. RNA-binding activities have been attributed to three members of the MSL complex, and both *roX* transcripts can be immunoprecipitated with anti-MSL antibodies (Richter et al. 1996; Akhtar et al. 2000; Meller et al. 2000; Smith et al. 2000). Mutation of both *roX* genes results in male lethality, but males are rescued by autosomal *roX* transgenes (Meller and Rattner 2002). These observations point to a role for *roX* RNA in assembly of functional MSL complexes.

The X-linked *roX* genes also overlap two male-specific DNase I hypersensitive sites (DHS; Kageyama et al. 2001; Park et al. 2003). These are proposed nucleation sites for assembly and spreading of MSL complexes into flanking chromatin. Autosomal insertions containing a *roX* DHS can recruit the MSL complex to chromatin in cis (Kelley et al. 1999). The *roX* DHS also appear to be 2 of ~35 sites on the X chromosome that retain binding of partial MSL complexes in some *msl* mutant backgrounds. These sites have also been termed chromatin entry sites (CES) to reflect their proposed role in MSL complex recognition of the X, but it is unknown if the sites not associated with *roX* genes can also recruit MSL complexes to chromatin in cis.

The location of both *roX* genes and their associated DHS on the X chromosome is believed to contribute to the spread of MSL complexes along X chromatin. It has recently been proposed that the ratio between MSL
proteins and roX RNA determines the extent of spreading from roX genes. If assembly of MSL proteins onto nascent roX transcripts occurs rapidly, functional complexes are formed before the release of roX transcripts and these complexes tend to accumulate on chromatin near the roX genes (Park et al. 2002; Oh et al. 2003). This artificial situation can be experimentally achieved by overexpression of MSL1 and MSL2 proteins or by mutation of the roX genes.

In theory, control of roX expression could regulate the rate of MSL complex formation and influence its localization. This prompted us to examine the transcriptional regulation of the roX genes in its native chromosomal context. roX transcripts are never detected in the salivary glands of female larvae or in Northern blots from female adults (Meller et al. 1997, 2000). roX accumulation in males does not depend on transformer (tra), an essential step in the pathway that controls all aspects of sexual differentiation in Drosophila (Amrein and Axel 1997; Meller et al. 1997). This suggests that a novel mechanism for the regulation of these genes may exist. In the absence of an entire set of MSL proteins, the condition in females, ectopically expressed roX produced from a transgene is unstable and never localizes to the X chromosome (Meller et al. 2000). Therefore, stabilization of the roX RNAs could cause their sex-specific accumulation, but the question of how transcription of the endogenous roX genes is controlled remains unanswered.

In this work, we provide evidence that the roX genes display male-specific transcription that depends on a single member of the dosage compensation complex, the MSL2 protein. MSL2 does not require any of the other MSL proteins for this novel activity and can promote roX expression even when mutated in its RING finger domain, a region essential for dosage compensation. Deletions of the roX1 DHS, the sequence that provides a binding site for incomplete MSL complexes, show that this region is dispensable for MSL2-mediated roX transcription. Our observations suggest a mechanism for the maintenance of a MSL/roX ratio that is favorable for spreading of the complex along the X chromosome.

MATERIALS AND METHODS

Drosophila stocks: Larvae and flies were raised on standard cornmeal-yeast-agar-molasses medium containing propionic acid in a humidified incubator at 25°C. Mutations in msl genes have been previously described as follows: missense mutation msl2 (Zhou et al. 1995), null mutation msl1 (Chang and Kuroda 1998), missense mof and nonsense mof mutations (Hilfiker et al. 1997; Gu et al. 1998), nonsense mle mutation (Rastelli and Kuroda 1998), and msl3 mutation (Lindsay and Zimm 1992). The roX1Δ750 and roX1Δ845 alleles have been reported previously (Meller et al. 1997; Kelley et al. 1999). Deletion of roX2 was accomplished by combining X chromosomes bearing a lethal roX2 deficiency with autosomal insertion of a cosmid that rescues essential functions but lacks roX2 (Df(1)52; w+ 4D4.3) described in Meller and Rattner 2002). The [h83m2-61] transgene was described in Kelley et al. (1995). The [h83m2ΔRING] transgene was generated by Lyman et al. (1997). The [h83s5/roX1] transgene contains a 908-bp roX1 fragment (base pairs 279–1187) (Meller et al. 2000), roX1Δ750 and roX1Δ845 were generated by imprecise excision of the P element from roX1Δ750 and removal of bases 283–2669 and 809–3159, respectively (S. Souter and V. H. Meller, unpublished results). Numbering is from Amrein and Axel (1997).

Fly genetics: Larvae were sexed by gonad size as viewed through the cuticle. Homozygous mle, msl1, and msl2 larvae were identified by the absence of a y+ marker on the Gt/y+ balancer chromosome. Homozygous msl3 [h83m2-61] larvae were identified by the absence of the Tb dominant marker, present on the TM6B balancer chromosome.

In situ hybridization and immunohistochemistry: Whole-mount in situ hybridization to third instar salivary glands was performed as previously described (Meller et al. 1997; Meller and Rattner 2002). Antisense digoxigenin-labeled roX2 and roX1 riboprobes were transcribed from linearized templates derived from a full-length roX2 cDNA (Amrein and Axel 1997) or from the roX1 genomic region. Antisense roX1 probes hybridizing to 1.4 kb of the 5′ end (PvuII-BglII fragment) and 0.8 kb of the 3′ end (XmnI-EcoRI fragment) were used (Meller 2003). Alkaline phosphatase substrate color development times were comparable with all riboprobes used, taking between 15 and 30 min. Individual experiments were repeated a minimum of three times, with 8–10 salivary glands per genotype. Immunohistochemical detection of MSL2 in whole-mount salivary glands was performed essentially as in embryos and was described previously (Rastelli et al. 1995; Meller 2003). Briefly, tissues were incubated with a rabbit anti-MSL2 antibody and visualized with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Visualization and photography was done with a Zeiss Axioscope 2 fitted with an Axio phot photography system.

RESULTS

Transcription of roX1 RNA is male specific: All five MSL proteins are required for stabilization of the roX transcripts and their accumulation on the X chromosome (Amrein and Axel 1997; Meller et al. 2000). Nevertheless, sex-specific differences at the level of transcription may also exist. The location of the genes on the X chromosome, whose structure is altered in males, may be a factor influencing their transcription. For this reason we wished to study regulation of the roX genes in as natural a chromosomal context as possible. The roX RNA covers the entire male X chromosome, preventing direct observation of transcription at the X-linked roX genomic loci by in situ hybridization to roX probes (Figure 1B). In contrast, no transcripts are visualized in salivary glands from female larvae (Figure 1C). The roX1Δ750 mutation was generated by insertion of a P element that disrupts the gene ~1.4 kb from its 5′ end. roX1Δ750 produces an unstable mutated roX1 transcript that never coats the X chromosome (Meller et al. 2000; Figure 1A). In the presence of a wild-type copy of the roX2 gene, roX1Δ750 flies show no detectable defects. In situ hybridization of roX1 probes antisense to the 5′ end of the transcript reveal a discrete spot of roX1 transcription in salivary glands from third instar roX1Δ750 males.
MSL2 Controls Sex Specificity of the roX Genes

Figure 1.—Transcription of roX1 is male specific and does not require the complete MSL complex. (A) Structure of the roX1ex6710 and roX1ex6 alleles. Thick lines represent roX1 transcripts produced by the alleles used in this study. The roX1ex6710 transcript terminates within the P element but only the transcribed portion of roX1 is indicated. [h83-5’troX1] is an autosomal transgene. The bar with diagonals indicates the antisense riboprobe used in these experiments. (B–I) roX1 transcripts revealed by in situ hybridization to salivary glands from third instar larvae. (B) roX1+/H11001 male. (C) roX1+/H11001 female. (D) roX1ex6710 male. (E) roX1ex6710 female. (F) roX1ex6710;[h83-5’troX1] male. (G) roX1ex6710;[h83-5’troX1] female. (H) roX1ex6710 mof male. (I) roX1ex6710; mle male.

(Figure 1D). This signal presumably represents nascent transcripts that are not stabilized and do not colocalize with MSL proteins on the X chromosome. The absence of roX1 hybridization signals in salivary glands from female larvae indicates that no artifactual hybridization to genomic DNA is detected under the conditions used in these experiments (Figure 1C).

To determine if transcription plays a role in sex-specific accumulation of roX1 RNA, we asked whether the roX1mb710 allele was differentially transcribed in males and females. No nascent transcripts were detected in roX1mb710 female salivary glands (Figure 1E), suggesting that the roX1 gene is transcribed only in males. An alternative explanation is that truncated roX1mb710 RNA fragments are transcribed equally in both sexes but are more rapidly degraded in females and, for that reason, were not visualized by in situ hybridization. To address this, we expressed transgenic roX1 RNA fragments of similar sequence to the transcribed portion of roX1mb710 in a roX1ex6 background. roX1ex6 was created by an imprecise excision removing 1.4 kb of roX1 sequence. Male larvae carrying the roX1ex6 allele show no evidence of roX1 transcription (Kelley et al. 1999). roX1ex6 flies carrying a transgenic construct that produces 900 bp of roX1 RNA under the control of the hsp83 promoter showed similar discrete spots of transcription in salivary glands from either sex (Figure 1, F and G), suggesting that this transcript is equally unstable in females and in males. This observation suggests that the difference observed between roX1mb710 females and males is achieved at the level of transcription and is unlikely to result from differential stabilization of the mutated roX1 RNA.

roX1 RNA transcription does not require a complete set of MSL proteins: How can sex-specific roX1 transcription be achieved? Male-specific factors might induce roX1 transcription or relieve constitutive repression. Alternatively, female factors might repress roX1 transcription. The female-specific Sex lethal protein (SXL) controls somatic sexual differentiation through a pathway involving the tra and tra2 genes (reviewed in Cline and Meyer 1996). SXL also blocks dosage compensation by inhibiting translation of MSL2 in females (Kelley et al. 1997; Gebauer et al. 2003). The roX RNAs can be induced in otherwise normal females by misexpression of the sex lethal gene (Amrein and Axel 1997; Meller et al. 1997). These observations prompted suspicion that the MSL proteins, which are necessary for sex-specific accumulation of the roXRNAs, might also regulate their transcription. Males carrying mutations in the msl genes survive to the third instar larval stage, allowing us to perform in situ hybridization to salivary glands from mutated males to determine if roX1 transcription can occur in the absence of individual MSL subunits. Males carrying the roX1ex6710 chromosome and mutations in mof, mle, or msl3, still revealed transcription of roX1ex6710 (Figure 1, H and I, and Table 1). This result indicates that neither the formation of intact MSL complexes nor the individ-
Inappropriate upregulation of female matin-binding activity (together with the roX RNAs) may play a role in directing the MSL protein into the complex. We have used females that are forced to express the MSL2 protein to their normal target sequences. In the absence of MSL2 expression and produce healthy larvae (Kelley et al. 1995; Meller and Rattner 2002). Females that constitutively express MSL2 but lack the MLE or MSL3 proteins still transcribed roX1 (Table 1). In this genetic background the absence of a complete set of MSL proteins prevents the stabilization of full-length roX1 transcripts and allows visualization of transcription. Unexpectedly, msl2-expressing females still sustained roX1 transcription even in the absence of MSL1 (Figure 2B). Transcription of roX2 could also be detected in these females, but rather than being limited to the site of synthesis, roX2 transcripts were visible throughout the nucleus (Figure 2D). This suggests that roX2 is more stable than roX1 in the absence of a complete MSL complex (compare Figure 2B with D). Females mutated for msl1 but lacking the msl2 transgene transcribe neither roX1 nor roX2 (see Figure 2A and C). MSL2-driven transcription could also be detected in msl1 females carrying the roX1a710 mutation (Table 1). Comparable results from these two alleles support the idea that transcription of roX1a710 reflects that of the wild-type gene. These observations suggest that the presence of MSL2 protein induces transcription from the roX genes. Transcription does not require any other known MSL protein and thus cannot depend on the formation of partial or complete dosage compensation complexes.

The roX RNAs play a role in directing the MSL proteins to their normal target sequences. In the absence of roX1 and roX2 the MSL proteins no longer localize to the X chromosome normally, but they do retain chromatin-binding activity (Meller and Rattner 2002). We asked if a roX transcript was also necessary for MSL2-mediated roX1 transcription. MSL2-expressing females carrying a complete deletion of the roX2 gene are able
The roX1 DHS is not essential for MSL2-driven transcription of roX1: MSL2 could regulate roX RNA transcription by direct or indirect interactions with a response element at the roX loci. The DHS is able to bind partial MSL complexes in males mutated for mle, mof, or msl3 (Kelley et al. 1999). This combination of features makes the DHS the most likely candidate for an MSL2-response element. Transcription of roX1 alleles carrying DHS deletions was examined in females expressing MSL2. These females were also mutated for msl3 so that roX1 transcripts were not stabilized (Figure 4B). The roX1+ and roX1lox alleles were generated by imprecise excision of the roX1lox P element and lack 2431 and 2350 bp of transcribed region (see Materials and Methods for breakpoints). An antisense riboprobe that hybridizes to an 800-bp region that is retained in roX1+ and roX1lox detected similar levels of transcript from wild-type chromosomes and both deleted alleles (Figure 4, B–D). Otherwise wild-type females carrying the roX1+ and roX1lox alleles do not transcribe roX1, indicating that removal of the deleted sequences does not relieve repression of transcription (data not shown). This suggests that the DHS is nonessential for MSL2 control of roX1 transcription.

**DISCUSSION**

The roX RNAs play crucial roles in male dosage compensation and their regulation is likely to be an integral part of their normal function. In this work we demonstrate that, even though the stability of the roX transcripts and their accumulation along the X chromosome are tightly dependent on the presence of the five male-specific lethal genes, male-specific transcription also occurs and is dependent only on MSL2. None of the other MSL proteins is essential for this function, as mutation in each of them does not prevent MSL2-driven transcription of the endogenous wild-type roX1 gene. Likewise, MOF-mediated acetylation of histone H4 at lysine 16 is not a prerequisite for roX1 transcription, nor is the activity of the RNA/DNA helicase, MLE. In contrast, these two activities are essential for the *in cis* spreading of MSL complexes from DHS and for the stability of roX RNA in males (Amrein and Axel 1997; Gu et al. 2000; Meller et al. 2000). The observation that MSL2 holds a function independent of MSL1 was unanticipated. MSL1 and MSL2 have been suggested to comprise the chromatin-binding activity of the MSL complex and to function together during the initiation of its association with the X chromosome. In addition, direct MSL2 interaction with MSL1 has been demonstrated *in vitro* (Copps et al. 1998). Ectopic expression of MSL2 in females appears to stabilize MSL1 (Kelley et al. 1995). These two proteins are mutually dependent for localization at ∼35 CES on the X chromosome in the absence of MSL3, MLE, or MOF (Lyman et al. 1997; Gu et al. 1998). The absence of an msl1 role in roX transcriptional regulation is supported by the demonstration that the MSL2 RING domain is dispensable for MSL2-driven transcription of roX1.
finger, a domain essential for dosage compensation and for the interaction between MSL1 and MSL2, is dispensable for roX1 transcription. This emphasizes that transcriptional regulation of the roX genes represents a novel role for MSL2 that is genetically and molecularly distinct from its function as an MSL complex subunit.

Expression of MSL2 in an otherwise normal female allows roX transcription. These females deploy the male dosage compensation system, but they are not otherwise sexually transformed and are presumed to retain normal expression of SXL. As SXL directs female gene expression patterns, this makes it unlikely that roX transcription is normally blocked in females by a sex-limited factor. However, it is possible that MSL2 acts by relieving a general transcriptional repression at the roX genes.

Alternatively, MSL2 may control roX sex specificity by binding to nascent transcripts, thus relieving a transcriptional pause. The present results do not allow us to distinguish between stimulation of transcription or a relief of an inhibition that occurs before transcriptional initiation or during early elongation.

The male-specific roX1 DHS has been shown to recruit MSL complexes to autosomes and to support spreading of these complexes into flanking chromatin (Kageyama et al. 2001). In spite of the overall lack of similarity between the roX genes, roX2 also overlaps a male-specific DHS that recruits MSL complexes (Park et al. 2003). The presence of these regions in two genes that are each regulated by MSL2 was highly suggestive. As the only sequence within roX1 known to interact with MSL proteins, the DHS is the primary candidate for the MSL2-responsive enhancer governing roX1 transcription. Surprisingly, transcription from roX1 alleles lacking the DHS reveals that MSL2 does not require this sequence to drive roX1 transcription. Furthermore, these roX1 excisions do not derepress roX1 transcription in females. If MSL2 acts to relieve a general repression of roX transcription, repression does not require the presence of the DHS or other internal sequences that have been excised. The roX1 transcription assay used in these studies is likely to reflect the input of all regulatory elements, including distant enhancers and local chromatin context. For this reason we expect that it provides an accurate indication of the transcriptional status of roX1 in its native context.

What could be the advantage of MSL2 having two roles in dosage compensation, one as a subunit of the MSL complex and another as the transcriptional regulator of RNAs in the same complex? A recent model proposes that the ratio between MSL proteins and roX RNA influences spreading from roX DHS (Park et al. 2002; Oh et al. 2003). This model posits that when the MSL/roX ratio is high (for example, due to reduced roX RNA in the nucleus), complexes are fully assembled before the release of the nascent roX transcripts from the DNA templates. These complexes can immediately bind to chromatin and tend to accumulate in the vicinity of roX genes. By contrast, if the MSL/roX ratio is low, final assembly of the complex occurs in the nucleoplasm following release of the roX transcript. The assembled complex, no longer associated with a particular region, is free to move throughout the nucleus and may travel in trans to other chromosomes. Although the molecular interactions that promote in cis spreading remain obscure, this model is supported by experimental manipulations of MSL and roX levels. For example, when one of the two roX genes is mutated and MSL1 and MSL2 are increased, males display a dramatic enrichment of MSL complex surrounding the remaining roX gene (Oh et al. 2003). These findings suggest that the normal distribution of MSL proteins along the length of the male X chromosome is at least in part due to maintenance of MSL/roX ratios. Regulation of roX transcription by MSL2 suggests a mechanism by which the level of available MSL2
The MSL2 protein dictates the supply of roX transcripts, thus maintaining a constant ratio between these two molecules.

We propose a model (Figure 5) in which MSL2 is in a dynamic equilibrium between two possible states. Most of the MSL2 in normal males is assembled into dosage compensation complexes. The amount of roX RNA in the nucleus will determine how much MSL2 can assemble into functional complexes and how much of the protein is available to drive transcription of more roX RNA. It is unknown if MSL2 that is assembled into complexes can stimulate roX transcription, but the vast majority of MSL2 in this form is bound along the length of the X chromosome and is not free to do so. Binding of partial complexes to the roXDHS was previously shown to require MSL2 and MSL1 (Lyman et al. 1997; Kelley et al. 1999). However, it is clear that MSL2 can stimulate roX transcription in the absence of any other MSL protein and that interaction with the roX1 DHS is not required for transcription of this gene. If roX transcription is driven only by free MSL2, transcription would keep pace with the available supply of precursor proteins, thus maintaining a stable MSL/roX ratio. This would hold some advantages for the fly. Small changes in the level of roX RNA could be rapidly corrected. Such an autoregulatory mechanism would ensure that the rate of roX transcription and the rate of MSL complex assembly onto nascent roX RNAs are optimal.

Although we have examined only roX transcription, it is possible that MSL2 directs the transcription of other male-specific genes. If this is the case, the number of MSL2-driven genes is anticipated to be small as females that misexpress MSL2 show no signs of sexual transformation (B. P. Rattner and V. H. Meller, unpublished observations). Nevertheless, the observation that MSL2 can drive the transcription of a male-limited gene is intriguing and raises the possibility that other sex-specific genes might be similarly controlled.

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