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 *m*ale-specific *l*ethal (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 *D*Nase I *h*ypersensitive *s*ites (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 *c*hromatin *e*ntry *s*ites (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

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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°. Mutations in *msl* genes have been previously described as follows: missense mutation $msl2^{1}$ (Zhou *et al.* 1995), null mutation $msl1^{L60}$ (Chang and Kuroda 1998), missense mof^{1} and nonsense mof^{2} mutations (Hilfiker *et al.* 1997; Gu *et al.* 1998), nonsense mle^{1} mutation (Rastelli and Kuroda 1998), and $msl3^{2}$ mutation (Lindsley and Zimm 1992). The $roX1^{mb710}$ and $roX1^{ex6}$ 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^{+}4Δ4.3]; described in Meller and Rattner 2002). The [*h83*-M2-6I] transgene was described in Kelley *et al.* (1995). The [*h83*-M2ΔRING] transgene was generated by Lyman *et al.* (1997). The [*h83*-5′*roX1*] transgene contains a 908-bp *roX1* fragment (base pairs 279–1187) (Meller *et al.* 2000). $roX1^{ex7B}$ and $roX1^{ex40A}$ were generated by imprecise excision of the *P* element from $roX1^{mb710}$ 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 *CyO* y^{+} balancer chromosome. Homozygous *msl3* [*h83*-M2-6I] 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 (*Pvu*II-*Bgl*II fragment) and 0.8 kb of the 3′ end (*Xmn*I-*Eco*RI 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. Immunhistochemical 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 Axiophot 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 $roX1^{mb710}$ mutation was generated by insertion of a *P* element that disrupts the gene ~1.4 kb from its 5′ end. $roX1^{mb710}$ 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, $roX1^{mb710}$ 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 $roX1^{mb710}$ males

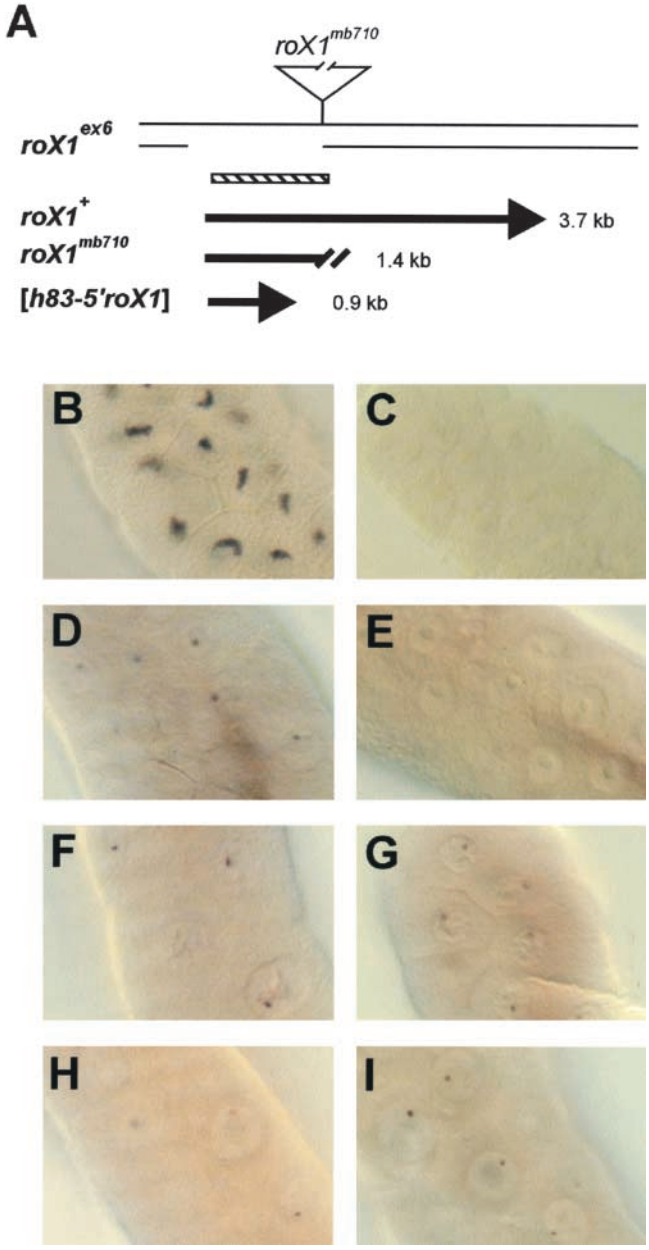

Figure 1.—Transcription of *roX1* is male specific and does not require the complete MSL complex. (A) Structure of the $roX1^{mb710}$ and $roX1^{ex6}$ alleles. Thick lines represent *roX1* transcripts produced by the alleles used in this study. The $roX1^{mb710}$ transcript terminates within the *P* element but only the transcribed portion of *roX1* is indicated. [*h83-5′roX1*] 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) $roX1^{+}$ male. (C) $roX1^{+}$ female. (D) $roX1^{mb710}$ male. (E) $roX1^{mb710}$ female. (F) $roX1^{ex6}$; [*h83-5′roX1*] male. (G) $roX1^{ex6}$; [*h83-5′roX1*] female. (H) $roX1^{mb710}$ *mof* male. (I) $roX1^{mb710}$; *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 $roX1^{mb710}$ allele was differentially transcribed in males and females. No nascent transcripts were detected in $roX1^{mb710}$ female salivary glands (Figure 1E), suggesting that the *roX1* gene is transcribed only in males. An alternative explanation is that truncated $roX1^{mb710}$ 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 $roX1^{mb710}$ in a $roX1^{ex6}$ background. $roX1^{ex6}$ was created by an imprecise excision removing 1.4 kb of *roX1* sequence. Male larvae carrying the $roX1^{ex6}$ allele show no evidence of *roX1* transcription (Kelley *et al.* 1999). $roX1^{ex6}$ 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 $roX1^{mb710}$ 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 MSL2, indicating that the expression of *roX* genes does not require the absence of SXL, nor is it blocked by other female-specific factors dependent on SXL, such as TRA (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 *roX* RNAs, 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 $roX1^{mb710}$ chromosome and mutations in *mof*, *mle*, or *msl3*, still revealed transcription of $roX1^{mb710}$ (Figure 1, H and I, and Table 1). This result indicates that neither the formation of intact MSL complexes nor the individ-

TABLE 1

Transcriptional status of *roX1* in different genetic backgrounds

Genotype	*roX1* transcription
Male	
roX1^{+}	+[a]
roX1^{mb710}	+
roX1^{ex6}	−
roX1^{ex6}; [*h83-5′ roX1*]	+
roX1^{mb710} *mof*	+
roX1^{mb710}; *msl3*	+
roX1^{mb710}; *mle*	+
Female	
roX1^{+}	−
roX1^{mb710}	−
roX1^{ex6}	−
roX1^{ex6}; [*h83-5′ roX1*]	+
roX1^{+}; *msl1*	−
roX1^{+}; *msl1*; [*h83*-M2]	+
roX1^{mb710}; *msl1*; [*h83*-M2]	+
roX1^{+}; *mle*; [*h83*-M2]	+
roX1^{+}; *msl3* [*h83*-M2]	+
roX1^{ex7B}; *msl3* [*h83*-M2]	+
roX1^{ex40A}; *msl3* [*h83*-M2]	+
roX1^{mb710} *roX2*$^{-}$; [*h83*-M2]	+
roX1^{+}; *msl2*; [*h83*-M2ΔRING]	+

Transcription was revealed by *in situ* hybridization to salivary glands from third instar larvae using *roX1* antisense riboprobes. −, the *roX1* transcript was never detected after a minimum of three experiments; +, visualization of nascent transcripts over the sites of synthesis in 60–100% of the nuclei (see MATERIALS AND METHODS for details of experimental procedures).

[a] *roX1* accumulates on the X chromosome of wild-type males and prevents detection of nascent transcripts.

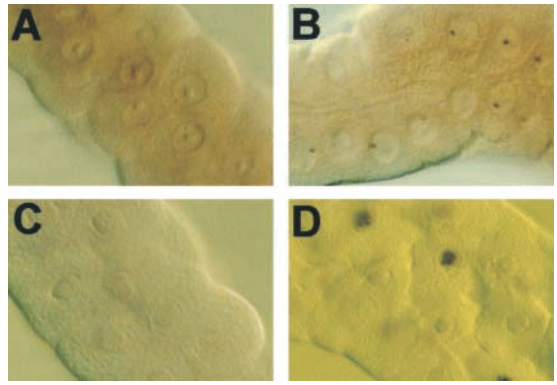

FIGURE 2.—Transcription of *roX1* and *roX2* is dependent on MSL2 but independent of MSL1. The presence of *roX1* transcripts was detected by *in situ* hybridization to salivary glands from third instar larvae. (A and B) *roX1* transcription. (C and D) *roX2* transcription. A and C are glands from *msl1* females; B and D are *msl1*; [*h83*-M2] females. Although the *roX2* transcript is detected in most cells in the experiment presented in D, differences in focal plane restrict the number of nuclei that can be shown in a single photograph.

ual activities of these three proteins are required to induce *roX1* transcription.

MSL2 is the only MSL protein required for *roX* transcription: Flies mutated for *msl1* or *msl2* present a more severe phenotype than other *msl* mutants do and show reduced numbers of male larvae. MSL1 and MSL2 have been proposed to form the core of the MSL complex. These two proteins require each other to bind at the ~35 CES along the X chromosome, and they interact directly with one another (LYMAN *et al.* 1997; COPPS *et al.* 1998). MSL2 expression is normally limited to males, whereas all the other MSL proteins, including MSL1, are produced in both sexes. To look at the roles of these two proteins in the regulation of *roX* transcription, we used females that are forced to express the MSL2 protein. In these females, the whole set of MSL proteins, together with the *roX* RNAs, is recruited to both female X chromosomes. Inappropriate upregulation of female X chromosomes leads to reduced viability and infertility (KELLEY *et al.* 1995). Females mutated for both *roX* genes or for any protein-encoding *msl* gene are rescued from the lethal effects 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 2, B with D). Females mutated for *msl1* but lacking the *msl2* transgene transcribe neither *roX1* nor *roX2* (see Figure 2, A and C). MSL2-driven transcription could also be detected in *msl1* females carrying the *roX1*^{mb710} mutation (Table 1). Comparable results from these two alleles support the idea that transcription of *roX1*^{mb710} 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

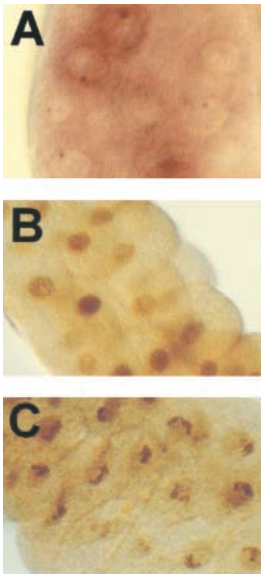

FIGURE 3.—The RING finger domain of MSL2 is dispensable for *roX1* transcription. (A) *roX1* transcripts in salivary glands from an *msl2*; [*h83*-M2ΔRING] female. (B and C) Anti-MSL2 immunostaining of salivary glands. (B) *msl2*; [*h83*-M2ΔRING] male. (C) *msl2*/+; [*h83*-M2ΔRING] male.

to transcribe the $roX1^{mb710}$ allele (Table 1). This result indicates that MSL2-driven transcription of *roX1* does not require a functional *roX* RNA.

The RING finger domain of MSL2 is not essential for *roX1* transcription: These observations indicate that MSL2 is involved in two fundamentally different processes. It is an essential subunit of a complex that mediates a male-limited upregulation of X-linked genes that are expressed in both sexes. Additionally, MSL2 regulates transcription of the male-specific *roX* genes. It is possible that the divergent functions of MSL2 require different portions of this protein. The RING finger domain of MSL2 is essential for dosage compensation, and males carrying an in-frame deletion that removes the RING finger, MSL2ΔRING, do not live (LYMAN *et al.* 1997). Whereas MSL2 is found normally localized to the X polytene chromosome in male salivary glands (Figure 3C), the MSL2ΔRING protein does not localize to the X chromosome and is instead found throughout the nucleus (LYMAN *et al.* 1997). Males mutated for *msl2* and carrying an MSL2ΔRING transgene show unlocalized expression of the mutant MSL2 protein (Figure 3B). This result indicates that the MSL2ΔRING protein is expressed in males but is unable to coat the X chromosome. As *msl2* male larvae are typically scarce, we looked at *roX1* transcription in females expressing transgenic MSL2ΔRING and homozygous for a null allele of the endogenous *msl2* gene. These females continue to transcribe *roX1* (Figure 3A). This result indicates that the RING finger domain, which is essential for assembly of the MSL complex and male dosage compensation, is dispensable for MSL2-driven *roX1* transcription.

The *roX1* DHS is not essential for MSL2-driven transcription of *roX1*: MSL2 could regulate *roX* 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 $roX1^{ex7B}$ and $roX1^{ex40A}$ alleles were generated by imprecise excision of the $roX1^{mb710}$ *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 $roX1^{ex7B}$ and $roX1^{ex40A}$ detected similar levels of transcript from wild-type chromosomes and both deleted alleles (Figure 4, B–D). Otherwise wild-type females carrying the $roX1^{ex7B}$ or $roX1^{ex40A}$ 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

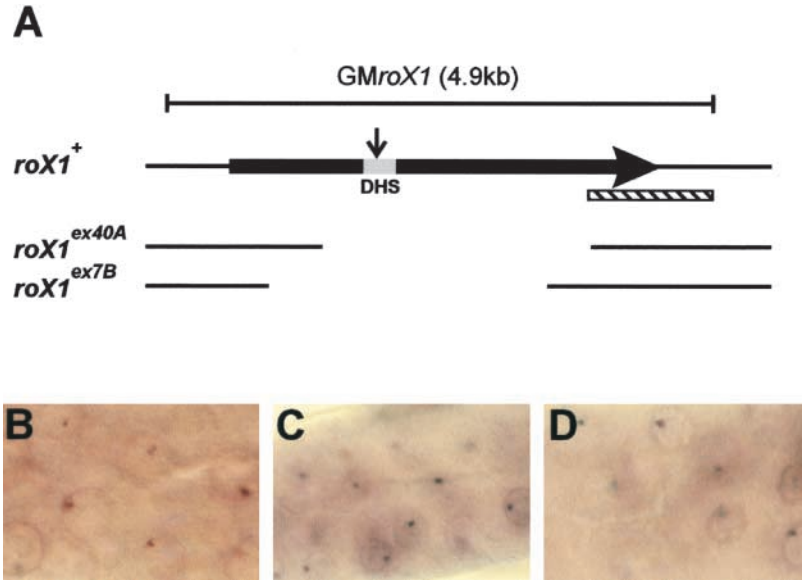

Figure 4.—The DHS is dispensable for MSL2-driven *roX1* transcription. (A) Schematic of *roX1* alleles. The top line represents a genomic transgene that rescues roX^{-} males. The thick horizontal arrow indicates the 3.7-kb transcribed *roX1* sequence and the position of the DHS. The vertical arrow in the DHS designates the $roX1^{mb710}$ *P*-element insertion site. The structures of the $roX1^{ex40A}$ and $roX1^{ex7B}$ excisions are shown. A bar with diagonals indicating the sequences recognized by the riboprobe used in this experiment is also shown. (B–D) *roX1* transcripts revealed by *in situ* hybridization to larval salivary glands. (B) $roX1^{+}$; *msl3* [*h83*-M2] female. (C) $roX1^{ex40A}$; *msl3* [*h83*-M2] female. (D) $roX1^{ex7B}$; *msl3* [*h83*-M2] female.

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

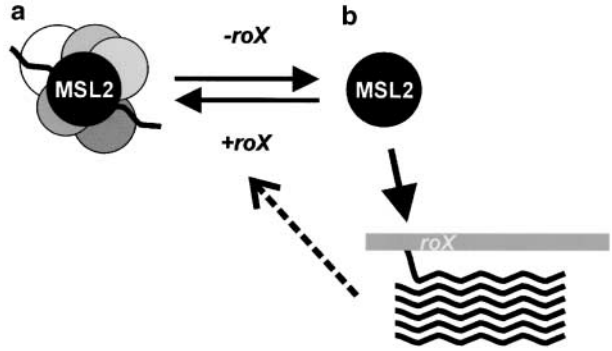

FIGURE 5.—Regulation of Drosophila dosage compensation by MSL2 and *roX* transcription. The MSL2 protein is in a dynamic equilibrium between dosage compensation complexes (a) and free MSL2 (b). The intact complexes are bound along the X chromosome, reducing the level of MSL2 protein available to regulate *roX*. *roX* transcripts, required for the assembly and localization of intact complexes, influence this equilibrium (dashed arrow).

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 *roX* DHS 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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