The *roX* genes encode redundant *male-specific lethal* transcripts required for targeting of the MSL complex

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The *roX1* and *roX2* genes of *Drosophila* produce male-specific non-coding RNAs that co-localize with the Male-Specific Lethal (MSL) protein complex. This complex mediates up-regulation of the male X chromosome by increasing histone H4 acetylation, thus contributing to the equalization of X-linked gene expression between the sexes. Both *roX* genes overlap two of ~35 chromatin entry sites, DNA sequences proposed to act *in cis* to direct the MSL complex to the X chromosome. Although dosage compensation is essential in males, an intact *roX1* gene is not required by either sex. We have generated flies lacking *roX2* and find that this gene is also non-essential. However, simultaneous removal of both *roX* RNAs causes a striking male-specific reduction in viability accompanied by relocation of the MSL proteins and acetylated histone H4 from the X chromosome to autosomal sites and heterochromatin. Males can be rescued by *roX* cDNAs from autosomal transgenes, demonstrating the genetic separation of the chromatin entry and RNA-encoding functions. Therefore, the *roX1* and *roX2* genes produce redundant, *male-specific lethal* transcripts required for targeting the MSL complex.
Keywords: dosage compensation/epigenetic regulation/*male-specific lethal*/non-coding RNAs/*roX* RNA

Introduction

Organisms with divergent sex chromosomes, such as X and Y, employ various chromatin-based mechanisms of gene regulation to equalize sex chromosome expression between the two sexes (Meller, 2000). *Drosophila melanogaster* males up-regulate most of the genes on their X chromosome using a male-limited protein complex composed of the five *male-specific lethal* gene products (MSL proteins) and JIL-1 kinase (Cline and Meyer, 1996; Hilfiker *et al.*, 1997; Jin *et al.*, 2000). This complex binds to the X chromosome and directs acetylation of histone H4 on lysine 16 (H4Ac16), a modification associated with the elevated expression of X-linked genes (Akhtar and Becker, 2000; Smith *et al.*, 2001). It is widely assumed that the non-coding, male-specific *roX1* and *roX2* RNAs play a role in dosage compensation. Both transcripts 'paint' the X chromosome of male, but not female *Drosophila*, and they colocalize with the MSL proteins in a finely banded pattern along the length of the polytenized salivary gland X chromosome (Meller *et al.*, 1997, 2000; Franke and Baker, 1999; Gu *et al.*, 2000). Both *roX* RNAs can be

co-immunoprecipitated with anti-MSL antibodies, indicating that they form a stable association with these proteins (Akhtar *et al.*, 2000; Gu *et al.*, 2000; Meller *et al.*, 2000). Three of the MSL proteins bind RNA or may be released from the X chromosome by RNase treatment (Richter *et al.*, 1996; Akhtar *et al.*, 2000). Taken together, these findings indicate that the *roX* RNAs participate in the MSL complex and are likely to serve a functional role in compensation. The absence of a phenotype associated with mutations in *roX1* further suggested that *roX1* and *roX2* might be redundant. Because the *roX* transcripts share almost no sequence similarity, the aspect of these molecules that might confer functional redundancy is still unknown (Amrein and Axel, 1997; Franke and Baker, 1999). A prior study using large, embryonic-lethal excisions to remove *roX2* suggested a requirement for the *roX* genes in formation of the MSL complex or in its localization to the male X chromosome, a step which precedes compensation during embryogenesis (Franke and Baker, 1999). Although this supports the previous notion that one, but not both, of these genes is essential, the *roX2* deletions used for this study also removed several essential genes including the largest subunit of RNA polymerase II, *RpII215*, situated <10 kb distal to *roX2* (Voelker, 1985). The resulting disruption of zygotic gene expression causes non-specific developmental defects that could mask the true *roX⁻* phenotype. Because dosage compensation depends on the expression of zygotic genes in response to signals from the sex determination pathway, these embryos may have a failure in this process which is unrelated to the absence of the *roX* RNAs. It also remained a possibility that other *roX* RNAs existed that were normally expressed later in development, and these had escaped detection due to the early death of the embryos used in this study.

Both *roX* genes are X-linked, and they overlap two of ~35 chromatin entry sites on the X chromosome. These sites retain partial MSL complexes after the bulk of MSL binding to the X has been eliminated by mutations in the *mle*, *msl3* or *mof* genes (Lyman *et al.*, 1997; Gu *et al.*, 1998). When moved to an autosomal location, both *roX* genes provide sites from which the MSL complex can spread into chromatin *in cis*, and the presence of these sites is believed to be the mechanism used to differentiate the X chromosome from the autosomes (Kelley *et al.*, 1999; Meller *et al.*, 2000). Although a non-*roX* chromatin entry site has not been isolated, an entry site within the *roX1* gene has been studied in detail, and it lies in a few hundred base pairs of DNA internal to the transcribed region. The entry function is independent of transcription, and thus cannot be a property of the *roX* RNA. It is instead a characteristic of the *roX* genomic region (Kageyama *et al.*, 2001). As the *roX1* and *roX2* genes correspond to two of ~35 X-linked chromatin entry sites, it remained an

intriguing possibility that additional *roX* RNAs were transcribed from other entry sites. Alternatively, the *roX1* and *roX2* entry sites could be uniquely associated with *roX* transcripts. Finally, the remaining entry sites might differ qualitatively from those identified through their association with the *roX* genes, and it is possible that simultaneous mutation of *roX1* and *roX2* entry sites would prevent *cis*-directed dosage compensation of the male X chromosome.

To address these questions we have created flies deleted for *roX2*, and have used these to show that neither the *roX2* chromatin entry site nor its transcript is essential. However, when combined with a *roX1* mutation, a striking male-specific reduction in viability is observed. Male lethality may be rescued by expression of *roX* cDNAs from autosomally located transgenes, demonstrating the genetic separation of the chromatin entry function, which acts *in cis*, from the *roX* transcript, which may originate from any chromosome. Mutation of both *roX* genes rescues the lethality of females that inappropriately express *msl2*, and would otherwise be forced to up-regulate both X chromosomes. MSL protein localization to the X chromosome is profoundly disrupted in *roX⁻* males, as is H4Ac16 enrichment. These experiments reveal that the transcripts produced from *roX1* and *roX2* are required for targeting of the MSL complex. The male requirement for *roX* transcripts allows the *roX* genes to be classified as redundant *male-specific lethals*. Surprisingly, ~5% of the *roX⁻* males emerge as developmentally delayed escapers. Their presence suggests that partial compensation can occur without any wild-type *roX1* or *roX2* transcripts.

Results

Generation of a roX2 deletion

roX2 lies in a region densely packed with essential genes (Figure 1). We surveyed P-element lines with insertions cytologically mapped to the 10C region and identified two, P1547 and P1877, close to *roX2*. Several transcribed regions and lethal complementation groups lie between *roX2* and the closest insertion, P1877. Consequently, an excision of *roX2* caused by P1877 mobilization would be lethal due to disruption of other genes, regardless of the *roX2⁻* phenotype. We mobilized P1877 to generate lethal deletions, and identified several that removed *roX2* (see Materials and methods). These were mapped by complementation with genomic cosmids from the region and a single deletion, Df(1)52, could be rescued by autosomal insertions of the *roX2*-containing cosmid [*w*⁺4] (Figure 1). Because development of Df(1)52 flies is arrested in mid embryogenesis, no larvae or adults will be obtained without restoration of a portion of the deleted region (not shown). A 4.3 kb fragment containing *roX2* was removed from [*w*⁺4] to create [*w*⁺4Δ4.3], and transgenic flies carrying the deleted cosmid were generated.

roX2 is non-essential

Females heterozygous for Df(1)52 and a balancer chromosome were mated to males carrying an autosomal insertion of the [*w*⁺4Δ4.3] cosmid, which can be followed in a *w⁻* genetic background by light yellow eye color. As anticipated, no deficiency males lacking [*w*⁺4Δ4.3] were recovered, but fertile male offspring carrying the Df(1)52
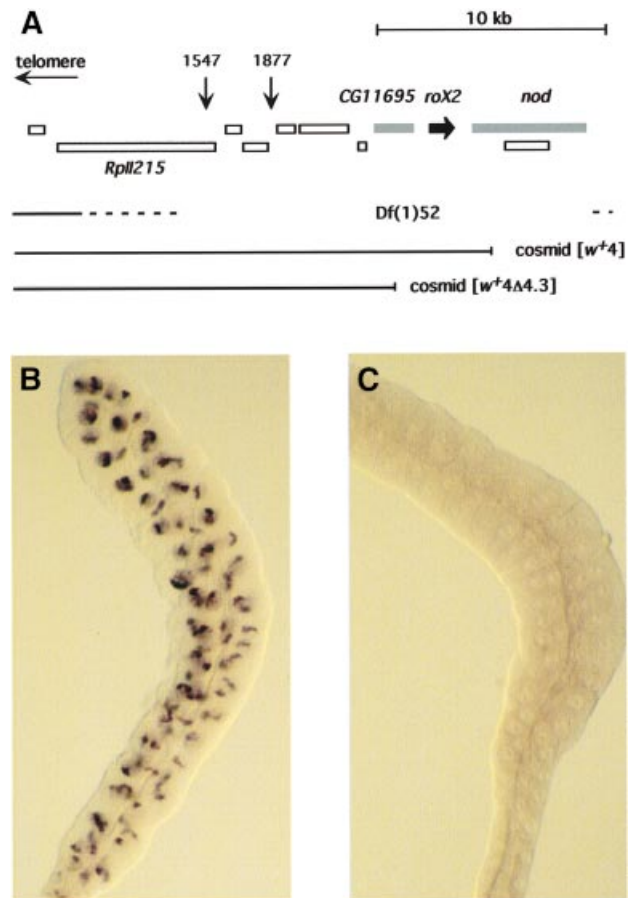

Fig. 1. Deletion of *roX2*. (**A**) Identified and predicted transcripts surrounding the *roX2* gene were compiled from the *Drosophila* genome project and Voelker *et al.* (1985). The insertion sites of P1877, P1547 and the proximal ends of the cosmids [*w*⁺4] and [*w*⁺4Δ4.3] are shown. The *roX2* gene is indicated in black; flanking genes disrupted by Df(1)52 and not restored by [*w*⁺4Δ4.3] are denoted in gray. (**B**) *In situ* detection of *roX2* in a wild-type male salivary gland. (**C**) *In situ* detection of *roX2* in a Df(1)52; [*w*⁺4Δ4.3]/+ male salivary gland.

chromosome and [*w*⁺4Δ4.3] were obtained. These males were not developmentally delayed, were recovered in numbers consistent with full viability and appeared completely normal in spite of their lack of *roX2*. *In situ* hybridization to salivary glands from Df(1)52/Y; [*w*⁺4Δ4.3]/+ males detected no *roX2* transcripts (Figure 1C). In addition to the deletion of *roX2*, these males also lack the *nod* gene, proximal to *roX2*, and the predicted zinc finger gene CG11695, distal to *roX2*. The *nod* gene product has not been reported in males and its disruption is unlikely to affect the outcome of this analysis (Zhang and Hawley, 1990). The overt normality of these males suggests that CG11695 sequences are also dispensable.

Male development is disrupted in roX double mutants

Recombinant X chromosomes carrying the mutations *roX1^mb710* or *roX1^ex6* and Df(1)52 were obtained. *roX1^mb710* is caused by a P-element insertion within the gene. Over 1 kb of *roX1* is transcribed from the *roX1^mb710* chromosome, but this fragment is unstable and can not accumulate (Meller *et al.*, 2000). *roX1^ex6* was created by an

Table I. Simultaneous mutation of both *roX* genes reduces male survival

Female parent	Male parent	Female progeny	Male progeny			
			Binsincy	*roX1⁻* Df(1)52	*roX⁻*; [*w⁺4Δ4.3*] survival (%)	*roX⁻*; [*w⁺4Δ4.3*] delay (days)
roX1^{ex6} Df(1)52 Binsincy	*yw*; [*w⁺4Δ4.3*] Y +	686	170	7	4.1	8
roX1^{ex6} Df(1)52 Binsincy	*yw*; [*w⁺4Δ4.3*] Y [*w⁺4Δ4.3*]	431	132	13	5.9	7
roX1^{mb710} Df(1)52 Binsincy	*yw*; [*w⁺4Δ4.3*] Y [*w⁺4Δ4.3*]	1257	390	33	5.3	3

Females heterozygous for a balancer chromosome and a *roX⁻* chromosome were mated to males carrying the [*w⁺4Δ4.3*] cosmid. Male survival is

Table II. Females are unaffected by loss of both *roX* RNAs

Female parent	Male parent	Female progeny	
		roX1^{ex6} Df(1)52 Binsincy	*roX1^{ex6}* Df(1)52 / *roX1^{ex6}* Df(1)52
roX1^{ex6} Df(1)52; [*w⁺4Δ4.3*] Binsincy +	*roX1^{ex6}* Df(1)52 Dp(1:Y)*B^{s}⁻v⁺y⁺*	218	117

Females heterozygous for a balancer chromosome and a *roX⁻* chromosome, and carrying one copy of the [*w⁺4Δ4.3*] cosmid were mated to males carrying a *roX⁻* X chromosome and a rescuing duplication on the Y chromosome. Because the [*w⁺4Δ4.3*] cosmid is present in one copy in the female parent, survival of *roX⁻* daughters will at best be 50% that of their sisters carrying the balancer chromosome.

imprecise excision of *roX1^{mb710}* and is deleted for 1.4 kb near the 5′ end of the transcript. When using *in situ* hybridization conditions optimized for detection of *roX1* in wild-type males, no transcripts could be detected in salivary glands from *roX1^{ex6}* males. Both *roX1* alleles allow normal male development in spite of the severity of their effect on transcript accumulation. Females heterozygous for either *roX1^{mb710}* Df(1)52 or *roX1^{ex6}* Df(1)52 and a balancer chromosome were mated to *yw* males carrying an autosomal [*w⁺4Δ4.3*] insertion. Males inheriting the *roX⁻* chromosomes were severely affected, with only 5% survival (Table I). The eclosion of these males was delayed, most markedly for the *roX1^{ex6}* Df(1)52 chromosome, which did not appear in progeny until at least a week after adult emergence began. In spite of the low numbers of adults recovered, doubly mutant males appear healthy and fairly abundant at the end of the third larval instar. Survival of males into the third instar is characteristic of the *male-specific lethal* mutations, but the presence of escaper adults is not (Belote, 1983; Baker *et al.*, 1994; Hilfiker *et al.*, 1997).

roX RNAs are not required in females
Females heterozygous for *roX1^{ex6}* Df(1)52 and a balancer chromosome and carrying an autosomal [*w⁺4Δ4.3*] transgene were mated to *roX1^{ex6}* Df(1)52/Dp(1:Y)-*B^{s}⁻v⁺y⁺* males that are rescued by duplication of the *roX2* region on the Y chromosome. Only half of the zygotes from this cross will inherit the [*w⁺4Δ4.3*] transgene, so *roX⁻* female survival will at best be 50% that of their sisters carrying the balancer chromosome. The *roX⁻* females were recovered at 54% the level of their sisters (Table II). Peak emergence days coincided for *roX⁻* females and their sisters (data not shown), suggesting that

removal of both *roX* RNAs has no effect on female development.

Restoration of either roX1 or roX2 in trans rescues roX⁻ males
The *roX* genes have two recognized functions: they contain *cis*-acting DNA sequences (chromatin entry sites) from which dosage compensation spreads into surrounding chromatin; and they produce the *roX* transcripts. The chromatin entry site internal to the *roX2* gene is removed by Df(1)52, and most of the *roX1* entry site is deleted in *roX1^{ex6}* (Kageyama *et al.*, 2001). We restored *roX* RNA to *roX1^{ex6}* Df(1)52; [*w⁺4Δ4.3*]/+ males to determine if the lethality associated with this X chromosome was the consequence of loss of the *roX* transcripts or of mutation of both *roX*-associated chromatin entry sites. Females homozygous for a *roX1^{ex6}* Df(1)52 chromosome and carrying one copy of the [*w⁺4Δ4.3*] transgene were mated to males homozygous for autosomal insertions of heat shock driven *roX1* or *roX2* cDNAs. Only half of the males will inherit the [*w⁺4Δ4.3*] cosmid that complements lethality, and consequently male survival can not exceed 50% even with full rescue of the *roX⁻* phenotype. Control crosses in which females were mated to males with no *roX* transgene displayed delayed emergence of male offspring (not shown) and survival of <4% (Table III). However, when *roX* transgenes were present, male survival increased and the eclosion time of rescued males approached that of their sisters. *Hsp83*-driven *roX1* cDNA allows recovery of 47% of males (Table III), thus it fully rescues mutant viability. Less dramatic rescue was observed with an *Hsp70-roX2* transgene, perhaps because these experiments were conducted at 25°C and the *Hsp70* promoter has low activity in the absence of heat shock. Two transgenes carrying *roX1* fragments driven by

Table III. Males carrying a *roX1ex6* Df(1)52 chromosome are rescued by *roX* cDNAs

Transgene	Progeny		
	Females	Males	% male survival
–	171	6	3.5
Hsp83-roX1	266	125	47
Hsp70-roX2	204	58	28
Hsp83-roX1 3′	207	7	3.4
Hsp83-roX1 5′	235	9	3.8

roX1ex6 Df(1)52; [*w*+4Δ4.3]/+ virgins were mated to males homozygous for autosomal insertions of the indicated transgenes.

the *Hsp83* promoter were also tested. Neither the 3′ 1 kb nor the 5′ 2.5 kb of *roX1* could rescue, indicating that the intact RNA is required.

Removal of both roX RNAs compromises dosage compensation

The sex-specificity of X chromosome up-regulation is achieved by tight repression of *msl2* expression in females (Bashaw and Baker, 1997; Kelley *et al*., 1997; Gebauer *et al*., 1998). Inappropriate up-regulation of X chromosomes can be induced by ectopic expression of *msl2* from the [*w*+H83M2-6I] transgene, and this is sufficient to recruit the rest of the MSL proteins to both female X chromosomes resulting in low viability, sterility and delayed development (Kelley *et al*., 1995). If the *roX* transcripts do play a vital role in dosage compensation, removal of these RNAs should block the MSL2-induced lethality of females carrying the [*w*+H83M2-6I] transgene. A cross introducing [*w*+H83M2-6I] into females with wild-type *roX* genes produced the anticipated reduction in viability and developmental delay of daughters inheriting this transgene (Figure 2A). We then performed a similar cross in a genetic background lacking *roX1* (not shown), *roX2* (Figure 2B) or mutated for both *roX* RNAs (Figure 2C; see Materials and methods for details). In the absence of wild-type *roX* RNA, female offspring carrying [*w*+H83M2-6I] were recovered in numbers equal to their sisters, and they display eclosion times similar to their sisters (Figure 2C). Elimination of only *roX1* or *roX2* resulted in an intermediate level of female lethality and developmental delay in response to the [*w*+H83M2-6I] transgene.

Localization of the MSL proteins and H4Ac16 to the X chromosome is disrupted in roX− males

Mutation of *mle, msl3* or *mof* results in a reduced pattern of X-chromosome binding by the remaining MSL proteins, and mutation of *msl1* or *msl2* leads to a complete loss of chromatin binding by the remaining members of the complex (Lyman *et al*., 1997; Gu *et al*., 1998). The removal of the *roX* RNAs might similarly disrupt formation of the MSL complex or affect its localization to the X chromosome. We tested this by immunolocalization of MSL1 (not shown) and MSL2 to chromosome preparations from males that were wild type, mutated for *roX1* or *roX2*, or mutated for both *roX* RNAs. No differences were discernible in immunolocalization to chromosomes from wild-type males (Figure 3B) or males mutated for only one
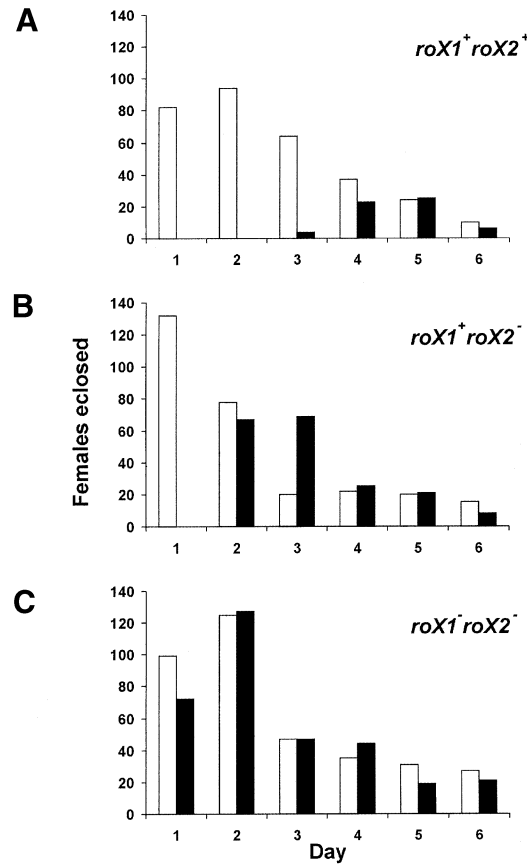

Fig. 2. Females are rescued from *msl2* expression by removal of the *roX* RNAs. (**A**) Females display reduced viability and developmental delay if they carry the *msl2*-expressing transgene [*w*+H83M2-6I] (filled bars). Open bars indicate their sisters lacking the transgene. (**B**) The deletion of *roX2* allows partial rescue of females carrying [*w*+H83M2-6I]. (**C**) Mutation of both *roX1* and *roX2* fully rescues females carrying [*w*+H83M2-6I]. See Materials and methods for a complete description of the genotypes used in these experiments.

of the *roX* genes (Figure 3D and F). However, males lacking any wild-type *roX* RNA have sharply reduced overall staining of the X chromosome (Figure 3H). A number of bands retaining MSL2 do remain on the X chromosome, and this pattern is reminiscent of the reduced pattern of X-chromosome binding seen after mutations in *mle, msl3* or *mof*. However, a few autosomal sites have strongly enhanced MSL2 binding (Figure 3H, asterisks). Notably strong staining is also seen on the fourth chromosome (arrowhead in Figure 3H), and diffuse anti-MSL2 staining appears in the heterochromatin of the chromocenter (arrow in Figure 3H). The alteration in chromatin structure associated with dosage compensation is linked to an increase in the width of the single male X chromosome to about the width of a pair of autosomes (Baker *et al*., 1994). Significantly, the X chromosome of *roX*− males appears thinner than the compensated X chromosomes from wild-type or singly mutated males.

Acetylation of histone H4 on lysine 16 (H4Ac16) is linked to elevated transcription, and enrichment of H4Ac16 on the male X chromosome is believed to be central to the mechanism by which X-linked genes are up-regulated. We examined the pattern of H4Ac16 enrichment by probing chromosome preparations with an
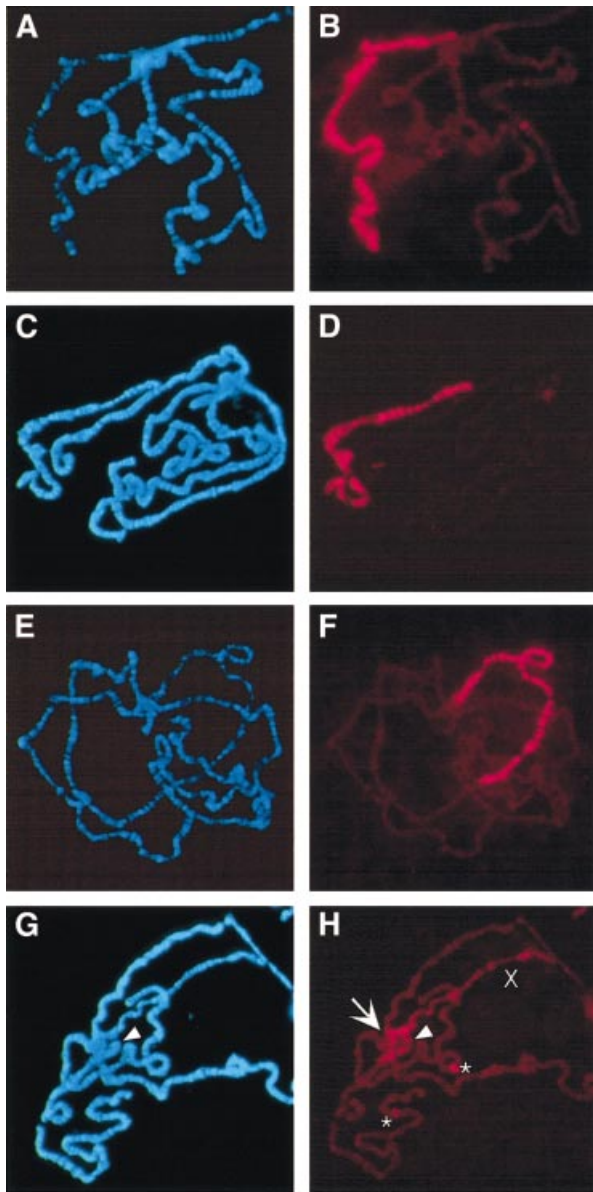
Fig. 4. Histone H4 acetylation at lysine 16 (H4Ac16) is disrupted in *roX⁻* males. (**A** and **B**) Wild-type male. (**C** and **D**) Male mutated for both *roX* RNAs (*roX1ᵉˣ⁶* Df(1)52/Y; [4Δ4.3]/+). (**A** and C) are chromosome preparations counter stained with Hoechst 33258. (B and D) are probed with an epitope-specific antibody that recognizes the H4Ac16 isoform of histone H4, detected in Texas Red. Exposure times for Texas Red is 8 s.

Fig. 3. Localization of MSL2 is disrupted in males lacking both *roX* RNAs. (**A** and **B**) Wild-type male. (**C** and **D**) Male mutated for *roX1* (*roX1ᵉˣ⁶*). (**E** and **F**) Male deleted for *roX2* (Df(1)52/Y; [4Δ4.3]/+). (**G** and **H**) Male mutated for both *roX* RNAs (*roX1ᵉˣ⁶* Df(1)52/Y; [4Δ4.3]/+). (A, C, E and G) Chromosome preparations counter stained with Hoechst 33258. (B, D, F and H) Chromosome preparations probed with antibodies against MSL2 and detected with Texas Red. In (G and H) the arrowhead points to the fourth chromosome; in (H) the arrow indicates the chromocenter and asterisks mark two strongly staining autosomal sites of MSL2 localization. 'X' indicates the X chromosome. Exposure times for Texas Red were between 6 and 8 s.

antibody specific to this acetylated isoform. Wild-type males show the anticipated pattern of H4Ac16 localization on the X chromosome (Figure 4B), but *roX1⁻* males display sharply reduced X chromosome staining and an overall increase of staining on the autosomes (Figure 4D). Similar to MSL2 localization in *roX⁻* males, there is an enrichment of H4Ac16 in the chromocenter (arrowhead in Figure 4D).

Autosomal sites of MSL2 binding frequently appear puffed in *roX⁻* males, and thus are presumably actively transcribed. These larvae produce poor quality chromo-
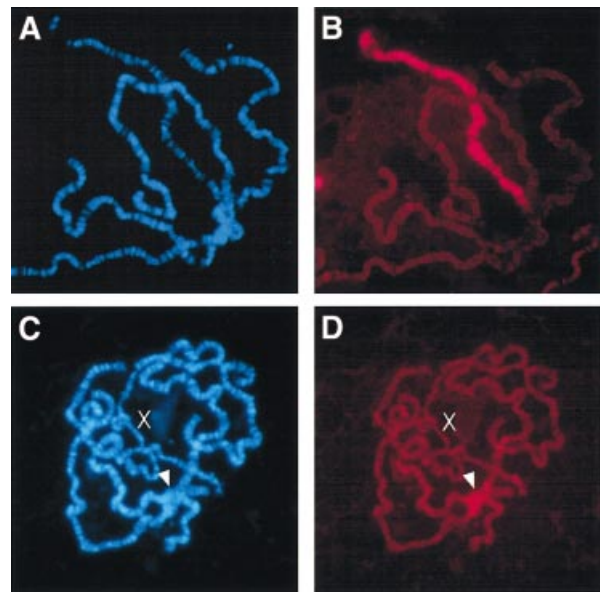
somes, complicating the accurate mapping of MSL2-binding sites. However, one prominent autosomal site is easily located due to its subtelomeric position at 21B. Visualization of MSL2 localization in chromosome preparations from wild-type males reveals weak signal at 21B, but in *roX⁻* males this region is often puffed and heavily labeled by anti-MSL2 antibodies (Figure 5).

Discussion

The roX transcripts are redundant male-specific lethals

The X-localization of *roX* transcripts, their male-specificity and regulation of *roX* RNA accumulation by the MSL proteins all have suggested a role for these RNAs in dosage compensation. However, failure to detect a phenotype in males mutated for *roX1* and the absence of precise mutations in *roX2* had previously precluded formal tests of this possibility. Furthermore, slight differences between the *roX* RNAs, such as the ability of transcripts from *roX2*, but not *roX1*, to move to all entry sites on the X chromosome in a fly mutated for *msl3,* and the identification of flies and cell lines lacking *roX1*, but none lacking *roX2*, supported the notion that *roX2* might be an essential gene in males (Meller *et al.*, 2000; Smith *et al.*, 2000). However, our mutation strategy, generation of a lethal deletion and restoration of essential gene functions with a genomic cosmid, produced healthy males lacking *roX2* RNA. When the *roX2* deletion was combined with a *roX1* mutation, male-specific lethality was revealed. Heat shock-driven expression of either *roX* RNA could restore the viability of doubly mutated males, indicating that the male-specific phenotype is due to lack of *roX* RNA only. These experiments show that *roX2* is not essential in males, and the synthetic lethality detected when both *roX*
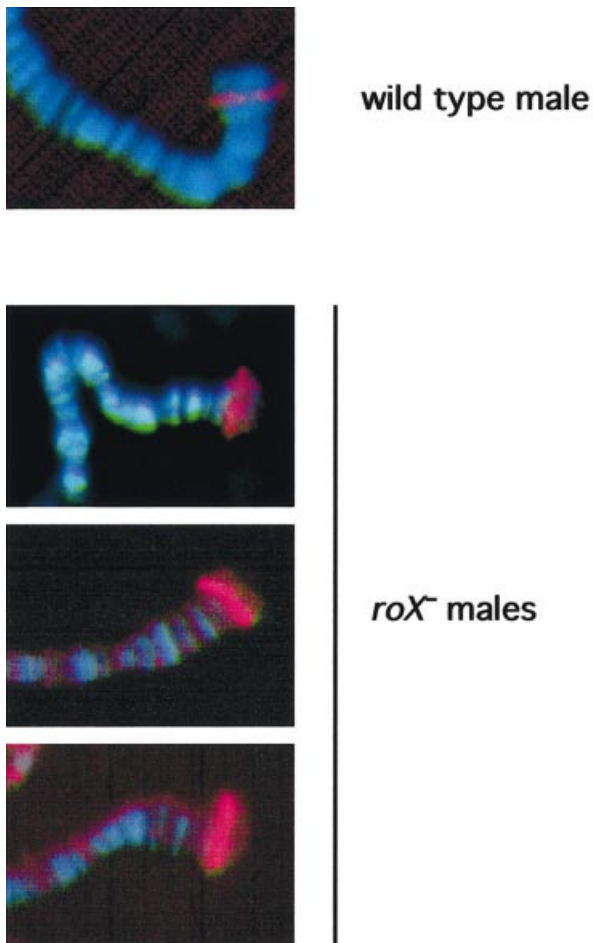
Fig. 5. Autosomal MSL2 binding is enhanced in *roX⁻* males. In wild-type males (top panel) a fine band of MSL2 binding can be detected at 21B. Upon elimination of both *roX* RNAs (*roX1^{ex6}* Df(1)52/Y; [4Δ4.3]) this site may be puffed and heavily stained by anti-MSL2.

genes are mutated eliminates the possibility of undetected *roX* transcripts with similar function. The *roX2* deletion removes the *roX2*-associated chromatin entry site at 10C, and all but 40 bp of the *roX1* site is removed in *roX1^{ex6}* (Kageyama *et al*., 2001). Because male viability is rescued by *roX* RNA *in trans,* male lethality is consequential to loss of the *roX* transcripts and not to mutation of the chromatin entry sites. We can conclude that the RNA products of the *roX* genes are redundant *male-specific lethals*. The *roX2* deletant that we have generated is still complex, as two genes flanking *roX2* are disrupted by Df(1)52 and not restored by cosmid [*w⁺*4Δ4.3]. The rescue of male viability by *roX* cDNAs also demonstrates that male lethality is not an unintended consequence of other mutations associated with the Df(1)52 chromosome or the rescuing [*w⁺*4Δ4.3] transgene.

The involvement of the *roX* RNAs in dosage compensation can be functionally demonstrated by the rescue of females forced to express *msl2*. Production of the MSL2 protein triggers the formation of intact MSL complexes that bind to the X chromosome and enhance the transcription of X-linked genes. Female expression of *msl2* leads to an inappropriate up-regulation of both X chromosomes resulting in a high level of lethality that can be blocked by

mutations in another of the protein-coding *msl* genes (Kelley *et al*., 1995). Removal of both *roX* RNAs similarly rescues *msl2*-expressing females. Partial rescue of female lethality by elimination of only one of the *roX* RNAs is consistent with previous results indicating that females expressing *msl2* from the [*w⁺*H83M2-6I] transgene are a sensitized genetic background in which changes in the level of components of the MSL complex may be detected (Kelley *et al*., 1995). These studies specifically demonstrate a block of dosage compensation when both *roX* RNAs are eliminated.

In contrast to the absence of escapers from mutations in the protein-coding *msl* genes, the doubly mutant X chromosomes used in this study did allow a low number of escaper males. This is a surprising finding in light of the disruption in MSL localization and loss of H4Ac16 enrichment on the X chromosome. It suggests that in spite of the clear importance of the *roX* RNAs for histone acetylation, compensation of the X chromosome is not completely compromised in the *roX⁻* males that we have generated. It is possible that the *roX* RNAs are peripheral to formation of the MSL complex. Residual MSL2 binding on the X chromosome of *roX⁻* males would be consistent with this. Partial complexes with reduced activity could also form in the absence of the *roX* RNAs, and the simultaneous enrichment at the chromocenter of MSL1, MSL2 and H4Ac16 in *roX⁻* males supports the idea that some or all of the MSL proteins may still assemble into a complex capable of acetylating histone H4.

It is possible that the available *roX1* mutations are not complete loss-of-function alleles. This initially seemed unlikely because mutant *roX1* transcripts can not be visualized painting the X chromosome in males carrying *roX1^{mb710}* or *roX1^{ex6}* (Meller *et al*., 1997, 2000). However, both mutations leave the 3′ end of *roX1* intact and it is possible that, in the absence of *roX2*, low levels of a mutated *roX1* transcript could support partial X chromosome compensation. Although survival is equivalent for doubly mutated males with either *roX1* allele, the delay to male emergence is most pronounced for *roX1^{ex6}* (Table I). Our detection of a subtle allelic difference between *roX1^{ex6}* and *roX1^{mb710}* implies that at least one of these mutations is not functionally null.

Function of the roX transcripts

Two lines of evidence support the idea that the MSL proteins, in the absence of the *roX* RNAs, can act to regulate gene expression. First, MSL1, MSL2 and H4Ac16 are similarly redistributed in *roX⁻* males, suggesting that a protein complex able to direct histone acetylation still forms in the absence of *roX* RNA, although its localization is disrupted. Secondly, the sites of autosomal accumulation of the MSL proteins in *roX⁻* males are often puffed, suggestive of locally high rates of transcription. Autosomal sites of MSL3 binding have been reported in wild-type males, and we have detected MSL2 at several sites, including three specifically noted as binding MSL3 (Gorman *et al*., 1995). The one we have focused on, 21B, displays fairly weak MSL2 staining in wild-type males but is often strongly stained and puffed in preparations from *roX⁻* males. We suggest that an MSL complex lacking *roX* transcripts normally functions at a few autosomal sites in wild-type males. It is plausible that these represent a

handful of genes with sex-specific expression. Removal of *roX* RNA releases the bulk of the MSL proteins from the X chromosome, and these are consequently available at high titers for association with the autosomal sites. Puffing of these sites suggests that elevated levels of the MSL proteins can hyper-activate transcription.

Alternatively, it is possible that all targeting of the MSL proteins to chromatin requires an RNA cofactor, but only *roX1* and *roX2* serve to direct the MSL proteins to the X chromosome. This would evoke the presence of other *roX*-like RNAs that specify a more restricted set of targets, perhaps a few autosomal genes. The potential utility of a system of gene activation that can be redirected by deploying an assortment of transcripts is quite attractive; however, in this instance the restriction to a single sex would limit the range of target genes considerably.

Mislocalization of MSL2 is strikingly different in *roX*⁻ males than in those lacking *mle, msl3* or *mof*, where residual MSL2 is observed binding only to ~35 chromatin entry sites. This points to a role for *roX* RNA in correct targeting of the intact complex to the X chromosome. The mechanism driving relocation of the MSL proteins to heterochromatin is at this point speculative. Two of the components of the MSL complex, MSL3 and MOF, contain variant chromodomain motifs (Lucchesi, 1996). Chromodomains are involved in targeting HP1 to heterochromatin, and localization depends on the interaction of this domain with histone H3 methylated on lysine 9, a modification found in heterochromatic regions (Bannister *et al.*, 2001; Nakayama *et al.*, 2001). A chromodomain found in the Polycomb protein is also involved in its localization (Platero *et al.*, 1995). The variant chromodomains of MSL3 and MOF have been shown to bind RNA *in vitro*, and it is likely that the *roX* RNAs are their normal ligands (Akhtar *et al.*, 2000). Removal of *roX* transcripts could allow these domains to engage in inappropriate protein–protein interactions that target the remaining members of the MSL complex to heterochromatin.

The chromatin entry and RNA-encoding functions of the roX genes are separable

The *roX* genes have two distinct functions in dosage compensation; they are the source of the *roX* RNA molecules, and they are chromatin entry sites that direct the MSL complex to chromatin *in cis* (Kelley *et al.*, 1999). Male-lethality upon elimination of both *roX* RNAs rules out the existence of other equivalent RNA-encoding genes. However, there are over 30 other proposed chromatin entry sites. Because of their number it will be impractical to mutate, or possibly even to identify, all of the non-*roX* sites. The *roX1ᵉˣ⁶Df(1)52* chromosomes display no problems in X localization of the MSL complex if *roX* transcripts are supplied *in trans*. This indicates that all necessary *cis*-acting information is present, presumably supplied by remaining entry sites.

The genetic separation of the *roX* transcripts, which can originate from any chromosome, and the chromatin entry site, which does not require transcription and acts *in cis*, poses the question of why these functions are found together in the *roX* genes. It is possible that the proximity of entry sites to *roX* RNA synthesis is important for the correct assembly of the MSL complex. For example, the *roX* RNAs are rapidly degraded in the absence of the MSL proteins (Meller *et al.*, 2000). If these proteins are recruited to the *roX* genes by the chromatin entry sites, they will be situated in the vicinity of the transcripts as they are produced, and this could be a factor in transcript stability. Rapid recruitment of the MSL proteins to the *roX* genes might also enhance nuclear retention of the transcripts. The *roX* cDNAs that were used to rescue *roX*⁻ males do contain chromatin entry sites, and therefore these would have been available to fulfill any necessary organizing role in assembly of the MSL complex at the site of synthesis. It is possible that other entry sites were at one time sources of *roX* RNA, but because *roX* RNA may be supplied *in trans*, there is little selective pressure to retain multiple genes encoding *roX* transcripts. One or a few genes could supply all the needed RNA, and this perhaps represents the situation now observed in *Drosophila melanogaster*. However, marking the X chromosome *in cis* for compensation might not be efficiently assumed by a small number of X-linked loci. *roX1* and *roX2* could represent genes whose abundant RNA production has enabled the degradation and loss of other *roX* transcripts from the X chromosome.

Materials and methods

Fly stocks

Flies were raised at 25°C, 75% humidity on standard cornmeal molasses fly medium. The *roX1ᵐᵇ⁷¹⁰* mutation and the *roX1ᵉˣ⁶* deletion have been described previously (Meller *et al.*, 1997; Kelley *et al.*, 1999), as have transgenes expressing *roX* cDNAs under control of heat shock promoters (Meller *et al.*, 2000).

Mutagenesis of roX2

Eight P-elements cytologically mapped to the 10C region were obtained from the Bloomington *Drosophila* Stock Center and tested for proximity to *roX2* by inverse PCR and hybridization to overlapping cosmids spanning the *roX2* region (Dalby *et al.*, 1995). Two lethal insertions were found near *roX2*: P1547 disrupts *RpII215* and is 9.8 kb distal to *roX2*; and P1877 is an insertion in CT4038, 6.8 kb distal to *roX2* (Figure 1). Excisions of the *roX2* region were generated by crossing dysgenic females of genotype P1877/Df(1)*nod*, FM7a; [*w*⁺4]; Sb *P[ry*⁺Δ2–3]/+ to X/Dp(1:Y)*Bˢ⁻v*⁺*y*⁺ males. Nineteen lethal *w*⁻ chromosomes were obtained; *in situ* hybridization to polytene chromosomes revealed that four of these were deleted for *roX2*. One deletion, Df(1)52, could be rescued by the cosmid [*w*⁺4] (Figure 1). Using Southern blotting and PCR the proximal breakpoint of Df(1)52 was mapped to a series of tandem repeats at the 3′ end of *nod*. Failure of Df(1)52 to complement the lethality of the P1547 chromosome indicates that it is also disrupted for *RpII215*.

Screening a library with a *roX2* probe identified cosmid [*w*⁺4], containing the 5′ end of *nod* and extending distally for 36 kb. Partial *Not*I digestion of [*w*⁺4] removed a 4.3 kb fragment containing *roX2*, producing [*w*⁺4Δ4.3].

Genetics

The survival of males carrying the *roX*⁻ chromosome was based on the number of females emerging from each cross. Testing of the *roX1ᵉˣ⁶* Df(1)52 chromosome produced 1439 adults, and testing of *roX1ᵐᵇ⁷¹⁰*Df(1)52 produced 1680 adults. We discounted the possibility of escapers resulting from recombinational repair of a *roX* gene because: (i) we observed no evidence of recombination between the *roX*⁻ chromosome and a multiply marked balancer; (ii) the developmental delay of escaping males indicates that they are severely challenged, unlike males with a single *roX* gene; and (iii) progeny of escapers mated to attached X females display similar male survival and developmental delay.

The survival of females carrying the [*w*⁺H83M2-6I] transgene was determined by crossing *yw* virgins to *yw*/Y; [*w*⁺H83M2-6I]/+ males (Figure 2A), *yw*Df(1)52; [*w*⁺4Δ4.3] virgins to *yw*Df(1)52/Dp(1:Y)*Bˢ⁻v*⁺*y*⁺; [*w*⁺H83M2-6I]/+ males (Figure 2B); and by crossing *yw*

roX1^{ex6} Df(1)52; [*w^+*4Δ4.3] virgins to *yw roX1^{ex6}* Df(1)52/Dp(1:Y)*Bs^-v^+y^+*; [*w^+*H83M2-6I]/+ males (Figure 2C). The [*w^+*4Δ4.3] insertion produces a pale yellow eye color readily distinguished from the deeper eye color of the [*w^+*H83M2-6I] transgene.

In situ hybridization and immunohistochemistry

Detection of transcripts by *in situ* hybridization has been described, with the exception that the current study used DIG-labeled antisense RNA probes and hybridization temperatures of 50°C (Meller *et al.*, 1997). Probes were transcribed from a full-length *roX2* cDNA (Amrein and Axel, 1997). Immunohistochemical detection of MSL2 and H4Ac16 on polytene chromosomes was done as described previously (Kelley *et al.*, 1999). Antibody to H4Ac16 was purchased from Serotec.

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