Molecularly Severe roX1 Mutations Contribute to Dosage Compensation in Drosophila

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Summary: Drosophila melanogaster males maintain a constant ratio of X-linked to autosomal gene products by increasing expression from their single X chromosome. This is achieved through the action of a complex composed of protein and roX RNA. This complex binds in the body of genes and increases expression through chromatin modification. The X-linked roX genes produce RNAs that are essential but redundant for recognition and modification of the male X chromosome. We report that some molecularly severe roX1 mutations with no detectable transcript accumulation contribute dramatically to male rescue by autosomal roX1 transgenes. We propose that this represents genetic complementation between a source of roX RNA (the autosomal transgene) and the severely mutated X-linked allele. genesis 0:1–6, 2009. © 2008 Wiley-Liss, Inc.

Key words: dosage compensation; roX RNA; noncoding RNA; complementation; MSL complex; DCC

INTRODUCTION

Drosophila males have a well-studied epigenetic system that increases expression of X-linked genes to maintain a normal ratio between autosomal and X-encoded gene products. Modulation of transcription is accomplished by the Male Specific Lethal (MSL) complex, composed of protein and RNA. A central feature of this system is the ability to selectively modify chromatin at hundreds of X-linked genes. The MSL complex is targeted to transcribed regions of the X chromosome by a combination of X-linked identity elements and co-transcriptional binding (Alekseyenko et al., 2008; Dahlsveen et al., 2006; Gilfillan et al., 2006; Larschan et al., 2007).

The large, noncoding roX1 and roX2 RNAs (RNA on the X) are essential components of the MSL complex. roX1 and roX2 are strikingly male-preferential in expression and redundant in function. roX transcripts assemble with the MSL proteins and are required for recognition of the X chromosome. Polytenic preparations from roX1 roX2 males show reduced MSL binding on the X chromosome and ectopic binding at autosomal sites (Meller and Rattner, 2002). A direct relationship is observed between the recovery of male roX1 roX2 adult escapers and the amount of MSL protein remaining on the X chromosome (Deng et al., 2005). roX RNA originating from an autosome enables the MSL complex to coat the X chromosome and rescue roX1 roX2 males (Meller and Rattner, 2002; Meller et al., 1997). roX RNA can therefore function from a site of transcription in trans to the X chromosome. However, both roX genes are X-linked and capable of directing MSL binding and gene regulation in chromatin flanking an autosomal roX insertion (Henry et al., 2001; Kelley and Kuroda, 2003; Kelley et al., 1999). The situation of the roX genes on the X chromosome is thus expected to contribute to X chromosome recognition. Many studies of this aspect MSL localization have relied on roX genes capable of producing functional RNA (Oh et al., 2003; Park et al., 2002). However, there is also evidence that recruitment does not require a functional roX gene (Kageyama et al., 2001).

We observed that the rescue of roX1 roX2 males by autosomal roX transgenes depends on the roX1 roX2 chromosome being rescued. roX2 is completely deleted from all roX1 roX2 chromosomes. Although no complete and viable deletions of roX1 exist, several partially deleted or rearranged roX1 alleles with no detectable transcript production are in common use. To determine whether roX1 alleles on the X chromosome influence male rescue by autosomal roX transgenes, we measured the survival of males carrying different roX1 roX2 chromosomes and roX1 transgenes. We find that the level of rescue is highly dependent on the roX1 allele present on the X chromosome. roX1 alleles with little capacity for RNA production have a dramatic influence on male rescue by autosomal roX1 transgenes and contribute to detectable differences in localization of the MSL.
Proteins. This suggests functional complementation between X-linked roX1 alleles and autosomal roX1 transgenes. The influence of roX1 roX2 chromosomes is most pronounced when rescue by mutated roX1 transgenes is measured. This is of practical importance as the activity of roX transgenes is almost always tested in roX1\textsuperscript{exo} roX2 males, which carry an X chromosome that contributes significantly to male rescue (Meller and Rattner, 2002; Park et al., 2007; Stuckenholz et al., 2003). Transgenes mutated for the 3' end direct considerable MSL protein to the X chromosome but fail to correctly modify chromatin and only partially rescue roX1 roX2 males (Kelley et al., in press; Park et al., 2008).

... the roX1 alleles tested in this study are presented in Figure 1a. No roX1 transcripts are detected in roX1\textsuperscript{exo} or roX1\textsuperscript{exo44} embryos or larvae by RNA blotting or quantitative reverse transcription PCR (qRT PCR), but a transcription start site at $-46$, representing a minor class of ESTs, is retained in roX1\textsuperscript{exo} (Deng et al., 2005). roX1\textsuperscript{SMC17A} lacks 94% of the roX1 gene, including the entire 3' end, but also retains this transcription start site. roX1\textsuperscript{SMC17A} is genetically the most severe roX1 mutation available, possibly due to insertion of LacZ and an SV40 poly(A)\textsuperscript{+} site between remaining 3' and 5' sequences. Although roX1\textsuperscript{exo} retains a transcription start site at $-46$ and some roX1 sequence, adult male escapers are rarely observed (Deng et al., 2005). We previously determined that whole mount in situ hybridization to roX1 was a more sensitive indicator of transcript accumulation than qRT PCR (Deng et al., 2005). Males carrying roX1\textsuperscript{exo} were compared to wild type males and females using this technique. Consistent with previous observations in our laboratory, a low level of roX1 is present in the female central nervous system (Fig. 2c). Although there is no known function of roX1 in females, the staining pattern suggests expression in mitotically active neuroblasts and cells of the developing optic lobe. In contrast, no roX1 is detected in the nervous system, imaginal discs or salivary glands (not shown) of roX1\textsuperscript{exo} males (Fig. 2e,f). This suggests that either the promoter at $-46$ is inactive in larvae, or that roX1\textsuperscript{exo} transcript is unstable or produced at such low levels as to be undetectable by in situ hybridization or qRT PCR (Deng et al., 2005).

The roX1 transgenes tested are presented in Figure 1b. [Hs83-roX1\textsuperscript{+}i], driven by the strong Hs83 promoter, fully rescues MSL localization in roX1 roX2 mutants. An identical transgene lacking bases 283-2669 ([Hs83-roX1\textsuperscript{exo7}]), and a 4.9 kb genomic fragment containing roX1 driven by the authentic promoter ([GM-roX1]) were also tested (Kelley et al., 1999). Hs83-driven roX1 cDNAs deleted for the 3' stem loop, [Hs83-roX1\textsuperscript{exo7} and [Hs83-roX1\textsuperscript{exo7}], and [Hs83-roX1\textsuperscript{exo7}], have been described (Stuckenholz et al., 2003).

As roX1 and roX2 are redundant, transgenes were tested in flies null for roX2 (Meller and Rattner, 2002). Because male survival is sensitive to environmental conditions that can vary between vials, large numbers of offspring from each cross were scored. Rescue of roX1 roX2 males by [Hs83-roX1\textsuperscript{+}] is high, and rescue by [GM-roX1] is intermediate (Table 1). These transgenes retain all essential roX1 sequences, but [Hs83-roX1\textsuperscript{+}] is driven by a stronger promoter. Although all roX1 roX2 X chromosomes are rescued, those carrying roX1\textsuperscript{exo} and roX1\textsuperscript{exo44} support higher male survival. For example, recovery of roX1\textsuperscript{exo44} roX2 [GM-roX1] males is 40% lower than roX1\textsuperscript{exo} roX2 [GM-roX1]. We conclude that even fully functional roX1 transgenes benefit from weak roX1 alleles on the X chromosome. roX1\textsuperscript{SMC17A} retains a
transcription start site, as well as all 3' and some 5' roX1 sequence. A valid concern is that this rearrangement produces antimorphic transcripts that inactivate the MSL complex or compete with roX1 from transgenes, leading to consistently poor rescue of roX1SMC17AroX2. If roX1SMC17A is antimorphic, rescue should be espe-

**FIG. 2.** roX1 transcript is unde-tectable in roX1ex6 male tissues. In situ hybridization using an anti-sense roX1 probe detects roX1 accumulation in the central ner-vous system (CNS: (a) and eye antenna imaginal disc (b) of wild type male. Some staining is observed in the CNS (c) of a wild type female. Mitotic cells in the developing optic lobe (arrowheads) are particularly strongly stained. No stain is detected in the eye antenna imaginal disc of a female (d). No stain is observed in the CNS (e) and eye antenna imaginal disc (f) of a roX1fne6 male. All tissues were processed in parallel and developed for 20 min.

**Table 1**

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<th>Male parent</th>
<th>Female progeny</th>
<th>roX1 roX2 Male progeny</th>
</tr>
</thead>
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<td>100 (1171)</td>
<td>83.0 (486) 3.8 (22)</td>
</tr>
<tr>
<td>wroX1fne6roX2</td>
<td>100 (883)</td>
<td>87.2 (385) 1.6 (7)</td>
<td></td>
</tr>
<tr>
<td>ywroX1fne6roX2</td>
<td>Binsincy/Y;[Hs83-roX1]/+</td>
<td>100 (1252)</td>
<td>72.8 (456) 0 (0)</td>
</tr>
<tr>
<td>wroX1fne6roX2</td>
<td>100 (1191)</td>
<td>66.0 (393) 0 (0)</td>
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Females carrying roX1 roX2 chromosomes were mated to males carrying autosomal roX1 transgenes. The presence of the transgene in offspring is detected by the w marker. The recovery of males (%) is based on the number of female progeny. In tests of roX1SMC17AroX2 survival, only female progeny carrying the roX1 roX2 X chromosome were counted. Rescue of ywroX1fne6roX2 males from mothers heterozygous for the Binsincy balancer was determined by crossing females to homozygous wroX1fne6roX2/Y males or to control (yw) males. Rescue from heterozygous mothers was similar to that from homozygous wroX1fne6roX2/Y males. XO males resulting from maternal non-disjunction are not included. The number of individuals of each genotype is given in parentheses. A chi-squared test was performed to determine whether the survival of roX1fne6roX2 males is significantly different from that of other roX1 roX2 males in the presence of a roX1 transgene. Significance level is indicated by * (P < 0.05), ** (P < 0.01), and *** (P < 0.001).
cially low for the [GM-roX1] transgene, driven by a weak promoter. In contrast, roX1\textsubscript{exoA} lacks all transcription start sites and makes no detectable transcript. [GM-roX1] rescues both chromosomes similarly, but slightly more roX1\textsubscript{SMC17A}roX2;[GM-roX1] males are recovered (Table 1). We conclude that roX1\textsubscript{SMC17A} is a severe hypomorph or null for roX function.

In spite of deletion of the 3' stem-loop, roX1\textsubscript{AOL} partially rescues all roX1 roX2 chromosomes (Table 1). Rescue of roX1\textsubscript{SMC17A}roX2 is lowest, yielding a quarter as many males as roX1\textsubscript{exoA}roX2 (Table 1). The roX1\textsubscript{AOL} transcript rescues salivary gland development and binds to a limited number of nuclear sites when expressed in roX1\textsubscript{SMC17A}roX2 males, but the pattern of localization differs from that observed in males that carry a wild type roX1 gene (see Fig. 3). Rather than binding to a single domain, roX1\textsubscript{AOL} accumulates at multiple sites (Fig. 3c). In polytenes from wild type males, roX1 is exclusive to the X chromosome (Fig. 3d). Although heavy accumulation of roX1\textsubscript{AOL} transcript is observed over the X chromosome of roX1\textsubscript{SMC17A}roX2;[Hs83-roX1\textsubscript{AOL}] males, ectopic binding of the transcript is also evident (Fig. 3e). Consistent with the idea that roX1\textsubscript{AOL} assembles with MSL proteins but is deficient in directing their localization, binding of MSL1 to the X chromosome when expressed in roX1\textsubscript{FSG}roX2 males (Table 1, Fig. 4d). The exclusivity of X chromosome binding is therefore influenced by the roX1 allele remaining on the X chromosome. The roX1\textsubscript{A10} transgene is similar to roX1\textsubscript{AOL} but lacks additional sequence adjacent to the stem loop. roX1\textsubscript{A10} has weaker rescue but displays a similar pattern of complementation. Greater disparity between roX1\textsubscript{FSG}roX2;[Hs83-roX1\textsubscript{A10}] (18% survival), and roX1\textsubscript{SMC17A}roX2;[Hs83-roX1\textsubscript{A10}] (2.4% survival) is observed with this transgene.

roX1\textsubscript{exoB} has an intact 3' stem loop but only 236 bp of 5' roX1 sequence. [Hs83-roX1\textsubscript{exoB}] rescues weakly but maintains a similar pattern of complementation, with roX1\textsubscript{exoB}roX2 males surviving in greater numbers. In no instance does survival exceed 15%, but rescue of the roX1\textsubscript{exoB}roX2 chromosome is almost nine-fold higher than rescue of the roX1\textsubscript{SMC17A}roX2 chromosome.

The apparent activity attributable to roX1\textsubscript{exo}, roX1\textsubscript{SMC17A}, and roX1\textsubscript{exo4A} alleles is surprising given the molecular severity of these mutations. Although they contribute to recognition of the X chromosome, they do so with little ability to produce functional transcripts. Furthermore, these roX1 alleles have little ability to support male survival on their own (Table 1, right column). We hypothesize that the situation of these alleles on the X chromosome is an important factor. For example, transcription from the roX1 locus may contribute to X recognition even if the transcript produced is unstable or nonfunctional, as may be the situation with the roX1\textsubscript{exo} and roX1\textsubscript{SMC17A} alleles. This would support a model of X

![Fig. 3](image-url)

**FIG. 3.** roX1\textsubscript{AOL} transcript partially rescues roX1\textsubscript{SMC17A}roX2 males but displays ectopic localization. In situ hybridization detects roX1 accumulation. (a) Salivary gland of a wild type male. (b) Salivary gland from a roX1\textsubscript{SMC17A}roX2 male. (c) Salivary gland from a roX1\textsubscript{SMC17A}roX2;[Hs83-roX1\textsubscript{A10}] male. All tissues were hybridized and developed under identical conditions and photographed using a ×40 objective. (d) In situ hybridization to a polytene preparation from a wild type male displays exclusive X chromosome localization of roX1. (e) Polytenic preparation from a roX1\textsubscript{SMC17A}roX2;[Hs83-roX1\textsubscript{A10}] male displays roX1\textsubscript{A10} binding to the X chromosome as well as ectopic localization (arrowheads).
chromosome recognition that involves co-transcriptional recruitment of chromatin to a nuclear region, such as a nuclear pore, where loading of the MSL complex can occur (Akhtar and Gasser, 2007; Deng and Meller, 2006). However, a functional RNA is also required for effective MSL complex recognition of the X chromosome. Since the influence of X-linked roX alleles is most apparent when a transgene supplies roX RNA, we propose that this represents a functional complementation between the roX1 allele on the X chromosome and RNA supplied from the roX1 transgene.

The contribution of X-linked roX1 alleles to male survival is most dramatic when deleted transgenes, such as [Hs83-roX1ex7B] are tested. As roX1ex6roX2 is most frequently used to assay the activity of mutated roX transgenes, studies relying on this chromosome should be viewed with caution.

METHODS

Flies were maintained at 25°C on standard cornmeal-agar fly food in a humidified incubator. The roX1ex6, roX1SMC17A, and roX1ex84 mutations have been described (Deng et al., 2005; Meller and Rattner, 2002; Meller et al., 1997). roX1SMC40A is an imprecise excision removing 3.6 kb of the roX1 gene. Elimination of roX2 is accomplished by combining a lethal deletion removing roX2 and essential flanking genes, Df(1)52, with a cosmid insertion carrying essential deleted genes but lacking roX2 ([w14D4.3]; Meller and Rattner, 2002). For convenience this combination is referred to as roX2.

FIG. 4. Rescue of MSL1 localization is affected by the roX1 allele on the X chromosome. Males carrying roX1ex6roX2 (a–d) and roX1SMC17AroX2 (e–f) are rescued by the fully functional [Hs83-roX1+] transgene (a,b) or by the partially functional [Hs83-roX1-OL] transgene (c–f). The X chromosome is indicated by arrowheads in panels showing Hoechst counterstaining of DNA (a,c, and e). Chromosomes are probed with MSL1 antibodies detected by a Texas Red conjugated secondary antibody (Kelley et al., 1999). Full genotypes are: roX1ex6Df(1)52; [w14D4.3];[Hs83-roX1+] (a–b), roX1ex6Df(1)52; [w14D4.3]; [Hs83-roX1SMC17A]CSF67 (c–d) and roX1SMC17A Df(1)52; [w14D4.3]; [Hs83-roX1SMC17A]CSF67 (e–f).
17% lactic acid, 4% formaldehyde, squashed and then flattened overnight beneath a weight. Preparations were frozen in liquid N$_2$, cover slips removed and then plunged into 95% ethanol. Rehydration in 70% and 30% ethanol and a PBST wash was followed by a 15 min fixation in PBST with 4% formaldehyde. Slides were washed 3 times in PBST, digested with 20 µg/ml Proteinase K for 4 min, rinsed twice quickly with 2 mg/ml glycerine, and twice with PBST before a 10 min fixation in 4% formaldehyde in PBST. Slides were rinsed 3 times in PBST and preabsorbed anti-DIG antibody conjugated to alkaline phosphatase (Roche). Five washes in PBST and two washes at room temperature in 1× SSC in PBST. Slides were blocked 2 h at room temperature with 5 mg/ml BSA in PBST and incubated 3 h in a 1:1,000 dilution of preabsorbed anti-DIG antibody conjugated to alkaline phosphatase (Roche). Five washes in PBST and two washes in alkaline phosphatase buffer were done before developing the color. DNA was visualized by Hoechst counterstaining.

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LITERATURE CITED


