Functional Redundancy Within *roX1*, a Noncoding RNA Involved in Dosage Compensation in *Drosophila melanogaster*

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ABSTRACT

Drosophila melanogaster males dosage compensate by twofold upregulation of the expression of genes on their single X chromosome. This process requires at least five proteins and two noncoding RNAs, *roX1* and *roX2*, which paint the male X chromosome. We used a deletion analysis to search for functional RNA domains within *roX1*, assaying RNA stability, targeting of the MSL proteins to the X, and rescue of male viability in a *roX1*$^{-}$ *roX2*$^{-}$ mutant background. We found that deletion of 10% segments of the RNA did not dramatically reduce function in most cases, suggesting extensive internal redundancy. The 3′ 600 nt of *roX1* were most sensitive to mutations, affecting proper localization and 3′ processing of the RNA. Disruption of an inverted repeat predicted to form a stem-loop structure was found partially responsible for the defects observed.

NONCODING RNAs are postulated to regulate gene expression in many different ways (reviewed in Eddy 2001). Some, such as *AIR*, are implicated in the regulation of imprinted expression of nearby genes (Sleutels and Barlow 2002; Sleutels *et al.* 2002). Noncoding RNAs also participate in dosage compensation, where they are important for the regulation of gene expression along the length of the X chromosome in mammals and Drosophila (reviewed in Franke and Baker 2000; Pannuti and Lucchesi 2000; Avner and Heard 2001; Lee 2002; Meller and Kuroda 2002). In mammals, females inactivate one of their two X chromosomes, thus equalizing gene expression to males with a single X. This process requires expression of a 15- to 17-kb noncoding RNA, *Xist*, from the future inactive X and results in silencing of thousands of genes. *Xist* is the central player in correct targeting of X inactivation, because when the *Xist* gene is moved to an autosome, its RNA spreads into flanking autosomal genes causing them to be silenced (Lee and Jaenisch 1997; Wutz and Jaenisch 2000).

Drosophila melanogaster achieve dosage compensation by the opposite mechanism, twofold upregulation of gene expression from the single male X chromosome (reviewed in Franke and Baker 2000; Pannuti and Lucchesi 2000; Meller and Kuroda 2002). Yet, two noncoding RNAs, *roX1* and *roX2* (*R*NA *o*n *X*), are required for this process as well (Amrein and Axel 1997; Meller *et al.* 1997; Franke and Baker 1999; Meller and Rattner 2002). These two RNAs are present in a complex with at least five proteins, collectively referred to as the *male-specific lethals* (MSLs; Meller *et al.* 2000; Smith *et al.* 2000). Targeting the X chromosome by the MSL complex is thought to occur, at least in part, through spreading *in cis* from *roX* genes (Kelley *et al.* 1999; Park *et al.* 2002). One of the MSLs, *males-absent on the first* (MOF), is a MYST histone acetyltransferase that specifically acetylates histone H4 at lysine 16 (H4Ac16; Turner *et al.* 1992; Hilfiker *et al.* 1997; Smith *et al.* 2000; Akhtar and Becker 2001). The histone kinase JIL-1 is also associated with the MSL complex and phosphorylates histone H3 at Ser10 (Jin *et al.* 1999, 2000; Wang *et al.* 2001). By restricting the MSL complex to the male X, JIL-1- and MOF-mediated histone modifications are enriched on X chromatin, presumably to mediate the twofold upregulation of X-linked gene expression.

All of the MSL proteins are required for male viability. Loss-of-function mutations in the *msl* genes cause lethality in males because of a lack of dosage compensation, but have no effect on female viability (Meller and Kuroda 2002). In contrast, available *roX1* mutations have no adverse effect on either sex (Meller *et al.* 1997; Kelley *et al.* 1999). A complete deletion of *roX2* is also male viable (Meller and Rattner 2002). However, removing both *roX1* and *roX2* dramatically reduces male viability with no apparent effect in females (Meller and Rattner 2002). The functional redundancy of the *roX* RNAs is surprising, given that the *roX1* RNA is ~3.7 kb, compared to only ~600 nucleotides (nt) for the major *roX2* transcript (Smith *et al.* 2000; Y. Park, personal communication). The two share little primary se-

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quence, with homology searches detecting only a 30-nt motif in common (Amrein and Axel 1997; Franke and Baker 1999).

In a *roX1*$^{-}$ *roX2*$^{-}$ double mutant, MSL complex localization is undetectable in embryos (Franke and Baker 1999). In mutant larvae, the complex binds weakly to a variable number of sites on the polytene male X chromosome, but can also be found at autosomal sites and the chromocenter (Meller and Rattner 2002). Therefore, one likely function of the *roX* RNAs is to facilitate X chromosome targeting or binding by the MSL complex.

We have used a genetic approach to search for functional domains within *roX1*. We created a series of *roX1* deletions and tested them for their ability to support dosage compensation *in vivo*. The RNAs were also assayed for their ability to target the MSL proteins to the X and modify chromatin. We found that the 3′ end of *roX1* is important for full activity and X localization. Surprisingly, most *roX1* transcripts carrying 10% deletions retained near normal activity. Larger deletions were almost nonfunctional, suggesting that multiple, redundant functional domains may be interspersed along the length of *roX1* and that the structure of the RNA may be quite flexible. As *roX1* lacks obvious repeated sequence motifs, functional domains may operate at the level of tertiary structure, not primary sequence. This is consistent with overlapping genetic function shared by *roX1* and *roX2* in the absence of primary sequence similarity.

MATERIALS AND METHODS

Flies were maintained on standard cornmeal-molasses food at room temperature. Transgenic flies were obtained using standard protocols, mapped, and crossed to homozygosity in a *y w roX1*^{ex6} background (Kelley *et al.* 1999).

We adapted the *roX1*$^{-}$ *roX2*$^{-}$ double-mutant X chromosome by meiotic recombination to include a w^{-} allele to track the presence of w^{+} marked transgenes and a y^{-} allele to track differentially marked X chromosomes. We also recombined an X chromosome insertion of the cosmid transgene [w^{+} 4Δ4.3] (Meller and Rattner 2002) onto the *roX1*$^{-}$ *roX2*$^{-}$ X chromosome. The cosmid [w^{+} 4Δ4.3] is required to rescue the embryonic lethality of flanking genes deleted in the *roX2* deficiency, Df(1)52. Df(1)52 flies carrying cosmid [w^{+} 4Δ4.3] still lack CG11695, a predicted Zn finger gene with no mutant phenotype, as well as *nod*, a kinesin motor required for fidelity of chromosome segregation during female meiosis (Zhang *et al.* 1990). Supplemental Figure 1 (at http://www.genetics.org/supplemental/) shows the crosses used to create the *y w roX1*^{ex6} Df(1) 52 [w^{+} 4Δ4.3] X chromosome. For all complementation tests, ~30 *y w roX1*^{ex6} Df(1) 52 [w^{+} 4Δ4.3] virgins were mated to ~30 *w*; [w^{+} *roX1*]/Balancer males. Bottles were flipped two times and progeny were counted for 2 weeks following eclosion of the first offspring.

roX1 deletions were created by long-range PCR amplification of the *roX1* cDNA c20, including its pBlueScript backbone, with primers pointing away from each other and carrying a unique *Nhe*I restriction site. PCR products were digested and recircularized. Deletions were subcloned into a pCaSpeR derivative carrying the hsp83 promoter and a 450-nt *Pst*I fragment containing polyadenylation signals from the *tra2* locus (Meller *et al.* 2000).

Site-directed mutants in the stem-loop were created by a two-step PCR scheme using overlapping primers in opposite orientation, each carrying the desired nucleotide changes. Independent PCR reactions were run with each mutated primer and an appropriate nonmutated primer that annealed outside the 1.2-kb *Hin*dIII fragment containing the *roX1* 3′ end. The two PCR fragments were purified, allowed to anneal where the mutated primers overlap, and used as template in a second PCR step, using only the outside primers. The resulting PCR product was cut with *Hin*dIII and used to replace the unmutated *Hin*dIII sequence in the c20 cDNA. Candidates were confirmed by sequencing. These mutant cDNAs were subcloned into the H83pCaSpeR vector and injected as above.

Transgenic RNA for Northern analysis and RNase protection was isolated from sexed third instar larvae homozygous for the transgene in a *y w roX1*^{ex6} background using Trizol (Invitrogen, San Diego). In Figure 6, larvae homozygous for the c20 wild-type cDNA were in a *y w roX1*^{MB710} background (Meller *et al.* 1997), but identical results were obtained for the *roX1*^{ex6} background (data not shown). Northern analysis was performed as previously described using 20 μg of total RNA (Kelley 1993; Meller *et al.* 2000).

Probes for RNase protection were generated by PCR (AAT GAACACAGCCAAGCAAG and TTGATTAACCCTTAGCATG TCC for transgenic probe, AAATGAACACAGCCAAAGCAAG and CCGAAAGCACATATTCCCAAC for genomic probe) and subcloned into pCR Topo II (Invitrogen) using the TOPO TA cloning kit (Invitrogen). Plasmid was digested with *Xba*I or *Bam*HI and transcribed using Ambion's (Austin, TX) MaxiScript kit with T7 or Sp6, depending on orientation of the insert. Because of the length of the transgenic probe, this transcription reaction was supplemented with 1 μl of 60 μM unlabeled UTP. Either 50,000 cpm of genomic or 100,000 cpm of transgenic probe and 10 μg total RNA were used for each reaction. RNase protection assays were carried out using Ambion's HybSpeed RPA kit per manufacturer's instructions, using only RNase T1. Reactions were run on a denaturing sequencing gel (8 M urea, 4% polyacrylamide) and exposed to film.

Larvae for squashes were obtained by crossing *roX1*^{ex6} Df(1) 52 [w^{+} 4Δ4.3] virgins (proximal recombination event only; Table 1 and Supplemental Figure 2 at http://www.genetics.org/supplemental/) to homozygous transgenic males with a *y w roX1*^{ex6} X chromosome, reared at 18° in uncrowded vials, and stained as described previously for MSL1 (Kelley *et al.* 1999) and for *roX1* (Meller *et al.* 2000). To exclude squashing nondisjunction males, only larvae with y^{+} mouthhooks were analyzed. The template for the *tra2* probe used to detect hybrid RNAs on the X chromosome (Figure 7) was made by PCR amplification using primers GCGACGATTAACTTGGTGA ATG and CTGCAGCTCATCGACCAATTCAGCAC.

RESULTS

Complementation of *roX1*$^{-}$ *roX2*$^{-}$ double mutants with *roX1* transgenes: Most males carrying a *roX1*$^{-}$ *roX2*$^{-}$ double-mutant X chromosome die, but can be rescued if one of the *roX* RNAs is expressed from a cDNA transgene inserted on an autosome (Meller and Rattner 2002). However, when either the 5′ 900 nt (construct 5′ roX1) or the 3′ 2.4 kb (construct 3′ roX1) is expressed, neither is sufficient for rescue (Meller and Rattner 2002). Therefore, to map functionally important domains within

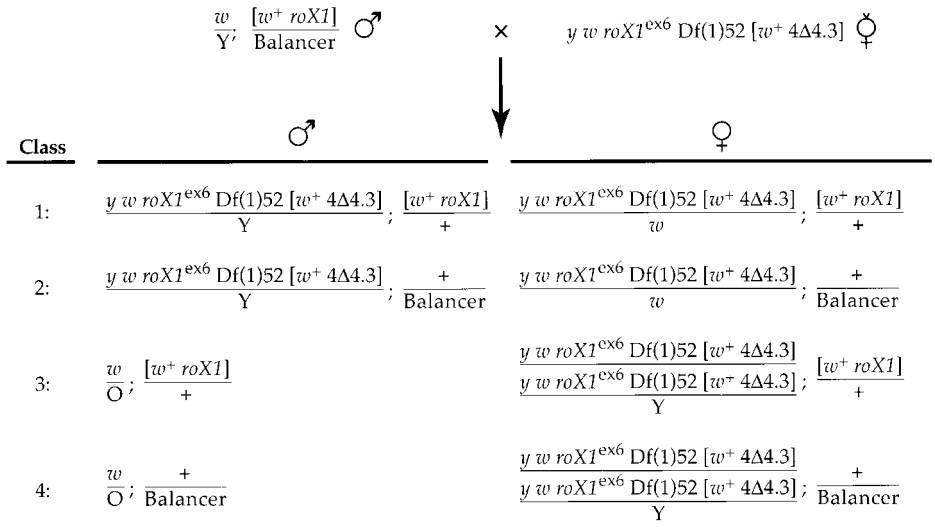


FIGURE 1.—Complementation test. Depending on the insertion site of the [w^+ *roX1*] transgene, fathers carrying either the CyO or the TM3 *Sb e* balancer were crossed to females homozygous for the *roX1*$^-$ *roX2*$^-$ double-mutant X chromosome. Four different classes of progeny are expected with males and females for each. The ratio of males of class 1 to females of class 1 is the rescue frequency. The ratio of males of class 2 to females of class 2 is the escaper frequency. Classes 3 and 4 represent progeny arising from X chromosome nondisjunction during meiosis of the *nod*$^-$ females.

the RNA, we constructed a series of *roX1* transgenes deleting ~10% segments and tested them for rescue of male lethality.

We set up the complementation test outlined in Figure 1 using a modified *roX1*$^-$ *roX2*$^-$ X chromosome (see MATERIALS AND METHODS and Supplemental Figure 1 at http://www.genetics.org/supplemental/). *roX1*$^-$ *roX2*$^-$ females were crossed to males carrying a y^+ X chromosome and a *roX1* transgene balanced by either CyO or TM3, depending on whether the insertion of the transgene was on the second or third chromosome. The balancer allowed us to distinguish between two classes of male offspring: rescued males, *i.e.*, males surviving because of expression of a *roX1* transgene, and escaper males, *i.e.*, males surviving despite a lack of *roX* RNAs. The frequency of either rescued males or escaper males is calculated as the ratio of these males to their respective sisters (class 1 males/class 1 females for the rescue frequency; class 2 males/class 2 females for the escaper frequency; Figure 1). A complication arises because the mothers in the complementation test are also mutant for *nod*, a kinesin motor required for the fidelity of nonrecombinant chromosome segregation during female meiosis (ZHANG *et al.* 1990). Females mutant for *nod* produce a high frequency of gametes carrying either zero or two X chromosomes (ZHANG and HAWLEY 1990). This enables abnormal father-to-son transmission of the paternal X chromosome, giving rise to male offspring with an X chromosome wild type for both *roX1* and *roX2*. By marking the paternal X chromosome with y^+, it is possible to identify such nondisjunction XO offspring, which occurred at a frequency of ~3% (classes 3 and 4 in Figure 1).

Since transgenes insert randomly into chromatin and are subject to position effects, we analyzed several insertions per construct. For each, we selected the transgene with the highest rescue frequency as being representative of its rescue potential and used it for further analysis. Our rationale for following this approach, rather than deriving an average value for each set of insertions, was that poor rescue should reflect a functional defect in the RNA, not low abundance of a fully active RNA. However, comparing the averaged rescue values for each construct yielded results very similar to those obtained by comparing only the highest rescue values (data not shown).

Using the complementation cross outlined above, we found that an unmutated *roX1* cDNA c20, expressed from the constitutively active Hsp83 promoter (MELLER *et al.* 2000), was capable of substantial rescue in the sense orientation, but not in the antisense orientation (66 *vs.* 4%; Figure 2). Likewise, both 5′ roX1 and 3′ roX1 were significantly impaired in their ability to rescue male lethality (3 and 6%, respectively; Figure 2). The frequency of escaper males was <1%. Repeating the complementation test with y^+ males without a transgene, we also found double-mutant males at <1% (Figure 2). These rare escapers died within a few days of eclosion (data not shown).

Since *roX1* and *roX2* share an essential function in dosage compensation, we tested whether the short stretch of primary sequence homology (25/30-nt match located at the 3′ end of each RNA; FRANKE and BAKER 1999) was required for function. We found that *roX1* RNA lacking the 30-nt motif (*roX1Δ30*) was able to rescue at a level similar to that of the wild-type cDNA construct (65 *vs.* 66%; Figure 2). The 30-nt motif was previously found neither necessary nor sufficient for MSL binding to the *roX1* gene (KAGEYAMA *et al.* 2001). Therefore, the function of the only identified sequence common to *roX1* and *roX2* remains elusive.

To scan the entire *roX1* gene, we created a series of 11 overlapping deletions in the *roX1* cDNA c20. Each deletion was between 260 and 400 nt in length (Figure 2). Since the *roX1* c20 cDNA misses ~200–300 nt of sequence from the *roX1* 5′ end (R. L. KELLEY, personal

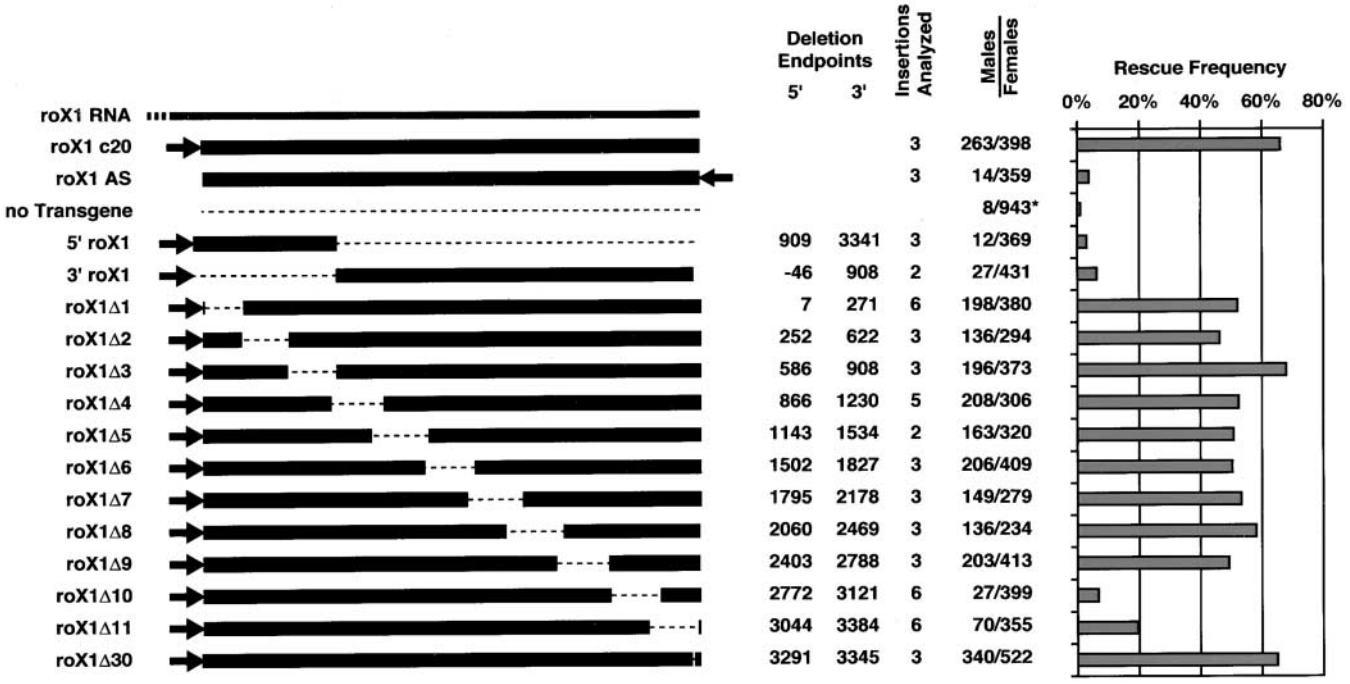


FIGURE 2.—Rescue by *roX1* deletion constructs. Overview of 5′ and 3′ roX1 constructs and deletions *roX1Δ1–roX1Δ30*. The arrow indicates the Hsp83 promoter and the orientation of the transgene. The top line shows the beginning and end of *roX1* RNA. The 5′ and 3′ ends of each deletion are shown; numbering is for c20 *roX1* cDNA (accession no. AB051842). Constructs 5′ roX1 and 3′ roX1 are based on the c3 cDNA and have been described previously (MELLER *et al.* 1997, 2000). Also shown are the number of independent insertions analyzed, the number of rescued males and females counted for the insertion with the highest rescue (class 1 in Figure 1), and a bar graph for the rescue frequency. (*) For the complementation test without a rescuing transgene, classes 1 and 2 (Figure 1) both represent escaper males and their sisters. These are combined and shown both in actual numbers and as ratios in the bar graph.

communication), it in itself represents a deletion. Figure 2 is a summary of our findings with the deletion constructs using the highest rescuing constructs. We placed these (and subsequent) mutants into three broad classes based on rescue of male viability. Most deletions, like *roX1Δ1–roX1Δ9*, rescued about as well as the wild-type c20 cDNA. Almost 80% of *roX1* sequence can be deleted in small intervals without obvious consequence. A second group, here represented by *roX1Δ11*, gave weaker rescue of ~20%, suggesting impaired function. Finally, the transgene *roX1Δ10* rescued only slightly better than the *roX1* antisense negative control and the 3′ roX1 construct.

We noted that the 3′ roX1 construct, which removes the 5′ 900 nt of *roX1*, was impaired in its ability to rescue, but that none of the small deletions in this region (*roX1Δ1–roX1Δ3*) showed a similar reduction in rescue ability. One explanation for these findings is that several redundant elements are contained within the 5′ 900 nt of *roX1*. *roX1Δ1–roX1Δ3* may remove only one of these elements and therefore encode an RNA that is functional. Whether these elements are important for RNA function or stability is unclear.

The deletion analysis pointed to the 3′ end of the RNA as playing an important role in *roX1* function. Both *roX1Δ10* and *roX1Δ11* had poor male rescue activity, with *roX1Δ10* showing the least rescue (7%). An analysis of the region deleted in these transgenes revealed a large inverted repeat in *roX1*, which was predicted to form a stem-loop structure by computer analysis with mfold (GCG; see Figure 3). Since stem-loops are important structural features of RNAs and are frequently the site of RNA-protein interactions, we analyzed the importance of this stem-loop by removing the sequence deleted in both *roX1Δ10* and *roX1Δ11* (Δ*Overlap*) or precisely removing the inverted repeat (Δ*Inverted Repeat*). In addition, we created site-directed mutants within the inverted repeat predicted to either disrupt the potential stem-loop structure (5′ and 3′ stem mutants) or restore the structure with a different primary sequence (double mutant). We also scrambled the sequence inside the seven-nucleotide loop (loop mutant).

All transgenes were assayed in the complementation test described in Figure 1. We found that the inverted repeat and its associated potential stem-loop structure, but not the actual nucleotide sequence of the inverted repeat, were important for full *roX1* function (Figure 3). All transgenes deleting a portion of the inverted repeat (Δ*Overlap*, Δ*Inverted Repeat*) resulted in impaired rescue frequency (27 and 22%, respectively) similar to

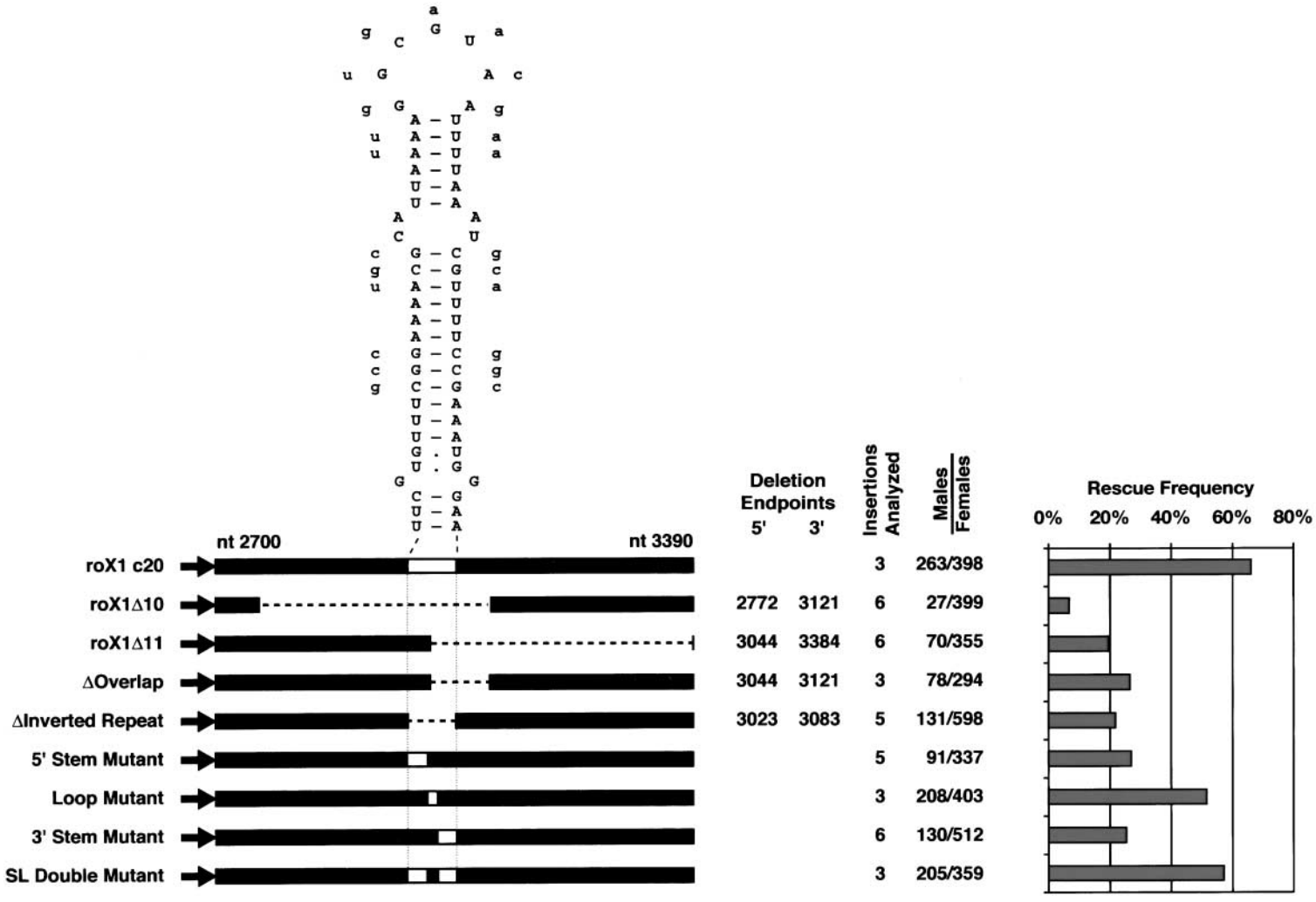


FIGURE 3.—Rescue by *roX1* stem-loop mutants. Overview of the predicted stem-loop region in the 3′ end of *roX1*. Shown are nucleotides 2700–3390 of the *roX1* cDNA c20. The sequence of the stem-loop is indicated in uppercase letters. Mutations in the stem-loop are indicated to the side in lowercase letters (indicated as open boxes in the diagrams of constructs). Also shown are the endpoints of each deletion (see Figure 2), number of independent insertions analyzed, and the actual number of males and females counted to calculate the rescue frequency for the insertion with the highest rescue.

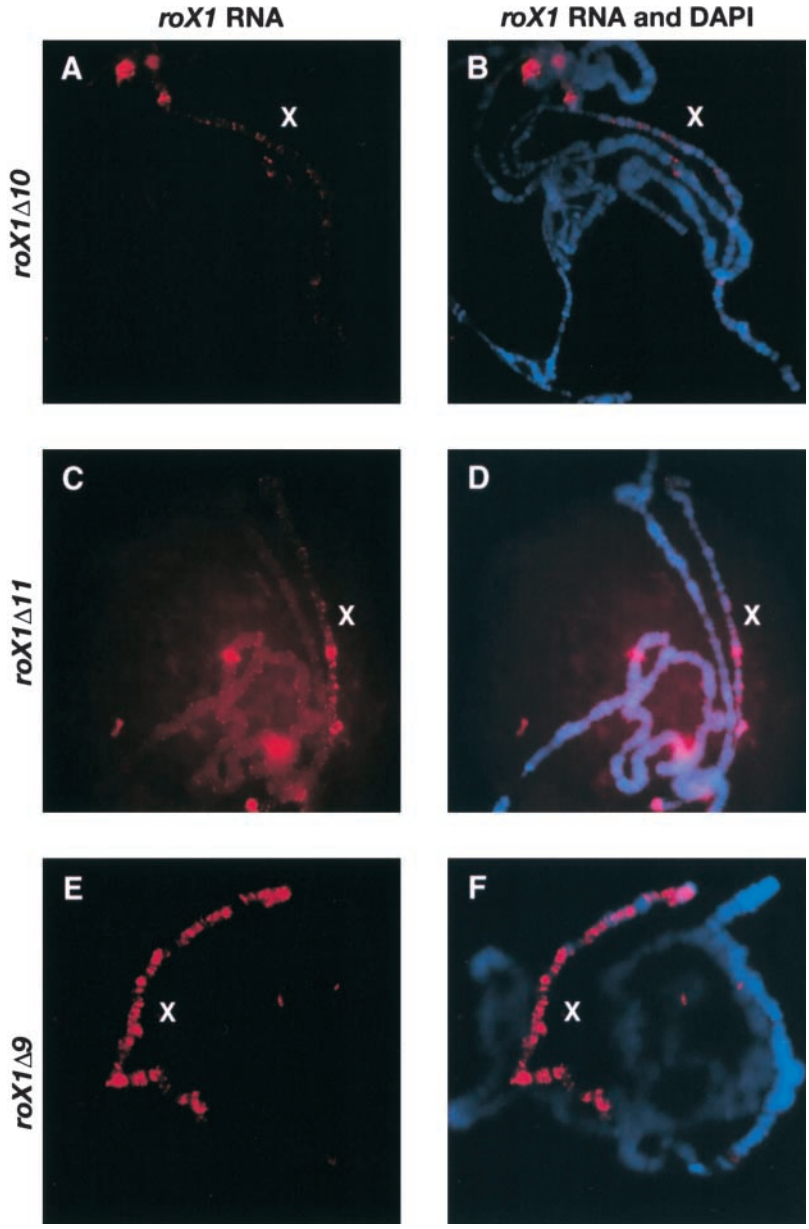


FIGURE 4.—RNA *in situ* hybridization to transgenic polytene chromosomes. Chromosome spreads from male transgenic larvae with a *roX1*$^{-}$ *roX2*$^{-}$ X chromosome. A and B are from a *roX1Δ10* transgenic; C and D, *roX1Δ11*; and E and F, *roX1Δ9*. A, C, and E show the *roX1* signal in Texas red. B, D, and F show the *roX1* signal merged with the DNA counterstain 4′,6-diamidino-2-phenylindole (DAPI; blue). In this and subsequent figures, X marks the X chromosome.

the rescue frequency obtained with *roX1Δ11* (20%), but not as low as for *roX1Δ10* (7%). Transgenes destroying the structure by site-directed mutagenesis also resulted in impaired rescue (27 and 25%), similar to *roX1Δ11*. In contrast, transgenes changing the sequence, but not the structure of the stem-loop (loop mutant and double mutant) rescued at levels similar to that of the wild-type cDNA (52 and 57% *vs.* 66%). We conclude that the inverted repeat and its associated stem-loop structure play an important role in *roX1* function. We propose that *roX1Δ10* removes a second upstream element in addition to the inverted repeat, as none of the stem-loop mutants are as severe as *roX1Δ10*.

Localization of mutant RNAs to the X chromosome: Since *roX1Δ10* and *roX1Δ11* were able to rescue only partially, we asked if the mutant transgenic RNAs could paint the X chromosome. Polytene chromosomes were prepared from male third instar larvae whose only source of *roX* RNA was the transgene. *roX1* RNA localization to the X chromosome was analyzed by *in situ* hybridization with digoxigenin-labeled antisense *roX1* riboprobes (MELLER *et al.* 2000). We found that neither *roX1Δ10* nor *roX1Δ11* was capable of painting the male X chromosome normally (Figure 4, A–D). We observed weak staining of the X chromosome, but also additional staining of the chromocenter and autosomes. This RNA *in situ* pattern is reminiscent of MSL staining in *roX1*$^{-}$ *roX2*$^{-}$ larvae without a rescuing transgene (MELLER and RATTNER 2002; Figure 5, A and B), but more X staining was observed. The significance of the ectopic association of mutant MSL complexes with autosomes and the chromocenter remains unknown. Although *roX1Δ11* rescued better than *roX1Δ10* (Figure 2, 20 *vs.* 7%), the *roX1Δ11* staining pattern did not appear significantly

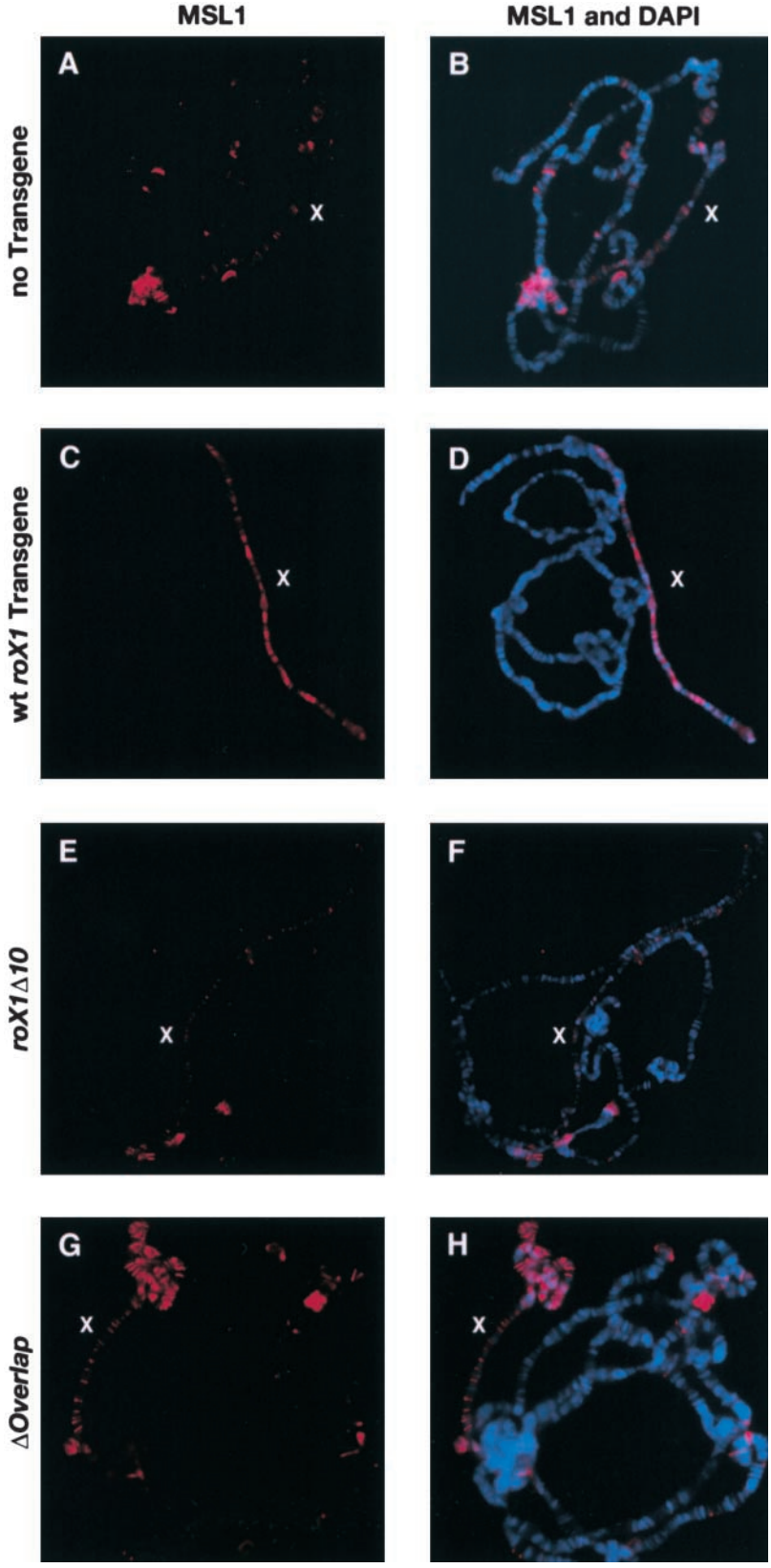


FIGURE 5.—MSL1 immunostain of transgenic polytene chromosomes. Chromosome spreads from male larvae with a *roX1*$^{-}$ *roX2*$^{-}$ X chromosome. A and B are from larvae without a *roX1* transgene; C and D, with a wild-type *roX1* transgene; E and F, *roX1Δ10*; and G and H, Δ*Overlap*. A, C, E, and G show the MSL1 signal in Texas red. B, D, F, and H show the MSL1 signal merged with the DNA counterstain DAPI (blue).

better than that of *roX1Δ10*. In contrast, *roX1Δ9*, a deletion that rescues well, showed strong staining of the mutant RNA on the X chromosome (Figure 4, E and F). All site-directed stem-loop mutant RNAs appeared to paint the X chromosome normally (data not shown). Therefore, the RNA *in situ* assay did not distinguish between transgenic lines that rescued well and ones that exhibited only intermediate rescue. However, RNA

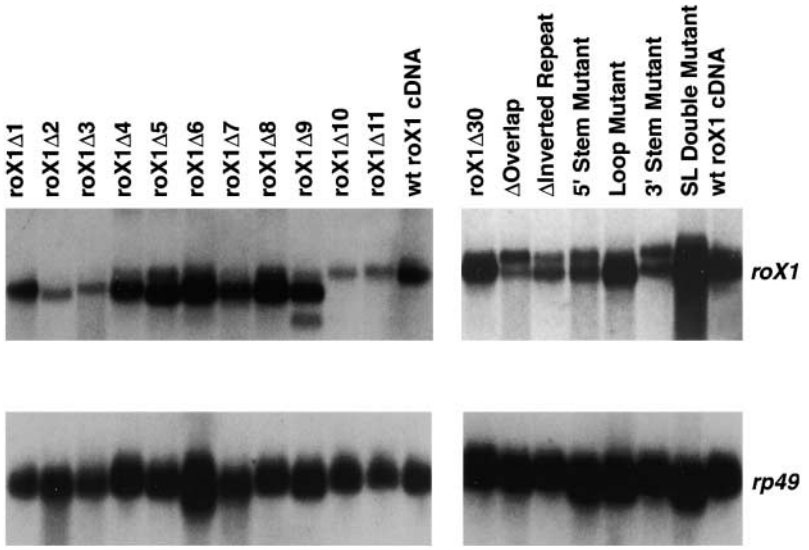


FIGURE 6.—Northern analysis of transgenic RNAs. Total RNA from *y w roX1*^{ex6}; [w^+ *roX1*] transgenic larvae was probed successively with a *roX1* and an *rp49* probe.

localization in the *roX1Δ10* and *roX1Δ11* transgenic lines was clearly weaker than that in all other lines tested.

Transgenic *roX1* RNAs restore MSL localization to the X chromosome: Since the MSL proteins are unable to bind the male X normally without *roX* RNA (MELLER and RATTNER 2002), we tested whether our set of mutant RNAs could direct the MSL complex to the X chromosome. In the *roX1*$^-$ *roX2*$^-$ double mutants, MSL proteins are found at only a few sites on the X chromosome and, aberrantly, at the chromocenter and at a number of autosomal sites (Figure 5, A and B). The wild-type *roX1* cDNA c20 expressed from a transgene is capable of restoring the normal X staining pattern of the MSL complex (Figure 5, C and D). Of the deletions, *roX1Δ1–roX1Δ9* showed normal MSL staining (data not shown), consistent with their ability to rescue *roX1*$^-$ *roX2*$^-$ male lethality at levels similar to that of the wild-type c20 cDNA. *roX1Δ10* and *roX1Δ11*, however, showed autosomal bands in addition to weak X staining, which appeared less dense than normal (for *roX1Δ10* see Figure 5, E and F; similar results were observed for *roX1Δ11*). The autosomal MSL1 staining pattern was reminiscent of that of *roX1*$^-$ *roX2*$^-$ larvae (Figure 5, A and B). We observed weak levels of H4Ac16 on the X chromosome in *roX1Δ10* and *roX1Δ11* (data not shown), suggesting that these mutant RNAs are capable of at least partially restoring functionality to the complex.

Δ*Overlap* showed increased X staining compared to *roX1Δ10* and *roX1Δ11*, but still showed more ectopic staining than wild type showed (Figure 5, G and H). *roX1Δ30*, Δ*Inverted Repeat*, and site-directed stem-loop mutants showed normal X staining (data not shown). Since Δ*Inverted Repeat* and 5′ and 3′ stem mutants rescue at a lower frequency than that of the wild-type cDNA, their apparently normal X staining pattern is puzzling. These RNAs may therefore paint the X chromosome and cause the MSL complex to localize in a wild-type pattern, without being fully functional. This has been observed for the *Xist* RNA, where the ability to silence the X chromosome and to paint the X chromosome was genetically separated (WUTZ *et al.* 2002). On the other hand, since MSL immunostaining is not quantitative, subtle differences in the level of MSL complex on the X chromosome would not be detected. Consistent with this possibility, Δ*Inverted Repeat* and 5′ and 3′ stem mutant RNAs are found at lower steady-state levels by Northern analysis (see below).

Alteration of 3′ processing in *roX1* 3′ deletion mutants: Previous work has shown that *roX1* RNA is unstable unless the MSL proteins are also coexpressed (AMREIN and AXEL 1997; MELLER *et al.* 1997, 2000). To determine whether mutant RNAs were capable of assembling with MSL proteins, we tested all *roX1* transgenics by Northern analysis for stable RNA accumulation and integrity. We found that all transgenes make stable transgenic RNA that can be readily detected (Figure 6), suggesting that these RNAs do assemble with MSL proteins. This was particularly surprising for *roX1Δ10* and to a lesser extent for *roX1Δ11*, since these transgenes rescued poorly and the RNA was impaired in painting the X chromosome. However, it was generally true that transgenic lines rescuing at a lower frequency exhibited less *roX1* RNA. All transgenes that removed or destroyed the stem-loop structure and had impaired rescue also showed lower levels of *roX1* RNA. On the contrary, it was not strictly true that lower RNA levels correlated with poor rescue. Both *roX1Δ2* and *roX1Δ3* had lower levels of *roX1* RNA, yet rescued at least 46%.

The unmutated *roX1* cDNA encodes an RNA of ~3.4 kb. Most of the deletions (*roX1Δ1–roX1Δ9*) showed the expected reduction in size of ~300 nt. However, *roX1Δ10* and *roX1Δ11* transgenic RNAs were abnormally long: the deletions of 349 or 341 nt, respectively (Figure 2), should reduce the size of these RNAs to a size similar to *roX1Δ1–roX1Δ9*. Instead, *roX1Δ10* and *roX1Δ11* encode transcripts of approximately the same length as the wild-

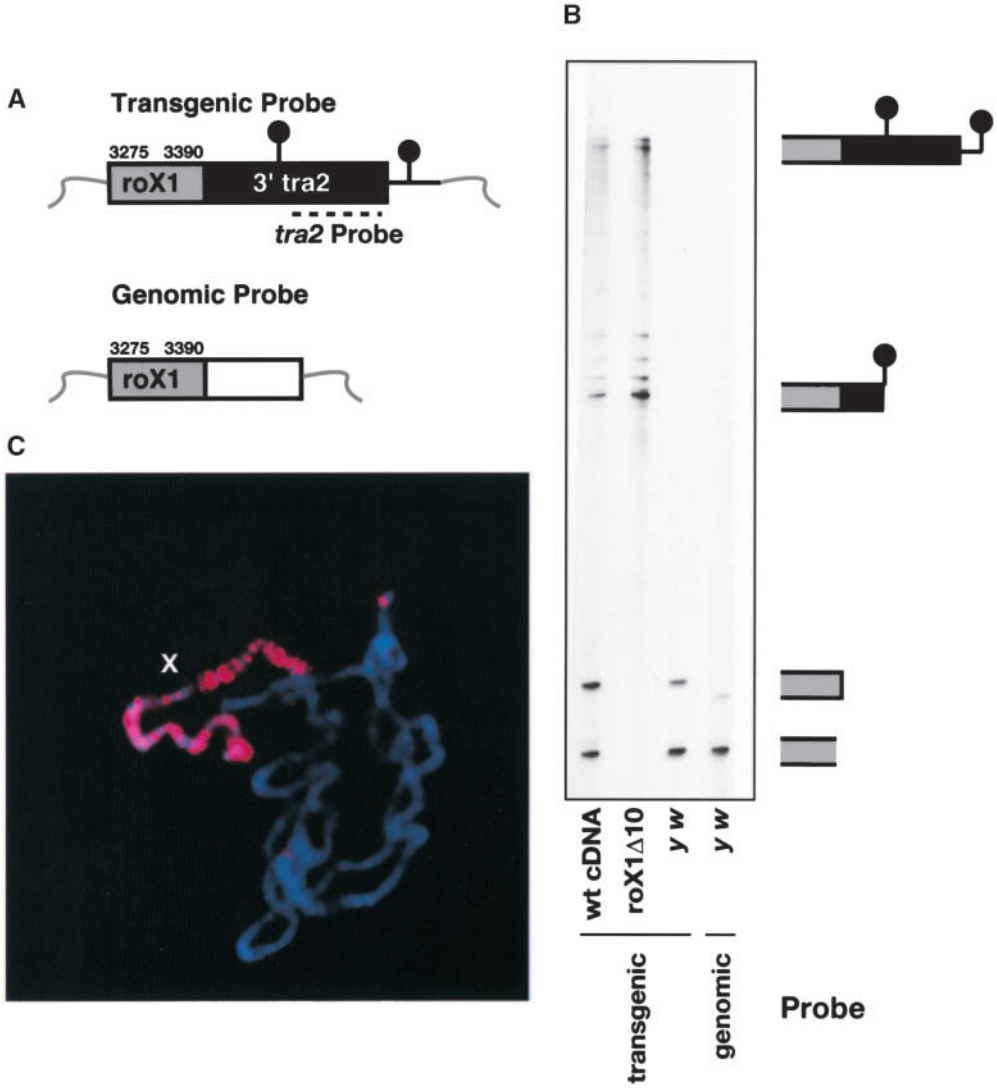


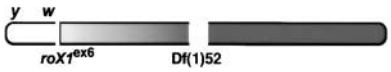
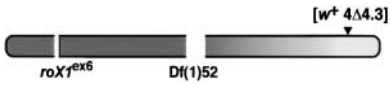
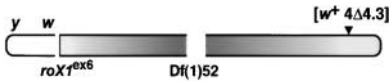
FIGURE 7.—Abnormal processing at the *roX1* 3′ end. (A) Overview of RNase protection probes used. Both probes have the same *roX1* sequence at their 5′ end (indicated by nt position from cDNA c20), but are fused to sequence from the injection vector (transgenic probe) or genomic sequence (genomic probe). Lollipops indicate predicted polyadenylation sites. The dashed line below the transgenic probe diagram indicates the *tra2* probe used for C. (B) RNase protection. Diagrams of the protected products are shown to the right of the autoradiograph. These correspond to the probe maps shown in A. (C) RNA *in situ* hybridization of *y w roX1*^{ex6}; [w^+ wt *roX1*] male larvae using the *tra2* probe indicated in A.

type cDNA transgene. The inverted repeat mutants also showed an upper band (~3.7 kb) in addition to the expected band (~3.4 kb).

Little is known about *roX1* RNA transcription or processing. All transgenes in this study use the Hsp83 promoter (a PolII promoter), but it is not known which RNA polymerase transcribes the endogenous *roX* genes. *roX* RNAs are spliced, but it is not clear if they are also capped and polyadenylated, since none of the isolated *roX1* cDNAs contain long poly(A) tails. Because the mutants at the 3′ end of the RNA showed the most prominent size discrepancies, we focused our analysis on the 3′ processing of the *roX1* transgenic RNAs. Shown in Figure 7A is a schematic of this region in the pCaSpeR vector. Downstream of the *roX1* 3′ end is the 3′ genomic sequence from *tra2* containing a polyadenylation site (MELLER *et al.* 2000). The pCaSpeR vector sequence also contains a potential polyadenylation site. One explanation for the longer mutant transcripts was failure to terminate at the *tra2* polyadenylation site and read through to the pCaSpeR polyadenylation site. Using RNase protection assays, we found that normal, unmutated *roX1* transgenes terminate at the 3′ end of the cDNA, even though they have been removed from their genomic context and the cDNA lacks a consensus polyadenylation site in this region (compare Figure 7B, transgenic wild-type *roX1* c20 RNA and *y w* RNA with both transgenic and genomic probes). *roX1Δ10*, however, completely failed to terminate at this site; its RNA was not processed until reaching the *tra2* polyadenylation sites. We noted that even wild-type cDNA transgenes encode some transcripts that read through the normal *roX1* 3′ processing site. Using an RNA probe complementary to the *tra2* sequence that would detect only readthrough transcripts (Figure 7A), we found that hybrid transcripts with this extra sequence were capable of painting the X chromosome with normal specificity (Figure 7C). We conclude that the presence of the additional *tra2* sequences at the 3′ end of some *roX1* transgenic RNAs does not interfere with their localization, although an effect on function cannot be assayed. The increased presence of heterologous 3′ sequences on

TABLE 1

Variation in *roX1*$^-$ *roX2*$^-$ escaper frequency maps to the proximal X chromosome

X chromosome	Recombination event	[w^+ 4Δ4.3] chromosome	Escaper frequency (%)	Escapers/ sisters
	Ancestral[a]	2	>5	
	Distal	2	9	50/543
		2	7	47/690
		3	6	22/403
	Proximal	X	0	1/440
	Distal and proximal	X	1	6/648
		X	1	8/974
		X	1	4/486

Females homozygous for the indicated X chromosome and, in some cases, also homozygous for an autosomal insertion of [w^+ 4Δ4.3] were crossed to *w*/Y; msl3/TM3 *Sb e* males [except for females with the proximal recombination event only (see Supplemental Figure 2 at http://www.genetics.org/supplemental/), which were crossed to *y w roX1*^{ex6}/Y; *msl3*/TM3 *Sb e* males to allow identification of nondisjunction males]. Escaper frequency was calculated as described for complementation tests using nontransgenic males in Figure 2.

[a] Meller and Rattner (2002).

mutant RNAs could be a contributing factor to the phenotype of the mutant RNAs. However, the localization of unmutated hybrid RNAs to the X chromosome suggests that the reduced localization and function of mutant RNAs is most likely a consequence of the altered or deleted *roX1* sequences.

***roX1*$^-$ *roX2*$^-$ escaper frequency variability:** Meller and Rattner (2002) reported 3.5% escaper males that appear healthy in the absence of *roX* RNAs. When these males are crossed to *roX* mutant females, the escaper frequency appears constant, suggesting that the recovery of mutants is stochastic rather than due to a genetic alteration. In our complementation tests, we consistently recovered smaller numbers of escapers than reported previously (<1%). When constructing the *roX1*$^-$ *roX2*$^-$ X chromosome for this study, we recombined *y*$^-$ and *w*$^-$ alleles onto the distal end of the original X isolated by Meller and Rattner (2002). In addition, we recombined a cosmid transgene [w^+ 4Δ4.3], inserted at position 18F-19A in the proximal X, to rescue the embryonic lethality of flanking genes deleted in the *roX2* deficiency, Df(1)52. By examining various intermediate recombinant chromosomes, we noted that the lower escaper frequency could be mapped to the replacement of the proximal X chromosome (Table 1). The insertion site of [w^+ 4Δ4.3] in the proximal X might be detrimental to subviable *roX1*$^-$ *roX2*$^-$ mutant males, although it is tolerated in homozygous females. Alternatively, other loci on the X chromosome proximal to the *roX2* locus might influence the ability of males to live without *roX* RNAs.

DISCUSSION

The ~3.7-kb *roX1* RNA is predicted to make up over half the mass of the MSL complex. Yet, surprisingly, our principal finding is that most of the *roX1* is dispensable for function, as constructs lacking almost any 10% of the sequence still rescued *roX1*$^-$ *roX2*$^-$ double-mutant male lethality similar to a wild-type *roX1* cDNA. Comparison of a 900-bp deletion construct (3′ roX1), with the overlapping 300- to 400-nt deletions *roX1Δ1–roX1Δ3* showed that this region contains at least two redundant elements. It is possible that several additional redundant elements are located within the region of *roX1* defined by deletions *roX1Δ4–roX1Δ9*. Our finding that most small domains are dispensable is consistent with the absence of primary sequence homology between the functionally interchangeable *roX1* and *roX2* RNAs. Whatever *roX* RNAs do, their functions likely depend on multiple, complex tertiary folds, not primary nucleotide sequence. The one short 30-nt sequence element shared between *roX1* and *roX2* could be deleted without obvious consequence. This finding is similar to results obtained with *Xist*, where conserved sequence elements were not necessarily important for RNA function (Wutz *et al.* 2002). The deletion in *roX1Δ4* is identical to a deletion called *roX1ΔDHS* that removes a male-specific DNaseI hypersensitive (DHS) site in the *roX1* gene (Kageyama *et al.* 2001). The DHS site is the principal DNA-binding site for the MSL complex within the *roX1* gene, but here we find that the sequence is dispensable in the *roX1* RNA.

In addition to functional redundancy in the 5′ end of *roX1*, we found that the 3′ end is most important for full function of the RNA. Deleting sequences within the last 600 nt of the RNA resulted in markedly lowered rescue efficiency and abnormal RNA and MSL complex localization. A predicted stem-loop structure in this region is partially responsible for the loss of rescue activity, but we suspect a second, partially redundant element is removed by the *roX1Δ10* deletion.

roX1 RNA may not undergo typical polyadenylation, but our studies do not address this in detail. Whether *roX1* RNA is polyadenylated or is enriched on oligo(dT) columns through internal A-rich stretches is not known. The absence of consensus polyadenylation sites raises the possibility that an unusual mechanism might be employed to terminate the RNA. This mechanism might be important for keeping the RNA in the nucleus and thus for localization of both the MSL proteins and the RNA to the X chromosome (Zhao *et al.* 1999).

No physical information about the structure of the MSL RNA-protein complex is available. We do not know whether both *roX1* and *roX2* are present in one MSL complex or if two different complexes exist, one with *roX1* and one with *roX2*. It is not known whether *roX1* RNA is flexible or adopts a rigid structure in the MSL complex, possibly stabilized by many weak RNA-protein interactions. However, our results are compatible with a model in which the RNA is more flexible and perhaps surrounds an MSL protein core. In one view of dosage compensation, the histone-modifying enzymes account for the altered chromatin found on the male X and the resulting twofold increase in transcription. The main function of the *roX* RNAs might therefore be to restrict MSL action to the X chromosome *in vivo*. This is consistent with our observations that *roX1Δ10* and *roX1Δ11* fail to rescue well and mislocalize the mutant MSL complexes to many autosomal sites. It might also account for the presence of escaper *roX1*$^{-}$ *roX2*$^{-}$ males: since MSL complexes still bind some sites on the X chromosome in the absence of any *roX* RNAs, enzymatically active MSL proteins might weakly act on the entire genome. Weak H4Ac16 histone acetylation has been observed in *roX1*$^{-}$ *roX2*$^{-}$ males (Meller and Rattner 2002); therefore, rare males might have sufficient levels of histone modifications on the X chromosome without fatal levels of increased autosomal activity.

Our finding of extensive redundancy within the *roX1* RNA is reminiscent of a recent study examining *Xist* using a similar deletion strategy. This study also found multiple redundancies within the *Xist* RNA (Wutz *et al.* 2002). Additional examples from other areas of RNA biology include redundant RNA localization signals in the 3′ untranslated region of *bicoid* in Drosophila and of Vg1 in Xenopus and interactions between HIV Rev and its cognate RNA element, the RRE (Karn *et al.* 1994; Gautreau *et al.* 1997; Macdonald and Kerr 1997). The interaction between Rev and the RRE may prove relevant: the Rev protein binds at a bulge in the RRE, a large RNA structure. Once the first Rev molecule is bound to the RRE, however, additional Rev molecules can load onto the same RNA (Daly *et al.* 1993; Karn *et al.* 1994). It is possible that the region within *roX1Δ10* is required for the efficient binding of a first MSL protein to the RNA. Once this first protein is bound, more MSL proteins can bind to the RNA and make it fully functional. In this model, most of the RNA would consist of secondary MSL-binding sites of lower affinity, which could result in inefficient loading of the MSL complex to the *roX1Δ10* mutant RNA.

roX1 and *roX2* are redundant with each other (Franke and Baker 1999; Meller and Rattner 2002) and we have demonstrated functional redundancy within *roX1*. If both RNAs exert their function through a common tertiary structure, this structure must be able to form despite completely different primary sequences. There are examples of proteins that fold into highly similar structures despite their lack of sequence identity (Perutz *et al.* 1965; Krishna *et al.* 1994; Mitton-Fry *et al.* 2002). The versatility in intramolecular interactions within RNAs may be instrumental in allowing them to assume many varied tertiary structures (Doudna 2000), but most RNA structures are still completely unknown. Presumably RNAs as different as *roX1* and *roX2* fold into a common tertiary structure necessary for dosage compensation in Drosophila.

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Note added in proof: A second conserved sequence element present in both *roX1* and *roX2* was recently reported (Y. Park *et al.*, 2003, Sequence-specific targeting of *Drosophila roX* genes by the MSL dosage compensation complex. Mol. Cell **11:** 977–986).

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