ONCODING 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 (RNA on 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 malespecific 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-
sequence, 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 chromatocenter (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 roX1flp 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 cosmids transgene [w− 44A.3] (Meller and Rattner 2002) onto the roX1− roX2− X chromosome. The cosmids [w− 44A.3] is required to rescue the embryonic lethality of flanking genes deleted in the roX2 deficiency, Df(1)52. Df(1)52 flies carrying cosmids [w− 44A.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. 1996). Supplemental Figure 1 (at http://www.genetics.org/ supplemental/) shows the crosses used to create the y w roX1flp Df(1)52 [w− 44A.3] X chromosome. For all complementation tests, ~30 y w roX1flp Df(1)52 [w− 44A.3] virgins were mated to ~30 w− /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 back bone, with primers pointing away from each other and carrying a unique Ndel restriction site. PCR products were digested and recircularized. Deletions were subcloned into the H83pCaSpeR derivative carrying the hsp83 promoter and a 450-nt Pdfl 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 HindIII 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 HindIII and used to replace the unmaturated HindIII 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 roX1flp background using Trizol (Invitrogen, San Diego). In Figure 6, larvae homozygous for the c20 wild-type cDNA were in a y w roX1flp background (Meller et al. 1997), but identical results were obtained for the roX1flp 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 GACGAGCGGAACGAAG and TTAGTATACCTTAGCATG TCC for transgenic probe, AATGACGACGCAAAAGGAAC and CCGAAAGCATAATCGCCAC for genomic probe) and subcloned into pCR Topo II (Invitrogen) using the TOPO TA cloning kit (Invitrogen). Plasmid was digested with Xbal or BamHI and transcribed using Ambion’s (Austin, TX) Maxi-Script 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 roX1flp Df(1)52 [w− 44A.3] virgins (proximal recombination event only; Table 1 and Supplemental Figure 2 at http://www.genetics.org/ supplemental/) to homozygous transgenic males with aby meiotic recombination to include a double-mutant X chromosome, reared at 18° and stained as described previously for MSL1 (Kelley et al. 1997), but identical results were obtained for roX2 (Meller et al. 2001). To exclude squashing nondisjunction males, only larvae with y− mouth hooks 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 CGGACGATTAACGTGGTGA ATG and CTGCGATCTATCGGACAAATTCACGAC.

**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
the RNA, we constructed a series of roX 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 roX- roX- X chromosome (see MATERIALS AND METHODS and Supplemental Figure 1 at http://www.genetics.org/supplemental/). roX- roX- females were crossed to males carrying a y+ X chromosome and a roX 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 roX 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 roX 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) is sufficient for MSL binding to the roX 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′/H11032 and 3′/H11032 roX1 constructs and deletions roX1/H9004. 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 roX1ΔL–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 (roX1ΔL–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. roX1ΔL–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 (ΔOverlap) or precisely removing the inverted repeat (ΔInverted 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 (ΔOverlap, ΔInverted 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.
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 roX1Δ11 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, ΔOverlap. 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
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 roX1 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.

ΔOverlap 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, ΔInverted Repeat, and site-directed stem-loop mutants showed normal X staining (data not shown). Since ΔInverted 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, ΔInverted 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 roX 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 transgens for 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-
type cDNA transgene. The inverted repeat mutants also showed an upper band (\( \sim 3.7 \) kb) in addition to the expected band (\( \sim 3.4 \) kb).

Little is known about \( \text{roX1} \) RNA transcription or processing. All transgenes in this study use the Hsp83 promoter (a PolIII promoter), but it is not known which RNA polymerase transcribes the endogenous \( \text{roX} \) genes. \( \text{roX} \) RNAs are spliced, but it is not clear if they are also capped and polyadenylated, since none of the isolated \( \text{roX1} \) 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 \( \text{roX1} \) transgenic RNAs. Shown in Figure 7A is a schematic of this region in the pCaSpeR vector. Downstream of the \( \text{roX1} \) 3’ end is the 3’ genomic sequence from \( \text{tra2} \) 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 \( \text{tra2} \) polyadenylation site and read through to the pCaSpeR polyadenylation site. Using RNase protection assays, we found that normal, unmuted \( \text{roX1} \) 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 \( \text{roX1} \) c20 RNA and \( y \) \( \text{w} \) RNA with both transgenic and genomic probes). \( \text{roX1} \Delta 10 \), however, completely failed to terminate at this site; its RNA was not processed until reaching the \( \text{tra2} \) polyadenylation sites. We noted that even wild-type cDNA transgenes encode some transcripts that read through the normal \( \text{roX1} \) 3’ processing site. Using an RNA probe complementary to the \( \text{tra2} \) 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 \( \text{tra2} \) sequences at the 3’ end of some \( \text{roX1} \) 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

<table>
<thead>
<tr>
<th>X chromosome</th>
<th>Recombination event</th>
<th>[(w^+ \Delta 4.3)] chromosome</th>
<th>Escaper frequency (%)</th>
<th>Escapers/sisters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancestral*</td>
<td>2</td>
<td>(\geq 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distal</td>
<td>2</td>
<td>9</td>
<td>50/543</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7</td>
<td>47/690</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6</td>
<td>22/403</td>
<td></td>
</tr>
<tr>
<td>Proximal</td>
<td>X</td>
<td>0</td>
<td>1/440</td>
<td></td>
</tr>
<tr>
<td>Distal and proximal</td>
<td>X</td>
<td>1</td>
<td>6/648</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>1</td>
<td>8/974</td>
<td></td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>1</td>
<td>4/486</td>
<td></td>
</tr>
</tbody>
</table>

Females homozygous for the indicated X chromosome and, in some cases, also homozygous for an autosomal insertion of \([w^- \Delta 4.3]\) were crossed to \(w^+Y\); ms3/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 \(y w^+ \Delta 9/\); 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.

* Meller and Rattner (2002).

**DISCUSSION**

The \(\sim 3.7\)-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 \(roX1\Delta 1^- roX1\Delta 9\) 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 \(roX1\Delta 4^- roX1\Delta 9\). 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 (Wurtz et al. 2002). The deletion in \(roX1\Delta 4\) is identical to a deletion called \(roX1\Delta DHS\) 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 roXI, 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.

roXI RNA may not undergo typical polyadenylation, but our studies do not address this in detail. Whether roXI 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 roXI and roX2 are present in one MSL complex or if two different complexes exist, one with roXI and one with roX2. It is not known whether roXI RNA is flexible or adopts a rigid structure in the MSL complex or if two different complexes exist, one with roX1 and one with roX2. The versatility in intramolecular interactions within MSL RNA-protein complex is available. We do not know structures despite their lack of sequence identity (Zhao et al. 1999). 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).

roXI and roX2 are redundant with each other (Franke and Baker 1999; Meller and Rattner 2002) and we have demonstrated functional redundancy within roXI. 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 (Perrutz 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 roXI and roX2 fold into a common tertiary structure necessary for dosage compensation in Drosophila.

We thank R. L. Kelley for numerous helpful suggestions in experimental design and X. Bai, R. L. Kelley, and Y. Park for critical reading of the manuscript. We are grateful to P. R. Gordadze, H. G. Kennedy, X. Chu, and C. Olson for excellent technical assistance. This work was supported by grants from the National Institutes of Health and the Welch Foundation. M.I.K. is an Investigator of the Howard Hughes Medical Institute.

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

LITERATURE CITED


Gautreau, D., C. A. Cote and K. L. Mowry, 1997 Two copies of a subelement from the Vg1 RNA localization sequence are sufficient to direct vegetal localization in Xenopus oocytes. Development 124: 5013–5020.


Communicating editor: B. J. Meyer