MLE functions as a transcriptional regulator of the \textit{roX2} gene

Chee-Gun Lee*, Trevor W. Reichman, Tina Baik, and Michael B. Mathews

Department of Biochemistry and Molecular Biology

Graduate School of Biomedical Sciences

University of Medicine and Dentistry of New Jersey

185 South Orange Avenue, Newark, NJ 07103

*To whom correspondence should be addressed:
Tel: 973-972-0130; Fax: 973-972-5594; E-mail: leecg@umdnj.edu

Running title: MLE interaction with the \textit{roX2} gene

Key words: RNA helicase, DNA helicase, MLE, MSL1, MSL2, \textit{roX2}
SUMMARY

Dosage compensation is a process that equalizes transcription activity between the sexes. In Drosophila, two noncoding RNA, roX1 and roX2 RNAs and at least 6 protein regulators, MSL-1, MSL-2, MSL-3, MLE, MOF, and JIL-1, have been identified as essential for dosage compensation. Although there is accumulating evidence of the intricate functional and physical interactions between protein and RNA regulators, little is known about how roX RNA expression and function are modulated in coordination with protein regulators. In this report, we find that a relatively short upstream genomic region of the roX2 gene (about 350 bp), Prox2, harbors an activity that drives transcription of the downstream gene. Our study shows that MLE can stimulate the transcription activity of Prox2 and that MLE associates with Prox2 through direct interaction with a newly identified 54 bp repeat, Prox. Our observations suggest a novel mechanism by which roX2 RNA is regulated at the transcriptional level.
INTRODUCTION

In higher eukaryotes, females contain an extra X chromosome. The transcription activity of the X chromosome must be equalized between the sexes during development, and any failure in this process leads to embryonic lethality (1,2). Diverse mechanisms have evolved to reconcile the differences in X chromosome dosage. Eutherian mammals suppress the transcription activity of one of their two X chromosomes in females (3,4). Hermaphrodite nematodes (XX) reduce the transcription activity of their X chromosomes by 50% (5,6). On the other hand, Drosophila males (XO) double the transcription activity of their single X chromosome (7-9).

In humans and Drosophila, dosage compensation is achieved by opposite processes, i.e., by transcriptional repression and activation, respectively. Despite this difference, it is intriguing to find that both processes involve non-coding RNAs that associate with the X chromosome. In humans, \textit{XIST} (X inactivation specific transcript), synthesized from the inactive X chromosome, physically coats the entire inactive X chromosome, conferring an inactive status (10-12). In contrast, in Drosophila, \textit{roX1} and \textit{roX2}, two RNAs originating from the male X chromosome, associate with hundreds of sites on the X chromosome, making it transcriptionally hyperactive (13,14).

To date, genetic analysis has identified 8 transacting factors that are necessary for the onset or maintenance of dosage compensation in Drosophila. Based on observations that loss of their functional allele leads to a male-specific lethal phenotype, these factors
are named the MSL (male-specific lethal) proteins. They include MSL1 (15), MSL2 (16,17), MSL3 (18), MLE (maleless) (19), MOF (male-absent on the first) (20), and a more recently identified factor JIL-1 (21). It is generally believed that dosage compensation occurs in several discernible steps, starting with the targeted association of roX RNAs and MSL complex with the X chromosome. Upon the expression of MSL2 protein, MSL proteins assemble a complex on approximately 35 sites of the X chromosome, called “chromatin entry sites” (22-24). Once the chromatin entry sites are fully occupied with the MSL complex in the presence of MLE, the flanking chromatin region becomes competent in binding the MSL complex. This “spreading” or “nucleation” process appears to require the histone acetyltransferase (HAT) activity of MOF (25).

We previously reported that mle<sup>GET</sup>, a mutant MLE defective in ATPase activity, led to a poor association of the MSL complex with the male X chromosome and lethality of male embryos (26). Recently, it has been shown that the expression of mle<sup>GET</sup> in place of the wild type MLE resulted in the formation of the MSL complex devoid of roX2 RNA (25). Furthermore, Mof<sup>Δ</sup>, which lacks HAT activity, was unable to support the spreading of MSL complexes on the X chromosome and also drastically reduced the association of MLE with the X chromosome (25). However, lack of HAT activity did not influence the association of MSL complex with roX RNAs in nucleoplasm and with the chromatin entry sites. Although the underlying mechanism remains unknown, it is likely that MLE plays a critical role in determining the intracellular level of roX2 RNA or its interaction.
with MSL complexes or the chromatin entry sites. In this report, we provide evidence indicating that MLE regulates \( \text{roX2} \) RNA at the transcriptional level.
EXPERIMENTAL PROCEDURES

Genomic cloning of Prox2 and construction of a reporter plasmid, Prox2-luciferase. About 1x10⁴ of Schneider 2 (S2) cells, grown in a serum-free medium (SFM, Invitrogen), were re-suspended in 200 µl of a solution consisting of Tween-20 (0.5%) and proteinase K (60 µg/ml), and incubated at 56°C for 4 hr, then at 95°C for 30 min. Using an aliquot of lysate (5 µl) as template, genomic PCR was performed in a mixture (50 µl) containing 2.5 mM MgCl₂, 1x PCR buffer II (Perkin Elmer), 0.25 mM dNTP, AmpliTaq polymerase (Perkin Elmer, 5 units), and a pair of primers, Prox2-5 and Prox2-3 (for sequences, see Fig. 1B). Reaction condition was as follows: 4 min at 95°C, 35 cycles of 15 sec at 95°C, 30 sec at 57°C, 1 min at 67°C, and 7 min at 72°C. PCR product, purified by spin column (Quigen), was digested by HindIII and NheI, and subcloned into pGL3-basic (Promega). Three independent positive clones, all containing 0.5 kb insert (Fig. 1C), were confirmed by DNA sequencing and named Prox2-luciferase.

Transient transfection assay. A construct pMt/Hy-MSL2-HA, expressing HA-tagged MSL2 upon copper sulfate treatment, was kindly provided by Dr. J. Lucchesi. pMT-MLE and pMT-MSL1, expressing flag-tagged proteins, were constructed as follows. Full-length cDNAs, coding for MLE and MSL1, were PCR-amplified using Pfu polymerase (Stratagene), and inserted into pMT/V5 vector (Invitrogen). Detailed procedure is available upon request. S2 cells, grown in 6-well culture plates at about 20% confluence, were transfected with 1 µg of Prox2-luciferase, 20 ng of pMT/V5-βGAL (Invitrogen), and varying amounts (0.4-1.2 µg) of the indicated pMT construct (Fig. 5A)
using the standard calcium phosphate method. After 24 hr incubation, cells were supplied with fresh media containing 0.5 mM copper sulfate. On the following day, cells were harvested in ice-cold phosphate-buffered saline (1x PBS), and resuspended in 200 µl of a lysis buffer consisting of 50 mM potassium phosphate buffer, pH 7.4, 1% Triton X-100, 5 mM β-glycerophosphophate, and 2 mM DTT. Lysates were cleared by centrifugation for 15 min at 15,000 rpm at 4°C. Luciferase and β-galactosidase activities were measured as described previously (27).

**Isolation of flag-tagged MSL1.** For experiments with flag-tagged MSL1 protein (Fig. 3), S2 cells, seeded on 10 culture plates (90 mm) at a density of 1x10^6 cells per plate, were transfected with 4 µg of pMT-MSL1. The total amount of DNA was kept constant (10 µg) using empty pMT vector. On the following day, cells were supplied with 0.5 mM copper sulfate. After 48 hr incubation, cells were harvested in ice-cold phosphate-buffered saline (1x PBS), and resuspended in two packed-cell volumes of lysis buffer consisting of 10 mM potassium phosphate buffer, pH 7.4, 0.1% NP-40, 1% deoxycholate, 10% glycerol, 0.5 mM PMSF, and 2 mM DTT. Cell lysate was sonicated for 5 sec, centrifuged at 15,000 rpm for 20 min at 4°C, and then mixed with 1/10th volume of 10x PBS and 100 µl of M2 affinity gel (Sigma). After 4 hr incubation on nutator at 4°C, followed by washing 4 times with 1x PBS containing 0.05% Tween-20 (1x PBST), M2 affinity gel was packed into a 1 ml pipette tip and bound proteins were eluted with 1x PBST containing the flag peptide (Sigma, 200 µg/ml) at a rate of 150 µl per fraction. This procedure yielded about 30 µg of flag-tagged MSL1, measured using Bradford reagent (Bio-Rad).
Electrophoresis mobility shift assay (EMSA). Two complementary primers, Prox-A and Prox-B, were synthesized to contain a 6 nucleotide overhang at the 5-end upon their annealing. Their sequences are 5'-TTGGAATCCCGCTATTTTCGGATTCATGCAGTTCCCATTATTTTATTCGGTA-3' and 5'-TACCGAATAAAAATATAATGGGAACTGCATGAATCCGAAAATAGCGGGA-3'. Annealing of Prox-A (20 pmol) and Prox-B (25 pmol) was performed in a solution (34 µl) containing 5 µl of 10x NEB buffer I (New England Biolabs) by incubating at 94°C for 5 min, 63°C for 8 hr, followed by slowly cooling down to 25°C. Subsequently, primer mixture was provided with 2 µl of 2 mM dATP/dGTP/dTTP, 1 µl of 0.1 mM dCTP, 10 µl of α-32P-dCTP (3000 Ci/mmol), 1 µl of 0.1 M DTT, and Klenow fragment (New England Biolabs, 10 units). After 30 min incubation at 25°C, 2 µl of 0.5 M EDTA was added to the above mixture. An aliquot (0.5 µl) was subjected to TCA precipitation to measure specific radioactivity of annealed Prox probe, and the rest was loaded onto a G50 column (5 ml) equilibrated with 1x TBS (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA). When eluted at a rate of 200 µl per fraction, annealed Prox probe was recovered in fractions 9-11. Unless otherwise described, EMSA was performed in a mixture (10 µl) containing 50 mM Tris-HCl, pH 7.4, 1 mM DTT, 0.2 mg/ml bovine serum albumin (BSA), 7 mM MgCl₂, 5% glycerol, 0.05% NP-40, 0.25 μg of 1 kb DNA ladder (Invitrogen), and 50 fmol of annealed Prox probe. After 30 min incubation at 37°C, reaction mixtures were resolved by electrophoresis for 90 min at 19 mA on a 4.5% polyacrylamide gel (30:1) containing 5% glycerol and 0.5x TBE. Complexes formed with Prox were visualized by autoradiography.
**RT-PCR analysis.** S2 cells were transfected with the indicated pMT/V5 construct (5 µg) as described above. On the following day, cells were provided with 0.5 mM copper sulfate and incubated for an additional 48 hrs. Cells were harvested by centrifugation at 800x g for 10 min, washed with ice-cold 1x PBS, and resuspended in 1 ml of Ultraspec reagent (Biotecx). Total RNA, isolated by the recommended procedure, was dissolved in 85 µl of DEPC-treated distilled water and mixed with 10 µl of 10x DNase I buffer and 5 µl of RNase-free DNase I (Promega). After 30 min incubation at 37°C, RNA was re-purified by phenol/chloroform extraction and ethanol precipitation. About 85-100 µg of RNA was obtained from 1x10^7 S2 cells.

Primary cDNA was prepared with an aliquot (1 µg) of total RNA using Superscript RT II (Invitrogen) following the recommended procedure. An aliquot of cDNA (1 µl) was added in a mixture (50 µl) containing 1x Advantage II PCR buffer, 0.25 mM dNTP mix, 30 µCi of α-32P-dCTP, Advantage II polymerase (Clontech), and mixture of 4 PCR primer sets (10 pmol/primer): MSL1-5, 5′-GAAGATCTATGAGCCCA-3′; MSL1-3, 5′-CTGCTTTAATTCTCATTCTGCG-3′; dSRPK1-5, 5′-GATGAGATCTATGAGCCCA-3′; dSRPK1-3, 5′-CATCCTTTTGGCAGCCTCCTTCTGCG-3′; MLE-5, 5′-GAAGATCTATGAGCCCA-3′; MLE-3, 5′-CTGCTTTAATTCTCATTCTGCG-3′; dSRPK1-5, 5′-GATGAGATCTATGAGCCCA-3′; dSRPK1-3, 5′-CATCCTTTTGGCAGCCTCCTTCTGCG-3′; MLE-5, 5′-GAAGATCTATGAGCCCA-3′; MLE-3, 5′-CTGCTTTAATTCTCATTCTGCG-3′. PCR was performed as follows: 1 min at 95°C, 25 cycles of 15 sec at 95°C, 30 sec at 55°C, 1.5 min at 68°C, and 7 min at 68°C. PCR products were purified using spin column (Quigen), and aliquots (10%, 2.5
µl) were resolved on a 2% agarose gel in 0.5x TBE. PCR products were visualized by ethidium staining and autoradiography, and quantified by Instant β-Imager (Packard).
RESULTS

Identification of the upstream genomic sequence of the roX2 gene

When ectopically expressed, MSL2 induces dosage compensation even in female flies (17,28). Since roX RNAs are critical for dosage compensation, transcription of the corresponding genes, which are suppressed in female flies, must be activated in these transgenic female flies expressing MSL2. To understand the mechanism underlying transcription activation of the roX2 gene, we attempted to identify an upstream genomic region potentially important for transcriptional regulation of the roX2 gene. The 14 kb chromosomal region containing the roX2 gene is depicted in Fig. 1A. Five genes, including A, B, C, roX2, and nod, are identified in the sense template, and two genes, D and E, are in the anti-sense template. A 350 bp intergenic region, which we title Prox2, is present between CG11695 and the roX2 gene and contains a T-rich region at position -331 to -150 and a 54 bp-repeated sequence at position -261 to -154.

To confirm the above sequence information obtained from the fly genome database, we performed PCR using two primers, Prox2-5 and Prox2-3, and genomic DNA isolated from S2 cells as a template. PCR primers were designed to contain restriction enzyme cleavage sites, NheI and HindIII (Fig. 1B). The PCR product was subsequently treated with NheI and HindIII, re-isolated (Fig. 1C, lane 2), and inserted into pGL3-basic that was pre-cleaved with BamH1 and HindIII (Fig. 1C, lane 1). Three independent clones, containing 0.5 kb insert, were sequenced, which verified the
identified sequence presented in Fig. 1A, including the 54 bp repeat present within the upstream region of the roX2 gene. We named this newly identified 54 bp repeat “Prox”.

**Detection of a Prox binding activity in S2 cell extracts**

The 54 bp repeat (Prox) present in the intergenic region upstream of the roX2 gene prompted us to determine whether it serves as a cis-acting element for transacting factors. We tested this possibility, employing electrophoresis mobility shift assay (EMSA) using whole cell extracts (WCE) prepared from S2 cells and ³²P-labeled Prox as probe. Indeed, WCE of S2 cells exhibited an activity to form stable complexes with ³²P-labeled Prox in the presence of 200-fold molar excess of non-specific dsDNA competitor (Fig. 2A, lane 2). To further explore the specificity of Prox-protein complexes, we supplemented the EMSA reactions with 25- and 100-fold molar excess of dsDNA competitors, as shown in Fig. 2B, in addition to 250 ng of non-specific DNA. Drastic reduction in the complex formation was observed with Prox (Fig. 2A, lanes 3 & 4). XorP, a 54 bp synthetic dsDNA containing reverse sequence of Prox, was less efficient in competing with ³²P-labeled Prox (lanes 5 & 6). Since (A+T) sequence accounts for about 64% of Prox, we also tested if the (A+T)-richness is an important sequence feature for the formation of Prox-protein complexes. For this purpose, a 24 bp dsDNA, TA:AT, containing solely (A+T) sequence, was synthesized and compared with Prox. As shown in Fig. 2A, lanes 7 & 8, TA:AT only weakly influenced the formation of Prox-protein complexes. These results indicate that S2 cells contain a factor that specifically recognizes and associates with Prox.
A direct interaction of MLE with Prox in vitro

Among all known transacting factors involved in dosage compensation, MLE is the only protein that possesses demonstrated DNA binding activity (26,29). However, MSL1 contains an acidic domain, which is often found among proteins possessing DNA binding activity (15). To determine whether any of these factors are capable of interacting with Prox, we expressed and purified MSL1 and MLE. A detailed procedure for the purification of recombinant MLE was described previously (26). MSL1 was over-expressed in S2 cells using pMT/V5 vector (Invitrogen) (Fig. 3). Expression of either flag-tagged MLE (lane 2) and flag-tagged MSL1 (lane 4) in S2 cells upon treatment of copper sulfate was confirmed by Western blot analysis, as summarized in Fig. 3A. It should be noted that although the estimated molecular weights of MSL1 is 105 kDa, it migrates more slowly than predicted on SDS-PAGE (lanes 4, asterisk), as reported previously (17,30,31).

Employing M2-affinity chromatography, MSL1 was successfully isolated from whole cell extracts (Fig. 3B). An additional protein of about 55 kDa in size is believed to be a major degradation product of MSL1 since it cross-reacts with anti-MSL1 antibodies (data not shown). To compare their activity to interact with Prox, either MSL1 (120 ng) or MLE (65 ng) was incubated in reaction mixture containing 50 fmol (approximately 1.8 ng) of $^{32}$P-labeled Prox and 250 ng of non-specific dsDNA in the presence or absence of ATP, and subsequently analyzed on native gel. As shown in Fig. 3C, MLE but not MSL1
exhibited an activity to form complexes with Prox in an ATP-independent manner. MSL1 did not react with Prox even in the absence of non-specific DNA competitor (data not shown). Our results indicate that at least MLE is capable of interacting with Prox in a sequence-specific fashion.

**Interaction of Prox and roX2-DHS with MLE in vitro**

It is generally accepted that MSL proteins assemble a complex on the male X chromosome in a sequence-specific manner, including approximately 35 sites of X chromosome, called “chromatin entry sites” (22-24). A recent study has identified consensus sequences common to the MSL binding sites within the roX1 and roX2 genes and named it roX-DHS (DNase-hypersensitive site) (32). Since there is no apparent sequence similarity between Prox and roX-DHS, we tested if roX2-DHS, a consensus MSL binding site identified within coding sequence of the roX2 gene (32), interacts with MLE. For this purpose, 135 bp roX2-DHS was amplified by PCR in the presence or absence of α-32P-dCTP, and used as competitor or probe in reactions containing 65 ng of MLE. As shown in Fig. 4A, molar excess (4- or 20-fold) of unlabeled Prox efficiently competed with Prox for MLE (compare lane 2 with 5 & 6). In contrast, poor competition was observed with either roX2-DHS (lanes 3 & 4), TA:AT (lanes 7 & 8), or xorP (data not shown). In addition, when incubated in the presence of only 250 ng of nonspecific DNA competitor, roX2-DHS was not able to form complexes with MLE (Fig. 4B, lane 2). Essentially the same results were obtained with a 143 bp DNA containing full 54 bp
repeat sequence. These results support a sequence-specific interaction between MLE and Prox.

**Activation of Prox2-driven transcription by MLE, MSL1, and MSL2**

Considering that induction of dosage compensation in female flies by ectopic expression of MSL2 should be accompanied with transcription activation of the roX genes (17), it is tempting to posit that more than one cis-acting element is involved in the process of dosage compensation or that MLS2, and perhaps MSL1, execute their function to initiate roX2 transcription through the activation of MLE (see Discussion).

Taking the above possibilities into consideration, we examined whether the interaction between MLE and Prox influences the transcription activity of Prox2, and whether MSL1 or MSL2 also alter the transcription activity of Prox2. For this purpose, 1 µg of Prox2-luciferase construct (Fig. 1D) was co-transfected with increasing amounts (0.4–1.2 µg) of pMT/V5 construct expressing MLE, MSL1, or MSL2. The presence of Prox per se increased luciferase activity up to 4-fold (data not shown). This result indicates that Prox functions as a cis-acting element and transcriptionally activates downstream genes such as luciferase. Although it appears that transacting factors reacting with Prox are not limited in S2 cells, ectopic expression of MLE further stimulated transcription activity of Prox about 2-fold (Fig. 5A, lanes 2 & 3). Though slightly less efficient, similar results were obtained with MSL1 and MSL2 (lanes 4-7). Interestingly, it appears that the extents of transcription activation are proportional to intracellular
levels of ectopically expressed MLE, MSL1, and MSL2 in S2 cells (compare Figs. 3A and 5A).

Next, we examined if exogenously expressed MLE or MSL proteins influence the endogenous roX2 gene. Considering that intracellular levels of endogenous MLE and MSL proteins are sufficiently high to support Prox-driven transcription, we anticipated only marginal change, if any, in the expression of the roX2 gene by exogenous MLE or MSL proteins. For this purpose, we employed transient transfection assays coupled with semi-quantitative RT-PCR. In brief, total RNAs were isolated from S2 cells transfected with pMT/V5 construct expressing MLE, MSL1 or MSL2. Subsequently, RT-PCR was performed in the presence of α-32P-dCTP and 4 pairs of PCR primers specific for MLE, MSL1, roX2, and SRPK1. Under the optimized condition, PCR reaction containing all 4 pairs of primers (Fig. 5B, lane 5), performed either for 25 cycles or 30 cycles, produced a mixture of products whose overall yield was seemingly comparable to a PCR reaction performed with only a pair of cognate primers (compare lanes 1-4 with lane 5). Treatment of total RNA with DNase I was essential for the reproducibility of semi-quantitative RT-PCR containing a mixture of primers (data not shown). The two PCR products for roX2, obtained with pre-mRNA containing an intron and a mature mRNA (24), are presented as roX2-1 and roX2-2, respectively.

PCR reactions were performed for 25 cycles using total RNAs, isolated from S2 cells expressing the indicated protein, as template in the presence of α-32P-dCTP. Aliquots (2 µl) were analyzed on 2% agarose gel. Subsequent to staining with ethidium
bromide, PCR products were visualized by autoradiography (Fig. 5C), which shows that the mRNA level for MLE (lane 2) or MSL1 (lane 3) was increased in S2 cells transfected with a cognate pMT/V5 construct. To determine the effect of increased MLE, MSL1, or MSL2 on roX2 expression, PCR products were quantified by Instant β-Imager and normalized to the level of SRPK1 mRNA, and compared to those obtained with total RNAs isolated from S2 cells transfected with empty pMT/V5 vector. As summarized in Fig. 5D, transfection of S2 cells with pMT-MLE or pMT-MSL1 led to increases in their own mRNA level of about 2-fold (lanes 2 and 3). In addition, over-expression of MLE or MSL1 caused noticeable changes in roX2 mRNA level, with about 12.5 or 11% increase, respectively (Fig. 5D, lanes 2 & 3, filled bars). Although far less efficient than those obtained with a reporter construct (Fig. 5A), the effect of MLE or MSL1 on the endogenous roX2 gene was reproducible. Interestingly, a much broader impact was observed with MSL2. Over-expression of MSL2 (see Fig. 3A, lane 6) increased both MSL1 and roX2 mRNAs about 20% and 4% respectively, but decreased MLE mRNA about 25% (Fig. 5D, lane 4).

It is intriguing to note that the relative extent of increase in endogenous roX2 gene expression by MLE, MSL1, or MSL2 is proportional to that of luciferase expression driven by Prox (compare Fig. 5A with Fig. 5D). These results suggest that the activation of Prox-driven transcription in either a reporter construct or in a chromosomal context might be mechanistically similar and that MLE plays an active role in that process as a transacting factor specific for Prox.
DISCUSSION

Two X-linked genes, \( \text{roX}1 \) and \( \text{roX}2 \), encode non-coding RNAs (13,14). It is generally believed that \( \text{roX}1 \) and \( \text{roX}2 \) RNAs exert their function through the interaction with MLE and MSL proteins. Although earlier studies, employing embryos lacking \( \text{roX}1 \), \( \text{roX}2 \) or both genes, show that they are functionally redundant (14,33,34), at least two lines of evidence support their independent functions. First, in the absence of MSL3 protein, \( \text{roX}2 \) RNA, but not \( \text{roX}1 \), spreads to other sites on the X chromosome, a cytological indicator of normal dosage compensation (34). Second, in embryos, \( \text{roX}1 \) RNA is transcribed in both sexes in the absence of MSL2, whereas transcription of \( \text{roX}2 \) RNA is preceded by MSL2 expression in males and activated by exogenous expression of MSL2 in females (35). These observations suggest that the \( \text{roX}1 \) and \( \text{roX}2 \) genes are differentially regulated at the transcription level during development and that MSL proteins play active roles in transcription of the \( \text{roX}2 \) gene, but not the \( \text{roX}1 \) gene.

At present, the molecular mechanisms underlying \( \text{roX} \) gene transcription remain unknown. It has been shown that mutant embryos, lacking MLE, normally synthesize \( \text{roX}1 \) RNA, but \( \text{roX}1 \) RNA appears to be concentrated at its site of synthesis (35). Based on this observation, it has been proposed that MLE is required for the stability of \( \text{roX}1 \) RNA and its movement from the transcription site but not for its synthesis. MLE has also been implicated in stable maintenance of the steady-state level of \( \text{roX}2 \) RNA. Expression of \( \text{mle}^{\text{GET}} \) in place of the wild type MLE results in drastic reduction in the \( \text{roX}2 \) RNA level and the formation of the MSL complex devoid of \( \text{roX}2 \) RNA (25). In addition to
this post-transcriptional function, the present study suggests a direct involvement of MLE in transcription regulation of the \(\text{roX2}\) gene. First, MLE interacts with the upstream promoter region (i.e., Prox2) of the \(\text{roX2}\) gene through association with a 54 bp repeat, Prox. Second, over-expression of MLE activates transcription driven by Prox2 either in a reporter construct or in chromosomal context.

Our study shows that ATP is not essential for the interaction of MLE with Prox. In addition, MLE ATPase activity is dispensable for transcriptional activation supported by Prox2 (data not shown). These results are consistent with the findings that MLE retains X chromosome binding ability in spite of various mutations introduced in the ATPase motifs (26,36) and that the ATPase activity is dispensable for transcriptional activation of the X-linked genes (37). Since mutations in the ATPase motifs of MLE affect the viability of male flies, the ATPase activity seems to be required for normal development of male flies (36). Then, by what mechanism does the ATPase activity of MLE influence dosage compensation? Based on poor binding of \(\text{roX1}\) RNA to the X chromosome in flies expressing \(\text{mle}^{\text{GET}}\), Kuroda and coworkers have proposed that MLE ATPase activity plays an early role, perhaps in packaging \(\text{roX2}\) RNA into growing MSL complexes (36). In support of this hypothesis, a recent study has shown that in the absence of an ATP-dependent function of MLE, MSL complex can be assembled but are devoid of \(\text{roX}\) RNA (25). Thus, it is likely that in addition to transcriptional regulation by an ATP-independent function of MLE, \(\text{roX2}\) RNA is post-transcriptionally regulated through association with MLS proteins, which require an ATP-dependent function of MLE.
REFERENCES

1. Pannuti, A., and Lucchesi, J. C. (2000) *Curr Opin Genet Dev* **10**, 644-650
2. Park, Y., and Kuroda, M. I. (2001) *Science* **293**, 1083-1085
3. Brockdorff, N. (2002) *Trends Genet* **18**, 352-358
4. Cohen, D. E., and Lee, J. T. (2002) *Curr Opin Genet Dev* **12**, 219-224
5. Hodgkin, J. (1990) *Nature* **344**, 721-728
6. Parkhurst, S. M., and Meneely, P. M. (1994) *Science* **264**, 924-932
7. Kelley, R. L., and Kuroda, M. I. (1995) *Science* **270**, 1607-1610
8. Mukherjee, A. S., and Beerman, W. (1965) *Nature* **207**, 785-786
9. Baker, B. S., Gorman, M., and Marin, I. (1994) *Annual Review of Genetics* **28**, 491-521
10. Hong, Y. K., Ontiveros, S. D., and Strauss, W. M. (2000) *Mamm Genome* **11**, 220-224
11. Brockdorff, N., Ashworth, A., Kay, G. F., McCabe, V. M., Norris, D. P., Cooper, P. J., Swift, S., and Rastan, S. (1992) *Cell* **71**, 515-526
12. Brown, C. J., Hendrich, B. D., Rupert, J. L., Lafreniere, R. G., Xing, Y., Lawrence, J., and Willard, H. F. (1992) *Cell* **71**, 527-542
13. Meller, V. H., Wu, K. H., Roman, G., Kuroda, M. I., and Davis, R. L. (1997) *Cell* **88**, 445-457
14. Amrein, H., and Axel, R. (1997) *Cell* **88**, 459-469
15. Palmer, M. J., Mergner, V. A., Richman, R., Manning, J. E., Kuroda, M. I., and Lucchesi, J. C. (1993) *Genetics* **134**, 545-557
16. Bashaw, G. J., and Baker, B. S. (1995) *Development* **121**, 3245-3258
17. Kelley, R. L., Solovyeva, I., Lyman, L. M., Richman, R., Solovyev, V., and Kuroda, M. I. (1995) *Cell* **81**, 867-877
18. Gorman, M., Franke, A., and Baker, B. S. (1995) *Development* **121**, 463-475
19. Kuroda, M. I., Kernan, M. J., Kreber, R., Ganetzky, B., and Baker, B. S. (1991) *Cell* **66**, 935-947
20. Hilfiker, A., Hilfiker-Kleiner, D., Pannuti, A., and Lucchesi, J. C. (1997) *Embo J* **16**, 2054-2060
21. Jin, Y., Wang, Y., Johansen, J., and Johansen, K. M. (2000) *J Cell Biol* **149**, 1005-1010
22. Kageyama, Y., Mengus, G., Gilfillan, G., Kennedy, H. G., Stuckenholz, C., Kelley, R. L., Becker, P. B., and Kuroda, M. I. (2001) *Embo J* **20**, 2236-2245
23. Kelley, R. L., Meller, V. H., Gordadze, P. R., Roman, G., Davis, R. L., and Kuroda, M. I. (1999) *Cell* **98**, 513-522
24. Meller, V. H., Gordadze, P. R., Park, Y., Chu, X., Stuckenholz, C., Kelley, R. L., and Kuroda, M. I. (2000) *Curr Biol* **10**, 136-143
25. Gu, W., Wei, X., Pannuti, A., and Lucchesi, J. C. (2000) *Embo J* **19**, 5202-5211
26. Lee, C. G., Chang, K. A., Kuroda, M. I., and Hurwitz, J. (1997) *Embo J* **16**, 2671-2681
27. Nakajima, T., Uchida, C., Anderson, S. F., Lee, C. G., Hurwitz, J., Parvin, J. D., and Montminy, M. (1997) *Cell* **90**, 1107-1112
28. Copps, K., Richman, R., Lyman, L. M., Chang, K. A., Rampersad-Ammons, J., and Kuroda, M. I. (1998) *Embo J* **17**, 5409-5417
29. Zhou, K., Choe, K. T., Zaidi, Z., Wang, Q., Mathews, M. B., and Lee, C. G. (2003) *Nucleic Acids Res* **31**, 2253-2260

30. Chang, K. A., and Kuroda, M. I. (1998) *Genetics* **150**, 699-709

31. Scott, M. J., Pan, L. L., Cleland, S. B., Knox, A. L., and Heinrich, J. (2000) *Embo J* **19**, 144-155

32. Park, Y., Mengus, G., Bai, X., Kageyama, Y., Meller, V. H., Becker, P. B., and Kuroda, M. I. (2003) *Mol Cell* **11**, 977-986

33. Franke, A., and Baker, B. S. (1999) *Mol Cell* **4**, 117-122

34. Meller, V. H., and Rattner, B. P. (2002) *Embo J* **21**, 1084-1091

35. Meller, V. H. (2003) *Mech Dev* **120**, 759-767

36. Richter, L., Bone, J. R., and Kuroda, M. I. (1996) *Genes Cells* **1**, 325-336

37. Chiang, P. W., and Kurnit, D. M. (2003) *Genetics* **165**, 1167-1181
FOOTNOTES

1Abbreviations used are: DTT, dithiothreitol; MSL, male-specific lethal; MLE, maleless; MOF, male-absent on the first; PBS, phosphate-buffered saline; Prox2, the upstream promoter of the roX2 gene; roX, RNA on the X.

ACKNOWLEDGEMENTS

We thank Drs Mitzi I. Kuroda and Andrew Parrott for comments and critical reading of the manuscript. We also thank Dr. John C. Lucchesi for providing expression vector for MSL2 and antibodies to MSL1 and MSL2, Dr. Maxwell S. Scott for FLAG-MSL1 construct, and Dr. Hubert Amrein for roX2 cDNA. This study was supported by a Research Grant from the University of Medicine and Dentistry of New Jersey (UMDNJ) Foundation to C. G. Lee and by NIH grant AI34552 to M. B. Mathews.
FIGURE LEGENDS

FIGURE 1. Identification and cloning of the upstream genomic region of the roX2 gene. (A) Genomic structure of the X chromosomal region harboring the roX2 gene. A to E represent 5 different open-reading frames designated as CG11697, CG11696, CG11695, CG15191, and CG1561. The roX2 and nod genes are indicated. Exons are presented as filled boxes. A hypothetical promoter region of the roX2 gene is indicated as Prox2 and its sequence is presented. A 54 bp-repeated sequence is bold-faced and a T-rich region is boxed. (B) Nucleotide sequences of the two PCR primers, Prox2-5 and Prox2-3. In (A) & (B), Numerals show the nucleotide position relative to the first nucleotide (+1) of the underlined sequence (A) that matches to the 5’-end of roX2 cDNA (U85981). (C) Genomic PCR cloning of Prox2 and its subcloning into pGL3-basic vector. Genomic PCR was performed as described in Materials and Methods. PCR product was subcloned into BamHII and HindIII sites of pGL3-basic. Aliquots (0.2 µg) of cleaved pGL3-basic (lane 1) and Prox2 (lane 2) were resolved on a 1.2% agarose gel and visualized by ethidium staining. (D) Schematic diagram of the reporter construct, Prox2-luciferase.

FIGURE 2. Detection of DNA binding activity in S2 cells specific for a 54 bp-repeat sequence (Prox). Reaction mixtures (10 µl) consisted of 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 7 mM MgCl2, 5% glycerol, 0.05% NP-40, 0.25 µg of 1 kb DNA ladder, 50 fmol (1.8 ng) of 32P-labeled Prox probe, indicated competitor, and 40 µg of whole cell extract (WCE). Lane 1, without WCE; lane 2, without competitor; lanes 3 & 5, 70 ng of competitor; lanes 4 & 6, 350 ng of competitor; lane 7, 200 ng of TA:AT; lane 8, 1 µg of
TA:AT. After 30 min incubation at 37°C, reaction mixtures were resolved on a 4.5% (30:1) polyacrylamide/5% glycerol composite gel. Prox-protein complexes formed were detected by autoradiography (A). Nucleotide sequences of Prox, xorP, and TA:AT are given only for their positive strands in (B).

**FIGURE 3.** MLE but not MSL1 specifically interacts with Prox. (A) S2 cells were transfected with various constructs expressing flag-tagged MLE (lanes 1 & 2), flag-tagged MSL1 (lanes 3 & 4), or HA-tagged MSL2 (lanes 5 & 6). WCE was prepared before (lanes 1, 3 & 5) and after treatment with copper sulfate (0.5 mM) for 48 hr (lanes 2, 4 & 6) and aliquots (20 µg) of WCE were subjected to Western blot analysis using antibodies specific for the flag (lanes 1-4) or HA epitope (lanes 5 & 6). (B) M2 affinity chromatography was performed to isolate flag-tagged MSL1, as described in Materials and Methods. Aliquots of the indicated fraction were subjected to 7.5% SDS-PAGE, and proteins were visualized by coomassie staining. Lane 1, 20 µg of WCE; lane 2, 20 µg of the flow-through fraction; lane 3, 120 ng of affinity-purified proteins. (C) In place of WCE, MSL1 (120 ng) (lanes 2-4) or MLE (65 ng) (lanes 5-7) was added in the reaction mixture (10 µl) containing 32P-labeled Prox as described in Fig. 2. In lanes 4 & 7, 1 mM ATP was provided.

**FIGURE 4.** MLE does not specifically interact with roX2-DHS. Prox (50 fmol) (A) or roX2-DHS (50 fmol) (B) was provided in the standard reaction mixture (10 µl) containing increasing amounts of the indicated competitor and 65 ng of MLE. Lane 1, without MLE; lane 2, without competitor; lanes 3 & 4, 0.2 & 1 µg of TA:AT; lanes 5 &
6, 0.2 & 1 pmol of unlabeled Prox; lanes 7 & 8, 0.2 & 1 pmol of unlabeled roX2-DHS. Nucleotide sequence of the roX2-DHS is presented in (C).

**FIGURE 5.** Influence of MLE, MSL1, and MSL2 on the transcription activity of Prox2. (A) S2 cells, seeded in 6 well plate, were transfected with Prox2-luciferase (1 µg, lane 1) and increasing amounts (0.4 -1.2 µg) of pMT/V5 vector expressing MLE (lanes 2 & 3), MSL1 (lanes 4 & 5), or MSL2 (lanes 6 & 7). On the following day, cells were treated with copper sulfate (0.5 mM), and 24 hr later, total cell lysate was prepared and used to measure both luciferase and β-galactosidase activities, as described in Materials and Methods. Luciferase activities in lanes 2-7 were normalized to β-galactosidase and presented as RLU (relative luciferase unit) in comparison with that of control in lane 1. (B) RT-PCR was performed in a mixture (50 µl) provided with the indicated primers, as described in Materials and Methods. Following either 25 or 30 cycles of reaction, aliquots (5 µl) of PCR products were resolved on a 2% agarose gel and visualized by ethidium staining. Predicted size of RT-PCR product is 737 bp for MLE mRNA, 408 bp for dSRPK1 mRNA, and 322 bp for MSL1 mRNA. Two RT-PCR products for roX2 represent unspliced (503 bp) and spliced form (233 bp) of mRNA. (C & D) RT-PCR was performed in the standard reaction mixture containing α-32P-dCTP (30 µCi) and cDNA (1 µl) prepared from S2 cells expressing the indicated protein. RT-PCR products were isolated by spin column, and aliquots (10%) were resolved on a 2% agarose gel, followed by autoradiography (C). All RT-PCR products were quantified by Instant β-Imager, normalized to that of Drosophila SRPK1 (dSRPK1) and presented as relative RNA abundance in comparison with cognate RT-PCR product of control cells (D). Statistical
analysis was performed using two-tailed student t-test, comparing control and test samples. *; p <0.05.
Figure 1

(A) Schematic representation of the genomic region including the Prox1 promoter. The positions of the restriction sites are indicated.

(B) NheI restriction enzyme sites are highlighted. Prox2-5: 5'-ctgctagc-3'. Prox2-3: 5'-caggccgcttccc-3'.

(C) Gel electrophoresis showing the digestion pattern with NheI enzyme.

(D) Diagram of the luciferase assay system with the Prox2 promoter construct and pGL3-basic vector.
Figure 2
Figure 3
Figure 4

A  

|   | TA-AT | Prox | 10X2-DHS | MLE (65 ng) |
|---|-------|------|----------|-------------|
| 1 | -     | -    | -        | -           |
| 2 | -     | +    | -        | +           |
| 3 | +     | -    | -        | +           |
| 4 | +     | -    | -        | +           |
| 5 | +     | -    | -        | +           |
| 6 | +     | -    | -        | +           |
| 7 | +     | -    | -        | +           |
| 8 | +     | -    | -        | +           |

B  

|   | TA-AT | Prox | 10X2-DHS | MLE (65 ng) |
|---|-------|------|----------|-------------|
| 1 | -     | -    | -        | -           |
| 2 | -     | +    | -        | +           |
| 3 | +     | -    | -        | +           |
| 4 | +     | -    | -        | +           |
| 5 | +     | -    | -        | +           |
| 6 | +     | -    | -        | +           |
| 7 | +     | -    | -        | +           |
| 8 | +     | -    | -        | +           |

C  

5'-CAATTGCGAAATATACAGATCGATTTAGAGCCATGAC
AAATAAGAGGGCGATCCTCACGGTTAGACATTTCCTTTAAAAAG
AAAGAGAGGGGAGAAGCTGCTGGCTTGAGAGAGATGCA
ATTACTAATTTACTGCT-3'

Downloaded from http://www.jbc.org/ by guest on March 25, 2020
Figure 5
MLE functions as a transcriptional regulator of the roX2 gene
Chee-Gun Lee, Trevor W Reichman, Tina Baik and Michael B Mathews

J. Biol. Chem. published online September 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408207200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts