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MSL *cis*-spreading from *roX* gene up-regulates the neighboring genes

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ABSTRACT

The male-specific lethal (MSL) complex in *Drosophila melanogaster* paints the male X chromosome in a manner that is both *cis* and *trans* to induce 2-fold hypertranscription of the X chromosome. To characterize the upregulation of gene expression by MSL *cis*-spreading, we measured the expressional change of neighboring genes by microarray when the genes were bound by MSL complexes that spread from an autosomal *roX* transgene. Genes within a 200 kb region that includes *roX* transgenes were upregulated concurrently with MSL *cis*-spreading. Conversely, there was almost no expressional change in genes from other regions. RT-PCR and ChIP analyses confirmed that the approximately 2-fold gene hypertranscription was due to MSL *cis*-spreading. We also demonstrated that upregulation of the neighboring gene could rescue haplo-insufficient phenotypes of the *Minute* mutant, such as short bristle, delayed adult eclosion and decreased viability. These results indicate that the hypertranscription by MSL *cis*-spreading is a general mechanism that occurs in several tissue types. Our molecular and genetic data suggest that *cis*-spreading of the MSL complex from high-affinity sites including the *roX* gene results in upregulation of the neighboring genes, which are targets for dosage compensation in the male X chromosome.

1. Introduction

One mechanism of epigenetic gene transcription regulation involves the chromatin-remodeling complex binding to a number of nucleation sites of specific DNA sequences and spreading *in cis* into the neighboring regions. This spreading mechanism occurs in several organisms, including heterochromatin silencing in yeast [1] and dosage compensation of the X chromosome in worms [2], fruit flies and mammals [3]. In *Drosophila*, the male-specific lethal (MSL) complex upregulates male X-chromosomal genes by 2-fold to normalize the level of X-chromosome gene transcription between both sexes [4,5]. This MSL complex contains at least five proteins (MSL1, MSL2, MSL3, MOF: histone acetyltransferase, and MLE: RNA helicase) and two noncoding RNAs (*roX1* and/or *roX2*), which are located on the X chromosome [6–8]. The MSL complex locally spreads *in cis* from the *roX* genes into the neighboring regions [9,10] and *roX* genes contain an MSL binding site that was discovered to be a male-specific DNase I Hypersensitive Site (DHS) [11,12]. *cis*-Spreading of the MSL complex from autosomal *roX* transgene results in recognizing histone H3K36 trimethylation of the neighboring genes [13].

When there is a *roX*$^-$ double mutant on the X chromosome, an autosomal *roX* transgene shows extensive *cis*-spreading of >1 megabase pair (Mbp) of the MSL complex in almost 100% of nuclei [9]. However, in wild type or each *roX*$^-$ single mutant, the same *roX* transgene exhibits less MSL spreading in terms of both frequency and extent, which can be partially recovered by overexpression of MSL1 and MSL2 proteins [9]. It has been suggested that nascent *roX* transcripts compete for MSL proteins to be assembled into the complete MSL complex and that the ratio of MSL protein to *roX* RNA determines the extent of MSL spreading from the *roX* gene [9,10,14].

In this study, using microarray, RT-PCR and ChIP analyses we were able to show that MSL *cis*-spreading resulted in an approximately 2-fold transcriptional increase of the neighboring genes. In addition, haplo-insufficient phenotypes of the *Minute* mutant were suppressed by neighboring *roX* transgenes only in males, indicating that MSL *cis*-spreading upregulates expression of the neighboring genes, which are targets for dosage compensation on the X chromosome.

2. Materials and methods

2.1. Microarray, RT-PCR, and ChIP analyses

Total RNA was extracted with the TRIzol Reagent (Invitrogen) from salivary glands of 3rd instar male *Drosophila* larvae carrying the GMroX2-86F or GMroX2-97F transgene in *roX*$^-$ mutant background. The extracted RNA was then used as probes for the hybridization reaction on Affymetrix *Drosophila* 2.0 microarray chips in

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the EOHSI facility (http://eohsi.rutgers.edu/). The expression levels of genes in the GMroX2-86F or GMroX2-97F transgenic were calculated by the MAS-5 (Affymetrix algorithm) normalization method. Microarray data are available from the corresponding author.

For RT-PCR, oligo dT-primed cDNAs were made from the same total RNA as used for microarray analysis, and then used as templates for the quantitative real-time PCR, which was performed with SYBR green PCR master mix (Applied Biosystems). The *rp49* gene was used as an internal reference for normalizing the quality of total RNA purified from each transgenic line (86F or 97F). The fold changes (ratio of 86F/97F) in gene expression (#1–6 candidates in Fig. 2C) were determined by comparative C_T method (Applied Biosystems).

For ChIP analysis, 200 pairs of salivary glands were isolated from the 3rd instar male larvae of each transgenic line (86F or 97F) and then cross-linked with 1.0% formaldehyde in PBST (1X PBS supplemented in 0.2% Triton X-100) for 5 min at room temperature. Chromatin immunoprecipitation was performed according to the manufacturer's instructions (Upstate) using 3 μL of rabbit IgG (SC2027, Santa Cruz Biotechnology) and anti-MSL-1 antibodies (Kuroda's lab). Real-time PCR was performed using 10 ng of the immunoprecipitated DNA as template. Fold increase of MSL binding was determined by calculating the MSL1/IgG binding ratio (Fig. 2D). Statistical differences (*P*-value) were determined using the Student's *t*-test.

2.2. Fly genotype, bristle, adult eclosion, and viability

The X chromosome genotypes were *y w* (wild type), *y w roX1*^{ex6} (*roX1*$^{-}$ mutant), and *y w roX1*^{ex6} *Df(1)roX2*^{52} P{w^{+} 4Δ4.3} (*roX*$^{-}$ double mutant) [9]. To compare bristle lengths of 2-day-old adults between heterozygous M99B mutant (M) and wild type (W) with the GMroX transgene (Fig. 3B), *y w*/Y; M99B/TM3, *Ser* males were crossed to *y w roX1*^{ex6} *Df(1)roX2*^{52} P{w^{+} 4Δ4.3}; [*roX* transgene] females, and then each sex of the resulting progenies [*roX* transgene]/M99B and [*roX* transgene]/TM3, *Ser* were compared to each other.

To measure the 2nd-day adult eclosion rate (%) of the M99B mutant [15] with the GMroX transgene (Fig. 3C), adult eclosion numbers of the above progenies [*roX* transgene]/M99B or [*roX* transgene]/TM3, *Ser* were counted everyday during 10 days in each sex. Because delayed and normal adults were concurrently eclosed since 3rd day, we measured the percentage of 2nd-day adults over total 10 days from each genotype. We then calculated the 2nd-day adult eclosion rate (%) by dividing the 2nd-day eclosion percentage of [*roX* transgene]/M99B by the 2nd-day eclosion percentage of [*roX* transgene]/TM3, *Ser*. For example, when the 2nd-day eclosion percentage of [GMroX1-99C]/M99B females and [GMroX1-99C]/TM3, *Ser* females were 8% and 24%, respectively, the 2nd-day adult eclosion rate was determined by 8%/24%, yielding 33% (Fig. 3C).

To measure the adult viability (%) of *Dl*^{1} M99B double mutants [15] with the GMroX transgene (Fig. 3D), *y w roX1*^{ex6}/Y; *Dl*^{1} [*roX* transgene]/[*roX* transgene] males were crossed with *y w roX1*^{ex6}; M99B/TM3, *Sb* females, and then the ratio (%) of *Dl*^{1} M99B double mutant (*Dl*^{1} [*roX* transgene]/M99B) to M99B single mutant ([*roX* transgene]/M99B) progenies was calculated from each sex.

3. Results and discussion

3.1. MSL cis-spreading from the roX gene upregulates the neighboring genes

To determine whether MSL *cis*-spreading results in upregulation of gene expression, we used a system that measured MSL spreading from the autosomal *roX* transgene into the neighboring genes [16]. In the *roX*$^{-}$ double mutant, the autosomal *roX* transgene showed MSL *cis*-spreading that was both extensive (up to a few Mbp) and consistent (close to 100%) in the polytene chromosomes of the salivary glands [9]. By microarray analyses using total

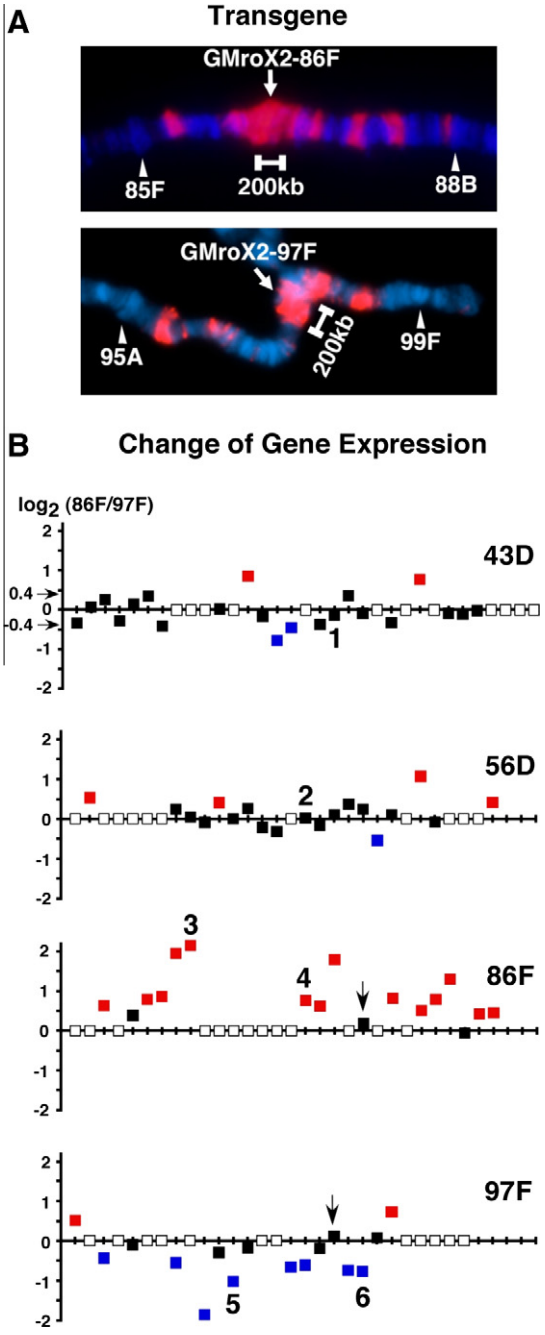

Fig. 1. MSL *cis*-spreading from the GMroX transgene results in transcriptional activation of the neighboring genes. (A) Polytene chromosomes (blue) immunostained with anti-MSL1 antibody (red) from salivary glands of male larvae carrying hemizygous GMroX2-86F or GMroX2-97F transgene in *roX*$^{-}$ mutant. A 200 kb region including *roX* transgene (arrow) is indicated with the cytological map. (B) Comparison of gene expression between GMroX2-86F and GMroX2-97F transgenics. Change of gene expression is indicated as $\log_2$ (86F/97F), which is averaged from three independent microarray analyses. Within 200 kb of four regions (43D, 56D, 86F and 97F), the white, black, red, and blue squares represent genes with unexpressed, non-changed, increased, and decreased expression, respectively. Six genes (#1–6) were further analyzed by RT-PCR and ChIP analyses (Fig. 2C and D). #1: CG30498; #2: CG9291; #3: CG6908; #4: CG14721; #5: CG5974; #6: CG3350; vertical arrow: transgene location. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)