

# A Plasmid Model System Shows that *Drosophila* Dosage Compensation Depends on the Global Acetylation of Histone H4 at Lysine 16 and Is Not Affected by Depletion of Common Transcription Elongation Chromatin Marks<sup>∇†</sup>

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**Dosage compensation refers to the equalization of most X-linked gene products between males, which have one X chromosome and a single dose of X-linked genes, and females, which have two X's and two doses of such genes. We developed a plasmid-based model of dosage compensation that allows new experimental approaches for the study of this regulatory mechanism. In *Drosophila melanogaster*, an enhanced rate of transcription of the X chromosome in males is dependent upon the presence of histone H4 acetylated at lysine 16. This chromatin mark occurs throughout active transcriptional units, leading us to the conclusion that the enhanced level of transcription is achieved through an enhanced rate of RNA polymerase elongation. We used the plasmid model to demonstrate that enhancement in the level of transcription does not depend on other histone marks and factors that have been associated with the process of elongation, thereby highlighting the special role played by histone H4 acetylated at lysine 16 in this process.**

Dosage compensation is a regulatory mechanism that ensures equal amounts of X-linked gene products in males and females, in spite of the difference in the number of X chromosomes present in these two sexes. In *Drosophila melanogaster*, this equalization is achieved by regulating the level of transcription such that the single X chromosome in males is hypertranscribed in relation to each X chromosome in females. Five positive regulators of dosage compensation were identified on the basis of the male-specific lethal phenotype of their loss-of-function alleles: maleless (*mle*; an ATPase/RNA-dependent helicase), male-specific lethal 1 (MSL-1) (*mSl-1*), MSL-2 (*mSl-2*), MSL-3 (*mSl-3*), and males absent on the first (MOF) (*mof*; a histone acetyltransferase). The products of these genes form a core complex (an MSL complex) that contains one of two noncoding RNAs (RNA on the X1 or X2, *roX1* or *roX2*) and are found at numerous sites along the single X chromosome in males (reviewed in reference 13). From these sites, the MSL complex associates with activated genes (20). As a consequence of this association, MOF acetylates histone H4 at lysine 16 (H4K16ac) throughout transcriptional domains, favoring the 3' end of transcription units over promoters (24).

To facilitate the study of the mechanism underlying dosage compensation, we developed an experimental model by repro-

ducing dosage compensation on a plasmid transfected and expressed transiently in cultured cells. This model represents a level of biological complexity that is intermediate between in vitro reconstitutions and chromatin extracted from embryos or other developmental stages. It is substantially more amenable to biochemical manipulations than experimental material from larval salivary glands, and the possibility of using RNA interference (RNAi) offsets the use of mutations. Lastly, because the compensated element is a circular DNA plasmid, it allows the study of topological changes that underlie the compensation process.

The function of the MSL complex is unlikely to be the initiation of gene activity. This conclusion derives from the observation that X-linked genes are activated in normal spatial and temporal fashions in wild-type females, where the complex is absent, and in mutant males, where the complex is inactive (in mutant males, X-linked gene products are present at half the normal level, eventually leading to lethality). Furthermore, the acetylation of H4K16 does not favor the promoter region of compensated genes; it occurs throughout transcriptional units, and its level actually tends to increase towards the 3' end (24). The distribution of H4K16ac in X-linked transcriptional domains, coupled with the observation that dosage-compensated genes exhibit an approximately twofold enhancement in steady-state levels of product irrespective of the strengths of their promoters, has led to the conclusion that the primary mechanistic result of the chromatin modifications responsible for dosage compensation is an enhancement in the rate of transcription elongation (24), i.e., an increase in the extension rate of the RNA polymerase. This conclusion was supported recently by Alekseyenko et al. (2) and Gilfillan et al. (7), who used chromatin immunoprecipitation (ChIP) with high-resolution microarrays to map the position of the MSL complex along the X chromosome.

We used the plasmid model to ask whether the rate of

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elongation of RNA polymerase II (RNAP II) on compensated genes depends on the presence of histone modifications associated with active genes and laid down by the process of transcription by testing the effect of the loss of several known marks and factors associated with the elongation function of RNAP II. We observed that the relative increase in the rate of transcription of dosage-compensated chromatin is not affected by a reduction in the levels of the histone methyltransferases Set1, Set2, or Mes-4 or of the PAF1 and FACT complexes but is affected by reducing the level of MOF. These observations emphasize the unique role played by acetylated H4K16 in this process.

## MATERIALS AND METHODS

**Plasmid construction.** The copia-*Renilla* luciferase (R) plasmid (5) consists of the *Drosophila* copia promoter inserted into pRL-null plasmid (Promega). The tTA plasmid (ptTA) has the transcriptional activator tTA encoding the tetracycline repressor protein (TetR in a Tet-Off system) driven by the *D. melanogaster* alpha-tubulin 1 promoter (cloned as an XhoI-EcoRI fragment) replacing the cytomegalovirus promoter in the plasmid pUHD15.1 (8). The firefly luciferase plasmid (FF) contains the tetracycline resistance operator (*tetO*) inserted upstream of the firefly luciferase gene of the pGL3-Basic plasmid (Promega). The roX-FF plasmid is the FF plasmid with a 1,087-bp fragment of the *Drosophila* roX2 gene (nucleotides 158 to 1244 of GenBank sequence U85981) inserted in the BamHI unique pGL3-Basic site. The Nesprin-FF plasmid (N-FF) is the FF luciferase plasmid with 1,140 bp from the Nesprin human intron (nucleotides 99300 to 100440 of the emb AL359235 sequence) inserted in the BamHI unique pGL3-Basic site to replace the roX2 gene sequence. The Sxl expression plasmid (Mtn-Sxl) was synthesized by inserting the female-specific *Sxl* sequence derived from the MS3 cDNA of Samuels et al. (19) into the pMK33/pMthY expression vector (12).

**S2 transfection and transcription assays.** *Drosophila* Schneider line 2 (S2) cells were grown in HyQ SFX-insect medium (HyClone) with penicillin-streptomycin antibiotic at 25°C without CO<sub>2</sub>. Cells were split 2 to 22 h prior to transfection to 30 to 60% confluence. Transfection was carried out following the QIAGEN Effectene protocol with 1.0 ng ptTA plasmid, 5.4 ng R plasmid, 1.2 μg pBluescript (Stratagene), and 15 ng supercoiled (or 30 ng relaxed) FF, roX2-FF, N-FF plasmid, or 100 ng of Mtn-Sxl plasmid, 10.8 μl enhancer, and 19.2 μl Effectene reagent for 5 × 10<sup>6</sup> cells. On the next day, the cells were split to a final concentration of 0.3 × 10<sup>6</sup> cells/ml. Three to 5 days after transfection, the cells were collected for the luciferase assay, RNA isolation, DNA isolation, and/or protein isolation. Luciferase activity was determined by using the dual luciferase reporter assay system (Promega). The firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample. At least three independent experiments were performed; error bars in the figures represent standard deviations of the means.

**ChIP analysis.** Chromatin from transfected S2 cells was immunoprecipitated following the method of Oberley and Farnham (18), with the following modifications. Sonication was performed with 25 sets of 4-s pulses; preclearing was performed with protein A-coated beads treated with tRNA, salmon sperm, and bovine serum albumin; and cross-linking was reversed at 65°C in 0.2 M NaCl. ChIP grade H4K16ac and H3K36me3 antisera were purchased from Serotec and Abcam, respectively; RFX antiserum, a gift of J. Boss, was used as a background immunoprecipitation control; the supernatant was used as input. DNA was isolated with a QIAquick PCR purification kit (QIAGEN). The level of pull down of the firefly luciferase gene was measured by quantitative PCR with a pair of primers spanning an internal region in the case of H4K16ac and with three pairs of primers spanning 5', internal, and 3' regions in the case of H3K36me3 (see Table S1A in the supplemental material for primer sequences).

**RNAi knockdown.** The day before transfection, 0.9 × 10<sup>6</sup> S2 cells were transferred to a six-well culture dish 3 to 5 h prior to pretreatment with 10 μg/ml double-stranded RNA (dsRNA). Cells were transfected 18 to 22 h later and, 1 day after transfection, were split to ~0.3 × 10<sup>6</sup> cells/ml. Additional dsRNA was added to the medium at that time to maintain the 10 μg/ml concentration. dsRNA was made following Ambion's MEGAscript protocol. The primers used to amplify the 500- to 600-bp regions of the dSet1, dSet2, Mes-4, dSrp, dSpt16, *mof*, *msl2*, and green fluorescent protein (GFP) genes are presented in Table S1B in the supplemental material.

**Immunoblot analysis.** Protein extracts were isolated from S2 cell pellets in Laemmli sample buffer, separated on Criterion Tris-HCl gels (Bio-Rad) and transferred to a polyvinylidene difluoride membrane by using 20% methanol-Tris-glycine transfer buffer, following Bio-Rad's Criterion protocol. Primary antibodies used were H3 (1:5,000, Abcam), H3K4me1 (Abcam; 1:500), H3K4me2 (Upstate; 1:2,000), H3K4me3 (Abcam; 1:2,000), H3K36me1 (Abcam; 1:1,000), H3K36me2 (Upstate catalog no. 07-369, 1:2,000), H3K36me3 (Abcam; 1:2,000), and *Drosophila* FACT subunits dSPT16 and dSSRP1 (received from S. Hirose [22]; 1:5,000). After secondary anti-rabbit horseradish peroxidase antibody labeling (Pierce; 1:20,000), immunoblots were developed by using enhanced chemiluminescence (ECL-Plus; GE Healthcare) and quantitated with a Bio-Rad Fluor-S Multi-Imager Max or ImageJ program on scanned developed films. The levels of knockdown expressed as a percentage of control (GFP RNAi) are listed in Table S2 in the supplemental material.

**Quantitative RT-PCR.** Total RNA was isolated using the QIAGEN RNeasy kit. Real-time reverse transcription-PCR (RT-PCR) measurements of the levels of firefly, *Renilla*, ribosomal protein 49 (RP49), glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), dSpt6, glycerol 3-phosphate dehydrogenase (GPDH), and dSpt4 transcripts were made using the QIAGEN QuantiTect SYBR green RT-PCR kit and the Bio-Rad iCycler. For the gene-specific primer pairs, see Table S1C in the supplemental material. The level of transcripts from each gene was normalized to the internal standard RP49. The number of normalized cycles of the compensated GFP-treated sample was set to 1, and the percentage of change from that number is plotted as the relative level of mRNA. Error bars represent standard deviations from the means of at least three different treatments/transfections.

## RESULTS AND DISCUSSION

**A plasmid model of dosage compensation.** We constructed a series of plasmids containing a reporter gene (firefly luciferase) with (roX-FF) or without (FF) a 1,087-bp fragment from the roX2 genomic sequence that nucleates the MSL complex. These plasmids are transfected into *Drosophila* S2 cells, together with another plasmid (R) containing a different reporter gene (*Renilla* luciferase) as a control for levels of transfection. The firefly luciferase gene is under the control of the tetracycline resistance *tetO* that can be induced to very high levels of transcription by a synthetic tTA. A plasmid containing the gene for the activator under the control of the constitutive alpha-tubulin promoter (ptTA) is cotransfected into S2 cells. The *Renilla* luciferase gene is under the control of a constitutive copia promoter (Fig. 1A). We obtained evidence of the topological organization of transfected plasmids into chromatin by using ChIP analysis with a general histone antiserum (data not shown) and by displaying the plasmids as a distribution of topoisomers in chloroquine gels (Fig. 1B); similar distributions of topoisomers are obtained whether supercoiled plasmids are transfected directly or after pretreatment with topoisomerase I to relax them. These experiments also provided evidence that the ratio of the firefly luciferase-bearing plasmids to the *Renilla* luciferase-bearing plasmid remains comparable after 4 days of cell culture (see Fig. S1 in the supplemental material). S2 cells are "male" cells in that they fail to express Sex-lethal (*Sxl*), the master regulatory gene responsible for female sex determination; furthermore, these cells elaborate a fully functional MSL core complex (25). In the presence of the roX2 sequence, there is an almost perfect twofold enhancement of transcription of the firefly luciferase gene (Fig. 1C). Within a relatively wide range, this effect is independent of the amount of transfected template; it is also independent of the orientation of the roX2 sequence (data not shown). The twofold enhancement of transcription of the roX-FF plasmid is maintained over a wide range of transcrip-

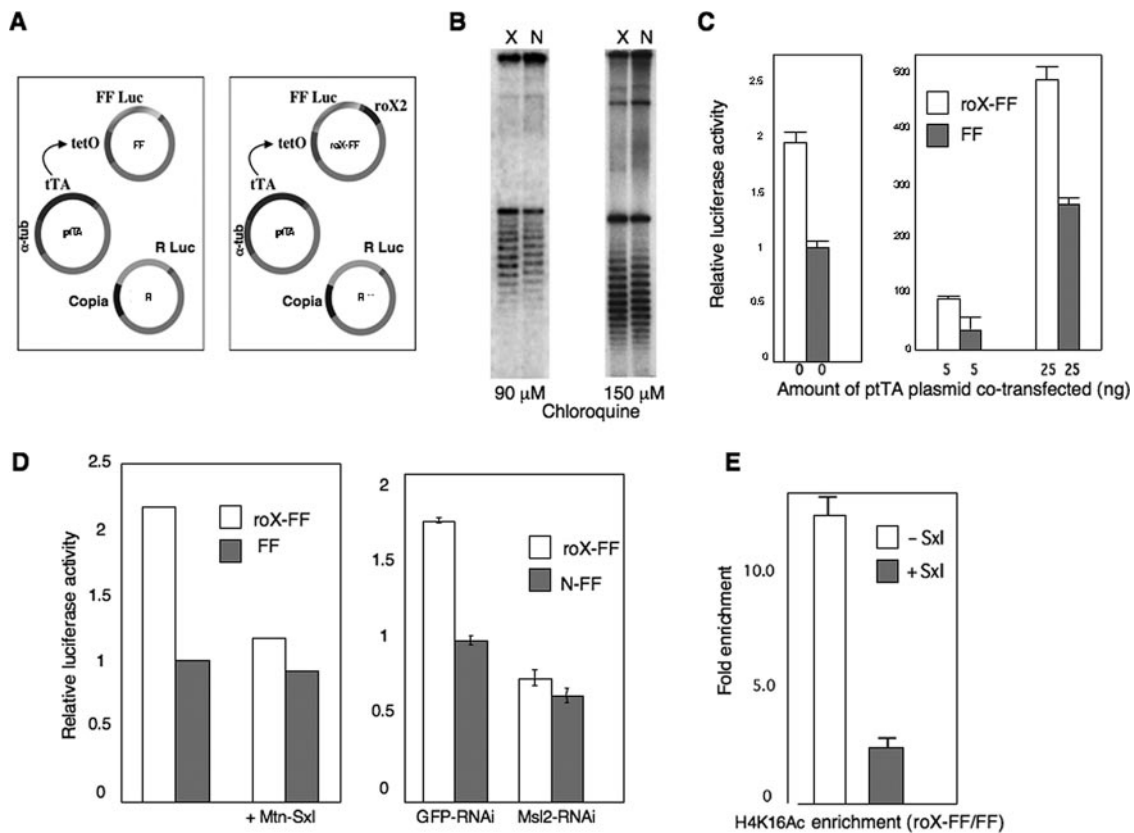


FIG. 1. The plasmid model of dosage compensation. (A) Diagram of the plasmids used to transfect S2 cells. The FF plasmid contains *tetO* inserted upstream of the firefly luciferase gene; the roX-FF plasmid includes the *roX2* gene sequence downstream of the firefly luciferase gene; the R plasmid contains the *Renilla* luciferase gene under the control of the *Copia* promoter; the pTA plasmid expresses the tTA transcriptional activator gene driven by the alpha-tubulin promoter. Experimental (right panel) and control (left panel) transfections are indicated. (B) Southern blots of DNA extracted from S2 cells at 4 days posttransfection run in agarose gels with two different concentrations of chloroquine and probed with a firefly luciferase sequence. X and N refer to roX-FF and N-FF plasmids, respectively (see Materials and Methods). (C) There is a twofold enhancement in firefly luciferase relative to *Renilla* luciferase activity due to the presence of the *roX* sequence in the absence of tTA because the *tetO* promoter exhibits some leakiness; the twofold difference is maintained over a wide range of cotransfection with the tTA-expressing plasmid. (D) The enhancement is abrogated by the presence of an SXL-expressing plasmid under the control of the *Drosophila* metallothionein promoter (left panel) or by RNAi to deplete MSL2 (right panel). GFP denotes an RNAi control with GFP dsRNA. (E) ChIP analysis reveals a better than 10-fold enrichment in H4K16ac on the *roX2*-bearing plasmid that is eliminated by cotransfection with SXL. Each experiment was repeated three times, and the error bars represent the standard deviations of the means (the experiment for panel D, left side, was performed twice).

tional levels achieved by increasing the amount of cotransfected tTA-expressing plasmid (Fig. 1C). This result is concordant with the fact that on the X chromosome in males, genes that are dosage compensated are up-regulated, on average, to a twofold level regardless of the strength of their promoters. Two lines of evidence indicate that the twofold enhancement in firefly luciferase activity is the result of the action of the MSL complex. First, the enhancement is reduced greatly if the cells are cotransfected with a plasmid that expresses SXL and prevents the formation of the complex (4, 10); similar results are obtained if RNAi is used to deplete MSL2 (Fig. 1D). Second, the chromatin of the roX-FF plasmid is highly enriched in histone H4 acetylated at lysine 16. This enrichment is, once again, diminished greatly by cotransfection with an SXL-producing plasmid (Fig. 1E). The response of the *tetO* promoter is directly proportional to the level of tTA-expressing plasmid transfected into the cells. It is possible, therefore, to set the level of transcription of the control FF plasmid to that of the roX-FF plasmid by doubling the amount of pTA in-

cluded in the control plasmid transfection (see Fig. S2 in the supplemental material). This feature of the system can be used to parse those modifications that are necessary for dosage compensation to occur from those modifications that are the result of its occurrence, i.e., that are the cause rather than a consequence of the enhanced level of transcription.

**Dosage compensation is not affected in cells lacking transcription-associated histone marks.** Gene activation involves the acetylation of various lysine residues in the N-terminal tails of histones H3 and H4 and, in particular, the methylation of histone H3 at lysine 4. These modifications are restricted largely to the promoter and 5' end of genes. Following assembly and activation of the transcription complex and the transition to productive elongation, a number of covalent histone modifications occur in the transcribed region. These modifications include the dimethylation of H3K4 (H3K4me2) predominantly in the middle of genes and the monomethylation of this residue (H3K4me1) towards the 3' end of genes; they also include the presence of trimethylated H3K36 (H3K36me3)

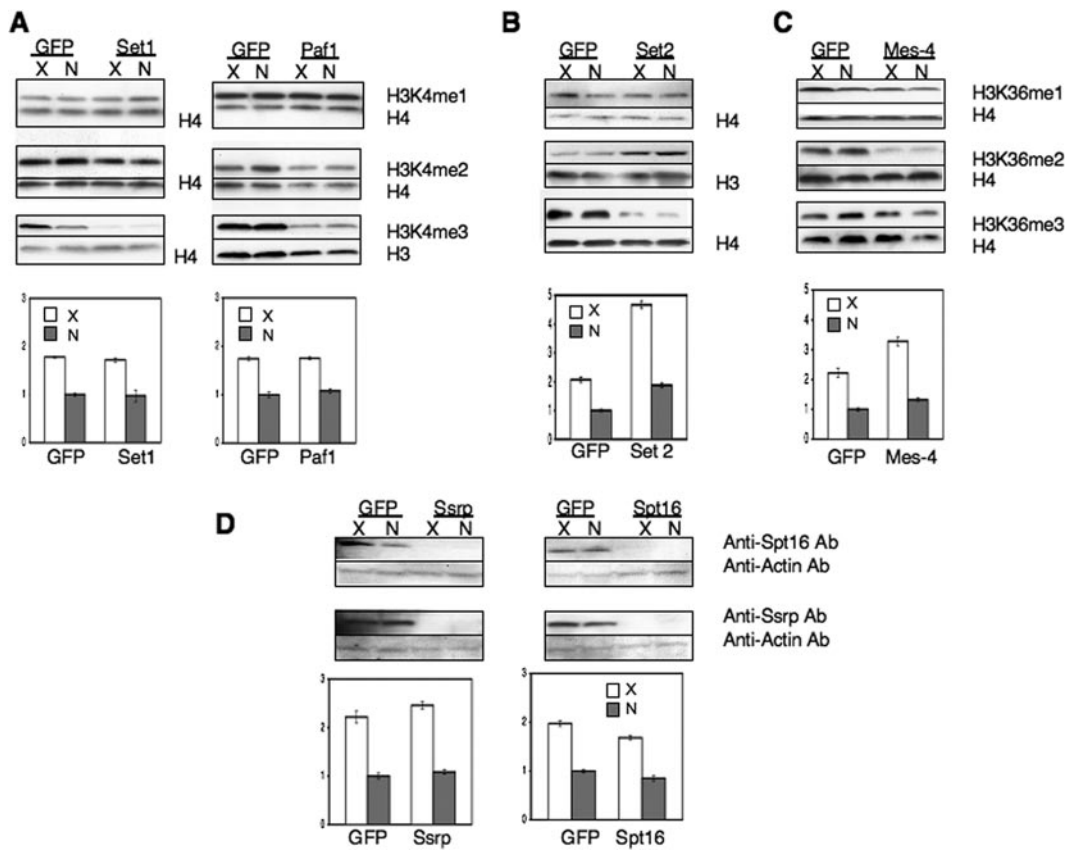


FIG. 2. RNAi-mediated depletion of selected transcription-associated histone marks. (A) Treatment with dSet1 or Paf1 dsRNA reduces the level of nuclear H3K4me3 (upper panels) but has no effect on dosage compensation (lower panels). This is evidenced by the approximate twofold increase in relative firefly to *Renilla* luciferase expression by the *roX*-bearing plasmid (white columns) relative to the control plasmid (gray columns). Similar results are obtained by reducing the levels of H3K36me3 (B) or of H3K36me2 (C) by RNAi of dSet2 or Mes-4, respectively. (D) Knockdown of the Ssrp or Spt16 subunits of the FACT complex has no effect on dosage compensation. Each experiment was repeated three times, and the error bars represent the standard deviations of the means. X and N refer to the roX-FF and N-FF (control) plasmids, respectively.

and dimethylated H3K36 (H3K36me2) throughout the length of the transcribed region (see reference 21 for a review; 26). In *Drosophila* and other organisms, these modifications are found predominantly on histone H3.3 rather than H3 (16). This is particularly true of the X chromosome, where the rate of replication-independent replacement of H3 is greater than on the autosomes because of the enhanced level of transcription of X-linked genes (17).

All of the evidence available to date leads to the conclusion that the MSL complex does not play a role in X-linked gene activation; rather, it modifies the chromatin organization of activated genes in order to enhance the level of their transcription. As noted above (Fig. 1D), depletion of the MSL complex by cotransfection with a Sxl-expressing plasmid or by RNAi targeted to the MSL2 subunit eliminates the transcriptional enhancement exhibited by the *roX*-bearing plasmid. This effect is thought to result from the absence of H4K16 acetylation. We tested this conclusion directly by RNAi depletion of MOF and observed that the level of dosage compensation of GFP-dsRNA-treated cells was reduced from  $2.0 \pm 0.07$  to  $1.44 \pm 0.15$  ( $n = 3$ ). We used RNAi to test whether other histone marks found downstream of the promoter region of transcribed genes play a role in enhancing the rate of RNAP II

elongation responsible for dosage compensation. We treated S2 cells with dsRNA homologous to CG1716, the *Drosophila* ortholog of yeast (*Saccharomyces cerevisiae*) SET2 that is responsible for the di- and trimethylation of H3K36, or CG40351, the *Drosophila* ortholog of yeast SET1 that is responsible for the di- and trimethylation of H3K4. We also depleted cells of PAF1 that is involved in the methylation of H3 at residues K4 and K79 and has been shown to interact physically with FACT in yeast. In *Drosophila*, PAF1 depletion diminishes the level of H3K4me3 greatly but has no effect on the di- or trimethylation of H3K79; in addition, the depletion of PAF1 results in significantly lower levels of FACT and Spt6 (1). Therefore, in order to parse the effects of depleting PAF1 on methylation and on the association of FACT with the transcript elongation complex, we targeted FACT specifically for RNAi. We used the plasmid system to assess the effects of all of these treatments on the mechanism of dosage compensation.

RNAi targeting of dSet1 or Paf1 reduced the level of H3K4me3 significantly in bulk chromatin without affecting the process of dosage compensation (Fig. 2A). Similar results were obtained by depleting dSET2, resulting in the overall decrease in the level of H3K36me3, with a concomitant increase in

H3K36me2 (Fig. 2B). We then searched the *Drosophila* database for additional homologs of dSet2 and discovered Mes-4. A histone methyltransferase with the same name exists in *Caenorhabditis elegans*, where it was shown to be responsible for dimethylated H3K36 (3). RNAi depletion of this enzyme in S2 cells substantially reduced the level of H3K36me2 without any significant effect on H3K36me3. Once again, there was no effect on dosage compensation by the depletion of MES-4 alone (FIG. 2C) or in combination with the depletion of dSET2 (see Fig. S3 in the supplemental material). Lastly, we achieved the depletion of FACT by using RNAi against the genes encoding its two subunits dSpt16 and dSsrp. Surprisingly, the depletion of either subunit resulted in the depletion of the other, suggesting that they depend on their association for stability. Once again, we noted no effect on dosage compensation (Fig. 2D).

To determine that RNA interference affects the chromatin of the reporter gene on the plasmid, we performed ChIP analysis with an antiserum that recognizes one of the methylation marks, H3K36me3. We carried out duplicate experiments, wherein RNAi against dSet2 reduced the nuclear levels of H3K36me3 to 18% and 20% of that in the control (GFP RNAi). This treatment resulted in a 2.6-fold decrease of the immunoprecipitated firefly luciferase gene as determined by using primers specific for the 5', internal, and 3' regions (see Fig. S4 in the supplemental material).

To establish that the results obtained with the plasmid system are an accurate reflection of the activity of endogenous chromosomal genes, we monitored the effects of dSET1, dSET2, and MES-4 depletion on the transcriptional activity level of three X-linked genes known to be dosage compensated. As a control, we measured the effect of knocking down the MSL complex on the same genes and on two autosomal genes that are not dosage compensated. The steady-state levels of transcripts of the X-linked *Pgd*, *Zw*, and *Spt6* genes (encoding 6PGD, G6PD, and the *Drosophila* homologue of yeast suppressor of Ty6 [dSPT6], respectively), as measured by real-time RT-PCR, confirm our previous observation that the depletion of these three histone methyltransferases has no effect on dosage compensation (Fig. 3).

Our results are concordant with those of Mason and Struhl (15), who reported that in yeast, the elongation rate of RNAP II is not affected in strains lacking each of a number of putative elongation factors, including Set1, Set2, and subunits of the Paf1 complex. Although these and the other histone marks and factors that we have tested have a clearly established association with the process of elongation, they are not used by the MSL complex to achieve the specific enhancement of transcription necessary for dosage compensation.

**Implications for targeting of the MSL complex and putative histone deacetylases.** As mentioned previously, the MSL complex normally forms on the X chromosome at the site of transcription of the *roX* genes and spreads by binding to numerous sites for which it has an entire range of affinities; it also binds to activated X-linked genes and enhances their rate of transcription in order to achieve dosage compensation. To date, the mechanism responsible for targeting of the complex to active genes is not understood. In yeast, methylated H3K36 recruits the histone deacetylase (HDAC) complex Rpd3S via the chromodomain of one of its subunits, Eaf3 (4, 9, 11). Eaf3 is a member of the MRG15/MSL3 family, leading to the

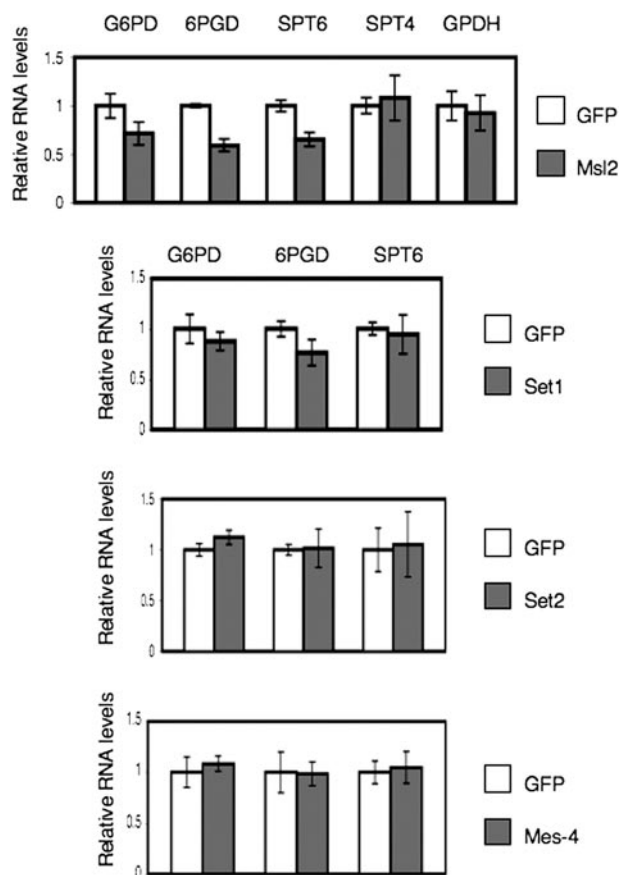


FIG. 3. The dosage compensation of endogenous X-linked genes is not affected by RNAi-mediated depletion of H3K4me3, H3K36me3, or H3K36me2. S2 cells were treated with dSet1, dSet2, or Mes-4 dsRNA; GFP dsRNA was used as a control. The steady-state levels of G6PD, PGD, and SPT6 were determined by real-time RT-PCR and corrected for the level of RP49 transcripts. The GFP control results are set at a value of 1, and an abrogation of dosage compensation would result in a decrease in the level of the three X-linked transcripts below this level. Such an effect is evident when RNAi is used to deplete the cells of the MSL complex by using dsRNA homologous to MSL2 (top panel, gray columns); SPT4 and GPDH are autosomal gene products whose levels are not affected by MSL2 RNAi.

thought that the MSL complex may be recruited to active genes via the chromodomain of MSL3. The data discussed in the previous section do not support this possibility, at least with regard to the specific methyl marks tested.

Yet, these same data may provide some circumstantial evidence for the recruitment of an HDAC activity to activated genes via the H3K36 methyl mark. As can be seen in Fig. 2B and C, the relative levels of firefly luciferase produced by the *roX*-bearing and control plasmids following SET2 and MES-4 RNAi are higher than those in the GFP knockdowns. This result could be explained by the failure to recruit an HDAC. The increase in the level of firefly luciferase produced by the *roX*-bearing plasmid is greater than the increase produced by the control plasmid. The reason for this difference in response of the two reporter genes is likely to be attributable to the enhanced acetylation of the *roX*-bearing compensated plasmid: while the acetylation marks characteristic of activated promoters should be present at comparable levels on FF and *roX*-FF

plasmids, the chromatin of the latter is highly enriched in H4K16ac (Fig. 1E).

**Possible mechanisms of transcriptional enhancement by H4K16ac.** Covalent modifications such as lysine acetylation and serine phosphorylation alter the charge differential between histones and DNA, thereby reducing the strength of their association and, at least in theory, facilitating the disassembly and removal of nucleosomes by the elongating RNAP II complex. A second mechanism of action of H4K16ac has been suggested by the discovery of an acidic patch formed by an H2A/H2B dimer with which the basic tail of histone H4 from a neighboring nucleosome can associate (6, 14). In reconstituted nucleosomal arrays, the acetylation of H4 at lysine 16 prevents the conversion of the array into a 30-nm fiber thought to represent a level of compaction unfavorable to transcription (23). The MSL complex may achieve the enhancement in the rate of transcription responsible for dosage compensation by either or both of these effects.

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