Targeting Dosage Compensation to the X Chromosome of *Drosophila* Males

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RNA has attracted increasing attention from biologists because of its versatile talents in biological processes. RNA's capabilities are not confined just within traditional models, as messenger RNA or scaffolds of a ribosome, but have been extended to catalysis and regulation of diverse biological functions, supporting a primordial "RNA world hypothesis." One of RNA's fascinating roles is regulation of gene expression as an RNP (ribonucleoprotein) complex. Examples include dosage compensation in *Drosophila* by *roX* RNAs, Xist/Tsix RNAs in mammalian female X-inactivation, and siRNAs/miRNAs in posttranscriptional gene regulation in various organisms. *roX* RNAs are required for correct targeting of dosage compensation complexes to the *Drosophila* X chromosome (Meller and Rattner 2002). We previously proposed that targeting occurs by spreading *in cis* from multiple, discrete initiation sites (Kelley et al. 1999; Park et al. 2002; Oh et al. 2003). Here we emphasize that spreading cannot be the sole mechanism for targeting of dosage compensation and suggest that the MSL (Male-Specific Lethal) complex has evolved multiple ways to recognize genes on the X (Oh et al. 2004).

MSL complexes bind the male X chromosome and induce twofold hypertranscription so that the amount of gene product from one male X chromosome equals that from both female X chromosomes. This requires at least five proteins—MLE (*maleless*); MSL1, MSL2, and MSL3 (*male-specific lethal 1, 2, and 3*, respectively); and MOF (*males absent on the first*)—and one of two *roX* (RNA on X) RNAs. MLE and MOF have enzymatic activities that are essential for dosage compensation: MLE is a DExH RNA helicase (Kuroda et al. 1991; Lee et al. 1997) and MOF is a MYST family histone acetyltransferase (Hilfiker et al. 1997; Akhtar and Becker 2000; Smith et al. 2000). JIL-1, a histone H3 kinase, also associates with the MSL proteins (Jin et al. 2000). The MSL proteins, JIL-1, and *roX* RNAs bind in a precise pattern along the length of the male X chromosome, resulting in enrichment of chromatin modifications associated with hypertranscription, such as histone H4 acetylated at lysine 16 and H3 phosphorylated at serine 10 (Turner et al. 1992; Wang et al. 2001).

The two noncoding RNAs, *roX1* and *roX2*, are functionally redundant (Meller and Rattner 2002) even though they have very little sequence homology and are distinct in size (3.7 kb for *roX1* RNA vs. 0.5–1.2 kb for

roX2 RNA) (Amrein and Axel 1997; Smith et al. 2000). Deletion of either *roX* gene has no effect on males. Missing both of them, however, results in male lethality (Meller et al. 1997; Meller and Rattner 2002). The MSL-binding pattern on the X chromosome is drastically disrupted in these *roX1 roX2* double mutant males, suggesting that *roX* RNAs are important for correctly targeting MSL complex to the X.

The targeting of hundreds of sites along the length of the X chromosome has been the subject of analysis and speculation for many years, but the mechanisms are still largely unknown. Initially, the prevailing view was that genes on the X evolved local *cis*-acting sequences for attracting the dosage compensation machinery. This was based on the observation that X-linked genes translocated to autosomes could retain dosage compensation. These studies demonstrated that the fly system is quite different from mammalian dosage compensation, in which a single region of the X (the X-inactivation center) controls the chromosome as a whole. Another key difference is that mammalian X-inactivation involves silencing one of two X chromosomes in females rather than up-regulation of the single X in males. However, several recent parallels suggest that the fly targeting mechanism might have mechanistic similarities to X-inactivation (Fig. 1). In both cases, noncoding RNAs (*roX1* and *roX2* in flies and *Xist* in mammals) are required for targeting the correct chromosome for regulation. Furthermore, in each case there is evidence for spreading of the dosage compensation process long distances along the chromosome from the sites of synthesis of those noncoding RNAs. Flies and mammals differ in that *Xist* is limited to action *in cis*, while *roX* RNAs and the MSL complex can clearly also act *in trans* (Meller et al. 1997).

MSL COMPLEX STRONGLY PREFERS TARGETING THE X CHROMOSOME

Large X to autosome transpositions retain both the ability to dosage compensate and the characteristic diffuse appearance of the male X chromosome (Offermann 1936; Aronson et al. 1954; Dobzhansky 1957). We analyzed several fly lines containing large X-ray-induced X to autosome transpositions obtained from the *Drosophila* stock center. The region of transposed X chromosome appears as wide as the paired autosomes that flank the in-

Figure 1. Spreading *in cis* of noncoding RNA from *Xist* and *roX* transgenes. (Left) In mammals, *Xist* RNA is transcribed from the XIC (X-inactivation center) and can spread *in cis* to “paint” the inactive X chromosome. An *Xist* transgene inserted on an autosome can cause *Xist* RNA to spread on the autosome. (Right) In flies, there are about 35 high-affinity sites (also called chromosome entry sites or CES) on the X chromosome including *roX1* and *roX2*. Like *Xist*, *roX1* and *roX2* transgenes also cause spreading *in cis* of noncoding RNAs on autosomes. (Adapted, with permission, from Park and Kuroda 2001 [©AAAS].)

section, suggesting that the transposed section of chromosome adopts a less compact chromatin structure similar to that of the intact male X chromosome. All X to A transposition stocks that we tested showed MSL binding within the transposed fragments (Fig. 2).

Figure 2. MSL complex strongly prefers binding to segments of the X chromosome, and avoids autosomes. Polytene chromosome squashes from male larvae containing large X to autosome (A–D) or autosome to X (E) transpositions, double stained with DAPI (blue) and antibodies specific for the MSL1 protein (red). (A) *Tp(1;3) sta* (1E1-2A:89B-C), (B) *Tp(1;3) JC153* (16E2-17A:99D), (C) *Tp(1;2) r⁺75C* (14B13-15A9:35D-E), (D) *Tp(1;2) rb⁺71g* (3F3-5E8:23A15), and (E) *Tp(3;1) O5* (88A-92C:4F2-4F6). Arrows indicate the site of transpositions. (Adapted, with permission, from Oh et al. 2004 [©Elsevier].)

Consistent with immunostaining of X to autosome transposition flies, an autosome to X transposition stock showed lack of MSL staining of a region of the third chromosome that was transposed to the X. MSL1 protein was detected at sites flanking the breakpoints of the transposition (Fig. 2, vertical arrows), but no staining was observed within the transposed section of the third chromosome. Thus, MSL immunostaining of translocation stocks supports a model featuring local control, suggesting that MSL complexes strongly prefer any piece of the X over the autosomes.

To characterize features of the X chromosome that attract the MSL complex, we isolated cosmids derived from X, inserted them on autosomes, and tested them for the ability to attract MSL complex to their ectopic site of insertion (Oh et al. 2004). In almost all cases, a 30-kb segment of X DNA appears sufficient to attract MSL complex, while smaller segments can be either positive or negative. Thus, it is possible that whatever marks the X might be dispersed on average every 20–30 kb, or that there is a cumulative effect of many very-low-affinity sites present on any 30-kb stretch of X-derived DNA. Ongoing transgenic studies should allow us to distinguish between these two possibilities.

Despite the global nature of regulation of the male X chromosome, not all genes on the X are dosage compensated (for review, see Baker et al. 1994), as is also the case for mammalian X-inactivation (Disteche 1995; Brown et al. 1997; Carrel et al. 1999). Genes that are sex-specific, present on both X and Y, or recently translocated to the X chromosome are not dosage compensated. This gene-by-gene adaptation for dosage compensation is likely to occur slowly, in response to the gradual degeneration of the Y chromosome. What could be the nature of *cis*-acting sequences? Molecular and evolutionary data have suggested that a unique sequence composition of the X chromosome correlates with dosage compensation (Huijser et al. 1987; Pardue et al. 1987; Lowenhaupt et al. 1989; Bachtrog et al. 1999; Gonzalez et al. 2002). Several repeated sequences (microsatellites), such as (CA)_n/

Figure 3. Local *cis* spreading of MSL complexes on the X chromosome. Polytene chromosomes were stained with anti-MSL1 antibodies, and visualized with Texas Red-conjugated secondary antibody (red). DNA was stained with DAPI (blue). Arrowheads and arrows indicate the locations of the *roX1* and *roX2* genes, respectively. The genotypes are (A) *msl3*[M1][M2] female, (B) wild-type male, (C) *roX1*^{ex6}; [M1][M2] male, (D) *Df(1)roX2*⁵²; [M1][M2] male, (E) *roX1*^{ex6} *Df(1)roX2*⁵² [*w*⁺ GMroX1-13A]; [M1][M2] male, and (F) *roX1*^{ex6} *Df(1)roX2*⁵² [*w*⁺ GMroX1-18C] male without extra MSL1 and MSL2. [M1][M2] stands for [*w*⁺ H83M1][*w*⁺ H83M2]/+ on the third chromosome. (G,H) Model for *cis*- vs. *trans*-interaction of MSL complexes with the X chromosome. (Adapted, with permission, from Oh et al. 2003.)

G. High ratio of MSL proteins / *roX*RNA (*cis* >> *trans*)

H. Normal ratio of MSL proteins / *roX*RNA

(GT)_n, (CT)_n/(GA)_n, (TA)_n, and (GC)_n, are more abundant on the X chromosome than on the autosomes. However, no apparent consensus sequence has been found near X-linked genes that retain dosage compensation when moved to autosomes (for review, see Baker et al. 1994). Clearly, a genomic rather than piecemeal approach will be needed to identify features in DNA that attract the MSL complex.

LOCAL SPREADING OF MSL COMPLEX FROM *roX* GENES

In the absence of individual MSL proteins (MSL3, MLE, or MOF), the core subunits MSL1 and MSL2 bind approximately 35 high-affinity sites (Fig. 3A), which are dispersed along the X chromosome (Palmer et al. 1994; Lyman et al. 1997; Gu et al. 1998). Two of these are the *roX* genes themselves, which encode RNA components of the MSL complex (Amrein and Axel 1997; Meller et al. 1997; Kelley et al. 1999). The spreading model for MSL targeting to the X is based on the observation that MSL complexes spread *in cis* from small *roX* transgenes inserted on the autosomes, regardless of neighboring sequences (Kelley et al. 1999). Although the identities of the remaining high-affinity sites were not known, we proposed that they might also behave as spreading initiation sites based on the behavior of the two *roX* genes. In this model, MSL complexes would first recognize high-affinity sites in a sequence-specific manner, followed by spreading *in cis* along the neighboring chromatin to up-regulate flanking genes (Kelley et al. 1999). Regulation would still be relatively local, since there are approximately 35 dispersed high-affinity sites. The evolution of specific *cis*-acting sequences at each dosage compensated gene might not be necessary, as the complex might spread

in cis from initiation sites to recognize something common to active genes.

Evidence for the *cis* spreading model came initially from studies of autosomal *roX* transgenes. Subsequently, we demonstrated that MSL complexes can spread locally from the endogenous *roX* genes on the X, the natural target of dosage compensation. We observed that wild-type males require a balance of MSL proteins and *roX* RNAs to evenly distribute MSL complexes both locally and at a distance along the X chromosome (Fig. 3B). When we artificially increased the amounts of MSL1 and MSL2, thought to be the limiting proteins (Kelley et al. 1997; Chang and Kuroda 1998; Park et al. 2002), and lowered the number of *roX* genes, MSL complexes concentrated predominantly over a local segment of the X surrounding a *roX* gene (Fig. 3C,D). More remote regions of the X were relatively depleted of MSL complex. This dramatically altered the morphology of polytene X chromosomes in many nuclei. When the *roX* genes were moved to new locations along the *roX*-deficient X chromosome, additional MSL1 and MSL2 produced bright MSL staining and diffuse chromosome morphology surrounding the re-located *roX*⁺ gene (Fig. 3E). Again, more distant regions of the X had much less MSL staining. Because a strong bias for local MSL spreading was seen at several different locations of *roX* transgenes on the X chromosome, the sequences surrounding the endogenous *roX* genes are unlikely to contain special spreading elements. Moreover, some *roX* transgenes displayed a strong preference for regional spreading from their site of insertion with only wild-type levels of MSL1 and MSL2 (Fig. 3F). This indicates that changes in the MSL protein:*roX* RNA ratio or chromatin environment can produce large shifts in the pattern of local MSL spreading. A model based on these observations proposes that if MSL proteins are abundant

and rapidly assemble onto growing *roX* transcripts, functional complexes will be completed before release of the nascent *roX* transcript from the DNA template. These complexes are postulated to immediately bind the flanking chromosome regardless of sequence and begin spreading *in cis* (Fig. 3G). When *roX* genes compete for a finite supply of MSL proteins, nascent *roX* RNA may be released from the template with an incomplete set of MSL subunits. After maturation is completed in solution, these complexes are postulated to diffuse through the nucleus until encountering the X (Fig. 3H).

These results suggest that the *roX* genes are the predominant spreading sites on the X chromosome. However, *roX*-deficient X chromosomes are painted by MSL complex as long as *roX* RNA is supplied from autosomes (Meller et al. 2000), suggesting that the nascent *roX* transcript can function as an assembly site for MSL proteins in any location (Park et al. 2003) and that other high-affinity sites might play a role in targeting MSL complex to the X chromosome.

TARGETING OF MSL COMPLEX THROUGH HIGH-AFFINITY SITES ON X: A COMPARISON OF *roX* GENES AND THE 18D REGION

We initially postulated that the approximately 35 high-affinity sites might contain a common sequence to attract the MSL complex. Therefore, we analyzed the MSL-binding sites within *roX* genes to look for targeting motifs. DNaseI hypersensitivity and transgenic deletion mapping identified an ~200-bp MSL-binding site in each *roX* gene, designated as DHS (DNaseI hypersensitive site) (Kageyama et al. 2001; Park et al. 2003). Sequence alignments revealed short stretches of evolutionarily conserved consensus elements in both sites and mutagenesis data have suggested that they are essential for MSL binding (Park et al. 2003). Unfortunately, this MSL-binding sequence has not led us to identify other high-affinity sites.

Surprisingly, we recently found that these strong MSL-binding sites within *roX* genes are not essential for spreading *in cis* from *roX* transgenes (Bai et al. 2004). Rather, the sites are utilized by MSL proteins to specifically up-regulate transcription of *roX* RNAs in males. How can spreading be initiated from a transgene in the absence of an observable MSL-binding site? Perhaps initial RNA binding can lead to a histone-binding/modification cycle in the absence of a specific DNA interaction, as proposed for HP1 spreading to form heterochromatin (Bannister et al. 2001; Lachner et al. 2001). This would be consistent with previous studies that *roX* RNA itself is the initial assembly target for MSL proteins and that copies of nontranscribed *roX* DNA cannot compete for assembly and spreading of MSL complexes (Park et al. 2002).

The idea that *roX* DHS sequences are dedicated to specific regulation of *roX* RNAs may explain why we were unable to find the MSL-binding consensus sequences at other sites on the X chromosome. Several other lines of evidence suggest that the majority of high-affinity sites are quite different from *roX* genes (Oh et al. 2003, 2004; and see below). First, the *roX* genes are highly dependent on MLE for recruiting MSL complex, unlike the other sites,

which still display MSL binding in *mle*⁻ mutants (Meller et al. 2000; Kageyama et al. 2001). Second, the two *roX* RNAs seem to be the only RNA components of the MSL complex, suggested by male-specific SAGE analysis (Fujii and Amrein 2002) and subtractive RNA analysis from immunoprecipitated MSL complex (Oh et al. 2003). Third, the *roX* genes appear to be the two major MSL-spreading initiation sites, where the balance between local spreading *in cis* and diffusion of soluble complex *in trans* to the X may be determined by the rate of assembly of MSL proteins onto nascent *roX* transcripts (Oh et al. 2003).

To understand what role, if any, the approximately 35 high-affinity sites play in MSL targeting, we characterized an additional high-affinity MSL-binding site (18D10). We screened a cosmid library, constructed an overlapping cosmid contig around 18D10, created transgenic lines for each of the cosmids, and tested them for MSL binding at their new sites of insertion. In an *msl3*⁻ genetic background in which the high-affinity sites are most easily monitored, only one out of the three cosmids showed a strong MSL signal, comparable to the endogenous 18D10 region on the X chromosome. Further analysis, including male-specific DHS assays and chromatin immunoprecipitation (ChIP) assays, narrowed it down to a 510-bp fragment, which showed quite different characteristics from the *roX* DHS. First, the 18D entry site does not share any apparent sequence similarity with the *roX* DHS. Second, the 18D region does not seem to be transcribed nor encode a noncoding RNA. Third, the 510-bp fragment from the 18D region must be multimerized, while *roX* DHS (~200-bp) monomer was sufficient to recruit partial MSL complexes. Finally, MSL spreading from an 18D transgene was very rare and limited, consistent with a lack of RNA product for assembly of MSL complexes. These results suggest the possibility that interaction of MSL complexes with other high-affinity sites requires multiple, relatively weak, and dispersed *cis*-elements, instead of a strong interaction within a relatively short (~200-bp) *roX* DHS.

What could be the consequence of recruitment of the MSL complex to high-affinity sites? First, the other sites could function as male-specific enhancers to induce male-specific transcription of neighboring genes, like the *roX* DHS (Bai et al. 2004). Second, if we assume that several X-enriched *cis*-elements are required for MSL complex binding, those sequences may have evolved to cluster around genes which are haplo-insufficient, thus requiring dosage compensation. How many genes are dosage-compensated by MSL complexes is not known currently, so it is possible that the high-affinity sites are located around especially important target genes. Third, the other sites, evenly distributed along the X chromosome, may have evolved to act as physical facilitators of MSL spreading, to make it efficient and balanced. Finally, although spreading from 18D10 transgenes appears rare and modest, even MSL spreading from *roX* genes was rare in the presence of other *roX* genes in the genome, presumably because of the limited availability of protein components (Park et al. 2002; Oh et al. 2003). Therefore, it is still possible that the 18D10 site and other sites act as weak spreading nucleation sites to help paint the X chromosome with MSL complexes.

Figure 4. Genes on the X may have evolved multiple ways to attract the MSL complex. (A) Segments without a high-affinity site may contain weak-affinity sites that cooperate to recruit the MSL complex, followed by spreading only when complex reaches a threshold concentration. (B) Sites of *roX* RNA production recruit enough MSL complex for variable spreading, through a high local concentration of MSL complex. Current evidence suggests that the nascent *roX* RNA alone can be sufficient to reach this concentration even in the absence of the high-affinity DHS site. (C) An intermediate-affinity site requires another intermediate- or weak-affinity site for recruiting and spreading of MSL complex. (Adapted, with permission, from Oh et al. 2004 [©Elsevier].)

A MODEL FOR MSL BINDING AND SPREADING ON THE X CHROMOSOME

Our current data can be interpreted in the following framework (Fig. 4A–C). Perhaps there are diverse DNA recognition elements on the X chromosome that have different affinities for MSL complex: high, intermediate, or weak. High-affinity *cis*-elements, such as within the *roX* genes, would not require additional *cis*-elements for recruiting MSL complexes and might be involved in multi-fold gene activation instead of twofold hypertranscription. This interaction might be strengthened by *roX* RNA (Fig. 4B). An intermediate-affinity *cis*-element like the 18D10 site might require additional intermediate- and/or weak-affinity elements for robust binding and would have the ability to attract partial MSL complexes with a minimal MSL1/MSL2 composition (Fig. 4C). Weak-affinity *cis*-elements might require interaction with several additional weak-affinity *cis*-elements, which might explain occasional autosomal MSL signals and how X fragments on the autosomes attract wild-type MSL complexes even without a high-affinity site (Fig. 4A).

Regulation of MSL complex binding to target genes by multiple relatively weak *cis*-elements might be the most efficient way to achieve twofold activation, or could reflect the simplest way to evolve MSL activity on the X chromosome. MSL spreading may be mainly dependent on the local concentration of functional complex regardless of the presence of specific sequences.

SUMMARY AND CONCLUSION

A fundamental difference between *Xist* RNA and *roX* RNA is that transcription of *Xist* RNA can lead to inactivation of a whole chromosome, only *in cis*, whereas the

presence of a *roX* gene is not sufficient to induce chromosome-wide gene regulation on a chromosome that it normally does not regulate. Instead, MSL complex prefers binding to the *roX*-deficient X chromosome, indicating that there is something else on the X chromosome that attracts MSL complexes. So far, we have identified a high-affinity site for MSL complex in the 18D10 region on the X chromosome showing male-specific DNaseI hypersensitivity, partial MSL complex recruitment in the absence of MSL3, and some MSL spreading from the high-affinity site. However, unlike our expectation that the other sites might share their own conserved sequence, no similar sequence to the 18D site was found in the rest of the genome. It is possible that conserved DNA sequences for MSL binding might be composed of short stretches of a few nucleotides, which makes homology search efforts very difficult. Another possibility is that MSL complex recognizes local chromatin structure, DNA topology, or a specific location within the nucleus, independent of nucleotide sequence. Given the fact that the Y chromosome has gradually degenerated over time and the X has evolved to overcome the deficiency of X-linked gene products in males, it is also possible that dosage-compensated genes evolved independently and ended up having a diversity of DNA sequences for MSL complex binding.

ISSUES FOR FUTURE STUDIES

Molecular and Functional Bases for Redundancy of *roX1* and *roX2*

Even though *roX1* (3.7 kb) and *roX2* (0.5 kb) are very different in size and sequence, they show a redundant

function. However, the exact role of these *roX* RNAs in targeting and spreading of MSL complex to the male X chromosome is unknown. Therefore, characterization of the function of these *roX* RNAs will be a key to understanding how this RNP complex regulates chromatin organization.

It was reported that MSL3 and MOF have RNA-binding activity in vitro (Akhtar et al. 2000). In addition, MSL3, MOF, and MLE were shown to be dissociated from the MSL complex with RNase treatment in vivo, suggesting that the *roX* RNAs interact with each of those three MSL proteins to keep the MSL complex structure intact and functional. How MSL proteins and *roX* RNAs are associated with each other remains to be solved. Therefore, it is important to define the key functional domains of *roX* RNAs.

MSL proteins and *roX* RNAs are present in several other *Drosophila* species and are thought to localize to the male X chromosome. To find functional domains of *roX* RNAs, a phylogenetic comparison method could be employed to find conserved sequences and/or structure of *roX* RNAs in several different *Drosophila* species. Secondary or tertiary structures should be important for the function of noncoding *roX* RNAs, and therefore it is likely that the structure of essential domains in *roX* RNAs is conserved among different fly species, despite the substantial divergence in primary sequences of noncoding RNA through evolution. In this respect, it is much easier to map the functional domains within *roX2* RNA instead of *roX1*, since *roX2* (0.5 kb) is smaller than *roX1* (3.7 kb) RNA and nevertheless they show functional redundancy. Once the conserved secondary structures are defined, mutation, deletion, or domain-swapping experiments could be performed to draw a functional map between *roX* RNA and MSL proteins, which will help us understand the mechanism of gene regulation by the RNP complex.

A Delicate Twofold Up-Regulation of Gene Expression in Male X-linked Genes

Proper gene expression in the process of development is often achieved by dynamic reversible modification of histones to change chromatin structure at each gene locus. The *Drosophila* male X chromosome is acetylated at H4K16 by MOF protein. It is not known how the acetylated H4K16 is regulated to achieve just twofold activation of X-linked genes. Compared to X-inactivation, dealing with twofold up-regulation (in male flies) or down-regulation (in hemaphrodite *Caenorhabditis elegans*) requires much finer control of a dosage compensation complex. One possible way to keep the level of regulation twofold could be achieved by weak interactions between *cis*-acting DNA sequences and the *trans*-acting protein complex. Even though no conserved *cis*-element has been found around dosage compensated genes either in *Drosophila* or *C. elegans*, the *roX* genes in flies and the *her-1* gene in worms interact with dosage compensation complexes to activate (*roX*) or repress (*her-1*), not just twofold but in a sex-specific way. In each case, those high-affinity sequences were not found in other regions in

either fly or *C. elegans*, indicating that the sequences for twofold regulation might be quite different. It is possible that some X-enriched repetitive nucleotide sequence might be responsible for weak interactions with the dosage compensation complex in both systems.

Another way of achieving just twofold regulation could be limiting the amount of dosage compensation complex in the nucleus. In metamorphosing (1X3A), transcription of X-linked genes is increased and more diffuse X morphology is observed compared to the normal male (1X2A) (Lucchesi et al. 1977). This observation suggests that *trans*-acting factors encoded by autosomes (e.g., MSL2) may be normally limiting in diploids, and responsible for additional hyperactivation of the X chromosome when overexpressed. In *C. elegans*, the SDC complex recruitment to the X chromosome is reduced in the presence of multicopy arrays of putative SDC-binding fragments (Csankovszki et al. 2004). If dosage compensation complexes are limiting, how is the amount of complex controlled? It is possible that the stability or activity of protein components can be regulated by reversible protein modifications such as ubiquitination, phosphorylation, and acetylation. It was recently shown that MSL3 and MSL1 can be acetylated by MOF, which is a H4 acetyltransferase in the MSL complex (Buscaino et al. 2003; Morales et al. 2004). MSL1 also has several PEST domains (Chang and Kuroda 1998), which may be a target for phosphorylation, often followed by ubiquitination and degradation. MSL2 has a Ring-finger domain, which might act in the ubiquitination pathway as a E3 ligase to degrade interacting proteins or to modulate transcriptional activity of the interacting complex. Even though Jil-1 was initially found as an H3 kinase, it is possible that Jil-1 might phosphorylate other MSL proteins to modulate activity of MSL complex to achieve the proper level of gene expression.

Since MSL1 and MSL2 are less conserved among different species and function as two core components in the MSL complex, it is possible that those two proteins are the most ancient members of the MSL complex that have managed to achieve a primitive but inefficient mechanism of dosage compensation. As more genes degenerated on the Y chromosome, perhaps a more efficient mechanism of dosage compensation was adapted by adding general histone modifying enzymes to a core MSL1/MSL2 complex. Perhaps *roX* RNAs came even more recently, like a virus infection, and landed on the X chromosome by accident, giving the local region an advantage with regard to dosage compensation by a spreading mechanism.

cis-acting Elements on the X Chromosome

It seems that multiple classes of MSL-binding sites target dosage compensation to the X chromosome in fly. Many questions remain in the field of dosage compensation with regard to the identities of *cis*-acting elements on the X chromosome. Now that the fly genome sequence is available, more systematic approaches can be employed to find those *cis*-acting sequences on the X. Since it has

been shown that there are X-enriched microsatellites such as (CA)_n/(GT)_n, (CT)_n/(GA)_n, (TA)_n, and (GC)_n (Huijser et al. 1987; Pardue et al. 1987; Lowenhaupt et al. 1989; Bachtrog et al. 1999; Gonzalez et al. 2002), the link between those repeated nucleotides and dosage compensation can be characterized by combining bioinformatics with molecular analyses such as in vivo MSL binding or chromatin immunoprecipitation assays. A microarray-based "ChIP on chip" analysis should be especially informative, although the availability of the proper microarray chips, which include intergenic regions, would be a key factor. Identification of a large number of small, specific *cis*-acting elements should greatly increase our understanding of how the MSL complex is recruited to achieve global twofold regulation of the male X chromosome.

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