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The Amino-Terminal Region of *Drosophila* MSL1 Contains Basic, Glycine-Rich, and Leucine Zipper-Like Motifs That Promote X Chromosome Binding, Self-Association, and MSL2 Binding, Respectively

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In *Drosophila melanogaster*, X chromosome dosage compensation is achieved by doubling the transcription of most X-linked genes. The male-specific lethal (MSL) complex is required for this process and binds to hundreds of sites on the male X chromosome. The MSL1 protein is essential for X chromosome binding and serves as a central scaffold for MSL complex assembly. We find that the amino-terminal region of MSL1 binds to hundreds of sites on the X chromosome in normal males but only to approximately 30 high-affinity sites in the absence of endogenous MSL1. Binding to the high-affinity sites requires a basic motif at the amino terminus that is conserved among *Drosophila* species. X chromosome binding also requires a conserved leucine zipper-like motif that binds to MSL2. A glycine-rich motif between the basic and leucine-zipper-like motifs mediates MSL1 self-association in vitro and binding of the amino-terminal region of MSL1 to the MSL complex assembled on the male X chromosome. We propose that the basic region may mediate DNA binding and that the glycine-rich region may promote the association of MSL complexes to closely adjacent sites on the X chromosome.

Significant progress has been made in understanding the regulation of transcription of individual genes in eukaryotes (9, 42). It has also become apparent that the transcription of many genes within a particular region of a chromosome can be coordinately regulated by mechanisms that involve changes in the local chromosome structure (15, 25). The most dramatic example of this is X chromosome dosage compensation, the equalization of X-linked gene transcription between XY males and XX females (1, 31). In mammals, this is achieved by compacting one X chromosome in females into an inactive heterochromatic structure (19). In *Drosophila melanogaster*, the male X chromosome is modified to a more open structure that somehow leads to a precise doubling of transcription of nearly all the predicted 2,240 X-linked genes (12, 14).

Dosage compensation in *Drosophila* requires the ribonucleoprotein male-specific lethal (MSL) complex (1). The complex binds to hundreds of sites along the male X chromosome (17, 26). The core protein components are MSL1, MSL2, MSL3, MLE, and MOF. Loss-of-function mutations in any of the genes encoding these proteins lead to male-specific lethality, due to a failure in dosage compensatation (5, 20). A sixth protein, JIL1, preferentially associates with the male X chromosome and has been shown to coimmunoprecipitate with components of the MSL complex (21). Loss-of-function *jil1* mutations are, however, lethal to both sexes, indicating a vital role for JIL1 in addition to X chromosome dosage compensation. The two noncoding RNA components of the complex, *roX1* and *roX2*, share little sequence similarity but are genetically redundant and appear to be functionally interchangeable (32). The MSL complex does not assemble in females as one protein component, MSL2, is absent (4, 24, 47).

MSL1 plays a central role in assembly of the MSL complex (41). The amino-terminal domain of MSL1 binds to MSL2 (8, 41). We previously suggested that the interaction between MSL1 and MSL2 was via predicted amphipathic coiled-coil α-helical regions that are found within the interacting domains (41). In addition, the carboxyl-terminal domain of MSL1 binds to both MSL3 and MOF (41). Subsequent studies have shown that MOF and MSL3 bind to adjacent regions in the MSL1 carboxyl-terminal domain (34). Further, formation of the MSL1/MSL3/MOF complex leads to a significant increase in the histone acetylase activity of MOF (34), which preferentially acetylates histone H4 at lysine 16 (2, 43). Both MSL3 and MOF have been shown to bind RNA nonspecifically in vitro and thus may have a role in incorporation of *roX* RNAs into the complex (3). Incorporation of MLE into the complex is presumably via interaction with the *roX* RNA, as MLE contains an RNA binding domain but does not appear to interact with any of the other protein components of the complex (8).

While progress has been made in understanding MSL complex assembly, how the complex specifically binds to hundreds of sites on the male X chromosome and then upregulates transcription so precisely remains poorly understood. One model for X chromosome binding is that the first step involves recognition of approximately 30 "high-affinity" or "chromatin entry" sites on the X chromosome by the MSL1/MSL2 dimeric

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complex (22). Additional binding to the high-affinity sites within the *roX1* and *roX2* genes requires MLE. Subsequently, the other components bind, and the complex then spreads along the chromosome to hundreds of other sites. One of the key observations that support this model is that the MSL1/MSL2 complex binds to the high-affinity sites in the absence of MSL3, MLE, or MOF (17, 27). MSL1 and MSL2, however, do not contain any of the well-characterized DNA binding domains (36, 47). We previously found that a deletion mutant of MSL1 that lacked the first 84 amino acids (aa) bound to MSL2, MSL3, and MOF but failed to bind to the X chromosome (41). This result suggested an important role for the amino-terminal region in X chromosome binding. Here, we show that a conserved basic segment at the amino terminus of MSL1 is essential for binding to the high-affinity sites on the X chromosome in the absence of endogenous MSL1. We also find that the adjacent region of MSL1 mediates MSL1 self-association. Lastly, we confirm the importance of the predicted coiled-coil region of MSL1 in binding to MSL2 and that this interaction is essential for binding of the amino-terminal region of MSL1 to the X chromosome.

MATERIALS AND METHODS

Plasmid construction and sequence analysis. An 0.8-kb HpaI/EcoRI (MSL1 aa 1 to 265) fragment from the *msl1* gene, together with an EcoRI/XbaI hemagglutinin (HA) tag was inserted into EcoRV/XbaI sites of pBluescript II KS (Stratagene). The EcoRI/XbaI HA tag was made by annealing the synthetic oligonucleotides 5′-AATTCTACCCCTACGATGTGCCCGATTACGCCTAA T-3′ and 5′-CTAGATTAGGCGTAATCGGGCACATCGTAGGGGTAG-3′.

The 0.8-kb NotI/XbaI fragment containing the HA tag was inserted into the NotI/XbaI sites of P transformation vector pCaSpeR-hs. Subsequently, this construct was used as a template for PCR to make the truncated and site-directed mutants. The sequences of the primers used to make these constructs and amplification conditions are available on request. The respective PCR fragments were digested with NotI/XbaI and inserted into pCaSpeR-hs. To make the MSL2 N terminus, PCR primers containing FLAG tag were designed. The PCR fragments were digested with EcoRI/XbaI and cloned to pCaSpeR-hs. All constructs were confirmed by DNA sequencing.

Multiple sequence alignments of MSL1 amino acid sequences were performed using Clustal W and then refined manually. Accession numbers for the sequences used are available upon request.

Fly crosses and transgenesis. Flies were grown at 25°C on standard cornmeal-yeast-sugar-molasses medium. To create homozygous $msl1^{L60}$ female larvae that express MSL2 and MSL1NHA, P[*MSL1NHA* w^+] *y w*; *Bc*/$msl1^{L60}$ males were crossed to *w*; $msl1^{L60}$; P[*H83M2-6I* w^+] females. For other *msl1* constructs, we first selected recombinants carrying the transgene and $msl1^{L60}$. Recombinant *y w*/Y; P[*MSL1NHAmut* w^+] $msl1^{L60}$/*Bc* males were crossed to *w*; $msl1^{L60}$; P[*H83M2-6I* w^+] females. Homozygous $msl1^{L60}$ female larvae were distinguished from their heterozygous siblings by the absence of the dominant black cell marker.

To make transgenic flies, plasmids were purified by CsCl-ethidium bromide gradient centrifugation and microinjected together with Δ2,3 helper plasmid into *y w* embryos by standard procedures (40). Transgenics were identified due to expression of the *white* marker gene.

Immunofluorescent chromosome staining, immunoprecipitation, and Western blotting. Polytene chromosome squashes and immunostaining procedures were as described by Lyman et al. (27). Transgenic larvae were grown at 25°C. Primary rat anti-HA (Roche), rabbit anti-MSL2, and anti-MOF antibodies were used at a dilution of 1:50. Fluorescein isothiocyanate-conjugated rabbit anti-rat (Sigma) and Alexa Fluor-594-conjugated donkey anti-rabbit (Molecular Probes) secondary antibodies were used at a dilution of 1:600 and 1:1,000, respectively. DNA was counterstained with 4′,6′-diamidino-2-phenylindole (DAPI).

To confirm protein expression of transgenic flies, adult flies were heat shocked at 37°C for 1 h, and then recovery took place at 25°C for 4 h. A protein extract was prepared, and Western blots were performed as described previously (41). For immunoprecipitation, approximately 50 heat-treated flies were homogenized in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 300 mM NaCl, 2 mM EDTA, 1% NP-40) containing protease inhibitor (Roche). The homogenate was incubated for 30 min on ice and then centrifuged at 12,000 × *g* for 10 min at 4°C to remove debris. Protein concentration was determined by the Bradford method with a protein assay kit (Bio-Rad). A total of 0.7 mg of protein extracts was incubated with 25 μl of anti-HA affinity beads (Roche) at 4°C for 4 h with constant rocking. The beads were washed three times with washing buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% NP-40). Bound proteins were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, and Western blot analysis was performed.

In vitro transcription and translation; immunoprecipitation pulldown. DNA templates for in vitro transcription and translation were prepared from pCaSpeR-hs templates by PCR as described previously (41). Coupled in vitro transcription-translation reactions were performed with the TNT system (Promega) according to the manufacturer's instructions. For immunoprecipitation reactions, approximately 0.7 mg protein extract from transgenic flies that express MSL2NFLAG was incubated with anti-FLAG affinity beads (Sigma), and the beads were then washed extensively. A total of 4 μl of [^{35}S]methionine-labeled in vitro-translated proteins was then mixed with the prebound beads in 200 μl of binding buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% NP-40) containing protease inhibitor at 4°C for 4 h. The beads were washed four times with a high-salt binding buffer (containing 500 mM NaCl) and then once with standard binding buffer. Bound proteins were analyzed on 10% SDS-PAGE gels, followed by autoradiography.

RESULTS

A comparison of *Drosophila* species MSL1 proteins identifies two conserved regions. Using standard bioinformatics methods, we identified sequences encoding probable homologs of *Drosophila melanogaster* MSL1 in the completed genome of *Drosophila pseudoobscura* (39) and in the draft genomes of five other *Drosophila* species (12). An alignment of the amino-terminal domain sequences of the seven MSL1 sequences is shown in Fig. 1. As anticipated, the greatest similarity was between the *D. melanogaster* MSL1 sequence and MSL1 sequences from the closely related species *Drosophila simulans*, *Drosophila yakuba*, and *Drosophila erecta*. However, there was also significant similarity with *Drosophila pseudoobscura* and *Drosophila virilis* MSL1s, which are more distantly related to *D. melanogaster*. Two well-conserved regions were apparent from the alignment. Eight of the first 16 amino acids were identical in all *Drosophila* MSL1s. In particular, the basic and aromatic amino acids were highly conserved. The predicted coiled-coil region (aa 96 to 159 of *D. melanogaster*) was also highly conserved. The region between the two conserved regions (aa 16 to 95) had few amino acids that were identical but was enriched for glycine, proline, histidine, and asparagine.

The amino-terminal domain of MSL1 binds to hundreds of sites on the male X chromosome. We previously found that a version of MSL1 missing the first 84 amino acids with a FLAG tag at the amino end did not bind to the male X chromosome (41). Full-length MSL1 with an amino-terminal FLAG tag did bind to the male X chromosome, although binding was not strong. This indicated that the first 84 amino acids were important for X chromosome binding and that adding a FLAG tag at the amino end may have interfered with binding. To determine if the amino-terminal domain was sufficient for X chromosome binding, we made transgenic *Drosophila* lines that expressed the domain with an HA epitope tag at the carboxyl end (MSL1NHA) (Fig. 2A). The domain includes the conserved N-terminal basic region, the predicted coiled coil, and an acidic region (aa 179 to 186). We predicted that this domain would be able to bind to the male X chromosome but only to the ~30 high-affinity sites. This is because the amino-terminal

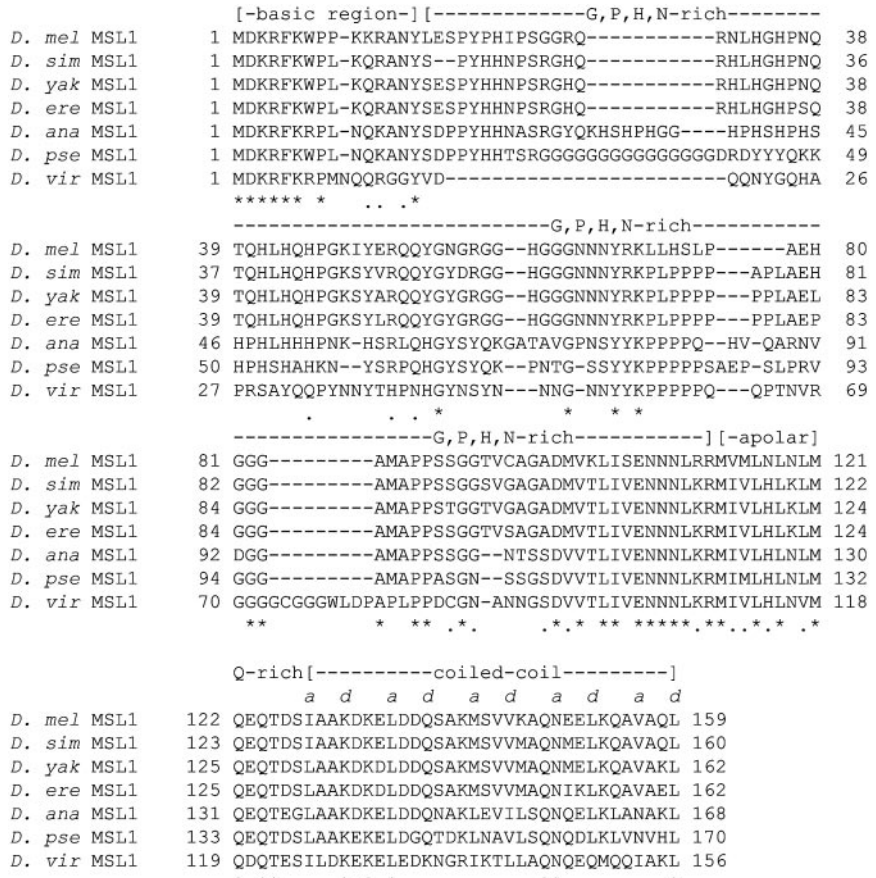

FIG. 1. Alignment of the amino-terminal domain sequences of MSL1 homologs from seven *Drosophila* species. Likely homologs of *D. melanogaster* MSL1 were identified from the completed genome sequence of *D. pseudoobscura* (*D. pse*) and the draft genome sequences of *D. simulans* (*D. sim*), *D. yakuba* (*D. yak*), *D. erecta* (*D. ere*), and *D. virilis* (*D. vir*). The basic region (aa 1 to 15), apolar region (aa 113 to 121), glutamine (Q)-rich region (aa 122 to 127), and coiled-coil domain (aa 128 to 159) are all well conserved. While few individual amino acids in the glycine (G)-, proline (P)-, histidine (H)-, and asparagine (N)-rich domain (aa 16 to 112) are well conserved, all *Drosophila* MSL1s contain a high proportion of G, P, H, and N in this region.

domain does not interact with MOF and MSL3, both of which are needed for the MSL1/MSL2 complex to bind to sites on the X chromosome other than the high-affinity sites (17, 37). However, we found that the HA-tagged amino-terminal domain of MSL1 (MSL1NHA) bound to hundreds of sites on the male X chromosome (Fig. 2C and D). Identical results were obtained if MSL1NHA expression was controlled by either the strongly heat-inducible *hsp70* promoter (Fig. 2C and D) or the constitutive *armadillo* promoter (Fig. 2E). Further, we found that with the *hsp70* construct, basal-level expression at 25°C was sufficient to detect X chromosome binding of MSL1NHA (Fig. 2D). Heat shock treatment to overexpress MSL1NHA did not lead to a significant increase in binding to the autosomes (Fig. 2C), nor did it disrupt X chromosome binding by other components of the MSL complex (data not shown). Since heat treatment was not necessary to detect X chromosome binding of MSL1NHA, all additional experiments in this study were performed with larvae raised at 25°C without heat shock. Surprisingly, daily heat-shock treatment of the progeny of an *MSL1NHA* line had little effect on male viability (85 male and 119 female progeny obtained), indicating that binding of MSL1NHA to the X chromosome did not significantly disrupt MSL complex activity. In contrast, we previously found that overexpression of a truncated version of MSL1 missing the first 84 amino acids that did not bind to the X chromosome was lethal to males (41). Δ84HA, which is identical to MSL1NHA but lacks the first 84 amino acids, did not bind to the male X chromosome (Fig. 2F). The lack of binding could be because the Δ84HA protein lacks a nuclear localization sequence. However, staining of whole salivary glands showed that Δ84HA was localized to the nucleus (Fig. 2H and I). Thus, the first 84 amino acids of MSL1 appear to play an essential role in X chromosome binding.

In the absence of endogenous MSL1, X chromosome binding of MSL1NHA is restricted to the high-affinity sites. The observed binding of MSL1NHA to hundreds of sites on the male X chromosome could be because the domain recognizes all the sites or because it associates with the MSL complex bound to the X chromosome. To distinguish between these two possibilities, we carried out the appropriate crosses to generate larvae that carried the MSL1NHA transgene but lacked endogenous MSL1. In the absence of MSL1, none of the components of the MSL complex bound to the X chromosome. As it can be difficult to obtain good-quality polytene chromosomes

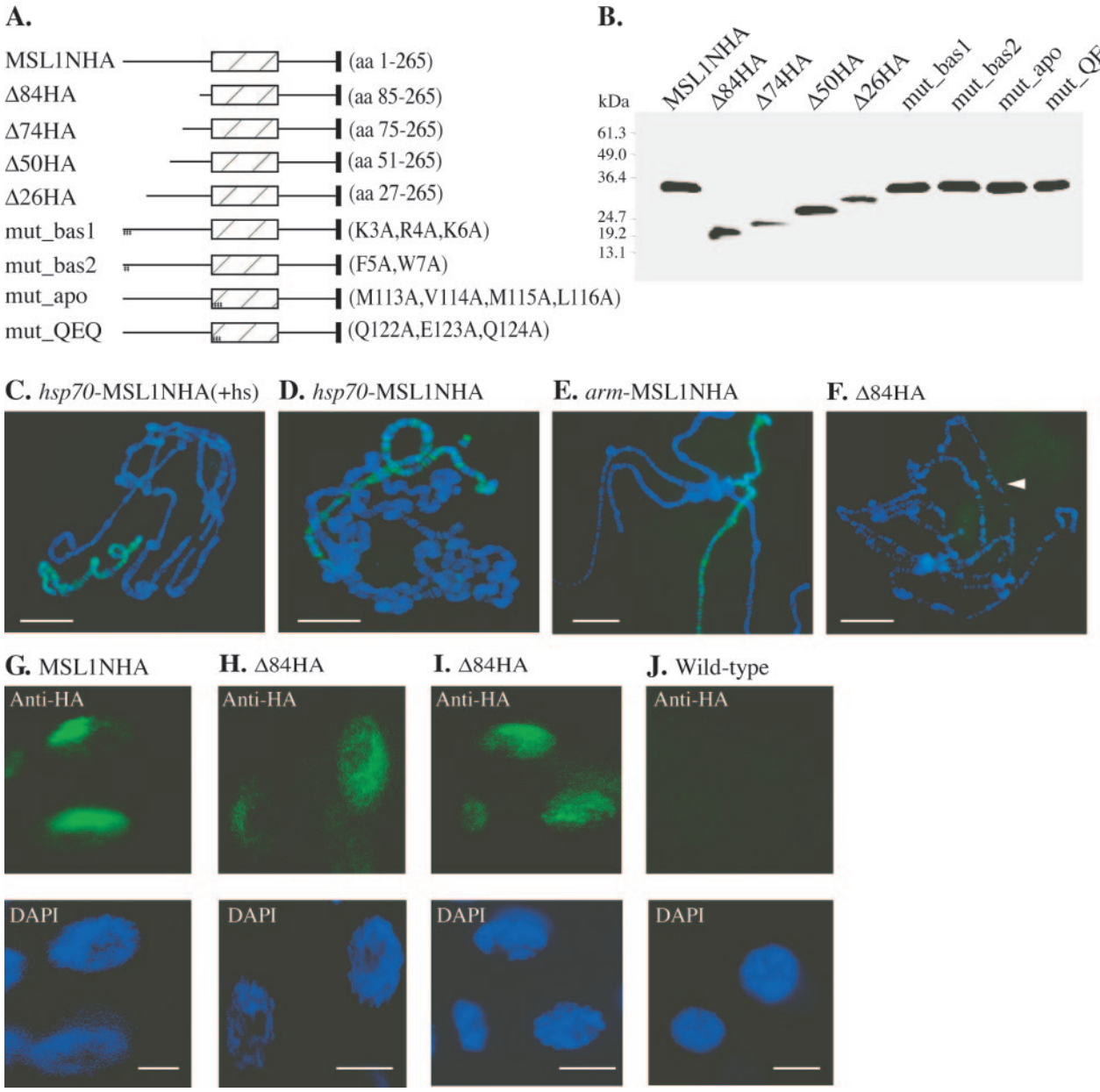

FIG. 2. MSL1NHA binds to hundreds of sites on the male X chromosome. (A) Schematic representation of the *msl1* constructs used to make transgenic flies. All constructs carry an HA epitope tag at the end of the open reading frame. Expression was controlled using the heat-inducible *hsp70* promoter. The boxed region indicates the apolar, Q-rich, and coiled-coil regions highlighted in Fig. 1. For the alanine replacement mutations, the amino acids that are changed are indicated in parentheses. (B) Western blot with anti-HA antibody and protein extracts from adult flies. Protein of the anticipated mass is detected in extracts from all lines. (C to F) MSL1NHA but not Δ84HA binds to the male X chromosome. Male salivary gland nuclei were stained with anti-HA antibody (green) and counterstained with DAPI (blue) to visualize all of the chromosomes. The *hsp70* promoter was used to control *MSL1NHA* and *Δ84HA* gene expression in all lines except the results shown in panel E, where the constitutive *armadillo* promoter (45) was used. All larvae were raised at 25°C and not heat shocked with the exception of the results shown in panel C, where larvae were treated at 37°C for 20 min and then left to recover at 25°C for 4 h. MSL1NHA was detected at hundreds of sites on the male X chromosome either with (C) or without (D) heat shock and with either promoter (C and E). In contrast, no binding of Δ84HA was detected to the male X chromosome (arrowhead) in both heat-treated (not shown) and unshocked (F) larvae. (G to I) Both MSL1NHA and Δ84HA are localized to the nucleus. Whole male salivary glands with intact nuclei were stained with anti-HA antibody (green) and counterstained with DAPI (blue). MSL1NHA (G) and Δ84HA (H and I) are detected in the nucleus. No nuclear staining was detected in the wild-type untransformed control (J). Scale bar, either 30 μm (C to F) or 10 μm (G to J).

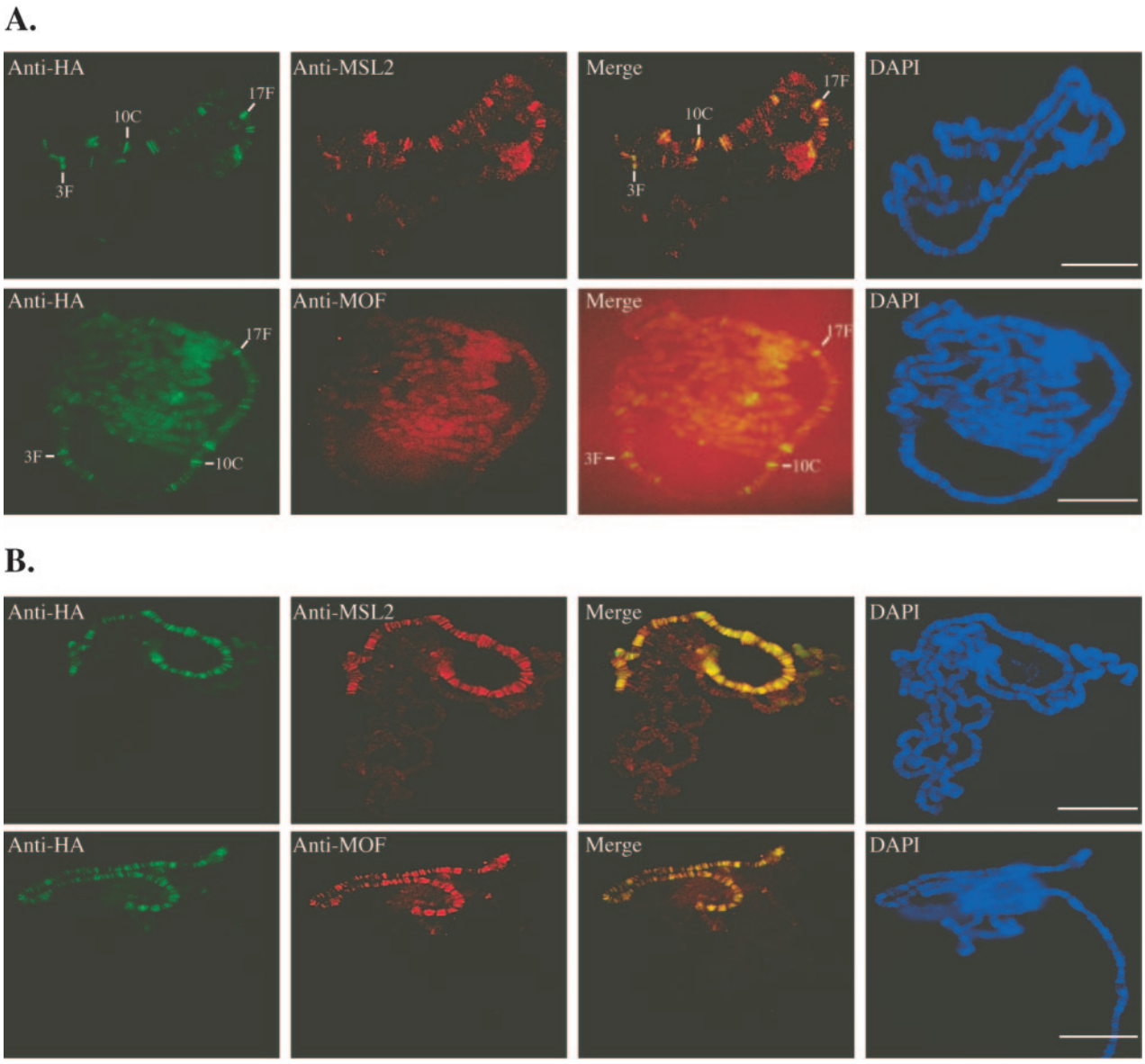

FIG. 3. MSL1HA binds to ~30 high-affinity sites on the X chromosome in larvae that lack endogenous MSL1. (A) Homozygous $msl1^{L60}$ female salivary gland nuclei that express MSL2 (*hsp83-msl2*) and MSL1NHA (*hsp70* promoter) were stained with anti-HA (green), anti-MSL2 (red) (top), or anti-MOF (red) (bottom) and counterstained with DAPI (blue). The high-affinity sites at 3F, 10C, and 17F are indicated. MSL2 but not MOF colocalizes with MSL1NHA to ~30 high-affinity sites in larvae that are homozygous for $msl1^{L60}$, a ~2.5-kb deletion of most of the *msl1* gene (R. Kelley, personal communication). (B) MSL1NHA binds to hundreds of sites on the X chromosome in female larvae that express MSL2 and are heterozygous for a null mutation in *msl1*. Heterozygous $msl1^{L60}$ female salivary gland nuclei that express MSL2 and MSL1NHA were stained with the antibodies, as in the results shown in panel A. Scale bar, 30 μm.

from dying *msl1* mutant males, we isolated salivary glands from female larvae that constitutively expressed MSL2. In the absence of *msl1*, MSL1NHA bound to about 30 sites, which corresponded to the previously mapped high-affinity sites (Fig. 3A) (27). MSL2 colocalized with MSL1NHA to the high-affinity sites. MLE also colocalized to the high-affinity sites with MSL1NHA (data not shown); however, MOF did not (Fig. 3A). The latter result was expected, as MOF binds to the carboxyl-terminal domain of MSL1 (41) and functional MOF is required for MSL complex binding to sites other than the high-affinity sites (18). In control sibling female larvae that were heterozygous for the $msl1^{L60}$ null mutation, MSL1NHA bound to hundreds of sites on the X chromosomes (Fig. 3B). MSL2 and MOF colocalized with MSL1NHA. These results show the amino-terminal domain of MSL1 complexed with MSL2 can specifically recognize the high-affinity sites on the X chromosome. However, in the presence of native MSL complex, MSL1NHA binds to hundreds of sites, presumably via association with the complex.

The conserved amino-terminal basic region of MSL1 is essential for X chromosome binding. Since Δ84HA does not bind to the male X chromosome, we made three additional smaller

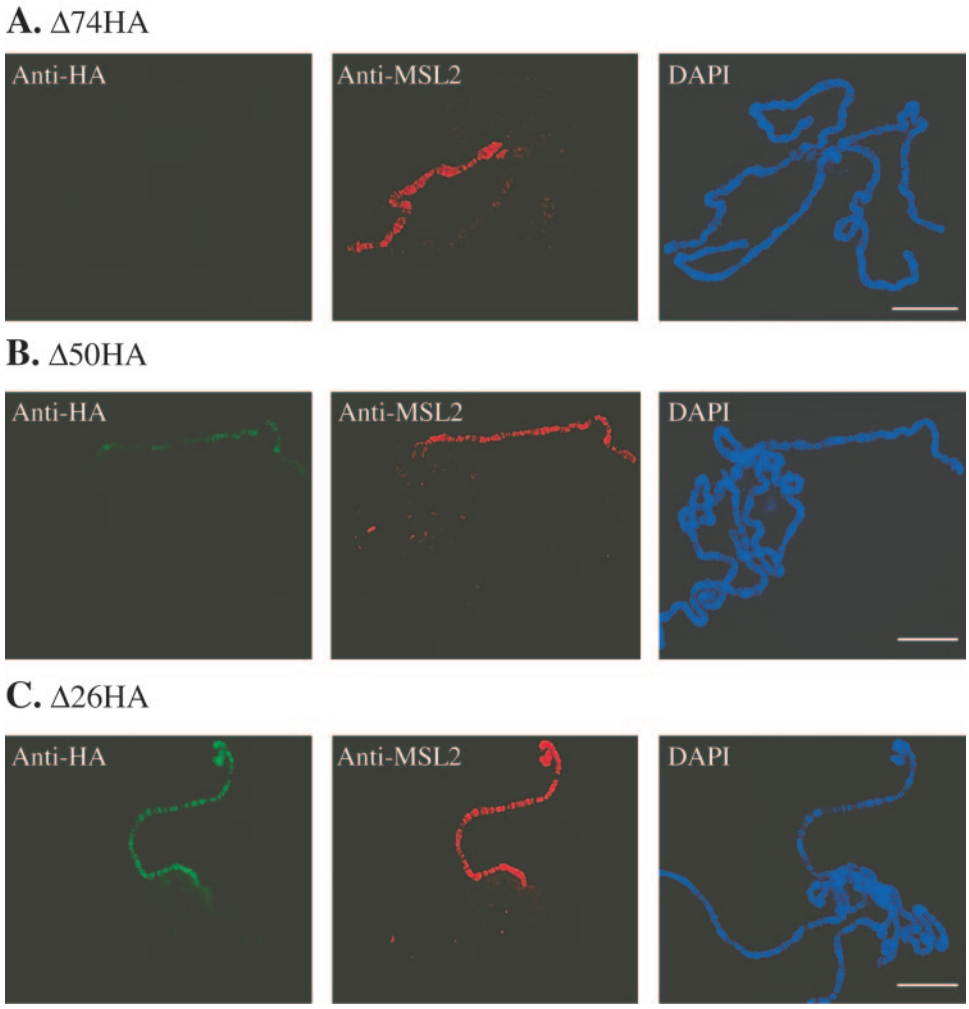

FIG. 4. Binding pattern of MSL1 amino-terminal deletion mutants to the male X chromosome. (A to C) Male salivary gland nuclei were stained with anti-HA antibody (green) and anti-MSL2 (red) and counterstained with DAPI (blue). Δ74HA does not bind to X chromosome (A), Δ50HA binds weakly (B), and Δ26HA binds more strongly to the X chromosome than Δ50HA (C); but staining intensity is consistently less than for MSL1NHA (Fig. 2C and D). Scale bar, 30 μm.

deletion mutants to identify the region important for X chromosome binding. Like Δ84HA, Δ74HA did not bind to the male X chromosome (Fig. 4A). Δ50HA, however, bound very weakly to the male X chromosome in approximately 50% of the nuclei examined (Fig. 4B). In the other 50% of nuclei, no staining of the X chromosome with the anti-HA antibody could be detected above background levels. In contrast, Δ26HA bound more strongly to the X chromosome but with less intensity than MSL1NHA (Fig. 4C).

Given that the binding of MSL1NHA to the X chromosome is restricted to the high-affinity sites in the absence of endogenous MSL1, we next asked if Δ26HA could bind to the X chromosome in a *msl1* null mutant background. We found that there was no binding of Δ26HA to the X chromosomes in homozygous $msl1^{L60}$ female larvae that expressed MSL2 (Fig. 5A). This demonstrates that the first 26 amino acids of MSL1 are essential for binding to the high-affinity sites. As discussed above, this region contains several well-conserved basic and aromatic amino acid residues. To test the importance of some of these conserved amino acids in X chromosome binding, we made two mutant versions of MSL1NHA. In mut_bas1, three of the conserved basic amino acids, lysine 3, arginine 4, and lysine 6, were all replaced by alanine. In a wild-type genetic background, this mutant version of MSL1NHA bound to hundreds of sites on the male X chromosome (data not shown). However, in the absence of endogenous MSL1, binding was restricted to only five of the high-affinity sites (Fig. 5B). Two of these sites mapped to the location of the *roX* genes, *roX1* at 3F and *roX2* at 10C. In the second mutation, mut_bas2, two of the conserved aromatic amino acids (phenylalanine 5 and tryptophan 7) were changed to alanine. This mutation did not appear to disrupt binding to the high-affinity sites in $msl1^{L60}$ null female larvae that expressed MSL2 (Fig. 5C). However, mut_bas2 bound to significantly more autosomal sites than MSL1NHA. Thus, it appears that three of the conserved basic amino acids are essential for binding to most of the high-affinity sites. In addition, two of the conserved aromatic amino acids appear to be important for distinguishing X from autosomes, that is, the specificity of binding.

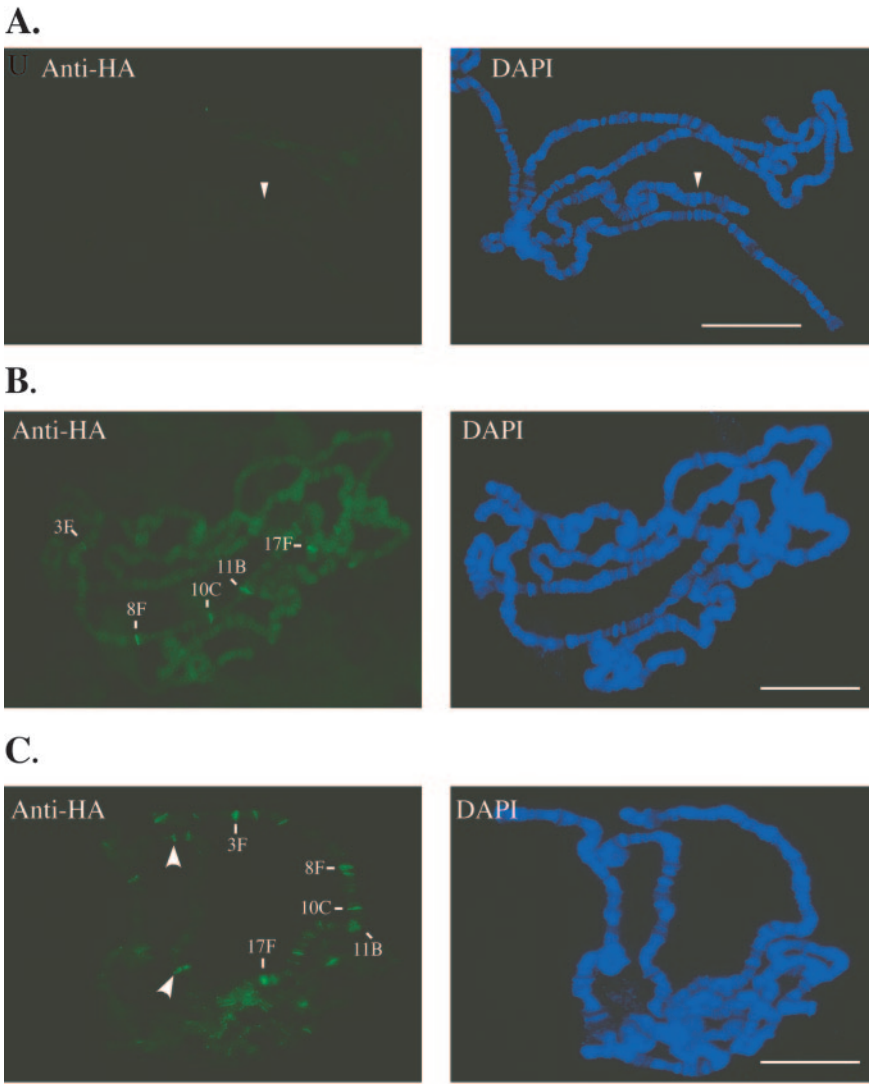

FIG. 5. The amino-terminal basic region is essential for binding to the X chromosome in the absence of endogenous MSL1. (A) Δ26HA does not bind to the X chromosome (arrowhead). Homozygous *msl1*^{L60} female salivary gland nuclei that express MSL2 (*hsp83-msl2*) and Δ26HA were stained with anti-HA (green) and counterstained with DAPI (blue). (B) Replacement by alanine of three conserved basic amino acids in the amino-terminal basic motif eliminates binding to most of the high-affinity sites. Homozygous *msl1*^{L60} female salivary gland nuclei that express MSL2 (*hsp83-msl2*) and mut_bas1 were stained with anti-HA (green) and counterstained with DAPI (blue). Binding was detected only to the high-affinity sites at 3F, 8F, 10C, 11B, and 17F as indicated. (C) Replacement by alanine of two conserved aromatic amino acids in the amino-terminal basic motif increases binding to the autosomes. Homozygous *msl1*^{L60} female salivary gland nuclei that express MSL2 (*hsp83-msl2*) and mut_bas2 were stained with anti-HA (green) and counterstained with DAPI (blue). mut_bas2 binds to all of the high-affinity sites on the X chromosome and also binds to more autosomal sites than MSL1NHA (arrowheads). Scale bar, 30 μm.

The glycine-rich region promotes MSL1 self association. The binding of MSL1NHA to hundreds of sites on the male X chromosomes appears to be in part due to association with the native MSL complex. Our observation that Δ26HA bound to these sites but Δ74HA did not indicated that the region between amino acids 26 and 74 is important for association with the MSL complex. This region is particularly rich in the amino acids glycine, proline, asparagine, and histidine in all *Drosophila* MSL1 proteins (Fig. 1). Glycine-rich domains are a common feature of many proteins including RNA binding proteins and can mediate protein-protein interaction (7, 11). The glycine-rich domain of the *Drosophila* Sex-lethal RNA binding protein, which is the master regulator of dosage compensation (38), promotes self-association (10). We therefore tested if the MSL1 glycine-rich domain would facilitate MSL1 self-association. We found that MSL1 coimmunoprecipitated from whole-fly protein extracts with MSL1NHA and Δ26HA but not Δ84HA, Δ74HA, or Δ50HA (Fig. 6A). There was a small variation in immunoprecipitation efficiency of the HA-tagged proteins, which were also detected with the MSL1 antibody (Fig. 6A). However, this was not sufficient to account for the lack of coimmunoprecipitation of MSL1 with the more truncated versions of MSL1NHA. MSL2 was not required for MSL1 self-association, as protein extracts were prepared from adult females, which normally do not make MSL2 protein. Δ26HA did not coimmunoprecipitate with MSL3 (Fig. 6B),

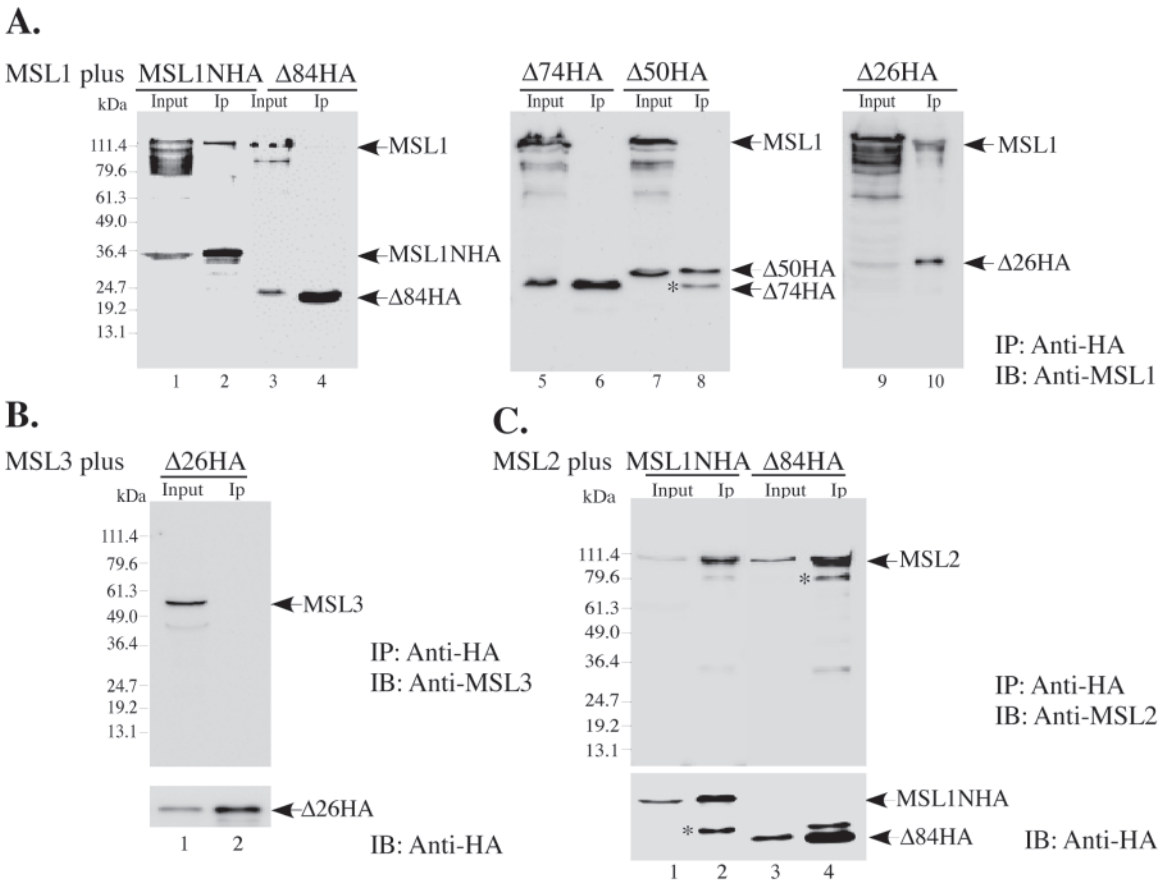

FIG. 6. The glycine-rich region mediates MSL1 self-association. Protein extracts from transformant flies that cooverexpressed an HA-tagged MSL1 amino-terminal domain and either MSL1 (A), MSL3 (B), or MSL2 (C) were immunoprecipitated with anti-HA affinity matrix (IP) and detected by Western blotting (IB) with indicated antibodies. Immunoprecipitated extracts (Ip) are shown in the even numbered lanes and 10% of the corresponding input is shown in the odd-numbered lanes (Input). (A) MSL1 coimmunoprecipitates with MSL1NHA but not Δ84HA. Protein extracts were from flies that cooverexpressed MSL1; either MSL1NHA (lanes 1 and 2), Δ84HA (lanes 3 and 4), Δ74HA (lanes 5 and 6), Δ50HA (lanes 7 and 8), or Δ26HA (lanes 9 and 10) was immunoprecipitated and detected by Western blotting with anti-MSL1. MSL1 (arrow) coimmunoprecipitated with MSL1NHA and Δ26HA but not with any of the other amino-terminal deletion mutants (arrows). (B) MSL3 does not coimmunoprecipitate with Δ26HA. Protein from flies that cooverexpressed MSL3 and Δ26HA (lanes 1 and 2) were immunoprecipitated and detected by Western blotting with either anti-MSL3 (top) or anti-HA (bottom). (C) MSL2 coimmunoprecipitates with both MSL1NHA and Δ84HA. Protein from flies that cooverexpressed MSL2 and either MSL1NHA (lanes 1 and 2) or Δ84HA (lanes 3 and 4) was immunoprecipitated and detected by Western blotting with either anti-MSL2 (top) or anti-HA (bottom). MSL2 (arrow) coimmunoprecipitates with both MSL1NHA and Δ84HA. (B and C) Western blotting with anti-HA antibody shows the efficiency of immunoprecipitation. Lower-molecular-weight bands were occasionally detected in immunoprecipitated protein (asterisk), which are presumably products of partial protein degradation.

showing the specificity of the interaction of Δ26HA with MSL1 (Fig. 6A, lane 10). Deletion of the first 84 amino acids did not, however, disrupt interaction with MSL2 (Fig. 6C), confirming previous studies (8, 41). Thus, MSL1NHA appears to interact with the native MSL complex via MSL1 self-association.

The predicted leucine zipper-like region of MSL1 is essential for interaction with MSL2. We previously suggested that the predicted leucine zipper-like region of MSL1 may interact with an predicted amphipathic α-helix at the amino terminus of MSL2 to form a coiled-coil structure (41). Likely orthologs of MSL1 and MSL2 have been identified from invertebrate and vertebrate genome sequences (29). Amino acid sequence alignments of MSL1 and MSL2 orthologs showed a high degree of conservation of the predicted α-helical regions (Fig. 7A and B). Inspection of the alignments showed that both MSL1 and MSL2 proteins contained a highly conserved region that is largely apolar and precedes the coiled coil. For MSL1, a glutamine-rich spacer separated the apolar and coiled-coil regions. Alanine substitution mutations were made in the apolar, glutamine-rich, and leucine zipper-like regions of MSL1 to investigate the relative importance of these regions in dimerization with MSL2 (Fig. 7C).

In vitro-translated [^{35}S]methionine-labeled MSL1NHA coimmunoprecipitated with the FLAG-tagged amino-terminal domain of MSL2 (aa 1 to 193) (MSL2NFLAG) from transformed whole-fly extract (Fig. 7D, lanes 1 and 2). MSL1NHA did not coimmunoprecipitate with control extract prepared from untransformed wild-type flies (Fig. 7D, lane 3). Immunoprecipitations were performed under stringent high-salt conditions (500 mM NaCl), and thus only specific interactions should be detected. This was confirmed by the lack of coimmunoprecipitation of the carboxyl-terminal domain of MSL1

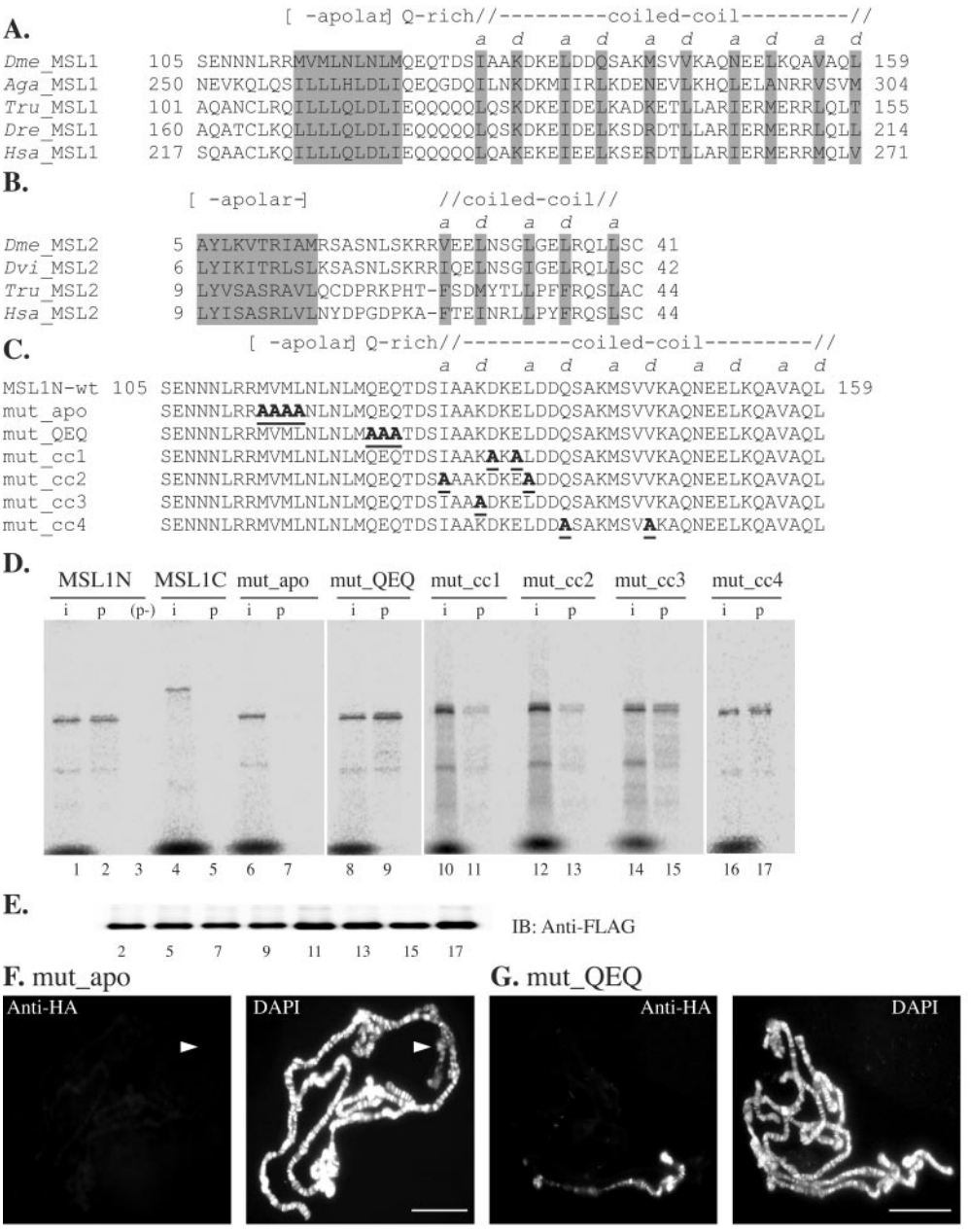

FIG. 7. The leucine zipper-like motif of MSL1 is required for binding to MSL2. (A) Alignment of the coiled-coil regions of orthologs of *D. melanogaster* (*Dme*) MSL1 from the mosquito *Anopheles gambiae* (*Aga*), from the fish *Takifugu rubripes* (*Tru*), zebra fish *Danio rerio* (*Dre*), and human *Homo sapiens* (*Hsa*). The apolar, glutamine (Q)-rich, and leucine zipper-like regions are indicated. The *a* and *d* positions of the coiled-coil motif, which are usually occupied by apolar amino acids, are indicated. The alignment is slightly different than that published previously for MSL1 orthologs (29). (B) Alignment of the amino-terminal region of orthologs of *D. melanogaster* (*Dme*) MSL2 from *Drosophila virilis* (*Dvi*), the fish *T. rubripes* (*Tru*), and humans (*Hsa*). The apolar and coiled-coil regions are indicated. (C) Alanine substitution mutations in MSL1NHA used in this study. (D) Coimmunoprecipitation of the amino-terminal domains of MSL2 and MSL1. In vitro-translated [^{35}S]methionine-labeled MSL1NHA, the carboxyl-terminal domain of MSL1 (aa 705 to 1039) (41), and alanine substitution derivatives of MSL1NHA were incubated with protein extracts from either wild-type flies or transgenic flies that had been prebound to anti-FLAG affinity beads. The transgenic flies overexpressed MSL2NFLAG, which is the amino-terminal domain of MSL2 (aa 1 to 193) with a FLAG tag at the C end. Bound proteins immunoprecipitated with anti-FLAG affinity beads, were separated by SDS-PAGE, and were detected by autoradiography. [^{35}S]methionine-labeled proteins coimmunoprecipitated with MSL2NFLAG (p) are shown in lanes 2, 5, 7, 9, 11, 13, 15, and 17; those coimmunoprecipitated with extract from control wild-type flies (p-) are shown in lane 3; the other lanes had 10% of the corresponding input (i). MSL1NA (lane 2), mut_QEQ (lane 9), mut_cc3 (lane 15), and mut_cc4 (lane 17) all efficiently coimmunoprecipitated with MSL2NFLAG. MSL1NHA did not coimmunoprecipitate with the negative control extract from untransformed wild-type flies (lane 3). Significantly less coimmunoprecipitation was seen with mut_cc1 (lane 11) and mut_cc2 (lane 13). MSL1C (lane 5) and mut_apo (lane 7) did not coimmunoprecipitate with MSL2NFLAG. (E) Western blot of coimmunoprecipitated samples with anti-FLAG antibody. Equivalent aliquots of immunoprecipitated protein used in the results shown in panel D were size separated by SDS-PAGE, and MSL2NFLAG was detected by Western blotting. The lane numbers correspond to the identical samples used above (D). (F and G) mut_QEQ binds to the male X chromosome but mut_apo does not. Male salivary gland nuclei that express either mut_apo (F) or mut_QEQ (G) were stained with anti-HA antibody and counterstained with DAPI. The arrowhead (F) points to the male X chromosome. Scale bar, 30 μm.

(aa 705 to 1039) with MSL2NFLAG (Fig. 7D, lanes 4 and 5). A derivative of MSL1NHA with mutations in the apolar region (mut_apo) did not coimmunoprecipitate with MSL2NFLAG (Fig. 7D, lanes 6 and 7) In contrast, mutations in the glutamine-rich region (mut_QEQ) did not appear to disrupt the MSL1:MSL2 interaction (Fig. 7D, lanes 8 and 9). This cannot be due to differences in immunoprecipitation efficiency, as recovery of MSL2NFLAG was similar (Fig. 7E, lanes 7 and 9). Consistent with these in vitro binding results, mut_QEQ bound to hundreds of sites on the male X chromosome (Fig. 7G). Further, we could not detect binding of mut_apo to the male X chromosome (Fig. 7F). Thus, the apolar but not the glutamine-rich region of MSL1 appears to be important for interaction with MSL2.

Dimerization of coiled-coil proteins is driven by interaction between apolar side chains in the *a* and *d* positions of the α-helix. The binding is enhanced by ionic interactions between charged amino acids in the *e* and *g* positions. Consequently we made alanine-substitution mutations in the *a*, *d*, *e*, and *g* positions in the leucine zipper-like motif that follows the glutamine-rich region. We found that all of the mutant versions of MSL1NHA coimmunoprecipitated with MSL2NFLAG (Fig. 7D, lanes 11, 13, 15, and 17). However, there appeared to be significantly less coimmunoprecipitation of two of the mutations, mut_cc1 and mut_cc2, with MSL2NFLAG (Fig. 7D, lanes 11 and 13). The efficiency of immunoprecipitation of MSL2NFLAG was similar for all four coiled coil mutant preparations (Fig. 7E, lanes 11, 13, 15, and 17). These results suggest that the mut_cc1 and mut_cc2 alanine substitution mutations have weakened the interaction between MSL1 and MSL2.

DISCUSSION

The amino-terminal basic motif of MSL1 is essential for binding to the high-affinity sites. It is almost 14 years since the first report that a component of the MSL complex selectively binds to hundreds of sites on the male X chromosome (26). Although neither MSL1 nor MSL2 has a readily identifiable DNA binding domain (36, 47), both are essential for binding to the high-affinity sites on the X chromosome (27). Here, we show that a conserved basic motif at the amino terminus of MSL1 is required for binding to high-affinity sites. Short stretches of basic amino acids are involved in DNA recognition by basic leucine zipper (bZIP) (46) and basic helix-loop-helix (28) proteins. By analogy, a possible role for the basic region of MSL1 is to recognize a DNA sequence within the ~30 high-affinity sites on the male X chromosome. Consistent with this possibility, we found that replacement of three of the conserved basic amino acids at positions 3, 4, and 6 by alanine eliminated binding of the amino-terminal region of MSL1 to all but five of the high-affinity X chromosome binding sites. Two of the five sites mapped to the location of the *roX* genes (*roX1* at 3F and *roX2* at 10C). It is possible that binding to these sites could be via association with the RNA components of the complex rather than via DNA recognition. Two conserved aromatic amino acids in the basic region appear to be important for binding specificity, as alanine substitution led to increased binding to the autosomes. Aromatic and nonpolar amino acids in the basic domain of bZIP protein C/EBPα are important for DNA recognition and binding specificity, respectively (33). Investigating these possibilities will require in vitro binding studies with DNA sequences from the three high-affinity sites that have been identified. It is also possible that the amino terminus of MSL1 could bind RNA, as several proteins bind to RNA via basic-rich motifs (30). If so, the MSL1/MSL2 complex would associate with the nascent RNA of genes transcribed within the high-affinity sites. However, binding of MSL1 to the X chromosome is not disrupted by RNaseA treatment (6). This suggests that it is more likely that the MSL1/MSL2 heteromeric complex recognizes DNA sequences within the high-affinity sites.

Role of the conserved apolar region of MSL1 in binding to MSL2. bZIP and basic helix-loop-helix proteins bind to DNA as dimers with bZIP dimers, forming coiled coil structures. Coiled coil domains contain a heptad repeat of the form $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, where positions *a* and *d* are commonly occupied by apolar residues (16). Oligomerization then occurs through the formation of a multistranded, α-helical coiled coil in which *a* and *d* residues become internalized and hence shielded from the aqueous environment. We previously proposed that the short heptad substructures observed in the sequences of both MSL1 and MSL2 (residues 128 to 143 and 25 to 40, respectively) could provide a simple means by which chain dimerization could be effected in vivo (41). Here, we identified highly conserved apolar regions that lay immediately N terminal to the heptad motif in both chains (residues 113 to 121 and 5 to 14, respectively). Alanine substitution of four amino acids in the MSL1 apolar region eliminated binding to MSL2 in vitro and in vivo. A possible explanation for the critical importance of the MSL1 apolar region is that this acts as a trigger motif that facilitates coiled-coil formation. In the case of long heptad-containing regions, trigger motifs are sometimes used in the sequence to provide a short length of highly stable coiled coil that acts as a nucleating point for subsequent coiled-coil formation (23, 44). For short lengths of coiled coil, however, other features may play an important role in either stabilizing or facilitating the formation of coiled-coil structure (13). If the apolar region of MSL1 does serve as a trigger for dimerization, then the first turn of the α-helix would be expected to be important in zipping together the two proteins. Consistent with this suggestion, we found alanine substitution of the first two apolar amino acids in the *a* position and of the charged amino acids in the *e* and *g* positions of the first heptad decreased binding to MSL2 in vitro. It should also be noted that the RING finger domain of MSL2, which immediately follows the short heptad motif, is also important for binding to MSL1 (8).

Significance of MSL1 self-association mediated by the glycine-rich motif. Remarkably we found that the amino-terminal domain of MSL1 lacking the basic motif (Δ26HA) bound to all sites on the male X chromosome. This appears to be because Δ26HA binds to full-length MSL1 incorporated into the complete MSL complex. We found that a glycine-rich region between the basic and coiled-coil motifs facilitated MSL1 self-association in vitro and binding to the MSL complex in vivo. The glycine-rich and leucine zipper-like motifs appear to function independently, as MSL1 self-association does not require MSL2 and deletion of the glycine-rich motif (e.g., Δ84HA) does not disrupt binding to MSL2. However, binding to the MSL complex in vivo does require interaction with MSL2, as a

mutation (mut_apo) that disrupted binding of MSL1 to MSL2 in vitro also eliminated binding to the male X chromosome, despite containing a complete glycine-rich motif. An explanation for these observations is that the MSL1NHA:MSL2 heterodimer binds to sites on the X chromosome immediately adjacent to sites occupied by the endogenous MSL complex and that the binding is stabilized by association of MSL1NHA with MSL1 in the complex. The 18D10 high-affinity site appears to consist of a cluster of sites of intermediate or weak affinity for the MSL complex (35). It is likely that stable binding to the X chromosome involves some cooperativity between MSL complexes bound to adjacent sites of differing affinity. MSL1 self-association may then be important in cooperative binding of MSL complexes to the male X chromosome, but testing this proposal will require evaluation of a series of alanine substitution mutations within the glycine-rich region. Alternatively, our results do not preclude the possibility that MSL1NHA is recruited to the male X chromosome by interaction with both MSL2 and MSL1 in prebound MSL complex. Interaction with MSL1 in the complex must also be necessary, as Δ84HA does not bind to MSL1 or male X chromosome, yet binds to MSL2.

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