Two Conserved Cysteine Residues Are Required for the Masculinizing Activity of the Silkworm Masc Protein

Susumu Katsuma, Yudai Sugano, Takashi Kiuchi, and Toru Shimada

Background: The functional domains of the silkworm masculinizing protein Masc are unknown.

Results: The essential region and residues involved in the masculinizing activity of the Masc protein are identified.

Conclusion: Masc functions as the masculinizing protein via its C-terminal region but not two zinc finger domains in the N-terminal region.

Significance: Our study suggests the mode of action of the masculinizing protein.

We have recently discovered that the Masculinizer (Masc) gene encodes a CCCH tandem zinc finger protein, which controls both masculinization and dosage compensation in the silkworm Bombyx mori. In this study, we attempted to identify functional regions or residues that are required for the masculinizing activity of the Masc protein. We constructed a series of plasmids that expressed the Masc derivatives and transfected them into a B. mori ovary-derived cell line, BmN-4. To assess the masculinizing activity of the Masc derivatives, we investigated the splicing patterns of B. mori doublesex (Bmdsx) and the expression levels of B. mori IGF-II mRNA-binding protein, a splicing regulator of Bmdsx, in Masc cDNA-transfected BmN-4 cells. We found that two zinc finger domains are not required for the masculinizing activity. We also identified that the C-terminal 288 amino acid residues are sufficient for the masculinizing activity of the Masc protein. Further detailed analyses revealed that two cysteine residues, Cys-301 and Cys-304, in the highly conserved N-terminal region are tightly associated with the masculinizing activity.

In a lepidopteran model insect, the silkworm Bombyx mori, females have WZ sex chromosomes, whereas males have ZZ sex chromosomes (1). The W chromosome has been shown to determine the B. mori femaleness, irrespective of the Z chromosome number, indicating the presence of a putative dominant feminizing gene Feminizer (Fem) on the W chromosome (2). Despite many efforts over the last 80 years, the Fem gene has not been cloned. The W chromosome is almost fully occupied by transposable elements or other repeat elements (3); therefore, it is very difficult for researchers to assemble long accurate contigs for this chromosome, which prevents the molecular identification of Fem. Given that transposons or other selfish elements are the precursors of PIWI-interacting RNAs (piRNAs), we previously employed a piRNA sequencing-based approach to identify the transcripts produced from the B. mori W chromosome (4). Based on deep sequencing of piRNAs from the gonads of B. mori mutant strains, each of which possesses a unique W chromosome structure (5), we identified female-enriched piRNAs, which are produced from the putative sex-determining region of the W chromosome (4). However, it was not clear whether these piRNAs play a role in sex determination or sexual dimorphism in B. mori until quite recently.

In 2014, we identified differentially expressed transcripts between female and male embryos by deep sequencing (RNA-seq) (6, 7). Among them, we found a single transcript that is expressed specifically in females throughout the embryonic stages. This transcript was produced from the W chromosome, and it was found to be a precursor of a female-specific piRNA using silkworm piRNA libraries (4, 8, 9). To determine the role of this female-specific piRNA, we designed an RNA inhibitor for this piRNA, injected it into female embryos, and examined the splicing patterns of B. mori doublesex (Bmdsx), a gene that acts at the end of the sex differentiation cascade (10, 11). Injection of the inhibitor RNA resulted in the production of male-type splice variants in female embryos, thereby indicating that the precursor of this piRNA is the long sought feminizing factor. Thus, we designated this precursor RNA as Fem (6).

piRNAs bind to PIWI proteins and silence the activity of transposons by cleaving transposon mRNA via the piRNA-PIWI complex (12). Using bioinformatic analysis, we identified the target gene Masculinizer (Masc), which is located on the Z chromosome. RNA interference-mediated depletion of Masc in male embryos led to the production of the female-type splicing of Bmdsx (6), which indicates that the Masc protein is required for silkworm masculinization. We also observed that knockdown of Masc mRNA resulted in the abnormal up-regulation of Z-linked genes but not autosomal genes (6), thereby indicating
that this masculinizing protein also controls dosage compensation in silkworm embryos.

Masc encodes a CCCH tandem zinc finger (ZF) protein, which is conserved among lepidopteran insects (6, 13, 14). Transfection of a B. mori ovary-derived cell line, BmN-4, with Masc cDNA produced the male-type splice variants of Bmdsx (6), as well as enhanced the expression of BmIMP (B. mori IGF-II mRNA-binding protein) (13, 14), the product of which is involved in the male-specific splicing of Bmdsx (15). These results indicate that the masculinizing activity of the Masc protein can be assessed in BmN-4 cells by transfecting Masc cDNA derivatives. Using this heterologous assay system, we recently detected the masculinizing activity of the Masc proteins from other lepidopteran insects, that is, Trilocha varians and Ostrinia furnacalis (13, 14). However, the domains and/or amino acid residues of the Masc protein that are required for the masculinizing activity are unknown. In this study, we investigated the structure-function relationship of the Masc protein in BmN-4 cells using a series of Masc cDNA derivatives. Our results clearly showed that two cysteine residues in the highly conserved region, but not the two ZF domains, are required for the masculinizing activity of the silkworm Masc protein.

Experimental Procedures

Plasmid Construction—Masc or Fem piRNA-resistant Masc (Masc-R) cDNA was cloned into the piZ/V5-His vector (Invitrogen), as reported previously (6). Plasmid mutagenesis was performed using a KOD-plus mutagenesis kit (TOYOBO) or overlapping PCR with the primers listed in supplemental Table S1. DNA sequences were verified by using an ABI Prism 3100 DNA sequencer (Applied Biosystems).

Transfection—The B. mori ovary-derived BmN-4 cells were cultured at 27 °C in IPL-41 medium supplemented with 10% fetal bovine serum (9). BmN-4 cells (2.5 × 105 cells per 35-mm diameter dish) were transfected with plasmid DNAs (2 μg) using FuGENE® HD (Promega). Three days after transfection, Zeocin (final concentration, 500 μg/ml) was added to the medium. Seven days after drug selection, we examined the splicing patterns of Bmdsx and the expression levels of BmIMP.

Reverse Transcription-PCR—Total RNA was prepared using TRizol reagent (Invitrogen) according to the manufacturer’s protocol and subjected to reverse transcription using avian myeloblastosis virus reverse transcriptase with an oligo(dT) primer (TaKaRa). PCR was performed with KOD FX-neo DNA polymerase (TOYOBO). Sex-specific splicing of Bmdsx was examined by RT-PCR with the primers listed in supplemental Table S1. Quantitative RT-PCR (RT-qPCR) of BmIMP and rp49 was performed using a KAPA™ SYBR Fast qPCR kit (Kapa Biosystems), and the specific primers are listed in supplemental Table S1.

Comparison of Masc Protein Sequences from Lepidopteran Insects—The cDNA sequences of the Masc genes from B. mori, T. varians, and O. furnacalis were reported previously (6, 13, 14). Full-length cDNA sequences of Samia cynthia ricini, Danausplexippus, and Heliconius melpomene were obtained from SilkBase and the Butterfly Genome Database. Amino acid sequences were aligned by CLUSTALW (16) using the MEGA 6 program (17) and drawn with the SeaView program (18).

Localization of EGFP-fused Masc Proteins—The EGFP gene was fused to the 3′ end of Masc-R using an In-Fusion HD cloning kit (Clontech) according to the manufacturer’s protocol and designated as Masc-R-EGFP (see Fig. 1A). Site-directed mutagenesis was performed using Masc-R-EGFP as the template, as described above. BmN-4 cells (2.5 × 105 cells per 35-mm diameter dish) were transfected with plasmid DNAs (2 μg) using FuGENE® HD. Three days after transfection, the localization of Masc-EGFP fusion proteins was examined using a FLoid™ cell imaging station (Life Technologies) (19). The splicing patterns of Bmdsx and BmIMP expression were examined as described above.

Results

The silkworm ovary-derived cell line BmN-4 expresses the female-type splicing variants of Bmdsx, but the male-type variants are barely detected (6). We recently reported that the over-expression of Masc cDNA in BmN-4 cells produces male-type splicing variants of Bmdsx and enhances the expression of BmIMP (6, 13, 14). In this study, using this cell-based assay system, we searched for the regions or amino acid residues required for the masculinizing activity of the Masc protein. First, we found that the exogenously introduced Masc cDNA with the Fem piRNA target sequence functions to some extent as a competitor for the endogenous Fem piRNA-Siwi complex, thereby exhibiting a moderate masculinizing activity in BmN-4 cells.3 Based on this observation, we mainly used the Masc-R-based cDNAs in this study. Masc-R is a Fem piRNA-resistant Masc with five nucleotide mutations in the Fem piRNA-Siwi cleavage site, which do not result in amino acid substitutions in the Masc protein (Fig. 1) (6). Therefore, Masc-R mRNA does not function as a competitor for the endogenous Fem piRNA-Siwi complex.

The ZFs Are Not Involved in Masc-induced Masculinization—We examined the importance of two ZFs in Masc-R-induced masculinization. We generated Masc-R derivatives where nucleotide mutations were introduced at each of (R-mut1 and R-mut2) or both of (R-mutx2) the CCCH-type ZFs (Fig. 1). These mutations resulted in amino acid substitutions (C53A, C85A, or both) in the first cysteine residue of the ZFs, which might diminish the functions of CCCH-type ZFs (20, 21). Transfection of BmN-4 cells with these plasmids resulted in the production of the male-type Bmdsx variants (Fig. 2, A and C) and enhanced the expression of BmIMP (Fig. 2, B and D), which were almost similar to the levels observed in Masc-R cDNA-transfected cells. These results indicate that functional ZFs are not required for the masculinizing activity of the Masc protein.

We also generated two plasmids, cd2 and cd3, which expressed the C-terminally truncated Masc proteins with two ZFs (Fig. 1) and examined their masculinizing activity in BmN-4 cells. Transfection and subsequent experiments indicated that both of these constructs did not induce masculinization in BmN-4 cells (Fig. 3, A and B). This demonstrates that the N-terminal ZF-containing region of the Masc protein alone is not sufficient for masculinization. When combined with the

3 S. Katsuma, unpublished results.
transfection results using Masc derivatives with the mutated ZFs, we conclude that both of the two ZFs are not involved in the masculinizing activity of the Masc protein.

**Determination of the Region Required for the Masculinizing Activity of the Masc Protein**—Next, we generated a series of plasmids containing the 5´-truncated Masc cDNAs (Fig. 1) and examined their masculinizing activity in BmN-4 cells. Transfection with plasmids R-nd2 and R-nd3 resulted in the production of the male-type Bmdsx (Fig. 4A) and enhanced BmIMP expression (Fig. 4B). By contrast, the masculinizing activity was not observed in BmN-4 cells when the R-nd4, R-nd5, and R-nd6 plasmids were transfected (Fig. 5, A and B). These results demonstrate that the 50-amino acid-long region from residues 276 to 325 is critical for the masculinizing activity of the Masc protein.

To narrow the region required for the masculinizing activity, we constructed three R-nd3-based truncated plasmids, that is, R-nd3.1, R-nd3.2, and R-nd3.3 (Fig. 6A), and examined their masculinizing activity. As shown in Figs. 6B and 7A, transfection with R-nd3.1 but not with R-nd3.2 and R-nd3.3 induced masculinization in BmN-4 cells, which indicates that the eight-amino acid region from residues 295–302 of the Masc protein is essential for the masculinizing activity.

To examine the importance of the C terminus of the Masc protein for masculinization, we generated R-nd3.1-based 3´-truncated plasmids (R-nd3.1-cd1, R-nd3.1-cd1.1, and R-nd3.1-cd1.2) (Fig. 6A) and examined their activity in BmN-4 cells. The male-specific variant of Bmdsx was barely detected in BmN-4 cells transfected with R-nd3.1-cd1, R-nd3.1-cd1.1, or R-nd3.1-cd1.2 (Fig. 6B), and BmIMP mRNA was not enhanced at all when each C-terminally deleted plasmid was transfected (Fig. 7B). Together with the observation that the C-terminally deleted Masc-R protein (residues 1–513) retains the masculinizing activity,3 this suggests that the C-terminal deletion from the N-terminally truncated, 294-amino acid Masc protein might result in an unstable or nonfunctional form of the Masc protein.

**Identification of Amino Acid Residues Essential for the Masculinizing Activity of the Masc Protein**—To identify the critical residues required for the masculinizing activity, which are located between residues 295 and 302 of the Masc protein, we generated four additional constructs, that is, R-nd3.11, R-nd3.12, R-nd3.13, and R-nd3.14 (Fig. 8A) and examined their masculinizing activity in BmN-4 cells. Transfection with R-nd3.11, R-nd3.12, and R-nd3.13 produced the male-type variants of Bmdsx and enhanced the expression of BmIMP (Figs. 8B and 9A). By contrast, transfection with R-nd3.14 did not obtain the masculinized phenotype in BmN-4 cells, thereby indicating that Cys-301 is essential for the masculinizing activity of the Masc protein (Figs. 8C and 9B). We also observed that transfection with two R-nd3.13 variants, that is, R-nd3.13S and R-nd3.13A, which expressed the truncated Masc protein with substitutions of Cys-301 by serine and alanine, respectively (Fig. 8A), did not lead to the male-type splicing of Bmdsx and BmIMP up-regulation (Figs. 8C and 9B). These results demon-
FIGURE 2. Effects of mutations in either or both of the zinc finger domains on the masculinizing activity of the Masc protein. A, splicing pattern of Bmdsx in BmN-4 cells transfected with R-mut1 or R-mut2. The splicing patterns of Bmdsx were examined by RT-PCR. The F and M indicate female- and male-type splicing of Bmdsx, respectively. B, expression of BmIMP in BmN-4 cells transfected with R-mut1 or R-mut2. The mRNA levels of BmIMP were examined by RT-qPCR. The BmIMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates. C, splicing pattern of Bmdsx in BmN-4 cells transfected with R-mutx2. D, expression of BmIMP in BmN-4 cells transfected with R-mutx2. The data shown are means ± S.D. of triplicates.

FIGURE 3. Effects of deleting the C-terminal regions of the Masc protein on the masculinizing activity. A, splicing patterns of Bmdsx in BmN-4 cells transfected with cd2 or cd3. The F and M indicate female- and male-type splicing of Bmdsx, respectively. B, expression of BmIMP in BmN-4 cells transfected with cd2 or cd3. The mRNA levels of BmIMP were examined by RT-qPCR. The BmIMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates.

FIGURE 4. Masculinizing activity of the truncated derivatives R-nd2 and R-nd3. A, splicing patterns of Bmdsx in BmN-4 cells transfected with R-nd2 or R-nd3. The Bmdsx splicing was examined by RT-PCR. The F and M indicate female- and male-type splicing of Bmdsx, respectively. B, expression of BmIMP in BmN-4 cells transfected with R-nd2 or R-nd3. The mRNA levels of BmIMP were examined by RT-qPCR. The BmIMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates.

FIGURE 5. Masculinizing activity of the truncated derivatives R-nd4, R-nd5, and R-nd6. A, splicing patterns of Bmdsx in BmN-4 cells transfected with R-nd4, R-nd5, and R-nd6. The Bmdsx splicing was examined by RT-PCR. The F and M indicate female- and male-type splicing of Bmdsx, respectively. B, expression of BmIMP in BmN-4 cells transfected with R-nd4, R-nd5, and R-nd6. The mRNA levels of BmIMP were examined by RT-qPCR. The BmIMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates.

Essential Domains of the Silkworm Masc Protein

strate the importance of Cys-301 for the masculinizing activity of the Masc protein.

Next, we examined whether Cys-301 is conserved among the Masc proteins of lepidopteran insects. We obtained full-length protein coding sequences of Masc genes from six lepidopteran insects and aligned their deduced amino acid sequences (Fig. 10). As shown in Figs. 10 and 11A, Cys-301 was conserved in all of the species examined, which indicates the importance of this residue. We found that the other cysteine residue Cys-304 was also identical among all species. To examine whether this residue is important for the masculinizing activity, we constructed R-nd3.13–304S, an R-nd3.13 derivative that expressed the truncated Masc protein where Cys-304 was substituted by serine in BmN-4 cells (Fig. 11B). Further-
Essential Domains of the Silkworm Masc Protein

A

| Masc | R-nd3 | R-nd3.1 | R-nd3.2 | R-nd3.3 | R-nd4 |
|------|-------|---------|---------|---------|-------|
|      |       |         |         |         |       |
| 1    |       |         |         |         |       |
| 47   | 74    | 79      | 106     |         |       |
| 588  |       |         |         |         |       |

B

vector Masc-R R-nd3.1 R-nd3.2 R-nd3.3

C

vector Masc-R R-nd3.1-cd1 R-nd3.1-cd1.1 R-nd3.1-cd1.2

FIGURE 6. Determination of the critical region ranging from residues 276 to 325, which is required for the masculinizing activity of the Masc protein. A, schematic drawing of $R$-nd3.1, $R$-nd3.2, $R$-nd3.3, and C-terminally truncated $R$-nd3.1 derivatives. B, splicing patterns of Bmdsx in BmN-4 cells transfected with $R$-nd3.1, $R$-nd3.2, and $R$-nd3.3. C, splicing patterns of Bmdsx in BmN-4 cells transfected with C-terminally truncated $R$-nd3.1 derivatives. The $F$ and $M$ indicate female- and male-type splicing of Bmdsx, respectively.

FIGURE 7. Determination of the critical region ranging from residues 276 to 325, which is required for the masculinizing activity of the Masc protein related to Fig. 6. A, expression of BmiMP in BmN-4 cells transfected with $R$-nd3.1, $R$-nd3.2, and $R$-nd3.3. The mRNA levels of BmiMP were examined by RT-qPCR. The BmiMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates. B, expression of BmiMP in BmN-4 cells transfected with C-terminally truncated $R$-nd3.1 derivatives. The mRNA levels of BmiMP were examined by RT-qPCR. The BmiMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates.

FIGURE 8. Fine mapping of the residues required for the masculinizing activity of the Masc protein. A, schematic drawing of the Masc derivatives $R$-nd3.11, $R$-nd3.12, $R$-nd3.13, $R$-nd3.13A, and $R$-nd3.14. B, splicing patterns of Bmdsx in BmN-4 cells transfected with $R$-nd3.11, $R$-nd3.12, or $R$-nd3.13. The $F$ indicates female-type splicing of Bmdsx, respectively. C, splicing patterns of Bmdsx in BmN-4 cells transfected with $R$-nd3.13S, $R$-nd3.13A, or $R$-nd3.14.

FIGURE 9. Fine mapping of the residues required for the masculinizing activity of the Masc protein related to Fig. 8. A, expression of BmiMP in BmN-4 cells transfected with $R$-nd3.11, $R$-nd3.12, or $R$-nd3.13. The mRNA levels of BmiMP were examined by RT-qPCR. The BmiMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates.

Experiments clearly showed that both of these mutant Masc proteins completely lacked the masculinizing activity (Figs. 11C and 12). Overall, these results indicate that two cysteine resi-
FIGURE 10. Alignment of Masc protein sequences from six lepidopteran insects. The highly conserved region is highlighted by the red square. Two CCCH-type ZFs are also indicated.
Essential Domains of the Silkworm Masc Protein

dues, that is, Cys-301 and Cys-304, play essential roles in the masculinizing activity of the Masc protein.

The alignment of Masc protein sequences showed that the amino acids around residues 305–310 are highly conserved, especially Glu-306, Arg-307, and Glu-308, which were identical in all of the species examined (Figs. 10 and 11A). To understand the contribution of these residues to the masculinizing activity, we performed R-nd3.13-based alanine scanning at residues 305, 306, 308, 309, and 311 and examined their masculinizing activity in BmN-4 cells by transfecting these mutant plasmids. As shown in Figs. 11D and 13A, the masculinizing activity was retained in the transfected cells, although the substitution of Leu-309 by alanine decreased the masculinizing activity. These results indicate that Val-305, Glu-306, Glu-308, Leu-309, and Ile-311 are not essential for the masculinizing activity of the Masc protein.

FIGURE 11. Identification of the residues essential for the masculinizing activity of the Masc protein. A, alignment of Masc protein sequences from six lepidopteran insects. The Masc sequences corresponding to residues 299–314 of B. mori Masc protein are shown. B, splicing patterns of Bmdsx in BmN-4 cells transfected with R-nd3.13–304S. The F and M indicate female- and male-type splicing of Bmdsx, respectively. C, splicing patterns of Bmdsx in BmN-4 cells transfected with R-nd3.1–305A or R-305A. D, splicing patterns of Bmdsx in BmN-4 cells transfected with R-nd3.1–306A, R-nd3.1–308A, or R-nd3.1–310N. E, splicing patterns of Bmdsx in BmN-4 cells transfected with R-nd3.1–307N, R-nd3.1–310K, or R-nd3.1–310N.

FIGURE 12. Identification of the residues essential for the masculinizing activity of the Masc protein related to Fig. 11 (B and C). Expression of BmIMP in BmN-4 cells transfected with R-nd3.13–304S, R-301S, or R-304S was examined by RT-qPCR. The BmIMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates.

FIGURE 13. Identification of the residues essential for the masculinizing activity of the Masc protein related to Fig. 11 (D and E). A, expression of BmIMP in BmN-4 cells transfected with R-nd3.1–305A, R-nd3.1–306A, R-nd3.1–308A, R-nd3.1–310, or R-nd3.1–311A. The mRNA levels of BmIMP were examined by RT-qPCR. The BmIMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates. B, expression of BmIMP in BmN-4 cells transfected with R-nd3.1–307K, R-nd3.1–307N, R-nd3.1–310K, or R-nd3.1–310N. The mRNA levels of BmIMP were examined by RT-qPCR. The BmIMP mRNA level was normalized to that of rp49. The data shown are means ± S.D. of triplicates.
Masc protein. We also generated mutants for two arginine residues, that is, Arg-307 and Arg-310, by changing arginine to lysine or asparagine. The two mutant Masc proteins with Arg-307 substitutions induced masculinization in BmN-4 cells (Figs. 11E and 13B). By contrast, changing Arg-310 to asparagine but not lysine obtained the decreased masculinizing activity, although the activity was not lost completely (Figs. 11E and 13B). The residue at 310 is serine instead of arginine in *O. furscinalis* (Fig. 11A), so we also generated *R-nd3.1–310S*, a R-nd3.1 derivative that expressed the truncated Masc protein where Arg-310 was substituted by serine. Transfection with *R-nd3.1–310S* obtained the masculinized phenotype in BmN-4 cells (Fig. 13, C and D). According to these results, the positive charges on residues 307 and 310 do not contribute greatly to the masculinizing activity of the Masc protein.

**Relationship between the Masculinizing Activity and Intracellular Localization of the Masc Protein**—Finally, we examined the localization of the Masc protein using a series of EGFP fusion constructs (Fig. 14A). Fluorescence microscopy showed that the Masc-R-EGFP fusion protein localized exclusively in the nucleus (Fig. 15B), whereas EGFP was localized in the cytoplasm (Fig. 15A), thereby indicating that the Masc protein is a nuclear protein. Both Masc-R-C301S-EGFP and Masc-R-C304S-EGFP exhibited distinct nuclear localization (Fig. 15, C and D). Transfection of BmN-4 cells with R-301S and R-304S did not obtain the masculinizing activity at all (Figs. 11C and 12), but the EGFP-fused derivatives possessed a weak masculinizing activity (Fig. 14, B and C).
We observed that this mutant localized to both the nucleus and cytoplasm in most of the EGFP-positive cells, whereas distinct nuclear localization was detected in some cells (Fig. 15E). By contrast, Masc-R-(1–300)-EGFP did not exhibit the masculinizing effects (Fig. 14, B and C), but it localized exclusively to the nucleus (Fig. 15F). This indicates that the N-terminal 1–300 region contains a nuclear localization signal(s). To examine whether functional ZFs are required for the nuclear localization, we generated three EGFP-fused Masc mutants where amino acid substitutions were introduced in each or both of the two ZFs (Fig. 14A). As shown in Fig. 15 (G–I), all of these mutant Masc proteins were localized in the nucleus, which demonstrates that functional ZFs are dispensable for the nuclear localization of the Masc protein, as well as for its masculinizing activity (Fig. 14, B and C). To narrow the region required for nuclear localization, we generated an additional C-terminally truncated mutant, Masc-R-(1–200)-EGFP, where EGFP was fused to the N-terminal 200 amino acid residues of the Masc protein (Fig. 14A). We observed that this mutant protein localized to both the nucleus and cytoplasm, which indicates that the nuclear localization signal occurs in the region ranging from residues 200 to 300 (Fig. 15J). Thus, we conclude that the masculinizing activity is not tightly associated with the nuclear localization of the Masc protein and that amino acids 200–300 are required for complete nuclear localization.
Discussion

The silkworm Masc protein is a recently discovered CCCH tandem ZF protein, which is required for both masculinization and dosage compensation (6). A homology search showed that this protein is conserved among lepidopteran insects (6). However, the domains or amino acid residues required for masculinization or dosage compensation have not yet been fully determined. In the present study, we utilized a cell-based assay system where the masculinizing activity was estimated on the basis of Bmdsx splicing and the BmIMP mRNA level, and we successfully identified the region and the residues in the Masc protein that are required for masculinization.

The overall homology of Masc proteins is not very high among lepidopteran insects (Fig. 10), but the residues from 301 to 311 are highly conserved (Figs. 10 and 11A). Among these 11 residues, we found that six residues are identical in six lepidopteran insects, that is, Cys-301, Cys-304, Val-305, Glu-306, Arg-307, and Glu-308. Site-directed mutagenesis showed that Cys-301 and Cys-304 are the essential residues required for the masculinizing activity of the Masc protein. What are the functions of these cysteines? Cysteine is frequently utilized as a disulfide bond donor for oligomerization or to facilitate intramolecular conformational stability. If this is true of the Masc protein, then these residues are important for the structure rather than for the masculinizing activity of the Masc protein.

We observed that both of the EGFP-fused cysteine mutant derivatives (Masc-R-C301S-EGFP and Masc-R-C304S-EGFP) had weak but significant masculinizing effects on BmN-4 cells (Fig. 14, B and C). Given that the GFP protein has a tendency to oligomerization (22, 23), we suggest that EGFP fusion could partially rescue the loss of the oligomerization activity in these Masc derivatives, thereby obtaining a weak masculinizing activity. Collectively, these cysteine residues are probably utilized as a disulfide bond donor to facilitate oligomerization, which is critical for the masculinizing activity.

The present study and previous reports (6, 13, 14) show that overexpression of Masc cDNA in female BmN-4 cells produces the male-specific splice variants of Bmdsx and enhances the expression of BmIMP. The male-type splicing of Bmdsx is regulated by BmIMP (15), so the Masc-induced increase in the BmIMP protein is considered to directly control the splicing of Bmdsx. Thus, our next goal is to unravel the signaling cascade from the Masc protein to BmIMP transcriptional induction. We have shown that Masc is a nuclear protein and that the C-terminal 288 amino acid residues can trigger the masculinizing signal. Together with the findings that some CCCH tandem ZF-containing proteins play biological roles by binding nucleic acids (24–26), we suggest that the Masc protein may function via binding genomic DNA or nuclear RNA. To determine the genomic and RNA targets of Masc protein, we are now trying to establish the chromatin immunoprecipitation and cross-linking immunoprecipitation systems using BmN-4 cells and anti-Masc antibody.

In our assay system, it may be difficult to assess the precise effects on dosage compensation because cultured silkworm cells maintain abnormal numbers of chromosomes during their immortalization (27). To determine the regions of the Masc protein that are involved in dosage compensation, we will have to perform new experiments using silkworm embryos or genetically engineered silkworm strains where Masc is partially disrupted or where mutant Masc derivatives have been introduced. In the present study, we showed that Masc is a nuclear protein, which is consistent with the fact that the Masc protein is required for dosage compensation, because dosage compensation should occur in the nucleus. In addition, we found that mutations in each or both of the ZFs had little effect on the masculinizing activity of the Masc protein in BmN-4 cells. If dual roles are regulated by each of the two different domains of the Masc protein, it is possible that the two ZFs might be involved in dosage compensation. Further studies using a new assay system will provide evidence for the roles of the two ZFs in the Masc protein.

Author Contributions—S. K. designed the study and performed most of the experiments; Y. S. generated the EGFP fusion constructs and performed the experiments of Figs. 14 and 15; T. K. performed the bioinformatic identification of the Masc proteins and their conserved regions; S. K., Y. S., T. K., and T. S. analyzed the data; and S. K. wrote the manuscript with intellectual input from all authors.

Acknowledgments—We thank Hikaru Koga and Jung Lee for some preliminary experiments and Munetaka Kawamoto for clerical assistance.

References

1. Tanaka, Y. (1916) Genetic studies in the silkworm. J. Coll. Agric. Sapporo 6, 1–33
2. Hasimoto, H. (1933) The role of the W-chromosome in the sex determination of Bombyx mori. Jpn. Genet. 8, 245–247
3. Abe, H., Mita, K., Yasukochi, Y., Oshiki, T., and Shimada, T. (2005) Retrotransposable elements on the W chromosome of the silkworm, Bombyx mori. Cytogenet. Genome Res. 110, 114–151
4. Kawaoka, S., Kadota, K., Arai, Y., Suzuki, Y., Fujii, T., Abe, H., Yasukochi, Y., MitA, K., Sugano, S., Shimizu, K., Tomari, Y., Shimada, T., and Katsuma, S. (2011) The silkworm W chromosome is a source of female-enriched piRNAs. RNA 17, 2144–2151
5. Abe, H., Fujii, T., Tanaka, N., Yokoyama, T., Kakehashi, H., Ajimura, M., Mita, K., Banno, Y., Yasukochi, Y., Oshiki, T., Neno, M., Ishikawa, T., and Shimada, T. (2008) Identification of the female-determining region of the W chromosome in Bombyx mori. Genetica 133, 269–282
6. Kiuchi, T., Koga, H., Kawaoka, M., Shoji, K., Sakai, H., Arai, Y., Ishihara, G., Kawaoka, S., Sugano, S., Shimada, T., Suzuki, Y., Suzuki, M. G., and Katsuma, S. (2014) A single female-specific piRNA is the primary determinant of sex in the silkworm. Nature 509, 633–636
7. Kawamoto, M., Koga, H., Kiuchi, T., Shoji, K., Sugano, S., Shimada, T., Suzuki, Y., and Katsuma, S. (2015) Sexually biased transcripts at early embryonic stages of the silkworm depend on the sex chromosome constitution. Gene 569, 50–56
8. Kawaoka, S., Arai, Y., Kadota, K., Suzuki, Y., Hara, K., Sugano, S., Shimizu, K., Tomari, Y., Shimada, T., and Katsuma, S. (2011) Zygotic amplification of secondary piRNAs during silkworm embryogenesis. RNA 17, 1401–1407
9. Kawaoka, S., Hayashi, N., Suzuki, Y., Abe, H., Sugano, S., Tomari, Y., Shimada, T., and Katsuma, S. (2009) The Bombyx ovary-derived cell line endogenously expresses PIWI/PIWI-interacting RNA complexes. RNA 15, 1258–1264
10. Suzuki, M. G., Funaguma, S., Kanda, T., Tamura, T., and Shimada, T. (2003) Analysis of the biological functions of a doublesex homologue in Bombyx mori. Dev. Genes. Evol. 213, 345–354
11. Suzuki, M. G., Funaguma, S., Kanda, T., Tamura, T., and Shimada, T.
(2005) Role of the male BmDSX protein in the sexual differentiation of *Bombyx mori*. **Evol. Dev.** 7, 58–68

Iwasaki, Y. W., Siomi, M. C., and Siomi, H. (2015) PIWI-interacting RNA: its biogenesis and functions. **Annu. Rev. Biochem.** 84, 405–433

Lee, J., Kiuchi, T., Kawamoto, M., Shimada, T., and Katsuma, S. (2015) Identification and functional analysis of a *Masculinizer* orthologue in *Trilocha varians* (Lepidoptera: Bombycidae). **Insect Mol. Biol.** 24, 561–569

Fukui, T., Kawamoto, M., Shoji, K., Kiuchi, T., Sugano, S., Shimada, T., Suzuki, Y., and Katsuma, S. (2015) The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by targeting the masculinizing gene. **PLoS Pathog.** 11, e1005048

Suzuki, M. G., Imanishi, S., Dohmae, N., Asanuma, M., and Matsumoto, S. (2010) Identification of a male-specific RNA binding protein that regulates sex-specific splicing of *Bmdsx* by increasing RNA binding activity of BmPSI. **Mol. Cell Biol.** 30, 5776–5786

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucleic Acids Res.** 22, 4673–4680

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. **Mol. Biol. Evol.** 30, 2725–2729

Gouy, M., Guindon, S., and Gascuel, O. (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. **Mol. Biol. Evol.** 27, 221–224

Ishihara, G., Shimada, T., and Katsuma, S. (2013) Functional characterization of *Bombyx mori* nucleopolyhedrovirus CG30 protein. **Virus Res.** 174, 52–59

Qu, J., Kang, S. G., Wang, W., Musier-Forsyth, K., and Jang, J. C. (2014) The *Arabidopsis thaliana* tandem zinc finger 1 (AtTZF1) protein in RNA binding and decay. **Plant J.** 78, 452–467

Kelly, S. M., Leung, S. W., Apponi, L. H., Bramley, A. M., Tran, E. I., Chekanova, J. A., Wente, S. R., and Corbett, A. H. (2010) Recognition of polyadenosine RNA by the zinc finger domain of nuclear poly(A)-RNA-binding protein 2 (Nab2) is required for correct mRNA 3′-end formation. **J. Biol. Chem.** 285, 26022–26032

Yang, F., Moss, L. G., and Phillips, G. N. Jr. (1996) The molecular structure of green fluorescent protein. **Nat. Biotechnol.** 14, 1246–1251

Zacharias, D. A., Violin, J. D., Newton, A. C., and Tsien, R. Y. (2002) Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. **Science** 296, 913–916

Blackshear, P. J., and Perera, L. (2014) Phylogenetic distribution and evolution of the linked RNA-binding and NOT1-binding domains in the tristetraprolin family of tandem CCCH zinc finger proteins. **J. Interferon Cytokine Res.** 34, 297–306

Brooks, S. A., and Blackshear, P. J. (2013) Tristetraprolin (TTP): interactions with mRNA and proteins, and current thoughts on mechanisms of action. **Biochim. Biophys. Acta** 1829, 666–679

Pomeranz, M. C., Hah, C., Lin, P. C., Kang, S. G., Finer, J. J., Blackshear, P. J., and Jang, J. C. (2010) The *Arabidopsis* tandem zinc finger protein AtTZF1 traffics between the nucleus and cytoplasmic foci and binds both DNA and RNA. **Plant Physiol.** 152, 151–165

Imanishi, S., and Ohtsuki, Y. (1988) Characteristics of cell lines established from embryonic tissues of several races of the silkworm, *B. mori*, cultured in vitro. **J. Seric. Sci. Ipn.** 57, 184–188