Transcriptional regulation by Modulo integrates meiosis and spermatid differentiation in male germ line

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Communicated by Daniel L. Hartl, Harvard University, Cambridge, MA, June 17, 2006 (received for review May 13, 2006)

Transcriptional activation in early spermatocytes involves hundreds of genes, many of which are required for meiosis and spermatid differentiation. A number of the meiotic-arrest genes have been identified as general regulators of transcription; however, the gene-specific transcription factors have remained elusive. To identify such factors, we purified the protein that specifically binds to the promoter of spermatid-differentiation gene *Sdic* and identified it as Modulo, the *Drosophila* homologue of nucleolin. Analysis of gene-expression patterns in the male sterile *modulo* mutant indicates that Modulo supports high expression of the meiotic-arrest genes and is essential for transcription of spermatid-differentiation genes. Expression of Modulo itself is under the control of meiotic-arrest genes and requires the DAZ/DAZL homologue Boule that is involved in the control of G_2/M transition. Thus, regulatory interactions among Modulo, Boule, and the meiotic-arrest genes integrate meiosis and spermatid differentiation in the male germ line.

Drosophila | spermatogenesis

Spermatogenesis is strikingly similar between *Drosophila* and mammals (1). Transcriptional activation in spermatocytes furnishes material that supports spermatocyte maturation and meiosis as well as further spermatid differentiation. Execution of the meiosis/differentiation program requires a number of general transcriptional regulators collectively known as the meiotic-arrest genes. These include the genes of the *aly* group that encode subunits of the putative chromatin-modifying complex (2–6) and the genes of the *can* group that code for the subunits of testes-specific TFIID, which is involved in initiation of transcription as part of the preinitiation complex (2, 7) and probably participates in displacement of the repressory Pc complexes from the testes-specific promoters (8). A number of meiotic regulators, such as *cyclin B*, *boule*, and *twine*, as well as many genes required for spermatid differentiation, are under the control of meiotic-arrest genes (2, 6).

Although the general regulators of transcription in testes have been extensively characterized, the gene-specific transcription factors have long been elusive. To characterize such factors, we sought to identify the protein that binds to the conserved positive regulatory element b2UE1/b2UE2/TSE that is necessary for activity of the *β(2)tubulin* promoter in *Drosophila* testes (9) and is present in the promoter of the testes-specific gene *Sdic* (10). Here, we report purification of the protein that specifically binds to the b2UE1/b2UE2/TSE motif and identification of it as Modulo, the *Drosophila* homologue of nucleolin. Modulo is required for transcription of a number of spermatid-differentiation genes, including *β(2)tubulin* and *Sdic*. Expression of Modulo itself in testes is positively regulated by the meiotic-arrest genes at the posttranscriptional level and requires the DAZ/DAZLA homologue Boule, the protein that also controls the G_2/M meiotic transition through posttranscriptional regulation of Cdc25/Twine (11). Thus, Modulo plays an important role in integration of meiosis and spermatid differentiation in *Drosophila* spermatogenesis.

Results and Discussion

Conserved Promoter Motif TSE Binds a Testes-Specific Protein. The promoter of the testes-specific gene *Sdic* contains the TSE motif that shows similarities to the conserved elements b2UE1 and b2UE2 found in other testes-specific promoters (9, 10, 12). An abundant TSE-binding protein was detected in protein extracts from *Drosophila* testes but not from gonadectomized males by using EMSA (Fig. 1*A*). Formation of the DNA–protein complex was completely inhibited by a 100-fold molar excess of the unlabeled TSE probe. At the same time, the presence of a 10^4-fold molar excess of the heterologous double-stranded oligonucleotide competitor 1 in all EMSA reactions did not inhibit formation of the DNA–protein complex, indicating that binding of the protein is sequence-specific. Further addition of the different oligonucleotide competitor 2 in 100-fold excess to the probe did not interfere with the complex formation (Fig. 1*A*). To corroborate this finding, we tested five more different heterologous oligonucleotides using the same conditions, and none of them impeded complex formation (data not shown). Thus, a protein that specifically binds to the conserved TSE promoter motif is up-regulated in testes and may be involved in transcriptional regulation of *Sdic*.

Purification and Identification of the TSE-Binding Activity. To identify the TSE-binding factor, we developed a multistep procedure for its biochemical purification from whole adult flies (Fig. 1*B*). The details are described in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. A representative result of the final step (the sequence-specific DNA-affinity purification) is shown in Fig. 1*C*. The eluted fractions contained only two major proteins, one of ≈150 kDa and another of ≈50 kDa. The elution profile of the 50-kDa protein (Fig. 1*C Lower*) matched that of the TSE-binding activity (Fig. 1*C Upper*), implying that this protein may represent the TSE-binding factor. Southwestern blot analysis of the purified protein supported this suggestion, revealing a single 50-kDa protein species capable of binding to the TSE probe (Fig. 1*C*). The 150-kDa protein, identified later by liquid chromatography (LC)/tandem MS (MS/MS) as the topoisomerase II, was not able to bind the TSE probe in EMSA and Southwestern blot assays.

The 50-kDa protein was further purified by SDS/PAGE and confidently identified by nano-LC/MS/MS as the DNA/RNA-binding protein Modulo with 30% overall sequence coverage. To further confirm the identity of the TSE-binding factor, affinity-

Conflict of interest statement: No conflicts declared.

Abbreviations: MS/MS, tandem MS; LC, liquid chromatography.

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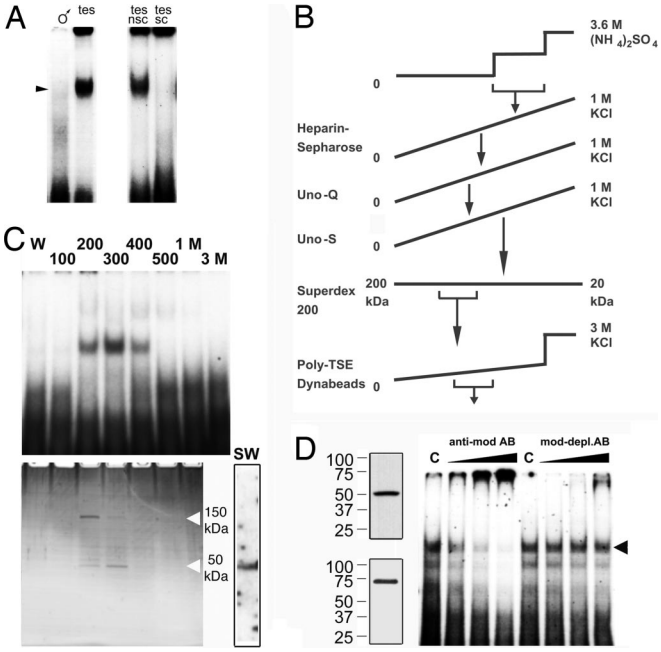

Fig. 1. Identification of the TSE-binding activity as Modulo. (*A Left*) TSE-binding activity in testes (tes) and in the whole gonadectomized males (as shown at the top) analyzed by EMSA using the TSE oligonucleotide probe. The prominent DNA–protein complex identified in testes is indicated by the arrowhead. (*A Right*) Reactions with protein extracts from testes performed in the presence of the unlabeled TSE oligonucleotide (SC), or the nonspecific oligonucleotide competitor 2 (nsc). (*B*) Outline of purification of the TSE-binding activity. The media used for purification are indicated at the left. (*C*) Final stage of purification of the TSE-binding protein. (*Upper*) Fractions eluted from the DNA-covered beads with the concentrations of KCl indicated at the top, in millimoles, were analyzed by EMSA using the TSE oligonucleotide probe. (*Lower*) The fractions as above analyzed by SDS/PAGE in 4–15% gradient gel, followed with silver staining. SW, Southwestern blot analysis of the fraction eluted with 300 mM KCl, using the ^{32}P-labeled TSE probe. (*D*) Immuno-EMSA of the purified TSE-binding protein confirms its identity as Modulo. (*Left*) Reactivity of the anti-Modulo IgY with the total adult fly protein (*Upper*) and the purified recombinant full-size 62-kDa Modulo protein (*Lower*). (*Right*) EMSA analysis of the purified TSE-binding protein preincubated with increasing amounts (0.1, 0.3, and 1.0 μg) of the anti-Modulo IgY (anti-mod AB) or Modulo-depleted IgY used as negative control (mod-depl.AB). The control reactions (C) did not contain any antibody.

purified polyclonal IgY was raised in chicken against the peptide derived from the Modulo sequence (SwissProt ID P13469). This antibody was tested for specificity by using Western blot analysis of the purified recombinant Modulo protein and of the whole-fly lysates. In both cases, a single major band of expected mobility was detected (Fig. 1*D*). In addition to the anti-Modulo IgY, affinity purification also yielded the sample of Modulo-depleted IgY as the flow-through from the column with immobilized antigenic peptide. Preincubation of the purified TSE-binding protein with the anti-Modulo IgY before EMSA interfered with formation of the DNA–protein complex in a dose-dependent manner; at the same time, identical concentrations of the Modulo-depleted IgY (used as the negative control) did not have a discernible effect (Fig. 1*D*). Thus, Modulo specifically binds to the conserved testes-specific promoter motif TSE.

Different Modulo Variants Are Expressed in Testes and in Somatic Tissues. Modulo is a broadly expressed protein that has been detected in ovaries (13), embryonic epidermis and mesoderm (14, 15), larval imaginal discs, salivary glands, and brain (16) and in cultured cells (17). Western blot analysis showed that the size of Modulo differs between testes and somatic tissues represented

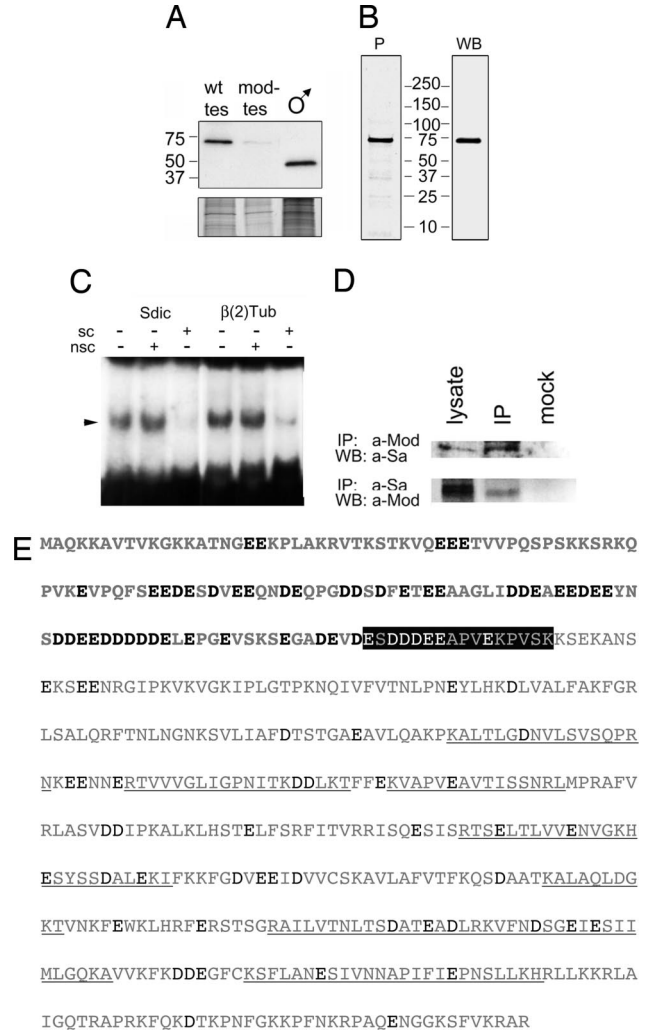

Fig. 2. Modulo variant expressed in testes carries an acidic activator domain. (*A Upper*) Western blot analysis shows the difference in size between the Modulo variants expressed in testes (wt tes) and in somatic tissues (gonadectomized males). Absence of the signal in testes of the male sterile *mod*^{07570} mutant (mod-tes) confirms specificity of analysis. (*A Lower*) Silver staining of the duplicate gel shows total protein loading on the lanes. (*B*) Staining of the SDS/PAGE gel with Coomassie blue (P) and Western blot analysis using anti-Modulo IgY (WB) show purity of the recombinant full-size His$_6$-tagged Modulo protein used for EMSA. (*C*) Binding of the recombinant full-size Modulo to testes-specific promoters is sequence-specific. PCR-amplified core promoter fragments of *Sdic* and *β(2)Tubulin* were used as the probes for EMSA. The complexes formed by Modulo are indicated by the arrowhead. The specific competitor (the TSE oligonucleotide, sc) and the nonspecific competitor 2 (nsc) were added to reactions as indicated at the top. (*D*) Modulo coimmunoprecipitates with the testes-specific TFIID subunit Sa. Samples of the total testes lysates, immunoprecipitated proteins (IP), and mock-precipitated samples (mock) analyzed by Western blotting. The antibodies used for immunoprecipitation (IP) and Western blot analysis (WB) are indicated at the left. (*E*) Characterization of the 50-kDa somatic Modulo variant using the nano-LC/MS/MS data. The full-size Modulo sequence is shown. The peptides identified by MS are underlined. The unusual peptide flanked with the trypsin cleavage site only at its C terminus is highlighted in black; this peptide probably marks the N terminus of the 50-kDa Modulo variant. The acidic domain present only in the full-size Modulo but not in the 50-kDa variant is shown in bold; the acidic residues (D, E) are highlighted.

by the gonadectomized males (Fig. 2*A*). In testes, mobility of the protein is more consistent with the predicted 60.3-kDa size of the Modulo polypeptide; no signal was detected in testes of the *mod*^{07570} male sterile mutant, thus confirming the specificity of

the assay. However, the apparent molecular mass of the Modulo variant expressed in somatic tissues is ≈50 kDa, which corresponds with the size of the TSE-binding protein that we purified from the whole-fly extracts and identified as Modulo.

To gain further insight into the structure of the 50-kDa somatic Modulo variant, we analyzed the LC/MS/MS data in more detail. Analysis of the MS spectra against conceptual translation of the 100-kb genome region encompassing *modulo* did not reveal any novel peptides, thus ruling out involvement of previously uncharacterized coding sequences. Instead, the analysis indicated that the 50-kDa protein is a truncated variant missing the N terminus of the full-size Modulo. All of the identified trypsin-generated peptides were located in the C-terminal portion of the molecule, whereas not a single peptide was identified near the N terminus (Fig. 2*E*). In addition, an unusual peptide flanked with the trypsin cleavage site at only one (the C-terminal) end was repeatedly identified with high confidence during the analysis. The N-terminal end of the peptide thus possibly represents the N terminus of the truncated 50-kDa Modulo variant, and its position is consistent with the size of the protein (predicted molecular mass, 46.2 kDa). In this case, the 50-kDa variant is missing the highly acidic N-terminal domain that is present in the full-size protein (Fig. 2*E*).

We have already shown that the 50-kDa somatic Modulo variant is capable of specific binding to the TSE motif. To confirm that the full-size Modulo variant present in testes has similar activity, we expressed recombinant full-size His_6-tagged Modulo in the *Schneider-2* cultured cells and purified it to near homogeneity (Fig. 2*B*). Binding of the purified recombinant Modulo to the 200- to 250-bp PCR-amplified core promoter fragments was analyzed by EMSA. The double-stranded TSE oligonucleotide was used as the specific competitor. Full-size Modulo was specifically binding to the promoters of *Sdic* and *β(2)Tubulin* (both of which possess the TSE/b2UE1/b2UE2 motif) (Fig. 2*C*). However, we did not observe appreciable binding to the promoters of the testes-specific genes *fzo* (18), *ocn* (19), and *dj* (20) that lack apparent similarities to the TSE sequence (data not shown).

Thus, a specific variant of Modulo is expressed in testes, where it is capable of specific binding to the TSE-containing promoters. This full-size Modulo variant contains the N-terminal acidic domain, the structure of which is characteristic for the acidic activators (21) that facilitate assembly of the core transcription machinery on the promoter and recruitment of chromatin-remodeling factors (22). Known acidic activators interact with the TFIID complex and facilitate interaction of TFIID with TFIIA and TFIIB (23–28). In testes, TFIID is represented by a specific variant encoded by the meiotic-arrest genes of the *sa* group (7). Our analysis showed that Modulo coimmunoprecipitates with the testes-specific TFIID subunit Sa (Fig. 2*D*) and, thus, probably interacts with the testes-specific TFIID during transcriptional activation of testes-specific genes.

The suggested activity of Modulo as transcriptional activator in testes is not consistent with its role in somatic tissues, where it is involved in multiple vital activities (13, 14, 16, 21, 29) that probably include chromatin-mediated transcriptional repression [based on the demonstrated *Su(var)* phenotype of the *modulo* mutants] (29). Structural differences between the Modulo variants may underlie this apparent discrepancy. The 50-kDa somatic Modulo variant is able to bind to DNA but is missing the N-terminal acidic domain and, thus, cannot establish the activating interactions observed in testes. Instead, the somatic Modulo variant may contribute to repression of testes-specific (as well as other) genes in somatic tissues.

Modulo Is Up-Regulated in Spermatogenesis Before Expression of the Spermatid-Differentiation Gene *Sdic*. The Modulo-binding element TSE is present in the promoter of the testes-specific gene *Sdic*

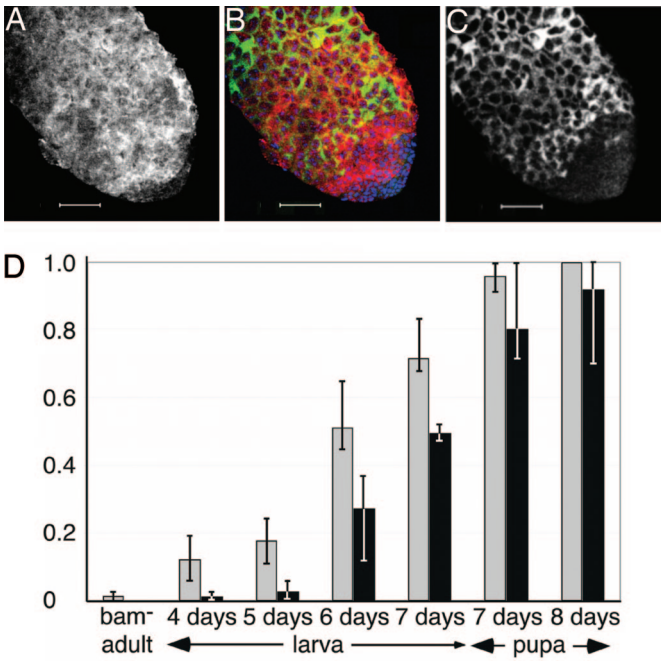

Fig. 3. Modulo is expressed in spermatogenesis before up-regulation of the spermatid-differentiation genes, similar to the general regulators of transcription. (*A–C*) Localization of Modulo in adult testes by immunofluorescense (red channel, *A*) shows that Modulo's up-regulation precedes activation of the *Sdic*::*GFP* transgene (green channel, *C*). The tip of the testis containing stem cells, spermatogonia, and spermatocytes is shown. (*B*) The red and the green channels merged with the signal from the chromatin DAPI stain (blue). Note condensation of small compact nuclei of spermatogonia and stem cells at the tip of the testis. (Scale bars, 30 μm.) (*D*) Up-regulation of the meiotic-arrest genes precedes transcriptional activation of the spermatid-differentiation genes in testis development. The bar graph shows the relative amounts of the transcripts of the meiotic-arrest genes (gray, average of the data for *aly*, *can*, and *mia*) and of the spermatid-differentiation genes [black, average of the data for *Sdic*, *dhod*, *β(2)Tubulin*, *fzo*, *ocn*, and *dj*] in testes of larvae and pupae of different ages (as indicated at the bottom). Error bars show the range of the real-time RT-PCR data for individual genes within each group.

that is up-regulated in primary spermatocytes (10). To regulate *Sdic* expression, Modulo has to be present at the same or earlier stage of spermatogenesis. Localization of the zone of up-regulation of Modulo in adult whole-mount testes using immunofluorescence showed that this zone, indeed, precedes and overlaps the zone of *Sdic* expression (Fig. 3). To visualize *Sdic* expression, we took advantage of the *Sdic*::*GFP* fusion transgene (10). Modulo is weakly expressed in early spermatogonia and stem cells located at the tip of the testis, but is up-regulated in late spermatogonia/early spermatocytes. Within the cells, Modulo is localized in both the nucleus and the cytoplasm (Fig. 3*A*). Specificity of the immunofluorescence staining was confirmed by the absence of appreciable signal in testes of the mod^{07570} and $achi^{1}$ mutants that show severe down-regulation of Modulo; also, no staining was observed in wild-type testes when the primary antibody was omitted (data not shown).

Thus, in spermatogenesis, up-regulation of Modulo precedes activation of its putative regulation target, *Sdic*. We sought to analyze whether this pattern is also present for the general transcriptional regulators (the meiotic-arrest genes) and their regulation targets (the spermatid-differentiation genes). To dissect the temporal order of expression of these genes, we quantitated transcript levels in testes dissected from developing larvae using real-time RT-PCR. In *Drosophila*, spermatogenesis begins early in larval development, and the first wave of meiosis commences at the time of pupation (30). We found that all of the

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Table 1. Transcription of spermatogenesis-related genes in testes of the male sterile mutants

Transcript	Amount of transcript in mutant testes compared with wild type (SD)				Fold down-regulation in the *modulo* mutant (probability of value being equal to 1.0)
	achi/vis	*rye*	*sa*	*mod*	
mod	1.425 (0.000)	0.919 (0.546)	1.391 (0.247)	0.177 (0.097)	5.7 (0.005)*
Ubiquitously expressed genes					
Act5C	0.267 (0.003)	0.415 (0.008)	0.408 (0.042)	1.533 (0.746)	0.7 (0.34)
Rpl9	1.071 (0.079)	0.665 (0.085)	1.082 (0.064)	0.966 (0.205)	1.0 (0.8)
Broadly expressed spermatogenesis-related genes					
twe	0.281 (0.018)	0.352 (0.142)	0.611 (0.104)	0.674 (0.356)	1.5 (0.25)
des	0.503 (0.022)	0.835 (0.012)	1.049 (0.285)	0.656 (0.306)	1.5 (0.19)
Meiotic arrest genes					
aly	0.324 (0.047)	0.223 (0.023)	0.327 (0.050)	0.205 (0.117)	4.9 (0.007)*
ach/vis	0.344 (0.050)	0.360 (0.067)	0.238 (0.026)	0.541 (0.483)	1.8 (0.24)
can	0.312 (0.014)	0.334 (0.051)	0.583 (0.000)	0.162 (0.174)	6.2 (0.014)†
mia	0.286 (0.010)	0.257 (0.070)	0.363 (0.037)	0.459 (0.655)	2.2 (0.29)
rye	0.687 (0.003)	0.065 (0.024)	2.137 (0.188)	0.135 (0.147)	7.4 (0.001)*
nht	0.234 (0.026)	0.437 (0.015)	0.482 (0.092)	0.135 (0.132)	7.4 (0.001)*
Testes-biased genes relatively insensitive to mutations in meiotic arrest genes and *modulo*					
CG13981	0.554 (0.135)	0.309 (0.008)	0.215 (0.101)	0.840 (0.085)	1.2 (0.082)*
Crtp	0.175 (0.052)	0.229 (0.040)	0.181 (0.018)	0.352 (0.130)	2.8 (0.013)†
Ku80	1.432 (0.035)	0.826 (0.093)	1.356 (0.100)	1.255 (0.316)	0.8 (0.30)
Yu	0.199 (0.062)	0.178 (0.041)	0.295 (0.100)	0.591 (0.268)	1.7 (0.12)
Testes-biased genes sensitive to mutations in meiotic arrest genes but moderately affected by *modulo* mutation					
Mst98Ca	0.003 (0.002)	0.014 (0.000)	0.020 (0.002)	0.236 (0.052)	4.2 (0.002)*
CG10934	0.030 (0.006)	0.041 (0.000)	0.108 (0.002)	0.294 (0.007)	3.4 (<0.0001)*
Pros28.1B	0.038 (0.002)	0.052 (0.013)	0.078 (0.019)	0.342 (0.148)	2.9 (0.016)†
Testes-biased genes sensitive to mutations in *modulo* but moderately affected in meiotic arrest mutants *rye* and *sa*					
Ssl	0.081 (0.006)	0.273 (0.012)	0.293 (0.023)	0.041 (0.030)	24.2 (0.0003)*
Testes-biased genes sensitive to mutations in meiotic arrest genes and *modulo*					
dj	0.000 (0.000)	0.006 (0.000)	0.007 (0.002)	0.113 (0.137)	8.8 (0.008)*
β(2)Tub	0.040 (0.005)	0.047 (0.002)	0.074 (0.002)	0.131 (0.042)	7.6 (0.0008)*
fzo	0.003 (0.000)	0.015 (0.003)	0.039 (0.002)	0.084 (0.041)	12.0 (0.0007)*
dhod	0.002 (0.002)	0.015 (0.005)	0.009 (0.001)	0.088 (0.057)	11.3 (0.0013)*
Sdic	0.001 (0.000)	0.095 (0.001)	0.048 (0.002)	0.058 (0.052)	17.2 (0.001)*

Testes were dissected from the $achi^1$, sa^1, $Taf12L^{KG00946}$ (rye), and mod^{07570} mutants and from the wild type, and transcripts were quantitated by real-time RT-PCR. The amounts of transcripts in mutants relative to the wild type are shown; the gene *Rp49* was used as the cDNA template loading reference. Transcripts of the six spermatid-differentiation genes most severely affected in the mod^{07570} mutant are in boldface type.

*Down-regulation in mod^{07570} highly significant ($P < 0.01$).

†Down-regulation in mod^{07570} significant ($P < 0.05$).

five tested spermatid-differentiation genes, including *Sdic*, show drastic up-regulation in late third-instar larvae, i.e., during spermatocyte maturation before meiotic divisions (Fig. 3*D*). This finding is consistent with the observed pattern of up-regulation of the *Sdic*::*GFP* transgene in adult testes (10). However, up-regulation of the three studied meiotic-arrest genes precedes up-regulation of the spermatid-differentiation genes (Fig. 3*D*). Hence, Modulo is up-regulated in the male germ line in a manner similar to known general transcriptional regulators, before the major wave of transcriptional activation that includes spermatid-differentiation genes.

Modulo Is Necessary, but Not Sufficient, for Transcription of a Number of Terminal Differentiation Genes in the Male Germ Line. Although knockout *modulo* mutations result in lethality (29), a male sterile hypomorphic mutation mod^{07570} has been described (31); thus, mutation, caused by a transposon insertion, results in testes-specific *modulo* knockdown (Fig. 2, Table 1). To analyze the role of Modulo in transcriptional regulation, we quantitated transcripts of a number of spermatogenesis-related genes in testes of the mod^{07570} mutant and of the wild type, using real-time RT-PCR (Table 1). The constitutive transcript of the ribosomal protein gene *Rp49* (32) was used as the cDNA template-loading reference. The ubiquitous transcripts *RpL9* (33) and *Act5C* (34) and the broadly expressed spermatogenesis-related genes *des* (31) and *twe* (35) were not significantly affected by the mod^{07570} mutation.

Conversely, a number of genes with testes-biased expression were down-regulated to various extents in the mod^{07570} mutant testes. In particular, several meiotic-arrest genes (including *aly*, *can*, *nht*, and *rye*) were down-regulated 5- to 7-fold. Among the 13 other testes-biased genes examined, 7 showed moderate 2- to 5-fold down-regulation. However, four testes-biased genes showed >10-fold down-regulation, and two more genes were down-regulated 7- to 9-fold. Thus, there is a subset of testes-biased genes [including *Sdic*, *Ssl*, *fzo*, *dhod*, *β(2)Tubulin*, and *dj*] that are specifically affected by the Modulo deficiency.

Because the meiotic-arrest genes themselves are involved in transcriptional regulation in testes, some effects of the Modulo deficiency may be mediated by their down-regulation. Such effects should be similar to the effects caused by mutations in the meiotic-arrest genes themselves. We addressed this possibility by analyzing gene expression patterns in testes of the meiotic-arrest mutants $achi^1$, sa^1, and $Taf12L^{KG00946}$ (*rye*), and by comparing them to the pattern of gene expression in the mod^{07570} mutant testes (Table 1). Among the 13 testes-biased genes analyzed, four genes were affected differently by the meiotic arrest and the *modulo* mutations. The genes *Mst98Ca* (36), *Pros28.1B* (37), and *CG10934* were very sensitive to mutations in meiotic-arrest genes but not in *modulo* and, conversely, the gene *Ssl* did not

show striking sensitivity to the mutations in the meiotic-arrest genes *sa* and *rye* but was severely affected in the *modulo* mutant. Therefore, the effect of Modulo deficiency on transcription in testes cannot be reduced to down-regulation of the meiotic-arrest genes. It is possible that such down-regulation leads to the subpar performance of the meiotic-arrest genes that is still sufficient to carry the germ line of the mod^{07570} mutant through the meiotic divisions (31) but results in moderate (e.g., 3- to 4-fold) down-regulation of testes-biased genes, such as *Mst98Ca*, *Pros28.1B*, and *CG10934*. However, a more severe effect of the Modulo deficiency on a subset of spermatid-differentiation genes probably reflects disruption of gene-specific transcriptional regulation and provides a molecular basis for the spermatid-differentiation failure observed in the mod^{07570} mutant (31).

The subset of genes strongly affected in the mod^{07570} mutant includes *Sdic* and *β(2)Tubulin*. These genes possess the TSE-like Modulo-binding motifs and, thus, probably represent the direct regulatory targets of Modulo. Interestingly, we were not able to detect specific binding of Modulo to the promoters of *fzo* and *dj* that are also strongly affected by the *modulo* mutation. At the same time, the studied *dj* promoter fragment contained all sequences necessary for efficient testes-specific transcription (38). This finding implies that Modulo has indirect target genes such as *dj* and, probably, *fzo* that may be regulated by transcription factors that are, in turn, under the control of Modulo. Our broad survey of 96 transcriptional regulators expressed in testes identified nine putative transcription factors that are down-regulated >10-fold in the mod^{07570} mutant testes (data not shown). Thus, mutation in *modulo* can lead to disruption of the downstream cascade of transcriptional regulation that includes Modulo-dependent transcription factors and their regulation targets.

To determine whether Modulo is sufficient to induce ectopic transcription of spermatid-differentiation genes, we expressed recombinant full-size Modulo in the *Schneider-2* cultured cells under the control of metallothionein promoter. Stable transfected clones were selected, and expression of the transgene was induced by various concentrations of Cu^{2+} in the culture media. Unexpectedly, we observed that the *Schneider-2* cells naturally express the full-size Modulo variant. Nevertheless, these cells do not show significant expression of the Modulo-dependent testes-specific genes *Sdic*, *Ssl*, and *dhod*, and increase of the Modulo dose by induced expression of the transgene did not affect the levels of these transcripts (data not shown). Therefore, other, presumably testes-specific, factors (such as the testes-specific TFIID) have to cooperate with Modulo to induce expression of spermatid-differentiation genes, thus defining tissue specificity of the Modulo-mediated transcriptional regulation.

Expression of Modulo in Testes Is Under the Control of Meiotic-Arrest Genes and the RNA-Binding Protein Boule. To analyze the regulation of Modulo expression in testes, we analyzed a number of mutants that control different stages of spermatogenesis. In testes of the *bam* mutant (39), both the Modulo protein (Fig. 4) and *modulo* transcript are severely down-regulated (real-time RT-PCR showed the transcript level in *bam* mutant testes at 10% of the wild type; SD 2%). Thus, high levels of Modulo expression in the testes require the onset of the meiosis/differentiation program. Furthermore, mutations in the meiotic-arrest genes *achi/vis*, *sa*, and *rye* result in severe down-regulation of Modulo protein in testes (Fig. 4); however, *modulo* transcription is not affected (Table 1). Therefore, Modulo expression in the testes is regulated by the meiotic-arrest genes at posttranscriptonal levels, similar to the regulation of the meiotic entry control protein Cdc25/Twine (2). Translation of Twine in the testes requires the RNA-binding protein Boule (11). To investigate whether a similar mechanism is involved in the regulation of Modulo, we analyzed testes of the *boule* mutants and found that Modulo expression in testes is severely affected by the Boule deficiency (Fig. 4).

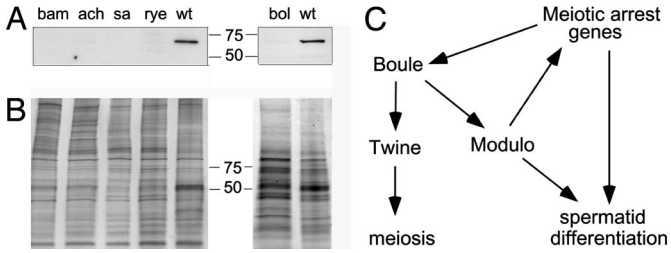

Fig. 4. Regulation of Modulo expression in testes by the meiotic-arrest genes and Boule links the pathways leading to meiosis and spermatid differentiation. (*A*) Modulo expression in testes of the wild type (wt) and of the $bam^{\Delta[supi]86}$ (bam), $achi^{1}$ (ach), sa^{1} (sa), $Taf12L^{KG00946}$ (rye), and bol^{1} (bol) analyzed by Western blotting. (*B*) The duplicate gels stained with silver (*Left*) or Coomassie blue (*Right*) show total-protein loading on the lanes. (*C*) Model for the role of Modulo in cross-communication between the pathways leading to the G_2/M transition and spermatid differentiation. Arrows indicate positive regulation.

Regulation of Modulo expression in testes by Boule provides a mechanistic link between meiosis and spermatid differentiation in the male germ line. The meiotic-arrest genes are required for expression of a number of spermatogenesis-related genes, including *boule* (2, 5). Boule is required for expression of Modulo, which, in turn, is necessary to maintain expression of several meiotic-arrest genes. These events establish a positive regulatory loop that sustains high levels of expression of Boule, Modulo, and the meiotic-arrest genes after the onset of the meiosis/differentiation program in spermatocytes (Fig. 4). Boule further regulates the G_2/M transition in meiosis by positive translational regulation of Cdc25/Twine (2, 11), and Modulo and the products of the meiotic-arrest genes are required for expression of a number of spermatid-differentiation genes. Thus, the pathways that lead to meiosis and to expression of the spermatid-differentiation genes in the male germ line are integrated in a single mechanism to ensure coordinated execution of meiotic divisions and spermatid differentiation.

Methods

Detailed descriptions of protein extraction and EMSA, purification of the TSE-binding activity, Western and Southwestern blot analysis, coimmunoprecipitation, and immunofluorescence and microscopy are included in *Supporting Materials and Methods*.

***Drosophila* Stocks.** *D. melanogaster* stocks were maintained on yeast–molasses media at 20°C. The wild-type flies used were Oregon-R for transcription assays and the mixture of Oregon-R and *y w* for biochemical purification of proteins. The stocks carrying the mutations $Taf12L^{KG00946}$, $bam^{\Delta 86}$ (39), aly^{1} (2), sa^{1} (2), bol^{1}, and mod^{07570} (31) were obtained from the Bloomington *Drosophila* Stock Center at Indiana University, Bloomington. The $achi^{1}$ mutant (5) was generously provided by Rob White (University of Cambridge, Cambridge, U.K.).

Protein Extracts from Dissected Tissues and EMSA. Tissues were manually dissected in PBS, and proteins were extracted with 1.5 M KCl in the presence of 0.4% Triton X-100. Double-stranded oligonucleotide AGCTTTGATCGTAGTGTGCCTTTGGGGGAAATTCTG (the TSE probe) or PCR-amplified core promoter fragments labeled with polynucleotide kinase and [γ-^{32}P]ATP were used as the probes. Specific competitor (the unlabeled TSE probe) and the nonspecific competitor 2 (the double-stranded oligonucleotide TTCGATCAAATCTA-

ACTTTATTCCATATAGTTGCTTATAC) were used in a 100-fold molar excess to the labeled probe.

Purification of the TSE-Binding Activity. Proteins extracted from homogenized adult *D. melanogaster* were fractionated by precipitation with $(NH_4)_2SO_4$. The proteins were resolubilized and separated by sequential chromatography on the heparin–Sepharose, Uno Q-1 and Uno S (Bio-Rad), and Superdex-200 HR (Amersham) columns; purification of the TSE-binding activity was monitored by EMSA. Partially purified fractions were pooled and incubated with the DNA affinity beads covered with concatenated TSE oligonucleotides. The beads were step-eluted with increasing concentrations of KCl (ranging from 0.1 to 3 M). Proteins were fractionated by SDS/PAGE in the 4–15% gradient gel and stained by using colloidal Coomassie blue, and the bands of interest were excised and sent for identification by nano-LC/MS/MS to Midwest Bio Services (Overland Park, KS).

Expression and Purification of Recombinant Modulo. The full-size Modulo ORF was amplified from the testes cDNA by PCR and cloned into the vector pMT/V5-HisA (Invitrogen) under the control of metallothionein promoter. The plasmid was transfected into the *Schneider-2* cells along with the blasticidin-resistance plasmid pCoBlast (Invitrogen) by using Tfx-20 reagent (Promega), and polyclonal stable lines were selected by using blasticidin. The His_6-tagged Modulo protein was induced by 500 μM $CuSO_4$ and purified on the immobilized cobalt column (Talon; CLONTECH) according to the manufacturer's recommendations.

Reverse-Transcription and Real-Time PCR. RNA was extracted from dissected tissues with Trizol reagent (Invitrogen). Reverse-transcription reactions were performed by using 1 μg of total RNA as a template with the PowerScript enzyme (CLONTECH). A total of 0.5% of the reverse-transcription reaction was used as a template for a 20-μl real-time PCR. Reactions were run in triplicate in the ABI 5700 Sequence Detector, by using SYBR green chemistry (Applied Biosystems).

Antibodies. The primary polyclonal antibody against Modulo was raised in chicken against the peptide SVSQPRNKEENNERT and affinity-purified at Aves Labs. The primary polyclonal affinity-purified antibody raised in guinea pig against Sa was a generous gift from Dr. X. Chen (Stanford University, Stanford, CA). Secondary HRP-conjugated and Alexa Fluor 594-conjugated goat anti-chicken antibodies were from Aves Labs, and HRP-conjugated goat anti-guinea pig antibody was from Abcam.

Coimmunoprecipitation. Testes dissected from the 1-day-old wild-type males were cross-linked with 1 mM dithiobis[succinimidyl propionate] (DSP; Pierce), and proteins were extracted with 1% SDS and immunoprecipitated by using primary antibody against Modulo or Sa and agarose beads conjugated with the goat anti-chicken antibody (PrecipHen; Aves Labs) or with the protein G (Upstate Biotechnology, Lake Placid, NY). The DSP cross-link was cleaved by boiling with 10 mM β-mercaptoethanol for 15 min and the proteins were analyzed by Western blotting.

We thank Irina Sorokina (Midwest Bio Services) for the very helpful, in-depth analysis of the LC/MS/MS results. This work was supported by National Institutes of Health Grant GM61549 (to D.I.N.).

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