The *Drosophila* Modifier of Variegation *modulo* Gene Product Binds Specific RNA Sequences at the Nucleolus and Interacts with DNA and Chromatin in a Phosphorylation-dependent Manner*

Laurent Perrin§, Pascale Romby¶, Patrick Laurenti‡, Hélène Bérenger*, Sacha Kallenbach‡, Henry-Marc Bourbon**, and Jacques Pradel‡ ‡‡

From the §Laboratoire de Génétique et de Biologie du Développement, Institut de Biologie du Développement de Marseille, Parc Scientifique de Luminy, CNRS Case 907, 13288 Marseille Cedex 9, France, the ¶Laboratoire de Biologie du Développement, Université de Paris 7, case 7077, 2 place Jussieu, 75251 Paris Cedex 5, France, **Institut de Biologie Moléculaire et Cellulaire UPR 9002 du CNRS, 15 rue René Descartes, 67084 Strasbourg, France, and the ‡‡Centre de Biologie du Développement, 118, route de Narbonne, 31062 Toulouse, France

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**modulo** belongs to the modifier of Position Effect Variegation class of *Drosophila* genes, suggesting a role for its product in regulating chromatin structure. Genetics assigned a second function to the gene, in protein synthesis capacity. Bifunctionality is consistent with protein localization in two distinct subnuclear compartments, chromatin and nucleolus, and with its organization in modules potentially involved in DNA and RNA binding. In this study, we examine nucleic acid interactions established by Modulo at nucleolus and chromatin and the mechanism that controls the distribution and balances the function of the protein in the two compartments. Structure/function analysis and oligomer selection/amplification experiments indicate that, in *vitro*, two basic terminal domains independently contact DNA without sequence specificity, whereas a central RNA Recognition Motif (RRM)-containing domain allows recognition of a novel sequence-motif-specific RNA class. Phosphorylation moreover is shown to down-regulate DNA binding. Evidence is provided that *in vivo* nucleolar Modulo is highly phosphorylated and belongs to a ribonucleoprotein particle, whereas chromatin-associated protein is not modified. A functional scheme is finally proposed in which modification by phosphorylation modulates Mod subnuclear distribution and balances its function at the nucleolus and chromatin.

Many eukaryotic gene products elicit remarkable functional diversity, even those involved in basic cellular processes. In particular, increasing evidence indicates that a number of ribosomal proteins fulfill a second function apart from ribosome and protein synthesis (1). Ribosomal biogenesis takes place in the nucleolus where rDNA is transcribed in a large precursor RNA which is then processed by nucleotide modifications and specific cleavages into mature rRNAs that eventually assemble with ribosomal proteins into ribosomal subunits (2, 3). This process requires a number of additional factors, such as small nucleolar RNAs (snoRNA) and non-ribosomal nucleolar proteins. Our knowledge of nucleolar proteins is limited. However, a shared structural feature is the presence of modules thought to represent functional domains (3). Such modules include basic and acidic stretches, which possibly interact with ribosomal proteins (4), and the so-called RNA Recognition Motif (RRM) (5), which could bind either rRNAs at different stages of maturation or snoRNAs.

Ribosome biogenesis is tightly regulated in a close relationship with cell growth or differentiation. It has long been shown that changing cell culture conditions in a way that either improves the growth rate or induces a differentiation program results in enhanced versus decreased ribosome biosynthesis, respectively (6). Moreover, mutations that disrupt gene functions necessary for ribosome assembly often result in growth defects. In yeast or mammalian cell lines, requirement for cell growth has been demonstrated for several snoRNAs (7) and for all the components of RNase MRP, the best characterized snoRNP (8). In *Drosophila*, several classes of mutations cause growth alteration during development, such as the *Minute* class (9), widely believed to affect ribosomal protein genes (10), and *mini* and *bobbed* mutations, which alter rRNA production (11).

The *Drosophila* *modulo* gene (*mod*) has first been characterized as a dominant suppressor of Position Effect Variegation (PEV) (12). PEV occurs in experimental strains where genes placed close to constitutive heterochromatin are randomly turned on or off, leading to mosaic adult structures. The products of genes that modify PEV are believed to change the local chromatin structure, which leads, when they are mutated, to an increased (suppression of variegation) or decreased (enhancement of variegation) expression of neighboring genes (13–16). The dominant PEV suppression phenotype of *mod*, therefore, suggests that its product participates in the formation of multimeric protein complexes that package the DNA, promoting chromatin compaction and inactivation (12). We have recently reported that *mod*, apart from its role in chromatin structuration, has a second cellular function and is required in nucleolus activity and protein synthesis capacity (17). First, cell clones deficient for *mod* express phenotypic traits characteristic of *Minute* mutations. Second, the protein, while

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*The abbreviations used are: snoRNA, small nucleolar RNAs; RRM, RNA Recognition Motif; PEV, Position Effect Variegation; GST, glutathione S-transferase; SELEX, selection-amplification procedure; NS, non-selected sequence; RNP, ribonucleoprotein; dsDNA, double-stranded DNA; PAGE, polyacrylamide gel electrophoresis.*
actually associated to condensed chromatin and heterochromatin sites, is also abundantly found at the nucleolus. Consistent with this localization, Mod displays a modular organization which is often found in nonribosomal nucleolar proteins, including an acidic stretch, two basic regions located at both ends of the molecule, and four reiterated RRM s in the remaining core portion (18, 19).

The goal of the investigation reported here was to gain insight into the nucleic acid interactions established by Mod at the nucleolus and chromatin and to determine the molecular mechanism that controls the distribution and balances the function of the protein in the two sub-nuclear compartments. The data show that the two basic domains independently contact DNA without sequence specificity, whereas RRMs provide a sequence-specific RNA-binding activity. We also report that nucleolar Mod is phosphorylated and associated to an RNA moiety, whereas the chromatin-bound Mod appears to be unmodified. A functional scheme is finally proposed in which modification by phosphorylation modulates Mod subnuclear distribution and balances its function at the nucleolus and chromatin.

**EXPERIMENTAL PROCEDURES**

**Production of Mod Variant Proteins**—Primers used to generate by polymerase chain reaction sequences encoding the Mod variants were: 1) AGCTGGATCCTCGAGCTGATCCAC; 2) AGCTGGATCCTCGAGCTGATCCAC; 3) AGCTGGATCCTCGAGCTGATCCAC; 4) AGCTGGATCCTCGAGCTGATCCAC. Two-modified polymerase chain reaction products cloned in pGEX-2T (Amersham Pharmacia Biotech), purified according to Smith and Johnson (20) and stored at −20 °C in NT2 buffer (20 mM Tris-HCl, 5 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% Nonidet P-40, 10% glycerol, pH 7.5).

**SDS-PAGE, Bidimensional Gel Analysis, and Western Blotting**—For SDS-PAGE, proteins were fractionated on 7.5% gels and electroblotted onto nitrocellulose. Bidimensional gel analysis was performed as described in (21). Nuclear proteins from embryos (0–18 h) were treated with BsaHI-digested polymerase chain reaction products cloned in pGEX-2T (Amersham Pharmacia Biotech), purified according to Smith and Johnson (20) and stored at −20 °C in NT2 buffer (20 mM Tris-HCl, 5 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% Nonidet P-40, 10% glycerol, pH 7.5).

**Nuclear Fractionation and Immunoprecipitation Procedure**—Nuclear fractions were prepared from either 1000 dechorionated embryos or 10^8 KT cells, diluted in Iysis buffer (20 mM Tris-HCl, 0.15 mM, 1 mM MgCl₂, 1 mM NaCl, 0.5% Nonidet P-40, pH 7.5) supplied with a mixture of protease inhibitors and preincubated for 1 h at 4 °C with protein A-Sepharose beads (Amersham Pharmacia Biotech). Depleted samples (1 ml) were then incubated overnight at 4 °C in the same buffer with 10 mg of Maleless (Mle) or BsaHI-digested polymerase chain reaction products cloned in pGEX-2T (Amersham Pharmacia Biotech), purified according to Smith and Johnson (20) and stored at −20 °C in NT2 buffer (20 mM Tris-HCl, 5 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% Nonidet P-40, 10% glycerol, pH 7.5). Western analysis of the recovered fractions was performed with a panel of monoclonal antibodies directed against chromatin-bound proteins like histone 2B, nucleosoluble components (S5 and P11 antigens), and Mod (17, 23). The Mod protein was detected in 0.15 M and 0.30–0.45 M NaCl fractions only. For Mod immunoprecipitation assays, sub-nuclear proteases were prepared from dechorionated embryos or 10^8 KT cells, diluted in Iysis buffer (20 mM Tris-HCl, 0.15 mM, 1 mM MgCl₂, 1 mM NaCl, 0.5% Nonidet P-40, pH 7.5) supplied with a mixture of protease inhibitors and preincubated for 1 h at 4 °C with protein A-Sepharose beads (Amersham Pharmacia Biotech). Depleted samples (1 ml) were then incubated overnight at 4 °C in the same buffer with 10 mg of Maleless (Mle) or BsaHI-digested polymerase chain reaction products cloned in pGEX-2T (Amersham Pharmacia Biotech), purified according to Smith and Johnson (20) and stored at −20 °C in NT2 buffer (20 mM Tris-HCl, 5 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% Nonidet P-40, 10% glycerol, pH 7.5). Cell fractions were analyzed on 20% polyacrylamide, 8M urea gels. RNase footprinting experiments were performed as described previously (23) but in the presence of 10 mM sodium pyrophosphate (10 mM pyrophosphate) and of phosphatase inhibitors (2 mM sodium vanadate, 0.1 mM sodium fluoride, 20 mM EDTA, and 20 mM EGTA). Mod immunoprecipitation was also performed in the presence of sodium pyrophosphate and phosphatase inhibitors.

**DNA Binding Assays**—Purified GST fusion proteins (5 μg) were added to dsDNA-cellulose beads (Amersham Pharmacia Biotech, 0.5 ml in NT2 buffer supplemented with 0.1 mg/ml bovine serum albumin) and incubated at 20 °C for 2.5 h. After centrifugation and three washes in NT2 (0.5 ml each), bound proteins were stepwise eluted from the beads with 0.5 ml of NT2 containing increasing amounts of NaCl. Fractions, including the three washes, were precipitated with 5% trichloroacetic acid, washed with acetone, and analyzed by Western blotting. For embryonic Mod, nuclei were isolated and fractionated as described previously (23) but in the presence of 10 mM sodium pyrophosphate (10 mM pyrophosphate) and of phosphatase inhibitors (2 mM sodium vanadate, 0.1 mM sodium fluoride, 20 mM EDTA, and 20 mM EGTA). Mod immunoprecipitation was also performed in the presence of sodium pyrophosphate and phosphatase inhibitors.

**SELEX Procedure**—We used the synthetic RNA pool first described in Tsi et al. (24). SELEX procedure was essentially as described in Ghisolfi-Nieto et al. (25), with the following modifications. Purified GST fusion proteins (5 μg) were incubated with 20 μl of 50% glutathione-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 20 °C in NT2 buffer (200 μl final volume). After three washes in 0.5 ml of NT2 buffer, beads were suspended in 50 μl of binding buffer (20 mM KCl, 150 mM NaCl, 50 mM Tris-HCl, 0.05% Nonidet P-40, 2.5 mM polyvinyl alcohol, 1 mM EDTA, 100 μg/ml tRNA, 125 μg/ml bovine serum albumin, 1 mM dithiothreitol, 0.8%, pH 7.4) and incubated with the RNA sample (0.5 μg) for 5 min at 20 °C. For the first two rounds, RNA samples were preincubated for 5 min at 20 °C with Sepharose protein prior to the selection step. After the third round, 0.5 μg were added in the last wash. Polymerase chain reaction products were cloned and sequenced after seven rounds of selection/amplification.

**Rna Structure Probing and Footprint**—RNAs were transcribed in vitro by T7 RNA polymerase, 5’-end-labeled with [γ-32P]ATP and T4 polynucleotide kinase, purified on a 10% polyacrylamide, 8 μl urea gel, eluted overnight, and precipitated with ethanol. Labeled RNA (50,000 cpm) was renatured in reaction buffer at 37 °C for 15 min and complex formation carried out at 20 °C for 15 min in the presence of increasing amounts of Mod. Enzymatic cleavage reactions by RNase T1 (10⁻² unit), RNase V1 (0.2 unit), RNase T2 (0.2 unit), or nucleic NC (0.2 unit) were performed at 20 °C for 5 min in 0.5 ml of 50 mM Tris-HCl, 5 mM MgCl₂, 150 mM NaCl, and 1 μM of carrier tRNA, pH 7.5. Iron-EDTA reactions were done as in (26). Reactions were stopped by phenol/ chloroform extraction, and RNA fragments recovered by ethanol precipitation were analyzed on 20% polyacrylamide, 8 μl gels. RNA bands were visualized by autoradiography.

**Polytene Chromosome Squashes and RNase Treatment**—Polytene squashes were performed on salivary gland tissue as described in Richter et al. (27). Briefly, salivary glands were preincubated in phosphate-buffered saline containing 0.5% Triton X-100, and then for 10 min in either phosphate-buffered saline alone (as a control) or phosphate-buffered saline supplemented with 0.5 mg/ml RNase A. Polytene chromosome squashes and staining were performed as described in Clark et al. (28). Mod was detected by the mAb LA9 at 20 μg/ml and Maleless (Mle) by affinity purified rabbit anti-Mle antibodies (gift of M. Kuroda) at a dilution of 1:500. Anti-
mouse-fluorescein isothiocyanate-conjugated and anti-rabbit-tetra-
methylrhodamine isothiocyanate conjugated secondary antibodies
(Jackson) were used at a dilution of 1/150. Slides were examined under
an Axiohot microscope (Zeiss).

RESULTS

Mod DNA-binding Is Mediated by the N- and C-terminal Domains—Mod has been previously shown to bind DNA both
in vitro and in vivo (12, 18). Cross-linking/immunoprecipitation
experiments moreover demonstrated interaction with
several repetitive elements, consistent with the protein asso-
ciation to heterochromatin and the dominant phenotype of PEV
suppression (17).

In order to identify the domains in Mod involved in DNA
binding, GST fusions of various truncated versions of the pro-
tein were tested on dsDNA-cellulose chromatography. The res-
ults showed that the deletion of the two positively charged N-
and C-terminal domains is required to abolish the ability of
Mod to bind DNA in this assay, whereas truncated mutant
proteins of only one of these domains still exhibit some activity
(not shown). We further develop SELECT experiments (29) to
ask the question of sequence specificity in Mod DNA binding.
No obvious consensual motif was revealed by sequence compar-
ison of 40 clones obtained after five rounds of selection/ampli-
ification performed using the full-length protein. Thus, the two
terminal domains in Mod likely interact with DNA without
sequence specificity.

Mod Is a Phosphoprotein—The Mod sequence contains a
number of putative phosphorylation sites (18). Metabolic incor-
poration of 32P in KC cells, followed by immunoprecipitation,
Western blotting and autoradiography, reveals that Mod gives
rise to a single band of 78 kDa (Fig. 1A, lane 2), indicating that
the protein actually is phosphorylated in vivo. Consistently,
treating Western blots with alkaline phosphatase progres-
sively reduces and finally abolishes the 32P labeling (Fig. 1A,
lanes 3 and 4). Next, by comparing the 32P incorporation in
starved and serum-stimulated cells, we noticed that serum
addition raised the specific incorporation in Mod by a factor of
16, whereas the increase in crude cell extracts and purified
nuclei was only 2.7- or 4.7-fold, respectively (Fig. 1B). As inter-
nal control to these experiments, we compared the Western
signals from serial dilutions of cell extracts grown in the pres-
ence or absence of 10% fetal calf serum to show that the serum
has no significant effect on Mod de novo synthesis (not shown).
These data strongly suggest that serum enhances Mod
phosphorylation.

To investigate for Mod phosphorylation in vivo, protein iso-
electric variants present in embryos were analyzed by dimen-
sional PAGE followed by a Western blot analysis. Nuclear
extracts were prepared either in the presence of a mixture of
phosphatase inhibitors or digested with phosphatases. Mod
from phosphatase-treated extracts runs as a single spot with
an apparent pI of 7.2, indicating that the multiple isoforms
differing by negative charges (pI from 7.2 to 5.2) observed from
non-digested nuclei, correspond to stepwise phosphorylation
events on several amino acid residues (Fig. 1C).

Mod Differential Phosphorylation at Chromatin and Nucle-
olus—We previously reported a procedure that combines step-
wise salt extraction with low and high speed centrifugations, to
isolate distinct subnuclear fractions from purified nuclei, and a
comparative Western analysis demonstrating that nucleo-
soluble proteins and components firmly bound to chromatin
were recovered in distinct subfractions; the former by extrac-
tion at low salt concentration (0–0.15 M and then 0.15–0.3 M
NaCl) and the latter by sonication after raising NaCl concen-
tration from 0.30 to 0.45 M (23). Mod was detected in nu-
cleosoluble (0–0.15 M NaCl) and chromatin (0.30–0.45 M) frac-
tions only. Most significantly, the protein has never been seen in
the intermediate salt extract (0.15–0.30 M), ruling out the
possibility of a cross contamination between nucleolar and
chromatin Mod fractions. This led us to conclude, when consid-
ered with immunolocalization and genetic data, that part of the
protein is firmly bound to chromatin, whereas the major frac-
tion is nucleolar and solubilized at low salt (17). To test

![Fig. 1. Phosphorylation of Mod in vivo. A, 32P in vivo labeling of
Mod. Immunoprecipitates obtained with mAb LA9 from nuclear prepa-
rations of 32P metabolically labeled KC cells were fractionated by
SDS-PAGE, blotted on nitrocellulose, and revealed by autoradiography.
Lane 1, crude nuclear extract; lanes 2, 3, and 4, immunoprecipitated
fractions. Prior to autoradiography, the nitrocellulose stripes corre-
spanding to lanes 3 and 4 were treated with alkaline phosphatase for 5
and 30 min, respectively. B, serum effect on Mod phosphorylation.
Ratios between 32P incorporation in KC cells grown in the presence and
in the absence of 10% fetal calf serum: total cell extract (a), purified
nuclei (b), and immunoprecipitated Mod (c). Values from three distinc-
t experiments were, in cpm for 5106 KC cells, 1.8107/6.7106 (2.7) for
bar a, 7.1107/1.5107 (4.7) for bar b, and 3.2106/2.0105 (16) for bar c. Serum
clearly induces increased 32P incorporation in Mod. C, bidimensional
Western analysis. Nuclear proteins from embryos (0–18 h) were first
separated by isoelectric focusing and secondly by SDS-PAGE. Corre-
sponding Western blots were probed with mAb LA9. Top panel, nuclear
extract prepared in the presence of phosphatase and protease inhibi-
tors. Bottom panel, nuclear extract digested for 2 h with alkaline phos-
phatase at 37 °C. Dephosphorylating the sample resumes the series of
native Mod isoforms into a single migrating species.
Phosphorylation Down-regulates Mod DNA-binding Activity—

In Vitro—These data also suggested the hypothesis that phosphorylation could control Mod DNA-binding activity. To test this hypothesis, nuclear and chromatin fractions prepared from embryos were assayed on dsDNA-cellulose chromatography. Data in Fig. 2B indicate that Mod extracted from chromatin strongly binds DNA and is eluted at NaCl concentrations of 0.5–1.0 M, whereas the nucleosoluble protein shows significantly weaker affinity and is eluted at NaCl concentrations of 0.2–0.3 M. Significantly, Mod from a nucleosoluble fraction digested by phosphatase exhibits a substantially improved DNA-binding activity (elution at NaCl concentrations of 0.3–1.0 M). We therefore conclude that phosphorylation likely down-regulates Mod DNA-binding activity in vitro.

Identification of RNA Aptamers of Modulo—The presence of four RRMs in Mod led us to investigate the RNA binding properties of the protein. We first tested the affinity of GST-Mod or of a truncated version consisting only of the four RRMs (GST-RRM) to homopolymeric oligoribonucleotides. Both are able to bind poly(G) and poly(U), to a lesser extent poly(A), but not poly(C) (not shown). Second, we used GST-Mod and GST-RRM proteins in a selection-amplification procedure known as SELEX (30) to look for nucleotide specificity in RNA binding. PCR products were cloned and sequenced after seven rounds of selection/amplification of a 25-nucleotide-long random sequence (24). Out of 43 sequences selected from the GST-Mod, 24 obey the consensus (UUAC(N)6GU(A/G)G(U/A)(M)N), where N and M are complementary nucleotides putatively able to form a stem, and “x” is comprised between 4 and 6 (Fig. 3). The other clones lack the consensus and do not efficiently bind Mod in gel shift controls (not shown). Their selection can originate from unspecified interaction with the basic terminal domains present in the full-length protein. This hypothesis is supported by the second SELEX experiment performed with GST-RRM. 10 clones from this selection were sequenced, and all were found to match with the consensus defined in Fig. 3. This experiment first confirms the consensual motif derived from the selection by GST-Mod and secondly indicates that RNA binding specificity is only provided by the RRM-containing domain.

Band shift assays were performed to confirm that selected sequences actually interact with Mod fusion proteins and to get an estimate of their affinities ($K_d$ values are given in Fig. 3). M8 and M12 were found to correspond to the aptamers of highest affinity, with $K_d$ values of 25 nM for GST-Mod and of 10 nM for GST-RRM. The other selected RNAs present weaker affinities, with $K_d$ values ranging from 100 nM to 1000 nM. Fig. 4 reports only the data obtained with M12 aptamer and the various controls done. The aptamer is actually shifted by GST-Mod and GST-RRM, whereas a ribonucleotide lacking the consensus (non-selected sequence, NS) does not (Fig. 4A). Fig. 4B provides other controls showing that anti-Mod mAb LA9 inhibits the interaction (lane 2), GST alone is ineffective (lane 3), and competition with unlabeled M12 progressively abolishes the shift whereas competition with ribonucleotide NS has no effect (lanes 4–8). Taken together these data indicate that Mod is a sequence-specific RNA binding protein and support the idea that the bipartite motif defined here constitutes a high affinity site for the protein.

Probing the RNA Aptamer Conformation and Mod Footprint—The conformation of M12 was investigated using several enzymatic probes (31) such as RNase V1 (specific for paired nucleotides; Fig. 5B, lane 9), RNase T1 (specific for unpaired guanines; Fig. 5B, lane 1), RNase T2 (preferential cut after unpaired adenines; Fig. 5B, lane 5), and nuclease from Neurospora crassa (specific for unpaired nucleotides; Fig. 6A). The results are reported on the secondary structure model derived from the enzymatic probing (Fig. 5C). The data support the existence of a stable hairpin structure presenting an external loop (nucleotides G27–U31) and two internal loops (nucleotides A13–C21/A38–A41 and A47–A55). The external loop is well defined by the presence of an RNase T1 cut at G30 and of several RNase T2 or nuclease NC cleavages at U29 and A28 (see Fig. 5, A and B). Interestingly, G27 is not accessible to RNase T1, indicating that this peculiar guanine is either stacked within the loop or base paired with U31. Many hairpin loops closed by non-canonical base pairs (G–A or G–U) have been described (32). In most of the selected aptamers, position U31 is predominantly U or A (see Fig. 3). Other single-stranded specific RNase cleavages are mainly located in the regions A15–U19, U48–A52, and G64–A67 (Fig. 5, A and B). The existence of helices I and II is supported by the presence of several RNase V1 cleavages at positions 9–11, 22–24, 36, 44–
These data indicate that the two conserved sequences (UUAC and GU(A/G)G(U/A)) are located likely in single-stranded regions. The Mod footprint was next studied using RNases T1, T2, V1, and nuclease NC as enzymatic probes. We also used iron-EDTA which generates, in the presence of hydrogen peroxide, reactive hydroxyl radicals cleaving ribose moieties irrespective of secondary structure (33). Experiments are shown in Fig. 5, A and B, and summarized on the predicted secondary structure of M12 depicted in Fig. 5C. Mod induces strong protection against single-stranded specific RNases at positions that correspond to the two conserved sequences, in the hairpin loop (at U28, A29, and G30) and in one internal loop (at U18, U19, and A20). Reduced RNase V1 cleavages are also observed on both sides of helix II and at positions 9–11, whereas significant enhancements occur at positions 44–45. Furthermore, iron-EDTA footprint experiments revealed protections at riboses 29 to 35 (Fig. 5C). It has to be noted that no information was obtained for the nucleotides A15–A20 and A59–A63 from iron-EDTA experiments, owing to the presence of several nonspecific cleavages. Mod does not induce reactivity changes in the bottom of helix I, indicating that the two non-random oligonucleotides used for the selection do not directly participate in the RNA binding site.

The folding program of Zuker (34) was used to generate suboptimally folded structures for the other selected aptamers. Interestingly, M8 RNA, which binds Mod with the same affinity as M12, is predicted to fold in a very similar structure. The hairpin loop GUGGA and the single-stranded sequence UUAC in M8 are separated by a stem formed by five base pairs (seeFig. 3, Selection of Mod aptamers. A, schematic representation of the Mod variants used in Selex experiments. Mod primary structure consists in the juxtaposition of a basic N terminus (light gray), a large acidic region (dark gray), a repetition of four RRMs (white) and a basic C terminus (light gray). B, the RNA template used in SELEX experiments, shown at the top, encompasses constant sequences and a core consisting of 25 randomly synthesized nucleotides. Sequences selected by GST-Mod (15) and by the GST-RRM (9) are aligned. When one sequence was isolated several times, occurrence is given in brackets. The derived consensus is at the bottom. Underlined nucleotides belong to flanking constant sequences of the RNA template. Conserved nucleotides are in bold, and nucleotides in italic correspond to putative stem region. N and M are complementary nucleotides. $K_d$ values of eight aptamers for the full-length protein are shown.
Fig. 3). For the other examined RNAs which present weaker binding affinities, helix I appears to be shorter (4 base pairs in M19) or is prolonged by several base pairings involving the conserved sequence UUAC (M4, M19, M29, and M37). M9 RNA is of particular interest because this aptamer lacks the conserved motif UUAC but efficiently binds Mod (Fig. 3). RNase probing and footprint experiments showed that M9 can adopt two conformations and that Mod binding to the GUGGU conserved motif stabilizes a stem-loop structure that resembles that of M12 (not shown).

These data indicate that the essential determinants for RNA recognition by Mod are located within the hairpin loop having the conserved sequence GU(A/G)G(U/A). The presence of the second conserved UUAC sequence located in a single-stranded region and at an appropriate distance from the hairpin loop increases the efficiency of binding. Our data also suggest that Mod makes specific contacts with nucleotides in the hairpin loop but also with the ribose-phosphate backbone.

**Mod Belongs to a Ribonucleoprotein Complex in Vivo**—We reasoned that if Mod is involved in a ribonucleoprotein (RNP) complex, the electrophoretic pattern in native conditions should be changed after digestion by RNase. The question was addressed using soluble nuclear extracts from Schneider cells. Western blot analysis of RNase-treated and non-treated extracts run on native PAGE (Fig. 6) actually shows that RNase treatment strongly modifies the migration of Mod, consistent with an association to RNA molecule(s). Moreover, only one migrating species is revealed, indicating that most of the nucleosoluble Mod protein is involved in a single complex. Note-wor thy, preincubation with an excess of M12 aptamer shifts Mod toward a faster migrating form (Fig. 6, lane 3). Thus, M12 can displace RNA molecule(s) associated to Mod in vivo. This effect is clearly sequence-specific because competition with a large excess of ribonucleotide NS does not change the Mod

**Fig. 5. Secondary structure probing of M12 and Modulo footprint.** Panels A and B provide examples of M12 structure probing and protection by Mod. A, N. crassa RNase digestion of M12 in the absence (lane 1) or the presence of Mod (lane 2, 10⁻⁶ M; lane 3, 10⁻⁷ M; lane 4, 10⁻⁸ M). Mod induces strong protection in two regions corresponding to nucleotides 18–20 and 29–32. B, digestion of M12 by RNase T1 (lanes 1–4), RNase T2 (lanes 5–8), and RNase V1 (lanes 9–12) in the absence (lanes 1, 5, and 9) or the presence of Mod (lanes 2, 5, 6; 10⁻⁶ M; lanes 3, 7, 11; 10⁻⁷ M; lanes 4, 8, 12: 10⁻⁸ M). Lanes C and C+, incubation controls of free or Mod-associated RNAs. Lane T1, guanosine-specific ladder generated by RNase T1 digestion under denaturing conditions; lane L, alkaline hydrolysis ladder. Protection by Mod is seen at various places, especially at positions 9–24 and 29–33. C, summary of RNase and iron-EDTA cleavages and protection by Mod on the secondary structure of M12.
migration pattern (Fig. 6, lane 2). These results suggest that M12 RNA aptamer binds to the same or overlapping site as the in vivo cognate RNA(s). We have previously reported that Mod only from nucleolus and not the chromatin-bound protein is recovered in soluble nuclear extracts (17). Thus, the evidence provided here, that Mod belongs to a RNP complex in vivo, regards the nucleolus-associated form only.

**RNase Treatment Does Not Prevent Mod Association to Chromatin**—On polytene chromosomes, Mod is associated to condensed chromatin sites, including a majority of bands on chromosome arms, and to the nucleolus (17). In order to test whether Mod at the chromatin might also interact with a RNA molecule, we analyzed the Mod pattern on polytene chromosomes from salivary glands previously digested with RNase. As a control, we simultaneously followed the distribution of the Mle protein, which is known to be released from the male X chromosome with RNase A (27). Male salivary glands digested or not by RNase A were squashed, and chromosome preparations were stained for both Mod and Mle. Comparison of panels A to C and B to D in Fig. 7, indicates RNase digestion actually induces efficient release of Mle from the X chromosome and of Mod from the nucleolus, but does not affect the Mod chromosomal pattern.

**DISCUSSION**

Previous studies indicated that Mod likely fulfills two distinct nuclear functions, because the dominant suppression of PEV (12) and recessive Minute-like (17) phenotypes are suggestive for roles in chromatin compaction and regulation of nucleolus activity, respectively. The present findings provide molecular evidence supporting first that the protein is involved in distinct networks at nucleolus and chromatin and second that phosphorylation down-regulates DNA-binding and therefore controls distribution and function of Mod in the two sub-nuclear compartments.

The structure/function analysis performed using Mod truncated isoforms has clearly identified the domains involved in nucleic-acid binding. One major conclusion is that Mod is a sequence/motif-specific RNA binding protein. Two lines of evidence indicate that this property is provided by the RRM-containing domain. First, the same consensus was derived from SELEX experiments performed with GST fusions of either the whole protein or the RRM moiety only. Second, the two protein variants show close $K_d$ values for M12 aptamer association. Structural probing and footprinting experiments predict that Mod stabilizes a hairpin-like structure and presumably establishes direct contacts within two single-stranded GU(A/G)U(U/A) and AUAC sequences corresponding to the hairpin loop and part of a bulged loop and with the ribose-phosphate backbone as well. This RNA motif appears quite different from that of the few targets of RRM-containing proteins identified so far, which are usually constituted of hairpin loops of 8 to 10 residues (Ref. 25, and references therein). More generally, a search in data bases failed to reveal any significant resemblance between the Mod cognate motif and a previously identified RNA sequence. A second conclusion is that the two basic terminal domains of Mod are able to bind DNA in a sequence-specific manner. The decreased affinity of protein variants truncated of either the N or C terminus indicates that each domain can act independently. It is, however, conceivable that the two domains function synergistically in the native protein to improve DNA-binding activity.

Mod therefore presents unique in vitro nucleic acid binding properties as it is able to directly contact DNA via the two tips and bind to a specific RNA motif via the central RRM domain. This leads up to consider three points regarding the situation in vivo. One can first wonder about the nature of the nucleic acids Mod is interacting with at the nucleolus and chromatin. Clear evidence that Mod is released from the nucleolus as a RNP complex is provided by the RNase-induced modification of nucleosoluble Mod migration in native gel electrophoresis. Also consistent with a direct interaction with a nucleolar RNA, M12 aptamer specifically displaces the protein from the RNP complex. This latter result in addition strongly suggests that the
Mod RNA target at nucleolus likely presents a structure similar to M12. However, we did not find any motif related to the aptamer in the repertoire of Drosophila RNA or snoRNA sequences available in data bases. Eukaryotic cells contain an extraordinarily complex population of snoRNAs (8, 35), but only a few have been cloned in Drosophila. It is therefore tempting to assume that the Mod RNA target at the nucleolus corresponds to snoRNA sequences not identified so far. As the protein was previously shown not to bind rDNA (17), we conclude that in this compartment Mod associates to specific RNA molecule(s) but does not contact DNA. Interactions of Mod with nucleic acids at chromatin appear to be quite different. On one side, it is well established that the protein directly contacts genomic DNA (12, 17). On the other side, digestion by RNase does not affect the Mod pattern on polytene chromosomes, while it induces efficient release of Mle from the X chromosome and of Mod from the nucleolus. The simplest explanation is that chromatin-associated Mod does not interact with RNA. Alternatively, in the process of chromatin compaction, Mod may recruit RNA together with additional protein factors to form highly condensed structures in which the RNA moiety is protected from digestion by RNase. Whether or not Mod requires a RNA partner in the process of chromatin compaction obviously needs further investigation.

The second point regards the molecular mechanisms that control Mod distribution between the two subnuclear compartments. We propose that posttranslational modification by phosphorylation down-regulates the DNA-binding activity and capacity of the protein to link chromatin and, therefore, modulates the equilibrium between chromatin versus nucleolus association. This model is supported by several lines of evidence: i) the chromatin-bound protein does not detectably incorporate \(^{32}P\) in cell culture assays; ii) the nucleolar fraction is highly modified and cannot bind DNA in vitro; and iii) the DNA-binding activity is restored after digestion by phosphatase. In contrast, phosphorylation is unlikely to affect the capability of Mod to bind RNA because competition experiments have shown that the M12 aptamer, selected by a recombinant protein produced in Escherichia coli (i.e. unmodified), binds phosphorylated nucleolar Mod as well.

The third point to consider is how the molecular data reported here correlate to, and possibly improve our understanding of, functional data obtained from genetics. The dominant suppression of PEV phenotype unambiguously indicates that the encoded protein participates in the local assembly of high order chromatin structure and transcriptional silencing of neighboring genes. This function is thus clearly related to chromatin rather than nucleolus and to the DNA-binding activity of Mod. The lack of sequence specificity in DNA recognition that is exhibited by the two basic terminal domains in vitro contrasts with the protein distribution at specific heterochromatic sites on mitotic chromosomes (17). This suggests that Mod might associate to unknown factors that could provide specificity and direct the complex toward particular chromatin sites. An interesting possibility is that the distal domains in Mod interact with sequences lying relatively far apart from each other on the DNA fiber, which could favor and stabilize the formation of condensed chromatin structures.

Total loss of mod function results in the expression of several recessive phenotypes. Some have clearly been related to defect in ribosome biogenesis and protein synthesis capacity, such as the “Minute-like” phenotype of mutant cell clones induced in a wild type background (17) and defect in cell growth and proliferation of mutant imaginal tissues.\(^2\) Other phenotypes, malonic tumor formation (12) and lymph gland hyperplasia,\(^2\) which are highly reminiscent to phenotypes caused by mutations in the ribosomal protein S6 gene (36), are also consistent with a role for Mod in the regulation of ribosome assembly and cell growth. Interestingly, Mod phosphorylation is enhanced when cell growth is stimulated in cultures supplemented with serum. As our bidimensional gel analysis revealed a multiplicity of Mod phosphoryforms in vivo, it is therefore tempting to assume that modification by phosphorylation at critical sites in Mod is required not only to prevent DNA-binding and chromatin compaction, but also to enhance nucleolus activity. Regarding the molecular function of Mod at the nucleolus, an involvement in rDNA transcription appears unlikely as the protein does not bind rDNA and is released from the organelle by low salt extraction of purified nuclei (17). Instead, it presumably participates in a subsequent step of the ribosome biosynthesis. Like most nucleolar proteins identified so far, Mod indeed presents a modular structure that combines four RRMs with acidic and basic domains and suggests interaction with nucleolar components, including ribosomal proteins, rRNA and/or snoRNAs, and possibly a chaperone function facilitating ribosome assembly (4).

Various aspects in the molecular function of Mod remain to be resolved, such as the nature of RNA target in vivo and its possible involvement in chromatin compaction, the identity of kinases/phosphatases that modify the protein or the nature of transcriptional chromatin targets. However, the results presented here, together with previously reported molecular and genetic data, led us to propose the following functional model for Mod. In response to physiological stimuli, mimicked by serum in cell culture assays, the protein becomes phosphorylated and is released from chromatin, inducing structural relaxation and PEV suppression, moves to the nucleolus where it associates RNA, and forms a RNP required to improve ribosome biogenesis and protein synthesis capacity. As far as we know, among ribosomal or nucleolar proteins thought to possess a second cellular activity apart from ribosome assembly and function (1, 37), Mod is the first example in which the bifunctional character is proven by genetics and in which a regulatory molecular mechanism of how the two functions are coordinated in vivo is proposed.

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