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The PRP4 (RNA4) Protein of *Saccharomyces cerevisiae* Is Associated with the 5′ Portion of the U4 Small Nuclear RNA

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We have combined oligonucleotide-directed RNase H degradation and immunoprecipitation in a study of the association of the *Saccharomyces cerevisiae* PRP4 protein with the U4-U6 complex. We have found that three oligonucleotides were able to direct nearly to completion the RNase H-specific cleavage of the target RNA molecules as they exist in splicing extracts. Immunoprecipitation of the degradation products with PRP4 antibody showed that the 5′ portion of U4 small nuclear RNA (snRNA) and the 3′ portion of U6 snRNA coimmunoprecipitated with the PRP4 protein. Micrococcal nuclease protection experiments confirmed further that the 5′ portion and 3′ end of U4 snRNA were very resistant to nuclease digestion, whereas the 3′ portion of U6 snRNA was protected to only a very small extent. We conclude that the PRP4 protein of *S. cerevisiae* is associated primarily with the 5′ portion of U4 snRNA in the U4-U6 small nuclear ribonucleoprotein (snRNP).

Nuclear mRNA processing in eucaryotes takes place in a complex ribonucleoprotein structure called the spliceosome. Spliceosomes are composed of at least four species of small nuclear ribonucleoproteins (snRNPs): U1, U2, U5, and U4-U6. These, in turn, are composed of both small nuclear RNAs (snRNAs) and proteins (for review, see references 18, 20, and 22). The structures of snRNAs have been the subject of considerable study, mostly through phylogenetic analysis and oligonucleotide-directed RNase H cleavage (for a review, see reference 11). In contrast, the structures of snRNPs, including their protein components, as well as the RNA-protein and protein-protein interactions involved in their formation and function, are much less well understood.

In recent years several polypeptides have been identified as constituents of snRNPs in both HeLa cells and *Saccharomyces cerevisiae* (2, 17, 18, 27). These fall into two general classes: proteins that are specific for one class of snRNP and those that are present, at least in HeLa cells, in all four of the splicing-related snRNPs (18).

The following information is currently available concerning the unique proteins. HeLa cell U1 snRNP contains three proteins called A, C, and 70k (18); all three bind directly to defined regions of U1 snRNA, although only A and 70K have an RNA recognition motif (13, 19, 20, 29). No unique U1 snRNPs are known in *S. cerevisiae*. HeLa cell U2 snRNP contains two unique proteins called A′ and B″; neither has been shown to bind directly to U2 snRNA, although B″ has an RNA recognition motif (19, 20, 29). Models of the U1 and U2 snRNPs have been proposed (19, 20, 24, 26). No unique U2 snRNPs are known in *S. cerevisiae*. HeLa cell U4-U6 contains no known unique proteins. The corresponding yeast snRNP contains at least one specific protein, the product of the *PRP4* gene (3, 27). It is not known whether PRP4 protein binds directly to either U4 or U6 snRNA; the protein has no known RNA-binding motif (3, 27). HeLa cell U5 snRNP has recently been shown to contain six unique proteins, identified only by their molecular weights (2); little else is known of them. In *S. cerevisiae* the *PRP8* gene product is associated with the U5 snRNP (17).

The proteins that are present in all four snRNPs in HeLa cells have been called the Sm proteins (18). These seven polypeptides (B′, B, D, D′, E, F, and G) are thought to be associated with the snRNPs in a common structural motif called the A domain (6). Micrococcal nuclease (MN) protection experiments have confirmed that an RNA sequence called the Sm domain is protected in U1, U2, U4, and U5 snRNAs (9, 15). It has been shown that *S. cerevisiae* also contains polypeptides that bear antigenic determinants that react with patient anti-Sm autoantibodies (35) and that Sm proteins from *Xenopus laevis* are able to bind to some yeast snRNAs (30). The nature of the Sm-like protein(s) of *S. cerevisiae* is not known. *S. cerevisiae* snRNAs U1, U2, U4, and U5 contain the putative Sm domain sequence (reviewed in reference 8).

Structural analysis of snRNPs is a key step in understanding the mechanism of pre-mRNA processing. One way to approach this problem is through assembly studies and structural analysis, as has been carried out with U1, U2 snRNPs of HeLa cells (12, 13, 15, 19, 20, 23–26, 29, 36, 37), and recently also U4-U6 (28) and U5 snRNPs (4). Our interest is in learning something of the structure of the yeast U4-U6 snRNP. The knowledge that PRP4 protein is associated with this snRNP (3, 27), coupled with the availability of an antiserum to this protein (27), prompted us to carry out a series of experiments that made use of oligonucleotide-directed RNase H and MN digestions to localize this protein in the U4-U6 snRNP. Our results indicate that the PRP4 gene product is associated with the 5′ portion of the U4 snRNA, although we cannot say that this interaction is through direct protein-RNA contact.

MATERIALS AND METHODS

Sera. The anti-PRP4 serum was raised and characterized as described previously (27). The 2,2,7-trimethylguanosine (m_3G) antibody was from Reinhard Lührmann.

Oligonucleotides. Oligonucleotides were synthesized by The Biotechnology Service Center, The Hospital for Sick Children, Toronto, Ontario, Canada. They were purified on 20% denaturing polyacrylamide gels followed by 0.5 M

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ammonium acetate extraction and SEP-PAK C18 Cartridge (Waters Associates, Inc.) chromatography and then were lyophilized. Lyophilized oligonucleotides were suspended in H_2O or 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA (TE) to a final concentration of 10 pmol/μl. The sequences of these oligonucleotides are as follows: U4a', GTATTTCC CGTGCATAAGGAT; U4a, ATATGCGTATTTCCCGTGC AT; U4b, ATCTCGGACGAATCCTCAC; U4c, TTTCAAC CAGCAAA; U4d; GAGACGGTCTGGTTTATAATT; U4e, AAAGGTATTCCAAAAATTCCCTA; U6a, GAAGGGTTA CTTCGCGAAC; U6b, TGCTGATCATCTCTGTATTGT; U6c, TCATCCTTATGCAGGGGAAC; U6d, TTTGTAAAA CGGTTCATCCTT; U6e, AAAACGAAATAAATCTCTTT GTAAA.

RNase H reactions. Yeast splicing extracts were prepared as described by Lin et al. (16). Yeast splicing extract (5 μl) was incubated in a mixture with final concentrations of 10 mM HEPES-K^+ (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid potassium salt; pH 7.4), 25 mM KCl, 10% glycerol, 10 mM $MgCl_2$, 1 mM dithiothreitol, 300 Units of RNasin per ml, and an oligonucleotide (10 pmol). In the control sample, H_2O was added instead of oligonucleotide. No exogenous RNase H was added, since yeast splicing extract has endogenous RNase H activity (31). The mixture was incubated at room temperature for 30 min, and the reaction was stopped by addition of proteinase K. Following a 30-min incubation at 37°C, snRNAs were extracted with phenol-chloroform and precipitated with ethanol. The RNAs were fractionated on a 10% polyacrylamide–7 M urea gel and blotted to a Hybond N membrane (Amersham Corp.), which was probed with ^{32}P-labeled oligonucleotides. For phenol-chloroform-extracted yeast splicing extract, the RNAs were heated with or without the relevant oligonucleotide(s) to 80°C for 5 min and cooled slowly to room temperature over a 2-h period. The oligonucleotide-directed RNase H cleavage was carried out as described above, except that exogenous RNase H (Bethesda Research Laboratories, Inc.) was added to 2 U per reaction.

Immunoprecipitation. Yeast splicing extract was treated with RNase H before immunoprecipitation. A 20-μl portion of the extract was incubated at room temperature for 15 min in a mixture of final concentrations of 10 mM HEPES-K^+ (pH 7.4), 25 mM KCl, 10% glycerol, 2 mM ATP, 10 mM $MgCl_2$ 1 mM dithiothreitol, 300 U of RNasin per ml, and 20 pmol of oligonucleotide(s) in a total volume of 30 μl. Immunoprecipitation was performed by the method of Lossky et al. (17). For anti-PRP4 and anti-m_3G antibodies, 6 and 2 μl of sera was used per reaction, respectively. The immunoprecipitated material was extracted with phenol-chloroform and fractionated on a 10% polyacrylamide–7 M urea gel.

RNA blot hybridization. RNAs were separated on a 10% polyacrylamide–7 M urea gel and electroblotted to a Hybond membrane (Amersham) in 0.5× TEB (45 mM Tris borate, 12.5 mM EDTA) at 70 V for 1 h. The blot was air dried for 10 min at 37°C, and the RNAs were UV cross-linked to the membrane as specified by the manufacturer. The membrane was prehybridized with 50 ml of hybridization buffer (50 mM sodium phosphate buffer [pH 7.0], 1 mM EDTA, 7% sodium dodecyl sulfate) for 1 h at 37°C and hybridized overnight at 37°C with 25 ml of hybridization buffer with a total of 10^7 to 10^8 cpm for ^{32}P-labeled oligonucleotide. The blot was washed with 1 liter of washing buffer (500 mM sodium phosphate [pH 7.0], 1 mM EDTA, 5% sodium dodecyl sulfate) at 55°C for approximately 20 min and air dried for about 40 min at room temperature before exposure. For reprobing, the blot was washed with 0.1% sodium dodecyl sulfate for 1 h at 65°C before prehybridization.

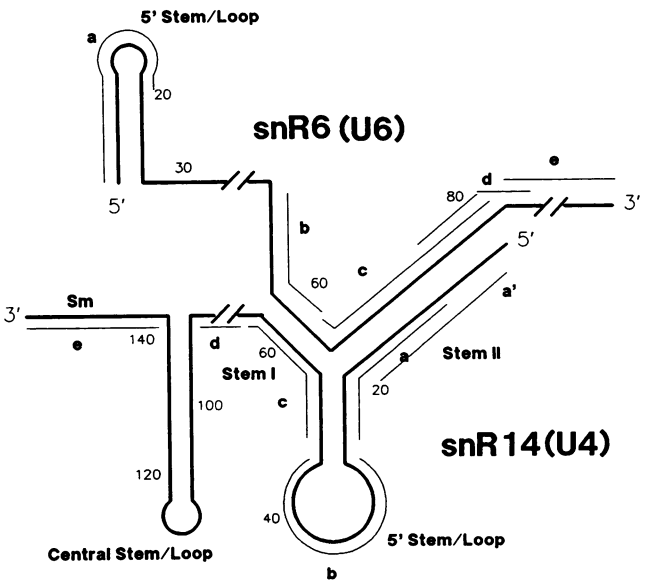


FIG. 1. Oligonucleotides complementary to portions of the U4 and U6 snRNA. Oligonucleotides complementary to U4 and U6 snRNAs are shown by thin lines and are positioned on the secondary structure of the U4-U6 snRNA molecules suggested by Brow and Guthrie (8). Oligonucleotides are complementary to nucleotides 1 to 21 (U4a'), 7 to 27 (U4a), 30 to 48 (U4b), 55 to 68 (U4c), 72 to 92 (U4d), and 138 to 160 (U4e) of U4 snRNA and 1 to 19 (U6a), 42 to 62 (U6b), 63 to 82 (U6c), 75 to 95 (U6d), and 88 to 112 (U6e) of U6 snRNA.

Protection from digestion by MN. A 10-μl portion of deproteinized or native yeast splicing extract in 20 mM HEPES-K^+ buffer (pH 7.4)–0.5 mM dithiothreitol–0.2 mM EDTA–50 mM KCl–20% glycerol was mixed with 5 μl of MN in 50 mM Tris-glycine (pH 9.2)–5 mM $CaCl_2$ at various concentrations for 30 min at 37°C or 60 min at 37°C. The resulting fragments were treated with proteinase K and phenol-chloroform and separated on a 10% polyacrylamide gel containing 7 M urea. The snRNA species were detected by probing with ^{32}P-labeled oligonucleotides as detailed in the figure legends.

RESULTS

Oligonucleotide-directed RNase H reaction. We wanted to define more closely the region(s) of U4 and/or U6 with which the PRP4 protein is associated. Attempts to immunoprecipitate U4 and U6 snRNAs separately after heating the splicing extract to 55°C were unsuccessful because of protein denaturation. High salt concentrations (350 to 750 mM NaCl) did not allow differential immunoprecipitation of U4 and U6 by PRP4 antibodies. Therefore, oligonucleotide-directed RNase H digestion was carried out in an attempt to degrade specifically U4 or U6 snRNA. Oligonucleotides complementary to regions of U4 and U6 were synthesized. Figure 1 shows the hypothesized structure of the U4-U6 complex as suggested by Brow and Guthrie (8) based on phylogenetic conservation. The thin lines along the U4-U6 complex structure indicate regions that are complementary to the oligonucleotides used in this work.

Eleven oligomers were tested for their ability to direct endogenous RNase H cleavage of native snRNAs in a yeast

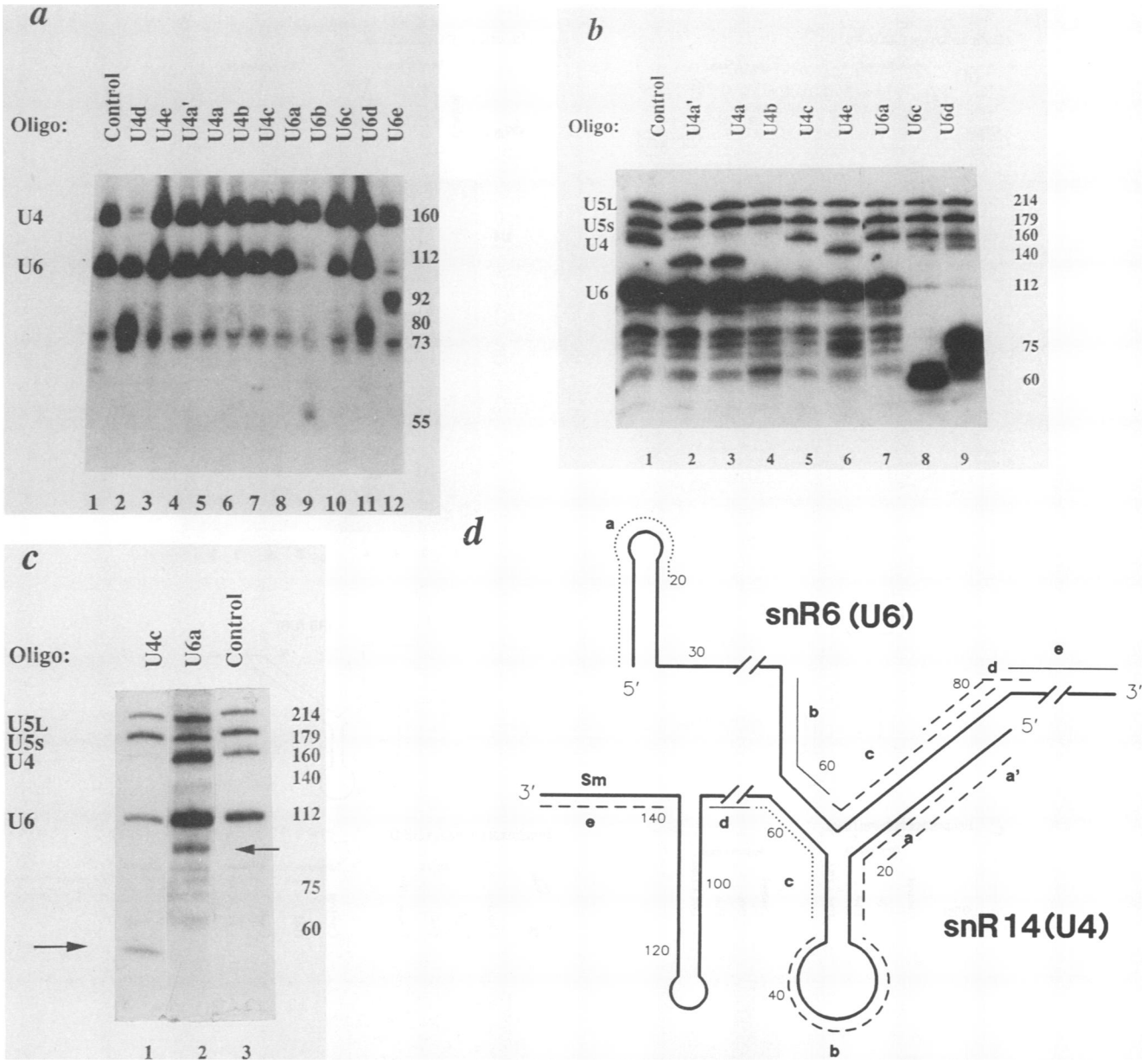


FIG. 2. Effect of various oligonucleotides on degradation of their target snRNA molecules. The oligonucleotide-directed RNase H reactions were carried out as described in Materials and Methods. (a) Whole yeast-splicing extract was treated with different oligonucleotides, as indicated at the top of each lane, in the presence of 2 mM ATP. Blotted snRNAs (see Materials and Methods) were probed with a mixture of ^{32}P-labeled U4a and U6c oligonucleotides. (b) Oligonucleotide-directed RNase H cleavage of deproteinized yeast splicing extract. Oligonucleotides used for the degradation are indicated at the top of each lane. The blot was probed with a mixture of ^{32}P-labeled U4d, U6b, and U5a (an oligonucleotide which is complementary to nucleotides 21 to 37 of U5 snRNA) oligonucleotides. (c) Same as in panel b, except that the oligonucleotide was incubated with snRNAs at 80°C prior to slow cooling and addition of RNase H. Oligonucleotides used are indicated at the top of each lane. The snRNAs were detected by probing the blot with a mixture of ^{32}P-labeled U4a, U5a, and U6b oligonucleotides. Arrows point to degradation products. (d) Summary of oligonucleotide-directed RNase H reaction of U4-U6 snRNAs in *S. cerevisiae*. ——, oligomers able to direct RNase H cleavage under native conditions; - - - -, oligonucleotides able to base pair with their target snRNA after the extract was phenol treated; ·····, inter- or intramolecular base-paired regions.

splicing extract prepared by the method of Lin et al. (16). Of all oligonucleotides tested, only U4d (complementary to nucleotides 72 to 92 of U4 snRNA [Fig. 2a, lane 2]), U6b (complementary to nucleotides 42 to 62 of U6 snRNA [Fig. 2a, lane 9]), and U6e (complementary to nucleotides 88 to 112 of U6 snRNA [Fig. 2a, lane 12]) caused nearly complete degradation of the complementary regions of their target snRNAs. The U6c and U6d oligomers (complementary to nucleotides 63 to 82 and 75 to 95 of U6 snRNA [Fig. 2a, lanes 10 and 11]) gave much less efficient cleavage. The degradation product from U6c treatment was not seen in Fig. 2a, because oligonucleotide U6c was used as the probe in the RNA blot analysis; when the blot was probed with ^{32}P-labeled U6a oligonucleotide, a degradation product approximately 65 nucleotides long was observed (data not shown).

All other oligonucleotides shown in Fig. 1 were tested, and none was observed to direct RNase H cleavage of native snRNAs, in either the presence or the absence of ATP.

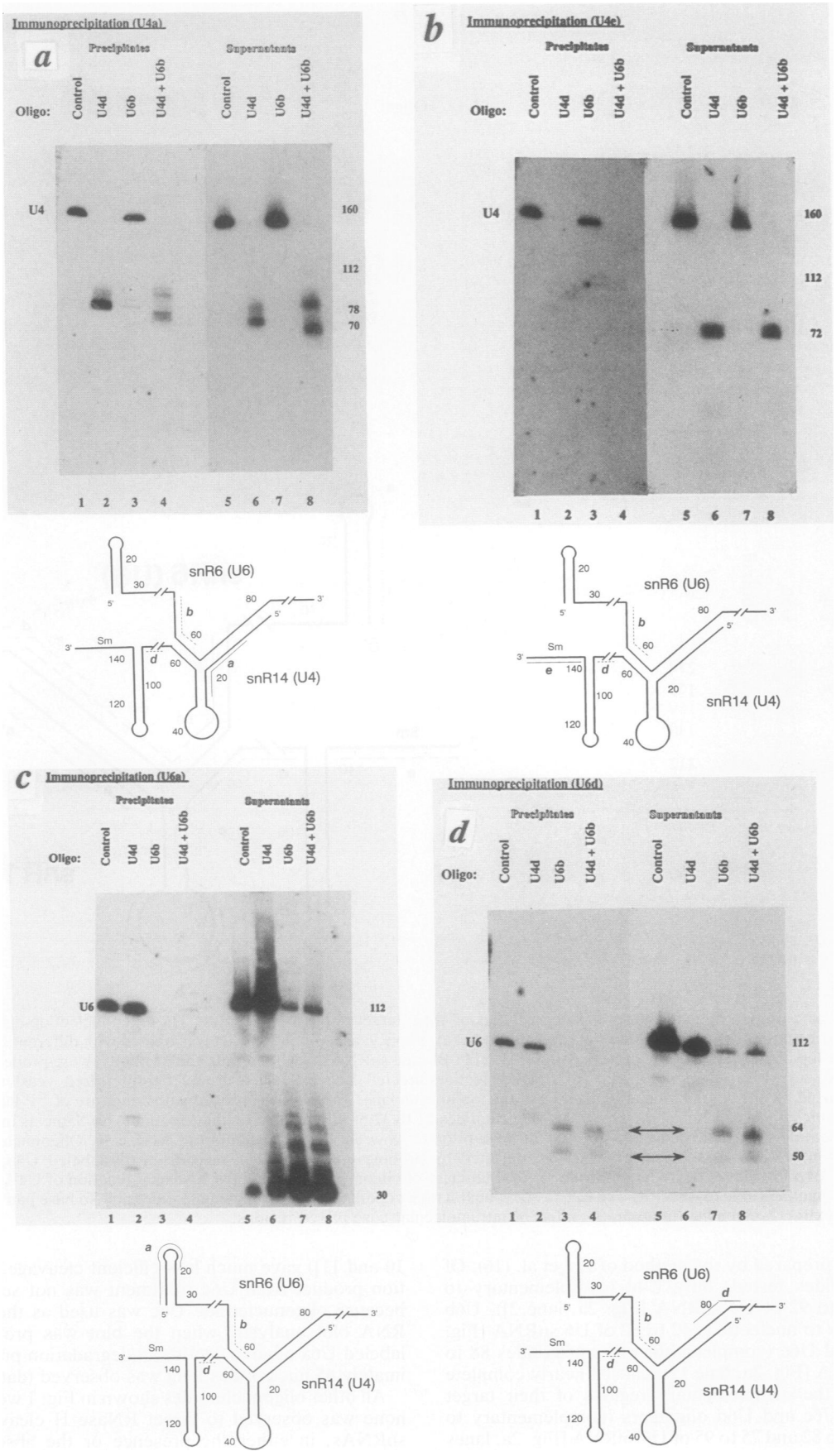

a
Immunoprecipitation (U4a)
Precipitates
Supernatants
Oligo:
Control
U4d
U6b
U4d + U6b
U4
160
112
78
70
1 2 3 4 5 6 7 8
snR6 (U6)
snR14 (U4)
b
Immunoprecipitation (U4e)
72
c
Immunoprecipitation (U6a)
U6
30
d
Immunoprecipitation (U6d)
64
50

Figure 2a shows the results in the presence of ATP. We observed similar cleavage in the absence of ATP (data not shown). These results indicate that the regions of U4 snRNA and U6 snRNA which are complementary to oligonucleotides U4a′, U4a, U4b, U4c, U4e, U6a, and U6e are not available for hybridization to the oligonucleotides, either because of the inter- or intramolecular base pairing of these snRNAs or because of protein protection. Alternatively, those regions are inaccessible to RNase H. When a yeast extract was deproteinized with proteinase K, extracted with phenol-chloroform, heated, and cooled slowly to room temperature prior to nuclease degradation, oligonucleotides U4a′, U4a, U4b, U4e, U6c, and U6d were observed to direct RNase H degradation almost to completion (>90% [Fig. 2b]). However, oligonucleotides U4c and U6a (Fig. 2b, lanes 5 and 7) were not able to direct cleavage unless either of them was heated together with the phenol-extracted splicing extract (Fig. 2c, lanes 1 and 2). This suggests that the regions in U4 and U6 snRNAs that are complementary to U4c and U6a oligonucleotides are intra- or intermolecularly base paired. The results shown in Fig. 2a to c are summarized in Fig. 2d.

Immunoprecipitation. To determine whether the PRP4 protein is associated with a specific region(s) of U4 and/or U6 snRNAs, we immunoprecipitated the U4 and U6 snRNA fragments with PRP4 antiserum following oligonucleotide-directed RNase H cleavage with U4d- and/or U6b-treated splicing extract. Both the U4d and U6b oligonucleotides cleave their target RNAs approximately in half. The degradation products were detected by probing with oligomers U4a, U4e, U6a, and U6d, which are complementary to the 3′ and 5′ portions of U4 and U6 snRNAs, respectively (Fig. 3a to d). (Note that in all of our immunoprecipitation experiments, regardless of which antibody was used, we were able to precipitate only 10 to 20% of the snRNAs in the total extract. We were unable to immunoprecipitate additional snRNA from the supernatant containing the remaining 80% of the snRNAs.) Both precipitates (lanes 1 to 4) and supernatants (lanes 5 to 8) are shown in Fig. 3. Following RNase H degradation of U4-U6 snRNAs, which was directed by U4d (Fig. 3, lanes 2 and 6), U6b (Fig. 3, lanes 3 and 7), and both U4d and U6b (Fig. 3, lanes 4 and 8), the 5′ portion of U4 (approximately 70 nucleotides [Fig. 3a, lanes 2 and 4]) and the 3′ portion of U6 (approximately 50 nucleotides [Fig. 3d, lanes 3 and 4]) can be immunoprecipitated by PRP4 antibodies. On the contrary, the 3′ portion of U4 (approximately 70 nucleotides [Fig. 3b, lanes 6 and 8]) and the 5′ portion of U6 (approximately 30 nucleotides [Fig. 3c, lanes 7 and 8]) remained entirely in the supernatant. These two observations strongly suggest that PRP4 protein is associated specifically with the 5′ portion of U4 snRNA and/or the 3′ cleavage product of U6 snRNA. Experiments have been carried out to show that all the oligonucleotide-RNase H-treated samples were not associated nonspecifically with protein A-Sepharose beads (data not shown).

Oligonucleotide U6e, complementary to nucleotides 88 to 112 of U6 snRNA, was used for further localization of the region of association between PRP4 protein and snRNA(s). When U6 snRNA was cleaved by U6e oligonucleotide-directed RNase H degradation, the product was an approximately 90-nucleotide, 3′-truncated U6 molecule that remained immunoprecipitable by PRP4 antibody along with the intact U4 snRNA (Fig. 4). The finding that oligonucleotide U6e is able to base pair to the 3′ end of U6 snRNA and direct RNase H cleavage, leaving the remainder of U6 snRNA associated with PRP4 in the presence of intact U4

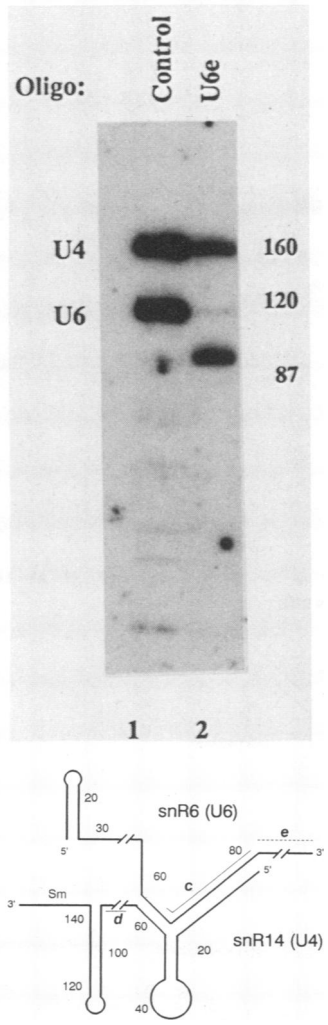
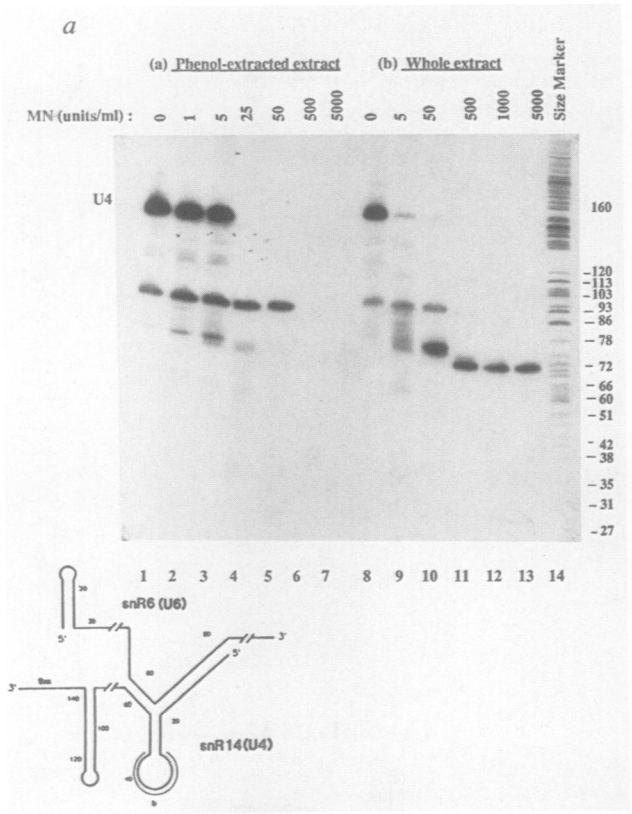
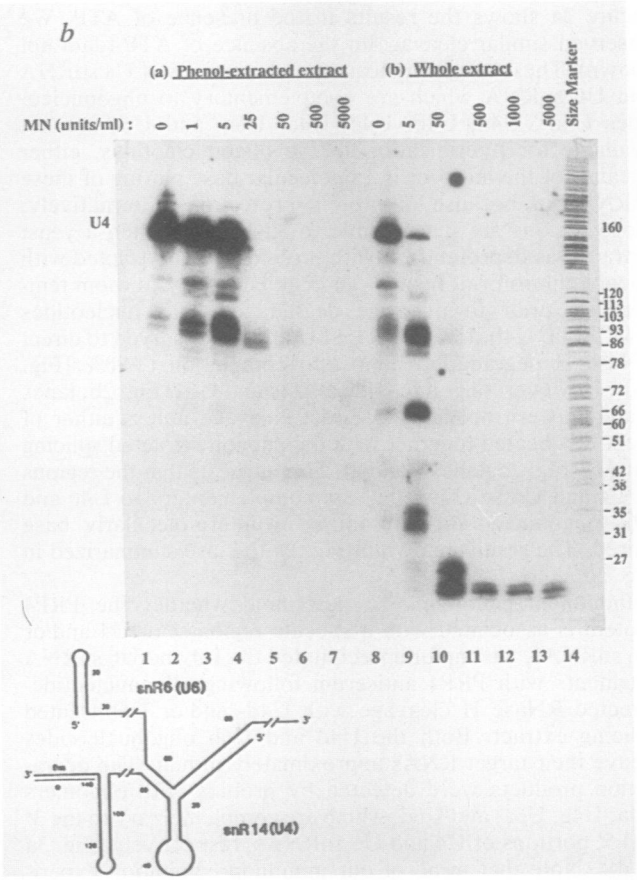
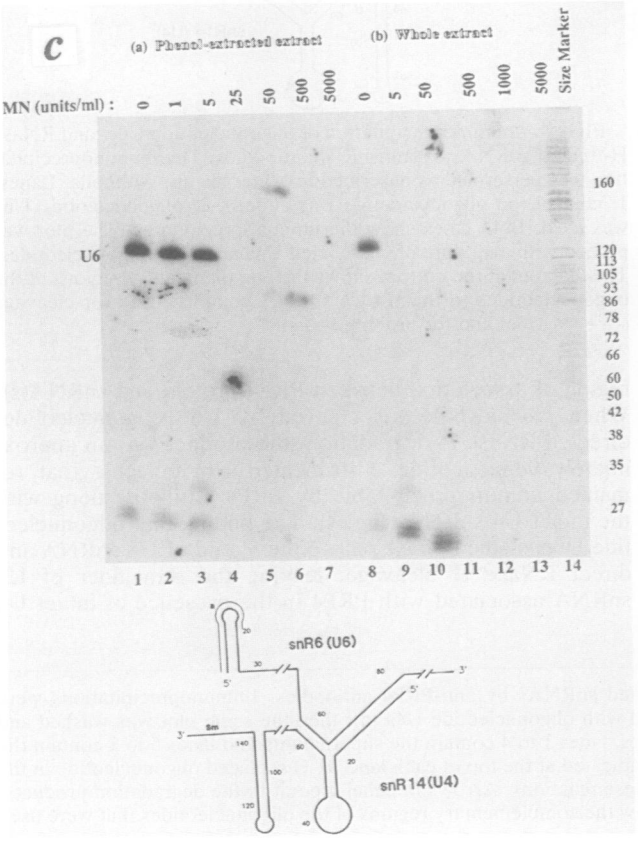


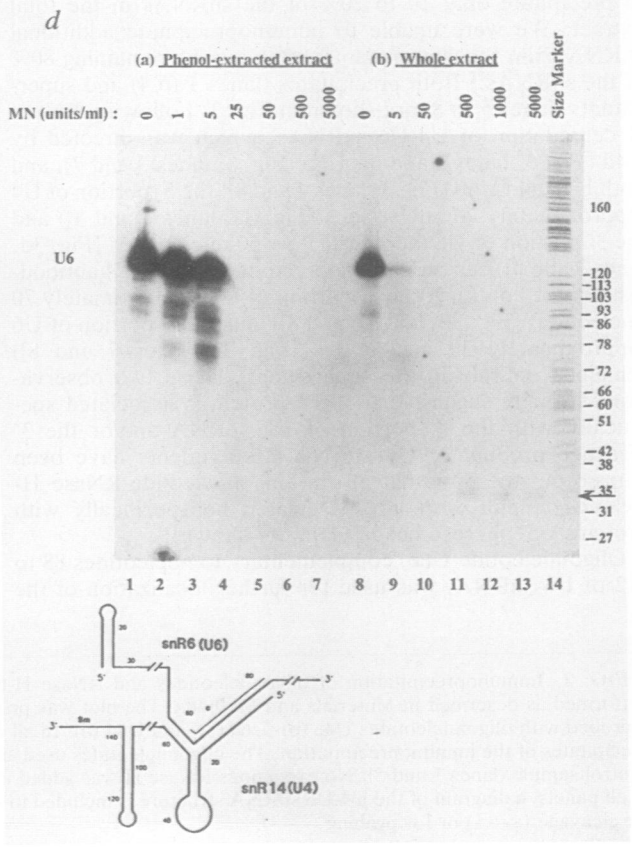
FIG. 4. Immunoprecipitation of oligonucleotide U6e- and RNase H-treated snRNAs by anti-PRP4 antibodies. The immunoprecipitation was performed as described in Materials and Methods. Lanes: 1, control (no oligonucleotide was added); 2, oligonucleotide U6e was used. Both lanes show the immunoprecipitates. The blot was probed with a mixture of ^{32}P-labeled U4a and U6d oligonucleotides. The diagram at the bottom shows the complementary regions of the oligonucleotides to the U4-U6 snRNA complex used for cleavage (- - - - -, U6e) and for probing (——).

FIG. 3. Immunoprecipitation of oligonucleotides and RNase H-treated snRNAs by anti-PRP4 antibodies. Immunoprecipitations were performed as described in Materials and Methods. The blot was probed with oligonucleotide U4a (a); then the same blot was washed and reprobed with oligonucleotides U4e (b), U6a (c), and U6d (d). In all cases, lanes 1 to 4 contain the supernatants and lanes 5 to 8 contain the precipitates of the immunoprecipitation. The oligonucleotides used are indicated at the top of each lane. H_2O replaced oligonucleotide in the control sample (lanes 1 and 5). No exogenous RNase H was added in these reactions. Arrows in panel d point to the degradation products. In all panels, a diagram of the U4-U6 snRNA structure is included to show the complementary regions of the oligonucleotides that were used for cleavage (- - - -) or for probing (——).


a
(a) Phenol-extracted extract
(b) Whole extract
MN (units/ml) : 0 1 5 25 50 500 5000 0 5 50 500 1000 5000
Size Marker
U4
160
120
113
103
93
86
78
72
66
60
51
42
38
35
31
27
1 2 3 4 5 6 7 8 9 10 11 12 13 14
snR6 (U6)
snR14(U4)


b
(a) Phenol-extracted extract
(b) Whole extract
MN (units/ml) : 0 1 5 25 50 500 5000 0 5 50 500 1000 5000
Size Marker
U4
160
120
113
103
93
86
78
72
66
60
51
42
38
35
31
27
1 2 3 4 5 6 7 8 9 10 11 12 13 14
snR6 (U6)
snR14(U4)


c
(a) Phenol-extracted extract
(b) Whole extract
MN (units/ml) : 0 1 5 25 50 500 5000 0 5 50 500 1000 5000
Size Marker
U6
160
120
113
105
93
86
78
72
66
60
50
42
38
35
27
1 2 3 4 5 6 7 8 9 10 11 12 13 14
snR6 (U6)
snR14(U4)


d
(a) Phenol-extracted extract
(b) Whole extract
MN (units/ml) : 0 1 5 25 50 500 5000 0 5 50 500 1000 5000
Size Marker
U6
160
120
113
103
93
86
78
72
66
60
51
42
38
35
31
27
1 2 3 4 5 6 7 8 9 10 11 12 13 14
snR6 (U6)
snR14(U4)

snRNA or its 5′ portion, indicates that PRP4 is not likely to be associated with the 20-nucleotide 3′ terminus of U6 snRNA.

MN protection experiment. The results reported in the previous section indicate that U4a′, U4a, and U4b oligonucleotides, which are complementary to the 5′ end of U4 snRNA (including the loop composed of nucleotides 29 to 45), were able to direct RNase H degradation only in a deproteinized yeast-splicing extract (Fig. 2b, lanes 2 to 4). Furthermore, the results from the immunoprecipitation experiment described above (Fig. 3) also suggested that PRP4 and/or other proteins might be associated with U4 snRNA in the 5′ region. To investigate this possibility further, MN protection was carried out to determine which regions of U4 might be protected by protein. MN degrades both single- and double-stranded DNA and RNA molecules in the absence of protection by protein (1). Splicing extracts, either native (Fig. 5, lanes 8 to 13) or phenol extracted (Fig. 5, lanes 1 to 7), were treated with increasing amount of MN as described in Materials and Methods. The resulting RNA blot was probed with ^{32}P-labeled oligonucleotides U4a (Fig. 5a), U4e (Fig. 5b), U6a (Fig. 5c), or U6c (Fig. 5d) or with a labeled whole-gene probe (data not shown). In deproteinized U4 snRNA (Fig. 5a and b, lanes 1 to 7), no U4 snRNA fragment longer than 20 nucleotides was seen when MN was used at concentrations greater than 50 U/ml (Fig. 5a and b). In contrast, in the native snRNP a 65- to 70-nucleotide region including the loop of U4 snRNA (Fig. 1, nucleotides 29 to 45) was resistant to digestion even when MN was used at 5,000 U/ml (Fig. 5a, lane 13); this indicates protection of this region of U4 snRNA by protein(s). The MN-resistant fragment from the 5′ portion of U4 snRNA (approximately 70 nucleotides) could be detected by hybridizing with oligonucleotide U4b (Fig. 5a) as well as other oligonucleotides spanning the region of nucleotides 1 to 68 of U4 snRNA (U4a′, U4a, and U4c; data not shown).

U6 snRNA, either native or deproteinized, was nearly completely degraded by MN at concentrations greater than 50 U/ml; the region that is proposed to base pair with the U4 snRNA (8) was only very weakly protected from MN digestion (Fig. 5d; the protected band is indicated by an arrow).

An immunoprecipitation of the MN-treated yeast splicing extract with anti-m_3G antibody (7) was performed to define the 5′ end of the fragment that was derived from the 5′ portion of the U4 snRNA following MN digestion (Fig. 6, lane 4). We have found that after a 10-min incubation with MN at 37°C, the snRNAs became associated with protein A-Sepharose beads nonspecifically in both the presence and absence of anti-m_3G antibody (Fig. 6, lanes 3 and 6). To avoid this problem, the yeast splicing extract was phenol extracted following MN treatment but prior to immunoprecipitation by anti-m_3G antibody; under these conditions, the beads were no longer associated with the (deproteinized) snRNAs (Fig. 6, lane 7), which were immunoprecipitated only in the presence of anti-m_3G antibody (Fig. 6, lane 4). This indicates that the 5′ cap of U4 snRNA is protected in the native snRNP.

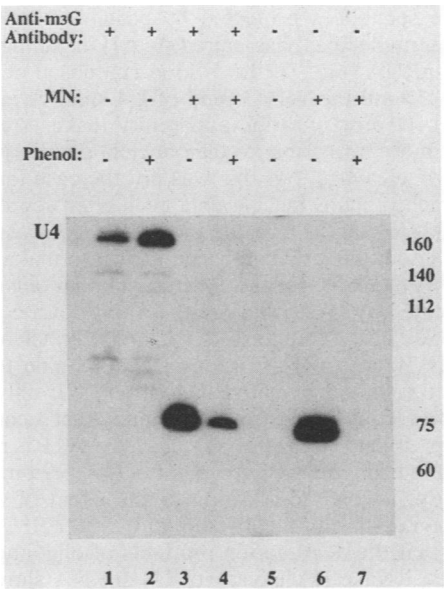


FIG. 6 Immunoprecipitation of MN-digested U4 fragment with anti-m_3G. A yeast splicing extract was digested with MN at a concentration of 5,000 u/ml for 10 min at 30°C. The reaction was stopped by the addition of 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Immunoprecipitations were performed as described for the experiment in Fig. 3. Lanes 1 to 7 show the precipitates. In lanes 1 to 4, anti-m_3G antibodies were coupled to protein A-Sepharose CL-4B; in lanes 5 to 7, only the beads were used as negative controls. Lanes: 1 and 5, extract without further treatment; 2, phenol-extracted splicing extract; 3 and 6, MN-digested splicing extract; 4 and 7, MN-digested and phenol-extracted (in that order) splicing extract. The blot was probed with ^{32}P-labeled U4a oligonucleotide.

A second region of U4 snRNA refractory to MN attack was near its 3′ end (approximately nucleotides 140 to 160 [Fig. 5b]), where a sequence containing a consensus binding site for the Sm antigen has been suggested to occur (6, 8, 32). It is possible that the yeast Sm antigen(s) is associated here.

DISCUSSION

The data from our oligonucleotide-directed RNase H cleavage of whole-yeast splicing extract or deproteinized extract shown in Fig. 1 support the structure of the U4-U6 snRNA complex as suggested by Brow and Guthrie (8); they are also consistent with the results of RNase H degradation of the U4-U6 snRNP in HeLa cells (see reference 5 and references therein). In the U4-U6 snRNP of *S. cerevisiae*, regions that are most accessible for base pairing with an oligonucleotide and cleavage by RNase H occur at nucleotides 72 to 92 in U4 snRNA and nucleotides 42 to 62 and 88 to 112 of U6 snRNA. Sequences that are accessible to oligonucleotide base pairing and RNase H cleavage only after the extract was deproteinized with phenol are candidates for points of interaction with protein(s). On the U4

FIG. 5. U4 and U6 snRNA protection from digestion by MN. Phenol-extracted yeast splicing extract (lanes 1 to 7) or whole extract (lanes 8 to 13) was treated with various amounts of MN for 30 min at 37°C (lanes 1 to 7) or 60 min at 37°C (lanes 8 to 13) in the presence of 2 mM $CaCl_2$. The numbers above the lanes refer to the final concentrations of MN (in units per milliliter). In panels a to d, the separated snRNA molecules were detected by probing or reprobing with ^{32}P-labeled U4b, U4e, U6a, and U6c oligonucleotides, respectively (only one blot was used). The diagrams at the bottom of the panels show the complementary regions of the oligonucleotides on the U4-U6 snRNA complex used for probing. In panel c, the blot probed with U6a was overexposed, and no RNA fragment longer than 25 nucleotides derived from the 5′ portion of U6 snRNA was observed.

snRNA, the sequences protected by protein(s) include the proposed intermolecular base-paired stem II including the cap on the U4 snRNA (Fig. 1), the 5′ loop composed of nucleotides 29 to 45, and the very 3′ end of U4 snRNA (about 20 nucleotides). Deproteinization apparently makes stem II of U4 snRNA more accessible to oligonucleotide hybridization and RNase H cleavage, but this was not the case for stem I (Fig. 1), which remains inaccessible even after deproteinization, probably owing to a stable intermolecular base-paired structure. In addition, ATP has been shown to make stem II of U4 snRNA available for base pairing with an oligonucleotide as indicated by Black and Steitz (5) for HeLa cells. The oligonucleotide complementary to the first 15 nucleotides of stem-loop II of U4 snRNA in HeLa cells is able to direct RNase H degradation of native U4-U6 snRNP only in the presence of added ATP (5); this might imply that a conformational change of the U4-U6 snRNP caused by ATP is required for the oligonucleotide to base pair to this region. In the present study, no characterization of the effect of ATP on RNase H degradation was carried out.

We note that the degradation products of oligonucleotide U4d-directed RNase H cleavage of U4 snRNA showed the expected lengths, assuming that RNase H cuts the U4 snRNA at the region to which U4d oligonucleotide hybridizes (approximately 70 nucleotides from both its 5′ and 3′ ends [Fig. 3a, lanes 2, 4, 6, and 8; Fig. 3b, lanes 6 and 8]). However, the nature of the double band derived from the 5′ portion of U4 snRNA (Fig. 3a, lane 6) is not clear. In the presence of U6b oligonucleotide, the products from the 5′ portion of the U4 snRNA by U4d oligonucleotide-directed RNase H degradation showed a different pattern (Fig. 3a, lanes 4 and 8) from that produced when only U4a oligonucleotide was used (Fig. 3a, lanes 2 and 6). Again, the reason for this observation is not clear. The expected products of U6b-directed RNase cleavage of U6 snRNA are a polynucleotide approximately 41 nucleotides from its 5′ end and approximately 50 nucleotides from its 3′ end. The results showed that two bands of approximately 45 and 55 nucleotides were derived from the 3′ portion of U6 snRNA (Fig. 3d, lanes 3, 4, 7 and 8); however, the product from the 5′ portion of U6 snRNA was shorter (approximately 30 nucleotides [Fig. 3c, lanes 7 and 8]). It is possible that the 5′ portion of U6 snRNA is rendered more susceptible to nonspecific RNase degradation following RNase H cleavage. We note that the 5′ end of U6 snRNA (about 30 nucleotides) might be selectively resistant to degradation because of a suggested hairpin structure (3) (Fig. 1, 5′ stem-loop of the U6 snRNA).

The apparent relative amounts of snRNAs in our experiments were usually approximately twofold excess of U6 snRNA over U4 snRNA. This is comparable to the finding of other laboratories when working with *S. cerevisiae* (10, 32).

The results from our MN protection experiments indicate that a fragment of approximately 65 nucleotides from the 5′ portion of the U4 snRNA was very resistant to MN digestion. In HeLa cells, on the contrary, only one fragment derived from the Sm-binding site of the U4 snRNA was protected from MN digestion (15), and no unique protein associated with the U4-U6 snRNP has been found in HeLa cells to date (18). This may suggest that *S. cerevisiae* has some different protein(s) associated with the U4-U6 snRNP; alternatively, it might mean that the counterparts of the PRP4 and/or other yeast proteins are loosely associated with the U4-U6 snRNP in HeLa cells and that the isolation of the U4-U6 snRNP results in a dissociation of these specific proteins from the particle. Immunoprecipitation of the MN digestion product(s) with PRP4 antibodies was not feasible because MN treatment caused snRNAs to be nonspecifically associated with protein A-Sepharose beads (Fig. 6, lane 6).

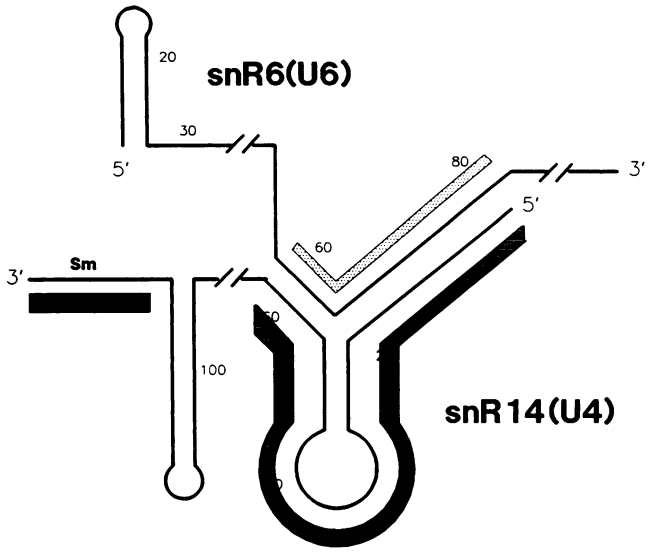


FIG. 7. Model of the yeast U4-U6 snRNP. The model summarizes the data presented in this work. Symbols: ■, region of a potential Sm-binding site; ▩. regions of strong protection by PRP4 protein and possibly other protein(s); ▩ , regions of weak protection.

Patton et al. (24, 26) have used a so-called antibody-mediated nuclease protection technique to show that only the 26-nucleotide Sm domain of the U1 snRNA in HeLa cells is resistant to MN digestion in the absence of an antibody or in the presence of an anti-Sm antibody. However, in the presence of certain other antibodies (for example, an anti-RNP antibody and some A- and C-protein autoantibodies), an additional fragment(s) derived from the U1 snRNA was protected. This phenomenon can be explained either by a tighter binding of the 70K, A, or C protein induced by a conformational change caused by an antibody, or by binding of the antibody itself to adjacent (previously naked) RNA when it binds to the protein (26). A similar phenomenon was not observed for the PRP4 antibody; no extended or additional band was protected by addition of this antibody to yeast splicing extract prior to MN digestion (data not shown).

A model of protein association with the yeast U4-U6 snRNP is proposed in Fig. 7. We conclude that the PRP4 protein is associated with the 5′ portion of U4 snRNA; the yeast Sm antigen(s) is likely to be associated with the 3′ end of U4 snRNA in the U4-U6 snRNP. It should be noted, however, that there is no indication of whether the PRP4 association represents direct binding of the protein to the U4 snRNA. General features of RNA-binding proteins have not been well studied until the recent recognition of an RNA-binding domain (usually composed of 80 to 90 amino acids) and an RNP consensus motif of 8 amino acids (see reference 21 for a review). However, these sequences cannot be a prerequisite for RNA binding per se, since they are not found in all RNA-binding proteins, for example, ribosomal proteins or viral RNA-binding nucleocapsid proteins (34). Moreover, the C protein in U1 snRNP lacks the RNP consensus sequence, as well as the RNA recognition motif (33), yet it has been shown that the C protein can bind U1 RNA directly (13). The sequence of PRP4 protein has been deduced from DNA sequencing (3, 27) and does not contain

any known RNA-binding motif. It is possible that more than one protein structural feature is involved in RNA binding. Very recently, another RNA-binding motif has been recognized as an arginine-rich motif, which is conserved among N proteins of bacteriophages, as well as many RNA-binding proteins from ribosomes and RNA virus capsids (14). This domain does not exist in the PRP4 protein.

Further mutational and biochemical studies will define more specifically the regions of interaction on both U4 snRNA and its bound proteins.

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