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The Low-Abundance U11 and U12 Small Nuclear Ribonucleoproteins (snRNPs) Interact To Form a Two-snRNP Complex

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A novel small nuclear ribonucleoprotein (snRNP) complex containing both U11 and U12 RNAs has been identified in HeLa cell extracts. This U11/U12 snRNP complex can be visualized on glycerol gradients, on native polyacrylamide gels, and by selection with antisense 2′-*O*-methyl oligoribonucleotides. RNase H-mediated degradation of the U12 snRNA confirmed a direct interaction between the U11 and U12 snRNPs. This snRNP complex is the first to be identified involving low-abundance snRNPs. Selection of the U11/U12 snRNP complex is sensitive to high salt, suggestive of a protein-mediated interaction. Secondary structure analyses revealed several regions of the U11 snRNP accessible for interaction with other RNAs or proteins but no detectable difference between the accessibility of these regions in the U11 monoparticle compared with the U11/U12 snRNP complex. There are also several accessible single-stranded regions in the U12 snRNP, and oligonucleotide-directed RNase H digestion identified nucleotides 28 to 36 of U12 as containing sequences required for the U11/U12 interaction. Both the U12 snRNP and the U11/U12 snRNP complex can be disrupted without altering the cleavage/polyadenylation activity of a nuclear extract.

U11 and U12 are two low-abundance small nuclear RNAs (snRNAs) found in HeLa cells at 10^4 and 5×10^3 copies per cell, respectively (23). U11 and U12 belong to the family of Sm small nuclear ribonucleoproteins (snRNPs), which are characterized by their immunoprecipitability with anti-Sm antibodies and by the presence of a 5′ trimethylguanosine cap on the RNA (reviewed in reference 27). Other Sm snRNPs include the highly abundant U1, U2, U4/U6, and U5 particles, all involved in the splicing of pre-mRNAs (reviewed in reference 33), and the low-abundance U7 snRNP, required for histone pre-mRNA 3′ end processing (reviewed in reference 3). The participation of each of these snRNPs in RNA processing events was originally proven by removing or masking the particle with specific antibodies or destruction of the snRNA component by oligonucleotide-directed RNase H digestion. So far these approaches have not been successful in assigning a function to either the U11 or U12 snRNP.

Recent detailed analyses have uncovered the importance of specific snRNP-snRNP interactions to RNA processing events. For instance, U4 snRNA base pairs extensively with U6 to form a U4/U6 snRNP that is very stable in extracts (6, 7, 16). Deletions which disrupt the U4-U6 interaction abolish spliceosome formation and splicing (2, 14, 21, 35). The U4/U6 snRNP itself interacts in an ATP-dependent manner with the U5 snRNP to form a U4/U5/U6 tri-snRNP complex (5, 9, 19). This interaction does not alter the accessibility of RNA sequences in U5 (5) and is sensitive to salt (1, 31), suggesting that it is protein mediated. In addition, U6 is known to interact with U2 through RNA base pairing, as demonstrated by psoralen cross-linking (17) and genetic suppression experiments (11, 38); this association may be only transient, perhaps occurring in the spliceosome. Analyses of all these snRNP-snRNP interactions have provided novel insights into the assembly of spliceosomes and the mechanism of pre-mRNA splicing. Thus, it is evident that identification of interactions between snRNPs and of regions in an snRNA that are potentially available for association with other RNAs can be invaluable in assigning functions to snRNP particles.

In this study, we used a number of techniques to demonstrate an interaction between the U11 and U12 snRNPs to form an 18S complex. We show that the association is probably protein mediated, and we locate sequences in U12 snRNA necessary for the interaction. We present structure mapping data that identify regions in U11 and U12 snRNAs potentially available for further molecular interactions. Although we do not detect a requirement for the U12 snRNP or the U11/U12 snRNP complex in polyadenylation complex or product formation in vitro, it is clear that whatever the function of U11 and U12 snRNPs in the mammalian cell, they act together.

MATERIALS AND METHODS

Nuclear extract and whole cell sonic extracts. Nuclear extract was prepared according to Dignam et al. (12) except that phenylmethylsulfonyl fluoride and $MgCl_2$ were omitted from all buffers. Whole cell sonic extract was prepared according to Lerner et al. (20).

RNase H digestion. RNase H digestion of snRNPs in nuclear extract was performed in 60% nuclear extract–2.2 mM $MgCl_2$–0.5 mM ATP–20 mM creatine phosphate–0.04 mg of oligonucleotide per ml–0.04 U of RNase H (Pharmacia) per μl at 30°C for 60 min (4). Treated samples were then analyzed by glycerol gradient fractionation, native gel electrophoresis, denaturing gel electrophoresis or for polyadenylation activity (as described below).

Glycerol gradients. Nuclear extract or RNase H reaction mixtures were diluted with 100 mM KCl–20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES;

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pH 7.9)–1 mM $MgCl_2$ to a final glycerol concentration of 10%. In all RNase H experiments, an aliquot of the reaction mixture was removed, and the RNA was isolated by PCA (phenol-chloroform-isoamyl alcohol, 50:50:1) extraction and ethanol precipitation for further analyses. All gradients were 10 to 30% glycerol and were run as previously described (23) except that centrifugation was typically for 15 h and fractionation was into approximately 25 samples. The RNA from each fraction was isolated by PCA extraction and ethanol precipitation and then detected by Northern (RNA) blot hybridization. An approximate S value for the U11/U12 snRNP complex was estimated from reported S values of the U5 and U2 snRNPs (5).

Native gel electrophoresis. RNase H or polyadenylation reaction mixtures were incubated with heparin (0.2 mg/ml) for 10 min on ice and then fractionated on native gels containing 4% polyacrylamide (80:1)–25 mM Tris–25 mM boric acid–0.75 mM EDTA as described by Skolnick-David et al. (30). The native gels were either dried prior to autoradiography or transferred to nylon membrane for Northern analyses.

2′-*O*-Me oligonucleotide selection. 2′-*O*-Methyl (2′-*O*-Me) oligoribonucleotides were synthesized, biotinylated, and purified according to Sproat et al. (32). Each 2′-*O*-Me oligonucleotide contains four 2′-deoxycytidines, either at the 3′ (U11-5′end and U12-5′end) or 5′ (U11-2loop, U11-3loop, and U12C) end, which are the sites of biotinylation. All oligonucleotides contained 2′-*O*-Me inosine instead of guanosine at sites complementary to cytosine. 2′-*O*-Me oligonucleotide selection of snRNPs from nuclear extract or whole cell sonic extract was done as described by Wassarman and Steitz (36) except that experiments in Fig. 3B used modified 250 mM wash buffer in which the concentration of NaCl was changed to 350, 450, or 550 mM as indicated. Selected RNAs were either 3′ end labeled with pCp and T4 RNA ligase (Pharmacia) (13) and fractionated on 10% polyacrylamide–7 M urea gels or detected by Northern blot analyses.

Chemical modification and enzymatic cleavage. Chemical modification [dimethyl sulfate (DMS), *N*-cyclohexyl-*N*′-β-(4-methyl morpholinium) ethylcarbodiimide *p*-toluenesulfonate (CMCT), or kethoxal] and enzymatic cleavage (RNase T_1, A, or V1) reactions were done on nuclear extract (36), the RNA was PCA extracted and ethanol precipitated, and the modification/cleavage sites were mapped by primer extension analysis. Primer extension reactions were carried out according to Montzka and Steitz (23), and dideoxy sequencing ladders were generated according to Zaug et al. (41). The primer extension products were fractionated on 8 to 12% polyacrylamide denaturing gels with an electrolyte gradient as described by Sheen and Seed (29).

Polyadenylation reactions. In vitro polyadenylation reactions were performed in a 25-μl total volume which contained 40% nuclear extract, 1 mM ATP, 20 mM creatine phosphate, 1 mM $MgCl_2$, 0.04 U of RNAguard RNase inhibitor (Pharmacia) per μl, and substrate at 30°C for the times indicated (24). The samples were either run on denaturing gels after PCA extraction and ethanol precipitation to assess the extent of polyadenylation of the substrate or fractionated on native gels to examine complex formation. The substrates used were in vitro-transcribed RNAs from plasmids pT3L3 (30) and pSV-141/+70 (39), which were gifts from Claire Moore and Marvin Wickens, respectively. For time course experiments analyzed on native gels, start times were staggered so that all reaction mixtures were loaded onto native gels at the same time. RNase H pretreatment of nuclear extract prior to polyadenylation was done under polyadenylation conditions minus substrate with 1 μg of oligonucleotide per 25 μl of reaction mixture for 45 to 60 min at 30°C. Polyadenylation reactions were started by addition of substrate followed by incubation at 30°C for the times indicated.

Northern blot analyses. RNA samples were fractionated on 10% polyacrylamide–7 M urea gels and transferred by electroblotting to a Zeta-probe or Zeta-probeGT nylon membrane (Bio-Rad). The membranes were probed with SP6- or T7-transcribed RNAs (22) complementary to U11, U12, U2, U4, U5, and U6 as described by the Zeta-probe manufacturer except that 10 to 100 times more probe was necessary to detect U11 and U12 from native gels compared with denaturing gels. Plasmids pSPU11 and pSPU12 to make full-length antisense U11 and U12, respectively, were constructed from two oligonucleotides cloned into the *Sma*I site in the polylinker of pSP64 (22) as described by Bruzik and Steitz (8). Plasmids used to synthesize U2, U4, U5, and U6 probes (5) were provided by D. L. Black.

RESULTS

Cofractionation of U11 and U12 snRNPs. Glycerol gradient fractionation of HeLa cell nuclear extract previously revealed multiple subpopulations of both U11 and U12 snRNPs, the fastest comigrating at ~18S (23). As seen in Fig. 1A, U11 snRNPs are found predominantly in two peaks, one at ~18S (fractions 9 to 11) and a lighter form (fractions 16 to 19). The U12 snRNP, averaging over many experiments, appears in three peaks: one at ~18S (fractions 9 to 11), one which migrates slightly slower than 18S (seen here as a shoulder; fractions 11 to 13), and a much lighter form (fractions 16 to 18). In this particular extract, most of the U12 snRNP is in the 18S peak while the other two peaks are barely visible. As indicated above, the relative distribution of U11 and U12 snRNPs among these subpopulations varies from extract to extract: the fraction of total in the 18S particle is from 20 to 40% for U11 and 50 to 90% for U12.

To establish that the appearance of the U11 and U12 snRNPs at 18S is due to an interaction between the two RNPs rather than fortuitous cosedimentation, the U12 snRNP was specifically degraded with a complementary deoxyoligonucleotide and RNase H prior to gradient fractionation. Incubation of nuclear extract with the 9C oligonucleotide (complementary to nucleotides 53 to 71 in U12; Fig. 1C) and RNase H resulted in complete degradation of the U12 snRNA (Fig. 1B, lane H) and the disappearance of the U11 snRNP from the 18S region (fractions 9 to 11). The U11 snRNP appeared instead in fractions 15 to 20 (compare Fig. 1B with Fig. 1A). Degradation of the U12 snRNP did not have a detectable effect on the sedimentation behavior of other snRNPs (U2, U5, and U6 were tested in this experiment). Also, incubation of nuclear extract with an oligonucleotide specific for the U2 snRNP (L15 or E15) (4) affected neither the U11 nor U12 snRNP sedimentation profile (data not shown). Thus, we conclude that the U11 and U12 snRNPs interact with each other.

An alternative method of examining higher-order snRNP complexes is native polyacrylamide gel electrophoresis. Again several subpopulations of both the U11 and U12 snRNPs are seen, with U11 and U12 snRNPs appearing together in the slowest-migrating form (Fig. 2, lanes 1 and 4). RNase H experiments analogous to those described above confirm that specific degradation of U12 results in the disappearance of most of the U11 signal from the U11/U12 region (Fig. 2, compare lane 3 with lane 2).

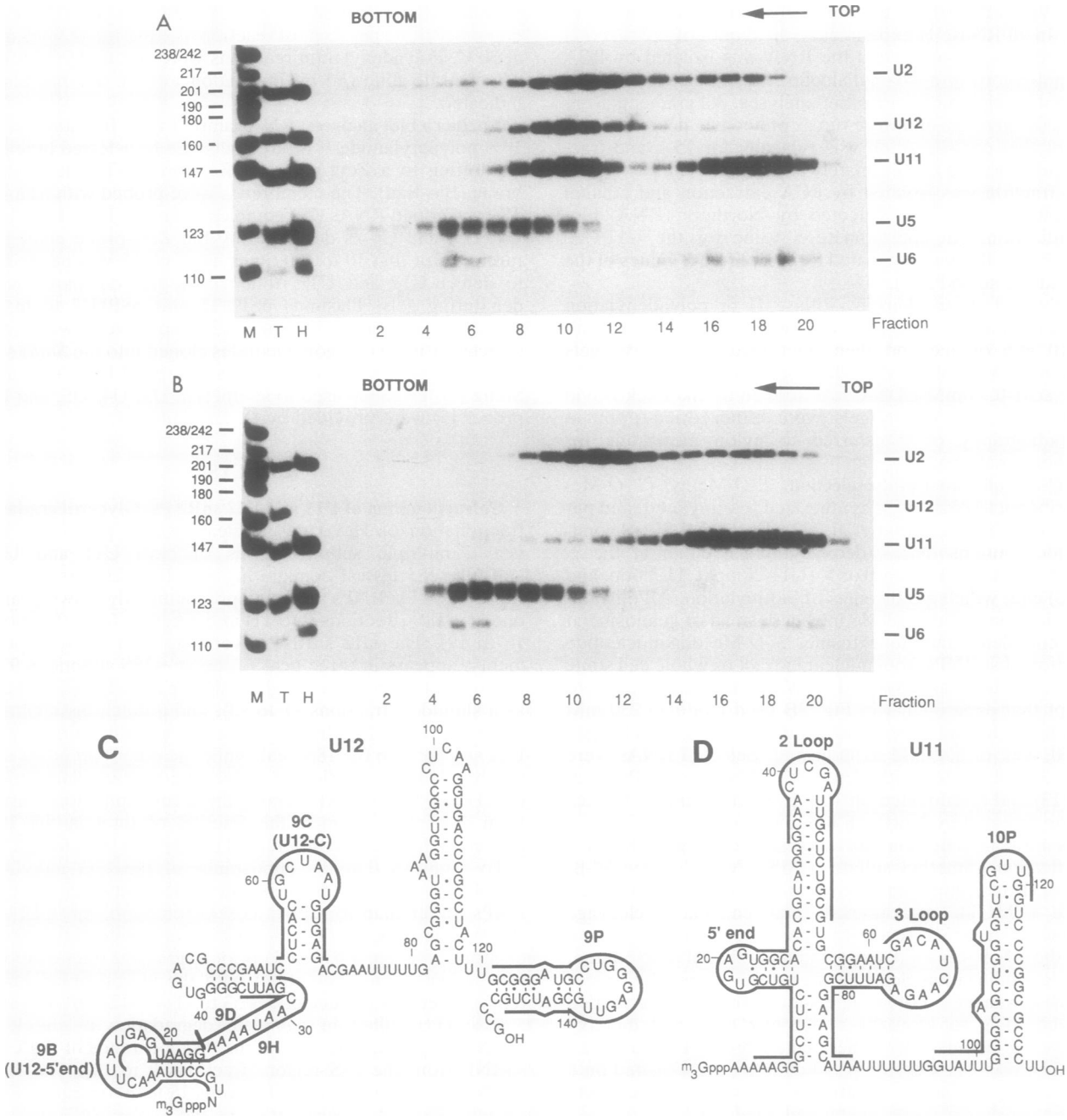

FIG. 1. Glycerol gradient analyses of U11 and U12 snRNPs before (A) and after (B) RNase H degradation of U12 snRNA. Nuclear extract was incubated at 30°C for 60 min with no (A) or 9C (B) oligonucleotide and run on 10 to 30% glycerol gradients as described in Materials and Methods. After fractionation of the gradients, RNA was isolated, run on 10% polyacrylamide gels, and analyzed on Northern blots probed for U11, U12, U2, U5, and U6 snRNAs. The direction of sedimentation is from right to left. Lane M, *Msp*I-digested pBR322 DNA markers; lane T, total RNA from nuclear extract; lane H, RNA isolated after RNase H treatment, before gradient fractionation. (C and D) Oligonucleotides complementary to U11 (C) and U12 (D) superimposed on their secondary structures. The 9B/D oligonucleotide contains both 9B and 9D sequences. The 9B deoxyoligonucleotide and U12-5'end 2'-*O*-Me oligoribonucleotide are complementary to the same sequences, as are the 9C deoxyoligonucleotide and U12-C 2'-*O*-Me oligoribonucleotide. The sequence of U11 snRNA (23) is modified to include additional nucleotides at the 5' end identified by Suter-Crazzolara and Keller (34) but not the extra U at the 3' end, which is not present on U11 snRNA in our extracts (data not shown). The N at the 5' end of U12 denotes at least one nucleotide and could represent several nucleotides.

Coselection of the U11 and U12 snRNPs. Further evidence for a direct interaction between the U11 and U12 snRNPs was obtained by using biotinylated 2'-*O*-Me oligoribonucleotides to select either U11 or U12 snRNA from nuclear extract on streptavidin agarose beads. Three 2'-*O*-Me oligonucleotides complementary to U11 (U11-5'end, U11-3Loop, and U11-2Loop; Fig. 1D) and two complementary to U12 (U12-5'end and U12-C; Fig. 1C) were synthesized. Each was

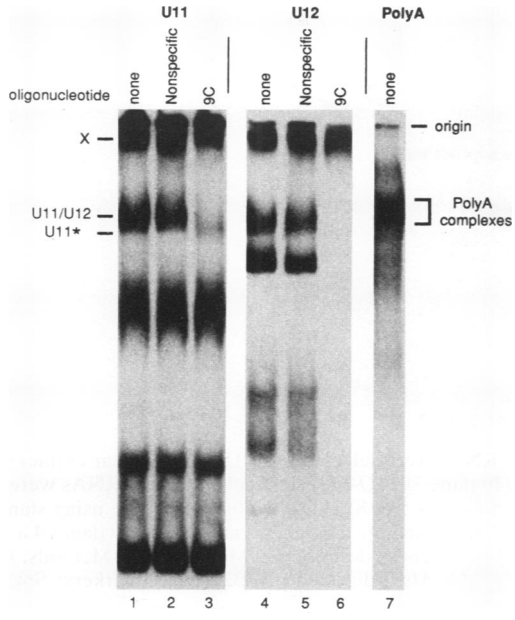

FIG. 2. Native polyacrylamide gel fractionation of U11 and U12 snRNP complexes. Nuclear extract was incubated with RNase H and no (lanes 1 and 4), nonspecific (lanes 2 and 5), or 9C (lanes 3 and 6) oligonucleotide for 60 min at 30°C, heparin (0.2 mg/ml) was added, and the extract was incubated for 10 min on ice. The samples were divided and run in two sets of lanes on a native gel (lanes 1 to 3 and 4 to 6). In addition, a 30-min polyadenylation reaction mixture containing ^{32}P-labeled adenovirus L3(102) substrate was loaded on the native gel for comparison (lane 7). The gel was transferred to a Zeta-probeGT nylon membrane and cut into three sections. One section was probed for U11 (lanes 1 to 3), one was probed for U12 (lanes 4 to 6), and the third was autoradiographed directly to examine polyadenylation complexes (lane 7). The nonspecific oligonucleotide is GCTAGAAACTCGAACCGATTCCTCTC.

shown to specifically select the appropriate RNA from nuclear extract (Fig. 3A; data not shown for U11-3Loop and U11-2Loop). Most notable in the patterns of selected RNAs with use of either the U11 or U12 specific oligonucleotide was the presence of untargeted U12 or U11 snRNA, respectively. In addition to the U11 and U12 RNAs, several other RNAs were evident after 2′-*O*-Me selection. In most cases, these can be ascribed to background since they appeared in the lanes with no oligonucleotide (Fig. 3A, lane 6) or in all lanes regardless of the oligonucleotide used (for example, 5S and 5.8S RNAs). In addition, a significant amount of U2 was present in the sample selected with the U12-5′end oligonucleotide (Fig. 3A, lane 4), but this is due to cross-hybridization of the U12-5′end oligonucleotide with U2 snRNA, as determined by the ability of this oligonucleotide to select gel-purified U2 snRNA (data not shown). To confirm that coselection of U11 and U12 was not due to similar cross-hybridization, each U11 and U12 oligonucleotide was likewise tested for its ability to select gel-purified U11 or U12 snRNA. The U11-5′end oligonucleotide did cross-hybridize with U12 to a limited extent, but the other oligonucleotides were highly specific (data not shown). We conclude that the ability of the oligonucleotides, with the exception of U11-5′end, to select the untargeted U11 or U12 snRNA can be ascribed to binding of the U11/U12 snRNP complex.

Each 2′-*O*-Me oligoribonucleotide was also analyzed for its efficiency in selecting its targeted snRNP from nuclear extract by comparing Northern blots of selected versus unselected RNA. The U12-5′end oligonucleotide binds over 90% of the U12 snRNP, while the U12-C oligonucleotide selects ~75%. The U11 oligonucleotides are less quantitative: the U11-5′end oligonucleotide selects 10 to 20% and the U11-3Loop and U11-2Loop oligonucleotides select less than 10% of the total U11 snRNPs. A U2 oligonucleotide (BU2b [36]) used as a control selected about 50% of the total U2 snRNPs (quantitative data not shown, but see Fig. 3A).

Structure mapping of the U11 and U12 snRNPs. Secondary structure analyses were carried out on both the U11 and U12 RNAs within their snRNPs to learn more about the nature of the particles and locate RNA sequences available for possible RNA-RNA or RNA-protein interactions. Single-stranded stretches were probed with DMS, CMCT, kethoxal, RNase T_1, and RNase A, while RNase V1 was used to identify double-stranded regions of RNA. Nuclear extract was treated with a chemical or enzymatic modifier, total RNA was isolated, and sites of modification or cleavage were mapped by primer extension analysis (Fig. 4). It should be noted that such experiments analyze only regions of RNA not covered by proteins.

A representative experiment is shown in Fig. 4. Figure 4A shows primer extension on U12 snRNA after treatment of nuclear extract with increasing concentrations of DMS (lanes 5 to 12). The primer used was 9P, which is complementary to nucleotides 121 to 146 of U12 (Fig. 1C); therefore, bases 3′ of nucleotide (nt) 115 were not analyzed. DMS modifies the N-1 of adenosine and to a lesser extent N-3 of cytosine, thereby blocking reverse transcription one base before the modification. Lanes 1 to 4 are dideoxy sequencing lanes used to map the stop sites. Figure 4B shows primer extension analysis of U11 after treatment with three different concentrations of RNase T_1 over time (lanes 1 to 4, 5 to 8, and 9 to 12) compared with dideoxy sequencing reactions (lanes 13 to 16). The primer used was 10P, which is complementary to nt 96 to 121 of U11 (Fig. 1D); therefore nucleotides 3′ of position 88 were not analyzed.

Chemical analyses proved to be most useful for U12, with the most information obtained from DMS experiments. In contrast, the single-strand-specific enzymes preferentially cleaved at only two sites (G-60 and U-62) (data not shown), so that it was impossible to obtain information on other regions of the U12 RNA. Preferential digestion was also seen with RNase V1, such that U12 was cleaved almost exclusively at nt 108 to 109, 109 to 110, 112 to 113, and 113 to 114 even at low and high concentrations of RNase V1 (data not shown). Therefore, little information concerning double-stranded regions of U12 was obtained.

Conversely, U11 was analyzed primarily by enzymatic methods because the U11 snRNP is an extremely tight particle that allows very few detectable chemical modifications under standard conditions. Increasing the amounts of chemical modifiers simply produced artifactual stops in the controls (data not shown). However, some data were obtained from DMS treatment, as exemplified by modification of adenosines (Fig. 4D). Even single-strand-specific enzymes required concentrations so high that recognition of U11 bases most probably in double-stranded regions (as judged by cleavage with RNase V1 and very strong RNA-RNA base pairing potential) occurred. The concentration of each enzyme was therefore titrated to avoid artifacts, as shown in Fig. 4B for RNase T_1. Here the highest concentration produced cleavage of U11 snRNA at nt 51, 53, and 55, which are most probably not single stranded. The sensitivities indicated in Fig. 4C and D are a compilation of many

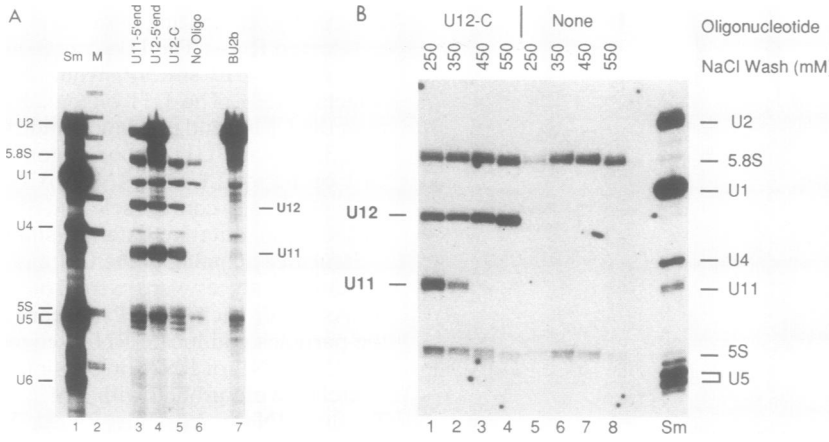

FIG. 3. 2′-*O*-Me oligoribonucleotide selection of U11 and U12 snRNPs. (A) RNAs were selected from 15 μl of nuclear extract with 0.25 μg of U11-5′end (lane 3), U12-5′end (lane 4), U12-C (lane 5), no (lane 6), or BU2b (lane 7) 2′-*O*-Me oligonucleotide. (B) RNAs were selected from 100 μl of whole cell sonic extract with 0.25 μg of U12-C (lanes 1 to 4) or no (lanes 5 to 8) 2′-*O*-Me oligonucleotide using standard 250 mM wash buffer (lanes 1 and 5) or modified 250 mM wash buffer containing 350 (lanes 2 and 6), 450 (lanes 3 and 7), or 550 (lanes 4 and 8) mM NaCl. Selected RNAs were 3′ end labeled and run on 10% polyacrylamide–7 M urea gels as described in Materials and Methods. Only half of the U2-selected sample (panel A, lane 7) was loaded relative to the others. M, *Msp*I-digested pBR322 DNA markers; Sm, anti-Sm immunoprecipitated RNA, 3′ end labeled.

experiments and may not relate directly to the data shown in Fig. 4A and B.

Figures 4C and D present proposed secondary structures for U11 and U12 based on the compiled data. Note that the only difference between these and the previously proposed computer-generated secondary structures (23) is a G · U base pair between bases 17 and 22 in U11, which is deduced because neither G-17 nor U-22 was recognized by RNase T_1 or A, whereas G-17 was cut by RNase V1. Interestingly, there is a region of U11, nt 64 to 80, which was recognized by both single-strand- and double-strand-specific nucleases. This same region of the U11 RNA often shows strong primer extension stops even without treatment (indicated by dots; Fig. 4B, lanes 1, 5, and 9). However, other experiments did not show these stops in the 0 time lanes and allowed the conclusions in Fig. 4D to be drawn. Conceivably, the third stem-loop region of U11 exists in several different configurations in nuclear extract, perhaps base pairing with another RNA.

Sequences involved in U11/U12 snRNP complex formation. To identify regions of U11 involved in U11/U12 complex formation, secondary structure analyses carried out on U11 snRNA in the U11/U12 complex versus the U11 monoparticle were compared. U11/U12 snRNP complexes were separated from the U11 mono-snRNP by two methods: glycerol gradient fractionation and 2′-*O*-Me oligonucleotide selection with the U12-C oligonucleotide. First, glycerol gradient fractions were analyzed by Northern blots to identify the positions of the U11/U12 complex and the U11 monoparticle. Then each fraction from a parallel gradient was treated with RNase T_1 or A, total RNA was isolated, appropriate fractions were pooled, and primer extension analysis was carried out to compare sites of modification on the U11 RNA in either the U11 snRNP or U11/U12 complex. To examine U11 in the U11/U12 complex isolated by 2′-*O*-Me oligonucleotide selection, nuclear extract was preincubated with biotinylated U12-C oligonucleotide and incubated with streptavidin agarose, and then the agarose beads were washed. The only RNAs selected should be U12, because of direct interaction with the oligonucleotide, and U11 through the U11/U12 snRNP complex. Selected RNAs were treated with RNase T_1 or A (under conditions known not to disrupt the U11-U12 interaction) while still bound to the 2′-*O*-Me oligonucleotide and streptavidin agarose, the RNA was isolated, and primer extension analysis was performed to compare the digestion pattern of U11 in the selected complex with the pattern of U11 in total nuclear extract. No differences were detected in the digestion patterns of U11 in the U11/U12 complex isolated by either of these methods compared with the U11 snRNP alone (data not shown).

A different approach was used to identify regions of U12 RNA that might be involved in the interaction with U11. This was necessary both because the U11/U12 complex and the U12 monoparticle peaks overlap in glycerol gradients (Fig. 1A) and because the efficiencies of selection of the U11/U12 complex by 2′-*O*-Me oligonucleotides complementary to U11 is so low. Instead, oligonucleotides that direct RNase H to trim progressively larger fragments from the 5′ end of U12 were used to determine which shortened U12 RNAs can be detected in U11/U12 complexes upon glycerol gradient fractionation. Figure 1B shows that when U12 was targeted for digestion with the 9C oligonucleotide, very little U12 signal remains, suggesting that the 9C oligonucleotide disrupts the core of the particle, leading to complete U12 degradation in the extract. Other oligonucleotides complementary to U12 do not result in total degradation but instead yield stable, shortened forms of the U12 snRNA. Figure 5A shows glycerol gradient fractionation of nuclear extract after digestion of U12 with the 9B oligonucleotide, which yielded a new 5′ end at nt 16 and 19. (For each oligonucleotide examined, the sites of digestion were determined by primer extension mapping; data not shown.) The shortened form of U12 (U12*) produced by 9B is the same size as full-length U11; hence, the Northern blot was first probed for U12 and then reprobed for U11 after the U12 signal had decayed. As can be seen, this shortened form of U12 still resided in the U11/U12 complex (Fig. 5A, fractions 9 to 11) as well as in the heavier U12 monoparticle (fractions 11 to 13). Conversely, the 9B/D oligonucleotide, which directed cleavage of U12 to bases 28 to 33, led to selective loss of the U11/U12 complex

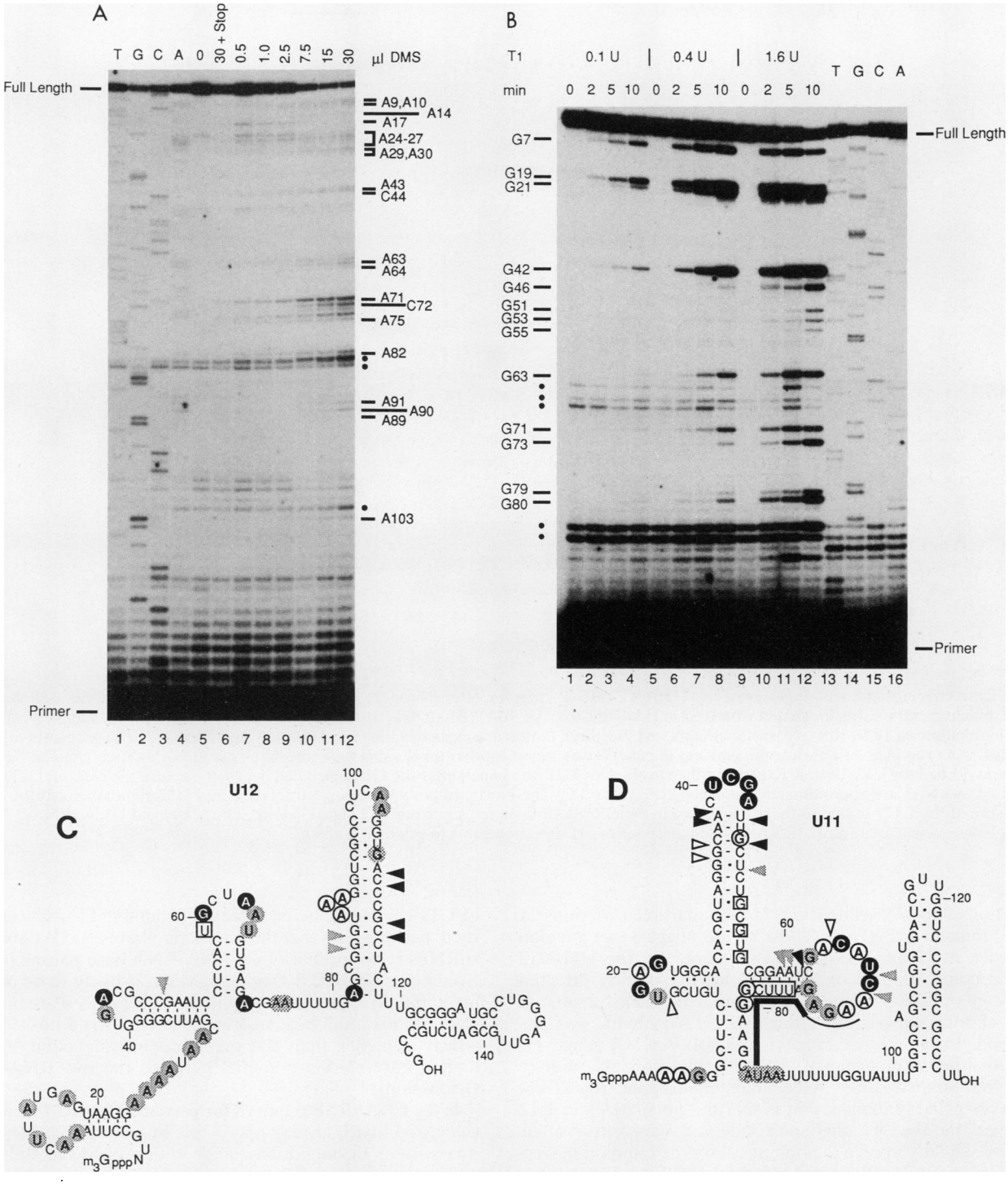

FIG. 4. Secondary structure analyses of the U11 and U12 snRNPs. (A and B) Primer extension analyses of U12 (A) or U11 (B) snRNA after treatment with DMS (A) or RNase T_1 (B). (A) DMS modification of U12. Forty microliters of nuclear extract in a 80-µl reaction was incubated for 15 min at room temperature. Lanes 5 and 7 to 12 show increasing amounts of DMS added; the amount of 5% DMS added is indicated at the top of each lane. Lane 6 is a stop control in which the stop solution was added prior to DMS to demonstrate the inactivation of DMS by the stop solution. (B) RNase T_1 cleavage of U11. Sixty microliters of nuclear extract in a 220-µl reaction was incubated with 0.1 (lanes 1 to 4), 0.4 (lanes 5 to 8), or 1.6 (lanes 9 to 12) U of RNase T_1 at 20°C. At 0, 2, 5, or 10 min, 75-µl aliquots were removed and PCA extracted to inactivate the RNase T_1. After treatment with DMS or RNase T_1, RNA was isolated and analyzed by primer extension with the 9P (A) or 10P (B) primer. Dideoxy sequencing ladders in lanes 1 to 4 (A) and 13 to 16 (B) were used to map the modification/cleavage sites. The letters and numbers indicate modified/cleaved bases; a dot indicates a strong primer extension stop site from untreated RNA. (C and D) Summary of all modification/cleavage data. Nucleotides modified or cleaved by single-strand-specific modifiers are marked by circles; positions cleaved by the double-strand-specific RNase V1 are marked by triangles; bars indicate regions which produced ladders when cleaved with RNase V1. Nucleotides cleaved only at very high concentrations of RNase T_1 are boxed. The extent of modification or cleavage is indicated by shading (black = strong; shaded = moderate; white = weak) or thickness (thick = strong; thin = weak).

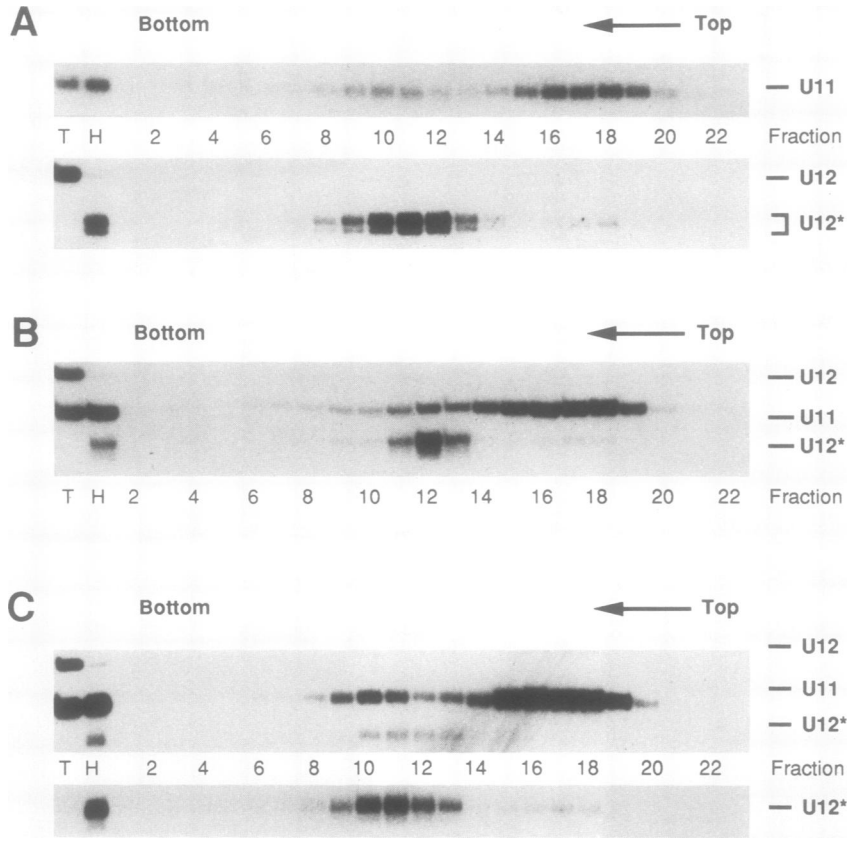

FIG. 5. Glycerol gradient analyses of the U11/U12 snRNP complex after RNase H digestion of U12 snRNA with different oligonucleotides. Nuclear extract was incubated with RNase H and the 9B (A), 9B/D (B), or 9H (C) oligonucleotide at 30°C for 45 min and then subjected to fractionation on 10 to 30% glycerol gradients and Northern blot analyses as described in Materials and Methods. The gradients in panels A and C were run in parallel, while the gradient in panel B was run at another time, using the same batch of nuclear extract. (A) The Northern blot was probed for U12 (bottom panel) and then probed for U11 (top panel) after the U12 signal had decayed because the cleaved U12 (U12*) and U11 snRNAs are approximately the same size. (B and C) The Northern blots were probed for U11 and U12 simultaneously. A darker exposure of the U12* region of the gel is shown in panel C (bottom panel) since the U12 signal is faint. U12*, cleaved U12 snRNAs; T, total RNA from nuclear extract; H, RNA isolated after RNase H digestion prior to gradient fraction.

(fractions 9 to 11), while the U12 monoparticle (fractions 11 to 13) remained (Fig. 5B). These results suggest that a region between nt 19 and 33 of U12 is required for the U11-U12 interaction. Another oligonucleotide, 9H, which directed cleavage of U12 at bases 24 to 30, yielded variable results: in two of four experiments, the U11/U12 complex was destroyed, while in the other two it was not (Fig. 5C); however, the positions of cleavage of U12 with this oligonucleotide in the four experiments were indistinguishable (data not shown). A likely explanation is that the nucleotides in U12 targeted by the 9H oligonucleotide are very close to or impinge slightly upon a region critical for the interaction with the U11 snRNP. We conclude that one boundary of the region of U12 required for the U11-U12 interaction is located between U12 nt 28 and 33.

Stability of the U11/U12 complex. Since the previously characterized interaction of the U5 snRNP with U4/U6 snRNP has been found to be sensitive to salt (1, 31), the U11/U12 complex was selected from whole cell sonic extract with the U12-C 2′-*O*-Me oligonucleotide and streptavidin agarose under different salt conditions. Figure 3B shows that selection with increasing concentrations of NaCl (250 to 550 mM) led to a marked decrease in the amount of U11 RNA selected (compare lanes 2 to 4 with lane 1), while the amount of U12 snRNA selected increased slightly. This susceptibility to salt suggests that the interaction between U11 and U12 snRNPs is not primarily via RNA-RNA base pairing (which would be stabilized by higher salt) but is likely to be protein mediated. We also find that the U11/U12 snRNP complex is stable to a 15-min heat treatment at 42°C (data not shown), which is higher than the predicted melting point for the limited base-pairing potential between the two RNAs (see Discussion).

Is the U12 snRNP required for polyadenylation? It has been suggested that U11 may play a role in polyadenylation since it copurifies through many steps with a cleavage and polyadenylation factor (10) required for in vitro cleavage and polyadenylation of pre-mRNA substrates. Although the U11 snRNP is not susceptible to oligonucleotide-targeted degradation with RNase H (23, 10), both the U12 snRNP and the U11/U12 snRNP complex can be readily destroyed. We therefore assayed polyadenylation in nuclear extract that had been treated with RNase H and oligonucleotide 9C (Fig. 6A, lanes 7 to 12), a nonspecific oligonucleotide (lanes 1 to 6), or no oligonucleotide (lanes 13 to 18). No effect on the rate or extent of polyadenylation was seen after complete degradation of U12 and the U11/U12 complex. Experiments shown in Fig. 6A used the simian virus 40 late polyadenyla-

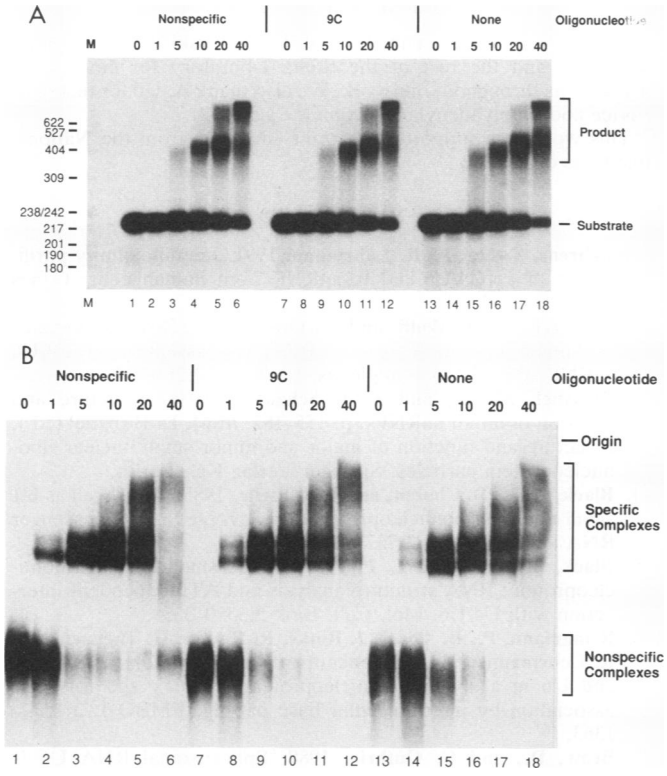

FIG. 6. Polyadenylation product and complex formation after RNase H digestion of U12 snRNA. Nuclear extract was preincubated with RNase H and a nonspecific (lanes 1 to 6), 9C (lanes 7 to 12), or no (lanes 13 to 18) oligonucleotide for 45 min at 30°C. Simian virus 40 late polyadenylation substrate was then added, and the mixture was incubated for 0 to 40 min, as indicated at the top. The samples were divided; RNA isolated from one half was run on a denaturing gel to assess the extent of substrate polyadenylation (A), while the other half was run on a native polyacrylamide gel to examine polyadenylation complexes (B). The nonspecific oligonucleotide was the same as the one used for Fig. 2.

tion site as a substrate, but similar results were obtained with the adenovirus L3 polyadenylation site (data not shown). Experiments that assessed the cleavage activity directly (39) also showed no effect of degradation of U12 (data not shown).

We further examined whether U11 and/or U12 snRNPs comigrate with polyadenylation complexes (15, 18, 30, 40), using the native gel system described above to examine U11 and U12 snRNPs. Intriguingly, the U11/U12 complex in the absence of substrate migrated in the same region of the gel as did active polyadenylation complexes (Fig. 2). Therefore, it was not possible to discern a shift of U11 and/or U12 into a complex with the substrate; however, the effect of degrading the U12 snRNP on the formation of complexes containing the labeled polyadenylation substrate could be assessed (Fig. 6B). Again, we found that degradation of the U12 snRNP or disruption of the U11/U12 snRNP complex had no effect on the assembly of the simian virus 40 substrate into polyadenylation complexes (compare lanes 7 to 12 with lanes 1 to 6). Comparable results were obtained with the adenovirus L3 substrate (data not shown). Note in Fig. 2 that although degradation of the U12 snRNP causes a loss of U11 signal from the U11/U12 complex, another form of the U11 snRNP (U11*) migrates just ahead of the U11/U12 complex, also in the vicinity of the polyadenylation complexes (Fig. 2; compare lanes 1 to 3 with lane 7). Because all complexes form broad, ill-defined bands in native gels, it is difficult to determine whether components precisely comigrate. Therefore, these experiments also do not rule out the possibility that the U11 snRNP is present in active polyadenylation complexes.

DISCUSSION

Fractionation of HeLa nuclear extract on glycerol gradients has revealed that the U11 and U12 snRNPs interact to form a U11/U12 complex sedimenting at ~18S. Since it is disrupted by high salt, the interaction is probably protein mediated. This is the first low-abundance snRNP-snRNP interaction to be identified.

Properties of the U11/U12 snRNP complex. The combined results of nuclease probing experiments and salt sensitivity of the complex argue against base pairing as the basis of the U11-U12 interaction. In particular, the third loop region of U11 snRNA was eliminated as a possible participant in base pairing with U12: this region had appeared especially attractive since it is recognized by both single- and double-strand-specific nucleases and also contains short stretches of complementarity to U12 (e.g., U11 bases 58 to 63 and U12 bases 31 to 36). On the other hand, we cannot rule out the 3′ stem-loop region of U11 RNA as a possible site of base-pairing interaction because our structure analyses used a primer extension assay and primers corresponding to the extreme 3′ end of U11 do not function (data not shown). There is in fact potential base pairing between U11 nt 113 to 120 and U12 nt 28 to 35, the latter coinciding with the boundary of the region that we defined in U12 snRNA as necessary for the U11-U12 interaction. Oligonucleotide-directed RNase H cleavage of U12 at nt 16 and 19 (9B) clearly did not disrupt the U11-U12 interaction, while cleavage at nt 28 to 33 (9B/D) did. Cleavage at sites between those two regions (nt 24 to 30 [9H]) sometimes interfered with interaction, arguing that the 9H oligonucleotide binds very close to a region important for U11-U12 interaction. Since the cleavage sites directed by the 9B/D and 9H oligonucleotides overlap, it may be that initial base pairing to U12 rather than the subsequent RNase H cleavage of U12 is the primary cause of U11/U12 disruption. The 9B/D oligonucleotide is complementary to nt 2 to 36 of U12, expanding the possible region of interaction in U12 through nt 36. In either case, it is clear that U12 nt 28 to 36 include a site critical for the U11-U12 interaction.

The 2′-*O*-Me oligonucleotide selection showed destabilization of the U11/U12 complex at high salt concentrations suggestive of a protein-mediated interaction. The U11-U12 interaction is therefore reminiscent of the interaction between the U5 snRNP and the U4/U6 snRNP to form the U4/U5/U6 complex. This U5 interaction is also thought to be protein mediated; it is disrupted by high salt, and structure probing of U5 also revealed no regions available in the U5 monoparticle compared with the tri-snRNP complex (5). In contrast to the U4-U5-U6 interaction, incubation in the presence or absence of ATP does not significantly affect the amount of the U11/U12 snRNP complex when analyzed on glycerol gradients (data not shown). However, when analyzed by complex gel electrophoresis, much of the U11/U12 snRNP complex shifts to a slower-migrating form after incubation with or without ATP (indicated by × in Fig. 2).

Several contrasts in the properties of U11 and U12 packaged into their respective snRNP particles are apparent. The

U11 snRNP was previously deduced to be a very tight particle on the basis of its inability to be cleaved by RNase H with a battery of complementary oligonucleotides (10, 23) and its resistance to digestion with micrococcal nuclease (23). Here we have provided further evidence of the inaccessibility of the U11 RNA in its particle; both chemicals and enzymes act only at very high concentrations, and 2′-*O*-Me oligonucleotides complementary to the most available regions (as determined by structure mapping) select, at most, 10 to 20% of the total U11 snRNPs. In contrast to U11, the U12 snRNP is extremely sensitive to micrococcal nuclease, highly accessible to chemical and enzymatic modifiers, efficiently selected with antisense 2′-*O*-Me oligonucleotides, and readily digested with RNase H in the presence of many complementary oligonucleotides. This is true in many extracts, although we previously reported that only the 9B oligonucleotide was able to direct RNase H cleavage of the U12 snRNP (23); we do not know the source of this variability.

Function of the U11/U12 snRNP. Since U11 and U12 coexist in a single snRNP complex, it follows that they function together in nuclear metabolism. It has been suggested that U11 may play a role in polyadenylation of pre-mRNAs because it cofractionates with the cleavage and polyadenylation factor through many steps of purification (10). Because the U11 snRNP is resistant to all modes of specific destruction and removal from extracts, it has been difficult to address this possibility directly. The contrasting sensitivity of the U12 snRNP to degradation has allowed us to determine that neither the U11/U12 complex nor U12 snRNP is required for polyadenylation as assayed in vitro.

However, several intriguing, albeit circumstantial, links persist between polyadenylation and the U11 snRNP. First, the purification scheme for the cleavage and polyadenylation factor yields an active fraction which contains U11 but no other snRNPs, indicating that the protocol selects for U11 monoparticles (10). Second, the U12 snRNA contains the sequence AAAAUAA (nt 24 to 30), which resembles the polyadenylation signal (AAUAAA); interestingly, this sequence is located just upstream of or possibly included in the sequence required for the U11/U12 interaction. Third, in vitro polyadenylation can be inhibited by micrococcal nuclease pretreatment of the extract, but only at very high concentrations of nuclease which are also required to completely degrade U11 snRNA (data not shown). Fourth, as more polyadenylation signals are examined, it has become increasingly clear that a number of ill-defined elements upstream and downstream of the AAUAAA sequence significantly affect polyadenylation in vivo (26, 37). Thus, it remains possible that U11 and/or U12 snRNPs might be involved in mediating the effect of these upstream and downstream elements. In addition, the purified polyadenylation systems lacking U11 do not respond to known downstream sequences in several polyadenylation sites (28).

Other conceivable roles for low-abundance snRNPs like U11 and U12 include coordinating the splicing and polyadenylation of a single transcript; it has been shown that polyadenylation is more efficient on transcripts that contain upstream splicing signals (25). Alternatively, U11 and U12 might modulate specific RNA processing events; perhaps the regulated splicing of some substrates requires a specific snRNP in addition to the general splicing machinery. What is needed are reliable in vitro systems that faithfully mimic in vivo processing and a method of specifically targeting the U11 snRNP for degradation or removal from extracts or cells.

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REFERENCES

1. **Behrens, S.-E., and R. Luhrmann.** 1991. Immunoaffinity purification of a [U4/U6.U5] tri-snRNP from human cells. Genes Dev. **5**:1439–1452.
2. **Bindereif, A., T. Wolff, and M. Green.** 1990. Discrete domains of human U6 snRNA required for the assembly of U4/U6 snRNP and splicing complexes. EMBO J. **9**:251–255.
3. **Birnstiel, M. L., and F. J. Schaufele.** 1988. Structure and function of minor snRNPs, p. 155–182. *In* M. L. Birnstiel (ed.), Structure and function of major and minor small nuclear ribonucleoprotein particles. Springer-Verlag KG, Berlin.
4. **Black, D. L., B. Chabot, and J. A. Steitz.** 1985. U2 as well as U1 small nuclear ribonucleoproteins are involved in premessenger RNA splicing. Cell **42**:737–750.
5. **Black, D. L., and A. L. Pinto.** 1989. U5 small nuclear ribonucleoprotein: RNA structure analysis and ATP-dependent interaction with U4/U6. Mol. Cell. Biol. **9**:3350–3359.
6. **Bringmann, P., B. Appel, J. Rinke, R. Reuter, H. Theissen, and R. Luhrmann.** 1984. Evidence for the existence of snRNAs U4 and U6 in a single ribonucleoprotein complex and for their association by intermolecular base pairing. EMBO J. **3**:1357–1363.
7. **Brow, D., and C. Guthrie.** 1988. Spliceosomal RNA U6 is remarkably conserved from yeast to mammals. Nature (London) **334**:213–218.
8. **Bruzik, J. P., and J. A. Steitz.** 1990. Spliced leader RNA sequences can substitute for the essential 5′ end of U1 RNA during splicing in a mammalian in vitro system. Cell **62**:889–899.
9. **Cheng, S.-C., and J. Abelson.** 1987. Spliceosome assembly in yeast. Genes Dev. **1**:1014–1027.
10. **Christofori, G., and W. Keller.** 1988. 3′ cleavage and polyadenylation of mRNA precursors in vitro requires a poly(A) polymerase, a cleavage factor, and a snRNP. Cell **54**:875–889.
11. **Datta, B., and A. M. Weiner.** 1991. Genetic evidence for base pairing between U2 and U6 snRNA in mammalian mRNA splicing. Nature (London) **352**:821–824.
12. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
13. **England, T. E., A. G. Bruce, and O. C. Uhlenbeck.** 1980. Specific labeling of 3′ termini of RNA with T4 RNA ligase. Methods Enzymol. **65**:65–74.
14. **Fabrizio, P., D. S. McPheeters, and J. Abelson.** 1989. In vitro assembly of yeast U6 snRNP: a functional assay. Genes Dev. **3**:2137–2150.
15. **Gilmartin, G. M., and J. R. Nevins.** 1989. An ordered pathway of assembly of components required for polyadenylation site recognition and processing. Genes Dev. **3**:2180–2189.
16. **Hashimoto, C., and J. A. Steitz.** 1984. U4 and U6 RNAs coexist in a single small nuclear ribonucleoprotein particle. Nucleic Acids Res. **12**:3283–3293.
17. **Hausner, T.-P., L. M. Giglio, and A. M. Weiner.** 1990. Evidence for base-pairing between mammalian U2 and U6 small nuclear ribonucleoprotein particles. Genes Dev. **4**:2146–2156.
18. **Humphrey, T., G. Christofori, V. Lucijanic, and W. Keller.** 1987. Cleavage and polyadenylation of messenger RNA precursors in vitro occurs within large and specific 3′ processing complexes. EMBO J. **6**:4159–4168.
19. **Konarska, M. M., and P. A. Sharp.** 1988. Association of U2, U4, U5, and U6 small nuclear ribonucleoproteins in a spliceosome-type complex in absence of precursor RNA. Proc. Natl. Acad. Sci. USA **85**:5459–5462.
20. **Lerner, M. R., J. A. Boyle, J. A. Hardin, and J. A. Steitz.** 1981.

Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. Science **211:** 400–402.

21. **Madhani, H. D., R. Bordonne, and C. Guthrie.** 1990. Multiple roles for U6 snRNA in the splicing pathway. Genes Dev. **4:**2264–2277.
22. **Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green.** 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. **12:**7035–7056.
23. **Montzka, K. A., and J. A. Steitz.** 1988. Additional low-abundance human small nuclear ribonucleoproteins: U11, U12, etc. Proc. Natl. Acad. Sci. USA **85:**8885–8889.
24. **Moore, C. L., and P. A. Sharp.** 1985. Accurate cleavage and polyadenylation of exogenous RNA substrate. Cell **41:**845–855.
25. **Niwa, M., S. D. Rose, and S. M. Berget.** 1990. In vitro polyadenylation is stimulated by the presence of an upstream intron. Genes Dev. **4:**1552–1559.
26. **Proudfoot, N.** 1991. Poly(A) signals. Cell **64:**671–674.
27. **Reddy, R., and H. Busch.** 1988. Small nuclear RNAs: RNA sequences, structure, and modifications, p. 1–37. *In* M. L. Birnstiel (ed.), Structure and function of major and minor small nuclear ribonucleoprotein particles. Springer-Verlag KG, Berlin.
28. **Ryner, L., Y. Takagaki, and J. L. Manley.** 1989. Sequences downstream of AAUAAA signals affect pre-mRNA cleavage and polyadenylation in vitro both directly and indirectly. Mol. Cell. Biol. **9:**1759–1771.
29. **Sheen, J.-Y., and B. Seed.** 1988. Electrolyte gradient gels for DNA sequencing. BioTechniques **6:**942–944.
30. **Skolnik-David, H., C. L. Moore, and P. A. Sharp.** 1987. Electrophoretic separation of polyadenylation-specific complexes. Genes Dev. **1:**672–682.
31. **Sontheimer, E. J., and J. A. Steitz.** 1992. Three novel functional variants of human U5 small nuclear RNA. Mol. Cell. Biol. **12:**734–746.
32. **Sproat, B. S., A. I. Lamond, B. Beijer, P. Neuner, and U. Ryder.** 1989. Highly efficient chemical synthesis of 2′-O-methyloligoribonucleotides and tetrabiotinylated derivatives; novel probes that are resistant to degradation by RNA or DNA specific nucleases. Nucleic Acids Res. **17:**3373–3384.
33. **Steitz, J. A., D. L. Black, V. Gerke, K. A. Parker, A. Kramer, D. Frendewey, and W. Keller.** 1988. Functions of the abundant U-snRNPs, p. 115–154. *In* M. L. Birnstiel (ed.), Structure and function of major and minor small nuclear ribonucleoprotein particles. Springer-Verlag KG, Berlin.
34. **Suter-Crazzolara, C., and W. Keller.** 1991. Organization and transient expression of the gene for human U11 snRNA. Gene Expression **1:**91–102.
35. **Vankan, P., C. McGuigan, and I. W. Mattaj.** 1990. Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes. EMBO J. **9:**3397–3404.
36. **Wassarman, D. A., and J. A. Steitz.** 1991. Structural analyses of the 7SK ribonucleoprotein (RNP), the most abundant human small RNP of unknown function. Mol. Cell. Biol. **11:**3432–3445.
37. **Wickens, M.** 1990. How the messenger got its tail: addition of poly(A) in the nucleus. Trends Biochem. Sci. **15:**277–281.
38. **Wu, J., and J. L. Manley.** 1991. Base pairing between U2 and U6 snRNAs is necessary for splicing of a mammalian pre-mRNA. Nature (London) **352:**818–821.
39. **Zarkower, D., and M. Wickens.** 1987. Formation of mRNA 3′ termini: stability and dissociation of a complex involving the AAUAAA sequence. EMBO J. **6:**177–186.
40. **Zarkower, D., and M. Wickens.** 1987. Specific pre-cleavage and post-cleavage complexes involved in the formation of SV40 late mRNA 3′ termini in vitro. EMBO J. **6:**4185–4192.
41. **Zaug, A. J., J. R. Kent, and T. R. Cech.** 1984. A labile phosphodiester bond at the ligation junction in a circular intervening sequence RNA. Science **224:**574–578.