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U5 Small Nuclear Ribonucleoprotein: RNA Structure Analysis and ATP-Dependent Interaction with U4/U6

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To understand how the U5 small nuclear ribonucleoprotein (snRNP) interacts with other spliceosome components, its structure and binding to the U4/U6 snRNP were analyzed. The interaction of the U5 snRNP with the U4/U6 snRNP was studied by separating the snRNPs in HeLa cell nuclear extracts on glycerol gradients. A complex running at 25S and containing U4, U5, and U6 but not U1 or U2 snRNAs was identified. In contrast to results with native gel electrophoresis to separate snRNPs, this U4/U5/U6 snRNP complex requires ATP to assemble from the individual snRNPs. The structure of the U5 RNA within the U5 snRNP and the U4/5/6 snRNP complexes was then compared. Oligonucleotide-targeted RNase H digestion identified one RNA sequence in the U5 snRNP capable of base pairing to other nucleic acid sequences. Chemical modification experiments identified this sequence as well as two other U5 RNA sequences as accessible to modification within the U5 RNP. One of these regions is a large loop in the U5 RNA secondary structure whose sequence is conserved from *Saccharomyces cerevisiae* to humans. Interestingly, no differences in modification of free U5 snRNP as compared to U5 in the U4/U5/U6 snRNP complex were observed, suggesting that recognition of specific RNA sequences in the U5 snRNP is not required for U4/U5/U6 snRNP assembly.

The most abundant small nuclear ribonucleoproteins (snRNPs) of mammalian cells, containing U1, U2, U4/U6, and U5 RNAs, function in pre-mRNA splicing as components of the spliceosome (13, 25, 30, 39). This large complex must be assembled from the pre-mRNA, the individual snRNPs, and accessory proteins before intron removal can occur. Early in the process, the U1 snRNP and the U2 snRNP (at least in *Saccharomyces cerevisiae*) interact through RNA base pairing with the 5′ splice site and the branch site of the pre-mRNA, respectively. The U5 snRNP appears to bind the 3′ splice site, apparently through an associated protein. The U2 snRNP may also require non-snRNP proteins to interact with the branch site (18, 38). It is not understood how the U4/U6 snRNP attaches to the spliceosome, although it may bind as a complex with the U5 snRNP. Beyond these interactions, the pathway of spliceosome assembly is only dimly understood. The U1, U2, and U5 snRNPs in HeLa cell nuclear extracts generally exist as free particles; the U4 and U6 snRNAs are contained within a single particle called the U4/U6 snRNP.

The U5 snRNP has also been reported to interact with the U4 and U6 RNAs to form an snRNP containing three snRNAs. This complex is observed in both *S. cerevisiae* and HeLa cell extracts upon electrophoresis through nondenaturing gels (10, 15, 16). When analyzed in this way, the complex seems to remain intact only in the absence of ATP. In contrast, Lossky et al. (24) have shown that an antibody against the *S. cerevisiae* rna8 protein, which specifically precipitates U5 (yeast snR7) from yeast extracts, coprecipitates U4 (snR14) and U6 (snR6) only when those extracts are incubated in the presence of ATP. In experiments following the time course of spliceosome assembly, the U4, U5, and U6 snRNPs appear to bind at the same time to a complex already containing the U2 snRNP, the pre-mRNA, and possibly the U1 snRNP (2, 10, 15, 22). Interestingly, the U4 snRNP is not present in later complexes when they are assayed by gel electrophoresis (10, 22, 33) but is present in similar complexes isolated by gel filtration chromatography (35).

Such observations lead to a picture of the initiation of splicing as an extremely dynamic process involving the formation and severing of multiple snRNP-snRNP contacts. A favored model suggests that these specific interactions involve snRNA-snRNA base pairing. The U4 and U6 snRNPs are already known to be bound together by RNA base pairs (7, 8, 14, 37). In developing models for how the snRNPs interact within the spliceosome, it is important to identify which RNA sequences within the snRNP structures are capable of interacting with other RNA molecules.

The snRNAs, like most nonmessenger RNAs, fold into stable secondary structures in which much of the RNA sequence is involved in intramolecular base pairing, leaving short runs of single-stranded nucleotides in loops and between stems. Some of the unpaired sequences are highly conserved across species (34) and are likely candidates for points of base-pairing interactions with other spliceosome components. Alternatively, they could serve as binding sites for snRNP proteins or engage in tertiary interactions within the snRNA.

Ribonuclease H (RNase H) cleavage has been useful for identifying the RNA sequences within an snRNP that are accessible for base pairing with other molecules. The sequences in U1 and U2 that are known to pair with the pre-mRNA are readily cleavable with RNase H and a complementary DNA oligonucleotide (3, 17, 19, 36). Other cleavable sequences in the snRNAs include nucleotides 1 to 14 in U2 (3, 17), nucleotides 1 to 15 and 63 to 83 in U4 (1, 4) and nucleotides 43 to 60, 57 to 78, and 78 to 95 in U6 (4; D. Black, unpublished observations). These regions include the most conserved sequences in each of these RNAs (34).

The U5 snRNP has so far not been analyzed for sequences which engage in base pairing with other RNA molecules. How the U5 snRNP interacts with other spliceosome com-

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ponents is not clear. In this paper, we have analyzed the assembly of U5 into a U4/U5/U6 snRNP complex and have determined the dependence of this assembly on ATP. We also present experiments which identify the sequences accessible for base pairing in the free U5 snRNP, as well as U5 incorporated in the U4/U5/U6 snRNP complex.

MATERIALS AND METHODS

Reagents. Oligonucleotides provided by J. Flory were synthesized on an Applied Biosystems DNA synthesizer and were purified by gel electrophoresis and chromatography on Sep-Pak C18 columns (Waters Associates). Dimethyl sulfate (DMS) and *N*-cyclohexyl-*N'*-β-(4-methyl morpholinium) ethylcarbodiimide *p*-toluenesulfonate (CMCT) were from Aldrich Chemical Co., Inc. Kethoxal was from U.S. Biochemical.

Nuclear extract and RNase H cleavage. Nuclear extract was prepared by the method of Dignam et al. (12) and was tested for splicing activity as previously described (4). RNase H cleavage of U5 was carried out as previously described for U2 and U4/U6 (3, 4).

Buffers and stock solutions. BMK buffer is 80 mM potassium borate (pH 8.1)–60 mM KCl–2 mM $MgCl_2$. HMK buffer is 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9)–60 mM KCl–2 mM $MgCl_2$. The DMS stock solution is 5% (vol/vol) DMS in dioxane. The kethoxal stock solution is 37 mg of kethoxal per ml in distilled water. The CMCT stock solution is 42 mg of CMCT per ml in BMK buffer. DMS stop buffer is 0.3 M sodium acetate–0.2 M β-mercaptoethanol–0.2 M Tris hydrochloride (pH 7.5)–5 mM EDTA. Kethoxal stop buffer is 0.3 M sodium acetate–0.2 M Tris hydrochloride (pH 7.5)–15 mM potassium borate–5 mM EDTA. CMCT stop buffer is 0.3 M sodium acetate–0.2 M PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] (pH 6.5)–5 mM EDTA. Hybridization buffer is 50 mM HEPES (pH 7.6)–5 mM potassium borate–100 mM KCl. Gradient dilution buffer is 140 mM KCl–20 mM HEPES (pH 7.9). Buffer D is 20 mM HEPES (pH 7.9)–20% glycerol–0.1 M KCl–0.2 mM EDTA–0.5 mM dithiothreitol.

Chemical modification. Prior to chemical modification, nuclear extract was incubated with hexokinase and glucose to reduce the concentration of ATP, thereby promoting the disassembly of U4/U5/U6 complexes. The hexokinase treatment was as follows. A 180-μl portion of nuclear extract was mixed with 90 μl of buffer D–150 μl of distilled water–18 μl of 55 mM $MgCl_2$–6 μl of glucose (0.375 M)–6 μl of hexokinase (2 U/μl). This mixture was incubated for 30 min at 30°C. After incubation, 150 μl of the reaction was dialyzed into BMK buffer for the CMCT modification and the rest was frozen for the DMS and kethoxal modifications.

For modification of the U4/5/6 complex, nuclear extract was preincubated for 30 min under splicing conditions (3), except that the ATP concentration was raised to 1 mM.

The modification reactions were carried out by adding the indicated amounts of stock solution (see Fig. 6) to 25-μl samples of HEPES-buffered extract for DMS and kethoxal reactions and to borate-buffered extract for CMCT reactions. For the RNA reactions, the total RNA in the nuclear extract was phenol extracted and ethanol precipitated. The RNA from 25 μl of extract was suspended in 25 μl of HMK buffer for DMS and kethoxal reactions and in 25 μl of BMK buffer for CMCT reactions. The $MgCl_2$ concentration was then raised to 12 mM to help stabilize the RNA secondary structure in the absence of proteins. After incubation with the modifying reagents for 15 min at room temperature, 300 μl of the appropriate stop buffer was added. The RNA reactions were immediately ethanol precipitated. The RNP reactions were phenol extracted and then ethanol precipitated.

The modified RNA was then subjected to reverse transcription from an oligonucleotide primer complementary to nucleotides 84 to 104 in U5 RNA. This primer was labeled by using [γ-^{32}P]ATP and polynucleotide kinase. In 5 μl of hybridization buffer, 1 ng of end-labeled primer was incubated with one half of the RNA from one modification reaction for 5 min at 90°C and then transferred to 65°C for 30 min. To each of these reactions was added 3 μl of a mixture containing 0.3 M Tris hydrochloride (pH 8.4 at 42°C), 22 mM $MgCl_2$, 44 mM β-mercaptoethanol, 44 μg of bovine serum albumin per ml, 900 μM each deoxynucleoside triphosphate, 22 mM dithiothreitol, and 10 U of reverse transcriptase. Reactions for the sequencing ladder also contained one dideoxynucleoside triphosphate (50 μM). Extension reactions were incubated at 45°C for 45 min, phenol extracted, and ethanol precipitated. Primer extension products were then separated on a denaturing polyacrylamide gel and detected by autoradiography.

ATP concentrations. ATP concentrations in the extract after various treatments were determined by the method of Condit (11), with a luciferin-luciferase kit from Sigma Chemical Co.

Gradients. All gradients shown were 10 to 30% glycerol in 100 mM KCl–20 mM HEPES (pH 7.9)–1 mM $MgCl_2$. Gradients in Fig. 1, 2 and 3 were run until bacterial 30S ribosomal subunits were near the bottom of a parallel marker gradient. In Fig. 4, the 30S marker was in the middle of the gradient. Figure 1 shows 5-ml gradients spun in an SW50.1 rotor for 6 h at 35,000 rpm at 4°C. Figures 2 and 3 show 12-ml gradients run in an SW41 rotor at 40,000 rpm for 12 h at 4°C. The gradients in Fig. 4 were similar to those in Fig. 2 and 3, except they were run for 6 h and 30 min. The gradient in Fig. 8 was spun in an SW50.1 rotor for 4 h and 30 min at 48,000 rpm. After fractionation of the gradients, the RNA from each fraction was phenol extracted, ethanol precipitated, and run on a denaturing 10% polyacrylamide gel. These gels were either silver stained (28) as in Fig. 1 and 8 or blotted to a Zetabind (BioRad Laboratories) membrane and probed according to the protocol of the manufacturer.

RNA probes. Blots were probed simultaneously with antisense RNA probes complementary to U1, U2, U4, U5, and U6 RNAs. Clones for generating the U1, U2, and U6 probes were kindly provided by K. Mowry, C.-Y. Yuo, and G. Das, respectively. These consist of the human (U1 and U2) or mouse (U6) snRNA genes which were cloned in opposite orientation to a phage promoter (SP6 or T7). The U4 and U5 probes were made by cloning long synthetic oligonucleotides into the polylinker of pSP64. These oligonucleotides were homologous to 90 and 79 nucleotides of U4 and U5, respectively. All of these plasmids were transcribed by the method of Melton et al. (27).

RESULTS

snRNP interactions characterized by gradient fractionation. As a step towards characterizing the interactions of the U5 snRNP, complexes containing the U5 RNA in the nuclear extract were analyzed. In particular, we were interested in the U4/5/6 snRNP complex and the effects of ATP on its assembly and disassembly. Nuclear extracts were therefore fractionated by glycerol gradient sedimentation, a technique which is more cumbersome than electrophoretic methods but might allow isolation of the U4/5/6 complex in quantities sufficient for structural and biochemical studies.

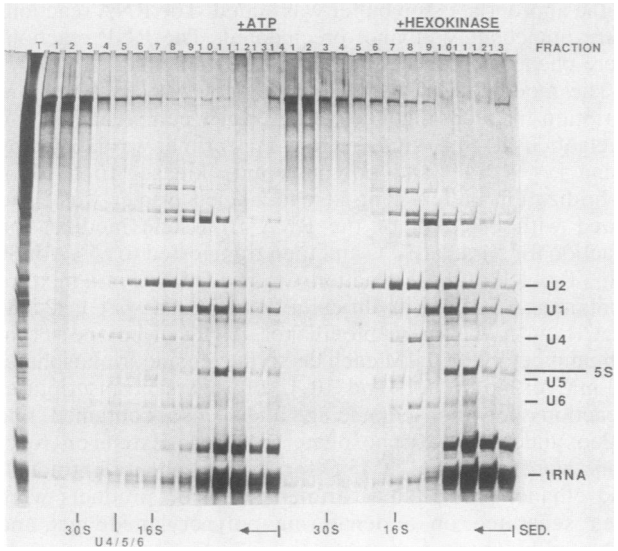


FIG. 1. Formation of the U4/U5/U6 complex in the presence or absence of ATP. Two 75-μl reactions containing 45 μl of nuclear extract and 2.2 mM $MgCl_2$ were incubated at 30°C for 30 min. One reaction contained 0.5 mM ATP and 20 mM creatine phosphate. The other reaction contained 4 U of hexokinase and 5 mM glucose. These reactions were diluted with 75 μl of gradient dilution buffer and were run on gradients as described in Materials and Methods. After separation, the gradient fractions were phenol extracted, ethanol precipitated, and run on a gel. This gel was silver stained to visualize the RNA. The fraction numbers are indicated along the top. The gradient on the left was loaded with extract incubated with ATP. The right-hand gradient had hexokinase-treated extract. The direction of sedimentation was from right to left. The major RNAs are indicated at the right. The position of the U4/U5/U6 complex and the positions of 30S and 16S markers run on a parallel gradient are shown at the bottom.

Nuclear extract was incubated either with 500 μM ATP and creatine phosphate or with hexokinase and glucose to remove any endogenous ATP from the extract. Our nuclear extracts contained about 3 μM residual ATP, which was reduced to about 0.6 to 0.7 μM upon treatment with hexokinase and glucose (data not shown). After fractionation on glycerol gradients, the RNA in each fraction was extracted, run on a denaturing polyacrylamide gel, and silver stained. In the extract incubated with hexokinase, each snRNP migrated at a characteristic position between 7S and 15S (Fig. 1, right side). Starting from the top, U1 peaked in fractions 9, 10, and 11, U4/U6 peaked in fraction 8, U2 peaked in fractions 7 and 8, and U5 peaked in fractions 6 and 7. A similar pattern was seen in an extract incubated with ATP (Fig. 1, left side). However, there was also a new peak containing U4, U5, and U6 RNAs sedimenting at about 25S (fractions 4 and 5).

It is possible that the U5 and the U4/U6 snRNPs, rather than binding each other, have each bound some other component of the extract that leads to their comigration. We tested this possibility by cleaving U4 or U6 with RNase H and complementary oligonucleotides; if U5 is truly complexed with the U4/U6 snRNP, its sedimentation should be altered by this treatment. After prior incubation with both oligonucleotides and ATP, the extract was gradient fractionated as before. RNA from each fraction was run on a gel and blotted to a nylon membrane, and the membrane was probed with RNA probes complementary to U1, U2, U4, U5, and U6 RNAs. Autoradiographs of such blots are shown in Fig. 2. In the control extract treated with an oligonucleotide which does not allow cleavage of any RNA (panel A, left side), the U4/5/6 snRNP peak was detected in fractions 6, 7, and 8. As anticipated, cleavage of U4 RNA alone (panel B, left side) or cleavage of both U4 and U6 RNAs (panel A, right side) caused the disappearance of the faster-sedimenting U5 peak, without affecting the slower U5 peak. Note that some of the cleaved U4 and (on darker exposure) the cleaved U6 comigrated with U5 in the leading edge of the slower U5 peak (panel A, right side). Cleavage of U6 RNA (panel B, right side) caused a smaller but reproducible shift in the position of the faster U5 peak (now in fractions 7, 8, and 9); in this case nearly all of the faster-sedimenting form of U4 still comigrated with the faster form of U5. This may suggest that the association of U5 with U6 is more dependent on the integrity of U4 than that its association with U4 is dependent on U6, although cleavage of other sequences in U4 or U6 may not have these effects. It should be noted that some of the cleaved U6 can also be observed in these fractions upon darker exposure. This truncated U6 might still be sufficient to stabilize the U4/5 interaction. In an attempt to eliminate these residual U6 RNA fragments, simultaneous RNase H digestion with a battery of U6-specific oligonucleotides (those complementary to nucleotides 18 to 29, 43 to 60, and 78 to 95) was performed. Gradient fractions were probed by Northern (RNA) hybridization as before. Although the majority of cleaved U6 RNA was found at the top of the gradient, some U6 fragments could still be observed comigrating with U4 and U5 near the bottom of the gradient.

A significant amount of U2 often appears in the 25S region of the gradient and sometimes repeaks with the U4/5/6 snRNP complex. To test whether U2 might also associate with the U4/U5/U6 snRNP complex, nuclear extracts in which U2 had been cleaved by oligonucleotide-mediated RNase H digestion were run on gradients (Fig. 3). Under conditions of virtually complete cleavage of U2 (Fig. 3, bottom panel), the mobility of the U4/5/6 complex did not shift at all from the normal (top panel); it appeared in fractions 6, 7, and 8 in both gradients. This indicated that the U4/U5/U6 snRNP complex probably did not contain U2 as well.

Finally, we asked whether ATP, instead of allowing the de novo assembly of the U4/5/6 snRNP complex, was preventing complexes already present in the extract from breaking down. Figure 4 shows the analysis of extracts which were incubated at 30°C for 30 min without added ATP; for 30 min without ATP, at which time ATP and creatine phosphate were added and the incubation was continued for 60 min; or for 90 min without ATP. After gradient fractionation, the RNAs were run on a gel, blotted, and probed as before. The extract incubated for 30 min without ATP showed a small amount of the U4/U5/U6 snRNP complex (Fig. 4, left side, fraction 16). (Although the U5 and U6 RNAs are artificially light in this blot due to problems in matching the specific activity of different probes, the U4 RNA clearly peaked in the U4/U5/U6 snRNP region of the gradient.) The addition of ATP and incubation for another 60 min produced a large increase in the amount of the U4/5/6 snRNP complex (middle gradient, fractions 16 and 17). That this increase was dependent on the addition of ATP is shown by the third gradient (right side), where continued incubation without ATP reduced the amount of complex until it was barely detectable (right gradient, fractions 16 and 17). This experiment indicated that ATP was necessary for the actual assembly of U4/U5/U6 snRNP complexes as analyzed in glycerol gradients. Different preparations of nuclear extract vary in the

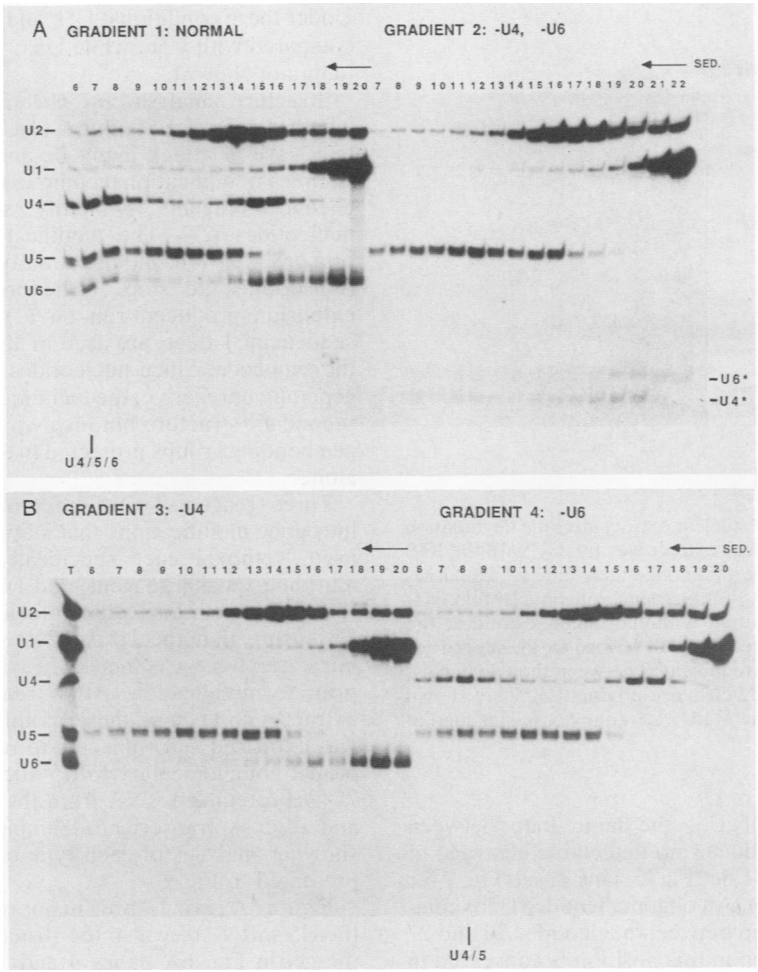


FIG. 2. Gradient analysis of extracts containing degraded U4 or U6 RNA. Extracts were treated with oligonucleotides and RNase H as described in Materials and Methods. The RNase H treatment was carried out in 125-μl reactions containing ATP and creatine phosphate. The extract in gradient 1 (panel A, left side) was treated with 10 μg of an oligomer which does not allow degradation of any RNA in the extract (oligonucleotide R5S; see reference 3). Gradient 2 (panel A, right side) contained extract treated with 5 μg each of oligonucleotides U4b2 (complementary to nucleotides 63 to 84 in U4) and U6b (complementary to U6 nucleotides 78 to 95). The extract in gradient 3 (panel B, left side) was treated with 6 μg of oligonucleotide U4b2. The extract in gradient 4 (panel B, right side) was incubated with 6 μg of oligonucleotide U6b. After incubation, the reactions were diluted with 125 μl of gradient dilution buffer and loaded on 12-ml 10 to 30% glycerol gradients, spun, and fractionated as described in the Materials and Methods. RNA from each fraction was run on a 10% denaturing polyacrylamide gel, blotted to a nylon membrane (Zetabind), and probed for U1, U2, U4, U5, and U6. Autoradiographs of such blots are shown. Fraction numbers and the direction of sedimentation are shown at the top. U2, U1, U4, U5, and U6 RNAs are indicated at the left and the RNase H cleavage products of U4 and U6 (U4* and U6*) are indicated at the right of panel A.

amount of endogenous U4/5/6 snRNP (compare Fig. 1 and 8B); in some cases it was possible to find virtually all of U5 in the larger complex. In all cases, however, ATP promoted assembly, and treatment with hexokinase resulted in disassembly.

U5 is relatively resistant to cleavage by RNase H. Our initial approach for identifying sequences available for base pairing in the U5 snRNP was through the use of oligonucleotide-directed RNase H cleavage. This had proven effective for locating accessible sequences in the U1, U2, U4, and U6 snRNPs (1, 3, 4, 17, 19, 36). A set of DNA oligonucleotides complementary to various regions distributed over the length of U5 RNA were tested, and their effects on the U5 RNA in its RNP form or as naked RNA are listed in Table 1. Each oligomer was incubated in a HeLa cell nuclear extract in the presence of RNase H. After extraction from the reactions, RNA was run on a gel and was stained with ethidium bromide to analyze the extent of cleavage of U5 RNA in the snRNP particle (Fig. 5). Under conditions whereby a U1 complementary oligomer (Fig. 5, lane 2) allows virtually complete digestion of U1 RNA in its snRNP, most of the U5 complementary oligonucleotides had no effect on the U5 snRNP (lanes 3, 4, 8, and 9). Only oligonucleotides U5d1, U5d2, and U5e (lanes 5, 6, and 7), complementary to positions 68 to 79 in the U5 sequence, targeted U5 in the snRNP for RNase H digestion. Of these oligomers, U5e, which has the longest complementarity to U5, gave the most efficient, but still incomplete, cleavage (lane 7). Note that oligomers U5d1 and U5d2 differ at position 12 (G in U5d1 and A in U5d2) and are designed to pair with the two forms of the reported sequence heterogeneity (C or U) in HeLa cell U5 RNA at position 68 (21). Although in this experiment U5d2 seems to have been more effective than U5d1 was, they usually produce about the same extent of cleavage.

Surprisingly, the oligonucleotide complementary to the

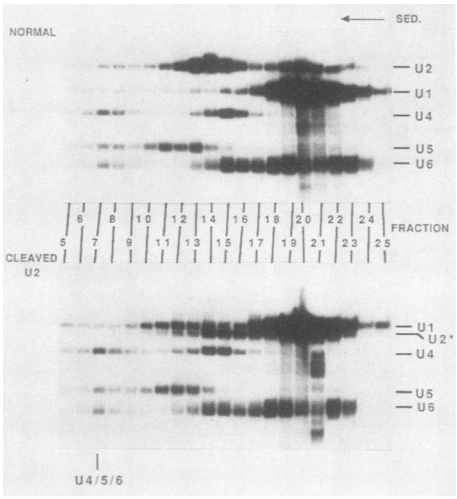


FIG. 3. Gradient analysis of nuclear extract after the degradation of U2 RNA. As in Fig. 2, nuclear extract was treated with the R5S oligonucleotide (top panel) or with an oligonucleotide which allows RNase H digestion of U2 RNA (bottom panel; oligonucleotide L15; see reference 3). After incubation with the oligonucleotides, the extracts and gradients were treated as described in the legend for Fig. 2. The gradient fractions are indicated between the two panels, and the small RNAs and the U2 cleavage product (U2*) are shown at the right. The position of the U4/U5/U6 complex is indicated at the bottom.

most conserved feature of U5, the large loop between nucleotides 36 and 46, produced no detectable cleavage of U5 in the snRNP (oligomer U5c, Fig. 5, lane 4; see Fig. 7 for the U5 secondary structure). An oligonucleotide (U5b) complementary to the bulge loop between nucleotides 20 and 27 was also tested; a bulge loop in this position is conserved in all known U5 species, whereas its sequence is not. As before, the U5b oligonucleotide produced no detectable cleavage of U5 in its RNP form (data not shown).

The oligonucleotides which did not target cleavage of U5 in the RNP were also tested with deproteinized U5 RNA. Under these conditions, U5b and U5f showed slight cleavage compared with U5e, while U5a, U5c, and U5g had no effect (data not shown).

Structure analysis by chemical modification. To test whether conserved features of U5 RNA that are insensitive to RNase H attack might be accessible to other probes, a chemical modification technique was used. This method uses chemical reagents to modify, selectively, single-stranded nucleotides (29). The modifications are then mapped by primer extension: modification of a base will cause reverse transcriptase to stop at the preceding base. The primer extension products, run on a gel adjacent to a dideoxy sequencing ladder, are used to align the modified bases with the sequence. Since nucleotides bound by protein are also generally unreactive, the technique probes not only the RNA secondary structure but also which bases have their hydrogen bonding groups protected in an RNP relative to the RNA alone.

Three reagents with different base specificities which introduce modifications that stop reverse transcriptase were used. Kethoxal reacts specifically with guanosine; CMCT is a uridine-specific reagent; and DMS modifies both A and C residues at positions which block reverse transcription (29). To ensure that the U5 snRNP was all in monomer form, nuclear extract was incubated with hexokinase and glucose prior to modification. After treating samples of a nuclear extract with each of these modification reagents, the RNA was extracted and subjected to reverse transcription from a primer complementary to U5 nucleotides 84 to 104 (see Fig. 7). Deproteinized RNA from the extract was also modified and reverse transcribed. Sample autoradiographs of gels showing analyses of each type of modification reaction are presented in Fig. 6.

Figure 6A reveals three major regions of DMS reactivity in the U5 snRNP (see also the structures in Fig. 7). Reading up the gel in Fig. 6A (lanes 4 and 7), the sequences between nucleotides C73 and A69, A44 and C36, and A29 and A19 show additional reverse transcriptase stops after modification. These correspond closely to the regions in U5 predicted to be single stranded in secondary structures derived from phylogenetic data or in nuclease sensitivity analyses of

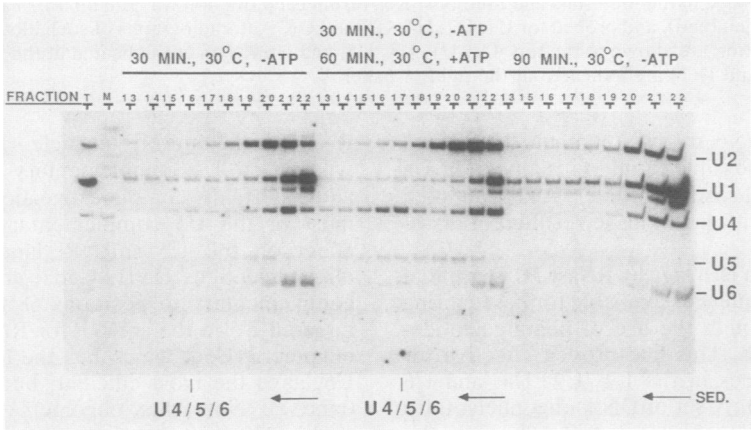


FIG. 4. Effect of ATP on formation of U4/U5/U6. Reaction mixtures containing 90 μl of nuclear extract and 2.2 mM $MgCl_2$ in a 138-μl volume were incubated at 30°C for 30 min. After 30 min, reaction 2 (center gradient) was brought to 0.5 mM ATP and 20 mM creatine phosphate in a 150-μl reaction and was incubated at 30°C for another 60 min. Reactions 1 and 3 were brought to 150 μl with distilled water. Reaction 1 (left side) was frozen until ready for loading on the gradient. Reaction 3 (right side) was incubated for another 60 min at 30°C. After incubation, the reactions were diluted with 150 μl of gradient dilution buffer, run on gradients, and assayed as described in the Materials and Methods and in the legend for Fig. 2. (Note that these gradients were run shorter than the previous ones; hence, the 30S marker was in fractions 13 and 14 and U4/5/6 was in fractions 16 and 17.)

TABLE 1. Oligonucleotides used in the RNase H cleavage experiments[a]

Oligonucleotide	Length in nucleotides	Sequence	Complementary nucleotides in U5	Digests RNA in	
				RNP	RNA
U5a	12	AAACCAGAGTAT	1-12	−	−
U5b	12	TTATGCGATCTG	18-29	−	(+)
U5c	19	CTTTAGTAAAAGGCGAAAG	32-50	−	−
U5d1	15	GACTCAGAGTTGTTC	65-79	+	NT
U5d2	15	GACTCAGAGTTATTC	65-79	+	NT
U5e	21	TTGGGTTAAGACTCAGAGTTG	68-88	+	+
U5f	12	TTGGGTTAAGAT	79-88	−	(+)
U5g	12	TTGCCAAGGCAA	100-111	−	−

[a] Oligonucleotides were tested for the ability to direct RNase H cleavage of U5 RNA in nuclear extracts where it is packaged as an RNP or cleavage of naked U5 RNA after deproteinization with phenol. −, No detectable cleavage; +, >50% cleavage; (+), <20% cleavage; NT, oligomer not tested under those conditions.

naked U5 RNA (6, 21, 32). The reactivity of nucleotides 69 to 73 was expected, since this region is susceptible to RNase H cleavage in both the RNP and the RNA. However, the sequence in the large loop, which was not cleaved by RNase H, is strongly modified by DMS (at A44, C39, C38, and C36) in both the RNA and RNP. (The reading of the C modifications is complicated by a strong reverse transcriptase stop and band compression at G37 in all lanes; nevertheless, one can read the C39 and C38 bands just below the strong stop and the C36 band just above it.) Although the modifications between A19 and A29 are better resolved on a gel which has been run longer (not shown), modifications at A19, A21, and C23 can be clearly seen in both the RNP and the RNA reactions (lanes 3, 4, 6, and 7), modification at A28 can be seen in the RNA lanes, and modification at A29 can be seen in the RNP lanes.

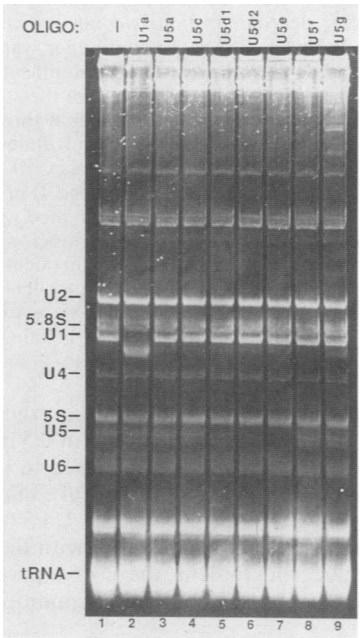


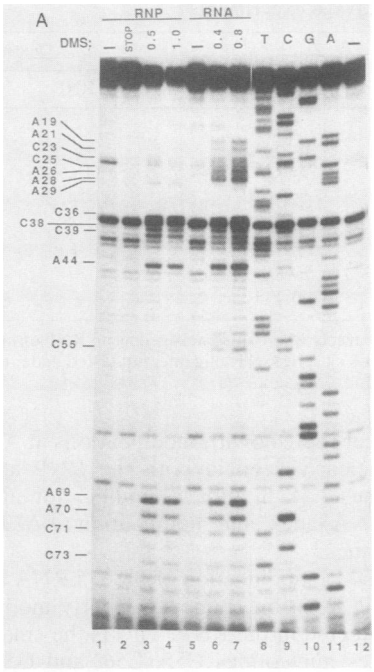
FIG. 5. RNase H cleavage of U5 RNA in nuclear extract. Lanes 1 through 9 each show the RNA from one RNase H reaction. Each 25-μl reaction contained 15 μl of nuclear extract, 1 μg of oligonucleotide, 0.5 mM ATP, 20 mM creatine phosphate, 2.2 mM $MgCl_2$, and 1 U of RNase H. After incubation for 1 h at 30°C, each reaction was phenol extracted and the RNA was ethanol precipitated and run on a 10% polyacrylamide denaturing gel. The gel was stained with ethidium bromide to visualize the RNA. The RNAs in the extract are indicated at the left, and the oligonucleotides in each reaction are indicated along the top. The extract in lane 1 contained no oligonucleotide. The extract in lane 2 contained an oligomer complementary to the first 12 nucleotides of U1 snRNA. The extracts in lanes 3 through 9 were each treated with an oligonucleotide complementary to a specific region in U5 RNA, as listed in Table 1.

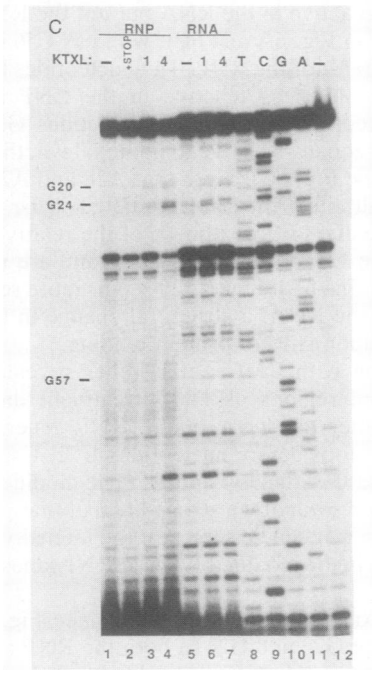
When the DMS modification of the U5 RNA and RNP are compared (Fig. 6A), one region is clearly modifiable in the RNA but protected in the RNP. This is the small bulge loop which includes nucleotides C55, C56, and G57. Both C55 and G57 are reactive in the RNA but not the RNP (compare lanes 4 and 7 in Fig. 6A and 6C), whereas C56 is reactive in neither the RNA nor the RNP. The nucleotides between A19 and A28 are also less reactive in the RNP and may be partially protected by protein. Nucleotides G20 and G24, however, are equally reactive to kethoxal whether in RNP or RNA form (see Fig. 6C). Note that A28 and A29 both react with DMS, although they are predicted to be base paired. This could be due to "breathing" of the relatively A/U-rich helix; yet, the U's on the opposite strand are not so easily modified (see Fig. 6B). Since A29 seems more reactive in the RNP than in the RNA, a structural change in the U5 RNA could occur upon snRNP protein binding.

Modification with CMCT at uridines revealed the same single-stranded regions as DMS treatment did (Fig. 6B). Modifying either the RNA or the RNP generated reverse transcriptase stops before nucleotides U72, U43, U42, U41, U40, U27, and U22 (lanes 4 and 7). (The modification at U40 is obscured somewhat by a strong band at that position in all lanes. However, it can be seen that the intensity of this band does increase in the modified RNA or RNP lanes [lanes 4 and 7].)

The kethoxal reaction with guanosine (Fig. 6C) shows modification of G20 and G24 in both the RNP and the RNA lanes. Similar to C55, G57 is modifiable only in the naked RNA. Unfortunately, any reactivity of G37 is completely obscured by the strong reverse transcriptase stop in that region in all lanes. Other gels, which were run longer to obtain better resolution toward the 5′ end of the RNA, showed that G8 is modifiable in both the RNP and the RNA.

Figure 7 summarizes the modification data for the U5 RNA and the RNP. The DMS and kethoxal modifications were also performed on gradient-purified U5 RNP monomer, yielding identical results to those obtained with nuclear extract (data not shown). Normal reverse transcriptase stops prevented measuring the modification of A1, U2, or U7 with any certainty. Unfortunately, we were also unable to obtain efficient extension of any of five different primers complementary to the stem loop region at the 3′ end of the RNA. This is apparently due to the extreme stability of the 3′ stem





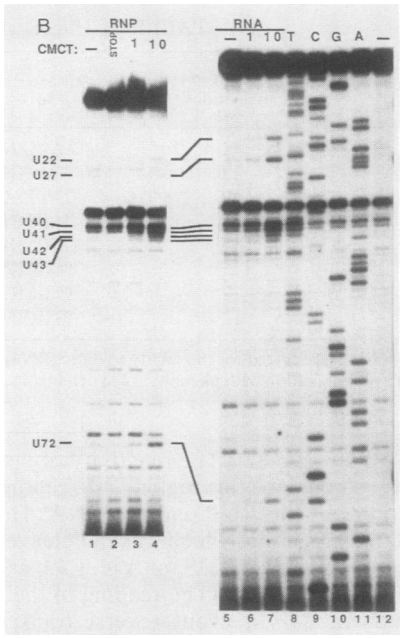


FIG. 6. Chemical modification of the U5 snRNP and the U5 snRNA. (A) DMS modification. Lanes 3 and 4 and lanes 6 and 7 show modification of the U5 RNP and RNA, respectively. Indicated at the top is the amount in microliters of DMS stock solution (5% DMS in dioxane) added to a 25-μl sample of nuclear extract or total extract RNA. Samples in lanes 1 and 5 received no DMS. To control for the possibility that DMS modification of the RNP reactions might be occurring after deproteinization, we ran the control in lane 2 marked STOP. In this reaction, the stop buffer was added prior to the incubation with DMS rather than after. Modifications in this lane would indicate that some DMS could survive the stop reaction and modify the RNA after phenolization. Lanes 8 through 11 show a dideoxy sequencing ladder of U5 RNA. Nucleotides showing modification by DMS are indicated at the left. (B) CMCT modification. Samples (25 μl) of nuclear extract (lanes 3 and 4) or nuclear extract RNA in borate buffer (lanes 6 and 7) were treated with 1 μl (lanes 3 and 6) or 10 μl (lanes 4 and 7) of CMCT stock solution prior to reverse transcription. All other lanes are equivalent to those in 6A. (C) Kethoxal modification. All lanes are equivalent to those in 6A except that the extract and RNA samples were treated with 1 μl (lanes 3 and 6) or 4 μl (lanes 4 and 7) of kethoxal stock solution.

loop, strongly disfavoring the formation of an intermolecular hybrid with the oligonucleotide. It should be noted that this portion of U5 RNA constitutes the "A domain," responsible for Sm antigen binding and common to all Sm snRNPs (5, 23). It is hence unlikely to execute any U5-specific function.

The DMS and kethoxal modification reactions were then performed on an extract that had been pretreated with 1 mM ATP to stimulate assembly of the U4/5/6 snRNP complex. In this particular extract, virtually all U5 was assembled into the U4/5/6 snRNP complex (Fig. 8B). Unfortunately, it was not possible to assess modification by CMCT because the U4/5/6 complex was not stable to dialysis into borate buffer; the U5 in the borate-dialyzed extract sedimented with the same mobility as the monomer in undialyzed extracts did. Interestingly, no significant differences in U5 modification in the U4/5/6 snRNP complex, as compared to its monomeric form, were observed (Fig. 8A). To ensure that modification was not promoting disassembly of the U4/5/6 snRNP complex, a sample of extract was treated with dioxane (to 6%) and run on a glycerol gradient; the U4/5/6 particle remained intact under these mock-modification conditions.

DISCUSSION

We report the results of a series of experiments analyzing the structure and interactions of the U5 snRNP. In characterizing the interactions between U5 and other snRNPs, we first looked for the reported U4/5/6 snRNP complex (10, 15, 16, 24). We observed a pool of U5 in our nuclear extracts which cofractionates with U4 and U6 on glycerol gradients. That this cofractionation is due to an association between these three snRNPs is strongly supported by the finding that cleavage of U4 and U6 by RNase H eliminates the faster-running U5 peak (Fig. 2).

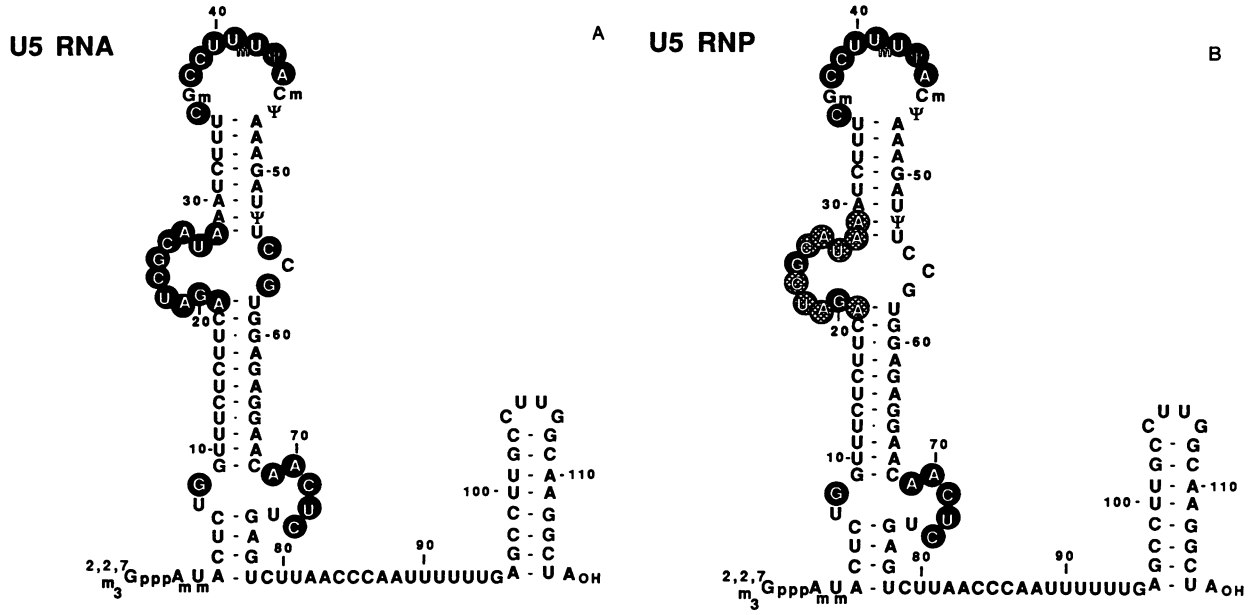


FIG. 7. Summary of chemical modification data. The structure shown is from Krol and Ebel (20). Chemically modifiable bases are indicated by black circles. Crosshatched circles indicate nucleotides whose reactivity is lower in the RNP than in the RNA. The reactivity of nucleotides 5′ to position 7 or 3′ to position 78 was not determined. Modification of naked U5 RNA is shown in panel A. Modification of the U5 snRNP in nuclear extracts is shown in panel B. Psi indicates a pseudouridine residue. N_m indicates a 2′-*O*-methyl nucleotide.

Interestingly, we observe that the appearance of the HeLa U4/5/6 tri-snRNP complex is dependent on the presence of ATP. Reducing the endogenous ATP concentration in the extract by 80% with hexokinase and glucose eliminates the complex. Conversely, the addition of ATP to the extract allows the de novo formation of U4/5/6 particles (Fig. 4). This dependence on ATP is similar to that reported by Lossky et al. (24), who showed that incubation of yeast extracts with ATP allowed the coprecipitation of yeast U4 and U6 (snR14 and snR6) with yeast U5 (snR7) by using an antibody directed against the rna8 protein. However, ATP-induced assembly of the U4/U5/U6 complex is in direct contrast to results in which gel electrophoresis is used to separate snRNP complexes. In both yeast and HeLa cell extracts, a U4/U5/U6 snRNP complex detected in native gels is broken down upon incubation with ATP (10, 16). Our results cannot be ascribed to a difference in the buffers used for gradient versus gel fractionation; after incubation with ATP, we observed equivalent amounts of the U4/U5/U6 snRNP complex in both HEPES (pH 7.9) (Fig. 4 and 8) and Tris glycine (pH 8.8) (the buffer used by Konarska and Sharp [16]) gradients. It also appears that the relative amounts of free U5 snRNP versus U4/5/6 snRNP in the initial extracts do not alter the ATP dependence, since we observed similar results in extracts with greatly different endogenous levels of the U4/5/6 snRNP complex (Fig. 4 versus Fig. 8B).

The relative positions of the slowest-migrating forms of snRNPs containing U1, U2, U4, U5, and U6 RNAs in the gradients also provide information on the nature of the snRNP monoparticles. In gradient analyses of affinity-purified snRNPs, U5 ran as the lightest of the four major snRNPs (U1, U2, U4/U6, and U5) (7), whereas in our gradients, U5 was the heaviest. It is tempting to speculate that this higher sedimentation velocity is due to the binding of the HeLa cell analog of the yeast rna8 protein. This protein has been shown to bind specifically to yeast U5 (snR7) and is large enough (260 kilodaltons) to add significantly to its S value (24). A HeLa cell analog of the yeast rna8 protein has been identified as a component of U5 snRNP and is similarly large (~200 kilodaltons; A. Pinto, unpublished results). The sedimentation pattern of U6 is also interesting in that there is free U6 but no free U4 sedimenting at positions above the U4/6 snRNP complex. Assuming that U4/U6 is a 1:1 dimer, this suggests a significant excess of U6 over U4 in these extracts (perhaps threefold) (see Fig. 3). Note also that there seems to be two forms of U6 (peaking in fractions 19 and 22 in Fig. 3), suggesting the binding of U6-specific proteins.

In analyzing the structure of the U5 snRNP, we first used RNase H cleavage in an attempt to identify U5 RNA sequences capable of engaging in base-pairing interactions with other RNA molecules. However, this strategy was much less successful than when it was previously applied to the U1, U2, and U4/U6 snRNPs. The most conserved sequence in U5 is in the large loop between nucleotides 36 and 46. It occurs in all known U5 RNAs from yeasts to humans (6, 20, 32), yet it is not cleavable by RNase H in either the RNP or the naked RNA. U5 also contains two bulge loops whose positions in the secondary structure, but not their sequences, are conserved in other species (6, 20, 32). One of these loops, between nucleotides 69 to 74, can be cleaved by RNase H in the RNP or deproteinized RNA, indicating that it can hybridize with a complementary nucleic acid molecule. The U5d1, U5d2, and U5e oligonucleotides, which allow cleavage of this sequence in U5, could be potentially useful for inactivating U5 in splicing extracts. Unfortunately, cleavage with these oligomers so far has not been sufficiently complete (about 80%; Fig. 5) to carry out such experiments successfully.

The inaccessibility of the U5 sequence from nucleotides 36 to 46 to RNase H cleavage was troubling since nearly all other sequences in the abundant U RNAs which are so highly conserved are cleavable at least in the RNA form (1, 3, 4, 17, 19, 36; D. Black, unpublished observations). One

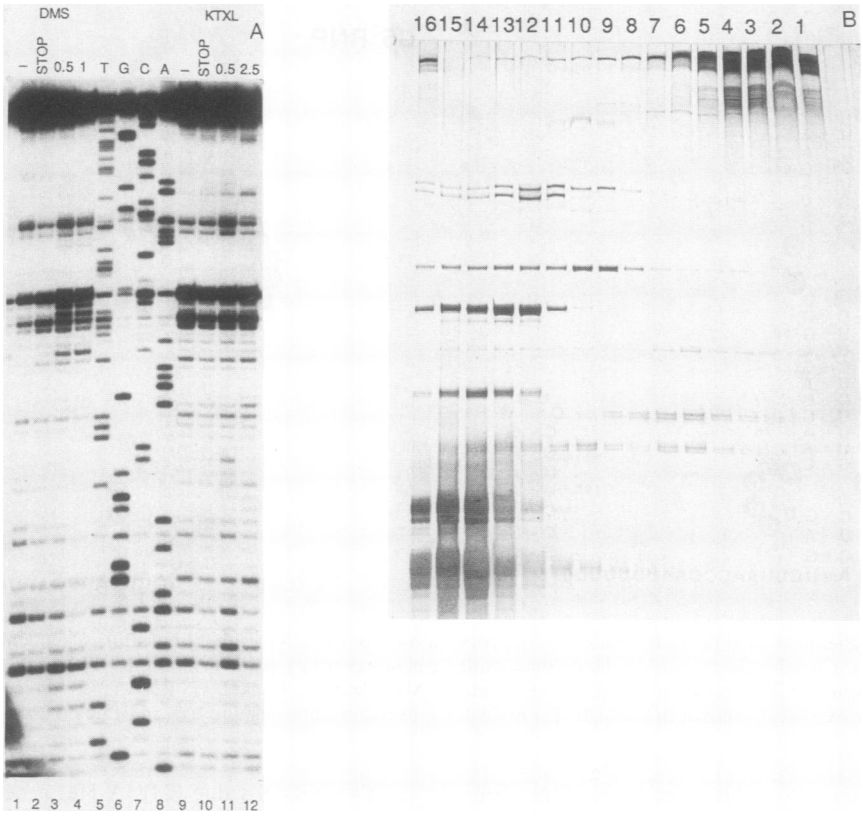


FIG. 8. Chemical modification of the U5 snRNP in the U4/5/6 complex. A 460-μl reaction containing 185 μl of nuclear extract was preincubated for 30 min under splicing conditions (3), with the exception that the ATP concentration was raised to 1 mM. The sample was then used either for modification or for a modification control gradient. (A) Lanes 1 through 4 and 8 through 12 show modification of the U5 RNP within the U4/5/6 complex with DMS and kethoxal, respectively. Indicated at the top is the amount in microliters of modifying agent (8% DMS in dioxane or 75 mg of kethoxal per ml in dioxane) added to a 25-μl sample of nuclear extract. Samples in lanes 1 and 9 received no modifying agent, as indicated. Modification was for 15 min at room temperature. Lanes marked STOP are as described in the legend to Fig. 6. Lanes 5 through 8 show a dideoxy sequencing ladder of U5 RNA. (B) A 125-μl sample of the initial nuclear extract was mixed with 8 μl of dioxane to give a final concentration of 6% dioxane, a higher value than in any of the modification reactions. The sample was then diluted 1:1 with gradient dilution buffer, spun, and fractionated as described in Materials and Methods. RNA from each fraction was run on a 10% denaturing polyacrylamide gel and silver stained. The bottom of the gradient is fraction 1. Note that the direction of sedimentation is from left to right, opposite to that shown in previous figures.

possibility is that this sequence hybridizes with the oligonucleotide in such a way that cleavage by the enzyme is somehow precluded. The block to cleavage cannot be due to bound proteins or the assembly into the U4/5/6 snRNP complex because it also occurs in phenol-extracted U5 RNA. Another possibility is that the lack of cleavage is due to the four consecutive U residues in the middle of the loop. Deoxy A:ribo U base pairs are extremely unstable (9, 26). Hence, even if the sequence in the loop is available for base pairing, stable heteroduplex formation enabling RNase H cleavage may not occur.

To look more closely at the accessibility of this sequence, we tested the reactivity of individual bases in U5 to various chemical reagents. We observed that nucleotides in the conserved loop were extremely reactive but were surprised to find no differences in the U5 snRNP and the U4/5/6 multi-snRNP complex. These results argue that intermolecular base pairing may not be required for assembly of the U5 snRNP into the U4/5/6 snRNP complex, although it is possible that U40 to U43 engage in interactions within this complex, as we were unable to determine their reactivity to CMCT in the U4/5/6 complex. The accessibility to modification of the large U5 loop even within the U4/5/6 snRNP complex suggests that it may interact with some other component of the spliceosome. Since there are no sequences in U1 or U2 snRNAs which exhibit extensive complementarity to the conserved U5 loop region, the sequence may represent a protein recognition sequence. Protein-binding sites are frequently those sequences that have been highly conserved in both primary sequence and secondary structure (31).

It is interesting to speculate that the U4/U5/U6 tri-snRNP complex is an early intermediate in spliceosome assembly and that it binds to other components as a single unit, perhaps using the conserved loop sequence. Kinetic studies of snRNP binding to the pre-mRNA suggest that these snRNPs bind simultaneously and interact with the U1 and U2 snRNPs (2, 10, 15, 22). Subsequently, there appears to be a change in the interaction of U4 with the rest of the spliceosome: loss of U4 snRNA has been observed in gel analyses of spliceosome complexes (10, 22, 33). Analysis of the complex interactions between the snRNPs in these larger multi-snRNP particles will require both their isolation in purer form and further analyses of the structures of the individual snRNPs.

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