MOLECULAR AND CELLULAR BIOLOGY, Aug. 1989, p. 3360–3368
0270-7306/89/083360-09$02.00/0

Vol. 9, No. 8

U1 Small Nuclear Ribonucleoprotein Particle-Specific Proteins Interact with the First and Second Stem-Loops of U1 RNA, with the A Protein Binding Directly to the RNA Independently of the 70K and Sm Proteins

JEFFREY R. PATTON,[1,2] WINAND HABETS,[3] WALTHER J. VAN VENROOIJ,[3] AND THORU PEDERSON[1]*

Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545[1]; Department of Pathology, University of South Carolina School of Medicine, Columbia, South Carolina 29208[2]; and Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands[3]

Received 16 December 1988/Accepted 10 May 1989

The U1 small nuclear ribonucleoprotein particle (U1 snRNP), a cofactor in pre-mRNA splicing, contains three proteins, termed 70K, A, and C, that are not present in the other spliceosome-associated snRNPs. We studied the binding of the A and C proteins to U1 RNA, using a U1 snRNP reconstitution system and an antibody-induced nuclease protection technique. Antibodies that reacted with the A and C proteins induced nuclease protection of the first two stem-loops of U1 RNA in reconstituted U1 snRNP. Detailed analysis of the antibody-induced nuclease protection patterns indicated the existence of relatively long-range protein-protein interactions in the U1 snRNP, with the 5′ end of U1 RNA and its associated specific proteins interacting with proteins bound to the Sm domain near the 3′ end. UV cross-linking experiments in conjunction with an A-protein-specific antibody demonstrated that the A protein bound directly to the U1 RNA rather than assembling in the U1 snRNP exclusively via protein-protein interactions. This conclusion was supported by additional experiments revealing that the A protein could bind to U1 RNA in the absence of bound 70K and Sm core proteins.

The first mRNA splicing cofactor identified was the U1 small nuclear ribonucleoprotein particle (U1 snRNP). The U1 snRNP binds to the 5′ splice site (25) and is required for the first step of splicing, cleavage at the 5′ end of the intron (4, 14). Since U1 RNA alone has no apparent specificity for the 5′ splice site (25), it is likely that the U1 snRNP is the active cofactor in splicing.

The U1 snRNP is a complex of at least nine proteins, termed 70K, A, B′, B, C, D, E, F, and G, bound to U1 RNA (reference 5 and references cited therein). Several of these proteins make up the so-called Sm core and are also constituents of the U2, U5, and U4/U6 snRNPs, but the 70K, A, and C proteins are associated only with U1 RNA (3, 5, 11, 13, 28, 35). It is therefore very likely that the 70K, A, and C proteins are related to the unique function of the U1 snRNP.

Knowing the structure of the U1 snRNP will be important in understanding how this cofactor functions in mRNA splicing. Toward this objective, we have developed a system for the in vitro assembly of U1 snRNP, using SP6-transcribed human U1 RNA added to a HeLa cell S100 fraction (26). The in vitro-assembled U1 snRNP is very similar to native U1 snRNP (26) and has been used to map the binding site of the 70K protein to the first stem-loop of U1 RNA (27). Other investigators, using *Xenopus* oocyte S100 fractions, have found that the first stem-loop of U1 RNA is necessary and sufficient for binding of the 70K, A, and C proteins (9, 10).

Human autoantibodies and mouse monoclonal antibodies (1, 2, 16, 17) have been used extensively to study the protein composition, assembly, and functions of the snRNPs (4, 20–23, 26–28, 35, 36). In this investigation, we used an antibody-mediated nuclease protection technique, previously used to identify the binding site of the 70K protein (27), to show that the site of interaction of the other two U1-specific proteins, A and C, includes not only stem-loop I but the entire second stem-loop as well. We also demonstrate that the A protein can be UV cross-linked to U1 RNA, establishing that it makes direct contact with the RNA, and we show that the A protein can bind to U1 RNA independently of bound 70K or Sm core protein.

MATERIALS AND METHODS

Assembly of U1 snRNP. Transcription of the human U1 RNA gene clone pHU1, assembly of U1 snRNP in a HeLa S100 fraction, and glycerol gradient purification were carried out as previously described (26, 27). U1 RNA was labeled with [α-^{32}P]GTP (4 mCi/ml) when the particles were used for RNA-protein mapping or with [α-^{32}P]GTP and UTP (each at 4 mCi/ml) for the UV cross-linking experiments.

Nuclease digestion of U1 snRNP in the presence of snRNP antibodies. Glycerol gradient-purified particles in 50 mM NH_4Cl–20 mM Tris hydrochloride (pH 7.5)–3 mM $MgCl_2$–5 mM 2-mercaptoethanol–50 μM phenylmethylsulfonyl fluoride (buffer A) were incubated with antibodies or nonimmune immunoglobulin G (IgG) at 4°C for 30 min and then digested with micrococcal nuclease (5,000 U/ml) at 37°C for 30 min in the presence of 1 mM $CaCl_2$ (27). Protein A-Sepharose was used to select the antigen-antibody complexes (37), and the RNA was isolated from the bound fractions. Control samples preincubated with no antibody were nuclease digested, and the RNA was isolated directly, omitting the protein A-Sepharose step.

Sequence analysis of protected RNA. RNA from antibody-bound, nuclease-digested U1 snRNP was electrophoresed on 10% polyacrylamide–8.3 M urea denaturing gels, and the bands were visualized by autoradiography. The bands were

* Corresponding author.

eluted from the gel and digested with RNase T_1 (20 U/ml) in 50 mM sodium acetate–2 mM EDTA for 1 h at 50°C. The reactions were extracted with phenol-$CHCl_3$ (1:1); after addition of calf liver tRNA (10 μg/ml) as carrier, the RNA was precipitated in 67% ethanol overnight at −20°C. The RNA was electrophoresed on 20% polyacrylamide–8.3 M urea gels. The prominent bands visualized by autoradiography were eluted from the gel and digested with RNase T_2 (20 U/ml) in 50 mM sodium acetate (pH 5.0)–1 mM EDTA–10 mM 2-mercaptoethanol at 50°C for 30 min. The reaction volumes were reduced to 5 μl by evaporation and spotted on thin-layer chromatography plates (cellulose; no. 13254; Eastman Kodak Co.). The solvent system was isobutyric acid-concentrated NH_4OH–H_2O (66:1:33, vol/vol/vol) (30).

RNA-protein cross-linking of U1 snRNP. U1 snRNPs were assembled in vitro, purified on glycerol gradients, dialyzed against buffer A, and concentrated fivefold in Centricon 10 miniconcentrators (Amicon Corp.). The particles were then irradiated (254 nm, 4,000 μW/cm^2) for 15 min at 4°C (24). The cross-linked samples were digested with micrococcal nuclease (400 U/ml) and RNase A (40 μg/ml) in buffer A containing 1 mM $CaCl_2$ and then reacted with antibodies that had been prebound to protein A-Sepharose. In other experiments, the particles were first selected with Sm antibody and then digested with nucleases after elution from protein A-Sepharose.

Proteins were precipitated with 9 volumes of acetone containing 50 mM HCl for 12 to 20 h at −20°C. The pellets were washed once with ethanol before preparation for electrophoresis on 15% polyacrylamide-sodium dodecyl sulfate gels (15). Sm antibody-selected, [^{35}S]methionine-labeled snRNPs were also run on the gel as markers.

snRNP antibody selection experiments. Antibodies were prebound to protein A-Sepharose, and the beads were washed thoroughly with 50 mM NaCl–10 mM Tris (pH 8.5)–1 mM $MgCl_2$. After incubation of the antibody–protein A-Sepharose with glycerol gradient-purified U1 snRNP, the antigen-antibody complexes were recovered as described previously (37), and the antibody-bound RNA was isolated and analyzed by gel electrophoresis.

Antibodies. Sm monoclonal antibody was obtained from the Y12 hybridoma line (16), kindly provided by Charles Janeway (Yale University School of Medicine); RNP monoclonal antibody (2) was obtained from hybridomas kindly provided by Sallie Hoch (Agouron Institute). Human autoantibodies B152, D18, H65, and O19 were obtained from human autoimmune patients in Nijmegen. HeLa cell nuclear extract proteins (8) were visualized by electrophoresis on a 13% polyacrylamide gel, and immunoblotting was performed as previously described (7).

RESULTS

We have previously shown that a U1 snRNP assembles in a HeLa S100 fraction and is very similar to native U1 snRNP with regard to buoyant density, sedimentation coefficient, nuclease sensitivity, Mg^{2+} dependence of conformation (29), and reactivity with Sm and RNP antibodies (26). These two antibodies have specificities for different snRNP proteins; Sm recognizes the B′, B, and D proteins (28), whereas RNP recognizes the U1 snRNP-specific 70K protein (2). In this investigation, we used human autoantibodies reacting with the A or C protein of the U1 snRNP. Antibody B152 reacted strongly with the A protein, moderately with C, and very weakly with B′ and B (Fig. 1A). D18 reacted with A, B′, and B and weakly with C and the 70K protein. H65 and O19 reacted strongly and specifically with the A protein. All four antibodies reacted with the reconstituted U1 snRNP (data not shown). Because we intended to use these antibodies in an immunoprecipitation assay of native proteins, we did not wish to rely on immunoblotting data alone. The reactivities of the antibodies were therefore evaluated by immunoprecipitation of [^{35}S]methionine-labeled native proteins translated in vitro. The mRNA for these translation reactions was synthesized in vitro with SP6 RNA polymerase from previously described cDNAs containing the complete coding sequence for the A (32) or C (31) protein. Sera B152, D18, and H65 contained antibodies against both the A and C proteins, whereas the O19 antibody was specific for the A protein (Fig. 1B).

Antibody-mediated nuclease protection. We recently discovered a phenomenon termed antibody-mediated nuclease protection and have exploited this finding to map the location of particular proteins in the U1 snRNP (27). The experimental strategy is as follows. If U1 snRNP is digested with micrococcal nuclease in the absence of an antibody or in the presence of nonimmune IgG, only the 26-nt Sm domain of U1 RNA is resistant to digestion (18, 26, 27; Fig. 2, lane 5). If U1 snRNP is incubated with Sm antibody before digestion, the same Sm domain region is nuclease resistant; i.e., binding of this antibody does not induce any additional nuclease-resistant regions (27). However, when certain other antibodies are allowed to bind to the U1 snRNP (for example, RNP monoclonal antibody [27]), additional regions of U1 RNA become protected against nuclease digestion. These U1 RNA fragments can then be recovered after nuclease digestion by virtue of the bound antibody, using protein A-Sepharose. The selected RNA is then subjected to sequence analysis.

Incubation of U1 snRNP with the A- and C-protein autoantibodies D18 (Fig. 2, lane 1) and B152 (lane 3) induced nuclease protection of several regions of the U1 RNA not observed when the particles were digested without antibody (lane 5). Neither autoantibody induced protection with protein-free U1 RNA (lanes 2 and 4). Since the immunoblotting and immunoselection data (Fig. 1) suggested that B152 and D18 may have very similar epitope reactivities, we also used antiserum H65, which was almost completely specific for the A protein in immunoblots (Fig. 1A) and therefore probably contained antibodies against a different epitope(s) on the A protein than did the B152 and D18 antisera. H65 induced a pattern of nuclease-protected fragments (Fig. 3, lane 3) very similar to that obtained with B152 (lane 4). Two of the antibody-bound protected fragments obtained with H65 (b and c, lane 3) were the same length as the major bound protected fragments (R1 and R2) observed with the RNP monoclonal antibody (Fig. 3, lane 2) (27), but, in addition, there was a major fragment >70 nt long (a in lane 3). Given its extreme specificity for the A protein (Fig. 1B), we were disappointed to find that antiserum O19 did not induce nuclease protection (data not shown).

Identification of nuclease-protected fragments. Fragments a to l in Fig. 3 were eluted and digested with RNase T_1, and the products were electrophoresed in 20% polyacrylamide gels. Figures 4A and B show the T_1 products from each of the fragments obtained with antibodies H65 and B152, respectively. The U1 RNA used for the initial U1 snRNP assembly was labeled with [α-^{32}P]GTP; therefore, each T_1 oligonucleotide (oligo), except those ending with a G adjacent to another 3′-ward G (fragments 1 and 4 in Fig. 5), contained only one ^{32}P. As a result, these two T_1 oligos were more

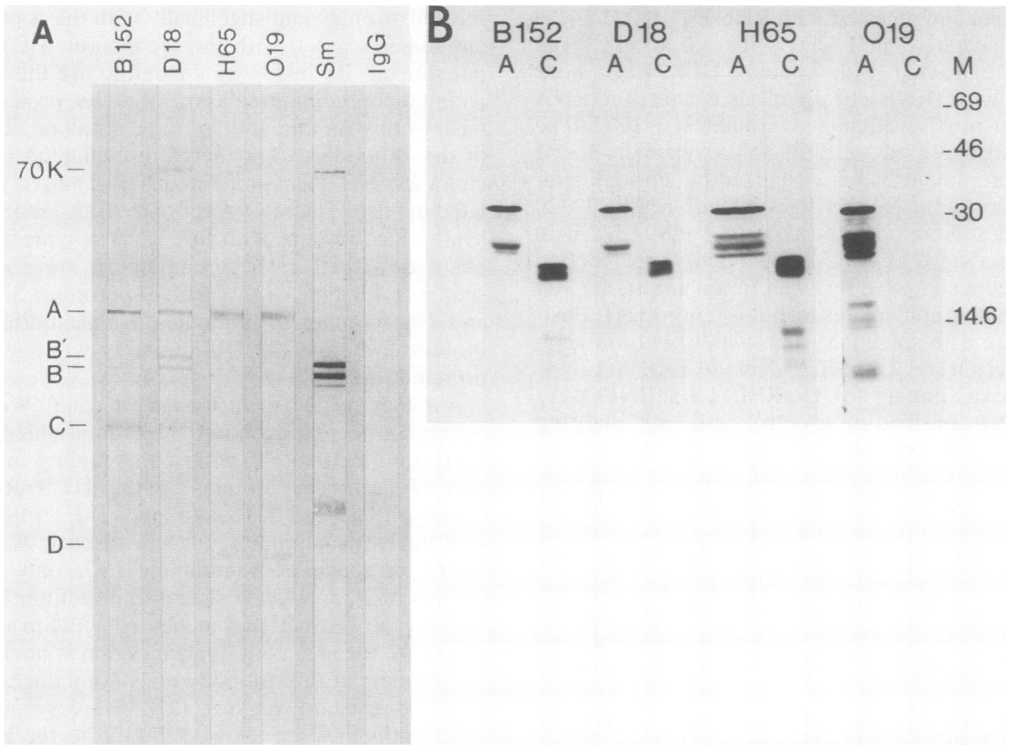

FIG. 1. (A) Immunoblot analysis of antibodies. HeLa cell nuclear extract proteins (8) were subjected to electrophoresis and immunoblotting as previously described (7), using the primary antibodies indicated. The Sm antibody was from a systemic lupus erythematosus patient. (B) Immunoselection of pure A and C proteins. SP6 transcripts of full-length A- or C-protein cDNA clones (31, 32) were translated in a wheat germ extract for 90 min at 25°C in the presence of [^{35}S]methionine. The translation products were then incubated with the antiserum indicated, antigenic complexes were recovered on protein A-Sepharose, and the ^{35}S-labeled proteins were analyzed by electrophoresis and fluorography. M, Molecular weight markers (in thousands).

intense on the autoradiogram, which facilitated their identification.

The nature of the U1 RNA primary nucleotide sequence and the absence of any short repetitive elements in this small RNA made it possible to assign, with considerable certainty, the protected fragments to particular U1 RNA sequences on the basis of the T_1 data alone (Fig. 4). However, we nonetheless confirmed the sequence identity of most of the T_1 oligos by secondary RNase T_2 digestion (Table 1). For illustration, consider fragment a, which yielded T_1 oligos that were 11, 9, 8, 7, and 6 nt long (Fig. 4A). The 9-nt T_1 oligo was more intense than the 11-, 8-, and 6-mers and must therefore have contained more than one labeled nucleotide. RNase T_2 digestion of the 9-mer revealed a labeled A and G (Table 1), allowing assignment of this 9-mer as T_1 oligo 4 (Fig. 5). The 7-nt T_1 oligo derived from fragment a (Fig. 4A) yielded, upon RNase T_2 digestion, equimolar ^{32}P-labeled U, C, and G, indicating that it must be a mixture of the two T_1 7-mers designated oligos 6 and 7 in Fig. 5. (T_1 oligo 9, also a 7-mer, is ruled out because it would have yielded twofold more labeled C.) The 11-mer from fragment a (Fig. 4A) is a unique T_1 oligo in U1 RNA and could thus be unambiguously assigned as fragment 5 in Fig. 5. T_2 digestion of the 8- and 6-nt T_1 oligos from fragment a (Fig. 4A) yielded only labeled U and C, respectively (Table 1), confirming assignment of these nucleotides as T_1 oligos 2 and 3 (Fig. 5). Therefore, fragment a must span at least nt 22 to 92 but could extend slightly farther on either end. However, since fragment a did not contain T_1 oligo 1 or 8, it follows that neither the extreme 5′ end of U1 RNA nor stem-loop III was present.

Data from the RNase T_1 and T_2 analyses are summarized in Table 1 and shown schematically in Fig. 6. It can be seen that antibodies H65 and B152 both induced protection of a region of U1 RNA that contained the first and all of the second stem-loops (fragments a and f). This is in contrast to finding with the RNP monoclonal antibody, which does not induce protection beyond nt 65 (27).

Although the H65 antibody had no apparent reactivity with the B′, B, or D proteins (Fig. 1), it nevertheless induced protection of sequences adjacent to the Sm core region (fragments c and d in Fig. 6). This is evidence for protein-protein interactions between the A and/or C proteins and the Sm core proteins, as was shown previously for the 70K protein (9, 27).

Identification of proteins cross-linked to U1 RNA. The antibody-induced nuclease protection data obtained previously with the RNP antibody (27) and with A- and C-protein antibodies (this report) indicate that the three U1 snRNP-specific proteins, 70K, A, and C, interact with regions of stem-loop I or II. To further define the structure of the U1 snRNP, we undertook UV-mediated RNA-protein cross-linking experiments. After UV irradiation and nuclease digestion, cross-linked ^{32}P-labeled nucleotides can be used to visualize, by electrophoresis and autoradiography, proteins that are in direct contact with the RNA.

Irradiation of ^{32}P-labeled U1 snRNP led to cross-linking of nucleotides to a set of several proteins (compare lane 2 in Fig. 7 with the nonirradiated control in lane 1). When cross-linked particles were reacted with Sm antibody, a set of proteins with a very prominent band at a molecular weight of ~11,000 (lane 3), which was probably the snRNP F protein (38), was selected. When the cross-linked particles

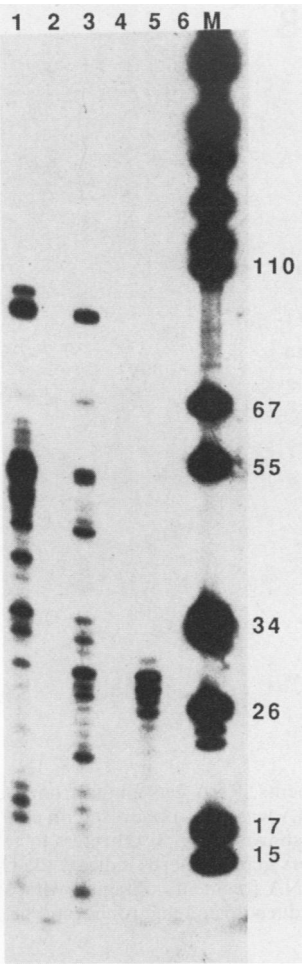

FIG. 2. Dependence of antibody-induced nuclease protection of U1 snRNP on bound proteins. Glycerol gradient-purified, in vitro-assembled U1 snRNP (lanes 1, 3, and 5) or deproteinized RNA isolated from these particles (lanes 2, 4, and 6) was incubated with antibody and then digested with micrococcal nuclease as described in Materials and Methods. The protein A-Sepharose-bound RNA was isolated and electrophoresed on an 11% polyacrylamide–7 M urea gel. Lanes: 1 and 2, D18 antibody; 3 and 4, B152 antibody; 5 and 6, no preincubation with antibody and no protein A-Sepharose selection; M, ^{32}P-end-labeled *Msp*I fragments from pGEM-1 plasmid DNA. Lengths (in nucleotides) are shown on the right.

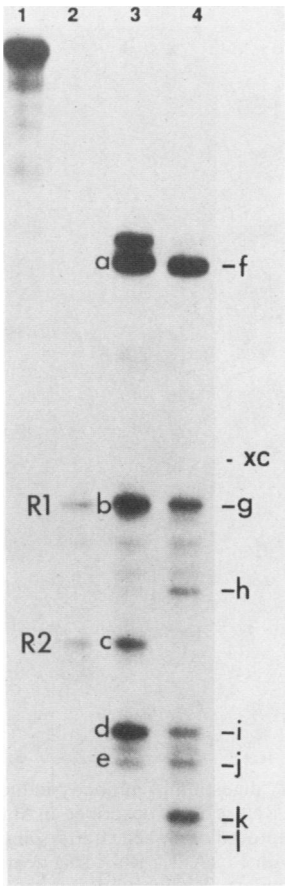

FIG. 3. Induction of comparable levels of nuclease protection by an antibody recognizing different A-protein epitopes. Glycerol gradient-purified U1 snRNP particles were incubated with RNP, H65, or B152 antibody before nuclease digestion. Protein A-Sepharose-bound RNA was electrophoresed on a 10% polyacrylamide gel containing 8.3 M urea. Lanes: 1, RNA from undigested U1 snRNP; 2, RNP monoclonal antibody; 3, H65 antibody; 4, B152 antibody. R1 and R2 denote the two major fragments obtained with the RNP monoclonal antibody (27). The letters a to e and f to l indicate RNA fragments obtained with H65 and B152, respectively, that were recovered for sequence analysis. xc, Position of xylene cyanol dye (55 nt).

were nuclease digested before reaction with the Sm antibody, a single protein with a molecular weight of approximately 21,000 was selected (lane 4). This may have been the snRNP D protein, with its slower migration relative to that of the non-cross-linked D protein (lane 9) reflecting the presence of a cross-linked nucleotide(s). Two antibodies that reacted with the A protein selected, after nuclease digestion, a ^{32}P-labeled protein with a molecular weight of approximately 34,000 (lanes 5 and 6), which was not observed with the Sm and RNP antibodies (lanes 4 and 7) or with nonimmune IgG (lane 8). The proteins in lanes 5 and 6 migrated somewhat more slowly than did non-cross-linked A protein (lane 9), again presumably reflecting the effect of cross-linked nucleotides. The lower intensity of the A protein than of the proteins cross-linked in lanes 3 and 4 likely reflects fewer cross-linked nucleotides and differences in the efficiencies of the reactions of the antibodies with free A protein as opposed to intact U1 snRNP.

Binding of the A protein to U1 RNA in the absence of 70K and Sm protein binding. The fact that the A protein could be UV cross-linked to U1 RNA (Fig. 7) shows that it is in direct contact with RNA in the U1 snRNP. We next inquired whether this protein can bind to U1 RNA in the absence of other U1 snRNP proteins. We considered it probable that the individual U1 snRNP proteins have different affinities for U1 RNA. Accordingly, we reasoned that it should be possible to assemble U1 snRNPs deficient in certain proteins by using more dilute extracts than in our standard U1 snRNP assembly system (26). This prediction proved to be correct.

Figure 8 shows the results of antibody selection experiments after U1 RNA was incubated in increasingly dilute HeLa S100 extracts. Binding of both Sm proteins and the 70K protein (RNP antibody) decreased as the extract was progressively diluted (Fig. 8, lanes 1 and 2 at each dilution). In contrast, binding of the A protein to U1 RNA was much less sensitive to extract dilution (lane 3 at each dilution). These results are given quantitatively in Table 2. At a

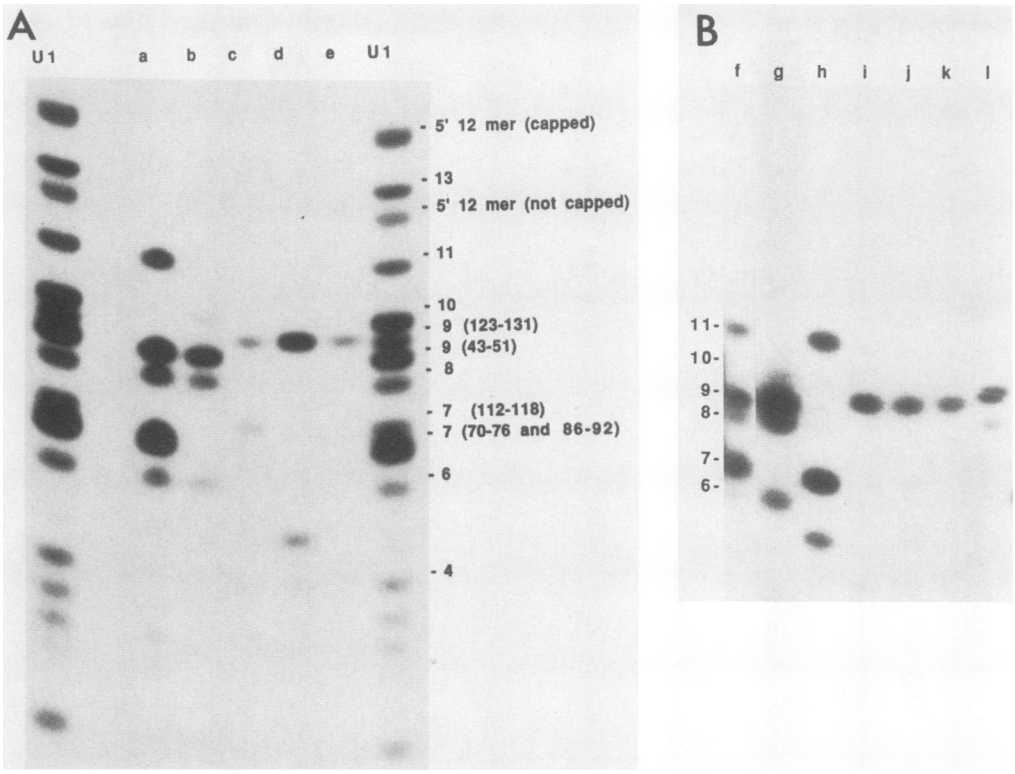

FIG. 4. RNase T_1 digestion of antibody-induced, nuclease-protected U1 snRNP fragments. RNA was eluted from the gel shown in Fig. 3 and digested with RNase T_1 as described in Materials and Methods. The reextracted RNA was electrophoresed on a 20% polyacrylamide–8.3 M urea gel and autoradiographed. Letters above the lanes refer to the RNA fragments shown in Fig. 3; U1 refers to gel-eluted, full-length U1 RNA digested with T_1. (A) U1 RNA fragments obtained with antibody H65. The numbers in parentheses indicate the U1 RNA nucleotides determined by secondary T_2 digestions for the indicated T_1 oligonucleotides. (B) U1 RNA fragments obtained with antibody B152. The numbers in the margins indicate the lengths (in nucleotides) of the RNA fragments as deduced from electrophoretic mobility and, in some cases, as confirmed by secondary RNase T_2 digestion (Table 1).

dilution of 1:120, binding of the Sm and 70K proteins was reduced two- and fivefold, respectively, whereas binding of the A protein was not appreciably reduced. At a 1:600 dilution, Sm and 70K protein binding was reduced 4- and 10-fold, respectively, whereas binding to the A protein was reduced less than 2-fold. These results established that the A protein can bind U1 RNA in the absence of bound Sm or 70K protein.

DISCUSSION

Our previous demonstration of the location of the 70K protein (27) and the results presented here on the A and C proteins reveal that all three of the U1-specific proteins are clustered on the 5′ end of the U1 RNA molecule, covering stem-loops I and II (Fig. 9). We have also established by UV cross-linking that the A protein interacts directly with U1

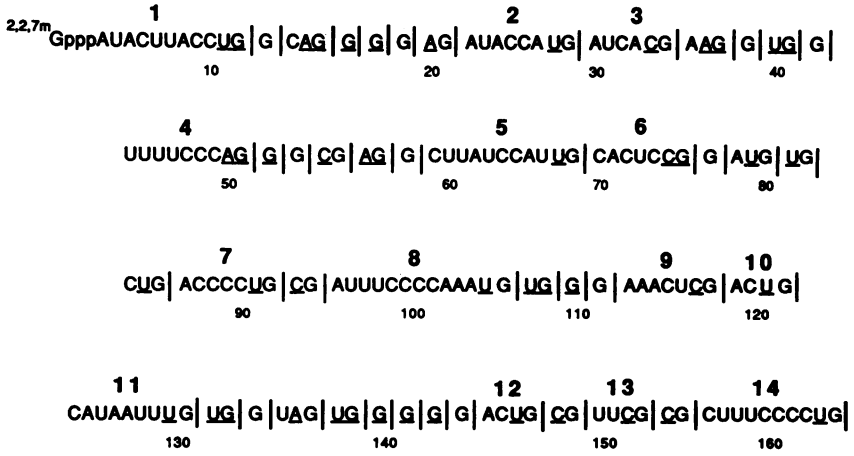

FIG. 5. Predicted RNase T_1 cleavage sites (|) in U1 RNA. Since [α-^{32}P]GTP was used during transcription of the U1 RNA, only residues 5′ to a G (underlined) will contain label after RNase T_1 digestion.

TABLE 1. Analysis of RNase T_1 data

Band on antibody protection gel[a]	Size (nt) of T_1 oligos[b]	Labeled nt upon RNase T_2 digestion	Assignment of T_1 oligos[c]
a	11	U	5
	9	A, G	4
	8	U	2
	7	U, C, G	6
	7	U, C, G	3
	6	C	3
b	9	A, G	4
	8	U	2
	6	nd[d]	3
c	9	U	11
	7	nd	9
	4	nd	10
d	9	U	11
	4	nd	10
e	9	U	11
	4	nd	10
f	11	U	5
	9	A, G	4
	8	U	2
	7	U, C, G	6
	7	U, C, G	7
	6	C	3
g	9	A, G	4
	8	nd	2
	6	C	3
h	11	U	5
	7	G, C	6
i	9	U	11
	4	nd	10
j–l	9	U	11
	4	nd	10

[a] See Fig. 3. Bands d and i contained, in addition to the T_1 oligos shown, a fragment 5 nt long that resulted from micrococcal nuclease digestion, in the U1 snRNP, of the 5′ 2 nt from T_1 oligo 9.
[b] See Fig. 4.
[c] See Fig. 5.
[d] nd, Not determined.

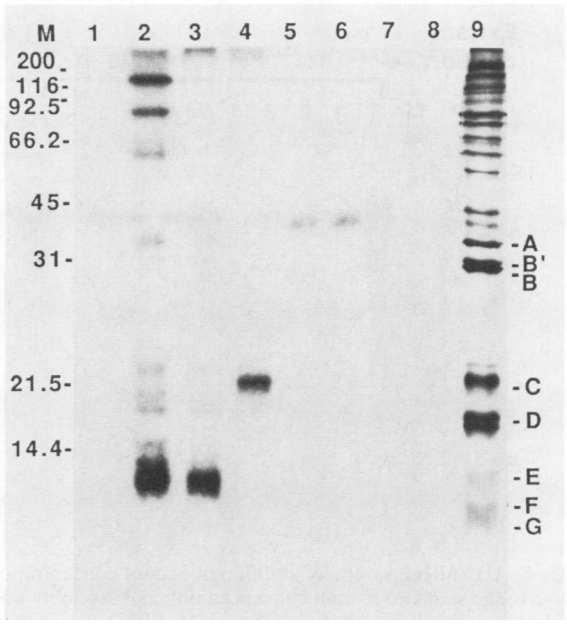

FIG. 7. UV cross-linking of snRNP proteins to U1 RNA. U1 snRNPs were isolated from glycerol gradients, dialyzed, and irradiated as described in Materials and Methods. After cross-linking, the RNA was digested with nucleases, and the proteins were recovered by acetone precipitation, electrophoresed on a 15% SDS-polyacrylamide gel, and autoradiographed. In lane 3, Sm antibody bound to protein A-Sepharose was used to select complexes before nuclease digestion. In lanes 4 to 8, the cross-linked particles were first nuclease digested, after which the proteins were selected with antibodies bound to protein A-Sepharose: lane 4, Sm; lane 5, O19; lane 6, H65; lane 7, RNP; lane 8, nonimmune human IgG. Other lanes: 1, non-cross-linked control; 2, total cross-linked proteins; 9, Sm antibody-selected proteins from [^{35}S]methionine-labeled HeLa nuclear extracts. The snRNP proteins are indicated by letters on the right. Positions of molecular weight markers (in thousands) are shown on the left.

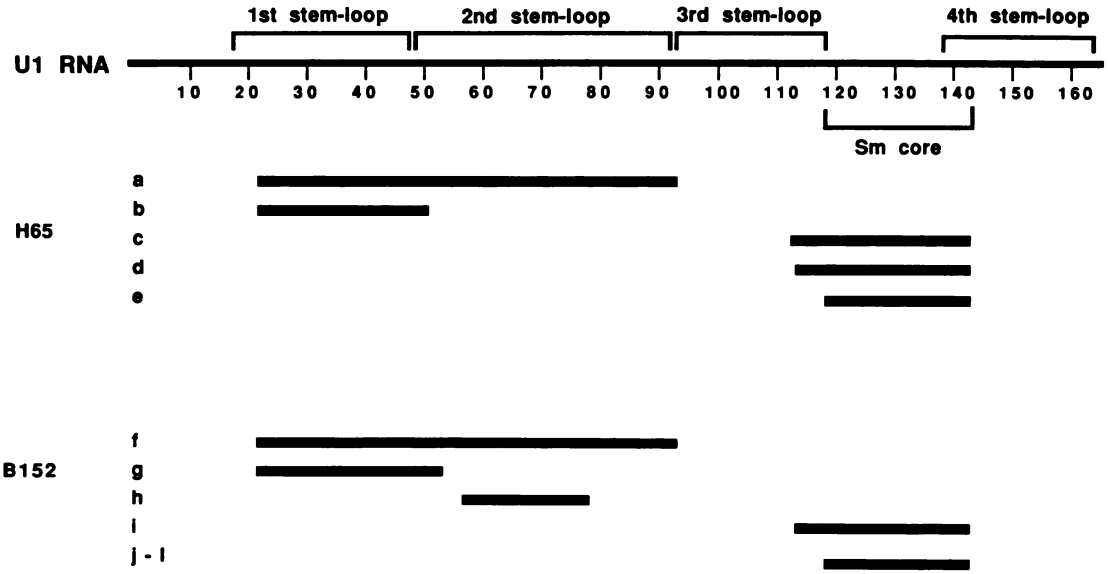

FIG. 6. Assignment of protected fragments to regions of U1 RNA. A linear diagram of U1 RNA with stem-loop regions and the Sm core (18, 19) is shown at the top. The horizontal bars below represent micrococcal nuclease-resistant fragments bound to the indicated antibody.

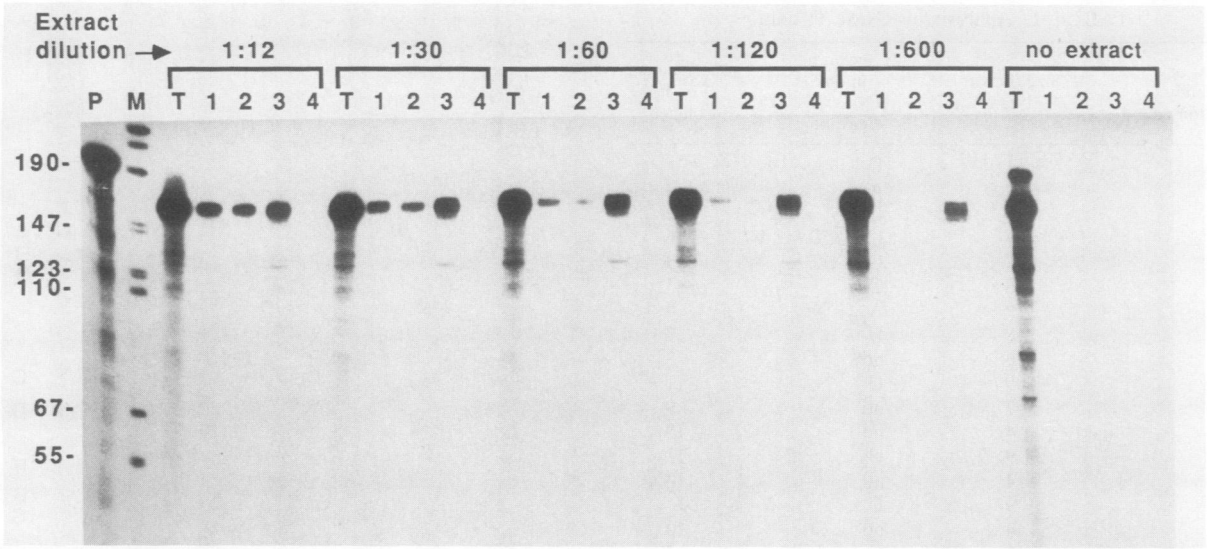

FIG. 8. U1 snRNP assembly at different extract concentrations. ^{32}P-labeled U1 RNA was incubated in extracts at the dilutions indicated (relative to the standard extract concentration), and assembly was allowed to proceed as usual (30 min, 37°C). Antibodies prebound to protein A-Sepharose were then added, and the selected RNA was isolated and electrophoresed on a 6% polyacrylamide–8.3 M urea gel. Lane P, U1 RNA used for assembly but not incubated in extracts (this U1 RNA transcript contains a 25-nt 3′ extension that is specifically removed by an oligodeoxynucleotide-RNase H cleavage step in the S100 assembly reactions [26]); lane T, total RNA from an equivalent portion of U1 snRNP; lane M, DNA size markers (lengths [in nucleotides] are shown on the left). Antibodies used: Sm (lane 1), RNP (lane 2), O19 (lane 3), and nonimmune human IgG (lane 4).

RNA. Moreover, U1 snRNP assembly experiments conducted at different protein concentrations demonstrated that the A protein can bind U1 RNA in the absence of bound 70K or Sm protein. These experiments extend recent studies (9, 10) indicating that stem-loop I is the only essential RNA sequence for binding of the U1 snRNP-specific proteins. Those experiments (9, 10) were obtained by mutating U1 RNA, an extremely conserved RNA, and defining the sequences necessary for the binding of U1 snRNP-specific proteins. Our experimental approach is different in that it addresses the U1 RNA sites that become bound by the U1-specific proteins once assembly has occurred. Thus, these two approaches yield complementary information on U1 snRNP structure; in broad terms, the results so far are quite congruent (9, 10, 27; this report). However, the temporal order of binding of the 70K, A, and C proteins remains unknown, as does the spatial relationships among these three proteins after the U1 snRNP has fully assembled. Since both the 70K protein (38) and the A protein (Fig. 7) can be cross-linked to U1 RNA, it is possible that they can each bind to U1 RNA independently of the other and of the C protein. It is interesting to note that the C protein lacks the RNP consensus sequence (31) that is present in both the 70K (33, 34) and A (32) proteins even though it has been suggested (10) that the C protein can bind U1 RNA directly. Finally, we note that neither the previous studies (9, 10, 27) nor those described here were designed to detect RNA-independent interactions among the 70K, A, and C proteins before binding to U1 RNA. Despite recent suggestions on the order of binding (10), it is our view that new experimental approaches will be required to clarify this important issue.

TABLE 2. Binding of the A protein to U1 RNA in the absence of Sm or 70K snRNP proteins

Extract dilution	% of U1 RNA bound to antibody			
	Sm	RNP	O19	IgG
1:12	11.1	9.8	21.6	0.2
1:30	8.2	6.5	20.7	0.1
1:60	5.3	3.5	21.4	0.1
1:120	4.9	2.0	19.3	0.1
1:600	2.4	1.1	13.3	0.7
No extract	0.0	0.2	0.9	1.2

The antibody-induced nuclease protection phenomenon is not well understood. It is possible that the antibody stabilizes a particular protein-RNA binding site so that it becomes more nuclease resistant than usual. Stabilization might arise from an antibody-induced reconfiguration of the RNA-bound protein (6), or it might be indirectly transferred via stabilization by the antibody of an existing protein-protein interaction on the RNA. It is intriguing that we have encountered several antibodies that bind efficiently to U1 snRNP but do not induce nuclease protection (unpublished results). This means that the mechanism of induced nuclease protection is more complicated than mere antibody binding causing the reacting protein to invariably bind the RNA more tightly. We also note that antibody-induced nuclease protection of U1 snRNP is observed not only with polyclonal antibodies (this report) but also with a monoclonal antibody (27). Therefore, the mechanism of this effect is not related to a multiple-antibody-binding phenomenon. Nor is it the case that all U1 snRNP-reactive polyclonal antibodies induce protection, since O19 does not (this report). We suspect that the phenomenon is related to the extent to which antibody binding to a particular epitope(s) induces a conformational change in that U1 snRNP protein, leading to a tighter, or broader, zone of nuclease protection.

Although the A and C proteins interact with stem-loops I and II, the H65 antibody also induced protection of U1 RNA sequences adjacent to the Sm domain (Fig. 3 and 9). We previously noted the same effect with the 70K monoclonal antibody (27). Independent evidence for interactions between the U1-specific proteins and the Sm core proteins has

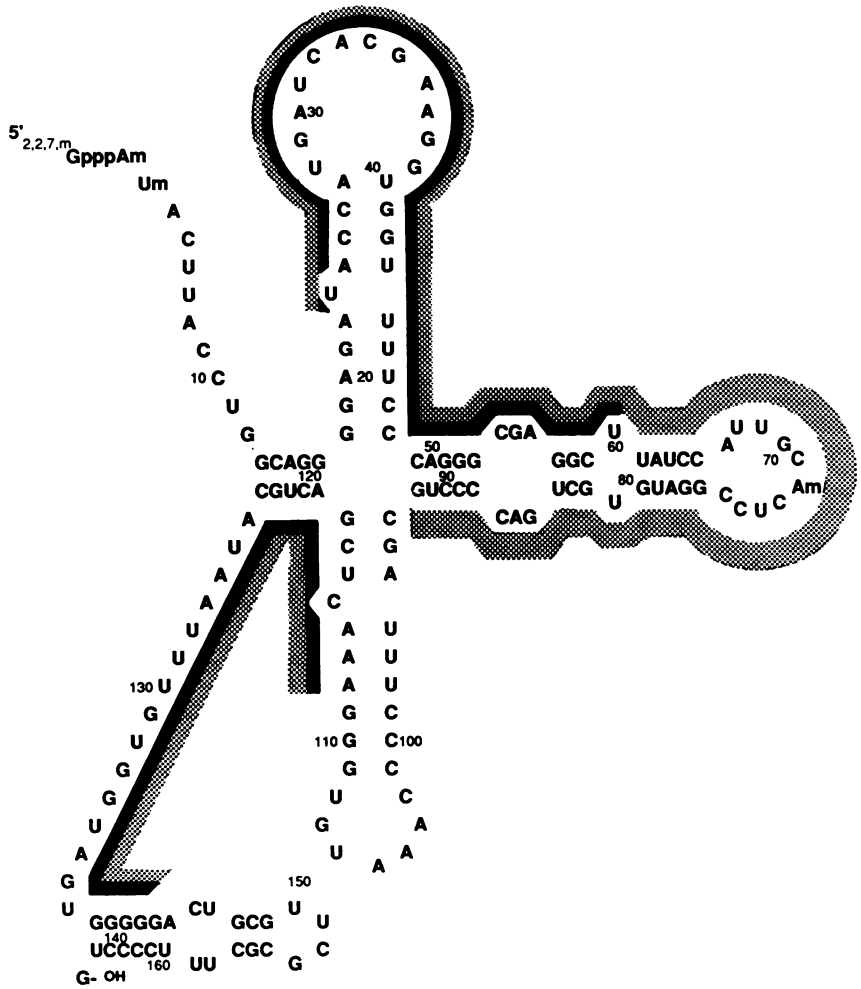

FIG. 9. Regions of U1 RNA protected from nuclease digestion in the presence of antibodies to snRNP proteins. Symbols: □, regions that become nuclease resistant after binding of antibodies that react with the A and C proteins; ■, regions over which nuclease protection is induced by the RNP monoclonal antibody (27).

been obtained (9). It is likely that these results are providing the first hints of the overall three-dimensional structure of the U1 snRNP. The fact that all three of the U1 snRNP-specific proteins are located on the 5′ end of U1 RNA suggests a functional cluster that may facilitate binding of U1 snRNP to the 5′ splice site in pre-mRNA. These results also indicate that a very substantial portion of the U1 snRNP mass, perhaps nearly half (70,000 + 32,000 [A protein] + 21,000 [C protein] = 123,000 daltons of an estimated total U1 snRNP mass of ~295,000 daltons), may lie quite close to the 5′ end of U1 RNA. This suggestion is strongly supported by a recent immunoelectron microscopy study of U1 snRNP, which indicates the presence of a large cluster of protein near the 5′ trimethylguanosine cap (12).

ACKNOWLEDGMENTS

J.R.P. and T.P. thank Ann Kleinschmidt, Gary Kunkel, John Maddalena, and Sandra Mayrand for advice and suggestions during the course of this work. We also thank Sandra Johnson and Susan Olszta for excellent secretarial assistance.

This work was supported by Public Health Service grant GM-21595-14 to T.P. and postdoctoral fellowship GM-11399 to J.R.P. from the National Institutes of Health.

LITERATURE CITED

1. **Agris, P. F., Y. Kikuchi, H. J. Gross, M. Takano, and G. C. Sharp.** 1984. Characterization of the autoimmune antigenic determinant for ribonucleoprotein (RNP) antibody. Immunol. Commun. **13**:137–149.
2. **Billings, P. B., R. W. Allen, F. C. Jensen, and S. O. Hoch.** 1982. Anti-RNP monoclonal antibodies derived from a mouse strain with lupus-like autoimmunity. J. Immunol. **128**:1176–1180.
3. **Billings, P. B., and S. O. Hoch.** 1984. Characterization of U small nuclear RNA-associated proteins. J. Biol. Chem. **259**: 12850–12856.
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. **Bringmann, P., and R. Luhrmann.** 1986. Purification of the individual snRNPs U1, U2, U5 and U4/U6 from HeLa cells and characterization of their protein constituents. EMBO J. **5**: 3509–3516.
6. **Davies, D. R., S. Sheriff, and E. A. Padlan.** 1988. Antibody-antigen complexes. J. Biol. Chem. **263**:10541–10544.
7. **Habets, W. J., J. H. den Brok, A. M. T. Boerbooms, L. B. A. van de Putte, and W. J. Van Venrooij.** 1983. Characterization of the SS-B (La) antigen in adenovirus-infected and uninfected cells. EMBO J. **2**:1625–1631.
8. **Habets, W. J., D. J. DeRooij, M. H. Salden, A. P. Verhagen, C. A. Van Eekelen, L. B. van de Putte, and W. J. van Venrooij.** 1983. Antibodies against distinct nuclear matrix proteins are characteristic for mixed connective tissue disease. Clin. Exp. Immunol. **54**:265–276.
9. **Hamm, J., M. Kazmaier, and I. W. Mattaj.** 1987. *In vitro* assembly of U1 snRNPs. EMBO J. **6**:3479–3485.

10. **Hamm, J., V. L. van Santen, R. A. Spritz, and I. W. Mattaj.** 1988. Loop I of U1 small nuclear RNA is the only essential RNA sequence for binding of specific U1 small nuclear ribonucleoprotein particle proteins. Mol. Cell. Biol. **8**:4787–4791.
11. **Hinterberger, M., I. Petterson, and J. A. Steitz.** 1983. Isolation of small ribonucleoproteins containing U1, U2, U4, U5 and U6 RNAs. J. Biol. Chem. **258**:2604–2613.
12. **Kastner, B., and R. Luhrmann.** 1989. Electron microscopy of U1 small nuclear ribonucleoprotein particles: shape of the particle and position of the 5′ RNA terminus. EMBO J. **8**: 277–286.
13. **Kinlaw, C. S., B. L. Robberson, and S. M. Berget.** 1983. Fractionation and characterization of human small nuclear ribonucleoproteins containing U1 and U2 RNAs. J. Biol. Chem. **258**:7181–7189.
14. **Kramer, A., W. Keller, B. Appel, and R. Luhrmann.** 1984. The 5′ terminus of the RNA moiety of U1 small nuclear ribonucleoprotein particles is required for the splicing of messenger RNA precursors. Cell **38**:299–307.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
16. **Lerner, E. A., M. R. Lerner, C. A. Janeway, and J. A. Steitz.** 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. Proc. Natl. Acad. Sci. USA **78**:2737–2741.
17. **Lerner, M. R., and J. A. Steitz.** 1979. Antibodies to small nuclear RNAs complexed with proteins are produced by patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. USA **76**:5495–5499.
18. **Liautard, J.-P., J. Sri-Widada, C. Brunel, and P. Jeanteur.** 1982. Structural organization of ribonucleoproteins containing small nuclear RNAs from HeLa cells. J. Mol. Biol. **162**:623–643.
19. **Lin, W.-L., and T. Pederson.** 1984. Ribonucleoprotein organization of eukaryotic RNA. XXXI. Structure of the U1 small nuclear ribonucleoprotein. J. Mol. Biol. **180**:947–960.
20. **Luhrmann, R.** 1988. snRNP proteins, p. 71–99. *In* M. L. Birnstiel (ed.), Small nuclear ribonucleoprotein particles. Springer-Verlag KG, Berlin.
21. **Mattaj, I. W.** 1986. Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. Cell **46**: 905–911.
22. **Mattaj, I. W.** 1988. UsnRNP assembly and transport, p. 100–114. *In* M. L. Birnstiel (ed.), Small nuclear ribonucleoprotein particles. Springer-Verlag KG, Berlin.
23. **Mattaj, I. W., and E. M. DeRobertis.** 1985. Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. Cell **40**:111–118.
24. **Mayrand, S., and T. Pederson.** 1981. Nuclear ribonucleoprotein particles probed in living cells. Proc. Natl. Acad. Sci. USA **78**:2208–2212.
25. **Mount, S. M., I. Pettersson, M. Hinterberger, A. Karmas, and J. A. Steitz.** 1983. The U1 small nuclear RNA-protein complex selectively binds a 5′ splice site in vitro. Cell **33**:509–518.
26. **Patton, J. R., R. J. Patterson, and T. Pederson.** 1987. Reconstitution of the U1 small nuclear ribonucleoprotein particle. Mol. Cell. Biol. **7**:4030–4037.
27. **Patton, J. R., and T. Pederson.** 1988. The M_r 70,000 protein of the U1 small nuclear ribonucleoprotein particle binds to the 5′ stem-loop of U1 RNA and interacts with Sm domain proteins. Proc. Natl. Acad. Sci. USA **85**:747–751.
28. **Pettersson, I., M. Hinterberger, T. Mimori, E. Gottlieb, and J. A. Steitz.** 1984. The structure of mammalian small nuclear ribonucleoproteins. J. Biol. Chem. **259**:5907–5914.
29. **Reveillaud, I., M.-N. Lelay-Taha, J. Sri-Widada, C. Brunel, and P. Jeanteur.** 1984. Mg^{2+} induces a sharp and reversible transition in U1 and U2 small nuclear ribonucleoprotein configurations. Mol. Cell. Biol. **4**:1890–1899.
30. **Silberklang, M., A. M. Gillum, and U. L. RajBhandary.** 1979. Use of *in vitro* ^{32}P labeling in the sequence analysis of nonradioactive tRNAs. Methods Enzymol. **59**:59–109.
31. **Sillekens, P. T. G., R. P. Beijer, W. J. Habets, and W. J. van Venrooij.** 1988. Human U1 snRNP-specific C protein: complete cDNA and protein sequence and identification of a multigene family in mammals. Nucleic Acids Res. **16**:8307–8321.
32. **Sillekens, P. T. G., W. Habets, R. P. Beijer, and W. J. van Venrooij.** 1987. cDNA cloning of the human U1 snRNA-associated A-protein: extensive homology between U1 and U2 snRNP-specific proteins. EMBO J. **6**:3841–3848.
33. **Spritz, R. A., K. Strunk, C. S. Surowy, S. O. Hoch, D. E. Barton, and U. Francke.** 1987. The human U1-70K snRNP protein: cDNA cloning, chromosomal localization, expression, alternative splicing and RNA-binding. Nucleic Acids Res. **15**: 10373–10391.
34. **Theissen, H., M. Etzerodt, R. Reuter, C. Schneider, F. Lottspeich, P. Agros, R. Luhrmann, and L. Philipson.** 1986. Cloning of the human cDNA for the U1 snRNA-associated 70K protein. EMBO J. **5**:3209–3217.
35. **Wieben, E. D., S. J. Madore, and T. Pederson.** 1983. Protein binding sites are conserved in U1 small nuclear RNA from insects to mammals. Proc. Natl. Acad. Sci. USA **80**:1217–1220.
36. **Wieben, E. D., S. J. Madore, and T. Pederson.** 1983. U1 small nuclear ribonucleoprotein studied by in vitro assembly. J. Cell Biol. **96**:1751–1755.
37. **Wieben, E. D., and T. Pederson.** 1982. Small nuclear ribonucleoproteins of *Drosophila*: identification of U1 RNA-associated proteins and their behavior during heat shock. Mol. Cell. Biol. **2**:914–920.
38. **Woppman, A., J. Rinke, and R. Luhrmann.** 1988. Direct crosslinking of snRNP proteins F and 70K to snRNAs by ultra-violet radiation *in situ*. Nucleic Acids Res. **16**:10985–11004.