U1 Small Nuclear Ribonucleoprotein Studied by In Vitro Assembly

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ABSTRACT The small nuclear RNAs are known to be complexed with proteins in the cell (snRNP). To learn more about these proteins, we developed an in vitro system for studying their interactions with individual small nuclear RNA species. Translation of HeLa cell poly(A)$^+$ mRNA in an exogenous message-dependent reticulocyte lysate results in the synthesis of snRNP proteins. Addition of human small nuclear RNA U1 to the translation products leads to the formation of a U1 RNA-protein complex that is recognized by a human autoimmune antibody specific for U1 snRNP. This antibody does not react with free U1 RNA. Moreover, addition of a 10- to 20-fold molar excess of transfer RNA instead of U1 RNA does not lead to the formation of an antibody-recognized RNP. The proteins forming the specific complex with U1 RNA correspond to the A, B_1, and B_2 species (32,000, 27,000, and 26,000 mol wt, respectively) observed in previous studies with U1 snRNP obtained by antibody-precipitation of nuclear extracts. The availability of this in vitro system now permits, for the first time, direct analysis of snRNA-protein binding interactions and, in addition, provides useful information on the mRNAs for snRNP proteins.

The small nuclear RNAs (snRNAs) are stable, abundant RNA species present in a wide spectrum of eucaryotic organisms from insects to humans (15). There is evidence that at least one snRNA, U1, may be involved in some aspect of RNA processing, perhaps mRNA splicing. This hypothesis is supported by the facts that a sequence near the 5′ end of U1 RNA is potentially complementary to splice junctions (1, 8, 10, 16), that U1 RNA is hydrogen-bonded to heterogeneous nuclear RNA in vivo (3), and that adenovirus mRNA splicing is inhibited when isolated nuclei are incubated with an antibody to U1 RNA-containing ribonucleoprotein particles (22).

The small nuclear RNAs are complexed with proteins in the cell (13, 18). It has recently been discovered that patients with autoimmune diseases such as systemic lupus erythematosus and mixed connective tissue disease often produce circulating antibodies against snRNA-protein complexes, or snRNPs (5, 9). One class of such antibodies, termed anti-Sm, precipitates snRNP complexes containing the five major, nonnucleolar snRNAs: U1, U2, U4, U5, and U6. Other patients produce antibodies, termed anti-RNP, which are essentially monospecific for U1 snRNP (9, 20, 21).

It is likely that these snRNA-protein complexes represent the functional forms of the snRNAs in vivo. The proteins may be enzymes important in RNA processing, as in the case of RNAse P, an *Escherichia coli* tRNA processing enzyme that is known to be a ribonucleoprotein complex (7). Alternatively, snRNP proteins may serve primarily a structural role, perhaps analogous to ribosomal proteins, which form a surface conducive to short RNA base-pairing interactions (codon:anticodon). It seems likely that understanding the function(s) of snRNPs will depend, at least in part, on defining the proteins that are bound to each of the individual snRNA species. In this paper we present a new approach to this problem. Cell-free translation of snRNP proteins and the use of autoimmune antibodies are combined to examine specific protein binding sites on snRNAs in vitro.

MATERIALS AND METHODS

Purification of snRNP and snRNAs: The isolation and analysis of snRNPs from log phase HeLa cell nuclei using IgG isolated from the sera of patients with autoimmune disease has been described previously in detail (20). snRNAs were recovered from antibody-selected snRNPs by phenol extraction and, where necessary, were further purified by electrophoresis in 10% polyacrylamide–7 M urea gels containing 50 mM Tris-borate, pH 8.3. Individual snRNA bands were visualized by brief staining with ethidium bromide, excised from the gel, and recovered by electroelution.

snRNA-protein Binding In Vitro: mRNA was prepared from HeLa cell cytoplasm by the guanidine-HCl-cesium chloride technique as described in detail previously (19) and was fractionated on oligo(dT)-cellulose. The mRNA-dependent rabbit reticulocyte lysate was prepared according to Pelham and Jackson (12) and was supplemented with 50 μg/ml calf thymus tRNA

(Boehringer Mannheim Biochemicals, Indianapolis, IN).

For snRNA-protein binding in vitro, 3.5–5.0 μg of polyadenylated RNA were added to 115 μl of the mRNA-dependent reticulocyte lysate. After 1 h at 30°C, 10–200 ng of purified snRNA or 1 μg of tRNA was added to the lysate, and the sample was incubated for an additional hour at 30°C. The translation-binding reactions were then chilled to 4°C, 1 M Tris-HCl, pH 8.5, was added to a final concentration of 10 mM, and 25 μg of the appropriate antibody or nonimmune IgG was added. After 30 min at 4°C, protein A-Sepharose was added, then washed with 0.15 M NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, and the antibody-antigen complexes were eluted with 0.1 M glycine-HCl, pH 3.0, as described in detail previously (20).

RESULTS

Antibody Specificities

The properties of human autoimmune antibodies reactive for nuclear RNA antigens, including snRNPs, have been described (5, 9, 20). The snRNP specificities of the particular antibodies used in this investigation are shown in Fig. 1. HeLa cells were labeled with [^{3}H]uridine or [^{35}S]methionine, and nuclear extracts were reacted with either anti-RNP or anti-Sm antibodies. Lanes *2* and *3* in Fig. 1 show the RNA species present in the RNP selected by anti-RNP and anti-Sm, respectively. Only U1 RNA-containing RNP is selected by anti-RNP, whereas anti-Sm selects RNPs containing U1, U2, U4, U5, and U6. None of these RNAs are selected from HeLa cell nuclear extracts by nonimmune IgG (20). As shown in lanes *5* and *6*, both antibodies select a similar set of eight proteins having molecular weights between 32,000 ("A") and 10,000 ("F"). These proteins are not selected by nonimmune IgG (Fig. 1, lane *4*). Anti-RNP, as its name implies, does not react with deproteinized U1 RNA or to an appreciable extent with free snRNP proteins (data not shown). This forms the basis for its use in the experiments to be described here.

Formation of Antigenically Active snRNP In Vitro

The experimental approach is shown in Fig. 2. HeLa cell poly(A)$^+$ messenger RNA is translated into [^{35}S]methionine-labeled protein in an exogenous mRNA-dependent rabbit reticulocyte lysate (12). U1 RNA or other purified snRNAs are then added, and their assembly into RNP complexes is monitored by the appearance of both the snRNA and specific ^{35}S-labeled proteins in antibody-recognizable structures.

We first examined the presence of endogenous rabbit U1 snRNP in the lysate itself. After addition of human anti-RNP antibody to an aliquot of lysate, followed by selection on protein A-Sepharose and elution, RNA was extracted and analyzed by gel blot hybridization with chicken U1 DNA probe. This probe hybridizes specifically with rabbit U1 RNA

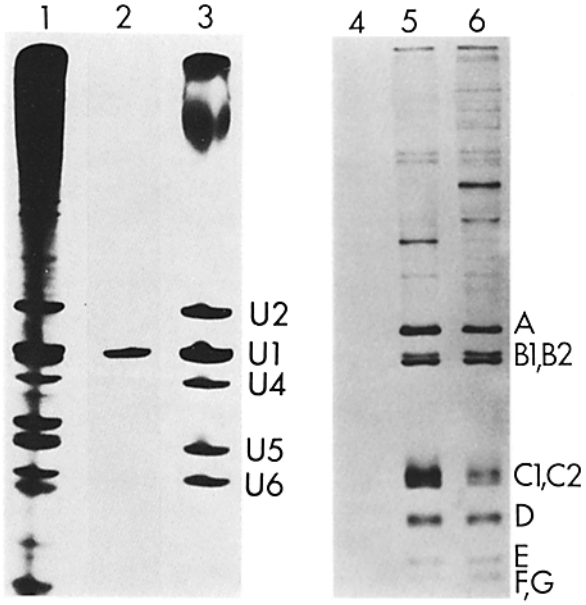

FIGURE 1 Specificities of the anti-RNP and anti-Sm antibodies. HeLa cells were labeled for 22 h with either [^{3}H]uridine (lanes *1–3*) or [^{35}S]methionine (lanes *4–6*). Nuclear extracts were reacted with antibody as designated. Lanes *1–3*: fluorographs of ^{3}H-RNA electrophoresed in 10% polyacrylamide gels containing 98% formamide and 40 mM sodium phosphate, pH 7.0. Lanes *4–6*: fluorographs of [^{35}S]methionine-labeled proteins electrophoresed in 11% SDS polyacrylamide gels. Lane *1*: Total snRNAs from HeLa cells. Lane *2*: RNA selected by anti-RNP. Lane *3*: RNA selected by anti-Sm. Lane *4*: Proteins selected by control (nonimmune) IgG. Lane *5*: Proteins selected by anti-RNP. Lane *6*: Proteins selected by anti-Sm. Proteins are designated as A–G according to Lerner and Steitz (9). We occasionally find that the lowest molecular weight protein runs as a doublet in this gel system (presumably *F* plus *G*). Accordingly, we designated this band as *F, G* in the figures, but for simplicity refer to it as *F* in the text.

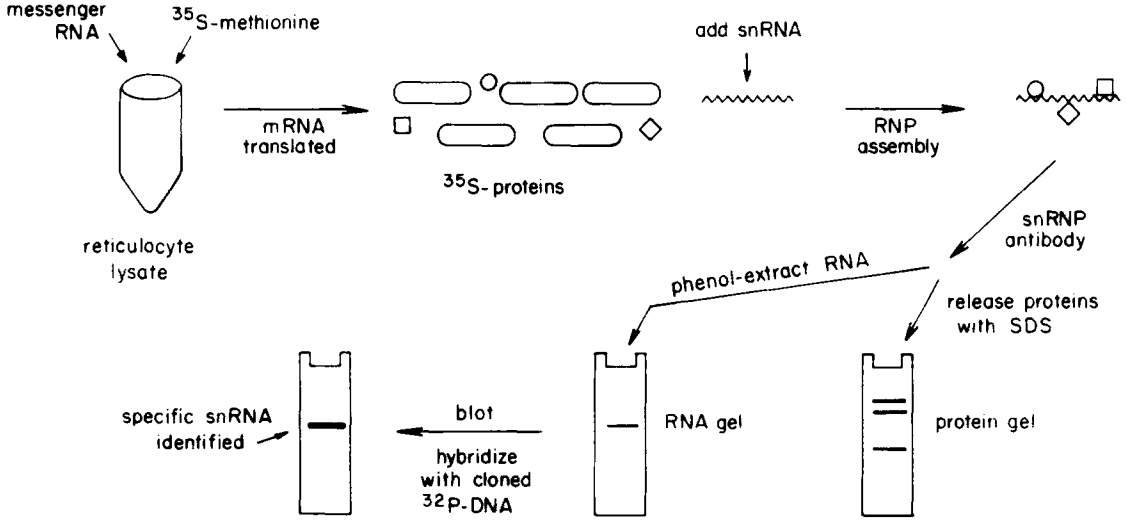

FIGURE 2 Analysis of specific small nuclear RNA:protein interactions in vitro.

because the sequences of avian and mammalian U1 RNAs are highly conserved (2). As can be seen in lane *1* of Fig. 3, a small amount of endogenous U1 RNA is indeed present in the lysate. This rabbit U1 RNA must be in RNP form because, as described above, the human anti-RNP antibody does not react with protein-free U1 RNA. Lane *2* in Fig. 3 shows that the endogenous U1 RNA is not selected from the lysate by nonimmune human IgG.

The reticulocyte lysate apparently also contains endogenous proteins capable of binding to exogenous U1 RNA, as shown by the greatly increased U1 blot hybridization signal observed when human U1 RNA is added (Fig. 3, lane *3*). None of the added human U1 RNA is recovered from the lysate by nonimmune human IgG (Fig. 3, lane *4*). The fact that this exogenous U1:endogenous protein complex is recognized by anti-RNP antibody suggests that the participating rabbit proteins are antigenically the same as those that are normally bound to human U1 RNA. The finding that the reticulocyte lysate, which is a cytoplasmic fraction, contains U1 RNA-binding proteins concurs with the results of a recent study demonstrating that U1 RNA microinjected into enucleated *Xenopus* oocytes forms RNP structures that are recognized by anti-RNP antibody (4).

Fig. 4 shows that snRNP proteins are synthesized when HeLa cell poly(A)$^+$ messenger RNA is translated in the reticulocyte lysate. Lane *2* in Fig. 4 shows the in vitro synthesized [^{35}S]methionine-labeled proteins that are selected from the translated lysate by anti-Sm antibody following the addition of small nuclear RNAs U1, U2, and U4–U6. It can be seen that the proteins synthesized correspond in molecular weight

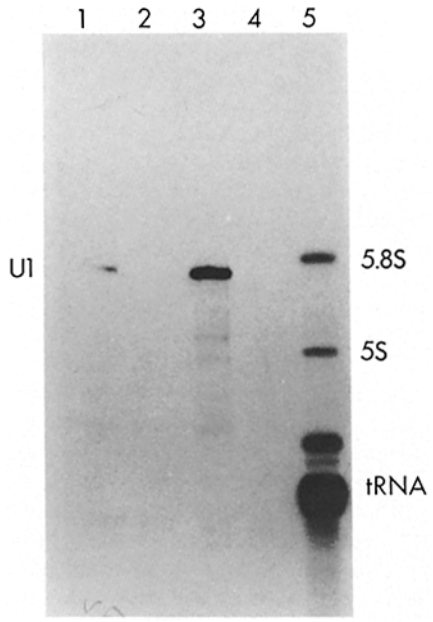

FIGURE 3 Assembly of purified human U1 RNA and endogenous proteins in the nontranslated rabbit reticulocyte lysate. U1 RNA (lanes *3* and *4*) or tRNA (lanes *1* and *2*) were added to an mRNA-dependent reticulocyte lysate in the absence of exogenous mRNA. After 60 min at 30°C, antibody selected RNAs were prepared and electrophoresed in a 10% polyacrylamide–7 M urea gel containing 50 mM Tris-borate, pH 8.3. Following electrophoretic transfer of the RNA to DBM paper, the blot was hybridized with a ^{32}P-labeled chicken U1 DNA clone (3, 17). Lanes *1* and *3*: RNA selected by anti-RNP. Lanes *2* and *4*: RNA selected by control (nonimmune) IgG. Lane *5* contains cytoplasmic RNA isolated from reticulocyte lysate and labeled in vitro with ^{32}P-pCp and T4 RNA ligase.

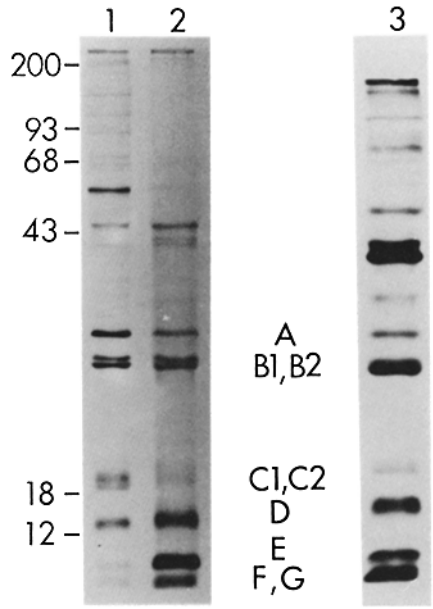

FIGURE 4 [^{35}S]Methionine-labeled HeLa proteins selected by anti-Sm. Lane *1*: Proteins selected by anti-Sm from the nuclei of HeLa cells labeled for 22 h with [^{35}S]methionine. Lane *2*: In vitro-synthesized proteins selected by anti-Sm from a reticulocyte lysate programmed with HeLa cell poly(A)$^+$ mRNA and supplemented with HeLa U1, U2, U4, U5, and U6 RNAs. Lane *3*: Proteins selected by anti-Sm from the cytoplasmic fraction of HeLa cells labeled for 30 min with [^{35}S]methionine. M_r ($\times 10^{-3}$) are designated to the left of lane *1*. The high molecular weight proteins (>35,000) present in this sample are also selected from HeLa cytoplasm by control serum (data not shown).

to the in vivo-labeled nuclear proteins selected by anti-Sm (Fig. 4, lane *1*). Noteworthy in the in vitro translation pattern (Fig. 4, lane *2*) is the relatively reduced amounts of A protein, the relatively increased amounts of the D–G proteins, and the almost total absence of C protein. It is of interest that this pattern is qualitatively very similar to that of proteins selected by anti-Sm from HeLa cytoplasm labeled with [^{35}S]methionine for 30 min (Fig. 4, lane *3*), where again the A and C proteins are reduced and D–G increased relative to the nuclear pattern.

When HeLa cell messenger RNA is translated in the reticulocyte lysate, subsequent addition of U1 RNA results in the formation of a RNA-protein complex that is specifically recognized by anti-RNP antibody. This U1 RNA-protein complex (Fig. 5, lane *2*) contains three newly translated proteins, with molecular weights of 32,000, 27,000, and 26,000. These proteins correspond in molecular weight to the A, B_1, and B_2 proteins found in vivo. As can be seen in lane *1* of Fig. 5, small amounts of these three proteins are recognized by the antibody without the addition of U1 RNA. This may reflect a low reactivity of the antibody for free proteins or, more likely, may be due to the small amount of U1 RNA present in the HeLa messenger RNA preparation used to program translation (data not shown). The absence of the C–F proteins from the U1 RNP complex (Fig. 5, lane *2*) is not due to their failure to be synthesized in the lysate, because they are readily detected by selection with anti-Sm antibody (Fig. 4), which recognizes free proteins as well as snRNP complexes (5).

The remote possibility that the presence of U1 RNA simply stabilizes these three proteins in the reticulocyte lysate is examined in the experiment shown in Fig. 6, where an excess of unlabeled methionine was added to the translation system after

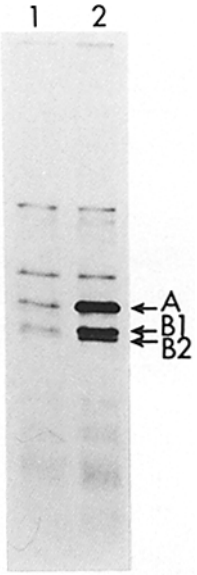

FIGURE 5 Specific binding of [^{35}S]methionine-labeled proteins synthesized in vitro with purified U1 RNA. tRNA (lane *1*) or anti-RNP antibody-purified, phenol-extracted U1 RNA (lane *2*) was added to [^{35}S]methionine labeled proteins that had been synthesized in a rabbit reticulocyte lysate programmed with HeLa cell poly(A)$^+$ mRNA. Antigenically active U1 RNP:protein complexes that assembled in vitro were recovered with anti-RNP.

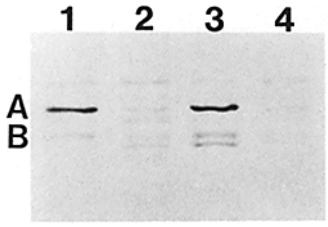

FIGURE 6 Stability of [^{35}S]-methionine labeled proteins binding to U1 RNA in the reticulocyte lysate. HeLa mRNA was translated in an mRNA-dependent reticulocyte lysate in the presence of [^{35}S]methionine. Lanes *1* and *2*: HeLa U1 RNA (lane *1*) or tRNA (lane *2*) were added to aliquots of reticulocyte lysate at the beginning of translation. After 45 min at 30°C, samples were chilled to 4°C and adjusted to pH 8.5. Antigenically active RNP complexes were isolated with anti-RNP. Lanes *3* and *4*: After an initial 45 min of translation in the absence of added snRNA, unlabeled methionine was added to a final concentration of 100 μM (20-fold excess of total methionine). After another 45 min at 30°C, U1 RNA (lane *3*) or tRNA (lane *4*) was added, and the incubation was continued for a final 45-min interval. Samples were then chilled to 4°C and adjusted to pH 8.5. Antigenically active RNP complexes were isolated with anti-RNP. The exposure time for this fluorogram was selected to ensure that the band of *A* protein was in the linear range of the dpm-grain exposure curve.

45 min of translation in the presence of [^{35}S]methionine. After a further 45 min of incubation to permit the postulated decay of the radioactive snRNP proteins synthesized during the initial incubation, U1 RNA (Fig. 6, lane *3*) or tRNA (Fig. 6, lane *4*) was added. After an additional 45 min, U1 RNA:protein complexes were isolated with anti-RNP antibody. No reduction in the intensity of the A or B protein bands was observed relative to a sample in which U1 RNA was added for the first 45 min of translation (Fig. 6, lane *1*). Hence the specific interaction between U1 RNA and these three proteins does not require the presence of U1 RNA at the time the proteins are synthesized.

DISCUSSION

The formation of antigenically active snRNA-protein complexes in vitro provides a new approach to the analysis of snRNP structure. When mRNA is translated in the reticulocyte lysate, subsequently added U1 RNA specifically interacts with the newly synthesized proteins in a manner that creates an antigenic site recognized by the anti-RNP antibody. The U1 RNA-protein complex assembled in vitro contains only three of the eight proteins found associated with U1 RNA in vivo. Thus, the five smallest snRNP proteins identified in vivo are apparently not required for the formation of the antigenic site recognized by this antibody. The three proteins which do bind to U1 under these conditions comigrate with the A, B_1, and B_2 proteins identified in vivo, and we suspect that they are identical to them. It is noteworthy that the in vitro assembly of an antigenically active U1 RNA-protein complex apparently does not require the short-lived, cytoplasmic precursor form of U1 RNA identified previously (6, 23). In addition, our results establish that at least some assembly of snRNP can occur in a specific manner in vitro in the absence of defined organelles or cellular architecture. Nor does in vitro assembly of snRNP from newly synthesized protein require deliberate "reconstitution" conditions such as the use of protein denaturants followed by slow reassociation of protein and RNA during dialysis. Finally, our results are compatible with the possibility that at least the initial steps in snRNP assembly in vivo occur in the cytoplasm (Madore, Wieben, and Pederson, manuscript submitted for publication; see also reference 4).

The approach we have developed provides new information about the mRNAs for snRNP proteins. The mRNAs for the A, B_1, and B_2 snRNP proteins contain poly(A) (Fig. 5). (The poly(A)$^-$ HeLa cytoplasmic RNA fraction has not been examined in detail, and therefore we cannot state whether or not these mRNAs are also present in the poly(A)$^-$ [oligo(dT)-nonbound] fraction.) Experiments with sucrose gradient-fractionated poly(A)$^+$ mRNA indicate that the mRNAs for the A, B_1, and B_2 proteins sediment at 15–20S and comprise 0.1–0.3% of the total HeLa cell poly(A)$^+$ mRNA (unpublished results).

It has been reported that U1 RNA inhibits protein synthesis in a wheat germ cell-free system (14). We find no such effect when the amounts of [^{35}S]methionine incorporation are compared in lysates run with HeLa poly(A)$^+$ mRNA plus or minus added U1 RNA (data not shown). It is possible that the concentrations of U1 we employed are below those required for the previously reported effect (14).

While the U1 RNA-dependent recognition of the A, B_1, and B_2 proteins in vitro constitutes a more rigorous identification of these proteins as being U1 RNA-associated than co-precipitation with a polyclonal antiserum, we have at present no reason to doubt that the five smaller proteins are also authentic components of U1 snRNP in vivo. It is probable that the assembly of U1 snRNP in this system is incomplete. Although the reasons for this are not understood at present, we have been able to rule out the trivial possibility that the C_1, C_2, D, E, and F proteins are not synthesized in the reticulocyte lysate. Experiments using Sm antibody, which recognizes free proteins as well as snRNA-protein complexes, confirm that all eight snRNP proteins are synthesized in the lysate (Fig. 4). Furthermore, these proteins (Fig. 4, lane *2*) are produced in amounts that approximate their relative abundance in the cytoplasm of HeLa cells after a short labeling period in vivo (Fig. 4, lane *3*). Complete assembly of snRNP in vitro might require the use of the precursors of snRNAs, co-factors not present in the reticulocyte lysate, or simply adjustments of the salt and temperature conditions used for assembly. Such factors have been shown to be very significant in the reconstitution of ribosomes from purified components (11).

The fact that specific binding of proteins to snRNA can occur in vitro opens the way for a more detailed analysis of the structure and assembly of snRNP than was heretofore possible. For example, the use of this procedure with other snRNA

species or defined snRNA fragments should lead to an understanding of the parameters regulating the assembly of snRNP, the proteins unique to individual snRNAs as opposed to ones common to all species (21), and the relative contributions of protein-RNA vs. protein-protein interactions. The in vitro assembly approach also provides a way of screening cDNA clone banks for snRNP protein mRNAs, after message selection. Such experiments are now in progress.

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