In vitro interaction of U2 snRNA with cytoplasmic 6S protein complexes

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Abstract Interactions of U2 snRNA with anti-Sm precipitable proteins in RNA-free cytoplasmic complexes were analyzed. U2 snRNA was found to bind specifically with proteins in the 6S complexes but not in the 20S complexes. The binding activity was preserved using U2 snRNA having a mutated Sm binding site. Label-transfer experiments indicate that snRNA makes direct contact with anti-Sm precipitable proteins in the 6S fraction with apparent molecular mass of about 16 kDa. These data corroborate that proteins in the 6S core particle are the first to interact with snRNA, and suggest that the proteins recognize snRNA structures in addition to the Sm site.

Key words: SnRNP; Sm protein; U2 snRNA

1. Introduction

Small nuclear ribonucleoprotein particles (snRNPs) are abundant components of the nucleus which function in RNA processing. The four major snRNPs, U1, U2, U5 and U4/U6 snRNPs, in conjunction with variety of non-snRNP factors, are required for pre-mRNA splicing [1]. These snRNPs are composed of an snRNA (U1, U2, U5 or dimer U4/U6), eight common core proteins (B/B′, D1, D2, D3, E, F, G) and a varying number of snRNA-specific proteins [2–4]. The common core proteins are also known as Sm proteins, after Smith, a systemic lupus erythematosus patient whose antisera recognizes these proteins [5], and for this reason, the snRNAs which complex with the Sm proteins are called Sm snRNAs in this study.

The contacts between the Sm proteins and Sm snRNA are not well understood. In vitro cross-linking studies of mature nuclear particles indicate that core protein G contacts the RNA's Sm site, a conserved, single-stranded region ($PaAU_{3-4}NUGPu$) found in all Sm snRNAs [6]. The Sm site is important to the association of Sm snRNA with mature particles [7]. However, snRNA-Sm protein interaction studies using snRNAs with mutations outside of the Sm site suggest that common core proteins make multiple contacts with snRNA [8].

A model for the cytoplasmic assembly of the snRNAs with Sm proteins has been proposed based on the results of in vivo kinetic studies [9]. After transcription by RNA polymerase II, snRNAs travel to the cytoplasm where they assemble with the common core proteins [10,11]. In the cytoplasm, snRNP proteins are stored in three RNA-free complexes: 2 S-5S (B/B′), 6S (D1, D2, E, F, G) and 20S (D3, B/B′, 69 kDa) [9,12]. Kinetic experiments suggest that the first step in assembly is the binding of the snRNA to the 6S complex in the cytoplasm [9–13]. The present study analyzes the specific interaction of U2 snRNA, as a representative of the Sm snRNAs, with proteins in the cytoplasmic 6S fraction. The in vitro results indicate that snRNA make direct contact with proteins in the 6S fraction which have apparent mobilities similar to D1 and D2 snRNP core proteins.

2. Materials and methods

2.1. Cell culture

HeLa cells and murine L929 cells (Tissue Culture Facility, Dept. of Microbiology, SUNY at Stony Brook) were maintained as suspension cultures in SMEM (Gibco-BRL) supplemented with 7% bovine calf serum (Atlanta Biologicals), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL) in a 5% CO_2 atmosphere at 37°C. L929 mouse fibroblast suspension cultures (Tissue Culture Facility, Dept. of Microbiology, SUNY at Stony Brook) were maintained under identical conditions, except that the medium was supplemented with 5% bovine calf serum and 2% fetal calf serum (Atlanta Biologicals). Cells in suspension culture were maintained between 4×10^5 and 5×10^5 cells per ml.

2.2. Aqueous cytoplasmic fractionation and sedimentation analysis

Aqueous cytoplasmic extracts for snRNA-Sm protein binding reactions were prepared as previously described [10], except that the buffer components differed slightly. HeLa cells (200 ml) were extracted in 2 ml HKM-Tx buffer (20 mM HEPES [pH 7.9], 100 mM KCl, 3 mM $MgCl_2$, 0.5% Triton X-100) with protease inhibitors added. The extract was resolved on a sucrose gradient containing the same buffer minus detergent and 1 ml gradient fractions were collected from the top, as described elsewhere [10]. Extracts prepared from HeLa cells and from L929 cells had identical RNA-binding activities in the assays used in this report (data not shown).

2.3. In vitro transcription

Several cDNAs were used as templates for in vitro synthesis of Sm snRNAs and 5S RNA with T7 or SP6 RNA polymerase. ^{32}P-labeled Sm snRNA was produced by in vitro transcription of linearized plasmid templates, as previously described [14]. Plasmid pGEM-U2 for transcription of wild-type U2 snRNA, and plasmid pGEM-U2-4 for transcription of U2 snRNA with a mutated Sm site, were both provided by Dr. Thoru Pederson of the Worcester Institute [14]. For transcription of human U1 snRAN, the pHU1A vector provided by Dr. Jeffrey Patton was used [15]. For transcription of U6 snRNA, T7 RNA polymerase was used to transcribe 20–50 ng DNA template produced by PCR as described [16]. All transcripts were gel purified as described [16].

To transcribe unlabeled snRNA for use in competition experiments, the reaction volume was scaled up 10-fold to yield 200–300 μg of transcript. After transcription, unincorporated nucleotides were removed by passing the sample over a Chroma Spin 30 column (Clontech). The amount of RNA in the eluate was determined by spectrophotometry.

2.4. SnRNA-Sm protein binding reaction and non-denaturing gel analysis

For snRNA-Sm protein binding reactions, 15 μl gradient fraction, 5 μl reaction buffer (10 mM DTT, 2 mM ATP, 100 mM creatine kinase, 1 mg/ml tRNA, 0.8 U/ml aprotinin, 4 mM leupeptin, 2 U/ml recombinant RNasin [Promega]), and 1 ng ^{32}P-labeled snRNA probe were incubated for 30 min in a 1.5 ml microcentrifuge tube

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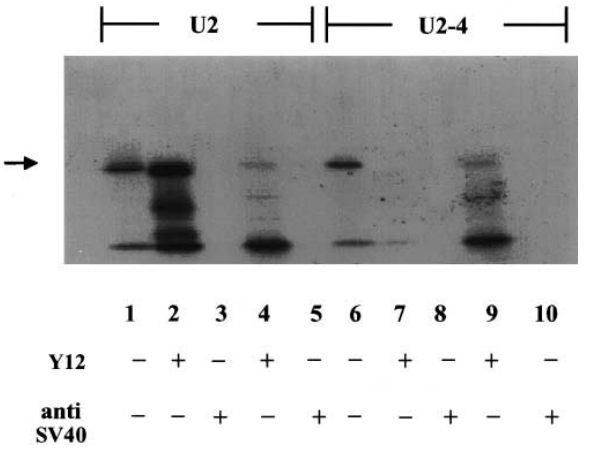

Fig. 1. Gel analysis of co-immunoprecipitation of wild-type U2 snRNA and of Sm site mutant U2-4 snRNA with Sm precipitable proteins from HeLa S100 extracts (lanes 2,3 and 7,8) and from sucrose gradient fraction 3 (lanes 4,5 and 9,10). Gel purified ^{32}P-labeled wild-type U2 snRNA (lanes 1–5) and ^{32}P-labeled Sm site mutant U2-4 snRNA (lanes 6–10) were used in the binding reactions. The migration of full length U2 and U2-4 snRNAs is indicated at left by an arrow. (Partial degradation of the RNA occurred during immunoprecipitation and subsequent steps which resulted in the appearance of faster migrating bands.) Uncomplexed wild-type (lane 1) and mutant (lane 6) U2 snRNAs (0.1 ng) are shown as markers. Completed reactions were immunoprecipitated with Y12 Sm monoclonal antibody (lanes 2,4,7,9) or with anti-SV40 large T monoclonal antibody (lanes 3,5,8,10).

at 30°C. 2 μl of loading buffer (50 glycerol, 10 μg/ml heparin) was added to each completed reaction. The reaction samples were run on a 5% polyacrylamide non-denaturing gel in a buffer with half the concentration of TBE (1/2 TBE) [16] and/or UV irradiated for cross-linking analysis (see below). Assembly in S100 extracts was performed as described [15].

2.5. Co-immunoprecipitation assay

Reactions to be assayed by immunoprecipitation were scaled up 3-fold. After an snRNA-Sm protein binding reaction or an assembly reaction, the Y12 monoclonal antibody was used to detect RNA-protein interaction by co-immunoprecipitation. The anti-SV40 T antibody was used as a control for non-specific binding during the immunoprecipitation. Protein A-Sepharose (50 μl) pre-bound to monoclonal antibody was added to each reaction in 1.5 ml microcentrifuge tubes and rotated at 4°C for 2 h. Immunoprecipitates were washed and displayed on SDS-PAGE gels as previously described [10].

2.6. Label transfer by UV cross-linking

RNP complexes formed as described above were also subjected to UV light (254 nm) for 8.3 min in a 'Stratalinker' from Stratagene for a total of 18 mJ/mm^2 [17], immunoprecipitated with a cocktail of three anti-Sm mAbs (Y12, 713, and KSm6) which together recognize several core proteins [18–20], and then digested with 1 μg/ml RNase A for 40 min at 37°C. The sample was boiled in Laemmli protein solubilizing solution before analysis by SDS polyacrylamide gel electrophoresis (SDS-PAGE) [21].

3. Results

3.1. Co-immunoprecipitation assay for interaction of snRNA with proteins in the 6S fraction

The cytoplasmic distribution of the Sm proteins into 6S and 20S complexes in sucrose gradient has been demonstrated previously [10]. Interaction of Sm snRNAs with cytoplasmic Sm proteins from the 6S fraction was assayed by co-immunoprecipitation of snRNA with Y12 Sm monoclonal antibody [18] (Fig. 1). Anti-SV40 T antigen monoclonal antibody was used as a control for non-specific interactions during immunoprecipitation. For a positive control for assembly, we reproduced the published snRNP assembly protocol using HeLa S100 extracts [15]. In vitro transcribed ^{32}P-labeled wild-type U2 snRNA and U2 snRNA with a mutated Sm site (U2-4) were compared in parallel assembly reactions. RNA from the immunoprecipitations was displayed by SDS-PAGE (Fig. 1). The same assay was performed using the S100 extract and the sucrose gradient fraction containing the 6S complex.

HeLa cell S100 is an unfractionated cytoplasmic extract,

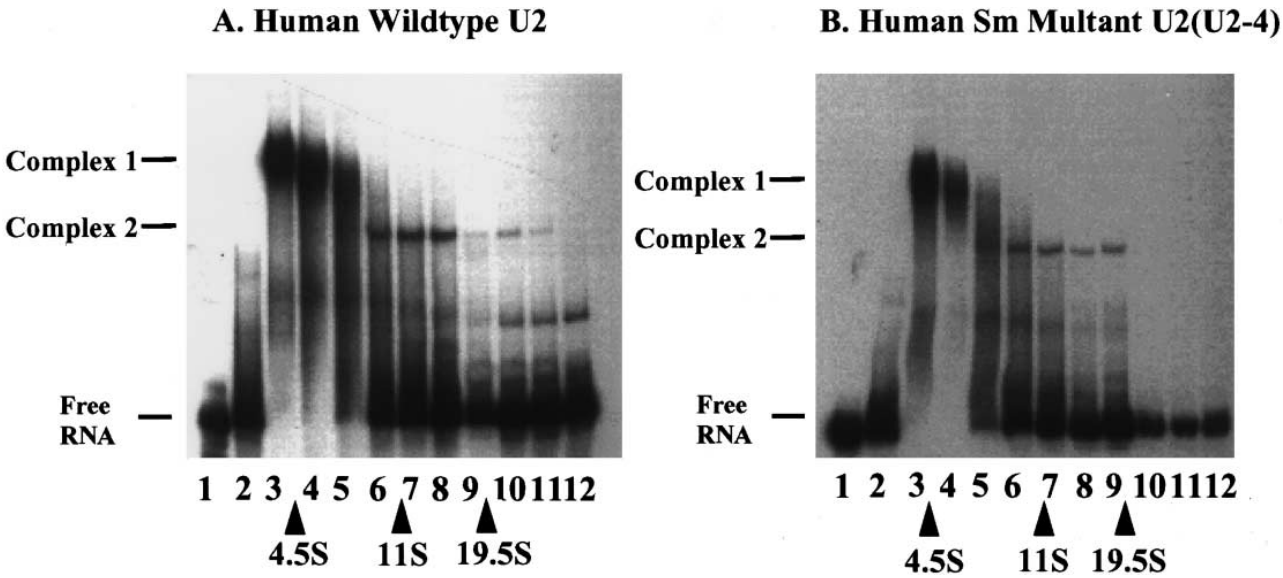

Fig. 2. Gel shift analysis of wild-type U2 snRNA and Sm site mutant U2-4 snRNA binding activities across a sucrose gradient. Sucrose gradient fractions in HKM buffer were used to analyze snRNA-Sm protein binding reactions across the gradient. ^{32}P-labeled U2 snRNA was incubated with gradient fractions 2–12 and analyzed on a 5% polyacrylamide non-denaturing gel (A). Identical reactions were performed with ^{32}P-labeled Sm site mutant U2-4 snRNA (B). Free snRNA was added as marker (lane 1, A,B). Lanes 2–12 indicate gradient fractions 2–12 (A,B). Free RNA, complex 1, and complex 2 are indicated. Sedimentation markers are given at the bottom.

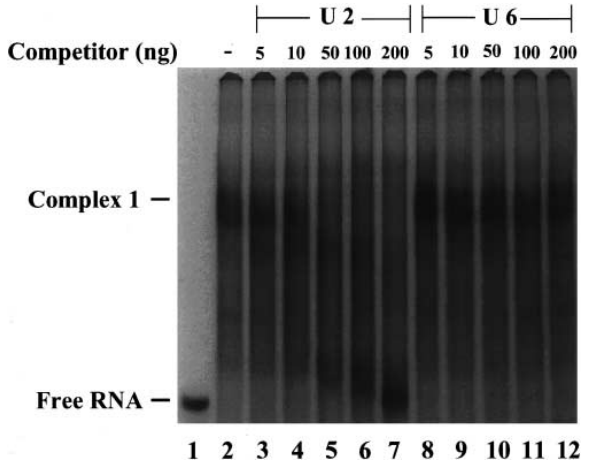

Fig. 3. U2 snRNA specifically competes for RNP complex 1. Cytoplasmic extracts were resolved on sucrose gradients. Fraction 4 was assayed for U2 snRNA binding activity. Mobility of ^{32}P-labeled U2 snRNA in a native gel was assayed after incubation with gradient buffer alone (lane 1) or with gradient fraction 4 (lane 2). The fraction 4/^{32}P-labeled U2 snRNA binding reaction was performed in the presence of increasing amounts of unlabeled U2 snRNA (lanes 3–7) or unlabeled U6 snRNA (lanes 8–12) and displayed on the gel. Competitor concentrations in ng are given at the top of each lane. All reactions were also performed in the presence of 2.5 μg/ml heparin. Free RNA and complex 1 are indicated at left.

and contains a pool of snRNP proteins as well as endogenous snRNAs. In HeLa S100 extracts, ^{32}P-labeled U2 snRNA interacts with proteins that immunoprecipitate with Y12 monoclonal antibody but not with anti-SV40 T antigen monoclonal antibody, as expected (Fig. 1, lanes 2,3). After assembly in S100 extracts, the Sm site mutant U2-4 snRNA co-immunoprecipitates, but at a much reduced level using the Y12 monoclonal antibody (Fig. 1, lanes 7,8). The co-immunoprecipitation assay was also used to determine if U2 snRNA interacts with anti-Sm-immunoprecipitable proteins in a fraction of sucrose gradient which has the cytoplasmic 6S complex and no detectable levels of snRNAs [10,22]. Y12 monoclonal antibody immunoprecipitates low levels of U2, while anti-SV40 T antigen immunoprecipitation detected nothing (Fig. 1, lanes 4,5) indicating that U2 snRNA interacts with anti-Sm-precipitable proteins in the fraction. In contrast to the S100 results, however, the Sm site mutant U2-4 snRNA co-immunoprecipitates with anti-Sm-precipitable proteins in the 6S fraction at the same level as wild-type U2 snRNA (lanes 9,10). This indicates that anti-Sm precipitable proteins in the snRNA-free 6S fraction can interact with U2 snRNA independent of the Sm site.

3.2. Gel mobility shifts

Nondenaturing gel analysis was used to determine U2 snRNA-binding activity across all fractions of the HKM sucrose gradients. In vitro synthesized ^{32}P-labeled U2 and U2-4 snRNAs were incubated on ice in the presence of excess nonspecific tRNA carrier with a small aliquot of each fraction from a sucrose gradient containing unlabeled HeLa cytoplasmic proteins. The incubation mixtures were subsequently electrophoresed on non-denaturing polyacrylamide gels at 4°C (Fig. 2). Gel shift analysis showed two major binding activities for in vitro transcribed human U2 snRNA across the gradient fractions (Fig. 2A). There was a strong RNA-binding activity in fractions 3–5 (complex 1), and a weaker activity in fractions 6–10 (complex 2) (Fig. 2A). A third activity in fractions 10–12 was not reproducible. U2-4 snRNA yielded a highly similar mobility shift pattern (Fig. 2B). Similar patterns of gel mobility shifts were seen if in vitro synthesized ^{32}P-labeled U1 or U4 snRNA were used or if gel purified snRNAs (U1, U2, U4 and U5) were isolated from cells labeled with inorganic [^{32}P]orthophosphate and used in the analyses (data not shown).

3.3. RNA specificity

The ability of complexes 1 and 2 to form in the presence of a specific competitor (cold U2 snRNA) or a non-specific competitor (cold U6 snRNA) was analyzed. [^{32}P]U2 snRNA was incubated with fraction 4 of the sucrose gradient to form only complex 1 (see Fig. 3). Complex 1 could be competed out with increasing amounts of cold U2 snRNA, but not with increasing amounts of cold U6 snRNA (Fig. 3). Therefore, complex 1 shows specificity for U2 snRNA. Complex 2 was formed by incubation of U2 snRNA with fraction 8. The U2 snRNA or U6 snRNA competition for complex 2 did not differ (data not shown) suggesting that complex 2 forms from non-specific RNA-binding protein(s).

3.4. Identification of the proteins in the complexes through UV cross-linking

To determine which proteins in the 6S region of the gradient are interacting directly with the snRNAs, in vitro synthesized ^{32}P-labeled U2 snRNA was incubated with an aliquot of each gradient fraction, and the samples were UV irradiated

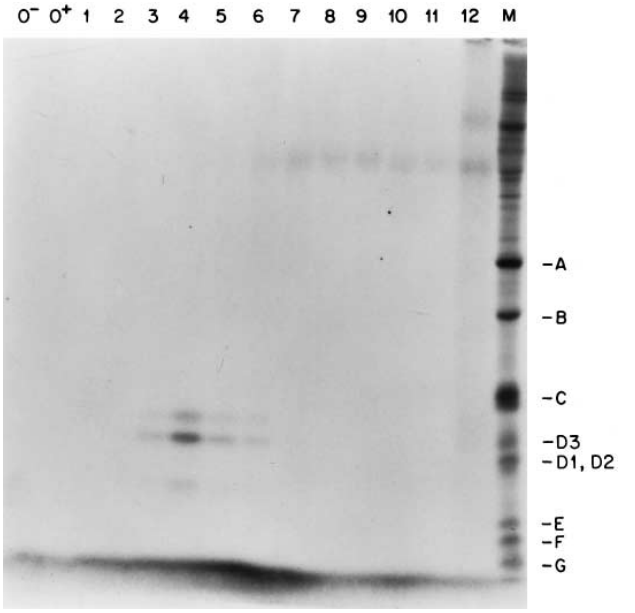

Fig. 4. U2 snRNA interacts with Sm precipitable proteins in the 6S fraction. ^{32}P-labeled U2 snRNA was incubated with gradient fractions 1–12 across the gradient, subjected to UV radiation, immunoprecipitated with a cocktail of anti-Sm mAbs containing Y12, 713 and KSm6, subjected to RNAse and SDS-PAGE. Lanes 1–12 are from fractions 1–12, respectively, from the top to the bottom of the gradient. Lanes 0⁻ and 0⁺ contain U2 snRNA incubated in buffer alone without UV radiation (−) and with UV radiation (+). Lane M contains snRNP marker proteins immunoprecipitated from ^{35}S-labeled nuclear extracts made as previously described [12]. The snRNP proteins in the marker lane are identified at the left.

to cross-link pyrimidine bases to neighboring amino acids in the proteins. Cytoplasmic proteins which UV cross-linked to the ^{32}P-labeled snRNA were immunoprecipitated with anti-Sm mAbs. The precipitates were treated with RNase and analyzed by SDS-PAGE (Fig. 4). Three anti-Sm precipitable cross-linked species appear in the lanes containing the 6S fractions of the sucrose gradient (Fig. 4, lane 3–5). The cross-linked species have similar gel mobility (at about 16 kDa) to the snRNP core proteins D1 and D2 found in the 6S fraction. Also another cross-linked species was detected with an apparent mobility of about 13 kDa.

As a control for the sensitivity and specificity of the label-transfer assay, interactions of other small RNAs with proteins in the sucrose gradient fractions were also analyzed. ^{32}P-labeled U6 snRNA forms a single RNP particle with proteins in the 4th fraction of the 6S region of the sucrose gradient (data not shown) and cross-links to one protein in that fraction with an apparent molecular mass of 45–50 kDa protein (data not shown), which almost certainly is La antigen [23]. For comparison, ^{32}P-labeled *Xenopus* 5S rRNA did not cross-link with any of the proteins in the gradient (data not shown). The results from these two RNAs support the observation that complex 1 activity in the 6S fraction has specificity for U2 snRNA, as representative of the Sm snRNAs.

4. Discussion

The present study has examined the interaction of U2 snRNA with the fractionated cytoplasmic core snRNP proteins using a variety of in vitro approaches. These included analyzing the binding of ^{32}P-labeled RNAs to snRNP proteins by (1) RNA/protein gel mobility shift analyses, (2) competition analysis, and (3) label transfer through UV cross-linking ^{32}P-labeled RNA to protein followed by immunoprecipitation of cross-linked proteins after label transfer. Our data suggest that the Sm snRNA initially interacts with proteins associated with the 6S cytoplasmic snRNP protein complex (D1, D2, E, F, G).

Analyses by label transfer through UV cross-linking of ^{32}P-labeled snRNAs to cytoplasmic proteins show that Sm snRNAs make direct contact with anti-Sm immunoprecipitable proteins in the 6S fractions of the sucrose gradient. The cross-linked species have mobility in SDS-PAGE gels similar to D1 and D2 core snRNP proteins, which are found in the 6S fraction. In addition, a smaller 13 kDa cross-linked species co-immunoprecipitates with the core snRNP proteins in the 6S fraction. These in vitro results are consistent with the hypothesis that the first step in snRNP assembly is the interaction of snRNA with proteins in the 6S complex.

The fact that the cross-linked species are immunoprecipitable by anti-Sm monoclonal antibodies, are in the 6S fraction and have similar mobilities to D1 and D2 polypeptide suggests that Sm snRNA makes direct contact with D1 and/or D2 proteins, however, we have not definitely identified the proteins involved. Another laboratory has shown that an immunoaffinity-purified sample containing an E/F/G trimer and the D1 and D2 proteins results in formation of an snRNP subcore particle containing snRNA and D1, D2, E, F, and G [24]. The E/F/G trimer, D1, and D2 independently assemble with the snRNA in the study, however, they do not bind snRNA as an intact 6S complex [24]. Heinrichs et al. [6] reported that ^{32}P-labeled snRNAs which contained the Sm-antigen binding motif could be directly cross-linked in situ by UV radiation to the snRNP G core protein. Their results indicate that the SnRNP G core protein is involved in the recognition of the common Sm-antigen binding motif on snRNAs in mature snRNP particles. Our results do not directly contradict this thought, but they open up other possibilities. It may also be that at early stages of snRNP assembly, another core protein is involved in recognition of the Sm snRNA in addition to or instead of the G core protein. The core proteins D1 and D2 are likely candidates for such recognition. In our hands, anti-Sm-immunoprecipitable proteins with mobility of D1 and D2 show RNA binding activity when denatured and immobilized on nitrocellulose (data not shown). Others have also observed RNA binding activity for D1 under similar conditions [25].

The results of the present study indicate that the presence of the Sm site, which is essential for mature snRNP particle assembly, may be dispensable for the early stages of snRNP assembly such as interaction of the Sm proteins in the 6S complex with Sm snRNA. Mutation of the Sm site from snRNA did not inhibit complex 1 formation although it did inhibit snRNP formation with unfractionated S100 extracts. The differences between the results from the two extracts may be due to the presence of endogenous snRNAs in the S100 extract. The Sm site mutant snRNA cannot compete successfully with the endogenous wild-type snRNAs in the unfractionated S100 extract for binding of snRNP proteins since it lacks an Sm site. In contrast, without competition, the Sm mutant snRNA can bind to the proteins in the 6S fraction, which does not have detectable endogenous competitor snRNAs [22]. The results suggest then that regions of the Sm snRNA other than the Sm site may be important to its early interaction with the Sm proteins. This is in agreement with other studies which suggest that snRNA sites other than the Sm site are involved in interactions with the common core proteins [8]. RNA structural elements such as helices have been implicated in protein recognition in numerous systems and are generally held to be essential for RNA/protein interactions [26]. It is likely that RNA structural elements also play some role in Sm snRNA/Sm protein interactions in addition to the Sm site. Sm snRNA structures such as helices may be involved in early interactions of the RNA with Sm proteins in the 6S complexes.

In summary, this in vitro study indicates that Sm snRNAs, in particular U2 snRNA, make direct contact with several anti-Sm-immunoprecipitable proteins in the 6S region of a sucrose gradient containing fractionated cytoplasmic proteins. The proteins have mobilities similar to D1 and D2 snRNP core proteins. The initial interaction of the snRNAs with these proteins may involve structures on the RNA in addition to the Sm site.

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