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U2 small nuclear RNP assembly *in vitro*

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ABSTRACT

Incubation of a SP6-transcribed human U2 RNA precursor molecule in a HeLa cell S100 fraction resulted in the formation of ribonucleoprotein complexes. In the presence of ATP, the particles that assembled had several properties of native U2 snRNP, including resistance to dissociation in Cs_2SO_4 gradients, their buoyant density, and pattern of digestion by micrococcal nuclease. These particles also reacted with Sm monoclonal antibody and a human autoantibody with specificity for the U2 snRNP-specific proteins A′ and B", but not with antibodies for U1 snRNP-specific proteins. In contrast, the particles that formed in the absence of ATP did not have these properties. ATP analogs with non-hydrolyzable β-γ bonds did not substitute for ATP in U2 snRNP assembly. Additional experiments with a mutant U2 RNA confirmed that nucleotides 154-167 of U2 RNA are required for binding of the U2 snRNP-specific proteins but not of the "Sm" core proteins. Pseudouridine formation, a major post-transcriptional modification of U2 RNA, was enhanced under assembly permissive conditions.

INTRODUCTION

Ribonucleoprotein complexes that contain the small nuclear RNAs U1, U2, U5 and U4/6 are cofactors in mRNA splicing (1). These small nuclear RNPs (snRNPs) each contain a core of seven "Sm" proteins denoted as B, B′, D, D′, E, F and G. In addition, there are U1 and U2 snRNP-specific proteins referred to as 70K, A and C, and A′ and B" respectively (2,3). These two snRNPs have distinct roles in the splicing of pre-messenger RNA (1), and it is likely that the different proteins bound to U1 versus U2 RNA reflect these specific functions. We and others have been developing systems for the in vitro assembly of mammalian snRNPs to facilitate understanding of their structures and functions (4-10). Here we report the in vitro assembly of human U2 snRNP.

MATERIALS AND METHODS

Both the SP6 promoter/human U2 DNA clone $pSPU2^{pre}$ (11) and a comparable T7 promoter/human U2 DNA clone, $pG2U2^{pre}$, were used for these experiments. U2 RNA was generated from BamHI-digested DNA by transcription with SP6 or T7 RNA polymerase in the presence of 1mM 7-mGpppG as previously described (11). An antisense U2 RNA was

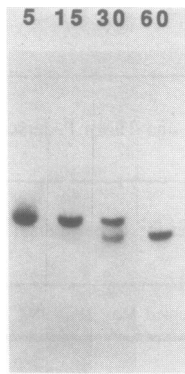

Figure 1: Assembly of U2 snRNP in HeLa S100. ^{32}P-U2 precursor RNA was incubated in the S100 assembly system for 5, 15, 30, or 60 min. The reaction mixture was layered on 1.25 g/cm^3:1.75 g/cm^3 Cs_2SO_4 gradients, which were centrifuged for 60 hr. at 32,000 revs/min. in a Beckman SW50.1 rotor (20^{o}C). The gradients were fractionated and the 1.4 g/cm^3 region was recovered and dialyzed against TE (10 mM Tris-HCl, pH 7.5, 1mM EDTA). The samples were deproteinized and the entirety of each was analyzed by electrophoresis.

generated from pG2U2^{pre} by SP6 RNA polymerase. The clone pG2ΔU2^{pre}, which contains a deletion of nucleotides 154-167 in the U2 coding region, was constructed by ligation of the HindIII-TaqI and RsaI-BamHI fragments of pSPU2^{pre} (11) and cloning of the resulting fragment into HindIII-BamHI digested pGEM-2 DNA. The in vitro assembly reactions were carried out in HeLa S100 fractions as described (7) except that no oligodeoxynucleotide-mediated RNase H cleavage was done. Unless otherwise noted, assembly reactions were for 60 min. at 37^{o}C. Antibody selection was performed using protein A-Sepharose to collect antigen:antibody complexes (12). The human autoantibodies V26 (2) and D18 were kindly provided by W. Habets and W. van Venrooij (University of Nijmegen).

RESULTS

Assembly of U2 snRNP precedes U2 RNA 3' processing

We have previously reported that the in vitro assembly of a U1 snRNP particle can occur on a mature length U1 RNA molecule (4,5,7,8). However, in vivo, snRNP assembly occurs on snRNA precursor molecules extended at their 3′ ends (13-16). In the present study we have used a precursor length U2 RNA that is 11 nucleotides longer than mature U2 RNA (11). As an initial assay for particle assembly on this precursor length molecule we employed fractionation in Cs_2SO_4 gradients. Cellular snRNPs withstand

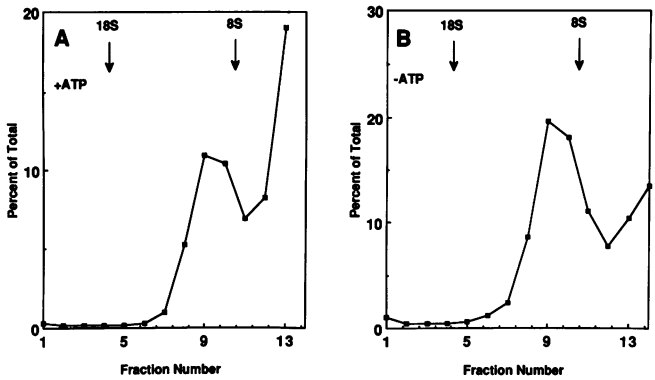

Figure 2: Glycerol gradient sedimentation of assembled U2 snRNP particles. ^{32}P-U2 RNA was incubated in the S100 assembly system for 60 min. and analyzed by centrifugation in 10%-30% linear glycerol gradients (containing 0.25M NH_4Cl, 20mM Tris-HCl, pH 8.1, 6.5mM $MgCl_2$ and 2mM dithiothreitol). Centrifugation was for 18 hr. at 40,000 revs/min. in a Beckman SW41 rotor (4°C). The arrows "8S" and "18S" denote the positions of alcohol dehydrogenase (7.6S) and apoferritin (17.6S) run as sedimentation markers. A. Particles assembled in the presence of 0.5 mM ATP and 20 mM creatine phosphate (CP). B. Particles assembled in the absence of ATP and CP.

isopycnic banding in cesium salts (7,17) and have a buoyant density of approximately 1.4 g/cm^3. ^{32}P-U2 RNA precursor was incubated in the S100 for increasing times, and the amounts and sizes of RNA banding at 1.4 g/cm^3 in Cs_2SO_4 were determined by denaturing gel electrophoresis (Figure 1). After 5 and 15 min., all of the U2 RNA banding at 1.4 g/cm^3 was precursor length. After 60 min., all of the U2 RNA banding at 1.4 g/cm^3 was mature length. After 30 min., both precursor and mature-length RNAs were present, indicating that U2 RNA processing is occurring in the assembled RNP particle, as is the case in vivo (16). To probe the sequence-specificity of the Cs_2SO_4-stable U2 RNP complex that assembles in the S100, we performed comparable experiments with an anti-sense U2 RNA (see Materials and Methods). This RNA was not assembled into a Cs_2SO_4-stable RNP in the S100 (data not shown).

ATP is required for assembly of the U2 snRNP complex

We have previously reported the in vitro assembly of a U1 snRNP particle that resembles native U1 snRNP in several respects (7). We noted that in the absence of ATP or creatine phosphate (CP) the efficiency of U1 snRNP assembly was decreased. In the present work we have explored the ATP requirement for snRNP assembly in greater detail, in this case using U2 RNA. Incubation of U2 RNA precursor in a HeLa S100 fraction in either the presence or absence of ATP and CP led to the formation of a ~10S particle as

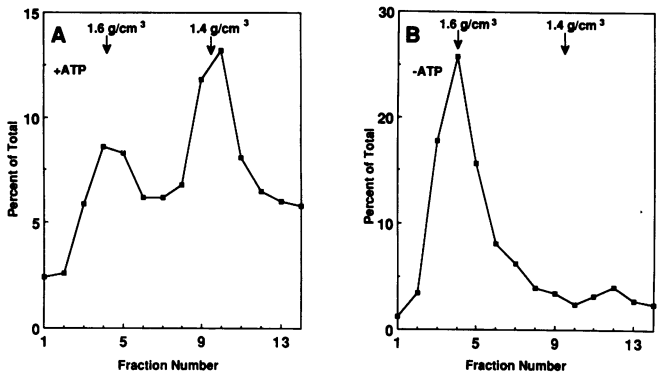

Figure 3: Cs_2SO_4 gradient centrifugation. ^{32}P-U2 RNA was incubated in S100 as in Figure 2, centrifuged in glycerol gradients and the ~10S region was recovered and dialyzed against SNURB (Small NUclear Ribonucleoprotein Buffer: 100mM NH_4Cl, 3mM $MgCl_2$, 20mM Tris-HCl, pH 8.0, 50 μM phenylmethylsulfonyl fluoride, 5mM 2-mercaptoethanol). Samples were analyzed on Cs_2SO_4 gradients as described in Figure 1. A. Assembly in the presence of ATP and CP. B. Assembly in the absence of ATP and CP.

analyzed by glycerol gradient centrifugation (Figures 2A and 2B). This sedimentation coefficient is the same as that of native U2 snRNP (data not shown). However, Cs_2SO_4 density gradient centrifugation revealed that the particles assembled in the presence or absence of ATP are different. A substantial fraction of the particles that assembled in the presence of ATP and CP withstood banding in Cs_2SO_4 (as do cellular snRNPs) and displayed the snRNP-characteristic density of ~1.4 g/cm^3 (Figure 3A). In contrast, particles assembled in the absence of ATP and CP did not withstand Cs_2SO_4 centrifugation, with the U2 RNA now banding at 1.6 g/cm^3, the density of protein-free RNA under these gradient conditions (Figure 3B). As was previously reported for U1 RNA (7), a significant but reduced amount of assembly was observed when CP alone was used in the U2 snRNP assembly reaction (not shown). The non-hydrolyzable ATP analogs adenylyl-imidodiphosphate (AMPPNP) and adenyl(β,γ-methylene)-diphosphonate (AMPPCP) did not substitute for ATP in the assembly of the Cs_2SO_4-stable U2 RNP particle, nor did they inhibit this process when present together with ATP. dATP was capable of supporting assembly, but CTP, GTP and UTP, individually, were not.

To further probe the structure of the U2 RNP particles assembled in the presence or absence of ATP, we employed the criterion of micrococcal nuclease digestion. Native HeLa cell U2 snRNP contains a 20-21 nucleotide long, nuclease hyper-resistant region, the so-called Sm domain, or Sm core, spanning nucleotides 94-114 of U2 RNA (17-24). As

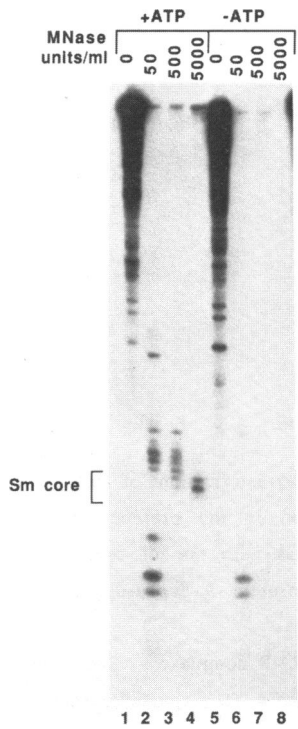

Figure 4: Nuclease digestion of assembled U2 snRNP particles. ^{32}P-U2 RNA assembled into particles in the presence or absence of ATP was centrifuged on glycerol gradients and the ~10S peak was recovered, dialyzed against SNURB and digested for 30 min. at 37°C with the concentrations of micrococcal nuclease shown. The samples were then deproteinized and analyzed by electrophoresis. The bracket labeled "Sm core" denotes the position of the 20-21 nt fragment that is resistant to digestion in native U2 snRNP.

can be seen in Figure 4, glycerol gradient-purified U2 RNP particles that assembled in the presence of ATP displayed the characteristic protected fragment after digestion with 5000 units of nuclease per ml (lane 4) whereas this fragment was not protected in the particles assembled in the absence of ATP (lane 8). In additional experiments, the 21 nucleotide fragment was digested with RNase H when incubated with an oligodeoxynucleotide complementary to nucleotides 110-127 of U2 RNA, but remained intact in the presence of RNase H and oligodeoxynucleotides complementary to other regions of U2 RNA (data not shown).

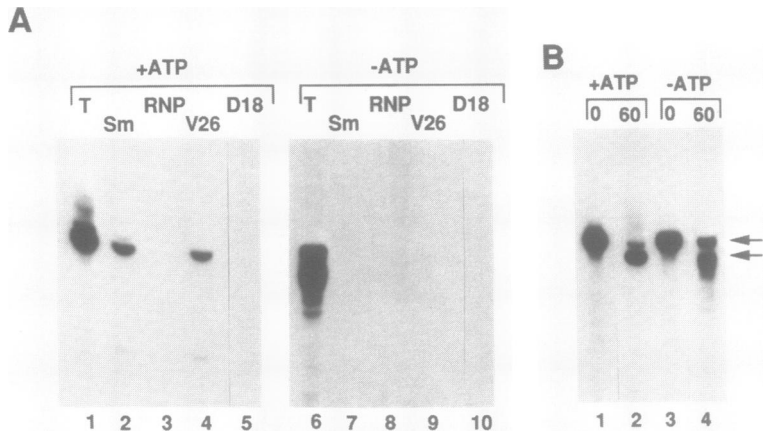

Figure 5: Immunochemical characterization of reassembled U2 snRNP particles. U2 snRNP particles were assembled in the presence or absence of ATP, recovered from glycerol gradients, and incubated with one of several snRNP antibodies. The antigenic complexes were recovered on protein A-Sepharose and the RNA was deproteinized and analyzed by electrophoresis.

A. T: total RNA from U2 RNP complexes. Sm: RNA from particles selected by Sm monoclonal antibody. RNP: RNA from particles selected by RNP monoclonal antibody. V26 and D18 antibodies are described in the text.

B. Total RNA isolated from the assembly reactions. "0" designates RNA isolated fom assembly reactions incubated at $0^{o}C$ for 60 min., and "60" designates RNA isolated following incubation at $37^{o}C$ for 60 min. The arrows to the right of lane 4 indicate the positions of the pre-U2 RNA (upper arrow) and the processed U2 RNA (lower arrow).

The U2 snRNP complex assembled in vitro contains both common and U2 snRNP-specific proteins

The presence of distinct proteins in the U2 snRNP particle was investigated with specific antibodies. As shown in Figure 5A, glycerol gradient purified particles assembled in the presence of ATP were selected by Sm monoclonal antibody (lane 2) and also by V26 (2), a human anutoantibody that reacts with the A′ and B" proteins of U2 snRNP and the A 70K proteins of U1 snRNP (lane 4). The particles assembled in the absence of ATP did not react with either of these antibodies (lanes 7 and 9). To ensure that the selection of V26 was due to its reactivity with U2 snRNP proteins two additional antibodies were used. These were RNP, which recognizes the 70K U1 snRNP protein, and D18, a human autoantibody that reacts predominately with the A and C U1 snRNP proteins and to a minor extent with the B and B′ proteins (Patton, J.R.,

Habets, W., van Venrooij, W. Pederson, T., manuscript submitted). Neither of these antibodies selected particles assembled in the presence (lanes 2 and 5), or in the absence (lanes 8 and 10), of ATP. Taken together, these immunochemical data establish that the particles assembled in the presence of ATP contain Sm-reactive proteins and at least one of the two U2 snRNP-specific proteins. They also establish that the U2 snRNP does not contain any of the three U1 snRNP-specific proteins.

We have consistently observed that U2 RNA recovered from particles assembled in the absence of ATP is slightly degraded (e.g. lanes 1 vs. 6 in Figure 5A). This is shown in more detail in Figure 5B, in which U2 RNA was isolated directly out of assembly reactions without glycerol gradient centrifugation of particles. In lane 4 it can be seen that although there is some degradation in the absence of ATP, the majority of the U2 RNA is intact precursor or mature-length U2 (arrows). Yet, none of these intact U2 RNA molecules assemble into Cs_2SO_4-stable or Sm/V26-antibody reactive particles in the absence of ATP. Therefore we suspect that the tendency for some U2 RNA degradation to occur in the absence of ATP is a result, rather than a cause, of its inability to assemble into a snRNP particle.

The selection of in vitro assembled U2 snRNP by U2-specific but not with U1-specific antibodies demonstrates the RNA sequence selectivity of the snRNP assembly reaction. To further investigate the sequence requirements for U2 snRNP assembly we constructed a U2 clone, $pG2\Delta U2^{pre}$, in which nucleotides 154-167 of U2 RNA are deleted (see Materials and Methods). This region of U2 RNA has been shown to be part of the binding domain of the U2 snRNP-specific proteins (22-24). We found that ΔU2 RNA was assembled into particles that were selected by Sm monoclonal antibody (Figure 6, lane 5), but not by the V26 antibody (lane 6). These results indicate that nucleotides 154-167 of U2 RNA, or sequences therein, are required for binding of the U2 snRNP-specific proteins A′ and B" in this in vitro system.

Pseudouridine formation in U2 RNA is enhanced under assembly-permissive conditions

One of the earliest recognized characteristics of U1 and U2 small nuclear RNAs was their content of pseudouridine residues (25). We have previously shown that pseudouridine formation is not required for U1 snRNP assembly (7). In the present study we have analyzed pseudouridine formation in more detail. As shown above, U2 snRNP assembly occurs only in the presence of ATP. We therefore compared the extent of pseudouridine formation in U2 RNA from particles assembled in the presence or absence of ATP. U2 RNA was recovered from the ~10S peaks of glycerol gradients, the RNA was digested with nuclease P1 and the resultant 5′-mononucleotides were analyzed by thin-layer chromatography (26). As can be seen in Table 1, the particles that assembled in the presence of ATP contained a 10-fold higher level of pseudouridine.

We also analyzed the extent of pseudouridine formation as a function of incubation

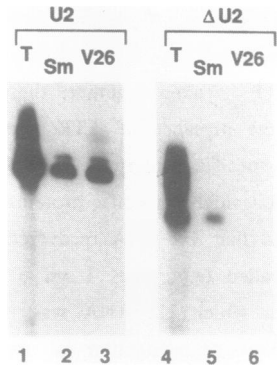

Figure 6: Sequences 154-167 of U2 RNA are required for binding of U2 snRNP-specific proteins in the assembly system.

U2 or ΔU2 RNP particles were assembled in the presence of ATP and CP, and reacted with antibodies. T: total RNA from U2 (lane 1) or ΔU2 (lane 4) RNP complexes. Sm: RNA from particles selected by Sm monoclonal antibody. V26: RNA from particles selected by V26 antibody.

time in the presence of ATP. Although the majority of assembly, as defined by Cs_2SO_4 stability, occurs within the first 5 min. (not shown), little or no pseudouridine formation is observed even at 15 min. (Table 1). Thus, U2 snRNP assembly does not lead to instantaneous pseudouridine formation in this system. At 60 min. (the standard asembly reaction time used for experiments in this paper), RNA in the 1.4 g/cm^3 region of the gradient had 13.7% of the theoretical pseudouridine content. This raises the possibility that pseudouridine formation occurs only on U2 RNA molecules that are present in snRNP complexes. To determine whether this is indeed the case, we took advantage of the fact that even in the presence of ATP not all of the U2 particles are native by the criterion of stability in Cs_2SO_4 gradients (see Figure 3A). Thus, the pseudouridine content of U2 RNA isolated from both the 1.4 and 1.6 g/cm^3 regions of a Cs_2SO_4 gradient was analyzed (Table 1). Although the extent of modification is higher in molecules from the 1.4 g/cm^3 region of the gradient, there is also significant modification of RNA from the 1.6 g/cm^3 region as well. Taken together these results show that, in this in vitro system pseudouridine formation is promoted under snRNP assembly-permissive conditions, but is not restricted to RNA that has formed a stable U2 snRNP particle.

DISCUSSION

The present experiments demonstrate that ribonucleoprotein particles similar to cellular U2 snRNP assemble when human U2 RNA is incubated in a HeLa cell S100 fraction

Table 1. Pseudouridine formation

	ψ (% of theoretical)[a]
60 min. +ATP[b]	11.0
-ATP[b]	1.5
15 min.[c] 1.4 g/cm^3	3.6
60 min.[c] 1.4 g/cm^3	13.7
60 min.[d] 1.4 g/cm^3	13.4
1.6 g/cm^3	8.4

[a]The pseudouridine content of human U2 RNA is unknown. The % of theoretical values were based on an assumed ψ content of 13 residues, the value for rat U2 RNA (26).
[b]RNA was isolated from the ~10S region of glycerol gradients
[c]+ATP
[d]Following P1 digestion the nucleotides were analyzed by one-dimensional thin-layer chromatography (27).

in the presence of ATP. The properties of this U2 RNP particle add to previous evidence (7,8) showing that this snRNP assembly system is RNA sequence-specific. The U2 snRNP particle that assembles in this system contains at least one of the two U2 snRNP-specific proteins but does not contain the three U1 snRNP-specific proteins (Figure 5), even though these latter proteins are present in this system in a U1 snRNP assembly-competent form (7,8, and Patton, J. R., Habets, W., van Venrooij, W. J., and Pederson, T., manuscript submitted). Furthermore, the ΔU2 mutant RNA results (Figure 6) show that specific U2 RNA sequences within nucleotides 154-167 are required for binding of the U2-specific proteins, confirming what has been observed in a Xenopus oocyte system (22,28).

Although the present experiments employed a U2 RNA precursor molecule, Cs_2SO_4-stable snRNP complexes were also assembled when a mature-length U2 RNA was added to this system (data not shown). Thus, although the assembly of U2 snRNP in vivo takes place on U2 precursor RNAs (16), the 3′ trailer sequence on the precursor is not necessary for snRNP assembly, at least in vitro. We also note that the in vitro synthesized U2 RNAs used as substrates for these experiments contain extra nucleotides at their 5′ ends (11). To the extent that the in vitro particle (assembled in the presence of ATP) resembles native U2 snRNP, it follows that these extra 5′ sequences do not interfere with proper RNP assembly.

One of the earliest recognized features of the U-snRNAs were their content of pseudouridine (25). In the present investigation, we have determined that in vitro

pseudouridine formation was enhanced under assembly permissive conditions but does not depend upon tight binding of the snRNP proteins. It is possible that this reflects a requirement of ATP for pseudouridine formation. However, no pseudouridine synthases characterized to date require ATP (29,30). Another interpretation of these results is that under assembly permissive conditions the sites on U2 that are to be modified become more accessible to the pseudouridine synthase. We also wish to point out that we have not analyzed the sites of pseudouridine formation in the reassembled U2 snRNP, nor have we determined whether the observed sub-stoichiometric level (11-14% of theoretical) represents pseudouridine formation at preferred, identical sites in each molecule or instead reflects a low level at all possible sites.

There is a striking difference between the U2 snRNP particles that assemble in the presence or the absence of ATP. In the absence of ATP, RNP complexes do form and sediment at approximately 10S in glycerol gradients (surprisingly the same S value as cellular snRNP's). Yet this U2 RNP assembled in the absence of ATP possesses none of the other characteristic properties of native U2 snRNP that were examined. In contrast, the particles assembled in the presence of ATP were similar to U2 snRNP in several respects, including banding in Cs_2SO_4 density gradients, pattern of digestion by micrococcal nuclease, reactivity with Sm antibody and content of U2 snRNP-specific proteins.

Although our HeLa S100 system requires exogenous ATP for U1 and U2 snRNP assembly, this is not the case for U1 snRNP assembly in Xenopus whole oocyte extracts (6). This may reflect differences in the amount of endogenous ATP present in the two extracts. A more intriguing possibility is that the snRNP proteins that are stockpiled in the oocyte (31) have already been "activated" for RNP assembly by a prior, ATP-dependent event during oogenesis. (In the HeLa S100 system, it is likely that the snRNP assembly observed reflects the participation of cytoplasmic snRNP proteins whose translation had just been completed in vivo prior to cell fractionation).

The assembly system we have developed will facilitate elucidation of additional structural aspects of the U2-snRNP particle. One potential disadvantage of this system however is the limited amount of U2 RNP that can be assembled. We estimate that in a typical reaction approximately 0.05 pmol of U2 snRNP is assembled, whereas there is approximately 1.5 pmol of endogenous U2 snRNP in this amount of S100 extract, and 15 pmol of endogenous U2 snRNP in a nuclear extract from a comparable number of cells. Thus, it will not be straightforward to assemble amounts of U2 snRNP sufficient to rescue splicing in a U2 snRNP depleted nuclear extract. Moreover, the presence of an approximately 30-fold excess of endogenous over assembled U2 snRNP in the S100 itself presents an additional difficulty. Alternatively, one can perform "tracer" experiments aimed at determining whether ^{32}P-labeled, assembled U2 snRNP can bind a

pre-mRNA. We have so far not detected specific binding of the assembled U2 snRNP to a human β-globin pre-mRNA under splicing-permissive conditions, nor has another group in a recent report (10). Therefore it is likely that the assembly system will need to be modified if binding- and/or splicing-competent U2 snRNP particles are to be assembled.

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