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Association of U2, U4, U5, and U6 small nuclear ribonucleoproteins in a spliceosome-type complex in absence of precursor RNA

(ribonucleoprotein complexes/small nuclear ribonucleoprotein complexes/pseudospliceosome/spliceosome)

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ABSTRACT **Small nuclear ribonucleoprotein particles (snRNPs) associate to form multi-snRNP complexes during splicing of mRNA precursors. A vast majority of the three snRNPs U4, U5, and U6 are present in a nuclear extract in a single complex, while U1 and U2 snRNPs exist as separate particles. Under conditions optimal for splicing *in vitro* the U4–U5–U6 (U4/5/6) complex dissociates to release free snRNPs, suggesting that the interactions between its components are dynamic. Several forms of splicing complexes assemble on precursor RNA during splicing *in vitro*. One of these forms, spliceosome B, contains U2, U4, U5, and U6 snRNPs bound to the precursor RNA. This same set of snRNPs associates efficiently in the absence of precursor RNA during incubation of the extract at high salt concentration. Formation of this U2–U4–U5–U6 (U2/4/5/6) complex, the pseudospliceosome, suggests that the basic structure of the spliceosome is specified by snRNP–snRNP interactions.**

Involvement of small nuclear ribonucleoprotein (snRNP) particles in splicing of nuclear precursor RNA (pre-RNA) is now well established (reviewed in refs. 1–4). Splicing of pre-RNA occurs in a large multicomponent complex, termed the spliceosome (5–7). Analysis of splicing complexes resolved by electrophoresis in native polyacrylamide gels has shown that the assembly of the spliceosome is based on sequential multiple interactions of snRNP particles and pre-RNA (refs. 8–11; Fig. 1). In particular, at least U2, U4, U5, and U6 snRNP particles interact stably with pre-RNA and/or with each other during spliceosome formation. The first stable splicing-specific complex to form, A, is generated by binding of the U2 snRNP to the region upstream of the 3′ splice site in pre-RNA. In the next step, a larger complex, B, is formed, apparently by association of complex A with a multi-snRNP particle containing U4, U5, and U6 snRNAs (11). Improved resolution of large splicing complexes has revealed the presence of another complex, C, which contains the splicing RNA intermediates, the 5′ exon, and the lariat form of intervening sequence–3′ exon (refs. 12 and 13; M.M.K., unpublished observations). Conversion of complex B to C is apparently correlated with the release of the U4 snRNP particle from the spliceosome (9, 13, 14). The final step of splicing results in release of the ligated exons and generation of complex I, which contains the lariat form of the intervening sequence bound to U2, U5, and U6 snRNPs (11). Here we provide evidence that, under certain conditions, U2, U4, U5, and U6 snRNPs, the identified components of the spliceosome, stably interact with each other in the apparent absence of pre-RNA, generating pseudospliceosome complexes.

MATERIALS AND METHODS

***In Vitro* Assays.** HeLa nuclear extracts were prepared as described by Dignam *et al.* (15) but were dialyzed against buffer D without KCl. A typical reaction mixture (10 μl) contained 3 μl of a HeLa nuclear extract, 2 mM $MgCl_2$, 1 mM ATP, 5 mM creatine phosphate, 6 mM Hepes (pH 7.6), 0.06 mM EDTA, 6% (vol/vol) glycerol, and 50 or 250 mM KCl or NH_4Cl. Splicing assays contained in addition ≈25 ng of unlabeled pre-RNA transcribed with T3 RNA polymerase from a *Sau*IIIA1-cleaved pBSAd1 plasmid (11). Incubations were for 1 hr at 30°C, unless indicated otherwise.

Separation of snRNP Particles. snRNP particles and splicing complexes were separated by electrophoresis in a nondenaturing 4% polyacrylamide gel, transferred to a nylon membrane, and detected by hybridization with snRNA or pre-RNA probes as described (11). For the gradient sedimentation analysis, the incubations were scaled up to 500 μl. Sedimentation was carried out in an SW41 rotor at 29,000 rpm for 16 hr at 4°C. Gradients were divided into 40 fractions (1–40, top to bottom; 450 μl each) and 30-μl aliquots of even numbered fractions were used for analysis in a native gel. Sedimentation coefficients of snRNP complexes were estimated by extrapolation from the positions of HeLa (28S, 18S, 5S) and *Escherichia coli* (23S, 16S) ribosomal RNAs separated in parallel gradients.

RESULTS AND DISCUSSION

Electrophoresis in native polyacrylamide gels combined with an RNA blot hybridization analysis permits a detailed description of associations between snRNP particles in nuclear extracts (11, 14). Two major snRNAs, U1 and U2, are found in discrete particles, which are apparently not stably associated with any other snRNPs. A vast majority of the U4, U5, and U6 snRNAs, on the other hand, are present in a single complex, U4–U5–U6 (termed U4/5/6) (11, 16). This large complex is remarkably stable under a variety of conditions, including a wide range of pH, temperature, and salt concentration. However, when samples of a HeLa nuclear extract are incubated in the presence of ATP under standard splicing conditions, this U4/5/6 complex disassembles to generate the U4–U6 (termed U4/6) particle and, finally, separate U4, U6, and U5 snRNPs (see Fig. 2*a*). The fact that under the same conditions the U4/5/6 complex participates in the spliceosome assembly to generate complex B (Fig. 1) and undergoes active disassembly to release separate snRNP particles suggests that interactions between snRNP components are quite dynamic.

To study the snRNP–snRNP interactions in more detail, we analyzed the stability of the U4/5/6 complex after incubation in the presence of ATP at various temperatures. As shown in Fig. 2*b*, when extract samples were incubated at 50 mM NH_4Cl, the U4/5/6 complex remained stable between 4 and 35°C, but further increases in temperature resulted in almost complete dissociation of the particle. When the same set of incubations was carried out at 250 mM NH_4Cl, a similar

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Abbreviations: snRNP, small nuclear ribonucleoprotein particle and all associated proteins; snRNA, small nuclear RNA; pre-RNA, precursor RNA.

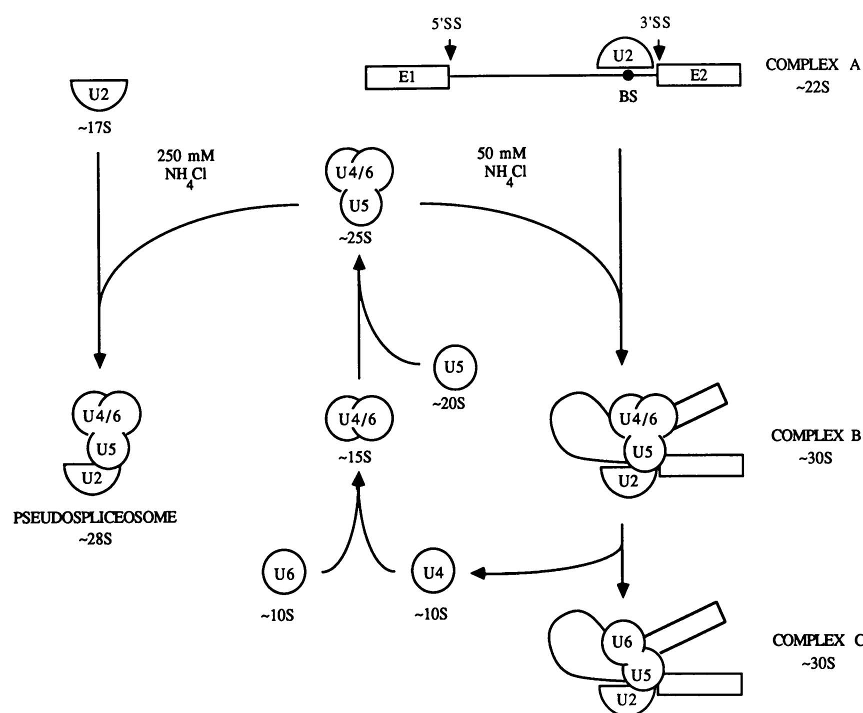

FIG. 1. Schematic representation of involvement of snRNP particles in formation of spliceosome and spliceosome-like complexes. Relative positions and molar ratios of snRNPs in various complexes are arbitrarily illustrated.

pattern of snRNPs was observed, except that at 30–35°C an additional slowly migrating complex was detected. Complete RNA blot analysis indicated that, in addition to U4, U5, and U6 snRNAs, this complex also contained the U2 snRNA (Fig. 3; data not shown). However, U1 RNA, the most abundant snRNA in the extract, was not detected in this complex (data not shown). Thus, the new complex has a snRNP composition characteristic of the splicing complex B. Formation of the spliceosome-like, or pseudospliceosome, complex occurs at comparable concentrations of other salts (KCl, NaCl, LiCl, KNO_3, $NaC_2H_4O_2$; data not shown), indicating that a specific ionic strength is the important factor. More detailed analysis of the effects of ionic strength on formation of multi-snRNP complexes suggests that the assembly of the U2–U4–U5–U6 (termed U2/4/5/6) pseudospliceosome complex and the authentic splicing complexes are separate processes (Fig. 3). In reaction mixtures containing nuclear extract, the U2/4/5/6 complex formed between 200 and 300 mM NH_4Cl and was not detected at lower salt concentrations. In contrast, in similar splicing reaction mixtures containing, in addition, pre-RNA, formation of the splicing complexes A, B, and C was detected under low but

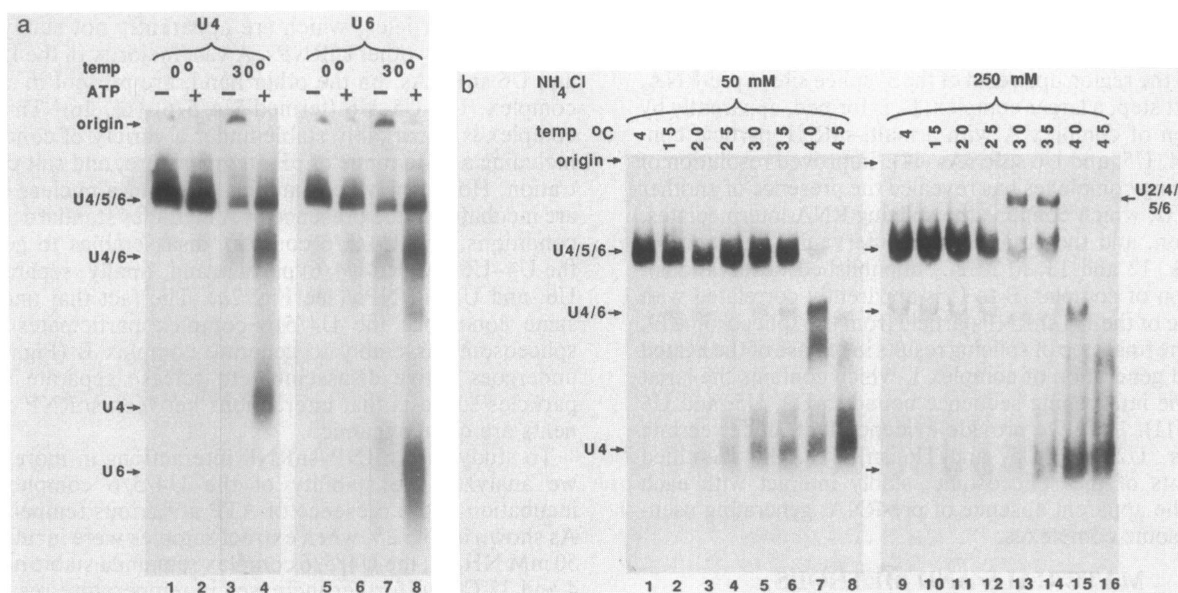

FIG. 2. Disassembly of the U4/5/6 snRNP complex and generation of pseudospliceosomes. (*a*) Reaction mixtures were incubated for 1 hr at 0°C or 30°C, in the presence or absence of ATP, as indicated. snRNP particles were separated by electrophoresis in a 4% polyacrylamide gel, transferred to a nylon membrane, and detected by hybridization of the blot with RNA probes complementary to U4 (lanes 1–4) and U6 (lanes 5–8) snRNAs, as described (11). (*b*) Reaction mixtures similar to those in *a*, but containing 50 mM (lanes 1–8) or 250 mM (lanes 9–16) NH_4Cl instead of 50 mM KCl, were incubated in the presence of ATP for 45 min at various temperatures, as indicated. snRNP complexes were detected by hybridization with a U4-specific RNA probe. Positions of major snRNP complexes are indicated.

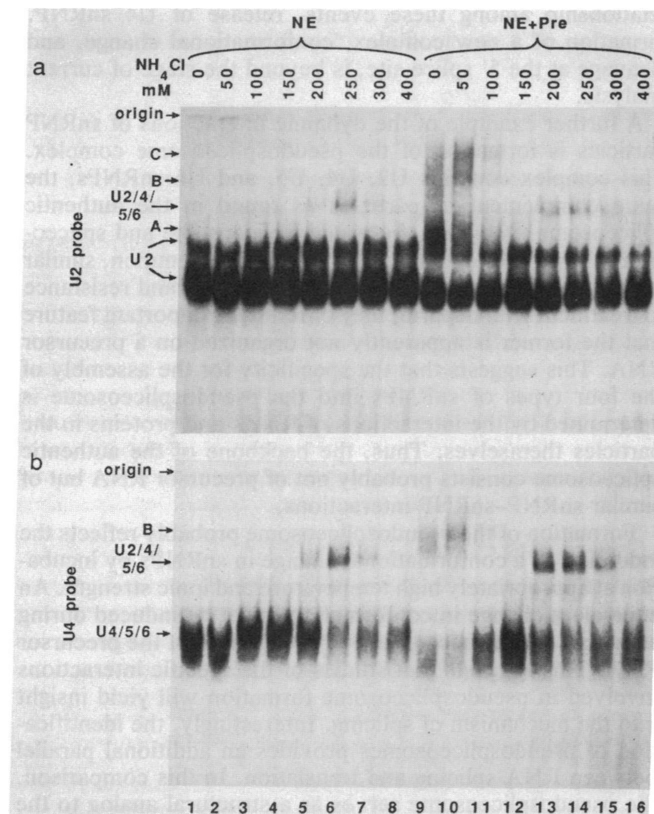

FIG. 3. Spliceosomes and pseudospliceosomes are generated by separate processes. Samples of HeLa nuclear extract were incubated in the presence of increasing concentrations of NH_4Cl (from 0 to 400 mM, as indicated) and ATP for 45 min at 30°C (lanes 1–8). Identical reaction mixtures containing, in addition, pre-RNA were analyzed in parallel (lanes 9–16). The single blot was sequentially hybridized with RNA probe complementary to U2 (*a*) and U4 (*b*) snRNAs. Positions of major snRNP and spliceosome complexes are indicated. The identity of spliceosome complexes A, B, and C was, in addition, confirmed by hybridization of the same blot with a pre-RNA-specific probe. This probe did not detect pre-RNA in pseudospliceosome complexes (data not shown).

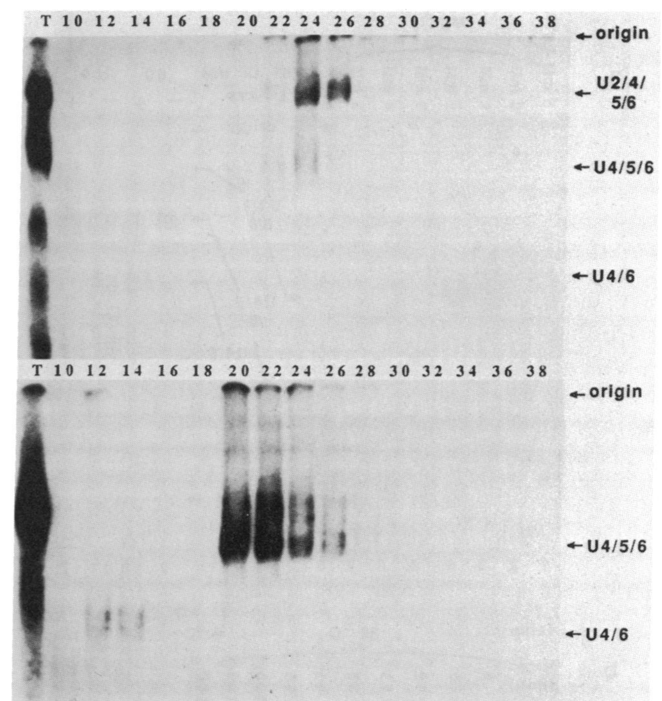

FIG. 4. Sedimentation analysis of multi-snRNP complexes. Samples of a HeLa nuclear extract were incubated in the presence of 250 mM (*Upper*) or 50 mM (*Lower*) NH_4Cl for 2 hr at 30°C and products were sedimented through a 10–30% glycerol, 50 mM Tris-glycine gradient. Aliquots (50 μl) of even-numbered fractions of the gradient (fractions 10–38; top to bottom) and of a total reaction (T) were resolved in a 4% polyacrylamide gel. Under these conditions, a vast majority of U4, U5, and U6 snRNA-containing particles was present in the U4/5/6 or U2/4/5/6 complexes; only small amounts of separate monomeric particles were detected in fractions 1–10 of the gradient (data not shown; see also Figs. 2*b* and 5*a*). U4 snRNP-containing particles were detected by hybridization with RNA probe complementary to U4 snRNA.

not high salt conditions (Fig. 3). The multi-snRNP complexes were also analyzed by sedimentation through a glycerol gradient. snRNP particles present in fractions of the gradient were then separated in a native gel and detected by the RNA blotting technique (Fig. 4). Samples of nuclear extract incubated at 250 mM NH_4Cl contained the U2/4/5/6 pseudospliceosome complex sedimenting at ≈28S (Fig. 4 *Upper*). It should be noted that after prolonged incubation (2 hr) under these conditions almost all of the U4/5/6 complex has been converted to the pseudospliceosome form (see also Fig. 5*b*). Similar reaction mixtures incubated at low salt contained the typical set of particles including the U4/5/6 (25S); U4/6 (15S); U2 (17S); U5 (20S); and U1, U4, and U6 (10S) snRNPs (Fig. 4 *Lower*). Splicing complexes formed in the presence of precursor RNA sedimented in a parallel gradient at ≈30S (complexes B and C), 29S (complex I), and 22S (complex A; data not shown; ref. 11).

The pseudospliceosome complex was optimally generated at low Mg^{2+} concentrations and, unlike the splicing complexes, formed in the absence of Mg^{2+} ions (Fig. 5*a*). This association of U2, U4, U5, and U6 snRNPs was typically stimulated by the addition of ATP, although the degree of stimulation varied with different preparations of nuclear extract (Fig. 5*a*; data not shown). Finally, the pseudospliceosome complex did not preexist in the extract; rather, it was generated *de novo* during incubations carried out under appropriate conditions. This is indicated by the time course shown in Fig. 5*b*, in which the pseudospliceosome complex was first detected after 5–10 min of incubation at 30°C and continued to accumulate for at least 2 hr. Accumulation of the U2/4/5/6 complex was not observed when the incubation was carried out for 3 hr at low temperature (4°C). Both spliceosomes and pseudospliceosomes were resistant to treatment with a polyanion, heparin (5 mg/ml; 5 min at 30°C), while the endogenous U4/5/6 snRNP complex was completely destroyed under these conditions (data not shown). This heparin resistance indicates a highly stable association of snRNPs in both spliceosome and pseudospliceosome complexes.

We cannot exclude the possibility that the pseudospliceosome complex is assembled on endogenous RNAs in the extract. However, we consider this possibility unlikely, since (*i*) pseudospliceosomes form under conditions that do not permit assembly of splicing complexes on exogenous pre-RNA, and (*ii*) pseudospliceosomes do not preexist in the nuclear extract. Furthermore, analysis of RNA cosedimenting in a glycerol gradient or comigrating in a native gel with the pseudospliceosome complex detected only the expected U2, U4, U5, and U6 snRNAs (data not shown).

We have previously proposed that the interactions between snRNP particles are quite dynamic (11). The observed dependence of the disassembly of the U4/5/6 complex on incubation at 30°C and in the presence of ATP (i.e., splicing conditions) is consistent with this suggestion. The first indication of the dynamic nature of snRNP interactions was the discovery that during splicing U4 snRNP was released as

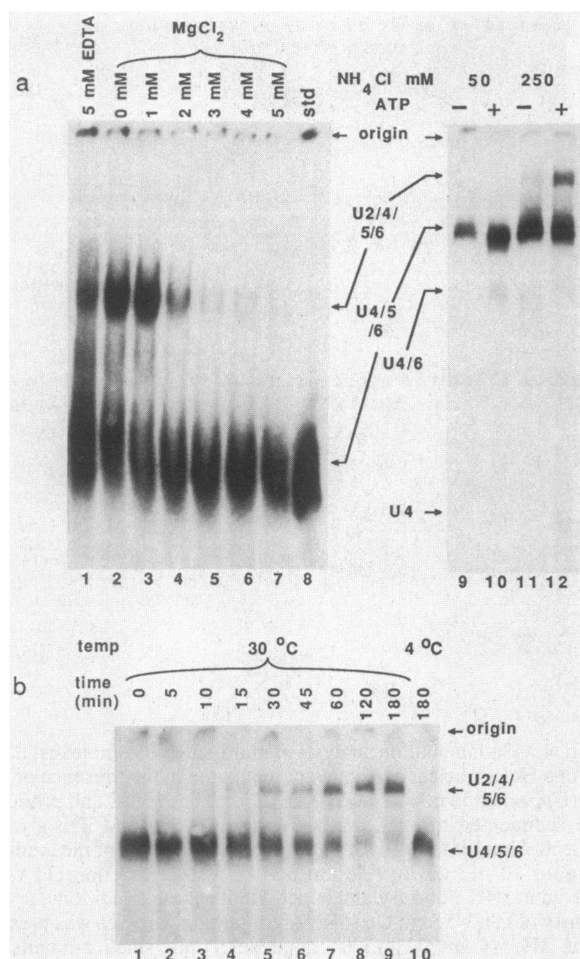

FIG. 5. Requirements for generation of pseudospliceosome complexes. (*a*) Samples of HeLa nuclear extracts were incubated in the presence of 50 mM (lanes 8–10) or 250 mM (lanes 1–7, 11, and 12) NH_4Cl for 30 min at 30°C. Reaction mixtures in lanes 2–7 contained increasing concentrations of $MgCl_2$ or EDTA, as indicated. Standard conditions (std, lane 8) are as in Fig. 2*a*. Reactions in lanes 9–12 were carried out in the presence of 2 mM $MgCl_2$, and in lanes 9 and 11 ATP was omitted. (*b*) Time course of formation of pseudospliceosome complexes. Reaction mixtures as in *a* (lane 4 or 12) were incubated in the presence of 250 mM NH_4Cl for the indicated period of time. All reactions were separated in 4% polyacrylamide gels and blots were hybridized with an RNA probe complementary to U4 snRNA. Positions of major snRNP complexes are indicated.

a separate particle from a complex containing the intron and U2, U5, and U6 snRNPs (11). Reassembly of the U4 snRNP into a U4/6 and, finally, into a U4/5/6 complex would be required for participation of this stable particle in a second splicing reaction. Release of U4 snRNP from the spliceosome occurs simultaneously with generation of a new slower migrating complex in native gels (13, 14), probably reflecting a conformational change within the multi-snRNP particle. Formation of this complex is also correlated with the first step in splicing, cleavage at the 5′ splice site and generation of the lariat intermediate. Establishment of the functional relationship among these events, release of U4 snRNP, formation of a new complex, conformational change, and cleavage at the 5′ splice site, is beyond the stage of current analysis.

A further example of the dynamic interactions of snRNP particles is formation of the pseudospliceosome complex. This complex contains U2, U4, U5, and U6 snRNPs, the same complement of particles as found in the authentic spliceosome. Although the pseudospliceosome and spliceosome show several additional properties in common, similar mobility in native gels and glycerol gradients and resistance to treatment with heparin, they differ in the important feature that the former is apparently not organized on a precursor RNA. This suggests that the specificity for the assembly of the four types of snRNPs into the pseudospliceosome is determined by the interactions of RNAs and proteins in the particles themselves. Thus, the backbone of the authentic spliceosome consists probably not of precursor RNA but of similar snRNP–snRNP interactions.

Formation of the pseudospliceosome probably reflects the induction of a conformational change in snRNPs by incubation at appropriately high temperature and ionic strength. An equivalent change in conformation might be induced during assembly of the spliceosome by recognition of the precursor RNA. This suggests that studies of the specific interactions involved in pseudospliceosome formation will yield insight into the mechanism of splicing. Interestingly, the identification of pseudospliceosomes provides an additional parallel between RNA splicing and translation. In this comparison, the pseudospliceosome serves as a structural analog to the free unbound 80S ribosomal particle.

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