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The Spliceosome Assembly Pathway in Mammalian Extracts

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A mammalian splicing commitment complex was functionally defined by using a template commitment assay. This complex was partially purified and shown to be a required intermediate for complex A formation. The productive formation of this commitment complex required both splice sites and the polypyrimidine tract. U1 small nuclear ribonucleoprotein (snRNP) was the only spliceosomal U snRNP required for this formation. A protein factor, very likely U2AF, is probably involved in the formation of the splicing commitment complex. From the kinetics of appearance of complex A and complex B, it was previously postulated that complex A represents a functional intermediate in spliceosome assembly. Complex A was partially purified and shown to be a required intermediate for complex B (spliceosome) formation. Thus, a spliceosome pathway is for the first time supported by direct biochemical evidence: RNA + U1 snRNP + ?U2 auxiliary factor + ?Y → CC + U2 snRNP + Z → A + U4/6,5 snRNPs + β → B

The spliceosome is the macromolecular enzyme that catalyzes the splicing of precursors to mRNAs (pre-mRNAs) (7, 11, 15). The assembly of the spliceosome in extracts of baker's yeast (*Saccharomyces cerevisiae*) and human HeLa cells has been postulated to involve at least two stable intermediates (8, 16, 20, 21, 30, 32, 36, 39). The proposed pathway is pre-mRNA → U1 small nuclear ribonucleoprotein (snRNP) complex (commitment complex) → prespliceosome (complex A) → spliceosome (complex B). The U1, U2, U4/6,5 and snRNPs, which have been shown to be required for spliceosome formation (1, 3–5, 25, 38), are added successively in each step in the proposed assembly pathway. Protein factors that are not intrinsic snRNP components have also been shown to be required for pre-mRNA splicing by using biochemical complementation assays in HeLa cell extracts (13, 22, 24, 43). Although this process has been intensely studied, formal biochemical proof for the proposed assembly pathway is lacking.

A splicing commitment activity in yeast nuclear extracts does not require exogenous ATP (27, 39). This commitment activity requires U1 snRNP as a *trans*-acting factor and requires the 5′ splice site and branch point sequence as *cis*-acting elements (39, 40). Formation of two stable U1 snRNP-containing complexes (CC1 and CC2) correlates with the presence of this activity, strongly suggesting that these complexes are chased into the spliceosome and contain the bulk of the commitment activity (27, 39, 40).

An ATP-independent commitment activity in HeLa nuclear extracts has been described recently (30). ATP-independent complexes have been shown to form in HeLa nuclear extracts by gel filtration (complex E) (30, 34) and by gel electrophoresis (−ATP complex) (19). Although fractions containing complex E have commitment activity, this activity has not been shown to copurify with complex E, and therefore the role of this complex is uncertain (30). The −ATP complex, which shares many properties with complex E, also may have some commitment activity; however, the −ATP complex does not copurify with the bulk of this activity (see below). Thus, although a commitment activity has been shown to exist in mammalian extracts (30), the complex in which this activity resides has not been formally identified.

The formation of complex A, which involves the binding of U2 snRNP to the branch site (2, 8, 11, 15, 16, 20, 32), is thought to be the step immediately following commitment complex formation, predicting RNA→CC→A (30, 39). Formation of complex A in HeLa extracts has been shown to require U2 snRNP (1, 3) and several protein factors (13, 22, 24, 43). It is not clear whether or not these non-snRNP factors are required for this step or for the prior formation of the commitment complex. The formation of this complex in these extracts requires a polypyrimidine tract (11, 12), a branch point sequence (17, 35), and usually a 3′ splice site (12, 26). The formation of complex A requires the hydrolysis of ATP (21, 32), a phenomenon that is not yet mechanistically understood.

Complex A has been postulated to be a required intermediate in the formation of complex B (spliceosome) (8, 21, 30, 32). Therefore, the proposed scheme to arrive at complex B in HeLa extracts is RNA→CC→A→B. In yeast extracts, it has been possible to identify other intermediates in the spliceosome pathway by using genetics and biochemistry (8, 32). In both yeast and mammalian extracts, the spliceosome is thought to form when the U4/6,5 multi-snRNP complex associates with complex A (21, 28). Fractions that contain partially purified complex A are known to have complex B-forming activity (30). It is not clear, however, whether this activity copurifies with complex A and whether all of complex B is derived from complex A.

In this report, we present evidence for the formation of a mammalian splicing commitment complex that can be partially purified in glycerol gradients. All of the commitment activity detected in nuclear extracts in the absence of ATP resides in this complex. The commitment complex requires wild-type 3′ and 5′ splice sites and a wild-type polypyrimidine tract of an adenovirus-derived intron for efficient formation. Furthermore, this complex can be chased into complex A and into spliceosomes. We show that formation of the commitment complex requires U1 snRNP but not the

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other U snRNPs in the spliceosome. Moreover, we show that all of the detectable complex B-forming activity resides in complex A. Thus, we present formal evidence for the RNA→CC→A→B spliceosome assembly pathway.

MATERIALS AND METHODS

Plasmids, RNAs, and nuclear extracts. pPIP7.A was described previously (14). pPIP3, pPIP3py5, and pPIP3py6 were all described previously (12). pPIP4 was constructed by cloning the complementary oligonucleotides 5′-GGACAAA CTCTTCGCGGTCTTTGCATG-3′ and 5′-CAAAGACCGC GAAGAGTTTGTCCTGCA-3′ into *Pst*I- and *Sph*I-linearized pPIP3. This introduced more exonic adenovirus -2-derived sequences into the second exon of pPIP4. pPIP4.2 was cloned from pPIP4 by replacing the *Kpn*I-*Bam*HI fragment with a mutant *Kpn*I-*Bam*HI fragment derived from an oligonucleotide that changes the wild-type 5′ splice site sequence from GGG:GUGAGU to GGG:AUGAAU. The sequences of all plasmid inserts were determined by dideoxy sequencing, using Sequenase 2.0 (U.S. Biochemical) (37).

All RNAs were synthesized as described before (12, 29) except that RNAs were labeled with [α-^{32}P]UTP at a final specific activity of 300 Ci/mmol in the labeling reaction. Competitor RNAs were labeled in a reaction mixture containing approximately 3.75 mCi of [α-^{32}P]UTP per mmol. All RNAs were transcribed with T7 RNA polymerase (New England Biolabs) from templates linearized with *Hin*dIII. Nuclear extracts were prepared by the method described by Dignam et al. (10).

Splicing reactions. Splicing reactions were carried out as described previously (12). Final concentrations were 33% (vol/vol) for nuclear extract, 56 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, and 5 mM creatine phosphate. Reactions were incubated at 30°C for various lengths of time, depending on the assay used (see diagrams and figure legends). Reactions were placed on ice and supplemented with heparin (Sigma catalog no. H9266) to a final concentration of 0.5 mg/ml and incubated at 30°C for 5 min. Splicing reactions (5 to 7μl) were loaded on nondenaturing polyacrylamide gels for visualization of splicing complexes (19, 20). Electrophoresis was at 200 V (12.5 V/cm) at room temperature for 3 h. Gels were dried at 80°C under vacuum for 1 h. Splicing complexes were visualized by autoradiography with XAR-5 film and one intensifying screen. Reactions carried out to identify splicing intermediates, products were incubated for 40 min at 30°C, and the RNA was extracted and loaded on a 15% denaturing polyacrylamide gel and visualized as described above.

Sedimentation analysis and chase assays. For each glycerol gradient, a 100-μl splicing reaction mixture containing 1 × 10^6 to 10 × 10^6 cpm of ^{32}P-labeled precursor was incubated for 10 min at 30°C, supplemented with 0.5 mg of heparin per ml, and further incubated for 5 min at 30°C. −ATP reactions contained no exogenous ATP or creatine phosphate. A 100-μl volume of the total reaction mixture was loaded on a 2.1-ml gradient of 10 to 30% (wt/vol) glycerol in 6.4 mM [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9)–2 mM $MgCl_2$–50 mM KCl. Centrifugation was carried out in a TLS55 rotor (Beckman) at 55,000 rpm for 2.5 h at 4°C, and 20 100-μl fractions were collected (20). The Cerenkov counts was determined by counting 12.5 μl of each fraction in a scintillation counter (LKB-Betarack). Gradient fractions were analyzed by nondenaturing polyacrylamide gel electrophoresis as described above; 12.5 μl of the gradient fractions was used in the commitment assay described above (see Fig. 2A).

snRNP-depleted extracts. The method of specific U snRNP depletion was previously described (1, 5, 25). Extracts were prepared in the laboratory of Angus Lamond (EMBL). The specific depletion of U1 and U2 by this method was determined by Northern blot analysis of native gels (19, 21). The U5-depleted extract was shown to also have very low levels of the U4/6 snRNP.

Nuclear extract fractionation. HeLa cell nuclear extract prepared by the method of Dignam et al. (10) was dialyzed against buffer D with 500 mM KCl; 1.6 ml (28.6 mg) of this extract was loaded on a poly(U)-agarose column (cross-sectional area, 0.39 cm^2; height, 2.6 cm; volume, 1.1 ml) and run at a flow rate of 2.5 ml/h/cm^2. The column had been equilibrated several hours before the nuclear extract was applied. The column was washed with buffer D with 500 mM KCl (fraction I) and then sequentially eluted with buffer D with 1.5 M KCl (fraction II) and with buffer D with 100 mM KCl and 2 M guanidine HCl (fraction III). Column fractions were screened for protein by using the Bio-Rad reagent. Column fractions containing the protein peaks from the wash and the two-step elutions were pooled and dialyzed against buffer D with 50 mM KCl, yielding fractions I, II, and III for use in the reconstitution assay described above.

RESULTS

A splicing commitment activity in HeLa nuclear extracts. The experimental scheme for the detection of splicing commitment activity in HeLa nuclear extract is presented in Fig. 1A. A pre-mRNA, PIP7.A, derived from the adenovirus major late promoter transcription unit, forms splicing specific complexes in the absence of added ATP (* in Fig. 1C) and in the presence of 1 mM ATP and 5 mM creatine phosphate (A and B in Fig. 1C; lanes 1 and 2). If, however, the HeLa nuclear extract was preincubated with a 1,000-fold molar excess of unlabeled PIP7.A pre-mRNA, the formation of all splicing complexes was inhibited by more than 90% (Fig. 1B and C, lanes 13 and 14). In contrast, if labeled PIP7.A pre-mRNA was incubated with nuclear extract in the absence of added ATP first, then incubated with a 1,000-fold molar excess of unlabeled PIP7.A pre-mRNA, and finally incubated with 1 mM ATP and 5 mM creatine phosphate, the labeled PIP7.A was able to form splicing complexes (Fig. 1C, lanes 7 and 8). These results show that a stable interaction between the splicing machinery and the pre-mRNA is established in the absence of added ATP, as was first described for yeast extracts by LeGrain et al. (27). This stable interaction is the commitment activity. We show below that all of the detectable commitment activity resides in a complex which we will refer to as a commitment complex.

Development of the commitment complex was rapid and clearly seen above the preincubation level of background after only 1 min of incubation (Fig. 1C, lane 4). The level of commitment complex that could be chased to complex A increased from 1 to 3 min of incubation (lanes 4, 6, 8, 10, and 12). The incubation being varied in this experiment is the first incubation shown in Fig. 1A. The commitment complex detected in these assays with the PIP7.A RNA was shown to be functionally competent by the detection of spliced products and intermediates (Fig. 1D).

The same commitment activity was shown with a rabbit β-globin pre-mRNA by detection both of complexes A and B and of spliced products (data not shown). Thus, the ATP-independent splicing commitment activity is most likely a general phenomenon of in vitro pre-mRNA splicing.

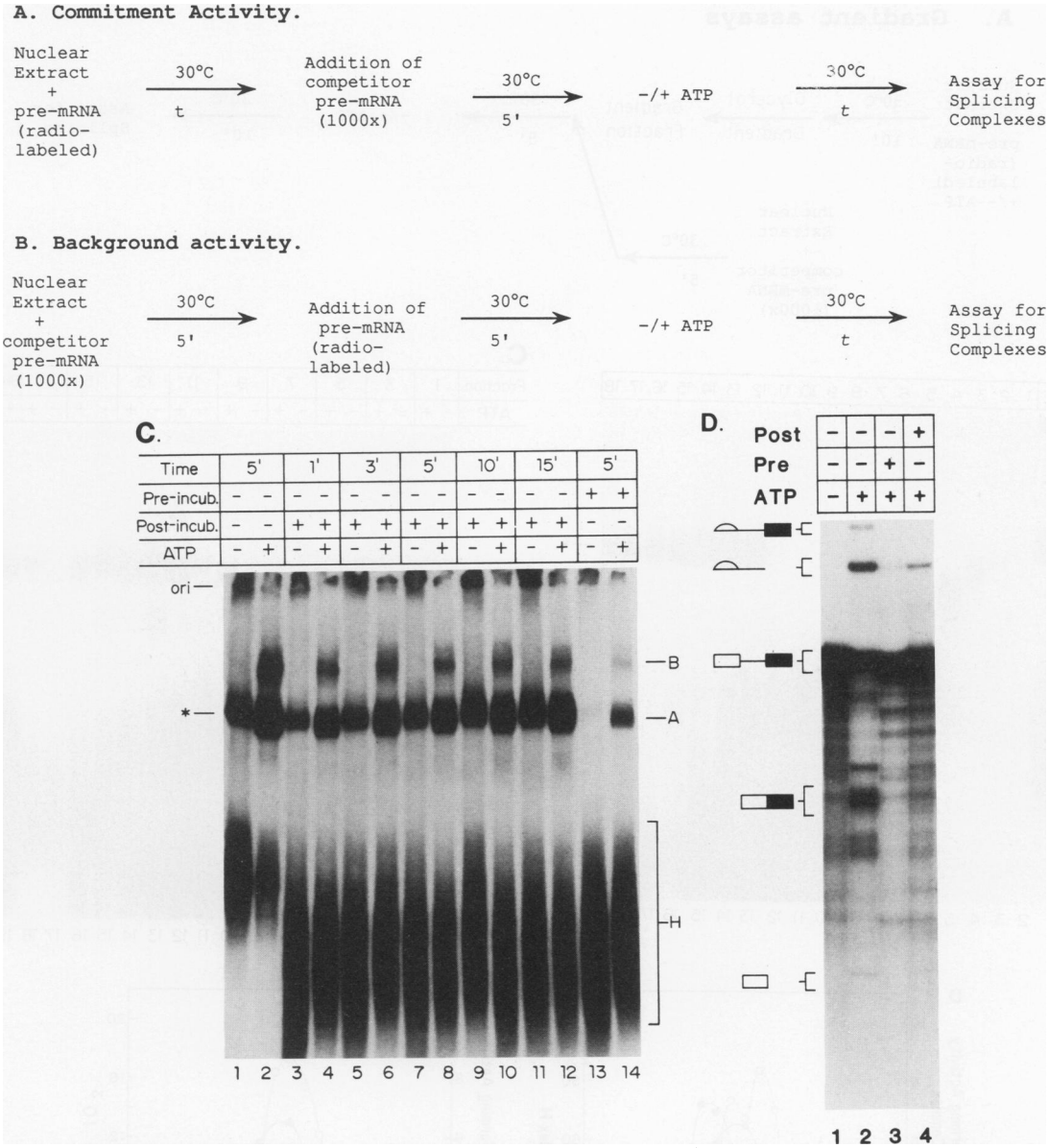


FIG. 1. Evidence that a commitment activity in HeLa nuclear extracts does not require addition of ATP. The adenovirus-derived PIP7.A pre-mRNA, which was uniformly labeled, was incubated with HeLa cell nuclear extract. The mixtures were supplemented with 1,000-fold unlabeled PIP7.A pre-mRNA as a competitor and incubated further in the presence of 1 mM ATP and 5 mM creatine phosphate. The level of commitment activity was determined by assaying for the formation of splicing complexes. (A) Schematic of the splicing commitment assay. For experiments shown in panel C, the time of the first incubation was varied (t); otherwise, it was 5 min. (B) Schematic of the control for the splicing commitment assay used to determine the background in the assay. (C) Formation of splicing complexes in a native gel. The last incubation, shown in schematics A and B, was carried out either in the absence of added ATP (odd-numbered lanes) or in the presence of 1 mM ATP and 5 mM creatine phosphate (even-numbered lanes). The levels of complexes in the absence of any competitor are shown in lanes 1 and 2. In the absence of added ATP, the −ATP complex (*) is formed; in the presence of ATP, complex A (prespliceosome) and complex B (spliceosome) are formed (A and B, respectively). The nonspecific hnRNP complexes (H) form under both conditions. The duration of the first incubation varied from 1 to 15 min, as indicated. The background is given by the level of complexes shown in lanes 13 and 14. ori, origin. (D) Denaturing gel showing spliced intermediates and products from the commitment assay described above. Postincubation with unlabeled RNA competitor as depicted in schematic A and preincubation as described in schematic B are indicated. Icons, from top to bottom, indicate migration of lariat intermediate, lariat product, pre-mRNA, mRNA (spliced product), and free 5′ exon.

The commitment complex is a required intermediate in complex A formation. Nuclear extract was incubated with labeled PIP7.A pre-mRNA under splicing conditions except that ATP was not added. The reactions were subjected to sedimentation in glycerol gradients. The gradient fractions were supplemented with precompeted nuclear extract and incubated in the presence of ATP. The reaction mixtures were then assayed for the formation of complexes A and B. This assay revealed which of the gradient fractions contained pre-mRNA committed to the spliceosome formation pathway (Fig. 2A).

The complexes that separated in glycerol gradients were

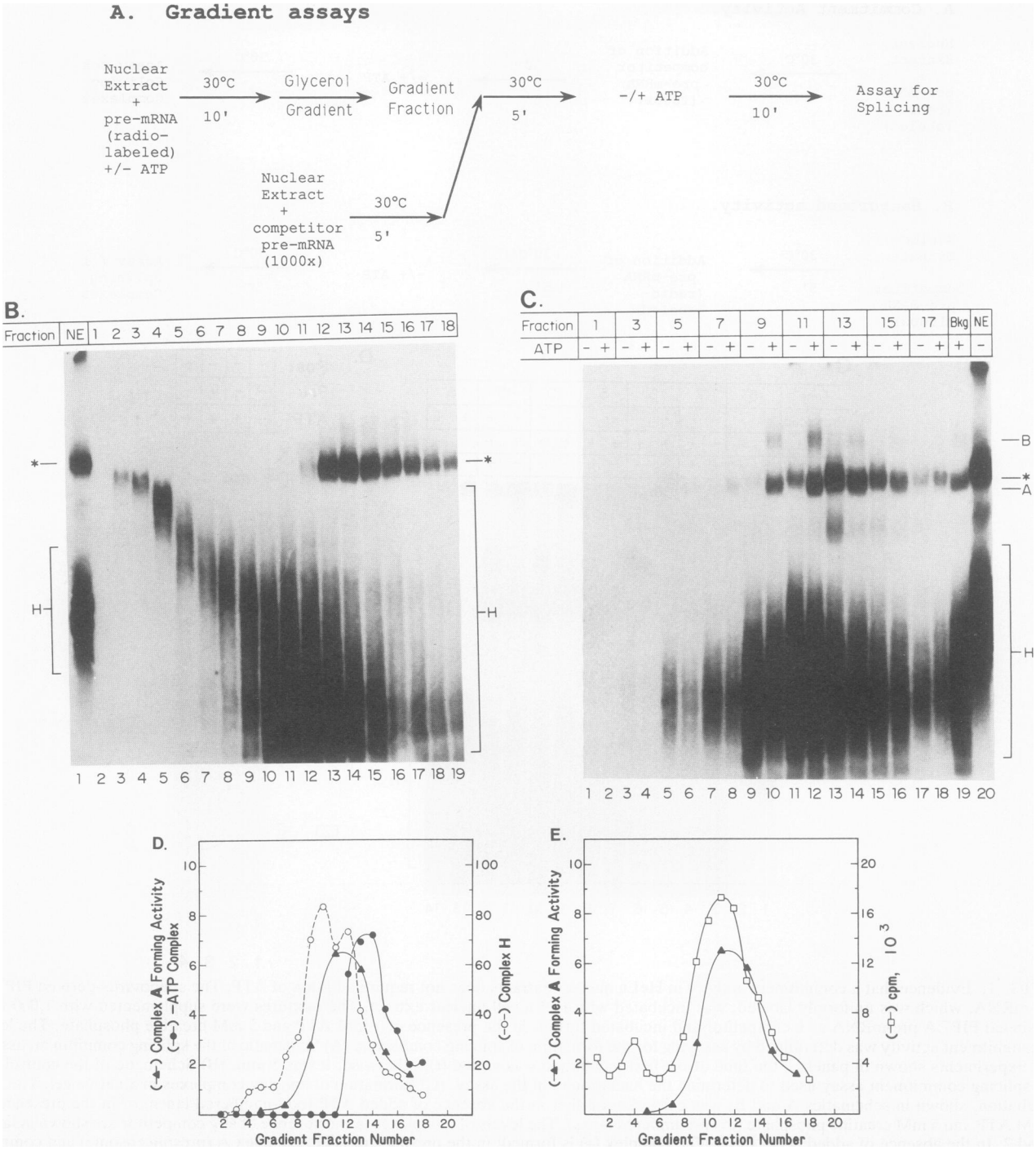


FIG. 2. Evidence that a commitment complex contains all of the commitment activity. Nuclear extracts were incubated with PIP7.A pre-mRNA in the absence of ATP as described for Fig. 1. The incubated reactions were subjected to glycerol gradient sedimentation, and the fractions were analyzed for commitment activity. (A) Schematic of the commitment assay. (B) Assay in which splicing complexes were separated on a glycerol gradient and visualized by native gel electrophoresis and autoradiography. Complexes formed in the absence of ATP in the reaction are shown in lane 1. Complexes separated in the glycerol gradient are shown in lanes 2 through 19 (fraction numbers are indicated above the lanes; NE, nuclear extract). Nonspecific hnRNA complexes (H) and the −ATP complex (*) are indicated. (C) Assay in which gradient fractions were chased as depicted in panel A. The chase of the odd-numbered glycerol gradient fractions is shown in lanes 1 through 18; 1 mM ATP and 5 mM creatine phosphate were added to chases shown in even-numbered lanes. Background levels (lane 19) were determined as shown in Fig. 1B. A splicing reaction containing 1 mM ATP and 5 mM creatine phosphate was run in lane 20 to serve as a positive control for splicing complexes A and B. (D) Quantification of gradient fractions. The complex A-forming activity (commitment complex), −ATP complex levels, and level of complex H are plotted versus glycerol gradient fraction number. The relative units were determined by scanning the autoradiographs with a laser scanner (LKB). (E) The complex A-forming activity (commitment complex) and total counts per minute plotted versus glycerol gradient fraction number.

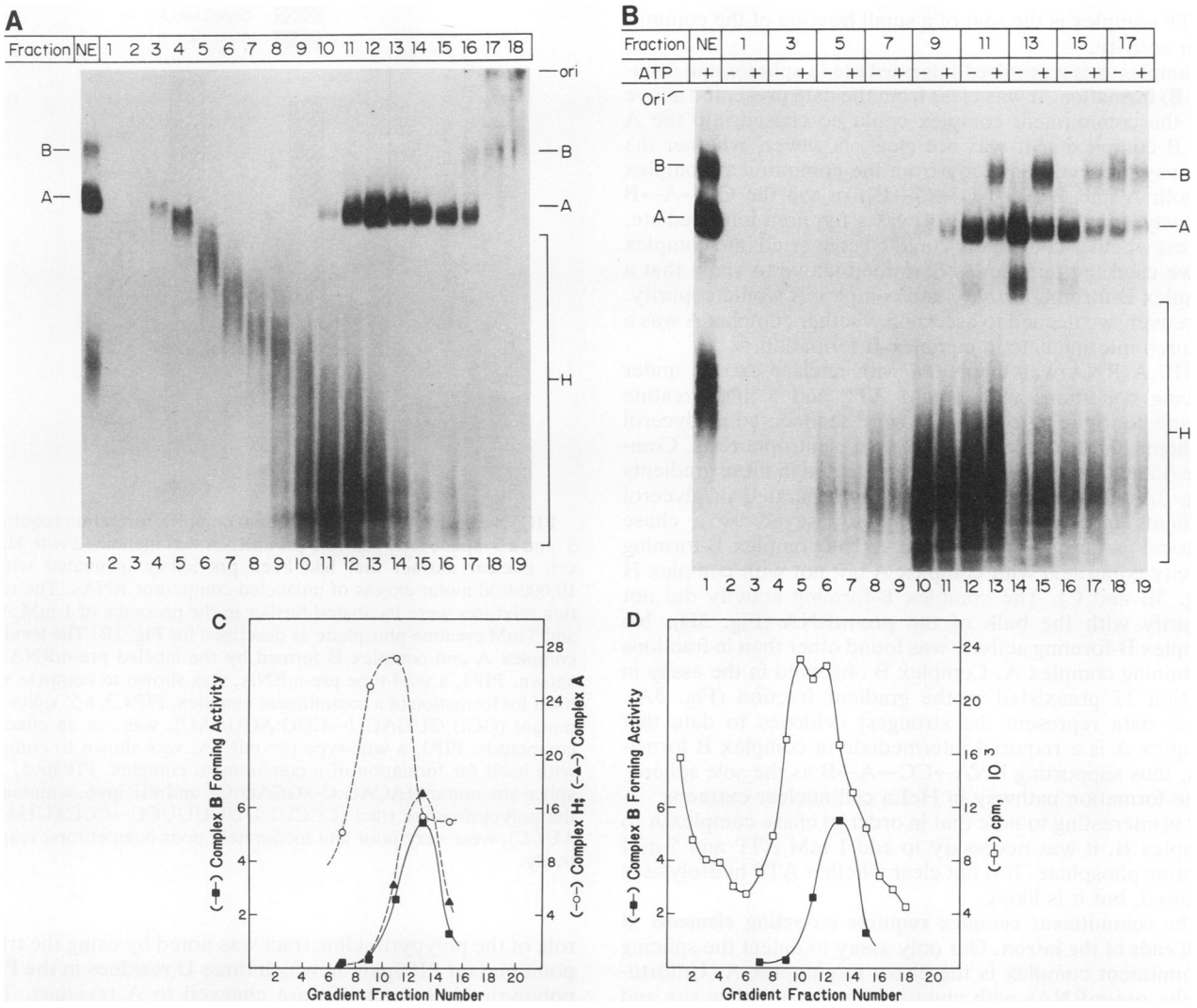


FIG. 3. Evidence that all of the complex B-forming activity resides in complex A. Nuclear extracts were incubated with PIP7.A pre-mRNA in the presence of added ATP and creatine phosphate as described for Fig. 1. The reaction mixture was subjected to glycerol gradient sedimentation, and gradient fractions were analyzed for the formation of splicing complexes on native polyacrylamide gels. (A) Assay in which splicing complexes separated in the glycerol gradient were visualized by native gel electrophoresis and autoradiography. An aliquot of the reaction mixture which was not subjected to glycerol gradient sedimentation is shown in lane 1. Lanes 2 through 19 show separation of the splicing complexes. Gradient fractions are indicated above the lanes. NE, nuclear extract. Complex B, complex A, and complex H are indicated. ori, origin. (B) Chase reactions of the odd-numbered gradient fractions. Reactions supplemented with 1 mM ATP and 5 mM creatine phosphate are shown in the odd-numbered lanes. Complexes A, B, and H are indicated. (C) The complex B-forming activity, level of complex A, and level of complex H plotted versus glycerol gradient fraction number. (D) The complex B-forming activity and total counts per minute plotted versus glycerol gradient fraction number. All odd-numbered fractions were assayed, and complexes were quantitated with a laser scanner.

resolved in native gels (Fig. 2B). The −ATP complex (19) (* in Fig. 2B) fractionated as a complex apparently larger than complex A (35S) (compare with migration of complex A in Fig. 3). The heterogeneous nuclear ribonucleoprotein (hnRNP) complexes (H in Fig. 2B) sedimented as a series of complexes of different sizes. Commitment activity, assayed as complex A-forming activity, was analyzed across the gradient. Complex A-forming activity sedimented as a complex of ~30S (Fig. 2C and D). The commitment complex did not copurify with the bulk distribution of the PIP7.A RNA, as determined by total counts per minute eluted from the gradient (Fig. 2E) or with the bulk of complex H (Fig. 2D). Given the heterogeneity of complex H, it is possible that a component of complex H copurified with the commitment activity. No significant commitment activity resided outside of the ~30S commitment complex, showing that under these conditions, the commitment complex was a necessary intermediate of complex A formation, as shown by RNA→CC→A. Complex B was also formed during these assays. Complex B-forming activity exactly copurified with complex A-forming activity, indicating that one commitment complex was capable of forming both complex A and complex B.

The data showed that although the peak of the −ATP complex (19) had considerable commitment activity, the −ATP complex did not copurify with the commitment activity. This finding does not exclude the possibility that the

−ATP complex is the seat of a small fraction of the commitment activity.

Complex A is a required intermediate in spliceosome (complex B) formation. It was clear from the data presented above that the commitment complex could be chased into the A and B complexes. It was not clear, however, whether the pathway proceeded directly from the commitment complex to both A and B (B←CC→A[→B]) or via the CC→A→B pathway, in which complex A was a required intermediate. To test whether complex A could be converted into complex B, we used the techniques described above to show that a complex B-forming activity and complex A would copurify. Moreover, we needed to ascertain whether complex A was a required intermediate in complex B formation.

PIP7.A RNA was incubated with nuclear extract under splicing conditions with 1 mM ATP and 5 mM creatine phosphate. Splicing complexes were sedimented in glycerol gradients and identified by native gel electrophoresis. Complex A and complex B were well separated in these gradients (Fig. 3A). The ability of complexes separated in glycerol gradients to form complex B was assayed by a chase protocol as described for Fig. 2A. The complex B-forming activity copurified with complex A but not with complex H (Fig. 3B and C). The complex B-forming activity did not copurify with the bulk of the pre-mRNA (Fig. 3D). No complex B-forming activity was found other than in fractions containing complex A. Complex B observed in the assay in fraction 17 preexisted in the gradient fraction (Fig. 3A). These data represent the strongest evidence to date that complex A is a required intermediate in complex B formation, thus supporting RNA→CC→A→B as the sole spliceosome formation pathway in HeLa cell nuclear extracts.

It is interesting to note that in order to chase complex A to complex B, it was necessary to add 1 mM ATP and 5 mM creatine phosphate. It is not clear whether ATP hydrolysis is required, but it is likely.

The commitment complex requires *cis*-acting elements at both ends of the intron. Our only assay to detect the splicing commitment complex is the chase to complex A. Unfortunately, pre-mRNAs with mutations in the 3′ splice site and the polypyrimidine tract do not form complex A; moreover, pre-mRNAs with mutations in the 5′ splice site form lower levels of complex A (19). Thus, direct examination of these mutants in the chase assay was not a productive way of determining which *cis*-acting elements are required for formation of the commitment complex. The assay used to test the importance of *cis*-acting elements involved preincubating nuclear extract with unlabeled competitor pre-mRNAs, either wild type or mutants, and determining the *cis*-acting element's ability to prevent formation of the commitment complex. The scheme is similar to that shown in Fig. 1B and to that used by LeGrain et al. (27). The rationale was that if a factor is required to form a stable commitment complex, mutants in the *cis*-acting element bound by this factor should not form this stable complex. Therefore, functional pre-mRNA should be able to form a commitment complex in nuclear extracts precompeted with mutant pre-mRNAs.

Precompetition with PIP4, a functional pre-mRNA, but not PIP4.2, a 5′ splice site mutant pre-mRNA, inhibited formation of the commitment complex by PIP4 (Fig. 4). Thus, the 5′ splice site must be recognized by factors necessary for establishment of the commitment complex. Precompetition with PIP3 pre-mRNA prevented formation of the commitment complex of PIP3. A 3′ splice site mutant, PIP3py5, was unable to do this (Fig. 4). Not surprisingly, the 3′ splice site is also required for splicing commitment. The role of the polypyrimidine tract was noted by using the triple point mutant PIP3py6, in which three U residues in the PIP3 polypyrimidine tract (12) are changed to A residues. This mutant had an intermediate phenotype, implying that the polypyrimidine tract had a role in formation of the commitment complex (Fig. 4).

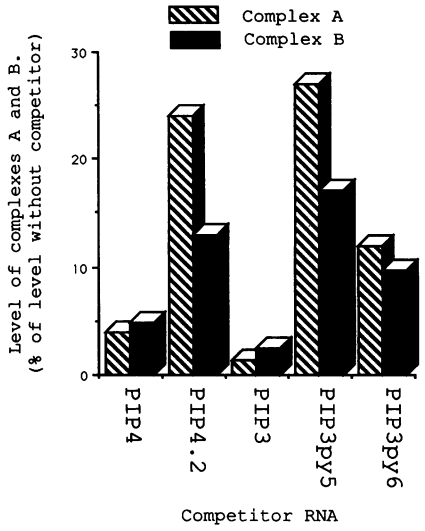
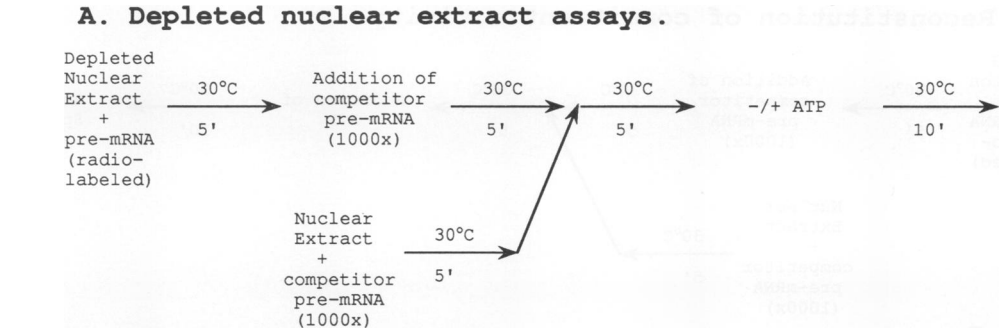


FIG. 4. Evidence that commitment complex formation requires a 5′ and a 3′ splice site. Labeled pre-mRNA was incubated with HeLa cell nuclear extract that had been previously incubated with a 10,000-fold molar excess of unlabeled competitor RNAs. The reaction mixtures were incubated further in the presence of 1 mM ATP and 5 mM creatine phosphate as described for Fig. 1B. The levels of complex A and complex B formed by the labeled pre-mRNA are shown. PIP4, a wild-type pre-mRNA, was shown to compete with itself for formation of a commitment complex. PIP4.2, a 5′ splice site mutant (GGG:GUGAGU→GGG:AUGAAU), was not an effective competitor. PIP3, a wild-type pre-mRNA, was shown to compete with itself for formation of a commitment complex. PIP3py5, a 3′ splice site mutant (ACAG:C→GGAG:C), and PIP3py6, a mutant in the polypyrimidine tract (CCCUUUUUUUUCC→CCCAUUAUU AUCC), were very poor and moderately poor competitors, respectively.

Commitment activity requires U1 snRNP and protein factors. To test the requirements for U snRNPs in HeLa nuclear extract, we used either mock-depleted, U1 snRNP-depleted, U2 snRNP-depleted, or U5 snRNP-depleted nuclear extracts (1). These extracts were depleted by using biotinylated antisense oligonucleotides as described by Lamond and colleagues (1, 5, 25). The scheme for these experiments is outlined in Fig. 5A. The mock-depleted extract was active in the commitment assay (Fig. 5B, lane 2). The U1 snRNP-depleted extract was inactive (lane 4) in the commitment assay, whereas the U2 snRNP-depleted (lane 6) and U5 snRNP-depleted (lane 8) extracts were active. The activity of these last two extracts was about 50% that of the mock-depleted extract. These results show that U1 snRNP and/or tightly associated factors are required for formation of the commitment complex.

Once the commitment complex has been formed, the chase to complex A requires U2 snRNP but not the U4/6 and U5 snRNPs. A U2 snRNP-depleted extract was capable of forming the commitment complex but not of chasing the commitment complex to complex A. A U5-depleted extract was shown to chase the commitment complex to complex A. To synthesize complex B, both U2 and U5 snRNPs were



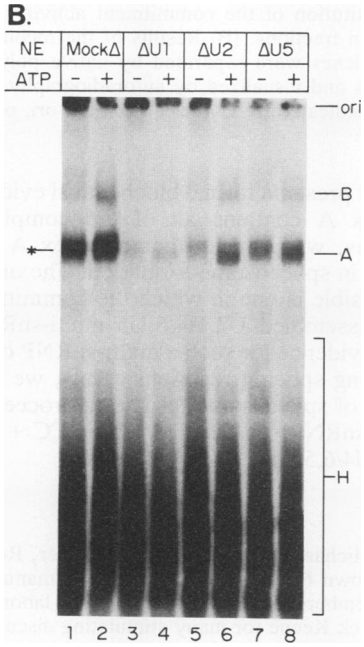


FIG. 5. Evidence that U1 snRNP is required for formation of the commitment complex. (A) Scheme for the use of depleted nuclear extracts in the commitment assay. (B) A commitment assay using either mock-depleted (MockΔ), U1 snRNP-depleted (ΔU1), U2 snRNP-depleted (ΔU2), or U5 snRNP-depleted (ΔU5) nuclear extracts (NE). Splicing complexes are defined as in Fig. 1. Addition of 1 mM ATP and 5 mM creatine phosphate is indicated by the plus sign in the ATP row. Splicing complexes were separated by native polyacrylamide gel electrophoresis and visualized by autoradiography. Complex B, complex A, −ATP complex (*), and complex H are indicated. ori, origin.

required. U4/6 snRNP was not tested; however, the U5 snRNP-depleted extract had very low levels of U4/6 snRNPs (data not shown).

To address the issue of non-snRNP factors required for formation of the commitment complex, we used a reconstitution assay. The HeLa nuclear extract was separated through poly(U)-agarose as described before (22, 31). Nuclear extract was supplemented with KCl to 500 mM and loaded on a poly(U)-agarose matrix. A flowthrough fraction (fraction I), a 1.5 M KCl salt eluate (fraction II), and a 2 M guanidine HCl eluate (fraction III) were collected, dialyzed, and used in the reconstitution assays. The scheme for the reconstitution of commitment activity is depicted in Fig. 6A.

Fraction I had commitment activity, albeit lower than that of nuclear extract (Fig. 6B). Fractions II and III did not have activity either alone or when added together (Fig. 6B and data not shown). The activity of fraction I was not enhanced by fraction II but was increased anywhere from 1.5- to 3-fold by addition of fraction III (data not shown). This enhancement by fraction III was not reproducible with all poly(U)-agarose runs, for reasons that are not clear but are discussed below.

To ascertain the factors that were present in the fractions, we used Northern (RNA) blots, Western immunoblots, and UV cross-linking assays. Analysis using Northern blots of native gels (21) to determine the presence of U snRNAs revealed that U1, U2, U4/6, and U5 snRNPs were found exclusively in fraction I (data not shown). This result was confirmed for U1 snRNP by Western blot analysis with an antibody to the U1 snRNP-associated 70-kDa protein. Thus, the requirement for fraction I is not surprising given the requirement for U1 snRNP shown above. Western blot analysis with anti-PTB rabbit antisera demonstrated that this protein was detectable only in fraction II (9a). This result was confirmed by UV cross-linking analysis that revealed a protein of around 62 kDa. Fraction III was variable and was shown to contain small amounts of a 69-kDa protein detected by Coomassie staining and UV cross-linking to labeled pre-mRNA. This protein could be the U2 snRNP auxiliary factor (U2AF) (43). UV cross-linking detected a protein of identical mobility in fraction I as well. All of these results lead us to believe that some U2AF was present in fraction I.

Therefore, components in fraction I (U1 snRNP and probably U2AF, at a minimum) are sufficient to commit the pre-mRNA to form complex A. It is clear that PTB (in fraction II) is not required for commitment complex formation.

DISCUSSION

In this report, we describe the biochemical characterization of the splicing commitment complex in HeLa cell nuclear extracts. The data show that the great majority, if not all, of the commitment activity described here and previously (30) resides within a complex of approximately 30S. This complex could not be identified as a complex visible on native gel electrophoresis, possibly because the complex was masked by complex H or was unstable to the electrophoretic conditions used. We showed that this commitment complex is converted to complex A upon addition of ATP. Furthermore, we showed that complex A is a required intermediate in spliceosome formation.

Not surprisingly, factors that recognize the 5′ splice site are required for commitment activity. This had been shown to be the case for yeast cells (39) and can be explained by the requirement for U1 snRNP. The requirement for the 3′ splice site is more controversial. Although it is known that 3′ splice

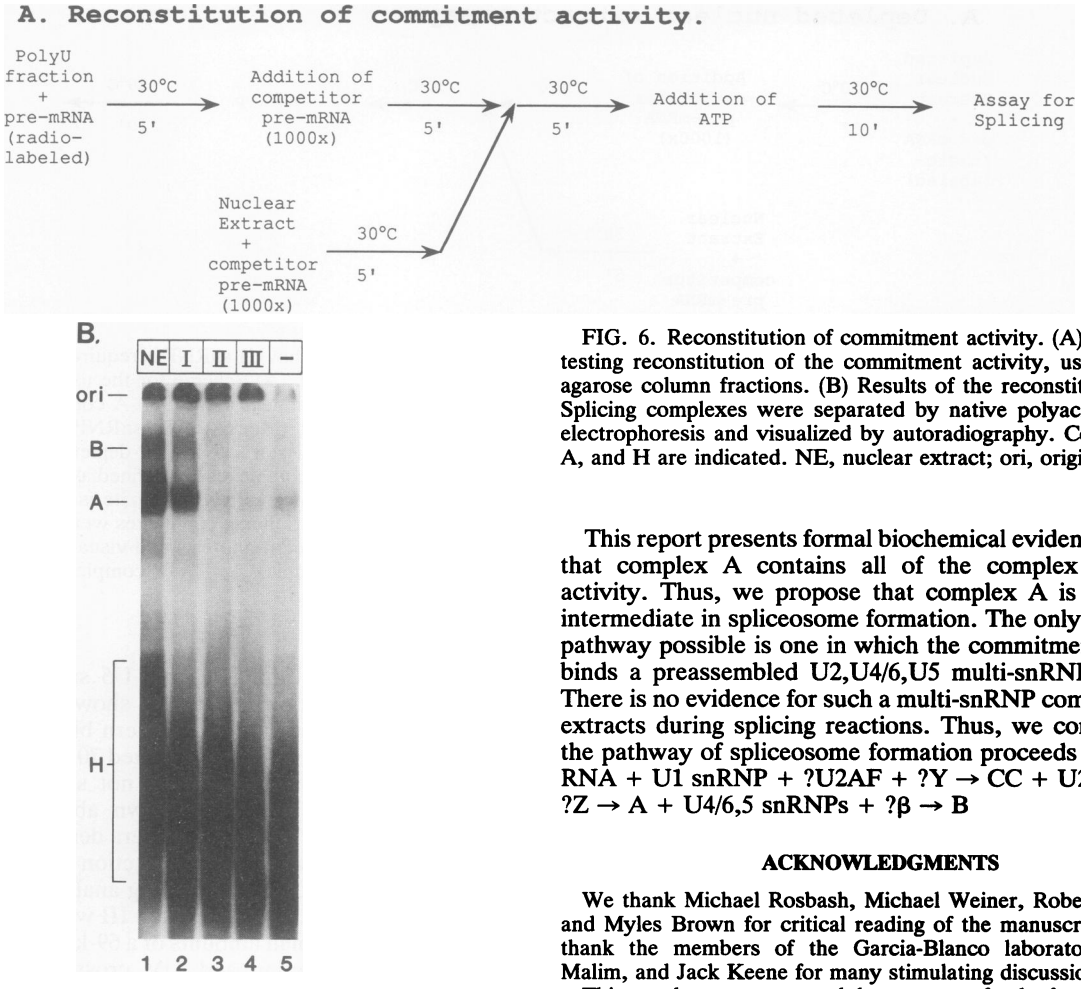


FIG. 6. Reconstitution of commitment activity. (A) Scheme for testing reconstitution of the commitment activity, using poly(U)-agarose column fractions. (B) Results of the reconstitution assay. Splicing complexes were separated by native polyacrylamide gel electrophoresis and visualized by autoradiography. Complexes B, A, and H are indicated. NE, nuclear extract; ori, origin.

site mutants do not form complex A in some introns (12, 26), in other introns these mutants do so and splice (18, 33, 41). The factor(s) that recognizes the 3′ splice site early in the splicing reaction has not been identified. hnRNP A1, however, may be such a factor (23, 39).

Factors that recognize the polypyrimidine tract play a role in commitment complex formation. A possibility is that the U1 snRNP directly interacts with the polypyrimidine tract of introns. Seraphin and Rosbash have proposed a more likely scenario in which a non-snRNP component (X) interacts with the polypyrimidine tract and also with U1 snRNP (39).

The candidates for the X component are factors that interact with the polypyrimidine tract, namely, PTB (12), U2AF (43), and hnRNP C (42). It was clear from our UV cross-linking and Western blot assays that fraction I did not contain any detectable PTB. Moreover, fraction II, which contains PTB, does not enhance the activity of fraction I. Thus, we conclude that our previous model (6) in which PTB was X is not correct. The role of hnRNP C in splicing in vitro was suggested by immunodepletion and neutralization experiments (9). Recent data for a functional pre-mRNA, containing a variant polypyrimidine tract, which does not bind hnRNP C argues persuasively that this protein does not have an essential role in vitro (35a). Therefore, it is unlikely that this protein is essential for commitment activity. Our data are most consistent with U2AF being X in the scheme proposed by Seraphin and Rosbash (39).

This report presents formal biochemical evidence showing that complex A contains all of the complex B-forming activity. Thus, we propose that complex A is a required intermediate in spliceosome formation. The only alternative pathway possible is one in which the commitment complex binds a preassembled U2,U4/6,U5 multi-snRNP complex. There is no evidence for such a multi-snRNP complex in our extracts during splicing reactions. Thus, we conclude that the pathway of spliceosome formation proceeds as follows: RNA + U1 snRNP + ?U2AF + ?Y → CC + U2 snRNP + ?Z → A + U4/6,5 snRNPs + ?β → B

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