Dynamics of the U1 Small Nuclear Ribonucleoprotein during Yeast Spliceosome Assembly*

(Received for publication, February 6, 1997, and in revised form, April 16, 1997)

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U1 small nuclear ribonucleoprotein (snRNP) may function during several steps of spliceosome assembly. Most spliceosome assembly assays, however, fail to detect the U1 snRNP. Here, I used a new native gel electrophoretic assay to find the yeast U1 snRNP in three pre-splicing complexes (δ, β1, α2) formed in vitro. The order of complex formation is deduced to be δ → β1 → α2 → α1 → β2, the active spliceosome. The δ complex is formed when U1 snRNP binds to pre-mRNA in the absence of ATP. There are two forms of δ: a major one, δun, unstable to competitor RNA; and a minor one, δcommit, committed to the splicing pathway. The other complexes are formed in the presence of ATP and contain the following snRNPs: β1, the pre-spliceosome, has both U1 and U2; α2 has all five, however, U1 is reduced compared with the others; and α1 and β2 have U2, U5, and U6. Prior work by others suggests that U1 is “handing off” the 5’ splice site region to the U5 and U6 snRNPs before splicing begins. The reduced levels of U1 snRNP in the α2 complex suggests that the handoff occurs during formation of this complex.

The U1 small nuclear ribonucleoprotein (snRNP) is one of several components including the U2, U4, U5, and U6 snRNPs and several non-snRNPs that form the spliceosome on pre-mRNA (for review, see Refs. 1–7). U1 is the first snRNP to bind to pre-mRNA during spliceosome assembly as studied by in vitro assays. Subsequently the U2 snRNP binds and the pre-spliceosome is formed. Next the U4/U5/U6 tri-snRNP binds, and the spliceosome is created. Finally, the spliceosome is activated for splicing by at least one conformational change in which the U4 and U6 snRNAs dissociate from one another.

Although the order of binding of snRNPs and some non-snRNP proteins during spliceosome assembly is known, the presence and function of U1 snRNP in several of the spliceosome assembly intermediates are controversial. The U1 snRNP has been thought to function in recognizing the intron primarily during the early steps of spliceosome assembly. As the first snRNP to bind to the pre-mRNA, U1 has an important, hierarchical role in spliceosome assembly and in recognition of the splice sites in the yeast Saccharomyces cerevisiae. The nucleotides 3–8 at the 5’ end of U1 snRNA base pair with the pre-mRNA 5’ splice site region (8). This interaction probably occurs when U1 binds to pre-mRNA because mutations in the pre-mRNA 5’ splice site decrease U1 snRNP binding and inhibit subsequent steps in spliceosome assembly (9, 10). RNase H degradation (9) or depletion (11) of the U1 snRNP prevents the other snRNPs from binding to the pre-mRNA as well.

In addition to base pairing with the 5’ splice site, U1 snRNP interacts directly or indirectly with several other spliceosomal components. A protein bridge may span the 5’ splice site and branchpoint region (12, 13) and contribute to U1 snRNP binding to the pre-mRNA (9, 14). U1 snRNP may also associate directly with the U2 snRNP as a U1/U2 snRNP complex can be induced in Hela cell extracts by an RNA complementary to the 5’ end of U1 snRNA (15). Finally, U1 snRNP may interact with additional spliceosomal components during the late steps of spliceosome assembly as suggested by the finding that a 2’O-methyl oligoribonucleotide complementary to U5 snRNA can induce the formation of a U1/U4/U5 snRNP complex in Hela cell extracts (16). Most of these interactions are probably important for spliceosome assembly but not for splicing catalysis because once a yeast spliceosome is formed on the pre-mRNA, it can lose U1 snRNA and still catalyze splicing (17).

These interactions suggest that the U1 snRNP is present in several intermediates in spliceosome assembly. At least in HeLa cell splicing extracts, this is the case. When pre-mRNA is added to HeLa cell extracts, the pre-splicing complexes E, A, and B form in that order as precursors to the active spliceosome, complex C (18–22). The U1 snRNP can be detected in all four complexes when purified by affinity chromatography. However, it is found predominantly in E, and its concentration decreases relative to the U2 and U4/U5/U6 snRNPs as these snRNPs bind during formation of the A and B complexes, respectively (20). These and other studies (23, 24) have proposed that the association of the U1 snRNP with the spliceosome changes sometime before splicing is catalyzed. In fact, U1 is supplanted by the U5 and U6 snRNAs at the pre-mRNA 5’ splice site before catalysis (for review, see Refs. 5 and 6). Recently, it has been suggested that the base pairing between U1 and pre-mRNA is disrupted even earlier, before U2 snRNP binds (25, 26).

Although the U1 snRNP has been thought to participate in several steps of spliceosome formation in yeast, its presence in most of the yeast pre-splicing complexes formed in vitro has not been shown. Pre-splicing complexes III, I, and II, as detected initially by one-gel electrophoretic assay (27, 28), and complexes B, A2–1, and A2–2, as identified by a different gel assay (29), form in that order and contain the U2, U2/U4/U5/U6, and U2/U5/U6 snRNPs, respectively. U1 snRNP binding to the pre-mRNA during the first step of yeast spliceosome assembly was discovered subsequently by affinity chromatography (9).
and by gel-electrophoresis (11). All gel electrophoretic assays, however, have failed to find U1 snRNA in pre-splicing complexes formed after this first assembly step (11, 18, 27, 29), although the U1 snRNP-specific 70K protein was found in pre-splicing complexes formed in HeLa extracts and resolved by gel electrophoresis (30).

To detect the U1 snRNP during yeast spliceosome assembly in vitro, I developed a new native gel electrophoretic assay. I found U1 snRNA in three pre-splicing complexes (δ, β1, and α2). One of these complexes, β1 (the pre-splicesome), contains both U1 and U2 snRNAs as had been proposed by previous studies (9, 11). The α2 complex contains the U2, U4, U5, and U6 snRNAs, as well as reduced amounts of U1. This reduction in the amount of U1 suggests that the association of U1 with pre-mRNA or other spliceosomal components that makes this snRNP stable in the pre-splicesome is disrupted about the time the α2 complex is formed. This change in U1 may indicate the “handing off” of the 5′ splice site region from the U1 to the U5 and U6 snRNPs.

**EXPERIMENTAL PROCEDURES**

**In Vitro Splicing Assays and Native Gel Electrophoresis—Plasmid for in vitro synthesis of Sp6-Wt (31) actin pre-mRNA was as described. The plasmid was cut with HpaII restriction endonuclease (New England Biolabs Inc.) for synthesizing the transcripts used in the native gel electrophoretic assays. Uncapped, radiolabeled pre-mRNAs for splicing and spliceosome assembly assays were synthesized in vitro with SP6 polymerase (Promega) and [32P]UTP (Amersham Corp.) as described previously (31). For Northern blot analyses, the pre-mRNA was synthesized with very low or no specific activity as described previously (29).

Whole cell splicing extract was made from strain EJ101 as described previously (31). A typical splicing reaction contained 0.4 mM radiolabeled pre-mRNA, 60 mM KPO4 (pH 7.4), 3 mM MgCl2, 2 mM ATP, 3% PEG8000, and 40% extract in 20 mM Hepes-K+ (pH 7.8 at 0 °C), 0.2 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol, and 0.2% glycerol. A 10-μl splicing reaction with radiolabeled pre-mRNA was incubated at 23 °C and then quenched by adding it to 10 μl of ice-cold 50 mM Hepes-K+ (pH 7.4), 2 mM (CH3COO)2Mg (R buffer; see Ref. 11) with 2 μg/μl carrier RNA. Carrier RNA was prepared from mouse intestine as described (32). The sample was incubated on ice for 10 min, and then 5 μl of loading buffer (200 mM Tris-phosphate (pH 8.0 at 0 °C), 50% (v/v) glycerol, 0.1% xylene cyanol, and 0.1% bromphenol blue) was added. The samples were fractionated on a 3.2% polyacrylamide gel (50:1, acrylamide: bisacrylamide) in TMAPS buffer (48 mM Tris-phosphate (pH 8.0 at 0 °C), 1.5 mM (CH3COO)2Mg) at 4 °C for 16–20 h at 5.5–6.7 V/cm. The gel was placed on 3MM Whatman paper, and the complexes were visualized by autoradiography with film or a Molecular Dynamics PhosphorImager.

The previously described prp6–1 mutant (33) was used for mutant extract. A UV-induced, temperature-resistant revertant of this prp6–1 mutant (prp6–1R1), was isolated and used for wild-type extract, as shown in Fig. 5.

**Northern Blot Analyses—**Ten-μl splicing reactions containing 4 μM pre-mRNA were run on native gels as described above. After electrophoresis, the gel was placed on 3MM Whatman paper and soaked in two changes of a 5-fold volume of TBE (8 μM Tris-borate (pH 8.2), 2 mM EDTA) with 8 x urea for 15 min each; this denaturation is essential for efficient transfer of the snRNAs in the next step. The RNAs in the gel were electrophoretically transferred to Gene Screen (New England Nuclear) in 0.25 x TBE at 120 V for 1 h at 4 °C in an apparatus described by Church and Gilbert (34). The wet membrane was then irradiated with three germicidal bulbs (General Electric, G15T8) at a distance of 35 cm for 15 min. The membrane was next simmered for 10 min in about 500 ml of boiling 0.1 x SSCP (120 mM NaCl, 15 mM Na citrate, and 20 mM NaPO4 (pH 7.0)) with 0.1% SDS in the microwave and then shaken for 10 min at room temperature. After prehybridization in hybridization buffer (50% formamide, 5 x SSCP, 0.1% SDS, 3 x Denhardt’s solution, and 100 μg/ml sonicated salmon sperm DNA) at 42 °C for at least 30 min, the radiolabeled probe was added in 15–20 mls of new hybridization buffer and incubated with the membrane overnight. The blot was washed three times at 23 °C for 10 min each in 3 x SSCP with 0.1% SDS, once for 10 min at 55 °C, and once in 0.1 x SSCP with 0.1% SDS for 10 min at 55 °C. The hybridization was detected by autoradiography with either film or a Molecular Dynamics PhosphorImager.

For radiolabeled snRNA probes, the DNA fragments encoding the snRNAs on plasmids pT7-U1 and pT7-U2 (from D. McPheeters, Case Western Reserve University, Cleveland, OH), pT7-U4 and pT7-U6 (from P. Fabrizio, Phillips University Marburg, Marburg, Germany), and pT7-U5 (from L. Krinke) encoding the snRNAs were amplified by the polymerase chain reaction (35). The sizes of amplified fragments are the following: U1, 575 bp; U2, 1200 bp, U4, 170 bp; U5, 180 bp; U6, 115 bp. The amplified DNAs were then radiolabeled by random oligodeoxynucleotide-primed extension (36).

**Deoxycylo nyucleotide-directed RNase H Degradation of snRNAs in Splicing Extracts—**Deoxycylo nyucleotides for targeted degradation of the U1 (oSR19), U2 (oSR20–also called SRU2), and U6 (dU) were as described (9, 37). For the experiment shown in Fig. 7, A and B, the deoxycylo nyucleotide oSR20 was added to a concentration of 1.3 μM in splicing extract with 1.6 mM MgCl2 and 2 mM dithiothreitol. After incubating the extract for 30 min at 30 °C, water was added to bring the extract to 40% of the volume, and KO1 (pH 7.6), MgCl2, ATP, and PEG8000 were added to final concentrations of 60, 3, and 2 mM and 3%, respectively. This brought the calculated concentration of the deoxy- cylo nyucleotide oSR20 to 600 nM for the splicing reactions incubated in Step 1. After addition of an equal volume of splicing reaction with active extract in Step 3, the final calculated concentration of oSR20 was 300 nM. The conditions for inactivating the U1 and U6 snRNAs in extracts were as described (9, 37).

**RESULTS**

**Detection of U1 snRNA in Pre-splicing Complexes—**As the U1 snRNA is not found in most pre-splicing complexes resolved by native gel electrophoresis, I specifically sought electrophoretic conditions that would retain the U1 snRNP in pre-splicing complexes. Among the gel buffer conditions tested, magnesium ion has a significant effect on U1 snRNP retention (Fig. 1). U1 snRNA, as detected by Northern blot hybridization, is present with pre-mRNA in two bands, δ and β1, in a gel run with or without magnesium acetate, but there is an average of 16-fold more U1 in the δ band (n = 4, standard deviation = 1.9) with magnesium ion than without it. Additional assays re-
vealed that the amount of U1 in the δ band is proportional to the magnesium ion concentration in the buffer up to 1.5 mM (data not shown). In a gel with or without magnesium ion, “free” U1 snRNP not bound to the added pre-mRNA migrates as two diffuse bands (designated by asterisks in Fig. 1). There is less free U1 snRNP with than without magnesium, and yet equivalent amounts of free U1 snRNP are detected in splicing reactions without added pre-mRNA. This suggests that most U1 snRNP bound to added pre-mRNA dissociates during electrophoresis without magnesium. The β₁ band migrates near the slower form of free U1 snRNP; however, it is distinct from free U1 snRNP as it is sharp and its formation depends on the presence of added pre-mRNA. That there is more δ band and less free U1 snRNP in gels with magnesium ion than without it suggests that magnesium ion stabilizes U1 snRNP bound to pre-mRNA. I included 1.5 mM magnesium acetate in all subsequent assays.

To begin to determine the order of formation of the pre-splicing complexes, I assayed their kinetics of formation in splicing reactions at the normal 23 or at 15 °C, which slows the splicing reaction (Fig. 2). Radiolabeled pre-mRNA was added to splicing reactions with or without added ATP, and at various times thereafter, samples were removed and analyzed by native gel electrophoresis. In the absence of added ATP at either temperature, there are two major bands, a rapidly migrating band and a more slowly migrating band (consists of two nearly unresolved bands appearing by 2–5 min). Later, there are low amounts of a slowly migrating band. The simplest interpretation of the temporal relationship of the formation of these bands is that one or more complexes in the δ band is the precursor of the complexes in the β and α bands. The β and α bands migrate close to one another and do not resolve well when formed on the amount, specific activity, and type of radiolabeled actin pre-mRNA used here. Subsequent experiments described below show that these two bands can be resolved.

If the δ band represents the first pre-splicing complex to form, then its formation should depend on the U1 but not the U2 snRNP (9, 11). I therefore analyzed its formation with radiolabeled actin pre-mRNA in splicing extracts in which either the U1 or U2 snRNA was inactivated by deoxyoligonucleotide-directed RNase H degradation (Fig. 3). The reaction with inactivated U1 snRNA forms no detectable pre-mRNA-specific bands (Fig. 3A). The reaction with inactivated U2 snRNA forms the δ band in the presence or absence of ATP but is unable to form additional complexes. Additionally, extracts pre-inactivated with the control deoxyoligonucleotide do not form the β and α bands unless ATP is added subsequently for the assay as the pre-incubation step depletes the endogenous levels of ATP (39). U2 snRNA in the gel was detected by Northern blot analysis with a U2-specific probe after sufficient time had elapsed for the radiolabel in the pre-mRNA to decay. As previously reported for the conditions used in this study (9, 40), greater than 97% of U2 snRNA was destroyed when targeted for degradation (Fig. 3B, lanes 9–12). This destruction, however, has no effect on the formation or migration of the δ band even though free U2 snRNP normally migrates close to the δ complex. Furthermore, the δ band has the same mobility when formed in extracts with either wild-type U2 snRNP or a smaller, but functional, U2 snRNP (41) despite the fact that the small U2 snRNP migrates nearly twice as far as wild-type U2 snRNP in the gel (data not shown). Thus, the formation and migration of the δ band requires U1 but not U2 snRNP while the formation of the α and β bands requires ATP and both U1
and U2 snRNPs. The properties of the δ band (that it contains U1 snRNA, forms in the absence of ATP and U2 snRNP, and forms first in the presence of ATP), as well as additional properties described below, indicate that the δ band contains a pre-splicing complex that I have called the δ complex.

To identify additional pre-splicing complexes that contain U1 snRNPs, a splicing reaction was incubated either with or without non-radiolabeled pre-mRNA, fractionated by native gel electrophoresis, and analyzed by Northern blot hybridization with the snRNA-specific probes (Fig. 4). This experiment resolved three slowly migrating complexes in addition to the δ complex. They are distinguished by their mobilities, kinetics of formation, and snRNA content, and are called β1, α, and β2. The δ and β complexes as detected with the U1 probe appear early in the splicing reaction. Of note is that both U1 and U2 snRNAs are present in the β complex at the early times (Fig. 4, lanes 1 and 6). At 30 min, however, U2, U5, and U6 snRNAs accrue, and U1 snRNA remains the same (lane 3) or decreases (data not shown). Furthermore, only pre-mRNA can be isolated from the β complex at early times in splicing reactions, whereas pre-mRNA and intermediates can be isolated from the β complex at late times (data not shown). These results suggest that there are two co-migrating β complexes that are kinetically distinguishable: β1, which forms early in the splicing reaction and contains U1 and U2 snRNPs, and β2, which forms later, contains U2, U5 and U6 snRNPs, and is the active spliceosome.

A third band formed on the pre-mRNA in the presence of ATP is also apparent in this assay (Fig. 4). The α band, as seen most clearly with the U4 snRNA probe, is identified by having 1) a slower migration than the β complexes, 2) a slower rate of formation than the β1 complex, and 3) the U4 as well as the U2, U5, and U6 snRNAs. A very small amount of U1 snRNA may also be present in this band. The α band is more readily identified by Northern blot hybridizations in part because the splicing reactions usually have 10-fold more pre-mRNA than those with radiolabeled pre-mRNA. Subsequent assays described below identified two complexes in this band.

To confirm the nature and composition of the β1 complex, I assayed its formation in a splicing extract in which the Prp6 protein was inactivated. The Prp6 protein is required for the U4/U5/U6 tri-snRNP to form and to be incorporated into the developing spliceosome (42). Active splicing extract made from a temperature-sensitive prp6–1 mutant grown at the permissive temperature has been shown to be temperature sensitive in vitro due to the mutant prp6 protein (33). The prp6–1 mutant extract is active for splicing in vitro at or below 26 °C but inactive at 30 °C, whereas the wild-type extract is active at all the temperatures tested (data not shown). When splicing reactions with mutant extract and radiolabeled pre-mRNA are analyzed by native gel electrophoresis, however, the reactions incubated at 23 °C look similar to those at 30 °C in that most of the radiolabeled pre-mRNA is in the β band (Fig. 5A). However, a defect in complex formation due to inactivation of the mutant prp6 protein at 30 °C is detected by Northern blot analyses (Fig. 5B). A wild-type reaction incubated at 30 °C forms all three bands (δ, β, and α) with the U2, U4, U5, and U6 snRNAs accumulating in the α band by 20 min. Again, a small amount of U1 snRNA may be present in the α band. The U2, U5, and U6 snRNAs also accumulate in the β band at 20 min. In contrast, a splicing reaction with mutant prp6 extract incubated at 30 °C accumulates U1 and U2 but not U5 and U6 snRNAs in the β band. Furthermore, little or no U2, U4, U5, or U6 is present in the α band at 20 min. Additionally, little or no tri-snRNP can be seen in the 30 °C reactions with mutant prp6 extract even in the absence of added pre-mRNA (Fig. 5B, lane 7, in U4, U5, and U6 panels), whereas this extract can form the tri-snRNP at 23 °C (data not shown). These results indicate that the reactions with the mutant prp6 extract at 30 °C form no tri-snRNP, α band, or β2 complex. They do, however, accumulate the U1 and U2 snRNAs in the β band, consistent with the properties of the β1 complex. Additionally, the results show that β1 is formed before the α and β2 complexes.

Inactivation of U6 snRNA by deoxyoligonucleotide-directed RNase H degradation has also been shown to prevent the U5, U6, and U6 snRNPs from forming the tri-snRNP and binding to the pre-spliceosome (37). I found that both control and U6-inactivated extracts form δ and β1 complexes with β1 containing both U1 and U2 snRNAs. The α and β2 complexes form only when U6 snRNA is intact (data not shown).

Given these data on the kinetics of formation, snRNA and pre-mRNA content, and formation of the pre-splicing complexes in inactivated extracts, I deduced the order of formation and composition of the δ, β1, and β2 complexes to be as follows: first δ (with U1 snRNA), then β1 (with U1 and U2 snRNAs), and eventually β2 (with U2, U5, and U6 snRNAs). The identities of complexes in the α band and their relationships to the other complexes were revealed in the experiments described below.

U1 snRNA in a Late Pre-splicing Complex—I next asked if U1 snRNA could be detected in any additional pre-splicing complexes. Two different approaches were used to increase the amounts of late-forming complexes.

In one approach, EDTA was added to the splicing reactions. Previously, Cheng and Abelson (29) added EDTA to splicing
**FIG. 5. Pre-splicing complex formation with wild-type and mutant prp6 splicing extracts.** A, splicing reactions with mutant prp6 extract but without splicing substrate were incubated for 2 min at the indicated temperatures. Radiolabeled actin pre-mRNA was then added. At 15 and 30 min, samples were removed, fractionated by native gel electrophoresis, and visualized by autoradiography as shown here. The bands formed by the pre-mRNA are indicated as $\alpha$, $\beta$, and $\alpha$. B, splicing reactions with non-radiolabeled pre-mRNA and either wild-type or mutant prp6 extract were incubated at 30°C as described in panel A. Samples were removed at 2, 10, and 20 min after pre-mRNA addition and assayed by native gel electrophoresis. The pre-splicing complexes detected in panel A are indicated. The symbol, $U4/U5/U6^*$, indicates the tri-snRNP that is formed in wild-type but not mutant prp6 extract at 30°C.

reactions to identify and distinguish in their native gel electrophoretic assay two complexes (A1 and A2–1) that form late in the assembly pathway. They showed that 5 mM EDTA in the yeast splicing reaction induces accumulation of A1 complex in particular and almost completely inhibits splicing. Complex A2–1 contains the U2, U4, U5, and U6 snRNAs and is most likely the precursor to complex A1, which contains the U2, U5, and U6 snRNAs. The A1 complex is the immediate precursor of the active spliceosome, designated A2–2 in the gel system of Cheng and Abelson (29). I assayed the effects of 5 mM EDTA on the active spliceosome, designated A2–2 in the gel system of Cheng and Abelson (29). I assayed the effects of 5 mM EDTA on the pre-splicing complexes by my native gel electrophoretic assay and Northern blot hybridizations (Fig. 6).

Six complexes are distinguishable in the reaction with 5 mM EDTA: $\alpha$, three $\beta$ complexes ($\beta_1$, $\beta_2$, and $\beta_2^*$), and two $\alpha$ complexes ($\alpha_1$ and $\alpha_2$). Three complexes that hybridize with the U1 probe form within 2 min after addition of pre-mRNA, $\delta$, $\beta_1$, and $\beta_2^*$. The $\beta_1$ complex is so designated in this reaction because it forms early, contains the U1 and U2 snRNAs, and migrates in the same position as the $\beta_1$ complex in reactions with no EDTA. A novel $\beta_2^*$ complex also contains U1 and U2 snRNAs and appears early but only in the presence of EDTA, and it migrates more slowly than $\beta_1$. The $\alpha_2$ complex, as most clearly seen with the U4 probe, contains U2, U4, U5, and U6 snRNAs like the A2–1 complex of Cheng and Abelson (29). Notably, a distinguishable, but small amount of U1 snRNP compared with the other snRNAs is also present in the $\alpha_2$ complex. Finally, two complexes containing the U2, U5, and U6 snRNAs accumulate late in the splicing reaction. The most abundant of these two probably corresponds to the A1 complex of Cheng and Abelson (29) as it accumulates in the presence of excess competitor pre-mRNA and can be chased into an active spliceosome in the presence of competitor and ATP (44). To test if the $\delta$ complex is the functional equivalent of CC, I determined if the $\delta$ complex could be chased into an active spliceosome in the presence of excess competitor pre-mRNA. Radiolabeled pre-mRNA was incubated in extract in the absence of ATP and an intact U2 snRNP (Fig. 7A, Step 1). After 10 min of incubation, a 25-fold molar excess of cold pre-mRNA was added (Fig. 7A, Step 2). One min later, additional extract and ATP were added and incubated for an additional 15 or 30 min (Fig. 7A, Step 3). Samples were removed at the various steps and assayed by native gel electrophoresis. When a 25-fold ex-
cess cold competitor pre-mRNA is added at Step 2, only a small fraction of the pre-mRNA in the δ complex is subsequently chased into the other complexes during the incubation in Step 3 (Fig. 7B, lanes 6–8). That these complexes are formed from the Step 1 δ complex is indicated by the datum that the 25-fold excess of cold competitor RNA is sufficient to block any pre-splicing complex formation if it is added in Step 1 before the radiolabeled pre-mRNA is added (lane 4). In contrast, the δ complex formed in Step 1 is stable in the extract in the absence of ATP and competitor RNA during the Step 3 incubation (Fig. 7B, compare lanes 6–8 with lanes 1–2). When ATP and complete extract are subsequently added in Step 3, the pre-mRNA can efficiently form additional complexes (lane 3). Thus, like the commitment complexes described by Rosbash and coworkers (10, 11, 44), some of the pre-mRNA in the δ complex is committed to the splicing pathway. Unlike the CC, however, a significant amount of pre-mRNA in the δ complex cannot be chased into the spliceosome by this assay.

One possible explanation for the fraction of unchaseable pre-mRNA in the δ complex is that some of the δ complex is not stable in the presence of excess cold competitor pre-mRNA. I, therefore, assayed the stability of the δ complex in the presence of competitor pre-mRNA. To measure the levels pre-mRNA in the δ complex only, I used splicing extract in which the U2 snRNPs had been inactivated by deoxyoligonucleotide-directed RNase H degradation. If the δ complex is formed first on the radiolabeled pre-mRNA, subsequent incubation with even an equal molar amount of cold pre-mRNA is sufficient to reduce the amount of radiolabeled pre-mRNA in the δ complex, with increasing reductions occurring with increasing amounts of cold competitor pre-mRNA (Fig. 7C). In contrast, at least a 10-fold excess of cold competitor pre-mRNA is required to block formation of the δ complex on radiolabeled pre-mRNA added after the cold competitor. Although this is the same extract used in the experiments in Fig. 7, B and C, I have found the same results with other extracts. Thus most of the radiolabeled pre-mRNA that cannot be chased into the spliceosome can be accounted for by being in a δ complex that is unstable in the presence of the excess cold competitor.

**DISCUSSION**

Detection of U1 snRNA in Three Pre-splicing Complexes—Although it has been proposed that the U1 snRNP has several functions in spliceosome assembly and in splice site recognition and selection, most spliceosome assembly assays have failed to detect the U1 snRNP. Here I used a new native gel electrophoretic assay to detect the yeast U1 snRNA in three pre-splicing complexes (δ, β2, α2) formed in an *in vitro* splicing reaction. I determined the most probable order of formation of the complexes to be δ → β2 → α2 → α1 → β2, where β2 is the active spliceosome. Unlike all previous electrophoretic gel assays, this new assay has detected the U1 snRNA together with other snRNAs in two complexes: 1) with U2 snRNA in the β1 complex and 2) with U2, U4, U5, and U6 snRNAs in the α2 complex. Although U1 is in the α2 complex, little of it is present compared with the other four snRNAs in the complex and to the levels of both U1 and U2 in β1. This reduction of U1 snRNA in α2 precedes the complete loss of U1 and U4 that occurs subsequently in the transition from α2 to α1. Both α1 and the active spliceosome as isolated by this gel electrophoretic assay contain the U2, U5, and U6 snRNAs.

As U1 snRNA is found in three pre-splicing complexes, the gel conditions used in this study most likely preserve several U1 interactions with other spliceosomal components. This gel assay will be useful for studying factors that affect these interactions during spliceosome assembly.

There Are Two Forms of δ Complex, One of Which Is Committed to the Splicing Pathway—A small fraction of the δ complex is probably the functional equivalent of the previously identified yeast commitment (11) and mammalian E (45) complexes. Like the CC and E complexes, the δ complex is formed in the absence of ATP. It is the first splicing-specific complex formed in the presence of ATP (Fig. 2). Its formation depends on the 5' end of U1 snRNP but not on other snRNPs (Figs. 3 and 5). Also, the δ complex (33) like CC (13, 46) forms in the absence of active Prp9 or Prp11 protein, each of which is required for pre-spliceosome assembly. Additionally, the effects of some mutations in both actin and RP51 pre-mRNAs on δ
A flow scheme for determining if pre-mRNA in the δ complex can be chased into the spliceosome. The scheme corresponds to the reactions shown in lanes 6, 7, and 8 in panel B. Step 1, radiolabeled pre-mRNA was incubated for 10 min at 23 °C in a splicing reaction with ATP and with the U2 snRNA inactivated by deoxyoligonucleotide-directed RNase H degradation. Step 2, a 25-fold excess of cold pre-mRNA was added to the reaction that was then incubated for 1 min at 23 °C. Step 3, an equal volume of a splicing reaction made with active whole cell extract and ATP was added to the reaction. After 15 and 30 min, samples were removed and assayed by native gel electrophoresis. The samples are from the following reactions: lane 1, radiolabeled pre-mRNA was incubated in Step 1 only; lane 2, radiolabeled pre-mRNA was incubated in Step 1 and Step 3 but with no ATP in Step 3; lane 3, radiolabeled pre-mRNA was incubated in Step 1 and Step 3; lane 4, 25-fold molar excess cold pre-mRNA was incubated in the splicing reaction in Step 1 and radiolabeled pre-mRNA and active splicing extract and ATP were added in Step 3; lane 5, radiolabeled pre-mRNA was added in Step 1, excess cold pre-mRNA was added in Step 2, and carrier RNA was added before active extract to quench the reaction; lanes 6, 7, and 8 are as diagrammed in panel A: lanes 9 and 10, radiolabeled pre-mRNA was added in Step 1, and active extract and ATP were added in Step 3; lane 11, “0” time point, radiolabeled pre-mRNA was added to the splicing reaction after the reaction had been stopped for native gel electrophoresis; and lanes 12 and 13, radiolabeled pre-mRNA was added to active splicing extract with ATP (Step 3 only), and samples were removed at 15 and 30 min, respectively.

The Yeast Pre-spliceosome Identified as the β Complex Contains the U1 and U2 snRNPs—The yeast pre-spliceosome has been thought to contain the U1 as well as the U2 snRNPs; however, previous gel electrophoretic assays have not found U1 snRNP in the B or III complex analogue of the pre-spliceosome (11, 27, 29). The mammalian homologs of the U1 and U2 snRNPs for its formation (Figs. 1–4). It does not as efficiently chased as the capped form from the commitment complex to the spliceosome in vitro in the presence of competitor RNA (49). However, the uncapped actin pre-mRNA used here in this study is spliced with equal efficiency as the capped form in vitro (31, 50). Thus, δ un may be a precursor of the pre-spliceosome or a precursor to δ commit. Alternatively, δ un may not be involved in splicing. Finally, I cannot exclude the possibility that there are more than two complexes in the δ band. Two forms of commitment complex formed on RP51 pre-mRNA migrate as two distinct bands, CC1 and CC2, in another gel assay system (11). Clearly, additional experiments are necessary to understand the parameters affecting the formation and stabilities of δ un and δ commit, the significance of these two complexes, and their relationship to each other and to the previously characterized commitment complexes, CC1 and CC2.

There are several possibilities to consider regarding the relationship between δ un and δ commit. The 5′ pre-mRNA cap and the nuclear cap binding complex may be pertinent to this relationship because the cap and complex help to stabilize interactions between the U1 snRNP and pre-mRNA (47, 48). Furthermore, an uncapped pre-mRNA of the RP51 gene is not as efficiently chased as the capped form from the commitment complex to the spliceosome in vitro in the presence of competitor RNA (49). However, the uncapped actin pre-mRNA used here in this study is spliced with equal efficiency as the capped form in vitro (31, 50). Thus, δ un may be a precursor of the pre-spliceosome or a precursor to δ commit. Alternatively, δ un may not be involved in splicing. Finally, I cannot exclude the possibility that there are more than two complexes in the δ band. Two forms of commitment complex formed on RP51 pre-mRNA migrate as two distinct bands, CC1 and CC2, in another gel assay system (11). Clearly, additional experiments are necessary to understand the parameters affecting the formation and stabilities of δ un and δ commit, the significance of these two complexes, and their relationship to each other and to the previously characterized commitment complexes, CC1 and CC2.

The Yeast Pre-spliceosome Identified as the β Complex Contains the U1 and U2 snRNPs—The yeast pre-spliceosome has been thought to contain the U1 as well as the U2 snRNPs; however, previous gel electrophoretic assays have not found U1 snRNP in the B or III complex analogue of the pre-spliceosome (11, 27, 29). β1, the second complex formed during spliceosome formation in this study, contains both the U1 and U2 snRNAs and most probably represents the pre-spliceosome. Like the mammalian A and yeast B and III complexes, β1 arises early during an in vitro splicing reaction and requires ATP and the U1 and U2 snRNPs for its formation (Figs. 1–4). It does not require a functional U4/U5/U6 tri-snRNP (Fig. 5). Furthermore, anti-Prp4 antibody that inhibits the U4/U5/U6 in yeast splicing extracts still allows formation of β1 (33) and B complexes (51). Finally, the Prp5, Prp9, and Prp11 proteins are necessary for β1 formation (33). The mammalian homologs of the Prp9 and Prp11 proteins are also required for pre-spliceo-

S. Ruby, unpublished data.
some formation in HeLa cell splicing extracts (52–54). Therefore, although I have not shown that pre-mRNA in the \( \beta_1 \) complex can be chased into an active spliceosome, the kinetics and other properties of its formation strongly suggest that the \( \beta_1 \) complex is the pre-spliceosome.

Recently, it has been proposed that disruption of base pairing between the U1 snRNA and 5' splice site of the pre-mRNA occurs after U1 snRNP binds to the pre-mRNA and before U2 snRNP binds during pre-spliceosome formation (25, 26). In support of this idea are the observations that less U1 than U2 snRNA is present in the mammalian pre-spliceosome (complex A) (20) and that U2 snRNP can bind to one mutant pre-mRNA without U1 snRNP and ATP in yeast splicing reactions (25). I have not observed any changes at this step of spliceosome assembly as evidence of this proposed alteration; in the absence of competitor pre-mRNA, the \( \delta \) complex is stable. Furthermore, there are only low levels of U1 snRNA in the \( \delta \) complex run in a gel without magnesium ion but higher levels of U1 snRNA in the pre-spliceosome (\( \beta_1 \) complex) (Fig. 1). This suggests that the U1 and U2 snRNPs stabilize each other in the pre-spliceosome.

I have also detected a second complex, \( \beta_1^* \), that, like \( \beta_1 \), contains pre-mRNA, and the U1 and U2 snRNAs. \( \beta_1^* \) appears early in splicing reactions with added EDTA and does not accumulate at later times. As its mobility is different than the \( \beta_1 \) complex formed in either the presence or absence of added EDTA, \( \beta_1^* \) is most likely a novel complex. \( \beta_1^* \) may be an assembly intermediate that, in the absence of added EDTA, co-migrates with \( \beta_1 \) or is short-lived. If it is an intermediate, then its existence may mean that additional non-snRNP factors bind to the pre-spliceosome or the conformation of the pre-spliceosome changes in preparation for the binding of the U4/U6/U5 tri-snRNP. Alternatively, it may be an aberrant complex formed as a result of the EDTA.

The Association of the U1 snRNP with the Developing Spliceosome Changes during or Shortly after the U4/U5/U6 Tri-snRNP Binds to the Pre-spliceosome—In the native gel electrophoretic assay described here, little \( \alpha \) band is present in splicing reactions with radiolabeled actin pre-mRNA, and the \( \alpha \) band migrates close to the \( \beta \) complexes. The \( \alpha \) band becomes apparent when more pre-mRNA is used in the reaction and it is assayed by Northern blot analyses. This band may have two origins: 1) the \( \alpha_2 \) complex that contains U2, U4, U5, and U6 snRNAs as well as a small amount of U1 snRNA (Fig. 4–6); and 2) the \( \alpha_1 \) complex that contains the U2, U5, and U6 snRNAs. Both of these complexes require a functional U4/U5/U6 tri-snRNP to form. Furthermore, splicing reactions blocked with anti-prp4 antibody that prevents the tri-snRNP from binding to pre-mRNA can only form the \( \delta \) and \( \beta_1 \) complex (33). These two complexes are distinguished by their behavior in reactions with 5 mM EDTA (Fig. 6). In the presence of 5 mM EDTA, the \( \alpha_1 \) complex migrates slightly faster than \( \alpha_2 \) and slower than \( \beta_2 \). They can also be differentiated under other experimental conditions. In the presence of low concentrations of added ATP, \( \alpha_2 \) accumulates and little \( \alpha_1 \) forms; and in heat-inactivated mutant prp2 extracts, mostly \( \alpha_2 \) accumulates.

Given the known order of snRNP binding to the pre-mRNA during splicing assembly (for reviews, see Refs. 1, 2, 7, and 55), the simplest interpretation of the data is that \( \alpha_2 \) is formed from the pre-spliceosome and that \( \alpha_1 \) is formed from \( \alpha_2 \).

The comparison of \( \alpha_2 \) and \( \alpha_1 \) with the previously described A2–1 and A1 complexes (29, 38) is also consistent with this interpretation. A2–1 and \( \alpha_2 \) are formed after the pre-spliceosome, contain the U2, U4, U5, and U6 snRNAs, and appear to be short lived but accumulate in the presence of low ATP. A1 and \( \alpha_1 \) contain the U2, U5, and U6 snRNAs and accumulate in reactions with 5 mM EDTA or inactivated Prp2 protein. The similarities of the \( \alpha_2 \) and \( \alpha_1 \) complexes to the A2–1 and A1 complexes suggests that \( \alpha_2 \) and \( \alpha_1 \) are intermediates in the spliceosome assembly pathway. However, that they are intermediates and equivalent to the A2–1 and A1 complexes remains to be shown.

The presence of the U1 snRNP with the other snRNPs in the \( \alpha_2 \) complex is supported by recent evidence that U1 and U5 snRNPs associate with each other in a complex. Recently, Ast and Weiner induced the formation of a U1/U4/U5 complex in HeLa extracts (16) as well as found that the U1 and U5 snRNAs can be cross-linked in a splicing related complex (56). Here I have observed that the U1 and U5 snRNPs co-migrate in a complex in the absence of added pre-mRNA although the function, significance, and origin of this complex has yet to be determined.

The amount of U1 snRNA is reduced relative to the other snRNAs in the \( \alpha_2 \) complex. This reduction suggests that the U1 snRNP dissociates from the \( \alpha_2 \) complex during gel electrophoresis or during spliceosome assembly about the time \( \alpha_2 \) is formed. A decrease in the levels of U1 relative to U2 in the transition from the pre-spliceosome to complex B in HeLa extracts has also been observed (57). Because studies in HeLa cell extracts have detected some U1 snRNP in late assembly intermediates (20, 23, 30, 57) as well as the formation of a U1/U4/U5 snRNP complex (16), I favor the first explanation that the loss of U1 is due to its instability in the \( \alpha_2 \) complex during gel electrophoresis. This instability may be due to a
disruption of the base pairing between U1 snRNA and pre-mRNA or of the U1 snRNP interactions with other spliceosomal components that make this snRNP stable to electrophoresis in the pre-spliceosome.

Genetic experiments in yeast have predicted a loss of base pairing between the pre-mRNA and U1 snRNA and the formation of base pairs between the 5' splice site and the U5 and U6 snRNAs sometime in the splicing pathway (for reviews, see Refs. 3 and 5). Subsequently, in vitro assays revealed that U5 and U6 snRNAs can be cross-linked to the 5' splice site region in a spliceosome formed in either yeast or HeLa cell extracts (for review, see Ref. 6). Furthermore, the U1 and U5 interact with the 5' splice site region during splicing assembly, whereas U5 and U6 associate with this region during splicing catalysis. These data support the notion that the U1 snRNP is "handing off" the 5' splice site region to U5 and U6 snRNPs. The low level of U1 snRNA in the spliceosome containing the U2, U5, and U6 snRNAs is formed shortly thereafter, the association of the U1 snRNP with the pre-spliceosome with the U1 and U2 snRNPs is formed. The U4/U5/U6 tri-snRNP binds to the pre-spliceosome, and the pre-spliceosome with the U1 and U2 snRNPs is formed.