The 35-kDa mammalian splicing factor SC35 mediates specific interactions between U1 and U2 small nuclear ribonucleoprotein particles at the 3' splice site

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ABSTRACT The splicing factor SC35 is required for the first step of the splicing reaction and for the assembly of the earliest ATP-dependent complex detected by native gel electrophoresis (A complex). Here we investigate the role of SC35 in mediating specific interactions between U1 and U2 small nuclear ribonucleoprotein particles (snRNPs) and the 5' and 3' splice sites of pre-mRNA. We show that U1 snRNP interacts specifically with both the 5' and 3' splice sites in the presence of ATP and that SC35 is required for these ATP-dependent interactions. Significantly, the SC35-dependent interaction between U1 snRNP and the 3' splice site requires U2 snRNP but not the 5' splice site. We also show that SC35 is required for the ATP-dependent interaction between U2 snRNP and the branch-point sequence. We conclude that SC35 may play an important role in mediating specific interactions between splicing components bound to the 5' and 3' splice sites.

Mammalian pre-mRNA splicing takes place in spliceosomes, which contain the small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4/U6, and U5 (for reviews, see refs. 1–4) and a large number of non-snRNP proteins (5). We previously raised a panel of monoclonal antibodies (mAbs) against partially purified spliceosomes as a means of identifying and characterizing functional components of the mammalian spliceosome (6). One of these mAbs, αSC35, specifically recognizes a 35-kDa protein doublet in Western blots. The SC35 antigen is required for the first step in the splicing reaction (i.e., cleavage at the 5' splice site and lariat formation). Native gel electrophoresis experiments showed that SC35 is required for the assembly of the earliest detectable ATP-dependent complex (A complex) (6). As shown (7), the A complex can be assembled on intact pre-mRNA as well as on RNA containing only the 3' splice site (7). The A complex was originally thought to contain U2 but not U1 snRNP (7), but recent studies have shown that U1 snRNP is specifically bound to the earliest known splicing complex formed in the absence of ATP (E complex; ref. 8), to the A complex (8), and to other ATP-dependent splicing complexes (8–10). In addition, A-complex formation was shown to be impaired in extracts in which U1 snRNP was depleted using a 2'-O-methylated RNA oligonucleotide (11).

In this paper we report the results of our analysis of the role of SC35 in spliceosome assembly using an RNase T1 protection/immunoprecipitation (RPI) assay (12, 13). This assay involves incubating labeled pre-mRNA substrate with nuclear extracts followed by RNase T1 digestion and immunoprecipitation using specific antibodies (12, 13). Interactions between splicing components and specific pre-mRNA sequences can then be detected by identifying the protected and immunoprecipitated RNase T1 fragments. Using this assay, we observed that αSC35 does not immunoprecipitate any

specific RNase T1 fragment when the substrate is incubated in the absence of ATP. However, when pre-mRNA is incubated in nuclear extracts in the presence of ATP and magnesium, specific RNase T1 fragments containing the 5' splice site or the branch-point sequence (BPS) at the 3' splice site are protected from RNase digestion. These fragments are immunoprecipitated with αSC35, αU1, and αU2 antibodies. Using this assay, we address the requirements of U1, U2, and SC35 for various RNase T1 protection events. Our results suggest that the role of SC35 in the early stages of spliceosome assembly is to mediate interactions between U1 and U2 snRNPs and the 3' splice site.

MATERIALS AND METHODS

Plasmids. The intact pre-mRNA and the 5' half RNA were transcribed from plasmid T7Hβ (14) linearized with BamHI in exon 2 or with Xho I in the middle of the intron. The 3' half plasmid was derived from T7Hβ by removing exon 1 and part of the intron upstream of the Xho I site (β3'; S. Michaud and R. Reed, personal communication). The 3' half RNA was transcribed from the BamHI-linearized plasmid.

Antibodies. mAb αU170K (15), mAb αSC35 (6), and human αU2 serum (gift from J. Bruzik and J. Steitz, Yale University Medical School) were used.

RNase Protection and Immunoprecipitation. The RPI assay was as described (12, 13, 16) with some modifications: A 25-μl reaction mixture was assembled in which 1–5 × 10⁶ cpm of RNA substrate was added. After incubation, 20-μl of RNase T1 (3.5 units/μl) was added and the digestion was carried out on ice for 30 min. For immunoprecipitation, 1.5 ml of mAb culture supernatant or 10 μl of serum was bound to 15 μl (packed volume) of protein G beads (Pharmacia). After washing with IP buffer (50 mM Tris·HCl, pH 7.9/150 mM NaCl/0.05% Nonidet P-40), the antibody–protein G beads in IP buffer [50% (vol/vol)] were added to the T1 digestion reaction mixture and the immunoprecipitation was carried out with rotation at 4°C for an additional 30 min. After washing the pellet with four 1-ml volumes of IP buffer, T1-resistant RNA fragments were isolated by phenol extraction followed by ethanol precipitation and analyzed on a 15% denaturing polyacrylamide gel.

RNase H Inactivation. RNase H inactivations of U1 and U2 snRNAs were as described (12). The anti-U1 oligonucleotide used in this study was 5'-CAGGTAAGTA-3'. The anti-U2 oligonucleotide used was 5'-GGCCGAGAAGCGAT-3'.

SC35 Depletion and Complementation. Depletion of SC35 and complementation were as described (6) with one modification: 25 μg of purified αSC35 was bound to 10 μl (packed volume) of protein G beads and the antibody–protein G beads

Abbreviations: snRNP, small nuclear ribonucleoprotein particle; mAb, monoclonal antibody; RPI, RNase protection/immunoprecipitation; BPS, branch-point sequence; IVS1, intervening sequence 1; nt, nucleotide(s).

were used to deplete SC35 in one 25-μl splicing reaction mixture containing 5–7.5 μl of nuclear extract.

RESULTS

SC35 Interacts with Spliceosomal Components Bound to 5' and 3' Splice Sites in Intact pre-mRNA. To determine whether SC35 interacts with spliceosomal components bound to specific regions of pre-mRNA, we carried out RPI experiments with the αSC35 mAb. A ^{32}P-labeled RNA containing the human β-globin intervening sequence 1 (IVS1) was incubated in nuclear extracts at 30°C in the presence or absence of ATP followed by digestion with RNase T1. The RNase T1 digestion products were immunoprecipitated with the αSC35, αU1, or αU2 antibodies and then fractionated by polyacrylamide gel electrophoresis (12, 13, 16). As reported, a 15-nucleotide (nt) RNase T1 fragment (A fragment) from the 5' splice site of β-globin pre-mRNA was immunoprecipitated with the αU1 snRNP mAb in the presence (Fig. 1, lane 3) or absence (Fig. 1, lane 8) of ATP or when the reaction mixture was kept on ice (Fig. 1, lane 3). Thus, U1 snRNP rapidly binds to the 5' splice site in the absence of ATP at temperatures at which functional prespliceosome complexes do not form (12, 13). A trace level of the A fragment was also immunoprecipitated with human αU2 autoimmune serum (Fig. 1, lanes 15–20). This result could be explained by weak interactions between U2 snRNP and a complex bound to the 5' splice site or by a low-level contamination of αU1 cross-reactivity in the αU2 serum. In contrast to the αU1 and αU2 snRNP antibodies, the A fragment was not

immunoprecipitated by αSC35 (Fig. 1, lanes 9–13). We conclude that SC35 is not directly associated with U1 snRNP, with the 5' splice site sequence, or with other components associated with the A fragment.

When intact pre-mRNA is incubated in nuclear extracts at 30°C in the absence of ATP and Mg^{2+}, the earliest detectable functional spliceosome precursor (E complex) is formed (5, 8). Analyses of affinity-purified E complex revealed that U1 snRNP is specifically associated (8). When we performed RPI assays under these conditions the αU1 snRNP antibody immunoprecipitated the A fragment as well as a number of other T1 fragments that were also immunoprecipitated by αU2 and by αSC35 but not by a control antibody (data not shown). Thus, these observations indicate that U1 snRNP binds specifically to the E complex, but specific RNase protection and immunoprecipitation with the αU2 and αSC35 antibodies require an ATP-dependent conformational change. If Mg^{2+}, but not ATP, was included in the reaction mixture, only nonspecific interactions were detected (Fig. 1, lanes 2, 8, 14, and 20).

A different pattern of RNase T1 protection was observed when the extracts were incubated for 5 min or longer in the presence of ATP. Two 42-nt fragments were immunoprecipitated with all three antibodies (Fig. 1): One of these fragments was previously shown to be derived from the 5' splice site (E' fragment), whereas the other includes the BPS at the 3' splice site (E fragment) (12). The identity of the fragments was confirmed by secondary RNase T1 digestion of the 42-nt
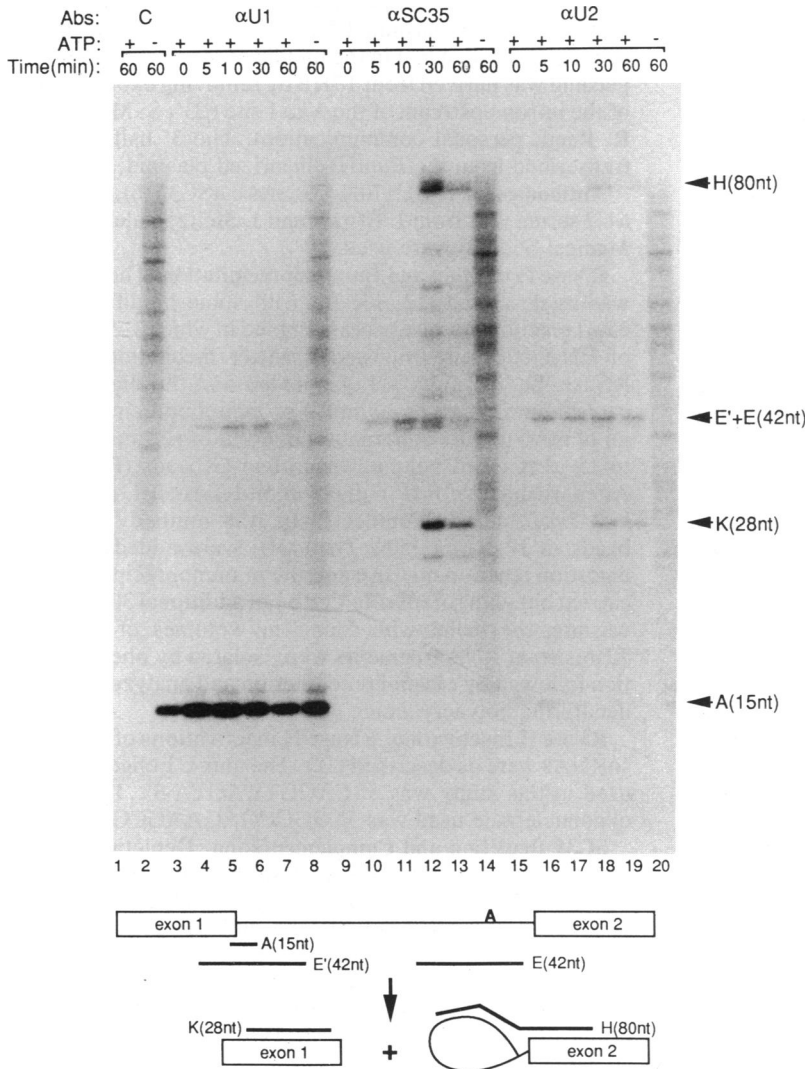
Fig. 1. (*Upper*) RNase T1 protection and immunoprecipitation assays using intact Hβ-globin IVS1. Splicing reactions were carried out for the indicated times in the presence (+) or absence (−) of ATP. After the RNase T1 treatment, immunoprecipitations were carried out with a control mAb (*C*) (lanes 1 and 2), αU170K mAb (lanes 3–8), αSC35 (lanes 9–14), and αU2 serum (lanes 15–20). (*Lower*) Protected RNA fragments. The A, E', and E fragments are from the RNA precursor, and the K and H fragments are from the splicing intermediates after the first step of the splicing reaction.

fragments immunoprecipitated by all three antibodies (data not shown; see also ref. 13). We conclude that SC35 is associated with factors that bind to the 5' and/or 3' splice sites in complexes assembled under splicing conditions.

After a 10-min incubation in the presence of ATP, a characteristic spectrum of protected T1 fragments was observed after immunoprecipitations with αU2 as reported (ref. 13; Fig. 1, lanes 18 and 19). The αSC35 antibody immunoprecipitated a set of T1 fragments similar to those precipitated by αU2 (Fig. 1, lanes 12, 13, 18, and 19). Of particular interest are H (80 nt) and K (28 nt) fragments, which are derived from splicing intermediates after the first step of the splicing reaction. The K fragment is from exon 1, which becomes associated with SC35 and U2 snRNP but not with U1 snRNP, suggesting that the 5' exon sequence has folded during spliceosome assembly in such a way that it contacts a factor(s), such as U2 snRNP, that interact with the 3' splice site.

SC35 Interacts with Spliceosomal Components Bound to the 3' Splice Site in RNAs Lacking a 5' Splice Site. To investigate interactions between SC35 and specific regions of pre-mRNA, we synthesized RNA substrates containing only the 5' splice site or the 3' splice site including the BPS. The RPI assay was carried out at the 5-min time point to detect interactions at an early stage of spliceosome assembly. We first tested the 5' half substrate. We found that αU1 immunoprecipitated the A fragment from the 5' splice site RNA with or without ATP (Fig. 2, lanes 3 and 4). However, the ATP-dependent 42-nt E' fragment from the 5' splice site could not be immunoprecip-

itated with αU1, αU2, or αSC35. Thus, the 3' splice site is required for the ATP-dependent protection and immunoprecipitation of the 5' splice site E' fragment.

Analysis of RNA containing only the 3' splice site revealed that αU2 immunoprecipitated two ATP-dependent T1 fragments: a 42-nt and a 35-nt fragment (Fig. 2, lanes 15 and 16) derived from the same region (data not shown). Although the 35-nt fragment was not detected with the intact pre-mRNA substrate under our RNase T1 digestion conditions, this fragment was detected when a higher concentration of RNase T1 was used (12). This observation suggests that interactions between spliceosomal components and the 3' splice site differ somewhat between intact pre-mRNA and RNA lacking a 5' splice site.

Significantly, both the 42-nt and 35-nt RNase T1 fragments were also immunoprecipitated with αU1 and αSC35, indicating that both of these spliceosomal components interact with factors bound to the 3' splice site in RNA lacking the 5' splice site. Again, these interactions were ATP-dependent (Fig. 2, lanes 11–14).

Specific Interactions Between SC35 and U1 snRNP and the 3' Splice Site Require U2 snRNP. We next investigated the requirements for specific interactions between U1 snRNP, U2 snRNP, SC35, and the 3' splice site. First, we analyzed the effects of removing the 5' end of U1 or U2 snRNAs by deoxyoligonucleotide-directed RNase H digestion. RNase H-inactivated U1 snRNP does not bind to the A fragment in intact pre-mRNA (13) or in the RNA containing only the 5' splice site (Fig. 3, lane 2). In contrast, RNase H digestion with a control deoxyoligonucleotide or with a deoxyoligonucleotide specific for the 5' end of U2 snRNA had no effect on U1 binding to the 5' splice site in the 5' half molecule (Fig. 3, lanes 1 and 3).

Different results were obtained when the 3' half molecule was used in this study. First, removal of the 5' end of U1 snRNA did not prevent RNase T1 protection and immunoprecipitation of the 42- and 35-nt fragments with either αU1 or αSC35 (Fig. 3, lanes 6 and 7). Thus, the assembly of a 3'-splice-site complex containing both U1 and SC35 does not
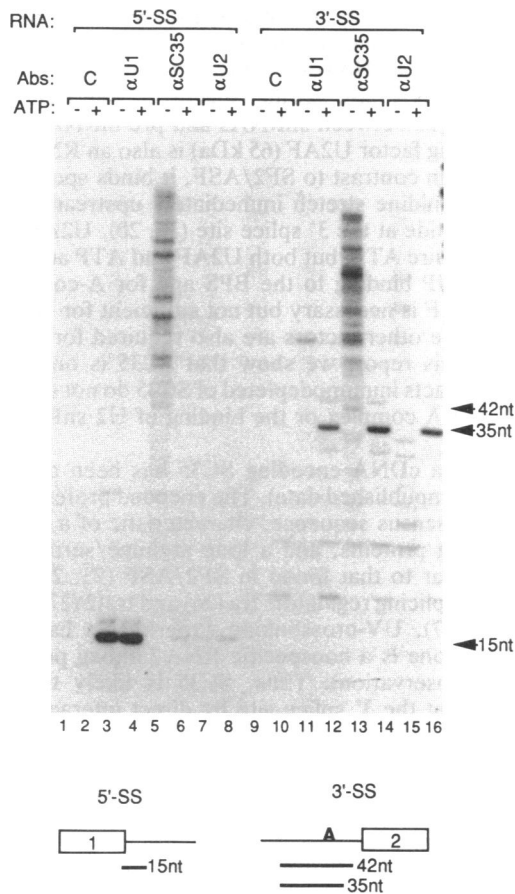

FIG. 2. (*Upper*) Detection of interactions of U1 snRNP, SC35, and U2 snRNP with 5' half (5'-SS) and 3' half (3'-SS) RNAs. The RPI assays were carried out in the presence (+) or absence (−) of ATP as indicated, with a control antibody (lanes 1, 2, 9, and 10), αU1 (lanes 3, 4, 11, and 12), αSC35 (lanes 5, 6, 13, and 14), and αU2 (lanes 7, 8, 15, and 16). (*Lower*) RNA substrates and the protected RNA fragments.
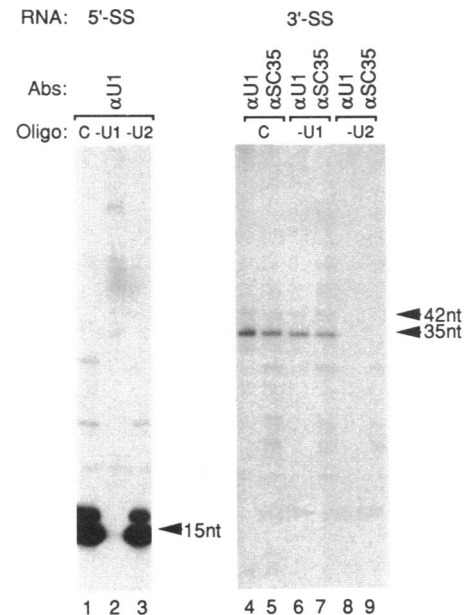

FIG. 3. Requirements of U1 and U2 snRNAs for the interaction of U1 with the 5' half (5'-SS) RNA and for the interactions of U1 and SC35 with the 3' half (3'-SS) RNA. Nuclear extracts were treated with RNase H plus a control oligonucleotide (lanes 1, 4, and 5), an anti-U1 oligonucleotide (lanes 2, 6, and 7), or an anti-U2 oligonucleotide (lanes 3, 8, and 9). Immunoprecipitations were performed using αU1 (lanes 1–3, 4, 6, and 8) and αSC35 (lanes 5, 7, and 9).

require the 5' end of U1 snRNA. Similarly, the removal of the 5' end of U1 snRNA did not prevent the immunoprecipitation of the 42- and 35-nt protected fragments by αU2 (data not shown; see also ref. 13). These observations are consistent with results from previous immunoprecipitation experiments (9), showing that the U1 snRNP interaction with the 3' splice site is independent of its interaction with the 5' splice site.

When the 5' end of U2 snRNA was inactivated, however, no protection of the BPS was observed. Neither αU1 nor αSC35 immunoprecipitated the 42- and 35-nt fragments containing the BPS (Fig. 3, lanes 8 and 9). Thus, specific interactions between U1 snRNP or SC35 with components bound to the BPS require the 5' end of U2 snRNA.

SC35 Is Required for Specific Interactions Between U1 snRNP and the U2 snRNP-Containing Complex Assembled at the 3' Splice Site. To determine whether SC35 is required for specific interactions between spliceosome components and sequences at the 5' and 3' splice sites, we carried out the RPI experiments in extracts depleted of SC35. As expected, removal of SC35 did not prevent the immunoprecipitation of the A fragment by αU1 (Fig. 4, lanes 1 and 2). Thus, SC35 is not required for the ATP-independent interaction between U1 snRNP and the 5' splice site. In contrast, removal of SC35 resulted in a significant decrease in the amount of 42-nt and 35-nt 3' splice site fragments immunoprecipitated by αU1 or αU2 antibodies (Fig. 4, lanes 6 and 8). Mock depletion with a control mAb had no effect on the binding of U1 and U2 to the 3' splice site (Fig. 4, lanes 5 and 7). These results are consistent with a recent observation that U1-depleted nuclear extracts are impaired in their ability to support the formation of a U2-containing A complex (11). We conclude that SC35 is required for the specific interactions between U1 and U2 snRNPs at the 3' splice site.

To demonstrate directly that SC35 can stimulate the U1 interaction with spliceosomal components bound to the 3' splice site, we performed an immunodepletion and reconstitution experiment. Nuclear extracts were first depleted of SC35 followed by complementation with recombinant SC35

Fig. 4. Role of SC35 in RNase protection. Nuclear extracts were depleted with a control mAb or αSC35. The nuclear extracts containing (+) or lacking (−) SC35 were used to perform the RPI assays on the 5'-half (5'-SS) or the 3'-half (3'-SS) RNAs with a control antibody (lanes 3 and 4), αU1 (lanes 1, 2, 5, and 6), and αU2 (lanes 7 and 8). The additional nonspecific RNA fragments from the 3'-SS RNA were not further characterized.

produced using a baculovirus expression system (unpublished data). As shown in Fig. 5B, SC35-depleted extracts were unable to splice β-globin IVS1 (lanes 1 and 3), and splicing activity could be reconstituted by the addition of recombinant SC35 (lane 4). When the parallel SC35-depleted and reconstituted extracts were used to perform the RPI assays with the αU1 antibody, it was clear that SC35 promotes the interaction between U1 snRNP and the complex assembled at the 3' splice site in a concentration-dependent manner (Fig. 5A, lanes 4–6). The control extract, in contrast, had no effect on U1 snRNP interaction with the 3' splice site (Fig. 5A, lanes 7–9). These experiments further substantiate the conclusion that SC35 is required for the specific interaction of U1 snRNP with the spliceosomal components bound to the 3' splice site.

DISCUSSION

Three mammalian splicing factors, SF2/ASF (17, 18), U2AF (19–21), and SC35 (6, 22), have been purified to homogeneity and shown to be required for the earliest steps of spliceosome assembly. The splicing factor SF2/ASF is a 33-kDa RNA binding protein containing an arginine/serine-rich region and an RNP-type RNA binding domain (23, 24). Although this factor can influence 5' splice site selection in pre-mRNAs containing competing 5' splice sites (18, 25), the purified factor does not bind specifically to U1 snRNP or to the 5' splice sites (17). Extracts lacking SF2/ASF are unable to assemble A complex but can do so after the addition of the purified SF2/ASF (17). The mechanism by which SF2/ASF is involved in splicing and influences 5' splice site selection is not understood. However, this factor is capable of catalyzing RNA·RNA base pairing, suggesting that it may mediate interactions between snRNAs and pre-mRNA (17).

The splicing factor U2AF (65 kDa) is also an RNA binding protein but, in contrast to SF2/ASF, it binds specifically to the polypyrimidine stretch immediately upstream from the AG dinucleotide at the 3' splice site (19, 20). U2AF binding does not require ATP, but both U2AF and ATP are required for U2 snRNP binding to the BPS and for A-complex formation. U2AF is necessary but not sufficient for U2 snRNP binding, since other factors are also required for this interaction. In this report we show that SC35 is one of these factors. Extracts immunodepleted of SC35 do not support the assembly of A complex or the binding of U2 snRNP to the BPS.

Recently, a cDNA encoding SC35 has been cloned and sequenced (unpublished data). The encoded protein contains an RNP consensus sequence, characteristic of a number of RNA-binding proteins, and a long arginine/serine-rich sequence similar to that found in SF2/ASF (23, 24) and the *Drosophila* splicing regulators tra (26) and tra-2 (27, 28). Like SF2/ASF (17), UV-crosslinking experiments have shown that SC35 alone is a nonspecific RNA binding protein (unpublished observation). Thus, SC35 is likely to mediate interactions at the 3' splice site by direct interactions with snRNA, pre-mRNA, or both in the presence of ATP and other splicing factors.

U1 snRNP plays a critical role in directing the splicing machinery to the correct 5' splice site. The 5' end of U1 snRNA is complementary to the 5' splice site consensus sequence, and base pairing between these sequences is required for splicing (29). It is known that U1 snRNP binds to 5' splice sites in the absence of ATP and protects a 15-nt fragment (A fragment) in β-globin pre-mRNA substrate from RNase T1 digestion (12). However, the functional significance of this interaction has not been demonstrated because U1 snRNP also binds to synthetic 5' splice sites that do not function in the splicing reaction (30). These studies indicate that additional pre-mRNA segments are necessary to select
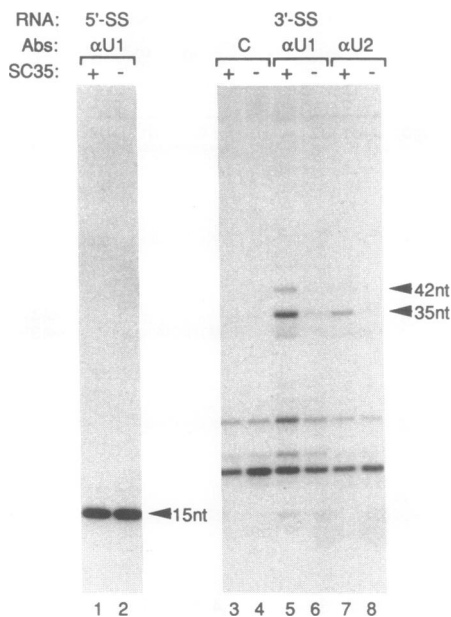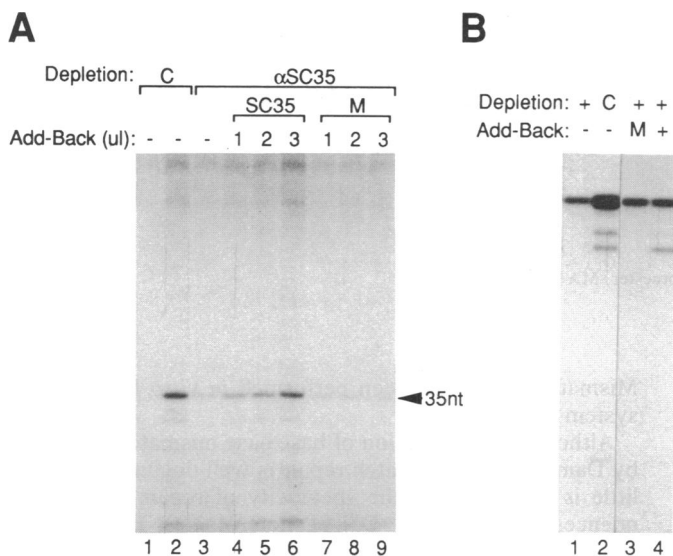
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FIG. 5. Reconstitution of the U1 interaction with the 3' splice site (*A*) and splicing (*B*) with recombinant SC35. (*A*) Nuclear extracts were depleted with a control antibody (*C*; lanes 1 and 2) or αSC35 (lanes 3-9) followed by complementation with insect cell extracts containing recombinant SC35 (lanes 4-6) or control extracts lacking SC35 (lanes 7-9). These extracts were used to perform the RPI assays on the 3' half (3'-SS) RNA with a control antibody (lane 1) and αU1 (lanes 2-9). (*B*) Nuclear extracts treated the same as *A* with a control antibody (lane 2) or with αSC35 (lanes 1, 3, and 4) were assayed for splicing activity after complementation with 3 μl of insect cell extracts containing recombinant SC35 (lane 4) or mock complementation (M) with 3 μl of control extracts lacking SC35 (lane 3). The pre-mRNA substrate, spliced product, and intermediates are indicated on the right.

the functional 5' splice site. In the present studies, SC35 is shown to be required for protection of the 42-nt E' fragment (which covers the A fragment), but not for the 15-nt A fragment, in the 5' splice site. The protection of the E' fragment, which is immunoprecipitated by αU1 and αSC35, requires ATP and incubation at 30°C. In addition, the E' fragment is not detected in the absence of an intact 3' splice site. Thus, the protection of the E' fragment correlates with the functional requirements for splicing and spliceosome assembly. The fact that E' can be immunoprecipitated by αSC35 indicates that SC35 is associated directly or indirectly with this RNase-resistant region of the pre-mRNA in assembled splicing complexes.

The step during spliceosome assembly at which SC35 is first involved has not been established. The earliest known functional spliceosome precursor is the E complex (5, 8). This complex is formed in the absence of ATP at 30°C, and it contains U1 snRNP (8). We do not know whether SC35 is required for E-complex assembly, but isolated E complex appears to contain SC35 (X.-D.F., T.M., and R. Reed, unpublished observation). The observations that SC35 is required for A-complex assembly and that the E complex appears to be the immediate precursor of the A complex (8) strongly suggest that SC35 is required for the E-to-A transition.

The pattern of RNase T1 protection and our current understanding of the composition of the A complex are summarized as follows. The protection of the 42-nt RNase T1 digestion fragments at the 5' and 3' splice sites requires ATP, SF2/ASF, SC35, U2AF, U1 snRNP, and U2 snRNP plus at least one additional splicing factor, SF1 (31). U1 snRNP binds specifically to the 5' splice site and interacts directly or indirectly with the 3' splice site. Conversely, U2 snRNP binds specifically to the branch-point sequence and interacts directly or indirectly with the 5' splice site. The splicing factor U2AF binds specifically to the pyrimidine tract at the 3' splice site and U2AF has been proposed to facilitate U2 snRNP binding to the BPS. SF2/ASF promotes proximal 5' splice site selection in cis-competition assays but does not affect 3' splice site selection, suggesting that it may function through components that interact with the 5' splice site. Finally, we show here that SC35 is required for U1 snRNP to interact with components bound to the 3' splice site, suggesting that it is one of the factors involved in bridging the 5' and 3' splice sites during spliceosome assembly.

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