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SR Proteins Promote the First Specific Recognition of Pre-mRNA and Are Present Together with the U1 Small Nuclear Ribonucleoprotein Particle in a General Splicing Enhancer Complex

DAVID STAKNIS AND ROBIN REED*

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115

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We show that addition of SR proteins to in vitro splicing extracts results in a significant increase in assembly of the earliest prespliceosomal complex E and a corresponding decrease in assembly of the heterogeneous nuclear ribonucleoprotein (hnRNP) complex H. In addition, SR proteins promote formation of the E5′ and E3′ complexes that assemble on RNAs containing only 5′ and 3′ splice sites, respectively. We conclude that SR proteins promote the earliest specific recognition of both the 5′ and 3′ splice sites and are limiting for this function in HeLa nuclear extracts. Using UV cross-linking, we demonstrate specific, splice site-dependent RNA-protein interactions of SR proteins in the E, E5′, and E3′ complexes. SR proteins do not UV cross-link in the H complex, and conversely, hnRNP cross-linking is largely excluded from the E-type complexes. We also show that a discrete complex resembling the E5′ complex assembles on both purine-rich and non-purine-rich exonic splicing enhancers. This complex, which we have designated the Enhancer complex, contains U1 small nuclear RNP (snRNP) and is associated with different SR protein family members, depending on the sequence of the enhancer. We propose that both downstream 5′ splice site enhancers and exonic enhancers function by establishing a network of pre-mRNA–protein and protein-protein interactions involving U1 snRNP, SR proteins, and U2AF that is similar to the interactions that bring the 5′ and 3′ splice sites together in the E complex.

Most pre-mRNAs in metazoans are highly complex, containing numerous 5′ and 3′ splice sites that are often alternatively spliced to generate multiple mRNAs from a single pre-mRNA. The splicing reaction requires sequence elements located at the 5′ and 3′ splice sites and at the branchpoint sequence. However, in most of the complex pre-mRNAs in higher eukaryotes, these intronic sequences are not well conserved and thus are not sufficient for specifying the 5′ and 3′ splice sites. A general role for exon sequences in splicing was revealed in studies showing that normal 5′ or 3′ splice sites are often not used when the exon sequences are substituted with a variety of random sequences (17, 44) or when the splice sites are inserted into different locations in the pre-mRNA (41). In addition to playing a fundamental role in the splicing of constitutively spliced pre-mRNAs, exon sequences are also essential for alternative splice site selection (6, 7, 12, 20–22, 26, 27, 34–36, 40, 46, 50, 52, 58).

Although a central role for exon sequences in splicing has been known for some time, only recently have these sequences begun to be defined (59, 61). Several pre-mRNAs contain an exonic element required for splicing the upstream intron; these elements, designated splicing enhancers, can potently stimulate splicing of pre-mRNAs containing poorly conserved splice site sequences (8, 33, 53, 55–57, 59, 61). All of the constitutive exonic enhancers characterized thus far consist of purine-rich sequences (55, 59), but the precise sequences required for enhancer activity are not yet defined. In addition to the exonic enhancers, enhancer activity can also be conferred by a 5′ splice site located on the downstream side of an exon (hereafter this element is referred to as a downstream 5′ splice site [Ds 5′ss] enhancer) (31, 47, 54). Both the exonic and the Ds 5′ss enhancers stimulate splicing of the upstream intron, and in general, it appears that increasing the match of the intronic 5′ or 3′ splice site sequences to the respective consensus sequences abrogates the requirement for the enhancers (24, 56). Moreover, under appropriate conditions, the different classes of enhancers can functionally substitute for one another (8, 33, 55, 59). It is not yet clear whether enhancers account for most of the general effects of exon sequences on 5′ and 3′ splice site use.

Members of the SR family of essential splicing factors have been shown to bind to three different exonic enhancers (33, 53, 57) and to be essential for enhancer function (53, 57). The SR proteins comprise a structurally related family of at least six proteins (SRp20, SRp30a [SF2/ASF], SRp30b [SC35], SRp40, SRp55, and SRp75) with partially overlapping functions (14, 16, 18, 29, 48, 57, 62, 63). U1 small nuclear RNA (snRNA) has been found to cross-link to another exonic enhancer (59), and the U1 small nuclear ribonucleoprotein particle (snRNP) binds to, and is required for the function of, Ds 5′ss enhancers (23, 31, 47). No studies have been done to determine whether both U1 snRNP and SR proteins are present on the same exonic enhancer. Similarly, it is not known whether SR proteins are involved in the function of Ds 5′ss enhancers.

Pre-mRNA is committed to the spliceosome assembly pathway in the prespliceosomal complex E (25, 38, 39, 49). The 5′ and 3′ splice sites are first functionally associated with one another in the E complex (25, 39, 49), and this interaction is thought to involve U1 snRNP bound to the 5′ splice site and U2AF bound to the 3′ splice site (39, 49, 64, 65). It has been proposed that SR proteins mediate the interactions between these components in the E complex (60). This proposal was based on the demonstration that SR proteins can interact via protein-protein interactions with one another (2, 60), with the U1 snRNP component U1 70K (28, 60), and with $U2AF^{35}$ (60).

* Corresponding author. Mailing address: Department of Cell Biology, 45 Shattuck St., Harvard Medical School, Boston, MA 02115. Phone: (617) 432-2844. Fax: (617) 432-1144.

In addition, SR proteins are required for assembly of the A complex (13, 29) and are thought to mediate interactions between the 5′ and 3′ splice sites in the A complex (15). However, it has not yet been determined whether SR proteins are present in the E complex or whether they play a role in assembly of the E complex. SR proteins have been shown to stimulate binding of U1 snRNP to the 5′ splice site (10, 28), but how this relates to the spliceosome assembly pathway is not known. Finally, in the presence of excess competitor RNA, pre-mRNA that is prebound to SR proteins is spliced more efficiently than naked pre-mRNA (11). However, the step in spliceosome assembly at which the prebound SR proteins first function was not addressed in these studies (11).

In this study, we show that SR proteins promote formation of the E complex and specifically UV cross-link to pre-mRNA in the E complex. We find that SR proteins not only promote U1 snRNP binding to the 5′ splice site (10, 28) but also promote U2AF binding to and U1 snRNP association with the 3′ splice site. These data, together with previous work (2, 10, 28, 39, 60, 65), support the proposal that the 5′ and 3′ splice sites are brought together in the E complex via a network of interactions involving U1 snRNP, U2AF, and SR proteins. These interactions involve not only protein-protein interactions of SR proteins (2, 28, 60) but also pre-mRNA–SR protein interactions. We also show that exonic enhancers and RNAs containing a 5′ splice site alone assemble into E-type complexes that contain both U1 snRNP and SR proteins (designated Enhancer [Enh] complexes). Thus, exonic enhancers and Ds 5′ss enhancers may function via the same type of RNA-protein and protein-protein interactions that bring the splice sites together in the E complex.

MATERIALS AND METHODS

Plasmids. Plasmids pAdML and pAd3′ have been described by Bennett et al. (3). DNAs were linearized with *Bam*HI for transcription. pAd5′, which contains exon 1 and the 5′ portion of the adenovirus major late (AdML) intron, was linearized with *Xho*I (39). pAdΔ5′ and pAdΔ3′, which contain substitutions of the 5′ and 3′ splice sites in pAd5′ and pAd3′, respectively, have been described by Michaud and Reed (39). The pAdΔ5′ 5′ splice site sequence is ATTGGAGCCAC. In pAdΔ3, eight consecutive T residues in the 3′ splice site (+155 to 162 in AdML) are changed to GTGATCAC. pAdΔ5′ and pAdΔ3′ were linearized with *Xho*I and *Bam*HI, respectively. pFP and pFPD (gifts from F. Rottman, Case Western Reserve University, Cleveland, Ohio) were linearized with *Bam*HI (53). pFtz (a gift from D. Rio, University of California, Berkeley) was linearized with *Xho*I. pASLV and pASLV-6U were constructed by ligating oligonucleotides (see Fig. 8 for sequences) into the *Bgl*II and *Eco*RI sites of pSP72 (Promega Biotec). Plasmids were linearized with *Eco*RI. pDsx (a gift from T. Maniatis, Harvard University, Cambridge, Mass.) was linearized with *Bam*HI. pDsx-ASLV and pDsx-ASLV-6U were constructed by ligating blunted oligonucleotides (see Fig. 8 for sequences) into the blunted *Bam*HI site of pDsx. Plasmids were linearized with *Mlu*I. Linearized DNAs were transcribed with T7 RNA polymerase. The nonspecific RNA lacking functional splice sites was an antisense transcript of a human β-globin derivative (39, 42). DNA was linearized with *Xho*I and transcribed with SP6 RNA polymerase.

Pre-mRNA synthesis and in vitro splicing reactions. For UV cross-linking, capped pre-mRNAs were synthesized in standard transcription reaction mixtures (37) containing 50 μCi each of ^{32}P-labeled ATP, GTP, CTP, and UTP (3,000 Ci/mmol), 100 μM unlabeled (cold) GTP and UTP, and 200 μM cold ATP and CTP. Assembly of splicing complexes was carried out under in vitro splicing conditions. For assembly of the B complex, reaction mixtures contained 30% nuclear extract and were incubated at 30°C for 20 min. For assembly of the E and Enh complexes, nuclear extracts were depleted of ATP as described previously (38). E and H complex assembly reaction mixtures lacked ATP, $MgCl_2$, and creatine phosphate. Reaction mixtures were incubated at 30°C for 15 min. For reactions using SC35-supplemented nuclear extracts, titrations were carried out to determine the levels of SC35 to add. We found that approximately 200 ng of purified recombinant SC35 in a 25-μl reaction resulted in maximal levels of complex stimulation. Recombinant SC35 was produced and purified from a baculovirus expression system as previously described (57).

Purification of splicing complexes and UV cross-linking. ^{32}P-labeled RNAs were incubated in splicing extracts to assemble complexes and then irradiated on ice with 254-nm UV light (Sylvania G15T8 lamp) for 5 min at a distance of 5.5 cm from the light source. The reaction mixtures containing the B complex were irradiated for 20 min because the ATP added for complex assembly absorbs UV light and thus significantly reduces cross-linking efficiency (data not shown). Cross-linked complexes were then fractionated by gel filtration as described previously (1, 45). In Fig. 3A and B (lanes 1 and 2), the H and E complexes were irradiated on ice for 5 min after gel filtration. Purified SR proteins from HeLa cells (a generous gift from J. Bruzik, Case Western Reserve School of Medicine, Cleveland, Ohio) were incubated with ^{32}P-labeled RNA, UV irradiated for 5 min, and immediately prepared for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) or two-dimensional (2D) gel electrophoresis analysis.

Preparation and analysis of proteins. To digest the ^{32}P-labeled RNA in the cross-linked, gel filtration-purified complexes, 3 μl of 10-mg/ml protease-free RNase A (Pharmacia) was added to 300 μl of gel filtration fractions containing the splicing complexes and incubated at 37°C for 30 min; 30 μl of 20% SDS and 3 μl of 2 M dithiothreitol were then added, and proteins were heated at 65°C for 5 min. Glycogen (2 μl as carrier) and 4 volumes of acetone were added, and samples were left at room temperature for 10 min and then spun for 10 min at room temperature. Recovered proteins were immediately dissolved in SDS or 2D sample buffer (8 M urea, 1.7% Nonidet P-40, 1.7% pH 3 to 10 ampholytes [Bio-Rad]) and stored at −70°C or loaded directly onto gels. 2D gel electrophoresis was carried out as described previously (3). The first dimension was nonequilibrium pH gradient gel electrophoresis (pH 3 to 10 ampholytes [Bio-Rad]), and the second dimension was SDS-PAGE (9.5% polyacrylamide gel) as indicated. Cross-linked proteins were detected by PhosphorImager analysis (Molecular Dynamics).

Immunoprecipitation of complexes. A U1 70K monoclonal antibody (MAb) was coupled to protein A-Trisacryl beads by using a rabbit anti-mouse secondary antibody. The coupled beads were then mixed for 4.5 h at 4°C with gel filtration fractions containing splicing complexes. The rabbit anti-mouse antibody was used as a control. After the immunoprecipitates were washed with 125 mM NaCl–20 mM Tris (pH 7.6), total RNA was prepared and analyzed on an 8% denaturing polyacrylamide gel.

RESULTS

SR proteins promote the earliest specific recognition of the pre-mRNA. To determine whether SR proteins play a role in E complex assembly, we compared E complex assembly in nor-

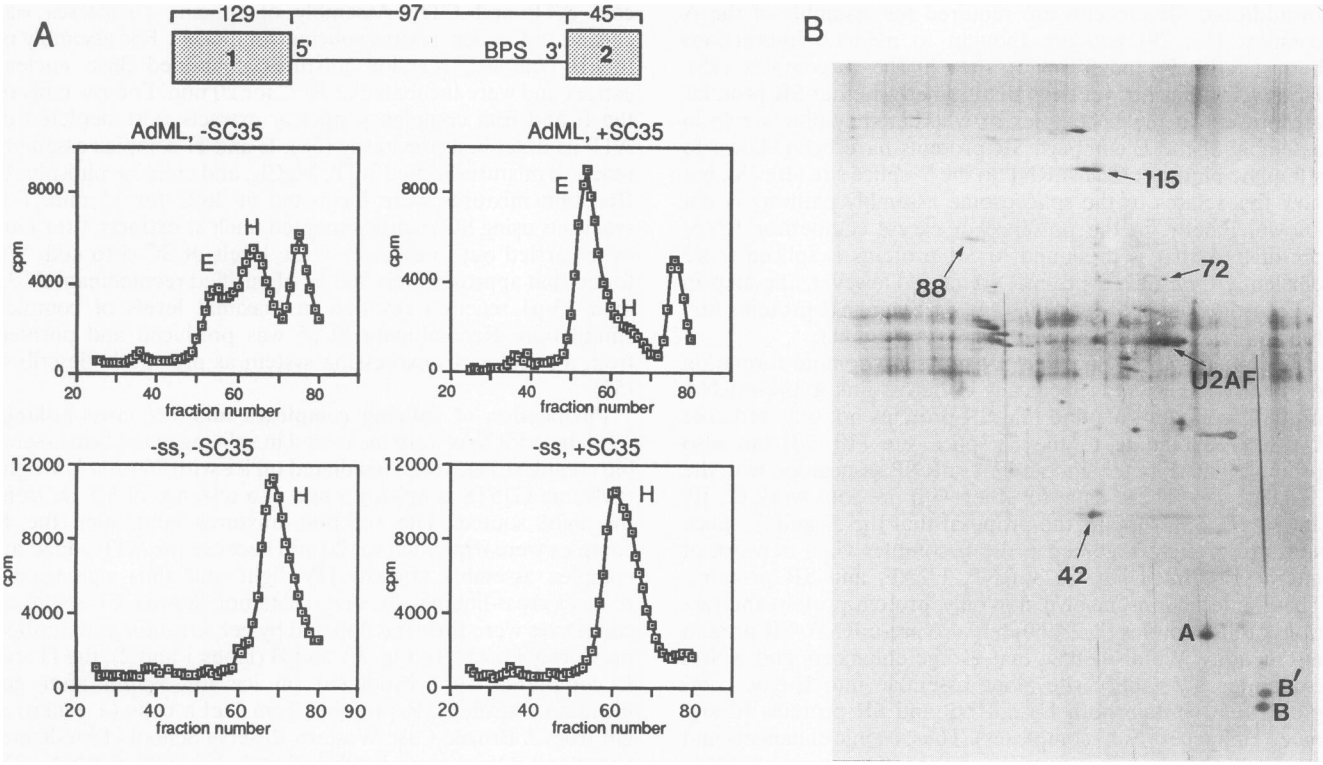

FIG. 1. SC35 promotes E complex assembly. (A) The sizes of the exons and intron in AdML pre-mRNA are shown. BPS, branchpoint sequence. For analysis of complexes, 200 ng of ^{32}P-labeled AdML pre-mRNA (AdML) or a pre-mRNA lacking splice sites (−ss) was incubated in normal nuclear extract (−SC35) or in extract supplemented with purified recombinant SC35 from baculovirus-infected cells (+SC35) in the absence of ATP and then fractionated by gel filtration. Complex assembly reactions were 100 μl. The same amount of SC35 was added to the −ss and AdML reaction mixtures (see Materials and Methods). The peaks containing the E and H complexes are indicated. The peaks to the left and right of the complexes are the void volume and degraded pre-mRNA, respectively. (B) The E complex was assembled in SC35-supplemented nuclear extracts and affinity purified, and total protein obtained from 200 ng of pre-mRNA was fractionated by 2D gel electrophoresis. Proteins were visualized by silver staining and are designated according to Bennett et al. (3). Sizes are indicated in kilodaltons.

mal nuclear extracts versus extracts supplemented with recombinant SC35 (Fig. 1). In the absence of added SC35, the E complex is detected as a shoulder on the H complex peak (Fig. 1A, AdML, −SC35 [38, 43]). In contrast, in SC35-supplemented extracts, the E complex is detected as a distinct peak, and the H complex is barely detected (Fig. 1A, AdML, +SC35). We did not observe a further increase in the levels of the E complex when more SC35 was added to the nuclear extract, and less stimulation was observed when less SC35 was added (see Materials and Methods). Importantly, analysis of the E complex assembled in SC35-supplemented extracts by 2D gel electrophoresis revealed that it has the same protein composition as that of the E complex assembled in normal nuclear extracts (Fig. 1B [39]). Thus, the complex assembled in SC35-supplemented extracts is the E complex and not, for example, an aggregation of SR proteins. Further support for this conclusion is the observation that AdML pre-mRNA incubated with SC35 alone (in the absence of nuclear extract) elutes between the H complex and the degraded RNA on the gel filtration column (data not shown).

The increase in E complex assembly is dependent on the addition of SC35, as no increase was observed when equivalent levels of other recombinant proteins, such as the A complex component SAP 61, were used (reference 5 and data not shown). On the basis of these data, we conclude that SC35 is a limiting factor for E complex assembly on AdML pre-mRNA. The significant stimulation of E complex assembly by SC35 (Fig. 1A) is observed reproducibly and in several independent preparations of nuclear extracts (data not shown). We note that we find no difference in the efficiency of E complex assembly whether the pre-mRNA is preincubated with SC35 or just added directly to nuclear extracts containing the additional SC35 (data not shown).

For technical reasons, we were unable to use SC35-immunodepleted extracts to demonstrate an absolute requirement for SC35 in E complex assembly. We found that mock-depleted extracts alone were unable to support significant levels of E complex assembly (data not shown), most likely because the treatment involved in immunodepletion partially inactivates other factors required for E complex assembly. This was probably not a problem in the reported immunodepletion-reconstitution studies because the splicing assays used previously are much more sensitive than the gel filtration assay used for E complex assembly (Fig. 1A [13]). S100 extracts were also not useful for our studies because spliceosomal complexes in general (A and B complexes) do not accumulate to high levels in S100 extracts supplemented with SR proteins (29) (data not shown).

SR proteins promote interactions at both the 5′ and 3′ splice sites. Only the H complex is detected when a nonspecific RNA lacking functional splice sites is incubated in SC35-supplemented nuclear extracts (Fig. 1A, −ss; compare +SC35 and −SC35 and see below). This observation indicates that the 5′ splice site, 3′ splice site, and/or exon sequences are required

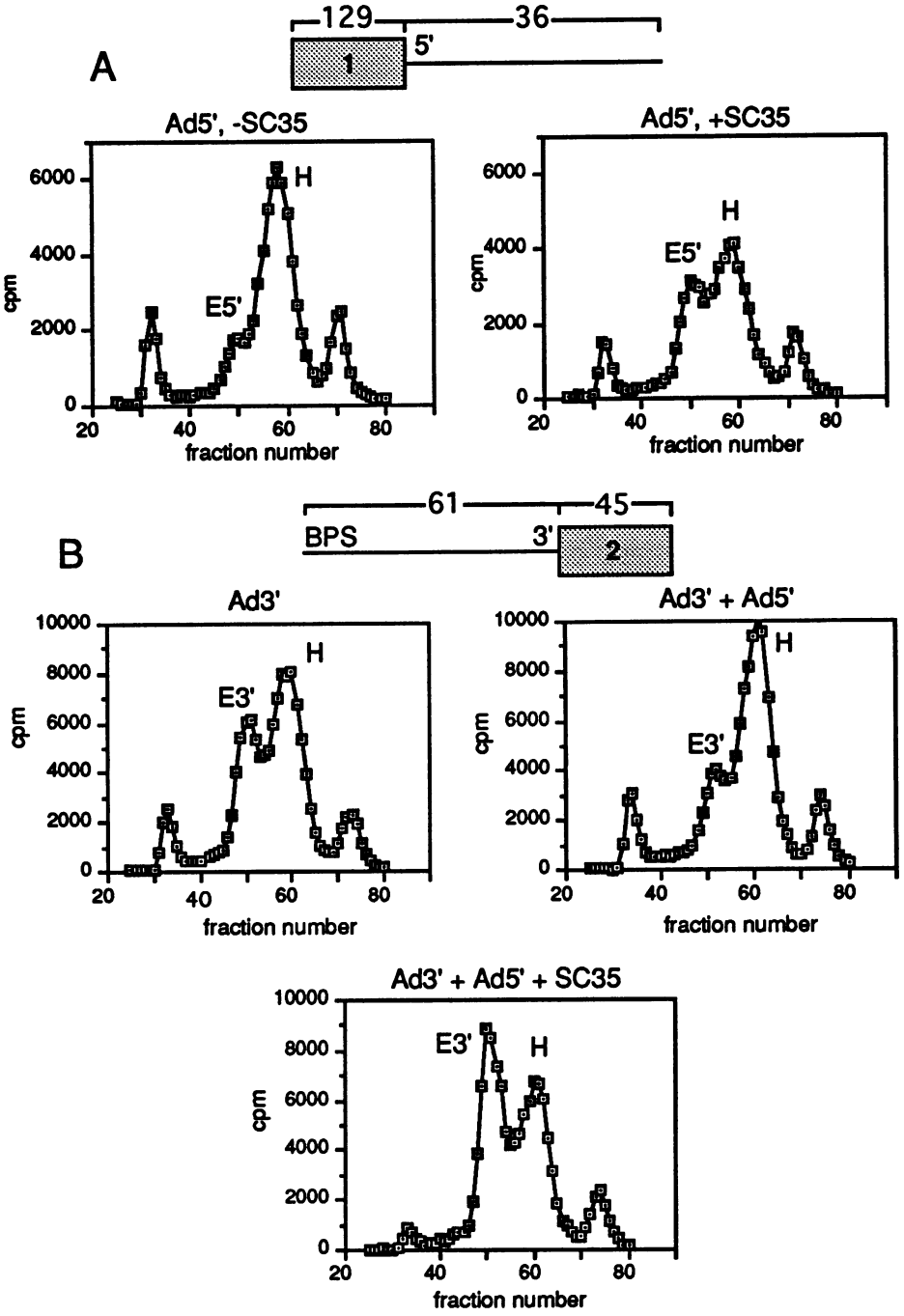

FIG. 2. SC35 promotes E5′ and E3′ complex assembly. (A) The sizes of the intron and exon in Ad5′ RNA are shown. For analysis of complexes, ^{32}P-labeled Ad5′ RNA (100 ng) was incubated in normal nuclear extracts (Ad5′, −SC35) or extracts supplemented with SC35 (Ad5′, +SC35). Complex assembly reactions were 100 μl. (B) The sizes of the intron and exon in Ad3′ RNA are shown. BPS, branchpoint sequence. ^{32}P-labeled Ad3′ RNA (20 ng) was incubated in normal nuclear extracts (Ad3′), extracts preincubated with 100 ng of cold Ad5′ competitor RNA (Ad3′+Ad5′), or extracts preincubated with cold Ad5′ competitor RNA (100 ng) and SC35 (Ad3′+Ad5′+SC35). Complex assembly reactions were 100 μl. The E3′, E5′, and H complexes are indicated.

for the function of SC35 in E complex assembly. Previous work showed that ATP-independent complexes, designated E5′ and E3′, assemble on Ad5′ and Ad3′ RNAs, which contain only a 5′ and only a 3′ splice site, respectively (39). The 5′ splice site is required for E5′ assembly, and the 3′ splice site is required for E3′ assembly. Thus, to identify the pre-mRNA sequences required for SC35 function, we analyzed E5′ and E3′ complex assembly in normal versus SC35-supplemented nuclear extracts (Fig. 2). Significantly, we observe that E5′ complex assembly is stimulated in SC35-supplemented extracts relative to normal nuclear extracts (Fig. 2A; compare Ad5′, −SC35 and +SC35). When similar experiments were carried out for the E3′ complex, only a small stimulation was observed in SC35-supplemented extracts (data not shown). This observa-

tion raised the possibility that another factor, such as U2AF, could be limiting for E3′ complex assembly. Thus, to determine whether SC35 is required for E3′ assembly, we used Ad5′ RNA as a cold competitor to titrate the SC35 in the E3′ complex assembly reaction (as shown in Fig. 2A, SC35 is the limiting factor for complex assembly on Ad5′ RNA). We first determined the amount of Ad3′ RNA that would assemble efficiently into the E3′ complex (Fig. 2B, Ad3′). We then examined whether the addition of Ad5′ RNA as a cold competitor would decrease the efficiency of E3′ complex assembly. Significantly, we found that the E3′-to-H complex ratio was indeed reduced by the addition of the cold Ad5′ RNA (Fig. 2B, Ad3′+Ad5′). Moreover, the addition of SC35 to the reaction mixture containing both the competitor and Ad3′ RNA resulted in a significant increase in the E3′-to-H complex ratio (Fig. 2B, Ad3′+Ad5′+SC35). We conclude that SC35 promotes E3′ complex assembly.

In extracts not supplemented with SC35, the ratio of the E-type complexes (E, E5′, and E3′) to H complex is much lower than in the SC35-supplemented extracts (Fig. 1 and 2). This observation indicates that SC35 promotes formation of the earliest detectable specific complexes during spliceosome assembly. Moreover, our data lead to the conclusion that in the absence of SR protein activity, only the H complex assembles on pre-mRNA.

Distinct sets of proteins cross-link to pre-mRNA in the E and H complexes. In previous work, we purified the H, E, A, B, and C spliceosomal complexes by using a two-step procedure in which complexes assembled on biotinylated pre-mRNA were isolated by gel filtration followed by avidin affinity chromatography (3, 4, 19, 43). Although several essential splicing factors and snRNPs are present in these complexes, no SR proteins were detected with UV cross-linking, silver staining, or Western blot (immunoblot) analysis as the assay (3, 51). Thus, it is possible that the SR proteins dissociate during purification of the complexes. As all of the complexes are functional when isolated by gel filtration (38, 45), we thought it possible that the SR proteins remain associated with the gel filtration-purified complexes. However, these complexes are only partially purified. Thus, to identify proteins specifically associated with complexes assembled on exogenously added pre-mRNA, we used UV cross-linking as an assay. Several studies have shown that purified SR proteins UV cross-link to naked RNA (29, 53).

In initial studies, in which the E complex was isolated by gel filtration, UV irradiated, and then affinity purified, we observed high levels of contamination with the H complex, which fractionates very close to the E complex and is affinity purified with much higher efficiencies than the E complex (data not shown). To try to circumvent this problem, we isolated the E complex by gel filtration, carried out the UV irradiation, but did not affinity purify the complex. Strikingly, comparison of the cross-linking patterns of the E and H complexes on an SDS-gel revealed little overlap (compare lanes 1 and 2 in Fig. 3A; see Fig. 3B, lanes 1 and 2, for a darker exposure). The bands corresponding to U2AF^{65} and heterogeneous nuclear ribonucleoproteins (hnRNPs) A and I were identified by 2D gel electrophoresis (Fig. 3C) on the basis of previous work (51).

To determine whether the differential cross-linking of the E and H complex components occurs when the pre-mRNA is incubated in the nuclear extract or occurs during the gel filtration step, we compared the cross-linking patterns of E and H complexes that had been UV irradiated prior to versus after the gel filtration step (Fig. 3A; compare lanes 3 and 4 with lanes 1 and 2). This analysis revealed that although the

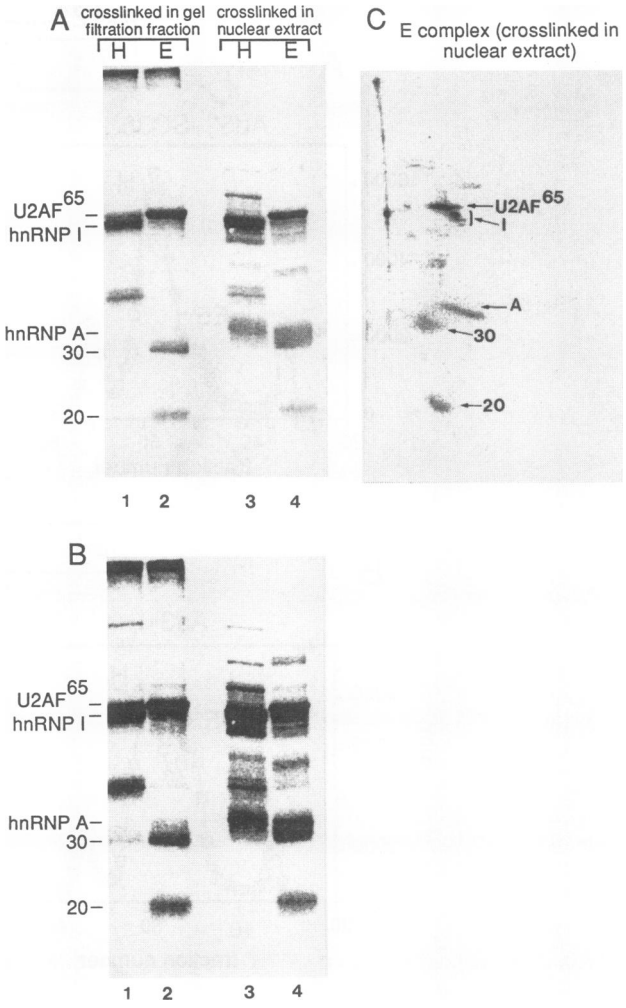

FIG. 3. Distinct sets of proteins UV cross-link to pre-mRNA in the E and H complexes. H and E complexes assembled on ^{32}P-labeled AdML pre-mRNA were UV irradiated prior to or after gel filtration as indicated. The complexes were then treated with RNase A, and total protein was fractionated on an SDS–9.5% polyacrylamide gel (A; panel B is a darker exposure of panel A) or on a 2D gel (C). Cross-linked proteins were detected by PhosphorImager analysis. U2AF^{65}, hnRNPs I and A, and novel E complex-specific 20- and 30-kDa proteins are indicated.

complexity of both the H and E complexes is greater when UV irradiated in the nuclear extract, the cross-linked H complex proteins are nevertheless largely excluded from the E complex and vice versa (see Fig. 3B for a darker exposure of the gel shown in Fig. 3A). We conclude that the exclusion of hnRNP cross-linking from the E complex occurs in the nuclear extract during complex assembly and not during purification of the complexes.

Previous work showed that hnRNPs UV cross-link in affinity-purified H complex but cross-link at much lower levels in affinity-purified B complex (51). Because of the technical difficulty of isolating affinity-purified E complex that lacks high levels of contaminating H complex (see above), we were previously unable to determine whether the hnRNPs were lacking in the E complex (51). However, our analysis of gel filtration-isolated complexes clearly shows that the loss of hnRNP cross-linking does indeed occur in the E complex.

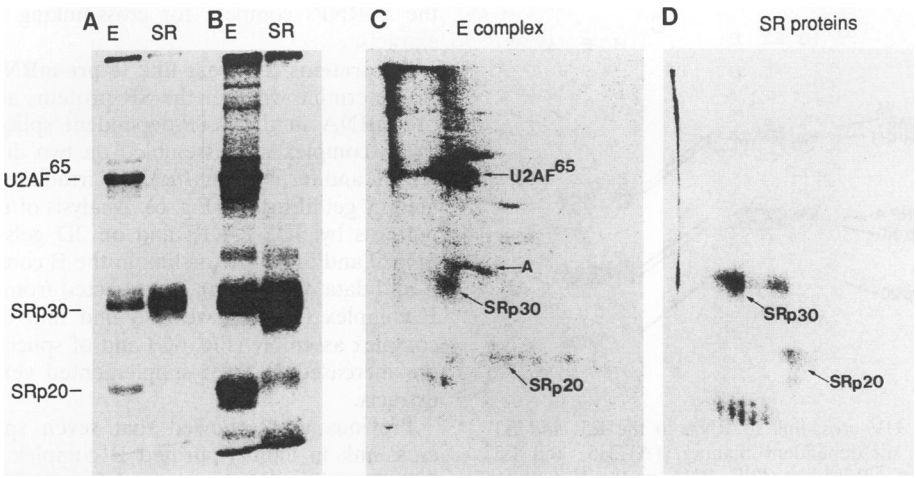

FIG. 4. SR proteins UV cross-link to pre-mRNA in the E complex. Cross-linked E complex or total SR proteins, as indicated, were fractionated by SDS-PAGE (A; panel B is a darker exposure of panel A) or 2D gel electrophoresis (C and D). Cross-linked proteins were detected by PhosphorImager analysis. U2AF^{65}, hnRNPs I and A, and SRp20 and SRp30 are indicated (3, 51).

Western analysis using antibodies to U2AF^{65}, U2AF^{35}, and U1 70K show that these splicing factors are lacking from affinity-purified H complex (4a). These data, together with previous work (4), indicate that the H complex contains hnRNPs and lacks splicing factors. We conclude that the RNA can be assembled into two largely distinct complexes (E and H) in the absence of ATP.

SR proteins cross-link to pre-mRNA in the E complex. Proteins of 20 and 30 kDa UV cross-link to pre-mRNA in the E complex, irradiated either before or after gel filtration, but do not cross-link in affinity-purified E complex (Fig. 3 [51]). On the basis of their absence from the affinity-purified complexes and their fractionation behavior on 2D gels (Fig. 3C [3]), we thought it possible that these proteins corresponded to SR proteins. To test this possibility, we UV cross-linked total purified HeLa cell SR proteins (SRp20, -30, -40, -55, and -75) to ^{32}P-labeled AdML pre-mRNA, treated the preparations with RNase, and then fractionated the proteins on an SDS-gel in parallel with cross-linked E complex proteins. These data show that purified SRp30 and SRp20 cross-link to naked AdML pre-mRNA with the highest efficiency (Fig. 4A, lane SR; see Fig. 4B, lane SR, for a darker exposure). Of these, the SRp30 band cofractionates with the 30-kDa E complex protein, whereas the SRp20 band fractionates slightly above the 20-kDa E complex protein (Fig. 4A and B; compare lanes E and SR).

Comparison of the cross-linked E complex and SR proteins on a 2D gel shows that the 30-kDa E complex protein cofractionates with SRp30 (compare Fig. 4C and D). The 20-kDa E complex protein fractionated as a streak in this experiment, making it difficult to compare it with SRp20 (Fig. 4C and D). However, in other experiments, the 20-kDa E complex protein appears as a discrete spot that fractionates in the same position as SR proteins in the isofocusing dimension (Fig. 3C and other examples below [3]). On the basis of these observations, we conclude that the 20- and 30-kDa proteins in the E complex are SR proteins. The difference in mobility between SRp20 and the 20-kDa E complex protein may be due to differences in the phosphorylation states of these proteins. Alternatively, as with the SRp30 band, which consists of at least two different proteins (SC35 and ASF/SF2), the SRp20 band may consist of more than one protein. In support of this notion, at least two bands in the region of SRp20 are detected in Western analysis of total nuclear extracts with a MAb to SR proteins (MAb 104 [48]; data not shown). We note that MAb 104, which recognizes a phosphoepitope on SR proteins (48), does not immunoprecipitate the cross-linked SR proteins in the E complex, most likely because of loss of the phosphoepitope during sample preparation (data not shown). Consistent with this notion, we have found that there is a phosphatase activity from the nuclear extract that dephosphorylates U2AF^{65}, U2AF^{35}, and U1 70K during gel filtration (51a).

SR protein cross-linking in the E complex is splice site dependent. To investigate the role of the 5′ and 3′ splice sites in SR protein cross-linking, we examined E5′ and E3′ complexes assembled on Ad5′ and Ad3′ RNAs, respectively (Fig. 5; see Fig. 2 for the structures of the RNAs). In addition, we examined the cross-linking of the H complexes assembled on these RNAs. As was observed with AdML RNA containing both 5′ and 3′ splice sites, the UV cross-linking patterns of the H and E complexes are distinct from one another (Fig. 5A [compare lanes H5′ and E5′] and B [compare lanes H3′ and E3′]). We found that SRp20 and SRp30 cross-link in the E5′ complex, whereas only SRp30 cross-links in the E3′ complex (Fig. 5A and B; compare lanes E5′ and E3′).

To determine whether the splice sites present in Ad5′ and Ad3′ RNAs are required for the SR protein cross-linking, we examined AdΔ5′ and AdΔ3′ RNAs, which contain substitutions of the 5′ and 3′ splice site sequences, respectively (see Materials and Methods). Previous work showed that these RNAs both assemble into complexes that fractionate in the position of the H complex (39). Significantly, neither SRp20 nor SRp30 UV cross-links in the H complexes assembled on AdΔ5′ or AdΔ3′ RNA (Fig. 5C, lanes Δ5′ and Δ3′). Thus, the 3′ splice site is essential for SR cross-linking to Ad3′ RNA, and the 5′ splice site is essential for SR cross-linking to Ad5′ RNA. The requirement of the respective splice sites for SR cross-linking in the E3′ and E5′ complexes strongly indicates that the cross-linking of the SR proteins is functionally significant. Purified hnRNPs alone (reviewed in reference 9) or purified SR proteins alone (reference 29 and this study) cross-link readily to RNA. Thus, the observation that SR protein cross-linking is specifically excluded from the H complex assembled on RNAs lacking splice sites indicates that the SR proteins and

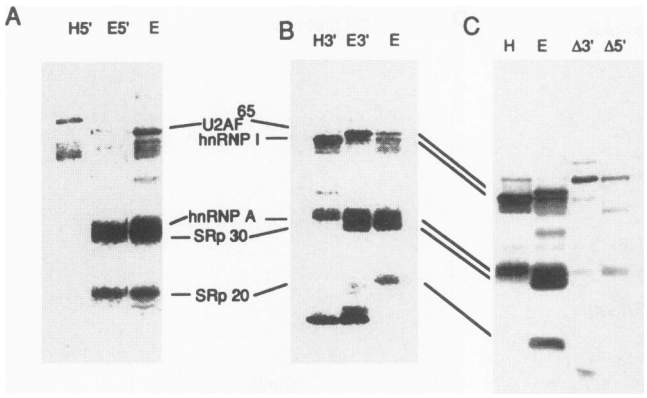

FIG. 5. SR proteins UV cross-link to RNA in the E5′ and E3′ complexes in a splice site-dependent manner. (A) H5′ and E5′ complexes assembled on ^{32}P-labeled Ad5′ RNA or the E complex assembled on ^{32}P-labeled AdML pre-mRNA were UV irradiated prior to gel filtration and then fractionated on an SDS–9.5% polyacrylamide gel. (B) H3′ and E3′ complexes assembled on ^{32}P-labeled Ad3′ RNA were UV irradiated prior to gel filtration and fractionated on an SDS–10.5% polyacrylamide gel. The E complex sample was prepared as for panel A. (C) ^{32}P-labeled AdΔ3′ RNA, which contains a 3′ splice site substitution (Δ3′), and AdΔ5′ RNA, which contains a 5′ splice site substitution (Δ5′), were incubated in nuclear extracts, UV irradiated, and then isolated by gel filtration. Most of the mutant RNA assembles into the H complex (reference 39 and data not shown). Total protein from the AdΔ5′ and AdΔ3′ H complexes were fractionated on an SDS–9.5% polyacrylamide gel. The E and H complexes were assembled on AdML pre-mRNA. Cross-linked proteins were detected by PhosphorImager analysis, and the bands corresponding to known proteins identified on 2D gels are indicated.

the hnRNPs compete for cross-linking to RNA in nuclear extracts.

SR proteins UV cross-link to pre-mRNA in the B complex. To determine whether the SR proteins also UV cross-link to pre-mRNA in the ATP-dependent spliceosomal complexes, the B complex was assembled on two different pre-mRNAs, AdML and fushi tarazu (*ftz*), UV irradiated, and then fractionated by gel filtration (Fig. 6). Analysis of the UV cross-linking patterns by SDS-PAGE and on 2D gels revealed that both SRp20 and SRp30 cross-link in the B complexes (Fig. 6A and B and data not shown). As expected from the results with the E complex (Fig. 2), we also find that the efficiencies of B complex assembly (Fig. 6C) and of splicing (data not shown) are increased in SC35-supplemented versus normal nuclear extracts.

Previous work showed that seven spliceosomal proteins cross-link in affinity-purified B complex (51). Of these, the 200-kDa U5 snRNP and the U2 snRNP-associated SAPs 49 and 155 are detected when cross-linking of the B complex is carried out in the nuclear extract (Fig. 6B). We are unable to conclusively identify the other proteins (SAPs 61, 62, 114, and 145). Thus, whether these SAPs cross-link only in affinity-purified complexes or cross-link in the nuclear extract, but are difficult to detect because of background, remains to be determined. It is possible, for example, that the loss of the SR proteins from the affinity-purified complex allows the other SAPs to cross-link. However, the observation that the SAPs cross-link under any conditions suggests that these proteins are likely to contact the pre-mRNA at some point in the splicing reaction.

Assembly of an Enhancer complex. Our analysis of the E3′ and E5′ complexes (Fig. 5) indicates that SR proteins UV cross-link to RNAs containing only a 5′ or only a 3′ splice site and that the splice sites are required for this interaction.

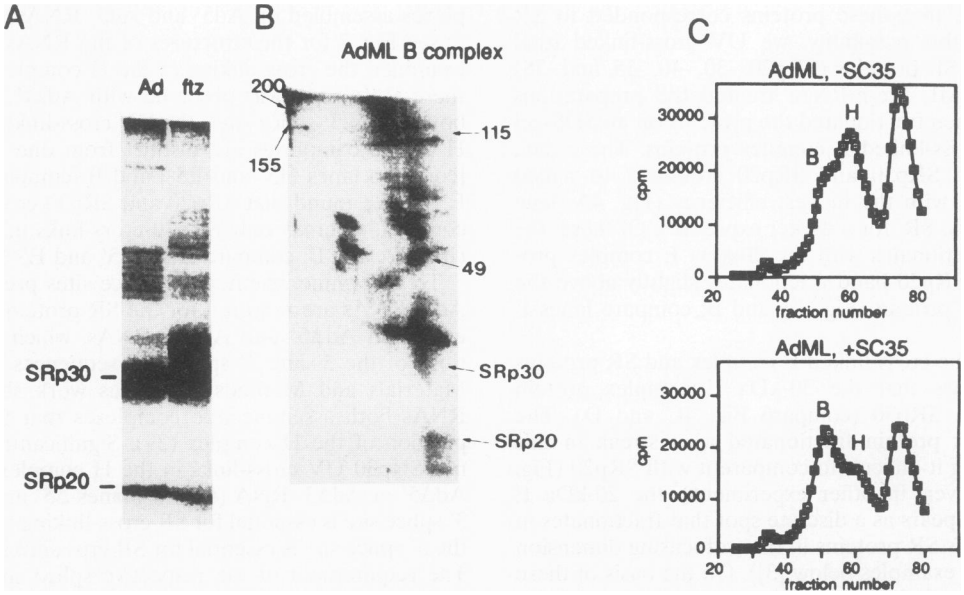

FIG. 6. SR proteins UV cross-link in spliceosomal complex B. (A) The B complex was assembled on *ftz* or AdML pre-mRNA and UV irradiated prior to gel filtration, and total protein was fractionated on an SDS–9.5% polyacrylamide gel and detected by PhosphorImager analysis. (B) Cross-linked proteins in AdML B complex were fractionated by 2D gel electrophoresis. Proteins were identified by comparison with 2D gels in which cross-linked proteins cofractionated with the corresponding silver-stained proteins in affinity-purified complexes (51). (C) AdML pre-mRNA (200 ng) was incubated for 20 min in normal nuclear extract (−SC35) or in extract supplemented with recombinant SC35 (+SC35) under splicing conditions (100-μl reaction mixtures). Complexes were fractionated by gel filtration. The B and H complexes are indicated. Sizes are indicated in kilodaltons.

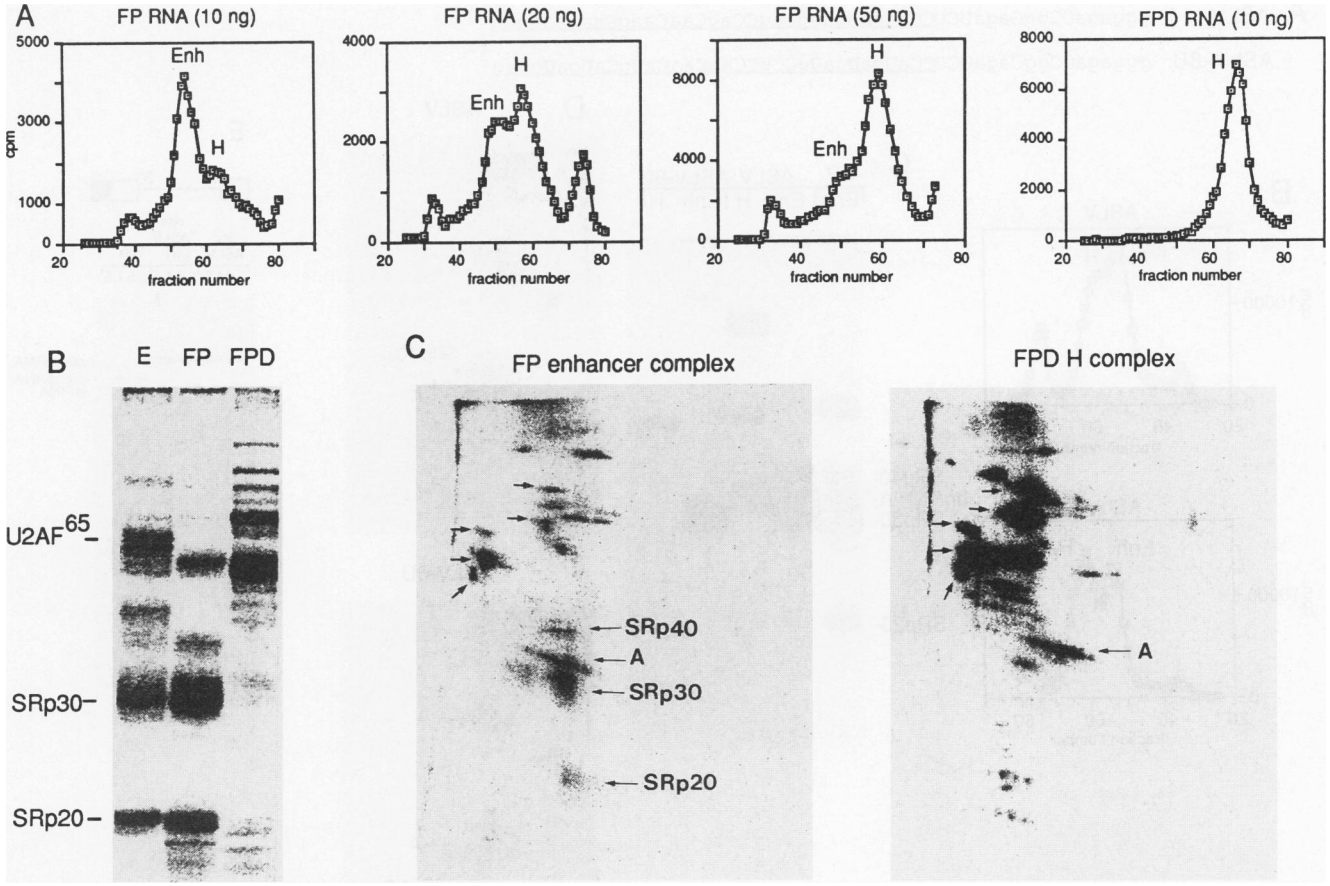

FIG. 7. Identification and characterization of an Enhancer complex that assembles on constitutive exonic enhancers. (A) The indicated amounts of ^{32}P-labeled RNAs containing either the bovine growth hormone enhancer (FP) or bovine growth hormone exon sequences lacking the enhancer (FPD) were incubated in nuclear extract (25-μl reaction) in the absence of ATP and then fractionated by gel filtration. The peaks containing the Enh and H complexes are indicated. (B) The E complex (E) assembled on AdML pre-mRNA, the Enh complex assembled on FP RNA (FP), and the H complex assembled on FPD RNA (FPD) were UV irradiated, fractionated by gel filtration, fractionated on an SDS–10% polyacrylamide gel, and detected by PhosphorImager analysis. (C) 2D gel analysis of UV cross-linked proteins in the FP Enh complex and FPD H complex.

However, previous work showed that SR proteins also bind specifically to purine-rich exonic enhancers and to the doublesex (*dsx*) enhancer, both of which lack splice sites (33, 53, 57). The observation that the SR proteins stimulate complex assembly on Ad5′ and Ad3′ RNAs raised the possibility that the SR proteins also stimulate complex assembly on purine-rich enhancers. To test this possibility, we incubated the exonic enhancer element from bovine growth hormone (53) (designated FP) or a control RNA containing exon sequences that lack the FP element (FPD) in nuclear extracts and fractionated the reactions by gel filtration (Fig. 7). Significantly, we found that the FP RNA (124 nucleotides [nt]) assembles into a complex that elutes in the same fractions as the E complex [Fig. 7A, FP RNA (10 ng)]. In contrast, the nonspecific FPD RNA (143 nt) assembles only into the H complex [Fig. 7A, FPD RNA (10 ng)]. We have designated the complex assembled on the FP RNA the Enhancer (Enh) complex.

Previous work showed that SRp30a (SF2/ASF) UV cross-links to FP RNA, but not to FPD RNA, and is essential for enhancer function (53). Our analysis of the cross-linked proteins in the Enh complex revealed the presence of SRp30 (our data do not distinguish between SRp30a [SF2/ASF] and SRp30b [SC35]) (Fig. 7B and C). In contrast to a previous study (53), we find that SRp20 and SRp40 also UV cross-link in the Enh complex (Fig. 7C; SRp40 was identified by comparison with purified SRp40 on 2D gels [3]). This discrepancy may result from differences in the levels of the SR proteins in different extracts. In addition to the SR proteins, hnRNP A (reference 53 and this study) and other hnRNPs (indicated by arrows in Fig. 7C) cross-link in the Enh complex. As expected, the levels of these hnRNPs are much lower than in the H complex assembled on FP RNA (data not shown). hnRNPs, but no SR proteins, cross-link to the FPD RNA in the H complex (Fig. 7B and C). We conclude that a discrete complex containing SR proteins assembles on an RNA containing a splicing enhancer. Moreover, the assembly of the Enh complex correlates with enhancer activity, as the FP RNA assembles the Enh complex and has enhancer activity, whereas the FPD RNA does not assemble the Enh complex and has no enhancer activity (Fig. 7A [53]). Addition of increasing amounts of FP RNA to splicing extracts decreases the ratio of the Enh to the H complex (Fig. 7A, FP RNA; compare 10, 20, and 50 ng). These data most likely indicate that, as observed with E complex assembly (Fig. 1), the SR proteins are limiting for Enh complex assembly. This results in the cross-linking of hnRNPs and formation of the H complex on the excess FP RNA.

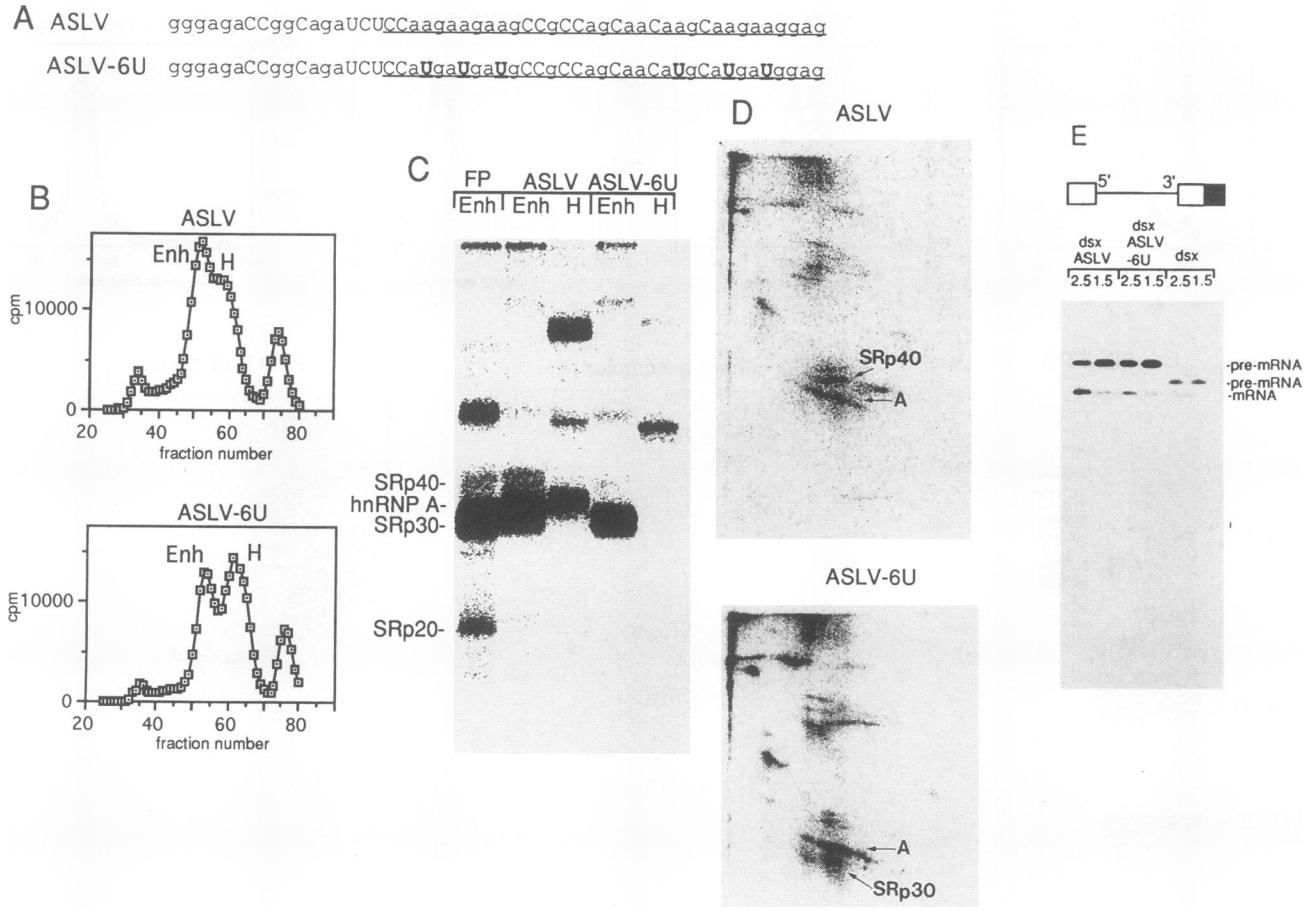

FIG. 8. Functional purine-rich or non-purine-rich enhancers assemble into Enh complexes that contain different SR proteins. (A) Nucleotide sequences of the ASLV and ASLV-6U RNAs used for complex assembly. The portion of the RNA that is present in ASLV pre-mRNA is underlined. The 5′ portion of the RNA is polylinker sequences. The six U substitutions in ASLV-6U are indicated in boldface. (B) ^{32}P-labeled ASLV and ASLV-6U RNAs (6 ng) were incubated in nuclear extract (25-μl reaction mixture) and then fractionated by gel filtration. The peaks corresponding to the Enh and H complexes are indicated. (C) The indicated complexes were assembled, UV irradiated, and fractionated by gel filtration, and cross-linked proteins were fractionated on an SDS–9.5% polyacrylamide gel. The labeled bands were identified on 2D gels (see panel D). (D) Enh complexes assembled on ASLV or ASLV-6U were fractionated on 2D gels. The SR proteins and hnRNP A are indicated. (E) The ASLV and ASLV-6U sequences shown in panel A were inserted at the 3′ end of *dsx* pre-mRNA (indicated by the black box in the schematic) to generate Dsx-ASLV and Dsx-ASLV-6U pre-mRNAs, respectively. These pre-mRNAs and *dsx* pre-mRNA lacking the enhancers were incubated under splicing conditions for the times indicated (in hours), and total RNA was analyzed on an 8% denaturing polyacrylamide gel. The bands corresponding to pre-mRNA and mRNA are indicated.

To determine whether assembly of the Enh complex occurs generally on purine-rich exonic enhancers, we examined complex assembly on the enhancer element from avian sarcoma-leukosis virus (ASLV) pre-mRNA, which is among the most potent of several purine-rich splicing enhancers (55). The sequence of this RNA (52 nt) is shown in Fig. 8A. Significantly, we found that the ASLV RNA also assembles into the Enh complex (Fig. 8B). However, in contrast to the FP Enh complex (Fig. 8C, FP, Enh), SRp40, and a small amount of SRp30 or SRp20, UV cross-links in the ASLV Enh complex (Fig. 8C and D, ASLV, Enh). We conclude that different SR proteins cross-link to the RNA in ASLV versus FP Enh complexes. The other major cross-linked band in the ASLV Enh complex is hnRNP A (Fig. 8D, ASLV).

To analyze the sequence requirements for assembly of the ASLV Enh complex, we introduced six U residues into the purine-rich sequence (Fig. 8A; compare ASLV-6U and ASLV). The rationale for the U substitutions was based on a previous study which showed that substitution of U residues for C residues in the immunoglobulin M purine-rich enhancer drastically decreases its activity (55). Unexpectedly, we found that the Enh complex assembles quite efficiently on ASLV-6U RNA (Fig. 8B; compare ASLV and ASLV-6U). However, in contrast to ASLV RNA, SRp30 cross-links to ASLV-6U RNA, and the levels of SRp40 cross-linking are greatly diminished (Fig. 8C and D; compare ASLV and ASLV-6U). Thus, the six U substitutions have a relatively minor effect on Enh complex formation but a major effect on the SR protein family member that cross-links. We note that all of the RNAs in the cross-linking studies were synthesized in transcription reaction mixtures containing all four ^{32}P-labeled nucleotides (G, A, U, and C). Thus, the differences in cross-linking are not likely to be due to differences in the distribution of labeled nucleotides in the RNA. In addition, competition studies using the ASLV and ASLV-6U RNAs to compete against one another for Enh complex assembly indicate that the differences in SR protein cross-linking reflect actual differences in SR protein binding (data not shown).

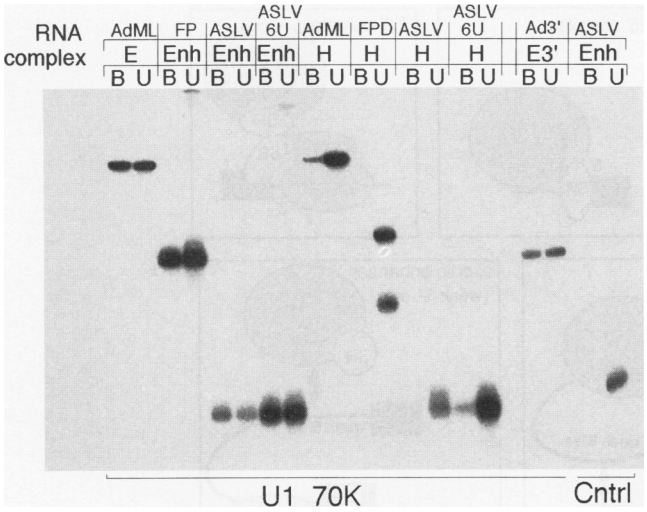

FIG. 9. The Enh complexes are specifically associated with U1 snRNP. The indicated complexes were isolated by gel filtration and incubated with U1 70K MAbs or with a nonspecific control (Cntrl) antibody bound to protein A-Trisacryl. After washing, total RNA was prepared from the bound (B) and unbound (U) fractions and fractionated on an 8% denaturing polyacrylamide gel.

To determine whether there is a correlation between enhancer function and Enh complex formation with ASLV and ASLV-6U RNAs, we inserted these sequences downstream of the 3′ exon in *dsx* pre-mRNA. This pre-mRNA contains a weak 3′ splice site, and in the absence of an enhancer, no spliced mRNA is detected after 1.5 or 2.5 h of incubation under splicing conditions (Fig. 8E, dsx, lanes 1.5 and 2.5; [55–57]). In contrast, as shown previously (55), dsx pre-mRNA containing the ASLV enhancer is efficiently spliced (Fig. 8E, dsx ASLV, lanes 1.5 and 2.5). Significantly, the dsx ASLV-6U pre-mRNA splices almost as efficiently as the dsx ASLV pre-mRNA (Fig. 8E, dsx-ASLV-6U, lanes 1.5 and 2.5). Thus, these data, together with the data on the FP enhancer, show a correlation between assembly of the Enh complex and splicing enhancer activity. Moreover, the data provide a striking example of differential cross-linking of SR protein family members to different RNA sequences: only a 6-nt substitution in the enhancer was sufficient to cause a switch from SRp40 to SRp30. Finally, the ASLV-6U enhancer lacks any obvious purine-rich stretches (Fig. 8A, ASLV-6U). Thus, sequences other than the purine-rich tracts previously identified (8, 33, 55, 59, 61) can function as constitutive splicing enhancers.

U1 snRNP is specifically associated with Enh complexes. The obvious similarities between the E and Enh complexes prompted us to determine whether U1 snRNP is also present in the Enh complex. Using MAbs to the U1 snRNP component U1 70K, we carried out immunoprecipitations of complexes isolated by gel filtration. The ^{32}P-labeled RNA in the bound versus unbound fractions is shown in Fig. 9. About 50% of the RNA is present in the bound fraction for the AdML E complex and for the Enh complexes formed on FP, ASLV, and ASLV-6U RNAs (Fig. 9, U1 70K). In contrast, only low levels of RNA were detected in the bound fraction for the H complex formed on AdML, FPD, ASLV, and ASLV-6U RNAs (Fig. 9, U1 70K). Moreover, E, Enh, or H complexes were not immunoprecipitated by a nonspecific control antibody (Fig. 9). In previous work, low levels of U1 snRNA were found to be specifically associated with affinity-purified E3′ complex (39). Consistent with this result, we find that the E3′ complex is also efficiently immunoprecipitated by U1 70K antibodies (Fig. 9, Ad3′, U1 70K). Finally, the E, E3′, and Enh complexes are also immunoprecipitated by anti-Sm antibodies which recognize core snRNP proteins (data not shown). We conclude that U1 snRNP is a component of E, Enh, and E3′ complexes but not of H complexes.

DISCUSSION

We have shown that a family of complexes related to the earliest complex (E) in the spliceosome assembly pathway assembles on RNAs containing exonic splicing enhancers (the Enh complex), RNAs containing only a 5′ splice site (the E5′ complex), or RNAs containing only a 3′ splice site (the E3′ complex) (Fig. 10). All of the complexes are ATP independent and are specifically associated with U1 snRNP and SR proteins. Moreover, UV cross-linking studies show that SR proteins directly contact the RNA in all of the complexes in a sequence-dependent manner. SR protein cross-linking does not occur in the hnRNP complex (H) that assembles concurrently with the E, Enh, E3′, and E5′ complexes, and conversely, hnRNP cross-linking is largely excluded from the E-type complexes.

SR proteins promote the initial recognition of 5′ and 3′ splice sites. Recent work showed that SR proteins (SF2/ASF) increase U1 snRNP binding to the 5′ splice site (10, 28). We observe that SR proteins (SC35) promote E5′ complex assembly, indicating that the stimulation of U1 snRNP binding (10, 28) is likely to occur at the time of E complex assembly. SR proteins interact with U1 snRNP via protein-protein interactions with the U1 snRNP component U1 70K (28, 60). SR proteins also interact with one another directly (2, 60). In addition to these protein-protein interactions, our data indicate that SR proteins (SRp30 and SRp20) UV cross-link to the RNA in the E5′ complex and that the 5′ splice site is essential for the cross-linking. These protein-protein and RNA-protein interactions in the E5′ complex are depicted in Fig. 10 (E5′ complex).

On the basis of the observation that SR proteins stimulate U1 snRNP binding to the 5′ splice site, it was suggested that SR proteins may be functionally similar to the splicing factor U2AF^{65}, which promotes U2 snRNP binding on the branch site (28). However, our data indicate that SR proteins play a more general role than this. We find that SR proteins stimulate E3′ complex assembly on the 3′ splice site as well as E5′ complex assembly on the 5′ splice site. Significantly, SR proteins have been shown to interact with U2AF^{35} (60), which forms a tight complex with U2AF^{65} (64, 66). Although direct proof that U2AF^{35} is an essential splicing factor is lacking, both U2AF^{35} and U2AF^{65} are present in the E complex (4, 4a). Thus, it is possible that the interactions between U1 snRNP and the 3′ splice site occur via protein-protein interactions involving SR proteins, U2AF^{35}, and U2AF^{65}. Our data show that SR proteins (SRp30) also UV cross-link to the RNA in the E3′ complex and that the 3′ splice site is required for the cross-linking. The proposed RNA-protein and protein-protein interactions in the E3′ complex are depicted in Fig. 10.

The levels of SR proteins in nuclear extracts can affect selection of both the 5′ and 3′ splice sites in pre-mRNAs containing alternative splice sites (16, 18, 30, 63). Our observation that increased levels of SR proteins in nuclear extracts stimulate E complex assembly on the 3′ as well as the 5′ splice site suggests that the function of SR proteins in alternative splice site selection occurs at the time of E complex assembly. In addition, previous work showed that in the presence of competitor RNA, pre-mRNA pre-bound with SR proteins is

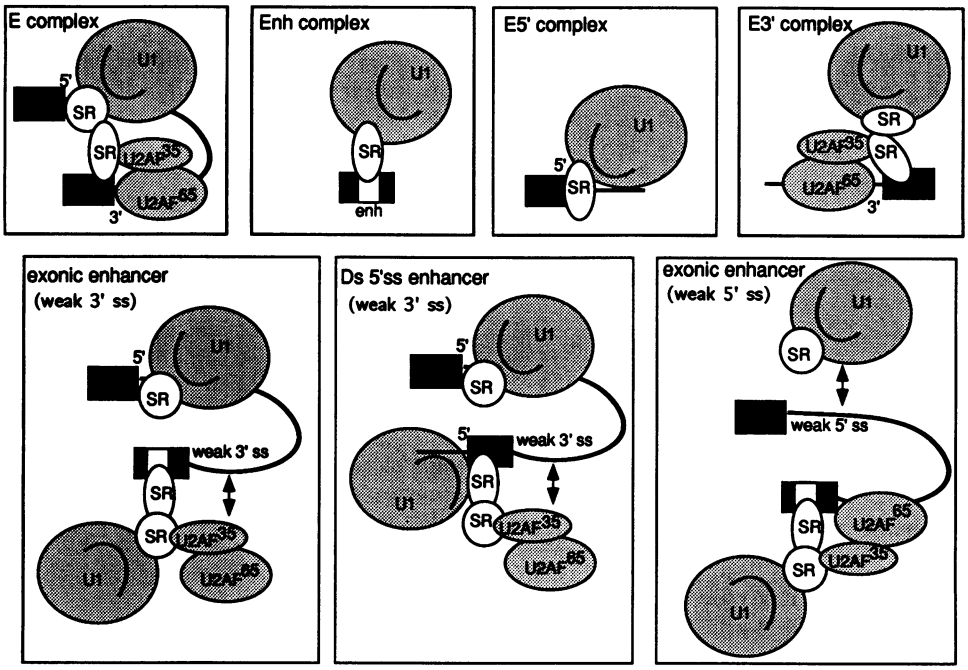

FIG. 10. Model for E complex and Enh complex structure and function. The RNA-protein (references 64 and 65 and this study) and protein-protein (2, 28, 60, 66) interactions of U1 snRNP, SR proteins, U2AF^{65}, and U2AF^{35} are depicted for each complex (see text). ss, splice site.

spliced with higher efficiency than naked pre-mRNA (11). Our data indicate that the increased splicing efficiency observed with the pre-mRNA pre-bound to SR proteins is due to the promotion of E versus H complex assembly.

Evidence that a general complex assembles on exonic and Ds 5′ss enhancers. Previous work showed that the SR protein SC35 is essential for the activity of a splicing enhancer involved in alternative splicing of *Drosophila dsx* pre-mRNA. SC35, together with two *dsx*-specific regulatory proteins (tra and tra 2) and several other SR proteins, form a large complex on the *dsx* exonic enhancer element (57). In another study, the SR protein SF2/ASF was shown to specifically bind and UV crosslink to a purine-rich enhancer (designated the FP element) in bovine growth hormone pre-mRNA and to be essential for enhancer function (53). Finally, at least one member of the SR family binds to the purine-rich enhancer in fibronectin pre-mRNA, though a role for these proteins in enhancer function has not been demonstrated (33). Other than the SR proteins, U1 snRNA was found to UV cross-link to another purine-rich exonic enhancer (59), and U1 snRNP binds to, and is required for the function of, Ds 5′ss enhancers (23, 31, 47).

Our data establish links between the previous observations by revealing that a specific complex containing both U1 snRNP and SR proteins assembles on purine-rich exonic enhancers (the Enh complex) as well as on RNAs containing a 5′ splice site alone (the E5′ complex). These data provide a likely explanation for the observation that purine-rich exonic enhancers and Ds 5′ss enhancers can functionally substitute for one another to stimulate splicing (8, 33, 59). Different SR proteins cross-link to different purine-rich enhancers (references 53 and 57 and this study). For example, SRp40 cross-links to the ASLV enhancer, whereas high levels of SRp30 and lower levels of SRp20 and SRp40 cross-link to the FP enhancer. The observation that different SR proteins cross-link to different enhancers correlates with previous data showing distinct activities for the various SR proteins (11, 53, 57, 63).

Model for splicing enhancer function. On the basis of our data and previous studies (2, 23, 31, 33, 47, 53–57, 59, 60), we propose that splicing enhancers function by promoting the assembly of a complex on pre-mRNAs containing weak 5′ or 3′ splice sites that resembles the E complex assembled on pre-mRNAs containing strong splice sites (Fig. 10). In pre-mRNAs containing strong splice sites, such as AdML pre-mRNA, U1 snRNP, U2AF, and SR proteins recognize and bring the splice sites together in the E complex, and enhancers are not required (Fig. 10, E complex). However, in pre-mRNAs containing a weak 3′ splice site and an exonic enhancer, the Enh complex assembles, and the same network of interactions that occurs between the 5′ and 3′ splice sites in the E complex takes place between the enhancer and the 3′ splice site [Fig. 10, exonic enhancer (weak 3′ ss)]. This functions to promote U2AF binding on the 3′ splice site. As depicted in Fig. 10, the Ds 5′ss enhancer could function via the same network of interactions. Consistent with this model, previous work showed that a Ds 5′ss enhancer promotes U2AF^{65} binding to the 3′ splice site (23, 31). A number of observations are consistent with the possibility that exonic enhancers also promote U2AF^{65} binding. Specifically, the immunoglobulin M purine-rich enhancer stimulates A complex assembly (59), and the fibronectin purine-rich enhancer promotes U2 snRNP binding (33), on pre-mRNAs containing a weak 3′ splice site. U2AF binding is known to be a prerequisite for A complex assembly and to occur inefficiently on weak 3′ splice sites (64, 65).

In the case of a weak 5′ splice site, interactions between the Enh complex and the 3′ splice site may also promote stable binding of U2AF on the 3′ splice site [Fig. 10, exonic enhancer (weak 5′ ss)]. According to this model, the interaction between the enhancer and the 3′ splice site would functionally substitute for the interaction that would normally occur between the

5′ and 3′ splice sites. Although our data show that U1 snRNP and the SR proteins can interact with the 3′ splice site in the absence of either a 5′ splice site or enhancer (Fig. 10, E3′ complex), it is clear from competition studies (32, 39) that the interactions at the 3′ splice site are stabilized by the presence of a 5′ splice site. Thus, it is reasonable to propose that the enhancer can stabilize interactions on the 3′ splice site when the 5′ splice site is too weak.

Different purine-rich enhancers vary considerably in their abilities to stimulate splicing of a heterologous pre-mRNA containing a weak 3′ splice site (55). This observation suggests that there may be an entire spectrum of enhancer strengths, ranging from potent enhancers in certain alternatively spliced pre-mRNAs with extremely weak splice sites to less potent enhancers in constitutively spliced pre-mRNAs with better splice sites. Our data indicate that, in addition to purine-rich enhancers, non-purine-rich sequences can function as constitutive splicing enhancers. Thus, taken together, the data indicate that exonic splicing enhancers, consisting of a variety of specific purine-rich and non-purine-rich sequences, vary in potency and function via differential interactions with members of the SR protein family.

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