Protein components specifically associated with prespliceosome and spliceosome complexes

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We have carried out a systematic analysis of the protein composition of highly purified mammalian spliceosomes. We show that >30 distinct proteins, including 20 previously unidentified components [designated spliceosome-associated proteins (SAPs)], are specifically associated with the spliceosome in a salt-resistant complex. In contrast to these spliceosome-specific proteins, we show that hnRNP proteins are not tightly associated with purified prespliceosome and spliceosome complexes. The splicing factor U2AF65, U1 snRNP-specific proteins, and several SAPs are present in the earliest prespliceosome complex (E). A set of 10 proteins is then added to the first ATP-dependent prespliceosome complex (A), and concomitantly, a significant decrease in the level of U2AF65 is observed. The fully assembled spliceosome is formed by the addition of 12 proteins in a reaction that requires ATP and both the 5' and 3' splice sites.

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Cellular processes requiring high levels of accuracy, such as DNA replication, protein translation, and pre-mRNA splicing, involve the assembly of large multicomponent complexes. To achieve a detailed understanding of these processes it is essential to identify and precisely define the functions of each of the components in the complexes. The interactions between the components and their order of assembly must also be established (for discussion, see Alberts 1984; Alberts and Miake-Lye 1992). Using a combination of genetic and biochemical approaches, the assembly and composition of both the ribosome and the "replisome" in prokaryotes have been largely established. At present, the spliceosome is not understood at the same level of detail. However, similar approaches are being used to identify splicing components and determine the pathway of spliceosome assembly (for review, see Green 1991). The five small nuclear RNA (snRNA) components of the spliceosome interact with pre-mRNA in a defined temporal order. U1 snRNA binds to the pre-mRNA first, followed by binding of U2 snRNA. Subsequently, U4, U5, and U6 snRNAs bind to the pre-mRNA, possibly in a tri-snRNP particle.

Specific functional interactions occur between the snRNAs and the pre-mRNA. Recently, U5 snRNA was shown to base-pair upstream of the 5' splice junction and downstream from the 3' splice junction [Newman and Norman 1992], whereas U6 snRNA was found to interact with regions downstream from the 5' splice site and near the branch site [Sawa and Shimura 1992]. In addition, U1 and U2 snRNAs base-pair to the 5' splice site and the branch site, respectively [Zhuang and Weiner 1986, 1989; Parker et al. 1987; Seraphin et al. 1988; Siliciano and Guthrie 1988; Wu and Manley 1989]. Functional associations also occur between the snRNAs themselves. U4 and U6 snRNAs are transiently base-paired [Bringmann et al. 1984], as are U2 and U6 snRNAs [Hausner et al. 1990; Datta and Weiner 1991; Wu and Manley 1991].

The numerous interactions that occur between the snRNAs and pre-mRNA suggest that several key steps in the splicing reaction, such as splice site recognition and catalysis at the splice junctions, may be mediated by RNA (for discussion, see Guthrie 1991). Each of the RNA–RNA interactions occurs in discrete prespliceosome and spliceosome complexes, and the protein components of these complexes are almost certainly critical to the function of the snRNAs (for review, see Green 1991; Guthrie 1991). Thus, an essential next step in understanding spliceosome assembly is to identify these protein components and determine their roles in the splicing mechanism.

Two non-snRNP splicing activities have been purified from mammalian cells and shown to be essential for spliceosome assembly. These are a 65-kD protein that binds to the 3' splice site [U2AF65; Zamore and Green 1991] and a family of functionally redundant proteins known as SR proteins [Mayeda et al. 1992; Zahler et al. 1992]. Included among the SR proteins are SC35 and SF2/ASF, which were identified as splicing factors before the discovery of the SR protein family [Fu and Maniatis...
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1990; Ge and Manley 1990; Krainer et al. 1990; Zahler et al. 1992]. An 88-kD protein has also been shown to be required for spliceosome assembly and splicing [Ast et al. 1991]. It is not known, however, whether this protein is a snRNP component. Several proteins bind specifically to the 3' splice site, including intron-binding protein [IBP] [Gerke and Steitz 1986; Tazi et al. 1986], heterogeneous nuclear ribonucleoproteins [hnRNP] A, C, and D [Swanson and Dreyfuss 1988], and hnRNP I/PTB [Garcia-Blanco et al. 1989; Gil et al. 1991; Patton et al. 1991]. HnRNP A, C, D, and I/PTB bind to the pre-mRNA as part of a larger hnRNP complex, and many of the other proteins in this complex also interact with the RNA in a sequence-dependent manner [Bennett et al. 1992]. The functional significance of the binding of IBP and the hnRNP proteins is not known, although one possible role for the hnRNP proteins is in splice site selection [Bennett et al. 1992; Mayeda and Kramer 1992].

The basis of genetic data in yeast [Vijayraghavan et al. 1989; for review, see Guthrie 1991] and biochemical studies in mammals [Reed 1990; Utans and Kramer 1990; Kramer and Utans 1991], the spliceosome is likely to contain additional non-snRNP proteins.

Each of the known splicing activities and pre-mRNA-binding proteins have, for the most part, been characterized individually. Thus, the relationships between the different proteins and the nature of their association with the pre-mRNA throughout the splicing reaction remains to be established. For example, both U2AF65 and hnRNP proteins A, C, D, and I/PTB bind to the 3' splice site. Do these proteins bind sequentially or simultaneously? Similarly, at what point in spliceosome assembly do the other known splicing factors first bind to the pre-mRNA, and what is the total protein complexity of the spliceosome?

To address these questions we have carried out a systematic analysis of the protein composition of the spliceosome and intermediate complexes in the spliceosome assembly pathway. These studies establish the temporal order of assembly of several of the known splicing factors. In addition, we have identified and determined the order of assembly of a set of novel spliceosome-associated proteins [SAPs].

Results

Purified spliceosomes were obtained in preparative amounts by use of a two-step purification scheme [Fig. 1; Reed 1990]. In the first step, biotinylated pre-mRNA was incubated under splicing conditions and fractionated by gel filtration [Fig. 1A]. In the second step, spliceosomes [B complexes] were affinity purified by binding to avidin–agarose [affinity purification was carried out in 250 mM salt; see below]. As a control, the same fractionation scheme was carried out with nonbiotinylated pre-mRNAs. In a previous study, a similar method was used to purify spliceosomes assembled on β-globin pre-mRNA [Reed 1990]. Optimization of the efficiency of spliceosome assembly revealed, however, that adenovirus major late [AdML] and α-tropomyosin pre-mRNAs assemble spliceosomes significantly more efficiently than β-globin [Bennett et al. 1992]. We therefore used AdML and α-tropomyosin pre-mRNAs in this study. The efficiency of spliceosome purification is shown in Figure 1B. Standard large-scale purifications yield ~1–4 μg of pre-mRNA assembled into spliceosomes.

As expected from previous studies [for review, see Green 1991], U1, U2, U4, U5, and U6 snRNAs are present in affinity-purified spliceosomes assembled on biotinylated AdML or tropomyosin pre-mRNAs [Fig. 1C, lanes 1[Ad] and 2 [Tm], respectively], whereas only background levels of U1 snRNA were detected when these pre-mRNAs lacked biotin [Fig. 1C, lanes 3,4]. Similarly, a large number of proteins were detected in spliceosomes assembled on biotinylated pre-mRNAs [Fig. 1D, lanes 1,2], but not on nonbiotinylated pre-mRNAs [Fig. 1D, lanes 3,4]. We note that higher levels of background are detected with tropomyosin pre-mRNA than with AdML [Fig. 1C,D]. This is observed because complexes assembled on tropomyosin pre-mRNA are not affinity purified as efficiently as those assembled on AdML [Fig. 1B]. Thus, tropomyosin complexes are bound to three to four times more avidin–agarose than are the equivalent amounts of AdML complexes. Because the background [e.g., U1 snRNP background] is derived from nonspecific binding to the avidin–agarose, three to four times higher background is observed with tropomyosin complexes. Finally, we note that the background binding of the nonbiotinylated pre-mRNA to the avidin–agarose is very low (0.2–2%; Reed 1990). Thus, the background protein bands that are observed in the nonbiotinylated control lanes [Fig. 1D, lanes 3,4] are most likely the result of endogenous spliceosome-like particles that copurify on the gel filtration column with the spliceosomes assembled on exogenously added pre-mRNA [see Reed 1990].

Our previous studies showed that spliceosomes affinity-purified in lower salt [100 mM; Reed 1990] have a much greater protein complexity than spliceosomes purified in 250 mM salt [Fig. 1D, lanes 1,2]. Most of this additional complexity, however, appeared to be the result of proteins also present in the H complex [Reed 1990]. The H complex assembles immediately upon addition of RNA to the in vitro-splicing reaction but does not require functional 5' or 3' splice sites. Thus, the H complex is not thought to be a functional intermediate in spliceosome assembly [Konarska and Sharp 1987; Michaud and Reed 1991; Bennett et al. 1992]. In recent studies, we found that the affinity-purified H complex consists primarily of the same hnRNP proteins that associate with nascent pre-mRNA in vivo [Bennett et al. 1992]. Polypyrimidine tract-binding protein [PTB], which was proposed to be a splicing factor that binds to the 3' splice site [Garcia-Blanco et al. 1989; Gil et al. 1991; Patton et al. 1991], corresponds to hnRNP I and first associates with the pre-mRNA as part of this larger hnRNP complex [see below; Bennett et al. 1992].

As observed in our previous studies [Bennett et al. 1992], the pattern of hnRNP proteins in the H complex is different on different pre-mRNAs [Fig. 2, cf. lanes 1 and 4]. The presence of lower levels of total hnRNP proteins
Figure 1. Two-step affinity purification of spliceosomes. (A) Gel filtration. Splicing reactions (11 ml) containing 44 µg of AdML (Ad) or 33 µg of tropomyosin (Tm) 32P-labeled pre-mRNA were incubated under splicing conditions. Reactions were fractionated by gel filtration [see Materials and methods]. The peaks containing the H and B complexes [spliceosomes] are indicated. The small peak between fractions 25 and 35 is the void volume of the column, and the peak between fractions 70 and 80 is degraded RNA. (B) Efficiency of purification. Shown are the data from a representative purification of spliceosomes assembled on AdML or tropomyosin pre-mRNA (affinity purification was carried out at 250 mM salt; see Materials and methods). We note that spliceosomes are both assembled and affinity-purified more efficiently on AdML than tropomyosin pre-mRNA. The amount recovered is the percent of the total micrograms of input RNA recovered. (C,D) (Lane 1) AdML + biotin; (lane 2) Tm + biotin; (lane 3) AdML − biotin; (lane 4) Tm − biotin. (C) RNA analysis. Total RNA was prepared from an aliquot of affinity-purified spliceosomes and end-labeled with [32p]pCp. The positions of the snRNAs and pre-mRNAs are indicated. Note that U6 snRNA does not end label efficiently but was detected on longer exposures of the film. (D) Protein analysis. Proteins from affinity-purified spliceosomes were fractionated on a 9% SDS gel and detected by silver staining. The molecular mass markers are indicated.

associated with AdML compared with tropomyosin pre-mRNA is the result, at least in part, of differences in their sequences (Bennett et al. 1992) but may also be related to their size differences (AdML is 213 nucleotides, whereas tropomyosin is 547 nucleotides; see Bennett et al. 1992).

Notably, the complexity of the H complex is reduced significantly by affinity purification in 250 versus 100 mM salt (Fig. 2, cf. lane 1 with 2 and lane 4 with 5). In fact, virtually all of the hnRNP proteins, including hnRNP I/PTB, dissociate from the AdML H complex in 250 mM salt (Fig. 2, lane 5; hnRNP I/PTB was identified in Bennett et al. 1992). We note that the levels of hnRNP I/PTB are higher in tropomyosin than in the AdML H complex most likely because the pyrimidine-rich sequences to which hnRNP I/PTB binds (Garcia-Blanco et al. 1989, Patton et al. 1991) is 65 nucleotides in tropomyosin (46% C, 49% U) and only 20 nucleotides in AdML (40% C, 45% U). The observation that many hnRNP proteins dissociate from single-stranded nucleic acids at 200-300 mM salt is consistent with previous studies (Piñol-Roma et al. 1988, S. Piñol-Roma, pers. comm.).

In contrast to the H complex, a large number of proteins remain bound to purified spliceosomes treated with 250 mM salt. This set of proteins appears to be sim-
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Figure 2. HnRNP proteins dissociate from the pre-mRNA in splicing complexes purified in 250 mM salt. The B and H complexes were affinity-purified in 100 or 250 mM salt, as indicated. The bands corresponding to hnRNP I/PTB are indicated. The proteins were fractionated on a 9% SDS gel and detected by silver staining. The sizes of molecular mass markers are indicated.

Protein components of the mammalian spliceosome

The protein composition of the spliceosome affinity-purified in 250 mM salt was analyzed by two-dimensional nonequilibrium pH gradient gel electrophoresis [NEPHGE; Fig. 3]. Several criteria were used to determine which of the proteins in the spliceosome are spliceosome specific. First, the protein must be present in spliceosomes assembled both on AdML and on tropomyosin pre-mRNAs. Second, the protein must be significantly enriched in the spliceosome (B complex) compared with the H complex (also affinity-purified in 250 mM salt). Third, the protein must be enriched in affinity-purified spliceosomes relative to gel filtration-isolated spliceosomes or the starting nuclear extract. Fourth, the protein must be present in spliceosomes assembled in different preparations of the nuclear extract.

Comparison of spliceosomes assembled on tropomyosin or AdML pre-mRNA shows that they have strikingly similar compositions [Fig. 3, cf. A and B]. Proteins in the spliceosomes are indicated by their molecular masses, or by their names in cases where the proteins were identified (see below for data pertaining to identification of known proteins). Interestingly, the main difference between the two types of spliceosomes is the proteins that are also present in the H complex (indicated by circles in Fig. 3, cf. A–D). For example, hnRNP I/PTB, J, K, P, and Q and other unidentified proteins are present in tropomyosin spliceosomes and are also detected in tropomyosin spliceosomes (Fig. 3A,C; nomenclature is according to Pifiol-Roma et al. 1988). Significantly, however, most of the H complex-specific proteins are present at much lower levels in the spliceosome than in the H complex (Fig. 3, cf. A and C). In contrast to tropomyosin, very few proteins are present in the AdML H complex (Fig. 3D; see also Fig. 2, lane 5). Thus, it is not surprising that few hnRNP proteins are detected in AdML spliceosomes (Fig. 3B).

To determine the level of enrichment of the proteins detected in affinity-purified spliceosomes, we compared them with similar amounts of total protein obtained from nuclear extract and from gel filtration-purified spliceosomes (Fig. 3, cf. A or B with E and F). The major proteins in common are indicated. This comparison identified two proteins, a 50-kD protein [marked with an asterisk] and the heat shock proteins [Hsc 70/Hsp 70, marked with a Δ], which are highly abundant in the nuclear extract and are also present in the affinity-purified spliceosomes. Therefore, we cannot rule out the possibility that these proteins may be present in the spliceosomes as contaminants. We note, however, that heat shock proteins play an essential role in the functions of other multicomponent complexes (for review, see Gething and Sambrook 1992), such as replication complexes, raising the interesting possibility that heat shock proteins are involved in spliceosome assembly (Yost et al. 1990). Other than the heat shock and 50-kD proteins, the proteins in the affinity-purified spliceosomes are significantly enriched compared with the gel filtration-purified spliceosomes and the nuclear extract. We note that the gel filtration-purified spliceosomes are enriched in hnRNPs [e.g., hnRNPs A, B, E, and I] and snRNPs [B, B', B''] relative to the nuclear extract (Fig. 3, cf. E and F).

On the basis of the comparisons presented above, we
Figure 3. Identification of spliceosome-specific proteins. Total protein from the B or H complexes assembled on AdML or tropomyosin pre-mRNAs (A–D, as indicated) and from nuclear extract or gel filtration fractions containing spliceosomes [E and F, as indicated] were fractionated by two-dimensional gel electrophoresis and silver stained [see Materials and methods]. Sizes of molecular mass markers are shown. HnRNP proteins A, B, and E (F) and G, I/PTB, J, K, P, and Q [A–F, as indicated] are designated according to the nomenclature of Piñol-Roma et al. (1988). Unidentified H complex proteins are indicated by arrowheads in A and C. H complex proteins present in the B complex are indicated by circles. The protein marked by an asterisk (*) in A, B, E, and F indicates the abundant protein in nuclear extract [see text] and A in A–F indicates the heat shock proteins. 70K and A are U1 snRNP-specific proteins, and A' and B' are U2 snRNP-specific proteins. We note that U1 70K is barely detected as a narrow streak by silver stain but was readily detected in two-dimensional gels of the spliceosome by Western blot analysis [see Fig. 4]. B and B' are snRNP core proteins. U2AF65 is a non-snRNP splicing factor (Zamore and Green 1991). We note that U2AF65 cofractionates with the 62-kD SAP on 9% SDS gels. However, we find that U2AF65 fractionates below the 62-kD SAP on a 7.5% SDS gel [see Fig. 4A inset]. The spliceosome-specific proteins are indicated by molecular mass. [For identification of known proteins, see Fig. 4.]
have identified 32 distinct proteins, ranging in molecular mass from 28 to 200 kD, as specific components of the spliceosome. The same set of proteins is present in spliceosomes assembled in different preparations of the nuclear extract (data not shown). Many of the high molecular mass proteins (e.g., 155, 130, 116, 115, and 114 kD) and some of the lower molecular mass proteins (e.g., 102, 88, 82, U2AF65) fractionate over a wide pH range in the isofocusing dimension (Fig. 3A, B). These proteins appear as streaks rather than as focused spots. This fractionation behavior occurs reproducibly and is typical of high molecular mass proteins or of modified proteins (Dunbar 1987). We note that we were not able to resolve any additional spliceosome-specific proteins by isoelectric focusing (IEF; data not shown).

The relative abundance of the different spliceosomal proteins varies (Fig. 3, A or B). The 62-, 61-, and 49-kD proteins are highly abundant, whereas many others, such as the 102-, 92-, 82-, and 68-kD proteins, appear to be lower in abundance. In some cases, these proteins may appear to be less abundant either because they fractionate as streaks or do not stain well with silver. It is also possible that some of the proteins are actually less abundant due to heterogeneity in the purified spliceosomes. The relative levels of some of the proteins differ between AdML and tropomyosin spliceosomes (Fig. 3, cf. A and B). For example, the 88-, 57-, and 42-kD proteins are more abundant in tropomyosin than in AdML spliceosomes, whereas the reverse is true with the 115-kD protein. This may reflect differences in how these proteins interact with the two different pre-mRNAs, or heterogeneity in the spliceosomes assembled on the two pre-mRNAs.

Identification of known proteins in the purified spliceosomes

To determine whether any known splicing factors or snRNP proteins are represented among our 32 spliceosome-specific proteins, we carried out Western blot analysis of AdML prespliceosome and spliceosome complexes (Fig. 4). In some cases, we compared the two-dimensional gel patterns of purified proteins and spliceosome complexes (Fig. 4). [The identification of known proteins is described here, and their order of assembly is discussed in the following section].

U2AF65 is a 65-kD non-snRNP splicing factor that binds to the 3' splice site (Zamore and Green 1991). U2AF65 has been detected in the ATP-dependent A3' complex that assembles on pre-mRNAs lacking a 5' splice site (Zamore and Green 1991). To identify U2AF65, we fractionated partially purified U2AF65 on two-dimensional gels and either stained with silver (Fig. 4A), or transferred to nitrocellulose and carried out Western blot analysis with polyclonal antibodies to U2AF65 (Fig. 4B). The nitrocellulose filter was gold stained after Western blot analysis to determine which of the proteins had bound to the antibody (data not shown). This analysis identified a protein of approximately the expected molecular mass in the basic region of the gel, consistent with the amino acid composition of U2AF65 (Zamore et al. 1992).

As indicated (e.g., Fig. 3A, B; Fig. 5A, B, D), we also identified U2AF65 in the affinity-purified prespliceosome and spliceosome complexes [We note that the levels of U2AF65 vary between these complexes (see below).] This was achieved by Western blot analysis of the affinity-purified splicing complexes with the U2AF65 polyclonal antibody and/or by cofractionation on two-dimensional gels. Western blot analysis identified a spot in the expected location on two-dimensional gels of the prespliceosome complex E (see below for description of the E complex, Fig. 4B inset). In addition, when partially purified U2AF65 (Fig. 4A) was fractionated in parallel with affinity-purified spliceosomes (see Fig. 3A), U2AF65 was identified near the 62-kD SAP in the spliceosomes. On lower percentage gels, however, U2AF65 migrates ahead of the 62-kD SAP, indicating that these two proteins are distinct (Fig. 4A inset).

The snRNP core proteins B and B' were readily detected in the spliceosome by use of an anti-Sm monoclonal antibody, and U1 snRNP-specific A and U2-snRNP specific A' and B" proteins were identified by use of EW patient sera (see below for identification of a protein that may correspond to IBP using Sm antibody; Fig. 4C, D, Fresco et al. 1991). Unexpectedly, the EW sera strongly react with the 62-kD SAP (Fig. 4D). This protein had not been reported previously as an antigen recognized by the EW antisera [Fresco et al. 1991].

Using a monoclonal antibody to the U1 snRNP-specific 70K protein (Billings et al. 1982), we detected a protein of the expected molecular mass in Western blots of the prespliceosome complex E (Fig. 4E) and of the spliceosome (data not shown). Some reactivity of the U1 70K antibody was also observed with U2AF65, which migrates ahead of the U1 70K protein (Fig. 4E). On the basis of other studies with this antibody, it appears that the U2AF65 signal is the result of nonspecific interactions (data not shown). We note that U1 70K, although readily detected by Western blot analysis, is barely detectable by silver stain (e.g., see Fig. 3A–B, Fig. 5A).

To determine whether the purified spliceosomes contained any of the known U5 snRNP-specific proteins (200, 116, 102, 100, 52, 40, and 15 kD [Bach et al. 1989]; purified U5 snRNP generously provided by R. Lehrmann, Institut für Molekularbiologie und Tumorforschung, Marburg, Germany), we fractionated purified U5 snRNPs and spliceosomes in parallel by SDS or two-dimensional gel electrophoresis (Fig. 4F). Superimposing the two-dimensional gels, we identified the 200-, 116-, 102-, 100-, and 40-kD U5 snRNP proteins in the purified spliceosomes. [We note that the 102- and 100-kD proteins migrate as 112- and 110-kD proteins on our gels.) As expected, the snRNP core proteins B and B' are also present in both the U5 snRNPs and the purified spliceosomes (Fig. 4F). Additional evidence that the 200-, 116-, 112-, 110-, and 40-kD proteins detected in the spliceosome correspond to the U5 snRNP proteins is the fact that these proteins were observed in the spliceosome,
Figure 4. Identification of known proteins in purified splicing complexes. (A,B) Partially purified U2AF65 was fractionated by two-dimensional gel electrophoresis and stained with silver [A] or transferred to nitrocellulose and probed with anti-U2AF65 polyclonal antibodies [B]. U2AF65 and the molecular mass markers are indicated. The inset in A is a portion of a 7.5% two-dimensional gel of affinity-purified spliceosomes that shows U2AF65 and the 62-kD SAP (spot to upper right of U2AF65) as distinct proteins [see text]. The inset in B shows a portion of a Western blot in which the U2AF65 polyclonal antibody was used to probe a two-dimensional gel of the affinity-purified E complex. (C,D) Affinity-purified spliceosomes assembled on AdML were fractionated by two-dimensional gel electrophoresis, transferred to nitrocellulose, and probed with anti-Sm monoclonal antibodies [C], snRNP core proteins B and B' are indicated or with EW patient sera [D], U1 A, U2 A', and B' and the 62-kD SAP are indicated. (E) The affinity-purified E complex was assembled on AdML, fractionated by two-dimensional gel electrophoresis, transferred to nitrocellulose, and probed with anti-U1 70K monoclonal antibodies [U1 70K is indicated; the asterisk (*) designates U2AF65, which reacted with the U1 70K monoclonal, see text]. (F) Purified U5 snRNP proteins [U5] or spliceosomes [B] were fractionated by two-dimensional [left] or SDS [right] gel electrophoresis. U5 snRNP-specific proteins [Lührmann et al. 1990] are indicated. (G) Western blot analysis of affinity-purified spliceosomes with Sm monoclonal antibody. The 110-kD protein is indicated by the arrow. The spots in the 70- to 80-kD range that react with the Sm antibody may correspond to the 70-kD protein described by Gerke and Steitz [1986] that is thought to be a breakdown product of IBP [Pinto and Steitz 1989; see text]. (H) Spliceosomes assembled on AdML were fractionated in parallel with SR proteins by two-dimensional gel electrophoresis. An SDS-gel lane of the SR proteins is shown to the right of the two-dimensional gel. The bands corresponding to SR proteins [Roth et al. 1991] were identified by Western blot analysis with a monoclonal antibody to these proteins [data not shown] and are indicated. The heat shock proteins are labeled on both gels, and the 62-, 61-, and 49-kD SAPs and snRNP proteins [B, B', B''] are indicated on the spliceosome gel. The molecular mass markers are shown.
Figure 5. Order of assembly of spliceosomal proteins. The E complex (A), A3' complex (B), A5' complex (C), and B complex (D) were affinity-purified and then fractionated by two-dimensional gel electrophoresis. A portion of a two-dimensional gel of the E3' complex is shown in the inset in A. The spliceosome-specific proteins that are enriched in each complex are indicated by molecular masses or names, if known. (For descriptions of labeled proteins, see legend to Fig. 3.) The proteins that first bind to the pre-mRNA in the A3' (B) or B (D) complex are circled. (We note that the 49-kD SAP fractionated as a streak in the isofocusing dimension in the gel shown in B; this was not observed in other gels with the same sample.) The 115-kD SAP and U1 snRNP-specific A protein that binds to the 5' splice site are circled in the A5' complex (C). The protein marked by an asterisk (*) indicates the abundant protein in the nuclear extract (see Fig. 3E); Δ indicates the heat shock proteins (see text).
which contains U5 snRNA, but not in the prespliceosome complexes, which lack U5 snRNA [see below]. Moreover, previous studies have shown that the 200-kD protein in the spliceosome corresponds to the 200-kD U5 snRNP protein [Anderson et al. 1989, Pinto and Steitz 1989, García-Blanco et al. 1990]. The U5 snRNP protein that we did not detect in the purified spliceosomes [52 kD] either may not be a component of the spliceosome or may have dissociated during purification. In addition, our analysis would not have detected the 15-kD U5 snRNP protein because it is below the molecular mass cutoff of our gels (<27 kD).

We find that the 112- and 110-kD U5 snRNP proteins fractionate differently from gel to gel. For example, we observe that the 110-kD protein migrates to a more basic position than the 112-kD protein on some gels [e.g., Fig. 4F], whereas on other gels the two proteins fractionate in a similar location in the isofocusing dimension [e.g., Fig. 4F, inset in U5]. Similar differences in the fractionation behavior of these proteins were observed in the purified spliceosomes [e.g., cf. Figs. 3B and 4F; (B)].

Previous studies identified a protein designated IBP, which binds to the 3' splice site and appeared to be a component of U5 snRNP [Gerke and Steitz 1986; Tazi et al. 1986]. In addition, IBP was also found to react with anti-Sm monoclonal antibodies [Gerke and Steitz 1986]. Although the reported size of IBP differs (70 vs. 100 kD; Gerke and Steitz 1986; Tazi et al. 1986), the 70-kD protein is thought to be a proteolysis product of the 100-kD protein [Pinto and Steitz 1989]. In another study, Bach and coworkers [1989] found that the 100-kD protein in U5 snRNP [110 kD on our gels] is also detected by anti-Sm antibodies, suggesting that this protein corresponds to IBP [Pinto and Steitz 1989]. We were unable to detect a protein of 70–100 kD using Sm antibodies to probe Western blots in which the second dimension of the twodimensional gels was 9% [data not shown]. By use of a 7.5% gel for the second dimension, however, the 110-kD U5 snRNP protein was detectable by the anti-Sm antibody [Fig. 4G]. In addition, the antibody reacted with two other proteins in the 70- to 80-kD region [Fig. 4G]. We conclude that the 110-kD protein present in our affinity-purified spliceosomes corresponds to the Sm-reactive U5 snRNP protein identified previously [Bach et al. 1989] and thus may be the same as IBP. The 70- to 80-kD proteins may correspond to the previously described breakdown products of IBP [Gerke and Steitz 1986; Pinto and Steitz 1989]. We note that in other preparations of nuclear extract the 70-kD protein is the predominant protein detected [data not shown]. Further studies are needed to determine whether these Sm-reactive proteins correspond to IBP.

Western blot analysis was used to identify hnRNP proteins [hnRNPs A, C, D, L, and I/PTB] in two-dimensional gels of the affinity-purified H complex or gel filtration-purified spliceosomes [data not shown]. The other hnRNP proteins indicated in Figure 3 (A–F) were identified previously [Bennett et al. 1992]. We have also identified the heat shock proteins [Hsc 70/Hsp 70] in all of the purified splicing complexes [see Fig. 3A–F]. The heat shock proteins were identified by supplementing affinity-purified spliceosomes with purified heat shock proteins and showing that the spots that increased in intensity corresponded to those of the heat shock proteins run individually on a parallel two-dimensional gel [data not shown].

The same methods were used to determine whether any members of the SR family of proteins [Mayeda et al. 1992], which includes SC35 and SF2/ASF [Fu and Maniatis 1990; Ge and Manley 1990; Krainer et al. 1990; Zahler et al. 1992], were present in the purified spliceosomes [Fig. 4H]. Previous studies have shown that antibodies to SC35 immunoprecipitate prespliceosome and spliceosome complexes [Fu and Maniatis 1990, 1992], suggesting that at least this SR protein is present in the spliceosome. Purified SR proteins [Zahler et al. 1992] fractionate on two-dimensional gels as highly basic and large diffuse spots [Fig. 4H, SR], consistent with the known amino acid composition of these proteins and the fact that they are multiply phosphorylated [Roth et al. 1991, X.-D. Fu, pers. comm.]. Unexpectedly, all of the SR proteins fractionate to the same position in the first dimension [Fig. 4H, SR]. Whether this is the result of an artifact from aggregation or reflects the high degree of similarity between the SR family members is not known. Purified SC35 cofractionates with SRp30 on two-dimensional gels as expected [data not shown; Zahler et al. 1992]. As reported previously [Roth et al. 1990, 1991], we readily detected all of the purified SR proteins by Western blot analysis with anti-SR protein antibodies [data not shown]. Surprisingly, however, we did not detect any of the SR proteins with anti-SR protein antibodies on Western blots of either SDS or two-dimensional gels of affinity-purified spliceosomes [data not shown]. Moreover, we did not detect the SR proteins [including SC35] in purified spliceosomes by comparisons on two-dimensional gels [Fig. 4H; cf. SR and B, with heat shock proteins as a reference]. Thus, it is possible that the SR proteins dissociate from, or are present at undetectable levels in, the purified spliceosomes. We note, however, that the SR proteins were also not detected in spliceosomes affinity-purified in lower salt [150 mM, data not shown]. These data suggest that the SR proteins do not correspond to any of the spliceosome-specific proteins that we have identified. It is possible, however, that the SR proteins, including SC35, were not detected in the spliceosome either by Western blot analysis or by comparisons with purified proteins because of differences in phosphorylation [Roth et al. 1990, X.-D. Fu pers. comm.]. Thus, further studies are required to determine the relationship between the SR family of proteins and the spliceosome.

In total, using all available reagents, we have shown that U2AF65, U1 snRNP-specific proteins [70K, A], U2 snRNP-specific proteins [A', B'], U5 snRNP-specific proteins [200, 116, 112, 110, and 40 kD], and snRNP core proteins [B and B'] are among the specific components of the spliceosome. In contrast, the hnRNP proteins and heat shock proteins that were detected in the spliceosome are also present or are enriched in the H complex.
We have designated the remaining previously unidentified spliceosomal components as SAPs.

We did not carry out a systematic analysis of the lower molecular mass proteins in the spliceosome (<27 kD), primarily because our spliceosome purification procedure leads to the presence of large amounts of avidin and RNase A in the lower molecular weight region of the gel (see Materials and methods; Reed 1990). On the basis of comparisons with published two-dimensional gels [Feeney et al. 1989; Woppmann et al. 1990], however, we do detect lower molecular weight proteins that are likely to correspond to the known snRNP core proteins [C, D, E, F, and G; data not shown]. Notably, other than these proteins, we do not detect significant levels of any other lower molecular weight proteins.

**Temporal order of assembly of spliceosomal proteins**

Association of the spliceosomal snRNAs with the pre-mRNA takes place in discrete prespliceosome and spliceosome complexes that assemble in a step-wise manner [for review, see Green 1991]. U1 snRNA binds to the pre-mRNA first, in the ATP-independent prespliceosome (E complex) [Seraphin and Rosbash 1989; Michaud and Reed 1991]. Subsequently, U2 snRNA binds to pre-mRNA in the ATP-dependent prespliceosome (A complex; for review, see Green 1991). The mature spliceosome (B complex) assembles by the addition of U4, U5, and U6 snRNAs [see Green 1991]. To determine the order of assembly of the 32 spliceosomal proteins that we have identified, we compared the composition of these prespliceosome and spliceosome complexes using two-dimensional gel electrophoresis (Fig. 5). However, the A3' complex, which assembles on pre-mRNAs lacking a 5' splice site, was analyzed instead of the A complex because this complex is so short-lived when assembled on intact pre-mRNA that it is difficult to purify reproducibly. Previous studies have shown that the A3' complex contains U2 snRNA and is similar to the A complex [Konarska and Sharp 1987].

In analyzing each of the different complexes, we have defined a protein as present in a particular complex only if that protein is significantly enriched in the complex relative to the other complexes. This definition was used in labeling the proteins in Figure 5. For example, the 49- and 62-kD proteins are more abundant in the A3' and B complexes than in the E or A5' complexes [Fig. 5, cf. A–D]. Thus, we define these proteins as present in the A3' and B complexes but not in the E or A5' complexes. At present, we do not know why certain proteins are present in lower abundance in a particular complex and more abundant in another. This could be the result of nonspecific associations of these proteins, heterogeneity in the purified complexes, or changes in the affinity of these components during spliceosome assembly.

U2AF65 first binds to the pre-mRNA in the E complex (Fig. 5A). This protein is also an abundant component of the E3' complex, an ATP-independent complex that assembles on RNA lacking the 5' splice site (Fig. 5A inset). This observation is consistent with previous studies showing that U2AF65 binds to the 3' splice site in the absence of ATP [Zamore and Green 1991]. The U1 snRNP-specific proteins [A and 70K], the snRNP core proteins [B and B'], and the 115-, 92-, 88-, 72-, and 42-kD SAPs also first bind to the pre-mRNA in the E complex. The other proteins in the E complex (unlabeled proteins in Fig. 5A) are either more abundant in other complexes or could not be identified reproducibly in the E complex.

Strikingly, we observe a significant decrease in the levels of U2AF65 in the A3' complex compared with the E or E3' complexes (cf. Fig. 5, B with A and A inset). Similarly, the levels of U2AF65 are lower in the B complex than in the E or E3' complex (cf. the AdML B complexes in Figs. 3B, 4F, 4H, and 5D with the E and E3' complexes in Fig. 5A and A inset). The observation that the levels of U2AF65 are lower in the A and B complexes relative to the E complexes indicates that U2AF65 is only transiently associated with the pre-mRNA during spliceosome assembly.

Concomitant with the decrease in U2AF65, a large number of SAPs first bind to the pre-mRNA in the A3' complex; these are proteins of 155, 145, 130, 114, 62, 61, 49, and 33 kD. In addition, the U2 snRNP-specific proteins A' and B' also first bind in the A3' complex (Fig. 5B; proteins that first bind in the A3' complex are designated by circles). We conclude that the binding of these proteins does not require a 5' splice site and is ATP independent. The latter conclusion is based on the observation that these proteins are not present in the ATP-independent E complex (Fig. 5, cf. A and B) nor in the ATP-independent E3' complex that assembles on RNA lacking a 5' splice site (S. Michaud and R. Reed, unpubl.).

The 92-, 88-, and 72-kD SAPs and B and B' are present in about the same levels in the A3' complex as in the E complex (Fig. 5, cf. A and B). This result indicates that these proteins do not require the 5' splice site for binding. Some of the proteins present in the E complex (115 KD, 42 kD and U1 A) are deficient, however, in the A3' complex compared with the E complex (Fig. 5, cf. A and B). To determine whether any of these proteins might interact with the 5' splice site, we examined the A5' complex (Fig. 5C). This complex, which was assembled in the presence of ATP on an RNA lacking the 3' splice site, contains U1 snRNA [S. Michaud and R. Reed, unpubl.]. This analysis revealed that the 115-kD protein and the U1 snRNP-specific A protein are enriched in the A5' complex compared with the A3' complex [Fig. 5, cf. B and C]. We note, however, that binding of the 115-kD protein and the U1 A protein is ATP-independent because these proteins are detected in the E complex and in the E5' complex, an ATP-independent complex that assembles on an RNA lacking the 3' splice site [S. Michaud and R. Reed, unpubl.].

The 42-kD protein that first binds in the E complex appears to be lower in abundance in the A3', A5', and B complexes compared with the E complex (Fig. 5, cf. A–D). This protein, however, is an abundant component of the B complex assembled on tropomyosin pre-mRNA (see Fig. 3A). Further studies are required to understand the significance of these observations.
The fully assembled spliceosome is formed by the binding of 12 proteins (indicated by circles, Fig. 5D) in addition to those already bound in the A5' and A3' complexes. These are the 200-, 116-, 112-, 110-, and 40-kD U5 snRNP-specific proteins and the 102-, 90-, 82-, 68-, 60-, 57-, and 55-kD SAPs. These proteins therefore require ATP and both the 5' and 3' splice sites for binding.

Table 1. 

| Ordered assembly of spliceosomal proteins | Molecular mass (kD) | Complex |
|-----------------------------------------|---------------------|---------|
| snRNP proteins                          |                     |         |
| U1 70K                                  | 70                  | E       |
| U1 A                                    | 34                  | A5'     |
| U2 A'                                   | 31                  | A3'     |
| U2 B"                                  | 28.5                | B       |
| U5                                      | 200                 |         |
| U5                                      | 116                 |         |
| U5                                      | 112                 |         |
| U5                                      | 110                 |         |
| U5                                      | 40                  |         |
| core B'                                 | 29                  |         |
| B                                        | 28                  |         |
| Non-snRNP proteins                      |                     |         |
| U2AF                                    | 62                  |         |
| SAPs                                    |                     |         |
| U2AF                                    | 62                  |         |
| 155                                     |                     |         |
| 145                                     |                     |         |
| 130                                     |                     |         |
| 115                                     |                     |         |
| 114                                     |                     |         |
| 102                                     |                     |         |
| 92                                      |                     |         |
| 88                                      |                     |         |
| 82                                      |                     |         |
| 72                                      |                     |         |
| 68                                      |                     |         |
| 62                                      |                     |         |
| 61                                      |                     |         |
| 60                                      |                     |         |
| 57                                      |                     |         |
| 55                                      |                     |         |
| 49                                      |                     |         |
| 42                                      |                     |         |
| 33                                      |                     |         |
| The spliceosome-specific proteins that are enriched in each complex are indicated. U1 70K was barely detected by silver stain in the complexes indicated but was readily detected by Western analysis with U1 70K monoclonal antibodies. (ND) Not determined. |

Discussion

We have carried out a detailed analysis of the protein composition of highly purified mammalian spliceosomes using two-dimensional gel electrophoresis. This analysis resulted in the identification of >30 distinct spliceosome-specific proteins with a combined molecular mass of 2.5 million (Table 1, B complex). Proteins designated as spliceosome specific are present in spliceosomes assembled on two different pre-mRNAs and are enriched in affinity-purified spliceosomes relative to hnRNP complexes (H), partially purified spliceosomes, and nuclear extract. Moreover, none of the spliceosome-specific proteins bind to RNAs lacking functional 5' and 3' splice sites (Bennett et al. 1992).

We identified both non-snRNP and snRNP-specific splicing factors by Western blot analysis and by analysis of purified or partially purified proteins by two-dimensional gel electrophoresis. These studies showed that U2AF65, U1 snRNP-specific-proteins (A and 70K), U2 snRNP-specific proteins (A' and B"), U5 snRNP-specific proteins [200, 116, 112, 110, and 40 kD], and snRNP core proteins (B and B') are present in purified prespliceosome or spliceosome complexes. The proteins that do not correspond to known splicing factors are designated SAPs (Table 1).

A comprehensive list of known splicing factors and those that we identified in the spliceosome is shown in Table 2. Those proteins classified as not present in the spliceosome [minus sign (~)] Table 2] were not detected in the purified splicing complexes with available reagents. For example, we did not detect any of the SR proteins [Mayeda et al. 1992] either by Western blot analysis or by comparisons with purified SR proteins on silver-stained gels. These non-snRNP proteins comprise a highly related family of functionally redundant proteins [Mayeda et al. 1992], and in vitro complementation studies showed that any one member of the SR protein family is required for assembly of the ATP-dependent prespliceosome complex [Mayeda et al. 1992; Zahler et al. 1992; X.-D. Fu, A. Mayeda, A. Krainer, and T. Maniatis, pers. comm.).

SC35 and SF2/ASF are two members of the SR family that were characterized independently (Fu and Maniatis 1990, Ge and Manley 1990, Krainer et al. 1990; Mayeda et al. 1992). Immunoprecipitation studies with antibodies to SC35 suggest that at least SC35 is in the spliceosome (Fu and Maniatis 1990, 1992). Thus, SC35 may dissociate from our splicing complexes during purification. Notably, however, we were also unable to detect the SR proteins in splicing complexes affinity-purified in low salt [data not shown]. We note that the SR proteins, as well as other spliceosomal proteins, may have eluded detection because they do not stain well with silver or do not fractionate as discrete spots on our two-dimensional gel system. Thus, the 32 spliceosomal proteins identified in our study may represent a minimal number of specific spliceosome components. Although it is possible that the EDTA and Triton X-100 present during our isolation of splicing complexes may result in dissociation of spliceosomal proteins, we note that prespliceosomes [E and A complexes] and spliceosomes have been isolated by gel filtration under these conditions and efficiently chased into spliced products by use of in vitro complementation assays [Abmayr et al. 1988; Michaud and Reed 1991].
Table 2. Known splicing factors and snRNP proteins

| Molecular mass (kD) | Spliceosomal proteins |
|------------------|----------------------|
|                  |                      |
| U1 snRNP 70K     | 70                   |
| A                | +                    |
| C                | +                    |
| U2 snRNP A'      | 31                   |
| B''              | +                    |
| U5 snRNP         | 200                  |
|                  | +                    |
| snRNP core B'    | 29                   |
|                   | +                    |
| snRNP core B      | 28                   |
|                   | +                    |
| SNR proteins     | 75, 55, 40, 30, 20   |
|                  | -                    |
| U2AF             | 62                   |
| 88 kD            | 88                   |
|                  | NI                   |

The snRNP proteins are described in Lührmann et al. (1990); the SR proteins are described in Roth et al. (1991). [ · ] The 30-kD SR proteins correspond to SC35 and SF2/ASF [Fu and Maniatis 1990; Ge and Manley 1990; Krainer et al. 1990; Mayeda et al. 1992; Zahler et al. 1992]. The 88-kD protein is described in Ast et al. [1991]. [ + ] Proteins identified among spliceosome-specific proteins. [ − ] Proteins not detected with available reagents. Proteins not identified [NI] were not detected because reagents were not available or because proteins are below the molecular weight cutoff of our gel system (<27 kD).

Among the proteins not identified [NI; Table 2] in the spliceosome were those below the molecular mass cutoff of our gel system (<27 kD) or those for which reagents were not available. Thus, it is possible that the latter class of proteins could be represented among the SAPs. For example, the 90- and 60-kD SAPs that first bind to the pre-mRNA in the B complex are candidates for the 90- and 60-kD proteins associated with U4, U5, and U6 snRNAs [Behrens and Lührmann 1991].

No hnRNP proteins were identified among the spliceosome-specific proteins. HnRNP proteins associate with the pre-mRNA in the first complex (H) assembled when RNA is added to splicing extracts; this complex is not thought to be a functional precursor to the spliceosome [Konarska and Sharp 1987; Michaud and Reed 1991; Bennett et al. 1992]. HnRNP I/PTB associates with the pre-mRNA at this time. Previous studies have shown that this protein binds to the 3' splice site, leading to the speculation that it may play a role in the splicing reaction [Garcia-Blanco et al. 1989; Gil et al. 1991, Patton et al. 1991]. Our studies show that in contrast to the spliceosome-specific proteins, hnRNP 1/PTB and most of the other hnRNP proteins are readily displaced from the pre-mRNA by affinity purification in 250 mM salt. Of the hnRNP proteins that are not dissociated by the salt treatment, we observe that these proteins are in significantly lower abundance in the spliceosome than in the H complex. Finally, there are large differences in the levels of hnRNP proteins associated with different pre-mRNAs [this study; Bennett et al. 1992]. Together, these observations suggest that rather than participating as essential splicing factors, hnRNP proteins may be involved in influencing the subsequent binding of splicing factors and/or in the mechanism of splice site selection. Indeed, HnRNP A1 has been shown to affect 5' splice site selection in vitro [Mayeda and Krainer 1992].

Dynamic pre-mRNA–protein interactions

The temporal order of assembly of the spliceosomal proteins is shown in Table 1. In the ATP-independent prespliceosome complex [E], the 5' splice site is recognized, either directly or indirectly, by the 115-kD SAP and U1 snRNP-specific proteins, whereas the 3' splice site is recognized by U2AF65. On the basis of previous studies [Zamore and Green 1991], U2AF65 most likely interacts directly with the 3' splice site. U1 snRNP proteins [70K and A], snRNP core proteins [B and B'] and 92-, 88-, 72-, and 42-kD SAPs also first interact with the pre-mRNA in the E complex. In the presence of ATP, eight SAPs associate with the 3' splice site in the A3' complex (see Table 1). As expected from previous studies [Konarska and Sharp 1987], U2 snRNP-specific proteins [A' and B''] are also present in the A3' complex. The fully assembled spliceosome is then formed by the addition of 12 proteins with binding that requires ATP and both the 5' and 3' splice sites. Among these components are the U5 snRNP-specific proteins and seven SAPs.

Our data support a model for dynamic interactions between the 3' splice site and proteins known to bind to this sequence element, including U2AF65, IBP, and hnRNP proteins A, C, D, and I/PTB [Gerke and Steitz 1986; Tazi et al. 1986; Swanson and Dreyfuss 1988; Gil et al. 1991; Patton et al. 1991; Zamore and Green 1991]. The hnRNP proteins [e.g., A, C, D, or I/PTB] first bind to the 3' splice site as part of a larger hnRNP complex that assembles immediately when RNA is added to splicing extracts [this study; Bennett et al. 1992]. Subsequently, U2AF65 binds to the pre-mRNA and is an abundant component of the first prespliceosome complex [E]. We note that this sequential order may not be absolute and may not reflect the situation in vivo. Because neither U2AF65 nor hnRNP proteins require ATP for binding, it is possible that the sequential order observed in vitro is simply determined by the relative levels of these two proteins in splicing extracts. In any case, our data show that hnRNP proteins such as hnRNP 1/PTB are clearly less tightly

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bound to the 3' splice site than U2AF65 and, in contrast to U2AF65, are not a major stable component of the earliest prespliceosome complex. Thus, it is possible that an exchange actually occurs on the 3' splice site between the hnRNP proteins and U2AF65 at the time of E complex assembly. Further studies are needed to establish this definitively. Unexpectedly, we found that the levels of U2AF65 decrease significantly in the first ATP-dependent prespliceosome complex. This suggests that U2AF65 dissociates from the pre-mRNA at this time. It is possible that another protein replaces U2AF65 in the prespliceosome complex. Finally, in the fully formed spliceosome, we detect a U5-specific protein that may correspond to the 3' splice site-binding protein IBP (Gerke and Steitz 1986; Tazi et al. 1986), suggesting that another exchange of 3' splice site-binding proteins may occur in the mature spliceosome. Thus, our study provides the first evidence for sequential and transient interactions of individual proteins with the pre-mRNA during spliceosome assembly.

Materials and methods

Plasmids

Plasmid pAdML was constructed by subcloning an EcoRI-Sau3A fragment from pBSAd10 (gift from M. Konarska, The Rockefeller Institute, NY) into the EcoRI and BamHI sites of SP72 (Promega Biotech). This fragment contains exon 1 (71 nucleotides), intron 1 (97 nucleotides), and exon 2 (45 nucleotides) derived from the adenovirus 2 major late transcription unit. DNA was linearized with BamHI (+213 in pAdML) for transcription of the whole pre-mRNA and with FspI (+107 in pAdML) for transcription of Ads' RNA. pAd3', which contains the 3' portion of the AdML intron and exon 2, was constructed by ligating the FspI-BamHI fragment from pAdML into the PvuII-BamHI sites of SP72 (Promega Biotech). pAdML and derivatives were transcribed with T7 polymerase. pGC + DX, which encodes exon 2 (143 nucleotides), intron 2 (218 nucleotides), and exon 3 (158 nucleotides) of rat α-tropomyosin pre-mRNA (547 nucleotides, Smith et al. 1989), was a gift from B. Nadal-Ginard (Harvard Medical School, Boston, MA). pGC + DX was linearized with BamHI for transcription with SP6 polymerase.

Pre-mRNA synthesis and in vitro splicing reactions

Biotinylated pre-mRNAs (Grabowski and Sharp 1986) were synthesized with SP6 or T7 polymerase in standard transcription reactions [Melton et al. 1984] that included 15 μM biotinylated UTP and 100 μM UTP. RNAs were capped during transcription as described (Konarska et al. 1984). In vitro splicing reactions were carried out according to Krainer et al. (1984), except that polyvinylalcohol (PVA) was omitted. Large-scale reactions used to obtain complexes for two-dimensional gel analysis were 11 ml and contained 44 μg of AdML or 33 μg of tropomyosin pre-mRNA. Reactions contained 30% nuclear extract, and the same preparation of nuclear extract was used for all of the data shown in this paper. Reactions were incubated at 30°C for 30 min to form the B, A3', and A5' complexes. We observed variability in the kinetics of assembly of the different splicing complexes in different preparations of nuclear extract. For example, in other nuclear extracts we incubated for only 15 min to form complexes that had the same protein compositions as the complexes shown here. These differences in kinetics are most likely the result of differences in the relative levels of splicing factors in the different preparations of nuclear extract.

For assembly of the H and E complexes, nuclear extract was depleted of ATP as described (Michaud and Reed 1991). Complex assembly reactions lacked ATP, MgCl2, and creatine phosphate. Reactions were incubated at 30°C for 30 min to form the E complex. The H complexes were formed by incubation for 1 or 5 min at 30°C (similar results were obtained by incubation for 5 min at 0°C). In some extracts, we observed that the H complexes formed on AdML pre-mRNA contained U2AF65 and U1 snRNP proteins. This may be the result of higher relative levels of these splicing factors in some extracts coupled with the fact that AdML pre-mRNA assemblies splice complexes very rapidly and efficiently relative to other pre-mRNAs, such as tropomyosin or β-globin (Bennett et al. 1992).

Purification of splicing complexes

For gel filtration of splicing complexes, in vitro splicing reactions were loaded directly onto 2.5 × 75-cm Sephacryl S-500 columns equilibrated in FSP buffer [20 mM Tris (pH 7.8), 0.1% Triton X-100, 60 mM KC1, 2.5 mM EDTA] (Abmayr et al. 1988; Reed et al. 1988). The peak fractions were pooled and the salt concentration was either left at 60 mM KC1 or adjusted with NaCl to a final concentration of 250 mM salt. Avidin–agarose (10 μl/ml) (Vector Labs) was added to the pooled fractions and mixed overnight at 4°C. Avidin beads were then washed four times with a volume of buffer equal to that of the pooled fractions (~50 ml for each wash). The wash buffer for the affinity selections that were carried out at 60 mM salt was made up of 20 mM Tris (pH 7.9), and 100 mM NaCl, whereas the wash buffer for the affinity selections that were carried out in 250 mM salt consisted of 20 mM Tris (pH 7.9), and 250 mM NaCl.

Preparation and analysis of protein and RNA samples

To remove RNA, 2 μl of 200 μg/ml protease-free RNase A (Boehringer Mannheim) was added to avidin–agarose-bound splicing complexes (0.5 ml) and incubated at 30°C for 10 min. Proteins were then eluted from the avidin beads in a buffer consisting of 20 mM Tris (pH 7.4), 20 mM DTT, and 2% SDS. Eluted proteins were heated at 65°C for 5 min. (This step was essential in reproducibly detecting all proteins by two-dimensional gel electrophoresis.) Glycogen (2 μl as a carrier) and four volumes of acetone were added, samples were left at room temperature for 10 min and then spun for 10 min at room temperature. (We determined that proteins were not lost during the acetone precipitation by comparing samples eluted directly in SDS–sample buffer with those that had been acetone precipitated first.) Recovered proteins were dissolved immediately in SDS or two-dimensional sample buffer [8 M urea, 1.7% NP40, 1.7% pH 3–10 ampholytes (Bio-Rad)] and stored at −70°C or loaded directly onto gels.

Two-dimensional gel electrophoresis was carried out as described (O’Farrell et al. 1977). The first dimension was NEPHGE, and the second dimension was 9% SDS–polyacrylamide gel electrophoresis. Proteins were visualized by silver staining (Morrissey 1981). The total protein obtained from splicing complexes assembled on 150–200 ng of pre-mRNA was loaded on one two-dimensional gel. For SDS gels, total protein obtained from 60–80 ng pre-mRNA was loaded.

RNA was isolated from the affinity-purified complexes by proteinase K digestion and phenol extraction, followed by ethanol precipitation with glycogen (Boehringer Mannheim) as a carrier (Reed 1990). RNA was end-labeled with [32P]pCp and
RNA ligase and fractionated on an 8% denaturing polyacrylamide gel.

For Western blots, samples (total protein isolated from splicing complexes assembled on 200–250 ng of pre-mRNA) were fractionated by two-dimensional gel electrophoresis, transferred to nitrocellulose, and probed with the antibodies indicated. Anti-mouse, anti-rabbit, and anti-human secondary antibodies were horseradish peroxidase conjugated, and the enhanced chemiluminescence detection system (Amersham) was used. The same blots of two-dimensional gels were probed sequentially with different antibodies using protocols provided (Amersham).

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