Presplicing complex formation requires two proteins and U2 snRNP

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Six fractions derived from a HeLa cell nuclear extract are necessary for the generation of spliced mRNA in vitro. To establish a function for the protein factors present in these fractions, their role in the formation of splicing complexes was analyzed by electrophoresis in native polyacrylamide gels. Two of the fractions are sufficient to assemble the adenovirus major late mRNA precursor into a presplicing complex with characteristics similar to the presplicing complex assembled in nuclear extract. One fraction supplies splicing factor (SF) 1 and at least one small nuclear ribonucleoprotein particle, U2 snRNP. The other fraction contains SF3. Extensive fractionation of this protein has revealed that it is essential for presplicing complex assembly and the splicing reaction.

[*Key Words*: Pre-mRNA splicing; splicing complex assembly; fractionation of splicing activities; snRNPs]

Received March 31, 1988; revised version accepted July 6, 1988.

Before nuclear mRNA precursors (pre-mRNA) can be spliced in mammalian cell-free extracts, the exon-intron junctions are recognized by a number of components, and the exons are brought into proximity to allow precise excision of the intron (for review, see Green 1986; Padgett et al. 1986; Maniatis and Reed 1987; Sharp 1987). This recognition/alignment proceeds in several steps, and specific structures are generated that can be separated by centrifugation in sucrose or glycerol gradients or by electrophoresis in native polyacrylamide gels (Friendewey and Keller 1985; Grabowski et al. 1985; Bindereif and Green 1986; Kaltwasser et al. 1986; Konarska and Sharp 1986, 1987).

Immediately upon addition of an RNA substrate to a nuclear extract, complex H is assembled. Its formation is independent of intron consensus sequences; therefore, this complex is believed to be nonspecific and presumably results from an interaction of general RNA-binding proteins with the RNA. Within 2–5 min of incubation at 30°C and in the presence of ATP, the pre-mRNA becomes sequestered into a presplicing complex (complex A) that is thought to represent an intermediate in the formation of the active splicing complex (also termed spliceosome or complex B). This assumption is based on the kinetics of the appearance of complexes A and B, both in the mammalian and yeast splicing systems (Friendewey and Keller 1985; Konarska and Sharp 1986; Pikielny et al. 1986; Cheng and Abelson 1987). It remains to be shown, however, whether the pre-mRNA present in complex A can be chased into the products of the splicing reaction. The primary recognition site in the pre-mRNA is the 3' splice site, as pre-mRNAs lacking this sequence are not assembled into a specific complex (Friendewey and Keller 1985; Bindereif and Green 1986; Konarska and Sharp 1986; Lamond et al. 1987). After a 10- to 20-min incubation, the spliceosome forms. The

cleavage and ligation reactions that lead to the generation of mature mRNA proceed within this complex (Friendewey and Keller 1985; Grabowski et al. 1985; Bindereif and Green 1986; Konarska and Sharp 1986; Friendewey et al. 1987). Assembly of the splicing complex is dependent on RNA sequences at both the 5' and 3' splice sites (Friendewey and Keller 1985; Konarska and Sharp 1986).

The components that associate with the pre-mRNA during complex assembly have been analyzed by a variety of methods (for review, see Maniatis and Reed 1987; Steitz et al. 1988). The U1 small nuclear ribonucleoprotein particle (snRNP) binds to the 5' splice site in an ATP- and temperature-independent fashion (Mount et al. 1983; Black et al. 1985; Chabot and Steitz 1987). This binding occurs via base pairing of U1 RNA with the 5' splice site (Zhuang and Weiner 1986), and additional mechanisms for early interactions with the pre-mRNA may exist (Zillmann et al. 1987).

The interactions occurring at the 3' end of the intron are more complex. A protein of 70–100 kD, called the intron-binding protein or IBP (Gerke and Steitz 1986; Tazi et al. 1986), binds to the 3' splice site in the absence of ATP and at 0°C (Black et al. 1985; Chabot et al. 1985). This binding depends on sequences immediately upstream of the intron-exon border: the AG dinucleotide and the polypyrimidine stretch. The protein can be found associated with U5 snRNP; however, this snRNP is not essential for the interaction. A second protein, the U2 snRNP auxiliary factor (U2AF), interacts with the same region of the pre-mRNA, thereby allowing the association of U2 snRNP with the branch point (Ruskin et al. 1988). The involvement of components other than U2 snRNP in the recognition of the 3' splice site is not surprising, because U2 snRNP binding to the branch point depends more on the sequences immediately up-

stream of the 3' splice site than on the branch point sequence itself (Ruskin and Green 1985b; Ruskin et al. 1985; Chabot and Steitz 1987). Binding of U2 snRNP occurs only in the presence of ATP and requires incubation at 30°C (Black et al. 1985; Chabot and Steitz 1987). The mode of its interaction with the branch point region has not been established for the mammalian system. Binding of U2 snRNP occurs via RNA–RNA base pairing (Parker et al. 1987) in *Saccharomyces cerevisiae*. Although this type of interaction could also apply to the mammalian system, interactions between U2 snRNP and the protein(s) that recognizes the 3' splice site may be important as well.

The association of components with the 3' splice site is a prerequisite for the assembly of complex A. U2AF mediates the U2 snRNP/branch point interaction (Ruskin et al. 1988), and U2 snRNP remains associated with the pre-mRNA in complex A as well as in complex B (Grabowski and Sharp 1986; Konarska and Sharp 1986, 1987; Bindereif and Green 1987). The evidence for the participation of IBP in complex formation is only suggestive (Chabot et al. 1985; Gerke and Steitz 1986; Tazi et al. 1986). In addition, it is not clear which role U1 snRNP plays in complex A assembly. Apparently there is no requirement for a functional 5' splice site during this process (Fredericewey and Keller 1985; Bindereif and Green 1986; Konarska and Sharp 1986; Lamond et al. 1987). Therefore, an interaction of U1 snRNP with a pre-mRNA lacking a 5' splice site would have to occur by means other than base-pairing to its complementary sequence. U1 snRNP can be detected in presplicing complexes assembled on an intact pre-mRNA (Bindereif and Green 1987; Zillmann et al. 1988); however, it has not been shown that the particle is also present in complexes assembled on an RNA that lacks a functional 5' splice site. In addition, a complex formed on an RNA that lacks a 5' splice site migrates slightly faster in a native gel than complex A assembled on an intact pre-mRNA (Zillmann et al. 1988). The difference in mobility may result from the absence or presence of U1 snRNP in the complex. Thus, it remains to be established whether U1 snRNP is essential for the interaction of the above-mentioned components with the 3' splice site.

The conversion of complex A to the spliceosome requires intact U4 and U6 snRNPs (Berget and Roberson 1986; Black and Steitz 1986; Fredericewey et al. 1987; Krämer 1987). U4, U5, and U6 snRNPs bind to the pre-mRNA simultaneously and are found in the active spliceosome in addition to U1 and U2 snRNPs (Grabowski and Sharp 1986; Bindereif and Green 1987; Chabot and Steitz 1987; Konarska and Sharp 1987). U4 snRNP is released from the spliceosome after the pre-mRNA has been cleaved at the 5' splice site and lariat formation has occurred (Konarska and Sharp 1987; Lamond et al. 1988).

Apart from the snRNPs, the class C hnRNP (heterogeneous nuclear ribonucleoprotein particle) proteins are necessary for the assembly of splicing complexes (Choi et al. 1986). In addition, chromatographic fractionation

of mammalian extracts has revealed the presence of factors (other than snRNPs) that participate in these early steps of the splicing reaction (Krainer and Maniatis 1985; Mayeda et al. 1986; Perkins et al. 1986; Tatei et al. 1987).

We have separated HeLa cell nuclear extracts into six fractions that contain components essential for pre-mRNA splicing *in vitro* (Krämer et al. 1987). Two of the fractions are enriched in the major snRNPs U1–U6. The remaining fractions contain protein factors, all of which are required for the production of splicing intermediates and products in the splicing reaction. Thus, each of these fractions contains at least one component that is involved in the events leading to cleavage of the pre-mRNA at the 5' splice site and lariat formation. To investigate the functions of these proteins in more detail, their participation in complex formation was analyzed. This study shows that two protein fractions are required to assemble a presplicing complex in addition to at least one snRNP (U2 snRNP).

Results

Formation of splicing complexes with fractionated components

The early steps in nuclear pre-mRNA splicing, the assembly of the substrate RNA into presplicing, and splicing complexes can be monitored by electrophoresis in native polyacrylamide gels (Konarska and Sharp 1986). This method was used to investigate the participation in complex formation of individual splicing activities fractionated from a HeLa cell nuclear extract (Krämer et al. 1987). The splicing activities are termed SF1 (splicing factor 1), SF2, SF3, and SF4. This designation does not imply that they represent single factors, as they have not been purified to homogeneity. The fractions used throughout this paper are designated by an abbreviation of the chromatographic material used and the splicing factor present (see Methods).

Figure 1A shows complex formation in the presence of nuclear extract and in the presence of the fractionated splicing factors. Free RNA (lane 1) associates with nuclear components to form two distinct complexes, A and B, in a 60-min splicing reaction (lane 2). Other slow-migrating complexes as described by Konarska and Sharp (1987), Lamond et al. (1987), and Zillmann et al. (1988), have not been observed under the conditions used here. When pre-mRNA is incubated in the presence of the fractions (lane 3), similar structures are apparent. Formation of complex A appears to be as efficient as in the nuclear extract, whereas only a small amount of complex B is made. In addition, complex H accumulates in the reaction. Hence, sufficient amounts of those components required for the formation of presplicing complexes are present among the fractionated splicing activities. Components involved in the conversion of complex A to complex B appear to be underrepresented, which may explain the extended lag phase and the reduced efficiency of splicing observed in reactions performed in the reconstituted system (Krämer et al. 1987).

Figure 1. Formation of splicing complexes in the presence of nuclear extract and of fractionated components. (A) Complex reactions (50 μ l) were performed for 60 min at 30°C in the absence of nuclear components (lane 1), in the presence of 10 μ l nuclear extract (lane 2), or in the presence of fractionated splicing components (lane 3). The fractions used were 5 μ l SF1 (DEAE–Sephacrose 100), 6 μ l SF2 (heparin–Sephacrose 150), 6 μ l SF3, 6 μ l SF4, 1 μ l U1 snRNP, and 1 μ l U2 snRNP (Mono Q). Reactions were terminated by addition of heparin, and 5- μ l samples were loaded onto a 4% composite gel (see Methods). The positions of free pre-mRNA and complexes A, B, and H are indicated at the right. (B) Complex reactions (20 μ l) were performed for 30 min at 30°C in the presence of the following components. Six microliters of nuclear extract (lane 1); complete reaction with 2 μ l SF1 (Bio-Rex 500), 2 μ l SF2 (Bio-Rex 50), 2 μ l SF3 (Mono Q), 3 μ l SF4 (Mono S), and 0.5 μ l each of U1 and U2 snRNP (Blue Sepharose) (lane 2); complete reaction minus SF1 (lane 3); complete reaction minus SF2 (lane 4); complete reaction minus SF3 (lane 5); complete reaction minus SF4 (lane 6). Samples (10 μ l) were analyzed in a 4% polyacrylamide gel.

To investigate the contribution of the fractionated components to splicing complex formation, individual fractions were omitted from the complete reaction that contained SF1–SF4, and the fractions enriched in the major snRNPs (Fig. 1B). Complex A is made efficiently within 30 min of incubation in the complete reaction (lane 2). Complex B is present in very low amounts, which can be explained by the shorter incubation time

as compared to that in Figure 1A. When SF1 or SF3 is omitted from the reaction, formation of complexes A and B is abolished (lanes 3 and 5). In the absence of SF2 or SF4, complex A is still made, but complex B is not detectable (lanes 4 and 6). Thus, both SF1 and SF3 are necessary for the generation of complex A, whereas SF2 and SF4 do not appear to participate in this step. Because the formation of complex B is very inefficient in the complete system under the experimental conditions used, it is difficult to assess whether SF2 and SF4 are required for the conversion of complex A to complex B or whether they act at a later step in the reaction.

When pre-mRNA is incubated in either BR-SF1 or Q-SF3 alone (Fig. 2), structures are formed that resemble complex H made in a nuclear extract, indicating that both fractions contain RNA-binding components. The degree to which the RNA is retarded in the native gel depends on the concentration of the fractions in the reaction. Complex A is assembled when BR-SF1 and Q-SF3 are combined. Therefore, these two fractions contain all the components necessary to sequester the pre-mRNA into complex A. In addition, the results obtained by titrating the fractions indicate that SF1 and SF3 represent separate activities.

Figure 2. Titration of SF1 and SF3. Complex reactions (20 μ l) were performed in the presence of increasing amounts of SF1 (Bio-Rex 500) and SF3 (Mono Q), as indicated at top. Samples of 10 μ l were analyzed in a 4% polyacrylamide gel.

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The kinetics of complex formation in fractions and in nuclear extract are compared in Figure 3. The pre-mRNA is converted to complex H immediately upon addition of nuclear extract. Complex A is apparent after 5 min at 30°C, followed by the formation of complex B after about 20 min of incubation. Components present in BR-SF1 and Q-SF3 bind to the pre-mRNA immediately, and structures similar to complex H are generated (Fig. 3). When pre-mRNA is incubated with both BR-SF1 and Q-SF3, complex A assembles within 5 min of incubation. This complex is not converted to complex B in the presence of the fractions during the reaction time or upon prolonged incubation (not shown). Thus, in the presence of BR-SF1 and Q-SF3, complexes H and A form with similar kinetics as in nuclear extract. Formation of complex H does not require ATP or any of the intron consensus sequences, whereas assembly of complex A needs ATP, elevated temperature, and sequences at the 3' splice site but not at the 5' splice site (not shown; Friendewey and Keller 1985; Grabowski et al. 1985; Konarska and Sharp 1986).

A minimum of two proteins and one snRNP are required for complex A assembly

U2 snRNP binds to the branch point within the intron and is involved in complex A formation (Black et al.

1985; Ruskin and Green 1985b; Konarska and Sharp 1986; Chabot and Steitz 1987; Friendewey et al. 1987; Ruskin et al. 1988). In addition, U1 snRNP binds to the 5' splice site very early during in vitro splicing (Mount et al. 1983; Black et al. 1985; Bindereif and Green 1987; Zillmann et al. 1987; 1988). Because it is not clearly established how U1 snRNP contributes to complex A assembly, i.e., to the binding of components to the 3' splice site (see introductory section), only the role of U2 snRNP in this process will be considered here.

Previous experiments have shown that the activities SF1 and SF3 are resistant to digestion by micrococcal nuclease, suggesting that they are proteins (Krämer et al. 1987). The Bio-Rex 70 fraction used as a source of SF1 still contains small amounts of all of the major snRNPs, whereas Q-SF3 is free of these particles (not shown; Krämer et al. 1987). To rule out the possibility that the complex-forming activity present in BR-SF1 merely represents U2 snRNP and not a protein factor, a mixing experiment was carried out. As shown in Figure 4, components present in a fraction enriched in U2 snRNP (Q-U2) do not associate detectably with the pre-mRNA (lane 4). Upon combination of Q-U2 with BR-SF1 or Q-SF3 (lanes 5 and 6), complex H but not complex A is formed, indicating that Q-U2 cannot substitute for either of these fractions. Complex A is assembled only in reactions that contain both BR-SF1 and Q-SF3 (lanes 7 and 8), demon-

Figure 3. Time course of complex formation. Complete reactions (10 μ l) were incubated for the indicated lengths of time in the presence of 3 μ l nuclear extract, 2.5 μ l SF1 (Bio-Rex 500), 2.5 μ l SF3 (Mono Q), or 2.5 μ l SF1 plus 2.5 μ l SF3, as indicated at top. Analysis of splicing complexes was in a 4% polyacrylamide gel.

Figure 4. Complex formation in the presence of different combinations of SF1, SF3, and U2 snRNP and after micrococcal nuclease treatment of these fractions. Complex reactions (20 μ l) contained 4 μ l SF1 (Bio-Rex 70), 4 μ l SF3 (Mono Q), and 2 μ l U2 snRNP (Mono Q), as indicated (+) at top. (Lanes 9–21) Fractions (×) were treated with micrococcal nuclease prior to the addition of the remaining constituents of the reaction. Samples of 10 μ l were analyzed in 4% polyacrylamide gels.

strating that SF1 and SF3 represent factors unrelated to U2 snRNP.

To investigate the role of snRNPs in complex A formation, fractions were treated with micrococcal nuclease, which will degrade the RNA component of snRNPs (Krainer and Maniatis 1985). Combinations of untreated and micrococcal nuclease-treated fractions (BR-SF1, Q-SF3, and Q-U2) were tested for their ability to assemble the pre-mRNA into complex A (Fig. 4). Nuclease treatment of the three fractions does not alter binding to the pre-mRNA significantly when the fractions are tested individually (lanes 9–11). Thus, formation of complex H (or related structures) is caused by an interaction between pre-mRNA and proteins. When micrococcal nuclease-treated BR-SF1 is incubated with untreated Q-SF3, complex A is not detected (lane 12), indicating that a nuclease-sensitive component necessary for assembly of complex A is present in BR-SF1. Upon addition of Q-U2 to this combination, the ability to form complex A is restored (lane 13). This effect is abolished when Q-U2 is treated with nuclease as well (lane 14).

These results demonstrate that BR-SF1 provides at least two factors that participate in complex formation: a nuclease-resistant activity, SF1, and a component that is dependent on an RNA moiety, most likely U2 snRNP.

Nuclease treatment of Q-SF3 has no effect on complex assembly, confirming the previous observation that this activity is resistant to micrococcal nuclease (Krämer et al. 1987). Reactions performed with nuclease-treated BR-SF1 and Q-SF3 (lanes 18–20) yield complexes similar to those obtained when only BR-SF1 is preincubated with nuclease. In summary, these results demonstrate that at least two protein factors, SF1 and SF3, are required to assemble the pre-mRNA into complex A in addition to a micrococcal nuclease-sensitive component, U2 snRNP. The results show clearly that the presence of either SF1 or SF3 and U2 snRNP is not sufficient to assemble specific complexes that can be distinguished from complex H.

The factors interact with the pre-mRNA at the 3' end of the intron

A convenient method to map the interactions between RNA and RNA-binding components is protection of the bound RNA from ribonuclease digestion (Black et al. 1985; Chabot et al. 1985; Ruskin and Green 1985b; Bindereif and Green 1986; Konarska and Sharp 1986; Chabot and Steitz 1987; Krämer 1987). To locate the site(s) of interaction between pre-mRNA and the factors described above, complexes were allowed to assemble, RNase T1 was added, and reaction products were analyzed in a native polyacrylamide gel (Fig. 5A). Of the two complexes (A and B) assembled in the nuclear extract, only complex A is resistant to digestion with RNase T1, whereas complex B is destroyed by the nuclease, consistent with previous results (Konarska and Sharp 1986). The RNase T1-resistant complex migrates slightly faster in the gel than untreated complex A, probably as a result of the reduced length of the protected RNA. Complex A formed in the presence of BR-SF1 and Q-SF3 is also resistant to digestion by RNase T1 and shows a comparable increase in mobility after RNase treatment. In contrast, RNA assembled into complex H is digested completely.

BR-SF1 contains both SF1 activity and snRNPs, whereas Q-SF3 contains only SF3 activity. No RNase T1-resistant complexes were observed when micrococcal nuclease-treated SF1 was tested in the absence or in the presence of SF3 and when Q-U2 was added to SF3 (A. Krämer unpubl.). Thus, the initial interactions (complex H) between pre-mRNA and fractionated activities appear to be disrupted upon RNase T1 digestion. However, if these interactions occur at random sites, they could not necessarily be monitored in a protection experiment. Once complex A is formed (either in nuclear extract or with the fractionated activities), the pre-mRNA has been assembled into a structure that is resistant to RNase T1 digestion.

To map the RNase T1-resistant region within the pre-mRNA, the RNA protected in the presence of SF1 and

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described in Methods. The resulting oligonucleotides were separated in a 20% polyacrylamide/8.3 M urea gel (lane 2). A complete RNase T1 digest of full-length RNA 10 was used for comparison (lane 1). (C) The nucleotide sequence of RNA 10 is displayed according to the fragments generated by digestion with RNase T1. The numbers below the sequence refer to the length of the individual digestion products. The protected RNA sequence is indicated by a broken line.

SF3 was isolated and digested to completion with RNase T1. The resulting oligonucleotides were separated in a denaturing gel and compared with a complete RNase T1 digest of full-length pre-mRNA. [For this experiment, RNA 10 was used, which lacks the nucleotide sequences of the 5' splice site (see Methods). The same result was obtained with RNA 1 (not shown).] As shown in Figure 5, B and C, the protected area in complex A covers a region of 46 nucleotides, including the polypyrimidine stretch (fragments 16–18), the branch point (fragment

Figure 5. Localization of interactions between pre-mRNA and fractionated components by RNase T1 protection. (A) Complex reactions (10 μ l) were performed in the presence of 3 μ l nuclear extract; 2.5 μ l SF1 (Bio-Rex 500), 2.5 μ l SF3 (Mono Q); or 2.5 μ l SF1 plus 2.5 μ l SF3 for 30 min at 30°C (top). To each reaction, 2 μ l of RNase T1 (units indicated at top) were added, and incubation was continued at room temperature for 10 min. After addition of heparin and further incubation at room temperature, the samples were analyzed in a 4% polyacrylamide gel. (B) RNA was recovered from the RNase T1-resistant complex assembled in the presence of SF1 and SF3, and digested to completion with RNase T1, as described in Methods. The resulting oligonucleotides were separated in a 20% polyacrylamide/8.3 M urea gel (lane 2). A complete RNase T1 digest of full-length RNA 10 was used for comparison (lane 1). (C) The nucleotide sequence of RNA 10 is displayed according to the fragments generated by digestion with RNase T1. The numbers below the sequence refer to the length of the individual digestion products. The protected RNA sequence is indicated by a broken line.

14), and another 22 nucleotides upstream of the branch point fragment. Two characteristic fragments derived from the second exon (9 and 12 nucleotides long) are absent from the protected RNA. The extent of the protected region agrees well with the length of the RNA present in the RNase T1-resistant complex, which was estimated to be ~50 nucleotides (not shown). These data show that the components present in BR-SF1 and Q-SF3 are sufficient to assemble the pre-mRNA into an RNase T1-resistant complex A. The components present in

these fractions protect the 3' part of the intron to the same extent as components present in a nuclear extract (Konarska and Sharp 1986; A. Krämer unpubl.).

SF3 is required for complex assembly and splicing

It has been shown previously that SF3 can be purified further by Mono S chromatography (Krämer et al. 1987). To correlate the activity that is essential for the formation of complex A with the activity involved in the overall splicing reaction, Q-SF3 was fractionated on a Mono S column. The resulting fractions were assayed for the generation of splicing products in combination with fractions containing SF1, SF2, SF4, and snRNPs and for complex formation in the presence and in the absence of BR-SF1. Figure 6A shows that splicing takes place when Mono S fractions 34–40 (lanes 10–13) are added to the remaining constituents of the complete reaction. Splicing activity elutes from the column at ~200 mM KCl (not shown), consistent with previous results (Krämer et al. 1987). Formation of complex A is apparent in the presence of BR-SF1 and Mono S fractions 32–40 (Fig. 6B) and thus mostly overlapping with the splicing activity found in the complete reconstituted system. Components in fractions 32–40 bind to the pre-mRNA in the absence of BR-SF1, resulting in a smear of radioactivity above the pre-mRNA in the native gel (Fig. 6C, lanes 6–10). Further purification is needed to determine whether or not the component(s) that is responsible for this reduction in electrophoretic mobility represents the activity involved in the formation of complex A. In summary, these results demonstrate that one of the components (SF3) involved in the assembly of complex A co-fractionates with an activity required for the overall splicing reaction. It can therefore be concluded that SF3 functions in pre-mRNA splicing at the level of complex A formation. A comparable analysis of the activity present in BR-SF1 has not been successful so far due to the lability of SF1 activity during chromatographic fractionation.

Two distinct structures that differ from complexes described previously in their migration in a native gel appear in the presence of Mono S fractions 28 and 30 (Fig. 6C, lanes 4 and 5) and fractions 38–44 (lanes 9–12). There is no evidence that the formation of these structures has any relevance to pre-mRNA splicing (A. Krämer unpubl.). Nevertheless, these results show that complex H (as formed in a nuclear extract) can be separated into multiple structures upon fractionation of RNA-binding components.

Comparison of SF1 and SF3 to other 3' splice site binding proteins

In addition to U2 snRNP, two protein factors have been described that interact specifically with a pre-mRNA substrate. The IBP recognizes the polypyrimidine stretch upstream of the 3' splice site (Gerke and Steitz 1986; Tazi et al. 1986). U2AF also binds to sequences at the 3' end of the intron, thereby allowing U2 snRNP to in-

teract with the branch point (Ruskin et al. 1988). According to these investigators, U2AF is present only in the nuclear extract, whereas IBP is present both in the nuclear extract and in the postnuclear supernatant (S100). As shown previously, two of the activities essential for splicing, SF1 and SF2, partially leak into the S100 during the preparation of nuclei (Krämer and Keller 1985). From these observations, it appeared that the S100 could substitute for SF1 in complex formation. As shown in Figure 7, only complex H is formed when pre-mRNA is incubated with S100 (lane 2). In addition, a combination of S100 and BR-SF1 does not support assembly of complex A (lane 3). However, this complex is generated when S100 and Q-SF3 are combined (lane 4). This result confirms the previous observation that SF1 (but not SF3) is present in S100. Thus, like IBP, SF1 fractionates into nuclear extract and S100, whereas SF3 can only be detected in nuclear extract and therefore parallels the distribution of U2AF. Although these observations are suggestive, more experiments are required to directly correlate these activities.

Discussion

To elucidate the function of partially purified splicing factors, their involvement in the early events of the splicing reaction was investigated. Two of six fractions required for the production of spliced RNA in vitro (Krämer et al. 1987) are sufficient to assemble the pre-mRNA substrate into a presplicing complex (complex A). These fractions (BR-SF1 and Q-SF3) contain at least two chromatographically distinct proteins. U2 snRNP, which is thought to interact with the branch point during the assembly of complex A (Grabowski and Sharp 1986; Konarska and Sharp 1986; Bindereif and Green 1987; Chabot and Steitz 1987; Frendewey et al. 1987; Krämer 1987; Zillmann et al. 1988), is present in BR-SF1. It can also be supplied by a purified snRNP fraction, which contains U2 snRNP and traces of U1, U4, U5, and U6 snRNPs. In the presence of SF1, SF3, and U2 snRNP, complex A is assembled with characteristics identical to those observed in reactions containing nuclear extract: Complex formation is ATP and temperature dependent, it occurs within 2–5 min of incubation at 30°C, and it requires nucleotide sequences at the 3' end of the intron but not a 5' splice site (Frendewey and Keller 1985; Grabowski et al. 1985; Konarska and Sharp 1986).

Factors in two additional fractions, SF2 and SF4, that are required for the generation of spliced RNA in vitro (Krämer et al. 1987) do not appear to contribute to complex A formation. A contamination of SF1 and SF3 with these components is unlikely, because splicing products have never been observed in the absence of SF2 or SF4 (Krämer et al. 1987). Also, addition of SF2 and/or SF4 to a reaction containing SF1 and SF3 does not improve complex A formation (results not shown). If these factors were essential for complex A assembly, they would have to act in very low concentrations, which are insufficient to support the complete splicing reaction.

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Figure 6. In vitro splicing and complex formation in the presence of Mono S-SF3 fractions. **(A)** Splicing reactions (50 μ l) contained 15 μ l of nuclear extract (NXT), or 5 μ l SF1 (DEAE-Sephacrose 100), 5 μ l SF2 (heparin-Sephacrose 150), 6.5 μ l SF3 (Mono Q or Mono S, as indicated), 6.5 μ l SF4 (Mono S), and 1 μ l each of U1 and U2 snRNPs (Mono Q). Only buffer was added to the control reaction (co). Reaction products were analyzed in a 12% denaturing polyacrylamide gel. (Lane M) *Hpa*II-digested DNA as a marker. The sizes of the fragments are shown on the left; the splicing products are explained on the right: (IVS-E2) Intron-exon 2 lariat; (IVS) intron lariat; (E1-E2) spliced product; (E1) cleaved exon 1. **(B)** Complex reactions (10 μ l) were performed in the presence of 1.5 μ l SF1 (Bio-Rex 500) and 1.5 μ l of the fractions indicated at top. **(C)** Complex reactions (10 μ l) were performed in the presence of 1.5 μ l of the fractions indicated at top. The broken brackets on the right indicate the positions of complexes A and H from B. Reaction products were analyzed in 4% polyacrylamide gels. **(D)** Elution profile of proteins from the Mono S column.

Figure 7. Substitution of a S100 for SF1/SF3. Complex reactions (10 μ l) were performed in the presence of the following components: 3 μ l nuclear extract (lane 1); 2 μ l S100 (lane 2); 2 μ l S100 plus 1.5 μ l SF1 (Bio-Rex 500) (lane 3); 2 μ l S100 plus 1.5 μ l SF3 (Mono Q) (lane 4). Reaction products were analyzed in a 4% polyacrylamide gel.

Therefore, these factors probably act subsequent to the assembly of complex A.

Consistent with the requirement for a 3' splice site in complex A formation (Frendewey and Keller 1985; Konarska and Sharp 1986), 46 nucleotides at the 3' end of the intron are protected from RNase T1 digestion within complex A (assembled in the presence of SF1 and SF3). The protected fragment encompasses the AG at the 3' splice site, the polypyrimidine stretch, the branch point, and 22 additional nucleotides upstream of the branch point. The same region of the pre-mRNA becomes resistant to RNase T1 digestion when complex A is generated in a nuclear extract (Konarska and Sharp 1986; A. Krämer unpubl.). According to these criteria, BR-SF1 and Q-SF3 contain all the components that are required for the generation of complex A as it is formed in a nuclear extract.

Due to the absence of G residues within the last 18 nucleotides upstream of the 3' splice site, the 3' border of the protected region cannot be determined accurately. Therefore, it remains unclear whether or not the 3' AG

is part of the binding site. This dinucleotide is required for efficient complex formation, because only a trace amount of complex A is assembled when a pre-mRNA missing the 3' AG is incubated in the presence of the fractionated components (not shown). Likewise, incubation of a pre-mRNA lacking the 3' AG in a nuclear extract leads to the generation of a small amount of mature spliceosomes and splicing intermediates (Frendewey and Keller 1985; Reed and Maniatis 1985; Ruskin and Green 1985a). In addition, the absence of the 3' AG from a pre-mRNA influences the binding of two proteins (IBP and U2AF) that interact specifically with the 3' end of the intron (Gerke and Steitz 1986; Ruskin et al. 1988). It also remains to be established whether the entire polypyrimidine stretch interacts with binding components. This sequence is absolutely required for complex formation, both in the presence of fractionated factors and in the presence of nuclear extract (results not shown; Frendewey and Keller 1985), and it apparently also represents the binding site for IBP and U2AF (Gerke and Steitz 1986; Tazi et al. 1986; Ruskin et al. 1988).

When pre-mRNA is incubated in BR-SF1 or Q-SF3 alone, it is assembled into a structure that resembles complex H formed in the nuclear extract. The interactions leading to the formation of this complex are sensitive to digestion with RNase T1. This result is in contrast to previous reports that investigated the binding of nuclear components to a pre-mRNA in the absence of ATP and at 0°C (Black et al. 1985; Chabot et al. 1985; Gerke and Steitz 1986; Chabot and Steitz 1987). In these studies, RNase T1 fragments containing the polypyrimidine stretch could be precipitated with anti-Sm antibodies indicating that components carrying anti-Sm determinants interact specifically with the pre-mRNA at the 3' end of the intron. One explanation for the failure to detect such interactions in the experiments presented here could be the low percentage of RNA (1–5%) that is bound by anti-Sm antigens (Black et al. 1985). Another reason could be the experimental conditions used. These reactions were carried out in the presence of polyvinyl alcohol and have to be treated with heparin prior to gel electrophoresis to avoid retention of complexed RNA at the origin of the gel. Heparin may abolish weak interactions of a component with an RNase T1 fragment(s), whereas binding of the same component to an intact pre-mRNA may be stable enough to resist heparin treatment.

In a preliminary experiment (not shown), RNase T1-treated complexes were resolved in a native gel without using heparin, and RNase T1-resistant structures were observed that migrated faster than complex A. Several such complexes formed in the presence of SF3 were found to contain sequences of the polypyrimidine stretch and fragments characteristic for exon 2. The branch point fragment was either not detected or it was present in less than stoichiometric amounts. Nuclease-resistant complexes formed in the presence of SF1 were heterogeneous and not observed consistently, which has precluded further investigation. Thus, specific interactions between SF3 and pre-mRNA apparently occur;

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however, they are obscured by other nonspecific interactions in complex H. These interactions are abolished when heparin is added after RNase T1 digestion of preformed complexes. Only interactions occurring in complex A are strong enough to withstand both RNase T1 digestion and incubation with heparin. This result suggests that interactions of single factors with the pre-mRNA are stabilized upon addition of the remaining components or that these interactions are altered when all factors are present at the same time.

During all steps of the fractionation of SF3 (chromatography on DEAE-Sephrose, heparin-Sephrose, Mono Q, and Mono S; results only shown for Mono S fractions), splicing activity and the activity required for complex A formation coelute. This result shows clearly that SF3 is involved in pre-mRNA splicing by participating in the assembly of complex A. In addition, SF3 activity coelutes with an RNA-binding component(s), which becomes apparent when reactions are performed with Mono S fractions only. This binding, however, shows no RNA substrate specificity (not shown). Although further purification is required to correlate SF3 activity unambiguously with the observed binding, it would be intriguing if SF3 could bind to an RNA substrate in the absence of other components required for complex A assembly. It might well be that SF3 by itself recognizes sequences related to its presumed binding site in a variety of RNA substrates but that efficient binding and/or correct positioning only occurs in the presence of the other factors.

Several proteins have been implicated in early events of the splicing reaction. The hnRNP C proteins bind to nascent RNA chains *in vivo* (for review, see Dreyfuss 1986) and also participate in splicing complex assembly *in vitro* (Choi et al. 1986). This class of proteins cannot be detected in SF3-containing fractions by immunoblotting, but it is present in the Bio-Rex 500 fraction used as a source of SF1 and in even higher quantities in BR-1000. Because BR-1000 cannot substitute for SF1 activity in complex formation (A. Krämer unpubl.), SF1 appears to be distinct from the hnRNP C proteins. However, it cannot be ruled out that these proteins are required for complex A formation in addition to SF1 and SF3.

Recently, two proteins have been shown to interact specifically with pre-mRNA substrates at the 3' end of the intron (Gerke and Steitz 1986; Tazi et al. 1986; Ruskin et al. 1988). As these sequences are a prerequisite for the formation of complex A in a splicing reaction (Frendewey and Keller 1985; Bindereif and Green 1986; Konarska and Sharp 1986) and because a minimum of two protein factors are involved in the assembly of this complex (results presented here), it is conceivable that SF1 and SF3 are related to these proteins. The IBP (Gerke and Steitz 1986; Tazi et al. 1986) interacts with the polypyrimidine stretch in β -globin and adenovirus major late pre-mRNAs (in the absence of ATP at 0°C), and the binding appears to be influenced by the 3' AG of the intron. IBP can be found associated with U5 snRNP (Tazi et al. 1986), and according to Gerke and Steitz (1986), it represents a 70-kD protein that carries an anti-Sm deter-

minant. In both SF1 and SF3 fractions, several polypeptides of $M_r \sim 70,000$ are detected that bind the monoclonal anti-Sm antibody Y12 used by these workers. SF3 coelutes from Mono S with three Y12 polypeptides; however, the activity is not retained by a Y12 affinity column under conditions where all Sm antigens are bound (A. Krämer unpubl.). Thus, it is unlikely that SF3 represents IBP. A relatively high lability of SF1 during chromatographic fractionation and antibody-depletion experiments has precluded further analysis of its antigenic properties so far.

Ruskin et al. (1988) have reported that IBP distributes between nuclear extract and S100, a characteristic that also applies to SF1 but not to SF3 (Krämer and Keller 1985; this paper). Although these observations suggest that IBP may correspond to SF1, the chromatographic properties of this protein cannot be reconciled with those observed for SF1 or SF3. Gerke and Steitz (1986) reported that IBP binds to DEAE-Sephrose in buffer containing 1 mM $MgCl_2$, but it is not retained by the column when fractionation is performed in buffer containing 15 mM $MgCl_2$. In contrast, SF1 fractionates into the DEAE-Sephrose flowthrough in the presence of 1 or 3 mM $MgCl_2$, and SF3 binds to the resin at 1, 3, and 25 mM $MgCl_2$ (Krämer et al. 1987; A. Krämer unpubl.). These observations taken together clearly distinguish SF3 from IBP. Although it is tempting to correlate SF1 with IBP, more information is required for a comparison of these two components.

A second protein that binds to the polypyrimidine stretch is U2AF, which is required for the U2 snRNP/branch point interaction (Ruskin et al. 1988). This protein binds to DEAE-Sephrose and elutes from Mono Q at about 300 mM KCl. SF3 exhibits a similar elution behavior in that it binds to both resins and is desorbed from Mono Q at 230–300 mM KCl. The chromatographic properties and the absence of SF3 from the S100 suggest that this factor represents U2AF. However, there is no evidence that SF3 and U2 snRNP interact with the branch point in the absence of SF1, as was shown for U2AF and U2 snRNP (Ruskin et al. 1988). An explanation for this discrepancy is that the methods used here might not allow detection of such an interaction; on the other hand, the results of Ruskin et al. (1988) could be explained if SF1 were present in either their U2 snRNP or U2AF fractions.

The characterization of SF1 and SF3 as proteins acting early during the splicing reaction allows them to be compared to splicing factors fractionated by other laboratories. Two of the factors described by Krainer and Maniatis (1985) are required for the production of splicing intermediates. One factor, designated SF4B, is not retained by spermine agarose, binds to CM-Sephrose, and is present in nuclear extract as well as in S100. A second protein (SF2), which binds to spermine agarose, is found only in nuclear extract. Therefore, SF1 might correspond to factor SF4B of Krainer and Maniatis (1985), whereas SF3 could be their SF2. Perkins et al. (1986) have separated splicing activities into three fractions. SF3 is probably one of the factors present in their

fraction II, which binds to DEAE-cellulose. The chromatographic properties of SF1 correlate best with fraction Ib of Perkins et al. (1986), which contains a component(s) required for spliceosome assembly.

In conclusion, the results presented here indicate that a minimum of two protein factors are necessary to assemble a presplicing complex in the presence of snRNPs. These proteins are present in fractions that support *in vitro* splicing, and one of the complex-forming activities, SF3, coelutes with a splicing factor. Both proteins are necessary for a productive association of U2 snRNP with the branch point region. The details of the interactions with the 3' end of the intron remain to be elucidated. Further experiments are also required to investigate the role of U1 snRNP in complex A formation. This question could not be resolved in this paper, due to a contamination of the U2 snRNP-enriched fractions with trace amounts of U1 snRNP. Preliminary data show that U1 snRNP is essential in this reaction in the presence of a 5' splice site; however, it is dispensable for complex A formation when an RNA lacking a 5' splice site is used as a substrate (A. Krämer unpubl.), suggesting that a stable interaction with the 3'-splice site can occur independently of the presence of U1 snRNP.

Having established the minimal factor requirements for presplicing complex formation, it should now be possible to assign functions to the remaining splicing factors, SF2 and SF4, in the subsequent steps of the splicing reaction. Supplementing preformed A complexes assembled with SF1, SF3, and snRNPs with additional fractions should also resolve the question of whether or not the presplicing complex is a true intermediate in spliceosome formation (see Maniatis and Reed 1987).

Methods

Buffers

Buffer A contains 10% (vol/vol) glycerol, 20 mM HEPES-KOH (pH 7.9), 3 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Buffer B is the same as buffer A but contains 50 mM Tris-HCl (pH 7.9). Buffers C and D are the same as buffers A and B, respectively, but contain 20% (vol/vol) glycerol.

Fractionation of splicing activities

Splicing activities have been partially purified from HeLa cell nuclear extracts (Dignam et al. 1983), as described (Krämer et al. 1987). SF1 activity was purified further from the DEAE-Sephacrose flowthrough by chromatography on Bio-Rex 70 (Bio-Rad). DEAE-flowthrough in buffer C + 50 mM KCl was loaded onto Bio-Rex 70 equilibrated with buffer A + 50 mM KCl. The column was washed with buffer A + 50 mM KCl, and bound proteins were step-eluted with buffer A + 200, 500, and 1000 mM KCl, respectively. The peak protein fractions of each step were pooled and dialyzed against buffer C + 50 mM KCl. To generate mRNA in an *in vitro* splicing reaction, both the 500 and 1000 mM KCl step fractions (designated BR-500 or BR-SF1 and BR-1000, respectively) had to be combined with SF2, SF3, SF4, U1, and U2 snRNP fractions. Only BR-500 was found to be

active in complex A formation (A. Krämer unpubl.). The component present in BR-1000 has not been characterized further. The activity present in the BR-500 fraction is referred to as SF1.

SF2 was fractionated on DEAE-Sephacrose and heparin-Sephacrose as described (Krämer et al. 1987). The heparin-Sephacrose flowthrough fraction was precipitated with ammonium sulfate (Krämer and Keller 1985), dialyzed, and chromatographed on Bio-Rex 70 as described above, except that the buffer contained Tris-HCl (pH 7.9). SF2 activity was found in the 50 mM KCl flowthrough fraction (A. Krämer unpubl.).

The fractionation procedure for SF3 and SF4 has been described (Krämer et al. 1987). The SF3-containing fractions used in most of the experiments presented are designated Q-SF3 (for Mono Q fractions).

All of the active fractions contain numerous proteins, as revealed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie blue. No protein bands could be detected that might correspond to any of the splicing activities.

U1 and U2 snRNPs were purified from Mono Q fractions (Krämer et al. 1987) by chromatography on Blue Sepharose (Pharmacia). Mono Q fractions enriched in either U1 or U2 snRNPs were individually loaded onto Blue Sepharose at 100 mM KCl in buffer D. Bound material was removed by step elution with 500 and 1000 mM KCl in buffer B. U1 snRNP distributed into both step fractions. The 1000 mM KCl step fraction contained mainly U1 and a small amount of U5 snRNP. U2 snRNP was quantitatively recovered in the 1000 mM KCl step fraction in addition to small amounts of U1, U4, U5, and U6 snRNPs. As judged by SDS-polyacrylamide gel electrophoresis, the preparation was >70% pure. The Mono Q-fraction enriched in U2 snRNP used in most experiments shown is referred to as Q-U2.

Preparation of [α -³²P]UTP-labeled pre-mRNA

Pre-mRNA derived from the adenovirus 2 major late transcription unit was synthesized *in vitro* with SP6 RNA polymerase (Boehringer-Mannheim) as described (Krämer 1987). RNA 1 was transcribed from plasmid pSP62Δ11 (Frederick and Keller 1985) and contains 102 nucleotides of exon 1, 113 nucleotides of intron, and 38 nucleotides of exon 2. RNA 10 was transcribed from plasmid Δ5' (Frederick and Keller 1985), which lacks a 5' splice site. It contains 91 nucleotides of pSP64 sequences plus sequences from the 3' part of the intron and 38 nucleotides of second exon.

In vitro complex formation

Reactions contained 0.4 mM ATP, 10 mM creatine phosphate, 2–2.5 mM MgCl₂, 10% (vol/vol) glycerol, 10 mM HEPES-KOH (pH 7.9) [or 25 mM Tris-HCl (pH 7.9), depending on the fractions tested], 25–50 mM KCl, 0.05 mM EDTA, 0.25 mM dithiothreitol, and 2.6% (vol/vol) polyvinyl alcohol (*M_w* 10,000; Sigma). Uniformly labeled pre-mRNA was used at 50,000 Cerenkov cpm/reaction (1.0–1.5 mM). The total volume of the reactions and the amount of extract or column fractions included are indicated in the figure legends. Unless stated otherwise, reactions were performed for 30 min at 30°C. Complex formation was terminated by addition of heparin (Sigma) to a final concentration of 200 μg/ml and incubation at room temperature for 10 min. Heparin treatment is necessary when reactions are carried out in the presence of polyvinyl alcohol, otherwise complexed RNA does not enter the native gel independent of the gel system used (e.g., Konarska and Sharp 1987).

Micrococcal nuclease digestion was performed as described (Krämer et al. 1987).

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Electrophoretic separation of splicing complexes

For the analysis of splicing complexes, reactions were loaded onto 4% polyacrylamide gels [acrylamide/bis-acrylamide = 80 : 1] in 25 mM Tris, 25 mM boric acid, and 1 mM EDTA, which had been preelectrophoresed at 300 volts for 30–60 min. Gel electrophoresis was performed at 20 mA at room temperature. Composite gels [4% polyacrylamide [acrylamide/bis-acrylamide = 250 : 1]/0.5% agarose] were used in some experiments. The buffer system and electrophoresis conditions were the same as above.

In vitro splicing

Splicing reactions were performed for 2 hr as described [Krämer et al. 1987], except that the reactions contained 2.6% polyvinyl alcohol [Sigma].

RNase T1 mapping

To map the site of complex formation within the pre-mRNA substrate, complexes were assembled in a total volume of 100 μ l in the presence of 25 μ l SF1 (DEAE–Sephrose 100), 25 μ l SF3 (Mono Q), and 1×10^6 cpm of RNA 10 (0.3 pmole) for 30 min at 30°C. RNase T1 (Calbiochem) was added to a final concentration of 20 U/ml, and the reactions were incubated for 10 min at room temperature. After addition of heparin to 200 μ g/ml, the reaction was loaded into a 5-cm-wide pocket of a 4% composite gel (see above).

The RNase T1-resistant complex was excised from the gel with the aid of the autoradiograph and incubated in 2.5 ml $1 \times$ TBE [Maniatis et al. 1982], 0.1% SDS, and 0.5 mg/ml proteinase K (Boehringer-Mannheim) for 15 min at 37°C. The gel slice was rinsed with $1 \times$ TBE twice and loaded onto a 20% polyacrylamide/8.3 M urea gel. The radioactive RNA was excised from the gel again and eluted into 800 μ l of 0.75 M ammonium acetate, 0.1% SDS, 0.1 mM EDTA, and 10 μ g tRNA at 37°C for 5 hr [Frederick and Keller 1985]. The RNA was phenol/chloroform-extracted and ethanol-precipitated.

Complete RNase T1 digestion of the RNA was performed in the presence of 200 U/ml RNase T1 for 30 min at 30°C. The resulting oligonucleotides were separated in a 20% polyacrylamide/8.3 M urea gel.

Acknowledgments

I would like to thank my colleagues, in particular W. Keller, for suggestions, criticism, and valuable discussions; M. Frick for superb technical assistance; G. Martin for assistance during the last stages of the experiments; S. Mähler for the preparation of the figures; W. Keller, G. Bilbe, G. Christofori, and K. Lang for critical comments on the manuscript; and J. Steitz for the Y12 hybridoma cell line. I am also grateful to many of the colleagues in the RNA processing field for communicating their results prior to publication. This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie, the Kantons of Basel, and the Schweizerischer Nationalfonds.

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Presplicing complex formation requires two proteins and U2 snRNP.

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Genes Dev. 1988, **2**:

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