Spliceosome assembly in yeast

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Precursors to mRNA become substrates for splicing by being assembled into a complex multisubunit structure, the spliceosome. To study the assembly of the yeast spliceosome, intermediate complexes were separated by electrophoresis on nondenaturing polyacrylamide gels. Four splicing-dependent complexes, A1, A2-1, A2-2, and B, were observed. The order of assembly of these complexes was determined to be B → A2-1 → A1 → A2-2. The assembly process can be blocked at complex A1 by addition of 5 mM EDTA or by carrying out the assembly process in heat-inactivated *rna2* extracts. The snRNA composition of the complexes was determined by hybridization with probes for five yeast snRNAs. snR14 (U4) was only found in complex A2-1, snR6 (U6) and snR7 (U5) were in complexes A1, A2-1, and A2-2, whereas snR20 (U2) was in all four of the complexes. snR19 (U1) was not present in any of the complexes. Hybridization with these probes was also employed to detect snRNPs present in yeast splicing extracts. We found that snR6, snR7, and snR14 were present together in a large complex. This complex underwent an ATP-dependent dissociation to give snR7 and snR6-snR14 complexes. snR19 and snR20 are present in distinct RNPs but the mobility of these is not affected by ATP. A mechanism for spliceosome assembly is proposed.

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mRNA precursors that contain introns are processed in a large particle called the spliceosome (Brody and Abelson 1985; Frendewey and Keller 1985; Grabowski et al. 1985). In the splicing reaction, the 5' and 3' splice junctions must be recognized and brought into proximity. Since the precursors themselves do not contain the requisite secondary and tertiary structure elements that could provide for the formation of the splicing substrate, it must be the process of assembling the spliceosome that folds the precursor RNA into a substrate that can be spliced.

The development of *in vitro* systems that carry out mRNA splicing has facilitated the study of the mechanism of mRNA splicing, the components required for the reaction and the pathway of assembly of the spliceosome (Kole and Weissman 1982; Hernandez and Keller 1983; Padgett et al. 1983a; Hardy et al. 1984; Krainer et al. 1984; Lin et al. 1985). A two-step mechanism for the splicing reaction has been established (Padgett et al. 1984; Ruskin et al. 1984), the first step being cleavage at the 5' splice site and formation of a lariat structure and the second step cleavage at the 3' splice site and ligation of the two exons.

The spliceosome is a multicomponent structure containing the precursor RNA and distinct subunits, the snRNPs. Five snRNAs together with their protein components are now known to be required for mRNA splicing in mammalian systems (Steitz et al. 1987). These are the abundant nuclear RNAs U1, U2, U4, U5, and U6. All of these except U1 have been shown to be components of the spliceosome (Grabowski and Sharp 1986). The U1 snRNP, however, is required for mRNA

splicing. It has been shown to be involved in the recognition of the 5' splice junction (Mount and Steitz 1981; Yang et al. 1981; Padgett et al. 1983b; Bozzoni et al. 1984; Fradin et al. 1984; Kraemer et al. 1984; Black et al. 1985; Krainer and Maniatis 1985; Zhuang and Weiner 1986). The yeast *Saccharomyces cerevisiae* also contains snRNAs (Wise et al. 1983), and recent work has identified the complete set of five snRNAs analogous to those required for splicing in mammalian systems. These are summarized in Table 1. For most, the identity was established by clear homology with portions of the mammalian counterpart. snR7 and snR20 have been shown directly to be required for mRNA splicing *in vivo* (Parker et al. 1987; Patterson and Guthrie 1987). snR20, the U2 analog, was shown by virtue of a compensatory mutation to recognize the conserved branch point sequence UACUAAAC (Parker et al. 1987). Some of the yeast

Table 1. Comparison of mammalian and yeast small nuclear RNAs involved in pre-mRNA splicing

designation	Mammalian		Yeast		References
	size	counterpart	size		
U1	164	snR19	569		Kretzner et al. (1987) Siliciano et al. (1987b)
U2	188	snR20	1175		Ares (1986)
U4	145	snR14	160		Siliciano et al. (1987a)
U5	115	snR7	179		Patterson and Guthrie (1987)
U6	107	snR6	112		Brow and Guthrie (pers. comm.)

snRNAs have been shown to be associated with the spliceosome (Pikielny et al. 1986; Z. Hostomsky et al., unpubl.; N. Riedel et al., pers. comm.), and we shall provide further evidence for such associations in this paper. The collection of yeast snRNAs listed in Table 1 have been further implicated in mRNA splicing because they are precipitated by a human autoimmune antiserum Sm (M. Jones and C. Guthrie, pers. comm.) as are their mammalian counterparts. This antiserum recognizes a protein component which is common to all five U-snRNPs involved in splicing.

The proteins required for mRNA splicing have been less studied, but in yeast a set of well-studied genes, the RNA genes, encode protein components of the mRNA splicing apparatus (Lustig et al. 1986). A yeast *ts* strain mutant in one of these genes, *rna2*, has provided some information about the assembly of the spliceosome. Using the *rna2* mutant extract, we have shown that the spliceosome is a true intermediate in the splicing pathway (Lin et al. 1987). These experiments also demonstrate that extrinsic proteins act on the fully assembled spliceosome. We will further show in this paper that heat-inactivated *rna2* mutant extracts accumulate an identifiable assembly intermediate.

Splicing complexes can be resolved by electrophoresis in nondenaturing polyacrylamide gels (Konarska and Sharp 1986; Pikielny et al. 1986; Konarska and Sharp 1987). In this paper we use that technique to identify four splicing-specific complexes in the assembly of the yeast spliceosome. Using the same gel system, we have discovered that three of the snRNAs found in these complexes, snR6, snR7, and snR14, associate in extracts

to form a large complex. This complex undergoes an ATP-dependent dissociation.

Results

Identification of two splicing complexes, A and B

To study the formation of the spliceosome, we have fractionated splicing reaction mixtures on nondenaturing polyacrylamide gels. The substrates in these experiments were ³²P-labeled SP6 RNA polymerase transcripts of a portion of the yeast actin gene. Substrates containing mutations in the actin intron were also used. These mutations were constructed in a background in which a cryptic TACTAAC sequence had been deleted ($\Delta 6$ actin) (Vijayraghavan et al. 1986). Heparin was added to terminate the splicing reaction and to eliminate non-specific binding of proteins to the RNAs (Konarska and Sharp 1986).

Initially, two complexes were observed during the course of the reaction (Fig. 1A). Complex B appeared after 1 min of incubation followed by complex A. Both of these complexes are specific for splicing because neither formed with an antisense actin transcript or with RNA containing an altered 5' splice site (A1 or C1) or TACTAAC sequence (A256, A257, or C259). With the 3' splice site mutation (ACAC), the first step of splicing can occur but the second step is blocked. This substrate can form spliceosomes as detected by glycerol gradient sedimentation (Vijayraghavan et al. 1986). When this substrate was used, both complexes B and A were observed with a pronounced accumulation of A. Since the

Figure 1. Formation of splicing complexes in the absence of EDTA. (A) Kinetics of complex formation using an actin precursor. ³²P-labeled actin transcripts were synthesized in vitro using SP6 polymerase. Splicing reactions were carried out under standard conditions and assayed at 1, 2, 5, and 10 min. Transcripts with various mutations in the intron consensus sequences were used. (WT) Wild-type actin transcript; ($\Delta 6$) wild-type actin gene with cryptic TACTAAC sequence deleted; (Antisense) $\Delta 6$ actin gene transcribed in reverse orientation; (ACAC) 3' splice site mutant; (A1, C1) 5' splice site mutants; (A256, A257, C259) mutants with single mutation in the TACTAAC sequence (Vijayraghavan et al. 1986). (B) RNA eluted from complexes A and B and analyzed on a denaturing polyacrylamide gel.

ACAC mutation blocks the second step of splicing, complex A probably represents the active form of the spliceosome, and complex B, a kinetic intermediate in its formation.

Wild-type RNA from complexes A and B was eluted from the gel and analyzed by electrophoresis under denaturing conditions (Fig. 1B). Complex B contained predominantly precursor RNA, whereas complex A contained the splicing intermediates, the lariat IVS, as well as the precursor. These are the same products that were found in the 40S complex seen in glycerol gradient sedimentation of the splicing reactions (Brody and Abelson 1985) and in the precipitate of a splicing reaction with the anti-(m³G)cap antibody (Cheng and Abelson 1986). Neither complex contained a significant amount of spliced mRNA. As splicing proceeds, the mature mRNA is released as a 20S particle (S. Goelz and J. Abelson, unpubl.). Consistent with this result, Figure 1A shows that the substrate RNA in complex A decreased after 5 min of incubation but accumulated in reactions with the ACAC RNA where mature mRNA was not made.

Identification of splicing complexes A1, A2-1, and A2-2

Complex A can be subdivided into three complexes, A1, A2-1, and A2-2. This subdivision can be seen when small amounts of EDTA are added to the reaction. The splicing reaction normally contains 3 mM Mg²⁺ as a required factor (Lin et al. 1985). When 1 mM EDTA was added to the reaction, a band migrating more slowly than A was seen (Fig. 2A). This band was called A1 and the original band A was renamed A2. Kinetically, A2 appeared first, immediately followed by A1. The substrate

RNA in A1 and A2 decreased after prolonged incubation in a reaction with wild-type precursor. However, with ACAC RNA as substrate complex A2 accumulated. RNA from complexes A1 and A2 was eluted from the gel, revealing that complex A2 contained only precursor at 2 min, both precursor and splicing intermediates at 5 min, and predominantly intermediates at 30 min (Fig. 2B). Complex A1 contained predominantly precursor at all times. The proximity of the two bands is likely to lead to some cross-contamination. We believe that complex A1 contains only precursor.

Complex A2 is the first species to appear and contains only precursor at early times. Complex A1 is an intermediate, appearing after A2 and disappearing with time. Complex A2 primarily contains intermediates and accumulates late in the reaction with ACAC RNA. We conclude that there are two forms of complex A2. The early form, designated A2-1, contains only precursor and is a transient intermediate. The late form, designated A2-2, is the active splicing complex, and therefore accumulates when the second step of splicing is blocked by the 3' splice site mutation, ACAC. A2 will be used to describe the complexes migrating at that position. The order of assembly of the four complexes appears to be: B → A2-1 → A1 → A2-2.

Accumulation of complex A1 at high concentrations of EDTA and in heat-inactivated rna2 extracts

We have found that EDTA blocks the transition from A1 to A2-2. Figure 3A shows the pattern of splicing complexes with increasing concentrations of EDTA. Each reaction contains 3 mM MgCl₂. The amount of complex

Figure 2. Formation of splicing complexes in the presence of EDTA. (A) Kinetics of complex formation. Splicing reactions were carried out in the absence or presence of 1 mM EDTA using wild-type or ACAC transcript, and assayed at 0, 1, 2, 5, 10, 20, and 30 min. (B) RNA from the ACAC reaction in A: bands B and A2 after 2 min reaction, bands A1 and A2 after 5 min reaction, bands A1 and A2 after 10 min reaction, and bands A1 and A2 after 30 min reaction. The RNA was eluted and analyzed on a denaturing polyacrylamide gel.

Figure 3. Titration of the splicing reaction with EDTA. Standard splicing reactions were carried out in the presence of 0, 0.3, 1, 2, 3, or 5 mM EDTA and assayed at 2 min and 10 min for complex formation (A), and at 10 min for splicing (B). The amount of complex A1 increased and the splicing activity decreased, with increasing EDTA. (IVS-E2) Intron-exon 2; (IVS) the excised intron; (p) precursor RNA; (m) the spliced message; (E1) the excised exon 1.

A1 increases with increasing EDTA concentrations, and at 5 mM EDTA very little complex A2-2 is seen. The transition from A1 to A2-2 must be especially sensitive to EDTA, and assembly is blocked at this step, causing

the accumulation of A1. As the concentration of EDTA is increased, splicing is also inhibited (Fig. 3B).

Complex A1 also accumulates in heat-inactivated *rna2* extracts. Figure 4 shows an experiment in which the splicing extract from an *rna2* strain was inactivated by increasing periods of preincubation at 30°C. Mutant substrate ACAC was used to facilitate observation of complex A2-2. Some splicing activity indicated by the appearance of intermediates is seen after 10 min of preincubation, and the activity is lost after 20 min of preincubation (Fig. 4, left panel). When these extracts were tested for complex formation (Fig. 4, right panel), both A1 and A2 formed in the control but only A1 accumulated in the heat-inactivated extracts. Apparently the transition from A1 to A2-2 requires the RNA2 gene product.

The 40S complex formed in heat-inactivated *rna2* extracts is known to be a functional intermediate in splicing (Lin et al. 1987). This complex contains only precursor, but it can be complemented (i.e., the splicing reaction takes place) by the RNA2 gene product together with other proteins supplied by extract fractions or heat-inactivated *rna5* extracts. (In what follows, we refer to the intermediate complexes containing only precursor as being "functional," and the complex containing the splicing intermediates and products as "active.") Complementation also requires ATP. We have done experiments to show that the complex accumulating at high concentrations of EDTA is also functional. Using the ACAC RNA, complex A1 was allowed to form in the presence of 5 mM EDTA and was isolated by glycerol gradient sedimentation. Figure 5 shows that the com-

Figure 4. Time course of heat inactivation of an *rna2* mutant extract. The *rna2* extract was incubated at 30°C for 0, 10, or 20 min as described in Lustig et al. (1986) and assayed at 15°C for 60 min for splicing activity (left) and complex formation (right) using the ACAC precursor.

Figure 5. Complexes were formed with the ACAC precursor RNA under standard splicing conditions in the presence of 5 mM EDTA using the wild-type extract (5 mM EDTA), in the absence of EDTA using the *rna2* extract (*rna2*), and were isolated from glycerol gradients [8–20% glycerol gradient in a buffer containing 20 mM KPO₄ (pH 7.4), 50 mM KCl] (lanes 1 and 4). Fraction I of the splicing extract was added to complement the complex as described in Lin et al. (1987). Fraction I alone does not splice pre-mRNA (Cheng and Abelson 1986) (lane 7). When Fraction I was added to the complexes isolated from the gradients, intermediates were formed in these complexes in the presence of Mg²⁺ (lanes 2 and 5) but not in the absence of Mg²⁺ (lanes 3 and 6).

plex contained almost exclusively precursor. Upon incubation with an ammonium sulfate fraction of the active extract (40P3, Fraction I, see Fig. 10A below), splicing intermediates formed (Fig. 5, lane 2), indicating that the complex A1 formed under such conditions is a functional intermediate in the assembly pathway. This reaction required Mg²⁺ (cf. lanes 2 and 3). Figure 5 shows that complementation of the 40S complex formed in heat-inactivated *rna2* extracts also requires Mg²⁺. It is likely that the complexes which form at high EDTA concentrations and in heat-inactivated *rna2* extracts are functionally identical. It is not known what changes occur in complex A1 to give the faster-migrating, active A2-2 complex. It is known from previous work (Lin et al. 1987) that at least the *RNA2* gene product and one other

protein factor are required for the formation of the active complex.

The requirement for ATP in the formation of splicing complexes

ATP hydrolysis is essential for mRNA splicing in both yeast and mammals (Hernandez and Keller 1983; Hardy et al. 1984; Lin et al. 1985). It is required in at least two steps of the mRNA splicing reaction—for the formation of the 40S complex (Brody and Abelson 1985) and for reactions that take place after this complex has formed (Lin et al. 1987).

Complex formation in the absence of ATP was examined by gel electrophoresis, as shown in Figure 6. Under these conditions only complexes B and A2 were seen (lanes 5 and 6). Presumably, this is complex A2-1 since no splicing occurred in the absence of ATP (lane 10). These complexes formed slowly and their formation was probably due to small amounts of endogenous ATP in the splicing extract. To show that ATP is absolutely required for complex formation, we formed complexes with partially purified extract fractions likely to be depleted of ATP. Ammonium sulfate fractions of the active extract, prepared as described earlier (Cheng and Abelson 1986; see also Fig. 10A), were used for this purpose. Combining fractions 40P3 and 40W (neither of which is active in splicing) reconstitutes splicing activity (lane 11). In the presence of ATP these fractions can form complex A2-2 after 20 min (lane 7). In the absence of ATP none of these complexes formed (lane 8). Therefore, ATP is absolutely required for at least the first step of the assembly process.

RNA components in the splicing complexes

To understand the mechanism of spliceosome assembly, it is important to identify the components of each complex in the assembly pathway. The small nuclear RNA content of each of the splicing complexes was investigated by electroblotting the complexes from the gel to GeneScreen membrane followed by hybridization with probes for actin and each of the relevant yeast snRNAs. The DNA probes were derived from clones of snR19 (U1), snR20 (U2), snR7 (U5), and snR14 (U4). The probe for snR6 (U6) was a 60-mer oligonucleotide complementary to residues 21–80 of *Drosophila* U6 RNA (Das et al. 1987). It is known that U6 RNA sequences are highly conserved in evolution (D. Brow and C. Guthrie, pers. comm.).

Figure 7 shows the results of this analysis. In this experiment, the splicing reactions were carried out in the presence of ATP for 2, 5, and 20 min and in the absence of ATP for 20 min. All reactions contained 0.5mM EDTA. Tenfold more substrate was used in these experiments to build up detectable levels of each complex. Splicing reactions were carried out in the presence (+S) or absence (–S) of the substrate. The RNAs in the complexes were electroblotted to GeneScreen membrane and subjected to hybridization analysis. The same blot

Figure 6. Formation of splicing complexes in the presence and absence of ATP. In the absence of exogenous ATP, no splicing reaction was seen using the whole-cell extract (WCE) or the reconstituted ammonium sulfate fractions (40P3 + 40W) (lanes 9–12). Smaller amounts of complexes B and A2 (presumably A2-1) formed in the absence of ATP with slower kinetics than when exogenous ATP was added using the whole-cell extract (lanes 1–6). Using the reconstituted ammonium sulfate fractions, which ATP contained less ATP, no complex formed in the absence of ATP (lanes 7 and 8).

was used successively for hybridization with the snRNA and actin probes. Hybridization with the actin probe (Fig. 7, Substrate) revealed the position and the relative amounts of complexes B, A1, and A2. Under these conditions complex B could be seen at all three time points. Complex A1 was most obvious at 5 and 20 min. Complex A2-1 was most easily detected after 2 min of incubation and in the $-$ ATP reaction. A2-2 was the predominant species in the A2 position after 20 min of incubation.

The snR20 (U2) probe hybridized to complexes B, A1,

and A2 in the $+$ S reaction and to very little in these complexes in the $-$ S reaction. The relative intensity of A2 at 2 and 20 min was identical to that of A2 when probed with actin at those times, indicating that both A2-1 and A2-2 contained snR20. A band migrating much faster than complex B was seen in both $+$ S and $-$ S reactions. The intensity of this band decreased with increasing incubation time in the $+$ S reaction, whereas it remained constant in the $-$ S reaction. This band may represent the endogenous snR20 RNP, since deproteinized snR20 migrated faster than this band in the same

Figure 7. Northern blot analysis of the splicing complexes. Splicing reactions were carried out in the presence of ATP for 2, 5, and 20 min, or in the absence of ATP for 20 min, with extremely low specific activity transcript ($+$ S) or no transcript ($-$ S). The complexes were separated by electrophoresis on a native polyacrylamide gel. RNA was then transferred electrophoretically to GeneScreen membrane and subjected to hybridization analyses with probes of actin, snR20, snR7, snR6, snR14, and snR19 as described in Materials and methods.

gel system (data not shown).

Neither the snR6 (U6), snR7 (U5), nor snR14 (U4) probe hybridized to complex B, indicating that none of these RNAs were present in this complex. snR6 and snR7 hybridized to complexes A1 and A2 in the same fashion as snR20 and are therefore components of complexes A2-1, A1, and A2-2. The snR14 probe hybridized only to the A2 complex. Hybridization was also seen to A2 in the $-$ ATP reaction, indicating that snR14 was present in A2-1. In the $+$ ATP reaction, the amount of snR14 that hybridized to A2 was small (note that the relative intensity of band A2 in the $+$ ATP reaction and in the $-$ ATP reaction is very low) and remained at approximately the same level throughout the reaction. This result indicates that snR14 is only present in complex A2-1 and that complex A2-1 is transitory in the assembly process. Part of the strong hybridization of snR14 in the $-$ ATP lane was also seen in the $-$ S control. This phenomenon will be discussed later in the paper.

The snR19 (U1) probe did not hybridize to any of the four splicing complexes but instead hybridized to bands identically in the $+$ S and $-$ S reactions. These bands may represent endogenous snR19 RNPs.

The order of assembly of the splicing complexes and their snRNA components is summarized in Figure 8.

Detection of snRNP complexes in splicing extracts

We have used the nondenaturing gel system to separate the various yeast snRNPs and to investigate possible interactions between them. Such interactions may be important for the assembly process. Aliquot of the extract containing heparin were separated by electrophoresis as in the experiments described above. snRNAs were analyzed by Northern blot analysis. The control for this experiment was deproteinized RNA prepared by proteinase K treatment of the splicing extract, followed by phenol-chloroform extraction and ethanol precipitation.

One feature apparent in the control RNA lane deserves special comment. snR14 and snR6 migrate anomalously slowly. snR14 is 160 bases long (Siliciano et al. 1987a), and snR6, 112 bases (D. Brow and C. Guthrie, pers. comm.). They migrate together and more slowly than snR7, which is found in two forms of 179 and 214 bases (Patterson and Guthrie 1987). The reason for this

Figure 8. Scheme for the assembly of the spliceosome. The small nuclear RNA content of each splicing complex is indicated.

peculiar behavior is that snR14 and snR6, like their U4 and U6 counterparts, form a stably hydrogen-bonded complex (D. Brow and C. Guthrie, pers. comm.) and in the mammalian case are found together in a single snRNP (Bringmann et al. 1984; Hashimoto and Steitz 1984). Confirming the results of Brow and Guthrie, we show that boiling the free snR6–snR14 complex for 5 min causes it to dissociate while the same heat treatment has no effect on the electrophoretic mobility of snR7 (Fig. 9B).

In the whole-cell extract, all of the snRNAs existed in several forms. snR14, snR7, and a portion of snR6 appeared to form a single large (slowly migrating) complex (Fig. 9A, forms I and II). The majority of snR6 was in a smaller complex, form V. When ATP was added to the whole-cell extract at 2 mM, forms I and II disappeared and forms III and IV appeared (Fig. 9A). The mobility of form III was the same when probed with snR6 and snR14, indicating that this complex contains both RNAs (III₁₄₊₆). The mobility of form IV was unique to each of the RNAs (IV₆, IV₇, IV₁₄). Thus, forms I and II seem to be complexes that contain the three snRNAs while III₁₄₊₆ contains snR14 and snR6.

The dissociation of forms I and II does not require incubation, but requires hydrolysis of ATP, since nonhydrolyzable ATP analogs failed to promote dissociation. dATP was almost as effective as ATP in promoting dissociation, while CTP, GTP, or UTP only resulted in the dissociation of form I but not form II and in the formation of III₁₄₊₆ but not IV₆ or IV₁₄. ATP at 0.2 mM gave similar results to that seen for GTP, CTP, or UTP (data not shown). Complex V₆ did not change mobility in the presence of any of the nucleoside triphosphates.

snR20 existed in two forms, both of which migrated slightly more slowly than free snR20 RNA. snR19 is in two major forms and one minor form. ATP has no effect on the snR19 or snR20 complexes.

The state of the various snRNPs was also investigated in ammonium sulfate fractions of the splicing extract. The fractionation scheme was as described previously (Cheng and Abelson 1986; see also Fig. 10A). The relevant fractions are 40P1, 40P3 and 40W, 40P3 being the 40% ammonium sulfate pellet washed two times with 40% ammonium sulfate.

snR7 and snR14 are greatly enriched in the 40P1 and 40P3 fractions. In 40P1, complex I, a major form in the crude extract containing snR7, snR6, and snR14, has disappeared. Instead, the majority of snR14 was in forms III₁₄₊₆ and IV₁₄ and II, the last of which still exhibited the ATP-induced dissociation effect. snR7 was primarily in forms IV₇ and II. In the 40P3 fraction, form II had mostly disappeared. The majority of snR14 was in a new form, III'₁₄₊₆ and snR7 also in an altered form III'₇. This change of mobility, an apparent result of the salt wash procedure, could be due to the dissociation of proteins causing the snRNP to adopt a more extended conformation with slower migration. The mobility of III₁₄₊₆ and III₇ was regained upon addition of the 40W fraction (Fig. 10C).

snR6 behaved the same as snR14 in the ammonium

Figure 9. (A) Analysis of snRNPs in the splicing extract. Nucleoside triphosphates as indicated and heparin (at 2 μ g per μ l of whole-cell extract) were added to whole-cell extract and the samples were subjected to the electrophoresis on a native polyacrylamide gel. The RNA was then transferred electrophoretically to GeneScreen membrane for Northern blot analysis. The same blot was used for probes of snR14, snR7, snR6, snR19, and snR20. (—RNA) Deproteinized whole-cell extract RNA. snR14, snR7, and snR6 formed large complexes I and II, which dissociated into complexes III and IV upon addition of ATP. snR6 also existed in another form V, which did not respond to ATP. snR19 and snR20 complexes did not change mobility in the presence of ATP. (B) Deproteinized whole-cell extract RNA was electrophoresed and transferred to GeneScreen membrane and probed with snR14, snR7, and snR6 probes as described in A. RNA was (HEAT +) or was not (HEAT –) boiled for 5 min before loading to the gel.

Figure 10. Analysis of snRNPs in the ammonium sulfate fractions of the splicing extract. (A) Scheme of fractionation. (B) Fractions 40P1, 40P3, and whole-cell extract (CE) were analyzed on a native polyacrylamide gel and blots were hybridized with probes for snR14, snR7, and snR6. (C) Fractions 40P3, 40W, and a combination of 40P3 and 40W (40P3 + 40W) were analyzed as described in Fig. 9 in comparison with the whole-cell extract.

sulfate fractionation scheme, except that V_6 did not fractionate with snR14 or snR7 in the 40P3 fraction. Instead, the majority of V_6 is found in the 40W fraction.

Thus, the large complexes I and II are dissociated not only by ATP but by high salt as well. Complex III₁₄₊₆ is more resistant to salt, indicating that there is a higher affinity between snR14 and snR6 and that snR7 is more easily dissociated from the large complexes.

Discussion

Identification of four splicing complexes

Four forms of the spliceosome have been detected by electrophoresis of splicing reaction mixtures in nondenaturing polyacrylamide gels. Three of these complexes are functional intermediates to the fourth, which is the active splicing complex. All four forms sediment at 40S (data not shown), so the spliceosome, as originally defined (Brody and Abelson 1985), is actually a mixture of all these complexes.

Complex B is the first detectable intermediate to form. We have not yet found either mutants or conditions that lead to the accumulation of this complex.

Complex A2-1 follows complex B in the assembly pathway and is detected after 2 min of incubation. A1, the next kinetic intermediate, is difficult to detect under our normal reaction and electrophoretic conditions, but can be seen with *rna2* extracts, which after heat treatment accumulate A1, or when the reaction contains EDTA. Complex A1 is seen with low EDTA concentrations (0.3–3 mM), at which only a portion of the Mg^{2+} in the reaction mixture (normally 3 mM) is chelated. At higher concentrations of EDTA, A1 accumulates. It is possible that the EDTA effect is due to the chelation of some cation other than Mg^{2+} , since there is enough Mg^{2+} in the extracts, as we prepare them, to support mRNA splicing without added Mg^{2+} (data not shown). In addition, the Mg^{2+} concentration required for formation of $B \rightarrow A2-1 \rightarrow A1$ seems to be extremely low, possibly in the nanomolar range. Complex A1, isolated either from splicing reactions at high concentrations of EDTA or from reactions with heat-inactivated *rna2* extracts, can be chased into the active form by addition of an extract fraction and ATP. This result argues that complex A1 is in fact a functional intermediate. We should emphasize, however, that only electrophoretic mobility connects A1/EDTA with A1/*rna2*. Given the limited resolution obtained in electrophoresis, it is possible that these complexes are different. They do, however, seem kinetically identical in the reaction scheme (data not shown).

Complex A2-2 is the active form of the spliceosome and accumulates when the 3' splice site mutant, ACAC, is used as substrate. A2-2 is the only complex that contains the splicing intermediates.

snRNA composition of the splicing complexes

The snRNA compositions of the different complexes were determined by Northern analysis of the native gels

using various snRNA probes. Complex B contains only snR20. Complex A2-1 contains, in addition to snR20, snR7, snR14, and snR6. Apparently, snR14 is lost as A2-1 is transformed into A1, since the latter complex contains only snR20, snR7, and snR6. Complex A2-1 appears to be extremely short-lived in the assembly pathway, since only small amounts of this complex are seen throughout the reaction (as indicated by the amount of snR14 in this complex). Complex A2-2, the active form of the spliceosome, contains the same snRNA constituents as A1.

The transient association of snR14 with the spliceosome is a fascinating and puzzling result. D. Brow and C. Guthrie (pers. comm.) have detected a stable complex between snR14 and snR6 RNAs analogous to the complex formed between their mammalian counterparts, U4 and U6 (Bringmann et al. 1984; Hashimoto and Steitz 1984). We have also detected a stable RNP containing both snR14 and snR6. The interaction between these two RNAs is mediated by hydrogen bonding between two complementary, internal loops (D. Brow and C. Guthrie, pers. comm.; Bringmann et al. 1984; Hashimoto and Steitz 1984). The proteinase K/SDS-treated snR14–snR6 complex can be separated by heat but the melting temperature is much higher than the temperature at which splicing occurs (D. Brow and C. Guthrie, pers. comm.). How then is the snR14 RNP expelled from the spliceosome during assembly? It may be that the hydrogen-bonding interactions seen between the free RNAs do not exist when the two snRNPs interact. More likely they do exist but the hydrogen bonds are broken in the assembly step from A2-1 to A1. Such a reaction might be mediated by an ATP-dependent RNA helicase. There is an apparent requirement for ATP in the A2-1 $\rightarrow$ A1 step. When assembly was monitored in the absence of ATP, complexes B and A2-1 slowly formed but not A1, implying a strong ATP dependence for this step. The slower migration of A1 following the loss of snR14 cannot be directly explained since we do not understand the factors that govern the mobility of these complexes in electrophoresis. One possible reason for the change in mobility might be a large conformational change in the complex attending the expulsion of snR14.

snR19, the yeast U1 analog, was not found in any of the complexes. It is possible that snR19 is only associated with the splicing complex as it is assembled and that, as for snR14, is a necessary but transient complex, which under our conditions is not detectable. In mammals, U1 is required for RNA splicing but has not been found in the spliceosome. There has been speculation that the reason for failure to find U1 in the spliceosome is due to the addition of heparin to the splicing reaction prior to electrophoresis. Perhaps the U1 snRNPs, interact weakly with the spliceosome and are removed from it by heparin. Affinity purification methods that do not employ either electrophoresis or heparin do yield complexes containing U1 (Bindereif and Green 1987) or snR19 (Rymond et al. 1987; S. Ruby et al., unpubl.). These affinity methods cannot at the moment distinguish between a transient interaction or a weak but nonetheless ubiquitous presence of U1/snR19

in the assembly pathway. Complex formed in heat-inactivated *rna2* extracts, treated with heparin and isolated by glycerol gradient sedimentation, can be complemented by addition of ATP and Fraction I (40P3) (data not shown). Thus, the addition of heparin had no detrimental effect in this assay.

Other workers have used similar assays to monitor assembly of splicing complexes in both mammalian and yeast systems (Konarska and Sharp 1986; Pikielny et al. 1986; Konarska and Sharp 1987). The snRNA composition of the complexes allows direct comparison of our results with those previously reported. Konarska and Sharp observed two complexes, "B" and "A". Complex A contained only U2 snRNA and formed rapidly. It therefore can be related directly to our complex B. The mammalian complex B is analogous to the collection of the three A complexes that we have described.

Pikielny et al. resolved three complexes I, II, and III in order of increasing mobility. Complex III contained only snR20 and formed rapidly and is therefore the same as our complex B. Complex II contained only snR7 and snR20. It also contained the splicing intermediates and is therefore the same as our A2-2. Pikielny et al. did not report snR6 in this complex but at the time snR6 was not known. Neither did they observe complex A1, which is difficult to detect unless EDTA is added to slow down the reaction. The complex I of Pikielny et al. is likely to be analogous to our complex A2-1, and complex II to our complex A2-2. These two complexes are resolved in their gel system.

Multiple roles for ATP in mRNA splicing

Previous results obtained in studying formation of the spliceosome in heat-inactivated *rna2* extracts proved that ATP is required in at least two stages of the reaction (Lin et al. 1987). It is required for the formation of a 40S complex that accumulates in these extracts, and it is required to chase the precursor in the 40S complex into products. These two steps correspond to formation of complex B, since it is the first 40S complex to form, and to step A1 → A2-2 in the assembly pathway. We can argue further that ATP is required for other steps of the assembly process. Formation of complex A2-1 involves association of snR6, snR7, and snR14 with the pre-mRNA. Although these three snRNPs associate in the splicing extract, snR7 dissociates from the snR6–snR14–snR7 particle upon addition of ATP. It is not clear whether this dissociation event is required for the formation of A2-1. If so, ATP is then required for step B → A2-1. ATP is also required for formation of complex A1, since A1 does not form in whole-cell extracts in the absence of ATP. We have suggested earlier that ATP-dependent helicases would be a possible source of the ATP requirement. These considerations make the search for such activities in our extracts an important agenda item.

Analysis of snRNPs in splicing extracts

Electrophoretic separation of complexes present in

splicing extracts (in the absence of added precursor mRNA) has provided evidence for the existence of yeast snRNPs and has suggested interactions between snRNPs that may be important in the assembly pathway, snR19 and snR20 are both observed by nondenaturing polyacrylamide gel electrophoresis as complexes that migrate more slowly than their respective free RNAs. Fractionation of the splicing extract by ammonium sulfate precipitation (Cheng and Abelson 1986) resulted in a separation of these two snRNPs. snR20 is in the 40% ammonium sulfate supernatant (40W) while snR19, along with snR7 and snR14, is in the precipitate (40P3). Despite the fact that complex B contains only snR20, the 40W fraction does not suffice to form complex B (data not shown). Apparently other components present in the precipitate are also required for assembly of this complex.

snR14, snR7, and snR6 can all be detected in a large complex. This complex dissociates in the presence of ATP or at high salt to give a free snR7 particle and a complex containing both snR6 and snR14. The existence of these complexes and the observation that snR14, snR6, and snR7 appear together in the assembly pathway suggest a functional role for these complexes. A similar particle containing U4/U5/U6 snRNPs has also been observed in mammalian extract (Konarska and Sharp 1987). There are differences between the yeast and the mammalian complexes. In the absence of ATP, a smaller amount of the U4/U5/U6 complex was observed but an increase was seen upon incubation with ATP. We observed an ATP-dependent dissociation of the corresponding particle in yeast.

Summary

Our results can be summarized in the assembly diagram shown in Figure 11. The formation of the early complex B requires the snR20 RNP. Parker et al. (1987) have shown by means of compensatory mutations that snR20 base-pairs with the conserved UACUAAC sequence near the 3' end of the intron. It is likely that many other components are also required for this reaction since the complex sediments at 40S. In addition, ammonium sulfate fraction 40W, which contains snR20, does not suffice to form complex B. By comparison with the mammalian system, it is likely that among the components necessary to form complex B is the snR19 (U1) complex.

In the next step B → A2-1, three snRNPs, snR7, snR14, and snR6, join the complex. These three snRNPs are seen as a large complex in splicing extracts, but in the presence of ATP, this complex dissociates, releasing snR7. It seems likely that the association of these snRNPs and their ATP-dependent dissociation is functionally relevant to this step in the assembly pathway. snR7 and snR6–snR14 particles may recognize different splicing signals, which are then brought to proper alignment by the same interactions seen between these two particles in the splicing extracts. It is interesting that in the mammalian system, U5 and U4/U6 RNPs have been proposed to interact with each other before associating

Figure 11. A proposed mechanism for spliceosome assembly.

with the pre-mRNA (Konarska and Sharp 1987). Although complex A2-1 (as defined by detection of snR6, snR7, and snR14 in the complex) is represented in the diagram as a folded particle, we have no evidence that folding takes place at this step. As we have pointed out earlier, complex A2-1 is transitory and only in small amounts of A2-1 can be detected throughout the reaction. We show snR7 associated with the 3' splice site in Figure 11 by analogy to the mammalian system in which U5 snRNP is implicated in the recognition of 3' splice site (Chabot et al. 1985). The way in which the U4/U6 snRNP interacts with the pre-mRNA is not yet established (Black and Steitz 1986).

In the next step $A2-1 \rightarrow A1$, snR14 leaves the spliceosome, and there is a change in mobility indicating, perhaps, a conformational change.

The transition $A1 \rightarrow A2-2$ generates the active splicing complex. This reaction requires extrinsic proteins: the RNA2 gene product (b_2), and at least one other protein, b_n , known to be heat stable (R.-J. Lin, pers. comm.). The splicing reaction occurs in two steps and additional proteins C_{III} and C_n are known to be required in the second step in which the intermediates are converted into products (Lin et al. 1987).

Once the reaction is completed, the mRNA leaves the spliceosome and appears as a 20S particle (S. Goelz and J. Abelson, unpubl.). The intron lariat sediments at 40S and so may be present as a particle that contains snRNPs as well. There is evidence for such a particle in mammalian systems where the intron lariat remains associated with U2, U5, and U6 snRNPs (Konarska and Sharp 1987).

This assembly diagram is similar to what has been proposed in the mammalian system (Sharp 1987). There are many superficial differences in splicing between yeast and man; notably the strong conservation of splice site sequences and the large size of some of the yeast snRNAs. Nonetheless, the essential feature of the assembly pathway, in its present, preliminary form, appear to be highly conserved.

Materials and methods

Preparation of the splicing extracts and substrates

Yeast whole-cell extracts were prepared according to Lin et al. (1985). Fractions 40P3 and 40W were prepared as described in Cheng and Abelson (1986). Actin precursors were synthesized *in vitro* using SP6 polymerase according to Lin et al. (1985) except that the UTP concentration was 100 μ M and the [α - 32 P]UTP was 2 mCi/ml. For Northern blot analysis, the ratio of the concentration of [α - 32 P]UTP to UTP was 1000-fold lower than in the usual transcription reaction. Actin antisense transcript was made from an SP6 clone containing the $\Delta 6$ -actin gene in reverse orientation.

In vitro splicing reaction and polyacrylamide gel electrophoresis

Standard splicing reactions were carried out in a buffer containing 60 mM KPO_4 (pH 7), 3 mM $MgCl_2$, 1 mM spermidine, 2 mM ATP, 3% PEG, 0.4 nM transcript, and 40% of the splicing extract, unless described otherwise. For Northern hybridization experiments, the reactions contained 4 nM transcript instead. Before electrophoresis on native polyacrylamide gels, splicing reactions were terminated by adding a solution containing 60 mM KPO_4 (pH 7), 3 mM $MgCl_2$, 3% PEG, 8% glycerol, and heparin at 2 μ g for each microliter of whole-cell extract in the reaction, and then kept on ice until loading to the gel. Electrophoresis was carried out in 4% polyacrylamide gels with an acrylamide-to-bisacrylamide ratio of 80:1, and a gel size 18 $\times$ 12 $\times$ 0.5 cm. Electrophoresis was at 25 volts/cm at 4°C for 1.5–4 hr in a buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA. For RNA analysis, RNA was eluted from the gel in a buffer containing 50 mM NaOAc (pH 5), 2 mM EDTA, and 0.1% SDS, at 37°C for 6–18 hr, followed by phenol-chloroform extraction and ethanol precipitation.

Hybridization analysis

After electrophoresis, RNA from the gel was transferred to a GeneScreen membrane (NEN) by electroblotting at 4°C for 2–4 hr. The electrophoretic transfer was performed at 40 volts in a buffer containing 25 mM $NaPO_4$ (pH 6.5). Membranes were subsequently irradiated with UV light (three UV bulbs, Sterilamp, G1578) at a distance of 35 cm for 10 min (Church and Gilbert 1984) and then subjected to prehybridization and hybridization with different probes. For probes made from clones of snRNAs, prehybridization and hybridization were carried out in a solution containing 25 mM $NaPO_4$ (pH 6.5), 6 $\times$ SSC, 5 $\times$

Denhardt's, 0.5% SDS, 0.1 mg/ml heat-denatured calf thymus DNA, and 50% formamide at 42°C. For the oligonucleotide probe, formamide was left out in the prehybridization and hybridization solutions. Prehybridization was at 65°C while hybridization was at 30°C. ³²P-Labeled probe (5–10 × 10⁶ cpm) was included in each 10 ml of the hybridization solution. After hybridization, membranes were washed sequentially in solutions containing 2 × SSC, 0.5% SDS at room temperature for 5 min, 2 × SSC, 0.1% SDS at room temperature for 5 min and 2 × SSC, 0.1% SDS at 50–65°C for 20–30 min. With the oligonucleotide probe, 6 × SSC was used and the last wash was at 50°C. DNA fragments containing the snR7 (Patterson and Guthrie 1987), snR14 (Siliciano et al. 1987a), snR19 (Siliciano et al. 1987b), or a portion of snR20 genes (Ares 1986) was used for synthesis of the probes by random primer extension method of Feinberg and Vogelstein (1983). A clone of snR20 was given by M. Ares, snR7 by B. Patterson, and snR14 and snR19 by P.G. Siliciano. A 60-mer oligonucleotide (5'-CGTGTATCCTTGC GCAGGGGCCATGCTAATCTTCTCTGTATCGTTCCAAT TTTAGTATA-3') complementary to residues 21–80 of *Drosophila* U6 snRNA (Das et al. 1987) was labeled at the 5' end by T4 polynucleotide kinase and used as a probe for snR6.

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Spliceosome assembly in yeast.

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