Assembly in an *in vitro* splicing reaction of a mouse insulin messenger RNA precursor into a 60–40S ribonucleoprotein complex

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ABSTRACT

An SP6/mouse insulin RNA precursor containing two exons and one intron can be spliced in a partially purified nuclear extract isolated from MOPC-315 mouse myeloma cells. We have detected the putative RNA splicing intermediate (intron-3'exon) in a lariat form, the excised intron in a lariat form, and the mRNA spliced product. The *in vitro* splicing reaction of gel-purified RNA precursors requires ATP and Mg^{2+} and was accompanied by the formation of a 60-40S ribonucleoprotein complex. The formation of the 60S complex requires ATP. At least two Sm snRNPs containing U1 and U2 RNAs are components of the 60-40S complex. The assembly of those snRNPs occurs early during the splicing reaction and it requires ATP and intron containing pre-mRNAs.

INTRODUCTION

Eukaryotic genes encoding proteins frequently contain intron sequences, which are first transcribed as parts of the pre-mRNAs and subsequently removed by splicing (1,3,7).

The cleavage and ligation reactions have been proposed to occur in a multicomponent precursor mRNA-ribonucleoprotein (RNP) complex, likely containing Sm small nuclear ribonucleoproteins (snRNPs) (12,17,18,21). Assembly of the pre-mRNA to the complex would position the cut exon and RNA intermediates to promote accurate ligation.

We have previously reported a partial purification of approximately 108 fold of a pre-mRNA splicing activity from nuclear extracts of MOPC-315 mouse myeloma cells (6). The splicing reaction of a non-gel purified SP6/adenovirus 2 EII (Ad2 EII) pre-mRNA occurred without a lag, did not require exogenously added Mg^{2+} and was dependent on ATP or GTP (9). In this study we show that when pre-mRNA's are purified on denaturing gels from contaminants of the transcription reactions, the *in vitro* splicing reactions required Mg^{2+} , ATP, and Sm snRNPs.

To facilitate the analysis of the *in vitro* processing products we have

now synthesized a short SP6/mouse insulin pre-mRNA (410 nucleotides) containing only one intron. We have detected: a) the putative splicing intermediate in a lariat form (intron-3'exon); b) the intron RNA in a lariat form; and c) the spliced mRNA product. Structural analysis of these RNA species provide direct evidence for the presence of an RNA branch in the lariat RNAs.

Analysis of the in vitro splicing reactions on glycerol gradients revealed two peaks, a 60S and a 40S ribonucleoprotein complex. The RNA products of the in vitro splicing reaction, however, were detected only in the 60S complex. The formation of the 60S complex required ATP and it was not detected under conditions known to inhibit the splicing reaction. Evidence is given that the assemble of U1 and U2 SnRNPs into the 40-60S complex are early events during the splicing reaction and it requires ATP and intron containing pre-mRNAs.

MATERIALS AND METHODS

Plasmid Construction, SP6 Transcriptions and In Vitro Splicing.

The plasmid SP64/MINS was constructed by ligating the AluI fragment (nucleotides 668-1028) of the mouse insulin gene into the SmaI site of pSP64.

The SP64/MINS DNA was linearized with EcoRI and transcribed with SP6 RNA polymerase in the presence of the four α -[32 P] nucleotides as described (6,9). Precursor RNAs were capped by including 2 mM of m⁷GpppG in the transcription reactions. The [32 P]-pre-mRNAs made in vitro were then purified by elution from a 5% polyacrylamide denaturing gel in a buffer containing 0.5 M ammonium acetate, 0.1% SDS and 0.001 M EDTA. RNA eluted from the gel was further purified by binding to DEAE-cellulose as described (11). The purified RNAs were then used as substrates for in vitro splicing.

Splicing reactions were carried out with a partially purified nuclear extract isolated from MOPC-315 mouse myeloma cells prepared as described (6,10). The in vitro reactions were as described (6,9) except that MgCl₂ (3 mM) and only ATP (0.5 mM) were included. The reactions were 150-300 μ l in order to obtain enough material for structural analysis.

The purification of the splicing activity was monitored at each chromatographic step (6) with both gel purified pre-mRNAs (SP6/Ad2 EII and the SP6/mouse insulin). The same result, as previously reported (6), of about 108-fold purification was obtained (data not shown).

Primer-extension Analysis

³²P-labeled RNA products of the in vitro splicing reactions were

purified by electrophoresis subsequently on a 5% and 8% polyacrylamide gels, eluted and further purified as described above.

The individual RNA species were hybridized (23) with 1 pmole of a 5'-³²P-end labeled of a synthetic 20-mer oligonucleotide. The DNA-RNA hybrids were extended with reverse transcriptase as previously described (6,9). The nucleic acids were then subjected to alkaline hydrolysis in 0.2 N NaOH for 16 hours at 37°C (to degrade all the ³²P-RNA), neutralized with HCl, extracted with phenol, and ethanol precipitated. The cDNAs were then analyzed on polyacrylamide denaturing gels as described in the legend to Figure 1.

Direct RNA Analysis

Individual RNA species purified as described above were subjected to complete digestion by nuclease P1 and the products fractionated by two-dimensional thin layer chromatography as described (16).

The branched oligonucleotide was eluted, digested with snake venom phosphodiesterase as described (15), and the products separated on PEI plate chromatography (24).

RESULTS

Time Course and Requirements of the In Vitro Processing Reaction For a Truncated SP6/Mouse Insulin RNA Precursor.

The structure of each RNA species shown schematically in Figure 1 was determined by primer-extension analysis of the individual gel-purified RNA species (Fig. 1B). Six major RNA splicing products are detected (Fig. 1A), including the unspliced pre-mRNA (RNA species 1), an RNA containing the intron-exon 2 lariat (RNA species 2), spliced exon 1-exon 2 (RNA species 3), intron-exon 2 (RNA species 4), the excised intron lariat (RNA species 5), and the exon 1 (RNA species 6). The RNA species 2 and 5 migrated as 400 and 150 nucleotides on a 5% polyacrylamide/urea gel, slower than expected for linear RNA species of 334 and 110 nucleotides, respectively (Fig. 1A). The RNA species 4 represents an aberrant cleavage within the intron (see Fig. 1B).

We have employed a 20 nucleotide synthetic 5'-³²P-labeled oligonucleotide that is homologous to sequence 45 and 25 nucleotides from the 3' splice site.

Primer-extension of the pre-mRNA (RNA species 1) produced a 232 nucleotide cDNA band, the distance from the primer to the 5' terminus of the SP6-mouse insulin transcript. Primer-extension of the lariat RNA (RNA

Figure 1. Time course and requirements of the *in vitro* splicing reaction.

A) ^{32}P -labeled SP6/MINS RNA precursor was synthesized, spliced *in vitro*, and then fractionated by electrophoresis on a 5% denaturing polyacrylamide gel. The reactions were terminated at the times indicated: 0 min (lane 1), 5 min (lane 2), 15 min (lane 3), 30 min (lane 4), 60 min (lane 5), and 120 min (lane 6). To study the requirements of the *in vitro* splicing reaction each reaction was incubated for 120 min as follows: plus CTP, UTP and GTP (lane 7), plus imidonucleotide AMP-PNP (lane 8), minus Mg^{2+} (lane 9), partially purified nuclear extract pre-incubated as described (6) in the presence of monoclonal anti-Sm antibody (lane 10), plus antibody control (lane 11). M; ^{32}P labeled markers of ϕX DNA digested with HaeIII. The structure of each numbered RNA species is schematically shown below the autoradiograms. The identity of each band is based on the predicted size of each RNA species and structural analysis presented in panel B. Boxes; exons; Lines; introns. Predicted sizes in nucleotides for linear RNA species are as follows: RNA specie 1:410, 2:334, 3:300, 5:110 and 6:76.

B) The RNA processing products of *in vitro* splicing reactions were purified on polyacrylamide gels as described in Materials and Methods and

used as templates for reverse transcriptase primer-extension analysis. The primer was an end-labeled synthetic oligonucleotide that is homologous to a sequence of 20 nucleotides located between 45 and 25 nucleotides from the 5' end of the exon 2. The RNA species used as templates for reverse transcriptase are indicated at the top of each lane. Lane 1: RNA specie 1, Lane 2: RNA specie 2, Lane 3: RNA specie 3, Lane 4: RNA specie 4, Lane 5: RNA specie 5. M, ^{32}P -labeled marker of ϕX DNA digested with HaeIII. The sizes of the cDNA products are indicated to the left of the autoradiogram. A diagram of the primer-extension products is shown below the autoradiogram. Boxes; exons; Lines; introns. The ^{32}P -end label of the oligonucleotide primer is indicated by an asterisk.

Approximately equal molar amounts of the purified RNA species were used for primer extensions. Therefore, the relative intensities of the cDNA bands do not reflect the relative abundance of the different RNA processing products. The relative abundance of the RNA spliced products is shown in Panel A.

C) Primer extensions of RNA species 5 and 6. The 20-mer oligonucleotide used for RNA species 5 is homologous to an intron sequence located after the first nucleotide of the branching sequence. The 20-mer oligonucleotides used for RNA species 6 are homologous to a sequence located at 16 and 26 nucleotides from the 3' of the first exon. Lane 1: RNA species 5, Lane 2: no RNA, Lane 3 and 4: RNA species 6 primer-extended with two oligonucleotides located at 16 and 26 nucleotides from the 3' of the exon, respectively.

species 2) generated a 63 nucleotide cDNA, indicating the presence of a structure that blocks the reverse transcriptase, with the block located at 18 nucleotides from the 3' of the intron. As expected, no primer extension product was observed with the intron (RNA species 5) or the exon 1 (RNA species 6) which lacks exon 2 sequences. Primer extensions with appropriate oligonucleotides confirmed the structure of RNA species 5 and 6 (Fig. 1C). The spliced mRNA (RNA species 3) generated a 120 nucleotide cDNA band, corresponding to the spliced product. The RNA species 4 produced a 55 nucleotide cDNA, corresponding to a product of aberrant cleavage event in the intron. Several aberrant RNA cleavage events, diminished by the use of capped RNA precursors, were also described for a β globin RNA precursor spliced in vitro (16,23).

The RNA species 2,4 (a very faint band) and 6 are detected at 30 min of incubation (Fig. 1A, lane 4). At 60 min (Fig. 1A, lane 5), two additional RNA processing products are observed: correctly spliced RNA (RNA species 3) and the excised intron (RNA species 5). The RNA species 5 (excised intron) and 6 (exon 1) are not in equal molar amounts with RNA species 3 (spliced RNA) and 2 (intron-exon 2), suggesting that RNA species 5 and 6 are less stable upon incubation. The same result was recently reported (21) for the in vitro splicing of the rat insulin II gene.

Some requirements for the in vitro splicing reaction with gel purified

Figure 2. Nuclease P1 digestion products of purified RNAs species generated from the in vitro splicing reaction.

A) SP6/MINS pre-mRNAs were synthesized in the presence of all four ^{32}P -ribonucleoside triphosphates, spliced *in vitro*, and the different RNA species purified by gel electrophoresis as described in Materials and Methods. The different RNA species were digested with nuclease P1 and subsequently the products fractionated by two-dimensional thin layer chromatography as described (14). pA, pC, pG, and pU indicate the four 5' nucleoside monophosphates. The nuclease P1-resistant structures (X) are marked. The different purified RNA species are indicated by the numbers at the top of each autoradiogram.

B) The spot marked X of RNA species 2 and 5 shown in Figure 4 was eluted and digested with snake venom phosphodiesterase as described (14). The products of digestion were then fractionated by one dimension chromatography on PEI plates (23). A, C, G, and U represent the four 5' nucleoside monophosphates. Lanes 1 and 2: pre-mRNA (RNA species 1) digested with nuclease P1 and ribonuclease T2, respectively, used as markers. Lane 3: spot X digested with snake venom phosphodiesterase.

A diagram consistent with the branched trinucleotide that yielded the nuclease-resistant component in RNA species 2 and 5 is shown to the right of panel B.

pre-mRNAs are demonstrated by the data in Figure 1A. The activities required magnesium and ATP (Fig. 1A, lanes 7 and 9), as reported for other *in vitro* splicing systems (13,16,19). The imidonucleotide analog AMP-PNP was not effective (Fig. 1A, lane 8), indicating that the splicing reaction requires the γ -phosphate moiety of ATP. The reaction also requires snRNPs since preincubation of the partially purified nuclear extract with monoclonal anti-Sm antibodies totally inhibited the reaction (Fig. 1A, lanes 10 and 11).

We have previously reported (6) that in vitro splicing of a non-gel purified SP6/Ad2 EII pre-mRNA required ATP or GTP but no exogenous added divalent cations. We have re-examined the requirements for in vitro splicing of gel purified SP6/Ad2 EII pre-mRNAs and they absolutely required Mg^{2+} and ATP (data not shown). We conclude that divalent cations carried with the non-gel purified pre-mRNAs from the transcription reactions were sufficient as cofactors for the in vitro splicing reaction.

To elucidate further the nature of the block of reverse transcriptase in RNA species 2 and 5, we directly analyzed the structure of the RNA processing products by nuclease digestion. As first described by Wallace & Edmonds (25), the 2'-5' phosphodiester bond of the branch trinucleotide makes the adjacent 3'-5' phosphodiester bond resistant to digestion by nuclease P1. The individual RNA species generated by in vitro RNA processing labeled with all four ^{32}P -ribonucleoside triphosphates were gel purified, digested with nuclease P1 and the products separated by two-dimensional, thin-layer chromatography. The four monophosphate nucleosides were produced from RNA species 1, 3, and 4 (Fig. 2A). However, in the case of the RNA species 2 and 5, an additional P1 nuclease-resistant oligonucleotide was resolved (spot X in Fig. 2A). The same nuclease-resistant oligonucleotide component was observed in RNA species 2 and 5 following phosphatase treatment (data not shown), suggesting that the modification in these RNAs is a 2'-5' phosphodiester bond. The nuclease-resistant oligonucleotide was eluted, purified, and then digested with snake-venom phosphodiesterase yielding radiolabeled pA, pG, and pC, but no pU (Fig. 2B). Therefore, the nuclease P1-resistant oligonucleotide is composed of A, G, and C residues in approximately equal molar quantities (the differences in intensities between the three monophosphate nucleosides are due to differences in specific activities). The nuclease resistant structure is consistent with its being a branch trinucleotide as shown in Figure 2B.

Ribonucleoprotein complex formed during the in vitro splicing reaction.

The SP6/mouse insulin pre-mRNA was incubated with the partially purified nuclear extract for different times at 30°C, the reaction was then immediately centrifuged through a 15 to 40% glycerol gradient. At 15 minutes of incubation two peaks of ^{32}P -labeled RNA were observed sedimenting at 40S and 60S (Fig. 3). The majority of the radioactivity sedimenting near the top of the gradient represents pre-mRNA (RNA species 1) that did not associate to the 60-40S complex (see Fig. 4).

Figure 3. Glycerol gradient analysis of the products of the *in vitro* splicing reaction.

^{32}P -labeled SP6/mouse *insulin* RNAs were spliced *in vitro* for the indicated times as described in Materials and Methods. The reactions at the end of the incubations were immediately layered over 12 ml, 15 to 40% glycerol gradients (4). The gradients were sedimented at 35,000 rpm for 16 hr in an SW41 rotor at 4°C. Fractions (0.5 ml) were collected, mixed with 0.5 ml of 0.02 M Tris-HCl pH 8, 7 M urea, 0.35 M NaCl, 2% SDS, 0.001 M EDTA, and then extracted twice with equal volumes of a phenol-chloroform mixture. The samples were ethanol precipitated twice. After three washes with 70% ethanol, the fractions were dried and the ^{32}P distribution measured by Cerenkov counting. The markers 60S and 40S ribosomal subunits, and the SP6/mouse *insulin* pre-mRNA, indicated by arrows, were run in a separate gradient. The brackets in the 15 and 90 min gradients represent the pooled fractions for analysis of U1 and U2 snRNPs. Splicing reactions were incubated for 90 min in the absence of ATP, and analyzed on glycerol gradients as described above. A splicing reaction incubated for 90 min was treated with Proteinase K (at 250 $\mu\text{g}/\text{ml}$) for 30 min at 37°C just before loaded on the gradient. Fraction 1: bottom and 23: top of the gradient.

Figure 4. Polyacrylamide-denaturing gel electrophoresis of the fractions from the glycerol gradient.

A) Fractions from the glycerol gradient containing 100 mM KCl of the splicing reaction incubated for 90 min in the presence of ATP (Fig. 3) were subjected to electrophoresis on 5% polyacrylamide, 7 M urea gels. The number at the top of the autoradiogram indicates the fraction number of the glycerol gradient. The numbers at the left of the autoradiogram indicate the different RNA species. The peak of the 60S and 40S markers sedimented in fractions 9 and 12, respectively.

B) ^{32}P -labeled RNA was synthesized from a plasmid DNA containing the SP6 promoter fused to the human insulin cDNA. The RNAs were gel purified, incubated for 90 min in a standard splicing reaction and loaded on a glycerol gradient as indicated above. The number at the top of the autoradiogram indicates the fraction number of the glycerol gradient. The peak of the 60S and 40S markers sedimented in fractions 9 and 13, respectively.

The 60S complex was detected after 15 min of incubation, increased at 45 min and by 90 min it decreased, suggesting a less stable complex after 90 min of incubation (Fig. 3). The 40S complex was detected immediately after incubation of the pre-mRNA with the extract on ice (data not shown). With increasing time of incubation this 40S complex is reduced (Fig. 3). Although this time course suggests a 40S-precursor-60S product relationship,

we cannot rule out that at least some of the 40S complex is not related to the formation of the 60S splicing complex. The 40S complex was detected very rapidly, with RNAs lacking introns and also under conditions known to inhibit the splicing reaction (see below).

Protease digestion of the splicing reaction prior to centrifugation through the glycerol gradient resulted in the disappearance of the complex and migration of the 32 P-RNA at the top of the gradient (Fig. 3).

Glycerol gradient sedimentation of reactions performed in the absence of ATP (Fig. 3) or in the presence of monoclonal anti-Sm antibodies (data not shown) shows the disappearance of the 60S peak and the formation of a 40S peak. We conclude that the association of the pre-mRNA in a 60S complex is ATP dependent and that conditions known to inhibit the splicing reaction affect the formation of the 60S peak in the same fashion.

The RNAs extracted from the glycerol gradient fractions, from a reaction incubated for 90 min in the presence of ATP, were fractionated by electrophoresis on polyacrylamide denaturing gels (Fig. 4A). The pre-mRNA is found throughout the gradient. However, the products of the in vitro splicing reaction are found almost exclusively in the fractions defining the 60S peak. Therefore, not only are the pre-mRNAs associated with the 60S complex but the intermediate RNA lariat forms and the spliced mRNA products were also in this complex.

To demonstrate the specificity of association of the pre-mRNA and splicing products with the 60S complex a 550 nucleotide capped insulin RNA was transcribed from a plasmid containing the SP6 promoter fused to the human insulin cDNA. The 32 P-labeled RNA lacking introns separates from the 60S peak sedimenting between the 40S region and almost the top of the gradient (Figure 4B).

The presence of U1 and U2 snRNPs in the 60S and 40S complex was directly assessed. The fractions of the gradients from reactions incubated for 15 and 90 min. were pooled as follows: pool II represents the 60S and pool III the 40S peaks; pool I the bottom and pool IV the top of the gradients (Fig. 3). The RNAs extracted from the gradient pools were hybridized to a 5' 32 P-end labelled U1 or U2 DNA probes. The hybrids were digested with S1-nuclease and the resistant DNAs analyzed on 12% denaturing polyacrylamide gels. Figure 5A and B shows that at 15 min. of incubation some U1 and U2 snRNPs are detected in the 60S complex but most of the particles sedimented as 40S and at the top of the gradient (10-11S). At 90 min. of incubation, however, most of the U1 and U2 snRNPs are associated with the 60S complex.

Figure 5. U1 and U2 snRNPs are assembled into a 60-40S complex during the splicing reaction.

Fractions of gradients from reactions incubated for 15 and 90 min. shown in Fig. 3 were pooled as follows: Pool I bottom fractions, Pool II fractions representing 60S, Pool III fractions representing 40S and Pool IV top fractions. The RNAs of the pools were extracted and hybridized to a 5' ^{32}P -end labeled U1 or U2 DNA probes. The DNA probes are shown schematically below the autoradiograms. Hybridizations to the U1 DNAs were for 16 hrs. at 55°C in 80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA and to the U2 DNAs were for 16 hrs at 37°C in the same buffer. The hybrids were digested with S1 nuclease (550 U/ml) at 30°C for 40 min and the resistant DNAs analyzed on 12% polyacrylamide gels.

A: Analysis of the U1 snRNPs at 15 and 90 min. of incubation (100 μl and 166 μl reactions respectively). The pools are indicated at the top of each lane. M: ^{32}P -labeled markers of ϕX DNA digested with HaeIII. A: RNA isolated from an extract (corresponding to 100 μl reaction) before the gradient. B: No RNA.

B: Analysis of U2 snRNPs (from 250 μl reactions) as described above. A: No RNA.

C. Analysis of U2 snRNPs (from 100 μl reactions) incubated for 90 min. in the absence of ATP.

In the absence of ATP the U1 and U2 snRNPs remained at the top of the gradient as expected for particles sedimenting at 10-11S (Figures 5C and 6). Therefore, the assemble of U1 and U2 snRNPs into a 60-40S complex

Figure 6. Analysis of U1 snRNPs from reactions incubated for 90 min in the absence of ATP or with RNA lacking introns.

Fractions of gradients (from 100 μ l reactions) incubated for 90 min. in the absence of ATP or with a 32 P-labeled RNA lacking introns (see Fig. 4B) were pooled as described in Fig. 5. The nucleic acids extracted from the gradient pools were then analyzed for U1 sequences as described in Fig. 5. The pools indicated at the top of each lane are as follows: Pool I bottom fractions, Pool II 60S, Pool III 40S and Pool IV top fractions. M: Markers of ϕ X DNA digested with HaeIII. A: No RNA. B: RNA isolated from an extract (corresponding to 100 μ l reaction) before the gradient.

absolutely requires ATP and intron containing pre-mRNAs. When an RNA lacking introns was incubated in a standard splicing reaction, the U1 (Fig. 6) or the U2 snRNPs (data not shown) were not detected in the 60S or 40S complex.

DISCUSSION

We have shown that the RNA precursors for the mouse insulin gene are accurately spliced in vitro by a partially purified nuclear extract via formation of a lariat RNA intermediate. This RNA (RNA species 2 in this study) results from cleavage at the 5' splice site and ligation of the 5' terminus of the intron to the 2' hydroxyl group of an adenine residue located at 18 nucleotides from the 3' splice site. The subsequent steps of the splicing reaction (11,15,16,20,23) are: cleavage at the 3' splice site with release of the intron (RNA species 5) in a lariat form, and ligation of the 5' and 3' exons generating a mature mRNA (RNA species 3).

The kinetics of in vitro splicing for the SP6/mouse-insulin RNA precursor was different from that for the SP6/Ad2-EII pre-mRNA. Whereas the RNA processing products of the mouse insulin pre-mRNA appeared after 30 min of incubation and the reaction was terminated between 60 and 90 min (Fig. 1A), for the SP6/Ad2-EII the reaction was terminated between 15 and 30 min (9). The kinetics and optimal splicing efficiency for different pre-mRNAs may depend on a complex set of parameters like: the ability of the RNA precursor to enter in a ribonucleoprotein complex, interactions between snRNPs with splice sites and branching sequences, and possibly interactions between the 5' splice site and the branchpoint sequence.

The ribonucleoprotein complex (spliceosome) for higher eukaryotes which we have observed in this study and also recently reported by others using Ad2 pre-mRNAs derived from the major late sequences (8,12) is a 60S ribonucleoprotein complex. The formation of the 60S complex requires ATP; substituting the imidonucleotide AMP-PNP for ATP was not effective (data not shown) indicating that the γ -phosphate of ATP was required for the 60S ribonucleoprotein complex formation.

The time course of the 40S-60S complex formation suggests a 40S-precursor-60S product relationship. The 40S complex, however, was detected under conditions that totally inhibited splicing (minus ATP, plus anti-sm antibodies) and also when RNAs without introns were used in the reaction. We conclude that at least some of the 40S complex is unrelated to the splicing reaction.

We have shown that at least two snRNPs the U1 and U2 are components of the 60-40S complex. The assemble of the snRNPs into a 40-60S complex occur early during the splicing reaction (Fig. 5A and B). At 15 min. of incubation (when no splicing products or intermediates were detected) most

of the U1 and U2 snRNPs sedimented as 10-40S and only some as 60S. By 90 min. however, most of the snRNPs were in the 60S complex. Our results also suggests that the U1 and U2 snRNPs are assembled first into a 40S and then into a 60S complex. Whether this 40S is indeed an intermediate that later generates a 60S complex remains unknown.

In a crude nuclear extract, there is about 100-fold excess of U1 and U2 snRNPs over the pre-mRNA substrate used in our splicing reactions (14). We have previously shown (14) that the partially purified nuclear extract contains about 18-fold excess of U1 and 8-fold excess of U2 snRNPs over the pre-mRNA substrate. Therefore, it was critical to use a partially purified extract to study the snRNPs distribution during assemble of the spliceosome complex. Since most of the snRNPs were associated into a 60S complex by 90 min of incubation then more than one U1 and U2 snRNP per pre-mRNA molecule (approximately five to ten) would have to be present in the complex. Moreover, this result suggests that U1 and U2 snRNPs are sequestered into the 60S spliceosome after prolonged times of incubation.

Recently (5), besides the U1 and U2 snRNPs a third Sm snRNP (probably containing U5 RNA) have been shown to specifically recognize the 3' splice site of a pre-mRNA. The expected S value for the three Sm snRNPs plus the pre-mRNA assembled in a complex, in the presence of ATP, is approximately 40S. Although much remains to be learned about spliceosome components and their exact order of assemble, it is tempting to speculate that other components of the splicing reaction (like hnRNP proteins) are later assembled to this 40S complex generating a 60S spliceosome.

We have shown in this study that the assemble of U1 and U2 snRNPs into a 60-40S complex requires ATP and intron containing pre-mRNAs. Binding of the U1 snRNPs to the 5' splice site and an Sm snRNP (probably containing U5 RNA) to the 3' splice site was reported to occur in the absence of ATP (2,5,18). The association of the U2 snRNPs with the branching consensus sequence however, it was not detected when ATP is omitted from the reaction mixture (2). The above observations are consistent with our results that in the absence of ATP the U1 and U2 snRNPs were not detected even in the 40S region (Figs. 5C and 6). Whether the U1 snRNP-pre-mRNA complex, formed in the absence of ATP, cannot interact with other splicing components because the U2 snRNPs were unable to associate with such a complex upon those conditions is not yet known. Further studies are in progress aimed to identify the spliceosome components and their order of assembly and disassembly.

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