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# Identification of a functional mammalian spliceosome containing unspliced pre-mRNA

**(mRNA splicing/small nuclear ribonucleoprotein/HeLa cells)**

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ABSTRACT **Functional 60S spliceosomes were assembled under conditions that block the first step of the mRNA splicing reaction. This block was imposed by carrying out the splicing reaction in nuclear extracts preincubated in 2.5 mM EDTA. Preparative amounts of the spliceosomes were isolated by gel filtration chromatography and shown to be functional by *in vitro* complementation assays. The unspliced pre-mRNA in the complex was converted to spliced products when incubated in cytoplasmic S100 extracts or in heat-treated or micrococcal nuclease-treated nuclear extracts. The latter result, in conjunction with the size of the complex, suggests that the spliceosome contains all of the small nuclear ribonucleoproteins (snRNPs) required for both steps of the splicing reaction. Biochemical characterization of the 5′ cleavage reaction revealed that ATP and $MgCl_2$ are required for this step in the splicing pathway. The presence of U1 snRNP in the blocked complex was demonstrated by quantitative immunoprecipitation of this complex by an anti-U1 snRNP monoclonal antibody.**

Pre-mRNA splicing occurs by a two-step mechanism in which the first step is cleavage at the 5′ splice site and "lariat" formation and the second step is cleavage at the 3′ splice site and exon ligation. These reactions take place within spliceosomes, 60S complexes containing pre-mRNA, small nuclear ribonucleoproteins (snRNPs), and other splicing components (reviewed in refs. 1–3). A pathway of spliceosome assembly and disassembly has been proposed based on the temporal appearance of discrete complexes during the *in vitro* splicing reaction (4–13). One form of the mammalian spliceosome, isolated by glycerol gradient sedimentation (14) or gel filtration chromatography (15), was shown to be a functional intermediate in this pathway. Evidence for a precursor–product relationship between other putative intermediate complexes has not been obtained.

A functional yeast spliceosome containing unspliced pre-mRNA was identified in extracts prepared from the RNA splicing mutant rna2 (10, 16). Spliced RNA is generated from this complex when it is incubated in partially purified extracts containing the wild-type *rna2* gene product. A similar, if not identical, complex is formed in extracts from wild-type yeast cells when EDTA is included in the splicing reaction mixture (10).

Although analysis of the splicing pathway in mammalian cells cannot be approached through genetics, the *in vitro* splicing reaction can be blocked biochemically at specific steps of the pathway (17). Functional intermediate complexes can then be purified in preparative amounts by gel filtration chromatography and tested for function by *in vitro* complementation experiments. This approach was recently used to characterize spliceosomes blocked after 5′ cleavage and lariat formation, but prior to 3′ cleavage and exon ligation (15). We designate this form of the spliceosome FS-I (functional spliceosome, intermediate), since it contains the RNA intermediates of the splicing reaction.

In this paper, we report the identification and purification of a functional spliceosome that is assembled in the presence of EDTA. Similar to the analogous complex in yeast (10), the mammalian complex is blocked prior to cleavage at the 5′ splice site, but after assembly of pre-mRNA into a spliceosome. We designate this complex FS-P (functional spliceosome, precursor), since it contains unspliced pre-mRNA. The preparative purification of this complex has allowed an analysis of the biochemical requirements of the 5′ cleavage reaction.

## MATERIALS AND METHODS

**Pre-mRNA Synthesis.** SP64-R$\beta$ and T7-H$\beta$ plasmids, containing rabbit and human $\beta$-globin gene sequences, respectively, have been described (15, 18). RNA precursors generated from the bacteriophage T7 promoter were synthesized and capped as described (19, 20), with modifications (15).

**Extracts.** Nuclear extracts and S100 extracts (100,000 × *g* supernatants) were prepared from HeLa cells as described (19, 21), with modifications (22).

***In Vitro* Splicing and Complementation Assays.** *In vitro* splicing reactions of HeLa cell nuclear extracts were assayed as described (15, 19) with the following exceptions. Splicing reaction mixtures (25 $\mu$l) contained 7.5 $\mu$l of nuclear extract, reducing the glycerol concentration to 6% (vol/vol). The final concentration of nuclear extract protein in the assay was still ≈4 mg/ml. RNA was precipitated by using glycogen as a carrier and subsequently analyzed by electrophoresis in 7 M urea/7% polyacrylamide gels. For the FS-P complex, nuclear extracts were first incubated with 2.5 mM EDTA on ice for 30 min and then formed by addition of T7-H$\beta$ pre-mRNA under standard splicing conditions in the presence of 2.5 mM EDTA and 3 mM $MgCl_2$.

*In vitro* complementation assays utilized micrococcal nuclease-treated nuclear extracts and S100 extracts (17). For the complementation assays, reaction mixtures (100 $\mu$l) contained 20 $\mu$l of extract and 40 $\mu$l of the SS-peak fraction from gel filtration (see Fig. 3*A*) and were incubated for 1.5 hr unless indicated otherwise.

**Density Gradient Sedimentation.** Splicing reaction mixtures (100 $\mu$l) were sedimented through 11-ml 10–30% (vol/vol) glycerol gradients (14) containing 100 mM KCl, 20 mM Hepes/KOH (pH 7.6), and either 1.5 mM $MgCl_2$ for a standard reaction or 2.5 mM EDTA for the blocked complex. These were centrifuged at 40,000 rpm in a Beckman SW41 rotor (197,000 × *g*) for 4 hr. Fractions of ≈400 $\mu$l were collected and analyzed by Cerenkov counting.

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Abbreviations: FS-I, functional spliceosome, intermediate; FS-P, functional spliceosome, precursor; snRNP, small nuclear ribonucleoprotein.

**Gel Filtration Chromatography.** Gel filtration chromatography was conducted as described (15). For these studies, a 2-ml reaction volume was applied to a Sephacryl S-500 column (1.5 cm × 110 cm) equilibrated with 20 mM Hepes/KOH (pH 7.6) containing 50 mM KCl, 0.1% Triton X-100, and 2.5 mM EDTA. Fractions of 1.6 ml were collected at a rate of 2 fractions per hour. Column fractions were stored at 4°C.

**Immunoprecipitation of Purified Spliceosomes.** Anti-Sm (gift from J. Steitz), anti-U1 (gift from S. Hoch), and rabbit nonimmune IgG were allowed to bind to protein A immobilized on Trisacryl (Pierce). Aliquots of the gel filtration SS peak were then incubated with the immobilized antibodies as described (15).

## RESULTS

**A Complex Containing Unspliced Pre-mRNA Is Formed in the Presence of EDTA.** In an effort to establish conditions that block the pathway of splicing-complex assembly at different steps, we examined spliceosome assembly under a variety of conditions. Based on the observation that EDTA treatment of the yeast *in vitro* splicing reaction results in the assembly of a spliceosome containing unspliced pre-mRNA (10), we examined the effect of EDTA on the mammalian splicing reaction. An mRNA precursor containing human β-globin exons 1 and 2 and intron 1 (T7-Hβ pre-mRNA; ref. 15) was incubated for 1 hr in HeLa cell nuclear extracts that had been preincubated with 0.5, 1, 2.5, or 4 mM EDTA on ice for 30 min. We found that 0.5 mM EDTA was insufficient to block the splicing reaction in the presence of 3 mM $MgCl_2$ (Fig. 1, lane 1). However, splicing was inhibited by higher concentrations of EDTA (lanes 2–4).

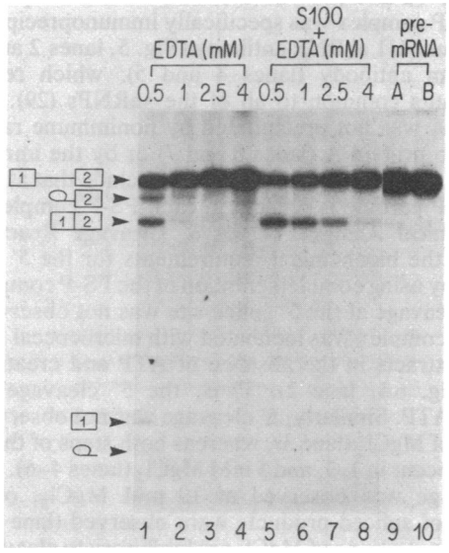


FIG. 1. EDTA blocks the splicing reaction but not the assembly of a functional splicing complex. Nuclear extracts were preincubated in 0.5, 1, 2.5, or 4 mM EDTA for 30 min on ice. $^{32}$P-labeled T7-Hβ pre-mRNA was then incubated in these extracts under splicing conditions for 1 hr at 30°C. A 2-μl aliquot of each reaction mixture was examined by electrophoresis in a denaturing polyacrylamide gel (lanes 1–4). Another 2-μl aliquot of each reaction was diluted into a 50-μl splicing mixture containing cytoplasmic S100 extract and incubated for an additional hour (lanes 5–8). Naked pre-mRNA was incubated under standard conditions for 2 hr in S100 extract (lane 9) or S100 extract containing 2 μl of nuclear extract (lane 10). Structures of the splicing intermediates and products are indicated at left (boxes represent exons 1 and 2; line represents the intron, which is looped to form a lariat structure).

To determine whether spliced RNA can be generated by complementing the EDTA-blocked splicing reaction, a 2-μl aliquot of each reaction mixture whose products are shown in Fig. 1, lanes 1–4, was diluted into a 50-μl splicing mixture containing the S100 extract and was incubated for 1 hr. As expected from previous studies (17), pre-mRNA was not spliced in the S100 extract alone (lane 9), or in an S100 extract containing 2 μl of nuclear extract (lane 10). In contrast, the splicing reactions blocked by 1 or 2.5 mM EDTA were complemented by the S100 extract (lanes 6 and 7), whereas reactions blocked by EDTA concentrations of 4 mM or higher could not be complemented by S100 (lanes 4 and 8). These results suggest that a stable splicing complex is formed in nuclear extracts preincubated in 1 or 2.5 mM EDTA.

**The FS-P Complex Is a 60S Particle.** To determine whether discrete splicing complexes were assembled in the presence of EDTA, the splicing reaction mixtures were analyzed by glycerol gradient sedimentation. As expected, a 60S spliceosome peak was observed in a standard splicing mixture (Fig. 2). A 60S peak was also observed in a splicing mixture blocked by 2.5 mM EDTA. Analysis of the $^{32}$P-labeled RNA species in the 60S peaks showed that the complexes formed in the normal splicing reaction contained unspliced precursor, splicing intermediates, and spliced product, whereas the complex formed in the EDTA-treated extracts contained only unspliced precursor (data not shown). We refer to the latter 60S complex as FS-P. We conclude that, as in yeast (10), the presence of EDTA in the splicing mixture allows spliceosome assembly but blocks 5′ cleavage and lariat information.

A peak corresponding to the previously described 30–40S complex (4, 14) was also observed in the standard splicing mixture, whereas a slightly larger complex was formed in the presence of EDTA (Fig. 2). The significance of this difference is not known.

**The FS-P Complex Purified by Gel Filtration Chromatography Is Functional.** The FS-P complex was fractionated on a 110-cm Sephacryl S-500 gel filtration column under condi-

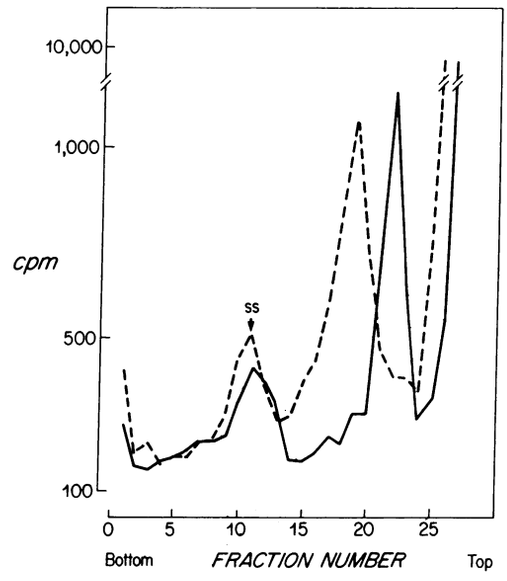


FIG. 2. A 60S spliceosome is assembled in EDTA-blocked splicing mixtures. $^{32}$P-labeled T7-Hβ pre-mRNA was incubated under standard splicing conditions at 30°C for 40 min (solid line), or under splicing conditions in the presence of 2.5 mM EDTA at 30°C for 1 hr (broken line). Reaction mixtures were then sedimented through glycerol gradients. Fractions were collected, and the distribution of $^{32}$P-labeled material was determined by Cerenkov counting. SS, spliceosome peak. The peaks in fraction 18 (broken line) and 22 (solid line) are probably analogous to the 30–40S complexes described previously (4, 14).

tions previously established for the purification of the FS-I complex (15). The distribution of $^{32}$P-labeled T7-H$\beta$ pre-mRNA in the column fractions is shown in Fig. 3*A*. Examination of the fractions by electrophoresis in denaturing polyacrylamide gels (*Inset*) revealed that unspliced precursor was present in the void volume ($V_o$) and in the peak designated SS. The peak designated $V_t$ contained free $^{32}$P-labeled nucleotides generated by RNA degradation during the splicing reaction. The distribution of protein in the column fractions is shown in Fig. 3*B*. Most of the protein was eluted as a single peak that was clearly separated from the SS peak. The distribution of RNA and protein in these fractions is similar to that observed when the FS-I complex was fractionated by gel filtration chromatography (15).

To determine whether the purified FS-P complex is functional, we incubated fractions from the SS peak with S100 extract or micrococcal nuclease-treated nuclear extract, conditions under which naked pre-mRNA is not spliced (17). Splicing intermediates and products were generated in either extract with the FS-P complex (Fig. 4*A*, lanes 2 and 3), but not with naked pre-mRNA (lanes 4 and 5). Spliced RNA was also generated by complementation of the FS-P complex isolated by glycerol gradient sedimentation (data not shown). These observations suggest that the FS-P complex is functional. Alternatively, however, it is possible that the pre-mRNA within the FS-P complex can dissociate and then reassemble with factors present in the SS peak and the complementing extract.

This possibility was tested by mixing experiments in which naked rabbit $\beta$-globin pre-mRNA was added to the comple-

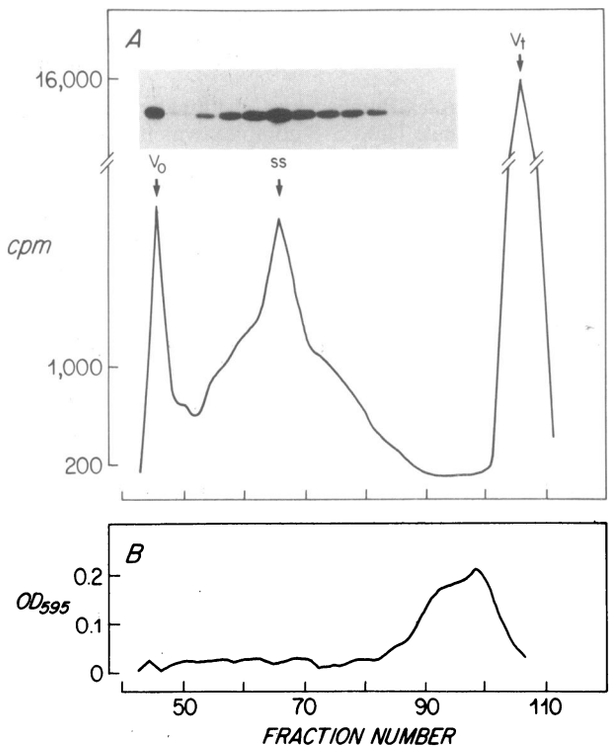


FIG. 3. Fractionation of the FS-P complex by gel filtration chromatography. Nuclear extract was preincubated with 2.5 mM EDTA on ice and then incubated with $^{32}$P-labeled T7-H$\beta$ RNA under splicing conditions. A 2-ml reaction volume was applied to a Sephacryl S-500 gel filtration column. (*A*) The profile of $^{32}$P-labeled material was determined by Cerenkov counting of 50-$\mu$l aliquots. $V_o$, void volume; SS, spliceosome; $V_t$, total column volume. $^{32}$P-labeled pre-mRNA in selected column fractions was analyzed by denaturing polyacrylamide gel electrophoresis (*Inset*). (*B*) The protein distribution was determined by Bradford (23) microassay of 20-$\mu$l aliquots of each fraction.

mentation assay. In control experiments, both the pre-mRNA in the FS-P complex and the naked pre-mRNA were spliced when incubated with the standard nuclear extract (Fig. 4*B*, lane 6). In contrast, only the RNA in the SS peak was spliced when the substrates were incubated with a micrococcal nuclease-treated extract (lane 7). We conclude that the FS-P complex is a functional intermediate in the splicing reaction. We note, however, that the FS-P complex does not contain all of the factors required for 5′ cleavage, since this activity was not observed when the complex was incubated under splicing conditions in the absence of extract (data not shown).

The complementation experiments (Fig. 4*A*) indicate that the factor(s) required for 5′ cleavage is present in the S100 and micrococcal nuclease-treated extracts. We found that this activity is relatively heat-stable, since it was resistant to incubation at 50°C for 10 min (Fig. 4*C*, lane 12). However, the heat treatment appeared to inactivate at least one factor that is required for spliceosome assembly, since splicing intermediates were not generated when naked pre-mRNA was incubated in the heat-treated extract (lane 17). An interesting consequence of this heat treatment is that the pre-mRNA is significantly more stable, possibly because a ribonuclease has been inactivated (Fig. 4*C*). This increase in stability provides a more sensitive complementation assay for the 5′ cleavage activity.

**The FS-P Complex Contains U1 snRNP.** U1 snRNP is associated with functional 60S spliceosomes purified by glycerol gradient sedimentation (14, 24) or by gel filtration chromatography (15). The presence of this snRNP in splicing complexes fractionated by nondenaturing gel electrophoresis or affinity chromatography depends on the conditions used (7–9, 12, 13, 25–27). To determine whether U1-snRNP is associated with the FS-P complex, we carried out immunoprecipitation experiments using a monoclonal antibody to the U1 snRNP 70-kDa protein (28). The $^{32}$P-labeled pre-mRNA in the FS-P complex was specifically immunoprecipitated by both the anti-U1 snRNP antibody (Fig. 5, lanes 2 and 3) and an anti-Sm antibody (lanes 4 and 5), which recognizes determinants common to all of the snRNPs (29). Labeled pre-mRNA was not precipitated by nonimmune rabbit IgG coupled to protein A (lanes 6 and 7) or by the immobilized protein A alone (lanes 8 and 9). We conclude that at least one U1 snRNP particle is present in each FS-P complex.

**Biochemical Analysis of the 5′ Cleavage Reaction.** We analyzed the biochemical requirements for the 5′ cleavage reaction by using complementation of the FS-P complex as an assay. Cleavage at the 5′ splice site was not observed when the FS-P complex was incubated with micrococcal nuclease-treated extracts in the absence of ATP and creatine phosphate (Fig. 6*A*, lane 2). Thus, the 5′ cleavage reaction requires ATP. Similarly, 5′ cleavage was not observed in the absence of $MgCl_2$ (lane 3), whereas both steps of the splicing reaction occur in 1, 3, and 5 mM $MgCl_2$ (lanes 4–6). Although 5′ cleavage was observed at 10 mM $MgCl_2$, only trace amounts of spliced products were observed (lane 7). Thus, high concentrations of $MgCl_2$ are inhibitory to cleavage at the 3′, but not the 5′, splice site. We also examined the effect of another divalent cation, $Mn^{2+}$, on the 5′ cleavage reaction. In contrast to 1 mM $MgCl_2$, which resulted in cleavage at both 5′ and 3′ splice sites (Fig. 6, lane 4), 1 mM $MnCl_2$ gave only 5′ cleavage (lane 8). However, the ability of $MnCl_2$ to replace $MgCl_2$ appeared to be extract-dependent. Therefore, we cannot exclude the possibility that $Mn^{2+}$ was simply displacing bound $Mg^{2+}$ from components in the crude extract, making it available for the 5′ cleavage reaction.

Variation of the KCl concentration in the complementation assay demonstrated that efficient 5′ and 3′ cleavage occurs at concentrations ranging from 15 mM to 135 mM (data not shown). Finally, analysis of the time course of the 5′ cleavage

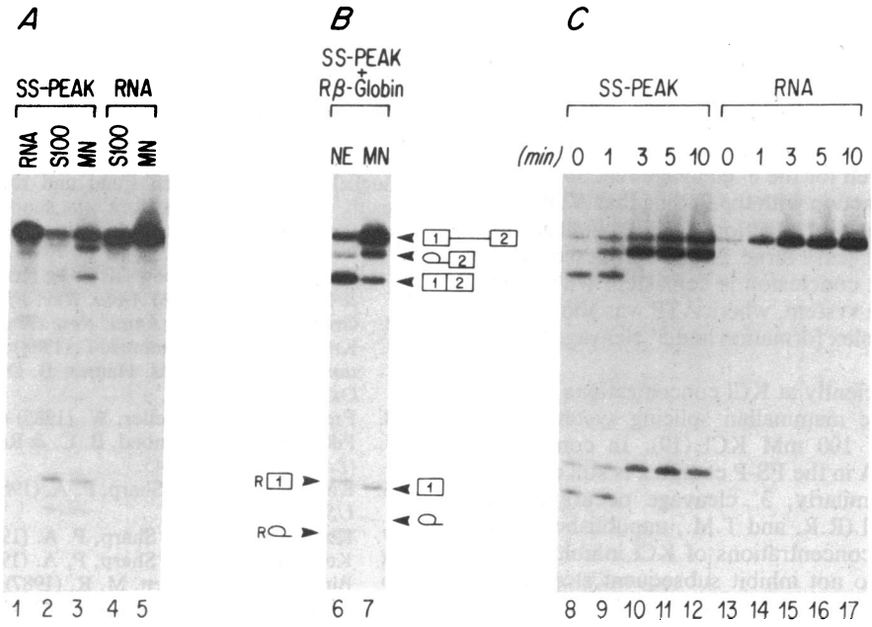


FIG. 4. The FS-P complex is a functional intermediate. (*A*) Aliquots of the SS peak from gel filtration were analyzed directly (lane 1), incubated with S100 extract (lane 2), or incubated with micrococcal nuclease-treated nuclear extract (MN, lane 3) under splicing conditions. Naked pre-mRNA was incubated under splicing conditions with S100 extract (lane 4) or micrococcal nuclease-treated nuclear extract (lane 5). Intact small nuclear RNAs (snRNAs) were not detected by ethidium bromide staining of nucleic acids prepared from micrococcal nuclease-treated nuclear extract (data not shown). (*B*) Aliquots of the SS peak were mixed with naked $^{32}$P-labeled rabbit β-globin (SP64-Rβ) pre-mRNA and incubated under splicing conditions with nuclear extract (NE, lane 6) or micrococcal nuclease-treated nuclear extract (lane 7). Structures of the splicing intermediates and products are indicated as in Fig. 1; R signifies rabbit. (*C*) Nuclear extract was heat-treated at 50°C for the indicated times. After centrifugation to remove precipitated protein, the extract was used to complement the SS peak (lanes 8–12) or naked RNA (lanes 13–17) under standard splicing conditions.

reaction revealed that splicing intermediates could be observed after incubation for only 10 min in micrococcal nuclease-treated nuclear extract. In contrast, spliced products were not detected until 60 min of incubation (Fig. 6*B*).

## DISCUSSION

We have identified an intermediate complex in the pathway of the mammalian splicing reaction. This 60S complex, which is formed in extracts treated with EDTA, contains unspliced pre-mRNA. Thus, EDTA blocks the first step of the splicing reaction but does not prevent spliceosome assembly. The FS-P complex is a functional intermediate in the splicing reaction, since it can be complemented by cytoplasmic S100 extract or micrococcal nuclease-treated nuclear extract. However, the complex does not contain all of the factors necessary for cleavage at the 5′ splice site, since no splicing was observed when the complex was incubated under splicing conditions in the absence of added extract.

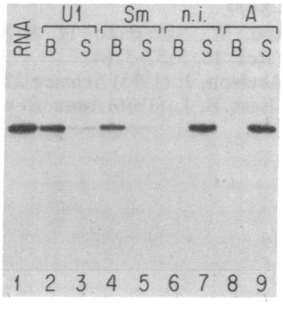


FIG. 5. The FS-P complex contains U1 snRNP. Aliquots of the SS peak were immunoprecipitated with the indicated antibodies, and total RNA was prepared from the bound (B) and supernatant (S) fractions. Lanes: 1, pre-mRNA from the SS-peak fraction; 2 and 3, anti-U1 snRNP; 4 and 5, anti-Sm; 6 and 7, rabbit nonimmune IgG; lanes 8 and 9, immobilized protein A alone.

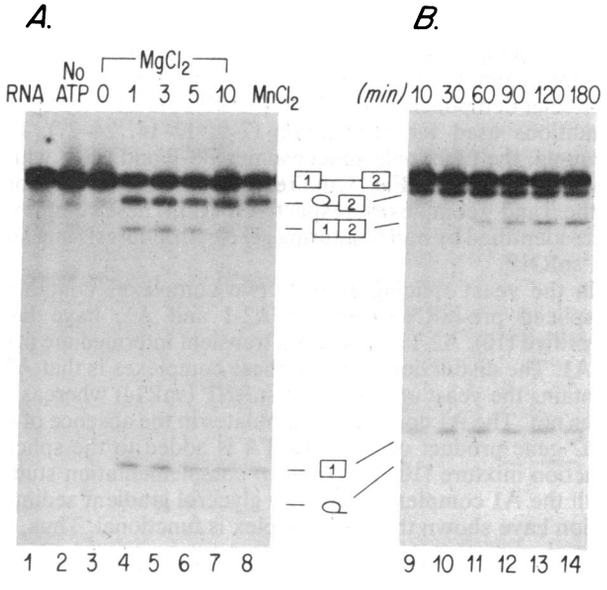


FIG. 6. The 5′ cleavage reaction requires ATP and $MgCl_2$ or $MnCl_2$. (*A*) Aliquots of the SS peak were analyzed directly (lane 1), or incubated with micrococcal nuclease-treated nuclear extract in the absence of ATP and creatine phosphate (lane 2). Fractions were complemented with micrococcal nuclease-treated nuclear extract in the absence of $MgCl_2$ (lane 3), in the presence of 1, 3, 5, or 10 mM $MgCl_2$ (lanes 4–7, respectively), or in the presence of 1 mM $MnCl_2$ (lane 8). (*B*) Aliquots of the SS peak were incubated with micrococcal nuclease-treated nuclear extract under splicing conditions for the indicated periods of time at 30°C. Structures of splicing intermediates and products are shown as in Fig. 1.

**Biochemical Requirements for Cleavage at the 5′ Splice Site.** Preparative purification and complementation of the FS-P complex provided the opportunity to study the requirements for the 5′ cleavage reaction independent of the requirements for spliceosome assembly. Previous studies have shown that ATP is required for spliceosome assembly (4, 14). We found that ATP is also required for the 5′ cleavage reaction. These observations, in conjunction with the finding that ATP is also required for the 3′ cleavage reaction (15, 30), indicate that ATP is required for at least three distinct processes in the splicing reaction. This conclusion is consistent with results from the yeast splicing system, where ATP was shown to be required for both complex formation and 5′ cleavage (10, 16, 31).

Splicing occurs efficiently at KCl concentrations between 10 and 60 mM in the mammalian splicing system but is severely inhibited by 100 mM KCl (19). In contrast, 5′ cleavage of pre-mRNA in the FS-P complex is still observed at 135 mM KCl. Similarly, 3′ cleavage occurs at high concentrations of KCl (R.R. and T.M., unpublished observations). Thus, high concentrations of KCl inhibit spliceosome assembly but do not inhibit subsequent steps in the splicing reaction.

Analyses of the divalent cation requirements for the first and second steps of the splicing reaction revealed significant differences. The 5′ cleavage reaction occurs over a broader range of $MgCl_2$ concentrations than 3′ cleavage, and $MnCl_2$ appears to satisfy requirements of 5′ but not 3′ cleavage. However, we cannot conclude that $MnCl_2$ is able to fulfill the requirements of 5′ cleavage, due to the complexity of the crude extracts. $MgCl_2$ and $MnCl_2$ are also able to satisfy the divalent cation requirement in self-splicing of *Tetrahymena* pre-mRNA and ribonuclease P cleavage of tRNA (32).

**Relationship Between FS-P and Previously Identified Splicing Complexes.** Putative intermediate complexes in the splicing pathway have been characterized by density gradient sedimentation, nondenaturing gel electrophoresis, affinity chromatography, and gel filtration chromatography. However, splicing complexes fractionated by these different methods cannot be compared directly, since the presence of particular components, such as U1 snRNP, is affected by the conditions used for purification (7–9, 12–14, 24–27). For example, the functional spliceosomes FS-P and FS-I, which contain U1 snRNP (Fig. 5 and ref. 15), probably correspond to the $\beta$ and $\gamma$ complexes, respectively (12). These complexes were identified by nondenaturing gel electrophoresis and lack U1 snRNP.

In the yeast splicing system, two complexes containing unspliced pre-mRNA, termed A2-1 and A1, have been identified (10). A2-1 appears as a transient intermediate prior to A1. The distinction between these complexes is that A2-1 contains the yeast analog of U4 snRNP (snR14) whereas A1 does not. The A1 complex accumulates in the absence of the *rna2* gene product or when EDTA is added to the splicing reaction mixture (10, 16). *In vitro* complementation studies with the A1 complex isolated by glycerol gradient sedimentation have shown that this complex is functional. Thus, the FS-P complex formed by EDTA treatment of mammalian splicing extracts is most likely analogous to the yeast A1 complex.

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