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Fractionation and characterization of a yeast mRNA splicing extract

(small nuclear RNAs/"spliceosome")

SOO-CHEN CHENG AND JOHN ABELSON

Division of Biology 147-75, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT **We have fractionated a yeast whole cell extract that can accurately splice synthetic actin and CYH2 pre-mRNAs. Three fractions, designated I, II, and III, have been separated by use of ammonium sulfate fractionation and chromatography on heparin agarose. Each fraction alone has no splicing activity. Fractions I and II allow the first step of the splicing reaction to proceed, giving rise to the splicing intermediates, free exon 1, and intron-exon 2. Addition of fraction III completes the reaction. Micrococcal nuclease treatment of the whole cell extract or of either fraction I or II abolished splicing activity, indicating that fractions I and II have RNA moieties that are required in the splicing reaction. The nature of the RNAs was examined using antibodies directed against the trimethylated cap structure unique to small nuclear RNAs. Preincubation of the whole cell extract with protein A-Sepharose coupled to trimethylated cap antibody abolished splicing activity. This indicates that at least one essential RNA component contains a trimethyl cap. Thus, in yeast as in mammalian systems, small nuclear RNAs are involved in mRNA splicing.**

The recent development of *in vitro* splicing systems has facilitated understanding of the mechanism of pre-mRNA splicing (1–7). Characterization of the spliced products and intermediates has led to a two-step model for mRNA splicing (8, 9). The first step involves cleavage at the 5′ splice site and the formation of a lariat structure in which the 5′ end of the intervening sequence (IVS) is joined in a 2′-5′ phosphodiester linkage to a specific residue a short distance upstream from the 3′ splice site (reaction 1). In the second step, the 3′ splice site is cleaved, and the two exons are joined (reaction 2). Analysis of mutant pre-mRNAs with specific intron mutations supports such a model (10, U. Vijayraghaven, R. Parker, J. Rossi, J. Tamm, Y. Iimuva, J. A. & C. Guthrie, unpublished work). Little further is known about the mechanism of mRNA splicing. ATP is required for the reaction to proceed (5–7), but its exact role has not been determined.

In mammalian systems some small nuclear ribonucleoproteins (snRNPs) are essential components of the splicing machinery (11–17). The U1 snRNP has been implicated in the recognition of the 5′ splice junction and the U2 snRNP with the site for branch formation near the 3′ splice junction (14). Since snRNPs contain an RNA molecule and at least 5 or 6 proteins (18), it could be anticipated that the mRNA splicing machine would be large and complex. Evidence that this is so has come from the discovery that the splicing intermediates are found on a large particle (40S in yeast) termed the "spliceosome" (19–21). The constituents of the spliceosome are as yet unknown.

To understand the precise mechanism of mRNA splicing, it will be necessary to identify the components involved in the splicing reaction. We have undertaken fractionation of a yeast whole cell extract and have separated three fractions essential for mRNA splicing. We also show that a small nuclear RNA (snRNA) is associated with at least one of the components and is required for splicing.

MATERIALS AND METHODS

Materials. Bacteriophage SP6 RNA polymerase and micrococcal nuclease were obtained from Boehringer-Mannheim. Protein A-Sepharose was from Pharmacia Fine Chemicals. Anti-trimethyl cap [(m_3G)cap] antibody was a generous gift of Joan Steitz (Yale University, New Haven, CT) and also Reinhard Lührmann (Max-Planck Institute of Molecular Genetics, Berlin).

Fractionation of Yeast Whole Cell Extract. The following $(NH_4)_2SO_4$ fractionation procedure was for a 1-liter yeast culture (2–3 × 10^7 cells/ml), and the percentage of $(NH_4)_2SO_4$ represents percentage saturation at 25°C. All $(NH_4)_2SO_4$ precipitations were performed at 0°C with stirring for 30 min followed by centrifugation at 4°C in a Sorvall SS-34 rotor at 17,000 rpm for 20 min. The yeast whole cell extract was prepared according to Newman *et al.* (10). Solid $(NH_4)_2SO_4$ was added to the supernatant from the 100,000 × *g* centrifugation to yield a 70% saturated solution. The precipitate was collected by centrifugation and resuspended in 10 ml of 40% saturated $(NH_4)_2SO_4$, prepared by dissolving 24.2 g of solid $(NH_4)_2SO_4$ in 100 ml of 20 mM Hepes (pH 7.9), 0.2 mM EDTA, and 0.5 mM dithiothreitol. After centrifugation, the soluble fraction (40W) was concentrated by precipitation with 70% saturated $(NH_4)_2SO_4$, and the pellet was dissolved in 4 ml of 20 mM Hepes (pH 7.9), 0.2 mM EDTA, 0.5 mM dithiothreitol, 50 mM KCl, 20% (vol/vol) glycerol (buffer D). The 40% saturated $(NH_4)_2SO_4$ insoluble fraction (40P1) was extracted twice more with 10 ml of 40% saturated $(NH_4)_2SO_4$. The final pellet (40P3) was then dissolved in 0.15 ml of buffer D. All fractions were then dialyzed for 3–4 hr against two changes of 1 liter of buffer D followed by centrifugation in an Eppendorf microfuge for 10 min to remove small amounts of insoluble material and then stored at −70°C in aliquots.

Fraction 40W (20 mg) was diluted by a factor of 8 with 20 mM Hepes (pH 7.9), 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% (vol/vol) glycerol (buffer I) and then applied to a 1-ml heparin-agarose column preequilibrated with 0.05 M NaCl in buffer I. After collection of the flow through fraction (0.05 M), the column was washed with 3–5 column volumes of 0.05 M NaCl in buffer I. The column was then eluted successively with 0.25 M and 0.5 M NaCl in buffer I. Fractions in 0.05 M and 0.5 M NaCl were then stored at −70°C in aliquots.

Splicing Assay. Splicing assays were according to Newman *et al.* (10) using uncapped actin pre-mRNA as substrate. In those assays containing several fractions, each fraction constituted 20% of the total reaction volume.

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Abbreviations: IVS, intervening sequence(s); snRNP, small nuclear ribonucleoprotein; snRNA, small nuclear RNA; trimethyl cap, (m_3G) cap.

Micrococcal Nuclease Reactions. Micrococcal nuclease digestions were carried out in a reaction mixture containing 2 mM $CaCl_2$, 8 units of micrococcal nuclease, and 10 μl of whole cell extract in a total volume of 18 μl. Reactions were incubated at 30°C for 45 min and stopped by adding 0.8 μl of 50 mM EGTA. To 14 μl of this reaction mixture was added 6 μl of the splicing reaction mix (10) and 5 μl of the complementation fraction or buffer D to assay splicing activity.

Immunoprecipitation. Antiserum or preimmune serum was coupled to protein A-Sepharose by incubation of 2 μl of serum (20 mg/ml) with 0.5 mg of protein A-Sepharose at room temperature for 1 hr in a total volume of 0.1 ml containing 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 0.05% Nonidet P-40. The material was then washed three times with 0.2 ml of the same buffer and twice with 0.2 ml of buffer without Nonidet P-40. For depletion assays, 25 μl of whole cell extract diluted 1:3 was incubated with the antibody-coupled protein A-Sepharose at 4°C for 1 hr. After removing protein A-Sepharose by centrifugation in a microfuge for 5 sec, the supernatant was assayed for splicing activity. For analysis of spliced RNA bound to antibody, splicing reactions were carried out at 23°C for 10 min in a total volume of 25 μl containing 5 μl each of fractions 40P3 and 40W. Reactions were then added to the antibody-coupled protein A-Sepharose on ice followed by incubation at 4°C for 1 hr. After removing the supernatant, the protein A-Sepharose was washed four times with 25 μl of 50 mM Tris·HCl (pH 7.4), 150 mM NaCl at 4°C and then subjected to phenol/chloroform extraction and ethanol precipitation followed by RNA analysis (10).

RESULTS

Fractionation of the Whole Cell Extract. The results of the fractionation experiments are summarized in Fig. 1 and are discussed in detail below. In summary, three fractions required for pre-mRNA splicing have been separated by use of ammonium sulfate fractionation and heparin-agarose chromatography. They are designated fractions I, II, and III. The fractionation scheme is shown in Fig. 1*A* and the involvement of these fractions in the splicing pathway in Fig. 1*B*. Each fraction by itself does not contain any splicing activity. A mixture of fractions I and II can carry out the first step of the splicing reaction, and a combination of all three fractions gives the complete reaction (Fig. 1*B*).

The whole cell extract was precipitated with 70% saturated $(NH_4)_2SO_4$ followed by back extraction of the pellet with 40% saturated $(NH_4)_2SO_4$. The insoluble fraction 40P1 gave a reaction in which the two splicing intermediates, free exon 1 and lariat intron-exon 2, accumulated (Fig. 2*A*). The reaction

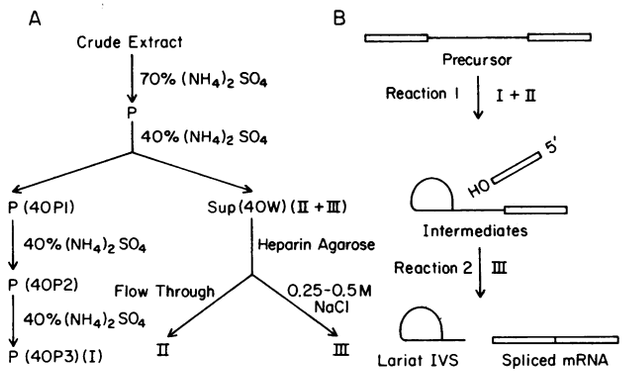

FIG. 1. (*A*) Scheme of fractionation of the mRNA splicing extract. (*B*) The mRNA splicing pathway and the involvement of fractions I, II, and III in the pathway.

in crude extract or 70P accumulated only small amounts of the intermediates (Fig. 2*A*). This indicates that the 40P1 fraction is deficient in some component responsible for the second step of the splicing reaction. Fraction 40W, which alone did not show any activity (Fig. 2*A*), when added to 40P1, restored complete splicing activity without accumulation of intermediates (Fig. 2*A*). To completely eliminate reaction 2 activity from 40P1, the fraction was extracted twice with 40% saturated $(NH_4)_2SO_4$, yielding the insoluble fraction 40P3. The 40P3 fraction did not have any splicing activity on its own (Fig. 2*A*). However, addition of 40W to 40P3 restored full activity (Fig. 2*A*), indicating both 40P3 and 40W were essential for splicing although each alone showed no activity. The fact that both reaction 1 and 2 activities in fraction 40P1 could be removed by two more 40% saturated $(NH_4)_2SO_4$ washings indicated that in the 40P1 fraction, only a small amount of some components required for reaction 1 and 2 remained. The accumulation of splicing intermediates in the 40P1 reaction suggests that the reaction 2 activity was more depleted than reaction 1 activity in the 40P1 fraction. This also indicated the two activities were conferred by at least two distinct components, the majority of which were present in the 40W fraction. We, therefore, named 40P3 fraction I and fractionated further the 40W fraction.

Heparin-agarose chromatography separated fraction 40W into two fractions (Fig. 2*B*). The flow through fraction (0.05 M NaCl) gave mainly reaction 1 when assayed in the presence of fraction I (lane 3), and was named fraction II. Addition of the fraction eluted from heparin-agarose column between 0.25 M and 0.5 M NaCl (fraction III) also gave reaction 2 (lane 6). As shown in Fig. 2*B*, the 0.5 M NaCl eluate gave a small amount of activity without accumulation of intermediates when fraction I but not fraction II was included in the assay (lane 5). When the flow through fraction (fraction II) was also added, it showed much higher activity (lane 6). Fraction III, therefore, contained small amounts of the activity required for reaction 1, allowing reaction 2 to proceed. On the other hand, fraction II also contained a small amount of reaction 2 activity (lane 3). It is possible that there is an affinity between essential components in fraction II plus III. As a consequence, the activities characteristic of fraction II plus III cannot be totally separated.

Involvement of RNA Components. In mammalian systems, snRNP are required for mRNA splicing (11–13, 15–17, 22). Although in yeast snRNPs have not been identified as such, snRNAs have been shown to exist by Wise *et al.* (23). To examine whether an RNA component is involved in yeast mRNA splicing, we first tested the sensitivity of the splicing extract to micrococcal nuclease treatment. Pretreatment of the whole cell extract with micrococcal nuclease in the presence of $CaCl_2$ abolished all the splicing activity (Fig. 3). In a control, preincubation was carried out in the presence of EGTA as well as $CaCl_2$, and the extract remained active. Thus loss of activity after micrococcal nuclease treatment was not due to an inhibitor present in the micrococcal nuclease fraction or incomplete inactivation of micrococcal nuclease by EGTA. Since splicing activity was not affected by pancreatic deoxyribonuclease at 10 μg/ml (76 units/μg of DNase I, Calbiochem), we conclude that the whole cell extract contains some RNA component essential for splicing. To determine which of the three fractions contains RNA, they were added to a micrococcal nuclease-treated extract to test for complementation (Fig. 3). Addition of fraction I alone or II plus III (40W) did not restore splicing activity, while addition of fractions I and II restored full activity. This indicated that both fractions I and II contain sensitive RNA moieties that were inactivated after micrococcal nuclease treatment, whereas fraction III was not. It is possible that fractions I and II contain components tightly associated in the whole cell extract, so that even though only one fraction was

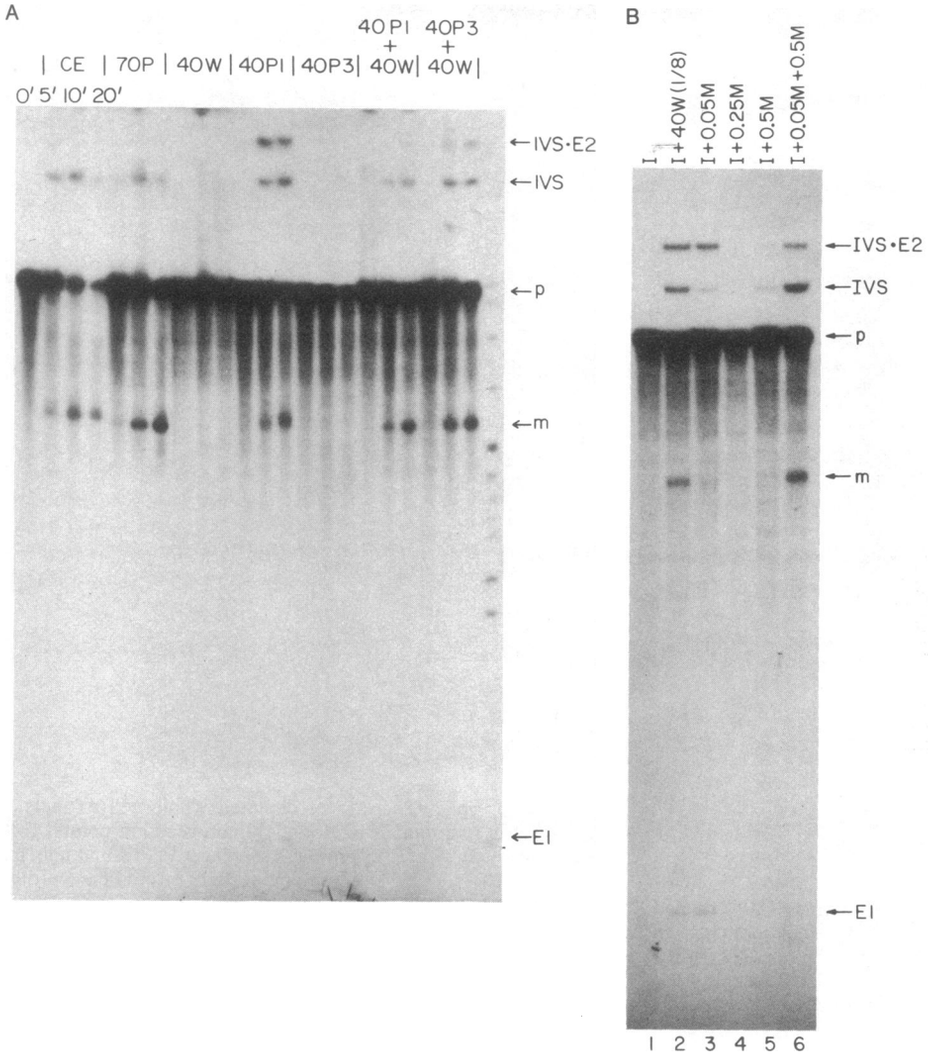

FIG. 2. Splicing assays of the fractions. (*A*) Ammonium sulfate fractionation of the whole cell extract. Each assay shows 5-min, 10-min, and 20-min reactions. CE, crude extract; 70P, 70% saturated $(NH_4)_2SO_4$ precipitate; 40P1, precipitate of 70P extracted with 40% saturated $(NH_4)_2SO_4$ once; 40W, supernatant of 70P extracted with 40% saturated $(NH_4)_2SO_4$; 40P3, precipitate of 70P extracted with 40% saturated $(NH_4)_2SO_4$ three times. (*B*) Heparin-agarose chromatography of 40W fraction. Lane 1: fraction I alone. Lanes 2–6 fraction I was added to the following: lane 2, 40W fraction diluted 1:8; lane 3, 0.05 M NaCl eluate; lane 4, 0.25 M NaCl eluate; lane 5, 0.5 M NaCl eluate; lane 6, 0.05 M NaCl eluate plus 0.5 M NaCl eluate. IVS·E2, intron-exon 2; IVS, intron; p, precursor; m, message; E1, exon 1.

inactivated, both fractions had to be added to restore activity. Thus it was necessary to test the micrococcal nuclease sensitivity with separated fractions I and II. Pretreatment of fraction I or II plus III (40W) with micrococcal nuclease abolished ability of each to yield spliced RNA or intermediates (data not shown). The activity could be restored by addition of the missing fraction. This suggests that both fractions I and II contain a micrococcal nuclease sensitive component, most likely an RNA molecule.

Depletion of Components Required for Splicing by Anti-(m_3G)cap Antiserum. It has been demonstrated that antibodies directed against U1 snRNP inhibit pre-mRNA splicing in a mammalian cell free system (11, 15). One of the features of the U-class of snRNAs is the presence of a distinctive cap, a trimethylated guanosine residue linked to the 5'-encoded residue by a 5'-5' triphosphate bridge. Antibodies directed against the (m_3G)cap inhibit splicing in the mammalian system (12). Guthrie *et al.* (24) have shown that the anti-(m_3G)cap antibody prepared by Chabot *et al.* (25) precipitates yeast small nuclear RNAs. These RNAs were shown to contain a mixture of dimethyl and trimethyl guanosine caps (23). Since both fractions I and II appeared to contain RNA moieties, it was possible that they were snRNAs. To investigate the possible involvement of snRNA in pre-mRNA splicing in yeast, we performed "depletion experiments" using anti-(m_3G)cap antibody (12). Preincubation of the whole cell extract with anti-(m_3G)cap antibody coupled to protein A-Sepharose followed by removal of the protein A-Sepharose precipitate resulted in a 80–90% loss of splicing activity (Fig. 4). Preincubation with the pre-immune serum did not show any significant effect. This suggests that in the whole cell extract, some component required for splicing reacted with the antibody and was removed from the extract after precipitation. To determine which fraction contains snRNAs, we performed experiments with fraction I and 40W (fraction II plus III). As in the whole cell extract, fraction I lost its splicing activity after preincubation with the antibody (data not shown), presumably due to depletion of snRNA from the fraction. Fraction II plus III (40W) showed no effect despite the fact that fraction II apparently contains an RNA moiety by the criteria of micrococcal nuclease sensitivity.

A Splicing Complex Associated with snRNA. As demonstrated by Black *et al.* (13), in mammalian systems both U1 and U2 snRNPs interact with the pre-mRNA during the splicing reaction. The complexes of pre-mRNA and U1 or U2 snRNP can be immunoprecipitated by antibodies directed against U1 or U2 snRNPs. Although snRNPs have not been characterized in yeast, it is likely that they exist since yeast has snRNAs. Anti-(m_3G)cap antibody was used to investigate a possible specific interaction between the snRNA and pre-mRNA and spliced products. Fig. 5*A* shows the experimental procedure. A splicing reaction mixture containing labeled pre-mRNA was incubated for 10 min and then added to protein A-Sepharose-coupled anti-(m_3G)cap antibody. After removal of the unreacted material, RNA species bound to the antibody were recovered and analyzed by gel electrophoresis. As shown in Fig. 5*B*, approximately 20% of the pre-mRNA was immunoprecipitated from the splicing reac-

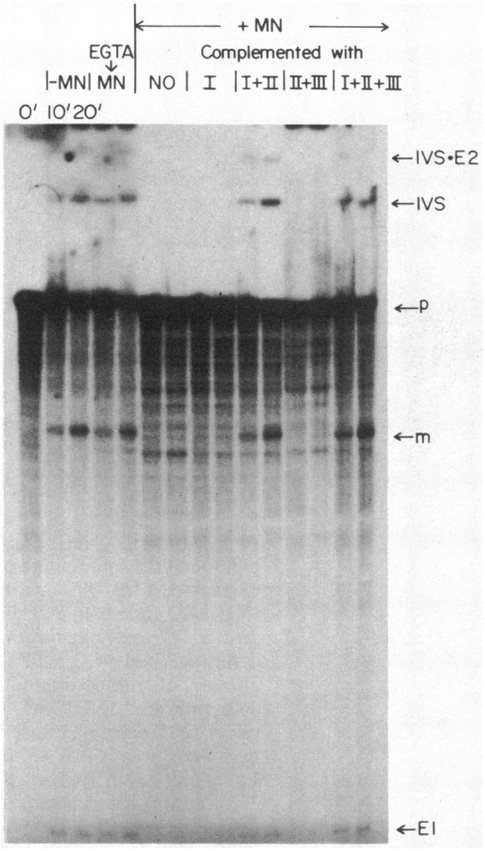

FIG. 3. Splicing assays of micrococcal nuclease (MN)-treated whole cell extracts. Each assay shows 10-min and 20-min reaction points. −MN, extract not treated with MN; EGTA→MN, extract treated with MN in the presence of EGTA; +MN, extracts pretreated with MN and complemented with fractions indicated.

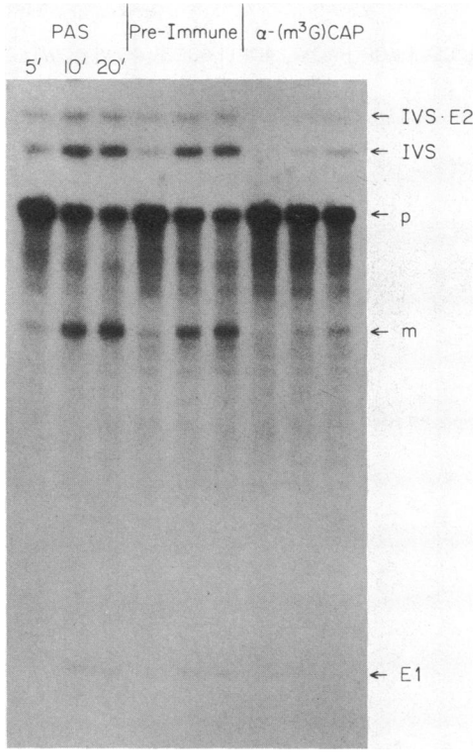

FIG. 4. Splicing assays of depleted whole cell extracts. Each assay shows 10-min, 20-min, and 30-min reaction points. Extracts were depleted with: PAS protein A-Sepharose; Pre-Immune, protein A-Sepharose-coupled pre-immune serum; α-(m_3G)cap, protein A-Sepharose-coupled α-(m_3G)cap antibody.

tion. The same percentage of the excised exon 1, lariat intron-exon 2, and IVS were also precipitated but only about 2% of mature message was precipitated. Formation of complexes specific to the mRNA splicing reaction has been shown by sedimentation analysis on glycerol or sucrose gradient in both yeast and mammalian systems (19–21). This complex, the spliceosome, contains pre-mRNA, exon 1, lariat IVS, and lariat IVS-exon 2. The spliced mRNA, however, is not found in the complex. Immunoprecipitation by anti-(m_3G)cap antibody of the same RNA species as found in the spliceosome suggests that the precipitate contained the complex or a related complex. These results suggest that a (m_3G)-capped snRNA, possibly as snRNP, forms part of the active complex.

DISCUSSION

By use of ammonium sulfate fractionation and heparin-agarose chromatography, we have identified three fractions required for yeast mRNA splicing. Each fraction alone does not show any splicing activity. Combination of fractions I and II allowed reaction 1 to proceed. Addition of fraction III allowed completion of the splicing reaction. Both fractions I and II are sensitive to micrococcal nuclease and so are likely to contain essential RNA moieties. The RNA in fraction I contains RNA with a cap structure since it can be depleted by preincubation with protein A-Sepharose-coupled anti-(m_3G)cap antibody. The possession of a (m_3G)cap by the RNA(s) in fraction I suggests that this fraction contains an snRNA (23). The fact that the pre-mRNA and splicing intermediates found in the 40S complex are also immunoprecipitable by anti-(m_3G)cap antibody suggests at least one snRNA possibly in the form of a ribonucleoprotein particle forms part of the 40S complex. In mammalian systems, both U1 and U2 snRNPs have been shown to be involved in pre-mRNA splicing (11–17). U1 snRNPs interact with the 5′ splice site (13, 26), and U2 snRNPs preferentially interact with a region of the pre-mRNA that includes the intron branch point (13). It is conceivable that in yeast snRNPs also interact with pre-mRNA and play a role in recognition of splicing signals.

Formation of the 40S complex requires that pre-mRNA undergo at least the first step of splicing reaction (20). Incubation of pre-mRNA with fraction I alone did not give the 40S complex on a glycerol gradient (G. McFarland and J.A., unpublished results), nor did it show significant immunoprecipitability by anti-(m_3G)cap antibody (unpublished results). Evidently, a component in fraction II is required together with components of fraction I for formation of the splicing complex. Since fraction II is required for the first step of the splicing reaction, it could be a multifunctional complex responsible for both recognition of splicing signals and catalytic cleavage of the 5′ splice site and formation of the lariat, or it could consist of several separable components that mediate those steps. The latter is not unlikely since at least six distinct factors necessary for pre-mRNA splicing *in vitro* have been identified in the mammalian system (14, 27). Alternatively, fraction II as well as fraction I may be required only for formation of the splicing complex, organizing the branch point and at least the 5′ splice site of the pre-mRNA into a proper configuration so that splicing can take place in an autocatalytic fashion.

Fraction II contains a micrococcal nuclease-sensitive component. The results of the depletion experiment, however, showed that fraction II could not be inactivated by preincubation with protein A-Sepharose-coupled anti-(m_3G)cap

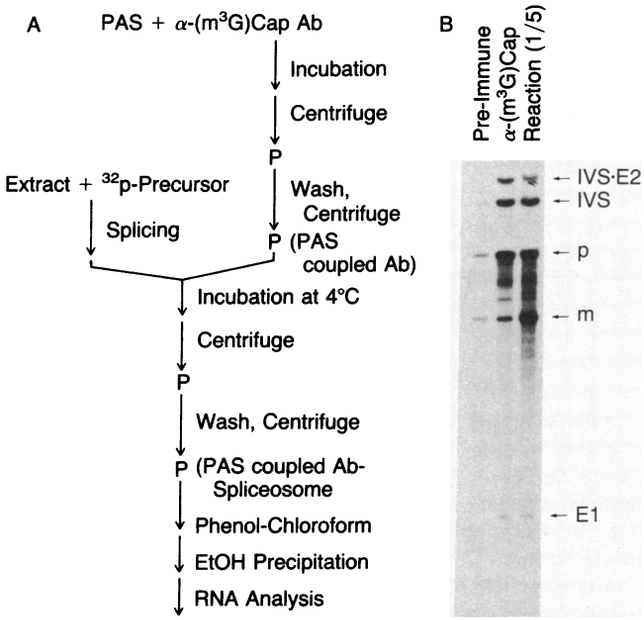

FIG. 5. (*A*) Scheme of immunoprecipitation of splicing reactions. (*B*) Splicing reactions. Pre-Immune, precipitated with protein A-Sepharose-coupled pre-immune serum; α-(m_3G)cap, precipitated with protein A-Sepharose-coupled α-(m_3G)cap antibody; Reaction(1/5), 1/5 volume of reaction not precipitated.

antibody, indicating that the RNA associated with it could not react with antibody. The nature of this RNA is not known. It is possible that the RNA is capped but is in such a configuration that it is not accessible to the antibody or that it is a molecule similar to snRNA but is not capped at the 5′ end as in the case of U6 snRNA (28, 29). One cannot exclude the possibility that this RNA represents a distinct species different from the U class snRNAs and plays a catalytic role in the splicing reaction. Catalytic RNAs have been demonstrated in ribonuclease P (30) and in self-splicing reactions (31, 32).

The activities that mediate steps 1 and 2 seem to be associated with each other as evidenced by their fractionation behavior: (*i*) They cofractionated from 40% to 70% saturated $(NH_4)_2SO_4$ (data not shown). (*ii*) The activities could be separated but never completely in several chromatographic steps. The amount of reaction 2 activity associated with fraction II diminished with increased dilution of the 40W fraction loaded on the column (data not shown). This suggested that reaction 2 activity dissociated from reaction 1 activity upon dilution of the 40W fraction and could then bind to the column. However, a small amount of reaction 2 activity that was still associated with reaction 1 activity also bound. We have not been able to completely separate the two activities.

The fact that a splicing complex could be precipitated by anti-(m_3G)cap antibody indicated that the complex contained a trimethylated cap accessible to the antibody. In the mammalian system, the 5′-terminal sequence of U1 snRNA has been proposed to base pair with the sequence of the intron of pre-mRNA at the 5′ splice site (12). Although the same phenomenon has not been demonstrated in yeast, by analogy it is likely that some yeast snRNA plays a role similar to U1 in recognition of the 5′ splice site. If this is the case, the accessibility of the cap to the antibody would indicate the 5′ terminus of this U1 like snRNA is not folded inside the complex and is still accessible to the antibody after pairing with the pre-mRNA, or else there may be some other capped snRNA in the complex providing the free cap for the antibody. In collaboration with C. Guthrie, N. Riedel, and B. Patterson (unpublished results), we have found that fraction I is greatly enriched in several of the yeast snRNAs. Investigation of these RNAs by both genetic and biochemical approaches should establish their role in the mRNA splicing reaction.

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