MOLECULAR AND CELLULAR BIOLOGY, Aug. 1987, p. 2877–2883
0270-7306/87/082877-07$02.00/0

Vol. 7, No. 8

U1 Small Nuclear Ribonucleoproteins Are Required Early during Spliceosome Assembly

MARTIN ZILLMANN, SCOTT D. ROSE, AND SUSAN M. BERGET*

Verna and Marrs McLean Department of Biochemistry, Baylor College and Medicine, Houston, Texas 77030

Received 22 October 1986/Accepted 6 May 1987

U1 small nuclear ribonucleoproteins (snRNPs) are required for in vitro splicing of pre-mRNA. Sequences within U1 RNA hybridize to, and thus recognize, 5′ splice junctions. We have investigated the mechanism of association of U1 snRNPs with the splicesome. U1-specific antibodies detected U1 association with precursor RNA early during assembly. Removal of the 5′ terminal sequences of U1 RNA by oligo-directed cleavage or removal of U1 snRNPs by immunoprecipitation prior to the addition of precursor RNA depressed the association of all snRNPs with precursor RNA as detected by immunoprecipitation of splicing complexes by either Sm or U1-specific antibodies. Assembly of the spliceosome as monitored by gel electrophoresis was also depressed after cleavage of U1 RNA. The dependency of Sm precipitability of precursor RNA upon the presence of U1 snRNPs suggests that U1 snRNPs participate in the early recognition of substrate RNAs by U2 to U6 snRNPs. Although removal of the 5′-terminal sequences of U1 depressed U1 snRNP association with precursor RNA, it did not eliminate it, suggesting semistable association of U1 snRNPs with the assembling spliceosome in the absence of U1 RNA hybridization. This association was not dependent upon 5′ splice junction sequences but was dependent upon 3′ intronic sequences, indicating that U1 snRNPs interact with factors recognizing 3′ intronic sequences. Mutual dependence of 5′ and 3′ recognition factors suggests significant snRNP-snRNP communication during early assembly.

Small nuclear ribonucleoproteins (snRNPs) recognize consensus sequence elements within pre-mRNA and target those sequences for cleavage and/or ligation by the splicing machinery (1, 3, 4, 6, 7, 9, 14–16). U1 and U2 snRNPs bind to splice junctions and branch point sequences, respectively; binding protects the consensus sequences from exogenous ribonuclease digestion (6, 7, 16). An Sm-reactive element, probably U5 snRNPs, recognizes 3′ splice junction sequences and protects the 3′ junction and adjacent polypyrimidine track against ribonuclease digestion (10, 23). The mechanism whereby individual snRNPs recognize their respective target sequences is under intensive investigation. Both splicing activity and protection of 5′ splice junctions of U1 snRNPs require the sequences of U1 RNA complementary to 5′ junction sequences (6, 7, 15, 24), leading to the suggestion that hybridization of U RNAs to precursor RNA is required for association of snRNPs with the spliceosome.

Studies of in vitro assembly of exogenous precursor RNA into spliceosomes (2, 5, 9, 11, 13, 19–21) indicate that assembly of exogenous precursor RNA into the active spliceosome is dependent on the presence of the consensus sequences recognized by U snRNPs. Substrates lacking all consensus sequences, however, assemble into RNP complexes, indicating that snRNPs may recognize splicing consensus sequences in precursor RNAs already complexed with heterogeneous nuclear RNP (hnRNP) RNP polypeptides (8, 22). Smaller assemblies are produced by using substrates lacking 3′ splice sites than by using substrates lacking 5′ splice sites, suggesting that sequences within the 3′ portion of the intron are recognized before those at the 5′ splice junction. Indeed, factors recognizing 3′ intronic sequences associate with precursor RNAs in the absence of association of U1 snRNPs with 5′ splice junctions (6, 7, 21).

The mechanism whereby U1 snRNPs remain a part of the spliceosome is also unclear. U1-specific antibodies are competent to immunoprecipitate both precursor and intermediate RNAs from active splicing extracts, suggesting stable association of U1 snRNPs with the spliceosome (2, 6, 7, 11). In contrast, certain separation techniques for purifying splicesomes have failed to indicate the presence of U1 snRNPs within isolated spliceosomes (12, 13). In the work reported in this communication we investigated the interaction of U1 snRNPs with precursor RNAs in in vitro splicing extracts. We document that U1 snRNPs are required to initiate stable association of snRNPs with the assembling spliceosome and that U1 snRNPs can associate with the spliceosome by mechanisms in addition to hybridization. We suggest that U1 snRNP proteins play an essential role in the assembly and maintenance of the spliceosome.

MATERIALS AND METHODS

Splicing reactions. Wild-type adenovirus precursor RNA of 442 nucleotides was produced by SP6 transcription of linearized IVS plasmid DNA (18) and contained the natural first and second exons from the late transcription unit separated by a shortened intron. A deletion substrate lacking the 5′ splice junction (denoted IVSΔ5′) was constructed by subcloning a segment of IVS beginning within the intron upstream of the lariat branch point. Truncation of the SP6 template DNA from this construction with *Bgl*I or *Sau*IIIa produced 193 or 133 (denoted IVSΔΔ5′) nucleotide transcripts, respectively, that contained or eliminated, respectively, the second 5′ splice junction beginning the truncated second intron in the precursor RNA. Substrate RNAs deleted for the 3′ half of the intron (denoted IVSΔ3′) were prepared by truncation of the IVS plasmid with *Sac*II to

* Corresponding author.

produce a 194-nucleotide transcript. Transcripts lacking all splicing consensus sequences were produced by SP6 transcription of *Pvu*II-digested SP6 DNA to produce a 235-nucleotide RNA.

Extract preparation and in vitro splicing reactions were performed as described previously (1). Oligonucleotide cleavage of specific U RNAs was done by incubation of extract under reaction conditions for 20 min in the presence of 0.02 OD_{260} of oligonucleotide and 10 U of RNase H per 0.015 ml of extract (1). Use of these conditions resulted in effective cleavage of over 95% of the target U RNAs and complete inhibition of splicing. Use of control oligonucleotides containing no sequences complementary to U RNAs had no effect on splicing even at 10-fold-higher concentrations. Cleavage of U2 and U4 RNA required the presence of ATP. U1 RNA was effectively cleaved in the absence of ATP. Cleavage reactions were substantially more reproducible if the final extract dialysis was into EDTA and extract was cleared with a 25,000 × *g* centrifugation for 30 min before storage.

Immunoprecipitation. Reaction mixtures were added to 0.001 to 0.005 ml of purified immunoglobulin G from either myeloma lines or serum samples from patients. The mixture was incubated for 30 min on ice. Prewashed Pansorbin (Calbiochem) was added for 5 min on ice. After addition of 0.20 ml of NET Mg: (0.05 M Tris hydrochloride [pH 7.9], 0.15 M NaCl, 0.05% Nonidet P-40, 0.5 mM dithiotreitol, 1.5 mM $MgCl_2$, the mixture was spun to collect immune complexes. Pellets were washed three times in NETM. RNA was prepared from the pellets and displayed on denaturing acrylamide gels. The control serum was a human immunoglobulin G fraction. Three U1-specific patient serum samples were used. Two of these (R-GD and R-PO) had activity against the U1-specific M_r 67,000 polypeptide on blots. No anti-Sm activity was detected with these serum samples even when they were used in substantially higher amounts than in the experiments described here. A third U1-specific serum sample (R-ED) had activity against the U1-specific A polypeptide. Used in the concentrations reported here, this antibody immunoprecipitated only U1 snRNPs. At 10-fold-higher concentrations, some anti-U2 activity was detected because of shared epitopes of U1- and U2-specific polypeptides. The Sm antibody was the Y12 monoclonal antibody. Anti-hnRNP C monoclonal 4F4 antibody was kindly provided by G. Dreyfuss (8). Used in the above amounts, each of these antibodies was competent to immunoprecipitate all of the appropriate U RNA and up to 30% of the radiolabeled precursor RNA added to the extract (see Fig. 1).

U1 depletion. To deplete extract for U1 snRNPs, we pretreated extract for 30 min on ice with RNP antibodies under reaction conditions except for the elimination of ATP and creatine phosphate. The amount of antibody used was slightly in excess of that required to immunoprecipitate all of the U1 RNA in the extract. Control human serum samples were used for pretreatment in control reactions. Immune complexes were collected by two cycles of Pansorbin treatment, with fresh Pansorbin washed with Roeder D Mg (20 mM Tris hydrochloride [pH 7.9], 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1.5 mM $MgCl_2$). Supernatant from Pansorbin treatment was cleared of trace Pansorbin by two cycles of centrifugation for 2 min in the Eppendorf centrifuge. Substrate RNA, ATP, and creatine phosphate were added to the depleted supernatants to initiate assembly. After the reaction mixtures had been kept for 10 min at 30°C, RNAs were immunoprecipitated with the anti-Sm monoclonal antibody as described above. Immunoprecipitated RNAs were displayed on denaturing acrylamide gels which were both stained and autoradiographed to detect U RNAs and precursor RNA, respectively. In complementation experiments, assembly was initiated by addition of substrate, ATP, creatine phosphate, and either untreated fresh extract or extract which had been subjected to U1 oligo cleavage.

RNP gel electrophoresis. Described in detail elsewhere (M. Zillmann and S. M. Berget, submitted for publication), the RNP gel system used a 2.5% acrylamide (acrylamide/bisacrylamide, 80:1)–0.5% agarose composite gel containing 10% glycerol, 25 mM Trisacetate, (pH 8.3), and 10 mM EDTA. Electrophoresis buffer contained 25 mM Trisacetate (pH 8.3) (the final acetate concentration at this pH is approximately 8.8 mM) and 10 mM EDTA. Splicing reactions were terminated by addition of heparin and EDTA to final concentrations of 2 mg/ml and 10 mM, respectively. The gels were electrophoresed at 10 V/cm for 3 h at 4°C.

RESULTS

Immunoprecipitation of complexes containing splicing precursors and intermediates. To visualize assembly intermediates, we analyzed splicing reactions by immunoprecipitation with snRNP-specific antibodies (Fig. 1). Observation of immunoprecipitated RNA indicates the presence of a stable assembly containing that RNA and the snRNP against which the antibody is directed. Immunoprecipitated RNAs (Fig. 1C) were correlated to the spectrum of splicing precursor and intermediate RNAs in the total reaction (Fig. 1A) and the appearance of large assemblies as assayed by native RNP gels (Fig. 1B). A single splicing reaction was used for experiments shown in all panels of Fig. 1. At early times, approximately 30% of the total RNA was immunoprecipitable by one or more antibodies. We assume that all of the radiolabeled RNA resides in snRNP-containing complexes and that the 30% value reflects the intrinsic stability of the complexes to immunoprecipitation. Once reaction intermediates began to appear, immunoprecipitability and visibility of complexes on RNP gels decreased, indicating spliceosome changes and disassembly accompanying the reaction.

Both RNP gels and immunoprecipitation detected the formation of large complexes containing U1 snRNPs at 0°C. Incubation at 30°C resulted in increased immunoprecipitation by U1-specific antibodies and the appearance of slower-moving complexes on RNP gels. The appearance of gel-visualized and immunoprecipitable complexes clearly preceeded the appearance of splicing activity. The ability of U1-specific antibodies to immunoprecipitate complex at early times in the reaction suggests the stable association of U1 snRNPs with the spliceosome at a very early stage in the assembly pathway.

Cleavage of U1 RNA decreases stable association of all snRNPs with precursor RNA. To investigate the mechanism of association of snRNPs with the spliceosome, we performed immunoprecipitations from splicing reactions in which U1, U2, or U4 RNA had been subjected to oligonucleotide-directed cleavage (1) (Fig. 2). All three cleavages abolished splicing activity and resulted in over 95% cleavage of the targeted U RNA. Cleavage of U2 or U4 RNA had no effect on the ability of either U1-specific or Sm-specific antibodies to immunoprecipitate precursor RNAs from a 5-min splicing reaction. In contrast, cleavage of U1 RNA within the sequences required for 5′ splice site recognition severely depressed the ability of both antibodies to immunoprecipitate precursor RNA, suggesting the requirement of

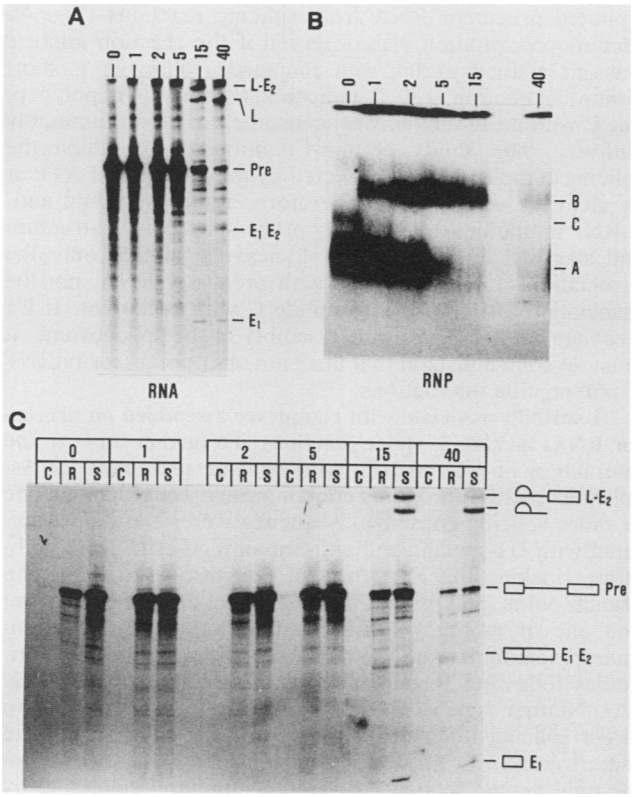

FIG. 1. Assembly of the spliceosome. A splicing reaction involving the use of wild-type adenovirus precursor RNA (see Fig. 5C) was sampled for total RNA (A), RNP complexes (B), and immunoprecipitation by snRNP-specific antibodies (C) at the times indicated. Products from a single reaction were subjected to all analyses. Equal amounts of reaction mixture were used for the total RNA analysis and for the RNP gel. A threefold-larger amount was used for each of the immunoprecipitations in panel C. Reaction intermediates and products first appeared at 15 min and are identified in panel A. The RNP gel resolves several complexes (Zillmann and Berget, submitted). Reaction intermediates could be isolated from complex B. Precursor RNA was located in all three complexes. RNA uncomplexed with proteins migrated off the bottom of the gel shown in panel B. Antibodies used for the immunoprecipitation included a control human serum (C), a Ul-specific serum sample from a patient (R-GD) with systemic erythematosus (R), and the Y12 Sm monoclonal antibody (S).

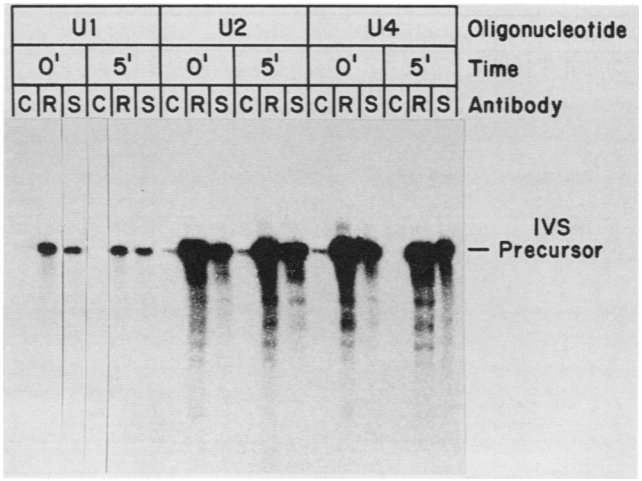

FIG. 2. Cleavage of U1 RNA alters spliceosome immunoprecipitability. Individual RNAs were cleaved with RNase H and oligonucleotides complementary to nucleotides 1 to 14 of U1 RNA, 10 to 25 of U2 RNA, and 66 to 85 of U4 RNA before precursor RNA was added. Each cleavage was judged to be more than 95% efficient by electrophoretic analysis of extract snRNAs and completely inhibited splicing even in long incubations. Antibodies included a control human serum sample (C), a U1-specific patient (R-GD) serum sample (R), and the Y12 monoclonal antibody (S). Treated extract was immunoprecipitated after incubation at 0°C (5') or 5 min at 30°C (5'). This experiment was performed as a part of the experiments in Fig. 1 and 3. The amount of utilized substrate was identical to that in the immunoprecipitions in Fig. 1, and immunoprecipitated signals in the two figures are comparable.

the removed U1 RNA sequences for antibody-mediated detection of stable complexes containing any snRNP. The inability of anti-Sm antibodies to immunoprecipitate complexes after U1 cleavage was surprising considering the ability of Sm antigens (possibly as a part of U5 snRNPs) to recognize sequences within the 3' portion of the intron.

Assemblies formed in the absence of intact U1 RNA were also analyzed by native RNP gels (Fig. 3). Cleavage of U1 RNA resulted in the inhibition of the formation of slow-migrating complexes normally seen after incubation of the splicing reaction mixture at 30°C. The coupled decrease of immunoprecipitability and spliceosome assembly as monitored by gel electrophoresis supports a pivotal role for U1 snRNPs in early assembly. Furthermore, because the removed sequences were those complementary to 5' splice junctions, stable U1-mediated recognition of 5' splice junctions must be an early step in spliceosome assembly.

U1 snRNPs associate with precursor RNA within the spliceosome by mechanisms in addition to hybridization. Cleavage of U1 RNA depressed the immunoprecipitability of precursor RNA by U1-specific antibodies, but did not abolish it (Fig. 2). We do not suspect that the residual immunoprecipitation resulted from a small amount of U1 RNA not undergoing cleavage. No trace of splicing activity was observed after cleavage even after extreme overexposure of the gels, suggesting that the observed immunoprecipitation signal was from complexes containing cleaved U1 RNA. Furthermore, anti-Sm immunoprecipitability also decreased

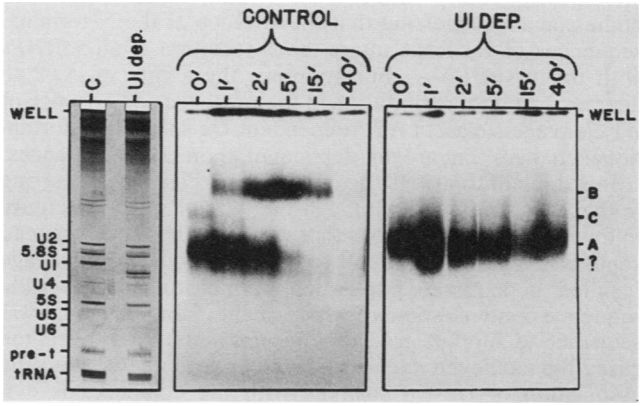

FIG. 3. Assembly in the absence of intact U1 RNA. Splicing reactions with control extract or extract in which U1 RNA had been cleaved (A) were subjected to neutral gel electrophoresis (Fig. 1). The splicing reactions analyzed in this figure are the same reactions analyzed in Fig. 1 and 2.

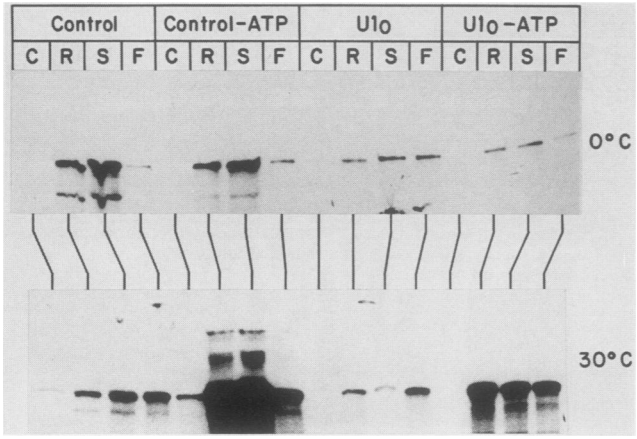

FIG. 4. ATP affects the level of immunoprecipitability of splicing complexes by U1-specific antibodies. Precursor RNA was immunoprecipitated from identical splicing reactions with normal extract (control), extract depleted for ATP (Control−ATP), extract treated to cleave U1 RNA ($U1_0$), or extract depleted for ATP and treated to cleave U1 RNA ($U1_0$−ATP). The reaction was carried out at 0 or 30°C for 5 min. Antibodies used included a control serum sample (C), a Ul-specific systemic lupus erythematosus patient serum sample (R), Y12 monoclonal antibodies (S), and the 4F4 monoclonal antobody (F) directed against the hnRNP C polypeptide. Equal exposures were used for all parts of the figure, resulting in extreme overexposure of the control-ATP samples. The bands above the precursor in these lanes are at the top of the gel, and a small amount of readthrough transcript is present in this RNA and only present in these lanes. No splicing was occurring in any sample.

upon U1 cleavage, suggesting the presence of an altered assembly in the absence of U1 5′ terminal sequences.

The level of immunoprecipitation observed following U1 cleavage was dependent upon the presence of ATP (Fig. 4). When extracts were simultaneously depleted for ATP and intact U1 RNA, not only was the formed assembly immunoprecipitable by both RNP and Sm antibodies, but the level of immunoprecipitation increased dramatically. Immunoprecipitation of precursor RNA from normal reactions also increased in the absence of ATP. The stimulatory effect of ATP depletion was observed only if the extracts were heated to 30°C. The absence of ATP affected immunoprecipitability by both U1-specific and anti-Sm antibodies, again suggesting that the absence of the 5′-terminal sequences of U1 RNA alters the association of all snRNPs with precursor RNA. Furthermore, the ability of ATP to depress immunoprecipitation in the presence and absence of U1 cleavage suggests ATP-dependent U1 snRNP conformational changes that are not dependent upon U1 5′ sequences.

It is difficult to assess the extent of U1 cleavage occurring in these experiments. Although the lack of splicing activity following cleavage suggests that very little U1 RNA escaped some cleavage, it is difficult to determine whether cleavage resulted in a mixed population of molecules of variable sequence content, some of which might maintain enough U1 sequence to support assembly but not activity. If this is the case, the achieved cleavages were adequate to alter the association of U2-U6 snRNPs with the complex, as evidenced by the co-inhibition of both RNP and Sm immunoprecipitability following cleavage.

An anti-hnRNP polypeptide antibody was also used to analyze spliceosome assembly. The monoclonal 4F4 antibody directed against the hnRNP C polypeptide immunoprecipitated precursor RNA from splicing reactions (Fig. 4). Immunoprecipitation was observed if the reaction mixture was maintained at 0°C but increased following a short (5-min) incubation at 30°C, indicating association of polypeptide C with the assembling spliceosome early in the assembly pathway. The ability of anti-C antibodies to inhibit the splicing complex was not affected by the presence of ATP or by cleavage of U1 RNA. Therefore, anti-hnRNP and anti-snRNP antibodies differentially probe spliceosome structure and stability. Furthermore, U1 cleavage altered only the association of other snRNPs with precursor RNA, not the association of hnRNP polypeptide C with precursor. If U1 cleavage results in aberrant assembly of the spliceosome, it must do so in a fashion that does not alter precursor-hnRNP C polypeptide interactions.

U1 snRNPs associate with complexes assembled on precursor RNAs lacking 5′ splice junctions. To further analyze the interaction of U1 snRNPs with splicing precursor RNAs, splicing reactions mixtures containing substrates lacking one or more splicing consensus sequence were immunoprecipitated with U1-specific and anti-Sm antibodies (Fig. 5). Substrates lacking the 3′ portion of the intron (including the branch point, polypyrimidine track, and 3′ splice junction) and one or more 5′ splice junctions (Fig. 5C) were immunoprecipitable with both U1-specific and anti-Sm antibodies following 10 min of incubation in the extract (Fig. 5A). Neither type of deletion substrate was competent to direct splicing (data not shown). Immunoprecipitation did require consensus sequences; a similar-length RNA containing only vector sequences was not immunoprecipitable by either antibody. Two different U1-specific antibodies directed against different U1-specific polypeptides were used. Both were able to immunoprecipitate substrate RNAs lacking 5′ splice junctions. Therefore U1 snRNPs are able to associate with precursor RNAs in the assembling spliceosome by means other than hybridization.

The level of immunoprecipitation of all substrates dropped upon cleavage of U1 RNA (Fig. 5B). Both U1-specific and anti-Sm antibodies were less competent to immunoprecipitate substrate RNAs after U1 RNA cleavage. Therefore U1 5′-terminal sequences are required for maximal association of all U snRNPs with the spliceosome, regardless of the structure of the substrate RNA. Residual immunoprecipitability remained after U1 RNA cleavage for substrates containing 3′ intronic sequences. Immunoprecipitation of substrate RNAs lacking the 3′ portion of the intron was most susceptible to cleavage of U1 RNA, indicating that sequences with the 3′ portion of the intron direct the association of U1 snRNPs with the spliceosome when U1 RNA has been cleaved even in the presence of valid 5′ splice junctions.

Depletion of extracts for U1 snRNPs inhibits association of other snRNPs with precursor RNA. Oligonucleotide cleavage of U1 RNA inactivates only U1 snRNPs; it does not eliminate U1 snRNPs from the extract. To assess the requirement for U1 snRNPs for assembly of precursor RNA into complexes containing other snRNPs, extract was depleted for U1 snRNPs before substrate RNA was added and before RNA was immunoprecipitated with anti-Sm antibodies (Fig. 6). Three different U1-specific antibodies were used. Antibody treatment removed 50 to 95% of the U1 RNA in the extract. Splicing activity was abolished in samples treated with U1-specific antibodies but not with control antibodies (the elimination of splicing in samples treated with antibody but retaining some U1 suggests that the remaining U1 snRNPs had bound U1-specific antibody). Immunoprecipi-

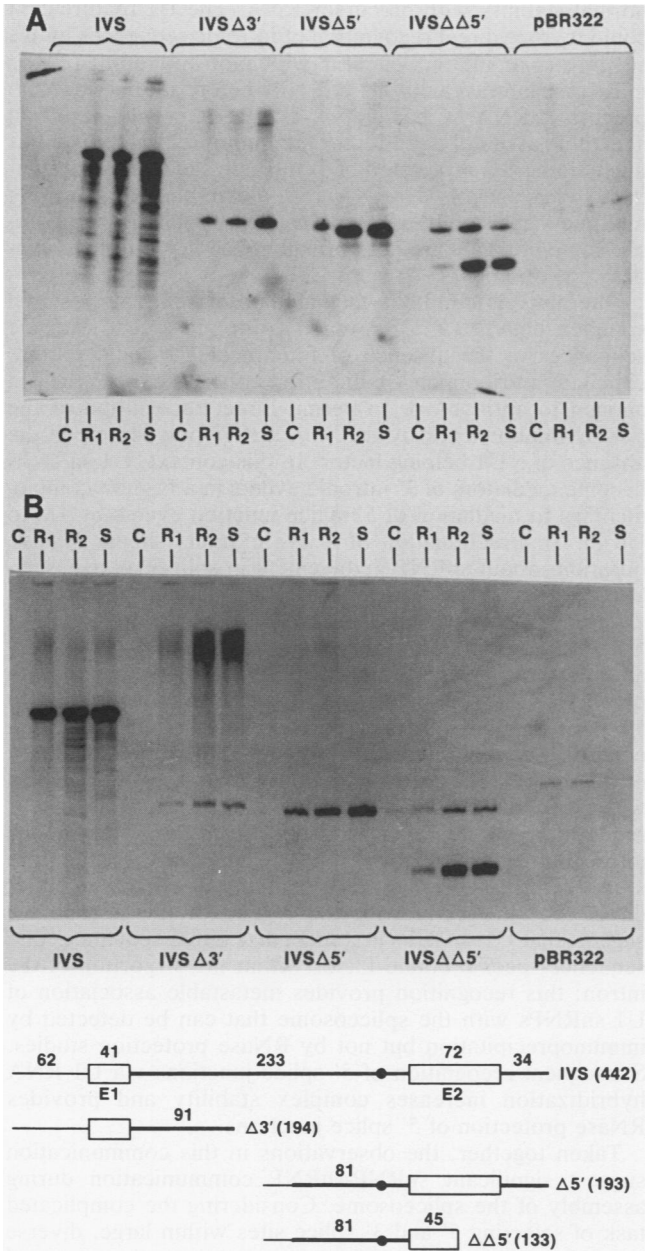

FIG. 5. U1 snRNPs associate with substrates lacking 5' splice junctions. Splicing reaction mixtures with untreated extract (A) or extract in which U1 RNA had been cleaved by oligonucleotide-mediated cleavage (B) were immunoprecipitated with the indicated antibodies after 10 min of incubation at 30°C. Antibodies included a control human serum (C), U1-specific patient serum sample directed against the U1-specific 67-kilodalton polypeptide (R_1) or the A polypeptide (R_2), and the Y12 Sm monoclonal antibody (S). Five substrates (C) were used, including IVS, a 442-nucleotide wild-type adenovirus substrate containing a single intron; IVSΔ3', a 194-nucleotide substrate truncated within the first intron upstream of the branch point; IVSΔ5', a 193-nucleotide substrate containing the 3' half of the intron and the entire second exon; IVSΔΔ5', a 133-nucleotide substrate containing the 3' portion of the intron and the 5' half of exon 2; and pBR322, a 235-nucleotide RNA containing only vector sequences. Equal amounts of each substrate were used. IVSΔΔ5' and IVSΔ3' contained some longer RNA resulting from incomplete cleavage of the template DNA used to synthesize precursor RNA. For IVSΔΔ5' this longer RNA is IVSΔ5', and for IVSΔ3' the longer RNA is produced by heterogeneous run-through transcription. This latter RNA has a relatively stronger immunoprecipitation signal in panel B because it contains 3' intronic signals. The autoradiograms in panels A and B were exposed for 16 and 60 h, respectively. The longer exposure time permitted comparison of the weaker immunoprecipitation signals observed when U1-cleaved extracts were used.

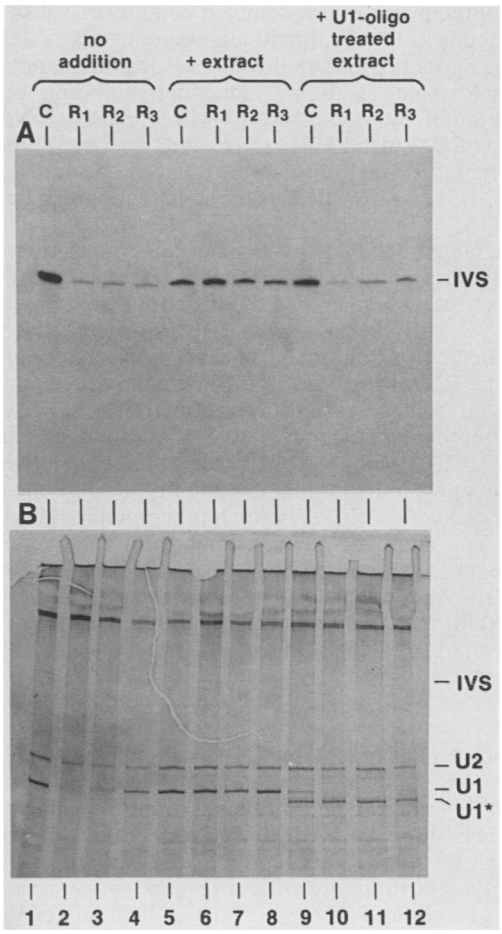

FIG. 6. U1 snRNPs are required for formation of stable complexes. Precursor RNA was immunoprecipitated from splicing reaction mixtures reconstituted with extract depleted for U1 snRNPs. To remove U1 snRNPs, extract was pretreated with either control (C) or U1-specific (R_1, R_2, R_3) antibodies, and the resulting immune complexes were removed with Pansorbin (Materials and Methods). Three U1-specific patient serum samples directed against the 67-kilodalton polypeptide (R_1 and R_3) or the A polypeptide (R_2) were used. Following pretreatment, assembly was initiated by the addition of substrate IVS RNA (lanes 1 to 4), IVS RNA plus untreated extract (lanes 5 to 8), or IVS RNA plus extract in which U1 RNA had been cleaved (lanes 9 to 12). Following incubation at 30°C for 10 min, RNA was immunoprecipitated with the monoclonal Y12 Sm antibody. Immunoprecipitated RNA was displayed on a denturing urea gel. Panel A is an autoradiogram of the silver-stained gel shown in panel B. Immunoprecipitated substrate and U RNAs are indicated. Some continued cleavage of U1 RNA occurred after mixing in lanes 9 to 12, causing a reduction in the total amount of U1 RNA.

tation affected the level of U1 snRNPs only (Fig. 6B); other snRNPs remained at normal levels and were still immunoprecipitable with anti-Sm antibodies.

Substrate RNA was added following antibody treatment, and association of substrate with snRNPs was assessed by

immunoprecipitation of assembled complexes with the anti-Sm antibody (Fig 6A). Immunoprecipitation was depressed by using extracts pretreated with U1-specific but not control antibodies (lanes 1 to 4), indicating inhibition of stable association of U2 to U6 snRNPs with precursor RNA in the absence of U1 snRNPs. U2 to U6 snRNPs were still present in the treated extract, as demonstrated by the appearance of U2, U4, U5, and U6 snRNAs in the immunoprecipitate (Fig. 6B).

To demonstrate that the extract component removed by antibody pretreatment was indeed U1 snRNPs, depleted extracts were complemented by extract containing either normal U1 snRNPs or U1 snRNPs in which U1 RNA had been cleaved. Addition of untreated extract, along with substrate RNA, restored anti-Sm immunoprecipitation of assembled complexes and appearance of U1 RNA in the immunoprecipitates (lanes 5 to 8). Addition of extract in which U1 RNA had been cleaved could not complement the defect in anti-Sm immunoprecipitability, despite the appearance of cleaved U1 RNA in the immunoprecipitates (lanes 9 to 12). Therefore the extract component removed by pretreatment of the extract with U1-specific antibodies was U1 snRNPs. We conclude that the presence of U1 snRNPs is required for the stable association of other snRNPs with the spliceosome.

DISCUSSION

Association of precursor RNAs with nuclear components to form active spliceosomes is a complicated process involving multiple nuclear factors including hnRNP polypeptides and at least four snRNPs. In this communication we document that U1 snRNPs are required very early in spliceosome assembly. Furthermore, the presence of functional U1 snRNPs is required before maximal association of U2 to U6 snRNPs with precursor RNA can be detected by immunoprecipitation with anti-Sm antibodies. Removal of the 5′ terminal sequences of U1 RNA is sufficient to depress both the association of Sm antigens with precursor RNA and the appearance of large complexes as monitored by native RNP gel electrophoresis, implicating U1 5′ RNA sequences in early assembly.

Studies in other laboratories have indicated that the first sequences recognized within a precursor RNA reside within the 3′ portion of the intron (6, 7, 10–13, 21, 23). Experiments involving the use of RNase protection or blot binding to probe snRNP associations with precursor RNAs implicated an Sm-reactive polypeptide of 70 to 100 kilodaltons as the factor recognizing the polypyrimidine track and 3′ splice junction (10, 23). This polypeptide does not appear to be a component of either U1 or U2 snRNP. Instead it most probably resides on U5 snRNPs. Protection of the polypyrimidine track by the Sm-reactive polypeptide is not altered by cleavage of U1 RNA, suggesting that this protein interacts with precursor RNA not already associated with U1 snRNPs (6, 7). From these experiments, it would have been predicted that cleavage of U1 RNA or depletion of splicing extracts for U1 snRNPs would depress the ability of U1-specific antibodies but not Sm antibodies to immunoprecipitate assemblies containing precursor RNA.

Our experiments failed to detect normal Sm immunoprecipitability of complexes containing precursor RNA following either cleavage of U1 RNA or depletion of the extracts for U1 snRNPs. Several possible explanations for this discrepancy exist. In the absence of functional U1 snRNPs, other snRNPs might fail to form complexes of normal stability with precursor RNA. The U1 requirement could involve direct recognition of intronic sequences by U1 snRNPs or an indirect effect in which intron-binding factors must first interact with U1 snRNPs before interacting with precursor RNA. Cleavage of U1 RNA would result in aberrant assembly, reducing immunoprecipitability but maintaining Sm protection of 3′ intronic sequences. Intriguingly, cleavage of U1 RNA resulted in the formation of multiple small complexes, as revealed by gel electrophoresis, some of which are not normally observed during assembly.

Alternatively, antibody-mediated observation of the initial complex between the Sm-reactive protein and 3′ intronic sequences in the absence of functional U1 could require concomitant ribonuclease digestion either to expose the Sm antigen to antibody or to permit direct recognition of the polypyrimidine track by the Sm-reactive polypeptide in the absence of a U1 helping factor. In this context, U1 snRNPs become mediators of 3′ intronic events in a fashion complementary to mediation of 5′ splice junction events by U2 to U6 (6, 7). Fractionation of active extracts should resolve questions about snRNP involvement in spliceosome assembly.

Although cleavage of U1 RNA depressed association of U1 snRNPs with the spliceosome, it did not completely abolish it, suggesting that U1 snRNPs can associate with precursor RNA by mechanisms other than hybridization to 5′ splice junctions. This association is less stable than that occurring normally, causing reduced immunoprecipitability of precursor RNA. The residual association of U1 snRNPs with precursor RNA in the absence of U1 RNA 5′ sequences required precursor RNA sequences from the 3′ end of the intron but did not require 5′ splice junctions. It therefore seems likely that U1 snRNP proteins can interact either directly or indirectly with the 3′ end of the intron. We suggest that U1 snRNPs first recognize either sequences or a sequence-specific bound factor within the 3′ portion of the intron; this recognition provides metastable association of U1 snRNPs with the spliceosome that can be detected by immunoprecipitation but not by RNase protection studies. Subsequent recognition of 5′ splice junctions via U1 RNA hybridization increases complex stability and provides RNase protection of 5′ splice junctions.

Taken together, the observations in this communication suggest significant snRNP-snRNP communication during assembly of the spliceosome. Considering the complicated task of selecting 5′ and 3′ splice sites within large, diverse precursor RNAs, it is perhaps not surprising that maximal associations at both ends of an intron require cooperation and proofreading by factors recognizing the other end of the intron.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants GM-35669 and GM-35670 to S.B. from the National Institutes of Health. M.Z. and S.R. are fellows of the Robert A. Welch Foundation (Q-821).

LITERATURE CITED

1. **Berget, S. M., and B. L. Robberson.** 1986. U1, U2, and U4/U6 small nuclear ribonucleoproteins are required for in vitro splicing but not polyadenylation. Cell **46**:691–696.
2. **Bindereif, A., and M. R. Green.** 1986. Ribonucleoprotein complex formation during pre-mRNA splicing in vitro. Mol. Cell. Biol. **6**:2582–2592.
3. **Black, D. L., B. Chabot, and J. A. Steitz.** 1985. U2 as well as U1

small nuclear ribonucleoproteins are involved in pre-mRNA splicing. Cell **42**:737–750.

4. **Black, D. L., and J. A. Steitz.** 1986. Pre-mRNA splicing in vitro requires intact U4/U6 small nuclear ribonucleoproteins. Cell **46**:697–704.
5. **Brody, E., and J. Abelson.** 1985. The "spliceosome": yeast pre-messenger RNA associates with the 40S complex in a splicing dependent reaction. Science **228**:963–967.
6. **Chabot, B., D. L. Black, D. M. LeMaster, and J. A. Steitz.** 1985. The 3′ splice site of pre-messenger RNA is recognized by a small nuclear ribonucleoprotein. Science **230**:1344–1349.
7. **Chabot, B., and J. A. Steitz.** 1987. Multiple interactions between the splicing substrate and small nuclear ribonucleoproteins in spliceosomes. Mol. Cell. Biol. **7**:281–293.
8. **Choi, Y. D., P. J. Grabowski, P. A. Sharp, and G. Dreyfuss.** 1986. Heterogeneous nuclear ribonucleoproteins: role in RNA splicing. Science **231**:1534–1539.
9. **Frendewey, D., and W. Keller.** 1985. Stepwise assembly of a pre-mRNA splicing complex requires U-snRNPs and specific intron sequences. Cell **42**:355–367.
10. **Gerke, V., and J. A. Steitz.** 1986. A protein associated with small nuclear ribonucleoprotein particles recognizes the 3′ splice site of premessenger RNA. Cell **47**:973–984.
11. **Grabowski, P. J., S. R. Seller, and P. A. Sharp.** 1985. A multicomponent complex is involved in the splicing of messenger RNA precursors. Cell **42**:345–353.
12. **Grabowski, P. J., and P. A. Sharp.** 1986. Affinity chromatography of splicing complexes: U2, U5, and U4/U6 small nuclear ribonucleoproteins in the spliceosome. Science **233**:1294–1299.
13. **Konarska, M. M., and P. A. Sharp.** 1986. Electrophoretic separation of complexes involved in the splicing of precursors to mRNA. Cell **46**:845–855.
14. **Krainer, A. R., and T. Maniatis.** 1985. Multiple factors including the small nuclear ribonucleoproteins U1 and U2 are necessary for pre-mRNA splicing in vitro. Cell **42**:725–736.
15. **Kramer, A., W. Keller, B. Appel, and R. Luhrmann.** 1984. The 5′ terminus of the RNA moiety of U1 small nuclear ribonucleoprotein particles is required for the splicing of messenger RNA molecules. Cell **38**:299–307.
16. **Mount, S. M., I. Pettersson, M. Hinterberger, A. Karmas, and J. A. Steitz.** 1983. The U1 small nuclear RNA-protein complex selectively binds a 5′ splice site in vitro. Cell **33**:509–518.
17. **Noble, J. C. S., C. Prives, and J. L. Manley.** 1986. In vitro splicing of simian virus 40 early pre-mRNAs. Nucleic Acids Res. **14**:1219–1235.
18. **Padgett, R. A., M. M. Konarska, P. J. Grabowski, S. F. Hardy, and P. A. Sharp.** 1984. Lariat RNAs as intermediates and products in the splicing of messenger RNA molecules. Science **225**:898–903.
19. **Perkins, K. K., H. M. Furneaux, and J. Hurwitz.** 1986. RNA splicing products formed with isolated fractions from HeLa cells are associated with fast-sedimenting complexes. Proc. Natl. Acad. Sci. USA **83**:887–891.
20. **Pikielny, C. W., and M. Rosbash.** 1986. Specific small nuclear RNAs are associated with yeast spliceosomes. Cell **45**:896–877.
21. **Ruskin, B., and M. R. Green.** 1985. Specific and stable intron-factor interactions are established early during in vitro pre-mRNA splicing. Cell **43**:131–142.
22. **Sierakowska, H., W. Szer, P. J. Furdon, and R. Kole.** 1986. Antibodies to hnRNP core proteins inhibit in vitro splicing of human beta-globin pre-mRNA. Nucleic Acids Res. **14**:5241–5254.
23. **Tazi, J., C. Alibert, J. Temsemani, I. Reveillaud, G. Cathala, C. Brunel, and P. Jeanteur.** 1986. A protein that specifically recognizes the 3′ splice site of mammalian pre-mRNA introns is associated with a small nuclear ribonucleoprotein. Cell **47**:755–766.
24. **Zhuang, Y., and A. M. Weiner.** 1986. A compensatory base change in U1 snRNA supresses a 5′ splice site mutation. Cell **46**:827–835.