Volume 17 Number 7 1989 Nucleic Acids Research

Evidence for nuclear factors involved in recognition of 5′ splice sites.

Maria L.Zapp and Susan M.Berget

Verna and Marrs McClean Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

Received December 14, 1988; Revised and Accepted February 22, 1989

ABSTRACT

To investigate soluble factors involved in pre-messenger RNA splicing we have fractionated nuclear extract by simple centrifugation to produce a supernatant pellet pair. Factors larger than 15S including U2, U4, U5, and U6 snRNPs fractionate with the pellet; U1 snRNPs distribute equally in pellet and supernatant. Each fraction is individually incompetent for splicing and spliceosome assembly; mixing restores wild type activity and assembly. The pellet fraction directs an aberrant assembly pathway in which proper 3', but improper 5' splice site recognition occurs. Complexes formed with the pellet fraction are distinguishable from wild-type complexes using native gel electrophoresis. Pellet complexes contain U1 snRNP antigens and their formation requires ATP, U1 snRNPs, U2 snRNPs, and sequences at the 3' end of the intron - properties shared with the initial steps of normal assembly and directed by sequences at the 3' end of the intron. In contrast, pellet complex assembly shows no dependence on the presence of a 5' splice junction within precursor RNA. Furthermore, binding of factors to the 5' splice junction is deficient in pellet assemblies. Thus, the pellet lacks a factor required for proper recognition of 5' splice sites. This factor can be supplied by the supernatant. Complementation occurs when supernatant U1 RNA is destroyed, suggesting that the supernatant factor recognizing 5' splice sites is not U1 snRNPs.

INTRODUCTION

Splicing of vertebrate pre-messenger RNA requires correct recognition of small exons of variable sequence content buried in non-desired, and much larger introns. Introns terminate with specific consensus sequences that define splice sites (1-4). Splice sites, and hence exons, are selected by recognition of these short sequences. Deletion studies indicate little or no requirement for distal intron or exon sequences (5-8). Thus, the splice site recognition machinery must have the ability to distinguish the appropriate 5'and 3' splice site consensus sequences from related sequences located within introns or exons. The consensus sequence CAG\GURAGU is located from -3 to +6 nucleotides at 5' splice sites, and is recognized via base pairing to U1 RNA (9-15). Mutations within this sequence that reduce complementarity to U1 RNA depress splicing efficiency (16-20), mutations that increase complementarity stimulate splicing (15,21). Mutation of the GU dinucleotide directly adjacent to the splice site is most deleterious. Mutation of other nucleotides has variable effects. A mutation at +5 has been rescued by a second site mutation of U1 RNA, providing direct proof for hybridization-mediated recognition of 5' splice sites (15). A mutation at +3, however, was not rescued by a complementary point mutation in U1 RNA (15), suggesting that factors other than hybridization play a role in recognition of the 5' splice site consensus sequence. Mutation of a 5' splice site frequently leads to efficient recognition of proximal cryptic sites (5-7,21,22). Experimental manipulation of 5' splice sites to investigate why cryptic sites are normally ignored indicates that parameters in addition to the sequence at the 5' splice site are influential (23-27). Analysis of precursor RNAs containing splice-site duplications suggests that the position of the splice site within the precursor affects utilization (20,28). Furthermore, examination of in vitro interactions of U1 snRNPs with wild type and mutant 5' splice sites indicates that U1 snRNPs bind consensus sequences that are not utilized (29,30), suggesting that binding of U1 snRNPs to 5' splice sites is necessary, but not sufficient, for splice junction selection. Therefore, it seems likely that productive U1 snRNP interactions with 5' splice sites leading to correct splice site recognition is more complicated than simple hybridization of U1 RNA to complementary sequences within a precursor RNA.

Investigation of interactions at the 3' end of the intron have indicated that non-snRNP factors participate in recognition of 3' consensus sequences (31,32). One report of a factor enhancing U1 snRNP interactions has appeared (33). In this communication, we have used in vitro splicing systems to search for soluble nuclear factors involved in the recognition of 5' splice sites. Simple size fractionation of splicing extract produced a fraction that directed assembly of aberrant complexes containing U1 snRNPs, but deficient in recognition of 5' splice sites. This fraction could be complemented for assembly and activity by the addition of a small nuclear factor(s), suggesting the existence of factors that assist U1 snRNPs in 5' splice site recognition.

EXPERIMENTAL PROCEDURES

Fractionation of nuclear extract

HeLa cell nuclear extract was prepared according to Dignam and Roeder (45) except that Roeder D buffer was altered to contain Tris, Cl; pH 7.9 (referred to as Roeder DT) instead of Hepes and dialysis was for 5 hours. Extract was cleared for 5 min in an Eppendorf centrifuge prior to fractionation. Cleared extract had a protein concentration of 7-10 mg/ml. Cleared extract was centrifuged in a Beckman airfuge at 106,000xg for 60 min at 25°C. Spinning produced a translucent pellet. The supernatant was carefully removed and used as the supernatant fraction. Supernatant contained 75-85% of the extract protein (final concentration was 5-8 mg/ml). The tube containing the pellet was washed several times with Roeder buffer DT to remove traces of supernatant. The pellet was resuspended by gentle repipetting in Roeder DT to the original volume of extract. Resuspended pellet was incubated at 30°C for 5 min to aid in resuspension. Heating helped produce a more reproducible pellet. No noticeable loss of necessary extract components was observed during heating. After heating, remaining insoluble material was removed by a 2 min spin in a cold Eppendorf centrifuge. The insoluble material was discarded; the soluble material was used as the pellet fraction. The pellet fraction contained 10-20% of extract protein (final concentration 0.8-2.0 mg/ml). Supernatant fractions were stable to extended storage at -70°C, pellet fractions deteriorated. Hence fresh pellet was prepared daily for experimental purposes.

Substrate RNAs

Wild-type adenovirus MINX precursor RNA contains a 58 nucleotide first exon (containing 25 nucleotides of SP6 polylinker and the nucelotides 39-72 of exon 2 from the major late adenovirus transcription unit), a 120 nucleotide intron (containing the first 43 nucleotides of intron 2 fused to the last 77 nucleotides of intron 1), and a 38 nucleotide truncated second exon from the adenovirus major late transcription unit plus 3 nucleotides of polylinker(39). IVPX was a longer (440 nucleotide) adenovirus substrate containing 45 nucleotides of SP6-derived polylinker, intact exon 1, an internally-deleted 224 nucleotide intron 1, the intact 72 nucleotide second exon, and 37 nucleotides of intron 2 from the adenovirus major late transcription unit (42). The 5' splice site deletion substrate RNA (Δ5) is a Hind III deletion of IVPX containing 9 nucleotides of SP6-derived polylinker fused to the last 77 nucleotides of intron 1 and the 72 nucleotide second exon. Δ5 contained the same branch point, pyrimidine track, and 3' splice junction as wild-type IVPX and wild-type MINX. All precursor RNAs were produced by SP6 transcription.

In vitro splicing

Extract preparation and in vitro splicing reactions were performed as previously described (40). Supernatant and pellet were mixed in a ratio of

1:1 for reconstituted splicing assays (total extract was 10ul in a 25ul reaction). To deplete extract for ATP, nuclear extract was preincubated under splicing conditions at 30^{o}C for 45 min in the absence of ATP and creatine phosphate and then added to 1mM β, γ ,-methylene ATP. Oligonucleotide-directed cleavage of individual U RNAs (13) was performed as described previously (40). Oligonucleotides were added at the level of .02 OD/10ul extract. Utilized U1 and U2-specific oligonucleotides were complementary to nucleotides 1-16 of each RNA. Cleavage of U RNAs in fractions required the addition of 0.8 U of RNase H per 0.010ul of unfractionated extract. These conditions resulted in complete inhibition of both splicing activity and complex assembly, and greater than 95% cleavage of the target U RNA. RNA reaction products were prepared as described previously and analyzed on 10% polyacrylamide denaturing gels containing 8M urea. U RNA cleavage was monitored by staining RNA gels with silver (46).

RNP gel electrophoresis

Splicing reaction were analyzed for complex assembly on native RNP gels (39). Reaction was terminated at desired times by addition of stop solution and quick freezing in liquid nitrogen. 6x stop solution contained 12.5 mg/ml heparin, 60mM EDTA, 35% glycerol. Samples were thawed at room temperature, and warmed to 30^{o}C for 5 min. Samples were loaded immediately onto a 3.5% polyacrylamide-bisacrylamide (80:1 vol/vol)-0.5%. agarose (Bio-Rad Laboratories) composite gel cast in 50mM Tris-Glycine, pH8.8; and 10mM EDTA. Electrophoresis was performed at 4^{o}C at 10 V/cm for 4 to 5 hrs. Gels were fixed in 50% methanol-12% acetic acid for 3 min prior to autoradiography.

Immunoprecipitation of whole and fractionated extract

Anti-RNP and control antibodies were from patients and have been previously characterized and described by this laboratory (42). The anti-Sm antibody was the Sm Y12 monoclonal antibody. Splicing reactions with whole or fractionated extract were incubated at 30^{o}C for 10 min. After this time, a 10ul aliquot was removed and added to 3ul of antibody. The mixture was placed at 0^{o}C for 30 min. Each reaction then received 15ul Pansorbin, which had been freshly washed with in NET(Mg) buffer (50 mM Tris,HCl,pH7.4; 150mM NaCl; 0.05% Nonidet P-40; 0.5mM dithiothreitol; and 1.5mM $MgCl_2$), and further incubated for 10 min at 0^{o}C. At the end of the incubation, 200 ul NET(Mg) was added and the reactions were spun in a microfuge for 15 sec. The supernatant was saved to monitor non-precipitated RNA. The pellet was washed three times with NET(Mg) buffer. The washed pellets were resuspended in 500ul of urea buffer (175mM NaCl; 5mM Tris,HCl,pH 7.4; 5mM EDTA; 0.5% sodium dodecyl sulfate). After a 5 min incubation at 80^{o}C, the Pansorbin was removed by centrifugation and the supernatant was extracted with phenol-chloroform and isopropanol precipitated with glycogen carrier. The RNA was analyzed by denaturing gel electrophoresis, and visualized by silver-staining (48) prior to autoradiography.

Oligonucleotide accessibility assays

To assay binding of factors to the 5' splice site (47), an oligonucleotide complementary to 12 nucleotides spanning the 5' splice site within the wild-type precursor RNA (MINX) was added at a concentration previously determined to insure total cleavage of the isolated substrate RNA (0.01SD). Reactions with fractions were supplemented with 0.8U of RNase H. Reactions were incubated for 10 min at 30^{o}C after the addition of oligonucleotide. The oligonucleotide cleaved MINX RNA to produce fragments of 55 and 153 nucleotides. Reaction RNA was isolated and analyzed on 8M Urea/8% acrylamide denaturing gels and exposed for autoradiography.

RESULTS

Fractionation of splicing extracts

We were interested in investigating nuclear factors involved in spliceosome assembly. The approach we took to search for such factors was extract fractionation. Because we were interested in small soluble factors, we size-fractionated nuclear extract to separate such factors from other, larger splicing factors such as U snRNPs and 30S hnRNP particles. To prepare size-fractionated material, we subjected nuclear splicing extract to ultracentrifugation under conditions that pelleted structures of 15S or larger. Such a simple fractionation scheme had several advantages. First, direct centrifugation produced a minimal number of fractions, eliminating the necessity of combining multiple fractions to regenerate splicing or spliceosome assembly activity. Second, by utilizing conditions that pelleted many of the required factors, complexes assembled with the pellet fraction should lack a minimal number of factors for assembly and/or splicing activity; thereby facilitating identification and ultimate purification of the factors. And finally, by analyzing the phenotype of a pellet fraction missing only a few factors, we hoped to learn something about the function of the missing factor.

Cleared nuclear extract (Experimental Procedures) was subjected to centrifugation for one hour at 106,000xg to produce a pellet -- supernatant pair. The utilized centrifugation condition was chosen both because of the 15S cut-off, and because it was the minimum centrifugation time sufficient to

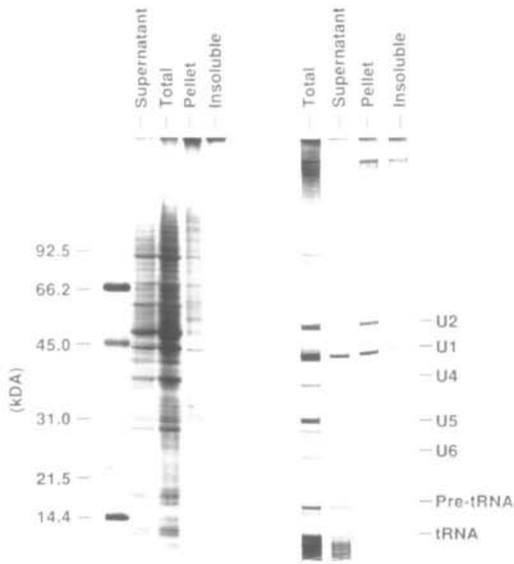


Figure 1. Fractionation of HeLa nuclear splicing extract by centrifugation. Extract was spun at 106,000 x g for 1 hour at 25^{0}C to generate a pellet and supernatant pair. Protein and small RNA profiles for equal volumes of total extract, the pellet fraction, the supernatant fraction, and a residual insoluble fraction that would not resuspend after centrifugation are shown. Each fraction was used in voluvmes equal to that of extract from which they were prepared. Therefore, the amount of material in the total lane is equal to the total material in the other lanes. Molecular weight markers are indicated for the protein gel. U RNAs are indicated for the RNA gel.

generate a supernatant -- pellet pair that were individually incompetent for splicing and spliceosome assembly but regained both activities upon mixing (see below). The pellet produced from the centrifugation was resuspended gently in extract dialysis buffer; resuspended pellet will be referred to as the pellet fraction in this communication. Some amount of pelleted material did not resuspend and was discarded before use of the pellet fraction. Supernatant, resuspended soluble pellet, and insoluble pellet were examined for protein and RNA content by gel electrophoresis (Fig. 1). The insoluble fraction from the pellet had a protein and RNA profile similar to that of total extract, provided no unique factor required for either assembly or activity, and was not included in this study.

Both the supernatant and pellet fractions had complicated protein compositions that could just be differentiated on one dimensional protein gels. Extract U RNAs and hnRNA fractionated with the pellet. Over 90% of the

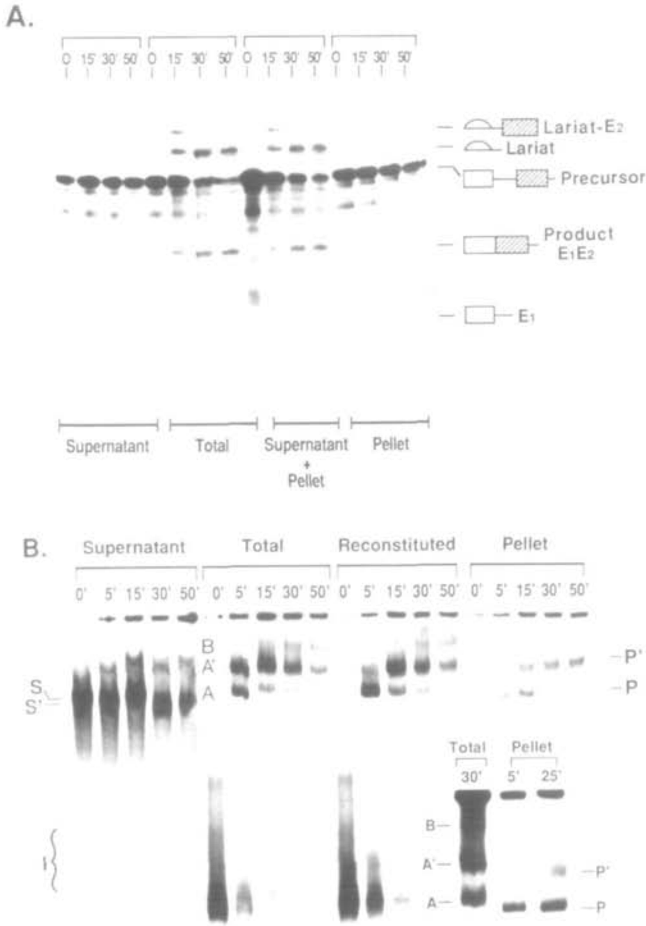


Figure 2. Splicing and assembly activity of supernatant and pellet fractions. Total, supernatant, pellet, and reconstituted supernatant and pellet fractions were used in standard splicing reactions with the MINX precursor RNA and sampled by gel electrophoresis for RNA (Panel A) and assembly (Panel B) at the indicated times (min). The RNA intermediates and products generated upon splicing are indicated for the RNA gel. A, A', and B RNP complexes formed by total extract on MINX pre-mRNA are indicated for the assembly gel. Pellet complexes P and P' and supernatant complexes S, and S' are indicated. The pellet non-specific I complex is hidden by the insert. The inset compares the mobility of complexes formed with unfractionated extract (total) and the pellet fraction on adjacent gel lanes.

U2, U4, U5, and U6 RNAs (and presumably their snRNPs) appeared in the pellet. U1 RNA distributed 50:50 into the supernatant and pellet fractions. Examination of U1 snRNP polypeptides by immunoprecipitation of pellet and supernatant fractions prepared from cells that had been labeled in vivo with ^{35}S-methionine indicated that the U1 snRNPs in both the pellet and supernatant contained a normal contingent of U1 snRNP proteins (data not shown). Thus, we interpret the equal distribution of U1 snRNPs to supernatant and pellet to be a property of both their small size (roughly 10S) and their association with larger structures, rather than as a result of the existence of sub-populations of U1 snRNPs.

Pellet and supernatant fractions neither splice nor assemble active spliceosomes.

The pellet and supernatant fractions was tested for the ability to splice an exogenous adenovirus precursor RNA containing a single intron. Each fraction was used in an amount equivalent to the amount of unfractionated extract from which it was prepared (Experimental Procedures). Neither fraction supported any splicing activity, even in extended reactions, as judged by the absence of reaction intermediate and product RNAs (Fig. 2A). Splicing activity was restored to wild type levels and at wild type kinetics when the pellet and supernatant fractions were mixed in equal amounts prior to the addition of substrate RNA. From this analysis we concluded that both fractions include factor(s) required for splicing and that no essential factor was lost or destroyed during fractionation.

The supernatant and pellet fractions were also examined for their ability to support spliceosome assembly, using a native RNP gel electrophoresis assay (Fig. 2B). Unfractionated extract assembles three complexes on substrates containing a single intron: A, A' and B. A and A' contain unreacted precursor RNA; B contains both precursor and reaction intermediates. Neither the supernatant or pellet fraction supported assembly of normal complexes. The pellet fraction directed assembly of two complexes, denoted P and P' in Figure 2B, containing unreacted precursor RNA. P and P' migrated reproducibly faster than the A and A' complexes assembled with the same substrate using unfractionated extract (Fig. 2B insert). During the course of this work, pellet fractions were analyzed from a number of extract preparations. The kinetics of appearance of complex P were very reproducible; however, the kinetics of appearance of P' varied. The profile in Fig. 2 represents an average result with P' appearing at 15-20 min; extremes of 5' and 45' were also observed. We do not know the reason for this variability.

Formation of P and P' required splicing consensus sequences in substrate RNA. No assembly of specific complexes was observed using substrates deleted for the 3' end of the intron and 3' splice junction (data not shown); a property similar to the assembly of identical deletion substrates using unfractionated extract (34-39). The similarity in pellet complexes to the complexes formed by unfractionated extract suggested that the pellet fraction directed the assembly of complexes that were similar, but not identical, to those formed with unfractionated extract. Furthermore, the pellet fraction offered a useful source of partially and/or aberrantly-assembled material to use in complementation experiments designed to identify and purify soluble supernatant factors required during spliceosome assembly.

The supernatant fraction rapidly assembled precursor RNA into a diffusely-migrating complex (Fig. 2B), denoted S. After extended incubation times, a supernatant complex of slightly faster mobility, designated S', was observed. Transition from S to S' complexes caused no apparent alteration in substrate RNA. Complex formation directed by the supernatant fraction was sequence-independent and observed gel migration was length-dependent (data not shown), suggesting the presence of general RNA-binding proteins in the supernatant that complex with any RNA to produce the observed S complexes.

The assembly factors missing in the pellet fraction, whose absence resulted in partial assembly, were present in the supernatant. Mixing equal amounts of supernatant and pellet before the addition of substrate resulted in assembly of three complexes phenotypically similar to the A, A',and B complexes formed using unfractionated extract. Furthermore, assembly using reconstituted extract was similar in extent and kinetics to that observed with unfractionated extract.

Pellet complexes have characteristics similar to normal complexes. To see if the pellet complexes were related to the A, A' and B complexes observed with unfractionated extract, we examined the characteristics of the pellet complexes using several different approaches. First, we asked if the P and P' complexes required ATP for assembly. Assembly of A, A', and B complexes using unfractionated nuclear extracts requires ATP (34,35). Fractions were depleted of ATP by preincubation at 30^{o}C and addition of the ATP analog, β,γ,-methylene ATP. The ability of the supernatant fraction to form complexes was unaffected by ATP depletion (Fig. 3), indicating that S complex formation was ATP-independent. A minor supernatant complex migrating slower than S or S' was only observed in the presence of ATP. It's identity has not been further investigated. In contrast, the ability to assemble P and P' complexes was lost when the pellet fraction was depleted for ATP. This result suggests that pellet complexes have an ATP requirement similar to that

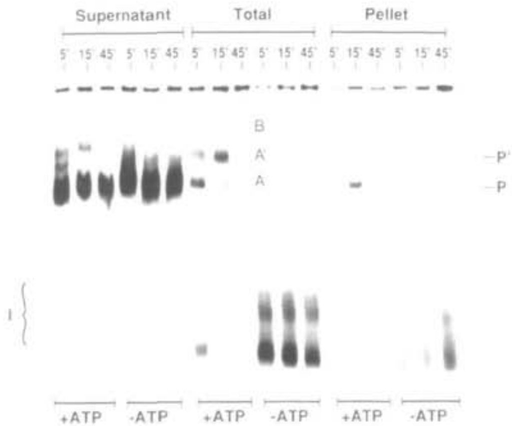


Figure 3. The formation of RNP complexes by the pellet fraction requires ATP. Splicing reactions using the supernatant, pellet, or total extract fractions were performed using MINX precursor RNA under conditions containing ATP (+ATP) or under conditions in which the extract has been depleted for ATP (-ATP). At the indicated times, reactions were sampled for complex formation on RNP gels. The supernatant S, pellet P and total extract A, A', and B complexes are indicated.

of the normal A, A' and B complexes; and that pellet assembly is arrested on the normal pathway of spliceosome assembly.

To ask if snRNPs are involved in the formation of pellet complexes, we explored the effect on complex formation of oligonucleotide-directed cleavage of individual U RNAs. To direct U RNA cleavage, an oligonucleotide complementary to an individual U snRNA was incubated with the pellet fraction prior to addition of substrate (40). Following addition of substrate, assembly activity was monitored on RNP gels (Fig. 4). The formation of pellet complexes P and P' was inhibited by cleavage of U1 RNA, suggesting that U1 snRNPs are required for assembly of pellet complexes. Cleavage of U2 RNA also inhibited the appearance of any pellet complexes (data not shown), consistent with the role of U2 snRNPs during early substrate recognition events. The demonstrated requirements for U snRNPs in pellet assembly suggested that complexes P and P' were related to the A, A' and B complexes normally seen in unfractionated extract.

Assembly of supernatant complex S was not inhibited by cleavage of U1 or U2 RNA (data not shown), suggesting that neither snRNP is required for formation of this complex. This lack of response to U RNA cleavage agreed with the lack of a requirement for consensus sequences for S formation, and further

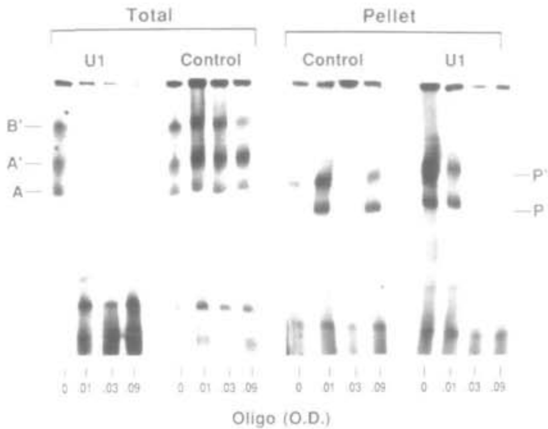
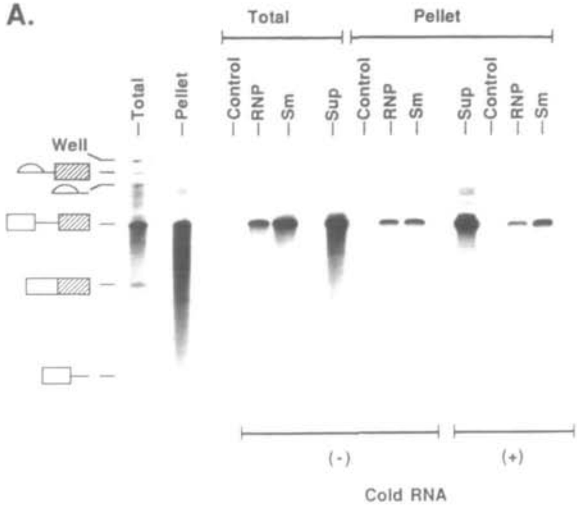


Figure 4. Pellet complexes require U1 RNA for formation. U1 RNA in total or pellet fractions was cleaved by the addition of a complementary oligonucleotide and RNAse H. Control lanes received an oligonucleotide complementary to a portion of the dihydrofolate reductase gene that caused no detectable RNA cleavage in the extract. Oligonucleotides were added in increasing amounts as indicated. Following cleavage, radiolabeled MINX RNA was added and reaction was allowed to proceed for 15 min. Samples were then analyzed for complex assembly on RNP gels. Total extract A, A', and B complexes and Pellet P, and P' complexes are indicated.

suggested that the supernatant complex is not directly related to normal assembly intermediates. Even though cleavage of U1 RNA did not abolish formation of S complex, the cleavage did have an observable phenotype. The shift in electrophoretic mobility form S to S' characteristically seen late during extended reactions with the supernatant fraction was not observed following U1 cleavage. It seems possible that U1 snRNPs associate with this complex despite its aberrant nature.

The presence of snRNPs within complexes formed during the early stages of spliceosome assembly has been well documented (1,3,41). We were interested in determining if snRNPs were present in complexes formed by the pellet fraction. To address this question, we analyzed assembly reactions by immunoprecipitation of complexes containing radiolabeled substrate RNA with anti-RNP and anti-Sm antibodies. U1 snRNPs were detected as components of the P and P' complexes, as determined by immunoprecipitation of substrate RNA by U1-specific antibodies (Fig. 5A). Anti-Sm antibodies also immunoprecipitated pellet complexes. Therefore U1 snRNP antigens, and presumably U1 snRNPs, stably associated with precursor RNA in the pellet fraction. The association of U1 snRNPs with precursor RNA was stoichiometric; i.e. in the presence of



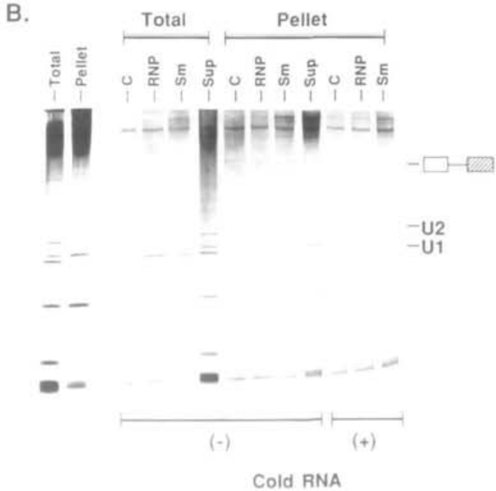


Figure 5. RNP complexes formed by the pellet fraction contain U1 snRNPs. Splicing reactions using radioactively labeled IVPX precursor RNA and pellet or total extract were incubated for 10 min. Unlabeled substrate RNA in amounts stoichiometric to the U RNAs present in the fraction was included in one set of pellet fraction reactions (+ lanes). At 10 min the reactions were divided into equal aliqouts which were sampled for total RNA (total and pellet lanes) or immunoprecipitated with control human antibodies (Control), U1-specific RNP antibodies (RNP), or monoclonal Sm antibodies (Sm). The supernatant from the control antibody immunoprecipitations are also displayed (Sup). The RNAs in the immunoprecipitates were displayed on denaturing gels. The gel was autoradiographed to detect radiolabeled substrate (Panel A). The gel was stained with silver to detect total precipitated RNA (Panel B). U RNAs andsubstrate RNAs are indicated.

high amounts of substrate RNA, equal amounts of precursor RNA and U1 RNA were immunoprecipitated (Fig. 5B). Although it is difficult to prove that the immunoprecipitated complexes are the same as those revealed on gel electrophoresis, the ability of U1-specific antibodies to immunoprecipitate stoichiometric amounts of substrate RNA suggests that the gel resolved pellet complexes contain U1 snRNPs.

Formation of pellet complex P is independent of the presence of 5' splice junctions.

We previously reported that unfractionated extract forms a specific complex with substrates deleted for 5' splice sites that we denoted the alpha complex (42). The alpha complex has a distinct electrophoretic mobility from that of the A complex formed with a wild type precursor RNA of approximately the same length and containing the same intron and second exon. The difference in A and alpha mobility is attributed to the presence of factors in the A complex that recognized 5' splice junctions, and which are missing from the alpha complexes formed with substrates lacking 5' splice sites. We noticed that the difference in gel mobility between the A and alpha complexes was very similar to that shown in Figure 2 for the difference between the A and P complexes, suggesting that the P complexes might be deficient in the recognition of 5' splice sites. We therefore asked whether the pellet fraction would respond to deletion of the 5' splice site to produce a new complex with altered electrophoretic properties compared to P. In fact, the pellet complex P formed with such a deletion substrate exhibited identical mobility to the P complex formed with wild-type substrate containing a 5' splice site (Fig. 6). This result suggests that formation of the P complex is independent of the presence of 5' splice sites within the precursor RNA, and that the pellet fraction is deficient in one or more factors required for proper 5' splice site recognition during assembly.

Deletion of the 5' splice site did affect assembly of the second pellet complex, P'. The P' complex, normally appearing at 15-20 min of reaction, was not observed in assembly reactions using deletion substrates even after extended incubation. Thus, the pellet fraction produced assemblies dependent upon 5' splice junctions, albeit at a much slower rate than that at which unfractionated extract produced complex A. Furthermore, these complexes had altered mobility compared to the normal A' complexes, and no third complex equivalent to B was ever observed.

Pellet P complexes lack factors protecting 5' splice sites

The lack of response of pellet assembly to the absence of a 5' splice site

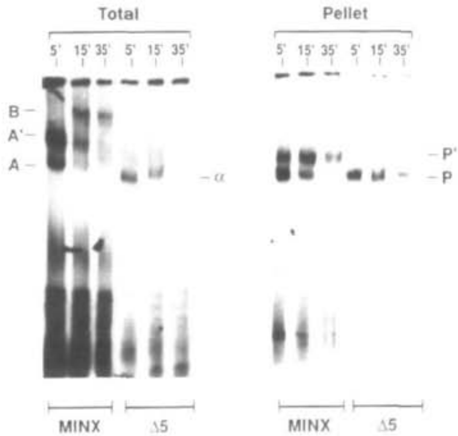


Figure 6. Assembly of the pellet P complex is independent of the presence of a 5' splice site. Wild type RNA (MINX) or a deletion lacking exon 1 and its 5' splice site (Δ5) were incubated in reactions using total extract or pellet fraction for the indicated times. Reactions were analyzed for complex formation on RNP gels. The A, A', B, and alpha complexes formed with total extract and the P, and P' complexes formed with the pellet fraction are indicated.

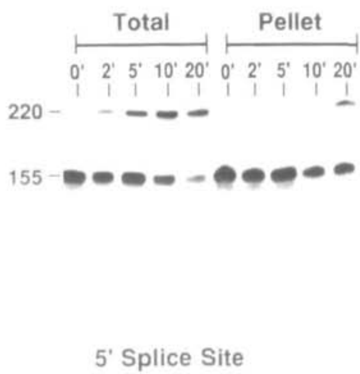


Figure 7. Pellet complexes lack factors normally bound to 5' splice sites. Splicing reactions of MINX precursor RNA with total or pellet fractions were incubated for the indicated times. An oligonucleotide complementary to the 5' splice site was added, and incubation was continued for another 10 min. RNA was analyzed on a denaturing gel. The appearance of full-length RNA (220 nucleotides) indicates protection of the 5' splice site by bound factors; the appearance of cleaved RNA (155 nucleotides) indicates no protection.

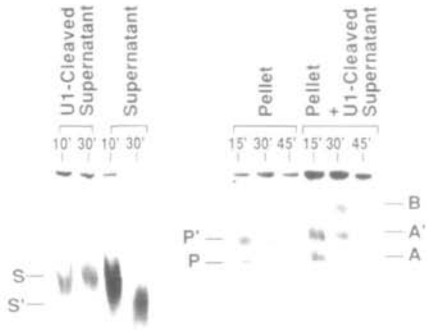


Figure 8. U1 cleavage does not destroy the ability of supernatant to complement the pellet fraction. U1 RNA in the supernatant was cleaved by incubation with a complementary oligonucleotide. Treated supernatant was mixed with 10 ul of the pellet fraction prior to the addition of substrate (pellet + U1-cleaved supernatant). Control reactions of untreated pellet (pellet), untreated supernatant (supernatant), and U1-depleted supernatant (U1-cleaved supernatant) were also performed. At the indicated times, each reaction was sampled for complex assembly on RNP gels. Supernatant S, and S' complexes, pellet P and P'complexes, and total A, A', and B complexes are indicated.

suggested that 5' splice sites were not properly recognized during pellet assembly. This idea was tested directly by assaying the accessibility of substrate RNA 5' splice sites to a complementary oligonucleotide and RNase H after assembly in the pellet fraction or in unfractionated extract (Fig. 7). Using unfractionated extract, 5' splice site protection occurs rapidly (47), indicating that factors recognizing 5' splice sites associate with precursor RNA during early assembly. In contrast, 5' splice site protection was not detected in pellet assemblies during the time interval in which P complexes were detected on RNP gels. Only after 20 min and the appearance of the P' complex, was protection observed. Furthermore, this protection never reached the levels observed with assemblies formed with unfractionated extract. We interpret the depressed 5' splice site protection in the P complex to indicate that P complexes lack factors required for normal recognition of 5' splice sites. In their absence, some slight level of recognition occurs resulting in P' formation and the observation of partial 5' splice site protection.

The complementing supernatant factor is not U1 snRNPs

Because P complexes appeared deficient in the recognition of 5' splice sites, we asked if the factor provided by the supernatant was U1 snRNPs. Although this seemed unlikely because of the ability of U1 snRNP-specific antibodies to immunoprecipitate substrate RNA in pellet complexes (and therefore the assumed presence of U1 snRNPs in the pellet complexes) the possibility remained that there were sub-populations of U1 snRNPs, only some of which were competent to recognize 5' splice sites. To test this possibility, supernatant fractions was treated with oligonucleotides complementary to U1 RNA to cleave supernatant U1 RNA prior to addition of supernatant to pellet in a complementation assay (Fig. 8). Cleavage of U1 RNA did not destroy complementation suggesting that U1 RNA (and its associated proteins) were not the factor missing in the pellet fraction.

We monitored the ability of oligonucleotide-mediated cleavage to destroy U1 RNA by gel electrophoresis of supernatant RNAs. By silver staining, we estimated that cleavage was effective to greater than 95%. Because it is difficult to prove that cleavage was efficient to reduce U1 levels to the nanogram levels of utilized substrate RNA, we also monitored the effect of cleavage on the ability of the treated supernatant to assemble S and S' complexes. The utilized cleavage permitted formation of S but eliminated the transition from S to S'. Therefore, enough U1 RNA was destroyed to affect the assembly properties of the supernatant fraction. Because this assay detects intact U1 at the stoichiometry of substrate RNA, we were assured that the complementation experiment used a supernatant fraction that contained no intact U1 RNA.

DISCUSSION

The mechanism whereby splice sites are unambiguously selected has always seemed puzzling considering the brevity of the consensus sequences containing splice sites and the complexity of the precursor RNAs in which they reside. Furthermore, the prevalence of differential splicing suggests that the cell frequently modulates splice site selection. Thus, it is perhaps not surprising that recognition of 5' splice sites may be more complicated than hybridization to complementary U1 RNA sequences. In this communication we have used extract fractionation to document the existence of at least one small nuclear factor required for recognition of 5' splice sites. Its absence results in aberrant spliceosome assembly in which recognition events at the 3' end of the intron occur, but proper recognition of the 5' splice site terminating exon 1 does not. As a result no "active" spliceosome appears and no splicing occurs.

Our fractionation approach utilized simple centrifugation in which many of the factors required for spliceosome assembly co-fractionated into the pellet. This produced a pellet fraction that directed an assembly pathway quite similar to that of unfractionated extract, facilitating identification of the step in spliceosome assembly altered because of removal of an essential factor by fractionation. Thus, the pellet fraction assembled a set of complexes of similar, but distinguishable, properties compared to normal complexes. The major deficiency in the pellet complexes was recognition of 5' splice sites. It is possible that other steps in assembly and/or activity were also altered. Purification of the missing factor will permit assays for other deficiencies. Simple fractionations of this sort should compliment more sophisticated, but complicated, fractionation schemes designed to isolate and purify all of the factors involved in pre-messenger RNA splicing.

It is always difficult to assess the relationship of gel-resolved complexes to those produced during in vivo assembly. Our gel-resolved pellet complexes had a set of characteristics common to assemblies produced using mammalian or yeast unfractionated extract. They require ATP, U1 snRNPs, U2 snRNPs, and 5' and 3' splice site consensus sequences within the RNA precursor for formation. Thus, they have properties similar to those of previously-characterized spliceosomes. They also do not appear to be dead-end complexes. Addition of supernatant resulted in appearance of normal assembly and the production of splicing intermediates even when added 15 min after intiation of pellet assembly (Zapp, unpublished data). Although it is difficult to prove the pathway whereby assembly is rescued in such an experiment until the pellet complexes have been removed from a gel and complimented with purified factors, it at least is consistent with the suggested relatedness of pellet and normal spliceosomes.

Interestingly, the aberrantly-assembled complexes lack factors bound to 5' splice sites but contain U1 snRNPs, as detected by the presence of U1 snRNP-specific antigens and a requirement for U1 snRNPs for formation. We have previously documented that U1 snRNPs are required for the first step in normal assembly -- an interaction dictated by sequences at the 3' end of the intron and occurring with substrates totally lacking a 5' splice junction (39,42). Furthermore, U1-specific antigens are components of assemblies formed with substrates lacking 5' splice sites (42). The complexes formed using unfractionated extract with substrates lacking 5' splice sites can be observed on native RNP gels (we denote them alpha complexes) and distinguished from the complexes formed with substrates containing 5' splice sites. Alpha complexes, therefore, appear very similar, and perhaps identical, to the pellet complex P described in this communication.

In a normal reaction, alpha complex formation cannot be observed; instead, complex A forms within a minute of incubation at 30^{0}C and contains factors bound to both 3' and 5' splice sites (37,39). Only in the absence of 5' splice sites, or a factor required for the recognition of 5' splice sites, can alpha-type complexes be observed. We postulate, therefore, that alpha complex formation can precede A complex formation when factors required for recognition of exon 1 are lacking. The factor missing from our pellet fraction and required for proper 5' splice site recognition is probably not a snRNP. All major snRNPs were detected in the pellet fraction at high concentration, including U1 snRNPs. The required factor could be supplied by a fraction in which U1 RNA had been stoichiometrically cleaved; therefore the missing factor was not an intact U1 snRNP. The possibility remains, however, that the supplied factor was a U1 snRNP polypeptide that easily dissociates from U1 snRNPs and was depleted in the pellet population. Analysis of the polypeptides present on pellet and supernatant U1 snRNPs indicated no differences although we did not assay for possible protein modifications. Preliminary observations indicate that the added factor is small (less that 100 kd), heat resistant, and NEM-sensitive properties that suggest it is a polypeptide. We are presently fractionating extract further to identify this polypeptide.

Other laboratories have suggested the existence of factors that enhance recognition of 5' splice sites by U1 snRNPs. Mayeda et al. (33) fractionated splicing extract through DEAE and noticed that the eluted U1 fraction recognized 5' splice sites (detected by a filter binding assay) better if the DEAE flow through lacking snRNPs was included, suggesting that factors were stripped from U1 snRNPs during chromatography. Chabot and Steitz (29) noticed that antibody-bound U1 snRNPs bound 5' splice sites well but lacked 5' splice site discriminatory properties possessed by the U1 snRNPs in unfractionated extract. It is difficult to assess how either chromatography or antibody association might alter the properties of U1 snRNPs. Both could strip weakly-associated factors. The approach used in this communication used direct centrifugal fractionation of extract; because the centrifugation was performed in extract-isolation buffer, minimal disassociation of U1-linked factors might be predicted. In fact, U1 snRNPs present in the pellet fraction were associated with substrate RNA even though 5' splice sites were not protected in an oligonucleotide-accessibility assay, suggesting that the factor facilitating this interaction in whole extract is a trans-acting factor not "associated" with U1 snRNPs in extract.

In vitro splicing requires the presence of a cap on the 5' end of precursor RNA (14,43-44). Interestingly, only the removal of intron 1 is cap-sensitive, suggesting that the first intron and its terminal splice site is recognized differently than internal introns and their 5' splice sites. It seems possible that the factor missing from our pellet fraction interacts with caps during recognition of exon 1 and its 5' splice site.

REFERENCES

1. Green, M.R. (1986) Annu. Rev. Genet. 20: 671-708.
2. Aebi, M. and Weissmann, C. (1987) Trends in Genetics 3: 102-107.
3. Maniatis, T. and Reed, R. (1987) Nature 325: 673-678.
4. Sharp, P.A. (1987) Science 235: 766-771.
5. Treisman, R., Orkin, S. and Maniatis, T. (1983) Nature 302: 591-596.
6. Treisman, R., Orkin, S. and Maniatis, T. (1983) In Globin Gene Expression and Hematopoietic Differentiation, G. Stamatoyannopoulos and A. Nienhuis, eds. (New York: Alan R. Liss) pp. 99-121.
7. Wieringa, B., Meyer, F., Reiser, J. and Weissmann, C. (1983) Nature 301: 38-43.
8. Wieringa, B., Hofer, E. and Weissmann, C. (1984) Cell 37: 915-925.
9. Lerner, M.R., Boyle, J.A., Mount, S.M., Wollin, S.L. and Steitz, J.A. (1980) Nature 283: 22-224.
10. Rogers, J. and Wall, R. (1980) Proc. Natl. Acad. Sci. USA 77:1877-1879.
11. Mount, S.M. (1982) Nucleic Acids Res. 10: 459-472.
12. Mount, S.M., Pettersson, I., Hinterberger, M., Karmas, A. and Steitz, J.A. (1983) Cell 33: 509-518.
13. Kramer, A., Keller, W., Appel, B. and Luhrmann, R. (1984) Cell 38: 299-307.
14. Tatei, K., Takemura, K., Mayeda, K., Fujiwara, Y., Tanaka, H., Ishihama, A. and Ohshima, Y. (1984) Proc. Natl. Acad. Sci. USA 81: 6281-6285.
15. Zhuang, Y. and Weiner, A.M. (1986) Cell 46: 827-835.
16. Solnick, D. 1981. Nature 291: 508-510.
17. Montell, C., Fisher, E.F.,Caruthers, M.H. and Berk, A.J. (1982) Nature 295: 380-384.
18. Montell, C. and Berk, A.J. (1984) Nucl. Acids Res. 12: 3821-3827.
19. Aebi, M., Hornig, H., Padgett, R.A., Reiser, J. and Weissmann, C. (1986) Cell 47: 555-565.
20. Eperon, L.P., Estibeiro, J.P. and Eperon, I.C. (1986) Nature 324: 280-282.
21. Aebi, M., Hornig, H. and Weissmann, C. (1987) Cell 50: 237-246.
22. Mitchell, P.A., Urlaub, G. and Chasin, L. (1986) Mol. Cell. Biol. 6: 1926-1935.
23. Pikielny, C.W., Rymond, B.C. and Rosbash, M. (1986) Nature 324: 341-345.
24. Somasekhar, M.B. and Mertz, J.E. (1985) Nucl. Acids Res. 13: 5591-5609.
25. Furdon, P.J. and Kole, R. (1986) Proc. Natl. Acad. Sci. USA 83: 927-931.
26. Oshima, Y. and Gotoh, Y. (1986) J. Mol. Biol. 195; 247-259.
27. Parent, A.S., Zeitlin, S. and Efstratiatis, A. (1987) J. Biol. Chem. 262: 11284-11291.

28. Reed, R. and Maniatis, T. (1986) Cell 46: 681-690.
29. Chabot, B. and Stetiz, J.A. (1987) Mol. Cell. Biol. 7: 698-707.
30 Nelson, K.K. and Green, M.R. (1988) Genes Devel. 2: 319-329.
31. Ruskin, B., Zamore, P.D. and Green, M. (1988) Cell 52:207-219.
32. Swanson, M.S. and Dreyfuss, G. (1988) EMBO J. in press.
33. Mayeda, A., Tatei, K., Kitayama, H., Takemura, K. and Ohshima, Y. (1985) Nucleic Acids Res. 14: 3045-3056.
34. Frendewey, D. and Keller, W. (1985) Cell 42: 355-367.
35. Grabowski, P.J., Seiler, S.R. and Sharp, P.A. (1985) Cell 42: 345-353.
36. Kaltwasser, E., Spitzer, S.G. and Goldenberg, C.J. (1986) Nucleic Acids Res. 14: 3687-3701.
37. Konarska, M.M. and Sharp, P.A. (1986) Cell 46: 845-855.
38. Perkins, K.K., Furneaux, H.M. and Hurwitz, J. (1986) Proc. Natl. Acad.Sci. USA 83: 887-891.
39. Zillmann, M., Zapp, L.M. and Berget, S.M. (1988) Mol. Cell. Biol. 8: 814-821.
40. Berget, S.M. and Robberson, B.L. (1986) Cell 46: 691-696.
41. Konarska, M.M. and Sharp, P.A. (1986) Cell 46: 845-855.
42. Zillmann, M., Rose, S.D. and Berget, S.M. (1987) Mol. Cell. Biol. 7: 2877-2883.
43. Edery, I. and Sonnenberg, N. (1985) Proc. Natl. Acad. Sci. USA 82: 7590-7594.
44. Ohno, M., Sakamoto, H. and Shimura, Y. (1987) Proc. Natl. Acad. Sci.USA 84: 5187-5191.
45. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11: 1475-1489.
46. Merrill, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981) Science 211: 1437-1438.
47. Ruskin, B. and Green, M.R. (1985) Cell 131: 131-142.