The 5' end domain of U2 snRNA is required to establish the interaction of U2 snRNP with U2 auxiliary factor(s) during mammalian spliceosome assembly

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ABSTRACT

Stable association of U2 snRNP with the branchpoint sequence of mammalian pre-mRNAs requires binding of a non-snRNP protein to the polypyrimidine tract. In order to determine how U2 snRNP contacts this protein, we have used an RNA containing the consensus 5' and the (Py)_n-AG 3' splice sites but lacking the branchpoint sequence so as to prevent direct U2 snRNA base pairing to the branchpoint. Different approaches including electrophoretic separation of RNP complexes formed in nuclear extracts, RNase T1 protection immunoprecipitation assays with antibodies against snRNPs and UV cross-linking experiments coupled to immunoprecipitations allowed us to demonstrate that at least three splicing factors contact this RNA at 0°C without ATP. As expected, U1 snRNP interacts with the region comprising the 5' splice site. A protein of approximately 65,000 molecular weight recognizes the RNA specifically at the 5' boundary of the polypyrimidine tract. It could be either the U2 auxiliary factor (U2AF) (Zamore and Green (1989) PNAS 86, 9243–9247), the polypyrimidine tract binding protein (pPTB) (Garcia-Blanco et al. (1989) Genes and Dev. 3, 1874–1886) or a mixture of both. U2 snRNP also contacts the RNA in a way depending on p65 binding, thereby further arguing that the latter may correspond to the previously characterized U2AF and pPTB. Cleavage of U2 snRNA sequence by a complementary oligonucleotide and RNase H led us to conclude that the 5' terminus of U2 snRNA is required to ensure the contact between U2 snRNP and p65 bound to the RNA. More importantly, this conclusion can be extended to authentic pre-mRNAs. When we have used a human β -globin pre-mRNA instead of the above artificial substrate, RNA bound p65 became precipitable by anti-(U2) RNP and anti-Sm antibodies except when the 5' end of U2 snRNA was selectively cleaved.

INTRODUCTION

In both mammalian and yeast splicing systems, U1, U2, U4-U6 and U5 snRNPs are the major components of functional spliceosomes (1–9). There is an ordered pathway of snRNP binding. U1 snRNP binds first. Indeed, recent results in yeast systems (10–13) have demonstrated that stable complexes containing U1 snRNP, referred as 'commitment complexes' (13), can be assembled in vitro. As a second step, a pre-spliceosome is formed containing U2 snRNP and additional proteins whose number remains to be determined. Finally the functional spliceosome is assembled (reviewed in ref. 14–16).

Several RNA binding proteins are candidates to be involved in the early stages of spliceosome assembly. They include the 100 Kda-70 Kda intron binding proteins (17, 18) that recognize the (Py)_n-AG sequence specifically. The 100 Kda protein is associated with U5 snRNP (17) which also contains an unexpectedly high number of specific proteins (19). Despite the failure to detect U5 snRNA in the pre-splicing complexes, saturation-complementation assays made with a purified IBP-U5 snRNP led us to consider that a form of U5 snRNP could have an early role in the spliceosome assembly pathway, thereby challenging the accepted model according to which U5 snRNP participates only after U1 and U2 are already bound to the pre-mRNA (20). Proteins different of IBP are the so-called U2 snRNP auxiliary factor (U2AF) (21) and the 62 Kd pPTB protein more recently described (22). Binding of U2AF to a part of the polypyrimidine tract is a prerequisite to the stable association of U2 snRNP to the branchpoint sequence (21, 23), while alterations in the polypyrimidine tract that reduce the binding of pPTB yield a corresponding reduction in the efficiency of formation of a U2 snRNP/pre-mRNA complex and splicing (22). Other factors whose function in spliceosome assembly remains to be elucidated include the mammalian equivalent of the yeast PRP8 protein found associated with U5 and U5/U4-U6 complexes (24–26) and the hnRNP proteins that bind preferentially to sequences typical of the polypyrimidine tract upstream from the 3' splice site (27–29). SF2 is now known as an activity necessary for

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5' splice site cleavage and lariat formation (30), but whether SF4B (31), or SF1 and SF3 (32) are or not related to the above better characterized factors is still unclear.

Recent results have emphasized the role of the branchpoint sequence in spliceosome assembly and splicing of mammalian pre-mRNAs. In contrast to yeast, mutations in the branchpoint sequence that decrease splicing efficiency do not affect spliceosome assembly (33). As in yeast, U2 snRNA base pairs with the branchpoint sequence (34, 35). A branchpoint can be specified independently of the 3' splice site if located at a long distance upstream (36) or if the pyrimidine tract is long (37). Branching on U residues downstream the normal A occurs at early times in the reaction most likely as a consequence of the early interaction of U2 snRNP with the factors recognizing the 3' splice site (38).

In this work, we show that the 5' end of U2 snRNA is essential to allow U2 snRNP to contact a 65 Kda protein that binds specifically to the 5' side of the polypyrimidine tract at 0°C without ATP. Whether the protein is U2AF (Ruskin et al. 1988; Zamore and Green 1989), pPTB (Garcia-Blanco et al. 1989) or both is unclear. Therefore, it will be referred as p65.

MATERIALS AND METHODS

Materials

SP6 RNA polymerase, RNasin, restriction enzymes and DNase I (RNase-free) were from Promega Biotec, RNase T1 from Calbiochem, E. Coli RNase H and ribonucleotides triphosphates from Boehringer Mannheim, m⁷G5'pppG5'cap and micrococcal nuclease from Pharmacia, α^{32} P triphosphates and rainbow protein molecular weight markers from Amersham. Oligodeoxynucleotides were synthesized in our laboratory using a Biosearch SAM I apparatus that uses phosphotriester chemistry. All other chemicals were of analytical grade.

Plasmids, SP6 transcription, Nuclear extracts and Oligodeoxynucleotide-directed cleavage of snRNAs

pSP64H β Δ 6, pSP64 β ¹¹⁰ and pSP64H β Δ 3' were generous gifts from Dr. T. Maniatis (Harvard University, Boston). The Δ BP Δ 3' plasmid was constructed by inserting an in vitro-synthesized DNA fragment containing a consensus 5' splice site and cohesive ends for the SalI and XbaI sites into the pGEMTM1 vector (5'-TCG-AACAGGTAAGTAT-3' and 5'-CTAGATACTTACCTGT-3'). The Δ BP Δ 5' plasmid was similarly constructed. It contains the last 24 nt of the first intron of the human β -globin gene and cohesive ends for the XbaI and EcoRI sites of the pGEMTM1 vector (5'-CTAGATTGGTCTATTTTCCCACCTTAG-3' and 5'-AATTCTAAGGGTGGGAAAATAGACCAAT-3'). The Δ BP mini-intron was obtained by inserting the above XbaI-EcoRI fragment into the corresponding sites of the Δ BP Δ 3' plasmid.

The Δ BP mini-intron and the Δ BP Δ 5' RNAs on one hand, the Δ BP Δ 3' RNA on the other hand were synthesized from respectively EcoRI and XbaI linearized plasmids. The β -globin RNAs were synthesized after cutting the plasmid at the BamHI site at the end of the second exon. Conditions for transcription were as previously described (7, 20, 38). Ten times more labeled RNAs were synthesized in experiments where immunoprecipitated cross-linked proteins were analyzed (Figure 7).

Preparation of HeLa cell nuclear extract was as originally described (39) in TEA buffer (20 mM TEA, pH 7.9, 20% [v/v] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT) (17). They were systematically incubated during 30 min at 30°C before utilization to hydrolyse endogenous ATP.

Oligodeoxynucleotides complementary to nucleotides 1–15 (counting the cap as nucleotide 0) of U1 and U2 snRNAs were synthesized and purified in 20% polyacrylamide gels. Conditions for RNase H reactions were as described (40) except that ATP and creatine phosphate were omitted. Although the oligonucleotides have not effect *per se* in either the formation of RNP complexes, the immunoprecipitations or the cross-linking assays, experiments were performed after the reactions were incubated at 30°C for additional 30 min with 20 units DNase I added. Examination of the snRNAs in polyacrylamide gels showed that either U1 or U2 were nearly completely cleaved unlike the other snRNAs. Control nuclear extracts with uncleaved snRNPs were similarly incubated.

Electrophoretic separation of RNP complexes

α^{32} P UTP-labeled RNAs were added to nuclear extracts in TEA buffer (10 μ l containing 30% nuclear extract, 3.2 mM MgCl₂ and 8 units of RNasin) and incubated at 0°C for 10 min. 5 μ l were withdrawn, added to 1 μ l of 20 mg/ml heparin and left for 20 min at room temperature. After addition of 2 μ l of loading buffer (50% glycerol, 1% bromophenol blue and 1% xylene cyanol) reactions could be kept at –20°C overnight. Electrophoreses were in 4% polyacrylamide/0.5% agarose composite gels as described (21)

UV Cross-linking

α^{32} P-labeled RNAs (5×10^4 cpm, ≈ 2.5 ng and 2.5×10^5 cpm, ≈ 7.5 ng [cerenkov counting] for the artificial and the human β -globin RNAs, respectively) were added to 30 μ l of 30% nuclear extract (either standard or with either U1 or U2 snRNAs cleaved) made 0.5 mM DTT and 3.2 mM MgCl₂ and containing 20 units of RNasin. The reaction mixtures were kept on ice for 10 min and irradiated during 10 min with a UV transilluminator at 254 nm (7 mW/cm² on the surface of the filter). The distance between the samples and the filter was 9 cm. The samples were diluted, made 2 mM in CaCl₂ and finally digested by RNase A (15 μ g/ml) and micrococcal nuclease (300 units/ml) during 30 min at 37°C (41). After nuclease digestion, protein adducts were precipitated by acetone and electrophoresed in 10% SDS-polyacrylamide gels followed by autoradiography on X-AR films.

Immunoselection of RNase T1 RNA fragments and cross-linked proteins

The patient anti-(U2) RNP (Ya) and the mouse monoclonal anti-Sm (7.13) and anti-RNP (2.73) antibodies were generous gifts respectively from Dr. J. Steitz (Yale University, New Haven) and Dr. Hoch (Agouron Institute, La Jolla). Immunoprecipitation/RNase T₁ digestions were as described (40, 42, 43) starting with 5×10^5 cpm (cerenkov counting) ≈ 4 ng of Δ BP mini-intron, 15 μ l of 30% nuclear extract (either standard or with either U1 or U2 snRNAs cleaved) made 3.2 mM in MgCl₂ and 2.5% in polyvinyl alcohol. Incubations were for 30 min at 0°C in the presence of 150 units of RNase T₁, 40 units of RNasin and an excess of antibody. Protein A-Sepharose-bound RNA fragments were processed as described (40) and analyzed on prerun 20% polyacrylamide-8 M urea gels in TBE buffer. Cross-linked proteins were selected similarly starting with 5×10^5 (2.5 ng) and 3.5×10^6 cpm (17.5 ng) of Δ BP mini-intron and human β -globin pre-mRNA, respectively. The RNAs were incubated for 10 min at 0°C in 30% nuclear extract made 3.2 mM MgCl₂ and 40 units of RNasin (final volume 30 μ l) and irradiated at 254 nm (see above) before addition of RNase T₁ and antibody.

Figure 1. Sequence of the DBP mini-intron. The sequence has been numbered counting the cap as nucleotide 0. A, A' and B segments refer to RNase T1 fragments described in Figure 6.

Washed protein A-Sepharose-bound material was subjected to RNase A and micrococcal digestion before to be eluted in Laemli buffer and electrophoresed in 10% SDS-polyacrylamide gels.

RESULTS

It is widely believed that the ATP-dependent interaction of U2 snRNP with the branchpoint sequence of mammalian pre-mRNAs requires at least one auxiliary protein whose binding to the polypyrimidine tract of pre-mRNA occurs at 0°C in the absence of ATP. How U2 snRNP contacts this protein, already bound to the pre-mRNA, be it U2AF or pPTB, is not known. At first glance, such an investigation would be feasible using an RNA containing a (Py)_n-AG 3' splice site, but lacking the branchpoint sequence so as to prevent base pairing with the internal U2 snRNA domain (nucleotides 28 to 42). As a matter of fact, it has been shown previously that a substrate that does not contain a branchpoint sequence but only the last 30 nucleotides of the first intron of the human β -globin sequence is able to bind both U2 snRNP and U2AF (21). In this work, we considered that the substrate should also contain a 5' splice site for two reasons. First, U1 snRNP precedes U2 in spliceosome assembly and, therefore, could have some cooperative effect on the binding of the auxiliary protein(s) or/and U2 snRNP. Second, the presence of a U1 snRNP binding site just upstream the polypyrimidine tract, instead of a free sequence even unrelated to splicing, should prevent any direct interaction involving the U2 snRNA sequence. Under these conditions, the contact between U2 snRNP and the RNA, if any, should be necessarily mediated by the auxiliary protein(s) bound to the polypyrimidine tract or both the auxiliary protein(s) and U1 snRNP.

The sequence of the artificial RNA we have constructed is shown in Figure 1. It is a 70 nt long molecule containing a mammalian consensus 5' splice site and the last 24 nucleotides of the first intron of the human β -globin gene. It lacks a branchpoint sequence and does not support splicing. It will be referred to as 'ΔBP mini-intron'. Two other substrates were also used in some experiments. One contains a consensus 5' splice site and the other one the last 24 nucleotides of the first intron of the human β -globin sequence. They will be referred to as ΔBPΔ3' and ΔBPΔ5' RNAs, respectively.

U1 snRNP binds to the ΔBP mini-intron

To see whether U1 snRNP stably binds to the ΔBP mini-intron, we performed electrophoretic separations of RNP complexes formed at 0°C without ATP in either standard (NE) or micrococcal nuclease-treated (MN-NE) nuclear extracts as well as in extracts in which U1 snRNA was subjected to oligonucleotide-directed cleavage by RNase H (Figure 2). Two

Figure 2. ΔBP mini-intron and ΔBPΔ3' RNAs form RNP complexes at 0°C without ATP. The following amounts of ³²P UTP-labeled RNA were used: 5×10⁴ cpm (2.5 ng) for the ΔBP mini-intron and the ΔBPΔ5'; 3×10⁴ cpm (1.5 ng) for the ΔBPΔ3' and the vector (pGEMTM1 linearized by XbaI). Lanes 7, 11 and 8, 12 correspond to U1 and U2 snRNAs cleaved (nt 1–15) nuclear extracts, respectively. In lanes 6 and 10, the nuclear extract was digested with 90 units micrococcal nuclease before utilization. Ct is a control assay with standard nuclear extract. Star indicates the slow-migrating RNP complex.

RNAs lacking the branchpoint sequence and either the 5' (ΔBPΔ5') or the 3' (ΔBPΔ3') splice sites were used (controls) as well as an RNA of similar size containing plasmid sequences (Materials and Methods). In brief, a slow-migrating complex was formed with the RNAs containing a consensus 5' splice site (lanes 1, 3 for the ΔBP mini-intron and the ΔBPΔ3' RNA, respectively) but not with those containing only the 3' splice site (lane 2) or sequences unrelated to splicing (lane 4). Micrococcal nuclease treatment of nuclear extract severely abolished the ability of the slow-migrating complex to form (lanes 6 and 10), indicating that at least one snRNP participates. Cleavage of U1 snRNA within the sequence required for 5' splice site recognition (nt 1–15) resulted in the inhibition of the formation of the slow-migrating complex (lanes 7 and 11), whereas cleavage of the 5' end of U2 snRNA (nt 1–15) remained without effect (lanes 8 and 12). We therefore concluded that at least U1 snRNP binds to the ΔBP mini-intron as well as to the ΔBPΔ3' RNA. Whether other snRNPs also bind is difficult to know using these electrophoretic

Figure 3. Proteins cross-linked to the artificial RNAs and authentic or mutants human β -globin pre-mRNAs. A) Proteins UV cross-linked to all the ^{32}P UTP-labeled artificial RNAs used in Figure 2. B) Competition assays using a 50-fold molar excess of unlabeled ΔBP mini-intron and either ^{32}P GTP and ^{32}P UTP-labeled human β -globin pre-mRNA (lane 3) or similarly labeled ΔBP mini-intron (lane 4). C) Proteins cross-linked to the ^{32}P UTP-labeled human β -globin mutants (ΔAG , lane 2) and ΔPyAG , lane 3). Protein markers are indicated. Star indicates the position of p65.

separations. Indeed, the slow-migrating complex is too poorly separated from free snRNPs, thus rendering illusory northern hybridizations with snRNA probes. Similarly, the complex migrated at 12–15S in sucrose gradients, i. e. the position expected for free snRNPs (not shown). In view of previous findings suggesting that U1 snRNP is loosely bound to the spliceosome and therefore can be lost before or during electrophoresis, we were surprised to see that most of the RNA introduced in the assay is found in the slow-migrating complex. Presumably, the perfect complementarity between this consensus 5' splice site and the 5' end of U1 snRNA is responsible for such a stability.

A 65 Kda protein interacts specifically with the polypyrimidine tract of the ΔBP mini-intron

Purified U2AF has been found to comprise roughly equimolar quantities of two polypeptides of 65 Kda and 35 Kda (44). Furthermore, the 65 Kda component of U2AF as well as the 62 Kda pPTB (22) readily cross-link upon UV irradiation to RNAs containing a polypyrimidine tract/3' splice site sequence. We therefore performed UV cross-linking assays to see whether these previously characterized proteins also contact specifically those of our artificial RNAs that have a polypyrimidine tract. Uniformly $\alpha^{32}\text{P}$ -UTP labeled ΔBP mini-intron, $\Delta\text{BP}\Delta 5'$, $\Delta\text{BP}\Delta 3'$ and RNA containing sequences unrelated to splicing were incubated with nuclear extracts at 0°C without ATP, i.e. under conditions where U2AF (44) and pPTB (22) bind efficiently to pre-mRNAs. Proteins were cross-linked to RNA by irradiation at 254 nm, then, after digestion by RNase A and micrococcal nuclease, protein adducts were separated by SDS-PAGE (Figure 3A). As expected,

several proteins were cross-linked in the 35 to 200 Kda range. Disregarding those cross-linking also to the vector and the 40 Kda polypeptides likely to be hnRNP proteins also known to bind polypyrimidine sequences, we focused our attention on a single 65 Kda species which appeared with the ΔBP mini-intron and, but to a lesser extent, with the $\Delta\text{BP}\Delta 5'$ RNA. As this suggested that the protein binds specifically to the polypyrimidine tract of these RNAs and, therefore, could be the 65 Kda component of U2AF, the 62 Kda pPTB or a mixture of both, we compared the proteins cross-linked to the ΔBP mini-intron to those cross-linked to the human β -globin pre-mRNA under identical conditions. We found that the pattern of bands was very similar and, most importantly, that a single species of approximately 65,000 molecular weight appeared with the two RNAs (Figure 3B, lanes 1, 2). To be sure that the 65 Kda polypeptide seen in the β -globin assay binds to the RNA with specificity, we performed cross-linking assays with the human ΔPyAG and ΔAG β -globin mutants (45). p65 was present in the ΔAG but not in the $\Delta\text{Py-AG}$ (Figure 3C). The polypyrimidine tract is therefore determining for p65 binding as reported for PPTB and U2AF. To see now whether the 65 Kda species cross-linked to the ΔBP mini-intron is identical to that cross-linked to the human β -globin pre-mRNA, we performed competition experiments using a 50-fold molar excess of unlabeled ΔBP mini-intron. As expected, all the bands disappeared in the homologous competition assay (Figure 3B, compare lanes 4 and 5). Cross-linking of p65 was competed also in the β -globin assay, but a # 100 Kda remained and a new faint band with an apparent molecular weight slightly lower than 65 Kd appeared (compare lanes 2 and 3). This last point was not further investigated. Finally, we narrowed down the binding site of p65. To this aim, RNAs progressively deleted in the

Figure 4. Delineating the binding site of p65. A) 32 P UTP-labeled RNA was synthesized after linearizing the Δ BP mini-intron plasmid by PvuII and subjected to RNase H digestion using the following oligos: a) AGGGAGACCGGAATTCTA; b) GAGACCGGAATTCTAAGG; c) GACCGGAATTCTAAGGGT; d) AATTCTAAGGGTGGGAAA. B, C) Electrophoresis of RNA fragments produced by cleavage with endogenous RNase H of nuclear extract (panel B) or purified E. coli RNase H (panel C). These fragments were eluted from the gels, analyzed by RNase T1 fingerprinting (not shown) and numbered 1 to 4 (see text for RNA 5 due to a redundant GGUCU sequence indicated ■ ■ ■ ■ ■ in A). The theoretical length of RNAs 1 to 5 is indicated in A. However, RNase T1 analysis denoted a heterogeneity at the 3' end from 0 to 2 nt. D) Similar amounts of RNAs 1 to 5 (2.5 ng) were used in standard cross-linking assays and compared to the full length Δ BP mini-intron.

polypyrimidine tract were generated from the Δ BP mini-intron by oligonucleotide-directed cleavage as detailed in Figure 4A. Following utilization of nuclear extract as a source of RNase H, the four expected RNAs (1 to 4 in Figure 4A) were quantitatively generated (Figure 4B) indicating that the sequence downstream position 51 is not covered by proteins. Using free RNase H instead of nuclear extract (Figure 4C), a new band was generated (RNA 5) due to the presence of a GGUCU sequence in the 45–49 positions (this sequence became inaccessible to RNase H in the nuclear extract) that also base pairs with the oligos a to c. This band represents an RNA having conserved only 3 of the 24 nt of the human β -globin sequence contained in the Δ BP mini-intron. All these RNAs (numbered 1 to 5) were excised from the gel shown in Figure 4C and used in cross-linking assays. Figure 4D shows that the 65 Kda protein cross-links to RNAs 1 to 3 (lanes 1–3) corresponding to progressive 3' deletions, noticeably less to RNA 4 (lane 4) lacking most of the polypyrimidine tract and not at all to RNA 5 (lane 5) terminating upstream of the Py-AG 3' splice site.

Figure 5. Binding of p65 to the Δ BP mini-intron depends of U1 snRNP. Either standard (lane 1), U1 snRNA cleaved (lane 2) or U2 snRNA cleaved nuclear extracts (lane 3) were used in cross-linking assays with the 32 P-UTP labeled Δ BP mini-intron as in Figure 3. Markers are indicated. Star points out p65.

Altogether these results demonstrate that the 65 Kda protein that binds to the polypyrimidine tract of the human β -globin pre-mRNA in crude extracts also binds to the Δ BP mini-intron specifically. We are at present unable to say whether the protein is U2AF, pPTB or a mixture of both. As a matter of fact, a single 65 Kda band reproducibly appeared in all our assays either performed with the human β -globin pre-mRNA or the Δ BP mini-intron. In this respect, it is noteworthy that Zamore and Green (44) on one hand, garcia-Blanco et al. (22) on the other hand have detected also a single species recognizing the polypyrimidine tract specifically. This was also the case in another more recently published work reporting similar experiments (46).

Binding of p65 to the Δ BP mini-intron depends on the integrity of U1 snRNA

We reproducibly observed that p65 cross-links to the Δ BP mini-intron more efficiently than to Δ BP Δ 5' RNA although the latter contains the same p65 binding site (Figure 3A). As this suggested that U1 snRNP, once bound to the 5' splice site, facilitates or stabilizes the binding of p65, we performed cross-linking assays using nuclear extracts in which the 5' end of U1 snRNA (nt 1–15) was subjected to oligonucleotide-directed cleavage by RNase H to prevent binding of U1 snRNP. The p65- Δ BP mini-intron cross-link was severely abolished while it remained unaffected in a control where the U2 snRNA 5' end was similarly removed (Figure 5, lanes 2 and 3, respectively). Therefore, it appears that U1 snRNP and p65 can be components of the same ribonucleoprotein complex formed at 0°C without ATP. Such a facilitating or stabilizing effect of U1 snRNP with respect to the binding of U2AF or pPTB was not reported in experiments with authentic pre-mRNAs. Indeed, we observed that binding of p65 to the human β -globin and adenovirus substrates occurred to a very similar extent with either normal or U1 snRNA-cleaved nuclear extracts (not shown). Therefore, binding of p65 at 0°C without ATP does not depend on U1 snRNP in pre-mRNAs, in which the binding sites of U1 snRNP and p65 are widely separated, in contrast to the Δ BP mini-intron in which they are juxtaposed.

Figure 6. Analysis of anti-(U1) RNP, anti-(U2) RNP and anti-Sm immunoprecipitated RNase T1 fragments from the Δ BP mini-intron. A) Δ BP mini-intron was incubated at 0°C without ATP in standard (lanes 1–3), U1 snRNA cleaved (lane 4) or U2 snRNA cleaved (lane 5) nuclear extracts in the presence of RNase T1 and one of the following antibodies: anti-(U1) RNP (lane 1), anti-Sm (lanes 2, 4, 5), anti-(U2) RNP (lane 3). Protected fragments were processed as described in Materials and Methods and separated on a pre-run 20% polyacrylamide-urea gel. B) RNase T1 analyses of the protected A, A' and B fragments from A) in a 20% polyacrylamide-urea gel. Markers were from free- Δ BP mini-intron digested by RNase T1.

U2 snRNP contacts p65 bound to the Δ BP mini-intron through the 5' end of its snRNA

Having established that U1 snRNP and p65 bind to the Δ BP mini-intron within the same complex, we asked whether U2 snRNP can in turn bind and, if so, how does the contact take place in the absence of a branchpoint sequence. To this aim, RNase T1 protection-immunoprecipitation assays (40, 42, 43) were carried out using anti-(U2) RNP as well as anti-Sm and anti-(U1) RNP antibodies. 32 P-labeled Δ BP mini-intron was incubated during 30 min at 0°C without ATP in a nuclear extract in the presence of one of the above antibodies and RNase T1. Immunoprecipitated RNase T1 fragments were deproteinized, separated through a 20% sequencing gel (Figure 6A) and the major ones subsequently identified by a secondary RNase T1 analysis (Figure 6B). This revealed that fragment B is the 3' terminal T1 oligonucleotide of the Δ BP mini-intron (19 nt), while A and A', in corresponding to the 25–41 and 21–34 positions, are derived from the consensus 5' splice site (see Figure 1). Anti-(U1) RNP antibody mainly precipitated fragment A and a small amount of A' (Figure 6A, lane 1). We calculated that about 50% of the Δ BP mini-intron is immunoprecipitated, a proportion commensurate to the fraction of input RNA engaged in a slow-migrating complex containing U1 snRNP in non-denaturing gels (Figure 2). The anti-Sm antibody precipitated fragments B, A and A' (Figure 6A, lane 2) indicating that another snRNP different from U1 binds to the 3' end of the Δ BP mini-intron. Immunoprecipitation with anti-(U2) RNP (lane 3) identified this snRNP as being U2. Interestingly, cleavage of the 5' end of U2 snRNA, which does not impair the immunoprecipitability of U2 snRNP, resulted in the absence of fragment B in an anti-Sm immunoprecipitation

Figure 7. Analysis of anti-(U1) RNP, anti-(U2) RNP and anti-Sm immunoprecipitated cross-linked proteins. A) Aliquots of 32 P UTP-labeled Δ BP mini-intron to high specific activity (Materials and Methods) were incubated during 10 min at 0°C without ATP in standard nuclear extracts, UV cross-linked and then further incubated as in Figure 6 in the presence of RNase T1 and one of the following antibodies: non immune human serum (lane 2), anti-(U1) RNP (lane 3), anti-(U2) RNP (lane 4), anti-Sm (lane 5). Antigen-antibody complexes were selected by protein A-Sepharose, digested with RNase A and micrococcal nuclease and analyzed in 10% SDS gels. Lane 1 shows a regular cross-linking assay made from Δ BP mini-intron labeled under standard conditions as in Figure 3. B) All conditions were as in A) except that only anti-Sm antibody was used and that nuclear extracts were either standard (lane 2), U1 snRNA cleaved (lane 3) or U2 snRNA cleaved (lane 4). C) Proteins cross-linked to the human β -globin pre-mRNA labeled to high specific activity and immuno-selected by anti-Sm antibodies under conditions identical to those used for the Δ BP mini-intron.

assay (lane 5), thereby demonstrating the participation of this U2 snRNA domain in the Δ BP mini-intron-U2 snRNP contact. As expected, cleavage of the 5' end of U1 snRNA dramatically reduces the immunoprecipitability of fragments A and A', but also that of fragment B. This is in keeping with our above observation that efficient binding of p65 to the (Py)_n-AG sequence of the Δ BP mini-intron requires U1 snRNP and demonstrates that U2 snRNP cannot interact with this RNA in a fashion not involving p65. We calculated that fragment B in either lane 2 or 3 in Figure 6A corresponds to about 2.5% of the input RNA, i.e. a low but significant recovery since greater than that obtained with authentic pre-mRNAs in similar experiments (40). This seems to be the bottom value representative of the amount of Δ BP mini-intron forming a complex containing U1 snRNP, p65 and U2 snRNP.

p65 Cross-linked to either the Δ BP mini-intron or an authentic pre-mRNA is immunoprecipitable by anti-(U2) RNP and anti-Sm antibodies

Considering that U1 snRNP and p65 bind to the Δ BP mini-intron directly and that binding of U2 snRNP is mediated by p65, we anticipated that cross-linked p65 should be detected by SDS-PAGE in anti-(U2) RNP and anti-Sm precipitates. Immunoprecipitations were carried out using anti-(U1) RNP, anti-Sm and anti-(U2)RNP after the Δ BP mini-intron was incubated in a splicing extract at 0°C without ATP, UV irradiated and finally subjected to RNase T1 digestion (Materials and Methods). Once eluted from protein A-Sepharose beads and digested by RNase A and micrococcal nuclease, protein adducts were separated by SDS-PAGE (Figure 7A). The anti-(U1) RNP precipitate mainly contained a 70 Kda band suggesting that it could be the U1 snRNP-specific 70 Kda protein. The anti-(U2) RNP precipitate

only contained a 65 Kda band, whereas all the main polypeptides seen with either anti-(U1) RNP or anti-(U2) RNP antibodies were present in the anti-Sm precipitate in addition to a 55 Kda band in an amount similar to the 65 Kda, and another polypeptide of smaller size. Assuming that the 65 Kda polypeptide seen in anti-(U2) RNP and anti-Sm precipitates is indeed p65, cleavage of the 5' ends of U2 and U1 snRNAs should lead respectively to the absence and the decrease of the 65 Kda polypeptide in the anti-Sm precipitate. Figure 7B shows that the protein is either almost absent (U2 cleaved in lane 4), or noticeably decreased (U1 cleaved in lane 3). However, we cannot explain at present why the 55 Kda polypeptide also disappeared in lane 4.

In a similar experiment, we used human β -globin pre-mRNA instead of Δ BP mini-intron (Figure 7C). Again, a 65 Kda band was immunoprecipitated by anti-Sm antibodies and cleavage of the 5' end of U2 snRNA impaired its immunoprecipitability.

DISCUSSION

We have exploited anti-(U2) RNP and anti-Sm antibodies to demonstrate that U2 snRNP binds an artificial RNA (Δ BP mini-intron) containing the canonical consensus 5' and (Py)_n-AG 3' splice sites but lacking the branchpoint sequence known to be essential for binding of U2 snRNP to pre-mRNAs. Binding to Δ BP mini-intron occurs at 0°C, does not require ATP and most likely represents the recognition of a 65 Kda non-snRNP protein already bound to the RNA at the 5' portion of the polypyrimidine tract in addition to U1 snRNP bound to the 5' splice site. We have no doubt that this 65 Kda species corresponds to either one of the previously characterized U2AF (21, 23, 44) and pPTB (22) proteins. Quite interestingly, we find that targeted cleavage of the 5' end of the RNA in U2 snRNP abolishes the interaction of U2 snRNP with the Δ BP mini-intron.

Are these findings representative of the early events of U2 snRNP binding to pre-mRNAs?

It is not surprising to find that a non-snRNP splicing factor recognizing specifically the polypyrimidine tract of pre-mRNAs, be it U2AF (44) or pPTB (22), interacts also with the artificial Δ BP mini-intron. As a matter of fact, the latter contains the polypyrimidine tract of the first intron of the human β -globin pre-mRNA. What is new is our observation that the protein bound to this RNA becomes precipitable by anti-Sm and anti-(U2) RNP antibodies unless the 5' end of U2 snRNA is cleaved. Since we have obtained the same results using an authentic pre-mRNA (Figure 7C), the possibility of artefactual binding of U2 snRNP to the artificial Δ BP mini-intron has to be ruled out. Therefore, the Δ BP mini-intron seems to be an useful substrate, even if it does not support splicing, to study the early ATP-independent interaction of U2 snRNP that precedes stable binding to the branchpoint sequence in spliceosome assembly. Under these conditions, it seems demonstrated that the 5' end domain of U2 snRNA is required to establish the U2 auxiliary protein-U2 snRNP interaction in pre-mRNAs.

Is the apparent U1 snRNP-dependence of p65 an important determinant in spliceosome assembly and splicing?

In yeast systems, it has been suggested that U1 snRNP is an important determinant to join the splicing sites (11) and that U1 snRNP may interact with a factor recognizing the branchpoint/3' splice site region (13), even if proteins typical of those recognizing the 3' splice site region in mammalian (17, 18, 21, 22, 27, 32) have not yet been identified in yeast. Also in mammalian systems,

it appears that U1 snRNP is required at early stages of spliceosome assembly (7, 32, 47). Quite recently, it was demonstrated that U1 snRNP has an additional role in mediating U2 snRNP binding independently of the U1 snRNA-5' splice site interaction (48), thus explaining why stable U2 snRNP-containing complexes can form with RNAs lacking a 5' splice site (2, 6, 47, 49). At first glance, this recent finding could agree with our observation that binding of p65 to the DBP mini-intron is controlled by U1 snRNP. However, what we observed seems to be due to the U1 snRNP bound to the 5' splice site, since dependent of the 5' end of U1 snRNA, and is therefore likely to be unrelated to this new function reported for U1 snRNP. A simple explanation to our observation, a situation not encountered in binding of either U2AF or pPTB to authentic pre-mRNAs, is to consider that the binding sites for U1 snRNP and p65 are juxtaposed in the Δ BP mini-intron and spaced out in a true pre-mRNA. We recall here that these results were obtained at 0°C without ATP, i. e. under conditions non-permissive for splicing. Under splicing conditions (30°C in the presence of ATP) the binding sites of U1 snRNP and p65 are brought closer to each other, most likely at an early stage of spliceosome assembly, so that U1 snRNP bound to the 5' splice site could serve to facilitate, stabilize or specify the interaction of the protein required for U2 snRNP binding. Testing this model with pre-mRNAs is difficult since cleavage of the 5' end of U1 snRNA would lead to the absence of recognition of the 5' splice site by U1 snRNP and therefore to the lack of interaction with the factor(s) recognizing the 3' splice site.

Is the 5' end domain of U2 snRNA directly involved in the RNA-bound p65-U2 snRNP interaction?

As a matter of fact, we have found that cleavage of the 5' end of U2 snRNA prevents the ATP-independent contact of U2 snRNP with the 65 Kda protein bound to the polypyrimidine tract equally well with the artificial RNA lacking the branchpoint sequence as with a pre-mRNA. In this respect, the results agree perfectly with previously reported conclusions regarding the structure/function relationship of different domains of the U2 snRNA sequence (8, 43, 50). Indeed, these authors reported that cleavage of the 5' end of the RNA in U2 snRNP prevents the formation of complex A (pre-spliceosome). However, more recent works with antisense 2'-OMe RNA oligonucleotides have led to the opposite conclusion (51, 52). Complex A but not functional spliceosome is formed suggesting that the 5' end of U2 snRNA is required only at a later step of spliceosome assembly, unless it can still function in the presence of the antisense RNA to promote complex A formation. To tentatively explain these contradictory data, Lamond and coworkers surmised that the consequences of RNase H cleavage and binding of an anti-sense 2'-OMe RNA on the structure of U2 snRNP are different. For example, cleavage of the 5' end of U2 snRNA could induce a structural modification in the sequence complementary to the branch site, so that base pairing and, of course, complex A cannot occur. In our case the problem is different since the Δ BP mini-intron does not contain a branchpoint sequence and therefore cannot form complex A. What we see is only the ATP-independent recognition of a protein bound to the polypyrimidine tract by U2 snRNP. However, the possibility exists that U2 snRNP proteins are involved in this recognition (protein-protein contacts) rather than the 5' end of U2 snRNA. Under such an hypothesis we have to consider that RNase H cleavage may induce changes in the ribonucleoprotein organization of the particle, so that the requirement for the 5'

end of U2 snRNA is only apparent. This seems rather unlikely since this sequence is accessible to the oligo in the absence of ATP. Furthermore, the common BB' and D-G proteins are located far from the 5' end and the specific A' and B' stay in the opposite region of the particle. In this respect the finding that much of U2 snRNA sequence including the site for A' and B' is dispensable for splicing (53) has to be recalled.

Finally, the finding that the 5' end of U2 snRNA is most likely involved in the pre-spliceosome-spliceosome transition (52) is not incompatible with a requirement for the same sequence to ensure the p65-U2 snRNP contact. Once complex A formed in the case of a true pre-mRNA, it is perfectly conceivable that the 5' end becomes available again to play another role later in spliceosome assembly and splicing.

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