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Involvement of U1 Small Nuclear Ribonucleoproteins (snRNP) in 5' Splice Site-U1 snRNP Interaction*

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U1 small nuclear ribonucleoprotein (snRNP) is an important ribonucleoprotein involved early in the spliceosome formation to commit pre-mRNAs to the splicing pathway. We have determined the association and dissociation kinetics of the 5' splice site-U1 snRNP interaction using purified U1 snRNP and a short RNA oligonucleotide comprising the 5' splice site (5'-SS) consensus sequence of pre-mRNAs (5'-SS RNA oligo). The association is rapid, does not require ATP, and is almost irreversible. Surprisingly, oligonucleotide-directed cleavage of the U1 small nuclear RNA (snRNA) 5' end sequence with RNase H has no significant effect on the rate of association of the 5'-SS RNA oligo, but it does lead to rapid dissociation. This provides evidence that U1-specific snRNP proteins are critical for the 5' splice site recognition while base pairing ensures the stability of the interaction. The recognition of the 5' splice site by U1 snRNP does not result from the individual action of one or more proteins but rather from their organization around U1 snRNA. A consequence of this organization is that the U1-C protein makes direct contacts with the site, as it becomes cross-linked to the RNA oligo upon exposition of the reactions to shortwave UV light.

The U1 small nuclear ribonucleoprotein particle (snRNP)¹ is abundant in eukaryotic cells and, along with three other members (U2, U4-U6, and U5), forms a class of factors that are required in spliceosome assembly and splicing (for a review, see Ref. 1). U1 snRNP is comprised of U1 snRNA and two kinds of proteins, the U1-specific proteins (70K, A and C) and the so-called Sm proteins (B', B, D1, D2, D3, E, F, and G) that are also present in the major U2, U4-U6, U5 snRNPs and in many other minor particles examined so far (for a review, see Ref. 2).

In view of the observed complementarity between the U1 snRNA 5' end sequence and nucleotides around the pre-mRNAs donor and acceptor sites (3, 4), U1 snRNP was hypothesized to be an important splicing factor. This hypothesis has been explored by biochemical and genetic analyses that have led to the conclusion that U1 snRNP is a splicing factor that base pairs with the 5' splice site and that commits the pre-mRNA to splicing (for a review, see Ref. 1). It is now evident that base pairing between the 5' end of U1 snRNA and

the 5' splice site is not sufficient to specify the site at which the nucleophilic attack takes place. Indeed, it is now clear that U5 (5–9) and U6 (9–12) snRNAs contribute to 5' splice site choices as a result of their presence in the tripartite structure resulting in the association of U4-U6 and U5 snRNPs (13, 14).

Other non-snRNP factors, conserved in metazoans and belonging to the so-called SR protein family including at least six related polypeptides (15), collaborate with U1 snRNP in the early identification of the 5' splice site during the commitment step in spliceosome assembly (16). SF2/ASF facilitates binding of U1 snRNP to the pre-mRNA by protein-protein interaction involving the RS domains of both SF2/ASF and the U1-specific 70K protein (16, 17) now known to be an SR-like protein. Another SR protein, SC35, has been proposed to act as a bridge between U1 snRNP bound to the 5' splice site and the 35-kDa subunit of the splicing factor U2AF bound to the 3' splice site (17). Finally, it now appears that SR proteins can compensate for the requirement of U1 snRNP (18, 19) but do not distinguish constitutive from alternative splice sites (19, 20).

Although U1 snRNP proteins have long been suspected to be involved in the mechanism leading to the spontaneous interaction of U1 snRNP with the 5' splice site of pre-mRNAs, very few studies have been undertaken to determine their exact function. The U1-C protein was the first reported to be required, since 5' splice site binding of particles lacking this protein is decreased by about 50–60% as compared with that of intact U1 snRNP (21). Very recently, it was found that U1-C is a requisite element of both the U1 snRNP-ASF/SF2 interaction and 5' splice site recognition (22). The U1-70K protein is thought to play an essential role as well, a role in which the phosphorylation state of the protein is determinant (23).

In this paper, we report kinetic studies of the 5' splice site-U1 snRNP interaction aimed at determining the respective roles played by the U1 snRNP proteins and U1 snRNA, independent of all the other splicing factors. Different forms of purified U1 snRNP were used along with an oligoribonucleotide comprised uniquely of a consensus 5' splice site. We have obtained evidence that the organization of U1 snRNP proteins around U1 snRNA is determinant for the recognition of the 5' splice site by U1 snRNP and that, as a consequence, the U1-C protein makes contact with the site, even when the 5' end of U1 snRNA is missing.

EXPERIMENTAL PROCEDURES

Materials—Micrococcal nuclease, RNase A, and protein A-Sepharose 4B were from Pharmacia Biotech Inc. RNase H and streptavidin-agarose were from Life Technologies, Inc. Proteinase K was from Boehringer Mannheim. Polynucleotide kinase and T7 RNA polymerase were from New England BioLabs. The antibodies used were the mouse monoclonal anti-(U1) RNP (2.73), a generous gift from S. Hoch (Agouron Institute, La Jolla, CA), and a patient serum identified in our laboratory as being mainly directed against the U1-specific C protein, although it also weakly recognizes the U1-specific 70K protein in blots at low dilution. In all the experiments described here, these antibodies were bound to

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¹ The abbreviations used are: snRNP, small nuclear ribonucleoprotein; mAb, monoclonal antibody; comprising the 5' splice site (5'-SS), 5'-splice site; oligo, oligonucleotide; RNA oligo, short RNA oligonucleotide.

protein A-Sepharose in NET buffer (25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 3.2 mM MgCl₂, 0.025% Nonidet P-40) as described (24). The amount of antibody was determined by titration assays in which immunoprecipitated material and supernatants were probed for their U1 snRNA content in Northern blots. All other chemicals were of analytical grade.

RNAs and U1 snRNPs—The undecamer RNA oligonucleotides 5' CAG ↓ GUAAGUAU3' and 5' AAG ↓ GUAAGUAT3' (↓ indicates a splice site) and the undecamer DNA oligonucleotides 5' CAGGTAAG-TAT3' were purchased from Genset and used according to the concentrations indicated by the manufacturer. These RNA oligos were 5' end-labeled using polynucleotide kinase and [³²P]ATP (3000 Ci/mmol) from Amersham Corp. Their specific activity was approximately 5 × 10⁵ cpm/ng. Several short RNAs devoid of consensus 5' splice site were used as controls to monitor nonspecific association (see legend to Fig. 1). Purified U1, ΔA U1, and ΔAC U1 snRNPs (21, 25) were generous gifts from Dr. R. Lührmann (Marburg University, Germany).

Nuclear Extracts and Oligodeoxynucleotide-directed Cleavage of U1 snRNA—HeLa cell nuclear extracts were prepared as originally described (26) in TEA buffer D (27) and, except when otherwise stated, were diluted three times by adding 1 volume of buffer D and 1 volume of 10 mM MgCl₂. These diluted extracts were incubated at 30 °C for 40 min before being used. In the case of U1 snRNA cleavage, 15 units/ml RNase H and 35 μg/ml undecamer DNA oligo complementary to U1 snRNA (nucleotides 1–11) were added. In one case, U1 snRNA was cleaved in purified U1 snRNP using 0.1 unit/ml RNase H and 10 μg/ml oligodeoxynucleotide.

Immobilization of U1 snRNPs on Antibody-bound Protein A-Sepharose—Either nuclear extract or purified snRNPs were added to the antibody-bound protein A-Sepharose and gently agitated for 2 h at 4 °C. Immobilized U1 snRNPs were extensively washed with NET buffer before use. In all experiments, the amount of antibody bound was such that the U1 snRNPs were quantitatively retained (at least 95%). We therefore considered in our calculations that the amount of bound U1 snRNP was equivalent to that of the initial extracts. This latter concentration was estimated by measuring the amount of U1 snRNA in ethidium bromide-stained RNA gels.

Kinetic Measurements—Binding of the 5' end-labeled 5'-SS RNA oligo to either intact or 5' end-deleted U1 snRNP was monitored using particles that were immobilized on protein A-Sepharose via the monoclonal antibody 2.73. This does not affect the capability of U1 snRNP to interact with the oligo, and the procedure is convenient for experiments with crude extracts as well as with purified U1 snRNP. Furthermore, it is easy to stop the reaction by dilution and to rapidly recover the complexes formed on the protein A-Sepharose beads by centrifugation.

The association and dissociation reactions were performed at 20 °C. Association was initiated by adding the 5' end-labeled 5'-SS RNA oligo to immobilized U1 snRNP suspended in 50 μl of NET buffer. After gentle agitation for the indicated times, the reactions were diluted with 1 ml of NET buffer and finally submitted to three rapid cycles of centrifugation-washing in an Eppendorf centrifuge. All the assays were done in triplicate by setting up different reactions for each time point. Protein A-Sepharose beads were counted to monitor bound material. To measure the rate of dissociation of preformed complexes, the washed beads were diluted and maintained under permanent agitation. Released radioactivity was counted as a function of time.

Miscellaneous—Biotinylation and immobilization of the T7 transcript, derived from the ΔBPΔ3' plasmid linearized by EcoRI (24) and used to retain U1 snRNP (Fig. 2), was performed as described previously (23). For UV cross-linking experiments, the reactions were kept on ice for the times indicated in the figure legends and then irradiated for 10 min with an UV transilluminator at 254 nm (7 milliwatts/cm² on the surface of the filter). The distance between the samples and the filters was 9 cm. Cross-linked material was immunoprecipitated and analyzed for the presence of adducts either directly or after digestion with RNase A (0.2 μg/ml for 30 min at 20 °C) or with proteinase K (200 μg/ml for 30 min at 37 °C). Micrococcal nuclease digestions of nuclear extracts (500 units/ml) were carried out in the presence of 1 mM CaCl₂ for 45 min at 30 °C and stopped by addition of 3 mM EGTA.

RESULTS

Oligodeoxynucleotide-directed Cleavage of the 5' End of U1 snRNA Does Not Prevent Specific Binding of U1 snRNP to a 5' Splice Site RNA Oligo—The reactions took place at 20 °C and contained the 5' end-labeled 5'-SS RNA oligo (5' CAG ↓ GUAAGUAU3') and U1 snRNP from nuclear extract that was immobilized on protein A-Sepharose via the mono-

clonal antibody 2.73 directed against the U1-specific 70K protein (see "Experimental Procedures"). U1 snRNP was either intact (U1 snRNP) or lacking the 5' end of its snRNA (cleaved U1 snRNP). The rates of complex formation with U1 snRNP (Fig. 1, *open circle*) and cleaved U1 snRNP (*open square*) were found to be very similar but the latter was reproducibly lower than the former (Fig. 1A). Rate constant values for association of the RNA oligo to both types of particles were calculated from several experiments and are given in Table I. They confirmed that the RNA oligo associates to cleaved U1 with a very similar rate as to intact U1 snRNP. As this suggested that the U1 snRNP proteins could have a more important role than base pairing in the association process, several kinds of experiments were performed to test whether this hypothesis was correct.

Since the above measurements were made with U1 snRNP isolated from nuclear extract by antibody-coupled protein A-Sepharose beads, we had to rule out the possibility of other components being retained with the antibody-bound U1 snRNP and with the antibody itself, which could be responsible for the retention of the RNA oligo. We therefore performed assays with purified U1 snRNPs, known to be free of contaminating proteins (25), which were immobilized to the antibody-bound protein A-Sepharose as above. This purified particle had the same behavior as that selected by the antibody from nuclear extracts, either when its U1 snRNA was intact or cleaved (*closed circle* and *square* in Fig. 1B), confirming that all components required for 5' splice site recognition are part of the U1 snRNP.

To ascertain the specificity of the interaction between the 5'-SS RNA oligo and intact or cleaved U1 snRNP, the following controls were carried out. Several RNA oligos (see legend to Fig. 1) with either unrelated sequences or containing the CAG ↓ GU motif from the consensus 5' splice site (*open inverted triangle* in Fig. 1) were tested. They all failed to bind to cleaved as well as to intact U1 snRNP.

We also had to rule out that no binding occurs with free forms of U1 snRNP components, for example the U1-70K protein which, if present in the nuclear extract, would certainly be selected by the mAb 2.73 bound to protein A-Sepharose. The 5'-SS RNA oligo did not bind when immuno-selection was carried out from a nuclear extract previously digested by micrococcal nuclease to destroy the U1 snRNP organization (Fig. 1A, *open triangle*), thus providing evidence that the U1-70K protein is not capable of binding to the 5'-SS RNA oligo by itself. This result suggests that binding of the 5'-SS RNA oligo to U1 snRNP is either promoted by another U1 snRNP protein or directly depends upon the assembly of several U1 snRNP proteins around U1 snRNA. To establish which of these hypotheses is correct, we devised an experiment in which a synthetic RNA, containing a consensus 5' splice site (24) and biotinylated at both ends, was immobilized on streptavidin-agarose beads (23) in order to recover U1 snRNP components by affinity chromatography. As expected, U1 snRNP was retained in such a biotinylated RNA (Fig. 2A, *lane 1*). In contrast, no U1 snRNP proteins were retained by this biotinylated RNA when the nuclear extract was digested with micrococcal nuclease to destroy the ribonucleoprotein organization of snRNPs (Fig. 2A, *lane 2*). This was true even when the amount of biotinylated RNA was increased from 1.5 μg (this experiment) to 15 μg (not shown). Only some non-U1 snRNP proteins, possibly heterogeneous nuclear RNP proteins, were retained, and these were identical to those retained from the nondigested nuclear extract. This substantiates the hypothesis that the specificity of the 5'-SS RNA oligo-U1 snRNP interaction is conferred by several proteins organized around U1 snRNA rather than by the action of individual snRNP proteins. As expected, cleaved U1 snRNP was retained on this biotinylated RNA (Fig. 2B, *lane*

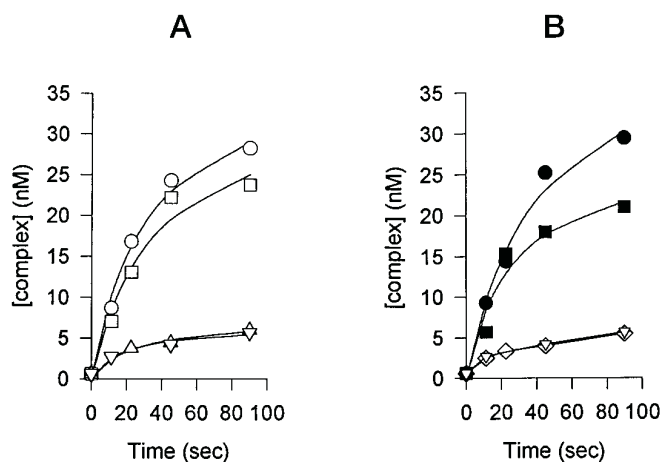


FIG. 1. Kinetics of the interaction between 5'-SS RNA oligo and U1 snRNP. *A* and *B* show association curves. U1 (circle) and cleaved U1 (square) snRNPs, either selected from nuclear extract (*A*) or from a preparation of purified particle (*B*), were immobilized on antibody-bound protein A-Sepharose and then were adjusted to 150 nM (*A* and *B*) in 50 μ l of NET buffer. In all cases the reaction was initiated by adding 1 μ l of 5' end-labeled 5'-SS RNA oligo (final concentration 40 nM). At the times indicated, 5'-SS RNA oligo-U1 snRNP complex was monitored by counting the radioactivity bound to the protein A-Sepharose beads. Nonspecific interaction of the 5'-SS RNA oligo with immobilized material from micrococcal nuclease-treated nuclear extract is shown (Δ in *A*). Another control used the 32 P 5' end-labeled RNA oligo 5'-GAAUA-CAAGCUUGGGCUGCAGGUCGA3' in place of the 5'-SS RNA oligo and exhibited nonspecific binding on immobilized U1 snRNP (∇ in *A* and *B*). Other labeled short RNAs with sequence unrelated to the 5' splice site were also tested and all showed nonspecific binding. Background binding of the 5'-SS RNA oligo to the antibody-coupled beads alone was also examined. It was not significant (shown in *B*, \diamond). All the assays in this and other experiments were carried out at 50 mM salt, a concentration found to be optimum to evaluate binding activity of U1 snRNP (21). Increasing the salt concentration to 100 mM to bring down nonspecific interactions would lead to dramatic decrease of U1 snRNP binding activity.

3 and 2C, lane 3), but efficient retention required more biotinylated RNA because, as we will see below and in Table I, the dissociation constant with cleaved U1 snRNP is higher than that with intact U1 snRNP.

Finally, our proposal that the U1 snRNP proteins have a more important role than base pairing for 5' splice site recognition by U1 snRNP is based on the observation that U1 snRNP cleared of the 5' end sequence of U1 snRNA is still able to interact specifically with the 5'-SS RNA oligo. As the length of U1 snRNA was reduced by oligodeoxynucleotide-directed cleavage with RNase H and as this procedure can leave some U1 snRNA molecules still containing a few nucleotides (28) that could base pair with the 5'-SS RNA oligo, it was necessary to rule out the possibility of retention of the 5'-SS RNA oligo by those of cleaved U1 snRNPs still containing incompletely cleaved U1 snRNA. Assuming that these incompletely digested U1 snRNPs could be responsible for the retention of the RNA oligo, adding an excess of unlabeled deoxyoligonucleotide complementary to the 5' end of U1 snRNA to antibody-bound cleaved U1 snRNP would lead to competition. This was not the case (Fig. 3B, compare circle and closed triangle), thus confirming our proposal that the U1 snRNP proteins are critical in specific recognition of the 5' splice site by U1 snRNP. As expected, binding of the RNA oligo to intact U1 snRNP was impaired by the deoxyoligonucleotide (Fig. 3A) in agreement with the results reported by Konforti *et al.* (29) who studied the formation of complexes in native gel electrophoresis. If the U1 snRNP proteins are so requisite in the binding process, then it seems rather surprising that the RNA oligo does not bind to intact U1 snRNP in the presence of DNA that blocks the 5' end

TABLE I
Kinetical parameters of the 5'-SS RNA oligo-U1 snRNP interaction at 20 $^{\circ}$ C

k_d is the rate constant for dissociation. It was expressed as an average of three determinations in which $\ln((\text{complex})_t/(\text{complex})_{t_0})$ was plotted as a function of time. The slope of the curve is $-k_d$. Standard deviation was 15%. No significant variation of k_d was found when the temperature of the reaction varied from 0 to 20 $^{\circ}$ C. K is the dissociation constant ($K = k_d/k_a$) and was calculated from the mean values of k_a and k_d . The dissociation constant of cleaved U1 snRNP can also be determined under equilibrium conditions from the saturation curve in Fig. 7A. The value is 14 nM and is in good agreement with that calculated from the data in the table. Note that the K constant with intact U1 snRNP cannot be calculated in this way because the interaction is practically irreversible under the conditions used.

	k_a $M^{-1} \text{min}^{-1}$	k_d min^{-1}	K nM
U1 snRNP	$0.7 \cdot 10^7 \pm 0.3 \cdot 10^7^a$ $1.3 \cdot 10^7 \pm 0.2 \cdot 10^7^b$	$2 \cdot 10^{-3}$	0.28 0.15
Cleaved U1 snRNP	$0.5 \cdot 10^7 \pm 0.3 \cdot 10^7^a$ $1.1 \cdot 10^7 \pm 0.2 \cdot 10^7^b$	$5 \cdot 10^{-2}$	10 4.5

^a k_a is the rate constant for association process. It was estimated by measuring the initial rate of numerous reactions ($v = k_a [\text{U1}] \times [5\text{'-SS RNA oligo}]$).

^b The k_a constant was also determined from half-time reactions under conditions where the concentration of U1 snRNP was in large excess compared with that of 5'-SS labeled RNA oligo ($t_{1/2} = 0.69/k_a [\text{U1}]$). This provided advantage to disregard the error about the concentration of labeled RNA oligo, therefore leading to more precise values. For $[\text{U1 snRNP}] = 150 \text{ nM}$, the half-times reaction was 21 s. \pm 2 and 24 s. \pm 2 for intact U1 and cleaved U1, respectively. When the 5'-SS RNA oligo was included in a longer sequence, the k_a values were slightly lower (not shown) due to RNA structure.

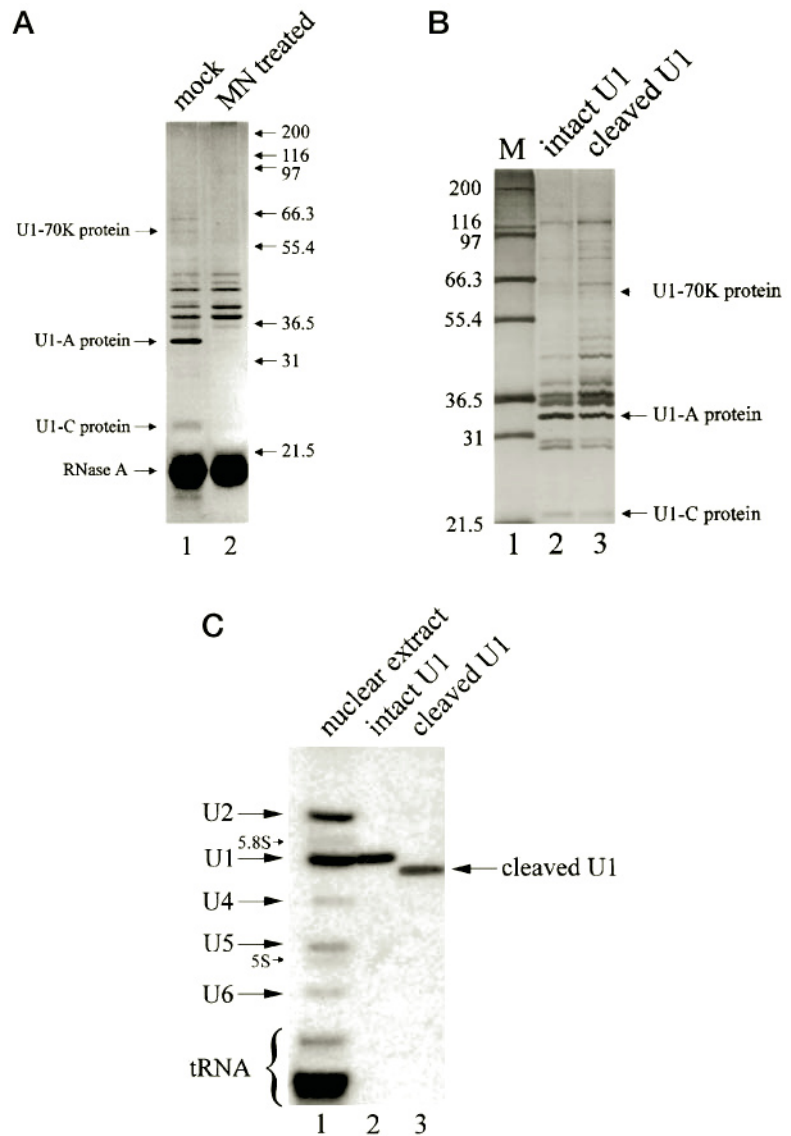
of U1 snRNA. We hypothesize that hybridization of the DNA oligo alters the structure of the particle in such a manner that the snRNP proteins can no longer cooperate efficiently. This is consistent with the above conclusion that the specificity is conferred by snRNP proteins as a result of their organization around U1 snRNA, rather than by the individual action of one or more snRNP proteins.

Base Pairing Prevents Dissociation of the U1 snRNP-5'-SS RNA Oligo Complex—The complex formed between the 5'-SS RNA oligo and cleaved U1 snRNP dissociates when diluted to 10 pM (Fig. 4, open square). In contrast, interaction of the 5'-SS RNA oligo to intact U1 snRNP is almost irreversible (Fig. 4, open circle). It appears, therefore, that base pairing is responsible for this strong stability (see in Table I the k_d value and the dissociation constant K). That the RNA oligo dissociates rapidly from cleaved U1 snRNP also explains the apparently lower rate of association of the 5'-SS RNA oligo to cleaved U1 than to intact U1 (Fig. 1, *A* and *B*). It is likely that some dissociation occurred during the three washes and centrifugation steps (about 2 min) required to recover the complex. We will therefore consider that the 5'-SS RNA oligo binds to the two types of particles with the same rate.

The U1-C Protein Makes Direct Contacts with the Consensus 5' Splice Site—In an attempt to evaluate the contribution of each U1-specific snRNP protein to the 5'-SS RNA oligo-U1 snRNP interaction, we have determined the association and dissociation rates using purified particles that were either lacking one (Δ A-U1 snRNP) or two (Δ AC-U1 snRNP) U1-specific proteins (21). A U1 snRNP lacking only the 70K protein does not exist, and the U1 snRNP preparation known as core U1, which lacks the A, C, and 70K proteins, does not result in the formation of a ribonucleoprotein complex with a pre-mRNA (22) and was not, therefore, examined here. The kinetics of association were measured without an excess of the particle, in order to minimize the contribution of contaminant intact U1 snRNP present in very small quantities in Δ A and Δ AC particles (21). Reproducibly, the rate constants for association to

FIG. 2. An immobilized RNA containing a consensus 5'-SS does not retain U1 snRNP proteins when isolated from their RNP context.

A, 170 μ l of diluted nuclear extract (U1 snRNP = 150 nM) were treated (lane 2) or not treated (lane 1) by 450 units of micrococcal nuclease (MN) for 45 min at 30 °C and added to 1.5 μ g of biotinylated RNA containing a consensus 5' splice site (23) immobilized on 20 μ l of streptavidin-agarose beads. After washing the beads with NET buffer, the bound proteins remaining were released by RNase A treatment (2 μ g/50 μ l) and separated in a 10% of polyacrylamide-SDS gel that was silver-stained. Protein marker sizes, RNase A, and U1-specific snRNP proteins are indicated. **B** and **C** show that intact U1 and cleaved U1 snRNP were retained on the immobilized RNA containing a consensus 5' splice site. 5 μ g of this biotinylated RNA (23) was coupled to the streptavidin beads, and then nuclear extracts with either intact (lane 2) or cleaved U1 (lane 3) were affinity chromatographed. **B**, proteins were analyzed by electrophoresis and stained as described in **A**. Note that RNase A runs out of the gel. The U1-70K protein is slightly visible in these gels, but it is known that it is poorly stained by silver. Its presence was currently confirmed by immunoblotting these and other gels with the mAb 2.73 (see Ref. 23). **C**, lanes 2 and 3 refer to a parallel experiment described in **B**, lanes 2 and 3, and shows the gel analysis of the RNAs retained. The experiment was carried out using a nuclear extract prepared from HeLa cells metabolically labeled with 32 P. The labeled RNA retained on the immobilized 5' splice site was recovered by proteinase K digestion, extracted with phenol, electrophoresed in a 12.5% urea gel, and autoradiographed. Lane 1 shows RNAs extracted from the metabolically labeled nuclear extract.



intact (circle) and Δ A-U1 snRNP (square) were identical (Fig. 5A), whereas for Δ AC-U1 snRNP (triangle) it was halved. Clearly the presence of U1-C but not that of U1-A is critical for the association of the RNA oligo to U1 snRNP. U1-C also seems to contribute to stabilize the interaction once base pairing has occurred, as dissociation was reproducibly two times more rapid with Δ AC-U1 snRNP than with Δ A or intact U1 snRNPs (Fig. 5B). In this series of experiments it would be interesting to measure the rates of association and dissociation of cleaved Δ AC-U1 snRNP. Unfortunately, digestion of this particle with RNase H leads to additional cleavages in the stem-loop II, normally protected by the U1-A protein (30), which exhibits some complementarities with the oligo used to cleave the 5' end of U1 snRNA. As a consequence this particle becomes incapable of interaction with the 5'-SS RNA oligo (not shown).

To examine whether the U1-C protein makes direct contact with the 5'-SS RNA oligo, reactions were exposed to UV light to induce RNA-protein cross-linking. In this experiment, the radioactive oligo added to nuclear extract was 5' 32 PAAAG \downarrow GUAAGUAT3', instead of 5' 32 PACAG \downarrow GUAAGUAU3'. The former has the same capability as the latter to bind to U1 snRNP (29) and allows the use of RNase A to analyze the protein adduct(s) formed upon irradiation of the reaction. As shown in Fig. 6A, the same radioactive bands were present using either intact or cleaved U1 snRNP (compare lanes 1 and 4), and no competition occurred when a large excess of

cold RNA with unrelated sequence was added to the reactions (not shown). Clearly, the band around 50 kDa present in lanes 1, 2 and 4 is a cross-linked RNA, as it is absent from lane 3 in which the sample was digested with RNase A, and its size is that of U1 snRNA. The presence of cross-linked cleaved U1 snRNA in lane 4 was unexpected. It cannot be due to the low amount of U1 snRNA molecules resisting cleavage with RNase H, since the size of the band corresponds well to cleaved U1 snRNA and since its amount is quite comparable with that of intact U1 snRNA in lane 1. It could result, for example, from an interaction between the RNA oligo and internal U1 snRNA sequence as described previously (29). Another more probable explanation is that residual undigested nucleotides, although being insufficient to base pair, still make contact with the RNA oligo and, therefore, lead to cross-linked material. The 22-kDa band is a protein since it is absent in lane 2 upon digestion of the reaction with proteinase K. Its size suggests that it is U1-C. The minor bands above the 50-kDa component disappeared upon treatment with proteinase K (lane 2) and RNase A (lane 3), respectively. They are, most likely, derived from complexes with multiple bridges containing labeled U1 snRNA and snRNP proteins.

To verify that the 22-kDa band is U1-C, 5'-SS RNA oligo-U1 snRNP complexes were selected from cross-linked reactions with an anti-U1-C antibody from a patient serum (see "Experimental Procedures") as well as with the mAb 2.73 directed against the U1-70K protein. After digestion with RNase A and

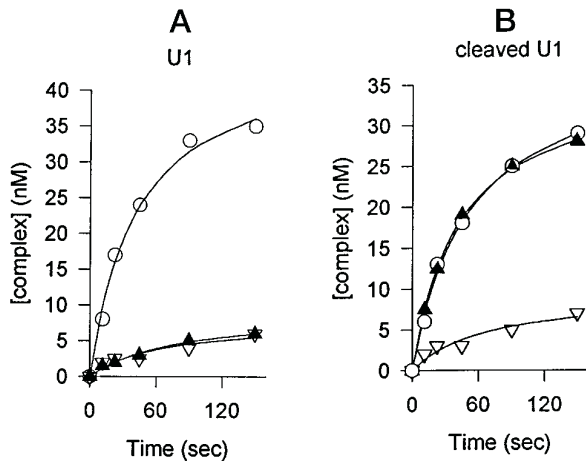


FIG. 3. A 5'-SS DNA oligo competes for the U1 snRNP-5' splice site interaction only when U1 snRNA is intact. The experimental parameters were as in Fig. 1. A, intact U1; B, cleaved U1. The assays without DNA oligo are represented by open circles. Before initiating the reaction by the 5' end-labeled RNA oligo, 25 μ l of 12 μ M DNA oligo solution (\blacktriangle) were added to 25 μ l of immobilized U1 snRNPs. Control (∇) was as in Fig. 1.

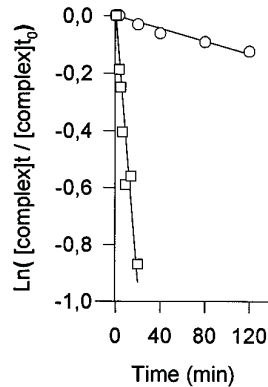


FIG. 4. Dissociation of the 5'-SS RNA oligo-U1 snRNP interaction. The dissociation curves (intact U1 snRNP \circ , cleaved U1 snRNP \square) were monitored by counting the radioactivity remaining bound to the beads as well as that released after dilution of the complex to 10 μ M in NET buffer.

disruption of bound complexes with 0.1% SDS, washed and remaining bound material were electrophoresed (Fig. 6B). The 22-kDa component was washed from the anti-U1-70K antibody (lane 1), whereas it remained bound to the anti-U1-C antibody (lane 4). This supports the conclusion that the 22-kDa component is the U1-C protein.

Finally, it was necessary to establish that cross-linked U1 snRNA and U1-C protein are representative of the 5'-SS RNA oligo-U1 snRNP interaction. As a first assay (Fig. 7A), we monitored how many complexes were formed using several concentrations of cleaved U1 snRNP, and we determined for each concentration the relative amounts of cross-linked U1-C protein and U1 snRNA. Radioactive material retained on protein A-Sepharose beads was counted, exposed to UV light, electrophoresed as in Fig. 6, and then the bands corresponding to U1 snRNA and U1-C protein were quantified by scanning. Fig. 7A shows that the three parameters measured were correlated. The *K* constant (14 nM), determined here under equilibrium conditions, is in good agreement with that determined from the association/dissociation curves (Fig. 1 and Table I). In a second assay, we titrated intact U1 snRNP by counting immunoprecipitated radioactive material from reactions containing increasing amounts of competing unlabeled 5'-SS RNA oligo, and again, we looked for cross-linked U1 snRNA and

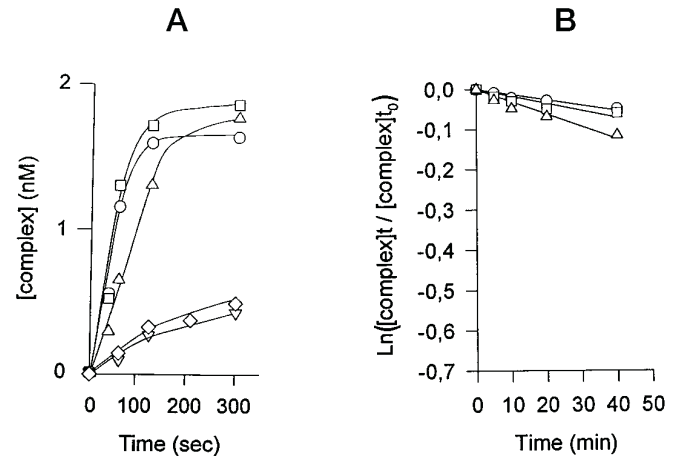


FIG. 5. The U1 snRNP C protein is directly involved in the 5'-SS RNA oligo-U1 snRNP interaction. All the assays shown here were carried out using preparations of purified U1 snRNPs depleted of diverse U1-specific proteins (\triangle A, \square , and \triangle AC, \diamond) as described (21) compared with intact U1 snRNP (\circ). The assays were monitored as in Fig. 1, and the concentrations of U1 snRNPs and 5' end-labeled RNA oligo in A were 6.3 and 12.5 nM, respectively. Controls (∇ and \diamond) were as in Fig. 1B. B shows dissociation kinetics using intact U1 (\circ), \triangle A (\square), \triangle AC (\triangle) snRNPs. They were determined as in Fig. 4.

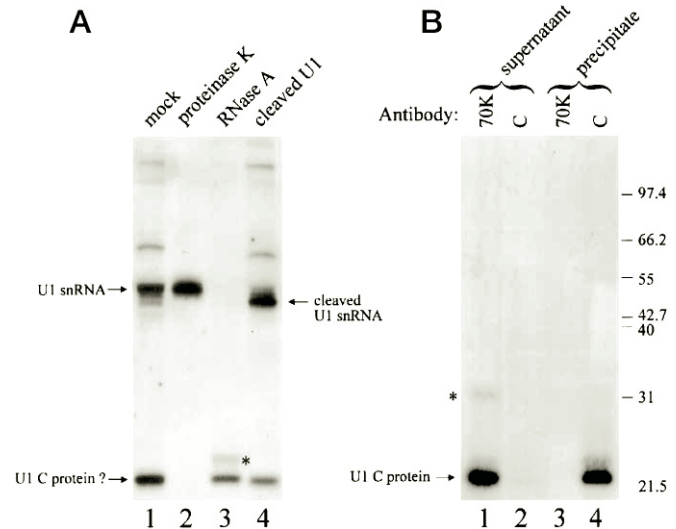


FIG. 6. The U1-specific C protein makes direct contact with the 5'-SS RNA oligo. A, samples containing 50 μ l of diluted nuclear extract and 3 ng of 5' end-labeled 5'-SS RNA oligo were incubated for 30 min at 0 $^{\circ}$ C, irradiated with shortwave UV light, selected by the anti-70K antibody bound to protein A-Sepharose, and then analyzed for the presence of adducts in 10% polyacrylamide-SDS gels that were autoradiographed. The analysis was either direct (lane 1), or after digestion by proteinase K (lane 2), or RNase A (lane 3). Lane 4 shows an assay similar to that in lane 1, performed with cleaved U1 snRNP. B, the cross-linking assay was identical to that shown in A, lane 3, except that two different antibodies were used and that immunoprecipitated material was washed with 0.1% SDS. Lanes 1 and 2 refer to SDS-washed material from anti-70K and anti-C antibodies, respectively. Lanes 3 and 4 correspond to material retained on the antibodies after the beads had been washed with SDS. * designates RNA material resisting RNase A digestion. Sizes of molecular weight markers are indicated.

U1-C protein. The three titration curves are superimposed (Fig. 7B). The third assay demonstrates that the formation of the 5'-SS RNA oligo-U1 snRNP complex and cross-linking of U1-C protein are inhibited in the same way when the nuclear extract used for immunoselection of U1 snRNP was digested by increasing amounts of micrococcal nuclease (Fig. 7C).

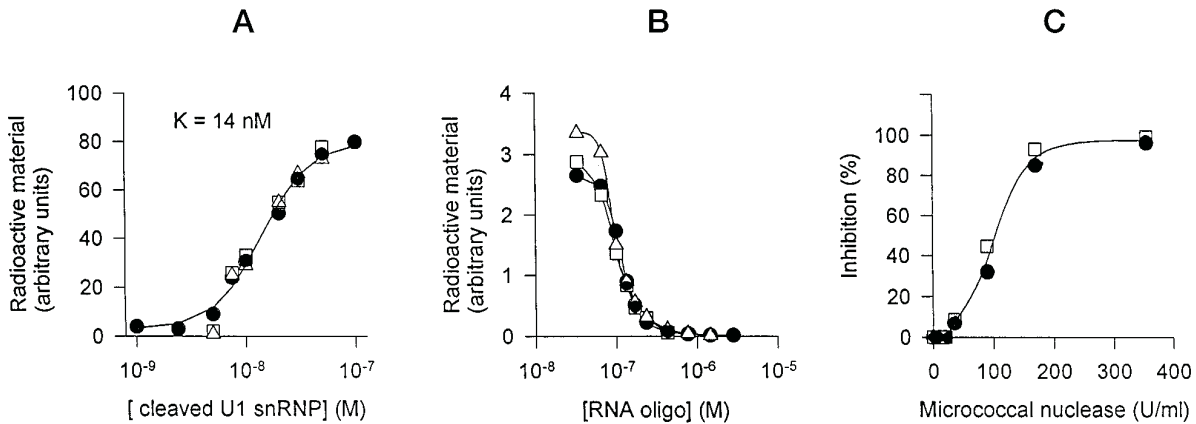


FIG. 7. Cross-linking of U1 snRNA and U1-specific C protein accounts for the 5'-SS RNA oligo-U1 snRNP interaction. In all cases, the 5'-SS RNA oligo was 5' end-labeled. The amount of complex formed was determined by counting immunoprecipitated radioactivity (●), and the relative amounts of cross-linked U1-specific C protein (□) and U1 snRNA (△) were estimated by scanning autoradiographs of gels similar to that shown in Fig. 6A. A shows that complex formation is dependent of the concentration of cleaved U1 snRNP. The reactions contained 2 nM of 5' end-labeled 5'-SS RNA oligo and increasing amounts (from 1 to 100 nM in 150 μ l of NET buffer) of cleaved U1 snRNP immobilized on protein A-Sepharose beads via the mAb 2.73. After incubation at 4 °C for 2 h with gentle stirring, the beads were washed, counted, and finally exposed to UV light before being diluted in Laemmli buffer and electrophoresed. B shows a titration curve of intact U1 snRNP. Nuclear extract was adjusted to 50 nM U1 snRNP by dilution with NET buffer and incubated at 4 °C for 2 h in the presence of 30 nM 5' end-labeled 5'-SS RNA oligo and increasing amounts of the same unlabeled RNA oligo ranging from 0.03 to 2.8 μ M. The reactions were exposed to UV light and then the complexes were selected, counted, and analyzed by gel electrophoresis. Note that the experiment was performed in the presence of competing proteins, thus explaining the high amount of 5'-SS RNA oligo required to titrate U1 snRNP. C shows how the formation of complex is inhibited as a function of micrococcal nuclease treatment of nuclear extract. Digestions were at 30 °C for 45 min in a final volume of 20 μ l containing 15 μ l of nuclear extract (370 nM U1 snRNP) and micrococcal nuclease concentrations varying from 0 to 360 units/ml. After stopping the digestions with EGTA, the samples were adjusted to 3.2 mM MgCl₂ and diluted to 50 μ l with NET buffer. After adding labeled 5'-SS RNA oligo (6 nM), the reactions were incubated at 0 °C for 30 min more and then submitted to the same analysis as in B.

DISCUSSION

In this study, we have examined the details of the interaction between the 5' splice site and U1 snRNP, independently of the other splicing partners and canonical sequences existing in pre-mRNAs. To do this, we have used an *in vitro* system comprised of U1 snRNP immobilized on antibody-bound protein A-Sepharose and an 11-nucleotide RNA oligo having the 5' splicing site consensus sequence (CAG \downarrow GUAAGUAU). Our conclusion is that the recognition of the 5' splice site by U1 snRNP likely depends on the structure resulting from the assembly of specific proteins around U1 snRNA, whereas base pairing engaging the 5' end of U1 snRNA serves to stabilize the interaction. This is based on our finding that a particle lacking the 5' end of U1 snRNA is still able to interact specifically with the RNA oligo but dissociates much more rapidly than with intact U1 snRNA. Although not being capable of interaction by itself, the U1-C protein makes physical contact with the RNA oligo, as it becomes cross-linked upon UV light irradiation.

The choice of a simple RNA oligo containing a consensus 5' splice site instead of an oligo corresponding to a natural 5' splice site or an authentic pre-mRNA was made as we anticipated that base pairing of U1 snRNA with such a 5'-SS RNA oligo would be more stable than with another RNA in the *in vitro* system we have used. We also considered that this 5'-SS RNA oligo was convenient to study a partial reaction in the spliceosome assembly pathway. Indeed, this oligo is sufficient to bind U1 snRNP (29) and to induce the formation of the U2-U4-U5-U6 complex (31). It also binds to U4-U5-U6 snRNP, cross-links to U6 snRNA when binding to U1 snRNP is inhibited (29, 32), and finally, it undergoes both steps of splicing when a second RNA containing the 3' splice site sequence is added in *trans* (33). We chose to use U1 snRNPs isolated from their nuclear extract context to establish the respective roles of U1 snRNP proteins and base pairing for two reasons. First, it was necessary to uncouple the 5' splice site-U1 snRNP interaction from subsequent recognition by the other spliceosomal components. Second, we judged that three kinds of particles (intact U1 snRNP, U1 snRNP cleared of the 5' terminus of its

U1 snRNA, and U1 snRNPs gradually depleted of the U1-specific proteins) would be useful to understand how snRNP proteins and base pairing contribute to the 5' splice site-U1 snRNP interaction. Another questionable point is the use of an antibody that binds to U1 snRNP via the U1-70K protein. Does the binding of an antibody induce some change in the particle that could explain binding of the RNA oligo to cleaved U1 snRNP? The result in Fig. 2 that cleaved U1 snRNP binds to an insolubilized biotinylated RNA argues against this possibility.

Our finding that the rate constant for the association of the 5'-SS RNA oligo to particles lacking the 5' terminus of U1 snRNA is practically identical to that for its association to intact U1 snRNP supports the conclusion that the U1 snRNA-associated proteins, but not base pairing, are responsible for the recognition event occurring in the binding process of U1 snRNP to the 5' splice site region. It is clear, however, that the U1 snRNP proteins are not capable of binding to the 5' splice site by themselves but rather that the recognition comes as a result of the ribonucleoprotein organization of these proteins around U1 snRNA. In support of this was our observation in Fig. 3 that a deoxyoligonucleotide complementary to the 5' end of U1 snRNA impairs binding of the RNA oligo to U1, in contrast to cleaved U1 snRNP, most likely as it induces some structural change in the intact particle. On the other hand, it seems clear that base pairing serves to stabilize binding, since the rate constant for dissociation with cleaved U1 snRNP was dramatically increased compared with that of intact U1 snRNP. In other words, the ability to base pair considerably increases the affinity of U1 snRNP for the 5' splice site, explaining why the association to U1 snRNP in a nuclear extract context without ATP is so exceptionally stable.

It was previously demonstrated by a filter binding assay under equilibrium conditions that the U1-C protein is likely to be involved in 5' splice site binding (21). Much more recently, Jamison *et al.* (22) characterized the elements required for U1 snRNP-ASF/SF2 interaction and 5' splice site recognition and reported that U1-C is required for proper 5' splice site recognition. Our results confirm and extend this point. We have

demonstrated that U1-C is involved in both the association and dissociation processes and that it makes specific physical contact with the 5' splice site. Indeed, it becomes cross-linked to the 5'-SS RNA oligo, but not to another RNA of unrelated sequence, upon exposition to shortwave UV light. It is known that the U1-C protein does not contain RNA recognition motif in contrast with the U1-70K and U1-A proteins (34), therefore explaining why it can only assemble to U1 snRNP when the other proteins are already associated with U1 snRNA (35). Gunnewiek *et al.* (36) have reported that the human U1-C protein is able to form homodimers and hypothesized that stable binding of U1 snRNP to the 5' splice site requires the presence of U1-C dimer. Perhaps such a U1-C dimer could be responsible for the contact with the RNA oligo leading to cross-linked U1-C. In fact, we have calculated from the kinetics shown in Fig. 5 that the affinity of U1 snRNP for a 5' splice site is only decreased 4-fold when U1-C is missing. Therefore, the U1-C protein cannot be the unique component involved in the binding of the RNA oligo to U1 snRNP. Unlike U1-A whose absence has no effect on the recognition mechanism, the U1-70K protein could be involved. Indeed, we previously showed that U1 snRNP becomes less tightly bound to an RNA containing a 5' splice site upon incubation of nuclear extract with ATP to introduce additional phosphate in U1-70K (37). We have also demonstrated that dephosphorylation is critical for the participation of the U1-70K protein in a pre-catalytic step of the splicing reaction (23). Finally, it must be borne in mind that the U1-C and, possibly, the U1-70K proteins operate efficiently only when assembled with U1 snRNA (this work) to form a structure in which they are interacting together and with the Sm proteins (35). It seems likely, therefore, that the exact function of these proteins in the 5' splice site recognition results from multiple interactions existing within the entire particle.

If base pairing is not the essential determinant, then what is the exact sequence motif required for the U1 snRNP to engage the recognition process? We have found that an RNA oligo that contained the first five nucleotides of the consensus sequence (-3 to +2) does not associate to U1 snRNP. It has been reported that compensatory mutations in U1 snRNA fail to restore accurate splicing of a mutated pre-mRNA at the +3 position in the intron (38). We propose that this be due to the inability of U1 snRNP proteins to recognize such a mutated 5' splice site sequence where a purine was replaced by a pyrimidine in spite of the possibility for its U1 snRNA to base pair. In this respect, Konforti and Konarska (32) have reported that changing A to U at the +3 position in the same RNA oligo as that used here leads to a dramatic decrease of U1 snRNP binding under equilibrium conditions. Maybe a purine at +3 position could be critical for U1 snRNP to be able to recognize the 5' splice site independently of base pairing.

Most of the 5' splice sites found in pre-mRNAs diverge from the consensus sequence, thus rendering less easy their selection by U1 snRNP. It has been proposed that a number of non-snRNP proteins, comprising the so-called SR proteins, cooperate with U1 snRNP for binding and are particularly effi-

cient in the case of alternative splicing (39, 40). One of the functions of these auxiliary factors could be to favor the recognition of one site instead of another and/or to stabilize the interaction. In brief, they could act as a means to control the U1 snRNP activity, although this does not exclude their involvement in other steps of splicing.

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