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Interaction of hnRNP A1 with snRNPs and pre-mRNAs: evidence for a possible role of A1 RNA annealing activity in the first steps of spliceosome assembly

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ABSTRACT

The *in vitro* interaction of recombinant hnRNP A1 with purified snRNPs and with pre-mRNAs was investigated. We show that protein A1 can stably bind U2 and U4 snRNP but not U1. Oligo-RNAse H cleavage of U2 nucleotides involved in base pairing with the branch site, totally eliminates the A1-U2 interaction. RNase T1 protection and immunoprecipitation experiments demonstrate that recombinant protein A1 specifically binds the 3'-end regions of both β-globin and Ad-2 introns. However, while on the β-globin intron only binding to the polypyrimidine tract was observed, on the Ad-2 intron a 32 nt fragment encompassing the branch point and the AG splice-site dinucleotide was bound and protected. Such protection was drastically reduced in the presence of U2 snRNP. Altogether these results indicate that protein A1 can establish a different pattern of association with different pre-mRNAs and support the hypothesis that this protein could play a role in the annealing of U2 to the branch site and hence in the early events of pre-splicing complex assembly.

INTRODUCTION

Pre-mRNA splicing *in vivo* occurs on a complex and still poorly defined ribonucleoprotein substrate. *In vitro* experiments with splicing extracts permitted to identify a macromolecular ribonucleoprotein assembly, the spliceosome, in which the splicing reaction takes place (reviewed in 1–4). The mayor small ribonucleoprotein particles U1, U2, U4/U6, U5 snRNPs are the best characterized functional components of the spliceosome along with several factors required for splicing (5–9). In addition, a number of RNA binding proteins, some of which exhibit sequence-specific binding, are probably associated to the spliceosome (10–16). Assembly of the spliceosome involves the stepwise binding of snRNPs and protein factors to the pre-mRNA through a mechanism which probably involves RNA-RNA, RNA-protein and protein-protein interactions.

The total population of pre-mRNA in the nucleus, known as heterogeneous nuclear RNA (hnRNA), is found in association with a discrete set of proteins to form repeated structures which can be visualized as a linear array of globular particles along the transcripts (17). Monomer structures, generated by RNase cleavage of linker RNA are isolated as 30S-40S particles, 20 nm in diameter (hnRNP particles or monoparticles). It was shown that a monoparticle packages about 700 nt of RNA in a sequence-indipendent way (18). The protein moiety of the hnRNP particles is composed of more than 20 different polipeptides that constitute a supramolecular complex with RNA (19). The six most abundant proteins (A1, A2, B1, B2, C1 and C2) named 'core proteins' and a few less abundant species have been extensively studied (20–26). Among hnRNP core proteins, protein A1 (M.W. 34 kd; pI 9.5) and protein C1 (M.W. 32 kd; pI 4.5) were characterized at a molecular level both in our and in G. Dreyfuss laboratory. These polypeptides belong to a family of RNA binding proteins that share an RNA binding motif (termed RRM) consisting of about 90 residues. The most conserved features of the RRM are an 8-aa sequence RNP-1 (RNP consensus sequence) and a less conserved 6-aa element RNP-2 (27, 28). An interesting feature of hnRNP proteins is a multi-domain modular structure. Typically they consist of one or more RRM domains and of an accessory domain that is different in different proteins. This general organization, which reminds that of transcription factors and of some splicing factors (27), suggests that also these proteins can establish multiple interaction.

Because of their abundance and their association with hnRNP complexes in a well defined stoichiometry (29) hnRNP proteins are thought to be involved in the packaging and processing of all pre-mRNAs. However, newly discovered properties and recently reported data, indicate for some of these proteins a more active and specific function. In fact, several hnRNP proteins, among which core proteins A1, C1/C2 and non core proteins such as D, I/PTB (30) were found to exhibit *in vitro* a binding preference for the pyrimidine-rich region at the 3'-end of introns (15, 16, 31–33). Proteins C were shown to play a role in spliceosome formation and splicing (10, 34). More recently protein A1 has been implicated in alternative splicing since it was demonstrated to antagonize *in vitro* the effect of splicing factor SF2/ASF in the 5'-splice site choice (35). Finally, protein A1 (like the essential splicing factor SF2/ASF) was found to promote renaturation of complementary strands (36–38) and we

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observed that the same is true for protein C1 (unpublished result). This property could be important *in vivo* to promote base-pairing interaction between pre-mRNA and snRNP.

Although the absolute requirement for hnRNP proteins in splicing has not been demonstrated, their potential involvement is supported also by their presence in early splicing complexes (39). Moreover other data indicate that specific interactions might take place between hnRNP and snRNP. For example, it was shown that in nuclear extracts, efficient UV-induced crosslinking of hnRNP proteins A1 and C to pre-mRNA requires the presence of U1 and U2 snRNP (33) and immunofluorescence studies of lamphrush loops of amphibian chromosomes suggest that the nascent transcripts are associated with hnRNP and snRNP perhaps in the form of unitary hnRNP/snRNP particles (40).

The experiments described in this paper demonstrate that recombinant hnRNP protein A1 can specifically bind *in vitro* purified U2 and U4 snRNPs. In addition our data suggest that protein A1 can establish different patterns of interaction with two different pre-mRNA. On the basis of these results a tentative model of pre-mRNA/hnRNP/snRNP interaction is proposed and its implications in the first steps of spliceosome assembly are discussed.

MATERIALS AND METHODS

Materials

Restriction enzymes, RNase H, SP6, T3, and T7 RNA polymerases were purchased from Boehringer-Mannheim, RNase-Free DNase, and RNasin from Promega,T4 RNA ligase from Biolabs, RNase T1 from BRL. Biotin-11-UTP and streptavidin agarose beads were purchased from Sigma, protein A sepharose from Pharmacia.

snRNP fractionation by CsCl density gradient centrifugation

Purification of snRNP by CsCl equilibrium density gradient centrifugation was as described (41). 4 ml of nuclear extract were centrifuged in 40% CsCl (w/w), 20 mM Tris-Cl pH 7.5, 15 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 150 mM NH_4Cl for 65 h at 33,000 rpm at 20°C in a Beckman SW40 rotor. The gradient was fractionated from the top into 17 (0.7 ml) fractions. 0.1 ml aliquots were used for density measurements. The remaining sample was dialyzed against buffer D (42) containing 3 mM $MgCl_2$. The RNA contained in each fraction was extracted with phenol, precipitated with ethanol and electrophoresed on a 12.5% denaturing polyacrylamide gel and then stained with ethidium bromide

Plasmids and RNA transcripts

Plasmid templates SP64-H$\beta\Delta$6 and SP64-H$\beta\Delta$3′ derived from human β-globin gene (kindly provided by T. Maniatis), were linearized with BamHI and transcribed with Sp6 polymerase. Truncated Ad-2 plasmid p5Eco0109 (kindly provided by W. Keller) was linearized with EcoRI and transcribed with T3 polymerase. pPIP4 plasmid was kindly provided by M.A. Garcia-Blanco. pPIP4Δ5′ constructed by removing the fragment EcoRI-SalI from pPIP4, was linearized with HindIII and transcribed with T7 polymerase. A1 cDNA (22) was linearized with HindIII and transcribed with T7 polymerase. UP1 cDNA derived from A1 cDNA by partial digestion of the coding region with XmnI was cloned in Bluescribe vector, linearized with BamHI and transcribed with T7 polymerase. High specific activity, ^{32}P-labeled RNAs (7.5×10^8 cpm/μg) used for RNase T1 protection and immunoprecipitation experiments were synthesized using either [α^{32}P]UTP or [α^{32}P]GTP and purified by 10% polyacrylamide-8M urea gel as previously described (43). Biotinylated ^{32}P-labeled RNAs at specific activity of 1.3×10^8 cpm/μg, were prepared as above except that 40 μM Biotin-11-UTP was added to the transcription reaction solution.

Oligonucleotides synthesis and purification

Oligonucleotides were synthesized on a 391 DNA synthesizer (Applied Biosystems), treated with NH_4OH at 55°C and purified by gel electrophoresis.

In vitro translation

Messenger RNA for A1 and UP1 were transcribed *in vitro* from the cDNA described above and translated in rabbit reticulocyte lysate as previously described (25).

Recombinat protein A1 purification

Recombinat hnRNP protein A1 and UP1 were produced and purified as previously reported (44).

Antibodies

Monoclonal antibody against protein A1 (4B10) was provided by G. Dreyfuss. Anti-Sm monoclonal antibody (Y12) and anti-U1/U2 snRNPs monoclonal antibody (11A1) were provided by W. J. Van Venrooij. Anti-U2 snRNP serum (GA) was a generous gift of J. Steitz. Polyclonal anti-A1 antibody was raised in rabbit inoculated with the recombinant protein A1.

RNase T1 protection and immunoprecipitation

The RPI assay was performed essentially as described (43). 6×10^5 cpm of transcripts and 700 ng of recombinant protein A1 were mixed and incubated at 25°C for 10 min in a 50 μl reaction mixture containing 10 mM Hepes-KOH pH 7.9, 10% glycerol, 50 mM KCl, 1.5 mM $MgCl_2$ 0.25 mM DTT. 500 U of RNase T1 were added and the incubation was continued for 30 min. Immunoprecipitations were performed by pre-binding 1μl of anti-A1 monoclonal antibody (4B10) to 30 μl of protein A sepharose in 10 mM Tris-Cl pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X100 (RSB-100) as previously described (31). After washing, beads were diluted to 300 μl with RSB-100 and then added to the 50 μl of binding/RNase T1 incubation reactions and the immunoprecipitation was carried out with rotation at 4°C for 30 min. The beads were pelleted by centrifugation. After washing the pellet with four 1ml volume of RSB-100 buffer, T1-resistant RNA fragments were isolated by phenol extraction followed by ethanol precipitation and analyzed on a 16% polyacrylamide-8M urea gel.

Indirect immunoprecipitations of snRNPs were carried out at 4°C for 45 min in a 50 μl reaction mixture containing: 30 μl of purified snRNP fractions, 700 ng of recombinant protein A1, 10 mM Hepes-KOH pH 7.9, 10% glycerol, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.25 mM DTT. Immunopurifications with anti A1-monoclonal antibody 4B10 were performed as described above. Bounds snRNPs were phenol-extracted, the snRNAs were ethanol-precipitated with 10 μg of glycogen carrier, 3′-end-labelled with [^{32}P] pCp and T4 RNA ligase (45) and analyzed on a 10% polyacrylamide-8M urea gel. Indirect immunoprecipitations of radiolabelled *in vitro* translated A1 and UP1 were performed in a 50 μl reaction mixture containig 30

μl of purified snRNP and 20 μl of each radiolabelled translation product. Samples incubated at 4°C for 1hr were immunoprecipitated with anti-U2 antibody (GA) prebound to protein A sepharose as described above.

Oligo directed RNAse H cleavage of U2 snRNP

RNase inactivation of U2 snRNA was as described (46). The anti U2 oligonucleotides used were 5′ AGGCCGAGAAGCG-AT 3′ and 5′ CAGATACTACACTTG 3′ (47). Reactions were incubated for 60 min at 30°C in a volume of 50 μl containing: 30 μl of purified snRNPs, 3 mM $MgCl_2$, 0.4 mM ATP and purified oligodeoxynucleotides at 2 μM. In order to degrade the excess oligonucleotide prior to the addition of recombinant A1 protein, the oligonucleotide/RNase H reactions were incubated for additional 15 min in the presence of 2 U of RNase-Free DNase. Indirect immunoprecipitation of snRNPs with anti-A1 antibody was as described above.

Streptavidin-agarose affinity selection assay

40 μl of streptavidin agarose beads suspension was pre-blocked for 30 min at 4°C by addition of 100 μg/ml glycogen and 1 mg/ml BSA (48) .The beads were pelleted and washed three times with 1ml of 100 mM NaCl, 10 mM Tris-Cl pH 7.5 and 0.05% NP-40 (IPP 100). After the final wash the beads were resuspended in 100 μl of IPP 100. Biotinylated RNA (Ad-2 pre-mRNA, 140,000 cpm), was mixed with non biotinylated RNA (pPip 4Δ5′ 140,000 cpm) in 10 μl of reaction mixture containing 20 mM Tris-Cl pH 7.5, 40 mM NaCl, 5% glycerol and incubated with 100 ng of recombinant protein A1 at 4°C for 30 min. Preblocked streptavidin agarose was added to the reaction and incubated for 1 h by rotation of the tube at 4°C. The beads were pelleted by centrifugation, the supernatant aspired, the pellet washed with 1ml of IPP100 five times, and resuspended in 100 μl of elution buffer containing 1% SDS, 1mM EDTA, 20 μg BSA and 10 μg tRNA. The bound RNA was eluted at 90°C over a 5 min period and the eluate was extracted with phenol and precipitated with ethanol. RNA species were resolved on a 10% polyacrylamide-8M urea gel.

RESULTS

Recombinant protein A1 binds *in vitro* to snRNP U2

When recombinant protein A1 was incubated *in vitro* with a snRNP preparation, the formation of stable A1-snRNP complexes was observed. snRNPs particles were purified from HeLa cell nuclear extract by CsCI density gradient centrifugation as described in Materials and Methods. Integrity of snRNP was deduced from the correct buoyant density pattern and was further confirmed by western blot analysis with anti-Sm (Y12) and anti U1/U2 antibodies (11A1) (data not shown).

Experiments were performed principally on two snRNP fractions containing U2, U1 and U4 in different relative amounts and traces of U5 and U6. Human recombinant protein A1 was prepared and purified to near homogeneity as previously described (44). The assay mixture contained a 2 to 5 fold molar excess of protein A1 over U1 and U2 snRNPs that were present at a concentration comparable to that in nuclear extract (0.1−0.2 μM; 47). Binding of protein A1 to snRNPs was assayed by indirect immunoprecipitation with a monoclonal anti-A1 antibody (4B10) or with anti-A1 polyclonal antibody raised against the

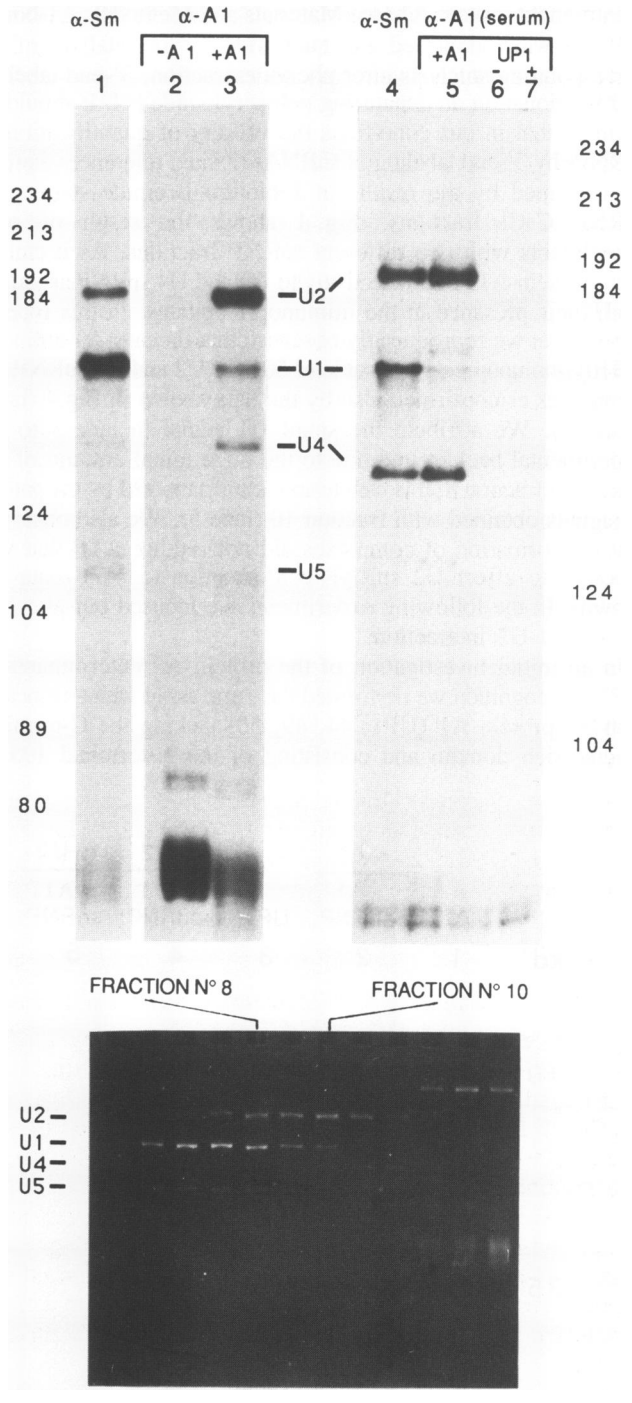

Figure 1. A1-snRNP interaction as detected by indirect immunoprecipitation. snRNPs from two CsCl-density gradient fractions were assayed (fraction 8 and 10). *Upper panel*: Recombinant protein A1 and snRNPs were mixed and complexes immunopurified with the indicated antibodies. Immunoprecipitated snRNPs were identified by the analysis of the corrisponding snRNAs after phenol extraction, 3′-end labeling with T4 ligase and fractionation on denaturing 10% polyacrylamide gel (see Material and Methods). Lanes 1 and 4: control immunopurification of snRNPs (fraction 8 and 10 respectively) with anti-Sm (Y12) antibody. Lanes 2, 3: immunopurification of snRNPs by anti-A1 (4B10) mAb in absence (−A1) or in presence (+A1) of recombinant protein A1. Lanes 5, 6 and 7: immunopurification of snRNPs by anti-A1 serum in presence (+A1) of recombinant protein A1 and in absence (−UP1) or in presence (+UP1) of recombinant protein UP1. Size markers are shown alongside. U1, U2, U4 and U5 snRNAs are indicated. *Lower panel*: Ethidium Bromide staining of CsCl density gradient fractions. Fractions 8 and 10, used in the upper panel experiments, are indicated.

recombinant protein A1 (see Materials and Methods). A1-bound snRNPs were identified and quantified by the analysis of the corresponding snRNAs after phenol extraction, 3'-end labeling and fractionation on denaturing polyacrylamide gel. It should be outlined that in our conditions the validity of quantification of snRNPs by 3'-end labeling of snRNAs (39 and references therein) is confirmed by the results of Ethidium Bromide staining of snRNP CsCl fractions. Fig. 1 shows the result of such experiments with two different snRNP fractions. As it can be seen, protein A1 binds selectively to U2 and U4 snRNP as judged from their presence in the immunoprecipitates. In this type of experiment we reproducedly observed that the anti-A1 antibody (4B10) immunoprecipitates at least 50% of U2 and U4 snRNP/A1 complexes as confirmed also by the data shown in Fig.4 (lanes 1 and 3). We attribute the small U1 signal in lane 3 to the experimental background due to the huge initial amount of U1 snRNP in fraction 8; this conclusion is substantiated by the pattern of signals obtained with fraction 10 (lane 5). We also observed that the formation of complexes did not require ATP that was reported to affect U2 snRNP conformation (5, 47) (data not shown). In the following experiments we focused our attention on the A1-U2 interaction.

In an initial investigation of the protein A1 determinants of snRNP recognition we performed the same assay with a truncated form of protein A1 (UP1; 44, 49, 50) lacking the C-terminal glycine-rich domain and consisting of the N-terminal 195 aa

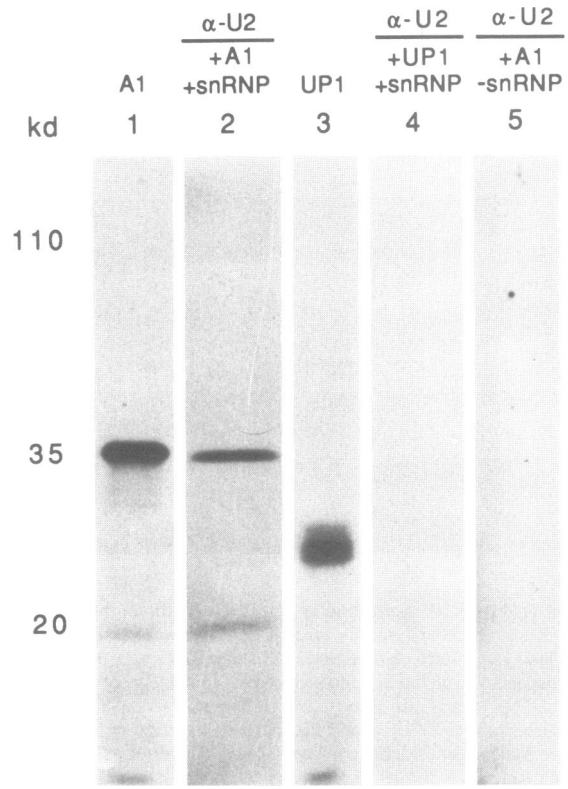

Figure 2. Binding of U2 snRNP to *in vitro* translated proteins A1 and UP1 as detected by indirect immunoprecipitation. A1 and UP1 cDNAs were translated in vitro as described in Materials and Methods. The [^{35}S] products were fractionated in SDS-PAGE (Lanes 1 and 3). Lanes 2 and 4: the proteins were mixed with snRNPs (fraction 8, see Fig. 1) and immunopurified with an anti-U2 antibody (GA). Lane 5: immunopurification of in vitro translated A1 protein with anti-U2 antibody in the absence of snRNPs. kd: molecular weight markers.

where two tandemly arranged RNA recognition motifs (RRM) are located (27, 28). Surprisingly, as the experiment in fig. 1 lane 7 shows, UP1 is unable to form complexes with snRNPs as indicated by the fact that no snRNP particles were immunoprecipitated with a polyclonal antibody directed against protein A1 that recognizes also protein UP1 (see Materials and Methods).

To further substantiate the previous results we performed indirect immunoprecipitation of *in vitro* translated A1 and UP1 proteins, using the anti-U2 antibody (GA; see Materials and Methods). As Fig. 2, lanes 2 and 4 shows, the anti-U2 antibody precipitates only the U2-A1 complex. This result confirms the specificity of binding and indicates that complex formation requires the integrity of protein A1. It should be outlined that, contrary to the previous one, this last assay entailed small amounts of protein (2ng vs 700ng) thus reducing the probability of artifacts due to spurius contaminants or to precipitation of the recombinant protein.

In conclusion these data indicate that protein A1 specifically binds U2 snRNP (and U4). Interestingly the failure of protein UP1 to interact with U2 and U4 suggests that also the C-terminal glycine-rich domain of the protein is involved in such a binding. It should be reminded that this domain was implicated in protein-protein interaction (44), as well as in establishing additional contacts with the nucleic acid with consequent increase of binding affinity (44, 51). The mode of protein A1 binding to U2 will be further analyzed in the next section.

Mapping of protein A1 binding site on U2 snRNP

As discussed above, the requirement of the C-terminal domain for the binding of A1 to snRNP U2 could suggest an important role of protein-protein interactions. On the other hand, given its described properties (44, 51) a binding of protein A1 (and UP1) to the naked and exposed regions of U2 RNA (5, 47, 52, 53) can be predicted. The experiment reported below indicates that complex formation between A1 and U2 actually involves protein-RNA binding. We performed the coprecipitation experiment described in Fig.1 in presence of an excess of a single-stranded oligonucleotide homologous to the 3' end of Adenovirus type 2 (Ad-2) major late transcript intron. In a previous paper (32) we showed that A1 has a high affinity for this sequence. As the result in Fig. 3 shows, excess of oligonucleotide completely abolishes the binding of A1 to U2 (and U4) (lanes 3 and 4) strongly suggesting that the binding is mediated by protein-RNA interactions and that protein-protein interactions play little or no role.

In order to identify the U2 snRNA sequences involved in A1 binding we explored, by oligo directed inactivation, the U2 snRNA regions that are known to be free of proteins and exposed. The best candidates are the first 50 nucleotides at the 5'-end that were reported to be available for base pairing with U6 snRNP and with a complementary sequence at the branch site of introns during spliceosome assembly (47, 54−58). Oligo directed RNase H cleavage of U2 snRNA was performed with oligonucleotides complementary to nt 1−15 (E15) and nt 28−42 (L15) (47) at concentrations calibrated to give complete digestion of the complementary snRNA sequences. After RNase treatment, the excess oligonucleotide was completely eliminated with DNaseI to avoid competition with snRNA for the binding to A1; the efficiency of DNaseI digestion was preliminary tested on the same oligonucleotide (data not shown). Site specific cleaved snRNPs were then mixed with a 5 fold molar excess of protein A1 and incubated for 1h at 4°C. Complex formation was detected by

indirect immunoprecipitation with 4B10 mAb as described before. After immunoprecipitation, the supernatants (containing unprecipitated snRNP) were phenol extracted, the snRNAs were 3′-end labeled and their size determined by gel electrophoresis. As expected, see Fig. 4 (lanes 2, 3) digestion with E15 and L15 oligonucleotides, shortens U2 snRNAs of 15 nt and 42 nt respectively while both U1 and U4 snRNAs are unaffected by the treatment. As show in Fig. 4 (lane 3) the removal of the first 15 nt of the 5′ end does not impair the binding of protein A1 to U2 as indicated by the substantial amount of snRNP in the immunoprecipitate. On the contrary the removal of nt 28 to 42 (Fig.4 lane 4) totally eliminates the binding. It should be observed that binding of A1 to U4 is unaffected by treatment with both oligonucleotides thus confirming the complete elimination of competition by residual oligonucleotide (see above).

In conclusion these results indicate that interaction of U2 snRNP with A1 is dependent on the integrity of the snRNA region comprised between nt 28 and 42 which, according to the predicted secondary structure (59) is in a single-stranded configuration. Interestingly this is the same sequence that was shown to be involved in base pairing with a complementary sequence at the branch site (54, 55). We cannot rule out the possibility that A1 might interact also with the first 28 nucleotides sequence although this seems unlikely also in view of their possible engagement in a hairpin structure that should reduce the binding affinity.

In the light of these results it remains difficult to explain why protein UP1 is unable to form precipitable complexes with U2 snRNA (see Fig.1 and 2), since it has been reported that UP1 binds to single-stranded nucleotides albeit with reduced affinity (44, 51). The different behaviour of A1 vs UP1 argues in favour of a role of the C-terminal domain in the stabilization of the binding.

RNase protection by protein A1 of the Ad-2 intron sequence between the branch point and the 3′ splice site

The ability of protein A1 to establish a specific association with U2 and U4 snRNPs prompted us to consider under a new light the implications of the previously reported specificity of protein A1 for the 3′ end of introns (31, 32). Taken altogether in fact these observations suggest a possible role of hnRNP in the

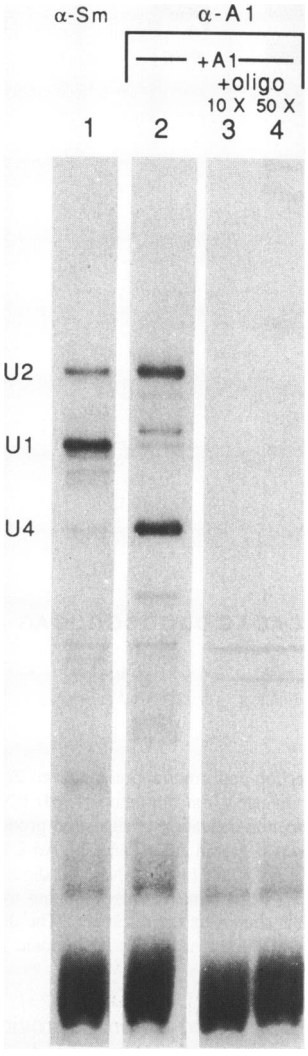

Figure 3. Inhibition of A1-U2 snRNP binding by excess of single stranded oligonucleotide. Indirect immunoprecipitations and analysis as in Fig. 1. Lane 1: control immunopurification of of snRNPs (fraction 8, Fig. 1). Lane 2: immunopurification of snRNPs by anti-A1 mAb (4B10) in presence of recombinant protein A1. Lane 3 and 4: same as lane 2 but in presence of 10× and 50× molar excess over snRNPs of a 21-mer oligonucleotide (see text). U1, U2 and U4 snRNAs are indicated.

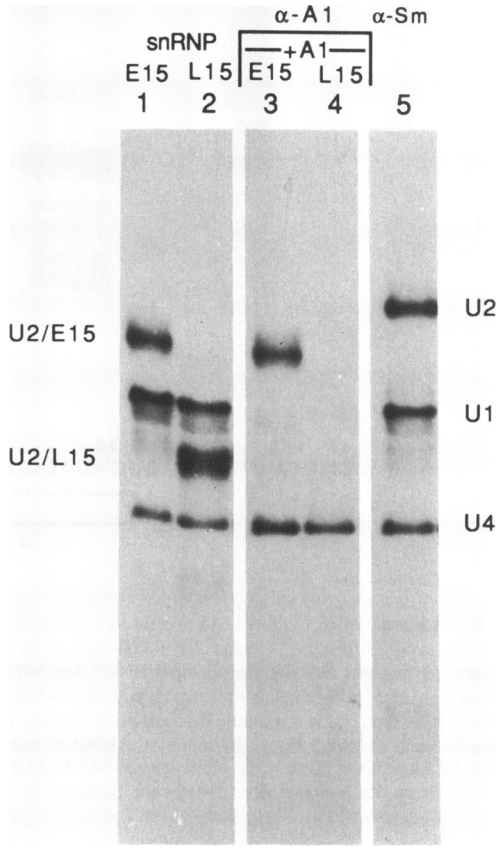

Figure 4. Mapping of protein A1 binding site on U2 snRNP by oligonucleotide directed RNase cleavage of snRNA. snRNPs (fraction 10) were incubated with oligonucleotides complementary to nt 1−15 (E 15) and nt 28−42 (L 15) of U2 RNA and digested with RNase H. After addition of recombinant protein A1, samples were immunoprecipitated with anti-A1 m Ab. Supernatants (lanes 1 and 2) and immunoprecipitates (lanes 3 and 4) were phenol extracted, and analyzed as in Fig. 1. Lane 5: control immunopurification of snRNPs with anti-Sm antibodies (Y12). U1, U2 and U4 snRNAs are indicated on the right side. Oligo-cleaved U2 snRNAs are indicated on the left side.

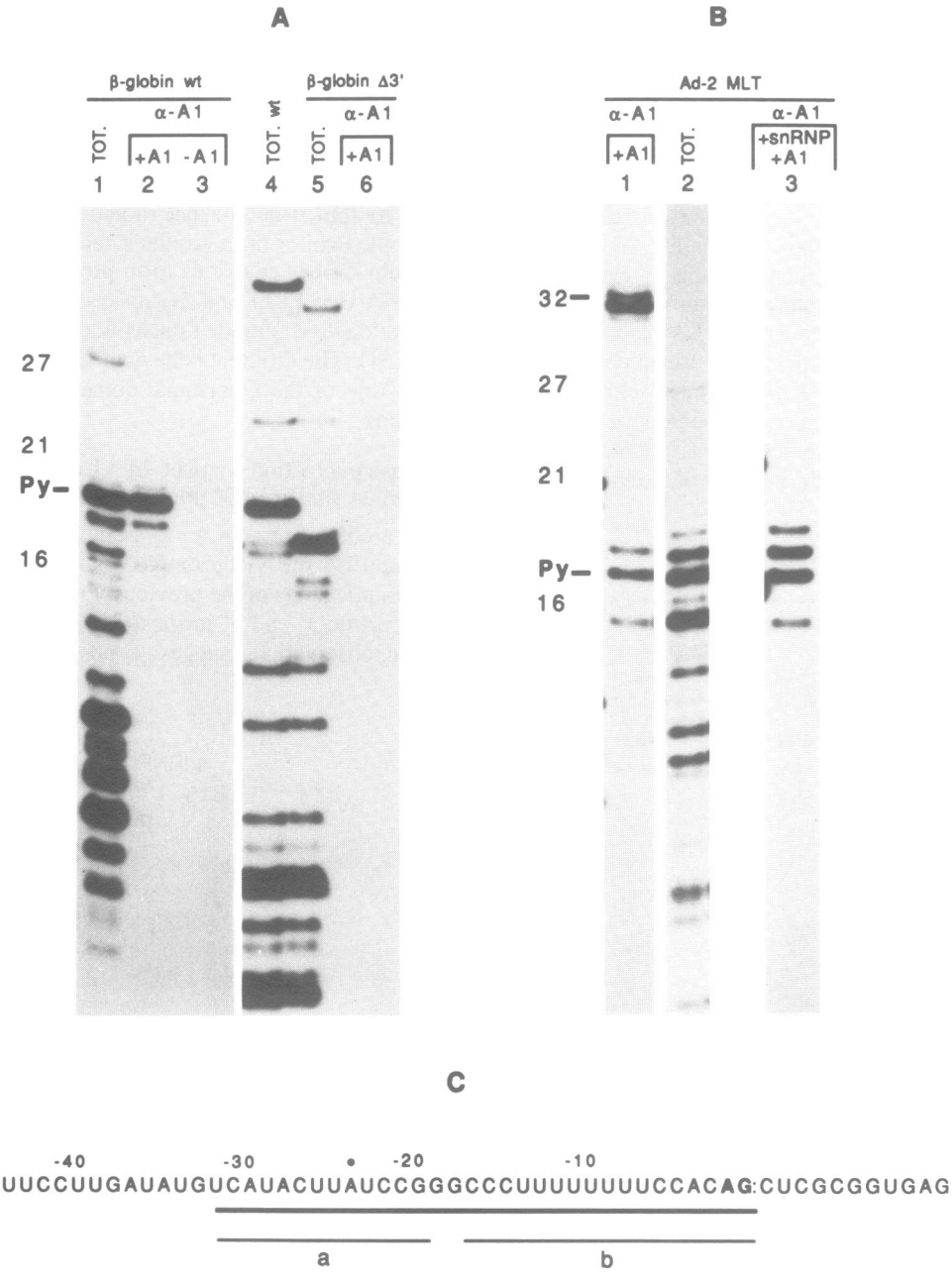

Figure 5. Binding assay of recombinant A1 protein to β-globin and Ad-2 pre-mRNAs by RNase T1 protection and immunoprecipitation. Panel A: human β-globin pre-mRNA wt (sP64-HβΔ6) and β-globin Δ3′ (sP64-HβΔ3′) (see text). Lanes 1 and 4: total RNase T1 digested (not immunopurified) RNA (β-globin wt). Lane 5: total RNase T1 digested (not immunopurified) RNA (β-globin Δ3′). Lanes 2 and 3: (β-globin wt), immunopurification of digestion products in presence (+A1) or in absence (−A1) of recombinant protein A1 with anti-A1 mAb (4B10). Lane 6: same as in lane 2 on β-globin Δ3′. Panel B: Ad-2 major late transcription unit p5Eco0109 (see text). Lane 1: immunopurification with anti-A1 mAb of digestion products in presence of A1. Lane 2: total RNase T1 digested non immunopurified RNA. Lane 3: same as lane 1 but in presence of snRNP (see text). Size markers are shown alongside. (Py): fragments corresponding to polypyrimidine tracts. Panel C: the location of the 32 nt protected fragment (see Panel B) within the Ad-2 MLT RNA sequence is shown by the thick line. The dot above the adenosine, 24 nt upstream from the 3′-splice site, denotes the branch point. a and b: RNase T1 digestion products of the 32 nt protected fragment (see text).

interplay of different factors in proximity of the 3′-splice site during the assembly of the splicing complexes. Such possibility is further supported by the experiments reported below concerning the RNase protection pattern of protein A1 on two different pre-mRNAs. The human β-globin 497 nt transcript, pSP64-HβΔ6, containing the first exon and intron and part of second exon (60) and a truncated version of Adenovirus type 2 mayor late transcription unit of 209 nt, p5Eco0109, containing shortened exon 1 and 2 and intron 1 (provided by W. Keller, unpublished data) were used. RNase T1 digestion and analysis of products were performed as described in Materials and Methods. As Fig. 5 panel A shows, the result obtained on the β-globin pre-mRNA confirms that recombinant protein A1 has selective affinity for the Py-rich fragment (19 nt) at the 3′ end of the intron since this digestion product is the major one immunoprecipitated by the anti-A1 antibody (see lane 2). In

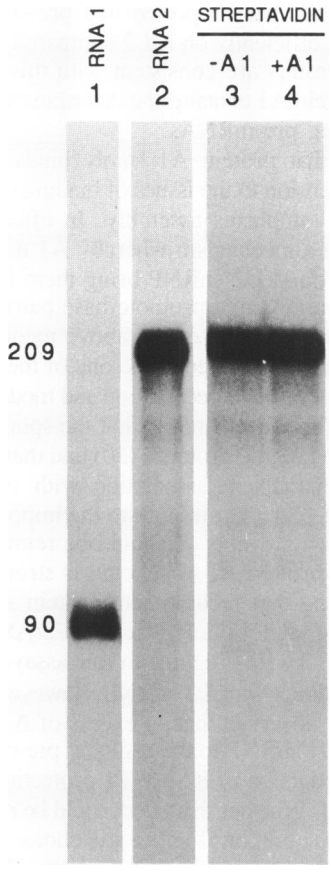

Figure 6. Assay of the ability of protein A1 to simultaneously bind two different RNAs. The assay was based on the streptavidin-agarose affinity selection method described in Materials and Methods. Recombinant protein A1 was incubated with a short non biotinylated Ad-2 pre-mRNA derivative (RNA 1; pPIP4Δ5′, see text) and with a biotinylated Ad-2 pre-mRNA (RNA 2; same as in Fig. 5) under optimal conditions for reannealing (see Materials and Methods). Both RNAs were labelled with [α^{32}P]UTP. Complexes were affinity purified with streptavidin agarose. RNAs were eluted and fractionated by gel electrophoresis. Lanes 1 and 2: RNA 1 and RNA 2 respectively. Lane 3 and 4: Streptavidin affinity purification of RNA 1+ RNA 2 mixture in absence or in presence of recombinant protein A1 respectively. The size of RNA 1 and RNA 2 is indicated.

agreement with this, when the Py-stretch deleted construct pSP64-HβΔ3′ was used (61) (lane 6) no fragment was immunopurified. It should be outlined that this result only demonstrates that protein A1 selects the Py-stretch out of all the fragment generated by RNase T1 digestion but does not necessarily indicate protection since the Py-stretch is the largest complete RNase digestion products.

On the contrary, in the experiment on the Ad-2 pre-mRNA (Fig. 5 panel B), in addition to the expected Py-stretch fragment (17 nt), two larger fragments (32−31 nt) are immunoprecipitated that must result from a true protection since they are not present among the control digestion products (lanes 1 and 2). To unambiguously determine the protected sequence, the resistant product was eluted from the gel and further digested with RNase T1. Separation of the digestion products on denaturing polyacrylamide gel evidenced two smaller fragments of 17 nt and 13 nt respectively (data not shown). On the basis of these results and of the inspection of the pre-mRNA nucleotide sequence it can be deduced that the protected sequence derives from the 3′ end of the intron and that the 17 and 13 nt fragments correspond respectively to the Py-stretch (fragment b) and the region overlapping the branch-site (fragment a) (see Fig. 5, panel C). Therefore in addition to confirming the binding specificity of A1, these data suggest that different RNAs could establish a different pattern of association with this protein (and perhaps with other hnRNPs). This point will be further addressed in the Discussion.

Does protein A1 affect the binding of U2 to the branch site?

It is well established that U2 snRNP specifically recognizes the branch site of introns and that its association is one of the first steps in spliceosome assembly. Stable U2 binding requires base pairing with the branch site sequence and interaction with other factors (e.g. the splicing factors SF1, SF2, U2AF) (7, 62, 63). On the basis of the results reported above we asked whether protein A1 could affect the interaction of U2 with the Ad-2 branch site. To this regard two possibilities can be envisaged a) A1 could stabilize the U2 binding, alike U2AF; b) A1 could 'bridge' U2 snRNP and intron sequences (i.e. the Py-stretch) during spliceosome formation. To determine whether A1 operates like U2AF we assayed its effect on RNase T1 branch-site protection by U2. The results did not evidence any increased protection by U2 indicating that protein A1 is not sufficient to stabilize the binding of U2 (data not shown). It should be reminded that RNase A protection of the branch site region, is the parameter commonly used to asses the stability of U2 binding and it was reported that this assay requires auxiliary factors including U2AF (64).

Since A1 is one of the first protein to became associated to the nascent transcripts it is tempting to hypothesize that, by virtue of its affinity for the Py-stretch and for U2 snRNA, it could be one of the factors that 'direct' the snRNP to its target site during spliceosome assembly. A1 could perform such a role by binding simultaneously the snRNP and the pre-mRNA. It should be mentioned that simultaneously binding of different RNA molecules to the multiple RNA binding domains of the protein (36, 51, 65) is the mechanism invoked by some authors (36−38) to explain the capacity of this protein to catalyze the reannealing of complementary strands.

In order to directly check this hypothesis a biotinylated Ad-2 pre-mRNA was incubated with snRNP in the presence of protein A1 and the formation of ternary complexes was assayed by affinity selection with streptavidin agarose. In such experiment however no pre-mRNA-A1-U2 complex formation was observed (data not shown).

This negative result suggest that, protein A1 cannot stably bind simultaneously two RNA molecules. Such an inability was further demonstrated by mixing protein A1 with a biotinylated Ad-2 pre-mRNA (209 nt, p5Eco0109) and with a shorter non-biotinylated Ad-2 derivative (90 nt, pPIP4Δ5′ derived from pPIP3; 66, 67), as described in Material and Methods. We proved that in the same conditions protein A1 efficiently binds to biotinylated RNA (data not shown). After 30 min incubation at 4°C, streptavidin agarose was added, the mixture was centrifugated, the RNA in the precipitate was eluted and resolved on a denaturing polyacrylamide gel. As shown in Fig. 6 only the biotinylated RNA was selected indicating that no ternary complex was formed in the presence of protein A1.

This result however does not role out the possibility that A1 proteins bound to both pre-mRNA and U2 snRNA could promote a transient association, driven by protein-protein interactions, not detectable in our type of assay since it requires additional factors to be stabilized. In effect, in another type of experiment, we could observe that an interplay of U2 snRNP and A1 at the branch

site of Ad-2 pre-mRNA actually does occur. As shown in Fig. 5 panel B, protein A1 protects the 3'-end intron region of Ad-2 pre-mRNA against RNase T1 digestion. However, when the same assay was performed in the presence of U2 snRNP (at a 1: 5 U2/A1 ratio) protection by A1 was drastically reduced (Fig. 5 panel B, lane 3). This result cannot be explained simply by a competition of U2 snRNP for the same intron sequence since A1 was in excess over U2 snRNP and in any case, in this assay, has a higher affinity for this region (as indicated by the higher protection capacity). On the other hand, any interference of the 100 kd (IBP) reportedly associated to U5 snRNP (12) can be ruled out since only trace amounts of this protein are present in the snRNP fraction used and in any case, not sufficient to cause RNase T1 protection of the Py-tract (not shown).

The most likely explanation for this effect is that an A1-U2 snRNP complex interacts by protein-protein binding with another A1 protein molecule bound at the branch-site. Here the strand-annealing activity of protein A1 might promote a transient reannealing between the 5' end of snRNA and the complementary sequence at the branch site. Since A1 is known to have very low affinity for double-stranded structures, this will result in detachment of the protein from the branch site with consequent loss of protection.

DISCUSSION

hnRNP protein A1 has been shown to bind intron sequences in and around the polypyrimidine tract of β-globin and adenovirus pre-mRNAs (31, 32).The results reported in this paper further confirm these findings and provide complementary information indicating that protein A1 might be functionally involved in the formation of early splicing complexes. To this regard the most relevant observation is the high affinity *in vitro* interaction between protein A1 and U2 snRNP that take place at the 5'-end portion of the snRNA known to be involved in base pairing with the branch point sequence. We also showed that a truncated form of protein A1 (UP1; also an RNA binding protein) is unable to bind U2 snRNP suggesting that the interaction involves all protein domains.

Protein A1 appears to interact also with U4 snRNP; this result could be expected since U4 snRNP (like U6 snRNP) has extended single-stranded RNA regions. Experiments to further characterize this interaction and a possible effect of A1 on the U4/U6 annealing are underway. On the contrary the finding that A1 does not bind U1 snRNP is surprising in view of the fact that also this snRNP has an exposed RNA tract that base-pairs with the 5' end of introns.

RNase T1 digestion-immunoselection experiments revealed the existence within two pre-mRNAs (β-globin and adenovirus) of high affinity sites for recombinant A1 binding located at the 3-'end of the introns. This result is particularly significant since it shows that the binding specificity is an intrinsic property of the protein and does not require additional components present in nuclear extracts (31, 33). In addition, on the adenovirus pre-mRNA the protein was found to bind and protect a region that extends from the 3'-splice site to beyond the branch point while on the β-globin pre-mRNA only the pyrimidine tract was protected. Thus, protein A1 can establish a different binding pattern on different introns. Recent experiments on the analysis of pre-splicing complexes assembled on several pre-mRNAs of different size and sequence as well as RNAs lacking functional splice sites show that different RNAs are associated with unique pattern of hnRNP proteins (69). In particular it has been reported that pre-splicing complex E assembles more efficiently on Ad-2 compared to β-globin intron (39, 69). Our results are consistent with this observations and suggest that protein A1 contains the determinants for a differential positioning along pre-mRNAs.

The finding that protein A1 stably binds U2 snRNP adds unexpected dimension to the issues of binding specificity and role in pre-splicing complexes assembly. In effect our results are compatible with a mechanism whereby A1 molecules bound to both pre-mRNA and U2 snRNP bring them in close contact at the branch site region and promote base-pairing. Therefore by virtue of this property and of the above mentioned differential intron affinity, protein A1 could be one of the early factors that favours the U2-branch site recognition and modulates the 3'-splice site selection. The recent finding that the splicing factor PTB is identifiable with hnRNP protein I (30) and that it can be purified from nuclear extracts in association with A1 and a 100 kd polypeptide (16, 68) adds strength to the importance of hnRNPs in the early splicing events. It should be reminded that a direct involvement of protein A1 in splicing is strongly supported by the recent finding that recombinant protein A1 antagonizes *in vitro* the activity of the splicing factor SF2/ASF in the 5'-splice site choice (35). To this regard, in our assays protein A1 does not appear to interact with U1 snRNP, however, in a preliminary experiment, we observed that an excess of A1 can compete for the binding of U1 snRNP to the β-globin pre-mRNA as revealed by the strong reduction of RNase T1 protection of 5'-splice site (data not shown). Whether this effect could be related to the above mentioned modulation of 5'-splice site choice still remains to be determined. In any case, the preferential binding of hnRNP A1 to 3'-splice site of introns is not contradictory with the above mentioned effect at 5'-splice site, since interactions between factors bound to these sites have been observed, during spliceosome formation (3, 70–72).

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