Structure-probing of U1 snRNPs gradually depleted of the U1-specific proteins A, C and 70k. Evidence that A interacts differentially with developmentally regulated mouse U1 snRNA variants

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Received November 15, 1989; Revised and Accepted January 4, 1990

ABSTRACT

The interaction of the U1-specific proteins 70k, A and C with U1 snRNP was studied by depleting gradually U1 snRNPs of the U1-specific proteins by Mono-Q chromatography at elevated temperatures (20 – 37°C). U1 snRNP species were obtained which were selectively depleted of either protein C, A, C and A, or of all three U1-specific proteins C, A and 70k while retaining the common proteins B′ to G. These various types of U1 snRNP particles were used to study the differential accessibility of defined regions of U1 RNA towards nucleases V_1 and S_1 dependent on the U1 snRNP protein composition. The data indicate that in the U1 snRNP protein 70k interacts with stem/loop A and protein A with stem/loop B of U1 RNA. The presence or absence of protein C did not affect the nuclease digestion patterns of U1 RNA. Our results suggest further that the binding of protein A to the U1 snRNP particle should be independent of proteins 70k and C. Mouse cells contain two U1 RNA species, U1a and U1b, which differ in the structure of stem/loop B, with U1a exhibiting the same stem/loop B sequence as U1 RNA from HeLa cells. When we used Mono Q chromatography to investigate possible structural differences in the two types of U1 snRNPs, we observed that protein A was always preferentially lost from U1b snRNP as compared to U1a snRNPs. This indicates that one consequence of the structural difference between U1a und U1b is a lowering of the strength of binding of protein A to U1b snRNP. The possible functional significance of this finding is discussed with respect to the fact that U1b RNA is preferentially expressed in embryonal cells.

INTRODUCTION

U1 snRNP is an essential cofactor of mRNA splicing. One of its major functions is the recognition of the 5′ splice site of the intron to be spliced (for reviews see 1–3). In addition to base-pairing between the 5′ end of U1 RNA and the 5′ splice junction (4, 5) U1 snRNP proteins are also needed for efficient [5′ splice site-U1 snRNP]-complex formation (6, 7). At least ten different proteins are associated with U1 RNA which can be divided into two classes. Seven proteins (B′, B, D, D′, E, F and G) are common to all major nucleoplasmic snRNPs U1, U2, U4/U6 and U5 (8–11; for review see 12). The remaining three proteins denoted 70k, A and C are specific for U1 snRNPs. Along the same lines U2 and U5 snRNPs also contain unique proteins. These are A′ and B″ for U2 (8, 9, 11) and seven proteins with molecular masses of between 40 and 200 kDa for U5 snRNP (13).

In the U1 snRNP the common proteins primarily interact with the so-called domain A of U1 RNA, a structural motif which consists of a single-stranded region $PuA(U)_nGPu$, with $n>3$ flanked by double-stranded stems (14–16). The domain A is shared by the snRNAs U1, U2, U4 and U5 (17), which explains why distinct snRNA molecules can interact with a common set of snRNP polypeptides. Binding of the U1-specific proteins 70k and A to U1 snRNP appears to be largely determined by specific RNA-protein interactions with U1 RNA stem/loop structures (18–25). Whether protein C interacts directly with U1 RNA is not yet clear.

In some cells more than one U1 RNA exists. Thus, both in mouse and Xenopus two major classes of U1 RNA can be distinguished, the so-called U1a and U1b RNAs. U1a RNAs are synthesized in all transcriptionally-active cells investigated while U1b RNAs accumulate only in cells that are capable of further differentiation, such as embryonic or germline cells (26). In mouse the sequence of U1b RNA differs from U1a in stem/loop B (27). The biological significance of the accumulation of particular U1 snRNAs during development is not clear, neither is it known whether snRNAs U1a and U1b interact differentially with U1 snRNP proteins.

A promising approach to learn more about the specificity of protein-protein and protein-RNA interactions is gradual depletion of a given RNP particle of individual proteins. This approach has previously been applied with success to study the structure of other RNP complexes such as ribosomes to name only one important example. Here we report that it is possible by Mono Q chromatography of purified snRNPs to isolate U1 snRNP particles which are specifically depleted of one or more of the

U1-specific proteins 70k, A and C, while still containing the common proteins. The U1 snRNP particles lacking defined sets of U1-specific proteins were subjected to limiting digestion with single- or double-strand specific nucleases. These data suggest that the 70k protein is primarily associated with stem/loop A of U1 RNA while the A protein is associated with stem/loop B of U1 RNA. The presence or absence of protein C on the U1 snRNP particle does not change the susceptibility of these regions of U1 RNA to nuclease attack, suggesting that binding of protein C to U1 snRNP may be dominated by protein-protein interactions. When we used Mono Q chromatography of UsnRNPs from mouse Ehrlich ascites tumor cells for the investigation of possible structural differences between the U1a and U1b snRNPs we observed that protein A was always lost preferentially from U1b snRNP as compared to U1a snRNP. This indicates that U1b RNA interacts less strongly with the A protein than U1a, most probably due to the structural changes of stem/loop B in U1b.

MATERIALS AND METHODS

Cells and nuclear extracts

Hela cells (S3) and Ehrlich ascites cells were grown in suspension as described (28): Two kinds of nuclear extracts were used as a source of UsnRNPs denoted NX-50 and splicing extracts. For the preparation of NX-50 extracts, the nuclei were obtained essentially as described by Zieve and Penman (29). The purified nuclei were resuspended in a buffer containing 10 mM Tris-HCl pH 7.4, 500 mM NaCl, 50 mM $MgCl_2$, 1 mM DTT and 1 mM PMSF at a density of 4×10^7 nuclei/ml, and then extracted by vigorous agitation on a Vortex mixer (rate 10) for 2 min. The resulting supernatant was freed from higher-molecular weight RNAs and RNPs by centrifugation for 90 min at 113,000 ×g. The high speed supernatant was taken as starting material for isolation of UsnRNPs. Splicing extracts were prepared from HeLa and Ehrlich ascites cell essentially as described by Dignam *et al.* (30).

Isolation of UsnRNPs over anti-m_3G chromatography

Immuno affinity purification of the snRNPs U1 to U6 form NX-50 and splicing extracts were performed using mAb H-20 (anti-m_3G) bound covalently to CNBr-activated Sepharose 4B, essentially as described (31).

Fractionation of the snRNPs U1 to U6 on Mono Q chromatography

A mixture of about 2 mg of snRNPs U1 to U6 from NX-50 extracts in ~10 ml of the m^7G elution buffer originally used for desorption of the snRNPs from the anti-m_3G immuno affinity column (300 mM KCl, 15 mM $MgCl_2$, 20 mM Tris-HCl, pH 8, 0.5 mM DTT, 1 mM PMSF and 15 mM m^7G) (31) was diluted with 2 volumes of Mono Q-0 buffer (20 mm Tris-HCl pH 7, 15 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin) in order to reduce the salt concentration. The snRNPs were then allowed to adsorb to a Mono Q column (1 ml bed volume), equilibrated with Mono Q-50 buffer (as Mono Q-0 buffer, but containing in addition 50 mM KCl), by passing the snRNPs solution over the column at a flow rate of 2 ml/min (pressure ~3 MPa). The column was washed with Mono Q-50 buffer untill the absorbance at 280 nm reached a steady value of zero (4 bed volumes). The snRNPs were then fractionated by elution in Mono Q-0 buffer containing added KCl at a concentration that rose stepwise from 50 mM to 1 M. Fractionation on the Mono Q column was carried out either at 4°C, 24°C or 37°C as indicated. The flow rate was 1 ml/min and about 55 1 ml fractions were taken. The fractionation of snRNPs U1 to U6 isolated by anti-m_3G affinity chromatography from splicing extracts followed the same protocol, except that the Mono Q buffer contained 1.5 mM $MgCl_2$ instead of 15 mM. The RNA and protein content of the fractions eluted from the Mono Q column were analysed as described (11).

Enzymatic cleavages and primer extension analysis

The wt U1 snRNPs and U1 snRNP particles lacking defined sets of U1-specific polypeptides were digested with RNAse V1 (Pharmacia) or RNAse S1 following the protocol of Krol and Carbon (32). An amount of U1 snRNPs equivalent to 1.3 μg of U1 RNA was mixed with 10 μg of *E.coli* tRNA in buffer A (10 mM Tris-HCl pH 7.5, 150 mM KCl, 2 mM $MgCl_2$, 5 % glycerol, 0.5 mM DTT and 1 mM PMSF) in a final reaction volume of 80 μl. As a control, 20 μl were withdrawn and incubated separately for 20 min at 25°C. 0.02 U/μg tRNA of RNase V1 were then added to the remaining 60 μl reaction volume which was incubated at 25°C. 20 μl aliquots were withdrawn after 5, 10 and 20 min and reaction was stopped by phenol extraction. The control reaction was stopped after 20 min by phenol extraction as well.

Limiting digestion of the various types of snRNPs with RNase S1 was carried essentially as described above except that 25 U/μg tRNA was used and that buffer A contained 1 mM $ZnCl_2$. Furthermore, timepoint aliquots were removed at 2, 5 and 10 min.

For the primer extension analysis the RNA from each timepoint aliquot and the control reactions was divided in three equal portions which were then separately annealed with one of the following three oligodeoxynucleotide primer: 5′-CCTTCGTGATCA-3′, 5′-CCGGAGTGCAATGG-3′, 5′-ACTACCACAAATTA-3′. These are complementary to the regions of U1 snRNA between residues 28–39, 64–77, 125–138, respectively. Annealing conditions and primer extensions with reverse transcriptase enzyme were as described (32). After incubation with reverse transcriptase for 30 min at 37°C, reactions were stopped by addition of 5 μl of formamide dye (32) and immediately subjected to electrophoresis in a 10 % denaturing polyacrylamide gel. For annealing of oligodeoxynucleotide 125–138 with U1 snRNA reaction volumes were heated at 65°C for 3 min, and incubated further at 37°C for 10 min prior to addition of reverse transcription.

RESULTS

Gradual depletion of HeLa U1 snRNPs of defined sets of U1-specific proteins by Mono Q chromatography

When we applied Mono Q anion exchange chromatography to the further fractionation of the mixture of anti-m_3G affinity-purified snRNPs U1, U2, U4/U6 and U5 we observed minor U1 snRNP peaks which were eluted from the column at slightly higher salt concentrations than the majority of U1 snRNP particles. While the U1 snRNA contained in these types of U1 snRNPs was intact, they were heterogeneous with respect to their content of U1-specific proteins (not shown). Whether such heterogeneous U1 snRNP populations may exist in the cell or not cannot be answered at present. We consider the possibility that the various protein-deficient U1 snRNP particles were due to *in vitro* manipulations of the snRNPs to be the more likely

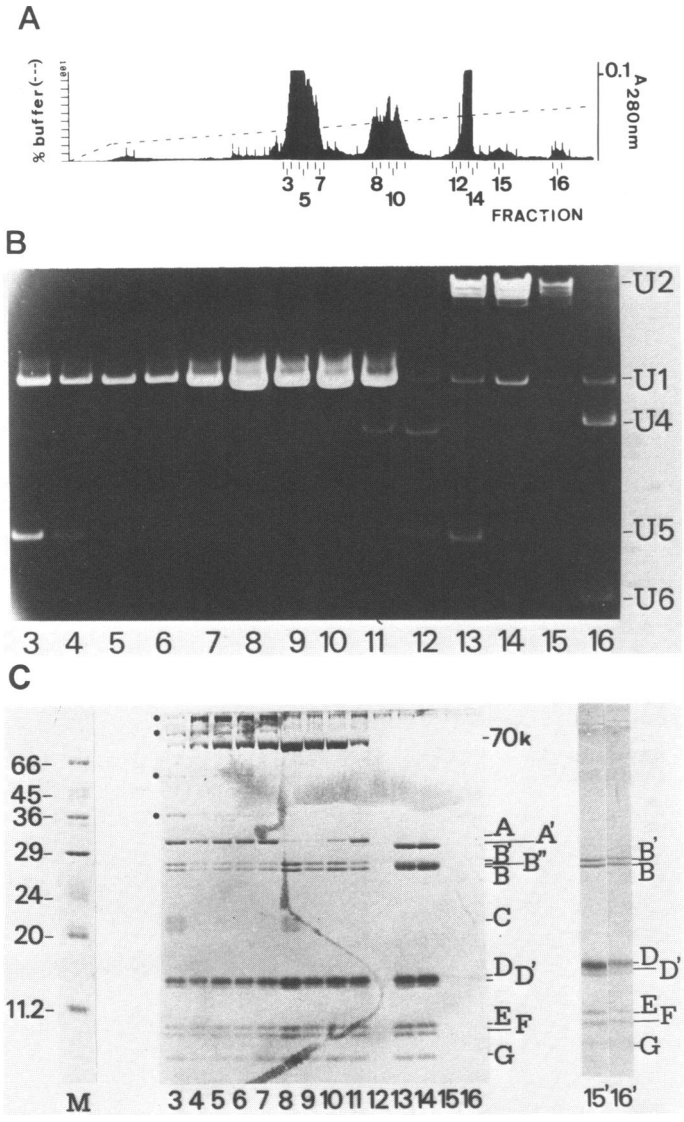

Fig. 1. Mono Q chromatography of snRNPs U1 to U6 affinity purified with anti-m_3G IgG from HeLa splicing extracts. Chromatography was performed at 24°C. (A) The elution profile of snRNPs from the Mono Q column is shown, where buffer B corresponds to the Mono-Q 1000 buffer (containing 1 M KCl). (B) RNA from the snRNP fractions indicated at the bottom of each lane was extracted and electrophoresed in a denaturing 10 % polyacrylamide gel and stained with ethidium bromide. (C) Proteins from the corresponding snRNP fractions as shown in panel B were extracted and separated in a 15 % SDS-polyacrylamide gel and stained with Coomassie blue. Dots on the left of lane 3 indicate positions in the gel of U5-specific proteins. Lanes 15'and 16'were loaded three fold more snRNP protein than lanes 15 and 16, respectively.

explanation . This notion was supported by our finding that the yield of the protein-deficient U1 snRNP particles were considerably increased when the Mono-Q chromatography step was carried out at higher temperatures instead of 4°C.

Figure 1 shows an example where a mixture of anti-m_3G affinity purified snRNPs U1 to U6 from HeLa nuclear splicing extracts were subjected to Mono Q chromatography at room temperature. On the basis of the protein gel electrophoresis pattern shown in Fig. 1C the following U1 snRNP populations may be distinguished. Fraction 3 from the first peak contains U1 snRNPs which have retained all three U1-specific proteins (denoted wildtype (wt) U1 snRNP). Note that the proteins of molecular masses 40, 52, 100 and 200 kDa which can be seen in this lane, represent U5-specific proteins from the 20S U5 snRNPs which elute at similar salt concentrations from Mono Q columns as wt U1 snRNPs (13). Fractions 5 to 7 contain U1 snRNPs lacking only protein C, denoted ΔC U1 snRNP. (The possibility that some of the U5-specific proteins may interact with ΔC U1 snRNPs can be excluded on the basis of sedimentation analysis in glycerol gradients where no co-fractionation of U5-specific proteins with ΔC U1 snRNPs has been observed (not shown)). U1 snRNP particles lacking only protein A(ΔA U1 snRNP) or both A and C(Δ[A,C]U1 snRNP) are contained in fractions 8 and 9, respectively (Figure 1C). Fraction 10 contains a U1 snRNP species which lacks protein C and, by comparison of the intensity of Coomassie stain, displays less protein A than U1 snRNPs in fractions 6 and 7, for example. The U1 snRNP particle in fraction 11 appears to be reduced in its 70k protein content. It should be noted that no differences in the composition of common snRNP proteins were apparent between the various U1 snRNP types eluting in fractions 3 to 11 (Fig. 1C).

Fractions 13 and 14 (Fig. 1) contain predominantly wt U2 snRNPs which in addition to the common proteins have retained the U2-specific proteins A′ and B″ (B″ migrates together with B in Fig. 1, see also ref. 31), as indicated by the high staining intensity and immunoblotting (not shown). Interestingly, fraction 15 contains U2 snRNP particles that completely lack the U2-specific proteins A′ and B″ (Δ[A′,B″]U2 snRNP) (Figure 1, lane 15′). Thus the same procedure can also be used to isolate protein-deficient U2 snRNPs.

Qualitatively similar results were obtained when snRNPs U1 to U6, isolated by anti-m_3G affinity chromatography from nuclear extracts prepared at high Mg^{++} concentrations (NX-50), were fractionated on Mono-Q columns under the same conditions as described above. Figure 2 (panels A and B) shows the RNA and protein composition of selected fractions from the gradient (the gradient profile is shown in Fig. 1A). wt, ΔC, ΔA and Δ[A,C] U1 snRNP particles are contained in fractions 2, 4, 6 and 7, respectively (Figure 2). It is important to note that unlike the situation with snRNPs from splicing extracts, U1 snRNP populations uncontaminated by 20S U5 snRNPs may be obtained by Mono Q chromatography of snRNPs from NX-50 extracts. This is due to the fact that during preparation of NX-50 nuclear extracts the 20S U5 snRNPs lose the U5-specific proteins (13). The resulting core U5 snRNPs containing only the common snRNP proteins B′ to G, elute at higher salt concentrations than the various U1 snRNP populations and are contained in an essentially pure form in fraction 10 of the gradient (Figure 2). As with snRNPs from splicing extracts, two U2 snRNP peaks were also obtained in this case, the first containing wt U2 snRNPs (fractions 12+13) the second Δ[A′,B″]U2 snRNPs (fractions 15+16, Figure 2).

It is further possible to produce U1 snRNP particles which lack all of the U1-specific proteins A, C and 70k (Δ[A,C,70k]U1 snRNPs). For this purpose, wildtype U1 snRNPs isolated from the Mono Q column at room temperature were re-chromatographed on the Mono Q column at 37°C. From this second fractionation step, a considerable amount (about 20 % of the original U1 snRNPs applied to the column) of Δ[A,C,70k]U1 snRNPs are recovered (Fig. 3, fractions 11−16) in addition to the A- and C-deficient U1 snRNP populations described above. Depletion of the U1-specific proteins from various U1 snRNPs preparations is not always quantitative and varies in individual experiments from 90 to 98% efficiency as estimated by Coomassie-staining of SDS-polyacrylamids gels and semi-quantitative immunoblotting (not shown).

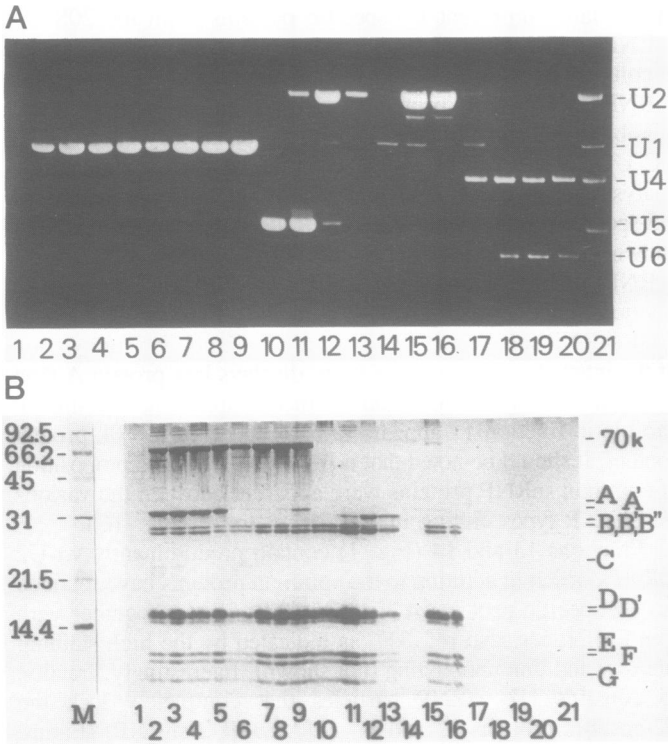

Fig. 2. Mono Q chromatography of snRNPs U1 to U6 affinity purified with anti-m_3G antibodies from HeLa NX-50 nuclear extracts. Chromatography was performed at 24°C essentially as described in Fig. 1. Panels A and B show respectively the RNA and protein composition of the various snRNP containing fractions obtained by elution of the Mono Q column with an increasing KCl gradient (from left to right). Extraction from snRNPs, gel fractionation and staining of snRNAs and snRNP proteins was performed as described for Fig. 1. Lane M, molecular weight markers.

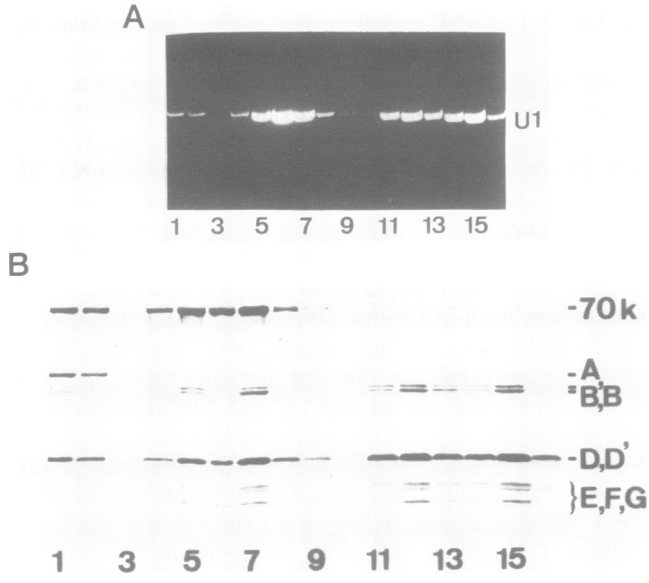

Fig. 3. Re-chromatography of purified U1 snRNPs on Mono Q resin at 37°C. Chromatography was performed essentially as described in Fig. 1 but at 37°C. The U1 snRNPs subjected to the second Mono Q chromatography step at 37°C were obtained from Hela NX-50 nuclear extracts as described in Fig. 2. They were derived from the first U1 snRNP peak eluted from the Mono Q column at 24°C (fraction 2 in Fig. 2) and contain all U1-specific and the common proteins. Panels A and B show respectively the RNA and protein composition of the various snRNP containing fractions obtained by elution of the Mono Q column with an increasing KCl gradient (from left to right). Extraction from snRNPs, gel fractionation and staining of snRNAs and snRNP proteins was performed as described for Fig. 1.

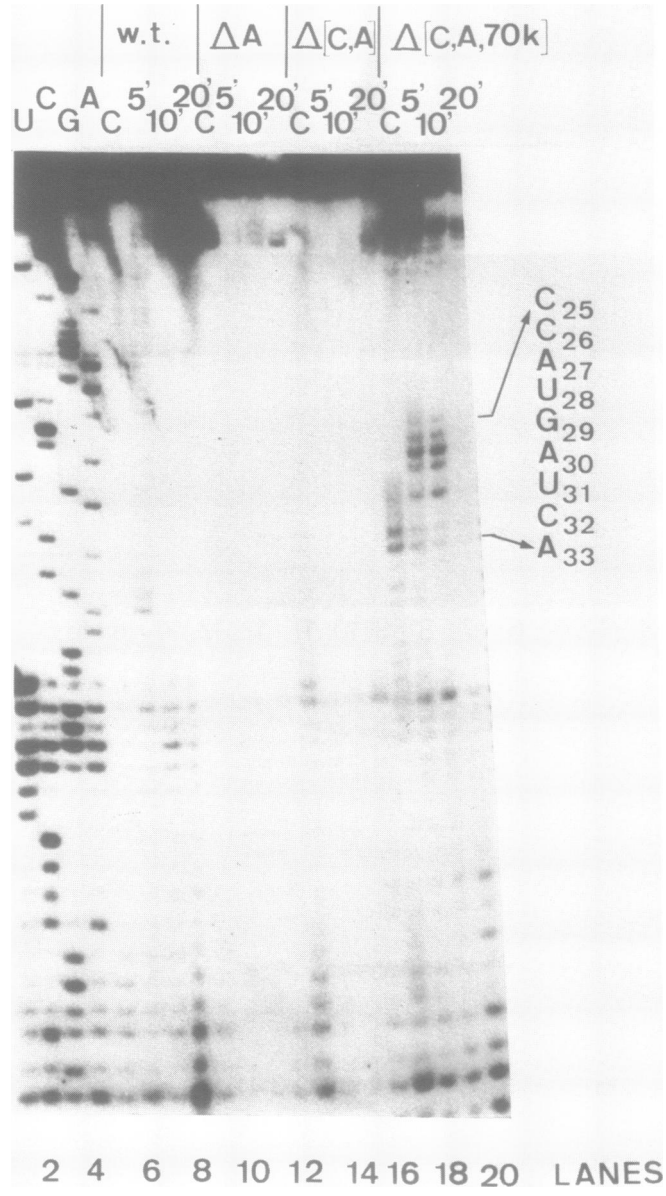

Fig. 4. RNase V1 cleavage of stem/loop A of U1 RNA in U1 snRNPs lacking the 70k, A and C proteins. Limited digestion of the indicated types of U1 snRNPs with RNase V1 and primer extension analysis with reverse transcriptase was carried out as described in Materials and Methods. Oligodeoxynucleotide 64–77 was used as a primer. In lanes 1–4 of the autoradiograph extension products of reverse transcriptase reactions are shown which were carried out on undigested phenolized U1 RNA in the presence of ddNTPs. Lanes 5–8, incubation of wt U1 snRNPs with RNase V1; lanes 9–12, incubation of ΔA U1 snRNP with RNase V1; lanes 13–16, incubation of Δ[C,A]U1 snRNP with RNase V1; lanes 17–20, incubation of Δ[A,C,70k]U1 snRNP with RNase V1. lanes denoted C represent control reactions carried out in the absence of RNase V1. 5, 10, 20 min indicate the time of incubation of the various U1 snRNP particles with RNase V1.

Differential accessibility of stem/loops A and B of U1 RNA towards nuclease attack in the various types of protein-deficient U1 snRNPs

Next we used the isolated U1 snRNP particles lacking defined sets of the U1-specific proteins to investigate the binding sites of these proteins on U1 RNA. For this purpose, the accessibility of certain regions of U1 RNA towards nucleases V1 and S1 in the protein-deficient U1 snRNP particles was compared to the wt U1 snRNPs. Reasoning that the U1 RNA binding site of a

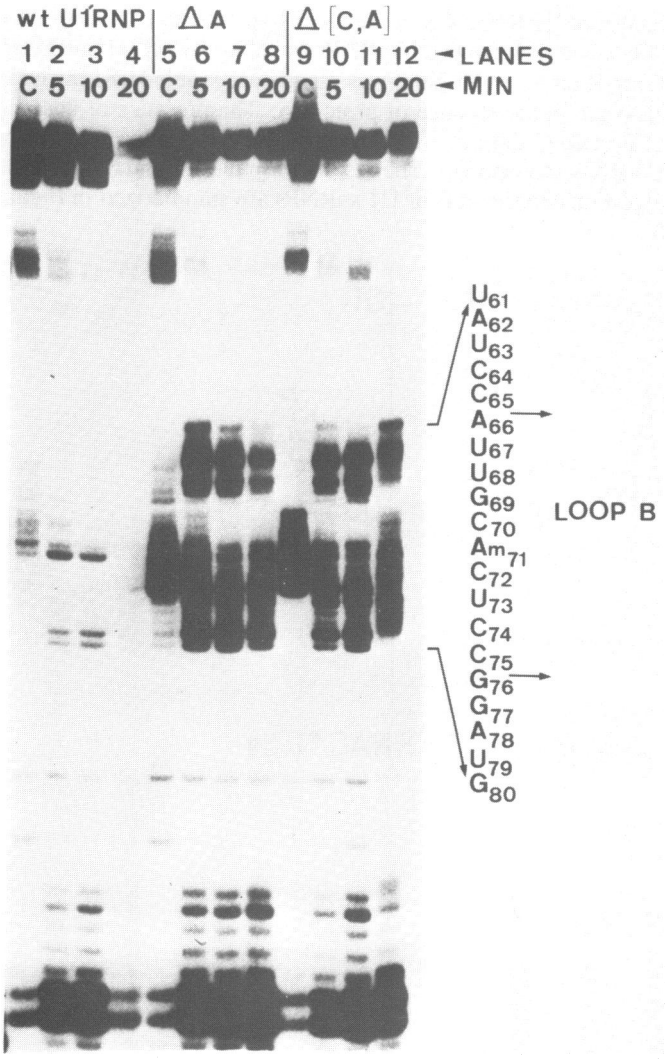

Fig. 5. RNase V1 cleavage in stem/loop B of U1 RNA in protein A-deficient U1 snRNPs. Limited digestion of U1 snRNPs with RNase V1 and primer extension analysis with reverse transcriptase was carried out as described in Materials and Methods. Oligodeoxynucleotide 125–138 was used as a primer. RNase V1 was incubated with wt U1 snRNP (lanes 1–4), ΔA U1 snRNP (lanes 5–8), Δ[A,C]U1 snRNP (lanes 9–12). Lanes denoted C represent control experiments, where the respective snRNPs were incubated in the absence of RNase V1. 5 , 10 and 20 min indicate the time of incubation of the U1 snRNP particles with V1 enzyme.

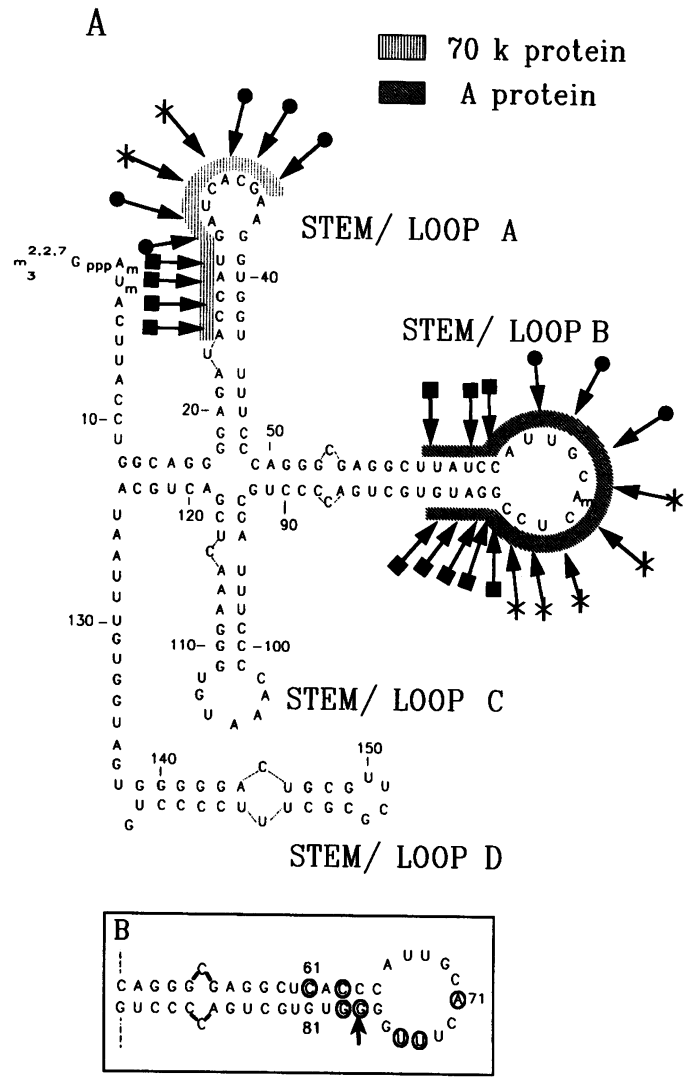

Fig. 6. Location of U1 RNA regions protected by of proteins 70k and A. A) Arrows indicate cleavage sites obtained by limited digestion of U1 snRNPs with RNase V1 (■→) or RNase S1 (●→). Cleavage of stem/loop A by the two nucleases was only observed when 70k protein was absent from U1 snRNPs in addition to A and C (Δ[70,A,C]U1 snRNPs). Cleavage of stem/loop B by the RNases was only observed in U1 snRNPs lacking protein A. Furthermore spontaneous cleavages in loops A and B of U1 RNA was also observed in protein-deficient U1 snRNPs (✱→). Stripped bar indicates the region of stem/loop A of U1 RNA protected by protein 70k in wt U1 snRNPs. The cross-hatched bar indicates the region of stem/loop B of U1 RNA protected by protein A in wt U1 snRNPs. The bars indicate minimal protein binding sites as indicated by our nuclease digestion experiments. Our data do not exclude more extended interaction of proteins 70k and A with stem/loop structures A and B, respectively. Panel B shows the structure of stem/loop B from mouse U1b RNA (taken from Lund *et al.* (27)). Circled nucleotides indicate differences in the nucleotide sequence between U1b and U1a. The structure of stem/loop B of U1a is identical with human U1 RNA (Panel A). The arrow indicates an additional G residue in U1b which is absent from U1a. A71 is methylated at the 2'hydroxil group in U1 RNA from man but not from mouse.

particular U1-specific protein should be more accessible to nucleases in U1 snRNPs depleted of this protein as compared to the wt U1 snRNP particle. The cleavage sites of the nucleases at defined regions of U1 RNA were detected *via* reverse transcriptase from defined DNA oligonucleotide probes. We have focussed our investigation on the differential nuclease accessibility of the region of U1 RNA upstream of the single-stranded domain A.

Figures 4+5 show typical cleavage patterns of U1 RNA obtained by treatment of the various U1 snRNP populations with the double-strand specific V1 nuclease. Figure 4 shows that stem A of U1 RNA between nucleotides 25 and 28 becomes accessible to cleavage by V1 only in U1 snRNP particles which lack the 70k protein in addition to A and C. Depletion of only proteins A and C does not change the nuclease sensitivity of this stem (Figure 4). This suggests that the 70k protein interacts directly with this part of U1 RNA. Figure 5 illustrates the differential accessibility of stem B of U1 RNA in the various types of U1 snRNPs. In this case, part of stem B, i.e. the phosphodiester bonds of nucleotides 61 to 65 and 76 to 80 are readily cleaved by V1 in the absence of protein A, suggesting that the A protein shields this region in the wt U1 snRNP. It should be noted that in protein A-deficient U1 snRNP particles the region between nt. 71 and 75 of loop B is particularly prone to spontaneous cleavages (compare lanes 1 to 5 and 9 in Fig. 5). These may have occurred during dialysis of the snRNP particles against the

buffer used for RNase digestions (not shown).

In contrast to the situation described for proteins 70k and A, we have not yet observed any differences in the V1 cleavage pattern of U1 RNA when wt and ΔC U1 snRNPs were compared (not shown).

When the various types of snRNPs were subjected to cleavage with the single strand-specific S1 nuclease, results complementary to those described above for V1 nuclease were obtained. Thus, loop A of U1 RNA was cleaved by S1 nuclease only when protein 70k was absent from U1 snRNPs (in Δ[A,C,70k] U1 snRNPs). Loop B on the other hand was readily accessible to S1 nuclease cleavage in the absence of protein A. The presence or absence of protein C did not affect the nuclease S1 cleavage pattern of U1 RNA (not shown). The results form the V1 and S1 nuclease digestion experiments of U1 snRNPs are summarized in Figure 6.

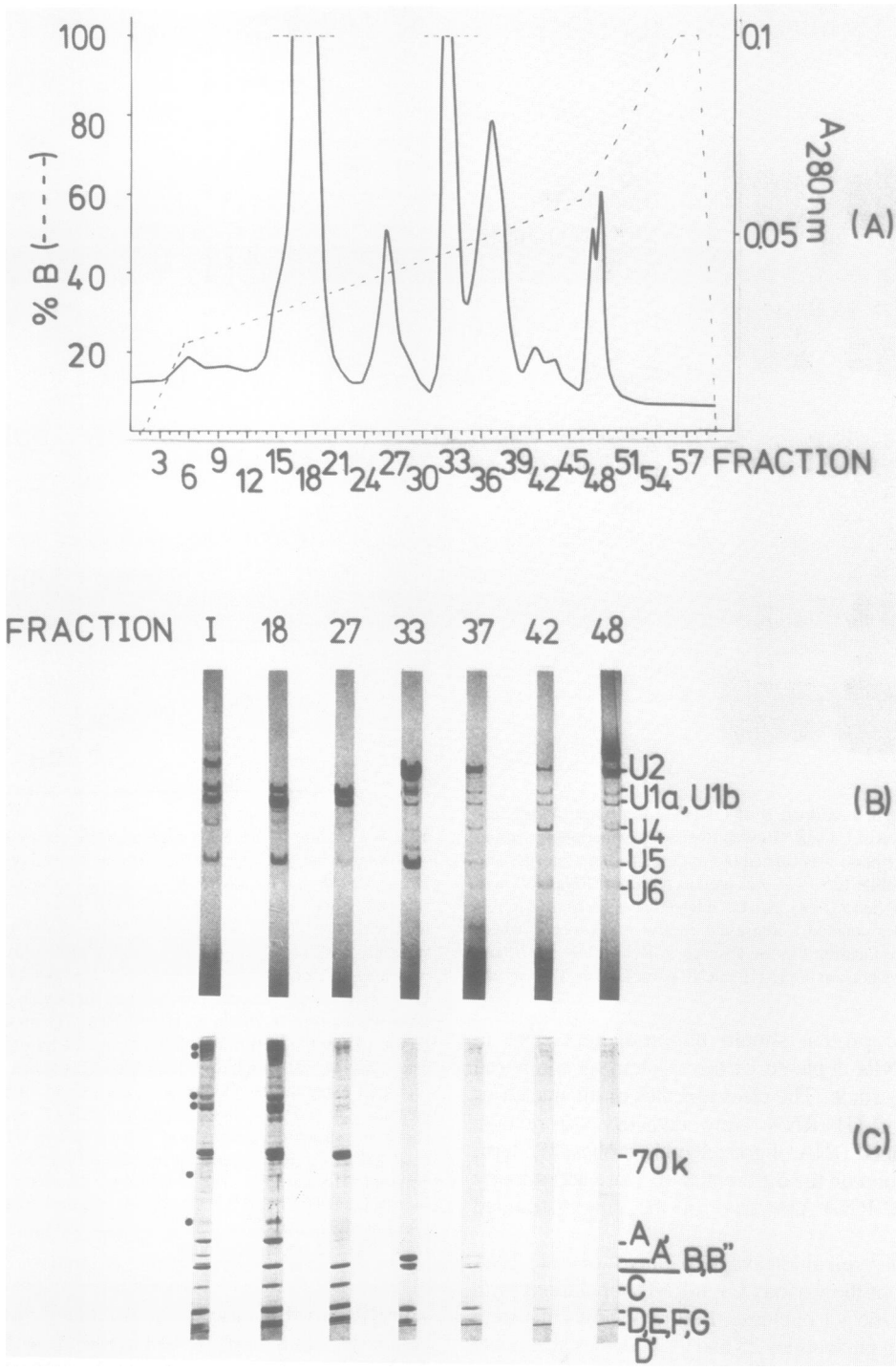

Fig. 7. Mono Q chromatography at 4°C of snRNPs U1 to U6 affinity purified with anti-m_3G antibodies from mouse Ehrlich ascites nuclear extracts. Chromatography was performed as described in Fig. 1 but at 4°C. (A) The elution profile of snRNPs from the Mono Q column is shown, where buffer B corresponds to the Mono-Q 1000 buffer (containing 1 M KCl). (B) RNA from the snRNP fractions indicated at the bottom of each lane was extracted and electrophoresed in a denaturing 10 % polyacrylamide gel and stained with ethidium bromide. (C) Proteins from the corresponding snRNP fractions, as shown in panel B, were extracted and separated in a 4–20 % gradient-polyacrylamide gel with SDS and stained with Coomassie blue. Dots on the left of lane I indicate positions in the gel of U5-specific proteins. Lanes I in panels B and C show RNA and protein compositions of the mixture of snRNPs U1 to U6 initially applied to the Mono Q column.

Preferential dissociation on Mono-Q columns of protein A from mouse U1 snRNPs containing U1b RNA as compared to U1a RNA

Mouse cells such as Ehrlich ascites tumor cells contain two types of U1 RNAs, U1a and U1b, which differ in the structure of stem/loop B (Fig. 6B). We were interested in investigating whether the two U1 RNAs differed with respect to their interaction with U1 specific proteins. For this purpose we have applied the mixture of anti-m_3G immuno affinity purified snRNPs from Ehrlich ascites tumor cells to Mono-Q chromatography, reasoning that possible differences in the strength of interaction of the U1-specific proteins with U1a and U1b RNAs might become visible in a differential distribution of the two U1 RNAs in certain protein-deficient U1 snRNP populations. On the basis of the protein gel electrophoresis patterns shown in Figures 7 and 8 it is clear that in principle the various types of U1 snRNPs lacking defined sets of U1-specific proteins may also be obtained from mouse cells. As with HeLa snRNPs the yields of the protein-lacking snRNP particles were also considerably increased when mouse snRNPs were fractionated at room temperature (Figure 8) instead of 4°C (Figure 7).

In view of the rationale for this experiment (discussed above) it is striking to notice that the two types of U1 RNA, U1a and U1b, partition unequally among wt U1 snRNPs and ΔA U1 snRNPs. At 4°C the ratio of U1a to U1b RNA in wt U1 snRNPs is about 3:1 (Figure 7, fraction 18). The reverse ratio is found in the ΔA U1 snRNPs contained in fraction 27 (Figure 7). This unequal division of U1a and U1b between the two types of U1 snRNP particles is even more pronounced when the snRNPs are fractionated at room temperature. Under these conditions wt U1 snRNPs consist only of U1a RNA; U1 snRNPs containing U1b RNA have completely lost their complement of protein A and are now quantitatively recovered as ΔA U1 snRNPs from the Mono-Q column (see Figure 8, fractions 2, 6+7). These results indicate that protein A is more strongly bound to U1 snRNPs containing U1a RNA as compared to those containing U1b RNA.

The data shown in Figures 7 and 8 further indicate that the content of proteins C and 70k in the various types of U1 snRNPs does not appear to vary with the ratio of U1a to U1b RNA in the respective particles. This indicates that the strength of binding of these two proteins is similar for both U1a and U1b RNA containing U1 snRNPs, and suggests further that the binding of protein A to the U1 snRNP particle should be independent of proteins 70k and C. One could speculate that protein C may interact with 70k in the U1 snRNP particle.

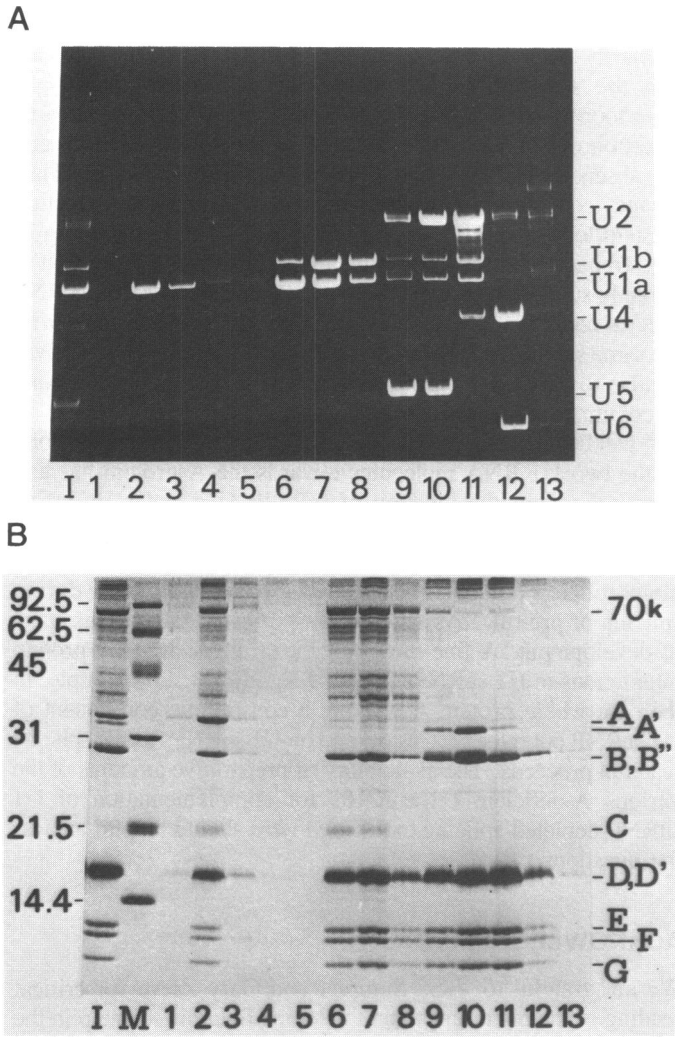

Fig. 8. Mono Q chromatography at 24°C of snRNPs U1 to U6 affinity purified with anti-m_3G antibodies from mouse Ehrlich ascites nuclear extracts. Chromatography was performed at 24°C essentially as described in Fig. 1. Panels A and B show, respectively, the RNA and protein composition of the various snRNP containing fractions obtained by elution of the Mono Q column with an increasing KCl gradient (from left to right). Extraction from snRNPs and staining of snRNAs and snRNP proteins was performed as described for Fig. 1. Lane M, molecular weight markers. Lanes I in panels A and B show RNA and protein compositions of the mixture of snRNPs U1 to U6 initially applied to the Mono Q column.

DISCUSSION

In this paper we have used Mono Q chromatography for the gradual depletion of U1 snRNPs of their specific proteins A, C and 70k and have used the various protein-deficient U1 snRNPs for the investigation of protein-RNA interactions in U1 snRNP particles. Apparently the U1-specific proteins are more loosely associated with the U1 RNA than the set of common snRNP proteins. This would explain the preferential dissociation of proteins C, A and 70k in the presence of the strong Mono Q anion exchange resin, which may compete with the U1 snRNP proteins for binding to negatively charged regions of either U1 RNA or other snRNP proteins. As an alternative explanation one might envisage a structural change in the U1 RNA during Mono-Q chromatography with the effect of lessening the affinity of the U1-specific proteins to U1 snRNP. While we cannot rigorously exclude this possibility, it is not very likely in view of the following findings. Taking advantage of the various protein-deficient U1 snRNP populations we recently observed that protein C is of prime importance for efficient complex formation between U1 snRNP and a 5′ splice site. More importantly, we could restore the ability of Δ[C,A]U1 snRNPs to bind to a 5′ splice site RNA by complementation with purified protein C (7). This data indicates that protein C may assemble with U1 snRNP in the absence of protein A in a functionally correct manner and shows further that depletion of one or more U1-specific proteins from U1 snRNP by Mono Q chromatography does not alter the integrity of the remaining snRNP particle.

Our results provide information on the interaction of the U1-specific proteins with U1 snRNP and to what extent their binding may be interdependent. The following types of U1 snRNPs were obtained in a reproducible manner: ΔC, ΔA,

Δ[C,A], Δ[C,A,70k]U1 snRNPs. The fact that proteins C and A may in principle dissociate from U1 snRNP in a non-concerted way indicates strongly that these two proteins should have independent binding sites on the U1 snRNP particle. Our failure to obtain Δ70k U1 snRNPs could indicate protein-protein interactions between 70k and the other two U1-specific proteins, in particular with protein C, which would allow the 70k protein to dissociate from U1 snRNPs only either together with or subsequent to proteins A and C. However, protein-protein interactions are not the only explanation for the apparent hierarchy of dissociation of the three proteins from U1 snRNP. Alternatively, this may simply indicate differences in the strength of binding of the three U1-specific proteins to the U1 snRNP particle, the 70k protein being most tightly bound. Interestingly, Verheijen *et al.* (33) during preparation of nuclear matrices by high-salt extraction observed soluble U1 snRNP particles which lacked only the 70k protein while retaining all the other common and U1-specific proteins. This indicates that binding of proteins A and C to U1 snRNP cannot be strongly dependent on the presence of the 70k protein.

Strong evidence that the major binding sites of at least proteins 70k and A on the U1 snRNP particle are distinct comes from our studies on the differential accessibility of U1 RNA towards nucleases V1 and S1 in the various protein-deficient U1 snRNP populations. This data indicates that the 70k protein interacts predominantly with stem-loop A while the primary binding site of the A protein is located on stem-loop B (see Fig. 6A). No differences were observed for the nuclease accessibility of stem-loop structures A and B when ΔC U1 snRNPs were compared with wt U1 snRNPs. This agrees with the other experimental evidence discussed above, for a protein C binding site distinct from the ones of A and 70k. This data does not exclude the possibility that protein C, like 70k and A, may also directly interact with U1 RNA in the U1 snRNP. More experiments are necessary before this point can be settled. In view of the finding that the complex formation of U1 RNA with proteins 70k and A shares a common feature, i.e. involvement of single-stranded loops as primary binding sites, it is interesting to note that both proteins also share an RNP binding domain with the characteristic RNP consensus sequence (34–37). Significantly, protein C does not contain this sequence (38).The RNP binding domain was recently discovered as an evolutionarily conserved structural motif characteristic of some RNA binding proteins contained in eucaryotic RNP particles (34; for reviews see 39–41). It will be interesting to see whether binding to single-stranded RNA loops is a characteristic feature of all RNA binding proteins containing the RNP binding domain. In agreement with such a possibility the U2-specific protein B″ which contains an RNP binding domain (42), also interacts with a single-stranded loop of U2 RNA (43).

How does our data relate to other published results? Evidence for interaction of protein 70k with stem-loop A of U1 RNA has been provided previously by other groups (18–21), in most cases by reconstitution experiments with mutated U1 RNA molecules and 70k containing nuclear extracts or *in vitro* translated 70k protein. With similar techniques a direct interaction between stem-loop B of U1 RNA with protein A was recently observed (22–25). Our data disagree with a report suggesting that protein C may play a dominant role in mediating the interaction of proteins 70k and A with stem/loop A of U1 RNA (19). The reason for this discrepancy is not known. These results were obtained by reconstitution experiments with mutated U1 RNA and free snRNP proteins contained in the ooplasm of *Xenopus* oocytes. It could be possible that the stockpiled U1-specific snRNP proteins in the ooplasm undergo protein-protein interactions, a situation which could give rise to apparent interdependent binding sites for the U1-specific proteins in the U1 snRNP.

When the ability of Mono Q-resin to dissociate preferentially the U1-specific proteins from U1 snRNP particles was used to investigate possible differences in the interaction of these proteins with the two types of U1 RNA, U1a and U1b, in mouse cells, an interesting aspect became apparent: U1a and U1b were unequally divided between wt U1 snRNP and ΔA U1 snRNP, i.e. U1b RNA was preferentially found in the ΔA U1 snRNP particles under all the conditions used for Mono Q chromatography. The most reasonable explanation for this is a higher strength of interaction of protein A with U1a snRNP particles as compared to U1b snRNPs.

Given the strong evidence provided by various independent experimental approaches (see discussion above) that protein A primarily interacts with stem/loop B of U1 RNA it is striking that the structure of U1b differs from U1a exclusively in stem/loop B (27). There are 5 base changes in addition to an insertion of one G residue between nucleotides 77 and 78 and the absence of 2′-0-methylation of A_{71} (see Figure 6B). Thus we may conclude that one consequence of the different structure of stem/loop B in U1b as compared to U1a is the lowering of the strength of interaction with the A protein. This notion is strongly supported by our observation that the mouse U1 snRNP species containing U1a RNA, i.e. the U1 RNA molecule with the same stem/loop B structure as HeLa U1 snRNA also behaves similar to HeLa U1 snRNP during chromatography on Mono Q columns (see Figure 7).

It will be interesting to elucidate whether the differential affinity of the two U1 RNA molecules towards the A protein has any consequence for the function of snRNPs U1a and U1b during splicing. This question is all the more interesting as U1b RNA is preferentially expressed in mouse embryonal cells (27) which might reflect a particular need for this type of U1 snRNP during splicing of pre-mRNAs specifically expressed during this stage of development. A fine tuning of the strength of RNA-protein interactions in U1 snRNPs could be significant, for example, in the case where protein A was not a constitutive component of U1 snRNP but rather cycles off and on U1 snRNPs as the splicing reaction proceeds. The availability of preparative amounts of the various A-deficient U1 snRNPs for complementation of U1 snRNP-depleted splicing extracts *in vitro* should help to answer the questions raised above.

ACKNOWLEDGEMENTS

We are grateful to Vicki Sumpter and Gary Zieve for critical reading of the manuscript and to Verena Buckow for help in the preparation of the manuscript. We would also like to acknowledge to expert technical assistance of Irene Öchsner-Welpelo. This work was supported by grants from the Deutsche Forschungsgemeinschaft to R.L. (SFB 272/A3). M.B. was supported in part by a fellowship from the Alexander-von-Humboldt-Stiftung.

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