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A New U6 Small Nuclear Ribonucleoprotein-Specific Protein Conserved between *cis*- and *trans*-Splicing Systems

KARSTEN GRÖNING,[1] ZSOFIA PALFI,[1] SHASHI GUPTA,[2] MIKE CROSS,[1] THORSTEN WOLFF,[1] AND ALBRECHT BINDEREIF[1]*

Max Planck Institut für Molekulare Genetik, Otto-Warburg-Laboratorium, Ihnestrasse 73, D-1000 Berlin 33, Federal Republic of Germany,[1] and Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030[2]

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Spliceosomal U6 small nuclear RNA (snRNA) plays a central role in the pre-mRNA splicing mechanism and is highly conserved throughout evolution. Previously, a sequence element essential for both capping and cytoplasmic-nuclear transport of U6 snRNA was mapped in the 5′-terminal domain of U6 snRNA. We have identified a protein in cytoplasmic extracts of mammalian and *Trypanosoma brucei* cells that binds specifically to this U6 snRNA element. Competition studies with mutant and heterologous RNAs demonstrated the conserved binding specificity of the mammalian and trypanosomal proteins. The in vitro capping analysis of mutant U6 snRNAs indicated that protein binding is required but not sufficient for capping of U6 snRNA by a γ-monomethyl phosphate. Through RNA affinity purification of mammalian small nuclear ribonucleoproteins (snRNPs), we detected this protein also in nuclear extract as a new specific component of the U6 snRNP but surprisingly not of the U4/U6 or the U4/U5/U6 multi-snRNP. These results suggest that the U6-specific protein is involved in U6 snRNA maturation and transport and may therefore be functionally related to the Sm proteins of the other spliceosomal snRNPs.

Small nuclear ribonucleoproteins (snRNPs) in conjunction with non-snRNP splicing factors are essential in the ordered assembly of splicing complexes and pre-mRNA splicing (for recent reviews, see references 6, 14, 21, 32, 37, and 42). The U1 and U2 snRNPs recognize the 5′ splice site and branch point region, respectively, whereas the U4/U6 and U5 snRNPs associate to form the U4/U5/U6 multi-snRNP before binding to the pre-mRNA-bound U1 and U2 snRNPs.

U6 small nuclear RNA (snRNA) differs in several respects from the other spliceosomal snRNAs. First, U6 snRNA is the most highly conserved snRNA, in particular with regard to the secondary structure and sequence of the U4-U6 interaction domain, which is organized as a so-called Y structure consisting of two U4-U6 intermolecular helices separated by a U4 intramolecular helix (11, 17). The 5′ end of almost every known U6 snRNA can be folded in a stem-loop of variable length and sequence, thus accounting for essentially all the variation in the overall length of U6 snRNAs (17, 33). In contrast to its structural conservation, this region of U6 snRNA shows relatively little sequence conservation except for an ACAU sequence at the 3′ end of the stem-loop and a GC base pair in the stem (see Fig. 1). In addition, the 5′ terminus of U6 snRNA follows the consensus GUPyC (39). Second, U6 snRNA has a unique cap structure. In contrast to U1, U2, U4, and U5 snRNAs, which are RNA polymerase II products and carry $N^{2,2,7}$-trimethylguanosine cap structures, U6 snRNA is transcribed by RNA polymerase III and capped by a γ-monomethylphosphate (35). Third, U6 snRNA is a component of several different snRNP complexes, namely, the U6 snRNP (8, 19), the U4/U6 snRNP, and the U4/U5/U6 multi-snRNP (20). In this context one should also note that U6 snRNA lacks a binding site for the Sm proteins (B′BDD′EFG; reviewed in reference 24), which are common to the other spliceosomal snRNAs. For U1, U2, U4, and U5 snRNAs, Sm protein binding is required for cytoplasmic cap trimethylation and cytoplasmic-nuclear snRNP transport (25, 26). In the case of U6 snRNA, a region near the 5′ stem-loop was identified as a sequence element essential for cytoplasmic-nuclear transport of U6 snRNA in *Xenopus* oocytes (19). Interestingly, mutations in the same region of human U6 snRNA also affected U6 snRNA capping (34). This correlation raises the question of whether U6 snRNA capping and snRNP transport might be interconnected in a manner similar to that previously shown for the other spliceosomal snRNAs (see above). To test the possibility that a protein factor may specifically recognize the 5′-terminal domain of U6 snRNA, we performed RNA-protein binding and competition assays. These experiments led to the identification of a novel mammalian U6 snRNA-specific protein which is a component of the nuclear U6 snRNP. However, we could not detect this protein in the U4/U6 snRNP or in the U4/U5/U6 multi-snRNP. Surprisingly, in extracts of *Trypanosoma brucei*, which processes pre-mRNAs exclusively through *trans* splicing (30, 38), we found a U6-binding protein with the same binding specificity. We propose that this newly identified U6 snRNA-specific binding protein functions in U6 capping and transport in a manner analogous to that of the Sm protein complex of the other spliceosomal snRNPs.

MATERIALS AND METHODS

Transcriptional templates. For T7 transcription from oligonucleotide templates (28), the two oligonucleotides were annealed and used at a concentration of 400 ng/25 μl of reaction mixture. The sequences of the synthetic RNAs deduced from the oligonucleotide DNA sequences are as follows (Fig. 1): HuU6-5′, nucleotides 1 to 31, with one extra G at the 5′ end of human U6 snRNA (13); HuU6-5′ΔACAU, like HuU6-5′, but with ACAU (nucleotides 18 to 21) deleted; HuU6-5′ΔGC, like HuU6-5′, but with the GC base pair of

* Corresponding author.

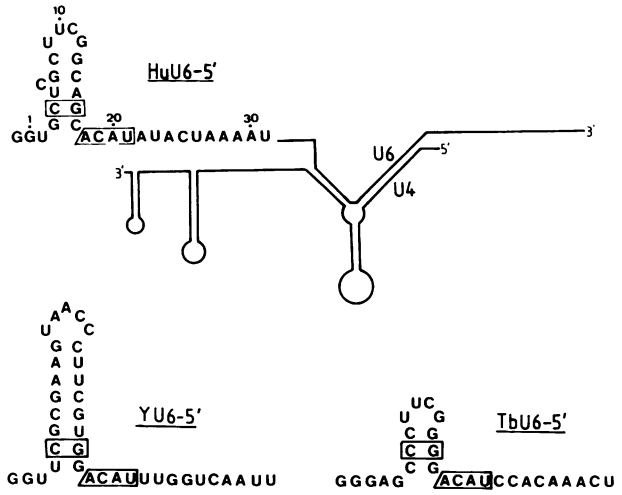

FIG. 1. Secondary structure of U6 RNAs. The sequence of the synthetic 5′ end of human U6 snRNA and of the corresponding synthetic RNAs of *T. brucei* (TbU6-5′; 29) and *S. cerevisiae* (YU6-5′; 11) are shown. All these synthetic RNAs have one extra G at their 5′ ends. The U4/U6 secondary structure is schematically outlined with the HuU6-5′ RNA (not drawn to scale). Two additional base pairs are possible at the base of the stem-loop of the human and *T. brucei* RNAs. The conserved positions in the 5′-terminal domain are outlined by boxes. Numbering refers to the human U6 snRNA sequence (13).

the stem (nucleotides 4 and 16) deleted; HuU6-5′Δstem-loop, like HuU6-5′, but with most of the 5′ stem-loop (nucleotides 5 to 15) deleted; HuU6-5′ins1(+17)6, like HuU6-5′, but with an insertion of 17 nucleotides (5′-GGG GAUCCUCUAGAGUC-3′) in place of nucleotides 2 to 5; YU6-5′, nucleotides 1 to 37, with one extra G at the 5′ end of *Saccharomyces cerevisiae* U6 snRNA (11); and TbU6-5′, nucleotides 1 to 27, with one extra G at the 5′ end of the *T. brucei* U6 snRNA (29).

Plasmid templates T7-TbU2 and T7-TbU6 were also used. The *T. brucei* U2 and U6 genes containing an upstream T7 promoter were amplified from genomic DNA by polymerase chain reaction and cloned into pUC19 digested with *Hin*dIII and *Xba*I. The sequence of each construct was confirmed by dideoxynucleotide sequencing with Sequenase (U.S. Biochemicals). Templates for T7 transcription were produced by *Xba*I digestion. The sequences of the synthetic RNAs deduced from the sequence of the DNA are as follows: for T7-TbU2, GGG/AUAU-U2 sequences-CGGU/CUAG; and for T7-TbU6, G/GGAG-U6 sequences-UUUU/CUAG (the extra nucleotides of the synthetic RNAs are separated from the natural snRNA sequence by the slash).

The templates for synthesis of SP6-U6/*Bam*HI RNA (7; denoted HuU6 in Fig. 2), MINX/*Bam*HI (43), and T7-U6 (16; denoted U6 in Fig. 5) have been described elsewhere. Gem RNA was transcribed from pGEM4/*Bam*HI by SP6 RNA polymerase. Transcriptions were done without the addition of cap analog, as described previously (5).

RNA-protein binding and capping analysis. To prepare mammalian U6-specific protein, 1 to 5 ng of ^{32}P-labeled HuU6-5′ RNA was incubated in 25 μl of reaction mixture containing 0.5 mM ATP, 20 mM creatine phosphate, 3.2 mM $MgCl_2$, 40 U of RNasin (Promega), 1 μg of tRNA, and 60% HeLa S100 extract (12) for 20 min at 30°C. RNP complex formation was analyzed by native gel electrophoresis and autoradiography as described elsewhere (31).

To prepare *T. brucei* U6-specific protein, 2.5 ng of ^{32}P-labeled TbU6-5′ RNA was incubated in 25 μl of reaction mixture containing 3.2 mM $MgCl_2$, 40 U of RNasin (Promega), 1 μg of tRNA, and 60% *T. brucei* S100 extract (11a) for 60 min at 37°C.

For capping analysis, T7 RNAs synthesized with [α-^{32}P]GTP label were incubated in HeLa cell extract as described elsewhere (16). The reaction mixture contained labeled RNA, 150 mM KCl, 50 mM Tris-HCl (pH 8.0), and 70% (vol/vol) HeLa cell extract. After incubation at 30°C for 30 min, the RNA was extracted and ethanol precipitated. The RNA was digested with nuclease P1 and alkaline phosphatase, electrophoresed on DEAE-cellulose paper at pH 3.5, and visualized by autoradiography.

UV cross-linking. To prepare mammalian U6-specific protein, 100 ng of ^{32}P-labeled, 5-bromouridine-substituted SP6-U6 3′Δ75 RNA (7) was incubated under standard binding conditions in HeLa S100 extract (25 μl of reaction mixture). The specific U6 complex was excised from an RNP gel and irradiated for 20 min with 302-nm UV light at an intensity of approximately 1,700 μW/cm^2 (Foto-Prep UV transilluminator). Then the gel slice was treated with RNase (1 mg of RNase A per ml for 30 min at 30°C) and laid over a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel (23).

To prepare *T. brucei* U6-specific protein, 20 ng of ^{32}P-labeled, 5-bromouridine-substituted TbU6-5′ RNA was incubated in *T. brucei* S100 extract under standard binding conditions in the presence of 1 μg of TbU6-5′ RNA as a specific competitor or 1 μg of T7-TbU2 as a nonspecific competitor (25 μl of reaction mixture). RNA-protein cross-linking was carried out for 20 min by 302-nm UV light. RNase A was added at 1 mg/ml and incubated for 20 min at 37°C. Protein analysis was done with a 12% SDS-polyacrylamide gel (23).

Affinity purification of snRNPs. Affinity purification of snRNPs from 500 μl of HeLa extract that had been adjusted to 0.4 M KCl was done with a biotinylated 2′-*O*-methyl RNA oligonucleotide complementary to the 3′ end of U6 snRNA (nucleotides 82 to 101; 5′-UXXXXAUGGAACGCUUCAC GAAUUU, where X denotes a biotinylated 2′-deoxycytidine; 36) at a concentration of 10 μg/ml at 30°C for 10 min. Then 100 μl of preblocked streptavidin agarose was added (4) and incubated at 4°C for 60 min. The depletion was controlled by analyzing aliquots of the total extract and the supernatant fraction (depleted fraction). The streptavidin pellet was washed three times with a buffer containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.0), 3 mM $MgCl_2$, 0.01% Nonidet P-40, 0.5 mM dithiothreitol, and 400 mM KCl. Proteins were released by incubating the pellet with 200 μl of 9 M urea at room temperature for 30 min. The resulting supernatant was dialyzed against buffer D (12) containing 0.1 M KCl at 4°C overnight and used in a standard binding assay. The affinity selection was controlled by analyzing the RNA content of the pellet.

HeLa nuclear extract (2 ml dialyzed against 20 mM HEPES [pH 8.0]–150 mM KCl–1.5 mM $MgCl_2$–5% glycerol) was fractionated through an 11-ml 10 to 30% glycerol gradient containing 20 mM HEPES (pH 8.0), 150 mM KCl, and 1.5 mM $MgCl_2$ (SW40 rotor, 29,000 rpm, 18 h, 4°C). Twenty-six fractions (0.5 ml each) were collected, and the following fractions were pooled: 5 to 9 (5 to 10S; U6 snRNP), 11 to 15 (10 to 15S; U4/U6 snRNP), and 20 to 24 (25S; U4/U5/U6 multi-snRNP). The KCl concentration was adjusted to 0.4 M. Half of each pool was used for U6-specific selection (using the U6-specific oligonucleotide), and half was used for

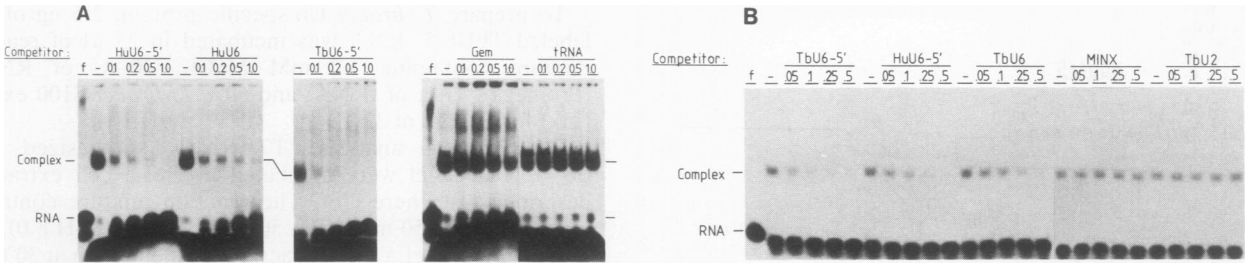

FIG. 2. Binding specificity and competition by heterologous RNAs. (A) Binding specificity of the mammalian protein. ^{32}P-labeled HuU6-5′ RNA (1 ng) was incubated in HeLa cytoplasmic extract under standard binding conditions in the presence of increasing concentrations of unlabeled competitor RNAs (HuU6-5′, HuU6, TbU6-5′, Gem, and tRNA). (B) Binding specificity of the *T. brucei* protein. ^{32}P-labeled TbU6-5′ RNA (2.5 ng) was incubated in *T. brucei* S100 extract under standard binding conditions in the presence of increasing concentrations of unlabeled competitor RNAs (TbU6-5′, HuU6-5′, TbU6, MINX, and TbU2). Complex formation was analyzed by native RNP gel electrophoresis and autoradiography. The positions of the complex and the RNA are marked at the left. The concentrations of competitor RNAs (0 to 1 μg/25 μl of reaction mixture) are indicated above the lanes. f, Free RNA.

control affinity selection (without oligonucleotide). Affinity selection and the release of bound protein were done as described above at an oligonucleotide concentration of 5 μg/ml.

RESULTS

U6 snRNA-binding protein with conserved binding specificity present in mammalian and *T. brucei* extracts. To identify new mammalian snRNP proteins, we have assayed small RNAs that represent isolated secondary-structure elements of snRNAs for specific protein binding. This approach makes use of a pool of unassembled, free snRNP proteins that usually exists in either the cytoplasmic or the nuclear fraction. The use of relatively small RNAs should facilitate the analysis of specific protein binding by native gel electrophoresis (20) under competitive binding conditions. We began our search for specific snRNA-binding proteins with the 5′-terminal domain of human U6 snRNA.

Using the first 31 nucleotides of human U6 snRNA as a ^{32}P-labeled probe (HuU6-5′; Fig. 1) and HeLa cytoplasmic extract, we detected efficient complex formation (Fig. 2A). In reproducible experiments, we found at least 10-fold-higher U6 snRNA-binding activity in cytoplasmic extracts than in nuclear extracts (see, for example, Fig. 6B). Optimal binding was observed during a 20-min incubation at 30°C; although ATP and $MgCl_2$ are not required for binding (data not shown), we usually performed binding reactions under the conditions of the in vitro pre-mRNA splicing reaction (22). Routinely, tRNA is added during complex formation at a concentration of 40 μg/ml, as it was found to reduce nonspecific RNA-protein interactions (data not shown). Competition experiments demonstrated that formation of a complex with HuU6-5′ RNA is due to specific protein binding (Fig. 2A). Competition with the 5′ end of human U6 (HuU6-5′, 32 nucleotides) and with full-length human U6 RNA (HuU6, 115 nucleotides) at a 100-fold molar excess reduced complex formation to less than 10% of complex formation in the absence of competitor (0.2 μg/25 μl of reaction mixture; Fig. 2A). In contrast, neither Gem RNA (42 nucleotides) nor tRNA (ca. 75 nucleotides) competed significantly, even at a 500-fold molar excess (1.0 μg/25 μl of reaction mixture; Fig. 2A).

To test whether there might be a conserved protein binding site in the 5′-terminal domain of U6 snRNA, we used the corresponding region of *T. brucei* U6 snRNA (nucleotides 1 to 27; Fig. 1) as a competitor. These two RNAs differ in sequence but can be folded into stem-loops of different lengths, and both contain the highly conserved ACAU sequence and the GC base pair (Fig. 1). Surprisingly, the *T. brucei* U6 snRNA competed as well as the corresponding human RNA (Fig. 2A), suggesting that the mammalian protein specifically recognizes a conserved element within the 5′-terminal domain of U6 snRNA.

Since the mammalian protein specifically bound the heterologous *T. brucei* RNA (TbU6-5′), we next asked whether we could also detect U6-specific protein binding activity in *T. brucei* extracts. Figure 2B shows that the 5′ end of *T. brucei* U6 snRNA (TbU6-5′) does form a specific complex in *T. brucei* cytoplasmic extract. The binding specificity was demonstrated by using as specific competitor RNAs the 5′ end of *T. brucei* U6 snRNA (TbU6-5′; 28 nucleotides), human U6 snRNA (HuU6-5′; 115 nucleotides), and full-length *T. brucei* U6 snRNA (TbU6; 105 nucleotides) and as nonspecific competitors two different RNAs, a mammalian pre-mRNA (MINX, 221 nucleotides) and *T. brucei* U2 snRNA (TbU2; 155 nucleotides). Complex formation in *T. brucei* extract could be specifically competed at lower competitor concentrations than those needed in mammalian extract. This may reflect a lower abundance of the binding protein in *T. brucei* extract (compare Fig. 2A and B). In summary, we conclude that mammalian and *T. brucei* extracts both contain a protein with conserved binding specificity for the 5′-terminal domain of U6 snRNA.

To further define the sequence requirements of protein binding, we synthesized several mutant U6 snRNAs. We compared the wild-type 5′ end of U6 snRNA (HuU6-5′; 32 nucleotides) with deletion derivatives lacking the ACAU sequence (HuU6-5′ΔACAU; nucleotides 18 to 21), the GC base pair in the stem (HuU6-5′ΔGC; nucleotides 4 and 16), or most of the 5′ stem-loop (HuU6-5′Δstem-loop; nucleotides 5 to 15). In addition, an insertion of 17 nucleotides 5′ of the stem-loop in place of nucleotides 2 to 5 was tested [HuU6-5′ins1(+17)6] as well as a heterologous RNA, the 5′-terminal domain of *S. cerevisiae* U6 snRNA (YU6-5′; nucleotides 1 to 37; Fig. 1). Finally, to further compare the specificities of the mammalian and trypanosomal U6 snRNA-binding proteins, we assayed complex formation of wild type (HuU6-5′) and two mutant derivatives (HuU6-5′ΔACAU and HuU6-5′ΔGC) in *T. brucei* extract (Fig. 3). Figure 3 shows that deletion of the highly conserved ACAU sequence (HuU6-5′ΔACAU) reduced binding of the mammalian U6-specific protein to very low levels (less than 5%

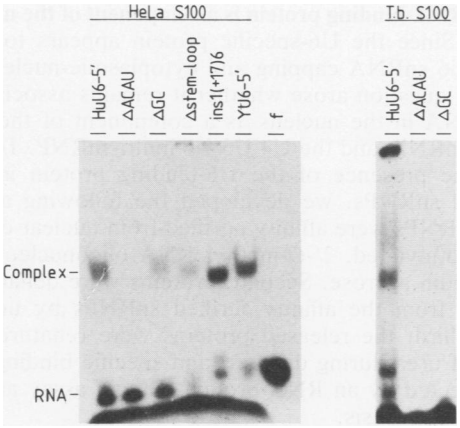

FIG. 3. Sequence requirements of RNA-protein binding. ^{32}P-labeled HuU6-5′ RNA and the mutant derivatives HuU6-5′ΔACAU, HuU6-5′ΔGC, HuU6-5′Δstem-loop, and HuU6-5′ins1(+17)6 as well as the corresponding *S. cerevisiae* RNA (YU6-5′) were assayed for protein binding in HeLa cytoplasmic extract (HeLa S100) and in *T. brucei* S100 extract (T.b. S100) under standard binding conditions. Complex formation was analyzed by native RNP gel electrophoresis and autoradiography. f, Free RNA.

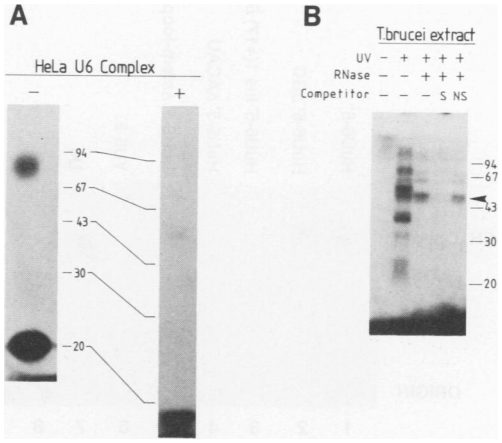

FIG. 4. Identification of mammalian and *T. brucei* U6-specific binding proteins by UV cross-linking. (A) UV cross-linking of the mammalian U6-binding protein. ^{32}P-labeled SP6-U6 3′Δ75/*Bam*HI RNA was incubated under standard binding conditions in HeLa cytoplasmic extract, and then the specific RNA-protein complex was separated by native RNP gel electrophoresis. After UV irradiation, the gel slice containing the specific complex was treated with RNase A (+) or was left untreated (−), and proteins were separated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. (B) UV cross-linking of the *T. brucei* U6-binding protein. ^{32}P-labeled TbU6-5′ RNA was incubated under standard binding conditions in *T. brucei* S100 extract in the absence of unlabeled competitor RNAs (competitor −) or the presence of specific (TbU6-5′; competitor S) and nonspecific (T7-TbU2, competitor NS) competitor RNAs and treated with RNase A (RNase +), and the cross-linked, labeled proteins were separated by SDS-polyacrylamide gel electrophoresis and autoradiography. As controls, a reaction without UV cross-linking (UV −) and a cross-linked reaction without RNase A treatment (RNase −) are also shown. The cross-linked protein is marked by an arrow. The sizes of the protein markers are indicated in kilodaltons.

that of wild type); deleting the GC base pair (HuU6-5′ΔGC) reduced binding to about 50% of wild-type levels. In addition to the ACAU sequence, the stem-loop was required for efficient protein binding since a deletion derivative lacking nucleotides 5 to 15 (HuU6-5′Δstem-loop), which should not allow folding of a stable stem-loop, was strongly reduced in binding (to ca. 10% of wild-type levels). An RNA with an insertion of 17 nucleotides near the 5′ end [HuU6-5′ins1(+17)6], which should still form the 5′ stem-loop, formed a complex with an efficiency similar to or higher than that of the wild-type U6. The corresponding heterologous RNA from *S. cerevisiae* (YU6-5′) can be folded into the conserved secondary structure of the 5′-terminal domain of U6 snRNA. Although the stem portion is longer than that of its mammalian counterpart, it does contain the ACAU sequence and the GC base pair at the conserved positions (Fig. 1). As expected, the yeast U6-5′ RNA efficiently formed a complex in mammalian extract (Fig. 3).

To compare the sequence requirements for RNA binding of the U6 snRNA-specific proteins from mammalian and *T. brucei* cells, the wild-type human U6 (HuU6-5′) and two deletion derivatives lacking the ACAU sequence or the GC base pair (HuU6-5′ΔACAU or HuU6-5′ΔGC, respectively) were assayed for binding activity in *T. brucei* extract (Fig. 3). The ACAU deletion had the same effect on binding of the *T. brucei* protein as was previously determined in mammalian extract. The GC deletion in the 5′-terminal stem was not bound by the *T. brucei* protein, whereas in mammalian extract there was still some residual binding activity. This slight discrepancy between the two extracts may reflect different stabilities in the two extracts of the base-pairing interaction in the stem region. Taken together, these mutational analyses further defined the sequence requirements for binding of the U6 snRNA-specific protein. Efficient binding depends on the ACAU sequence and on the presence of a stem-loop structure 5′ to the ACAU sequence.

Identification of mammalian and *T. brucei* U6 snRNA-binding proteins. To identify the U6-specific binding protein from both mammalian and *T. brucei* extracts, we used UV cross-linking followed by RNase A digestion and gel electrophoresis. Thereby internally ^{32}P-labeled RNA (human SP6-U6 3′Δ75 or *T. brucei* TbU6-5′) was first incubated in HeLa or *T. brucei* extract under optimal binding conditions. In the case of the mammalian protein, RNA-protein complexes were then separated on a nondenaturing gel, and the U6-specific complex was localized, excised from the gel, irradiated with 302-nm UV light, and RNase treated. In a control reaction, the RNase treatment was omitted. Cross-linked ^{32}P-labeled proteins were then separated by denaturing gel electrophoresis. Figure 4A shows that a single polypeptide became cross-linked in the specific RNP complex of SP6-U6 3′Δ75 RNA. Without the RNase treatment, it migrated with an apparent electrophoretic mobility of approximately 75 kDa; RNase treatment reduced the molecular mass of the cross-linked product to 55 kDa.

In the case of the *T. brucei* protein, UV cross-linking was performed in a crude binding reaction containing ^{32}P-labeled TbU6-5′ RNA and *T. brucei* cytoplasmic extract. Without RNase treatment, several cross-linked proteins were visualized by the ^{32}P label transfer procedure; after RNase treatment, however, a major polypeptide of molecular mass 50 kDa remained (Fig. 4B). Competition experiments with TbU6-5′ RNA as a specific competitor and T7-TbU2 RNA as a nonspecific competitor demonstrated that this polypeptide binds U6 snRNA specifically. The minor polypeptides of higher molecular weights may represent cross-linked prod-

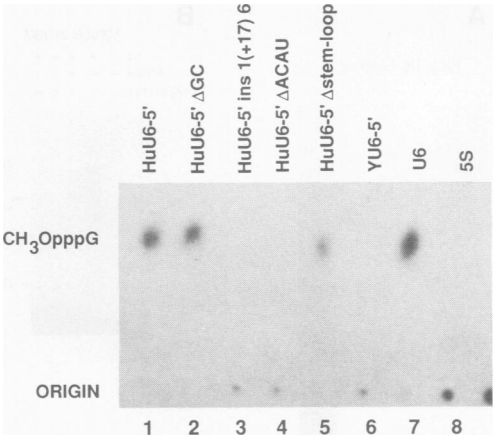

FIG. 5. Analysis of cap structure formed in vitro with different U6 snRNA derivatives. The RNAs were synthesized with [α-^{32}P] GTP label and capped posttranscriptionally under in vitro conditions (16). These RNAs (3,000 cpm for U6 and 5S; 100,000 cpm for HuU6-5′ and each of the mutants) were analyzed for their 5′ termini by digestion with nuclease P1 and alkaline phosphatase and then electrophoresed on DEAE-cellulose paper (13).

ucts with incompletely digested RNA components. In sum, we have identified two proteins that bind specifically to the 5′-terminal domain of U6 snRNA, a 55-kDa mammalian and a 50-kDa *T. brucei* polypeptide.

U6 snRNA capping requires protein binding. To correlate the sequence requirements for protein binding and U6 capping by γ-methylphosphate, we performed an in vitro capping analysis with the U6 snRNA mutant derivatives which had been assayed for protein binding (Fig. 3). HuU6-5′ and the mutant derivatives HuU6-5′ΔACAU, HuU6-5′ΔGC, HuU6-5′Δstem-loop, and HuU6-5′ins1(+17)6 as well as a heterologous RNA, the 5′-terminal domain of *S. cerevisiae* U6 snRNA (YU6-5′), were synthesized with [α-^{32}P]GTP label and capped posttranscriptionally under in vitro conditions (16). These RNAs were analyzed for their 5′ termini by digestion with nuclease P1 followed by alkaline phosphatase (13). Figure 5 shows that, in comparison with full-length U6 snRNA (lane 7), HuU6-5′ RNA was accurately capped at the 5′ end (lane 1). Although the capping efficiency of HuU6-5′ RNA (ca. 5%) was lower than that reported for full-length U6 snRNA (30 to 80%; 16), it was sufficient to assay the effect of mutations. The capping efficiencies of the HuU6-5′ΔACAU deletion and HuU6-5′ins1(+17)6 insertion derivatives and of the heterologous *S. cerevisiae* RNA (YU6-5′) were undetectable (lanes 3, 4, and 6, respectively). In contrast, deleting the GC base pair (HuU6-5′ΔGC, lane 2) did not change the capping efficiency significantly compared with that of wild type (HuU6-5′, lane 1); deleting the stem-loop (HuU6-5′Δstem-loop) reduced the capping efficiency to approximately 50% (lane 5). The capping efficiencies of these mutant derivatives and of the heterologous yeast RNA are consistent with a mutational analysis performed with the full-length U6 mutant derivatives (34). For most mutants, the effects on protein binding and capping correlated (HuU6-5′ΔACAU, HuU6-5′ΔGC, HuU6-5′Δstem-loop; compare Fig. 3 and 5). In contrast, for the HuU6-5′ins1(+17)6 and YU6-5′ RNAs, protein binding was efficient (Fig. 3), whereas capping in vitro was undetectable (Fig. 5). Therefore we conclude that protein binding is necessary but not sufficient for capping.

U6 snRNA-binding protein is a component of the nuclear U6 snRNP. Since the U6-specific protein appears to function during U6 snRNA capping and cytoplasmic-nuclear migration, the question arose whether it remains associated with U6 snRNA in the nucleus as a component of the U6, the U4/U6 snRNP, and the U4/U5/U6 multi-snRNP. To demonstrate the presence of the U6-binding protein in nuclear localized snRNPs, we developed the following approach. First, snRNPs were affinity purified from nuclear extract by using biotinylated, 2′-*O*-methyl RNA oligonucleotides and streptavidin agarose. Second, proteins were denatured and released from the affinity-purified snRNPs by urea treatment. Third, the released proteins were renatured by removal of urea during dialysis, and specific binding activity was detected by an RNA-protein-binding assay and native gel electrophoresis.

This approach relies on an efficient and selective affinity purification of snRNPs and on the successful renaturation of urea-released snRNP protein components. 2′-*O*-Methyl RNA oligonucleotides, which are extremely stable against nucleases (36), have been developed to deplete extracts of specific snRNPs. Depletion of nuclear extract with an oligonucleotide complementary to the 3′ end of U6 snRNA led to efficient selection of the U4/U6 snRNP (10; see below). In initial experiments, we found that most or all of the U6 snRNA-binding activity could be regained from urea-treated cytoplasmic extract by dialysis, indicating that the U6 snRNA-binding protein can in fact be renatured (data not shown).

First, we assayed snRNPs affinity purified by the U6-specific oligonucleotide from crude nuclear and cytoplasmic (S100) extract for the presence of the U6-specific protein (Fig. 6). Although in this experiment the snRNP depletion was not quantitative (Fig. 6A, compare lanes T and D), RNA analysis showed that the affinity-bound material contained both U4 and U6 snRNAs, indicating that under these conditions most of the selected complexes are U4/U6 snRNPs (Fig. 6A). A small part of the affinity-selected complexes may be U6 snRNPs (see below). Selection of both U4 and U6 snRNAs depended on the U6-specific oligonucleotide (data not shown; see below; Fig. 7). In contrast to results with cytoplasmic extract, only low amounts of U6-binding activity could be detected in crude nuclear extract (Fig. 6B, compare NE and S100, lanes T). After U6-specific depletion of snRNPs, U6-binding activity was reduced in both the nuclear and the S100 extracts (Fig. 6B, NE and S100, lanes D). After release and renaturation of affinity-selected material, however, binding activity specific for the 5′-terminal domain of U6 snRNA could clearly be detected from both nuclear and S100 extracts (Fig. 6B, NE and S100, lanes S). The slightly faster mobility of the complex formed with affinity-purified proteins from nuclear extract (NE, lane S) is probably caused by different ionic conditions during the binding assay. Using affinity-purified material, we sometimes observed an additional complex of faster mobility (see, for example, S100, lane S), which may have resulted from degradation of the binding protein during the selection procedure. This suggests that nuclear U6, U4/U6, or U4/U5/U6 snRNPs contain the U6-binding protein, which we previously identified in the free form from cytoplasmic extract (see above and Fig. 2, 3, and 4). Since the S100 extract is to some extent contaminated with nuclear material, we cannot draw any conclusions about the presence of the U6 protein in cytoplasmic U6 RNP complexes.

Second, since the U4/U5/U6 multi-snRNP appears to be the final assembly stage of U4, U5, and U6 snRNAs before

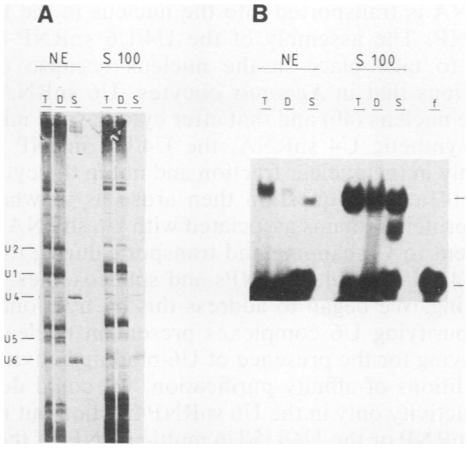

FIG. 6. U6-specific affinity purification of snRNPs from nuclear and S100 extracts. (A) RNA analysis of snRNPs affinity purified from HeLa nuclear (NE) and cytoplasmic (S100) extracts. RNA analyses of an aliquot of each of the extracts before (total, lane T) and after (depleted, lane D) affinity selection are shown. In addition, RNA bound to streptavidin agarose (selected, lane S) was analyzed. RNAs were visualized by silver staining. (B) RNA-binding analysis of affinity-purified protein fractions. Aliquots of nuclear extract (NE) and cytoplasmic extract (S100) were assayed for U6-specific binding before (total, lane T) and after (depleted, lane D) the affinity selection. For each of the extracts, bound material was released from the streptavidin agarose, renatured, and assayed for U6-specific binding (selected, lane S). Complex formation was analyzed by native RNP gel electrophoresis and autoradiography, using ^{32}P-labeled HuU6-5′ RNA as a binding substrate. f, Free RNA. The positions of the snRNAs are indicated at the left.

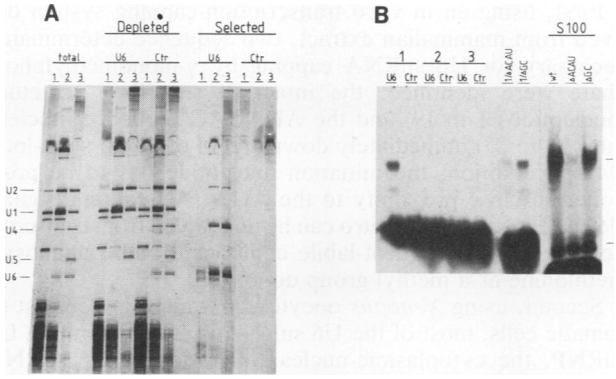

FIG. 7. Detection of U6-specific protein in affinity-purified U6, U4/U6, and U4/U5/U6 snRNPs. (A) RNA analysis of affinity purification of U6, U4/U6, and U4/U5/U6 snRNPs. snRNPs present in HeLa nuclear extract were fractionated by sedimentation through a glycerol gradient, and fractions containing U6 snRNPs (pool 1), U4/U6 snRNPs (pool 2), and U4/U5/U6 multi-snRNPs (pool 3) were collected. The RNA analysis of an aliquot of each of the fractions (pools 1 to 3, lanes 1 to 3) are shown before (total) and after (depleted) affinity selection. In addition, RNA bound to streptavidin agarose (selected) was analyzed. RNAs were visualized by silver staining. The positions of the snRNAs are indicated at the left. (B) RNA-binding analysis of affinity-purified protein fractions. Affinity-purified proteins were released from the streptavidin agarose, renatured, and assayed for U6-specific binding (pool 1, U6 snRNPs; pool 2, U4/U6 snRNPs; pool 3, U4/U5/U6 multi-snRNPs; each was selected in the presence [U6] or absence [Ctr] of the U6-specific oligonucleotide). In addition, U6-selected proteins from pool 1 were also assayed for binding of mutant substrates HuU6-5′ΔACAU and HuU6-5′ΔGC (lanes 1/ΔACAU and 1/ΔGC). For comparison, complexes formed with HuU6-5′, HuU6-5′ΔACAU, and HuU6-5′ΔGC RNAs in HeLa cytoplasmic extract (S100; lanes wt, ΔACAU, and ΔGC) were analyzed. Complex formation was analyzed by native RNP gel electrophoresis and autoradiography, using ^{32}P-labeled HuU6-5′ or the mutant RNAs as binding substrates.

they enter the spliceosome, we asked whether the U6-specific protein is present in all three known U6 complexes (U6, U4/U6, and U4/U5/U6 snRNPs) or only in a subset thereof. Therefore we first fractionated snRNPs present in nuclear extract by sedimentation through a glycerol gradient. In our preparations of nuclear extract, the majority of U4, U5, and U6 snRNAs cosedimented in the form of the 25S multi-snRNP complex (Fig. 7A; data not shown). A fraction of U4, U5, and U6 snRNAs sedimented also in smaller complexes ranging from 5S to 20S. These represent free U6 snRNPs, U4/U6 snRNPs, and 20S U5 snRNPs (2). Affinity purification of U6-containing snRNPs was followed by analysis of RNAs from the total pooled fractions and depleted fractions and from material released from the streptavidin beads after affinity selection (Fig. 7A, lanes total, depleted, and selected). When the U6-specific oligonucleotide was used, only U6 snRNA was selected from the 5S to 10S region, whereas equimolar amounts of U4 and U6 snRNAs were coselected from both fractions with larger complexes (10S to 15S and 25S, respectively). U5 snRNA was not coselected in the 25S region, because under the ionic conditions of affinity selection (0.4 M KCl), U5 is dissociated from the multi-snRNP complex (3). Significantly, the co-selection of approximately equimolar amounts of U4 and U6 snRNAs from the 25S region indicates that the stable U4-U6 interaction is retained in the U4/U5/U6 multi-snRNP. The small amount of U2 snRNA present in the affinity-purified material may result from cross-hybridization of the U6 oligonucleotide to U2 snRNA. RNA analysis of the fractions after affinity selection confirmed that more than 90% of the respective snRNAs had been depleted from the pooled gradient fractions (Fig. 7A). We then applied our procedure of urea release and renaturation of affinity-purified components to each of the three affinity-selected U6 complexes. Surprisingly, U6-binding activity could be recovered only from the U6 snRNP but not from the U4/U6 or the 25S multi-snRNP fractions (Fig. 7B). Detection of binding activity depended on the addition of U6-specific oligonucleotide (Fig. 7B, compare lanes U6 and Ctr). The specificity of the binding activity released from the U6 snRNP fraction was assessed by using mutant binding substrates: the ACAU deletion derivative (HuU6-5′ΔACAU), which showed no binding activity, and the GC base pair deletion (HuU6-5′ΔGC), in which U6 binding was strongly reduced (Fig. 7B). In conclusion, we detected a protein with U6-specific binding activity as a component of the U6 snRNP. According to its sequence requirements for U6 snRNA binding, the nuclear U6 snRNP-bound protein appears to be identical with the binding protein that we have previously identified in the free form from HeLa cytoplasmic extract (see above; Fig. 2, 3, and 4).

DISCUSSION

Previous studies of the mammalian and *Xenopus* systems provided evidence for a role of the 5′-terminal domain of U6 snRNA in both U6 snRNA capping mechanism and cytoplasmic-nuclear transport of U6 snRNA.

First, using an in vitro transcription-capping system derived from mammalian extract, two sequence determinants necessary for U6 snRNA capping by γ-monomethylphosphate were identified: the intact 5′ stem-loop structure (nucleotides 1 to 19) and the AUAUAC sequence (nucleotides 20 to 25) immediately downstream of the 5′ stem-loop (34). In addition, the initiation nucleotide has to be positioned in close proximity to the AUAUAC capping signal. U6 snRNA capping in vitro can be uncoupled from transcription and requires a heat-labile component and *S*-adenosylmethionine as a methyl group donor (16).

Second, using *Xenopus* oocytes, in which, in contrast to somatic cells, most of the U6 snRNA is in the form of a U6 snRNP, the cytoplasmic-nuclear migration of U6 snRNA was studied (19). A sequence element essential for transport maps in the region required for capping. Furthermore, a U6-associated protein of 50 kDa has been identified in *Xenopus* oocytes (19).

To test whether protein binding might be involved as a signal for both capping and transport processes, we assayed short synthetic RNAs containing the 5′-terminal domain of U6 snRNA and mutant derivatives thereof for specific protein binding. This binding analysis was facilitated by the fact that such short U6 snRNA derivatives cannot interact with U4 snRNA (7). No mammalian U4- or U6-specific proteins have so far been identified. There is, however, some suggestive evidence that in mammalian extract, U6 snRNA is protected near the 5′ stem-loop against RNase H (9). Since we expected a protein involved in transport to be localized in the cytoplasm, we searched for U6 snRNA-binding proteins first in HeLa cytoplasmic extract. A relatively abundant cytoplasmic protein of molecular mass 55 kDa was identified, the binding of which depended strongly on the conserved ACAU sequence (nucleotides 18 to 21). Significantly, this sequence overlaps the region essential for U6 snRNA capping and transport. Therefore we also analyzed our mutant constructs derived from the 5′-terminal domain of U6 snRNA for capping in vitro. The relatively low capping efficiency of the HuU6-5′ RNA is most likely caused by the extra nucleotide at the 5′ end, since the first 25 nucleotides of U6 snRNA are sufficient for capping at wild-type efficiency and since an insertion of 8 nucleotides at the 5′ end drastically reduces the capping efficiency (34). It is also possible that these short transcripts are more susceptible to nucleolytic degradation, which may destroy the capping signal. Mutations weakening or removing the 5′ stem-loop (HuU6-5′ΔGC and HuU6-5′Δstem-loop) reduced binding to levels between 10 and 50% that of wild type. This correlates well with the effect on capping in vitro of linker-scan mutations disrupting the 5′ stem-loop (34). In contrast, an insertion of 17 nucleotides between the initation nucleotide and the 5′ stem-loop significantly enhanced protein binding but reduced the capping efficiency to very low or undetectable levels (34; this study). In sum, the effect of mutations in the 5′-terminal domain are consistent with the notion that protein binding is necessary but not sufficient for capping. Protein binding and capping can therefore be uncoupled from each other. However, we cannot distinguish whether the capping enzyme is separate from the binding protein or whether both activities reside in the same protein or protein complex. Resolving this question will require a biochemical fractionation of the two activities.

Since the sequence requirements for capping and transport are similar or identical, protein binding appears to be necessary not only for capping but also for cytoplasmic-nuclear transport of U6 snRNA. Therefore, we assume that U6 snRNA is transported into the nucleus in the form of a U6 snRNP. The assembly of the U4/U6 snRNP has been thought to take place in the nucleus because of recent observations that in *Xenopus* oocytes, U6 snRNA did not leave the nucleus (40) and that after cytoplasmic microinjection of synthetic U4 snRNA, the U4/U6 snRNP could be found only in the nuclear fraction and not in the cytoplasmic fraction (41a). The question then arose as to whether the binding protein remains associated with U6 snRNA at stages subsequent to U6 capping and transport, during the assembly of U4/U5/U6 multi-snRNPs and spliceosomes, and during splicing. We began to address this by fractionating and affinity purifying U6 complexes present in nuclear extract and assaying for the presence of U6-binding activity. Under our conditions of affinity purification, we could detect U6-binding activity only in the U6 snRNP fraction but not in the U4/U6 snRNP or the U4/U5/U6 multi-snRNP. If the nuclear U6 snRNP represents a precursor in the U4/U5/U6 multi-snRNP assembly, this result suggests that the U6-protein is released before U4/U6 snRNP assembly. Alternatively, binding of the U6-protein in the larger snRNP complexes may be destabilized under the fractionation conditions, indicating a conformational change in protein binding before U4/U6 snRNP assembly. We cannot rule out the possibility that the U6 snRNP represents a nuclear storage complex normally not recruited for multi-snRNP assembly. Since we detected the U6-binding protein in a cell extract, association of this protein during cell disruption or extract preparation can in principle not be eliminated. However, on the basis of the differential distribution of the protein in U6 complexes, it seems unlikely.

The detection of the U6-binding protein points to an analogy with the Sm protein complex of the other spliceosomal snRNPs. First, both the Sm proteins and the U6-specific protein are present as a cytoplasmic pool of free protein and in a snRNP-bound form in the nucleus. Second, in both cases, protein binding appears to be required for capping and nuclear migration. In the case of U6, however, we do not know where U6 snRNA capping takes place. If U6 capping is nuclear, it might provide the signal for release of the binding protein. A recent study on the cytoplasmic-nuclear transport of snRNPs (18) suggested that the nuclear targeting signal of snRNAs is bipartite, consisting of the cap structure (m_3G; U6 γ-monomethylguanosine) and an internal sequence element (Sm-binding site; U6 nuclear localization signal). Third, binding of the Sm proteins and of the U6 protein appears not to be essential for spliceosome assembly, since both a U4 snRNA mutant lacking the Sm domain and a U6 snRNA mutant with the 5′-terminal domain deleted assemble into spliceosomes (7, 41).

The detection in *T. brucei* extracts of a U6 snRNA-binding protein of the same binding specificity as the mammalian protein underlines the strong conservation of snRNP structure and function even in very distantly related eucaryotic systems. Except for a recent identification of several proteins of the spliced leader RNP and of U2 snRNP (27), very little is known about trypanosomal snRNPs (for a recent review, see reference 1). In comparison with other eucaryotic snRNAs, the *T. brucei* snRNAs deviate probably most significantly from the eucaryotic consensus (17). Yet the identification of a U6-binding protein from *T. brucei* suggests that U6 maturation and transport processes might follow similar principles in all eucaryotes. Also in line with this suggestion is the recent finding that the characteristic U6 snRNA cap structure is not restricted to human RNA but occurs also in plant U6 snRNA (15).

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