cDNA Cloning and Characterization of the Human U3 Small Nucleolar Ribonucleoprotein Complex-Associated 55-Kilodalton Protein

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Received 11 July 1997/Returned for modification 12 August 1997/Accepted 13 October 1997

The eukaryotic nucleolus contains a large number of small RNA molecules (snoRNAs) which, in the form of small nucleolar ribonucleoprotein complexes (snoRNPs), are involved in the processing and modification of pre-rRNA. The most abundant and one of the best-conserved snoRNAs is the U3 RNA. So far, only one human U3 snoRNA-associated protein, fibrillarin, has been characterized. Previously, the U3 snoRNP was purified from CHO cells, and three proteins of 15, 50, and 55 kDa were found to copurify with the U3 snoRNA (B. Lübben, C. Marshallsay, N. Rottmann, and R. Luhrmann, Nucleic Acids Res. 21:5377–5385, 1993). Here we report the cDNA cloning and characterization of the human U3 snoRNP-associated 55-kDa protein. The isolated cDNA codes for a novel nucleolar protein which is specifically associated with the U3 snoRNA. This protein, referred to as hU3-55k, is the first characterized U3 snoRNP-specific protein from humans. hU3-55k is a new member of the family of WD-40 repeat proteins and is conserved throughout evolution. It appears that the C-terminal end of hU3-55k is required for nucleolar localization and U3 snoRNA binding.

Eukaryotic cells contain a large number of small nuclear RNAs (snoRNAs) which, in the form of small nucleolar ribonucleoprotein complexes (snoRNPs), are involved in the various steps of ribosome synthesis (reviewed in references 30 and 46). Several snoRNAs have previously been shown to be required for pre-rRNA processing (12, 30, 46), and a large set of snoRNAs are involved in ribose methylation and pseudouridylation of rRNA (8, 15, 24, 33, 34, 49). snoRNAs are heterogeneous in size, structural elements, and protein association. They are produced by two biosynthetic pathways. Most snoRNAs are encoded within the pre-mRNA introns of ribosomal or nucleolar proteins. The processing of such pre-mRNAs via endo- and exonucleolytic cleavages results in the generation of mature non capped snoRNAs (30). Other snoRNAs, e.g., U3 snoRNA, are transcribed from independent genes and typically possess a modified 5′terminus, usually a 5′trimethylguanosine (TMG) cap (30).

snoRNPs can be divided into four groups, which appear to be functionally distinct (1, 46). Methylation guide snoRNPs direct the site-specific formation of 2′-O-methyl groups in mature rRNA. All snoRNAs of this class contain two conserved sequence elements, referred to as box C and box D (30), and contain an extended region (10 to 21 nucleotides) of base complementarity to mature rRNA (8, 24, 34, 49). Members of the second group of snoRNAs, which encompasses U3, U8, U14, and U22 snoRNAs, also contain the conserved box C and D elements and are involved in pre-rRNA processing reactions (reference 46 and references therein). All box C- and D-containing snoRNAs, including methylation guide snoRNAs, are associated with the conserved nucleolar protein fibrillarin, which thus is a common snoRNP component (30). Members of the third class of snoRNAs lack the box C and D elements but share another conserved sequence element, referred to as the ACA box (1). Such snoRNAs have been implicated in the site-specific synthesis of pseudouridine in rRNA (15, 33). The last group of snoRNAs consists of only one snoRNA, RNase MRP. RNase MRP is an endoribonuclease involved in the processing of pre-rRNA at site A3 in the internal transcribed spacer 1 (27).

Although many snoRNAs have been identified in a wide range of eukaryotes, very few snoRNP proteins have been identified so far. For yeast, containing more than 50 snoRNAs, only eight snoRNA-associated proteins have previously been described (1, 30). So far, only two human snoRNP proteins have been characterized, fibrillarin (30) and hPop1, a component of the human RNase MRP particle (28).

U3 snoRNA, one of the most conserved snoRNAs, is the most abundant snoRNA in cells. All reported U3 snoRNA sequences contain five evolutionarily conserved sequence elements, the A, B, C, C′, and D boxes (20, 31, 47, 48). The presence of an intact box C sequence has previously been shown to be essential for the efficient binding of fibrillarin to U3 snoRNA (2). U3 snoRNA is not exported to the cytoplasm but is retained in the nuclei of cells. Mature U3 snoRNA contains a trimethylguanosine cap structure at its 5′end. The box D sequence element is required for efficient nuclear hypermethylation of U3 snoRNA both in vivo and in vitro (44, 45).

Eukaryotic rRNA is transcribed as a large 47S precursor and subsequently cleaved in a series of complex processing steps to generate mature 18S, 5.8S, and 28S rRNA species. U3 snoRNP is required for correct processing of the 18S rRNA and is involved in cleavages at site A0 in the 5′external transcribed spacer (27). Furthermore, a recent report suggests that U3 snoRNA facilitates the correct folding of the 18S rRNA (18).

As for other snoRNPs, our knowledge about the protein composition of U3 snoRNP is still very limited. Human U3 snoRNP is reported to contain at least six proteins with mo-
lecular masses of 74, 59, 36 (fibrillarin), 30, 13, and 12.5 kDa (35). Of these proteins, only the common snoRNP protein fibrillarin has been characterized. In yeast, besides fibrillarin, only one U3 snoRNP-associated protein, SOFI, has previously been characterized (21). SOFI is a specific U3 snoRNP protein, since it is not associated with other snoRNAs.

Previously, the U3 snoRNP particle was purified from CHO cells by anti-m 3G-immunoaffinity and Mono Q anion-exchange chromatography (26). Three proteins of 55, 50, and 15 kDa copurified with U3 snoRNA. These proteins may represent core U3 snoRNP proteins whose binding mediates the association of other proteins, such as fibrillarin, which are lost during high salt purification (26). By using a rabbit antiserum raised against the 55-kDa protein, the binding site of this protein on U3 snoRNA was localized. Stable binding of the 55-kDa protein requires sequences located between nucleotides 97 and 204 of human U3 snoRNA, including the conserved box B and C sequence elements (26).

In this report, we describe the cDNA cloning and characterization of the human U3 snoRNP-associated 55-kDa protein. We show that the isolated cDNA codes for a novel nucleolar protein which is associated with U3 snoRNA. This protein, referred to as hU3-55k, is a specific U3 snoRNP component from which the C-terminal end appears to be required for U3 snoRNA binding and nucleolar localization.

MATERIALS AND METHODS

Peptide sequence analysis. Purified 55-kDa protein for sequence analysis was isolated from CHO cells by anti-m 3G-immunoaffinity and Mono Q anion-exchange chromatography (26). The peptides obtained from the 55-kDa protein, AFEEDQVAGRLK and VWNVAEN, were used to design the following two peptides for trypsin digestion and microsequencing.

\[ \begin{align*}
\text{5' end} & \quad \text{AAG-CTT-C} \\
\text{3' end} & \quad \text{-CGC-AAG-CTT-C} 
\end{align*} \]

Sequence analysis revealed that the clones obtained were not full-length; three nucleotides were missing from the 5' end of the cDNA, PCR primers 5'-CTT-CTC-TGT-GAC-ATC-CCC-CTG-GTG-3' and 3'-CTT-CTC-TGT-GAC-ATC-CCC-CTG-GTG-3' (Promega) and sequenced by the dideoxynucleotide chain termination method (39).

In vitro transcription and translation. In vitro transcription was performed with T7 RNA polymerase and full-length hU3-55k cDNA cloned in vector pGEM-3Z+i (+) essentially as previously described (40). In vitro translation of hU3-55k was performed by incubating T7 mRNA with [35S]methionine (ICN) and wheat germ extract essentially as previously described (40). In vitro-translated hU3-55k protein was immunoprecipitated in IPP500 (100 mM NaCl, 10 mM Tris-Cl [pH 7.5], 0.5 mM MgCl2, 0.5% NP-40) with 10 μl of rabbit anti-55 kDa antiserum or 50 μl of normal rabbit serum coupled to protein A agarose beads as previously described (28).

Transfection constructs. To obtain a construct in which hU3-55k cDNA is cloned in frame behind the GFP coding sequence, hU3-55k cDNA was mutagenized by PCR to introduce an Ndel site at the start codon and a BarnHI site directly behind the stop codon. The PCR product was cloned in the PCR-II vector (Invitrogen), digested with BarnHI, and ligated in pGFP-C1 vector (Clontech) which was digested with EcoRI and Ndel, resulting in pGFP-C1 (BarnHI/PvuII) (35). The constructs were then used to transfect 293 cells. The resulting plasmid, GFP-55k, was linearized with NotI and used to transfect 293 cells.

RESULTS

Identification of U3-55k peptide sequences. As described previously (26), the U3 snoRNP particle was purified from CHO cells via anti-m 3G-immunoaffinity and Mono Q anion-exchange chromatography. U3 snoRNA copurified with at least three proteins, with molecular masses of 55, 50, and 15 kDa. A protein preparation containing the 55-kDa protein was fractionated by SDS-PAGE and blotted onto nitrocellulose, and the region containing the 55-kDa protein was

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FIG. 1. cDNA and deduced amino acid sequences of hU3-55k. (A) The obtained peptide sequences of the purified 55-kDa protein from CHO cells are shown in boldface. The putative bipartite NLS is double underlined, and the glutamic acid-rich region in the N-terminal part of the protein is shown in bold italics. The five WD-40 repeat regions are underlined and numbered. (B) The WD-40 repeat regions of hU3-55k were aligned manually with the consensus WD-40 repeat sequence (32). Amino acids which correspond to the consensus sequence are shaded. Parts A and B of WD-40 repeats are indicated.
excised. The nitrocellulose-bound 55-kDa protein was subjected to trypsin digestion, and the resulting peptides were sequenced. Two peptide sequences of 12 and 7 amino acids in length, AFEEDQVAGRLK and VWNVAEN, were obtained.

**Cloning of hU3-55k cDNA.** The peptide sequences obtained from the purified U3 snoRNP-associated 55-kDa protein from CHO cells were employed in designing two degenerate oligonucleotides (see Materials and Methods) that were used to screen a human teratocarcinoma cDNA library. Although these oligonucleotides were derived from hamster sequences, we speculated that as for other sn(o)RNP proteins (e.g., fibrillarin), the U3-55k sequence is highly conserved among mammals and chose a human cDNA library for screening. Several clones that hybridized with both oligonucleotides were selected, strongly suggesting that cDNAs coding for a protein containing both peptide sequences were found. Subcloning and sequence analysis of these clones (1.4 kb in length) revealed an open reading frame (ORF) starting at nucleotide 35 and extending to the 3' end of the cDNA coding for 458 amino acids. No in-frame stop codon was present at the C-terminal end of the ORF, suggesting that the cloned cDNA was missing the 3' end.

A comparison of this cDNA sequence with the nucleic acid sequences in databases revealed that three human EST sequences (HS705108, HS081224, and HS892) overlapped with our cDNA (overlap starting at nucleotides 1227, 1235, and 1250, respectively) and extended further to the 3' end. An oligonucleotide was designed on the basis of these EST sequences and used to amplify the complete 3' end from HeLa cell RNA by reverse transcription-PCR. An internal EcoRI restriction site close to the 3' end of the cDNA appeared to be responsible for finding only 3'-truncated cDNAs during screening of the teratocarcinoma cDNA library.

A complete cDNA (1,521 bp) was constructed by combining the teratocarcinoma cDNA with the 3' end obtained by reverse transcription-PCR. The combined cDNA encodes a protein of 475 amino acids, with a predicted molecular mass of 51.8 kDa and a pI of 7.8. The cDNA and deduced amino acid sequences are shown in Fig. 1A. The size of the cDNA was confirmed by Northern analysis of HeLa cell RNA, which revealed an ~1.7-kb mRNA (data not shown). The polypeptide encoded by the cDNA, hU3-55k, does contain both peptide sequences derived from the 55-kDa U3 snoRNP protein isolated from CHO cells. The first peptide sequence can be found from amino acids 102 to 113, and the second peptide sequence can be found from amino acids 267 to 273 (Fig. 1A).

To test whether the hU3-55k cDNA indeed represents the human homolog of the copurifying U3-55k protein from CHO cells, hU3-55k was translated in vitro and the resulting protein was immunoprecipitated with a rabbit antiserum generated against the purified 55-kDa CHO protein. This rabbit serum has been shown previously to recognize the native CHO and human 55-kDa proteins (26). The in vitro-translated hU3-55k protein was indeed immunoprecipitated by this rabbit antiserum (Fig. 2, lane 2), confirming that the protein encoded by hU3-55k cDNA is similar to the purified CHO protein. Serum from a nonimmunized rabbit was not able to immunoprecipitate the hU3-55k protein (Fig. 2, lane 3).

**hU3-55k is a member of the family of WD-40 repeat proteins.** A comparison of the hU3-55k protein sequence with the protein sequences in databases showed that hU3-55k is a novel human protein. A putative bipartite nuclear localization signal can be identified at the N terminus of hU3-55k (amino acids 8 to 40). Furthermore, a search for known protein motifs revealed the presence of five so-called WD-40 repeat units between amino acid positions 142 and 352 of hU3-55k (Fig. 1A). A WD-40 repeat consists of two conserved elements, A and B, separated by regions variable in both sequence and length (32, 50). The most characteristic features of a WD-40 repeat are the GH residues in part A and the WD residues in part B. The number of WD-40 repeats in a particular protein may vary from four to eight repeating units, spanning either the entire length of the protein or the N-terminal.
C-terminal, or central part of it. A multiple alignment of the hU3-55k WD-40 repeat units and the WD-40 consensus sequence is depicted in Fig. 1B. Another interesting feature of hU3-55k is the glutamic acid-rich stretch between amino acids 64 and 73. A similar stretch of glutamic acid residues is present in a number of nucleolar and nonnucleolar proteins and may be involved in protein-protein interactions.

In addition to identifying protein sequence motifs, the database search with the hU3-55k protein sequence revealed two putative yeast homologs. Along with the SOF1 protein (21), which is partially homologous to hU3-55k (17% identity and 42% similarity), there is yeast polypeptide with a higher degree of homology to hU3-55k. This *Saccharomyces cerevisiae* protein, which is encoded by the eighth ORF of cosmid 9659 (U40829), is 33% identical and 58% similar to hU3-55k, and like hU3-55k, it contains a glutamic acid-rich region in the N-terminal part of the protein, a feature lacking in the SOF1 protein sequence. We therefore conclude that the polypeptide encoded by the yeast U40829 ORF represents the true homolog of hU3-55k. This finding implies that the yeast U3 snoRNP particle contains two related proteins, with each one containing several WD-40 repeat units.

hU3-55k is a specific U3 snoRNP component. To determine whether the hU3-55k protein is a component of the human U3 snoRNP particle, immunoprecipitation experiments were performed with a tagged hU3-55k protein. hU3-55k cDNA with a 3' VSV tag sequence (25) was cloned into the mammalian expression vector pCI-neo and expressed in transiently transfected HeLa cells. As controls, a construct containing hU3-55k cDNA without the VSV tag and the pCI-neo vector were used. Two days after transfection, cells were lysed and the resulting total cell extract was used for immunoprecipitation with anti-VSV, antifibrillarin, and anti-U2B0 monoclonal antibodies. RNAs were extracted from immunoprecipitates, supernatants, and total cell extracts; fractionated by gel electrophoresis; and analyzed by Northern blot hybridization with probes specific for U3 snoRNA, U8 snoRNA, and U2 snRNA.

As is shown in Fig. 4A, lane 3, U3 snoRNA was coprecipitated by anti-VSV antibodies from cell extract containing VSV-tagged hU3-55k protein (55k-VSV), showing that the hU3-55k protein indeed is able to associate with the U3 snoRNP particle. The specificity of this result was established by the lack of U3 snoRNA coprecipitation with (i) anti-VSV antibodies for extracts from control cells (nontagged 55-kDa protein [55k] and pCI-neo vector) (Fig. 4A, lanes 2 and 4, respectively) and (ii) anti-U2B0 antibodies (Fig. 4A, lanes 8 through 10). U3 snoRNPs were immunoprecipitated from all three extracts (55k-VSV, 55k, and pCI-neo vector) by antifibrillarin antibodies (Fig. 4A, lanes 5 through 7). No U2 snRNA could be detected in anti-VSV or antifibrillarin precipitates.

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snoRNP protein or a common snoRNP protein (like fibrillarin), the same blot was hybridized with a number of snoRNA probes. Neither U8 snoRNA (Fig. 4A) nor U13 snoRNA (data not shown) was detectably coprecipitated with anti-VSV antibodies from extract containing the 55k-VSV protein, indicating that the hU3-55k protein is not able to associate with these snoRNP. Three classes of snoRNAs other than box C and D snoRNAs, such as U3 and U8, exist; they are methylation guide snoRNAs (e.g., U24), ACA box-containing snoRNAs (e.g., U17), and RNase MRP RNA (1, 46). To investigate whether the hU3-55k protein may be a component of these other classes of snoRNP, the Northern blot of the immunoprecipitations was hybridized with U24, U17, and RNase MRP RNA probes (data not shown). In all of these cases, anti-VSV antibodies failed to detectably coprecipitate the respective RNAs from extract containing the 55k-VSV protein, indicating that hU3-55k is indeed not associated with these other classes of snoRNAs.

To further establish hU3-55k as a specific U3 snoRNP component, coprecipitating RNAs from cell extracts containing 55k-VSV and 55k (nontagged) proteins were 3'end labeled with [32P]pCp and fractionated on an 8% polyacrylamide gel. As shown in Fig. 4B, lane 3, anti-VSV antibodies were able to specifically coprecipitate U3 snoRNA from cell extract containing the VSV-tagged hU3-55k protein. In contrast, fibrillarin antibodies were able to immunoprecipitate a large number of snoRNAs from cell extracts containing 55k-VSV and 55k proteins and from nontransfected HeLa cells (Fig. 4B, lanes 5 through 7, respectively). Taken together, these results strongly suggest that the hU3-55k protein is specifically associated with U3 snoRNP.

hU3-55k is localized in the nucleolus. To investigate the subcellular localization of hU3-55k, a plasmid in which hU3-55k cDNA was fused to the GFP sequence in the mammalian expression vector pEGFP (9) was constructed. The resulting construct (GFP-55k) and pEGFP as a control were used to transiently transfect HeLa cells, and 2 days after transfection, the localization of the GFP-55k fusion protein was determined via direct fluorescence microscopy, i.e., in vivo with nonfixed cells. GFP alone gave a strong fluorescence distributed throughout the HeLa cell (Fig. 5A), indicating that GFP is uniformly distributed over the cell and does not localize to a specific compartment of a HeLa cell. Expression of the GFP-55k fusion protein, however, resulted in strong nucleolar fluorescence (Fig. 5B), strongly suggesting that hU3-55k (and thus GFP-55k) accumulates in the nucleolus of a HeLa cell. Cells with a relatively high level of GFP-55k expression showed in addition to nucleolar fluorescence weak or moderate nucle-
oplasmic fluorescence, suggesting that when the nucleolus is saturated with GFP-55k, the remaining GFP-55k molecules reside in the nucleoplasm.

To more precisely assess the nucleolar localization of hU3-55k, GFP-55k-transfected cells were fixed with methanol-acetone 2 days after transfection and immunostained with a monoclonal antifibrillarin antibody. Fibrillarin is associated with a number of snoRNAs, including U3 snoRNA, and is localized to the dense fibrillar compartment of the nucleolus (37). As is shown in Fig. 6A through C, GFP-55k gave a somewhat more diffuse fluorescence of the nucleolus than did fibrillarin, which gave a more clumpy staining pattern of the nucleolus. However, superimposition of the two images showed that GFP-55k largely colocalized with fibrillarin, which might have been partially due to the fact that both fibrillarin and hU3-55k are associated with U3 snoRNA and therefore present in the same particle.

Many snRNAs, snoRNAs, and their associated proteins, including fibrillarin, are present in a nuclear organelle termed the coiled body (5, 36). To determine whether hU3-55k can also be found in this nuclear organelle, GFP-55k-transfected cells were immunostained with a monoclonal antibody against U2B', a component of the U2 snRNP particle, which exhibited strong staining of coiled bodies and weaker speckled nucleoplasmic staining (Fig. 6E). As shown in Fig. 6D through F, GFP-55k did not accumulate in coiled bodies.

hU3-55k elements essential for nucleolar localization and U3 snoRNA binding. The data discussed above show that the hU3-55k protein is a nucleolar protein which is specifically associated with U3 snoRNA. To determine which parts of hU3-55k are essential for nucleolar localization and U3 snoRNA binding, VSV-tagged deletion mutants of hU3-55k were constructed and expressed in HeLa cells by transient transfection. Two days after transfection, cells were lysed and the resulting total cell extracts were analyzed. The expression of tagged mutant proteins was checked by Western blotting of total cell extracts with anti-VSV antibodies. Only two of the eight mutants we used showed detectable expression of hU3-55k protein on a Western blot (data not shown). In particular, mutants from which larger parts of the hU3-55k protein were deleted could not be detected by anti-VSV antibodies. These mutant proteins are probably not expressed very well or are rapidly degraded.

The cellular localization and U3 snoRNA binding capacities of the two hU3-55k deletion mutants that had detectable protein expression were investigated. In the first mutant, 55kΔN-VSV, the N-terminal 44 amino acids of hU3-55k are deleted and a C-terminal VSV tag is added. This mutant protein thus lacks the putative bipartite nuclear localization signal at positions 8 to 40. The second mutant, VSV-55kΔC, lacks the C-terminal 17 amino acids of hU3-55k and contains an N-terminal VSV tag. The stop codon of full-length hU3-55k is lost in this construct, and the resulting protein is terminated by the first in-frame stop codon in the pCI-neo vector, giving rise to an additional 15 amino acids after the hU3-55k protein sequence.

The localization of full-length and mutant proteins was investigated by immunofluorescence assays of transiently transfected cells with anti-VSV antibodies. Nontransfected HeLa

![FIG. 5. Nucleolar accumulation of GFP-55k. GFP (A) and GFP-55k (B) were transiently expressed in HeLa cells. Two days after transfection, GFP and GFP-55k localization was examined by fluorescence microscopy in vivo with nonfixed cells.](http://mcb.asm.org/)

![FIG. 6. Colocalization of GFP-55k. GFP-55k was transiently expressed in HeLa cells. Two days after transfection, cells were fixed with methanol-acetone and incubated with antifibrillarin antibodies (A through C) or anti-U2B' antibodies (D through F), followed by incubation with Texas Red-labeled goat anti-mouse antibodies, and visualized by confocal microscopy. (A and D) GFP-55k localization (green); (B and E) fibrillarin localization and U2B' localization, respectively (red); (C and F) superimposition of panels A and B and panels D and E, respectively, with regions of colocalization shown in yellow.](http://mcb.asm.org/)
cells and cells transfected with the nontagged hU3-55k construct (55k) gave weak background staining of whole cells (data not shown). Full-length hU3-55k proteins containing N- and C-terminal VSV tags, VSV-55k and 55k-VSV, respectively, exhibited strong nucleolar staining (Fig. 7A and D). As described above for GFP-55k, cells with relatively high levels of 55k-VSV and VSV-55k expression showed not only nucleolar staining but also nucleoplasmic staining, whereas no protein was detected in the cytoplasm (Fig. 7A). The mutant hU3-55k protein in which the putative nuclear localization signal is deleted, 55kΔN-VSV, showed strong nucleolar staining. Cells with a relatively high expression level of this protein showed both nucleolar staining and cytoplasmic fluorescence, but no staining was observed in the nucleoplasm of these cells (Fig. 7C). The second mutant, VSV-55kΔC, showed nuclear accumulation, but no staining was found in the nucleoli of these cells (Fig. 7B). In cells with a relatively high expression level of VSV-55kΔC, this localization pattern remained unchanged.

To investigate whether these mutant hU3-55k proteins are still able to associate with U3 snoRNA, we performed immunoprecipitation experiments with extracts from transfected HeLa cells. As shown in Fig. 8A, both full-length hU3-55k proteins, 55k-VSV and VSV-55k, and the N-terminal deletion mutant, 55kΔN-VSV, were able to associate with U3 snoRNA (Fig. 8A, lanes 1, 3, and 4). In contrast, the C-terminal deletion mutant, VSV-55kΔC, seemed to have lost the ability to associate with U3 snoRNA (Fig. 8A, lane 2). As a control, U3 snoRNPs were immunoprecipitated from all cell extracts by antifibrillarin antibodies (Fig. 8A, lanes 6 through 10), whereas no U3 snoRNA could be detected in anti-U2B* immunoprecipitates (Fig. 8A, lane 11 through 15). A deletion of only 17 C-terminal amino acids of hU3-55k thus appears to have a drastic effect on U3 snoRNA binding and nucleolar localization. However, 44 N-terminal amino acids can be removed without significant loss of U3 snoRNA binding capacities and a deletion of the putative nuclear localization signal has only a limited effect on nucleolar localization.

**DISCUSSION**

By anti-mG-Imunoaffinity and mono Q anion-exchange chromatography, Lübben et al. (26) previously identified three proteins of 55, 50, and 15 kDa from CHO cells which copurified with U3 snoRNA. In this report, we have described the isolation and characterization of a CDNA encoding the human U3 snoRNA-associated 55-kDa protein, hU3-55k. Immunoprecipitation of in vitro-translated hU3-55k with a rabbit serum raised against the purified 55-kDa protein from CHO cells (26) confirmed that the cloned human protein is similar to the purified CHO protein and that this protein is conserved between humans and rodents.

A sequence analysis of the hU3-55k protein revealed that this protein is a new member of the family of WD-40 repeat proteins. This group of proteins is characterized by four to eight conserved repeating units that usually end with WD, thus forming a small b-strand–turn–b-strand–turn–b-strand and ending with WD, thus forming a small b-structure (32). This most likely not very stable structure could be stabilized by contact with other WD-40 repeats, leading to the formation of intramolecular dimers or tetramers (32). Interestingly, not only hU3-55k but also the SOF1 protein, a component of yeast U3 snoRNP, is a member of the WD-40 repeat family (21). However, the 56-kDa SOF1 protein is probably not the yeast homolog of hU3-55k. An alignment of hU3-55k with sequences in the EMBL and GenBank databases revealed that another yeast protein, the eight ORF of cosmid 9659, is more homologous to hU3-55k. This finding implies that at least in yeast, U3 snoRNP possesses two related proteins that contain several WD-40 repeat units; as mentioned above, these proteins may even form heterodimers. It will be interesting to find out if this is also the case in mammals. A candidate for a second WD-40 repeat family protein with a size similar to those of hU3-55k and SOF1 is the copurifying 50-kDa U3 snoRNP protein from CHO cells (21).

To confirm that hU3-55k is a component of the U3 snoRNP, we expressed a VSV-tagged version of hU3-55k in HeLa cells and after cell lysis immunoprecipitations with anti-VSV antibodies were performed. The results of these experiments showed that hU3-55k is indeed a component of U3 snoRNP. We could not detect other snoRNAs, such as U8, U13, U17, U24, or RNase MRP RNA, in immunoprecipitates by Northern blot hybridization. Since these RNAs are typical examples of the various snoRNA classes, these results strongly suggest that the hU3-55k protein is a specific U3 snoRNP protein.
addition, we were not able to detect snoRNAs other than U3 in the anti-VSV immunoprecipitate after 3'-end labeling with [32P]pCp. In contrast, the immunoprecipitate obtained with antifibrillarin antibodies contained a large number of different snoRNAs. These results corroborate our conclusion that hU3-55k is specifically associated with U3 snoRNA.

Lu¨bben et al. (26) proposed that the 55-kDa protein is a core U3 snoRNP protein and may bind directly to the RNA. With a rabbit antiserum raised against the 55-kDa protein, they showed that stable association of the 55-kDa protein with the U3 snoRNP requires sequences located between nucleotides 97 and 204 of human U3 snoRNA, including the conserved box B and C sequence elements. However, in addition to the WD-40 repeat units and the putative bipartite NLS sequence in the N-terminal part of the protein, no known RNA-binding or other protein motifs could be found in hU3-55k. Additional experiments have to be performed to find out whether hU3-55k is bound directly to the RNA and which parts of the protein are involved in the binding to U3 snoRNP.

By using a fusion protein of GFP with hU3-55k (GFP-55k), we showed that hU3-55k localizes to the nucleolus of a HeLa cell. An immunofluorescence assay of GFP-55k-transfected cells with antifibrillarin antibodies showed that hU3-55k largely colocalized with fibrillarin in the nucleolus, as expected for a U3 snoRNP component. We could not detect any GFP-55k in the cytoplasm, but in cells with a relatively high level of GFP-55k expression, the protein was also present in the nucleoplasm of HeLa cells. It has been proposed that nucleolar accumulation is a two-step process. First, a nucleolar protein is transported from the cytoplasm to the nucleoplasm due to its NLS, and then one or several functional domains that interact specifically with other nucleolar components allow it to accumulate within the nucleolus (16, 41, 53). The subcellular localization of GFP-55k is in agreement with such a two-step process, with...
U3 snoRNP carrying the site of interaction with the hU3-55k protein.

Most nucleoplasmic snRNPs reveal a speckled pattern in immunofluorescence assays and appear to concentrate in coiled bodies, a putative storage compartment for snRNPs (5). Coiled bodies also contain fibrillarin (36), but the presence of U3 snoRNA is still discernible. Although in most previous studies U3 snoRNA was not found in coiled bodies (7, 29), Jimenez-Garcia et al. (22) reported a low level of U3 snoRNA in coiled bodies. An immunofluorescence assay of GFP-55k-expressing cells with anti-U2B′ antibodies revealed that GFP-55k did not localize to coiled bodies. These results indicate that there are no or very few GFP-55k-bound U3 snoRNP particles in coiled bodies and that the fibrillarin present in coiled bodies represents probably free (non-U3 snoRNP-bound) protein or is associated with a different subset of U3 snoRNP particles.

We found that sequences in the C-terminal end of hU3-55k are required for nucleolar localization and most likely U3 snoRNA binding. In contrast, a deletion of the first 44 amino acids from hU3-55k, which removes the putative NLS, had no dramatic effect on nucleolar localization and U3 snoRNA binding. 55kΔN-VSV still accumulated in the nucleolus and was able to associate with U3 snoRNA. However, in contrast to the results for full-length hU3-55k, HeLa cells that showed a relatively high expression level of the mutant protein also accumulated 55kΔN-VSV in the cytoplasm and no nucleoplasmic accumulation was observed. This suggests that only when 55kΔN-VSV can associate with U3 snoRNA is it retained in the nucleolus and that the overexpressed mutant protein is not able to stay in or enter the nucleus by itself, possibly due to deletion of the putative NLS. How does this protein enter the nucleolus? A possible explanation is that the mutant protein enters the nucleus with another U3 snoRNP protein or a transport protein, binds to U3 snoRNA, and then is retained in the nucleolus. Another possibility is that a second NLS in hU3-55k does not localize to coiled bodies. These results indicate that there is or no very few GFP-55k-bound U3 snoRNP particles in coiled bodies and that the fibrillarin present in coiled bodies represents probably free (non-U3 snoRNP-bound) protein or is associated with a different subset of U3 snoRNP particles.

A deletion of 17 C-terminal amino acids of hU3-55k had a more severe effect on nucleolar localization and U3 snoRNA binding. VSV-55kΔC localized to the nucleoplasm of HeLa cells and could not be detected in the nucleolus. In addition, this mutant seemed to be unable to associate with U3 snoRNA. These results are in accord with the idea that binding to U3 snoRNP is essential for nucleolar localization. Further experiments are needed to establish whether certain C-terminal amino acids are required for U3 snoRNP binding or whether a C-terminal-end deletion induces a conformational change in the hU3-55k protein which leads to the loss of U3 snoRNA binding capacities and therefore the loss of nucleolar retention.

This study is a further step in resolving the complexity of the human U3 snoRNP particle. Detailed knowledge of interactions among the components of U3 snoRNP and of the functions of snoRNPs proteins is the next goal in the endeavor to understand the rRNA processing steps in which U3 snoRNP is involved.

ACKNOWLEDGMENTS

We thank G. J. M. Pruijn for helpful discussions and critical reading of the manuscript and J. M. H. Raats and A. van der Kemp for advice on tissue culture and immunofluorescence techniques. We are grateful to B. Lübben for isolation of the 55-kDa protein from CHO cells, K. M. Pollard and E. M. Tan for providing antifibrillarin (72B9) antibodies, and T. Kiss for providing plasmids of human U8, U13, and U24 snoRNAs.

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