Isolation of U3 snoRNP from CHO cells: a novel 55 kDa protein binds to the central part of U3 snoRNA

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ABSTRACT

U3 snoRNP, the most abundant of the small nucleolar ribonucleoprotein particles (snoRNPs), has previously been demonstrated to participate in pre-rRNA maturation. Here we report the purification of U3 snoRNP from CHO cells using anti-m₃G-immunoaffinity and mono Q anion-exchange chromatography. Isolated U3 snoRNPs contain three novel proteins, of 15, 50 and 55 kDa respectively. These proteins may represent core U3 snoRNP proteins whose binding mediates the association of other proteins, such as fibrillarin, that are lost during purification. Using a rabbit antiserum raised against the 55 kDa protein, and an *in vitro* reconstitution assay, we have localised the 55 kDa protein binding site on the U3 snoRNA. Stable binding of the 55 kDa protein requires sequences located between nucleotides 97 and 204 of the human U3 snoRNA, including the evolutionarily conserved B and C sequence motifs.

INTRODUCTION

All eukaryotes examined so far possess a distinctive set of small nuclear RNAs (snRNAs). These snRNAs can be divided into two classes on the basis of their localisation in the nucleoplasmic or nucleolar compartments. The nucleoplasmic snRNAs are primarily involved in the splicing of pre-mRNA (U1, U2, U4, U5, U6) and histone pre-mRNA 3'-end formation (U7), although for some snRNAs (U9–U12) a function remains to be established (1). In contrast, the nucleolar snRNAs, also referred to as snoRNAs, are believed to participate in ribosome biogenesis (2,3).

To date, more than 16 different snoRNAs have been identified in mammalian cells, of these U3 snoRNA is the most abundant with 2×10^5 copies per cell (2,3). The other snoRNAs U8, U14, U15, U16, RNA Y and 7-2/MRP RNA are considered to be minor snoRNAs, with only $1-4 \times 10^4$ copies per cell (2,3). In yeast, where 12 snoRNAs have been identified, the snoRNAs tend to be larger than their metazoan counterparts and are even less abundant with 200–1000 copies per cell (2,3). *In vivo*, snoRNAs are found assembled into small nucleolar ribonucleoprotein particles (snoRNPs), which themselves

associate with high molecular weight complexes (2,3) similar to those identified for spliceosomal snRNPs (4). We have only a vague knowledge of the protein composition of the snoRNPs (2,3).

U3 snoRNP has been proven to participate in the primary cleavage event of the 5'-external transcribed spacer (ETS) of the 47S rRNA precursor molecule in a mouse *in vitro* rRNA-processing system (5). Similar results were also obtained *in vivo*, by the depletion of the U3 snoRNA, the RNA component of U3 snoRNP, in *Xenopus laevis* (6) and yeast (7). In mammals, U3 snoRNA is 217 nucleotides long and has a 2,2,7-trimethylguanosine-cap (m₃G-cap) structure (2). All reported U3 snoRNA sequences contain five evolutionarily conserved sequence motifs (or boxes) designated A, A°, B, C, and D (2,8,9). Motifs A and B are only found in U3 snoRNAs (2,8,9), whereas motifs A° (also referred to as C'), C and D are also found in other snoRNAs such as U8 and U13 (10,11).

Mammalian U3 snoRNA is reported to associate with at least six proteins with molecular weights of 12.5, 14, 31, 36 (referred to as fibrillarin), 59 and 74 kDa (12). However it remains to be established whether these proteins are indeed integral components of U3 snoRNPs. In yeast, besides the fibrillarin homologue NOP1 (16), only one U3 snoRNP-associated protein, SOF1, has been reported (13).

The conserved U3 snoRNA sequence motifs have been proposed to be involved in conserved RNA–RNA interactions or alternatively in protein binding (2,3,12). Consistent with the latter proposal, the presence of an intact C box has been shown to be essential for efficient fibrillarin binding to U3 snoRNA *in vitro* and *in vivo* (14,15). Furthermore anti-Fib antibodies were shown to co-precipitate the RNAs U3, U8, U13, U14, U16 and RNA Y from mammalian cellular extracts, all of which share the C and D sequence motifs (10,11). In yeast, antibodies against NOP1 immunoprecipitate many nucleolar RNAs, including some which do not contain the conserved A°, C and D sequence motifs (16,17).

The functional importance of the RNA and protein components of U3 snoRNP was demonstrated using gene disruption experiments in yeast, where defective rRNA processing, in particular of the 18S rRNA, was observed when any one of U3 snoRNA, SOF1, and NOP1 were genetically depleted (13,16,17).

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An investigation of the U3 snoRNP structure is therefore central for our understanding of its function.

In this report we describe the isolation of U3 snoRNP from CHO cells using anti-m₃G-immunoaffinity and mono Q anion-exchange chromatography. We show that purified U3 snoRNP contains three proteins, with molecular weights of 55, 50, and 15 kDa. Using a rabbit antiserum raised against the 55 kDa protein and an *in vitro* reconstitution assay, we have localised the 55 kDa protein binding site on the U3 snoRNA.

MATERIALS AND METHODS

Extract preparation

Chinese hamster ovary (CHO) cells were grown in suspension culture as described (18) except that the medium was complemented with 8% new born calf serum and 0.3% (w/v) tryptone. Nuclei from approx. 5×10^9 cells were isolated as described (19). S2 nuclear extracts were then prepared from these isolated nuclei (20). HeLa cells were grown in suspension culture as described (18). HeLa whole cell extracts were prepared from approx. 5×10^7 cells as described (11).

Anti-m₃G-immunoaffinity and mono Q anion-exchange chromatography of CHO cell S2 nuclear extracts

Anti-m₃G-immunoaffinity chromatography was performed as described (21). Essentially CHO cell S2 nuclear extracts, from approx. 2×10^{10} cells, were adjusted so as to correspond to SP-300 buffer (SP-300 is 50 mM Tris-HCl [pH 7.4], 300 mM KCl, 4 mM MgCl₂, 5% (v/v) glycerol, 0.5 mM DTE and 4 µg/ml leupeptine). These extracts were then passed over the affinity column, bed volume 1.5 ml, equilibrated in SP-300 buffer. Washing and elution with m⁷G nucleoside (15 mM) was as described (22) except that all steps were performed with SP-300 buffer. Mono Q anion-exchange chromatography of m⁷G-eluates from the anti-m₃G-immunoaffinity column was performed using a 100 µl column and a Smart FPLC system (Pharmacia) as described (23).

Antibody preparation

55 kDa protein for immunisation was obtained by SDS-PAGE (24) of m⁷G-eluates from the anti-m₃G-immunoaffinity column and transferred onto nitrocellulose. The 55 kDa band was identified using Ponceau S stain, excised and strips corresponding to approx. 10 µg of protein used for subcutaneous immunisation of rabbits. Rabbits were repeatedly boosted at two week intervals.

Immunoblotting

For immunological investigation, proteins pre-fractionated using SDS-PAGE were transferred to nitrocellulose as described in (24). Specific immunocomplex formation with the cognate antigen was visualised using the alkaline phosphatase reaction (24).

Immunoprecipitations

Protein A Sepharose (Pharmacia) was pre-swollen in PBS buffer (130 mM NaCl, 20 mM NaPO₄, pH 8.0). 20 µl aliquots of a 50% suspension of beads were mixed with either 20 µg of purified H20 (21), 20 µl Y12 (25), or 100 µl of anti-55 kDa antiserum. The volume was adjusted to 400 µl with PBS and incubated overnight at 4°C with continuous end-over-end rotation. The beads were then washed four times with PBS, the tube changed once, before the antigen was added and the reaction was allowed to proceed in a 400 µl volume of Net-buffer (Net buffer is: 50

mM Tris-HCl [pH 7.4], 0.05% NP-40) containing 300 mM NaCl for the anti-55 kDa antibody and 150 mM for other antibodies for 2–3 h at 4°C. The beads were then washed four times with Net buffer containing either 500 mM NaCl for anti-55 kDa antibody or 150 mM NaCl for other antibodies. The RNA was then extracted using PCA, ethanol precipitated, and 3'-end-labelled using T4 RNA ligase and ³²P-pCp (Amersham) (26). Labelled RNAs were then analysed using denaturing 6M Urea-7% PAGE.

In vitro generation of human U3 snoRNAs

A human U3 snoRNA cDNA clone was placed under the control of a T7 polymerase promoter using PCR-amplification with a human U3 gene (27) as template and appropriate mutagenic oligonucleotides as described (28). Wild type and 3'-truncation mutants of U3 snoRNA were obtained by template digestion with *Rsa*I, *Bst*U1, *Fnu*4H1, and *Dde*I (see Figure 5B for a schematic diagram). The 5'-end deletion mutant U3Δ70, lacking the 5'-terminal 70 nucleotides was obtained using appropriate mutagenic oligonucleotides and PCR-amplification. All transcripts possess three additional 5'-guanosine residues and natural 3'-end sequences.

Oligonucleotides and RNase H digestion of U3 snoRNAs

Oligonucleotides were kindly synthesised on an Applied Biosystems DNA synthesiser by M.Krause. The oligonucleotide sequences were 64–80: CGTGGTTTTCGGTGCTC and 80–96: CGCTACCTCTCTTCCTC. For RNase H digestion, appropriate RNAs were incubated with 0.5 µg of oligonucleotide and 1 Unit of RNase H (Boehringer Mannheim) at 37°C for 30 min. Digested RNAs were used directly, without purification, for reconstitution assays.

In vitro reconstitution

In vitro reconstitution was carried out as described (29) using affinity purified snRNP proteins for U1 snRNP reconstitution. U3 snoRNP reconstitution was carried out using CHO cell m⁷G-eluate proteins (0.1 mg/ml) obtained by digestion of m⁷G-eluate (approx. 1 µg) with 10 Units of micrococcal nuclease for 30 min at 30°C. Digestion was stopped by addition of 10 mM EGTA. Free proteins were then incubated with ³²P-radiolabelled U3 snoRNA (0.1 pmol) for 30 min at 30°C and 15 min at 37°C. All reconstitutions were performed in the presence of a 100-fold molar excess of yeast tRNA. After reconstitution 1 µg heparin was added and samples incubated 10 min at RT. Reconstitution assays were then analysed using electrophoresis on 6% polyacrylamide-TAE gels run at 4°C.

RESULTS

Preparation of CHO cell nuclear extracts containing native U3 snoRNP

A pre-requisite for the biochemical characterisation of U3 snoRNP from mammalian cells was the extraction of preparative amounts of that particle from isolated nuclei or nucleoli. For this purpose we have used a protocol originally described for the isolation of nucleolin from CHO cells (20). Essentially, this method involves the homogenisation of cultured cells in the presence of collagenase and non-ionic detergents, resulting in the release of intact nuclei. These nuclei are then extracted with EDTA, and the non-soluble nuclear and nucleolar material are separated from the soluble fraction by high speed centrifugation.

Figure 1. CHO cell S2 nuclear extracts contain native U3 snoRNP. The RNA and protein content of CHO cell S2 nuclear extracts (S2), the CHO nuclei pellet (C2) are shown. For analysis, the pellet was re-extracted with the original volume of SP-300 buffer. Aliquots were extracted with phenol-chloroform-isoamylalcohol (PCA) and the RNA, from the aqueous phase, fractionated using denaturing PAGE and visualised using silver stain (A). Proteins, from the organic phase, were fractionated using SDS-PAGE and visualised using Coomassie Blue R250 stain (B). Lane M, protein molecular weight markers (kDa). Nuc, Nucleolin; Fib, fibrillarin.

The RNA and protein contents of such CHO cell nuclear extracts (designated S2 nuclear extracts) (20) are shown in Figure 1. S2 nuclear extracts contain U3 snoRNA as well as many nucleoplasmic snRNAs such as U1 and U2 (Panel A, lane 1). Consistent with previous reports (20), these S2 nuclear extracts are enriched in nucleolar proteins such as fibrillarin and nucleolin (Panel B, compare lanes 1 and 2). The identity of these proteins was confirmed by probing western blots with specific antibodies (data not shown). U3 snoRNA present in S2 nuclear extracts was immunoprecipitable with anti-Fib and anti-m³G-cap antibodies, indicating that this RNA associates with fibrillarin and possesses an m³G-cap (data not shown). Based on these criteria the S2 nuclear extracts contain intact U3 snoRNP.

Purification of U3 snoRNPs by anti-m³G-immunoaffinity and mono Q anion-exchange chromatography

We have exploited the presence of an m³G-cap structure on the mammalian U3 snoRNP and by using anti-m³G-immunoaffinity chromatography as a purification step. As shown in Figure 2A, when CHO cell S2 nuclear extracts (lane 1) were applied to an anti-m³G-immunoaffinity column, the bulk of the U3 snoRNP remained bound to the column and only minimal amounts were found in the flow through (lane 2). After the column had been washed (lane 3), bound material was eluted with m⁷G nucleoside (lane 4). Anti-m³G-immunoaffinity chromatography effectively enriched the U3 snoRNP, as judged by the RNA content (compare lanes 1 and 4). In contrast, the levels of nucleolin and fibrillarin in the m⁷G-eluate was dramatically reduced (Fig. 2B, compare lanes 2 and 5) and these proteins were instead found in the flow through (Fig. 2B, lane 3). The presence of fibrillarin in the flow through may result from a salt induced disassembly of the U3 snoRNP or alternatively reflect the

Figure 2. Anti-m³G-immunoaffinity enrichment of CHO cell S2 nuclear extracts. CHO cell S2 nuclear extracts, prepared from 2×10^{10} CHO cells, were passed over an anti-m³G-immunoaffinity column. After the column had been washed, bound material was eluted with m⁷G nucleoside. 0.2% of S2 extract applied, 0.2% of flow through, 5% of washing buffer, and 5% of m⁷G eluate was analysed as described in legend of Figure 1. (A) RNA. (B) Protein, protein molecular weight markers (left, size in kDa) and specific proteins are indicated (right: Nuc, nucleolin; U1 snRNP specific 70k protein; Fib, fibrillarin; 55, 50, and 15, the respective U3 snoRNP-specific proteins with these molecular weights).

presence of non-capped, fibrillarin associated, snoRNPs. After this step, significant amounts of nucleoplasmic snRNPs were found in the m⁷G-eluate as judged by the presence of the respective U1 and U2 snRNAs (Fig. 2A, compare lanes 1 and 4) and snRNP-specific proteins (Fig. 2B, compare lanes 2 and 5).

U3 snoRNP was then separated from the nucleoplasmic snRNPs by mono Q anion-exchange chromatography. The mono Q column was loaded with m⁷G-eluate and washed, before elution of bound material using a three-step salt gradient from 50 mM to 1M KCl. Figure 3A shows the elution profile of the individual fractions during such a salt elution gradient. The RNA content of individual fractions is shown in Figure 3B. U1 snRNA is eluted in peaks 1 and 2 at approx. 500 mM KCl (Fig. 3B, lanes 1–3). U2 snRNA is eluted next in peak 3, at approx. 550 mM KCl (Fig. 3B, lanes 5–7), followed by U3 snoRNA at approx. 650 mM KCl in peaks 4 (Fig. 3B, lanes 10–11) and at approx. 700 mM KCl in peak 5 (Fig. 3B, lane 12–13). The mono Q column therefore efficiently separates the U3 snoRNA from contaminating nucleoplasmic snRNAs.

U3 snoRNA co-purifies with at least three proteins, with molecular weights of 15, 50 and 55 kDa

The protein contents of the respective mono Q chromatography fractions indicated in Figure 3A are shown in Figure 3C. As expected from the RNA elution profile (Fig. 3B), the U1 (Fig. 3C, lanes 1–3) and U2 (Fig. 3C, lanes 5–7) snRNP fractions contain the common Sm proteins, D_{1–3}, E, F, G and also U1

and U2-specific proteins. For reasons not known, the B and B' protein levels are very low in these preparations. U3 snoRNP fractions corresponding to peak 4 (Fig.3C, lanes 11–12) contain three proteins with apparent molecular weights of 55, 50 and 15 kDa. As estimated from the Coomassie blue and silver stained gels (Fig.3C, lanes 11–12 and 18–19 respectively) the 55 kDa protein is the most abundant, followed by the 15 kDa protein. In contrast, the 50 kDa protein band is barely visible after staining with Coomassie blue, but is seen clearly in silver stained gels (Fig. 3C, compare lanes 11–12 with lanes 18–19). The co-elution of these three proteins with the U3 snoRNA at high salt (approx. 650 mM KCl) concentrations suggests that they are tightly associated. In contrast, only minimal amounts of fibrillarin are present in these U3 snoRNP fractions (Fig.3C, lanes 18–19). No proteins are seen in peak 5 (Fig.3C, lanes 13–14) although large quantities of U3 snoRNA are present in these fractions (Fig.3B, lanes 12–13). This result suggests that material eluted at salt concentrations higher than approx. 700 mM KCl probably corresponds to naked RNA. Peaks 4 and 5 may simply reflect the presence of both U3 snoRNP and free U3 snoRNA in the input CHO cell S2 nuclear extracts or alternatively result from the stringent conditions employed for the purification of the U3 snoRNP causing a gradual snoRNP disassembly.

A 55 kDa protein is also observed in earlier fractions containing U1 and U2 snRNAs. Whether this protein corresponds to the 55 kDa protein of U3 snoRNP is unclear. It is possible that this protein dissociates partially from U3 snoRNP during mono Q chromatography, or, alternatively, it could be a different protein with the same molecular weight. Likewise, other weak protein bands seen in silver stained gels are similar in size to the Sm proteins B, B', D₁₋₃, E, F and G. We cannot at present exclude the possibility that these also represent novel U3 snoRNP proteins. However, in contrast to the 55, 50 and 15 kDa proteins, these polypeptides do not clearly co-elute with U3 snoRNA.

We conclude that after anti-m₃G-immunoaffinity and mono Q anion-exchange chromatography that U3 snoRNA co-purifies with at least three proteins with respective molecular weights of 55, 50 and 15 kDa. These proteins probably represent core U3 snoRNP proteins. Other less tightly bound proteins are presumably lost during this isolation procedure.

Preparation of rabbit antibodies against the U3 snoRNP 55 kDa protein

Although our fractionation data suggest that the 55, 50 and 15 kDa proteins are U3 snoRNP specific, we sought additional evidence supporting this conclusion. The availability of specific antibodies would allow a direct association of these proteins with

Figure 3. Three proteins of 55, 50 and 15 kDa co-purify with CHO U3 snoRNA. m₃G eluate from the anti-m₃G-immunoaffinity column (approx. 200 µg) was fractionated over a mono Q anion exchange column (100 µl bed volume). After washing, elution of snRNPs was performed using a stepwise gradient of 50 mM to 1M KCl. During elution, aliquots of 100 µl were collected. (A) Absorbance at 280 nm. The fractions are indicated at the bottom. Percent buffer B (1 M KCl) relative to buffer A (50 mM KCl) is shown on the right and the gradient profile is indicated by the black line. RNA (B) and proteins (C) were extracted and analysed as given in Figure 1. The numbers above the lanes indicate the fractions in panel A. Panel C, lanes 18 and 19: silver stain of lanes 11 and 12 containing the U3 snoRNP-specific proteins. U3 snoRNP-specific proteins are indicated on the right, sizes given are in kDa. Left side: spliceosomal snRNP proteins. Fibrillarin is indicated with a triangle.

the U3 snoRNA to be established. We therefore exploited our observation that these proteins are easily detected in the m⁷G-eluate of the anti-m⁷G-immunoaffinity column (Fig. 2B, lane 5). In particular we focused our efforts on the 55 kDa protein because of its abundance. m⁷G-eluate proteins were fractionated using SDS-PAGE, blotted onto nitrocellulose, and the region containing 55 kDa protein was excised. This nitrocellulose-bound 55 kDa protein was then implanted subcutaneously into three rabbits, one of which produced specific anti-55 kDa protein (anti-55 kDa) antibodies after the second boost. As seen in Figure 4A, Western blotted m⁷G-eluates containing 55 kDa protein as shown using Ponceau S stain (lane 2), was specifically recognised by anti-55 kDa antiserum (lane 3), but not by control anti-U1 70k protein antibody (lane 4) or pre-immune serum (lane 5). A weaker immunoreaction, with a protein of approximately 50 kDa molecular weight, is unlikely to be significant since affinity purified anti-55 kDa antibodies did not recognise a 50 kDa protein in western blots of m⁷G-eluate proteins. The anti-55 kDa antiserum therefore specifically recognises western blotted 55 kDa protein.

The interaction of the 55 kDa protein with U3 snoRNA is stable and evolutionarily conserved

We next investigated whether our anti-55 kDa antiserum, which was raised against denatured protein, could immunoprecipitate native U3 snoRNP from CHO cell m⁷G-eluates. As seen in Figure 4B, anti-55 kDa antiserum (lane 1), but not pre-immune serum (lane 2), efficiently co-precipitated U3 snoRNA from CHO cell m⁷G-eluates. The anti-55 kDa antiserum also recognised native human 55 kDa protein, since anti-55 kDa antiserum (lane 4), but not pre-immune serum (lane 3) also co-precipitate U3 snoRNA from HeLa whole-cell extracts. We conclude that the anti-55 kDa antiserum recognises native 55 kDa protein in U3 snoRNPs, and that this protein is structurally conserved between humans and rodents.

These results also illustrate the strong association of the 55 kDa protein with U3 snoRNP since these immunoprecipitations were carried out in buffers containing 300 mM NaCl, and washes were performed at 500 mM NaCl. Interestingly, when these immunoprecipitations are performed at lower salt concentrations, the U3 snoRNP is precipitated less efficiently (data not shown). We interpret this as reflecting a salt-induced loosening of the U3 snoRNP particle, possibly including the loss of constituent proteins, but resulting in improved recognition by the antibodies.

Assembly *in vitro* of U3 snoRNA-specific complexes containing the 55 kDa protein

The above results establish that the 55 kDa protein is strongly associated with, possibly even directly bound to, U3 snoRNA in native U3 snoRNPs. We were therefore interested in elucidating the structural features of U3 snoRNA that are responsible for binding the 55 kDa protein. For this purpose we developed an *in vitro* U3 snoRNP reconstitution assay. Essentially, CHO cell m⁷G-eluates were digested with micrococcal nuclease to generate a pool of free proteins, after which the nuclease was inhibited by the addition of EGTA. This protein mixture was then incubated with either wild-type or mutant ³²P-radiolabelled U3 snoRNA transcripts, obtained by *in vitro* transcription of a human U3 snoRNA cDNA. U3 snoRNAs obtained by this procedure possess the wild type 3'-end and three additional guanosine residues at the 5'-end. Interspecies differences are unlikely to be a problem in our experiments, since

U3 snoRNA sequences are known to be highly conserved between rodents and humans (30), and 55 kDa protein appears also to be structurally conserved, as our anti-55 kDa antiserum reacts with the protein from both species (Fig.4B). The products of *in vitro* reconstitution were analysed by fractionation on native polyacrylamide gels.

As seen in Figure 5A, *in vitro* reconstitution of full-length human U3 snoRNA transcripts with free m⁷G-eluate proteins resulted in a band shift on native gels, indicating complex formation (compare lanes 1 and 5), presumably of U3 snoRNP. All reconstitutions were performed in the presence of a 100-fold molar excess of unlabelled tRNA to avoid non-specific complex formation. The observed band shift is U3 snoRNA-specific, since addition of a 200-fold molar excess of unlabelled U3 snoRNA (lane 11) abolished the band shift. In contrast, the same excess of U1 snRNA had no effect (lane 9). Complex assembly was only observed with free m⁷G-eluate proteins, since untreated or mock treated m⁷G-eluates gave only weak band shifts (data not shown). *In vitro* complex assembly did not require energy, as reconstitutions were performed successfully without the addition of ATP.

By immunoprecipitation with anti-55 kDa antiserum, we have established the presence of 55 kDa protein in U3 snoRNA-specific complexes reconstituted *in vitro*. Precipitated RNAs were analysed using denaturing-PAGE and autoradiography. As seen

Figure 4. The U3 snoRNP 55 kDa protein is specifically recognised by anti-55 kDa antiserum and is conserved between humans and rodents. (A) Denatured CHO U3 snoRNP 55 kDa protein is recognised by anti-55 kDa antiserum. Nitrocellulose strips containing approx. 10 µg CHO cell m⁷G eluate proteins were incubated with anti-55 kDa antiserum (1:100 dilution; lane 3), anti-U1 70k antibody (H386 at 1:20 dilution; lane 4), pre-immune antiserum (1:100 dilution; lane 5). Coomassie R250-stained gel prior to blotting (lane 1) and Ponceau S-stained nitrocellulose after blotting, showing proteins present in m⁷G eluate. (B) Native 55 kDa protein from CHO and HeLa cells is specifically recognised by anti-55 kDa antiserum. An autoradiograph of gel fractionated ³²P-pCp labelled RNAs from various immunoprecipitations is shown. m⁷G eluate, from approx. 2 × 10⁷ cells, was used for immunoprecipitation, and the RNA content of precipitates analysed. Lane 1, anti-55 kDa antiserum; Lane 2, pre-immune serum; Lane 6, RNA content from 0.5% of material used for immunoprecipitations. HeLa whole cell extracts, from approx. 5 × 10⁶ cells, were used for immunoprecipitation and the RNA content of precipitates analysed. Lane 3, pre-immune serum; Lane 4, anti-55 kDa antiserum; Lane 5, RNA content from 0.5% of material used for immunoprecipitations.

Figure 5. Structural requirements for *in vitro* reconstitution of U3 snoRNP. (A) 32 P-radiolabelled U3 snoRNA wild type (WT) and 3'-truncated U3 snoRNAs lacking 13 (*Bst*U1), 79 (*Fnu*4H1), and 104 (*Dde*1) nucleotides were reconstituted *in vitro* with free m^7 G-eluate proteins. The products of reconstitution were then analysed using non-denaturing PAGE. Lanes 1–4, non-reconstituted RNA transcripts. Lanes 5–8, reconstitution products. Reconstitution products obtained in the presence of 200-fold molar excess of, U1 snRNA (lanes 9,10) and U3 snoRNA (lanes 11,12). (B) Sequence and structure of the human U3 snoRNA indicating restriction sites used to cleave the plasmid or PCR-amplified product to make truncated U3 snoRNA transcripts (*Rsa*I, wild type: *Dde*1, *Fnu*4H1, *Bst*U1). Evolutionarily conserved sequence motifs A, A°, B, C, D (2–5) are boxed. Start site of the U3Δ70 deletion mutant transcript is marked with a triangle.

in Figure 6, reconstituted U3 snoRNP complexes could be immunoprecipitated with anti-55 kDa antiserum (lane 9), but not with pre-immune-serum (lane 6). Immunoprecipitation required assembly of U3 snoRNP, since free U3 snoRNA was not precipitated by anti-55 kDa (lane 10) or pre-immune sera (lane 5). Reconstituted U3 snoRNPs do not contain fibrillarin since anti-Fib antibodies do not precipitate reconstituted U3 snoRNPs (lane 3). This is not surprising, as m^7 G-eluates contain only minimal amounts of fibrillarin (Fig.2B, lane 5) and fibrillarin is known to disassociate from U3 snoRNP in the presence of 300 mM KCl, as used during *in vitro* reconstitution (Fig.2B). Likewise, the failure of anti-Sm protein antibodies to immunoprecipitate reconstituted U3 snoRNP (lane 7) demonstrates that Sm core proteins do not assemble with U3 snoRNA *in vitro*. In a control experiment, the same anti-Sm antibodies efficiently co-precipitate U1 snRNAs reconstituted *in vitro* as described by Sumpter *et al.* (29) (lane 8).

To summarise, we conclude that the 55 kDa protein, and possibly also other proteins, but not fibrillarin or Sm proteins, specifically bind U3 snoRNA *in vitro*.

***In vitro* assembly of U3 snoRNP requires sequences between nucleotides 97 and 204 of U3 snoRNA, including boxes B and C**

We have begun to identify the U3 snoRNA structures required for U3 snoRNP assembly *in vitro* using 3'-truncation mutants. Truncated RNAs were obtained by run-off transcription of the cDNA template after digestion with *Bst*U1, *Fnu*4H1, and *Dde*1 resulting in U3 snoRNAs lacking 13, 79, and 113 nucleotides from the 3'-terminus (see Fig. 5B for a schematic representation).

Figure 6. *In vitro* reconstituted U3 snoRNP contain the 55 kDa protein but not fibrillarin or Sm proteins. Immunoprecipitation of reconstituted 32 P-radiolabelled U3 snoRNPs with, anti-Fib (fibrillarin) antibodies (lane 3), anti-Sm antibodies (lane 7), pre-immune serum (lane 6), anti-55 kDa antiserum (lane 9). Immunoprecipitation of non-reconstituted U3 snoRNAs with, anti-Fib antibodies (lane 4), pre-immune serum (lane 5), anti-55 kDa antiserum (lane 10). Immunoprecipitation of reconstituted U1 snRNP with anti-Sm protein antibodies (lane 8). Lanes 1 and 2, marker U1 and U3 RNA transcripts respectively.

Figure 7. U3 snoRNA structures, including the sequence motifs B and C, are required for U3 snoRNP assembly *in vitro*. Reconstitutions were performed as described in the legend of Figure 5, except that ^{32}P -radiolabelled U3 snoRNA transcripts had been pre-digested with oligonucleotide-directed RNase H. Oligonucleotides were complementary to sequences between 64–80 and 80–96 respectively. Input digested U3 snoRNAs, lanes 1, 2; Reconstituted U3 snoRNAs, lanes 3, 4; Reconstitution of U3 snoRNAs in the presence of 200-fold molar excess of U3 snoRNA, lanes 5, 6, and U1 snRNA, lanes 7, 8. Reconstitutions performed using the mutant U3 snoRNA transcript lacking 70 5'-terminal nucleotides. Free U3Δ70 RNA, lane 9; Reconstituted U3Δ70 RNA, lane 10; U3Δ70 RNA reconstituted in the presence of 200-fold molar excess of either U3 snoRNA, lane 11, or U1 snRNA, lane 12.

As seen in Figure 5A, deletion of thirteen 3' nucleotides had no effect on the reconstitution of U3 snoRNP (compare lanes 2 and 6). This reconstitution was U3 snoRNA specific since a 200-fold molar excess of U3 snoRNA (lane 12), but not U1 snRNA (lane 10), abolished complex formation. In contrast, truncated U3 snoRNAs lacking either 79 or 113 3'-terminal nucleotides did not form complexes (compare lanes 3, 4 with lanes 7, 8).

We therefore conclude that the thirteen 3'-terminal nucleotides of the U3 snoRNA, including the D box motif, are not essential for U3 snoRNP assembly *in vitro*. In contrast, deletion of 77 or 113 nucleotides from the 3'-terminus, including the C box, prevented complex assembly, suggesting the C box is required for U3 snoRNP reconstitution. These experiments do not show whether the binding requires the intact C box as such, or depends upon the complete tertiary structure of the central stem-loop region, including boxes B and C (Fig.5B).

The importance of the central stem-loop region is further emphasised by the observation that a 5'-truncation mutant, U3Δ70, in which 70 5'-terminal nucleotides of U3 snoRNA are deleted, was capable of complex formation. As seen in Figure 7, U3Δ70 transcripts assemble U3 snoRNPs efficiently *in vitro* (compare lanes 9 and 10). This assembly is U3 snoRNA-specific since the presence of a 200-fold molar excess of unlabelled U3 snoRNA (lane 12), but not U1 snRNA (lane 11), hindered complex formation. Therefore sequences between nucleotides 71 and 217 of the U3 snoRNA, including the A°, B and C boxes

Figure 8. The 55 kDa protein specifically binds to U3 snoRNA sequences located between nucleotides 97 and 204. Reconstitutions were performed as described in Figure 5, except that either wild-type or 5' and 3'-truncated U3 snoRNA transcripts were used. ^{32}P -radiolabelled wild-type U3 snoRNA transcripts were reconstituted *in vitro* and then immunoprecipitated with, anti-55 kDa antiserum (lane 3), pre-immune serum (lane 4). ^{32}P -radiolabelled U3 snoRNA transcripts, encompassing sequences between nucleotides 97 and 204 of the human U3 snoRNA were obtained using RNase H and oligonucleotide (80–96) directed digestion of U3 *Bst*U1 transcripts. Transcripts were reconstituted *in vitro* and then immunoprecipitated with anti-55 kDa antiserum (lane 5) or pre-immune serum (lane 6). Marker wild type (lane 1) and truncated U3 snoRNA (lane 2) transcripts.

are sufficient for specific complex formation with U3 snoRNA *in vitro*.

Additional evidence supporting this conclusion was obtained from the *in vitro* reconstitution of U3 snoRNA 5'- and 3'-halves. These halves were generated using oligonucleotide-directed RNase H digestion of transcripts. For this purpose, two oligonucleotides complementary to U3 snoRNA sequences between nucleotides 64–80 and 80–96 were used, resulting in two U3 snoRNA fragments (Fig.7, lanes 1, 2). Reconstitution of both U3 snoRNA digests gave identical results and will therefore be described together. The larger 3'-regions were efficiently reconstituted into specific complexes (lanes 3, 4), but not the smaller 5'-regions (lanes 3, 4). These complexes were U3 transcript specific since a 200-fold molar excess of unlabelled U3 snoRNA (lanes 5, 6), but not U1 snRNA (lanes 7, 8), completely abolished the observed band shifts. Together with our observations described in the foregoing paragraph, these results suggest that sequences between nucleotides 97 and 204 of the U3 snoRNA, including the B and C boxes, but not the A, A°, and D boxes, are sufficient for specific U3 snoRNA binding *in vitro*.

We have used immunoprecipitation with the anti-55 kDa antiserum to confirm that the 55 kDa protein is also present in complexes, reconstituted *in vitro*, containing truncated U3 snoRNAs. Truncated U3 transcripts, encompassing nucleotides 97–204, were obtained using oligonucleotide-directed RNase H

digestion of U3 snoRNA transcripts lacking thirteen 3'-end nucleotides. As shown in Figure 8, wild type U3 snoRNA (lane 1) and truncated U3 transcripts (lane 2), were reconstituted *in vitro* and immunoprecipitated with anti-55 kDa antiserum or pre-immune serum. As seen in lane 3, reconstituted wild type and, lane 5, mutant U3 snoRNP complexes were efficiently immunoprecipitated by the anti-55 kDa antiserum, but not by pre-immune serum (lanes 4, 6 respectively). Precipitation was dependent on prior complex assembly, since free RNAs (data not shown) or the U3 snoRNA 5'-half (lane 5), were not immunoprecipitated. These results clearly indicate that the 55 kDa protein binding requires sequences between nucleotides 97 and 204, including only the B and C sequence motifs.

DISCUSSION

Using anti-m₃G-immunoaffinity and mono Q anion-exchange chromatography we have identified three previously undetected proteins of 55, 50 and 15 kDa from CHO cells which co-purify with U3 snoRNA (Fig.3C). These polypeptides, which bind U3 snoRNA even in the presence of high salt concentrations (approx. 650 mM KCl or 500 mM NaCl), are probably core U3 snoRNP proteins. In contrast, the association of other U3 snoRNP proteins, such as fibrillarin, is salt-sensitive (Figs.2, 5). The identification of these latter proteins will require alternative purification procedures.

Parker and Steitz previously reported that six HeLa proteins with molecular weights of 12.5, 13, 30, 36 (fibrillarin), 59 and 74 kDa are co-precipitated together with U3 snoRNA by anti-Fib antibodies (12). Purely on the basis of size, the U3-specific 55 and 15 kDa proteins identified in this work could correspond to the HeLa 59 and 12.5 or 13 kDa proteins. However, the same authors also reported that the association of these proteins with U3 snoRNA was lost in the presence of 250 mM NaCl. In contrast, the proteins identified in this report remain bound to U3 snoRNA even at 650 mM KCl and 500 mM NaCl. Furthermore, the HeLa 59 kDa protein was also reported to associate with the 5'-half of the U3 snoRNA. In contrast, the 55 kDa protein identified in this report is clearly associated with the central part of the U3 snoRNA (Fig.8). Based on these criteria, the proteins identified in this report do not correspond to those described by Parker and Steitz (12).

Similarly, a 59 kDa protein was also found to associate with U3 snoRNA in yeast (13). This protein, SOF1, was functionally identified as a suppressor of temperature-sensitive fibrillarin mutants, and is physically associated with the yeast homologue of fibrillarin. It is tempting to speculate that SOF1 and the 55 kDa protein might be functional homologues in yeast and mammals. However, the observation that antibodies against SOF1 do not recognise the mammalian 55 kDa on western blots or in immunoprecipitation experiments (B.L., R.L., E.Hurt, D.Tollervey, unpublished data), argues against this possibility.

We have raised specific antibodies against the 55 kDa protein from CHO cells. This antiserum recognises both denatured (Fig.4A) and native 55 kDa protein in intact U3 snoRNPs (Fig.4B). The antiserum also immunoprecipitates HeLa U3 snoRNP, suggesting that this protein is structurally conserved between humans and rodents.

Using the anti-55 kDa antiserum and an *in vitro* reconstitution assay (Fig.5) we have studied the structural requirements for 55 kDa binding to the U3 snoRNA. Using 5'- and 3'-truncation mutants we have localised the 55 kDa binding site to sequences

between nucleotides 97 and 204. These sequences include the evolutionarily conserved B and C boxes but not the A, A^o and D boxes.

Given the complex nature of the RNA structure encompassed within these sequences (Fig.5A) (10,12), further experiments to define the structural determinants of 55 kDa protein binding will require the use of more subtle mutations which are less likely to cause gross structural changes. Since based on their co-fractionation during mono Q chromatography only the 55, 50 and 15 kDa proteins are required for stable U3 snoRNA binding (Fig.3C), 55 kDa protein binding to U3 snoRNA must be either direct or, alternatively, indirect via the 15 or 50 kDa proteins. Interestingly, fibrillarin binding was recently shown by such an approach, to require the conserved box C sequence (14,15). It is conceivable that the binding of the U3 snoRNP core proteins reported here could mediate the subsequent binding of fibrillarin via protein-protein interactions. In this case, the U3 snoRNA C box mutational studies may have defined the structural requirements for the binding of these proteins and only indirectly those of fibrillarin binding. Furthermore, if indeed the C box motif is part of the U3 snoRNP 55 kDa protein binding site, then, other C box containing snoRNAs may also share this protein component (see introduction) (2,3). Experiments are underway to test these, and other possibilities.

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REFERENCES

1. Moore,M.J., Query,C.C., and Sharp,P.A. (1993) In: Gesteland, R. and Atkin,J. (Eds) *The RNA world*, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (In press).
2. Filipowicz,W. and Kiss,T. (1993) *Mol. Biol. Rep.* **18**, 149-156.
3. Fournier,M.J. and Maxwell,E.S. (1993) *TIBS*, **18**, 131-135.
4. Lührmann,R., Kastner,B. and Bach,M. (1990) *Biochim. Biophys. Acta Gene Struct. Expression*, **1087**, 265-292.
5. Kass,S., Tyc,K., Steitz,J.A. and Sollner-Webb,B. (1990) *Cell*, **60**, 897-908.
6. Savino,R. and Gerbi,S.A. (1990) *EMBO J.*, **9**, 2299-2308.
7. Hughes,J.M.X. and Ares,M.,Jr. (1991) *EMBO J.*, **10**, 4231-4239.
8. Hughes,J.M.X., Konings,D.A.M. and Cesareni,G. (1987) *EMBO J.*, **6**, 2145-2155.
9. Marshallsay,C., Connelly,S. and Filipowicz,W. (1992) *Plant Mol. Biol.*, **20**, 973-983.
10. Tycowski, K., Shu, M.-D., and Steitz, J.A. (1993) *Genes Devel.*, **7**, 1176-1190.
11. Tyc,K. and Steitz,J.A. (1989) *EMBO J.*, **8**, 3113-3119.
12. Parker,K.A. and Steitz,J.A. (1987) *Mol. Cell. Biol.*, **7**, 2899-2913.
13. Jansen,R., Tollervey,D. and Hurt,E.C. (1993) *EMBO J.*, **12**, 2549-2558.
14. Baserga,S.J., Yang,X.W. and Steitz,J.A. (1991) *EMBO J.*, **10**, 2645-2651.
15. Baserga,S.J., Gilmore-Hebert,M. and Yang,X.W. (1992) *Genes Dev.*, **6**, 1120-1130.
16. Schimmang,T., Tollervey,D., Kern,H., Frank,R. and Hurt,E.C. (1989) *EMBO J.*, **8**, 4015-4024.

17. Tollervey,D., Lehtonen,H., Carmo-Fonseca,M. and Hurt,E.C. (1991) *EMBO J.*, **10**, 573–583.
18. Bringmann,P., Rinke,J., Appel,B., Reuter,R. and Lührmann,R. (1983) *EMBO J.*, **2**, 1129–1135.
19. Zalta,J., Zalta,J.-P. and Simard,R. (1971) *J. Cell Biol.*, **51**, 563–568.
20. Bourbon,H.M., Bugler,B., Caizergues-Ferrer,M., Amalric,F. and Zalta,J.P. (1983) *Mol. Biol. Rep.*, **9**, 39–47.
21. Bochnig,P., Reuter,R., Bringmann,P. and Lührmann,R. (1987) *Eur. J. Biochem.*, **168**, 461–467.
22. Bringmann,P. and Lührmann,R. (1986) *EMBO J.*, **5**, 3509–3516.
23. Bach,M., Bringmann,P. and Lührmann,R. (1990) *Methods Enzymol.*, **181**, 232–257.
24. Lehmeier,T., Foulaki,K. and Lührmann,R. (1990) *Nucleic Acids Res.*, **18**, 6475–6484.
25. Lerner,E.A., Lerner,M.R., Janeway,C.A.Jr. and Steitz,J.A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2737–2741.
26. England,T.E. and Uhlenbeck,O.C. (1978) *Nature*, **275**, 560–561.
27. Yuan,Y. and Reddy,R. (1989) *Biochim. Biophys. Acta*, **1008**, 14–22.
28. Marshallsay,C., Kiss,T., and Filipowicz,W. (1990) *Nucleic Acids Res.*, **18**, 3459–3466.
29. Sumpter,V., Kahrs,A., Fischer,U., Kornstädt,U. and Lührmann,R. (1992) *Mol. Biol. Rep.*, **16**, 229–240.
30. Mazan,S. and Bachelierie,J.-P. (1988) *J. Biol. Chem.*, **263**, 19461–19467.