The EMBO Journal vol.8 no.10 pp.3113-3119, 1989

U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleolus

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Communicated by J.A.Steitz

Using anti-(U3)RNP autoantibodies, we have isolated and characterized two additional small nucleolar RNAs from HeLa cells, which are less abundant than U3 RNA. Both RNAs possess a trimethylguanosine cap as judged by precipitation with anti-TMG antibody, but are not precipitated by either anti-Sm or anti-La antibodies. In addition, both RNAs are not precipitable by anti-Th serum, which recognizes another nucleolar RNP autoantigen. Sequence analysis revealed that one of these RNAs, 136 nucleotides long, is the human U8 homolog; while the other, 105 nucleotides long, represents a novel species which we designate U13. Both RNAs share with U3 two conserved sequences (boxes C and D). The role of one or both of these boxes in binding the common 34 kd antigenic protein, otherwise known as fibrillarin, is discussed. Fractionation of HeLa cells revealed that U8 and U13, like U3, reside in the nucleolus. In glycerol gradients both RNAs cosediment with larger structures possibly representing ribosomal precursors. We propose that U3, U8 and U13 comprise a new subset of mammalian snRNPs whose roles in ribosome biogenesis are discussed.

Key words: antibodies/HeLa cells/nucleolus/RNA processing/ snRNA

Introduction

Small nuclear ribonucleoproteins (snRNPs) are a class of metabolically stable RNA-protein complexes found in the nuclei of all types of eukaryotic cells [reviewed by Busch *et al.* (1982) and Reddy and Busch (1988)]. snRNP particles of the Sm class each contain an RNA component, called a U RNA, and a set of up to nine different proteins. All U RNAs except U6 possess a 2,2,7-trimethylguanosine (m_3G) cap structure at their 5′ ends. The highly abundant snRNPs containing U1, U2, U4/U6 and U5 RNAs are present in the nucleoplasm at 10^5-10^6 copies/cell and are precipitated by anti-Sm antibodies from patients with autoimmune diseases such as systemic lupus erythematosus (Lerner and Steitz, 1979). Their roles in pre-mRNA splicing have recently been established [reviewed by Maniatis and Reed (1987) and Steitz *et al.*, (1988)]. In addition, a number of low abundance Sm snRNPs have been reported (Strub *et al.*, 1984; Reddy *et al.*, 1985; Kramer, 1987; Tollervey, 1987b; Montzka and Steitz, 1988). One of these particles, U7, functions in histone pre-mRNA 3′ end formation [reviewed by Birnstiel and Schaufele (1988) and Mowry and Steitz (1988)] and another, U11, has been implicated in polyadenylation (Christofori and Keller, 1988).

Another abundant snRNP, U3 ($\sim 2 \times 10^5$ copies/mammalian cell), has been found in all organisms studied [reviewed by Busch *et al.* (1982) and Reddy and Busch (1988)]. It has been characterized as a distinct species which does not belong to the Sm class. Instead it is precipitated by sera from scleroderma patients which are directed against a 34 kd protein (Lischwe *et al.*, 1985), otherwise known as fibrillarin (Ochs *et al.*, 1985). The U3 RNP is located in the nucleolus (Zieve and Penman, 1976; Reddy *et al.*, 1981) and has been implicated in rRNA processing (Bachellerie *et al.*, 1983; Crouch *et al.*, 1983; Tague and Gerbi, 1984; Parker and Steitz, 1987). However, experimental evidence supporting this hypothesis is still lacking. In addition, another snRNA, U8, isolated from rat cells has also been reported to reside in the nucleolus (Reddy *et al.*, 1985).

In yeast, where the number of snRNAs seems to be much higher than in mammals [$\sim 20-30$ (Riedel *et al.*, 1986)], additional non-Sm snRNA species have been identified. They include snR3, snR4, snR5, snR8, snR9, snR10 (Tollervey, 1987a; Parker,R. *et al.*, 1988), snR128 and snR190 (Zagorski *et al.*, 1988). All these RNAs appear to be located in the nucleolus since they have been shown to be associated by RNA-RNA hybridization with various ribosomal RNA precursors.

Here, we describe two novel small nucleolar RNA species from human cells, which are less abundant than U3 RNA. One of them is $\sim 85\%$ homologous to the rodent U8 RNA and we refer to it as human U8 while the other represents a new species which we designate U13.

Results

Immunoprecipitation with anti-(U3)RNP antibodies reveals additional snRNAs in HeLa cells

snRNAs were isolated from whole cell extracts by immunoprecipitation with monoclonal 72B9 anti-(U3)RNP antibodies or scleroderma patient sera and labeled at their 3′ ends with ^{32}pCp. Figure 1A, lane 3 shows a typical pattern of immunoprecipitation by the 72B9 antibody. In addition to U3, a number of other small RNAs were precipitated. The RNAs precipitated by the 72B9 antibody were phenol extracted and re-precipitated with anti-TMG antibody directed against the m_3G cap structure (lanes 4 and 5). This re-precipitation revealed, in addition to U3, two small RNA species. Both RNAs were subjected to enzymatic sequencing (Donis-Keller *et al.*, 1977; Donnis-Keller, 1980). The sequence of their 3′ ends was further confirmed by wandering spot analysis (Silberklang *et al.*, 1979). The combination of both methods permitted identification of ~ 30 nucleotides at the 3′ end. The comparison of the obtained sequences with known snRNAs revealed that the

longer RNA is nearly identical to the 3′ portion of rodent U8 RNA (Reddy *et al.*, 1985) and we refer to it as human U8. The other represents a novel species which we shall call U13. These two RNAs may correspond to m_3G-capped RNAs precipitated with $HgCl_2$-induced anti-(U3)RNP murine sera, reported by Reuter *et al.* (1989).

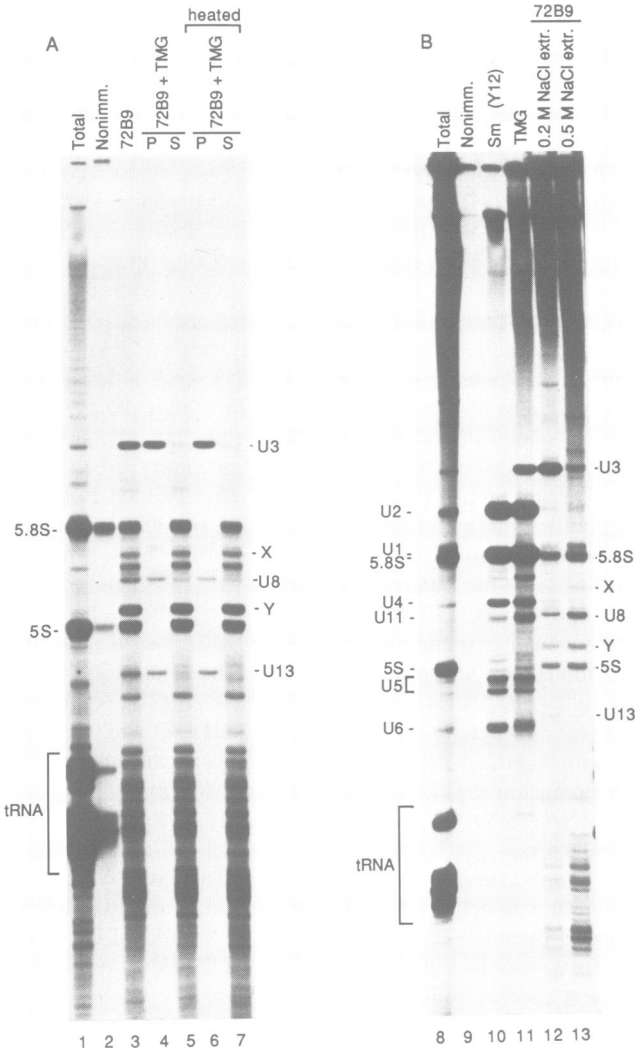

Fig. 1. Immunoprecipitation of snRNAs from HeLa cells by anti-(U3)RNP antibody. (**A**) After precipitation RNAs were 3′-end-labeled and fractionated in 8% denaturing polyacrylamide gels either directly (**lanes 2** and **3**) or after re-precipitation with anti-TMG antibody (**lanes 4–7**). Lanes are: **1**, total RNA; **2**, precipitation with nonimmune human serum; **3**, precipitation with monoclonal 72B9 anti-(U3)RNP antibody; **4**, RNA from the preparation shown in lane 3 re-precipitated with anti-TMG antibody; **5**, supernatant after precipitation shown in lane 4; **6**, RNA from the preparation shown in lane 3 heated for 5 min at 80°C then snap cooled and re-precipitated with anti-TMG antibody; **7**, supernatant after precipitation shown in lane 6. Total RNA represents 1/100 the amount of cells used in other lanes. (**B**) Cells were labeled with [^{32}P]orthophosphate *in vivo*. Lanes are: **8**, total RNA; **9–11**, precipitation with nonimmune human serum, monoclonal anti-Sm and anti-TMG antibody respectively; **12** and **13**, precipitation with monoclonal 72B9 anti-(U3)RNP antibody from extracts prepared in standard buffer containing 200 mM NaCl and from extracts prepared in buffer containing 500 mM NaCl respectively. Total RNA and the anti-Sm precipitate represent 1/100 and 1/5 the amount of cells used in other lanes respectively. RNA was fractionated on an 8% denaturing polyacrylamide gel. The positions of U8, U13 as well as other major U RNAs are indicated. The bands X and Y represent RNAs which have only been partly characterized in this paper.

In addition to U3, U8 and U13 RNAs, 72B9 monoclonal antibody (Figure 1A, lane 3) as well as patient anti-(U3)RNP sera (data not shown) reproducibly precipitated multiple RNAs which were not re-precipitated by anti-TMG antibody. These included bands designated X and Y for which partial sequences have been determined, at least two other small RNAs which run as distinct bands in an 8% polyacrylamide gel and a set of RNAs which run in the tRNA region.

To test whether precipitation of U8 and U13 by anti-TMG antibody was due to their hybridization to U3 RNA, which is known to possess an m_3G cap (Reddy *et al.*, 1979), we heated the RNA preparation for 5 min at 80°C and quick-cooled it before immunoprecipitation. This treatment, aimed at disruption of the possible complexes, did not eliminate the precipitation of U8 and U13 (Figure 1A, lanes 6 and 7) suggesting that both RNAs possess a m_3G cap.

Since labeling with ^{32}pCp can seriously misrepresent the relative abundance of RNAs, we quantitated U8 and U13 by immunoprecipitation of RNA labeled *in vivo* with [^{32}P]-orthophosphate. The quantitation of U8 and U13 relative to U3 may be inaccurate since these three snRNAs show different salt optima for extraction from the nucleus and immunoprecipitation by an anti-(U3)RNP antibody. The efficiency of U8 extraction increased gradually with increasing salt concentration (up to 500 mM NaCl studied, data not shown). This was also the case for U13 RNA. The efficiency of immunoprecipitation of U13 from extracts prepared with >400 mM NaCl, however, dropped significantly (Figure 1B, lane 13, and data not shown). The efficiency of U3 extraction was only slightly salt dependent. However, the precipitation of U3 from extracts containing >300 mM salt decreased dramatically (Figure 1B, lane 13, and data not shown). The decrease in efficiency of U3 and U13 precipitation was probably due to the dissociation of the 34 kd antigenic protein from their RNP particles. This phenomenon was reported previously by Parker and Steitz (1987). Therefore, to quantitate the amounts of U8 and U13, precipitation with 72B9 antibody was performed after sonication of HeLa cells in buffer containing either 200 mM (optimal precipitation of U3 and U13) or 500 mM (optimal for U8) salt (Figure 1B, lanes 12 and 13). The U3 and U13 bands from lane 12 and U8 from lane 13 were cut from the gel and Cherenkov counted. We estimate U8 RNA to be one fifth as abundant as U3 (4×10^4 copies/cell) and U13 about one twentieth that of U3 (10^4 copies/cell).

Since the U8 RNA from rat Novikoff hepatoma cells was surprisingly reported to be precipitated by monoclonal Y12 anti-Sm antibody (Reddy *et al.*, 1985), we tested whether this is the case for human U8 and U13 RNAs. We used the Y12 monoclone and three different patient anti-Sm sera and probed the precipitates for U8, U13 and control U3 RNA by reverse transcription using U8-21, U13-21 and U3-15 primers (see Materials and methods) respectively (Figure 2, lanes 3–6). None of these antibodies precipitated either U8 or U13. Likewise none of the three RNAs was precipitated by either anti-(U1)RNP or anti-(U2)RNP serum (lanes 7 and 8). U8 and U13, like U3, do not appear to be associated with the 50 kd La protein that binds the 3′ ends of RNA polymerase III transcripts since they were not precipitated by anti-La serum (lane 14). It is noteworthy that the human anti-Th serum which precipitates nucleolar 7-2 RNA (Hashimoto and Steitz, 1983) did not precipitate U8 and U13 (lane 13). Surprisingly, the precipitation of U13 by anti-TMG antibody from cell sonicate was very inefficient (lane 9),

perhaps because of inaccessibility of its 5′ end to antibody in a rapidly sedimenting complex [~40S (see below)].

Primary and secondary structures of U8 and U13 RNAs

About 30 nucleotides at the 3′ ends of U8 and U13 RNAs were determined by a combination of enzymatic RNA sequencing and wandering spot methods. This allowed the design of deoxyoligonucleotides complementary to the 3′ end of U8 (oligo U8−24) and U13 (oligo U13−21) which served as primers for reverse transcription. Primer extension analysis in the presence of dideoxynucleotides and chemical degradation of the full-length cDNAs then elucidated complete sequences for both RNAs. Comparison of the sequences obtained with known snRNAs revealed ~85% homology between human U8 and its rodent counterpart (Kato and Harada, 1984; Reddy *et al.*, 1985). The U13 sequence did not show significant homology to any known snRNA.

Computer modeling was used to generate secondary structures for human U8 and U13 RNAs, which are shown in Figure 3. Comparable secondary structures can be drawn for human and rat U8. Except for the third (from the 5′ end) stem−loop structure (boxed in Figure 3), nearly all nucleotide changes between human and rat sequences are located in single-stranded regions. Note that this is also the case for mouse U8 since the rat and mouse sequences are nearly identical (Kato and Harada, 1984; Reddy *et al.*, 1985).

The U8, U13 and U3 RNAs contain two highly conserved sequences, a 9-nucleotide box C and a 6-nucleotide box D (the names for these sequences have been adopted from U3).

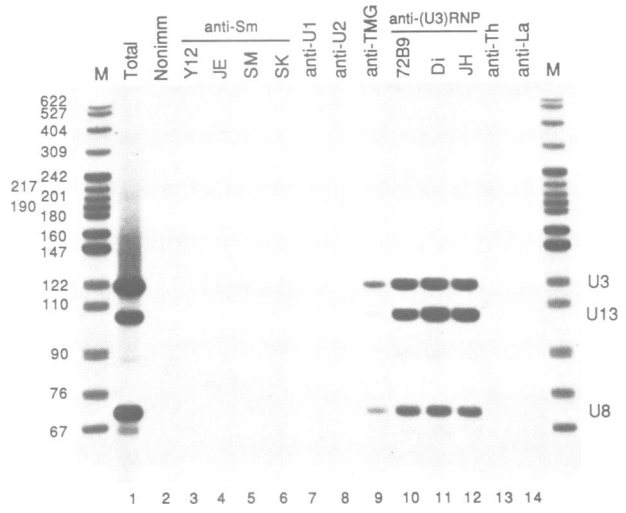

Fig. 2. Immunoprecipitation of U8, U13 and U3 RNAs by various autoantibodies. The precipitated RNA was probed for U8, U13 and U3 by reverse transcription using U8−21, U13−21 and U3−15 primers respectively. The reverse transcription products were analyzed on a 10% denaturing polyacrylamide gel. The names and specificities of the antibodies are indicated at the top. M stands for DNA markers, whose lengths are indicated at the left.

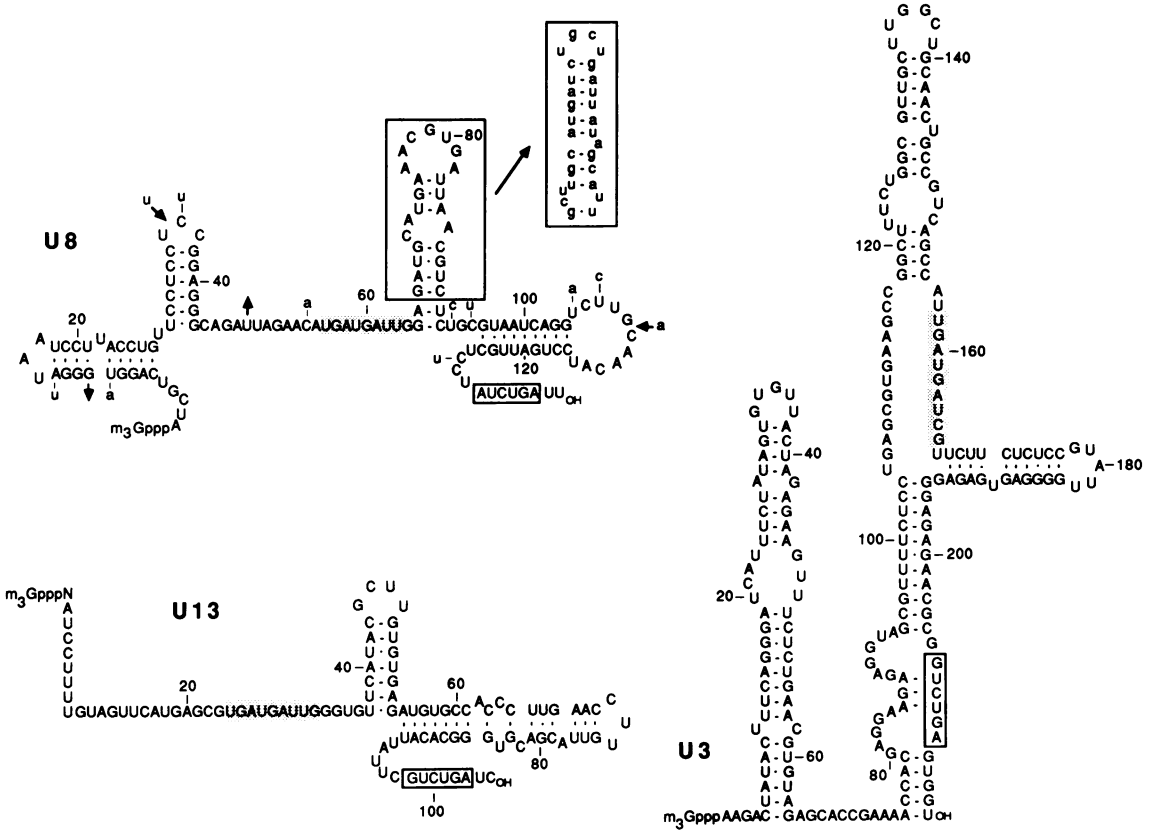

Fig. 3. Primary and possible secondary structures of human U8, U13 and U3 RNAs. The secondary structures of U8 and U13 were generated with help of the Fold and StemLoop Programs of the University of Wisconsin Genetics Computer Group. The structure of U3 is based on structural analysis (Parker and Steitz, 1987). The conserved boxes C and D have been shaded and boxed respectively. The lower-case letters mark changes between human and rat U8 RNAs. Arrows indicate deleted and inserted nucleotides in rat U8. The inset shows the boxed stem−loop as it appears in rat (Reddy *et al.*, 1985).

Table I. Compilation of 3′ terminal sequences of nucleolar snRNAs containing box D

snRNA	Organism	3′-end sequence
U3	Human	CGCG GUCUGA GUGGU
	Rat (U3B)	CGCA GUCUGA GUGGA
	X.laevis	ACAA G-CUGA GUGG
	S.cerevisiae	GGCA GUCUGA C
	S.pombe	UGUU UUCUGA CGUGU
	Broad bean	GGUG AUCUGA CAGAC
	B.mori	CGCU GUCUGA GGGGU
	D.discoideum	GGGA UUCGUA CUGGCU
U8	Human	UCCU AUCUGA UU
	Rat	UUCU AUCUGA UU
	Mouse	UCCU AUCUGA UU
U13	Human	AUUC GUCUGA UC
snR128	*S.cerevisiae*	GGAU GUCUGA GUGA
snR190	*S.cerevisiae*	GCAG AUCUGA GCC
4.5S hybRNA	Mouse	GGAU GUCUGA GCGAA
RNA X	Human	NNNN GUCUGA GAAGG
RNA Y	Human	NNNN GUCUGA GGGA
Box D consensus		PuUCUGA

The sequences were aligned around box D. They were taken from: U3; human (Suh *et al.*, 1986), rat (Reddy *et al.*, 1979), *Xenopus laevis* (Jeppesen *et al.*, 1988), yeast *Saccharomyces cerevisiae* (Hughes *et al.*, 1987), yeast *Schizosaccharomyces pombe* (Porter *et al.*, 1988), broad bean (Kiss *et al.*, 1985), *Bombyx mori* (Adams *et al.*, 1985), *Dictyostelium discoideum* (Wise and Weiner, 1980); rat U8 (Reddy *et al.*, 1985), mouse U8 (Kato and Harada, 1984), snR128 and snR190 (Zagorski *et al.*, 1988), 4.5S hybRNA (Maxwell and Martin, 1986; E.S.Maxwell, personal communication).

Both sequences are also conserved among U3 RNAs from non-mammalian organisms (Jeppesen *et al.*, 1988; Reddy, 1988; Porter *et al.*, 1988). Partial sequence analysis has also revealed the presence of box D in RNA X and RNA Y, which were precipitated by anti-(U3)RNP but not anti-TMG antibodies, as well as in some other snRNAs which seem to reside in the nucleolus (see Table I). Boxes C and D occupy similar positions in all three RNAs, namely box C is always followed by a hairpin structure and box D is situated only a few nucleotides from the 3′ end.

The U8 and U13 RNAs reside in the nucleolus

Immunoprecipitation of U8 and U13 by anti-(U3)RNP but not by anti-Sm antibodies suggested that these RNAs, like U3, might be located in the nucleolus. To test this hypothesis we fractionated HeLa cells into cytoplasmic and nuclear fractions (crude nuclei called 'nuclei I'). More highly purified nuclei ('nuclei II', see Materials and methods) were then further subfractionated into nucleoplasm and nucleoli. RNA was isolated from each fraction and probed for U8 and U13 by reverse transcription using U8 and U13 specific primers (Figure 4). As a control, each fraction was also probed for U2 and U3 snRNAs. Under the hypotonic conditions used for isolation of nuclei, U8 and U13 were retained in the nuclear fraction more efficiently than nucleoplasmic U2 RNA (compare lanes 2 and 3). Moreover, both RNAs were efficiently retained in the nuclei during their extraction with 420 mM KCl; consequently they are completely absent from nuclear extracts prepared according to Dignam *et al.* (1983), which are used for *in vitro* splicing (lane 5). Finally,

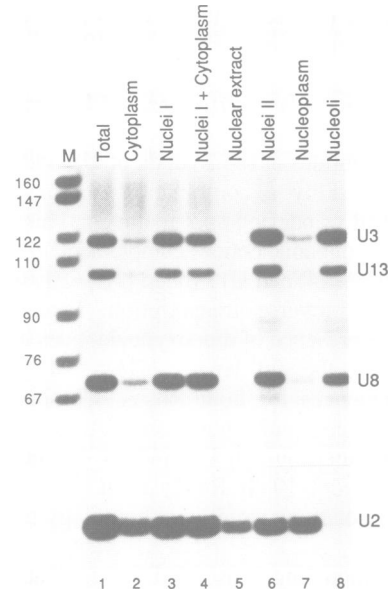

Fig. 4. Subcellular localization of U8 and U13 RNAs in HeLa cells. HeLa cells were fractionated into cytoplasmic and nuclear fractions, then the crude nuclei (nuclei I) were further purified (nuclei II) and subfractionated into nucleoplasm and nucleoli. RNA was isolated from each fraction and probed for U8 and U13 by reverse transcription using U8-21 and U13-21 primers, respectively. As controls, each fraction was also probed for U3 and U2 using U3-15 and U2-15 primers respectively. Lanes are: **1**, U8, U13, U3 and U2 in total HeLa RNA; **2**, the cytoplasmic fraction; **3**, the crude nuclear fraction; **4**, pooled nuclear and cytoplasmic fractions; **5**, 420 mM NaCl nuclear extract prepared according to Dignam *et al.* (1983); **6**, purified nuclear fraction; **7**, nucleoplasmic fraction; **8**, nucleolar fraction. M stands for DNA markers, whose lengths are indicated at the left.

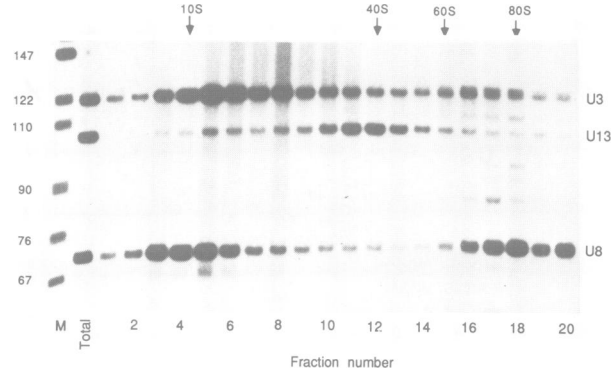

Fig. 5. Sedimentation of U8, U13 and U3 RNAs in glycerol gradients. Whole HeLa cell sonicate was fractionated in a 10–30% glycerol gradient which then was divided into 20 fractions. RNA was isolated from each fraction and probed for U8, U13 and U3 by reverse transcription using U8-21, U13-21 and U3-15 primers respectively. Products of reverse transcription were analyzed in a 10% denaturing polyacrylamide gel. 40S, 60S and 80S ribosome and 10S U1 RNP markers were run in parallel gradients. Total RNA represents one fifteenth the amount of sonicate applied to the gradient. M stands for DNA markers.

subfractionation of the purified nuclei showed that U8 and U13 were efficiently recovered in the nucleolar fraction (lane 8), whereas U2 cofractionated with the nucleoplasm (lane 7). Note that U8 and U13 fractionated in a manner simliar to U3 RNA, which has been well documented to reside in the nucleolus (Zieve and Penman, 1976; Reddy *et al.*, 1981).

Higher order complexes of U8 and U13 RNPs in glycerol gradients

The nucleolar location of U8 and U13 suggested a possible role for these RNAs in ribosome biogenesis, potentially ribosomal RNA maturation. Therefore, we asked whether U8 and U13 RNPs are associated with higher order complexes by fractionation of HeLa cell extracts in glycerol gradients. Both U8 and U13 sedimented in two populations of particles (Figure 5). About half of the U8 RNP migrated as anticipated for a monoparticle cosedimenting with U1 snRNP (~10S), while the other half migrated in a large complex cosedimenting with the 80S ribosome marker. In the case of U13 only a very small fraction was in the form of a putative monoparticle. The vast majority of U13 was present in a complex comigrating with the 40S ribosome marker. A significant amount of U3 RNA was also detected in higher order complexes (~70S) under these conditions. U3 RNA has been previously shown to be complexed with rRNA precursors (Calvet and Pederson, 1981; Epstein *et al.*, 1984).

Discussion

Immunoprecipitation of HeLa cell small RNAs with monoclonal anti-(U3)RNP antibody or scleroderma patient sera has revealed many species of small RNAs of lower abundance than U3. These newly discovered small RNAs fall into two categories: (i) RNAs possessing a trimethylguanosine cap as judged by immunoprecipitation with anti-TMG antibody; and (ii) RNAs which are not precipitable with anti-TMG antibody and thus contain either a cap other than m_3G or an unblocked 5′ end. The only known mammalian snRNA which possesses a cap other than m_3G is U6 (Epstein *et al.*, 1980); the structure of the U6 cap as a γ-monomethyl phosphate ester has only recently been identified (Singh and Reddy, 1989). The U8 and U13 RNAs, which appear to possess an m_3G cap, have been thoroughly studied in this paper. Partial sequence analysis of the other two RNAs which are not anti-TMG precipitable (bands X and Y in Figure 1) shows that they are previously uncharacterized species.

Reddy *et al.* (1985) reported that rat U8 RNA can be precipitated by anti-Sm antibodies suggesting that the sequence AUUUCCG might act as an Sm antigen-binding site. However, this sequence is not conserved in human U8 RNA. No other mammalian Sm U RNA is known to have C residues interrupting the U tract in the Sm binding site (Reddy, 1988). Moreover, recent studies by Bruzik *et al.* (1988) indicate that C residues are not tolerated in the mammalian Sm-binding site. Finally, we failed to precipitate human as well as rat (data not shown) U8 with either monoclonal Y12 or patient anti-Sm antibodies.

Sequence analysis has revealed that U8 and U13 share two conserved sequences (boxes C and D) which are also present in U3 RNA. The 9-nucleotide box C in all three RNAs is followed by a stem−loop structure. The 6-nucleotide box D is positioned only a few nucleotides from the 3′ end (Table I). Box D is also present in RNA X, RNA Y (this paper), yeast snR128 and snR190 (Zagorski *et al.*, 1988) and mouse 4.5S hybRNA (Maxwell and Martin, 1986; Trinh-Rohlik and Maxwell, 1988). All of these RNAs seem to reside in the nucleolus and some have been implicated in ribsomal RNA maturation. RNAs X, snR128, snR190 and 4.5 S hybRNA (not known for RNA Y) also possess a conserved UGAUGA sequence, which appears within and may correspond functionally to box C in U3, U8 and U13. Another human nucleolar RNA, 7-2, which is not precipitated by anti-(U3)RNP antibody, contains neither box C nor D (H.Gold and J.Craft, personal communication).

Boxes C and D had earlier been shown to be protected from nuclease digestion in the U3 RNP particle (Parker and Steitz, 1987); after digestion, fragments of U3 containing box C as well as D were retrieved by immunoprecipitation with anti-(U3)RNP antibodies, which recognize the 34 kd antigenic protein also known as fibrillarin (Lischwe *et al.*, 1985; Ochs *et al.*, 1985). This suggests that either or both boxes might serve as binding site(s) for this protein. The high conservation of boxes C and D among U3, U8 and U13 supports this hypothesis. Since box C appears within a single-stranded region adjacent to a stem−loop structure, its recognition by the 34 kd protein would parallel the situation found in nucleoplasmic Sm snRNAs, where the sequence $AU_{n\geq 3}G$ followed by a stem−loop appears to constitute a binding site for the common Sm antigen (Mattaj and De Robertis, 1985). Box D alternatively may play a role in the formation of the mature 3′ end of the RNAs in which it occurs. U3 is known to be transcribed as a precursor a few nucleotides longer at its 3′ end than the mature form (Stroke and Weiner, 1985).

The discovery of new nucleolar snRNAs poses the question of their function. The only previously known mammalian small nucleolar RNA, U3, has been implicated in pre-rRNA processing and a number of specific models have been proposed (Bachellerie *et al.*, 1983; Crouch *et al.*, 1983; Tague and Gerbi, 1984; Parker and Steitz, 1987). Recently, Stroke and Weiner (1989) have identified in rat cells an *in vivo* psoralen cross-link between U3 RNA and the 47S ribosomal precursor within 350 nucleotides from the processing site in the external transcribed spacer (ETS) (Kass *et al.*, 1987). Since the processing of pre-rRNA is a multi-step reaction, multiple snRNAs may be involved. The association of U8 and U13 with higher order complexes, which possibly contain pre-rRNA, supports this hypothesis. Alternatively, U8 and U13 may function in the assembly of ribosome particles or their transport to the cytoplasm. Interestingly, Parker,K.A. *et al.* (1988) have found an *in vitro* interaction of U3 RNP with mature 28S rRNA.

Recently, in addition to a U3 homolog, several yeast non-Sm snRNAs have been characterized (Tollervey, 1987a; Parker,R. *et al.*, 1988; Zagorski *et al.*, 1988). They are likely to be nucleolar since they are retained in the nucleus under conditions which allow leakage of Sm snRNAs and pre-mRNA and some have been found associated with rRNA precursors (Tollervey, 1987a; Zagorski *et al.*, 1988). Among eight of these RNAs studied only one, snR128, is necessary for the viability of yeast cells (Zagorski *et al.*, 1988). Yeast strains lacking another, snR10, show impaired growth and accumulate rRNA precursors (Tollervey and Guthrie, 1985; Tollervey, 1987a). It is conceivable that the dispensable RNAs perform functionally overlapping roles in essential pathways; alternatively they may mediate processes nonessential for viability. Whether similar dispensability occurs among mammalian non-Sm snRNAs remains to be established. Sequence comparisons have not revealed significant homologies between yeast non-Sm snRNAs and human U8 and U13. Thus, it is not yet clear

whether lower eukaryotes possess U8 and U13 homologs.

The U3, U8 and U13 RNPs appear to comprise a new subset of mammalian snRNPs. The U3 RNP contains at least six proteins, two of them phosphorylated (Parker and Steitz, 1987). The U8 and U13 particles share at least one of these proteins, the 34 kd antigen; the other protein components of the U8 and U13 RNPs have not yet been characterized. Intriguing questions remain to be answered concerning the assembly pathway of small nucleolar RNPs. It is conceivable that the m_3G cap acquisition by their RNAs and their cellular localization may be dictated by binding of the 34 kd antigen. This would mimic the role of the Sm-antigenic proteins in the biogenesis of the Sm snRNPs (Mattaj and De Robertis, 1985; Mattaj, 1986).

Materials and methods

Antibodies

Patient anti-(U3)RNP sera were obtained from Kathy Parker (Yale University). The monoclonal 72B9 antibody came from G.Reimer and E.Tan from the Scripps Institute (Reimer *et al.*, 1987) and patient anti-(U3)RNP sera were originally from G.Reimer (Di) and R.Sontheimer, University of Texas, Dallas (JH). The monoclonal anti-TMG antibody was donated by A.Krainer (1988). The monoclonal anti-Sm antibody (Y12) was prepared by Mei-Di Shu (Lerner,E.A. *et al.*, 1981). Additional patient sera with the following specificities were used: anti-(U1)RNP (Pettersson *et al.*, 1984), anti-(U2)RNP (Mimori *et al.*, 1984), anti-La (Hendrick *et al.*, 1981) and anti-Th (Hashimoto and Steitz, 1983).

Oligonucleotides

Oligonucleotides provided by Dr John Flory (Yale University) were synthesized on an Applied Biosystems DNA synthesizer and purified by gel electrophoresis. The following oligonucleotide primers were used: U3-15 complementary to nucleotides 106–120 in human U3 RNA, U8-21 and U8-24 to nucleotides 51–71 and 111–134 in human U8 respectively, U13-21 to positions 85–105 in U13 and U2-15 complementary to the human U2 RNA sequence in positions 28–42.

Preparation of extracts and snRNP immunoprecipitation

Preparation of HeLa cell extracts and immunoprecipitations were performed essentially as described (Lerner,M.R. *et al.*, 1981) except that cells were sonicated in NET200 buffer (40 mM Tris–HCl pH 7.5, 200 mM NaCl and 0.05% Nonidet P-40) or as otherwise stated. The sonication was performed 5 × 30 s with 30 s intervals at setting 1.5 using a Branson microtip. All immunoprecipitations and washings of the precipitates were performed in NET200 buffer. The recovered RNA was 3′ end labelled with ^{32}pCp and T4 RNA ligase (Pharmacia) (England and Uhlenbeck, 1978) and fractionated on 8% denaturing polyacrylamide gels. To prepare uniformly labeled RNA, cells were labeled with [^{32}P]orthophosphate (Mimori *et al.*, 1984). The precipitation of naked RNA with anti-TMG antibody was carried out in the presence of *Bacillus subtilis* 16S and 23S rRNA as a carrier at a concentration of 10 μg/5 × 10^6 cells.

RNA sequencing

The 3′-end-labeled RNA was subjected to partial digestion with base-specific ribonucleases T1, U2, Phy M, *Bacillus cereus* and CL3 (Pharmacia) (Donis-Keller *et al.*, 1977; Donis-Keller, 1980). Digestion products were fractionated on 20% denaturing polyacrylamide gels. The 3′ end sequences were also analyzed by the wandering spot method after partial alkali hydrolysis (Silberklang *et al.*, 1979) and the 3′ terminal nucleotides were determined by complete digestion of RNA with RNase T2 followed by TLC analysis (Silberklang *et al.*, 1979).

Primer extension and cDNA sequencing

Total cellular RNA was used as a source of templates for reverse transcription of U8 and U13 except when otherwise stated. Primer hybridization, extension reactions and sequencing by the dideoxynucleotide method were performed as described (Parker and Steitz, 1987). The full-length primer extension products were gel purified and sequenced by the chemical cleavage method (Maxam and Gilbert, 1977).

Cell fractionation

Fractionation of HeLa cells was performed according to Warner (1979) with slight modifications. Cells (1.5 × 10^8) were collected by centrifugation and washed with 100 ml TBS buffer (40 mM Tris–HCl, pH 7.5, 150 mM NaCl). After centrifugation the cells were suspended in 4 ml of buffer A (10 mM HEPES KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT) and swelled for 10 min on ice and then disrupted by 12–15 strokes in a tight-fitting Dounce homogenizer. The extract was centrifuged for 10 min at 3000 r.p.m. in a Sorvall SS34 rotor to pellet nuclei. The supernatant was considered the cytoplasmic fraction and the pellet as a crude preparation of nuclei called 'nuclei I'. Both nuclear and cytoplasmic fractions were sonicated as described earlier and centrifuged for 10 min at 10 000 r.p.m. (SS34 rotor). The supernatant was used in each case for RNA isolation.

For further subfractionation, 'nuclei I' were suspended in 10 ml of buffer containing 10 mM Tris–HCl, pH 7.5, 3.3 mM $MgCl_2$, 0.25 M sucrose and centrifuged for 5 min at 1000 r.p.m. The pellet was suspended in 2.5 ml of 10 mM $MgCl_2$ and 0.25 M sucrose, layered over an equal volume of 0.35 M sucrose and 0.5 mM $MgCl_2$ and centrifuged for 10 min at 2500 r.p.m. The supernatant was discarded and the nuclei were suspended in 2.5 ml of 0.35 M sucrose and 0.05 mM $MgCl_2$. To prepare nucleoli the nuclei were disrupted by sonication (4–5 × 15 s) with a Branson Sonifier equipped with a microtip (setting 1). The nuclei were checked microscopically for lysis after staining with azure C (Muramatsu *et al.*, 1963). The sonicate, called 'Nuclei II', was layered over a 2.5 ml cushion of 0.88 M sucrose and 0.5 mM $MgCl_2$ and centrifuged for 10 min at 4000 r.p.m. The upper two thirds was considered the nucleoplasm and the pellet contained nucleoli. Both fractions were used for RNA isolation. The fractionation was performed at 4°C and all centrifugations were carried out in Sorvall SS34 rotor.

RNA isolation

Each cellular fraction, as well as total cell sonicate, was made to contain 20 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.5% SDS and 500 μg/ml proteinase K (Beckman). Digestion was carried out at 37°C for 30 min, then nucleic acids were extracted twice with PCA (phenol:chloroform:isoamyl alcohol = 50:48:2), ethanol precipitated and resuspended in buffer containing 40 mM Tris–HCl, pH 8.0, 10 mM NaCl, 6 mM $MgCl_2$, 5 mM DTT and RNasin (Pharmacia) at 10 U/ml. DNase (Promega) was added to a final concentration 10 U/ml and DNA digestion was carried out for 30 min at 37°C. RNA was extracted with PCA and ethanol precipitated.

Glycerol gradients

The whole cell sonicate was fractionated on 10–30% glycerol gradients containing 20 mM HEPES KOH, pH 7.9, 60 mM KCl and 1 mM $MgCl_2$. Centrifugation was performed at 25 000 r.p.m. for 15 h at 4°C in a SW41Ti rotor. Gradients were fractionated into 20 samples, RNA was extracted with PCA, ethanol precipitated and analyzed by primer extension. Primers U8-21, U13-21 and U3-15, specific for U8, U13 and U3 respectively, were used.

Acknowledgements

We thank K.Parker, G.Reimer, E.Tan, R.Sontheimer and A.Krainer for generously providing antibodies, K.Parker, S.Lee, J.Bruzik, K.Montzka, S.Baserga and other members of the Steitz lab for helpful advice and discussions. We also thank A.Pinto, U.Bond and D.Wassarman for critical reading of the manuscript. This work was supported by grant GM26154 from the National Institutes of Health.

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Received on June 14, 1989; revised on July 4, 1989