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Association of the lupus antigen La with a subset of U6 snRNA molecules

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ABSTRACT

U6 snRNA is a component of the major class of small RNA-protein complexes, the Sm snRNPs, present in mammalian cell nuclei. Here we report that a substantial fraction (about 10%) of U6 RNA from human and mouse cells is associated with another lupus antigen, the 50 kd La protein. The La-bound U6 subpopulation is characterized by 3' end heterogeneity and partial undermethylation. These U6 molecules have U-rich 3' termini that could be responsible for their selective association with the La protein. The question of whether they are precursors to the major U6 RNAs found in Sm snRNPs is discussed.

INTRODUCTION

Antibodies from patients with autoimmune disorders have widely been used to study the structure and function of different classes of small ribonucleoprotein (RNP) complexes from eucaryotic cells (1-5).

One class of RNP complexes is recognized by anti-La sera (2), also known as anti-SS-B or anti-Ha (6,7). Anti-La antibodies specifically react with a mammalian cell protein of 50 kd (8-11), which is at least transiently associated with every known RNA polymerase III transcript (9,12). La RNPs therefore contain precursors to 5S and tRNAs (12), 7-2 RNA (4), 7SL RNA (13), two mouse 4.5S RNAs (3), Ro small cytoplasmic RNAs (3), and *in vitro* transcripts of Alu family DNA sequences (14). RNA polymerase III transcripts like VA I and II and EBER 1 and 2 RNAs encoded by adenovirus and Epstein Barr virus, respectively (2,15, 16), are likewise contained in La RNPs from infected cells.

Recently, association of the La antigen with small RNAs transcribed by other RNA polymerases has also been reported. Leader RNAs of vesicular stomatitis virus (17,18), which appear to be synthesized by the viral polymerase, are La-bound to a high degree. A small fraction of U1 RNAs [made by RNA polymerase II (19)] is precipitable by anti-La sera and is enriched in longer U1 RNA molecules (20).

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Here we show that a heterogeneous subset of snRNA U6 is likewise associated with the La antigen. The majority of U6 RNA molecules are known to be constituents of the small nuclear RNP (snRNP) class reactive with anti-Sm antibodies (1). The minority of U6 molecules that are La-associated are partially undermodified and have 3' end heterogeneity. The relationship of these U6 RNAs to the U6 RNAs found in Sm snRNPs is discussed.

MATERIALS AND METHODS

Cell Lines and Antibodies

HeLa and Ehrlich ascites cells were derived from standard laboratory stocks. Both cell lines were maintained on RPMI1640 (Gibco), supplemented with 5% heat-inactivated bobby calf serum (Gibco), 60 µg/ml penicillin and 100 µg/ml streptomycin. Cells were maintained at 37°C in 5% CO_2 at a density of approximately 3 x 10^5 cells/ml. Sera from patients with systemic lupus erythematosus or related autoimmune disorders were provided by Dr. John Hardin (Yale University). Antibodies were prepared from sera and characterized as described previously (3). Anti-La antibodies from three different patients gave identical results.

Preparation of ^{32}P-labeled RNA

To prepare ^{32}P-labeled RNA, usually 250 ml of HeLa or Ehrlich ascites cells at a density of 2 x 10^5 cells/ml in phosphate-free minimal essential medium (Gibco) were exposed to 40 µCi/ml $^{32}PO_4$ for 17 h. Cell sonicates, prepared according to the method of Lerner et al. (16) were subjected to either immunoprecipitation (1) or direct phenol extraction and subsequent ethanol precipitation of the RNAs. Such RNAs were used for hybridization experiments or fractionated directly by gel electrophoresis (see below).

Recombinant DNA

The recombinant λ phage U6.7 containing sequences complementary to U6 RNA was isolated from a genomic library of 15 kb partial EcoRI fragments of human placental DNA in the λ vector Charon 4A kindly provided by Dr. S.M. Weissman. This clone was originally selected by screening plaques by the method of Benton and Davis (21) using as probes fractions of immuno-precipitated HeLa cell La RNAs that had been labeled *in vitro* with 5' ^{32}P-pCp and T4 RNA ligase. The 4.6 kb EcoRI fragment carrying the sequences complementary to U6 RNA were subcloned into pBR325 (22),

designated recombinant plasmid pJU6. The recombinant plasmid pJU6.2 was derived from pJU6 by subcloning into pBR322 the 1.7 kb genomic fragment which contains the U6 pseudogene and an Alu family sequence (H. Theissen, J. Rinke, C. Traver, B. Appel, manuscript in preparation).

Plasmid DNA from the U1 pseudogene U1.15 and the U2 pseudogene U2.7 respectively were gifts from Dr. A. Weiner and colleagues (23,24). The recombinant DNA clone containing an Alu-family sequence subcloned from the human β-like globin gene cluster was a gift from Dr. C. Duncan (25).

RNA Analyses

Hybridization of RNA to purified DNA immobilized on nitrocellulose filters has been described previously (12). Generally 2 µg of recombinant plasmid and 20 µg of recombinant phage DNA were used in hybridization experiments.

RNAs were fractionated on 10% polyacrylamide gels containing 7 M urea (3). Individual bands were extracted by the crush and soak method (26). RNase T_1 fingerprints of eluted RNAs were performed as described by Barrell (27) with thin layer homochromatography on PEI plates (Brinkmann) in the second dimension (1).

Transfer of RNAs from gels to diazobenzyloxymethyl-paper was performed according to a modified method of Alwine et al. (28), as described by Ciechanover et al. (29). Hybridization of nick-translated plasmid to the RNA covalently attached to the paper was performed as described (28). The probe consisted of 1 µg nick-translated (30), linearized plasmid pJU6.2.

Quantitation of RNAs in gels and Northern blots was accomplished by densitometry of the appropriate bands, having selected the exposure times for autoradiography to ensure that the curve of dpm versus silver grains was always in the linear range.

RESULTS

Identification of U6 RNA Species in the La RNA Spectrum

The majority of the complex mixture of La RNAs from mammalian cells fall in the size range of 80-110 nucleotides. Examination of individual RNAs from such a heterogeneous population is facilitated by hybrid selection with appropriate DNA sequences (12). To ask whether U6 RNA (106 nucleotides) might be present in the La RNA mixture, La RNAs obtained by immune precipitation from *in vivo* ^{32}P-labeled HeLa cells extracts were hybridized to recombinant DNA containing sequences complementary to human

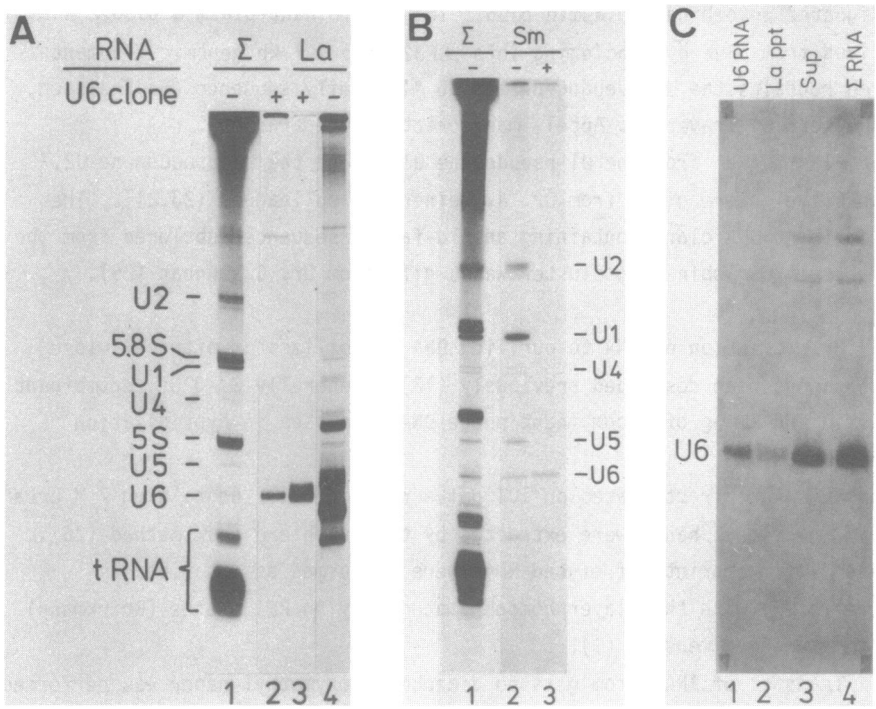


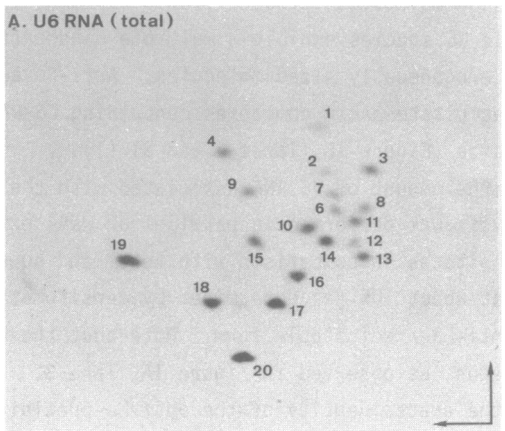
Figure 1. Detection of anti-La reactive U6 RNAs by hybridization to a U6 RNA pseudogene.
In vivo ^{32}P-labeled total HeLa cell RNAs, anti-La reactive RNAs, and anti-Sm reactive RNAs were hybridized to immobilized recombinant DNA from the λU6.7 clone as described in Materials and Methods. U6 RNAs selected from total RNAs (A, lane 2), from La RNAs (A, lane 3) and from Sm RNAs (B, lane 3) were separated on a 10% polyacrylamide gel. Total small RNAs (A and B, lanes 1), La RNAs (A, lane 4) and Sm RNAs (B, lane 2) represent 1% of the RNA amount used in the respective hybridization. All lanes in A and in B, respectively, are from the same gel with the same exposure time, except for lane A1 (Σ) which was exposed 1/10 as long as the other lanes. (C) Extracts of HeLa cells were precipitated with anti-La antibodies. The anti-La reactive RNAs (lane 2), supernatant (lane 3) and an equivalent amount of the original extract (lane 4) were electrophoresed together with gel-purified U6 RNA (lane 1) on a 10% polyacrylamide gel. The gel was transferred to diazobenzylmethyl paper, probed with nick-translated plasmid pJU6.2, and the bands quantitated as described in Materials and Methods. (The nature of the higher molecular weight RNAs in lanes C3 and 4 is currently under investigation. The two bands migrating faster than U6 in lane B2 are most likely breakdown products of the Sm RNAs.)

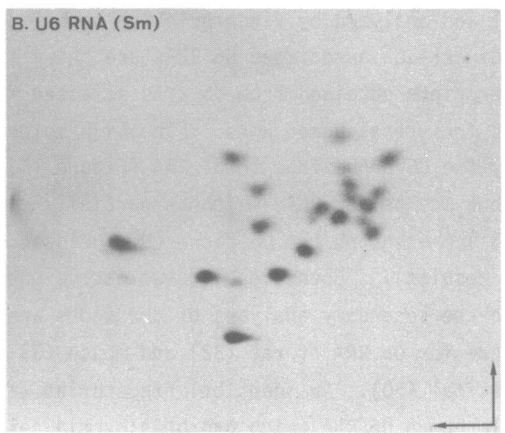
U6 RNA. The selected RNAs were eluted and displayed on a polyacrylamide gel (Figure 1A, lane 3) adjacent to U6 RNA selected by hybridization of total in vivo-labeled RNA to the U6 DNA probe (Figure 1A, lane 2). In

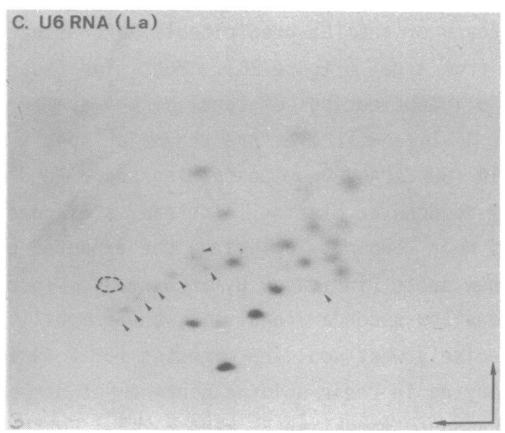
contrast to the U6 RNA molecules selected from total RNA, the anti-La-precipitable U6 species exhibit a multiple band pattern indicating the presence of heterogeneously sized molecules. Anti-Sm antibodies, on the other hand, precipitate snRNP complexes containing U6 RNA of predominantly one size (Figure 1B, lanes 2 and 3) (1).

To quantitate the amount of U6 RNA associated with the La antigen, northern blot analysis was performed in parallel on RNAs extracted from anti-La immunoprecipitates in comparison with total and supernatant RNAs. Figure 1C shows that about 10% (as determined by densitometry) of the U6 RNA exists in an anti-La-precipitable form. Note that the band is again somewhat heterogeneous, as observed in Figure 1A, lane 3.

To determine the exact identity of the anti-La-precipitable U6 RNA population, hybrid selected RNAs were extracted after gel fractionation (Figure 1A, lane 3) and analyzed by fingerprinting techniques. RNase T_1 fingerprints of HeLa cell La-associated U6 RNAs are shown in Figure 2 in comparison to fingerprints obtained from U6 RNAs selected from Sm-precipitable and from total human RNAs. [The high molecular weight RNA species selected by the U6 probe from total RNA (Figure 1A, lane 2) was not further analyzed, but may be 7SL RNA, which is partially complementary to Alu-sequence DNA (31) present on the U6 clone (H. Theissen, J. Rinke and B. Appel, unpublished results).] Both the oligonucleotide pattern of total U6 RNA (Figure 2A) and the secondary analyses of the spots are consistent with the reported sequence for U6 RNA of rat (32) and mouse (33), recently reported to be identical (50). An identical fingerprint was obtained for anti-Sm-precipitable human U6 RNA which had been hybrid selected and gel fractionated (Figure 2B). Several differences, however, are seen in the oligonucleotide pattern of anti-La-precipitable RNAs selected by the U6 clone and then cut from a gel (Figure 2C). Most significantly, although all other RNase T_1 oligonucleotides of total U6 RNA are present (demonstrating that U6-like molecules are the major species selected by the cloned DNA), spot 19 ($UUCCAUAUUUU_{OH}$) containing the 3' end of the molecule is greatly reduced or missing. Instead, a diagonal stretch of minor spots appears near (but <u>not</u> including) the expected position of spot 19. Although the new spots (indicated by arrowheads, Figure 2C) were too low in abundance to allow secondary analysis, their mobilities relative to one another are precisely what would be expected for a family of 3' oligonucleotides varying in their uridine-content [as has been documented for the 3' end of La-bound mammalian 5S rRNA (12)]. Further differences in


A. U6 RNA (total)
1
2
3
4
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20


B. U6 RNA (Sm)


C. U6 RNA (La)

the fingerprint can be ascribed to the underrepresentation of oligonucleotides containing modifications (e.g. 2'-O-methylation); U6 RNA possesses a high number of modified nucleotides in the center of the molecule (32,33). For example, oligonucleotide AmAG (spot 7) is clearly reduced in comparison to its unmethylated analogue AAG (spot 6). Likewise, the new spot (also indicated by an arrow) below oligonucleotide 14 (CmCCmCmUG) has a position expected for an undermethylated variant of this T_1 oligonucleotide. Such partial undermethylation as well as 3' end heterogeneity demonstrate that the U6 RNAs precipitable by anti-La antibodies comprise a distinct subset of the total population and represent a mixture of variant molecules.

Reaction of Anti-La Sera with U6 RNA Complexes from Mouse

The association of the La antigen with a mixture of U6 RNA variants was confirmed for mammalian cells other than human. Anti-La-precipitable U6 RNAs from Ehrlich ascites cells also exhibit a multiple band pattern (Figure 3, lane 2) while U6 RNA selected from total mouse RNA migrates as a single sharp band (Figure 3, lane 1). The RNA running below U6 RNA (Figure 3, lanes 1 and 2) is most likely 4.5S RNA (34) selected by the Alu-family sequence present on the U6 clone (H. Theissen, J. Rinke and B. Appel, unpublished results). This RNA is found in rodent cells (34,35) but not in human cells. It has extensive homology to Alu-type DNA sequences (36) and has been shown to be bound by the La antigen (3).

Do U RNAs Other Than U6 RNA Associate with the La Antigen?

To examine whether subsets of other abundant snRNAs are also bound by the La protein, *in vivo* ^{32}P-labeled La RNAs from HeLa cells were simultaneously hybridized to recombinant DNA carrying sequences complementary to U1, U2 and U6 RNAs, respectively (Figure 4, lanes 2-4).

Figure 2. RNase T_1 fingerprints of U6 RNA species selected from La RNAs, Sm RNAs and from total HeLa cell RNAs.
U6 RNA species selected by hybridization of *in vivo*-labeled total RNA (A), Sm RNA (B) or La RNA (C) to clone λU6.7 (see Figure 1A, lanes 2 and 3, and 1B, lane 3) were extracted from a 10% gel and subjected to RNase T_1 digestion. The digests were separated by electrophoresis on cellulose acetate (horizontal dimension) and by homochromatography on PEI thin-layer plates (vertical dimension). Nomenclature of the oligonucleotides is according to Epstein et al. (32). Sequence of oligonucleotides of U6 RNA isolated from total RNAs (A) is as follows:
1: G. 2: AG. 3: CG. 4: UG. G: AAG. 7: AmAG. 8: CAG. 9: AUG. 10: CUCG. 11: CAAmG. 12: AACG. 13: ACmACG. 14: CmCCmCmUG. 15: CUUCG. 16: AΨACm^{6}AG. 17: CAAAUΨCG. 18: AUUAmGmCAUG. 19: UUCCAUAUUUU$_{OH}$. 20: CACAUAUACUAAAAΨUG.

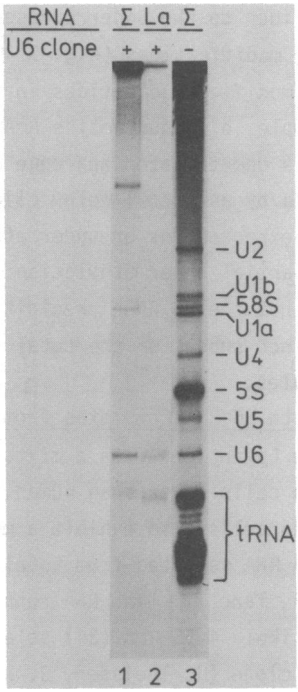


Figure 3. Analysis of anti-La reactive U6 RNAs from mouse.
An experiment similar to that described in Figure 1 was performed using in vivo ^{32}P-labeled Ehrlich ascites cells. RNAs selected by λU6.7 DNA from total mouse cell RNAs (lane 1) and from La RNAs (lane 2) were separated on a 10% polyacrylamide gel, along with 1% the amount of total mouse small RNAs (lane 3).

Since the amounts of DNA coding regions for each of these snRNAs were equivalent and were present in vast excess over the RNAs during the hybridization, the amounts of the various snRNAs selected (Figure 4) gives a rough estimate of their relative abundance in the La RNA mixture. Based on our prior finding (Figure 1C) of about 10% U6 RNA in the anti-La precipitate and on the 10-fold higher abundance of U1 relative to U6 RNA, we calculate that less than 0.1% of U1 RNA is anti-La precipitable. [Although differential hybridization efficiencies of various small RNAs to their complementary DNAs makes this number approximate, it agrees well with the amount of the U1 band seen in anti-La precipitates (compare lane 4 with lane 1, Figure 1).] No detectable U2 RNA or in vivo transcripts of Alu-family sequences (37,38) can be detected in the La RNA spectrum (Figure 4, lanes 3 and 5).

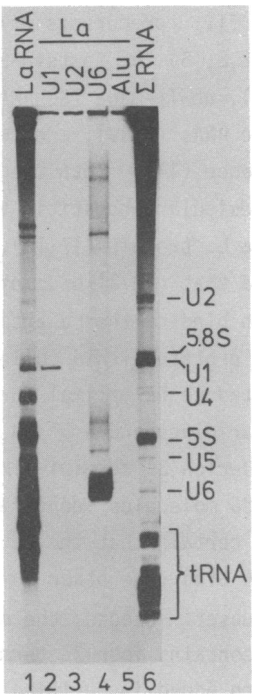


Figure 4. Hybridization of La RNAs from HeLa cells with DNA sequences complementary to snRNAs U1, U2 and U6 and to Alu DNA.
In vivo ^{32}P-labeled RNAs from anti-La immune precipitates were hybridized to recombinant DNA containing pseudogenes for U1, U2 and U6 RNA and to an Alu family sequence as described in Materials and Methods. RNAs selected by the U1 clone (lane 2), the U2 clone (lane 3), the U6 clone λU6.7 (lane 4) and the Alu family sequence (lane 5) were separated on 10% polyacrylamide gel. Lanes 1 and 6 show La RNAs and total small RNAs from HeLa cells, respectively. Total La RNAs (lane 1) represent 1% the amount used in the hybridization experiments (lanes 2-5).

DISCUSSION

In this report we show that a subset of U6 RNAs from human and mouse tissue culture cells are associated with the 50 kd protein carrying the La antigenic determinant(s). This U6 RNA subpopulation contains molecules of different size, many longer than the total or anti-Sm precipitable U6 RNAs (Figure 1). Fingerprint analysis of the La-associated U6 RNA species from HeLa cells (Figure 2) revealed heterogeneous 3' ends (apparently U-rich) and partial undermethylation.

All RNAs previously shown to be associated with the La protein have oligo(U) tracts at their 3' ends. These include many well-analyzed RNAs

transcribed by RNA polymerase III: precursors of 5S rRNA and of tRNA (12), adenovirus VA RNAs I and II (2,8,15) the Epstein-Barr virus encoded small RNAs EBER 1 and EBER 2 (15,16), newly-made 7SL RNA (13), two mouse 4.5S RNAs (3), Ro small cytoplasmic RNAs (3,39), and two small RNAs transcribed in vitro from an Alu DNA sequence (14). Both the plus-strand and minus-strand leader RNAs of vesicular stomatitis virus terminate in U-rich sequences and are bound by the La protein (17,18). Moreover, in vitro binding studies have specified that the 3' ends of VA I RNA (8,40) and $4.5S_I$ RNA (41) are involved in binding the La antigen. Interaction of the biochemically purified La protein with a collection of model tRNA precursors has been demonstrated to be optimal for molecules possessing at least three or four terminal uridines and a 3' OH group (11).

Do their heterogeneous, U-rich 3' termini likewise account for the association of the subset of U6 molecules identified here with the La protein First, we cannot be certain that the La antigen binds directly to these RNAs. Instead, it could bind some other component that selectively associates with this U6 RNA subset. Second, the majority of U6 molecules present in Sm snRNPs already contains four 3' terminal U residues and can be bound by the La protein when added in purified form to cell extracts (41). Hence, we might ask instead why all U6 RNAs are not in La RNPs. Third, the length of time La associates with various RNA polymerase III transcripts is highly variable. Therefore, what remains to be concluded from the current study is that a distinct subset of U6 molecules associates (directly or indirectly) with the La protein either more tightly or more stably than the majority of U6 RNAs.

How do the aberrant U6 RNA molecules associated with the La protein arise? 1) They could be transcribed from expressed U6 pseudogenes or variant genes and remain undermodified perhaps because they cannot be properly assembled into particles; to date, several U6 pseudogenes, but only one real gene, have been isolated from mammalian DNA (42,43; H. Theissen, J. Rinke, C. Traver, B. Appel, in preparation). 2) Alternatively, they could arise by post-transcriptional addition of U residues to slightly truncated 3' ends on a fraction of normal U6 RNAs. Such U addition has been observed for a 4.5S RNA from murine cells (44) and could occur because of improper protein packaging of these U6 molecules. 3) They could conceivably be authentic precursors to the major U6 RNA, but this seems unlikely since spot 19 (the normal 3' end) is not included in the trail of 3' terminal oligonucleotides (Figure 2C). Longer forms of U1 RNA

found in association with the La protein have previously been suggested to be U1 precursor molecules and to be selectively present in the cytoplasm (20). We have confirmed that U1 RNA can be precipitated by anti-La sera, but to a considerably lower extent than U6 RNA species (about 0.1% as compared to 10%, Figure 4). Moreover, we find that virtually all La protein-bound RNAs leak rapidly into the cytoplasm during aqueous cell fractionation procedures (unpublished observations), making conclusions concerning their in vivo localization difficult. Our observation that no U2 RNA is detected in La RNPs from HeLa cells is in accord with the previous report (20).

In general, U6 RNA has been regarded as a member of the snRNP class recognized by anti-Sm antibodies, which also includes U1, U2, U4, and U5 snRNAs (1). Yet, it exhibits some unusual features with respect to these other U RNAs. 1) U6 lacks both the so-called domain A, a single-stranded region bounded by two hairpins near the 3' terminus (45), which has been suggested to be the primary binding site for one or more of the antigenic snRNP proteins (46), and the 5' cap structure containing 2,2,7-trimethylguanosine (32,33). Instead, the 5' end of U6 RNA is blocked in some other way (32). 2) Recent work shows that U6 is not contained in a separate anti-Sm-precipitable RNP, but rather in a particle together with snRNA U4 (47,48). 3) It remains unclear whether U6 is synthesized by RNA polymerase II, as are the other U RNAs (19), or by RNA polymerase III; U6 RNA contains sequences that resemble other class III genes (49) and lacks a standard 5' cap (32). To this list can now be added our current finding that a significant fraction of U6 RNA exists as La RNPs. Clearly, many further studies will be required to elucidate the biosynthesis and functioning of this particular snRNA.

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