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Three small nucleolar RNAs of unique nucleotide sequences

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ABSTRACT **Three small RNA species were detected in human cells, and their cDNAs were synthesized and cloned. These RNAs are nucleolar, are 207, 154, and 135 nucleotides long, and are named E1, E2, and E3, respectively, and their unique nucleotide sequences suggest that they may belong to an additional family of small nucleolar RNAs. The 5′ ends of these three RNAs do not appear to have a trimethylguanosine cap or another type of cap. Apparent homologs of these three RNAs were detected in mouse, rabbit, and frog cells, suggesting their universal importance. They are housekeeping RNA species, since they are present in all rabbit tissues analyzed.**

The formation of cytoplasmic ribosomes in eukaryotic cells involves a complex series of synthesis, processing, assembly, and transport steps, many of which are poorly understood (reviewed recently in refs. 1 and 2). Some small nucleolar RNA (snoRNA) species, such as U3 RNA and yeast snR10 and U14 RNA, are required for rRNA precursor (pre-rRNA) processing (3–7). It has been difficult to study some of the low-abundance small nuclear RNAs in the absence of specific DNA or antibody probes. We have made cDNA probes for three snoRNAs and report some of the properties of these snoRNAs.‡

MATERIALS AND METHODS

General Methods. The following procedures were done as described: preparation of nuclear and cytoplasmic fractions from HeLa cells (8); isolation of nucleoli (9); isolation of RNA (10); hybrid selection of RNA (8); 10% polyacrylamide gel electrophoresis (8); electrophoresis of RNA in 1% agarose and 2.2 M formaldehyde (11); RNA sequencing by the dideoxynucleotide method, using HeLa cell nucleolar RNA, avian myeloblastosis virus reverse transcriptase, and sequence-specific, antisense radioactive primers (12); and immunoprecipitation assays (13). The recommendations of the corresponding manufacturers were followed for (*i*) hybridization with ZetaProbe membranes (Bio-Rad) and (*ii*) sequencing of supercoiled DNA by dideoxy chain termination using Sequenase (United States Biochemical) or *Taq* I DNA polymerase (Promega). RNA was transferred to ZetaProbe membranes by electroblotting from polyacrylamide gels and by capillary blotting from agarose gels.

Synthesis and Cloning of cDNA. Nuclear RNA from HeLa cells was fractionated by 40-cm-long, 10% polyacrylamide gel electrophoresis. The RNA corresponding to the sections of RNA bands 1, 2, and 3 (lane 1 in Fig. 1) was eluted, and poly(A) tails were added to the 3′ ends. After synthesis of double-stranded cDNA, it was dC tailed and annealed with *Pst* I-cut, dG-tailed pBR322. Competent DH5α bacteria (GIBCO/BRL) were transformed with these plasmids, generating three cDNA libraries. The cDNA libraries were screened with probes made from the starting gel fractions of small nuclear RNA (reverse transcribed with random primers) and DNA probes for known RNA species (e.g., U1, U2, U3, U4, U5, 7SL, 5S, 5.8S RNAs, and the gene for 47S pre-rRNA).

Antibodies and DNA Clones. We were kindly supplied with anti-N^2,N^2-7-trimethylguanosine (anti-m_3G) antibodies by R. Lührmann (Philipps University, Marburg, F.R.G.), plasmid pHU1-1, which carries a human U1 RNA gene, by E. Lund and J. E. Dahlberg (University of Wisconsin), and plasmid pU3B.4, which bears a rat U3 RNA sequence, by R. Reddy (Baylor College of Medicine).

Radioactive Hybridization Probes. The E1, E2, and E3 sequences in the corresponding cDNA clones, minus the tails that had been added for cDNA synthesis and cloning, were amplified by the polymerase chain reaction (14). The amplified products were then used as templates for extension of E1-, E2-, and E3-specific, antisense 3′ end primers with the Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP to generate the respective labeled, sequence-specific DNA probes. The radioactive DNA probe for U1 RNA was made by extension of a U1-specific primer using as template the human U1 gene plasmid, linearized near the 5′ end of the U1 gene. The radiolabeled DNA probe for U3 RNA was made by random primer labeling (15) using the rat U3 RNA sequence plasmid as template.

RESULTS

There were some minor small RNA bands in HeLa cell nuclear RNA (bands labeled 1, 2, and 3 in lane 1 of Fig. 1) that could not be hybrid selected with any of the available DNA clones for small RNAs. RNA from these three sections of the gel was used to make three cDNA libraries. Clones from these libraries were used to probe Northern blots of HeLa whole cell RNA that had been fractionated by 10% polyacrylamide gel electrophoresis. One of the clones isolated from each cDNA library hybridized to a single RNA band whose electrophoretic mobility was similar to that of the RNA used to make the corresponding library (Fig. 1, lanes 2–4). These probes did not hybridize to any high molecular weight RNA from whole cells (Fig. 1, lanes 5–7), implying that the RNAs named E1, E2, and E3 in this report are not breakdown products of large RNA.

The primary structure of E1, E2, and E3 RNAs first was determined by sequencing their cDNA clones and then was confirmed by sequencing RNA from a HeLa cell nucleolar fraction (Fig. 2). The nucleotide sequences of these three small RNAs do not have any obvious homology with those of any other small RNAs, large RNAs, and genes of any species, according to searches in sequence data banks. Based on sequencing, E1, E2, and E3 RNAs are 207, 154, and 135

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Abbreviations: snoRNA, small nucleolar RNA; pre-rRNA, rRNA precursor; m_3G, N^2,N^2,7-trimethylguanosine.

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L07382, L07383, and L07384 for the sequences of E1, E2, and E3 RNAs, respectively).

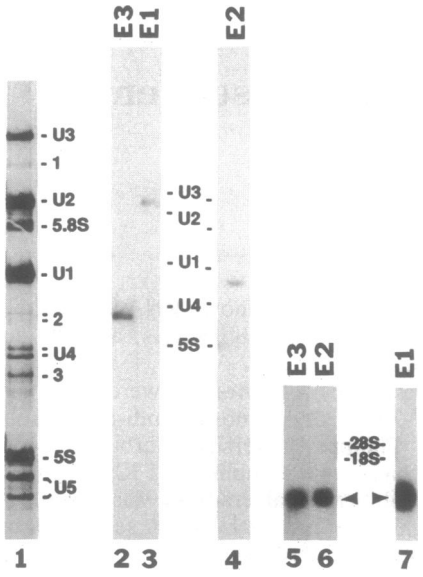

FIG. 1. RNAs that hybridize to cDNA clones E1, E2, and E3. Lane 1 is a fluorogram of HeLa cell nuclear RNA that was labeled *in vivo* with [^{3}H]uridine for 1 day and then was fractionated by electrophoresis in a 40-cm-long, 10% polyacrylamide gel; some RNA bands that could not be hybrid selected by the available DNA clones for small RNAs (U1, U2, U3, U4, U5, U6, U7, 7SL, 7SK, 5S, 5.8S, 4.5S$_I$, 4.5S$_H$, hY1, hY3, tRNA, and Alu DNA) are indicated with the numbers 1, 2, and 3. Northern blots of HeLa whole cell RNA after 10% polyacrylamide gel electrophoresis (lanes 2–4, 50 μg of RNA per lane) and formaldehyde/agarose gel electrophoresis (lanes 5–7, 100 μg of RNA per lane) were hybridized with radioactive DNA probes made with cDNA clones E1, E2, and E3. In lanes 2–4, the migration of U1, U2, U3, U4, and 5S RNAs was determined by probing their adjacent blot lanes. Lanes 2 and 3 are from a gel electrophoresis separate from that of lane 4. Lanes 5–7 show the electrophoretic mobility of 28S and 18S rRNAs and small RNAs such as tRNA (arrowheads) in their adjacent gel lanes. Lanes 5 and 6 are from a gel electrophoresis separate from that of lane 7.

nucleotides long, respectively, and these appear to be their full lengths, since they are comparable to the sizes estimated by polyacrylamide Northern blots. Thus, E1, E2, and E3 RNAs are not polyadenylylated species.

E1, E2, and E3 RNAs were present in the nuclear fraction but not in the cytoplasmic fraction of HeLa cells and were

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   E1
5' CCAACGUGGAUACACCCUGGGAGGUCACUCUCCCCGGGCUCUGUCCAAGU   50
   GGCGUAGGGGAGCAUAGGGCUCUGCCCCAUGAUGUACAAGUCCCUUUCCA  100
   CAACGUUGGAAAUAAAGCUGGGCCUCGUGUCUGCGCCUGCAUAUUCCUAC  150
   AGCUUCCCAGAGUCCUGUCGACAAUUACUGGGGAGACAAACCAUGCAGGA  200
   AACAGCC 3'                                           207

   E2
5' UGUGCACAUUGUUAGAGCUUGGAGUUGAGGCUACUGACUGGCCGAUGAAC   50
   UCGCAAGUGUAGGUAGUGUGCUACAUGAGGGGCAAGUUUUCGCUAACACC  100
   ACAAGGGUCUCUGGCCCAAUGAGUGGAGUUUGAUAGUAAUUCUUGCUACA  150
   AGUA 3'                                              154

   E3
5' AAAGCAGGAUUCAGACUACAAUAUAGCUGCUAAGUGCUGUGUUGUCGUUC   50
   CCCCUGCUUAAAAUAAAGUUGUUUCUUAACUAUACCUGUCUGCUAUUCUC  100
   CUGUAGCAGCCAGGGACGCUUGGUCUCAUACAUGU 3'               135
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FIG. 2. Nucleotide sequences of human E1, E2, and E3 RNAs. They were determined by sequencing cloned cDNAs and were confirmed by sequencing RNA from HeLa cell nucleoli.

detected in the nucleolar fraction but not in the nucleoplasmic fraction (Fig. 3). Subsequent reprobing of the same Northern blots with probes for U1 and U3 RNAs (as internal controls of nucleoplasmic and nucleolar RNA species, respectively) indicated that the nucleolar/nucleoplasmic fractionation in these experiments was good (Fig. 3, lanes 7 and 8). The DNA of the cells used had been prelabeled *in vivo* with [^{3}H]thymidine to follow the partition of chromatin during cell fractionation. The nucleolar pellet had only ≈15% of the trichloroacetic acid-precipitable ^{3}H cpm, indicating that most of the chromatin had not simply precipitated with this pellet. E1, E2, and E3 RNAs are not cleavage products of pre-rRNA because their sequences are not present in the human 47S pre-rRNA primary transcript.

There is a m$_3$G cap at the 5′ end of some of the snoRNAs, such as U3, U8, U13, and yeast U14/snR128, and nucleoplasmic small RNAs, such as U1, U2, U4, U5, and U7 (16–18). Anti-m$_3$G antibodies immunoprecipitate these RNAs (19, 20) but do not immunoprecipitate E1, E2, and E3 snoRNAs (Fig. 4*A*). Hybridization of the same Northern blot with additional probes showed appreciable levels of U3 and U1 RNAs in the anti-m$_3$G immunoprecipitate. The cap of U3 RNA is known to be perhaps the least accessible to anti-m$_3$G antibodies among the RNA species that have a 5′-end m$_3$G cap. Thus, accessibility of 5′ termini to anti-m$_3$G antibodies did not seem to be a problem in these experiments. The 5′ ends of E1, E2, and E3 snoRNAs do not appear to have m$_3$G caps.

For further analysis of the 5′ ends of these snoRNAs, HeLa cell nucleolar RNA was digested with alkaline phosphatase followed by incubation with T4 polynucleotide kinase and [γ-^{32}P]ATP, was hybrid selected with membrane-immobilized cDNA clones E1, E2, and E3, and was finally fraction-

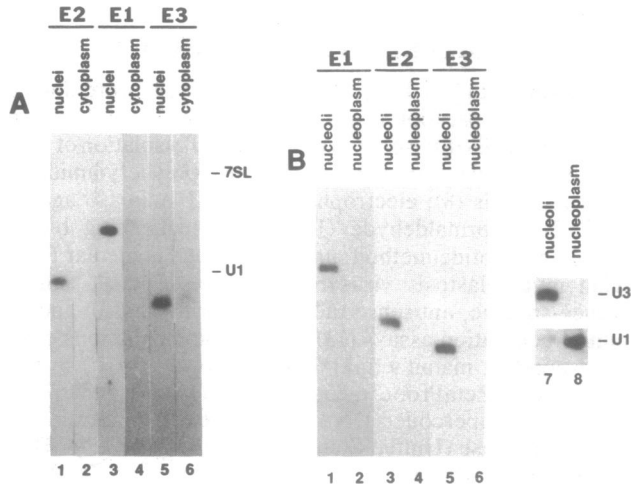

FIG. 3. E1, E2, and E3 RNAs are located in the nucleolus. HeLa cells were fractionated into nuclear, cytoplasmic, nucleolar, and nucleoplasmic fractions. RNA extracted from these fractions was analyzed by 10% polyacrylamide Northern blots. (*A*) Nuclear RNA (25 μg per lane) and cytoplasmic RNA (75 μg per lane) were analyzed. The blots were hybridized with cDNA probes for E2 (lanes 1 and 2), E1 (lanes 3 and 4), and E3 (lanes 5 and 6) RNAs. (*B*) Nucleolar and nucleoplasmic RNA samples from similar numbers of cell equivalents were analyzed. The blot lanes that contained nucleolar RNA (lanes 1, 3, 5, and 7) and the blot lanes that contained nucleoplasmic RNA (lanes 2, 4, 6, and 8) were sequentially hybridized with radioactive DNA probes for E2 (lanes 3 and 4), E1 (lanes 1 and 2), E3 (lanes 5 and 6), U3 (upper panel of lanes 7 and 8), and U1 (lower panel of lanes 7 and 8) RNAs. The probes were removed after each set of x-ray film exposures, except that probe E1 was not removed before hybridization with probe E3 (that is why there is a small amount of labeled band E1 in lane 5). The x-ray film exposures for lanes 1–6 were longer than for lanes 7 and 8.

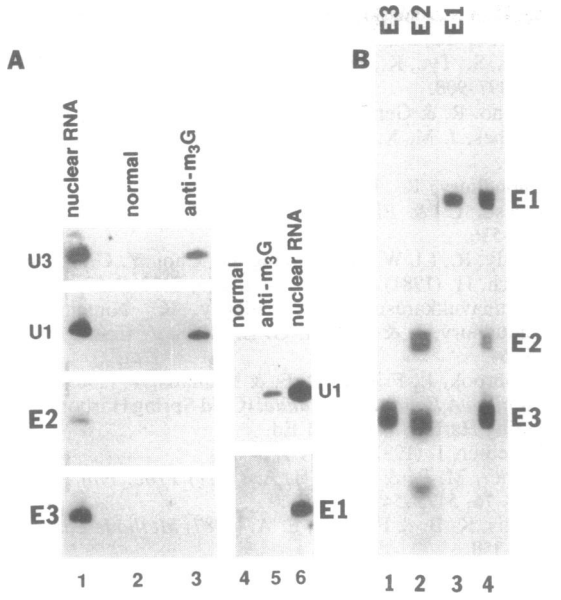

FIG. 4. E1, E2, and E3 snoRNAs are not immunoprecipitated by anti-m_3G antibodies (*A*) and are phosphorylated after incubation with alkaline phosphatase followed by polynucleotide kinase (*B*). (*A*) Northern blot analysis of HeLa cell nuclear RNA that was immunoprecipitated with anti-m_3G antibodies (lanes 3 and 5) or nonimmune immunoglobulin (lanes 2 and 4). The blots, which included the original total nuclear RNA (lanes 1 and 6), were hybridized sequentially with radioactive DNA probes for E2, E3, U1, and U3 RNAs (lanes 1–3), or they were hybridized sequentially with probes for E1 and U1 RNAs (lanes 4–6). The x-ray film exposures for the U1 and U3 probes were shorter than for the E2, E3, and E1 probes. (*B*) HeLa cell nucleolar RNA was incubated with calf intestinal alkaline phosphatase and deproteinized. It was then incubated with T4 polynucleotide kinase and [γ-^{32}P]ATP and hybrid selected with membrane-immobilized cDNA clones E3, E2, and E1, and the specific hybrids were analyzed by 10% polyacrylamide gel electrophoresis (lanes 1–3). The presence of some partially degraded E2 RNA molecules in the hybrid-selected samples did not obscure the detection of the full-length molecules (lane 2). The electrophoretic mobility of intact E1, E2, and E3 RNAs of untreated nuclear RNA in an adjacent gel lane was determined by Northern blot analysis (lane 4).

ated by 40-cm-long, 10% polyacrylamide gel electrophoresis. Radioactive E1, E2, and E3 RNA bands were detected, which comigrated with the corresponding snoRNAs from untreated nuclear RNA in an adjacent gel lane that was analyzed by Northern blotting (Fig. 4*B*). This suggests that ^{32}P labeling occurred at the 5′ end of the original molecules in the sample, instead of labeling of molecules whose 5′ termini had been partially degraded during handling. These results support the conclusion that E1, E2, and E3 snoRNAs do not have caps at their 5′ end, such as monomethylguanosine, trimethylguanosine, or γ-monomethyl phosphate caps (16, 21).

Apparent homologs of E1, E2, and E3 snoRNAs were detected in Northern blots of mouse myeloma C-66 cells and *Xenopus laevis* liver (Fig. 5*A*). The electrophoretic mobility of the frog homolog of E3 RNA was higher than that of its human counterpart, whereas the reverse was true for the *Xenopus* homologs of E1 and E2 RNAs. E1, E2, and E3 RNAs were present in Northern blots of every rabbit tissue analyzed (Fig. 5*B*). These results indicate that E1, E2, and E3 RNAs, in addition to being conserved during evolution, are housekeeping RNA species. The ratio of the levels of some small RNAs varied between some tissues. For example, the E1/U1 RNA ratio was higher in spleen than in brain, and the E1/E2 and E1/E3 RNA ratios in cerebellum were higher than in white skeletal muscle (Fig. 5*B*). The data from rabbit and

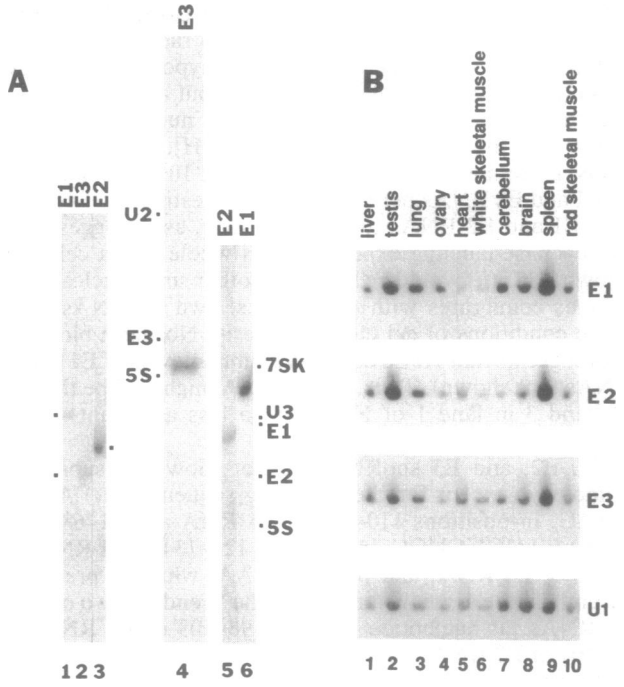

FIG. 5. Apparent homologs of E1, E2, and E3 snoRNAs are present in mouse and frog cells (*A*) and in every rabbit tissue tested (*B*). (*A*) Mouse myeloma C-66 whole cell RNA (lanes 1–3) and *X. laevis* liver nuclear RNA (lanes 4–6) were analyzed by 10% polyacrylamide Northern blots, which were hybridized with DNA probes for human E1 (lanes 1 and 6), E2 (lanes 3 and 5), and E3 (lanes 2 and 4) RNAs. The electrophoretic mobility of human E1, E2, and E3 RNAs is shown by dots in lanes 1–3, and the migration of human U2, E1, E3, E2, 5S, and 7SK RNAs in adjacent blot lanes is indicated in lanes 4–6. Lanes 5 and 6 are from a separate gel electrophoresis than lane 4. (*B*) RNA from freshly excised rabbit tissues was analyzed by 10% polyacrylamide Northern blots that were hybridized sequentially with DNA probes for human E1, E2, and E3, and U1 RNAs. The x-ray film exposures for the E1, E2, and E3 probes were longer than for the U1 probe. Different amounts of RNA were loaded on each lane of *A* and *B*.

frog tissues, plus the nucleolar localization of E1, E2, and E3 RNAs, indicate that these small RNAs are not derived from contamination of cells in culture.

DISCUSSION

E1, E2, and E3 snoRNAs may represent an additional class of snoRNA species, according to the following: (*i*) they are not capped by m_3G, as opposed to snoRNAs U3, U8, U13, and yeast U14/snR128 (16–18); (*ii*) they do not have a box C sequence (UGAUGAUYG, where Y indicates pyrimidine), which is present in snoRNAs U3, U8, and U13 (17) and is needed for binding of the nucleolar protein fibrillarin (22); (*iii*) they lack a box D sequence (RUCUGA, where R indicates purine), which is located near the 3′ end of snoRNAs U3, U8, U13, 4.5S hybRNA, X, Y, and yeast snR128 and snR190 (17); and (*iv*) they do not show any substantial sequence homology with any of the mammalian snoRNAs, such as U3, U8, U13, 4.5S hybRNA, and 7-2/MRP (16–18, 23–25), with any of the *Saccharomyces cerevisiae* small RNA species that are apparently nucleolar, U3/snR17, snR3, snR4, snR5, snR8, snR9, snR10, snR189, snR190, and U14/snR128 (3, 18, 26, 27), or with any of the *Tetrahymena* snoRNAs, such as snoRNA01, snoRNA02, snoRNA03, and snoRNA04 (28). The 3′-terminal 13 nucleotides of U3 snoRNA are required for its nuclear import (29). This sequence is absent in E1, E2, and E3 RNAs, suggesting that these snoRNAs have unique nucleolar or nuclear targeting signals.

There are about 3×10^4 molecules of U4 RNA per HeLa cell (30). In lane 1 of Fig. 1, the level of radioactivity in the putative E1 RNA band (band 1) and the hypothetical E2 RNA band (one of the two labeled 2) was about a tenth of that of the sum of the two U4 RNA bands in nuclear RNA from HeLa cells that had been labeled with [^{3}H]uridine for 1 day. Thus, there may be no more than about 10^3 molecules each of E1 and E2 RNAs per HeLa cell. This estimate (*i*) assumes that E1 and E2 RNAs turn over slowly, as is suggested by pulse–chase labeling experiments in whole HeLa cells (not shown), and (*ii*) would be lower if another small nuclear RNA species comigrates with one of these two snoRNAs under these conditions of gel electrophoresis. Northern blot analysis suggests that HeLa cells have similar levels of E1 and E3 RNAs (not shown). Thus, E3 snoRNA might not be the bulk of band 3 in lane 1 of Fig. 1 but a less abundant species migrating with or near band 3.

E1, E2, and E3 snoRNAs do not show any substantial homology, except for the following sequences: (*i*) AAAUAAAG, in positions 110–117 of E1 RNA and 61–68 of E3 RNA; (*ii*) UGUCUGC, in positions 128–134 of E1 RNA and 87–93 of E3 RNA; and (*iii*) CCACAA, which is present at essentially the same distance from the 5′ ends of two of these snoRNAs, at nucleotide positions 98–103 of E1 RNA and 99–104 of E2 RNA. Some of these sequences might be sites of intermolecular interaction occurring in more than one snoRNA species. If E1, E2, or E3 RNAs were synthesized by RNA polymerase III, it could be noted that they do not have RNA polymerase III intragenic promoter sequences, such as a type 1 internal control region box A or a type 2 internal control region box A or box B (reviewed in ref. 31).

The apparent conservation of these three snoRNAs between frog and human and their presence in various tissues are compatible with universal functions. The detection of E1, E2, and E3 small nuclear RNAs only in the nucleolar fraction suggests that they may function in ribosome formation.

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