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# U20, a Novel Small Nucleolar RNA, Is Encoded in an Intron of the Nucleolin Gene in Mammals

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**We have found that intron 11 of the nucleolin gene in humans and rodents encodes a previously unidentified small nucleolar RNA, termed U20. The single-copy U20 sequence is located on the same DNA strand as the nucleolin mRNA. U20 RNA, which does not possess a trimethyl cap, appears to result from intronic RNA processing and not from transcription of an independent gene. In mammals, U20 RNA is an 80-nucleotide-long, metabolically stable species, present at about $7 \times 10^3$ molecules per exponentially growing HeLa cell. It has a nucleolar localization, as indicated by fluorescence microscopy following in situ hybridization with digoxigenin-labeled oligonucleotides. U20 RNA contains the box C and box D sequence motifs, hallmarks of most small nucleolar RNAs reported to date, and is immunoprecipitated by antifibrillarin antibodies. It also exhibits a 5′-3′ terminal stem bracketing the box C-box D motifs like U14, U15, U16, or Y RNA. A U20 homolog of similar size has been detected in all vertebrate classes by Northern (RNA) hybridization with mammalian oligonucleotide probes. U20 RNA contains an extended region (21 nucleotides) of perfect complementarity with a phylogenetically conserved sequence in 18S rRNA. This complementarity is strongly preserved among distant vertebrates, suggesting that U20 RNA may be involved in the formation of the small ribosomal subunit like nucleolin, the product of its host gene.**

While the function of the abundant nucleoplasmic small nuclear RNAs (snRNAs) U1, U2, U4/U6, and U5 in pre-mRNA splicing is understood in great detail, our knowledge of the small nucleolar RNAs (snoRNAs), which represent most nonsplicing snRNAs, is much less advanced. Because of the specialization of the nucleolus in ribosome biogenesis, most snoRNAs are assumed to participate in rRNA maturation (16, 61). However, direct evidence linking individual snoRNAs to processing of pre-RNA has been obtained for only a limited subset of this class: U3, which is by far the most abundant and has been one of the best studied (7, 23, 24, 26, 53), U14 (22, 25, 35, 36, 56), U8 (50), snR10 (60), and snR30 (43). Identification of snoRNA functions faces a major experimental limitation: no cell-free system is yet available that reproduces faithfully the numerous steps of rRNA processing, except for the early cleavage of the primary transcript in mammals, for which an involvement of U3 has been clearly established (26). Moreover, snoRNAs now appear to represent an increasingly complex class. In yeast cells, more than 15 snoRNA species have been identified (15, 16, 61). Until recently, it was generally considered that yeast cells contained a richer set of snoRNAs than metazoan cells. However, further studies of vertebrate snoRNAs have shown that these two groups of organisms possess a similarly complex population, with a large number of species likely uncharacterized so far (15). Thus, it is noteworthy that thus far, only two yeast snoRNAs, U3 and U14, have an identified homolog in vertebrates. Surprisingly, gene disruption experiments with yeast cells have demonstrated that most snoRNAs are nonessential, with the sole exceptions of U3 (24), U14 (64), and snR30 (5). Accordingly, rather than acting as obligatory factors at definite steps of pre-rRNA processing, most snoRNAs could participate in a modulation of the rate and efficiency of ribosome production in a variety of cell growth conditions. Their involvement in ribosome biogenesis is indeed supported by several lines of indirect evidence: not only do all yeast snoRNAs associate with pre-rRNAs, but most of them also interact, directly or indirectly, with fibrillarin (16, 61). This essential nucleolar protein (54), the expression of which is coupled with the level of rRNA synthesis during development in *Xenopus laevis* (11), appears involved in multiple aspects or ribosome biogenesis (62).

A new and intriguing dimension to the question of snoRNA function(s) has been recently introduced by the finding that in vertebrates, several of them are encoded in introns of ribosomal or nucleolar proteins, as reported for U14 (34, 37), U15 (63), U16 (17), U17 (27), and U18 (48). Obviously, this peculiar form of gene organization could provide the basis for a coordinated expression between the snoRNA and the host gene (see references 15 and 57 for reviews). In agreement with this possibility, the observation that such intronic snoRNAs result from a novel form of intronic RNA processing (17, 27, 34, 48, 63) and not from transcription of an independent gene in the intron reinforces the notion that snoRNAs may serve as a link between the expression of rRNA and ribosomal or nucleolar protein genes. So far, the only exception to this direct connection between intronic snoRNA host gene and ribosome biogenesis is U17, which is encoded in two introns of the human cell cycle regulatory protein gene *RCC1* (27). However, this departure may be only apparent since *RCC1* appears to function in the GTPase cycle for nuclear import of proteins, a key step in ribosome biogenesis (19). It is noteworthy that U17 RNA also differs from most of the other vertebrate snoRNAs reported to date by the absence of the box C and box D sequence motifs and by its apparent lack of interaction with fibrillarin. The above-mentioned vertebrate intron-encoded snoRNAs represent a highly unusual subset of intronic sequences on the basis of their high phylogenetic conservation (17, 27, 34, 48), which is likely to reflect their functional importance. Initially guided by this criterion of remarkable

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intronic sequence conservation, we have identified a novel vertebrate snoRNA species, termed U20, which is encoded in an intron of the nucleolin gene in mammals. Like the other recently reported intronic snoRNAs, U20 RNA appears to result from intronic RNA processing. This novel species provides an attractive system for further analyzing both the precise role of a snoRNA and the biological significance of its intronic localization. Thus, the presence of a phylogenetically conserved, very extensive complementarity with mature 18S rRNA sequence points to an obvious target for a direct dissection of U20 RNA function which seems likely to involve a transient pairing with pre-rRNA. On the other hand, the characterization of U20 RNA opens a new opportunity to test the possibility of a direct link between the functions of the snoRNA and the host gene protein, since nucleolin is a major nucleolar protein which appears to play multiple roles at different stages of ribosome biosynthesis (10, 28, 29, 31).

## MATERIALS AND METHODS

**Oligonucleotide probes.** All U20 RNA antisense oligodeoxynucleotides (sequences are given in Fig. 1) and the U3 RNA-specific antisense oligonucleotide (complementary to positions 1 to 24 of the mouse [40] or human U3 RNA sequence) were synthesized by Y. de Préval (Laboratoire de Biologie Moléculaire Eucaryote du CNRS, Toulouse, France) either on an Applied Biosystems 391-01 DNA synthesizer or on a Pharmacia Gene Assembler. They have been used for end labeling according to standard procedures (52) without further purification. Probes for Northern (RNA) analysis or primer extension were 5′ end labeled as follows. Routinely, 30 pmol of oligonucleotide was incubated (45 min, 37°C) in the presence of 50 μCi of [γ-$^{32}$P]ATP (specific activity, 3,000 Ci/mmol) and 10 U of T4 polynucleotide kinase, resulting in probes with specific radioactivities of $1.0 \times 10^8$ to $1.2 \times 10^8$ cpm/μg.

For Southern analysis of genomic DNA, probes were labeled to a higher specific radioactivity ($1.8 \times 10^8$ to $2.8 \times 10^8$ cpm/μg) by utilization of [γ-$^{32}$P]ATP at 6,000 Ci/mmol. For identifying the 3′ terminus of U20 RNA, a 3′-end-labeled oligonucleotide was obtained by incorporation of a $^{32}$P-labeled ddA at the 3′ end of oligonucleotide probe 3A, using [α-$^{32}$P] ddATP (specific activity, 3,000 Ci/mmol) and terminal transferase (Bethesda Research Laboratories). The probe (specific radioactivity, $0.6 \times 10^8$ cpm/μg) was purified by electrophoresis on a 10% acrylamide–7 M urea gel before utilization for the S1 nuclease protection experiment.

**Nuclei isolation, RNA extraction, and Northern hybridization analysis.** Nuclei were isolated from HeLa S3 cells by a previously described procedure (2) involving mechanical disruption in an isotonic sucrose medium, in the presence of collagenase and nonionic detergents.

Total cellular or nuclear RNA was isolated by the guanidinium thiocyanate method (12) followed by phenol-chloroform extraction. Electrophoresis on 6 or 10% acrylamide–7 M urea gels was carried out as described previously (39). Electrotransfer onto nylon (Hybond N) membranes was followed by a short UV irradiation (250 mJ with a Stratalinker apparatus) before hybridization in 5× SSPE (1× SSPE is 150 mM NaCl, 1 mM EDTA, and 10 mM $NaH_2PO_4$, pH 7.4)–1× Denhardt's solution–1% sodium dodecyl sulfate (SDS)–150 μg of tRNA carrier per ml. Unless otherwise stated, hybridization was performed at a temperature 15°C below the denaturation temperature ($T_m$) calculated for a perfectly matched duplex (42). Filters were washed first with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS (15 min at room temperature) and then twice with 0.1× SSC–0.1% SDS (15 min at room temperature).

**Primer extension and S1 nuclease protection experiment.** Extension of a $^{32}$P-5′-end-labeled primer by reverse transcription was carried out as previously described (3), using 15 μg of total cellular RNA from HeLa cells. The S1 nuclease protection experiment was performed with about 1 pmol of U20 RNA purified from HeLa cell nuclear RNA by electrophoresis on a 10% acrylamide–7 M urea gel. U20 RNA was mixed with about 3 pmol of $^{32}$P-3′-end-labeled oligodeoxynucleotide 3A in 30 μl of a hybridization mixture containing 30% formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4), 400 mM NaCl, and 2 mM EDTA in the presence of 5 μg of tRNA carrier. The mixture was heated at 75°C for 10 min, and hybridization was carried out at 40°C for 4.5 h before addition of 300 μl of chilled S1 nuclease buffer (50 mM $NaCH_3CO_2$ [pH 5.0], 4.5 mM $ZnSO_4$, 280 mM NaCl) containing 6 μg of heat-denatured salmon sperm DNA. The mixture was divided into aliquots, which were submitted to different conditions of S1 nuclease digestion as specified in the legend to Fig. 2B. Digestion was stopped by addition of 16 μl of chilled 50 mM EDTA–4 M $NaCH_3CO_2$ (pH 5.1) containing 5 μg of tRNA carrier. Control reaction mixtures prepared in the absence of U20 RNA were processed in parallel in identical conditions of S1 nuclease treatment. Protected DNA fragments were analyzed on a 10% denaturing acrylamide gel.

**Genomic DNA hybridization.** Nylon (Hybond N) membranes containing transferred HeLa cell DNA digested by *Eco*RI were hybridized (at 50°C for 16 h) with different $^{32}$P-5′-end-labeled oligodeoxyribonucleotide probes in 5× SSPE–1× Denhardt's solution–1% SDS–150 μg of tRNA carrier per ml in the presence of different concentrations of formamide (16% with probe 3, 21% with probe 4+5, and 34.5% with probe 3A; for probe 1, hybridization was performed at 40°C in the absence of formamide). After a first washing in 2× SSC–0.1% SDS (15 min at room temperature), membranes were washed twice in 0.1× SSC at a temperature 5°C below the calculated $T_m$ of the hybrid (42) and exposed for autoradiography with an intensifying screen for 3 weeks.

**Preparation of small nuclear ribonucleoprotein (snRNP) extracts and immunoprecipitations.** Preparation of HeLa cell extracts and immunoprecipitation with antifibrillarin monoclonal antibody 72B9, obtained from G. Reimer and E. Tan (51), were performed essentially as described previously (33). Cells were sonicated in NET buffer (40 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Nonidet P-40) at a concentration of $4 \times 10^7$ cells per ml. Sonication was performed (five 30-s pulses with 30-s intervals) with a Branson microtip (setting of 1.5). The sonicated extract was rendered 300 mM in NaCl, and immunoprecipitation was carried out on 0.5-ml aliquots in the presence of 10 μl of serum. The same buffer was used for binding and washing. Immunoprecipitation was for 90 min at 4°C. The RNA was recovered from protein A-Sepharose by phenol-chloroform extraction and run on an 8% acrylamide-urea gel before Northern analysis.

Immunoprecipitation with the anti-trimethylguanosine (TMG) cap monoclonal antibody, obtained from R. Lührmann (38), was performed on purified nuclear RNA (dissolved in NET buffer) after a heat denaturation step.

**In situ hybridization.** Hamster (CHO) cells or mouse NIH 3T3 cells grown on coverslips were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min on ice and dehydrated successively in 30, 50, 70, and 95% ethanol. Permeabilization treatments were performed with 0.5% saponin–1 mM EGTA in PBS for 3 min on ice and then with cold (−20°C) methanol for 10 min. After prehybridization in 6×

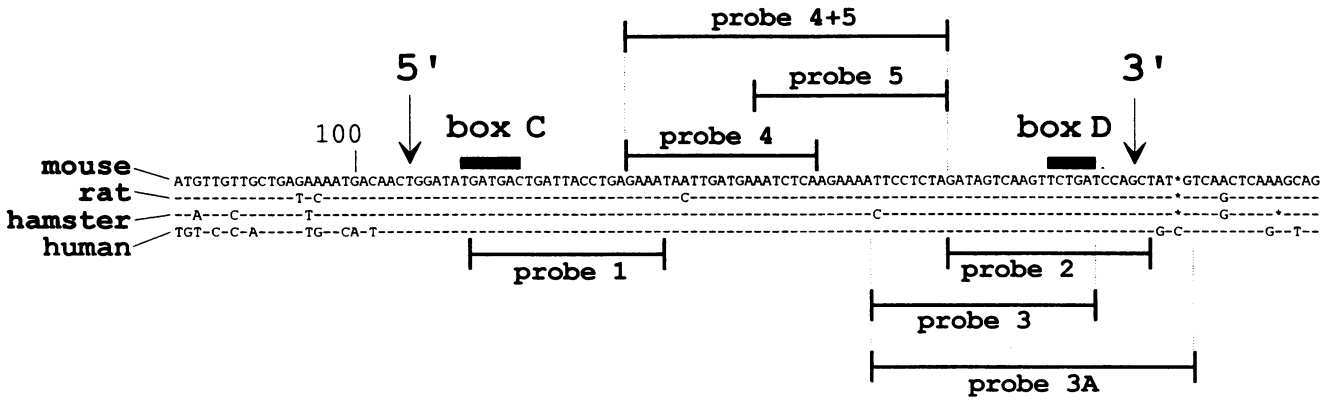


FIG. 1. The conserved region in intron 11 of the nucleolin gene in mammals. Reported sequences (9, 59) are aligned by reference to the mouse sequence (numbered starting from the 5′-terminal nucleotide of intron 11), with dashes denoting sequence identity. The alignable portion of each intronic sequence is represented in its entirety. The 5′- and 3′-terminal nucleotides of the snoRNA (identified as described in Materials and Methods) are shown by vertical arrows. The box C and box D motifs are overlined by a thick bar. The locations of the different oligodeoxynucleotide probes used in this work are also depicted above and below the alignment.

SSC–5× Denhardt's solution–10% dextran in the presence of 10 ng of *Escherichia coli* DNA per μl, oligonucleotide probes, labeled by addition of digoxigenin-11-dUTP (Boehringer Mannheim) by terminal transferase, were added to a final concentration of 2 ng/μl, and incubation was performed overnight at 37°C in a humidified chamber. Following hybridization, coverslips were washed successively in 6×, 2×, and 0.2× SSC at room temperature. Hybridization sites were immunolabeled with a rhodamine-antidigoxigenin conjugate (Boehringer Mannheim) diluted 1/20 in 0.1 M Tris-HCl (pH 8.5)–150 mM NaCl–5% bovine serum albumin. After washing, coverslips were mounted in Mowiol containing 4′,6-diamidino-2-phenylindole (DAPI; 25 ng/μl) as a DNA counterstain. Cells were examined with a Leitz Ortholux microscope equipped with standard excitation and emission filters for tetramethyl rhodamine isothiocyanate and DAPI fluorescence, using an NPL Fluotar 100×/1.3 oil objective. Photographs were taken automatically with Kodak Tmax 100 film.

**Nucleotide sequence accession number.** The EMBL accession number of the human U20 RNA sequence is Z34290.

## RESULTS

**A conserved intronic sequence in the mammalian nucleolin gene shares structural motifs with snoRNAs.** Sequence analysis of the nucleolin gene in three rodents, mice, rats, and hamsters, had revealed the intriguing conservation of three intronic sequence elements, each extending over about 100 nucleotides (nt), located in introns 1, 11, and 12 (9). However, the significance of this observation was limited by the close phylogenetic relationships among these three species. A better assessment of the conservation degree was later provided by the derivation of the human nucleolin gene sequence (59). Human-rodent comparisons definitely show that the sequence conservation of these intronic regions is surprisingly high, particularly for the element located in intron 11, termed CS2 (9), on which we have focused our attention. As shown in Fig. 1, this intronic region exhibits an uninterrupted block of 85 nt which are almost perfectly conserved among the four mammals. Remarkably, the box C and box D sequence motifs, hallmarks of several snoRNAs (15, 16), are present within this conserved tract, near its 5′ and 3′ boundaries, respectively. It is also noteworthy that this 85-nt-long core of strongly conserved intronic sequence is flanked by two segments (about 30 nt upstream and 25 nt downstream) of lesser but still significant conservation, which exhibit only a few differences among rodents and remain highly similar between humans and rodents. Heretofore, the most outstanding case of intronic sequence conservation concerned the vertebrate U14 RNA, a snoRNA, which is produced from hsc70 pre-mRNA by a novel mechanism of intron processing (34). In addition, U14 RNA contains the box C and box D motifs, in the vicinity of its 5′ and 3′ termini, respectively. These observations prompted us to test the possibility that the box C-box D-containing conserved sequence in intron 11 of the nucleolin gene in mammals also encoded a yet unidentified small cellular RNA.

**The conserved intronic sequence hybridizes to a stable small RNA, termed U20.** Several oligodeoxynucleotides complementary to the conserved sequence in intron 11 of the mammalian nucleolin gene (Fig. 1) have been synthesized and used as probes in Northern blot hybridization of total cellular RNA or nuclear RNA, either from human HeLa cells or from mouse NIH 3T3 cells, after separation by electrophoresis on 6% acrylamide–7 M urea gels. With the different oligonucleotide probes and for hybridizations performed at high stringency, the same result is always obtained; i.e., the radioactivity is essentially restricted to a single band, migrating like an 80-nt-long RNA.

The cellular content in this 80-nt-long RNA has been evaluated by quantitation of Northern hybridization signals, using a series of lanes loaded with increasing amounts of HeLa cell total cellular RNA (Fig. 2A). Membranes were hybridized in parallel with probe 3 (Fig. 1) and with a U3 RNA-specific oligodeoxynucleotide probe. Taking as a reference the U3 RNA signal levels and assuming the presence of about $2 \times 10^5$ copies of U3 RNA per HeLa cell (50), our data are consistent with the presence of about $7 \times 10^3$ molecules of this novel small RNA per HeLa cell.

When total HeLa cell RNA was separated on a 1% agarose denaturing gel and analyzed by Northern blot hybridization with the same oligonucleotide probes, no substantial signal was detected over the high-molecular-weight RNA areas of the membrane, indicating that the fraction of this intronic sequence present in the form of unspliced (or partially spliced) nucleolin pre-mRNA is very minor (representing definitely less than 5%) compared with the 80-nt-long RNA in exponentially growing HeLa or mouse 3T3 cells (result not shown).

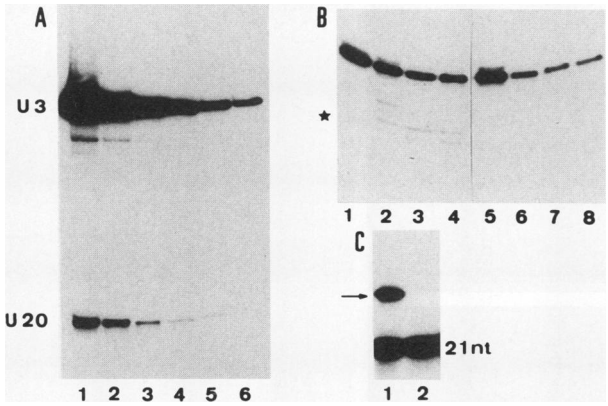


FIG. 2. Abundance of U20 RNA and identification of its termini. (A) Determination of U20 RNA cellular content by reference to U3 RNA by Northern hybridization. Decreasing amounts of HeLa total nuclear RNA were separated on a 6% acrylamide–7 M urea gel (10, 5, 2, 1, 0.5, and 0.25 μg in lanes 1 to 6, respectively). After electrotransfer, the membrane was cut into two halves, which were hybridized in parallel with either a U3 RNA-specific oligonucleotide (upper half) or a U20 RNA probe 3 (lower half). Both probes had been 5′ end labeled to the same specific radioactivity, and hybridizations were performed in identical conditions (stringency and probe concentrations) in the presence of probe excess. After hybridization, the two parts of the membrane were rejoined before autoradiography. Intensities of the hybridization signals were quantitated on a Fuji Bas-1000 Imager. (B) Identification of the 3′ terminus of HeLa cell U20 RNA by S1 nuclease protection. The fraction of HeLa cell nuclear RNA migrating around 80 nt was purified by gel electrophoresis and hybridized to a 3′-end-labeled U20 antisense oligodeoxynucleotide overlapping the 3′ end of the U20 RNA sequence in the human nucleolin gene. This 35-mer probe was obtained by addition of 3′-terminal [α-$^{32}$P]ddA to oligonucleotide 3A (depicted in Fig. 1) by terminal transferase. After hybridization of the probe with U20 RNA (lanes 1 to 4), aliquots were submitted to various conditions of S1 nuclease treatment at 20°C and analyzed on a 10% acrylamide–7 M urea gel together with control reactions performed in the absence of U20 RNA (lanes 5 to 8). Lanes 1 and 5, control without S1 nuclease; lanes 2 and 6, S1 nuclease for 30 min at 250 U/ml; lanes 3 and 7, S1 nuclease for 2 h at 250 U/ml; lanes 4 and 8, S1 nuclease for 30 min at 1,000 U/ml. Aliquots loaded in lanes 1 and 5 correspond to half the amount of material analyzed in the other lanes. The doublet of protected 3′-end-labeled fragments (29 and 28 nt) is denoted by a star. (C) Identification of the 5′ end of U20 RNA. The 21-nt-long antisense oligodeoxynucleotide 1 (see Fig. 1) was 5′ $^{32}$P labeled, a primer extension was carried out in the presence of HeLa cellular RNA, and the reaction mixture was analyzed on a 10% acrylamide–7 M urea gel (lane 1). The 28-nt-long extension product is denoted by an arrow. A control assay performed in the absence of HeLa RNA is shown in lane 2.

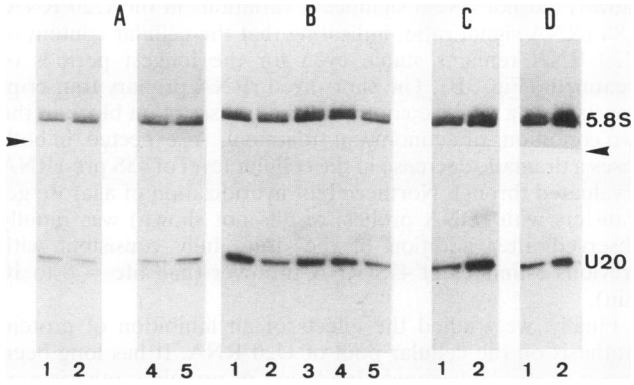


FIG. 3. Accumulation of U20 RNA after actinomycin D and cycloheximide treatments, measured by Northern analysis. (A and B) HeLa cells were treated with actinomycin D at 5 μg/ml (A) or 0.1 μg/ml (B) for 0.5 h (lanes 2), 1 h (lane 3), 3.5 h (lanes 4), or 9.5 h (lanes 5) before cell recovery and RNA isolation. RNA from untreated cells was analyzed in lanes 1. (C and D) HeLa cells were treated with cycloheximide at 10 μg/ml (C) or 100 μg/ml (D) for either 0.5 h (lanes 1) or 3.5 h (lanes 2) before recovery and RNA extraction. RNA aliquots were analyzed on a 6% acrylamide–7 M urea gel. After electrotransfer, the membranes were cut in two parts (as denoted by arrowheads). The lower parts were hybridized with U20 antisense probe 3, and the signals were normalized for equal amounts of loaded RNA in each lane, using 5.8S rRNA as an internal reference (the upper parts of the membranes were hybridized with a 5.8S rRNA-specific labeled oligonucleotide).

Termini of the small RNA species (as denoted in Fig. 1) have been directly identified by using an acrylamide gel-purified fraction from HeLa cells (Fig. 2B and C). The 3′-terminal nucleotide of the small RNA species was identified by an S1 protection experiment using a 35-nt-long synthetic oligonucleotide (Fig. 1) labeled at its 3′ end by the addition of a U20 RNA-complementary radioactive dideoxynucleotide (see Materials and Methods). Two protected labeled DNA fragments (denoted by a star in Fig. 2B), the sizes of which differ by a single nucleotide, are detected. Their relative intensities vary according to the U20 RNA preparation in a way suggesting that the shorter band mainly reflects a nibbling by exonuclease(s), during RNA purification and storage, of the 3′-terminal nucleotide which is protruding in the U20 RNA secondary structure (as shown below). The protection of the longer fragment corresponds to the 3′-terminal nucleotide denoted in Fig. 1. The 5′ end was mapped by primer extension with probes 1 (Fig. 2C) and 3. These end determinations fit precisely with the observed mobility of the small RNA in an acrylamide-urea gel, with the major termini being actually 80 nt apart along the conserved intronic sequence (as shown in Fig. 1). This novel small RNA species, which exhibits a 30% uridine content, will be referred to henceforth as U20.

To evaluate the stability of U20 RNA, its cellular accumulation was monitored by Northern hybridization with oligodeoxynucleotide probe 3 following a complete blockage of RNA synthesis achieved by actinomycin D (5 μg/ml) treatment of HeLa cells (Fig. 3A). To avoid systematic errors arising from fluctuations in cell numbers, RNA yields, or volumes of gel loadings among the different experimental points, the amount of 5.8S rRNA was used as an internal standard for each gel lane, taking into account the fact that rRNAs do not undergo a significant turnover in exponentially growing mammalian cells (1). Accordingly, the membranes were hybridized in parallel with the U20 probe and with a 5.8S rRNA-specific labeled oligonucleotide as detailed in the legend to Fig. 3. Whatever the duration of the actinomycin D (5 μg/ml) treatment, no significant change was detected in the U20 RNA/5.8S rRNA signal ratio, even for the most prolonged period of treatment, i.e., 9.5 h (Fig. 3A). It is therefore clear that U20 RNA represents a stable RNA species, with a half-life probably exceeding 20 h, i.e., similar to what is measured for U3 RNA.

In the same experiment, we also analyzed the effects of a much lower concentration of actinomycin D (0.1 μg/ml) on the cellular pool of U20 RNA. Such a treatment is known to rapidly result in a selective and complete inhibition of rRNA gene transcription, while RNA polymerase II and III transcription remains essentially unaffected (46). In these conditions also, densitometric analyses of the autoradiographs (not

shown) did not reveal significant variations in the U20 RNA/5.8S rRNA signal ratio, indicating that the cellular content of U20 RNA remains stable even for the longest periods of treatment (Fig. 3B). The short-lived rRNA primary transcript was used as a positive control of the transcription block in the two conditions of actinomycin treatment. As expected, in both cases a dramatic decrease in the cellular level of 45S pre-rRNA (evaluated through Northern blot hybridization of agarose gel transfers with rDNA probes; results not shown) was rapidly observed after addition of the drug, fully consistent with previous estimates of 45S RNA turnover (half-life = 8 to 10 min).

Finally, we studied the effects of an inhibition of protein synthesis on the cellular pool of U20 RNA. It has long been known that cycloheximide inhibition of protein synthesis rapidly impairs the production of mature rRNAs in eukaryotes (20). Following treatments of HeLa cells by cycloheximide concentrations which are inhibitory for protein synthesis, we again observed that the cellular level of U20 RNA remains stable relative to that of 5.8S rRNA, even after 3.5 h of treatment (Fig. 3C and D).

**U20 is an uncapped snoRNA interacting with fibrillarin.** The parallel analysis of nuclear and cytoplasmic RNAs by Northern hybridization with the different oligodeoxynucleotide probes indicated to us that U20 RNA does accumulate in nuclei and not in the cytoplasm (results not shown). To look for a possible nucleolar localization, two different approaches were used. First, nucleoli were isolated from HeLa cells, and RNA purified from the nucleolar fraction was analyzed by Northern hybridization with a U20-specific probe by reference with total nuclear RNA, which revealed that U20 RNA is strongly enriched in the nucleolar fraction (not shown). The same membranes were subsequently reprobed with a U3 RNA-specific oligodeoxynucleotide, and autoradiographs were analyzed by densitometry. The two RNA species exhibited very similar levels of enrichment in nucleolar RNA, strongly suggesting that U20 RNA, like U3 RNA, is essentially located in the nucleolus. To confirm this indication, we performed an in situ hybridization followed by indirect immunofluorescence, using as probes digoxigenin-labeled oligodeoxynucleotides complementary to U20 RNA. As shown in Fig. 4, the immunofluorescence is essentially restricted to the nucleoli regardless of the U20 oligonucleotide probe used for hybridization.

Taking into account the presence in the U20 RNA sequence of the box C motif, which has been involved in the binding of fibrillarin (6), the protein common to a major family of nucleolar snRNPs, we have looked for a potential interaction between fibrillarin and the novel snoRNA. Immunoprecipitations with antibodies against fibrillarin were performed on HeLa cell extracts, after which Northern hybridization with the different oligonucleotide probes specific for U20 was performed. As shown in Fig. 5, U20 is precipitated by antifibrillarin even more efficiently, at this ionic strength (300 mM), than U3 RNA, which was used as a positive control in this experiment.

Unlike the small nuclear or nucleolar RNAs independently transcribed from RNA polymerase II-specific genes, U14 and the other, subsequently identified intronic snoRNAs (17, 27, 34, 48, 63) do not possess a 5′ TMG cap. To ascertain the nature of the U20 RNA 5′ terminus, immunoprecipitation with an anti-TMG cap antibody (38) was performed on purified HeLa cell nuclear RNA extract, after which a Northern analysis of the immunoprecipitated fraction with U20 RNA probes was performed. Transfers of total and immunoprecipitated RNAs were subsequently rehybridized with a U3-specific

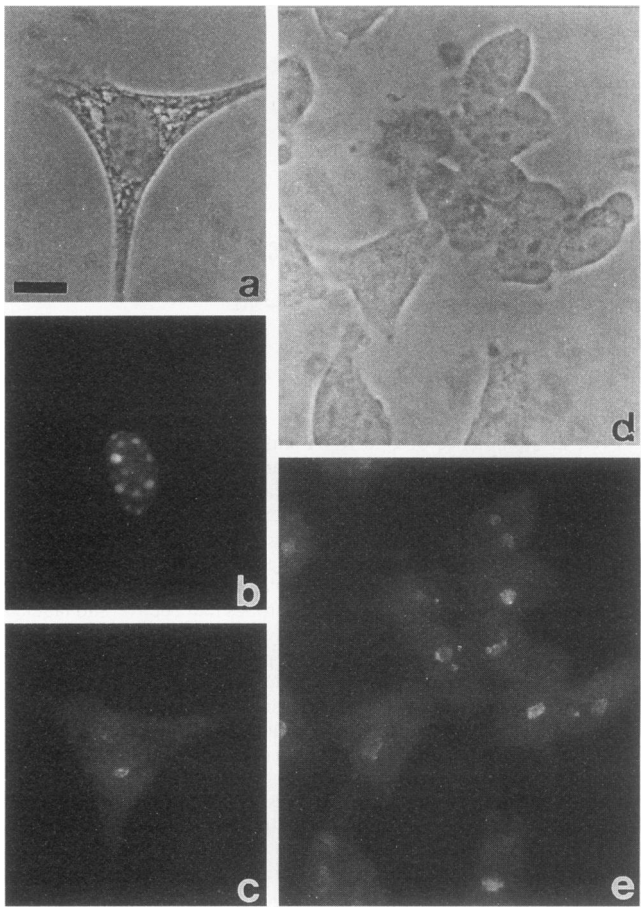


FIG. 4. Intranuclear distribution of U20 RNA in rodent cells. Fixed cells were permeabilized and observed either by phase-contrast microscopy (a and d), after DAPI counterstaining (b), or by fluorescence microscopy following in situ hybridization with U20 RNA antisense oligodeoxynucleotides (an equimolecular mixture of probes 1 and 2, delineated in Fig. 1) labeled with digoxigenin (c and e). Fluorescence detection was obtained by use of a tetramethyl rhodamine isothiocyanate-antidigoxigenin conjugate. (a to c) NIH 3T3 mouse cells. (d and e) A view of several hamster (CHO) cells displaying nucleolar fluorescence. Bar, 10 μm.

oligonucleotide probe in order to check the specificity of the immunoprecipitation assay (Fig. 5). In contrast to U3 RNA, U20 RNA is not immunoprecipitated (Fig. 5, lane 3), indicating that U20 RNA, unlike U3 RNA, does not possess a 5′ TMG cap.

**U20 snoRNA is not encoded elsewhere in the human genome.** To test the possibility that U20 RNA was transcribed from a genomic DNA sequence other than the one located in intron 11 of the nucleolin gene, we carried out Southern blot analyses of human genomic DNA, using various U20-specific oligonucleotide probes. HeLa cell genomic DNA was digested with *Eco*RI and hybridized either with different $^{32}$P-5′-end-labeled oligodeoxynucleotides (Fig. 6) or with a random-prime-labeled DNA clone obtained by PCR amplification of hamster genomic DNA over the U20 RNA-coding portion in intron 11 of the nucleolin gene (results not shown). Some of these data turned out to be more complex to interpret than anticipated because of an extensive cross-hybridization of some U20 probes with rRNA genes. As detailed below, the U20 RNA sequence shows in its 3′ half an extended comple-

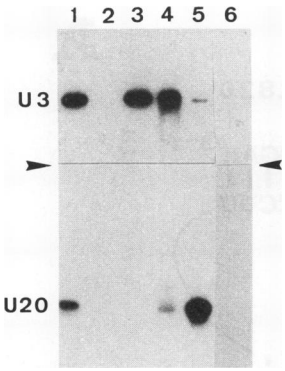


FIG. 5. Northern analysis of nuclear RNA immunoprecipitated by anti-TMG cap and antifibrillarin. Immunoprecipitation with anti-TMG cap was carried out on purified HeLa cell RNA, and immunoprecipitation with antifibrillarin was performed on a HeLa cell extract prepared by sonication in 150 mM NaCl after the NaCl concentration was increased to 300 mM. HeLa cell RNAs corresponding to control and immunoprecipitated fractions were analyzed on an 8% acrylamide–7 M urea gel. After transfer, the membrane was hybridized with U20 RNA antisense probe 3 and autoradiographed (bottom). It was subsequently rehybridized with a U3 RNA-specific antisense oligonucleotide before a second autoradiographic exposure (top). Lane 1, 10 μg of total RNA; lane 2, 10 μg of total RNA immunoprecipitated with a rabbit nonimmune serum; lane 3, 10 μg of total RNA precipitated by anti-TMG cap antibodies; lane 4, RNA isolated from the cell extract (corresponding to $2 \times 10^6$ cells); lane 5, RNA isolated after immunoprecipitation of an aliquot of the cell extract (corresponding to $2 \times 10^7$ cells) by antifibrillarin antibodies; lane 6, RNA isolated after precipitation with a mouse nonimmune serum of an aliquot of the cell extract (corresponding to $2 \times 10^7$ cells).

mentarity with a long tract near the 3′ end of the 18S rRNA sequence. Accordingly, antisense probes spanning this region of the U20 sequence result in a significant signal with the corresponding rDNA fragment, all the more as rRNA genes are present at about 160 copies per haploid genome in humans. Thus, with probe 3 (Fig. 6, lane 1), in addition to the expected 3.1-kb fragment carrying the U20 sequence in intron 11 of the nucleolin gene (56), a much stronger signal is observed at the position of the 7.1-kb *Eco*RI fragment of human rDNA, which spans the 3′ end of the 18S rRNA sequence, internal transcribed spacer (ITS) 1, 5.8S rRNA, ITS 2, and most of the 28S rRNA sequence (14). The relative intensity of the 7.1-kb band compared with the 3.1-kb signal, although significantly decreased at higher hybridization stringencies, nevertheless remained high with probe 3 (not shown). A weaker cross-hybridization is also observed for a 3.9-kb band (Fig. 6, lane 1) which turned out to correspond to a minor variant fragment of rDNA spanning the same 3′-terminal region of the 18S rRNA gene, as revealed by utilization of several rDNA probes (results not shown). Cross-hybridization with the 7.1-kb rDNA band was still observed with probe 4+5 and probe 3A (Fig. 6, lanes 2 and 3) but at a dramatically lower level. By contrast, with probe 1, which, unlike the other probes, spans the 5′-terminal region of the U20 RNA sequence, a unique hybridization signal is detected at the position of the 3.1-kb band (Fig. 6, lane 4). Finally, the only band recognized in common by all of the U20 probes does correspond to the expected 3.1-kb fragment, with the intensity of the signal consistent in all cases with the presence of a single copy of U20 RNA-coding sequence per haploid human genome, i.e., the one located within intron 11 of the nucleolin gene.

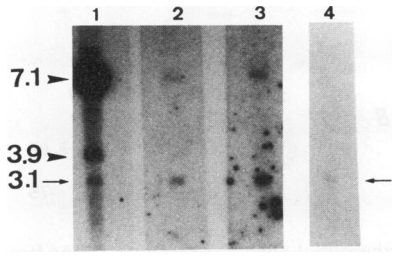


FIG. 6. Hybridization analysis of HeLa cell genomic DNA. *Eco*RI restriction digests of purified HeLa cell DNA (30 μg per lane) were fractionated in a 0.9% agarose gel and transferred to a nylon membrane. Strips of the membrane, each corresponding to a single lane, were hybridized with different oligodeoxynucleotide probes spanning the U20 RNA-coding region: probe 3 (lane 1), probe 3A (lane 2), probe 4+5 (lane 3), and probe 1 (lane 4). The position of the 3.1-kb *Eco*RI fragment of human DNA encompassing intron 11 of the human nucleolin gene (59) is denoted by an arrow. Cross-hybridizing rDNA fragments mentioned in the text are denoted by arrowheads. Sizes in kilobases are indicated on the left.

**The U20 RNA sequence exhibits an outstanding complementarity to a phylogenetically conserved segment of 18S rRNA.** We performed a search of the EMBL-GenBank data library for sequences homologous or complementary to the mammalian U20 RNA sequences. The match which is by far the most impressive corresponds to an outstanding complementarity between U20 RNA and a phylogenetically conserved segment in the 3′-terminal region of mammalian 18S rRNA. The potential base pairing, which includes 21 consecutive Watson-Crick base pairs without any irregularity, is shown in Fig. 7A. Obviously, such a match is statistically highly significant: this length of complementarity between any segment of U20 and a randomized sequence is expected to be found only about every $3 \times 10^{11}$ nt.

The corresponding segment in the secondary structure model of the 3′-terminal region of mouse 18S rRNA is delineated in Fig. 7B. It is part of an extended stem structure, universally conserved in both eukaryotic and prokaryotic small subunit rRNAs, and strongly supported by comparative evidence, with the occurrence of multiple compensatory base changes among very distant eukaryotes such as protists, fungi, or metazoans (49). It is noteworthy that the portion of the 18S rRNA stem involving the U20 RNA-complementary segment is thermodynamically rather unstable, with the presence of several noncanonical nucleotide appositions which have been precisely maintained in all eukaryotes and prokaryotes (49).

The U20 RNA-18S rRNA pairing is perfectly conserved for the four mammalian species sequences now available for comparison, since all of these sequence specimens are identical over the two matching segments. Accordingly, it cannot be tested on a comparative basis pending the derivation of nonmammalian U20 RNA sequences. However, it is already clear that this extended complementarity is largely, if not entirely, preserved among distant vertebrates, considering that the amphibian *X. laevis* and mammals differ by a single nucleotide change over the 18S rRNA segment (44) and that the corresponding sequence tract in U20 RNA also appears strongly conserved among the different vertebrate classes (as reported below).

**Identification of a U20 RNA homolog in all vertebrate classes.** U3 RNA exhibits a sufficient degree of conservation to allow for a substantial cross-hybridization among distant vertebrates (40), and the same holds true for U14 RNA (56). The conservation observed for U20 RNA sequence between rodents and humans exceeds what is observed for U3 or U14

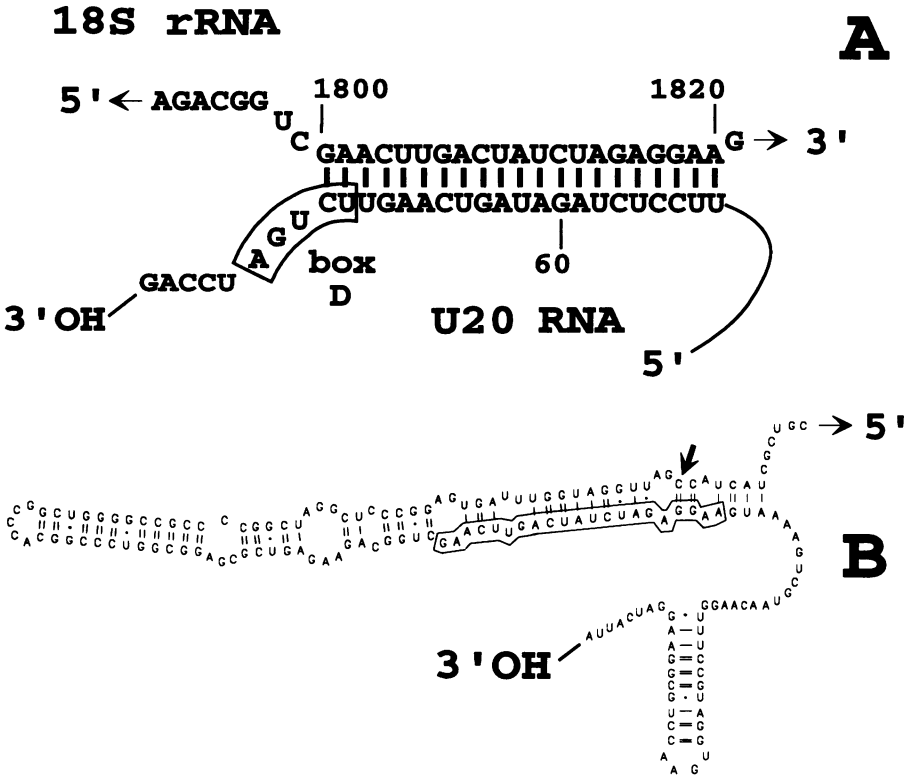


FIG. 7. Extended complementarity between U20 RNA and 18S rRNA sequences in mammals. The sequences represented in panel A are identical for both molecules in mice, rats, and humans. The location of the corresponding sequence in the secondary structure of the 3′-terminal domain of 18S rRNA is depicted in panel B. The arrow denotes a base pairing whose disruption in *E. coli* (41) impairs ribosomal subunit association (for details, see Discussion).

among the same species. We therefore have tried to identify a U20 homolog in vertebrates outside the mammalian class by Northern analysis through cross-hybridization with different mammalian oligonucleotide probes. We used moderately stringent conditions of hybrid formation, i.e., an incubation temperature about 30°C below the $T_m$ calculated for the homospecific duplex. A single radioactive signal with a mobility identical to or very similar to that of mammalian U20 RNA can be detected in representatives of all vertebrate classes, with the size of the U20 RNA homolog ranging from 75 to 80 nt, depending on the species. This band is labeled with all U20 probes for some species, whereas for other species, the signal may be strong with one probe but weak or even absent for the other probes. Results obtained with probes 2 and 3 are shown in Fig. 8A and B, respectively. For birds (ducks and pigeons), a single radioactive band migrating at 77 nt is revealed with all probes. However, the relative intensity of the signal, taking HeLa U20 RNA as a reference, is much lower with probe 1 (not shown) than with probes 2 and 3, indicating that the corresponding U20 segment is relatively less conserved. This is confirmed by the analysis of more distant vertebrate classes, with no signal observed for these species with probe 1. By contrast, with mammalian probes 2 and 3, which are located over the 3′ half of the mammalian U20 sequence, the U20 RNA homolog is also detected in a reptile and in the more distant amphibian and fish vertebrate classes. It is noteworthy that these two probes encompass most (for probe 2) or all (for probe 3) of the portion of U20 sequence complementary to 18S rRNA, thus pointing to the strong phylogenetic conservation of this particular segment of U20 RNA.

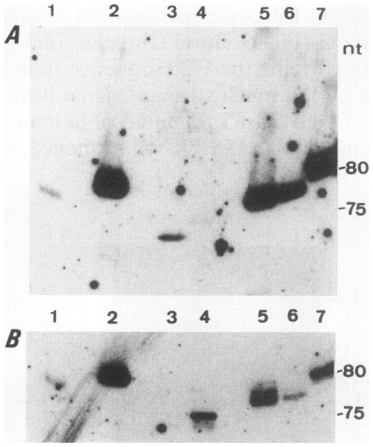


FIG. 8. Northern blot analysis of cellular RNAs from representatives of the different vertebrate classes. For each species, 10 μg of total cellular RNA per lane was separated on two 6% acrylamide–7 M urea gels. Lane 1, amphibian (*X. laevis*); lane 2, snake (*Natrix viperina*); lane 3, rainbow trout (*Salmo gardneri*); lane 4, carp (*Cyprinus carpio*); lane 5, pigeon (*Columba palumbus*); lane 6, duck (*Anas anserina*); lane 7, human HeLa cells. After electrophoretic transfer, two identical membranes were hybridized with $^{32}$P-5′-end-labeled oligonucleotide probes 2 and 3 (A and B, respectively) complementary to the mammalian U20 RNA sequence (see Fig. 1 for location). Hybridizations were performed at a temperature 30°C lower than the theoretical $T_m$ of the perfectly matched duplex (42).

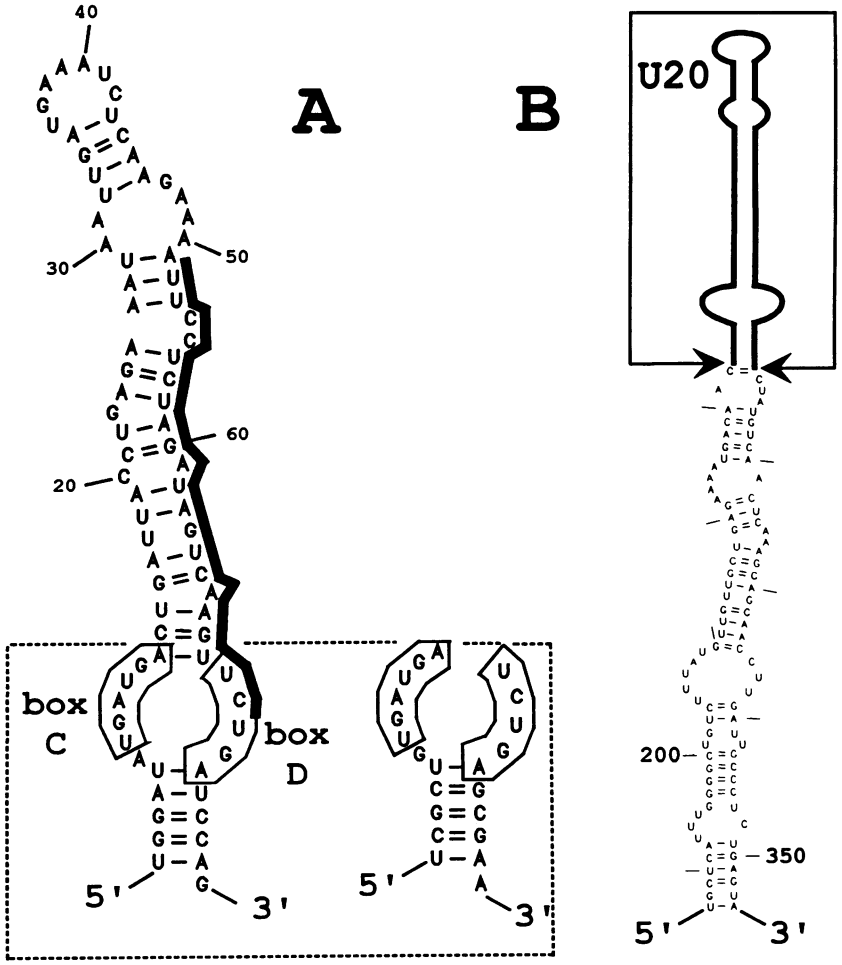


FIG. 9. Secondary structure models for mouse and human U20 RNAs. These structures represent computer predictions on a thermodynamic basis. (A) The sequence tract complementary to 18S rRNA is overlined. The domain in the dotted box has a striking homolog in U14 RNA, which is represented at the right. The long stem extension involving the sequences flanking U20 in the mouse intronic RNA is depicted in panel B.

**The U20 RNA secondary structure has features in common with other snoRNAs.** A secondary structure of mammalian U20 RNA has been derived on a thermodynamic basis (Fig. 9A). It exhibits a striking similarity with U14 RNA with regard to the organization of the molecule termini. Thus, for each snoRNA, both ends of the molecule are held together by a 5-bp stem which includes the 5′-terminal nucleotide but leaves an overhanging 3′-terminal nucleotide. This structure brings in close proximity the box C and box D motifs, which map at identical nucleotide positions relative to the stem in both U20 and U14 RNAs (Fig. 9A).

It is also noteworthy that for humans and mice (the two species for which the nucleolin gene intron 11 has been entirely sequenced), the intronic sequences immediately upstream and downstream from the U20 RNA-coding region are able to base pair. They form a long but imperfect duplex (shown in Fig. 9B for the mouse intronic RNA) which extends the 5′-3′ terminal stem of U20 RNA. However, the details of this secondary structure are not precisely conserved between mouse and human intronic RNAs.

## DISCUSSION

**U20 RNA, a novel intron-encoded snoRNA.** This study was prompted by the first report on an intron-encoded snoRNA in vertebrates, U14 (34), after we noticed that structural features typical of several snoRNAs were present in a surprisingly conserved intronic sequence in the mammalian nucleolin gene. Our present data show that this intronic sequence does encode a novel snoRNA, termed U20, which puts the number of vertebrate snoRNAs reported so far at 12 (15). The list is clearly far from complete, and we have more recently identified two additional vertebrate snoRNAs, termed U21 and U22, which are also intron encoded, in the ribosomal protein L5 gene and in another intron of the nucleolin gene, respectively (our unpublished results). The nucleolar localization of this novel U20 species in exponentially growing mammalian cells has been unambiguously established by both subcellular fractionation and in situ detection approaches. By the presence of the box C and box D sequence motifs and its direct or indirect association with the nucleolar protein fibrillarin, U20 belongs to the same subclass of snoRNAs as U3, U8, and U13 RNAs, which are transcribed from independent genes, and U14, U15, U16, and Y RNAs, which are also intron encoded in vertebrates (17, 34, 63). Like U3 RNA, U20 RNA is metabolically very stable. It is noteworthy that except for the most abundant U3 RNA ($2 \times 10^5$ copies per cell in HeLa cells) and U8 RNA ($4 \times 10^4$ copies per cell), the other members of this snoRNA

family including U20 have a roughly similar number of copies per cell, about $10^4$ (57).

Secondary structure comparisons indicate that within this snoRNA class, U20 RNA is more particularly related to U14, U15, U16, and Y RNAs (17, 22, 63) by the presence of a terminal stem structure bracketing the single-strand box C and box D motifs. This characteristic structural motif has been shown to represent a major determinant of the snoRNA stability in the case of U14, which has been functionally dissected in yeast cells (22). The presence of a structurally related domain has also been recently recognized within U3 RNA (63).

**Potential role of U20 RNA.** Except for vertebrate U8 RNA, recently shown to be required for correct maturation of 5.8S and 28S rRNAs (45), the other snoRNAs shown to participate in pre-rRNA processing all appear to be involved in the formation of 18S rRNA (15, 61). Remarkably, one of those snoRNAs, U14, contains two long (13- and 14-nt) sequence complementarities with 18S rRNA in both mouse and in yeast cells, and mutations in U14 disrupting the 18S rRNA complementarity alter the processing of yeast 18S rRNA (25). Taken together with the particularly close structural relationships observed between U14 and U20, the outstanding complementarity between U20 and a phylogenetically conserved sequence in 18S rRNA might therefore also reflect a direct involvement of U20 RNA in the production of mature 18S rRNA. Whatever its potential role, the biological significance of this complementarity seems very likely, not only from statistical considerations (the probability for such a long match between two molecules of these sizes to occur by mere chance is less than $2 \times 10^{-8}$) but also because it involves two highly conserved sequences on both RNAs. Thus, it is already clear that the complementarity is largely, if not entirely, preserved among distant vertebrates, taking into account first that the amphibian *X. laevis* and mammals differ by a single nucleotide change over the 18S rRNA segment (44) and second that the complementary sequence in U20 RNA appears strongly conserved among the different vertebrate classes, as indicated by the Northern cross-hybridization signals obtained with probes 2 and 3 (Fig. 8). In fact, our very recent sequence analysis of the U20 RNA homolog in chickens and the amphibian *X. laevis* (48a) fully supports this interpretation. It is noteworthy that the 21-nt-long segment of 18S rRNA complementary to mammalian U20 exhibits five nucleotide differences between mammals and the yeast *Saccharomyces cerevisiae*. Accordingly, the identification of a potential U20 RNA homolog in yeast cells should provide a straightforward comparative test of the preservation of the pairing among fungi and vertebrates. However, none of the many yeast snoRNAs sequenced so far (4, 16) exhibits a significant homology with mammalian U20.

The proximity of the U20-complementary sequence from the 3′ end of mature 18S rRNA might suggest an involvement of the pairing in the 3′-terminal processing of the small subunit rRNA, a possibility that we are presently testing with the aid of an in vivo processing system, by transfection of mouse cells with rRNA minigenes (21) in which this particular processing step (and other pre-RNA cleavages) can be faithfully reproduced, using severely truncated pre-rRNAs. It also seems noteworthy that the complementarity with U20 is located in a domain of crucial importance for the function of the mature rRNA, the decoding site, which is easily accessible in the free ribosomal subunit but which participates in direct contacts with mRNA and the large subunit (41, 49, 58, 65). Accordingly, whereas the U20–pre-rRNA pairing could be involved in a variety of rearrangements of pre-rRNA structure during transcript elongation and ribosomal protein assembly, it could also prevent the appearance of a reactive conformation of the decoding site during the nucleolar steps of ribosome biogenesis, thereby shielding the nascent small subunit from unwanted interactions, either with the nascent large subunit (which exhibits a much longer lifetime in the nucleolus) or even with some mRNAs which show a surprising level of nucleolar accumulation (8). Our ongoing analysis of the interactions of U20 RNA with various pre-rRNAs and pre-ribosomal RNP particles at different stages of their biogenesis in the nucleolus should provide further insight into U20 function.

**U20 results from a processing of intronic RNA.** Southern analysis of genomic DNA shows that the sole copy of U20 RNA-coding sequence in the human haploid genome is the one found in the single-copy nucleolin gene. Moreover, all of the available evidence indicates that U20 RNA is not encoded by an independent transcription unit located within the nucleolin gene intron but derives from a novel form of intronic RNA processing, similar to several other recently identified vertebrate snoRNAs, U14 (34), U15 (63), U16 (17), and U17 (27). Like these other novel species, U20 RNA does not possess a 5′ TMG cap, strongly suggesting that its 5′-terminal nucleotide does not represent the 5′ end of the primary transcript. In line with this result, inspection of the U20 RNA-coding and flanking sequences in intron 11 of the nucleolin gene does not reveal the presence of classical transcription signals recognized by either RNA polymerase II or III. In fact, we have recently observed that U20 RNA can be faithfully processed from an in vitro T7 transcript encompassing part of exon 11 and the 566 5′-terminal nucleotides of intron 11 of the mouse nucleolin gene when injected into *Xenopus* oocytes (16a). U20 RNA can also be processed in vitro from the same T7 transcript by incubation in presence of a HeLa cell extract (26a), like U17 RNA (27). Finally, using transient transfection assays allowing the expression of the same transcript in cultured mouse cells, we have observed that U20 RNA is faithfully and efficiently processed in vivo from the transfected intronic sequence (11a).

**Potential significance of the intronic location of U20.** Most gene hosts of the previously reported intronic snoRNAs encode proteins which are ribosomal, nucleolar, or ribosome associated (15, 57), supporting the notion that this particular genomic organization is functionally significant and might provide the basis for regulatory linkages during ribosome biogenesis or function. The detection of U20 RNA in a nucleolin gene intron fits well into this emerging pattern. It also provides a particularly attractive specimen for further analyzing the functional implications of this novel form of gene organization and expression. Although its function is far from being fully understood, nucleolin, a major nucleolar protein, appears to possess multiple roles in the synthesis, processing, and packaging of pre-rRNA (13, 18, 30, 47, 55). The likely yeast homolog of nucleolin, NSR1 (32), is directly involved in ribosome biogenesis (28, 29, 31): an *nsr1* mutant is defective in its ability to produce 18S rRNA, and the defect results in an unbalanced content of the cells in mature ribosomal subunits (31). Thus, U20 RNA, the structure of which strongly suggests a preferential interaction with the small rRNA moiety of the rRNA precursor(s), is located in an intron of the gene of a protein which is itself involved in the production of the small subunit of the ribosome. This additional connection lends further support to the notion that the intronic location of the snoRNA may have some relevance to the function of the host gene protein. In this perspective, the presence of a second intron-encoded snoRNA, termed U22 (our unpublished results), in the nucleolin gene of vertebrates may reflect the multiplicity of the regulatory circuits of ribosome production

involving this multifunctional protein throughout the cell cycle and in different conditions of cell growth (10, 30, 47).

Obviously, the biological significance of the intronic location of U20 and other snoRNAs could be better assessed if the functional relationships between splicing of the pre-mRNA and production of the mature snoRNA could be clearly defined. In the case of U16, it has been proposed that the production of the ribosomal protein L1 messenger and that of the intronic snoRNA are antagonistic processes (17). It is noteworthy that the exon 11-intron 11 junction, unlike all other exon-intron junctions of the nucleolin gene, exhibits a suboptimal splice site, with the presence of a C, an unusual splicing donor nucleotide, which is maintained among rodents (9) and humans (59). This structural peculiarity could provide the basis for an antagonism between the production of U20 and the splicing of nucleolin mRNA, and it will certainly be of interest to determine how the rates of formation of the two mature RNA species compare in different conditions of cell growth and during development.

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