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Xenopus Ro ribonucleoproteins: Members of an evolutionarily conserved class of cytoplasmic ribonucleoproteins

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ABSTRACT **Ro small ribonucleoproteins consist of a 60-kDa protein and possibly additional proteins complexed with several small RNA molecules. The RNA components of these particles, designated Y RNAs, are about 100 nt long. Although these small ribonucleoproteins are abundant components of a variety of vertebrate species and cell types, their subcellular location is controversial, and their function is completely unknown. We have identified and characterized the Ro RNPs of *Xenopus laevis*. Three of the four distinct *Xenopus* Y RNAs appear to be related to the previously sequenced human hY3, hY4, and hY5 RNAs. The fourth *Xenopus* Y RNA, xYα, does not appear to be a homologue of any of the human Y RNAs. Each of the human and *Xenopus* Y RNAs possesses a conserved stem that contains the binding site for the 60-kDa Ro protein. *Xenopus* and human 60-kDa Ro proteins are 78% identical in amino acid sequence, with the conservation extending throughout the entire protein. When human hY3 RNA is mixed with *Xenopus* egg extracts, the human RNA assembles with the *Xenopus* Ro protein to form chimeric Ro ribonucleoproteins. By analyzing RNA extracted from manually enucleated oocytes and germinal vesicles, we have determined that Y RNAs are located in the oocyte cytoplasm. By examining the distribution of mouse Ro ribonucleoproteins in cytoplast and karyoplast fractions derived from L-929 cells, we have determined that Ro ribonucleoprotein particles also primarily reside in the cytoplasm of mammalian cells.**

All eukaryotic cells contain many small RNA–protein complexes that play crucial roles in cell metabolism. These ribonucleoproteins (RNPs) are classified based on their subcellular location: small nuclear RNPs, small cytoplasmic RNPs, and small nucleolar RNPs. The best characterized small nuclear RNPs are the U RNPs, which are involved in pre-mRNA processing (1). Two small nucleolar RNPs, U3 and U14, function in rRNA processing (2). The most abundant small cytoplasmic RNP is the signal-recognition particle, which is important for targeting nascent secretory proteins to the endoplasmic reticulum membrane (3).

There are many additional small RNPs in cells whose functions remain obscure. One class of these particles, the Ro RNPs, were discovered because they are recognized by anti-Ro antibodies from patients with systemic lupus erythematosus (4–6). Although these RNPs have been best characterized in human HeLa cells, they are present in a variety of vertebrate species and cell types (7–9). In human cells, the Ro RNPs consist of four small RNA molecules of 85–112 nt, each of which is complexed with a 60-kDa protein (10–13). The human 60-kDa Ro protein (14) contains a domain found in many other RNA-binding proteins, known as the RNA recognition motif (RRM) (15). Ro RNPs may also contain an additional 52-kDa protein (16).

The RNA components of the Ro particles, known as Y RNAs, vary in number, depending on the species examined. While human cells contain four distinct Y RNAs, designated hY1, hY3, hY4, and hY5 (hY2 is a processing or degradation product of hY1), many species contain only two or three Y RNAs (7–9). The human Y RNAs, which are transcribed by RNA polymerase III, exhibit homologies in their sequences and secondary structures (11, 13). Each Ro RNP is present in about 10^5 copies per cell, or about 1% the number of ribosomes.

The subcellular location of the Ro RNPs is controversial. Human reticulocytes (which lack nuclei) contain two Ro RNPs, hY1 and hY4, indicating that at least a subset of the Ro RNPs is present in the cytoplasm (13). Immunofluorescence experiments using patient autoantibodies have given conflicting results, with different groups finding nuclear (17) and cytoplasmic (7) fluorescence. By aqueous fractionation techniques, the particles appear cytoplasmic (6); however, many components leak out of the nucleus in these fractionation protocols.

Both to identify conserved features of Ro RNPs and to allow us to use amphibian oocytes as a system to test potential functions of these particles, we have characterized the Ro RNPs of the African clawed toad *Xenopus laevis*. We report that the 60-kDa Ro protein and one Y RNA, Y3, are highly conserved between humans and amphibians. Surprisingly, the other Y RNAs appear less conserved. In addition, using fractionation techniques which minimize leakage of nuclear components, we have localized Ro RNPs to the cytoplasms of both amphibian and mammalian cells.

MATERIALS AND METHODS

Cell Extracts and Immunoprecipitations. *Xenopus* tissue culture cells were washed in 7 mM Tris·HCl, pH 7.4/105 mM NaCl and resuspended in NET-2 (40 mM Tris·HCl, pH 7.4/150 mM NaCl/0.05% Nonidet P-40) containing 10 mM vanadyl ribonucleoside complexes. After sonication, the extract was sedimented at 100,000 × *g* in a Beckman TLA 100.3 rotor for 20 min. Following immunoprecipitation (12), extracted RNAs were end-labeled with [^{32}P]pCp.

Isolation of the *Xenopus* 60-kDa Ro cDNA. The cDNA encoding the human Ro 60-kDa protein (14) was used to screen a library of *X. laevis* ovary cDNA in phage λgt11 (18). Hybridization was in 6× SSPE (1× SSPE is 0.15 M NaCl/0.01 M NaH_2PO_4, pH 7.4/1 mM EDTA) at 50°C followed by washing in 5× SSPE at 50°C. A single clone encoding the 3′-terminal third of the 60-kDa Ro protein was isolated. The 5′ end of this cDNA was used to design a primer which, in conjunction with a vector-specific primer, was used to amplify the remaining 5′-terminal cDNA from a *Xenopus* embryonic cDNA library in λgt11 (19). To identify errors

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Abbreviations: RNP, ribonucleoprotein; RRM, RNA recognition motif.

*The sequences reported in this paper have been deposited in the GenBank data base [accession nos. L15430 (Ro cDNA), L15431 (xY3), L15432 (xY4), L15433 (xY5), and L15434 (xYα)].

introduced during PCR amplification, products from three independent reactions were sequenced.

RNA Sequencing. Immunoprecipitated RNAs were 5′- or 3′-end-labeled with ^{32}P and sequenced with base-specific ribonucleases (20–22). The 5′- and 3′-terminal nucleotides were determined by digestion of end-labeled RNAs followed by thin-layer chromatography. The presence of 5′-terminal triphosphates was confirmed by labeling immunoprecipitated RNAs with guanylyltransferase and [α-^{32}P]GTP (23). To resolve ambiguities, genes encoding each of the *Xenopus* Y RNAs were isolated by the inverse PCR method (24). For each gene, products from three independent amplification reactions were sequenced.

Cell Fractionation. Mouse L-929 cells were enucleated (25, 26) on Ficoll (Pharmacia) gradients. Although the 17%/25% interface fraction was described as containing whole cells, we obtained a second karyoplast fraction at this position. This fraction was reduced in tRNA and enriched in small nuclear RNAs when compared with unfractionated cells. For RNA analysis, each fraction was resuspended in NET-2 and sonicated and the RNA was extracted (12). For protein analysis, fractions were resuspended in SDS/PAGE sample buffer (3.5% SDS/14% glycerol/40 mM Tris base/120 mM dithiothreitol).

Xenopus stage VI oocytes were enucleated by manual dissection under mineral oil (27). RNA was prepared from germinal vesicles, enucleated oocytes, or whole oocytes by homogenization in 50 mM NaCl/5 mM EDTA/0.5% SDS/50 mM Tris·HCl, pH 7.5 containing proteinase K at 200 μg/ml. After a 30-min incubation at 37°C, RNA was extracted with phenol/chloroform (1:1) and ethanol-precipitated.

Northern Blots. RNAs fractionated in 5% polyacrylamide/8 M urea gels were transferred electrophoretically to Hybond-N (Amersham) in 45 mM Tris borate, pH 8.0/1 mM EDTA. Hybridizations with ^{32}P-labeled random primed DNA, SP6 RNA, or T7 RNA probes were carried out as described (28). The U1 and 7SK plasmids were gifts of D. Wassarman (Yale University).

RNP Reconstitution in *Xenopus* Egg Extracts. We used PCR to place the hY3 coding sequence behind a T7 promoter. The 5′ primer contained 10 nt of hY3 sequence preceded by a T7 promoter and an *Eco*RI site. The 3′ primer contained 10 nt complementary to the 3′ end of hY3, preceded by a *Dra* I and a *Bam*HI site. After PCR with the cloned hY3 RNA gene (11) as template, the product was digested with *Eco*RI and *Bam*HI and inserted into the *Eco*RI/*Bam*HI sites of pSP64 (Promega). After cleavage with *Dra* I, transcription by T7 RNA polymerase yielded an RNA identical to hY3. Constructs that allow transcription of U3 RNA and Epstein–Barr virus-encoded EBER1 RNAs were gifts of S. Baserga and D. Toczyski (29, 30).

^{32}P-labeled U3, EBER1, and hY3 RNAs were synthesized with T7 RNA polymerase (31), using 2 μg of plasmid and 50 μCi of [α-^{32}P]CTP (Amersham; 400 Ci/mmol; 1 Ci = 37 GBq) in place of CTP. RNAs were suspended in 10 μl of water, and 2 μl of each was added to 25 μl of *Xenopus* egg extract (ref. 32; a gift of M. Solomon, Yale University) and incubated for 1 hr at 22°C. Immunoprecipitations were as described (12) except that 20 mM Hepes pH 7.5/150 mM NaCl/0.1% Triton X-100 was substituted for NET-2.

RESULTS

Four Small RNAs Are Components of Ro RNPs in *X. laevis*. To identify Y RNAs in *X. laevis*, we performed immunoprecipitations from *Xenopus* tissue culture cell sonicates, using several different anti-Ro sera. RNAs present in the immunoprecipitates were extracted and labeled at their 3′ ends with [^{32}P]pCp. Two different patient anti-Ro sera immunoprecipitated RNPs containing four small RNAs ranging from 69 to 98 nt (Fig. 1, lanes 3 and 4). Immunoprecipitation using a rabbit anti-bovine Ro serum (9) produced an identical pattern (lane 5). Two significantly smaller bands were also present in the anti-Ro immunoprecipitates (Fig. 1, asterisk). As RNAs vary in the efficiency with which they are labeled with [^{32}P]pCp, it was possible that other RNAs were present in the immunoprecipitates but not labeled. However, when the immunoprecipitated RNAs were visualized by silver staining, an identical pattern of small RNAs was obtained (data not shown).

The sequences of the four larger RNAs were determined by enzymatic cleavage of end-labeled RNA with base-specific ribonucleases (Fig. 2*A*). RNA sequences were confirmed and ambiguities were resolved by amplifying the genes for each of the four RNAs by inverse PCR (24). Only one amplification product was obtained for each RNA. In each case, the genomic DNA sequence was completely consistent with the RNA sequence. A TATA-like sequence was located 25–30 nt upstream from each of the RNA-coding regions (Fig. 2*A*, bar). Such sequences occur 20–30 nt upstream of many genes transcribed by RNA polymerase III (33).

We compared the sequences of the four *Xenopus* Y RNAs both with each other and with the previously sequenced human Y RNAs. The four *Xenopus* Y RNA genes share four regions of identity within the coding sequences (Fig. 2*A*). Comparison of the *Xenopus* and human Y RNAs revealed that the largest *Xenopus* Y RNA was 86% identical to hY3 RNA. The second largest *Xenopus* RNA was most similar to hY4 (75% identical), while the shortest species most resembled hY5 (70% identical). We have therefore named these RNAs xY3, xY4, and xY5. As the remaining *Xenopus* Y RNA did not resemble any particular human Ro RNA, we have designated this RNA xYα. Sequence analysis of the two smallest RNAs (Fig. 1, asterisk) revealed that they were 5′ truncated versions of xY5 RNA, beginning between nt 30 and 32 of the xY5 sequence. Although these truncated versions of xY5 RNA may be generated by nuclease degradation during isolation, they are invariably present in our immunoprecipitates.

Possible secondary structures for the human and *Xenopus* Y RNAs are shown in Fig. 2*B*. We have indicated two regions, designated A and B, which are conserved among all of the Y RNAs. In our proposed structures, these two conserved regions anchor the ends of an 18- to 20-bp stem formed by the 5′ and 3′ ends of the RNAs. In addition, all of the Y RNAs can be folded to contain a pyrimidine-rich internal loop.

The 60-kDa Ro Protein Is Highly Conserved Between Humans and Frogs. We obtained a cDNA clone encoding the *Xenopus* 60-kDa Ro protein. The sequences of the human and *Xenopus* Ro proteins are compared in Fig. 3. Both proteins are 538 amino acids in length and possess 78% amino acid

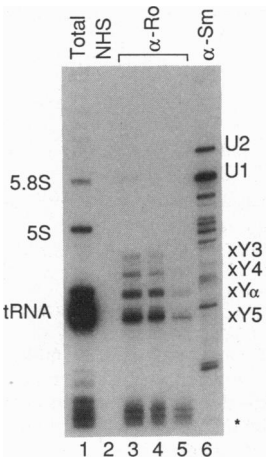

FIG. 1. *Xenopus* Ro RNPs contain four distinct Y RNAs. RNAs contained within immunoprecipitates were labeled with [^{32}P]pCp and fractionated in a 5% polyacrylamide/8 M urea gel. Shown are RNAs present in immunoprecipitates obtained with nonimmune human serum (lane 2), two patient anti (α)-Ro sera (lanes 3 and 4), rabbit anti-bovine Ro serum (lane 5), or patient anti-Sm serum (lane 6). Lane 1 contained total cellular RNA.

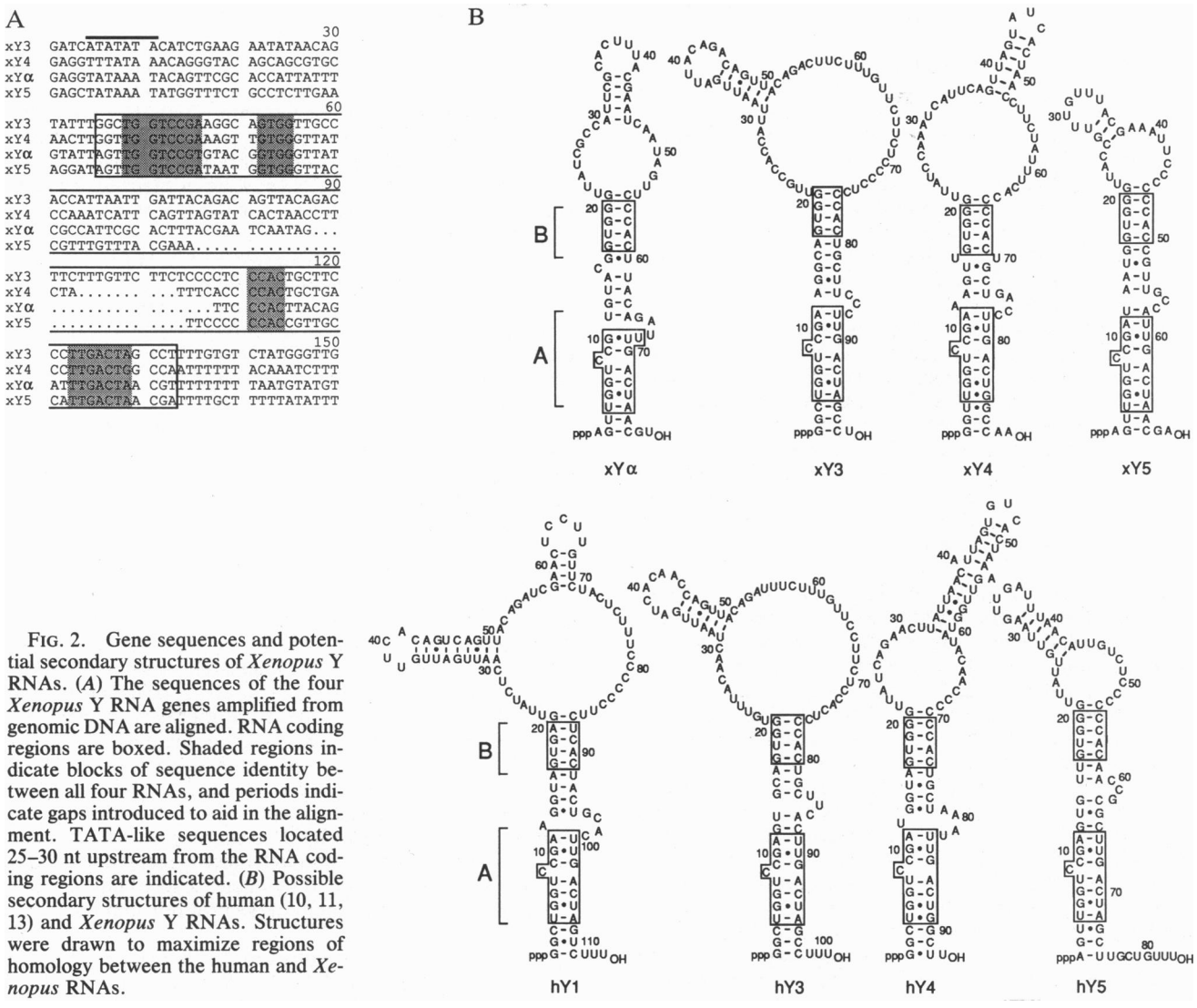

FIG. 2. Gene sequences and potential secondary structures of *Xenopus* Y RNAs. (*A*) The sequences of the four *Xenopus* Y RNA genes amplified from genomic DNA are aligned. RNA coding regions are boxed. Shaded regions indicate blocks of sequence identity between all four RNAs, and periods indicate gaps introduced to aid in the alignment. TATA-like sequences located 25–30 nt upstream from the RNA coding regions are indicated. (*B*) Possible secondary structures of human (10, 11, 13) and *Xenopus* Y RNAs. Structures were drawn to maximize regions of homology between the human and *Xenopus* RNAs.

identity. While the homology extends over the length of the protein, the RNP consensus motif is especially conserved (84%, indicated by the line in Fig. 3). However, a zinc finger motif noted in the human sequence (amino acids 305–323; ref. 14) is not conserved.

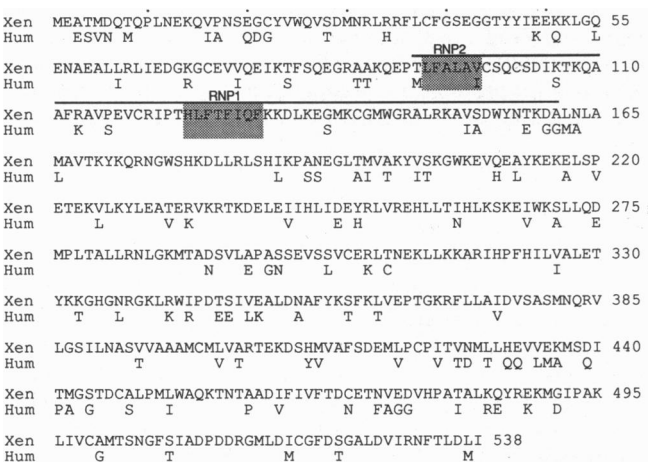

FIG. 3. Comparison of *Xenopus* (Xen) and human (Hum) 60-kDa Ro proteins. Amino acids that differ between the *Xenopus* and human (14) 60-kDa Ro proteins are indicated. The 70 amino acids comprising the RRM are indicated by the line. Two conserved sequence motifs, RNP1 and RNP2, are shaded.

Assembly of Chimeric Ro RNPs. The ability of human hY3 RNA to assemble with *Xenopus* Ro protein was tested by synthesizing hY3 RNA *in vitro* with T7 RNA polymerase and incubating the labeled RNA in a *Xenopus* egg extract. As controls we included the U3 nucleolar RNA and the Epstein–Barr virus-encoded EBER1 RNA. A portion of the mixture was then subjected to immunoprecipitation with human anti-Ro antibodies. Human hY3 RNA, but not EBER1 or U3 RNAs, assembled into immunoprecipitable Ro RNPs (Fig. 4, lane 5). The hY3 and EBER1 RNAs were also bound by a second protein, the *Xenopus* La protein (lane 4). The La protein is a 50-kDa nuclear protein which associates with all RNA polymerase III transcripts, at least transiently, via the 3′-terminal UUU_{OH} (34). As both the hY3 and EBER RNAs terminate in UUU_{OH}, they assemble with the *Xenopus* La protein (lanes 3 and 4).

Ro RNPs Reside in the Cytoplasm of Both Amphibian and Mammalian Cells. To determine the subcellular location of the *Xenopus* Ro RNPs, we manually separated oocytes into nuclear and cytoplasmic fractions (27). Northern analysis of RNAs extracted from the fractions (Fig. 5*A*, lanes 1–3) revealed that xY3 RNA resides exclusively in the cytoplasmic fraction. Repeated probing of the blot for the remaining Y RNAs revealed that all four of the *Xenopus* Y RNAs were exclusively cytoplasmic (data not shown). In contrast, *Xenopus* U1 small nuclear RNA was found primarily in the

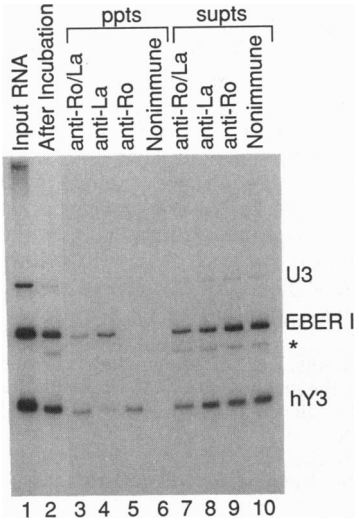

FIG. 4. Human hY3 RNA assembles into immunoprecipitable Ro RNPs in *Xenopus* egg extracts. ^{32}P-labeled U3, EBER, and hY3 RNAs were synthesized *in vitro* and incubated with *Xenopus* egg extracts (32). The extract was then extracted with phenol (lane 2) or immunoprecipitated with human serum containing mixed anti-Ro and anti-La antibodies (lanes 3 and 7), anti-La antibodies (lanes 4 and 8), anti-Ro antibodies (lanes 5 and 9), or a nonimmune serum (lanes 6 and 10). RNAs contained in immunoprecipitates (ppts) are shown in lanes 3–6, and the resulting supernatants (supts) in lanes 7–10. The band designated with an asterisk probably represents a degradation product of U3 RNA.

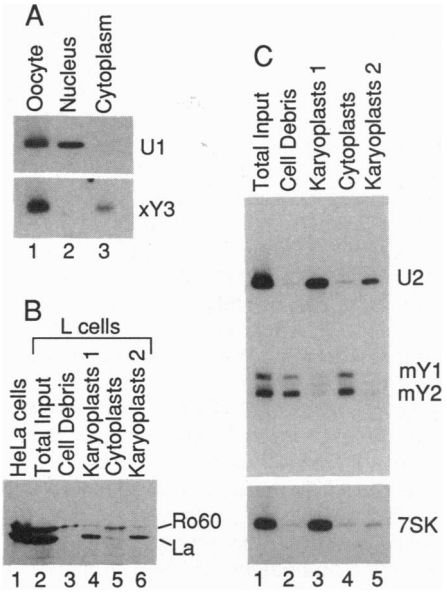

FIG. 5. Ro RNPs are primarily found in the cytoplasm of vertebrate cells. (*A*) RNAs extracted from equal numbers of whole stage VI oocytes (lane 1), germinal vesicles (lane 2), and the enucleated oocytes (lane 3) were analyzed by Northern blotting. The blot was probed with an SP6-transcribed RNA complementary to human U1 and a 17-nt oligonucleotide complementary to *Xenopus* Y3 RNA. (*B*) Mouse L-929 cells were fractionated into cytoplast and karyoplast fractions as described (25, 26). Gradient fractions consisting of cytoplasts (lane 5), the two karyoplast fractions (lanes 4 and 6), and cell debris (lane 3) were assayed by Western blotting with a patient antiserum containing both anti-Ro and anti-La antibodies. An equivalent number of cells as that placed on the gradient were analyzed (lane 2). A HeLa cell extract was also analyzed (lane 1). (*C*) RNAs contained in cytoplasts (lane 4), the two karyoplast fractions (lanes 3 and 5), and the cell debris fraction (lane 2) were analyzed by Northern blotting. Filters were probed for two mouse Ro RNAs, mY1 and mY2. As controls, the filters were probed for the U2 and 7SK RNAs.

nuclear fraction, indicating that minimal leakage of nuclear contents occurred during the enucleation procedure.

As the subcellular location of the Ro RNPs is controversial (7, 17), we sought to extend these findings to mammalian cells. We separated mouse L-929 cells into karyoplasts and cytoplasts by a previously described procedure (25, 26). After treatment with cytochalasin B, cells were enucleated by centrifugation through Ficoll gradients. We obtained four fractions: cytoplasts, two karyoplast fractions (which differed in the amount of cytoplasm remaining around the nuclei), and a cellular debris fraction at the top of the gradient. [As the debris fraction consisted of cytosol as judged by ethidium bromide staining of the extracted RNA (data not shown), this fraction was presumably generated during the extrusion of nuclei through the plasma membrane.]

To determine the distribution of the 60-kDa Ro protein in the gradient fractions, we probed protein immunoblots with a patient serum that contained both anti-Ro and anti-La antibodies. We could therefore also examine the distribution of the nuclear La protein, which leaks out of the nucleus during cell fractionation in aqueous buffers (6, 34). The majority of the Ro protein was detected in the cytoplast and cellular debris fractions (Fig. 5*B*, lanes 3 and 5), whereas the La protein was found predominantly in the two karyoplast fractions (lanes 4 and 6).

RNAs contained in each fraction were analyzed by Northern blotting. Mouse cells contain two Y RNAs, mY1 and mY2, which hybridize with the cloned genes for human hY1 and hY3 RNA (11). The two Y RNAs were largely found in the cytoplast and cellular debris fractions (Fig. 5*C*, lanes 2 and 4). The two karyoplast fractions, which contained a thin rim of cytoplasm surrounding the nuclei, contained <15% of the Y RNAs. In contrast, the U2 small nuclear RNA was predominantly found in the karyoplast fractions. We also probed the gradient fractions for 7SK RNA, a nuclear RNA that leaks out of the nucleus during standard fractionation procedures (35). The majority of the 7SK RNA was detected in the karyoplast fractions (Fig. 5*C Lower*), indicating that little nuclear leakage had occurred.

DISCUSSION

To use *Xenopus* oocytes as a system for probing the function of the Ro RNPs, we have identified and characterized the Ro RNPs of *X. laevis*. This analysis has revealed that certain features of Ro protein and RNA structure are highly conserved between humans and amphibians. In addition, we have determined that Ro RNPs, whose subcellular distribution has long been controversial, reside primarily in the cytoplasm.

Conservation of Ro RNPs. Our sequences of the *Xenopus* Y RNAs and 60-kDa Ro protein have allowed us to identify conserved features of Ro RNPs. All eight sequenced Y RNAs can be drawn as structures containing a pyrimidine-rich internal loop and a long stem formed by pairing the 5′ and 3′ termini. Within this stem are two conserved regions. The first motif (region A in Fig. 2*B*) is within the region most highly protected by Ro protein(s) from nuclease digestion. This region, which contains a single bulged cytidine within a conserved helix, was assigned as the likely binding site of the 60-kDa Ro protein (12). Mutations in this structure in which the bulged cytidine is deleted or replaced with adenine severely reduce binding by the 60-kDa Ro protein (36). The second motif (region B in Fig. 2*B*) consists of four identical base pairs at the top of the stem. Although this structure is not

protected from nuclease digestion by Ro protein, it may be important for the binding of Ro or other proteins.

While all the Y RNAs share certain structural features, the individual RNAs vary in the extent to which they are conserved across species. Although the human and *Xenopus* Y3 RNAs are 86% identical, the remaining Y RNAs are not nearly so conserved. One *Xenopus* Y RNA, xYα, has no apparent homologue in humans. Conversely, there does not appear to be a homologue of human Y1 in *Xenopus*. In humans, the genes encoding Y1 and Y3 are adjacent in the genome, indicating that these RNAs probably arose from duplication of a single ancestral gene (11). The finding that Y3 is strongly conserved, whereas no Y1 homologue apparently exists in the frog, suggests that Y1 may have arisen through duplication of the Y3 gene.

Although homologues of hY4 and hY5 do not exist in mice and rats, two of the *Xenopus* Y RNAs appear to be related to these two human RNAs. While the *Xenopus* Y4 and Y5 RNAs are only 75% and 70% identical in primary sequence to the respective human Y RNAs, each can be drawn to form a structure that resembles the human counterpart. If these RNAs are genuine homologues of the respective human species—that is, if they evolved from common ancestral molecules—then Y4 and Y5 RNAs have been dispensed with in some rodent species. Alternatively, similar functional constraints might have caused the *Xenopus* and human Y4 and Y5 RNAs to independently evolve to form similar structures.

Our reconstitution experiment has demonstrated that the *Xenopus* 60-kDa Ro protein can bind human hY3 RNA, indicating that the regions in the protein and RNA required for binding have been conserved between humans and amphibians. The 60-kDa Ro protein is also conserved throughout its length. It has been observed that small deletions in the human protein eliminate the ability of the protein to bind its target RNA (36), suggesting that sequences throughout the protein are critical for folding to form a functional RNA binding site. This is in contrast to many RRM-containing proteins in which a fragment of the protein containing the RRM can be identified that is sufficient for specific RNA binding (15). However, a potential zinc finger motif previously noted in the human protein (14) is not conserved in the *Xenopus* sequence.

Cytoplasmic Location of Ro RNPs. The location of Ro RNPs within the cell has been controversial (7, 17). Previous attempts to localize these particles relied largely on the use of patient sera in immunofluorescence experiments. As such sera often contain multiple antibody specificities, misleading results can be obtained. By fractionating *Xenopus* oocytes and mouse cultured cells into nuclear and cytoplasmic fractions under conditions where nuclear leakage is minimal, we have shown that Ro RNPs reside primarily in the cytoplasm. It remains possible that Ro RNPs are present in the nucleus at low levels or that they shuttle between the nucleus and the cytoplasm. However, our results from two organisms and two very different cell types indicate that the majority of the Ro RNPs are located in the cytoplasm.

Possible Functions for Ro RNPs. As Ro RNPs are conserved and abundant components of vertebrate cell cytoplasm, they presumably function in a basic cellular process, such as mRNA stability, mRNA localization, or translation. It is curious that different vertebrate species contain different subsets of distinct Y RNA species. In this respect, the Y RNAs are different from the best characterized class of small nuclear RNAs, the U RNAs. For the five major spliceosomal U small nuclear RNAs, clear homologues of each RNA exist in virtually all eukaryotic species. Each of these U RNAs functions in a distinct step in the splicing pathway, and the different U RNAs are not functionally interchangeable (1). As the Y3 RNA is strongly conserved between humans and frogs, it may similarly play a unique role in the normal functioning of vertebrate cells. The fact that the other Y RNAs are less conserved and vary in their distribution across species may indicate that these RNAs perform very similar or overlapping functions.

Although Ro RNPs are present in a wide variety of vertebrate species and cell types, they have not been found in nonvertebrate species. Based on their conservation and ubiquitous distribution in vertebrates, it is likely that these RNPs exist in other species. The reconstitution assay described here may be useful for identifying Y RNAs from lower eukaryotes.

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