The Heterogeneous Nuclear Ribonucleoproteins I and K Interact with a Subset of the Ro Ribonucleoprotein-associated Y RNAs in Vitro and in Vivo*

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The hY RNAs are a group of four small cytoplasmic RNAs of unknown function that are stably associated with at least two proteins, Ro60 and La, to form Ro ribonucleoprotein complexes. Here we show that the heterogeneous nuclear ribonucleoproteins (hnRNP) I and K are able to associate with a subset of hY RNAs in vitro and demonstrate these interactions to occur also in vivo in a yeast three-hybrid system. Experiments performed in vitro and in vivo with deletion mutants of hY1 RNA revealed its pyrimidine-rich central loop to be involved in interactions with both hnRNP I and K and clearly showed their binding sites to be different from the Ro60 binding site. Both hY1 and hY3 RNAs coprecipitated with hnRNP I in immunoprecipitation experiments performed with HeLa S100 extracts and cell extracts from COS-1 cells transiently transfected with VSV-G-tagged hnRNP-I, respectively. Furthermore, both anti-Ro60 and anti-La antibodies coprecipitated hnRNP I, whereas coprecipitation of hnRNP K was not observed. Taken together, these data strongly suggest that hnRNP I is a stable component of a subpopulation of Ro RNPs, whereas hnRNP K may be transiently bound or interact only with (rare) Y RNAs that are devoid of Ro60 and La. Given that functions related to translation regulation have been assigned to both proteins and also to La, our findings may provide novel clues toward understanding the role of Y RNAs and their respective RNP complexes.

Small ribonucleoprotein (RNP)1 complexes are usually composed of one molecule of a small RNA and several proteins that bind either directly to the RNA or indirectly via protein-protein interactions (1, 2). Many of these complexes exert essential functions that are often indispensable for survival, such as the small nuclear RNPs, which are major components of the spliceosomal machinery, or the signal recognition particle, which plays a key role in protein export. In contrast to these well-defined complexes, the structure of the cytoplasmic Ro RNPs is still not fully resolved, and their function has remained enigmatic (3, 4). They are composed of one molecule of a small Y RNA (transcribed by RNA polymerase III) and at least two proteins, the 60-kDa protein Ro60 and the 48-kDa phosphoprotein La. However, although Ro60 and Y RNAs are present in comparable stochiometric amounts, La is ~50-fold more abundant, and therefore the vast majority of La molecules is not bound to Y RNAs, in contrast to Ro60 (5).

Y RNAs are highly conserved in evolution (6) and have been found in all multicellular eukaryotic organisms and may also be present in some bacteria (7) but, remarkably, have so far not been detected in yeast. Interestingly, the genome of the nematode Caenorhabditis elegans contains only one functional Y RNA gene, whereas in humans and other vertebrates four closely related Y RNA species exist. Unlike other RNA polymerase III transcripts, Y RNAs retain the oligo(U) stretch at their 3’ end that forms the binding site for La; therefore these RNAs remain permanently associated with La (5, 8). On the other hand, nuclear Y RNAs do not seem to be associated with Ro60, and it was suggested that Ro RNPs assemble upon export to the cytoplasm (3, 9).

Several functions have been proposed for La, including regulation of RNA polymerase III transcription (10–12), involvement in internal ribosome entry site-dependent viral and cellular translation (13, 14), and a role in the assembly of small nuclear RNPs (15), but it is still not entirely clear which role this abundant nuclear and cytoplasmic protein plays in vivo. In particular, it is completely unknown whether a unique function can be attributed to Y RNA-associated La. Much less is known about biological activities of Ro60 (4). In the cytoplasm it may be involved in the regulation of translation as recently demonstrated for ribosomal protein L4 in Xenopus laevis oocytes (16) and in the nucleus Ro60 may be implicated in a discard pathway of 5 S rRNA by recognizing incorrectly processed and misfolded molecules (17, 18). However, these (proposed) functions do not seem to be dependent on the presence of Y RNAs in acid; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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§ The abbreviations used are: RNP, ribonucleoprotein; hnRNP, heterogeneous nuclear ribonucleoprotein; IPP, immunoprecipitation buffer; PTB, polypyrimidine tract-binding protein; SLE, systemic lupus erythematosus; VSV, vesicular stomatitis virus; β-gal, β-galactosidase; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; CHAPS, 3-[3-cholamidopropyl]dimethylammoniom]-1-propanesulfonic acid; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.
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*X. laevis* nor in *C. elegans*. Interestingly, a prokaryotic homologue of Ro60 was recently discovered in the bacterium *Deinococcus radiodurans* that seemed to contribute to the remarkable resistance of this organism to ultraviolet radiation (7). This may lead to speculation about a RNA chaperoning function of Ro60 as has been recently proposed for La (19).

Although the stable association of Ro60 and La with Y RNAs is beyond experimental doubt, the presence of other components in addition to Ro60 and La is still a matter of debate. Thus, the suggested associations of a 52-kDa protein (Ro52) and the Ca\(^{2+}\)-binding protein calreticulin have remained controversial (20–25), and recently reported interactions of two novel proteins with Ro60 observed in yeast two- and three-hybrid systems need to be confirmed (26, 27). In a previous study, we demonstrated in *vitro* binding of several proteins contained in a HeLa S100 extract to hY RNAs (28). These proteins (with molecular masses between 53 and 80 kDa) bound specifically to hY1 and hY3 RNA but only weakly or not at all to hY4 and hY5 RNA. Interestingly, autoantibodies to Ro60 observed in yeast two- and three-hybrid systems need to be confirmed (26, 27). In a previous controversial (20–25), and recently reported interactions of two novel proteins with Ro60 observed in yeast two- and three-hybrid systems need to be confirmed (26, 27).

**Experimental Procedures**

**Sera and Antibodies**—For immunodetection of proteins binding to hY1 and hY3 RNA in *vitro*, an anti-Ro positive serum from a patient (BM) with SLE containing antibodies to these proteins was employed (28). Sera from healthy persons and from patients with rheumatoid arthritis (which do not contain anti-Ro or anti-La autoantibodies) were used as negative controls. Monoclonal antibodies used were anti-La SW5 (30), anti-Ro60 2G10 (31), anti-hnRNP K 2G14 (Ref. 32; kind gift of D. M. Helft, Cold Spring Harbor Laboratory), and an anti-human interleukin-6 antibody (Janssen Biochimica, Denmark) as control.

**Cellular Extracts**—HeLa S100 extracts for immunoblot analyses were prepared essentially as described (28). Briefly, 1 × 10\(^8\) HeLa cells (Computer Cell Culture Center, Mons, Belgium) were washed twice in isotonic buffer (10 mM Tris-HCl, pH 7.9, 140 mM KCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 25% glycerol), resuspended in 2 pellet volumes of buffer A (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1.5 mM EDTA, 25% glycerol), and disrupted by Dounce homogenization. Nuclei were separated by centrifugation (3 min at 3,000 × g), and the supernatant was first centrifuged for 20 min at 20,000 × g and then for 1 h at 100,000 × g. After measuring the protein concentration the S100 extract was stored at −70 °C.

**Immunoprecipitation**—Immunoprecipitations with monoclonal antibodies to Ro60, La, and hnRNP I and K were performed as described with antibodies coupled to protein A-Sepharose beads (Amersham Pharmacia Biotech) using dimethylpimelimidate (Sigma) as a cross-linking agent (20). Twenty µl of a HeLa cell extract was diluted in 0.5 ml of immunoprecipitation buffer (IPP-150 (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40) and incubated for 1 h at 4 °C with immobilized antibodies (20 µl of packed bead volume). Subsequently, the immunoprecipitates were washed three times with IPP-150 and resuspended in 400 µl of IPP-150 containing 0.5% SDS. RNA was extracted with phenol/chloroform, precipitated with ethanol using 10 µg of glycogen as carrier, and analyzed by Northern blot hybridization. For isolation of proteins 200 µl of a HeLa cell extract was applied to 1 ml of anti-Ro or anti-La immunoaffinity columns using a peristaltic pump at a low flow rate. After washing with at least 10 bed volumes of IPP-150, proteins were eluted with IPP-1000 (10 mM Tris-HCl, pH 7.9, 1500 mM NaCl, 0.05% Nonidet P-40).

For immunoprecipitations carried out with extracts from transfected cells monoclonal antibodies (anti-VSV-G-tag+anti-Ro60, La, and hnRNP I and K) were coupled to protein A-agarose beads by incubating overnight at 4 °C in IPP-500 buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.05% Nonidet P-40). Subsequently, the antibody-coated beads were equilibrated with IPP-150 and incubated with cell extracts for 2 h at 4 °C. Then the beads were extensively washed with IPP-150, and coprecipitated RNAs were isolated by phenol/chloroform extraction and ethanol precipitation.

**In Vitro Transcription of Y RNAs and Northern Blot Analyses**—In *vitro* transcription of human sense and antisense hY RNAs and of hY1 RNA deletion mutants was performed as described (28). To generate biotinylated RNAs in vitro, yeast Mo6 Biochemicals) was used at 75 µM concentration, for preparation of radiolabeled RNAs 0.5 mM UTP and 40 µCi of [α-\(^32\)P]UTP (PerkinElmer Life Sciences) were employed. The plasmid encoding 5 S RNA was a kind gift of Dr. K. Nierhaus (Max Planck Institute for Molecular Genetics, Berlin, Germany). For detection of Y RNAs by Northern blot hybridization RNAs were separated on 10% polyacrylamide, 7 x urea gels, electroblotted onto nylon membranes (Zeta-probe; Bio-Rad), fixed by UV cross-linking, and hybridized with \(^32\)P-labeled antisense hY RNA transcripts by hybridization overnight at 65 °C as described (28, 34).

**Reconstitution and Purification of Y RNPs**—These procedures were performed essentially as described (28). To dissociate existing complexes, the salt concentration of the HeLa extract was first increased to 1 M KCl followed by 30 min of incubation on ice. Then 10 µg of biotinylated hY RNA or biotinylated control RNA (5 S RNA) and 200 µg yeast tRNA (Roche) was added and the KCl concentration was readjusted and the reaction was allowed to reconstitute by incubating at 30 °C for 20 min at 30 °C. Reconstituted hY RNPs were isolated by adding 20 µl of NeutrAvidin beads (Pierce) and rotating for 1 h at 4 °C. Beads were then washed five times with 1 ml of IPP-150, and bound proteins were isolated employing elution buffer (20 mM Tris-HCl, pH 7.9, 20 mM DTT, 2% SDS). Eluted proteins were heated for 5 min at 65 °C and precipitated by adding 1 µl of glycogen (20 mg/ml) and 4 volumes of acetone. Samples were left at −70 °C for at least 30 min and subsequently centrifuged for 15 min at 15,000 × g. Recovered proteins were dissolved either in SDS sample buffer for SDS-PAGE or in two-dimensional lysis buffer for application in two-dimensional electrophoresis.

**Two-dimensional Gel Electrophoresis**—Proteins binding to biotinylated hY RNAs in *vitro* were dissolved in 20–40 µl of lysis buffer containing 9 x urea, 2% CHAPS (Sigma), 0.8% amido black 3–10 (40% solution, Fluka, Switzerland), 1% DTT. High resolution two-dimensional gel electrophoresis on immobilized pH gradients was carried out on ready-cut I PG Immobiline strips, pH 3–10 nonlinear, 18 cm long (Amersham Pharmacia Biotech). After overnight incubation in rehydration buffer (8 x urea, 0.5% CHAPS, 15 mM DTT, 0.2% amido black 3–10), the strips were focused on a horizontal electrophoresis apparatus (Amersham Pharmacia Biotech) in a stepwise fashion: 0.5 h at 300 V, 1 h at 500 V, 1 h at 1500 V, and 15 h at 2500 V (total 40 kVh) at room temperature. The IPG strips were subsequently incubated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 8 x urea, 30% glycerol, 2% SDS, traces of bromophenol blue) containing 10 mg/ml DTT and for 15
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Fig. 1. Characterization of hY1 RNA-binding proteins by two-dimensional gel electrophoresis and immunoblotting. Proteins from HeLa cell extracts binding to biotinylated hY1 RNA in reconstitution assays were separated by two-dimensional electrophoresis, transferred to a nitrocellulose membrane, and stained with serum BM from a patient with SLE recognizing several hY1 RNA-binding proteins in addition to Ro60 and La (marked by arrows). The two stained spots migrating below La presumably represent La degradation products. Positions of Ro60 and La were determined in a separate experiment using monoclonal antibodies to these proteins. The hnRNP K protein was identified by tandem mass spectrometry, the identity of hnRNP I (migrating as a doublet) was deduced from its characteristic position on the two-dimensional gel and immunologically confirmed using a monoclonal antibody against hnRNP I (see Fig. 2C).

RESULTS

Identification of Novel hY RNA-associated Proteins—Recently, we have described a set of five proteins with molecular masses between 53 and 80 kDa that in addition to Ro60 and La bound to human hY RNAs in vitro, particularly to hY1 and hY3 RNA (28). Interestingly, autoantibodies to these proteins were detected in several sera of patients with SLE who commonly develop antibodies to Ro and La proteins. To further characterize these hY RNA-binding proteins Ro RNP complexes were reconstituted in vitro by incubating HeLa S100 extracts with biotinylated hY1 RNA. Reconstitution reactions employing biotinylated 5 S rRNA were performed in parallel to control for nonspecifically binding proteins. All reactions were supplemented with a 20-fold excess of yeast tRNA as a nonspecific competitor. Reconstituted complexes were purified by NeutAvidin affinity chromatography followed by two-dimensional gel electrophoresis and subsequently analyzed by immunoblotting using SLE serum BM known to recognize all five novel hY RNA-binding proteins as well as Ro60 and La (Ref. 28; see also Fig. 2A). As can be seen in Fig. 1, several protein spots on the membrane were stained by serum BM. The spots corresponding to proteins binding specifically to hY1 RNA are indicated by arrows. The positions of Ro60 and La with its typical isoelectric isoforms (37–39) were confirmed in a separate experiment using monospecific sera and monoclonal antibodies to these proteins (not shown); the two spots visible below the La spots presumably represent La degradation products. Apart from the spots corresponding to Ro60 and La proteins, four major protein spots can be detected migrating above Ro60. The most acidic of these proteins, which hydropically migrated at approximately 68 kDa.

min in equilibration buffer containing 48 mg/ml iodoacetamide (Sigma). Equilibrated IPG strips were immersed in SDS running buffer for a few seconds and placed on top of a 1-mm vertical SDS 10% polyacrylamide gel. SDS-PAGE was run for 4–5 h at 20 mA, and the gels were then stained with Coomassie Blue R-250 or electroblotted onto nitrocellulose membranes for immunodetection. Coomassie-stained spots corresponding to proteins binding to hY1 RNA (and not to 5 S rRNA) were excised, completely destained, and subjected to sequence analysis by tandem mass spectrometry, the identity of hnRNP I (migrating as a doublet) was deduced from its characteristic position on the two-dimensional gel and immunologically confirmed using a monoclonal antibody against hnRNP I (see Fig. 2C).

Transfection Experiments—VSV-G-tagged cDNAs were constructed as follows. For the human La protein the N-terminal VSV-G tag (MEITYDEMNRLLK) was introduced via PCR using the following primers: La-1 (5'-GAATTCGCCCAGCTGAGATTTATACAGACATAGAGATGAAACCCGGACCTTGGAAGGGCGCCGCATTGTGGAAA-ATTGGTATAATG-3') and La-2 (5'-CTCGAGCTACTGCTGCTACGCACCCATT-3') using a full-length La cDNA (35) as template. Indicated in bold type are the introduced EcoRI, NotI, and XhoI sites. The VSV-G tag encoding sequence is underlined. The PCR product was digested with EcoRI/XhoI and cloned into the corresponding sites of the pcDNA3 vector (Invitrogen). The cDNAs encoding hnRNPK and hnRNP I were kindly provided by Gideon Dreyfuss (Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, PA). The human hnRNPK cDNA was modified by PCR to introduce flanking NotI and XhoI sites, allowing replacement of the La cDNA with the hnRNPK cDNA, which resulted in an N-terminally VSV-G-tagged hnRNPK construct in pcDNA3. An N-terminally Myc-tagged version of a human hnRNPI cDNA in pcDNA3 was modified to replace the Myc tag with a VSV-G tag. The integrity of the constructs was confirmed by DNA sequencing. As a control, the empty pcDNA3 vector was used in transfection experiments.

African green monkey cells (COS-1) were transiently transfected with the expression constructs. Briefly, COS-1 cells were grown to 80% confluency in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin in 5% CO2 at 37 °C. Cells were trypsinized and resuspended in phosphate-buffered saline. Approximately 5 × 106 cells were transfected with 20 μg of plasmid DNA in a total volume of 400 μl of phosphate-buffered saline. Electroporation was performed at 300 V and a capacity of 125 microfarads with a Gene Pulser II (Bio-Rad). Subsequently, the cells were seeded in 75-cm² culture flasks and cultured overnight.

Yeast Three-hybrid System—To investigate the interaction of hnRNPI and hnRNPK with hY RNAs in vivo, a three-hybrid system (36) was used (RNA-protein hybrid reporter kit; Invitrogen, Groningen, The Netherlands). The cDNAs encoding hY RNAs and hY1 RNA deletion mutants were introduced into the Smal and AvrII sites of the pRHS' hybrid RNA vector downstream of two copies of the MS2 RNA sequence. hnRNPI and hnRNPK hybrids were constructed by ligating the PCR amplified fragments into the EcoRI and XhoI sites of the pYEASTrip2 hybrid protein vector. The sequence and orientation of all recombinant DNAs was verified by sequencing. The yeast strain L40uraMS2, which stably expresses the LexA DBD-M52 coat protein was double transformed with plasmids containing the hybrid RNAs and the hybrid proteins, respectively. Transformants were selected on synthetic medium plates lacking uracil and tryptophan. Expression of the bait proteins was checked by Western blotting using appropriate monoclonal antibodies. Double transformants were then assayed for β-galactosidase (β-gal) expression on a filter using X-gal as a substrate and for growth on selective medium without histidine. For quantitative determination of β-gal activity cells were disrupted with glass beads, and, after determination of protein concentration, enzyme activity was measured photometrically at 420 nm using 2-nitrophenyl-β-D-galactopyranoside as a substrate. As a positive control the established IRE-IRP interaction was used (36).
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Fig. 2. Detection of hnRNP K and hnRNP I in reconstituted hY RNA complexes. Proteins binding in reconstitution assays to biotinylated hY1 RNA (lanes 1) or 5 S rRNA (lanes 2) were isolated by streptavidine affinity chromatography, separated by SDS-PAGE, and probed by Western blotting with patient serum BM (A), a monoclonal anti-hnRNP K antibody (B), and a monoclonal anti-hnRNP I antibody (C).

kDa, was excised from a Coomassie-stained two-dimensional gel run in parallel, digested with trypsin, and microsequenced by tandem electrospray mass spectrometry. This analysis provided a 19-amino acid tryptic peptide sequence (GSYGDLGGPIITQVITPK), which completely matched a sequence contained in the human hnRNP K protein (residues 378–396). This result was compatible with the migration of the proteins in our two-dimensional gels, which largely corresponded to the molecular mass (66 kDa) and isoelectric point values (6.1–6.4) reported for hnRNPK (40).

The second protein that we could identify was a basic protein visible as a 62/60-kDa doublet. Previous experiments had already suggested the presence of a 60-kDa protein in reconstituted hY1 RNPs that comigrated with Ro60 in SDS-PAGE and appeared to have similar RNA binding properties as the 62-kDa protein (28). Because of its migration behavior and characteristic doublet appearance, we hypothesized that this protein was hnRNP I, the PTB, which is known to migrate as a double band of 60 kDa in SDS-PAGE and has a reported pI of 8.5 (40, 41).

To confirm the presence of hnRNP I and hnRNP K in reconstituted complexes hY1 RNA-binding proteins were separated by SDS-PAGE and stained with either serum BM or monoclonal antibodies to hnRNP K or hnRNP I, respectively (Fig. 2, lanes 1). Compatible with our previously published data serum BM recognized proteins of 60, 68, 65, 62, and 53 kDa in addition to Ro60 and La (28). In contrast to serum BM, the anti-hnRNP K antibody stained solely the 68-kDa band, whereas the anti-hnRNP I antibody recognized two bands of 62 and 60 kDa. Neither the serum nor the monoclonal antibodies recognized a protein in the control lanes containing proteins binding to 5 S rRNA (Fig. 2, lanes 2).

Of the three other hY1 RNA-binding proteins recognized by the serum, the 53-kDa protein was horizontally streaked and only weakly reactive with the serum on two-dimensional immunoblots and could therefore not be sequenced. For the 65-kDa protein migrating as a double spot at neutral pI and for the basic protein(s) migrating at ~70–72 kDa (presumably comigrating with hnRNP K in SDS-PAGE) no unambiguous data could be obtained, and the 80-kDa protein was not visible on Coomassie-stained two-dimensional gels.

Interaction of hnRNP I and hnRNP K with hY RNAs in Vitro—To study the interaction of hnRNP I and hnRNP K with hY RNAs in more detail, reconstitution reactions were performed with all four hY RNAs as well as with several deletion mutants of hY1 RNA. Proteins binding to hY RNAs were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and subsequently probed with monoclonal antibodies to hnRNP I and K as described above (Fig. 3); both proteins showed pronounced binding to hY1 RNA, but only hnRNP I efficiently bound to hY3 RNA, whereas the interaction of hnRNP K with hY3 RNA was relatively weak. In this assay, neither of the two proteins interacted detectably with hY4 or hY5 RNA, confirming previous data (28).

To map the regions of hY1 RNA involved in binding of the two proteins, deletion mutants of (biotinylated) hY1 RNA were used. Truncation of the Ro60 binding site did not have any effect on binding of either hnRNP I or hnRNP K (Fig. 3B, lane 5), whereas mutation of the La binding site (3′-terminal UU to AG) significantly decreased binding of both proteins (Fig. 3B, lane 6). The strongest effect was observed with a mutant lacking the pyrimidine-rich central loop 2b, which bound hnRNP I very weakly and hnRNP K not at all (Fig. 3B, lane 7). Deletion of stem2-loop1 or of stem3-loop3 had no or only little effect on binding of hnRNP I, whereas binding of hnRNP K appeared to be reduced by ~50% and 80%, respectively (Fig. 3B, lanes 8 and 9). Finally, the binding of both proteins appeared to be somewhat increased with a mutant lacking the stem4-loop4 region (Fig. 3B, lane 10). A comparable result was obtained with 35S-labeled hnRNP I and K proteins translated in vitro in a wheat germ system (data not shown).

Taken together, these results (i) clearly confirmed the in vitro binding of hnRNP I and K to hY1 RNA; (ii) showed the interaction of the two proteins with hY3 RNA to be slightly (hnRNP I) or considerably (hnRNP K) weaker than with hY1 RNA; (iii) demonstrated the central loop 2b to be indispensable for efficient binding of both proteins to hY1 RNA; (iv) suggested that La but not Ro60 is required for efficient binding; and (v) indicated that the binding sites for the two hnRNPs are closely spaced but not necessarily identical.

Interaction of hnRNP I and K with Native hY RNAs—To investigate whether and which hY RNAs are associated with the hnRNP I and K proteins in vivo, a HeLa cell extract was subjected to immunoprecipitation using specific monoclonal antibodies to hnRNP I, hnRNP K, Ro60, and La. RNAs were isolated from the immunoprecipitates by phenol-chloroform extraction and probed with radiolabeled antisense hY RNAs by Northern blot hybridization. As shown in Fig. 4A, coprecipitation of hY1 and hY3 RNA by the anti-hnRNP I monoclonal antibody was clearly observed although at a lower level as compared with the precipitates obtained with the anti-La and anti-Ro60 antibodies, which efficiently precipitated all four hY RNAs. RNA bands visible just below hY1 RNA and hY3 RNA presumably corresponded to previously reported degradation products of these two hY RNAs known as hY2 and hY3* RNAs (22, 42), which was consistent with the lack of coprecipitation of these molecules by the anti-La antibodies. These results not only confirmed the specific binding of hnRNP I to hY1 and hY3 RNA observed in vitro but also strongly suggested that hnRNP I is associated with hY1 and hY3 RNA in vivo. In contrast, coprecipitation of hY4 RNA by the anti-hnRNP K antibody was not detectable (lane 4).

Although these data suggested that the association of hnRNP I with hY1 and hY3 RNA occurs also in vivo, they did not allow us to conclude that this protein was present also in Ro RNPs (i.e. hY RNA complexes containing both Ro60 and La). To address this question, Ro and La RNPs were isolated from a HeLa cell extract using anti-Ro60 and anti-La micropreparative immunofluorescence columns. Proteins were eluted with 1 M NaCl, separated by SDS-PAGE, and identified by immunoblotting using monoclonal antibodies against hnRNP I and hnRNP K; proteins isolated from in vitro reconstituted hY1 RNPs served as controls (Fig. 4B, lane 1). In these experiments the presence of hnRNP I in both anti-La and anti-Ro60 eluates was reproducibly observed (Fig. 4B, lanes 2 and 3), demonstrating...
that this protein was contained in Ro RNP complexes. On the other hand, and in agreement with the RNA precipitation data, hnRNP K could not be detected in these eluates (not shown).

To confirm these data and to account for the possibility that the epitope recognized by the anti-hnRNP K monoclonal antibody might be inaccessible when the hnRNP K is complexed with hY RNA, we expressed both hnRNP proteins and, as a control, La as VSV-G-tagged fusion proteins in transiently transfected COS-1 cells. Western blot analysis with a monoclonal antibody directed to the VSV-G tag demonstrated that the three tagged proteins are expressed to similar amounts (Fig. 5A). This antibody was then used to immunoprecipitate lysates of transfected cells that were subsequently analyzed for the presence of (coprecipitated) Y RNAs by Northern blot hybridization. Also by this approach hnRNP I was found to associate with Y1 and Y3 RNA (Fig. 5B, lane 1), and no Y RNAs were detectable when cells were transfected with an “empty vector” (lane 11). All four Y RNAs were also coprecipitated by the anti-La (lane 3) and anti-Ro60 (lanes 6, 9, and 12) antibodies.

**Interaction of hnRNP I and K with hY1 and hY3 RNA in a Yeast Three-hybrid System**—To investigate the interactions of hnRNP I and hnRNP K with hY RNAs in a living cell, we made use of the yeast three-hybrid system (36). In analogy to the widely used two-hybrid system, this system is based on transcriptional activation of the reporter genes his3 and lacZ upon interaction of RNA with the protein of interest. In our case the first (protein) hybrid consisted of the DNA-binding domain of the transcriptional activator LexA fused to the RNA-binding viral MS2 coat protein (LexA DBD-MS2), the second (RNA) hybrid consisted of hY RNA (or hY1 RNA deletion mutants) cloned downstream of the MS2 RNA sequence (MS2 RNA-hY RNA), and the third (protein) hybrid was composed of the transcription activation domain of Gal4 fused to either hnRNP I or hnRNP K (B42AD-hnRNP protein) (Fig. 6A).

To examine the interaction of hY1 and hY3 RNA with hnRNP I or hnRNP K, a yeast strain expressing the LexA DBD-MS2 coat protein hybrid was cotransformed with the hybrid plasmids encoding these RNAs and proteins. Transformants lacking any of the (RNA or protein) hybrid components were not able to grow on a medium deficient of histidine (not shown) and showed no or only little β-gal activity when grown on a synthetic medium lacking uracil and tryptophan (Fig. 6B). In contrast, elevated β-gal activity was clearly seen in trans-
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**Fig. 5. Association of VSV-tagged hnRNP I with Y RNAs in transiently transfected cells.** COS-1 cells were transfected with constructs encoding VSV-G-tagged La, hnRNP I, and hnRNP K, and an empty vector (mock), and 24 h after transfection cell lysates were prepared and analyzed by Western blotting (A) or Northern blotting (B). 

A, expression of the VSV-G-tagged proteins La, hnRNP I, and hnRNP K analyzed by Western blotting using a monoclonal anti-VSV-G tag antibody. The positions of the molecular mass markers are indicated on the right. B, Northern blot analysis of Y RNAs coprecipitating with VSV-G-tagged proteins. The lysates were subjected to immunoprecipitation with monoclonal anti-VSV-G tag, anti-La, or anti-Ro60 antibodies, respectively. RNA isolated from the total lysates (input) and from the immunoprecipitates was analyzed by Northern blot hybridization using antisense hY RNAs as probes. The positions of Y1, Y3, Y4, and Y5 are indicated. The band indicated with ** probably represents a degradation product of Y3, previously designated Y3** (42).

**DISCUSSION**

Although data from several laboratories suggest that Ro RNP complexes may contain a number of other proteins in addition to Ro60 and La (20–28), unambiguous evidence for this has not been presented yet (3, 4). Here we have identified two of the proteins previously described by us to associate with hY RNAs in vitro (28) as hnRNP I and hnRNP K. The two proteins interacted strongly with hY1 RNA and to a lesser extent with hY3 RNA but not with hY4 or hY5 RNA, which is in agreement with our previous findings. Immuno precipitation experiments performed with extracts from HeLa cells and transiently transfected COS-1 cells provided substantial evidence for in vivo association of hnRNP I with hY1 and hY3 RNA and indicated the existence of a subpopulation of Ro RNPs containing hnRNP I in addition to Ro60 and La. The observation that the anti-hnRNP I antibody precipitated significantly smaller amounts of hY RNAs than the anti-Ro60 antibody (although comparable amounts of Ro60 and hnRNP I appeared to be bound in the reconstitution assays) indicates that, in contrast to Ro60, hnRNP I is associated with a minor portion (10–20%) of hY1 and hY3 RNAs. Although these assays did not provide any evidence for hY RNA-hnRNP K interactions in these cellular extracts, a clearly positive result was obtained in a yeast three-hybrid system that was comparable with that obtained with hnRNP I. Thus, hnRNP K may bind only to a rather minor subset of hY RNAs that are devoid of Ro60 (there is no Ro60 homologue in yeast) and therefore difficult to detect, or, alternatively, the interaction with hY RNAs may have been disturbed during preparation of the cellular extracts or upon antibody binding in immunoprecipitation assays.

The importance of an intact La binding site for efficient binding of hnRNP I and hnRNP K was remarkable and leads us to speculate that the function of La in hY RNP assembly may be that of an RNA chaperone being required for correct folding of hY RNAs, thus enabling binding of other proteins (with the notable exception of Ro60). This would be consistent with recent reports on the chaperoning role of La in small nuclear RNP assembly and pre-tRNA processing (12, 15, 19).

The results obtained with hY1 RNA deletion mutants showed that the internal pyrimidine loop (71–86 nucleotides) was indispensable for efficient association of the two hnRNP proteins with hY1 RNA and demonstrated their binding sites to be clearly different from the Ro60 binding site. Both hnRNP I and K are known to bind to pyrimidine-rich sequences (43, 44). Thus, hnRNP I, which is commonly known as PTB, binds to the polypyrimidine stretch present near the 3' splice site of many introns and also to pyrimidine-rich sequences of mature mRNAs (45). For hnRNP K, which shows increased affinity for poly(rC) sequences but does not bind to the polypyrimidine tract, several cytosine-rich recognition motives have been described, such as the CT element (CCCTCCCCC) of the c-myc gene (46) and CU repeats (CCCCACCUCUUCCUC) present in the 3'-untranslated region of erythroid 15-lipoxygenase mRNA (47). These sequences as well as those recognized by hnRNP I (PTB) show significant similarities with the central loop 2b region of hY1 RNA (UACUCUUUUCACCUUCU), supporting the assumption that this region may directly interact with both proteins as suggested by the results obtained with the deletion mutants. Remarkably, an internal pyrimidine-rich loop is present also in hY3 RNA, whereas it is lacking in hY4 and hY5 RNA, which would be consistent with their failure to bind the two hnRNP proteins.

The heterogeneous nature of Ro RNPs has been postulated by several researchers based on biochemical fractionation and in vitro reconstitution data (22, 23, 28). The number of Y RNAs has increased throughout the evolution from C. elegans, which expresses only one Y RNA, to vertebrates with four Y RNAs (6, 48). Interestingly, the Ro60 binding site and the pyrimidine-rich loop sequence represent the most conserved regions, being already present in C. elegans RNA, which shows the greatest
Interaction of hnRNP I and K Proteins with hY RNAs

FIG. 6. Interaction of hnRNP I and hnRNP K with hY1 and hY3 RNA in a yeast three-hybrid system. The yeast three-hybrid system was used to study interaction of the two hnRNP proteins with hY RNAs in vivo by measuring β-gal activity of transformed cells. A, schematic diagram showing gene activation by specific interaction between hY-RNA and hnRNP I or K proteins in the yeast three-hybrid system. Expression of reporter genes lacZ and his3 is activated upon positive interaction between hnRNP I or K and hY1 RNA dragging the following two domains of the transcriptional activator to a close vicinity: the DNA-binding domain of LexA (DBD) and the activation domain of Gal 4 (AD). B, analysis of β-gal activity. Transformants were grown on synthetic medium lacking uracil and tryptophan, and β-gal activity was tested in the blue color X-gal filter assay (right) and quantitatively determined by direct measurement of the enzymatic activity in units/mg yeast mass (left).

homology to hY3 RNA (49). This strongly suggests that the pyrimidine-rich element is important for Y RNA functioning, including interactions with other proteins and/or RNAs. Furthermore, the internal loop of hY1 RNA was shown to be resistant to enzymatic and chemical cleavage (50), an observation also made with pyrimidine-rich loops of other small RNAs transcribed by RNA polymerase III (51–53). Because the four vertebrate Y RNAs show a high diversity in the central region, this part of the molecule might determine the fate (i.e. localization and function) of Y RNPs or Ro RNPs, respectively, because of differential association with proteins of (more or less) diverse function (such as hnRNP I and hnRNP K).

Several RNA polymerase III transcripts including RNase P RNA, RNase MRP RNA, and hY RNAs (except hY4 RNA) have been localized to the perinucleolar compartments together with two hnRNP proteins, hnRNP I (41, 54), and CUG-binding protein/hNab50 (55, 56). Importantly, the presence of hnRNP I in the perinucleolar compartments was found to be sensitive to RNase A treatment (57), which may be considered a further (though rather indirect) indication for in vivo interactions of this protein with hY1 and hY3 RNAs. Remarkably, the presence of Ro60 and La could not be detected within the perinucleolar compartments (54), which may suggest the existence of hY RNA complexes containing hnRNP I (as well as other proteins yet to be identified) but devoid of both Ro60 and La.

hnRNP I and K are both shuttling proteins that belong to a group of multifunctional RNA-binding proteins exerting regulatory roles at the post-transcriptional level, including RNA processing and export as well as regulation of mRNA stability and translation (44, 58–61). Given that Ro RNPs are predominantly, if not exclusively, localized in the cytoplasm, it is important to mention reported functions of hnRNP I and K that are related to translational events. Thus, hnRNP I has been shown to bind specifically to several viral internal ribosome entry sites, thereby promoting ribosome binding in a cap-independent manner (62–64). Remarkably, a similar activity was also found for La (13), and cooperation of these two proteins in the regulation of internal ribosome entry site-dependent translation has been recently suggested (65). Furthermore, these proteins may be also required for efficient and correct initiation of cap-dependent translation by inhibiting translation of uncapped mRNAs (66). Apart from its role as transcription factor (44, 67, 68), hnRNP K protein has been demonstrated to act as a differentiation regulator by virtue of its binding to the control elements at the 3′ end of the erythroid 15-lipoxygenase mRNA, thereby inhibiting translation (47). In a similar manner translation of papilloma virus late mRNAs is inhibited by hnRNP K and the poly(rC)-binding protein (69). Furthermore, two translation-related functions have been assigned to the Ro60 protein, namely participation in a degradation pathway for misfolded 5 S rRNA (17) and a role in the regulation of translation of ribosomal protein L4 in concert with La and cellular nucleic acid-binding protein by binding to a polypyrimidine stretch in the 5′-untranslated region of L4 mRNA (16, 70).

Combining our experimental data and those of others one may speculate about the cellular role of Ro RNPs or hY RNPs, respectively: newly transcribed hY RNAs associate with La and move through the nucleoplasm toward the nuclear membrane where Ro60 associates just prior to export into the cytoplasm, being indispensable for nuclear export of hY RNAs (3, 9). During their intranuclear migration subsets of hY RNPs are formed by virtue of association with various proteins including hnRNP I and K, Ro52, and presumably other proteins including those reported to interact with hY RNA in vitro or with Ro60 in yeast two- and three-hybrid systems (26–28). All these proteins may be multifunctional and upon binding to hY RNAs...
become destined to exert specific functions either in the nucleus (particularly in the perinucleolar compartment) or in the cytoplasm. It is intriguing that for all four hY RNA-binding proteins identified to date (La, Ro60, and hnRNP I and K) regulatory roles in translation of certain cellular and viral mRNAs have been described, although these proteins were originally identified as nuclear proteins with (nuclear) functions mainly related to RNA processing and transcription. Thus, hY RNAs may serve as carriers for (nuclear) proteins involved in regulation of translation. Taken together, we think the roles of Ro RNP (or hY RNP) complexes but also may be involved in regulation of translation of certain cellular and viral proteins identified to date (La, Ro60, and hnRNP I and K).

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