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Interaction cloning and characterization of RoBPI, a novel protein binding to human Ro ribonucleoproteins

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ABSTRACT

Human Ro ribonucleoproteins (RNPs) are autoantigenic particles of unknown function(s) that consist of a 60-kDa protein (Ro60) associated with one hY RNA (hY1–5). Using a modified yeast three-hybrid system, named RNP interaction trap assay (RITA), we cloned a novel Ro RNP-binding protein (RoBPI), based on its property to interact in vivo in yeast with an RNP complex made of recombinant Ro60 (rRo60) protein and hY5 (rhY5) RNA. RoBPI cDNA contains three conserved RNA recognition motifs (RRM) and is present as a family of isoforms differing slightly at their 5′ end. The 2.0-kb RoBPI mRNA was detected in all human tissues tested. Highly homologous cDNA sequences were found in banks of expressed sequence tags (ESTs) from mice. Two-hybrid, three-hybrid, and RITA experiments respectively established that 60 kDa RoBPI did not interact in yeast with rRo60 alone, with rhY5 RNA alone, or with bait RNPs consisting of rRo60 and recombinant hY1, hY3, or hY4 RNAs. RoBPI coimmunoprecipitated with Ro RNPs from HeLa cell extracts and partially colocalized with Ro60 in nuclei of cultured cells. Because hY5 RNA and Ro^{hY5} RNPs are recent evolutionary additions seen only in primates, but RoBPI seems more conserved, their interaction may represent a gain of function for Ro RNPs. Alternatively, interaction of Ro^{hY5} RNPs with RoBPI may have no functional bearing, but may underlie some of the unique biochemical and immunological properties of these RNPs.

Keywords: autoimmunity; hY RNA; interaction assay; RNA-binding protein; Ro RNP

INTRODUCTION

Ro RNPs are low-copy RNPs present in all mammalian cells, including mature (anuclear) human erythrocytes (Rader et al., 1989) and platelets (Itoh & Reichlin, 1991). Ro RNP homologs are also found in *Caenorhabditis elegans* (Labbé et al., 1995; van Horn et al., 1995) and *Xenopus laevis* (O'Brien et al., 1993). Y RNAs, the nucleic acid component of Ro RNPs, are small noncoding RNAs transcribed by RNA polymerase (pol) III (Hendrick et al., 1981). The size and number of RNAs of the Y family vary somewhat between cell types and species (Mamula et al., 1989a; Rader et al., 1989; Itoh & Reichlin, 1991). The subcellular localization of Ro RNPs is controversial (Hendrick et al., 1981; Ben-Chetrit et al., 1988; Peek et al., 1993; Kelekar et al., 1994). In all species, a moderately conserved 60-kDa (Ro60) protein binds to Y RNAs by interacting with a highly conserved duplex structure resulting from base pairing between their 5′ and 3′ ends (Wolin & Steitz, 1984). In humans, Ro RNPs comprise one of the four hY RNAs (hY1, hY3, hY4, or hY5) associated with Ro60 and, at least in a fraction of Ro RNPs, with a 48-kDa protein called La (Mamula et al., 1989b; Boire & Craft, 1990). The La protein binds to the 3′ oligouridylate end of RNA pol III transcripts (Stefano, 1984; Slobbe et al., 1992) and is an RNA pol III transcription termination factor (Gottlieb & Steitz, 1989). La protein also plays a role in transcript release, reinitiation, and even processing of nascent transcripts (Maraia, 1996; Goodier et al., 1997; van Horn et al., 1997; Yoo & Wolin, 1997; Fan et al., 1998), and is implicated in the regulation of translation of some viral (Meerovitch et al., 1993; Chang et al., 1994; Svitkin et al., 1994; Ali & Siddiqui, 1997) and cellular (Pellizzoni et al., 1996, 1998) mRNAs. Both

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Abbreviations: AD: activating domain; CNBP: cellular nucleic acid binding protein; DTSSP: dithiobis[sulfosuccinimidyl propionate]; GAPDH: gluceraldehyde-3-phosphate dehydrogenase; hnRNA: heterogeneous nuclear RNA; rhY RNA: recombinant hY RNA; RITA: RNP interaction trap assay; RNA pol III: RNA polymerase III; X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

the oligouridylate tail and the associated La binding are usually lost during maturation of pol III transcripts (Maraia et al., 1994). For unknown reasons, but similar to some viral RNAs, the oligouridylate tail of hY RNAs is not cleaved and La protein remains a stable component of a fraction of Ro RNPs. In contrast, mature Y RNAs from *X. leavis* and *C. elegans* have no 3′ oligouridylate tail, suggesting a possible shift or gain of function through evolution.

Based on immunological evidence, it has been suggested that Ro RNPs interact stably or transiently with other intracellular proteins, such as a 52-kDa protein (Ro52) (Ben-Chetrit et al., 1988; Pruijn et al., 1991; Slobbe et al., 1992; Keech et al., 1996). Such an interaction has not been observed using classical biochemical methods (Kelekar et al., 1994; Boire et al., 1995) as well as interaction assays in yeast (authors' unpubl. data). A role for Ro60 in a quality control pathway of defective 5S RNAs before incorporation in ribosome in *X. leavis* oocytes (O'Brien & Wolin, 1994; Shi et al., 1996) and in *C. elegans* (Labbé et al., 1999) has been proposed. No evidence for a similar process in humans has yet been provided. Recently, a role for Ro60 protein (and an unidentified RNA) as a cofactor in the regulation of the translational fate of *X. laevis* ribosomal protein mRNAs has been proposed (Pellizzoni et al., 1998). Although *C. elegans* clones in which both copies of the Ro60 homolog had been disrupted (double knockouts) exhibited no phenotypic abnormalities, the expression of Y RNA was severely curtailed, suggesting a role for Ro60 in the expression of its cognate RNA (Labbé et al., 1999). Clearly, much remains to be found about the functions of Ro RNPs.

We previously reported that biochemical and immunological properties of Ro RNPs vary depending on the hY RNA they contain. Ro RNPs containing hY5 RNA (Ro^{hY5}) could easily be purified away from other Ro RNPs by anion-exchange and size-exclusion chromatography (Boire & Craft, 1989, 1990). Ro^{hY5} RNPs were also targeted by RNP-specific antibodies present in most if not all autoimmune sera containing anti-Ro RNP antibodies (Boire & Craft, 1989, 1990); specific antibodies to other hY RNA-containing RNPs (Ro^{hY1}, Ro^{hY3}, and Ro^{hY4}) have yet to be described. More recently, we observed that Ro^{hY1-4} RNPs from HeLa cells were almost exclusively cytoplasmic, whereas most Ro^{hY5} RNPs were found in the nucleus (M. Gendron, D. Roberge, & G. Boire, submitted). Transfection experiments in mouse cells showed that a specific sequence in hY5 RNA was essential for maintaining the nuclear localization of hybrid Ro^{hY5} RNPs formed between mouse Ro60 protein and hY5 RNA (M. Gendron, D. Roberge, & G. Boire, submitted). These observations suggested that Ro^{hY5} RNPs might possess unique interactions with intranuclear proteins or structures. Attempts to isolate putative partners of Ro^{hY5} RNPs using classical biochemical methods were repeatedly unsuccessful, however.

To identify partners of Ro RNPs, we adapted in vivo interaction assays in yeast (Fields & Sternglanz, 1994; SenGupta et al., 1996) to allow for the reconstruction of tripartite (protein–RNA–protein) RNPs. We named our modified system "RNP interaction trap assay" or RITA. During screening of a HeLa cDNA library using as bait an RNP made of recombinant Ro60 and hY5 RNA, we identified a family of highly homologous cDNAs encoding a previously unknown protein that we named Ro-binding protein I (RoBPI). In yeast, RoBPI bound only to RNPs made of recombinant Ro60 (rRo60) protein and hY5 (rhY5) RNA, but not to naked rRo60 or to recombinant hY RNAs or to Ro RNPs containing recombinant hY RNAs other than rhY5. Biological relevance of the interaction identified in yeast was suggested further as RoBPI partially colocalized with Ro60 in nuclei of cultured HeLa cells, where Ro^{hY5} RNPs are also found, and coprecipitated with Ro RNPs from chemically crosslinked HeLa cell extracts.

RESULTS

Construction of the RNP Interaction Trap Assay

To screen for novel partners of Ro RNPs, we developed an adaptation of the yeast three-hybrid assay called "RNP interaction trap assay" (RITA) (Fig. 1; Bouffard et al., 1999). The yeast strain L40-*ura3* requires histidine, tryptophan, leucine, and uracil to grow. In our Ro adaptation of RITA, hybrid proteins and the recombinant RNA are each expressed by separate plasmids that carry a different nutritional marker: TRP1 for Ro60 (part of the bait RNP), LEU2 for the prey protein, and URA3 for the hY RNA. In RITA, when RNA acts as a bridge between the two hybrid proteins, reporter genes (*lacZ* and HIS3) are activated, allowing the yeast to gain the capacity to grow on a medium lacking histidine and to turn blue when 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) is added to the medium. Ro60 binding to hY RNAs is dependent on the presence of a bulged cytosine at position 9 of the RNA (Pruijn et al., 1991; Green et al., 1998). La protein binds hY RNAs at their 3′ oligouridylate tail, whether Ro60 is present or not on the RNA (Stefano, 1984; Granger et al., 1996a). To check the correct in vivo expression of our constructs, we used as prey a hybrid La protein fused to the activating domain (AD) of GAL4 (rLa). When rRo60 and rLa proteins were coexpressed with recombinant hY RNAs in yeast, reporter genes were activated, indicating that a tripartite RNP was reconstituted, the rhY RNA acting as a bridge linking rRo60 and rLa proteins. Unrelated RNAs (e.g., IRE-MS2) as well as hY5ΔC9 RNA (lacking the essential bulged cytosine at position 9) failed to activate reporter genes (Bouffard et al., 1999).

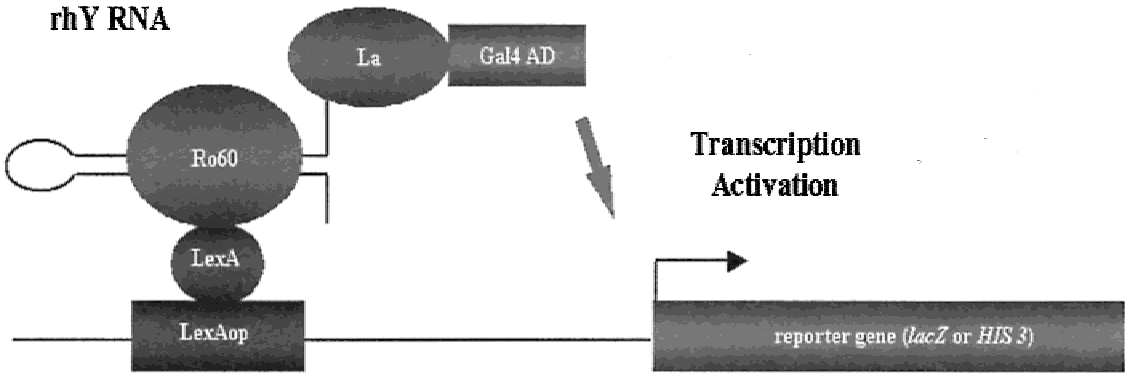

FIGURE 1. Reconstitution of a RNP (Ro60-hY RNA-La) in RNP interaction trap assay (RITA). Hybrid LexA-Ro60 (rRo60) protein binds both the LexA binding site on yeast genomic DNA and the Ro60-binding site on coexpressed hY RNA. Hybrid GAL4 activating domain (AD)-La (rLa) protein binds the 3′ oligouridylate tail of recombinant hY (rhY) RNA, and activates transcription of reporter genes.

Cloning of RoBPI using RITA

Because we had some evidence that, among Ro RNPs, those containing the hY5 RNA were most likely to have unique intracellular partners, we used as bait an RNP made of recombinant Ro60 (rRo60) protein and hY5 (rhY5) RNA. Two hundred and twenty-six transformants were able to grow on His− medium and to turn blue in the presence of X-gal, and were considered positive. One hundred and thirty-three positive clones contained highly homologous cDNAs encoding a novel RNA-binding protein that we named Ro binding protein I (RoBPI, Fig. 2). The sequence of the first of these clones (clone 2.1) was over 95% identical to a partial cDNA sequence stored in GenBank under the name Siah binding protein I (SiahBPI; GenBank accession number U51586). Differences in the sequence of clone 2.1 relative to SiahBPI were mostly found in its 5′ region: the 87-bp insertion (sequence A) and the 51-bp deletion (sequence B) did not change the reading frame of the predicted protein, suggesting that they resulted from alternative splicing of the same pre-mRNA. These sequences were thus called alternative exons A and B, respectively. Further RT-PCR analysis revealed the existence of at least three isoforms of RoBPI cDNA: A+/B−, A−/B+ and A+/B+, of which the A+/B− and the A−/B+ isoforms were also present among the RoBPI cDNA clones isolated during screening. The existence of an A−/B− isoform was not completely ruled out, as our attention was focused on larger PCR fragments, to get full-length RoBPI cDNAs. Using a 5′RACE inverse PCR approach, we also obtained a short (64 bp) G/C rich 5′ extension ahead of a Kozak consensus sequence (ANNATGG) for initiation of translation (Fig. 2).

Tissue distribution of RoBPI mRNA

To characterize the expression of RoBPI, we performed Northern blot analyses on mRNA isolated from 16 human tissues. Hybridization with a 739-nt-long probe derived from the 3′ region of RoBPI cDNA (i.e., not containing alternative exons A and B) showed a single reactive band at about 2.0 kb that was present in every tissue tested (Fig. 3). The identification of a unique 2.0-kb mRNA strongly suggested that the cloned RoBPI cDNA was full length. Spatial resolution on Northern blots would be insufficient to discriminate between RoBPI mRNAs containing both A and B exons (A+/B+; 1,946 bp) from those not containing one of them (A−/B+: 1,859 bp; A+/B−: 1,895 bp). Interestingly, qualitative standardization of mRNA levels with GAPDH mRNA suggested an apparent overexpression of RoBPI in testes and ovaries.

Specificity of interaction of RoBPI with recombinant Ro60-hY5 RNA RNPs in yeast

To determine the specificity of the interaction between RoBPI and recombinant Ro RNPs and their protein and RNA components, we used RoBPI as prey in two-hybrid and three-hybrid experiments where baits were rRo60 and various recombinant RNAs, respectively (Fig. 4). The two-hybrid assay demonstrated that RoBPI did not interact with rRo60 protein alone, in the absence of recombinant hY RNA (Fig. 4, lane 5). In three-hybrid experiments, RoBPI was shown not to be a nonspecific RNA-binding protein, as it did not interact with MS2-hY5 RNA alone (Fig. 4, lane 6), nor with other recombinant RNAs (data not shown). In RITA, no interaction was observed between RoBPI and recombinant rRo60-rhY1, rhY3, or rhY4 RNPs (Fig. 4, lanes 1–3). To summarize these results, RoBPI interacted only with recombinant RNPs consisting of rRo60 and rhY5 RNA (Fig. 4, lane 4), and not with their individual components nor with other recombinant Ro RNPs.

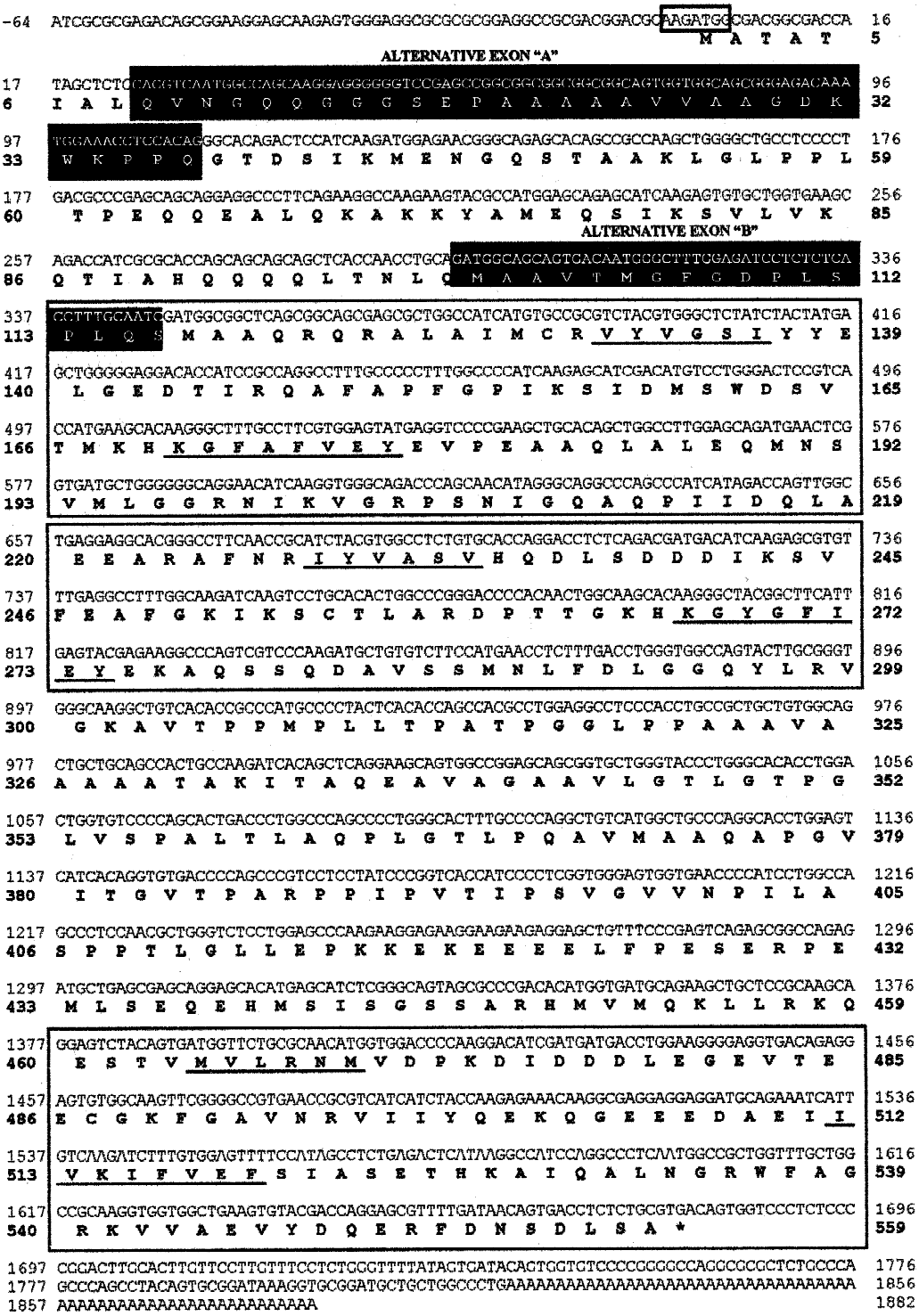

FIGURE 2. Nucleotide and predicted amino acid sequence of human RoBPI. The two black boxes contain alternative exons of 87 bp (exon A) and 51 bp (exon B). The three RRMs are shaded, and each one has its two conserved RNP motifs underlined. The boxed sequence of 7 nt is the Kozak consensus sequence determining the starting point of translation. The complete nucleotide sequence has been submitted to GenBank under accession number AF114818.

Coprecipitation of RoBPI with Ro RNPs from HeLa cells using anti-Ro60 antibodies

To confirm that the interaction observed in yeast between recombinant RoBPI and recombinant rhY5-containing Ro RNPs also occurred with native proteins and RNPs in living human cells, we used anti-Ro60 antibodies to immunoprecipitate Ro RNPs from HeLa cell extracts. The immunoprecipitated proteins were then separated on SDS-PAGE and detected by immunoblot-

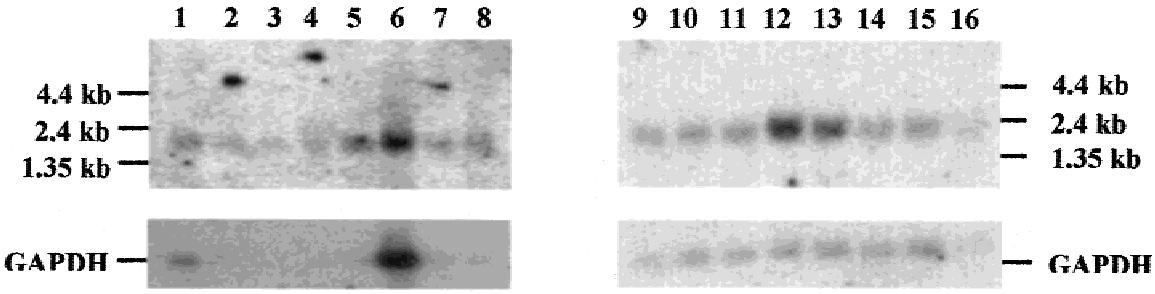

FIGURE 3. Northern blot analysis of human RoBPI expression in 16 human tissues. Approximately 2 μg poly A+ RNA are present in each lane. The upper panel shows hybridization with a RoBPI probe. The bottom panel shows hybridization with a GAPDH probe. Left panels: Lanes 1: heart, 2: brain, 3: placenta, 4: lung, 5: liver, 6: skeletal muscle, 7: kidney, 8: pancreas. Right panels: lanes 9: spleen, 10: thymus, 11: prostate, 12: testis, 13: ovary, 14: small intestine, 15: colon, 16: peripheral blood leukocytes.

ting using anti-Ro60 and anti-La antibodies, as well as a chicken anti-Poly U Factor (PUF) serum (gift from Drs. Patrick S. McCaw and Philip A. Sharp). The 60-kDa RoBPI protein migrated as a 65-kDa protein and, at higher concentrations, as a 130-kDa dimer (P.S. McCaw and P.A. Sharp, pers. comm.; pers. observations) under SDS-PAGE conditions used (Fig. 5D, lane RoBP1). Although Ro60 and La proteins were readily detected in standard anti-Ro immunoprecipitates (Fig. 5A, lanes Ro60 and Ro/La), little or no RoBPI was found (Fig. 5A, lane RoBP1). Several explanations for this inconclusive result are possible. First, because the interaction we were looking for implied only a fraction of Ro RNPs, that is those (or a fraction of those) containing hY5 RNA, the amount of immunoprecipitated RoBPI could be below the detection threshold of our assay.

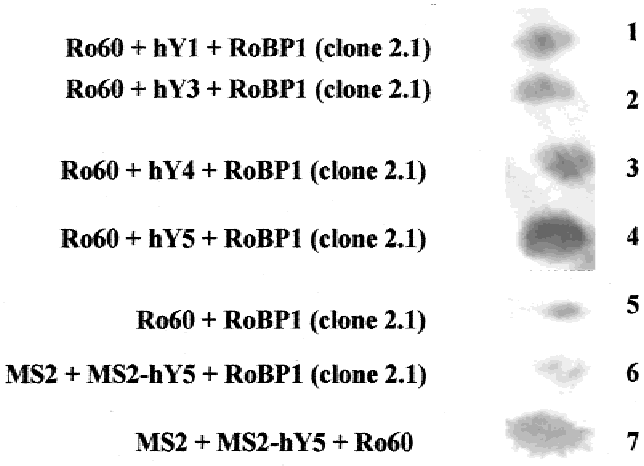

FIGURE 4. Interaction of RoBPI with Ro RNPs, characterized in yeast two-hybrid, three-hybrid, and RITA assays. RoBPI (clone 2.1) was transfected in yeast, along with rRo60 alone (two-hybrid; lane 5), with MS2-hY5 RNA (three-hybrid; lane 6), and with rRo60 and each individual recombinant hY RNA (RITA; lanes 1–4). As a positive control in three-hybrid, we used the interaction between rRo60 and MS2-hY5 RNA (lane 7). Yeasts were grown on selective media, and tested using a X-gal filter assay.

Second, the interaction of RoBPI with Ro^{hY5} RNPs in human cells might be transient, or so weak as to be disrupted during immunoprecipitation or, alternatively, it might occur only at specific times in the cell cycle. Third, the interaction observed in yeast might have no equivalent in human cells.

To verify the second hypothesis, we used a reducible crosslinking agent [dithiobis[sulfosuccinimidyl propionate] (DTSSP)] to stabilize protein interactions occurring with Ro RNPs in digitonin-permeabilized cells. DTSSP has a short armlength of 12 Å and was thus unlikely to crosslink proteins nonspecifically to our target RNPs. Although, similar to sonicated HeLa cells, little or no RoBPI was detected using digitonin-permeabilized cells (without crosslinking) (Fig. 5B, lane RoBP1), the use of both DTSSP and digitonin resulted in the detection of the 65-kDa RoBPI band in anti-Ro immunoprecipitates (Fig. 5C, lane RoBP1). The RoBPI band was not seen in immunoprecipitates from normal human sera (NHS) and from sera with antinuclear specificities other than anti-Ro (data not shown).

Partial nuclear colocalization of RoBPI and Ro60 in cultured HeLa cells

Chicken anti-PUF antiserum could not be used in immunofluorescence and immunoprecipitation assays. In an attempt to improve our serological tools, we prepared a rabbit RoBPI antiserum. Anti-PUF and anti-RoBPI sera recognized a protein of identical size in immunoblots of recombinant RoBPI (Fig. 6A, lanes 1–4) and of crude HeLa cell extracts (Fig. 6B, lanes 1–4). Unfortunately, RoBPI antisera failed to immunoprecipitate significant amounts of RoBPI from HeLa cell extracts, making impossible the detection of any Ro60 protein or hY RNA coprecipitating with RoBPI. When RoBPI antisera were used in immunofluorescence assays, however, RoBPI was decorated in a granular nucleocytoplasmic distribution (Fig. 7A) similar but distinct from the distribution of Ro60 protein (Fig. 7B) in these paraformaldehyde-

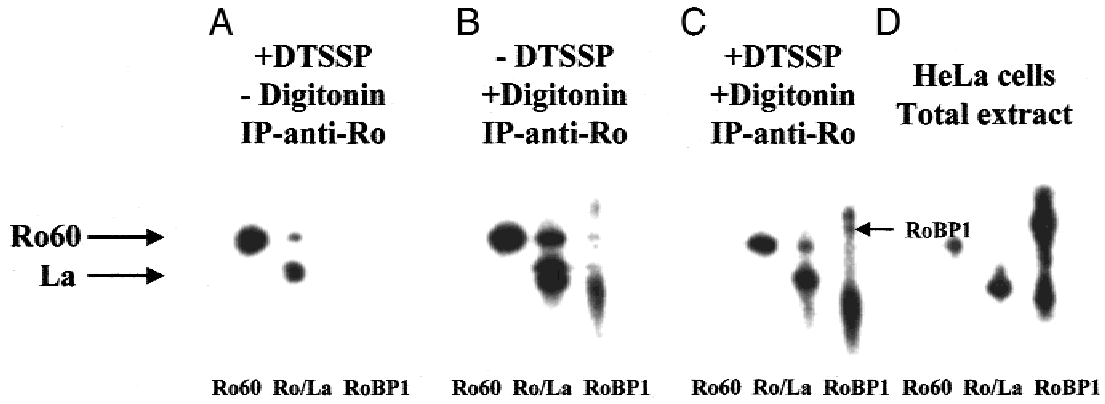

FIGURE 5. Coprecipitation of RoBPI and Ro RNPs from HeLa cell extracts using anti-Ro60 antibodies. HeLa cells were incubated on ice with DTSSP alone without treatment with digitonin (untreated; **A**), permeabilized with digitonin but without crosslinking (**B**), permeabilized with digitonin and crosslinked with DTSSP (**C**), before immunoprecipitation of soluble extracts using a human anti-Ro60 serum (the same used in immunoblot in lane Ro60 of each panel). Immunoprecipitated proteins were run in SDS-PAGE, electrotransferred to nitrocellulose, and blotted using antisera against Ro60 (lane Ro60), Ro60/La (lane Ro/La), and PUF (lane RoBP1). As a molecular weight control, total HeLa cell extracts were run in parallel (**D**) and blotted with the same antisera. In addition to the 65-kDa band corresponding to RoBPI, chicken anti-PUF serum also decorated other antigens at about 40–50 kDa present in cell extracts and in immunoprecipitates from anti-Ro sera and from other autoimmune sera (data not shown). These antigens were not further defined, as they were not recognized by RoBPI antisera (see Fig. 6).

fixed cells (Wahren et al., 1996). Distribution of control autoantigens $tRNA_{his}$ synthetase (Jo-1; cytoplasmic) and U1 RNP (nuclear) was appropriate (data not shown).

To explore the degree of overlap between RoBPI and Ro60, double immunofluorescence staining experiments were performed. Rabbit RoBPI antiserum and a human monospecific anti-Ro serum were used. These experiments revealed an extensive but incomplete colocalization of the intranuclear Ro60 and RoBPI antigens (Fig. 8A–C).

DISCUSSION

Identification of a protein partner of Ro^{hY5} RNPs

The aim of the present study was to identify novel intracellular partners of Ro RNPs. We now report the identification of a novel member of the RNA-binding protein, that we called Ro RNP binding protein I, based on its property to specifically interact in vivo in yeast with RNPs made of recombinant Ro60 and hY5 RNA. Some endogenous RoBPI protein was subsequently shown to coprecipitate with Ro RNPs from HeLa cell extracts, using specific anti-Ro60 antibodies. In immunofluorescence experiments, RoBPI colocalized predominantly to the nuclei of HeLa cells, where most Ro^{hY5} RNPs are found (M. Gendron, D. Roberge, & G. Boire, submitted). Incomplete colocalization between RoBPI and Ro60 proteins in nuclei was observed. In addition, antibodies present in a significant number of anti-Ro sera recognize in immunoblots recombinant RoBPI protein (data not shown). The prevalence of anti-RoBPI antibodies in sera of patients with various connective tissue diseases, with and without anti-Ro60 antibodies, is currently being studied.

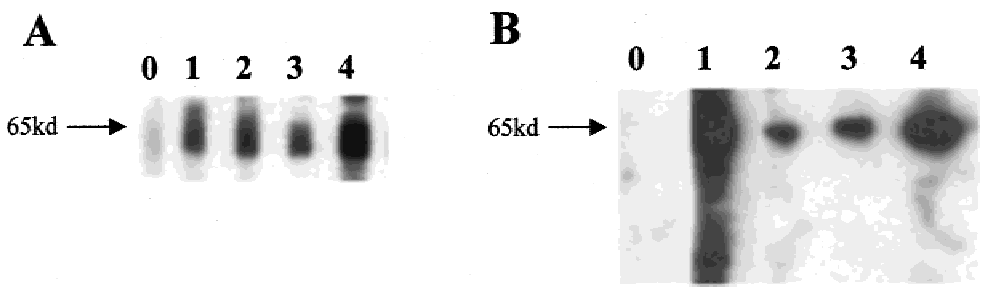

FIGURE 6. Recognition of recombinant and native RoBPI by anti-PUF antibodies and RoBPI antisera. His-tagged recombinant RoBPI (**A**) and native RoBPI from crude HeLa cell extracts (**B**) were separated in 10% SDS-PAGE and electrotransferred to nitrocellulose. The proteins were incubated with antisera and bound antibodies detected by chemiluminescence. Lane 0: pre-immune rabbit serum. Lane 1: anti-PUF antibodies. Lanes 2–4: RoBPI antisera from three different rabbits immunized with RoBPI. Anti-PUF antibodies and RoBPI antisera recognized the same protein bands.

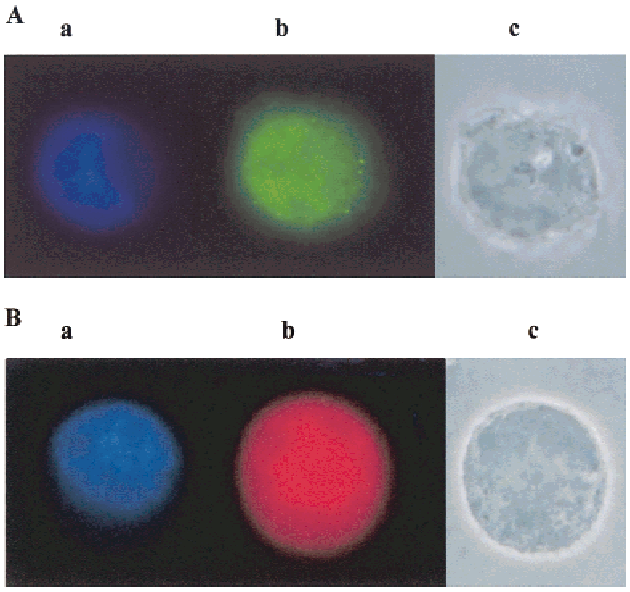

FIGURE 7. Indirect immunofluorescence. Immunofluorescence staining of cultured HeLa cells fixed with paraformaldehyde 4% using a RoBPI antiserum (**A**) and a monospecific human anti-Ro serum (**B**). a: Hoechst DNA fluorescence; b: staining with anti-RoBPI (**A**) and anti-Ro60 (**B**); c: phase contrast. Both sera gave predominantly nuclear staining, but RoBPI antisera gave a more coarsely granular distribution.

A 52-kDa protein dubbed Ro52 was previously reported as a component of Ro RNPs (Ben-Chetrit et al., 1988). Denatured Ro52 is recognized in immunoblot by antibodies present in most anti-Ro sera. Recognition of the native protein is far more controversial, however, because Ro52 is not immunoprecipitated by anti-Ro sera from ^{35}S-labeled cell extracts (Buyon et al., 1994), even after prolonged (40 h) labeling of exponentially growing cells (data not shown). Although there is indirect immunological evidence of an association of Ro52 with Ro60 or Ro RNPs (Slobbe et al., 1992; Peek et al., 1994; Topfer et al., 1995; Tseng et al., 1997), no direct biochemical demonstration of an association has been provided using either classical (Kelekar et al., 1994; Boire et al., 1995) or yeast interaction assays (authors' unpubl. data). RoBPI thus stands distinctly as the first *bona fide* Ro RNP partner.

Ro60 was recently shown to be an essential cofactor for the alternative interaction of La and cellular nucleic acid binding protein (CNBP) with the 5′ UTR of *X. laevis* ribosomal protein mRNAs, affecting the translational fate of these mRNAs (Pellizzoni et al., 1998). Ro60, as well as an unidentified RNA component, was essential for binding of La and CNBP to specific regions of the 5′ UTR. It remains unknown whether Ro60 directly interacts with La or CNBP proteins or with ribosomal protein mRNAs, or whether Ro60 is part of a macromolecular complex implicated in the alternative binding of La and CNBP. It is also currently unknown whether Y RNAs are implicated in the activation of the cofactor, acting as a putative link between Ro60 and La. One has to recall that mature *Xenopus* Y RNAs found in Ro RNPs have no 3′ oligouridylate extension that would allow binding of La. If Y RNAs are indeed part of the required cofactor, however, this would represent an example of the importance of an intact conformation of Ro RNPs for correct function, similar to the one we suggest for interaction with RoBPI.

Characterization of RoBPI

RoBPI protein is encoded by a single 2.0-kb mRNA that is expressed in all 16 human tissues tested, with an apparent relative overexpression in gonads. We presume that the poor band resolution seen in Northern blots prevented the detection of (at least) three potentially coexisting mRNA isoforms detected by RT-PCR from cDNA libraries. These mRNA isoforms would only differ according to the presence or absence of two short exons (87 and 51 bp) close to the 5′ end of the coding region. The exact significance of the resulting differences between putative RoBPI isoforms remains to be determined. Both A+/B− and A−/B+ isoforms were identified during RITA screening of a HeLa library; both had similar binding properties to Ro RNPs (data not shown). Immunoblot detection with anti-RoBPI antibodies on HeLa cells extracts showed reactivity with a tight doublet at about 65 kDa, suggesting the coexistence of more than one RoBPI isoform. Partial degradation or posttranslational modification of a single transcribed RoBPI protein were not excluded, however. RoBPI also appears relatively conserved through evolution, as the major part of the cDNA can be reconstructed from mouse expressed sequence tags (dbEST) (data not shown), and segments with significant homology can be found in *drosophila* dbEST banks (data not shown).

RoBPI cDNA encodes a 559-amino-acid protein, containing three complete RNA recognition motifs (RRM). RoBPI is thus a new member of the RNA-binding protein family with striking similarities with splicing factors such as U2AF65 (data not shown). Not surprisingly, RoBPI was predominantly found in the nucleoplasm of cultured HeLa cells where it partially colocalizes with Ro60 protein. Whether RoBPI can bind to RNAs or RNPs other than Ro^{hY5} RNPs is presently unknown. Currently available antibodies to RoBPI did not immunoprecipitate the native protein, hampering our efforts to better characterize the protein's binding properties. No small RNA other than hY RNAs were immunoprecipitated by anti-Ro60 sera that coprecipitate RoBPI, suggesting that none is associated stably with the RoBPI-containing Ro particles, at least under usual conditions for immunoprecipitation (that is, without previous crosslinking).

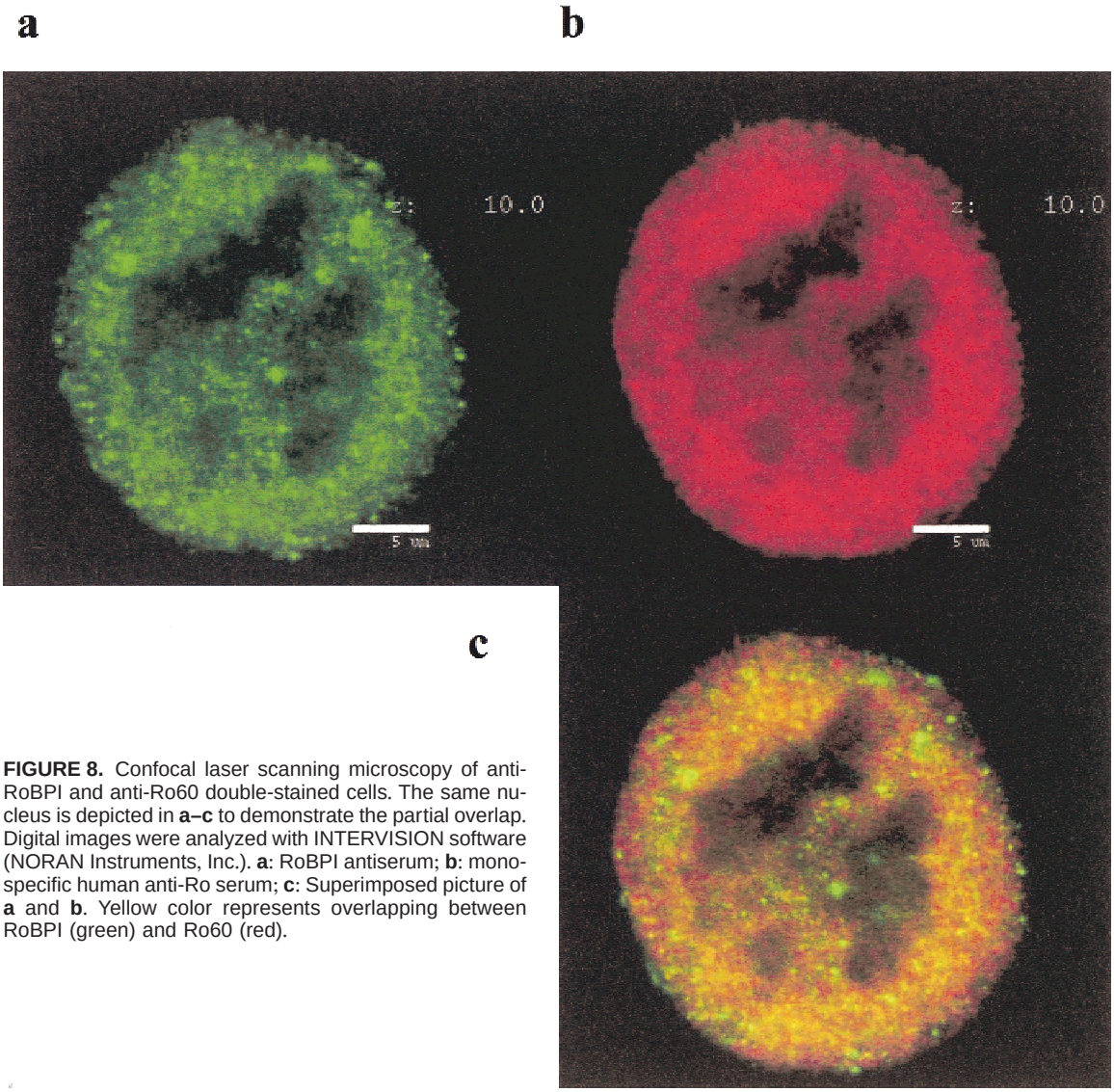

FIGURE 8. Confocal laser scanning microscopy of anti-RoBPI and anti-Ro60 double-stained cells. The same nucleus is depicted in **a–c** to demonstrate the partial overlap. Digital images were analyzed with INTERVISION software (NORAN Instruments, Inc.). **a**: RoBPI antiserum; **b**: monospecific human anti-Ro serum; **c**: Superimposed picture of **a** and **b**. Yellow color represents overlapping between RoBPI (green) and Ro60 (red).

Potential applications of RITA

Repeated failures to find partners using Ro60 protein as bait in two-hybrid and with hY RNAs in three-hybrid lead us to hypothesize that RNPs would be better partners for interaction than isolated proteins or RNAs. Identification of RoBPI would not have been possible without the specific properties of a RITA assay (Bouffard et al., 1999). The relevance of the interaction detected in RITA was confirmed by the demonstration of a RoBPI/Ro interaction in HeLa cells. Using RITA, binding of RoBPI was only detected when rRo60 and rhY5 RNA were coexpressed, possibly because binding of RoBPI to rRo60 alone and to rhY5 alone was too weak to be detected. Alternatively, interaction of RoBPI and Ro^{hY5} RNPs may be highly dependent on a specific tridimensional conformation induced on rRo60 and/or rhY5 RNA upon binding of RNA to protein. This possibility is strengthened by the recent documentation of conformational changes occurring outside the binding area in hY RNAs upon binding to Ro60 (Green et al., 1998), as well as by specific autoantibody binding to Ro^{hY5} RNPs (Boire & Craft, 1989). Using RITA, determinants of binding of RoBPI to recombinant Ro60-hY5 RNP could be further pinpointed to a short nucleotide stretch on rhY5 RNA that does not participate to binding of hY5 to Ro60 and/or La (M. Gendron, D. Roberge, & G. Boire, submitted).

Putative significance of the interaction of RoBPI with Ro^{hY5} RNPs

Ro^{hY5} RNPs have properties that are unique among Ro RNPs, and defining an interaction specific for these RNPs is very suggestive. Ro^{hY5} RNPs are easily purified away from other Ro RNPs (Boire & Craft, 1989, 1990). Ro^{hY5} RNPs from cultured HeLa cells are also

predominantly nuclear under conditions where other Ro RNPs are exclusively cytoplasmic (M. Gendron, D. Roberge, & G. Boire, submitted). Specific nucleotide sequences in hY5 RNA are required to maintain the nuclear localization of hybrid mouse/human Ro^{hY5} RNPs in transfected mouse cells (M. Gendron, D. Roberge, & G. Boire, submitted). It is of interest that Ro^{hY5} RNPs, but not other Ro RNPs, are targeted by specific anti-RNP antibodies present in the sera of most, if not all, patients with anti-Ro antibodies. In addition, among all hY RNAs, hY5 is the only one that is targeted by specific anti-RNA antibodies in sera of patients with anti-Ro antibodies (Boulanger et al., 1995). Although this is highly speculative, we propose that some of the unique biochemical properties of Ro^{hY5} RNPs, and potentially their interaction with RoBPI, may underlie their unique immunological specificities. Evidence has been presented that modifications in the tertiary or quaternary structure of an antigen affect the processing of the antigen and the resulting presentation of peptides to the immune cells (Rouas et al., 1993; Simitsek et al., 1995; Fatenejad & Craft, 1996). Preferential selection of recently acquired antigenic determinants by autoantibodies (Jemmerson & Blankenfeld, 1988) and the particulate nature of the autoantigen (Craft & Fatenejad, 1997) would favor, under appropriate conditions, autoimmune targeting of the Ro RNPs with the most recent evolutionary modifications. This would make Ro^{hY5} RNPs a likely driving source for the development or the perpetuation of the anti-Ro autoimmune response.

RoBPI appears highly conserved through evolution and ubiquitously expressed, with possible overexpression in gonads. By comparison, Ro60 protein is also expressed in all human cells and tissues tested, but its relative expression in human tissues has not been reported yet. In *C. elegans*, expression of the Ro60 homolog appeared constant through all developmental stages, whereas increased levels of Y RNA were found in embryo and in adult gonads (Labbé et al., 1999). Genetic or immunological homologs of Ro60 protein were not found in yeast cells, and its deletion was not lethal in *C. elegans*, rendering the identification of its function(s) problematic. In addition, hY5 RNA is expressed in all nucleated cells but not in mature erythrocytes or platelets. The hY5 gene has the properties of a retroposon and should have appeared through gene duplication after or around the primate divergence, about 75 million years ago (Maraia et al., 1996). Ro^{hY5} RNPs are thus very recent evolutionary additions, but their subsequent conservation suggests an advantage to the host, possibly through the development of novel function(s). Identification of an interaction between conserved RoBPI and recent Ro^{hY5} RNPs may be an indication of such a novel function, although this remains to be confirmed. From available data, RoBPI likely participates in a physiologic pathway distinct from the canonical (presumably cytoplasmic) function(s) of Ro RNPs. First, as alluded to previously, RoBPI presents striking structural similarities with splicing factors such as U2AF65. Second, RoBPI interacts specifically with the predominantly nuclear Ro^{hY5} RNPs, whereas all mouse and frog Ro RNPs as well as human $Ro^{hY1–hY4}$ RNPs are cytoplasmic. Third, only a small fraction of cellular RoBPI appears to interact with Ro^{hY5} RNPs, and the interaction is either transient or unstable. Experiments are being carried out to delineate the functional properties of RoBPI.

MATERIALS AND METHODS

Plasmids and yeast strain

Plasmids for RNA transcription in yeast pIIIEx426RPR, pIII/MS2-1 and pIII/MS2-IRE (Good & Engelke, 1994; SenGupta et al., 1996), yeast plasmids pLexA-MS2 and pAD-IRP (SenGupta et al., 1996) and yeast strain L40-ura3 (*MATα, ura3-52, leu2-3112, his3Δ200, trp1Δ1, ade2, LYS2::(lexAop)-HIS3, ura3::(lexAop)-lacZ*) (SenGupta et al., 1996) were generously provided by Dr. Marvin Wickens (University of Wisconsin, Madison, Wisconsin). Plasmid pBTM116 (Bartel & Fields, 1995) was a gift from Dr. Rolf Sternglanz (State University of New York (SUNY), Stony Brook, New York). Plasmid pGAD424 was purchased from Clontech Laboratories Inc. (Palo Alto, California). cDNA for Ro60α (p60-4) (Deutsher et al., 1988) was a gift from Dr. E.K.L. Chan (The Scripps Research Institute, La Jolla, California). The coding region of the Ro60 cDNA was amplified by PCR from plasmid p60-4 and ligated into the Lex-A containing plasmid pBTM116 to generate pBTM-Ro60. The cDNA of La was amplified from total RNA prepared from HeLa cells with Trizol Reagent (Gibco/BRL; Burlington, Ontario, Canada) using the protocol from the manufacturer. First-strand cDNA synthesis was primed with random hexamers, and the cDNA of La was amplified by nested PCR. The PCR product was then subcloned into the GAL4 AD-containing plasmid pGAD424 to generate pGAD-La. hY cDNAs were amplified by RT-PCR from hY RNAs to generate cDNAs flanked by *Eco*RI (or blunt *Sma*I and *Dra*I) sites, and then subcloned in pIIIEx426RPR to give pEx-hY1, pEx-hY3, pEx-hY4, and pEx-hY5, respectively, and in pIII/MS2-1 to give pIII/MS2-hY5. Mutagenesis by PCR was used to delete the cytosine at position 9 in hY5 cDNA to generate pEx-hY5ΔC9. All PCR products subcloned in plasmids were sequenced before use in yeast and did not contain PCR-generated mutations.

RNP interaction trap assay screening of a HeLa cell cDNA library

A human HeLa S3 MATCHMAKER cDNA library was purchased from Clontech Laboratories. All two-hybrid, three-hybrid, and RNP interaction trap (RITA) assays were performed in yeast strain L40-*ura3*. Details of the RITA assay have been described elsewhere (Bouffard et al., 1999).

RITA screening of the HeLa cDNA library was performed using a complex made of both LexA-Ro60 (encoded by plasmid pBTM-Ro60) and rhY5 RNA (encoded by plasmid pEx-

hY5) as bait (Fig. 1). Yeast transformation was performed using the lithium acetate transformation method (Transy & Legrain, 1995). Transformants were selected on SD plates lacking histidine, leucine, tryptophan, and uracil (His−, Leu−, Trp−, Ura−) containing 1 mM 3-amino 1,2,4-triazole (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) and 80 mg/mL X-gal. Transformants (2×10^6) were screened through two separate rounds of RITA. Positive clones were defined by the development of both reporter phenotypes (growth on His− media and expression of β-galactosidase). Plasmids were prepared from positive clones by yeast plasmid rescue after enzymatic lysis of yeast (Golemis et al., 1997). Isolated plasmid DNA was then used to transform HB101 *Escherichia coli* competent cells. Transformed cells were plated on M9 minimum plates lacking leucine and containing 50 μg/mL ampicillin. Plasmid isolation, DNA sequencing and Southern blotting followed standard methods (Granger et al., 1996a, 1996b).

Cloning of full-length RoBPI cDNA using 5′ RACE-PCR

Cloning of the 5′ end of RoBPI cDNA was performed using the 5′-Full RACE PCR kit according to manufacturer's instructions (TaKaRa Biomedicals, PanVera Corp., Madison, Wisconsin). For first-strand cDNA synthesis, the use of a snap cooling step was necessary because the terminal 5′ sequence of RoBPI cDNA was over 70% G/C rich (Fig. 2). Thus, 3 μg total RNA isolated from human testis (Clontech Laboratories) and 30 pmol primer SIAH300-REV (5′-CCCA GCTCATAGTAGATAGAGCC-3′) previously phosphorylated using T4 polynucleotide kinase (Amersham Pharmacia Biotech, Baie d'Urfé, Québec) were melted in a final volume of 16 μL at 90 °C for 3 min, incubated on ice for 2 min, and then 2 μL 10× reverse transcriptase buffer, 40 U RNAse inhibitor (TaKaRa Biomedicals), and 5 U AMV reverse transcriptase were added on ice in a final volume of 20 μL. The transcription reaction was started at 30 °C for 10 min and completed at 50 °C for 1 h. After heat inactivation of reverse transcriptase, the RNA template was digested using 60 U RNase H, and first-strand cDNA was precipitated using sodium acetate. Single-stranded cDNA was resuspended in 8 μL of 5× ssDNA ligation buffer (TaKaRa Biomedicals), 40% PEG 6000, and 12 μL of water (40 μL final volume), and ligated to form concatemeres at 16 °C during 16–24 h using 40 U T4 RNA ligase. Finally, two rounds of inverse PCR were performed on ligated cDNA using forward primer 217-1 (5′-CAGTGACAA TGGGCTTTGGA-3′) and reverse primer SIAHRACE-REV (5′-TATGGTCGCCGTCGCCATCTTGCG-3′) and 5 U Taq polymerase (Amersham Pharmacia Biotech). PCR products were separated in 1.5% agarose, selected bands were cut and extracted using the QiaExII kit (Qiagen Inc., Mississauga, Ontario, Canada), and subcloned in pGEM-T vector (Promega Corp., Madison, Wisconsin). Inserts were then sequenced in both orientations.

Northern blot analysis

Multiple tissue Northern blots were hybridized with ^{32}P-labeled probes using manufacturer's instructions (Clontech Laboratories). A 739-nt-long RoBPI probe was generated by RT-PCR from total RNA isolated from HeLa cells. First-strand cDNA synthesis was primed using an oligo dT(12–18), and was performed using Superscript Reverse Transcriptase (Gibco-BRL). PCR amplification of the probe from HeLa cell cDNA used primers 217-3 (5′-TCCCACTGCCGCTGC TGT-3′) and 213-1 (5′-CAGTGACAATGGGCTTTGGA-3′). Approximately 50–100 ng of PCR product were labeled with ^{32}P-dCTP using a random priming kit (Amersham Pharmacia Biotech). Between each hybridization, membranes were stripped twice by agitation at room temperature in 0.5% SDS previously heated at 100 °C.

Crosslinking and immunoprecipitation assay

HeLa cells (2×10^7) were washed in PBS (200 mM NaCl, 3 mM KCl, 5.4 mM $Na_2HPO_4 \cdot 7H_2O$, 1.8 mM KH_2PO_4, pH 7.4), resuspended in 1.7 mL of ice-cold PBS, and transferred into a clean 50-mL chilled plastic beaker. PBS (300 μL) containing 100 μg/mL (10 μL of a 20 mg/mL solution) of digitonin, 4 mmol of DTSSP, a cocktail of protease inhibitors (50 μg/mL each of chymostatin, leupeptin, antipain, pepstatin) and 40 U/mL of RNase inhibitor (Amersham Pharmacia Biotech) were added to the beaker. The resulting 2.0 mL suspension was slowly stirred on ice for 2 h using a small flat magnetic bar. The crosslinking reaction was stopped by adding 1/10 volume of 1 M Tris-Cl, pH 8.0. The suspension was transferred to 1.5-mL tubes and centrifuged at 13,000 × *g* during 15 min at 4 °C. The soluble fraction from about 7×10^6 cells was mixed with 10 mg of protein A-sepharose beads (Amersham Pharmacia Biotech) previously saturated with human anti-Ro60 or anti-Ro/La antibodies crosslinked with dimethylpimelimidate (Sigma-Aldrich Canada Ltd.), and rotated at 4 °C for 90 min. Beads were then washed five times with NET-2 buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% NP-40), and retained proteins were eluted by boiling for 5 min in 50 μL of SDS sample buffer (50 mM Tris-Cl, pH 6.8, 20% glycerol, 4.6% (w/v) SDS) containing 10% (v/v) 2-mercaptoethanol. Eluted proteins were loaded on a 15% SDS-PAGE minigel (172:1; acrylamide:bis) and transferred to a Hybond C nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was incubated overnight in blocking buffer (10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5% gelatin), and blotted with multiple sera using a miniblotter apparatus (Immunetics, Cambridge, Massachusetts). Bound antibodies were detected using ECL reagents (Amersham Pharmacia Biotech).

Anti-RoBPI antibodies

A protein (dubbed PUF for "Poly U Factor"), whose amino acid terminal sequence was highly homologous to the amino acid sequence predicted for a cDNA sequence identified as Siah binding protein I (SiahBPI; GenBank accession number U51586) was identified during characterization of proteins retained on poly(U) columns (P.S. McCaw and P.A. Sharp, pers. comm.). Antibodies recognizing an RRM region of the PUF protein were raised in chicken. Chicken anti-PUF antiserum recognized recombinant and HeLa cell PUF protein in immunoblots, but did not recognize the protein in more native assays such as immunoprecipitation and immunofluorescence. In parallel, we had observed almost complete sequence homology between SiahBPI protein and RoBPI. Drs. McCaw and Sharp agreed to share PUF antiserum with us.

We observed that anti-PUF antibodes recognized recombinant and HeLa cell RoBPI protein, suggesting that PUF and RoBPI were very similar or identical.

In parallel, 5-week-old New Zealand white rabbits were immunized both with recombinant PUF (rPUF) protein and RoBPI cDNA. His-tagged rPUF was produced in *E. coli* from pET15b vector (Novagen, Madison, Wisconsin) containing the PUF cDNA (generous gift from Drs. McCaw and Sharp, Massachusetts Institute of Technology) and purified on Ni-NTA agarose (Qiagen Inc.). One hundred micrograms of purified rPUF were run in SDS-PAGE, and the corresponding band was stained with Coomassie blue and cut out from the gel. The slice of polyacrylamide gel was crushed and vigorously mixed with an equal volume of Freunds' adjuvant (Gibco-BRL). The resulting 500-μL suspension was injected subcutaneously in rabbits every month. The first immunization used complete adjuvant, and the three following ones used incomplete Freunds' adjuvant. RoBPI cDNA was subcloned into expression vector pcDNA3.1/V5-His TOPO (Invitrogen), under transcriptional control of a CMV promoter. After three rounds of immunization with rPUF protein, 400 μg of the RoBPI plasmid DNA were suspended in 600 μL distilled water and injected intramuscularly every week for four weeks (La Cava et al., 1997). Blood was collected through the ear veins before each immunization and at the end of the immunization protocol. The resulting antiserum was called anti-RoBPI antiserum.

Indirect immunofluorescence and confocal microscopy

Cell fixation, permeabilization, and incubations with antibodies were done with rotational mixing at room temperature. For each experiment, 3×10^6 HeLa cells were washed twice in PBS (8 mM Na_2HPO_4, 1 mM NaH_2PO_4•$7H_2O$, 150 mM NaCl, pH 7.4). Cells were fixed in PBS containing 4% paraformaldehyde (Sigma-Aldrich Canada Ltd.) for 30 min. Fixed cells were carefully washed in PBS then permeabilized for 30 min with PBS containing 0.1% Triton X-100 (J.T. Baker Chemical Co., Phillipsburg, New Jersey). Cells were incubated sequentially for 30 min with primary antibodies (rabbit anti-RoBPI and/or human anti-Ro60, anti-Jo-1, or anti-U1 sera) diluted 1:200 in PBS, then incubated with secondary antibodies: goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC; Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania) and goat anti-human IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC; Jackson ImmunoResearch). Cells were washed in PBS between incubation with each different antibody. For fluorescence microscopy, 10 μM of the DNA marker Hoechst 33258 (Boehringer Mannheim, Laval, Québec, Canada) was also added. Finally, cells were mounted on a microscope slide with a PBS/glycerol/octane solution [4 mL PBS, 16 mL glycerol, 500 mg 1,4-diazabicyclo(2.2.2(octane (DABCO; Fluka Chemical Corp., Ronkonkoma, New York)].

Indirect immunofluorescence was examined without counterstain using an Axioscop 2 fluorescent microscope (Carl Zeiss Inc., Thornwood, New York) equipped with band pass filters for fluorescence of FITC (Excitation D480/30: Emission D535/40) or TRITC (Excitation D560/40: Emission D630/60), both from Chroma Technology Corp. Photomicrographs were obtained using a digital camera (Empix Imaging Inc., Niagara, New York) and enhanced with SPOT software.

Cells were also examined with a confocal laser scanning microscope (NORAN Instruments Inc., Middleton, Wisconsin) equipped with a krypton/argon laser and coupled to an inverted microscope with a 100X oil immersion objective (Nikon). Specimens were excited at 488 nm and 568 nm. Emitted FITC and TRITC fluorescence was measured at wavelengths 525–550 and $>$590 nm, respectively. Optical sections were collected at 0.2 μm intervals with a 10-μm pinhole aperture. Digitized images were obtained with 256$\times$ line averaging and enhanced with INTERVISION software (NORAN Instruments) on a Silicon Graphics 02 workstation.

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