Identification of Ribonucleoprotein (RNP)-Specific Protein Interactions Using a Yeast RNP Interaction Trap Assay (RITA)

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INTRODUCTION

Major intracellular processes are driven by ribonucleoproteins (RNPs), complexes resulting from specific noncovalent interactions between protein and RNA molecules (4,24). RNPs play a major role in DNA and RNA transcription, DNA replication, RNA posttranscriptional modifications, RNA transport and protein translation. Characterization of RNA-protein and protein-protein interactions occurring in RNPs has most often relied on in vitro models, but these tools have limitations. Recent DNA and RNA transcription, DNA replication, RNA posttranscriptional modifications, RNA transport and protein translation. Characterization of RNA-protein and protein-protein interactions occurring in RNPs has most often relied on in vitro models, but these tools have limitations. Recent two-hybrid experiments made by our group (unpublished results) and by others did not demonstrate any direct protein-protein interactions using Ro60 as bait. hY RNAs used as baits in three-hybrid experiments failed to bind specifically proteins other than Ro60 and La (unpublished results). Based on these repeated failures and on observations that binding of a protein to RNA may modify the tertiary structure of both the protein and the RNA (4,11,14) and alter their affinity for potential partners (2,16,19), we hypothesized that the use of baits made of RNPs rather than of isolated proteins or RNAs might allow the scoring of additional interactions. Using RITA, recombinant Ro60 (rRo60) protein and recombinant hY (rhY) RNA were found to interact efficiently and specifically in yeast. In addition, the La protein and a novel protein (RoBPI) binding specifically to RNPs containing rhY5 RNA were recovered at high frequency from a HeLa cell cDNA expression library using as bait a RNP.
made of rRo60 and rhY5 RNA. RITA can be used to reconstruct in *S. cerevisiae* mammalian RNPs composed of a single RNA associating with two specifically binding proteins. RITA thus represents a useful tool to characterize interactions occurring between proteins and RNP complexes.

**MATERIALS AND METHODS**

**Plasmids and Yeast Strains**

Plasmids for RNA transcription in yeast pIIIEx426RPR (8), pLexA-MS2, pIII/MS2.1 and pIIIIMs2-IRE, yeast plasmid pAD-IRP (20) and yeast strain L40-ura3 [MATa, ura3-52, leu2-3112, his3Δ200, trp1Δ1, ade2, LYS2::(lexAop)-HIS3, ura3::(lexAop)-lacZ] were gifts from Dr. Marvin Wickens (University of Wisconsin, Madison, WI, USA). Plasmid pBTM116 (1) was a gift of Dr. Rolf Sternglanz (SUNY, Stony Brook, NY, USA). Plasmids pGAD424 and pAS2 were purchased from CLONTECH Laboratories (Palo Alto, CA, USA). cDNA for Ro60α (p60-4) (3) was a gift of Dr. E.K.L. Chan (Scripps Research Institute, La Jolla, CA, USA). The cDNA of Ro60 was amplified by polymerase chain reaction (PCR) from plasmid p60-4, using primers Ro60-5′ (5′-ACCCGGGATGCCGAACCTTAAG-3′) and M13-Forw (5′-AGGGTTTTCCCAGTCACGACGTTG-3′). The product was then subcloned in plasmid PCR-Script™ (Stratagene, La Jolla, CA, USA) to generate plasmid pCR-Ro60. Ro60 cDNA was excised from pCR-Ro60 by digesting with Smal and Sall, and ligated into the LexA-containing plasmid pBTM116 to generate pBTM-Ro60, into the GAL4 DNA binding domain (DBD)-containing plasmid pAS2 to generate pARo60 and into the GAL4 activating domain (AD)-containing plasmid pGAD424 to generate pGAD-Ro60. The cDNA of La was amplified from total RNA prepared from HeLa cells with TRIzol® Reagent (Life Technologies, Burlington, ON, 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 first PCR was primed with oligonucleotides La1-5′ (5′-CTG-TGGCCCGGAACCTTAAG-3′) and La1-3′ (5′-TTCNACAGGTOGACAT-TGAAGTG-3′) giving a 1368-bp PCR product. The nested PCR was primed with oligonucleotides La2-5′ (5′-CCGGAAATTCATGGCTGAATGGT-GATAATG-3′) and La2-3′ (5′-CCGG-AAATCTACTGTTCTCCAGCACCAt-3′) giving a 1243-bp PCR product flanked by *EcoRI* sites at both ends. The PCR product was then subcloned into the *EcoRI* site of pGAD424 and pBTM116 plasmids and selected for correct orientation.

Plasmids for hY RNA transcription in RITA were made by subcloning DNAs encoding RNAs in *EcoRI* site of pIIIEx426RPR. All hY cDNAs were amplified by RT-PCR from hY RNAs to generate cDNAs flanked by *EcoRI* sites and then subcloned in pIIIEx426RPR to give pEx-hY1, pEx-hY3, pEx-hY4 and pEx-hY5, respectively. Mutagenesis by PCR was used to delete the cytosine at position 9 in hY5 cDNA to generate pEx-hY5ΔC9. Plasmids for hY RNA transcription in three-hybrid were produced by introducing the appropriate hY sequence in pIII/MS2.1 plasmid. All PCR products subcloned in plasmids were sequenced before use in yeast and did not contain PCR-generated mutations.

**Construction and RITA Screening of a HeLa Cell cDNA Library**

A human HeLa S3 MATCHMAKER cDNA library was purchased from CLONTECH Laboratories. The library contained 6.0 × 10^6 independent clones ranging from 0.4–2.0 kb in size, with an average insert size of approximately 1.5 kb.

Yeast transformations were performed using lithium acetate (23). The two plasmids encoding LexA-Ro60 (pBTM-Ro60) and recombinant hY5 RNA (pEx-hY5) were first co-transformed in yeast L40-ura3. Co-transformants were selected by culturing the cells on yeast agar plates (SD) lacking tryptophan (Trp-) and uracil (Ura-) at 30°C. A double transformant yeast clone was then transformed with the PGal GH plasmid library. Screening was done using manufacturer’s protocols. Transformants were selected on
RESULTS

Two- and Three-Hybrid Assays Using Components of Ro RNPs

We first set up a standard yeast two-hybrid system to detect proteins that would interact with Ro60 fused to the DBD of GAL4. We made sure that the expressed Ro60-GAL4DBD fusion protein present in yeast extracts was still able to bind to in vitro transcribed hY RNAs in EMSA (9). Furthermore the shifted material was shown to contain the fusion protein by immunological supershift experiments (Figure 1). Since interaction of Ro60 with hY RNAs is highly dependent on correct protein conformation (11,15), the Ro60 segment of the fusion protein appeared to assume a conformation close to the one adopted by native Ro60. However, we did not find any convincing protein partner of Ro60 during screening of a HeLa cell cDNA library (data not shown).

Positive clones were defined as growing on His-media and expressing β-galactosidase (β-gal).

Plasmids were recovered from positive clones by yeast plasmid rescue after enzymatic lysis of yeast (7). Isolated plasmid DNA was then used to transfact HB101 Escherichia coli competent cells. Transfected cells were plated on M9 minimum plates lacking leucine and containing 50 µg/mL ampicillin, to select for pGAD GH plasmids containing cDNAs from the library. Plasmid isolation, DNA sequencing and Southern blotting followed standard protocols (5). Electrophoretic mobilities shift assays (EMSA) were performed as described (9).

To test the hypothesis that Ro RNPs, rather than Ro proteins or isolated hY RNAs, would display specific protein interactions, we modified the three-hybrid system to allow for the production of baits consisting of a preformed RNP. The system should also be able to determine whether Ro RNPs interact with different intracellular proteins, depending on the identity of the hY RNA that they contain. The general outline of the system we designed and called RITA is shown in Figure 3A. Yeast strain L40-ura3 is auxotrophic for histidine, tryptophan, leucine and uracil. Hybrid proteins and the recombinant RNA are each expressed by separate plasmids that carry a different nutritional marker [TRP1 for the protein (e.g., Ro60) that is part of the bait RNP, LEU2 for the prey protein and URA3 for hY RNA]. This system comprises two hybrid proteins and their naturally interacting RNA partner acting as a bridge to reconstitute transcription of reporter genes (HIS3 and lacZ), allowing yeast to grow without histidine and to develop a blue color in the presence of X-gal, respectively.

In our model RITA assay, the bait consisted of a hybrid Ro60 protein fused to LexA (a bacterial DNA-binding protein) non-covalently bound to co-expressed rhY RNAs. Hybrid LexA-Ro60 protein from transformed yeast bound in vitro transcribed hY RNAs in EMSA assays (Figure 1). Yeast-transcribed rhY RNAs were still attached to RPR1 trailer and/or leader sequences (derived from the termination signal and from the promoter region of the RPR1 gene, respectively), as detected by northern hybridization (data not shown). However, the predicted secondary structure of recombinant hY RNAs suggested that the RNA structure was not affected, except at their extreme 5′ and 3′ ends, by the presence of RPR1 leader or trailer sequences (Figure 3B). In addition, binding of Ro60 to in vitro transcribed hY RNAs and to the corresponding rhY RNA was lost when a single C at position 9 was deleted in both (data not shown), suggesting that rhY RNA adopted a conformation similar to that of native hY5 RNA.

To further verify the correct in vivo expression of our rRo60 and rhY constructs, we used as prey a hybrid GAL4AD-La. When hybrid Ro60 and La proteins were co-expressed with any of the recombinant hY RNAs, reporter

![Figure 1. EMSA using recombinant Ro60 and La proteins produced in yeast. Protein extracts were mixed with in vitro transcribed 32P-labeled hY5 RNA to reconstitute hY5-containing RNPs. The resulting mixture was then run in a non-denaturing polyacrylamide gel. All lanes contained radiolabeled hY5 RNA. Lane 1: no protein extracts. Lanes 2–4: yeast extracts containing GAL4AD-La. Lanes 5–6: yeast extracts containing GAL4DBD-Ro60. Lanes 7–8: yeast extracts containing LexA-Ro60. Lanes 9–10: HeLa cell extracts. In some lanes, specific anti-Ro60 (lanes 3, 5 and 7) or anti-Ro60/La (lanes 4 and 9) antibodies were incubated with the mixture of protein extracts and radiolabeled hY5 RNA (supershift).]
genes were activated (Figure 4, A–D), indicating that a tripartite RNP was reconstituted, the recombinant hY RNA acting as bridge linking Ro60 and La hybrid proteins. No interaction was seen between LexA-Ro60 and GAL4AD-La in the absence of recombinant hY RNA (data not shown). Unrelated RNAs (e.g., IRE-MS2) and rhY5ΔC9 RNA (lacking the essential bulged cytosine at position 9 of hY5 RNA) could not supplement for the absence of rhY5 RNA (Figure 4, E and F, respectively). The specificity of the interaction was thus confirmed since activation of reporter genes required the presence of an intact Ro60 binding site on rhY RNAs.

Screening of a cDNA Library Using Recombinant Ro60-hY5 RNP as Bait

We had shown that tripartite RNPs could be readily reconstructed in vivo. We then screened a HeLa cell cDNA library using as bait a preformed RNP consisting of rRo60 and rhY5 RNA. Our assumption was that one of the most frequently recovered interacting partners would be La protein. As expected, more than 10% (26 out of 226) of yeast clones testing positive in RITA contained pGAD GH plasmids (from the library) encoding cDNAs that hybridized with La cDNA probes in Southern blots (data not shown). During the same library screening, a protein interacting specifically with recombinant Ro60-hY5 RNP (that we called RoBPI) was identified in 60% of the clones (unpublished). RoBPI did not bind recombinant RNPs containing recombinant hY RNAs other than rhY5. Similarly, no interaction was observed between LexA-Ro60 and GAL4AD-RoBPI in the absence of recombinant hY RNA (data not shown). RoBPI is a previously unidentified 60-kDa protein containing 3 distinct RNA recognition motifs. RoBPI could be co-immunoprecipitated by anti-Ro60 antibodies from HeLa cell extracts (unpublished).

DISCUSSION

Using Ro RNPs as models, we have shown that physiologically relevant tripartite RNPs can be readily reconstructed in vivo in yeast. The specificity of the interaction of Ro60 and La proteins through hY RNA binding was demonstrated by the activation of reporter genes when intact hY RNAs were used and by the lack of activation when no RNA was present or when either an unrelated RNA or a single-base mutant hY RNA containing a nonfunctional Ro60 binding site were used. The specificity of the interactions was also demonstrated by the recovery at high frequency of La cDNA and of a specific recombinant Ro60-hY5 binding protein (RoBPI) from a HeLa cDNA library.

A correct interpretation of the failure, in two-hybrid, to define protein interactions with Ro60 was difficult because no true positive controls were available. Similarly, we failed to identify partners of hY RNA, other than Ro60 and La, using the three-hybrid assay. Nonetheless, we hypothesized that the failure to identify interactions in these assays resulted from the presence, in the bait, of only one of the components of Ro RNPs. We reasoned that binding of a hY RNA to Ro60 might alter their affinity for potential partners. This hypothesis was partly supported by two earlier observations. First, autoantibodies specifically target Ro RNPs containing hY5 RNA (RohY5) (2). Second, RohY5 RNPs are found predominantly in the nuclear compartment of cultured HeLa cells (unpublished data) while RNPs containing
other hY RNAs are exclusively cytoplasmic. Thus, the type of hY RNA bound to Ro60 appeared to modulate both expression of antigenic epitopes and intracellular localization of the resulting RNP, presumably through reciprocal conformational changes in the protein and/or RNA (4,11,14) or through stabilization of weak interactions with both the RNA and the protein.

Although it presents the important advantage of using in vivo reconstituted RNPs as baits for protein interactions, RITA has limitations inherent to yeast-hybrid systems. First, fusion proteins may have altered conformations impeding interactions seen with native proteins or, alternatively, favoring interactions that are not seen with native proteins. Second, recombinant RNAs are transcribed under the regulation of RPR1, a strong RNA pol III promoter. Although transcripts may be matured or processed, the extent of maturation cannot presently be predicted in advance (8,20). Transcribed rhY RNAs still attached to sequences derived from the promoter gene were detected by northern hybridization in transformed yeast. These extraneous sequences apparently did not affect the structure responsible for binding to Ro60. Third, transcription by RNA pol III (in threecombination and in RITA) renders inevitable the binding of La protein to RNA transcripts, due to the presence of an oligouridy late stretch at their 3′ end. In RITA, however, rLa may be used as a control when RNPs without known interacting prey proteins are studied. Binding of rLa would then confirm correct in vivo expression of, and interaction between, LexA-bait protein and recombinant RNA transcripts.

Despite these limitations, the yeast RITA system offers significant advantages over classical biochemical and immunological methods to identify partners of RNPs. The use of a “cognate protein anchor” instead of a “fixed protein anchor” introduces a qualitative change in the interactions that are looked for, even if the reagents are essentially the same as in three-hybrid. In theory, any RNP comprised of (at least) one protein strongly interacting with the RNP-specific RNA could be used as bait in the assay. Interactions detected with RITA may result either from protein-RNA, protein-protein or protein-RNP interactions. When combined with three-hybrid, RITA can discriminate protein interactions dependent on the presence of preformed RNPs from mere RNA-protein interactions. In addition, when specific RNA is removed from the assay, RITA becomes a classical two-hybrid system, ensuring discrimination between interactions specific for the preformed RNP and simple protein-protein interactions. As a consequence, specific protein interactions dependent upon the simultaneous presence of both protein and RNA in the bait RNP can be readily detected.

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Figure 3. (A) Schematic representation of the RITA system using Ro RNPs as model. Hybrid LexA/Ro60 (rRo60) protein binds both the LexA binding site on yeast DNA and the Ro60 binding site on co-expressed rhY RNA. Hybrid X/GAL4 AD protein binds the resulting Ro60-rhY RNP and activates transcription of reporter genes. (B) Recombinant hY RNA expressed in yeast. From 5′ to 3′, the recombinant rhY RNA consists of the 5′ leader sequence of RNase P RNA, a single complete hY RNA motif and the 3′ trailer sequence of RNase P RNA (not present in a significant portion of transcribed rhY RNAs).

Figure 4. RITA using recombinant Ro60 (rRo60) and La (rLa) proteins and recombinant hY (rhY) RNAs. β-gal expression in yeast colonies is indicated by blue color development (dark gray; A–D); its absence by yellow color (light gray; E and F). Yeast colonies contain co-transformed plasmids expressing hybrid LexA/ro60 and GAL1-AD-La proteins and one of the following recombinant RNAs: (A) rhY1 RNA; (B) rhY3 RNA; (C) rhY4 RNA; (D) rhY5 RNA; (E) rhY5ΔC9 RNA and (F) rMS2-IRE RNA.
suggest that RITA represents a useful tool to identify novel partners of known RNPs or to study the structure requirements for interaction of the proteins and RNA components of these RNPs.

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REFERENCES


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