The Yeast RNA-binding Protein Rbp1p Modifies the Stability of Mitochondrial Porin mRNA

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The Saccharomyces cerevisiae RNA-binding protein Rbp1p was initially identified as a negative growth regulator; however, its function is still obscure. Here, we show that Rbp1p in cells is associated with structures that sediment at 10,000 as well as 100,000 × g. It appears microscopically as punctate signals partially localized to the perinuclear region. Over-expression of Rbp1p in yeast resulted in growth defects on nonfermentable carbon sources, suggesting a function for Rbp1p in mitochondrial biogenesis. Absence of Rbp1p increased the level of mitochondrial porin, whereas over-expression of Rbp1p, but not an N-terminally truncated form, decreased porin levels. Over-expression of Rbp1p also decreased the level of mitochondrial porin mRNA by enhancing its degradation, an effect that was dependent on all three of the Rbp1p RNA recognition motifs. In cells, the porin mRNA is associated with Rbp1p-RNP (ribonucleoprotein) complexes. In vitro binding assays showed that Rbp1p most likely interacts with a (C/G)U-rich element in the porin mRNA 3′-UTR. Based on these observations, we infer that Rbp1p has a role in negatively regulating mitochondrial porin expression post-transcriptionally.

Eukaryotic messenger RNAs (mRNAs) are produced in the nucleus and undergo sequential processing reactions that typically include capping, pre-mRNA splicing, and polyadenylation. Cellular mRNA is associated with protein from the time of its synthesis to its degradation. With growing recognition of the complexity of gene regulation, interest in the mechanism of mRNA degradation has increased rapidly during the past decade. The mRNA turnover is a means to permit timely adjustments to changes in growth conditions or to genetically controlled programs of expression (reviewed in Refs. 1 and 2). In eukaryotic cells, production of a particular gene product can be temporally and spatially regulated, allowing different cell types or different developmental stages of cells to fine-tune their patterns of protein expression. In fact, a number of developmental events, such as pattern formation and terminal differentiation, can be regulated post-transcriptionally by controlling mRNA stability, localization, and translation (reviewed in Refs. 3 and 4).

RNA-binding proteins play key roles in the post-transcriptional regulation of gene expression; many of them contain an RNA recognition motif (RRM; also called RBD (RNA-binding domain)) (reviewed in Refs. 5 and 6). RRM-containing proteins include small nuclear ribonucleoproteins (snRNP), polyadenylate-binding proteins (PAPB), pC36, and heterogeneous nuclear ribonucleoprotein particle (hnRNP) protein. Although the ubiquity of these RNA-binding proteins suggests a general housekeeping role, some of them may have specific functions. For example, the Drosophila ELAV protein plays a role in neuron-specific RNA splicing (7, 8) and vertebrate ELAV-like ribonucleoproteins (elA, B, C, and D) have been implicated in early development and neuronal differentiation (9).

RNA-binding proteins also play a role in mRNA stability. The human Hu protein inhibits AU-rich element (ARE)-mediated RNA decay in vivo and stabilizes deadenylated RNA intermediates in vitro (10–15). The AU-rich binding protein (AUF1) stabilized the parathyroid hormone mRNA in an in vitro degradation assay and is also involved in regulation of cyclooxygenase-2 mRNA stability (16, 17). In Xenopus, estrogen induces the stabilization of the transcripts of the yolk precursor protein vitellogenin, which is a result of the different affinities of the 155-kDa RNA-binding protein, vitilogenin, for cis-acting stability determinants (18). Furthermore, RNA-binding proteins may have functions in translational processes. For example, PABP is essential to stabilize the 3′-end of mRNA; however, PABP interacts with the cap-associated eukaryotic initiation factors eIF4G (in yeast and plants) and eIF4B (in plants). In mammals PABP interacts with a novel PABP-interacting protein that also binds eIF4A (19, 20). The Fragile X mental retardation syndrome protein, FMR1, and its homologous FXR proteins are associated with ribosomes, predominantly with 60 S large ribosomal subunits (21). These observations demonstrate that RNA-binding proteins serve as regulatory molecules.

A putative RNA-binding protein gene (RBP1, also called NGR1) was identified in Saccharomyces cerevisiae (22). Rbp1p contains three RRMs, two glutamine-rich sequences, and a C-terminal asparagine-methionine-proline-rich region. Although RBP1 is not an essential gene, over-expression of Rbp1p in yeast yields a slow-growth phenotype, suggesting that Rbp1p is functionally critical in certain biochemical processes. We undertook an analysis of the yeast Rbp1p with the aim of elucidating its cellular role. We report here that the level of mitochondrial outer membrane porin was lower in the Rbp1p yeast strains than it was in an rbp1 mutant. We further dem-
onstrate that Rbp1p can accelerate porin mRNA turnover, possibly through binding to the 3′-UTR of porin mRNA. These data provide the first evidence that Rbp1p might be involved in post-transcriptional regulation of porin expression in intact cells.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Plasmid Construction—The S. cerevisiae strain YTC345 (MATa ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-3,112) was the parental strain used for manipulation and gene disruption. The S. cerevisiae strain YTC345 (Δ YRP582, MATa rpb1-1 ura3 leu2) was a kind gift from Dr. Tien-Hsien Chang (Ohio State University) and was used for mRNA turnover studies. Yeast strains were cultured to early or mid-logarithmic phase. Ribonuclease (RNase) inhibitor was added to cultures to a final concentration of 50 μg/ml followed by incubation at 30 °C for 10 min and cooling on ice. Cells were harvested, washed once with TNN buffer (Tris-HCl, pH 7.5, 10 mM, NaCl, 100 mM, MgCl2, 30 mM, heparin, 200 μg/ml, and cycloheximide, 100 μg/ml, dissolved in diethyl pyrocarbonate-treated H2O), suspended in 0.4 ml of TNN buffer, and broken by vortex mixing with an equal volume of glass beads for 3 min. After the addition of 0.6 ml of TNN buffer, the lysate was clarified by centrifugation (5,000 × g for 5 min at 4 °C), and the supernatant was centrifuged at 12,000 × g for 10 min at 4 °C. Supernatant representing about 8 OD500 units were applied to a continuous 4.5 ml of 7–47% sucrose gradient (containing, 50 mM Tris, pH 7.5, 50 mM NaCl, 12 mM MgCl2, and fractionated by centrifugation in a Hitachi S-50T2–403 rotor at 29,000 rpm for 3.5 h at 4 °C. After centrifugation, the gradients were analyzed by measurement of absorbance at 254 nm in an ISCO UA-6 absorbent monitor.

RNA Blot Analysis and RNA Turnover Analysis—Yeast total RNA was isolated by the hot acid phenol method (31). RNA blot analysis was carried out as described previously (22). Yeast strain YTC345 carrying a temperature-sensitive RNA polymerase II allele (rpb1-1Δ) (22) to study the RNA turnover rate. The RBP1 gene of YTC345 was disrupted by hisG-URA3-hisG construction as described previously (22). YTC345 and YTC345 rbp1Δ:URA3 were grown in synthetic medium containing 2% galactose and 0.1% glucose with shaking overnight at 30 °C for 3 days, and photographed. The RBP1 gene of YTC345 was disrupted by hisG-URA3-hisG construction as described previously (22). RNase A and RNase T1 were added to cultures to a final concentration of 10 μg/ml. After incubation at 37 °C for 1 h, they were diluted serially 10-fold to 1 × 10−4. Samples (5 μl) of each cell suspension were spotted on synthetic medium containing 2% glucose or 2% galactose as the carbon source, incubated at 30 °C for 3 days, and photographed.

Ribosome-Polysome Profile Analysis—Yeast strains were grown with shaking at 30 °C in 50 ml of SC medium containing 2% glucose or 2% galactose. Yeast strain A608−1, these were diluted serially 10-fold to 1 × 10−4. Samples of each suspension were spotted on SC medium containing 2% glucose or 2% galactose as the carbon source, incubated at 30 °C for 3 days, and photographed.

Yeast strains were cultured in 50 ml of synthetic medium containing 2% galactose or 2% glucose with shaking at 30 °C overnight. Cells were pelleted and brought into spheroplasts by lyticase treatment. Spheroplasts were disrupted on ice with 1 ml of proteinase K (200 μg/ml) as described (39). As a second method, cells were suspended in lysis buffer as described above and broken by vortexing with glass beads (30). Homogenates were incubated at 4 °C with gentle rocking for 20 min and then centrifuged for 5 min at 1,600 × g (P4). The supernatants were centrifuged for 10 min at 10,000 rpm to yield mitochondria-rich pellets (P10). The supernatants were further centrifuged at 100,000 × g for 1 h to produce microsome-rich pellets (P100) and supernatants (S100) containing soluble proteins.

Growth Phenotype Analysis—Yeast over-expressing Rbp1p or its truncated forms was streaked on synthetic medium containing 2% glucose. After incubation at 30 °C for 3 days, cells were harvested and suspended in sterile double distilled H2O. After adjustment of the cell suspensions to A600 = 1, these were diluted serially 10-fold to 1 × 10−4. Samples (5 μl) of each cell suspension were spotted on synthetic medium containing 2% glucose or 2% galactose as the carbon source, incubated at 30 °C for 3 days, and photographed.

Ribosome-Polysome Profile Analysis—Yeast strains were grown with shaking at 30 °C in 50 ml of SC medium containing 2% glucose or 2% galactose. Yeast strain A608−1, these were diluted serially 10-fold to 1 × 10−4. Samples of each suspension were spotted on SC medium containing 2% glucose or 2% galactose as the carbon source, incubated at 30 °C for 3 days, and photographed.
10-μl reaction mixture contained of 10 mM Hepes buffer, pH 7.4, 3 mM MgCl2, 40 mM KCl, 5% glycerol, 1 mM dithiothreitol, 100 ng of recombinant GST-RBP1 or recombinant GST, 1 × 104 cpm of RNA probe (~10 ng), 2 μg of poly(I-C), and 50 μg of heparin. After 30 min at room temperature, 1 unit of RNase T1 was added to the mixture, and incubation was continued for 15 min. Loading dye was added, and the reaction components were resolved in 5% acrylamide-bis-acrylamide (39:1) mini-gel at 90 V for about 2 h in Tris-glycine buffer. After drying, the gel was used to expose the gel on an x-ray film. For the competition assays, an excess of the indicated unlabeled competitor RNAs was added to the labeled probe before the reaction.

**In Vivo Pull-down Assay and RNA Detection by RT-PCR**—For pull-down of the Rbp1p-RNA complex cross-linked in intact cells, we employed the tandem affinity purification (TAP) tag method (33). A DNA cassette encoding the TAP tag was integrated into the genome of a haploid cell in-frame with the C-terminal region of the Rbp1p without altering its natural level of expression. Yeast cells expressing Rbp1p-TAP tag were grown overnight in synthetic medium containing 0.2% glucose and 2% galactose, harvested, and washed twice with double distilled H2O. For intracellular in vivo cross-linking of the protein-RNA complex (34), cells were suspended in fixing solution (50 mM Hepes, pH 7.4, and 100 mM NaCl) with 0.3% (v/v) formaldehyde and incubated at room temperature for 10 min. Cells were harvested by centrifugation, washed twice with fixing solution, and dispersed in TST buffer (50 mM, Tris-Cl, pH 7.5, 150 mM, NaCl, 0.05%, Tween 20) containing protease inhibitors. Cells were broken by vortex mixing with glass beads, debris was removed by centrifugation (5,000 rpm for 10 min), and the supernatant was incubated with IgG beads (rocking, 4 °C for 2 h). The IgG beads were pelleted, washed five times with TST buffer, and suspended in 50 mM Tris-Cl, pH 7.5, 100 mM NaCl containing 2 units of DNase I. After rocking at room temperature for 20 min, the IgG beads were pelleted, washed twice with TST solution, and incubated at 70 °C for 45 min in TEDS solution (50 mM Tris-Cl, pH 7.5, 5 mM EDTA, 10 mM dithiothreitol, and 1% SDS) for reversal of cross-linking. The IgG beads were removed, the supernatant was extracted with phenol/chloroform, and RNAs were precipitated with salt and ethanol at −20 °C. Porin and control mRNAs were identified by RT-PCR using specific primers.

**RESULTS**

**Rbp1p Is Associated with Heavily Sedimented Structures and Is Localized in Part to the Perinuclear Region**—To investigate the function of Rbp1p, we raised polyclonal antibodies against recombinant His-tagged Rbp1p (see “Experimental Procedures”). Among total cellular proteins from wild-type yeast and a strain over-expressing Rbp1p, anti-Rbp1p antibodies mainly reacted with an ~90-kDa protein, the size expected for Rbp1p (Fig. 1A, lanes 1 and 2). This protein was not detected in an rbp1 mutant (Fig. 1A, lane 3) or by reaction with preimmune serum (data not shown). Two nonspecific signals were eliminated after affinity purification of anti-Rbp1p antibodies (Fig. 1A, lane 4). Affinity-purified antibodies of Rbp1p were used in all of the later experiments. To determine the subcellular distribution of endogenous Rbp1p, we fractionated spheroplast-homogenized lysates into three fractions, mitochondria-rich (P10), microsome-rich (P100), and soluble fractions (S100), by sedimentation centrifugation. Rbp1p was equally represented in both the P10 and P100 fractions but was barely detectable in the soluble fraction (Fig. 1B), indicating that Rbp1p was co-fractionated with heavily sedimented materials. The subcellular localization of Rbp1p was also assessed by indirect immunofluorescence. The signals were too weak to allow localization of Rbp1p in wild-type strains, which may be because of the low affinity of the Rbp1p antibody for native Rbp1p. For this reason, cells over-expressing epitope-tagged Rbp1p, HA-Rbp1p, were used for subsequent immunofluorescence experiments. As shown in Fig. 1C, most of the Rbp1p immunoreactivity was, like that of Kar2p, concentrated in the perinuclear region. In addition, some punctate signals were distributed in the cytoplasm. In other experiments, HA-Rbp1p appeared to be colocalized with mitochondrial porin in some
Overexpression of Rbp1p Affects Mitochondrial Porin Expression and Impairs Mitochondrial Function

As shown in Fig. 2, over-expression of Rbp1p affects mitochondrial porin expression and impairs mitochondrial function. A, immunofluorescence microscopy shows less porin staining in cells over-expressing HA-Rbp1p. Spheroplasts of strain rbp1/HA-RBP1 were prepared and reacted with affinity-purified anti-Rbp1p or anti-porin antibodies. Nuclear and mitochondrial DNA were stained with H33258 (rightmost panel). Phase-contrast image is also shown. Arrows indicate cells over-expressing HA-Rbp1p. B, mitochondrial porin in Rbp1p strains (wild-type, rbp1, rbp1/high copy over-expressing HA-Rbp1p, rbp1/high copy over-expressing HA-Rbp1p, rbp1/low copy (CEN-RBP1) expressing Rbp1p (24)). Cells were grown in synthetic galactose medium and disrupted, and protein extracts were prepared as described under “Experimental Procedures.” The distribution of Rbp1p and other marker proteins was evaluated by Western blot analysis with specific antibodies (lower panel). Upper panel, SDS-PAGE stained with Coomassie Blue. C, over-expression of Rbp1p inhibited cell growth. Yeast strains were grown and prepared as described under “Experimental Procedures.” 10-Fold serial dilutions of each cell suspension were spotted on synthetic medium containing glucose or glycerol as the carbon source and incubated at 30 °C for 3 days before being photographed.

Rbp1p Affects Steady-state Levels of Mitochondrial Porin mRNA

To determine whether Rbp1p altered the steady-state levels of porin mRNA, we compared different yeast strains expressing Rbp1p. Fig. 4 shows that the levels of porin mRNA in rbp1 and RBP1-dN-overexpressing cells were considerably

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higher than in the wild-type and Rbp1p-overexpressing strains, regardless of the carbon source. Amounts of the actin mRNA, however, were similar in all cells grown in galactose-containing medium. These results indicate that Rbp1p did affect levels of mRNA for the nuclear-encoded mitochondrial protein porin.

**Rbp1p Is Involved in the Regulation of Porin mRNA Stability**—To determine whether Rbp1p regulates porin mRNA levels post-transcriptionally, we used yeast strain YTC345, which carries an RNA polymerase II temperature-sensitive (37°C) mutation, to evaluate the mRNA degradation rates. YTC345 and YTC345 rbp1 strains were cultured at 25°C overnight, and samples were taken at several time points after raising the temperature to 37°C. Isolated RNAs were subjected to Northern blotting. As shown in Fig. 5A, the turnover of porin mRNA, but not of actin, COX3, CTY1, PGK1, or STE2, was slower in the rbp1 strain (half-life = 57 min) than in wild-type cells (half-life = 37 min), suggesting that Rbp1p is involved in the regulation of porin mRNA stability.

To determine whether over-expression of Rbp1p destabilized porin mRNA, YTC345 rbp1 yeasts carrying either pVT101U/HA-RBP1 or pVT101U were cultured overnight at 25°C and transferred to 37°C before harvesting at the indicated times. Degradation of porin mRNA was faster in cells over-expressing Rbp1p (half-life = 23 min) than in rbp1 cells (half-life = 55 min) (Fig. 5B). At least three genes (POR1, POR2, and TOM40) encode mitochondrial porins (35); however, the stability of POR2 and TOM40 mRNA was not affected by the amount of Rbp1p (data not shown). In addition, actin mRNA, but not COX3, CTY1, or PGK1, appeared more unstable in the strain over-expressing Rbp1p than in rbp1 strain, suggesting that Rbp1p may influence decay of more than one mRNA.

Rbp1p contains three copies of the RRM. To determine which of the RRMs is involved in destabilizing porin mRNA, two conserved phenylalanine (F) residues in the RNP1 domain of each RRM were replaced with aspartic acid (D). Mutants of RRM1, RRM2, and RRM3 were designated as Rbp1-rrm1, Rbp1-rrm2, and Rbp1-rrm3, respectively (Fig. 6A). Expression of these Rbp1-rrm mutants in YTC345 rbp1 was confirmed by Western blotting (Fig. 6B). mRNA turnover was measured in these mutants (Fig. 6C) as it was in Fig. 5. Over-expression of each of the RRMs in YTC345 rbp1 was without significant effect on the half-life of porin mRNA (55–60 min), in contrast to the effect of wild-type Rbp1p (half-life = 23 min in Fig 5B). This result indicates that each of the three RRMs in Rbp1p is essential for the regulation of porin mRNA stability.

**Rbp1p Interacted with Porin mRNA in Intact Cells**—To determine whether Rbp1p interacts with porin mRNA in cells, we employed the TAP tag to purify a Rbp1p complex and associated mRNA, as described under “Experimental Procedures.” RNP complexes containing Rbp1p-TAP were first isolated by binding to IgG beads (Fig. 7A). As shown in Fig. 7B, porin
mRNA was detected in association with Rbp1p-TAP but not with Rbp1p by RT-PCR with specific primers. The control CYT1 was not detected in either precipitate. Detection of the porin mRNA fragment in the Rbp1-TAP pull-down complexes suggests that Rbp1p interacts with porin mRNA in vivo.

Rbp1p Interacts with Porin mRNA 3’-UTR in Vivo—We employed a REMSA (Fig. 8) to determine whether Rbp1p could interact directly with porin mRNA in vitro. A number of well defined cis-elements appear to mediate the stability of specific mRNAs by interaction with specific trans-acting factors. With few exceptions, cis-elements are positioned within the 3’-UTR (36). To test whether Rbp1p could interact with the 3’-UTR of porin mRNA, we constructed a 192-bp fragment of the porin 3’-end ORF (por3’), a 345-bp fragment (por3’-1) containing the 192-bp por3’ plus 153 bp of the porin mRNA 3’-UTR, and a 177-bp fragment (por3’-2) containing the last 24 bp of por3’ plus the 153 bp of the porin mRNA 3’-UTR (Fig. 8A). Purified recombinant GST-tagged Rbp1p (GST-Rbp1p) and GST proteins were used in the REMSA. Fig. 8B shows that the migration of por3’-1 and por3’-U, but not por3’, fragments was substantially retarded when incubated with GST-Rbp1p (Fig 8B). The intensity of these shifted bands increased with increasing Rbp1p concentration (data not shown).

The binding specificity was further examined in a competition experiment. The 32P-por3’-U fragments were incubated with GST-Rbp1p in the presence of excess unlabeled por3’-1 or por3’-U fragments. The retarded migration signals of 32P-labeled por3’-1 containing the last 24 bp of por3’ plus the 153 bp of the porin mRNA 3’-UTR (Fig. 8A). Purified recombinant GST-tagged Rbp1p (GST-Rbp1p) and GST proteins were used in the REMSA. Fig. 8B shows that the migration of por3’-1 and por3’-U, but not por3’, fragments was substantially retarded when incubated with GST-Rbp1p (Fig 8B). The intensity of these shifted bands increased with increasing Rbp1p concentration (data not shown).

To determine which RRM of Rbp1p is important for interacting with the 3’-UTR of porin mRNA in vitro, recombinant GST-fused Rbp1p-rrm-mutant proteins (GST-Rbp1p-rrm1, GST-Rbp1p-rrm2, and GST-Rbp1p-rrm3) were synthesized in E. coli and purified for REMSA (Fig. 8C). Both RRM1 and RRM2 mutants (GST-Rbp1p-rrm1 and GST-Rbp1p-rrm2) failed to bind 32P-por3’-1; the RRM3 mutant (GST-Rbp1p-rrm3), however, like wild-type Rbp1p (GST-Rbp1p) still interacted with por3’-1 (Fig. 8D). This result demonstrates that both RRM1 and RRM2, but not RRM3, are necessary for Rbp1p binding to the 3’-UTR of porin mRNA in vitro.

**Fig. 4.** Rbp1p affects the mRNA level of porin. Yeasts were grown in synthetic medium containing 2% glucose or 2% galactose. Total RNAs were isolated, and samples (10 μg) were separated in denaturing agarose gels followed by blotting analysis. The upper panel shows an ethidium bromide-stained gel; 25 S and 18 S rRNAs are indicated. The RNA blot was hybridized with [32P]dCTP-labeled DNA probes for porin or actin as indicated.

**Fig. 5.** Rbp1p regulates the mRNA stability of porin. A, wild-type YTC345 and YTC345 rbp1-1 strains were cultured overnight in synthetic medium containing 2% glucose at 25 °C. After cultures were transferred to a 37 °C water bath, and cells were harvested at 15-min intervals for 60 min. Samples (10 μg) of total RNAs were subjected to electrophoresis, blotted to polyvinylidene difluoride membrane, and hybridized with [32P]dCTP-labeled DNA probes as indicated on the left. 25 S and 18 S rRNA are shown in the ethidium bromide-stained gel. STE2 was used as internal mRNA turnover control. B, over-expressing Rbp1p accelerated the turnover of porin and actin mRNAs. RNA from both YTC345 rbp1-1pVT101U and YTC345 rbp1-1pVT101U-HA-RBP1 strains was prepared and analyzed as described in A.

Rbp1p Preferentially Interacts with (C/G)U-rich Sequences—To identify the region of the porin mRNA 3’-UTR involved in Rbp1p binding, we constructed four fragments of the porin 3’-UTR as illustrated in Fig 9A. Unlabeled RNA fragments were generated by in vitro transcription and quantified. In competition assays with purified recombinant GST-Rbp1p and 32P-labeled por3’-1, virtually no Rbp1p-32P-labeled por3’-1 retarded complex was seen with a 10-fold excess of unlabeled por3’-3 fragment but not with 50-fold concentrations of unlabeled por3’-2, -4, or -5 (Fig. 9A, and data not shown). The por3’-2 element contains several repeats of C/G(U)2-3 sequence near its 5’ end, and por3’-5 contains a UUAUUUAUA sequence, which is a typical AU element. Hence, the 3’-UTR of porin mRNA contains a target sequence (+48 to +70) with which Rbp1p could interact.

To confirm the existence of that binding sequence in Rbp1p, 20 nucleotides (+50 to +69) near the 5’-end of por3’-1 were deleted to generate the por3’-1(d3) as illustrated in Fig. 9B. In competition assays (Fig. 9C), the Rbp1p-32P-labeled por3’-1 retarded complex was essentially abolished by a 10-fold concentration of unlabeled por3’-1 but not by a 50-fold concentration of unlabeled por3’-1(d3). These results suggested that the Rbp1p preferentially interacts with the (C/G)U-rich sequence rather than the typical AU element.

**DISCUSSION**

We serendipitously found that over-expression of Rbp1p impaired mitochondrial function and affected the expression of mitochondrial porin. The half-life of porin mRNA was greater in rbp1 mutants than in wild-type cells, whereas in cells over-expressing Rbp1p from a multicopy plasmid, the stability of
porin mRNA was decreased. We have presented several pieces of evidence suggesting that Rbp1p binds to the 3′-UTR of porin mRNA in vitro and that the three RRMs of Rbp1p are each involved in destabilizing porin mRNA. To our knowledge, this is the first demonstration of specific regulation of a nuclear-encoded mitochondrial gene transcript in intact cells, and it also identifies the trans-acting factor involved in regulating porin mRNA stability.

The biochemical characterization of Rbp1p revealed that it was associated in cells with structures sedimenting at P10 and P100 fractions. Rbp1p, like Rpl3p, was present in the P100 fraction, which perhaps is due to its association with ribosome-bound mRNA. Analysis of the ribosome-polysome profiles of Rbp1p, however, revealed that most of the Rbp1p was not associated with ribosome-bound mRNA. An alternative possibility is that Rbp1p may sediment when it is present in mitochondrial RNP granules, which contain mRNA and RNA-binding proteins. Consistent with this notion, we found that on fluorescence microscopy Rbp1p displayed punctate or granule signals, which were concentrated in part in the perinuclear region and also scattered through the cytosol. Moreover, our preliminary two-hybrid screening identified several Rbp1p-interacting molecules, including an RNA-binding protein involved in RNA degradation. Elucidation of the functional relationship of Rbp1p with its interacting proteins may reveal a novel selective RNA regulation.

Mitochondrial biogenesis is a complex process involving the concerted regulation of expression of the two genomes that encode organellar proteins. The majority of the mitochondrial...
proteins are encoded in nuclear DNA and synthesized on cytoplasmic polysomes as precursor molecules with short N-terminal extensions. Importation of mitochondrial proteins occurs, for the most part, post-translationally, although some precursors may also be imported co-translationally (37–39). Mitochondrial proteins that are encoded in nuclear DNA must be reliably delivered to the right place. This could be accomplished by transport of the appropriate mRNA from the nucleus to the site of protein synthesis near the mitochondria. It was suggested that organelle-specific mRNA-binding factors might serve to cluster and anchor specific mRNAs in the vicinity of the mitochondria (40). More recently, a subset of nuclear-encoded mRNAs was reported present adjacent to mitochondria (41). To date, no organelle-specific RNA-binding factors have been identified, although we found that a truncated Rbp1p lacking two glutamine-rich regions was localized to mitochondria (unpublished observation). It will be important to determine whether Rbp1p can shuttle between the endoplasmic reticulum-perinuclear region and the mitochondria and whether this transit is an obligatory aspect of its function in selective nuclear-encoded mitochondrial RNA metabolism.

The level of porin mRNA was decreased when Rbp1p was expressed, and its turnover rate was higher in the Rbp1p-overexpressing strain. The destabilization of porin mRNA by overexpression of Rbp1p was observed when a 192-bp fragment (porin 3’-end ORF), a 345-bp fragment (porin 3’U) containing the 192-bp porin3’ plus 153 bp of 3’-UTR, and a 177-bp fragment (porin 3’-1) containing 24-bp porin3’ plus 153 bp of 3’-UTR, B, were cloned in pBluescript II KS(+)-1 RNA fragments were generated by in vitro transcription. 100 ng of purified recombinant GST-Rbp1 or GST protein were incubated with the indicated [32P]-labeled RNA probe (~10 ng, 1 × 10^6 cpm), and REMSA was performed as described under “Experimental Procedures.” C, the recombinant GST-tagged Rbp1-rrm-mutated proteins (GST-Rbp1-rrm1, GST-Rbp1-rrm2, and GST-Rbp1-rrm3) were synthesized in E. coli and purified for REMSA. D, 100 ng of purified recombinant GST-RBP1, GST-Rbp1-rrm1, GST-Rbp1-rrm2, or GST-Rbp1-rrm3 protein was incubated with [32P]-labeled porin3’-1 RNA, and REMSA was performed. Data are representative of at least three experiments.
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Fig. 9. Rbp1p preferentially interacts with a (G/C)U-rich element in 3′-UTR of porin mRNA. A, competition assays defined the Rbp1p binding sequence in the por3-1 mRNA. A diagram of 3′-UTR constructs of porin mRNA is shown. 100 ng of purified recombinant GST-RBP1 protein was incubated with 32P-labeled por3-1 RNA, and REMSA was performed. For the competition assay, a 20- or 50-fold higher concentration of unlabeled por3-1 RNA, -2, -3, -4, or -5 was mixed with the labeled RNA before addition of protein. The competition efficiency of each construct is shown on the right. Data are representative of at least three experiments. B, the por3-1(d3) fragment was generated by deleting 20 nucleotides (+50 to +69) from por3-1. An AU element (+63 nt to 105 nt) in the 3′-UTR of porin mRNA is indicated. C, 100 ng of purified recombinant GST-RBP1 protein was incubated with 32P-labeled por3-1 RNA, and REMSA was performed. For the competition assay, the indicated excess concentration of unlabeled competitor por3-1 or por3-1(d3) was mixed with labeled RNA before protein was added. Data are representative of at least three experiments. D, structure of full-length 3′-UTR of porin mRNA as predicted by RNAdraw (version 1.1b2), the Rbp1p binding sequence (+50 to +69 nucleotides) occupies two-thirds of an open loop.
to +69 nucleotides) appears to occupy two-thirds of an open loop (Fig. 2D). It is possible that the formation of a stem-loop structure surrounding the Rbp1 recognition sequence facilitates the Rbp1-RNA interaction.

In summary, we have shown that Rbp1 could promote porin mRNA decay by binding to its 3′-UTR, or, alternatively, modulate the conformation of the porin mitochondrial RNP to allow degradation. The molecular mechanisms by which Rbp1 regulates the degradation of porin mRNA are still elusive. The possibility that Rbp1 recruits other RNA-binding proteins that, in turn, regulate porin expression may exist. Further studies on in vitro binding analyses would help us to unveil how regulatory proteins and mRNA binding regulate the production of nuclear-encoded mitochondrial proteins. Is the activity of Rbp1 limited to mRNA decay, or does it have other functions? Further exploration into the mechanisms and details of Rbp1-RNP granule formation and function should provide insights into the choreography of mRNA translation and decay.

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