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REF, an evolutionarily conserved family of hnRNP-like proteins, interacts with TAP/Mex67p and participates in mRNA nuclear export

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ABSTRACT

Vertebrate TAP and its yeast ortholog Mex67p are involved in the export of messenger RNAs from the nucleus. TAP has also been implicated in the export of simian type D viral RNAs bearing the constitutive transport element (CTE). Although TAP directly interacts with CTE-bearing RNAs, the mode of interaction of TAP/Mex67p with cellular mRNAs is different from that with the CTE RNA and is likely to be mediated by protein–protein interactions. Here we show that Mex67p directly interacts with Yra1p, an essential yeast hnRNP-like protein. This interaction is evolutionarily conserved as Yra1p also interacts with TAP. Conditional expression in yeast cells implicates Yra1p in the export of cellular mRNAs. Database searches revealed that Yra1p belongs to an evolutionarily conserved family of hnRNP-like proteins having more than one member in *Mus musculus*, *Xenopus laevis*, *Caenorhabditis elegans*, and *Schizosaccharomyces pombe* and at least one member in several species including plants. The murine members of the family directly interact with TAP. Because members of this protein family are characterized by the presence of one RNP-motif RNA-binding domain and exhibit RNA-binding activity, we called these proteins REF-bps for RNA and export factor binding proteins. Thus, Yra1p and members of the REF family of hnRNP-like proteins may facilitate the interaction of TAP/Mex67p with cellular mRNAs.

Keywords: Aly; Mex67p; hnRNP; nuclear export; TAP; Yra1p

INTRODUCTION

Messenger RNAs are exported from the nucleus as large ribonucleoprotein complexes (mRNPs). To date, proteins implicated in this process are distinct from the nuclear transport receptors of the Importin β-related, Ran Binding Protein family and include several nucleoporins and RNA binding proteins, an RNA helicase of the DEAD-box family (Dbp5), and the NPC-associated proteins Gle1p, TAP/Mex67p, and RAE1/Gle2p (reviewed by Mattaj & Englmeier, 1998; Görlich & Kutay, 1999; Nakielny & Dreyfuss, 1999). Gle1p and Mex67p are essential for mRNA export in *Saccharomyces cerevisiae*, whereas RAE1/Gle2p is essential in *Schizosaccharomyces pombe* (Brown et al., 1995; Murphy & Wente, 1996; Murphy et al., 1996; Segref et al., 1997). Their vertebrate homologs, hGle1p, TAP, and hRAE1/mrnp41 have been implicated in the export of cellular mRNAs (Bharathi et al., 1997; Kraemer & Blobel, 1997; Grüter et al., 1998; Watkins et al., 1998; Braun et al., 1999; Katahira et al., 1999; Pritchard et al., 1999). Moreover, TAP has also been implicated in the export of simian type D retroviral RNAs bearing the constitutive transport element (CTE) (Grüter et al., 1998; Bear et al., 1999; Braun et al., 1999; Kang & Cullen, 1999).

Recently, several Mex67p/TAP partners have been identified. These include various nucleoporins (Katahira et al., 1999; Bachi et al., 2000); p15, a protein related to the nuclear transport factor 2 (Katahira et al., 1999); E1B-AP5 (Bachi et al., 2000), an hnRNP-like protein that may play a role in the export of cellular and adenovirus mRNAs (Gabler et al., 1998), and transportin, which mediates TAP nuclear import (Bachi et al., 2000). Nucleoporin binding by TAP is mediated by its NPC-binding domain located at the very C-terminal end

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Abbreviations: CTE: constitutive transport element; FG-repeats: phenylalanine-glycine dipeptide repeats; NPC: nuclear pore complex; polyA$^+$ RNA: polyadenylated RNA; REF-bps: RNA and export factor binding proteins.

of the protein (residues 508–619) (Bachi et al., 2000; see Fig. 4C). This domain directly interacts with multiple FG-repeat containing nucleoporins in vitro whereas in vivo it targets TAP to the NPC and interferes with multiple nuclear export pathways (Bachi et al., 2000). TAP binding to p15 is mediated by the middle domain of the protein (residues 371–551) (Katahira et al., 1999; Bachi et al., 2000). The N-terminal domain of TAP (residues 1–372) is involved in direct binding to CTE-bearing RNAs (Braun et al., 1999; Kang & Cullen, 1999) and interacting with transportin and E1B-AP5 (Bachi et al., 2000).

Although TAP directly interacts with the CTE, its mode of interaction with cellular mRNAs remains to be elucidated and is likely to be different from that with the CTE RNA for the following reasons. First, most cellular mRNAs do not contain sequences similar to those present in the CTE. Second, although TAP exhibits a low, nonspecific affinity for RNA (Grüter et al., 1998; Braun et al., 1999), its affinity for the CTE RNA in vitro is approximately three orders of magnitude higher than that for the DHFR mRNA (Braun et al., 1999). Third, although an excess of CTE RNA saturates export of mRNAs, an excess of mRNA does not interfere with CTE export (Pasquinelli et al., 1997; Saavedra et al., 1997). Fourth, microinjection of recombinant TAP directly stimulates CTE-dependent export but not the export of cellular mRNAs in *Xenopus* oocytes (Braun et al., 1999; Bachi et al., 2000). Based on these observations, we previously hypothesized that the CTE bypasses several steps in the mRNA export pathway and directly interacts with TAP, whereas cellular mRNAs may recruit TAP via protein–protein interactions (Saavedra et al., 1997; Grüter et al., 1998; Braun et al., 1999; Bachi et al., 2000).

Here we report that Mex67p interacts with Yra1p, an essential yeast hnRNP-like protein (Portman et al., 1997). Yeast cells expressing Yra1p under the control of the GAL1 promoter accumulate polyA$^+$ RNAs within the nucleus when transferred to a repressing medium containing glucose. This nuclear accumulation of polyA$^+$ RNAs parallels the decrease of Yra1p protein levels, suggesting that Yra1p is directly implicated in the export of cellular mRNAs in yeast cells. Database searches revealed that Yra1p belongs to an evolutionarily conserved family of hnRNP-like proteins that includes more than one member in *S. pombe*, *Caenorhabditis elegans* and *Mus musculus*. By RT-PCR we have cloned three members of this family in mouse and shown that these proteins directly interact with TAP. Because members of this family contain one RNP-motif RNA-binding domain and exhibit RNA binding activity, and Yra1p also copurifies with yDbp5p (Schmitt et al., 1999), we called these proteins REF-bps for RNA and Export Factor binding proteins. Thus, members of the REF family of hnRNP-like proteins may facilitate the interaction between Mex67p/TAP and cellular mRNPs.

RESULTS

Mex67p interacts with Yra1p, an essential yeast hnRNP-like protein

To promote export, TAP/Mex67p must interact with other components of the nuclear transport machinery as well as with the mRNP-export cargo. Recently, several TAP/Mex67p partners have been identified; however, aside from E1BAP5, none of these could mediate the interaction between TAP/Mex67p and cellular mRNPs (see Introduction). To identify additional TAP/Mex67p-interacting proteins, we have used the tandem affinity purification strategy (for clarity, we will refer to this method as *tap* to distinguish it from TAP, the protein name), that allows efficient purification of protein complexes from cell extracts (Rigaut et al., 1999). Because of the high sequence homology between human TAP and yeast Mex67p, their similar localization and function, we expected that identification of Mex67p interacting proteins in yeast would also lead to the identification of TAP partners in vertebrate cells. The *tap* tag was fused to the C-terminus of Mex67p by integrating a DNA cassette into the genome of a haploid cell (Puig et al., 1998). The *tap* tag consists of a calmodulin binding peptide followed by a TEV protease cleavage site and two IgG-binding units of protein A from *Staphylococcus aureus* (zz-tag). Mex67p with its associated proteins were purified from the tagged strain following the two-step affinity purification of the *tap* method. Figure 1A shows a representative sample of the eluted proteins from the calmodulin-affinity resin. Most of the putative interacting proteins were found in substoichiometric amounts. This does not result from overexpression of the Mex67p protein that is expressed from its natural promoter, but rather suggests that Mex67p is not a subunit of a stable multimeric complex. Bands obtained in several selections were excised from the gels, the proteins were in-gel digested with trypsin, and the tryptic peptides were identified by mass spectrometry. The peptide map unambiguously identified Mtr2p (Kadowaki et al., 1994; Santos-Rosa et al., 1998) and Yra1p (Portman et al., 1997) as the proteins migrating with an apparent molecular weight of 20 and 26 kDa, respectively.

Because Yra1p was previously found to copurify with yeast Dbp5p (Schmitt et al., 1999), we investigated whether its interaction with Mex67p was direct or mediated by an additional protein present in the yeast lysate. To this end, we immobilized bacterially expressed GST-Mex67p or GST on glutathione agarose beads. Beads were then incubated with total lysates from *Escherichia coli* expressing Yra1p fused to an hexa-histidine tag. Figure 1B shows that 6xHis-Yra1p could be selected from *E. coli* lysates on GST-Mex67p coated beads (lane 5), but not on beads on which GST was immobilized (lane 3). Moreover, Yra1p could also

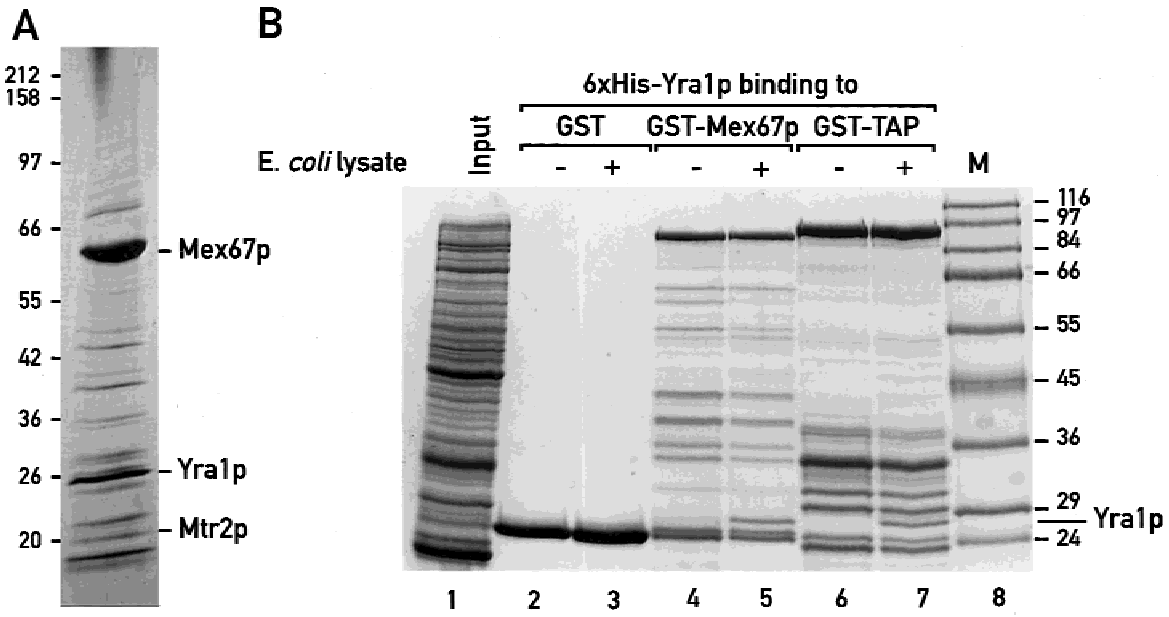

FIGURE 1. Mex67p interacts with Yra1p. **A**: Tandem affinity purification of Mex67p partners. Fractions eluted from the calmodulin affinity resin were pooled and analyzed on SDS-PAGE followed by silver staining. Proteins indicated on the right of the gel were identified by mass spectrometry. **B**: Lysates from *E. coli* expressing 6xHis-Yra1p were incubated with glutathione agarose beads precoated with GST (lane 3), GST-Mex67p (lane 5), or GST-TAP (lane 7). Bound proteins were eluted with SDS-sample buffer. One fiftieth of the input (lane 1) and one fifth of the bound fractions (lanes 2–7) were analyzed on SDS-PAGE followed by Coomassie staining. For each GST pull-down assay the background obtained in the absence of lysates is shown (lanes 2, 4, and 6). The position of 6xHisYra1p is indicated on the right of the gel.

be selected on GST-TAP coated beads (Fig. 1B, lane 7). These results suggest that the interaction between Mex67p/TAP and Yra1p is evolutionarily conserved and is not mediated by additional proteins.

Identification of the REF-family of Yra1p-related proteins

Because Yra1p interacts with TAP, we then searched for vertebrate Yra1p homologs. The murine protein Aly (Bruhn et al., 1997) exhibits a high degree of similarity to Yra1p. Because the similarity extends beyond the RNA binding domain (RBD/RRM; reviewed by Burd & Dreyfuss, 1994; Nagai et al., 1995) and both proteins have the same overall domain organization (Fig. 2), we attempted to clone Aly by RT-PCR using total RNA isolated from mouse embryos. Surprisingly, in addition to Aly cDNA, three highly related murine cDNAs encoding Yra1-like proteins were amplified and cloned. For reasons described above, these proteins were

FIGURE 2. REF: an evolutionarily conserved family of hnRNP-like proteins. Upper panel: Multiple alignment of members of the REF-family of proteins. Species names are indicated in front of the protein names (bm: *Bombyx mori*; ce: *Caenorhabditis elegans*; dm: *Drosophila melanogaster*; le: *Lycopersicon esculentum*; hs: *Homo sapiens*; mm: *Mus musculus*; rn: *Rattus norvegicus*; sc: *Saccharomyces cerevisiae*; sm: *Schistosoma mansoni*; sp: *Schizosaccharomyces pombe*; xl: *Xenopus* laevis; zm: *Zea mays*). Database accession numbers are indicated at the end of the sequences. Conserved charged residues are shown in red; conserved hydrophobic residues are shown in blue; other conserved residues are shown in green. Dots represent the lack of sequence information and dashes represent gaps in the alignment. This multiple sequence alignment was constructed by CLUSTAL_X (Thompson et al., 1997) and manually refined on the SEAVIEW alignment editor (Galtier et al., 1996). The RRM/RBD domain and the conserved REF-N and REF-C motifs flanking the RBD domain are shown in color above the sequences. The secondary structure prediction (Ros et al., 1994) is indicated below the sequences (H,h: α-helix; E,e: β-strand). Capital letters indicate prediction with expected average accuracy $<$82%. mREF1-I corresponds to Aly (Bruhn et al., 1997). The sequence data for mREF1-II and mREF2-I and II are available from GenBank/EMBL/DDBJ under accession numbers AJ252140 and AJ252141. Accession numbers for ESTs corresponding to additional murine REFs are AA623854, AI505206, AA030202, AA015345. The accession numbers for the putative homologs in *Arabidopsis thaliana*, *Brassica rapa*, *Citrus unshiu*, *Oryza sativa*, and *C. carpio* are AI994825, L38537, C24300, D15385, and AU052074, respectively. Lower panel: Domain organization of the REF proteins. The conserved N- and C-terminal motifs that define the REF subfamily of RRM/RBD proteins are shown (REF-N and REF-C, respectively). N-vr and C-vr represent the N-and C-terminal variable regions specific to each member of the family. These regions have a high content in glycine, serine, and positively charged residues.

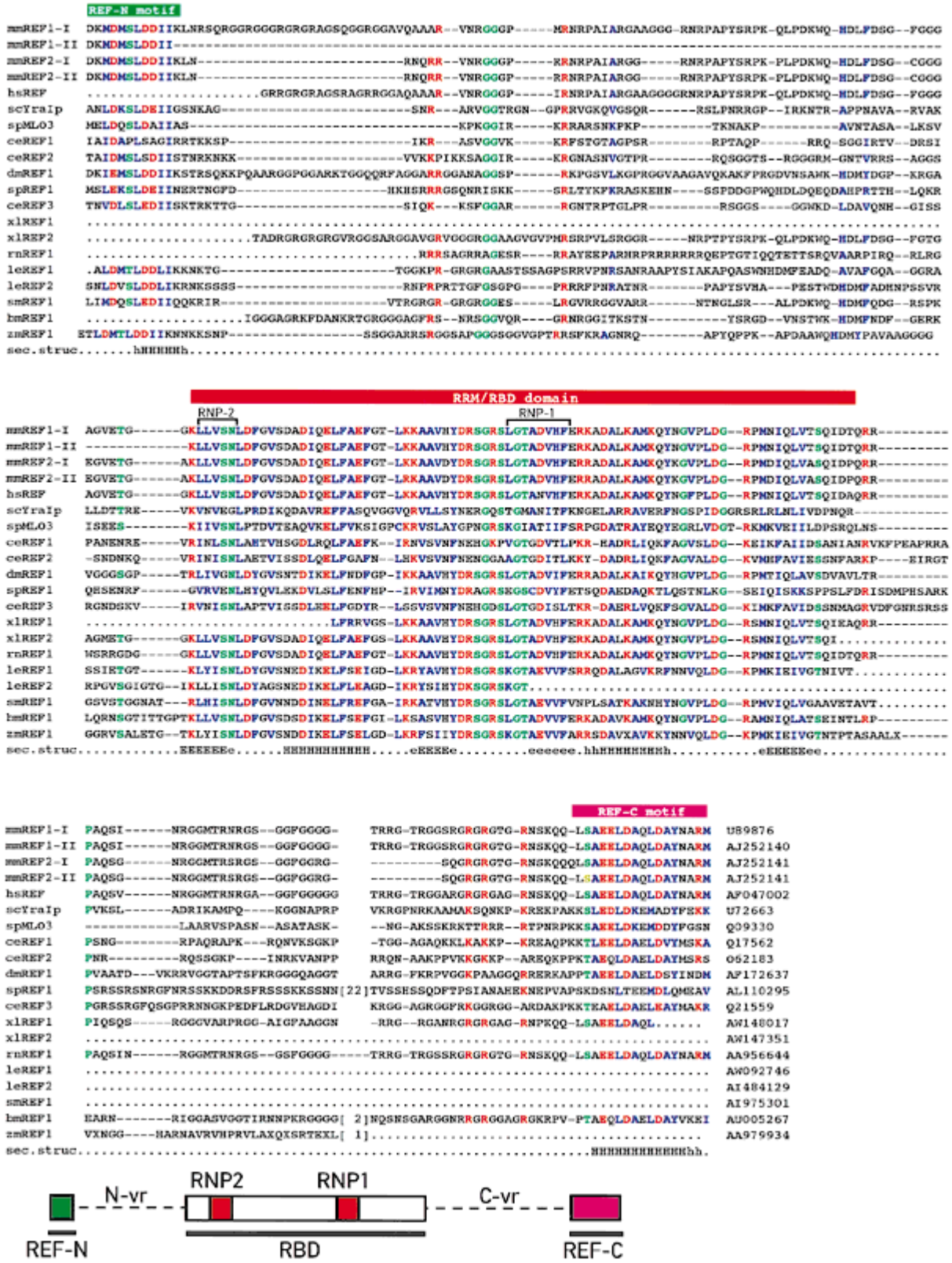

FIGURE 2. (*Figure legend on facing page.*)

named murine REF bps. As shown in Figure 2, mREF1-II is identical to Aly (mREF1-I), but has a deletion of 92 amino acids at its N-terminus. Murine REF2-I and REF2-II differ from each other by one single amino acid insertion in mREF2-I (Q198); however both proteins differ from Aly at multiple positions by the presence of several deletions or one amino acid change. Thus it is likely that mREF1-II and 2-II arise from alternative splicing of mREF1-I (Aly) and mREF2-I, respectively. The existence of these multiple variants was confirmed by the identification of ESTs specific to each form (Fig. 2). Additionally, several ESTs corresponding to at least two other murine REF variants were found (see legend to Fig. 2).

PSI-BLAST searches (Altschul et al., 1997) with Yra1p and murine REFs revealed the presence of homologous proteins in several species including *S. pombe*, *C. elegans*, *Drosophila melanogaster*, and *Homo sapiens* (Fig. 2), and allowed us to define the REF subfamily of RRM proteins. Additional Blast and Wisetool profile searches against EST databases (Birney et al., 1996) detect other REF-like proteins in plants (*Arabidopsis thaliana*, *Lycopersicon esculentum*, *Oryza sativa*, and *Zea mays*), insects (*Bombyx mori*), and several species including *Cyprinus carpio*, *Xenopus laevis*, *Gallus gallus* and *Rattus norvegicus* (Fig. 2). Thus Yra1p and Aly are members of an evolutionarily conserved family of hnRNP-like proteins characterized by the presence of one RNP-motif RNA-binding domain in the middle part of the protein and two highly conserved sequences at their N- and C-termini that we refer to as the REF-N and REF-C motifs (Fig. 2). Members of the REF family differ by the presence of insertions of different lengths between these conserved motifs. These variable regions (N-vr and C-vr in Fig. 2) are characterized by a high content of glycine, serine, and positively charged residues and are related to the RGG boxes described in many RNA binding proteins (reviewed by Burd & Dreyfuss, 1994). These regions may confer different protein or RNA-binding specificities to the various members of the family. The C-terminal variable region, C-vr, has been proposed to encode a nuclear localization signal in Yra1p (Portman et al., 1997).

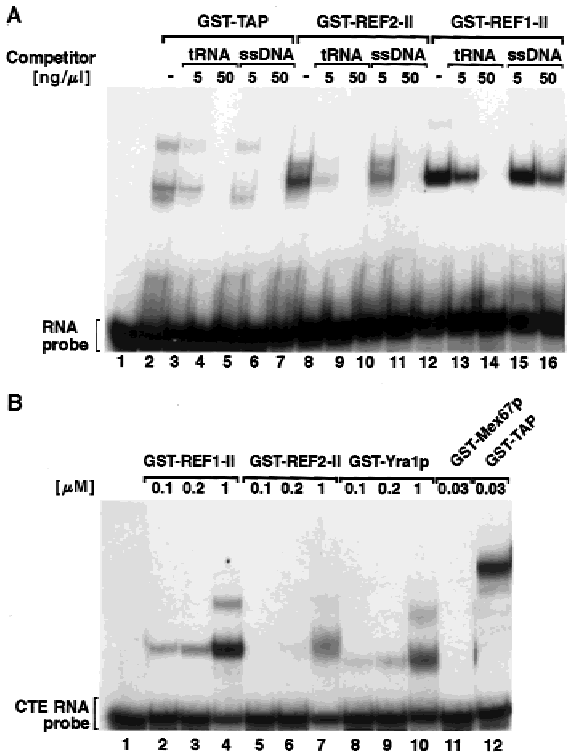

FIGURE 3. REF proteins exhibit RNA binding activity. **A**: An electrophoretic mobility retardation assay was performed with a labeled RNA probe (U1SII-; Sherly et al., 1989) and the purified recombinant proteins indicated above the lanes. For each protein, binding reactions were performed in the absence of competitor (lanes 2, 7, and 12) or in the presence of competitor tRNA or Herring sperm single-stranded DNA (ssDNA) as indicated. The concentration of the competitors is shown above the lanes. The concentration of the recombinant REF proteins in the binding reactions was 0.1 μg/μL and the concentration of TAP was 0.2 μg/μL. The position of the free RNA probe (lane 1) is shown on the left. **B**: An electrophoretic mobility retardation assay was performed with a labeled CTE RNA probe (Grüter et al., 1998) and the purified recombinant proteins indicated above the lanes. The concentrations of the recombinant proteins in the binding reactions are indicated above the lanes. The position of the free RNA probe (lane 1) is shown on the left.

REF proteins exhibit RNA-binding activity

The presence of an RNA-binding domain suggests that REFs proteins bind RNA directly. Indeed, Yra1p was shown to have RNA annealing activity in vitro (Portman et al., 1997). We therefore tested murine REF proteins for their ability to bind RNA in vitro. RNA binding was assayed by an electrophoretic gel mobility retardation assay. A 77-nt-long ^{32}P-labeled RNA probe (U1SII-; Sherly et al., 1989) was incubated with purified recombinant mREF1-II and mREF2-II fused to GST, the resulting complexes were resolved in a native polyacrylamide gel and visualized by autoradiography (Fig. 3A). As a control, binding of GST-TAP to the RNA probe was tested in parallel. The binding specificity of the proteins was assayed by adding unlabeled competitor tRNA or single-stranded DNA to the binding reactions. All proteins tested bound to the RNA probe although with different efficiencies (Fig. 3A, lanes 2, 7, and 12). Under the same conditions, GST did not bind to the RNA probe, whereas Yra1p exhibited RNA-binding activity (data not shown and Fig. 3B), in agreement with its RNA annealing activity in vitro (Portman et al., 1997). Formation of the protein–RNA complexes was competed by tRNA and single-stranded DNA at the higher concentration tested (Fig. 3A, lanes 4, 6, 9, 11, 14, and 16). At the lowest concentration, tRNA competed protein binding to the RNA probe more efficiently than single-stranded DNA (Fig. 3A, lane 8 versus 10, and lane 13 versus 15). When homoribopolymers were

used as competitors, poly(rG) efficiently prevented binding of TAP, Yra1p, and mREF proteins to the RNA probe, whereas, at the same concentration, poly(rA), poly(rU), or poly(rC) had no effect (data not shown).

In contrast to the results obtained with the U1SII-RNA probe, and as previously described (Grüter et al., 1998; Braun et al., 1999), when a CTE RNA probe was used in the binding assays TAP bound with high affinity, as 25 ng of recombinant protein were sufficient to observe the formation of TAP/CTE RNA complexes (Fig. 3B, lane 12). Under the same conditions, an ~30-fold molar excess of Yra1p or mREF proteins compared to GST-TAP was required to shift an equivalent amount of CTE RNA (Fig. 3B, lanes 4, 7, and 10). Furthermore, binding of REF proteins to the CTE RNA was nonspecific (data not shown), as these proteins did not discriminate between the wild-type CTE and the export-deficient mutant M36 (Grüter et al., 1998). Similarly, Mex67p did not specifically bind to the CTE RNA (Fig. 3B, lane 11). These results show that members of the REF family of proteins display a general affinity for RNA and single-stranded (ss) DNA as reported previously for many hnRNP proteins (Piñol-Roma et al., 1990).

REF-bps directly interact with TAP

To determine whether the murine members of the REF-family interact with TAP we performed in vitro binding assays. [^{35}S]methionine-labeled TAP was synthesized in vitro in rabbit reticulocyte lysates and assayed for binding to glutathione agarose beads coated with either GST or GST-REF1-II (the smallest member of the family). Figure 4A shows that TAP could be selected on glutathione agarose beads coated with GST-REF1-II (lane 3) but not on beads coated with GST (lane 2). Addition of micrococcal nuclease or of RNase A to the binding reactions did not affect TAP/REF1-II interaction (Fig. 4A, lanes 4 and 5). Thus, it is unlikely that TAP/REF1-II interaction represents nonspecific binding of these proteins tethered on RNA. Moreover, addition of in vitro-synthesized DHFR mRNA or of CTE RNA had no effect on TAP binding to immobilized REF1-II (Fig. 4A, lanes 6 and 7). Interestingly, although TAP binds Yra1p and mREFs with similar efficiencies (see Fig. 1B), Mex67p binds to mREFs much less efficiently than to Yra1p (data not shown).

In preliminary experiments we noticed that TAP binding to REF bps was mediated by its N-terminal domain (fragment 1–372; see Fig. 4C). This observation was confirmed by the experiment shown in Figure 4B. In this assay, we immobilized bacterially expressed GST fusions of Yra1p, mREF1-II, or mREF2-I on glutathione agarose beads. Beads were then incubated with total lysates from *E. coli* expressing the N-terminal domain of TAP (fragment 1–372). The TAP fragment could be selected from *E. coli* lysates on immobilized GST-Yra1p (Fig. 4B, lane 7), and on the immobilized murine REF1-II and -2-I (Fig. 4B, lanes 10 and 13) but not on GST (Fig. 4B, lane 4). Furthermore, binding of TAP fragment 1–372 to Yra1p or to the murine REFs was not affected by RNase A treatment (Fig. 4B, lanes 8, 11, and 14). These results indicate that TAP binding to the REFs is direct and mediated by its N-terminal domain.

Yra1p is involved in mRNA export

Its interaction with TAP/Mex67p and its RNA binding activity, suggests that Yra1p may be involved in mRNA nuclear export. To investigate the role of Yra1p in mRNA export, the chromosomal YRA1 gene of the W303 wild-type strain was placed under the control of the repressible GAL1 promoter and concomitantly tagged at its 5′ end with a sequence encoding three HA epitopes using a PCR-based gene modification approach (Longtine et al., 1998). This modified strain, called GAL-HA-YRA1, grows on YEPD plates containing galactose but not glucose, consistent with the essential nature of Yra1p (Portman et al., 1997, and data not shown). In liquid, the growth of the GAL-HA-YRA1 strain started to drop 6 h after transfer from a medium containing galactose to a medium containing glucose (Fig. 5A). To monitor the decrease in the levels of HA-Yra1p, Western blot analysis was performed on extracts of GAL-HA-YRA1 cells grown in the repressing (glucose) medium for different periods of time (Fig. 5B). Within 4 h of growth in glucose, the level of HA-Yra1p was reduced to ~5% of its level in cells grown in galactose. After 6 h in glucose, HA-Yra1p was barely detectable; the depletion of the protein therefore correlates with the cell growth arrest.

To determine whether depletion of HA-Yra1p resulted in an mRNA export defect, *in situ* hybridization with an oligo(dT) probe was performed on the GAL-HA-YRA1 cells at various times after transfer to glucose (Fig. 5C). After 2 h of depletion, a significant fraction of cells exhibited weak nuclear accumulation of polyA$^+$ RNA. By 4 h in glucose, most of the cells showed a strong nuclear signal, consistent with a block of mRNA export. The role of Yra1p in mRNA export is likely to be direct, as the polyA$^+$ RNA export defect, the depletion of the HA-Yra1p, and the decrease in growth rate show comparable kinetics.

Yra1p is a nonshuttling hnRNP-like protein

In yeast cells Yra1p has been localized to the nucleoplasm (Portman et al., 1997). Similarly, T7 epitope-tagged mREF1-I (Aly) was detected primarily in the nucleus of transfected COS7 cells (Bruhn et al., 1997). Indirect immunofluorescence on human Hep-2 cells using antibodies directed against hsREF showed a fine speckled nuclear pattern (Wichmann et al., 1999). Thus, the subcellular localization of REF proteins appears to be evolutionarily conserved. However, as is the case

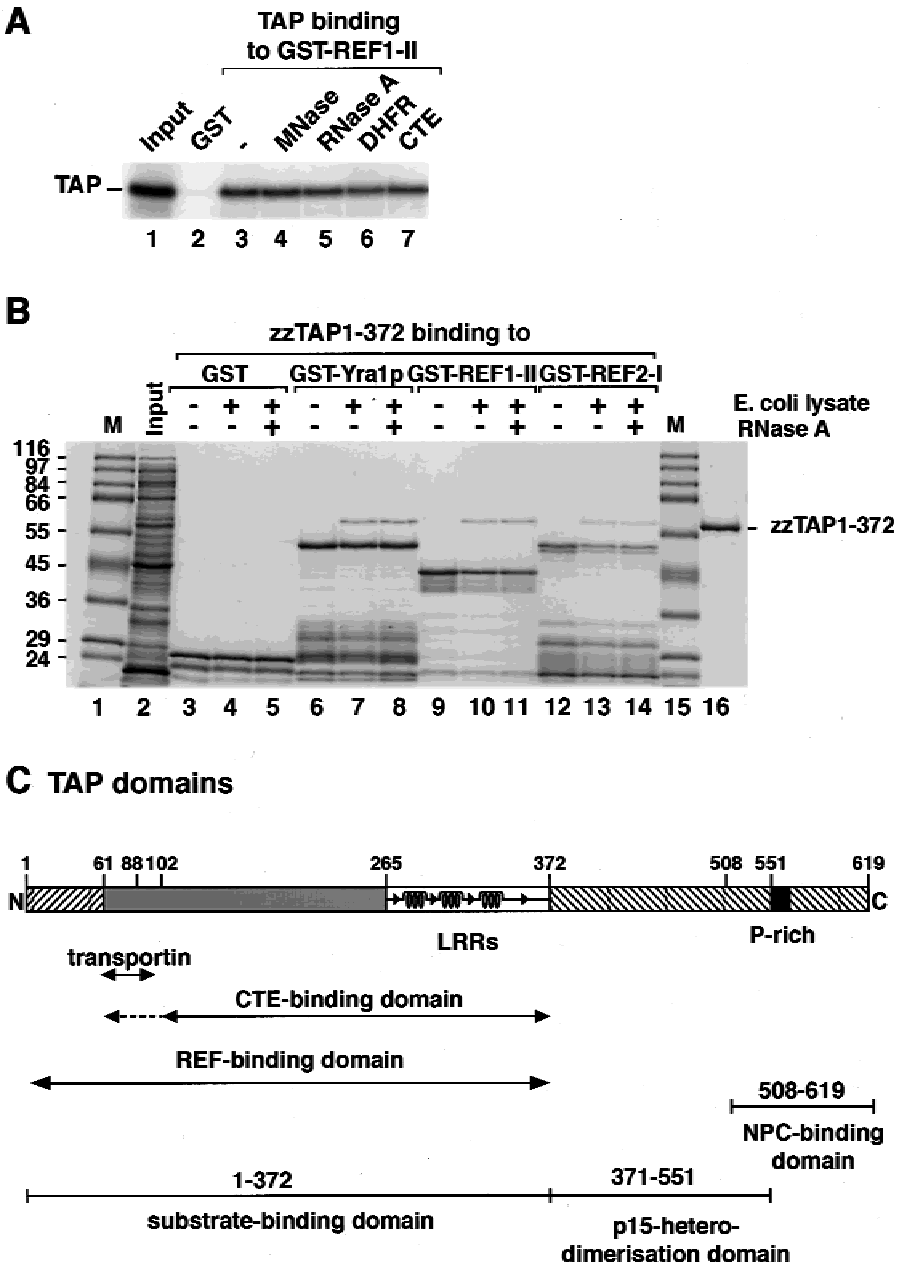

FIGURE 4. TAP directly interacts with members of the REF family of proteins. **A**: [^{35}S]methionine-labeled TAP was synthesized in vitro in rabbit reticulocyte lysates. Five-microliter samples from the lysates were incubated with glutathione agarose beads precoated with GST (lanes 2) or GST-REF1-II (lanes 3–7). In lane 4, ~3 units of micrococcal nuclease were added. In lane 5, binding was performed in the presence of 50 μg of RNase A. In lanes 6 and 7, in vitro-synthesized DHFR mRNA or CTE RNA were added to a final concentration of 0.3 μg/μL. One tenth of the input (lane 1) and one quarter of the bound fractions (lanes 2–7) were analyzed on SDS-PAGE followed by fluorography. **B**: A lysate from *E. coli* expressing zzTAP1-372-6xHis was incubated with glutathione agarose beads precoated with GST (lanes 4, 5), GST-Yra1p (lanes 7, 8), GST-mREF1-II (lanes 10, 11), or GST-mREF2-I (lanes 13, 14). Binding reactions were supplemented with 2 μg/μL of yeast RNA (lanes 4, 7, 10, 13) or with 50 μg of RNase A (lanes 5, 8, 11, 14). Bound proteins were eluted with SDS-sample buffer. One fiftieth of the input (lane 2) and one fifth of the bound fractions were analyzed on SDS-PAGE followed by Coomassie staining. For each GST pull-down assay the background obtained in the absence of lysates is shown (lanes 3, 6, 9, and 12). Lane 16 shows zzTAP1-372-6xHis purified on Ni-NTA agarose beads. The position of zzTAP1-372-6xHis is indicated on the right of the gel. **C**: Domain organization of human TAP protein. TAP domains defined in this and previous studies (Braun et al., 1999; Bachi et al., 2000) are indicated. The predicted folding within the LRRs is shown diagramatically.

for many hnRNP proteins, their nuclear localization at steady state does not exclude the possibility that they may rapidly shuttle between nucleus and cytoplasm. We therefore tested whether Yra1p is a shuttling protein in yeast cells using the export assay previously described by Lee et al. (1996). This assay takes advantage of the properties of the nucleoporin *nup49-313* temperature-sensitive strain (Doye et al., 1994). The

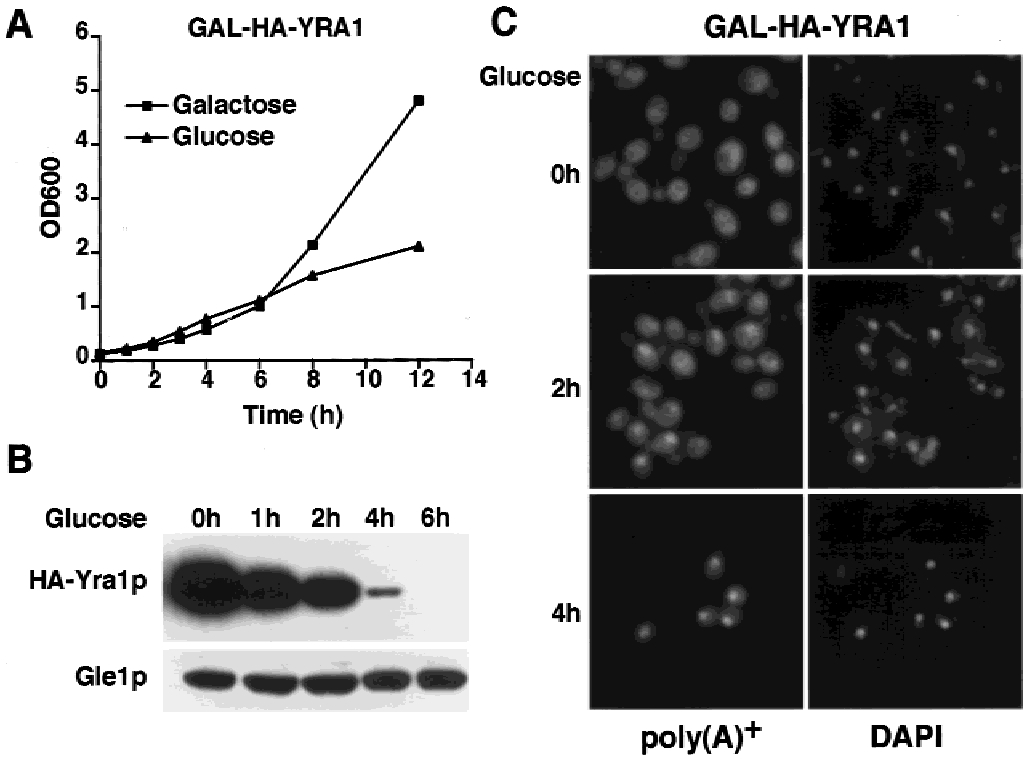

FIGURE 5. GAL depletion of Yra1p induces a block in polyA$^+$ RNA export. **A**: Growth curves of the GAL-HA-YRA1 strain in media containing galactose or glucose. **B**: Western blot analysis of the HA-tagged Yra1p protein after transfer of the GAL-HA-YRA1 strain to glucose-containing medium. For each time point, protein extracts were prepared from comparable cell equivalents and probed with an anti-HA antibody; the same extracts were probed with an anti-Gle1p antibody to control for equal loading. **C**: Localization of polyA$^+$ RNA in the GAL-HA-YRA1 strain grown for various times in glucose-containing medium. PolyA$^+$ RNA was detected by *in situ* hybridization with a digoxigenin-labeled oligo(dT) probe and an FITC-conjugated anti-digoxigenin antibody. The location of the nuclei was determined by staining the same cells with DAPI.

nup49-313 mutant exhibits a block in nuclear import at the nonpermissive temperature whereas export from the nucleus is not affected. Therefore, if Yra1p is able to leave the nucleus, it should accumulate within the cytoplasm of *nup49-313* cells shifted to 37 °C, as shown earlier for the shuttling hnRNP-like proteins Npl3p and Hrp1p (Lee et al., 1996; Kessler et al., 1997). To examine the localization of Yra1p, a construct expressing a galactose-inducible GFP-Yra1p fusion was introduced into the *nup49-313* strain. The GFP-Yra1p fusion is functional, as it can rescue a YRA1 disruption (data not shown). The GFP-Yra1p fusion was entirely nuclear both at 25 °C and 37 °C supporting the hypothesis that Yra1p does not exit the nucleus (Fig. 6a,c). In contrast, the GFP-Npl3p fusion examined in parallel was exclusively nuclear at 25 °C, but showed substantial cytoplasmic accumulation at 37 °C (Fig. 6e,g), consistent with the export of this hnRNP-like protein from the nucleus. Although, we cannot exclude that the reimport of GFP-Yra1p is unaffected by the *nup49-313* allele, our data suggest that Yra1p is not a shuttling protein or that its import is distinct from the import of other hnRNP-like proteins in yeast.

DISCUSSION

Previously, we have shown that TAP mediates export of CTE-bearing RNA substrates by directly interacting with the CTE RNA via its N-terminal domain (Grüter et al., 1998; Braun et al., 1999). Although TAP binds directly to the CTE RNA, its interaction with cellular RNAs is likely to be facilitated by protein–protein interactions (see Introduction). To investigate the mechanism by which both TAP and its yeast ortholog Mex67p are recruited by cellular mRNPs, we searched for Mex67p interacting proteins. Using the tandem affinity purification approach (Rigaut et al., 1999) we identified Yra1p as a putative Mex67p partner. Database searches and cDNA amplification by RT-PCR revealed the existence of a large, evolutionarily conserved family of Yra1p-related proteins. For reasons mentioned above these proteins were named REFs. We show that binding of REFs by Mex67p/TAP is direct and can occur in the presence of RNA. Thus REFs may facilitate the interaction of TAP/Mex67p with the mRNP-export complexes. More generally, the domain organization of the REF proteins (Fig. 2), their affinity for RNA (Fig. 3), and

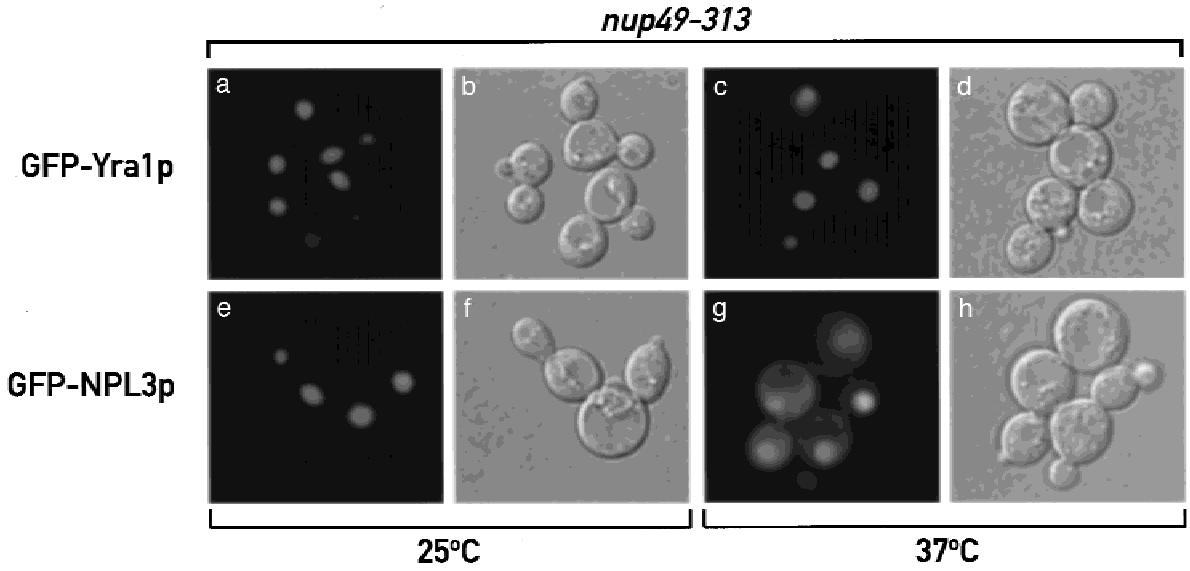

FIGURE 6. Yra1p does not shuttle between the nucleus and the cytoplasm. The *nup49-313* temperature-sensitive strain was transformed with a plasmid coding for the galactose-inducible GFP-Yra1p fusion protein. As a control, the mutant strain was also transformed with a construct expressing the shuttling GFP-Npl3p fusion from a GAL1 promoter (Lee et al., 1996). The transformed cells were grown in galactose-containing medium to induce the expression of the GFP fusions and subsequently transferred to glucose-containing medium at 25 °C for 2 h. Cells were then kept at 25 °C (a, b, e, and f) or shifted to 37 °C (c, d, g, and h) for 5 h and examined for GFP fluorescence (a, c, e, and g) or photographed with interference contrast optics (b, d, f, and h).

their subcellular localization (Bruhn et al., 1997; Portman et al., 1997; Wichmann et al., 1999) indicate that these factors function as hnRNP proteins, as previously suggested for Yra1p (Portman et al., 1997).

Yra1p: an essential hnRNP-like protein involved in mRNA export

Yra1p is an essential yeast protein previously identified as a major RNA-annealing activity in yeast whole-cell extracts (Portman et al., 1997). Because RNA annealing activity was observed for many of the human hnRNP proteins (Portman & Dreyfuss, 1994), and Yra1p is uniformly localized within the yeast nucleus, it has been suggested that Yra1p functions as an hnRNP protein having an essential role in pre-mRNA metabolism (Portman et al., 1997). Here we show that Yra1p is involved in mRNA nuclear export. Indeed, galactose depletion of Yra1p resulted in accumulation of polyA$^+$ RNAs within yeast nuclei. This mRNA export block exhibits the same kinetics as the reduction of Yra1p protein levels and the cell growth arrest (Fig. 5), suggesting that it is a primary effect of Yra1p depletion. However, the exact nature of the polyA$^+$ RNA signal observed by *in situ* hybridization in the GAL1-HA-YRA1 strain remains unclear. For instance, it is not clear whether Yra1p is involved in the export of a complex population of mRNAs or participates in the export of a small subset of very abundant mRNAs. The identification of the RNA substrates of Yra1p will allow a better understanding of its function in mRNA export.

Yra1p belongs to an evolutionarily conserved family of hnRNP-like proteins that interacts with TAP/Mex67p

Our data show that Yra1p belongs to a large, evolutionarily conserved, family of proteins (Fig. 2) having several of the characteristic features of hnRNP proteins (reviewed by Burd & Dreyfuss, 1994). These proteins were named REFs. Their sequences contain two highly conserved motifs at their N- and C-termini and one RNP-motif RNA-binding domain (RRM/RBD; reviewed by Burd & Dreyfuss, 1994; Nagai et al., 1995). The N- and C- terminal motifs, named REF-N and REF-C (Fig. 2), define the REF subfamily of RRM proteins. Between these conserved motifs and the RBD/RRM domain, REF proteins have regions of variable lengths (N-vr and C-vr) that are related in sequence to the RGG boxes described in many RNA-binding proteins (Burd & Dreyfuss, 1994). We show that Yra1p and REF proteins bind both RNA and ssDNA in vitro (Fig. 3). As mentioned above, their domain organization, their subcellular localization, and their affinity for RNA and ssDNA suggest that REF proteins may function as hnRNP proteins.

Some members of the REF family, such as Yra1p and hsREF, have been isolated previously through biochemical approaches (Portman et al., 1997; Wichmann et al., 1999) and others (such as MLO-3 and murine Aly) have emerged from genetic screens (Javerzat et al., 1996; Bruhn et al., 1997). Although our results are consistent with previously published data on Yra1p and

hsREF, currently it is difficult to reconcile our findings with the functions attributed to *MLO-3* and Aly in previous reports. For instance, overexpression of Mlo3 protein resulted in a failure to segregate chromosomes in *S. pombe*, and the *MLO3* gene was proposed to play an important role in chromosome transmission fidelity during mitosis (Javerzat et al., 1996). Using a yeast two-hybrid assay, Aly was identified as a protein interacting with LEF-1, a transcription factor that participates in the regulation of the T-cell receptor α (TCRα) enhancer (Bruhn et al., 1997). In this context, Aly was proposed to facilitate the functional collaboration of multiple proteins in the TCRα enhancer complex. One possibility is that some of the phenotypes resulting from depletion or overexpression of REF proteins are a secondary consequence of their primary function in mRNA export. Alternatively, REF proteins may have another function in addition to their general role in mRNA export. Finally, REF proteins may act as RNA (or ssDNA) chaperones which modulate RNA–RNA or RNA–protein interactions (Portman & Dreyfuss, 1994; Portman et al., 1997), and can therefore participate in multiple steps of mRNA biogenesis including transcription, processing, and transport.

Members of the REF family of proteins interact with both TAP and Mex67p. Binding of Yra1p/REFs to Mex67p/TAP is direct and mediated by the N-terminal domain of TAP (Figs. 1 and 4). Furthermore, this interaction can occur in the presence of RNA, although RNA binding is not strictly required for the interaction to occur (Fig. 4). It is therefore likely that REF-bps facilitate the recruitment of TAP/Mex67p to the mRNP complexes. However, in higher eukaryotes, other RNA-binding proteins may also facilitate TAP binding to the mRNPs as in vitro TAP interacts with E1B-AP5 (Bachi et al., 2000) and a subset of hnRNP proteins (E. Izaurralde, unpubl.). Note that TAP/Mex67p may also directly contact the mRNA as both proteins exhibit RNA-binding activity in vitro (Santos-Rosa et al., 1998; Braun et al., 1999; Katahira et al., 1999; Fig. 3). These multiple interactions may contribute to the formation of the RNP-fiber. Thus, TAP may associate with cellular mRNPs through the interaction of its N-terminal domain with hnRNP-like proteins or directly with the mRNA, whereas its NPC-binding domain may bridge the interaction between the mRNP-export cargo and the NPC (see Fig. 4C and Bachi et al., 2000).

The observation that Yra1p does not accumulate in the cytoplasm of *nup49–133* cells at 37 °C suggests that Yra1p is stripped from the mRNPs just prior to or immediately after translocation through the NPC, as is likely to be the case for the nonshuttling hnRNP proteins. In contrast, TAP shuttles (Bear et al., 1999; Braun et al., 1999; Kang & Cullen, 1999) even in the absence of mRNA synthesis (E. Izaurralde and Maria Carmo-Fonseca, unpubl. results). The mechanism by which TAP/Mex67p and REFs proteins are dissociated from their substrates is unknown. One possibility is that binding of import receptors on the cytoplasmic side of the pore would promote the dissociation of these proteins from the RNA and their recycling back to the nucleus. Alternatively, Dbp5 may also play an important role in disrupting mRNA–protein interactions as the mRNP emerges from the NPC (Snay-Hodge et al., 1998; Tseng et al., 1998; Schmitt et al., 1999). Interestingly, Yra1p was also selected with yDbp5p using the *tap* affinity purification (Schmitt et al., 1999), and hsREF can be selected from HeLa extracts on immobilized GST-hDbp5 (C. Schmitt and E. Izaurralde, unpubl. results). Thus Dbp5 may recognize directly or indirectly REF proteins bound to the mRNP-export substrates and facilitate their dissociation. Further biochemical studies in conjunction with the use of genetic tools are required to characterize the precise function of TAP/REF or Mex67p/Yra1p complexes in mRNA export, and their possible recognition by Dbp5.

MATERIALS AND METHODS

Plasmids, recombinant protein expression, and purification

Full-length murine REF1-II, REF2-I, and REF2-II cDNAs were obtained by PCR using an Expand™ high-fidelity PCR system (Boehringer), mouse cDNA as a template, and primers containing the appropriate restriction sites. Primers were designed based on the published coding sequence of mouse Aly (Bruhn et al., 1997). PCR fragments were cloned into the *Nco*I-*Bam*HI sites of vectors pGEXCS and pBSSK-HA, a derivative of pBSSK+ vector (Stratagene) having the β-globin 5′ untranslated region inserted between the *Hin*dIII and *Eco*RI sites. Plasmids pBSSK-HA REFs were used as templates for generation of [^{35}S]-labeled proteins with the combined in vitro transcription/translation (TnT) kit from Promega.

Mex67p was cloned by PCR using an Expand™ high-fidelity PCR system (Boehringer), yeast genomic DNA as a template, and primers containing the appropriate restriction sites. Primers were designed based on the published coding sequence of Mex67p (Segref et al., 1997). Yra1p was cloned into the *Bam*HI-*Hin*dIII sites of pRSETA (Invitrogen), Mex67p was cloned into the *Eco*RI-*Not*I sites of pGEX-5X2 (Pharmacia). All TAP constructs used in this study were previously described (Braun et al., 1999; Bachi et al., 2000). GST fusions were expressed in *E. coli* BL21(DE3) pLysS and purified as described by Grüter et al. (1998).

Protein identification

Proteins eluted from the affinity columns were analyzed on SDS-PAGE followed by silver staining. Bands of interest were excised, in-gel digested with trypsin (Shevchenko et al., 1996), and analyzed by peptide mass mapping on a Bruker REFLEX MALDI time-of-flight mass spectrometer (Bruker-Franzen, Bremen, Germany). A nonredundant protein database containing more than 300,000 entries was searched with the peptide masses. In cases when the identification was not certain, the

peptide mixture was extracted, desalted on a 100-nL Poros R2 column, eluted directly into a nano electrospray needle, and analyzed on a triple quadrupole tandem mass spectrometer (API III, PE-Sciex, Ontario, Canada; Wilm et al., 1996).

Pull-down assays

About 5 μg of GST-tagged recombinant protein immobilized on 20 μL of packed glutathione agarose beads was used per binding reaction. Following binding of the GST-tagged proteins, beads were washed three times with 0.5 mL of binding buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). Between 2 and 5 μL of in vitro-synthesized proteins were used per binding reaction in a final volume of 200 μL of binding buffer containing 0.1% BSA and protease inhibitors. Alternatively between 0.2–1 mL of *E. coli* lysates were used; in this case BSA was omitted. Binding was for 1 h at 4 °C. Beads were washed three times with 0.5 mL of binding buffer and once with 0.5 mL of binding buffer without Triton X-100. Bound proteins were eluted with SDS-sample buffer and analyzed by SDS-PAGE followed by fluorography or Coomassie staining. When indicated, about 3 U of micrococcal nuclease (Sigma) or 50 μg of RNase A were added per binding reaction. Ribonuclease activity was assessed by including in vitro-synthesized, ^{32}P-labeled U1 snRNA or tRNA, in the binding reactions.

In vitro RNA-binding assay

Binding to the CTE RNA probe was performed as described by Braun et al. (1999). For the RNA-binding assay shown in Figure 3A, a 77-nt RNA probe was employed (UISII-; Sherly et al., 1989). Yeast tRNA or Herring sperm single-stranded DNA were used as unlabeled competitors. Reactions were carried out in binding buffer (15 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.05 mg/mL BSA, and 0.05% NP40). Final sample volumes were 10 μL. Recombinant proteins were added to the reaction mixtures from a diluted stock solution in the same buffer. After 30 min at room temperature, 1 μL of a solution containing 0.05% bromophenol blue was added to the reaction mixtures. Samples were applied to a 5% nondenaturing polyacrylamide gel (19:1, acryl:bisacryl ratio). Electrophoresis was carried out at a constant voltage of 12 V/cm at 4 °C in 0.5× TBE buffer. Complexes were visualized by autoradiography. The amount of recombinant proteins and of competitors used are indicated on the figure legends.

Tandem affinity purification of Mex67p and associated proteins

The standard protocol for the tandem affinity purification (*tap*) of Mex67p and associated partners was used (Rigaut et al., 1999). Briefly, yeast cells were grown at 30 °C in 2 L of YPD medium to an OD_{600} = 2. Cells were harvested by centrifugation and frozen in liquid N_2. All subsequent steps were carried out at 0–4 °C. The pellet was resuspended in 1 vol of 10 mM K-HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM PMSF, 2 mM benzamidine, 1 μM leupeptin, 2 μM pepstatin A, 4 μM chymostatin, 2.6 μM aprotinin before lysis by two passages in a French-press (Sim-Aminco) at 8.27 MPa. The KCl concentration was adjusted to 200 mM and the lysate was cleared by two consecutive low and high speed centrifugation steps. The resulting whole cell extract was dialyzed for 3 h against 2 L of 20 mM K-HEPES, pH 7.9, 50 mM KCl, 0.2 mM EDTA, pH 8.0, 0.5 mM DTT, 0.5 mM PMSF, 2 mM benzamidine, 20% (v/v) glycerol before storage at −80 °C. Affinity purification steps, but TEV protease cleavage, were carried out at 0–4 °C. Two hundred microliters of IgG sepharose bead suspension (Pharmacia) were washed in a 10-mL Econocolumn (Biorad) with 5 mL of IPP150/IgG buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% NP40). Ten milliliters of extract, corresponding to approximately 2 L of cell culture, were adjusted to IPP150/IgG buffer concentrations for Tris-HCl, NaCl and NP40. This solution was incubated with the IgG agarose beads in the column by rotating for 2 h. Beads were washed with 30 mL IPP150/IgG buffer followed by 10 mL of TEV cleavage buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT). Beads were resuspended, in the column, in 1 mL of TEV cleavage buffer and 30 μL of TEV protease (about 10 U, kindly provided by G. Stier) and rotated at 16 °C for 2 h. The eluate was recovered by gravity flow and mixed with 3 mL of IPP150/calmodulin binding buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% NP40, 10 mM β-mercaptoethanol) and 3 μL of 1 M $CaCl_2$ per mL of eluate to neutralize the EDTA present in the eluate. This solution was incubated by rotation for 1 h in an Econocolumn with 200 μL of calmodulin beads slurry (Stratagene) previously washed with 5 mL IPP150/calmodulin binding buffer. After washing with 30 mL of IPP150/calmodulin binding buffer protein complexes were eluted in 5 fractions of 200 μL with IPP150/calmodulin elution buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM Mg-acetate, 1 mM imidazole, 2 mM EGTA, 0.1% NP40, 10 mM β-mercaptoethanol). Samples were frozen in dry ice and stored at −80°C.

Yeast strains and culture

The GAL-HA-YRA1 strain (**a**, *ade2*, *his3*, *leu2*, *trp1*, *ura3*, *Kanr-GAL-HA-YRA1*) expressing a galactose-inducible and N-terminally HA-tagged Yra1p protein was obtained by insertion of a DNA cassette in front of the chromosomal YRA1 gene in the wild-type haploid strain W303 using Kan^r as a selectable marker as described (Longtine et al., 1998).

To establish the growth curves, the GAL-HA-YRA1 strain was grown overnight in synthetic complete (SC) medium containing 3% galactose/1% raffinose at 30 °C to an OD_{600} = 0.15. Cells were split in two, washed with water, and resuspended in an equal volume of SC medium plus 3% galactose/1% raffinose or 2% glucose; growth at 30 °C was then monitored by measuring the optical density at 600 nm of both cultures at regular time intervals. The disappearance of the HA-Yra1p in the presence of glucose was examined by Western blot analysis with an anti-HA antibody (Roche Diagnostics, dilution 1:2,000). Total protein extracts were prepared as described (Stutz et al., 1997) from equal numbers of cells at various times after transfer to glucose-containing medium. As a control for loading, the same samples were probed with an anti-Gle1p antibody (a gift from Laura Davis; dilution 1:2,000).

In situ hybridization

To follow nuclear accumulation of polyA$^+$ RNA during Yra1p depletion, GAL-HA-YRA1 cells were grown to early-log phase (OD_{600} = 0.15) in SC medium containing 3% galactose/1% raffinose, washed with water, resuspended in an equal volume of YEPD (2% glucose), and further grown at 30 °C. Cells were fixed at various times after transfer to glucose and processed for *in situ* hybridization with a digoxigenin-labeled oligo(dT) probe as described (Strahm et al., 1999).

Protein shuttling assay

The construct GAL-GFP-YRA1 expressing a galactose-inducible GFP-Yra1p fusion was obtained by cloning the YRA1 cDNA as a *Sal*I PCR fragment into pCGF-1A (URA, 2μ), a galactose-inducible expression vector for fusion to the C-terminus of GFP (Lee et al., 1996). Plasmid GAL-GFP-YRA1 and a comparable construct expressing a GFP-Npl3p fusion (Lee et al., 1996) were transformed into the temperature-sensitive strain *nup49-313* (Doye et al., 1994). The nuclear export assay was performed essentially as described (Lee et al., 1996). Briefly, transformants were grown overnight at 25 °C to early-log phase in selective medium lacking uracil and containing 3% galactose/1% raffinose. Cells were washed with YEPD (medium containing glucose), resuspended in an equal volume of YEPD and incubated for 2 h at 25 °C. Cultures (10 mL) were then either shifted to 37 °C or remained at 25 °C for an additional 5 h. Cells were spun, resuspended in SC medium plus glucose and examined for GFP fluorescence signal under a Zeiss Axioplan microscope equipped with a 100× objective lens and a cooled CCD camera (Kappa). The cells were also photographed using interference contrast optics. Identical exposure times were used for comparable images, and composites were prepared using Adobe Photoshop.

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