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Dbp5p/Rat8p is a yeast nuclear pore-associated DEAD-box protein essential for RNA export

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To identify *Saccharomyces cerevisiae* genes important for nucleocytoplasmic export of messenger RNA, we screened mutant strains to identify those in which poly(A)$^+$ RNA accumulated in nuclei under non-permissive conditions. We describe the identification of *DBP5* as the gene defective in the strain carrying the *rat8-1* allele (*RAT* = ribonucleic acid trafficking). Dbp5p/Rat8p, a previously uncharacterized member of the DEAD-box family of proteins, is closely related to eukaryotic initiation factor 4A(eIF4A) an RNA helicase essential for protein synthesis initiation. Analysis of protein databases suggests most eukaryotic genomes encode a DEAD-box protein that is probably a homolog of yeast Dbp5p/Rat8p. Temperature-sensitive alleles of *DBP5/RAT8* were prepared. In *rat8* mutant strains, cells displayed rapid, synchronous accumulation of poly(A)$^+$ RNA in nuclei when shifted to the non-permissive temperature. Dbp5p/Rat8p is located within the cytoplasm and concentrated in the perinuclear region. Analysis of the distribution of Dbp5p/Rat8p in yeast strains where nuclear pore complexes are tightly clustered indicated that a fraction of this protein associates with nuclear pore complexes (NPCs). The strong mutant phenotype, association of the protein with NPCs and genetic interaction with factors involved in RNA export provide strong evidence that Dbp5p/Rat8p plays a direct role in RNA export.

Keywords: DEAD-box RNA helicase/*in situ* hybridization/nucleocytoplasmic transport/nucleoporin/*Saccharomyces cerevisiae*

Introduction

The nucleus is a defining feature of eukaryotic cells. The compartmentation of the genetic material within the nucleus, combined with the location of the translational machinery in the cytoplasm, creates the requirement that mRNA molecules synthesized in the nucleus be exported to the cytoplasm for translation. Defining the cellular components required for RNA export and determining the mechanisms by which they act is an area of intense investigation at present (for reviews see Corbett and Silver, 1997; Nakielny *et al.*, 1997; Nigg, 1997).

All exchange of macromolecules between the nucleus and the cytoplasm takes place through nuclear pore complexes (NPCs). These extremely large proteinaceous structures perforate the double-membraned nuclear envelope, have a mass of ~125 MDa in metazoan cells and 66 MDa in *Saccharomyces cerevisiae*, and contain ~40–80 different polypeptide species (Rout and Blobel, 1993; Rout and Wente, 1994). Transport through NPCs is an energy-dependent process which is saturable and requires specific signals within molecules being transported (Nigg, 1997). For example, proteins imported into nuclei contain nuclear localization signals (NLSs) which are recognized by soluble receptor proteins. For most nuclear proteins, the receptor is a heterodimeric complex which in yeast contains the proteins Srp1p and Kap95p/Rsl1p. Some proteins contain distinct minor classes of NLSs and interact with other receptors that are related to Kap95p (Aitchison *et al.*, 1996; Pollard *et al.*, 1996; Rout *et al.*, 1997; Siomi *et al.*, 1997). A complex containing the NLS-protein and the receptor docks at specific sites on the NPC and is subsequently translocated into the nucleus by an as yet poorly understood mechanism that requires Ran/Gsp1p, a small GTPase which also moves between the nucleus and the cytoplasm. Various studies to determine whether Ran is the only GTPase required for nuclear import have reached different conclusions (Sweet and Gerace, 1996; Weis *et al.*, 1996), so this remains an open question. Similarly, it is not known how many times Ran hydrolyzes GTP during the import or export of a single substrate molecule or complex.

Messenger RNA molecules are exported from nuclei as ribonucleoprotein particles. Current evidence supports the idea that at least some of the signals for RNA export reside in the protein components of RNP particles (Michael *et al.*, 1995; Izaurralde *et al.*, 1997a). Proteins that are exported from nuclei, either alone or in complex with mRNA, contain nuclear export signals (NESs) which have been shown to interact with soluble receptor molecules related to Kap95p, including Crm1p (Fornerod *et al.*, 1997; Seedorf and Silver, 1997; Stade *et al.*, 1997). There is also evidence suggesting that the capped structure found at the ends of mRNAs and U snRNAs plays a role in export (Hamm and Mattaj, 1990; Izaurralde *et al.*, 1992, 1995; Visa *et al.*, 1996), probably through binding to the cap of specific cellular proteins which may contain NESs. Both yeast Npl3p and the closely related mammalian hnRNP A1 protein shuttle between the nucleus and the cytoplasm, bind mRNA molecules within the nucleus, and contain information that directs their export (Flach *et al.*, 1994; Wilson *et al.*, 1994; Lee *et al.*, 1996; Pollard *et al.*, 1996). Studies in yeast indicate that Npl3p is required for mRNA export since mRNA export is blocked when strains bearing temperature-sensitive alleles of *NPL3* are shifted to 37°C. Similarly, the export of Npl3p requires ongoing

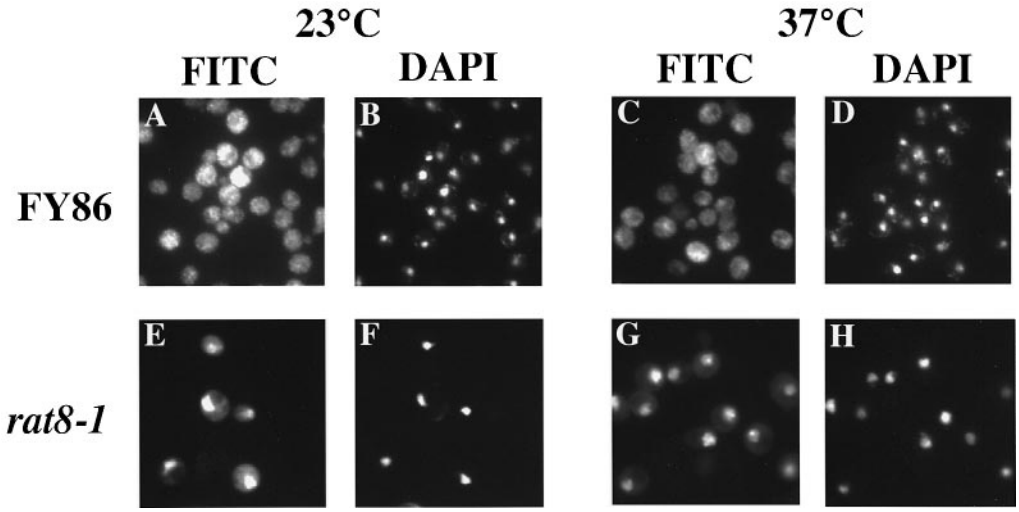

Fig. 1. *rat8-1* cells show a block in poly(A)$^+$ RNA export. Wild-type (FY86) and *rat8-1* cells were grown to mid-log phase at 23°C and subjected to a 1 h incubation at 37°C before fixation and *in situ* hybridization with a digoxigenin-tagged oligo $(dT)_{50}$ probe for localization of poly(A)$^+$ RNA. Panels A, C, E and G show the fluorescence signal after the cells were probed with FITC-conjugated anti-digoxigenin antibody. Panels B, D, F and H show DAPI staining of the same cells, respectively, and indicate the positions of the nuclei.

RNA synthesis, suggesting that Npl3p can be exported only when bound to RNA (Lee *et al.*, 1996).

Among the other types of factors which might be expected to play a role in RNA export are RNA helicases (for reviews see Schmid and Linder, 1992; Fuller-Pace, 1994). The types of RNA molecules transported through NPCs range in size from <100 nucleotides (e.g. tRNA) to rRNAs and mRNAs containing thousands or tens of thousands of nucleotides. NPCs are able to transport particles up to ~25 nm in diameter (Feldherr *et al.*, 1984; Dworetzky and Feldherr, 1988). Since RNAs are known to fold into complex and highly ordered structures, a role for RNA helicases in nuclear transport seems quite possible. High-resolution electron micrographs show that the very large transcripts found in Balbiani ring mRNP particles are restructured prior to translocation through NPCs and move through NPCs with their 5′ ends exiting the nucleus first (Mehlin *et al.*, 1992, 1995; Kiseleva *et al.*, 1996).

RNA helicases are a diverse superfamily of proteins characterized by the presence of seven canonical motifs. In yeast, there are at least 30 proteins that are members of this family, though very few of these have actually been shown to possess RNA helicase activity (Pause and Sonenberg, 1992; Pause *et al.*, 1993; Chuang *et al.*, 1997). The protein synthesis initiation factor, eukaryotic initiation factor 4A (eIF4A) is the RNA helicase that has been studied in the greatest detail, and it is the defining member of the DEAD-box subgroup of RNA helicases. RNA helicases have been shown to play key roles in most aspects of RNA metabolism, including tRNA (Winey and Culbertson, 1988; De Marini *et al.*, 1992) and pre-mRNA splicing (Company *et al.*, 1991; Schwer and Guthrie, 1991), rRNA processing (Venema and Tollervey, 1995; Venema *et al.*, 1997), translation (Linder and Slonimski, 1989; Schmid and Linder, 1991; Chuang *et al.*, 1997) and mRNA turnover (Czaplinski *et al.*, 1995; Weng *et al.*, 1996).

In our analysis of *RAT* (ribonucleic acid trafficking) mutants of *S.cerevisiae* with defects in mRNA export, we have identified Dbp5p/Rat8p, a putative DEAD-box RNA helicase closely related to eIF4A, as an essential factor for the nucleocytoplasmic export of mRNA. Cells carrying temperature-sensitive alleles of *DBP5/RAT8* display an extremely rapid and synchronous onset of the nuclear accumulation of poly(A)$^+$ RNA when they are shifted to 37°C and subsequent disappearance of poly(A)$^+$ RNA from the cytoplasm. We localized Dbp5p/Rat8p to the cytoplasm and to the region of the nuclear rim. Two-color indirect immunofluorescence analysis and analysis of the location of a functional green fluorescent protein (GFP)–Dbp5p/Rat8p fusion protein indicated that Dbp5p/Rat8p is associated with NPCs. Genetic synthetic lethal interactions were observed between mutant alleles of *DBP5/RAT8* and three nucleoporins required for efficient mRNA export. Synthetic lethality was also observed between *dbp5/rat8* mutants and temperature-sensitive alleles of *gle1/rss1*, an NES-containing protein required for efficient mRNA export (Del Priore *et al.*, 1996; Murphy and Wente, 1996) and *rna1*, encoding a GTPase activating protein (GAP) for Ran/GSp1p. Together, these results suggest that Dbp5p/Rat8p plays an essential, direct role in RNA export.

Results

To identify gene products required for nucleocytoplasmic export of mRNA, we initiated a genetic screen in *S.cerevisiae* several years ago to identify temperature-sensitive yeast strains which accumulated poly(A)$^+$ RNA in their nuclei following a shift to the non-permissive temperature of 37°C. The mutated genes in several of these strains encode nucleoporins. This paper describes studies that grew from our isolation of the *rat8-1* mutant strain. Figure 1 shows the distribution of poly(A)$^+$ RNA in the nuclei of wild-type and *rat8-1* cells, both at 23°C and shifted to 37°C for 1 h. *rat8-1* cells showed substantial accumulation of poly(A)$^+$ RNA in their nuclei, even at 23°C, and all cells displayed strong nuclear accumulation within 20 min of a shift to 37°C. In addition, this mutant strain grows considerably less rapidly than wild-type cells at 23°C

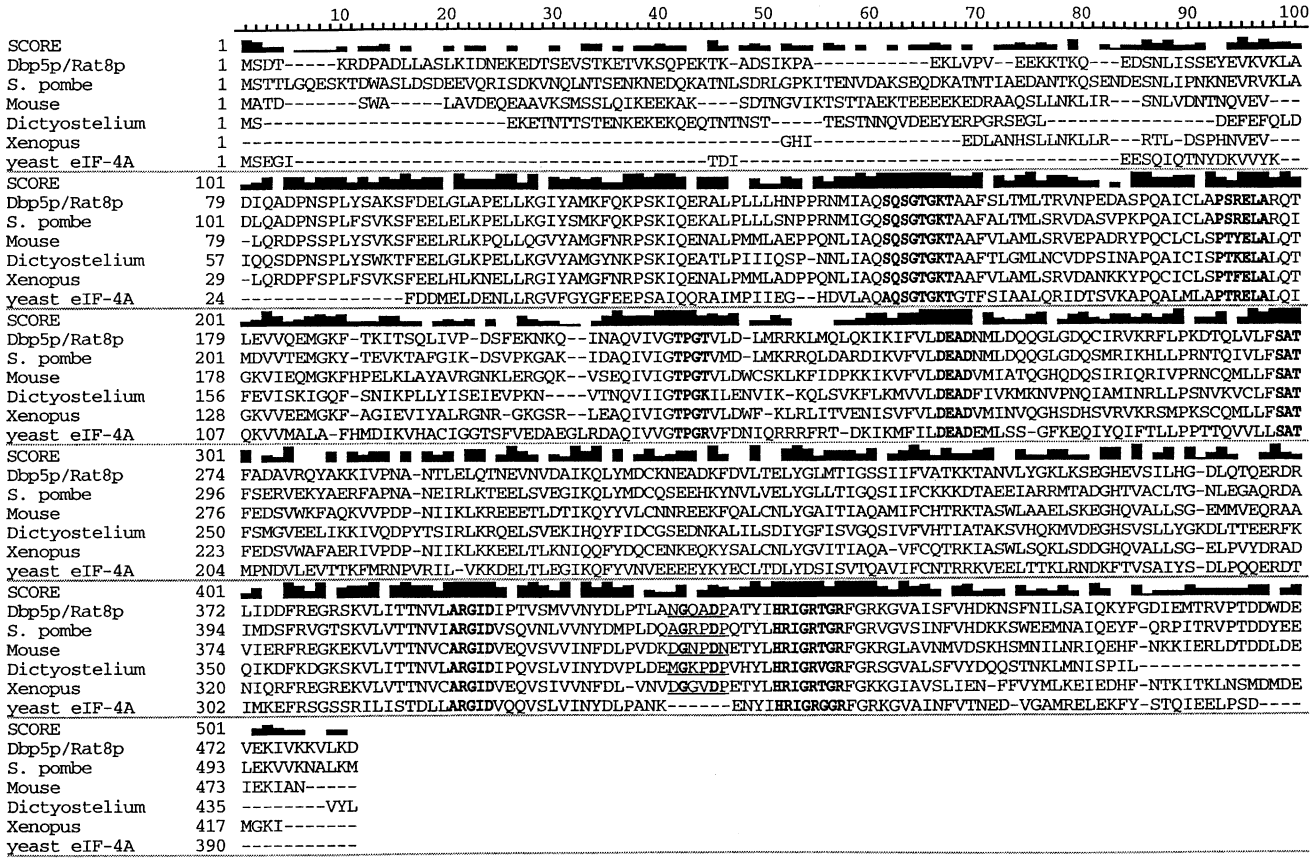

Fig. 2. Proteins very closely related to Dbp5p/Rat8p are found in various species. BLAST database searches (Altschul *et al.*, 1990, 1997) with the Rat8p sequence revealed potential homologs in several other species. An alignment of those sequences with Rat8p and with eIF4A was generated using the Gene Inspector program (Textco Inc., W. Lebanon, NH) which also calculated a similarity score at each position, as shown. The conserved motifs of DEAD-box family proteins are shown in bold. The six-amino-acid insertion that distinguishes the Rat8p sub-family from eIF4A is underlined. The accession numbers for these sequences is as follows: *S.pombe* (Z54140), *Mus musculus* (L25125), *Dictyostelium discoideum* (AF002677), *S.cerevisiae* eIF4A (*TIF1*) (X12813), *X.laevis* (M.L.King, personal communication).

(data not shown). We cloned the *RAT8* gene by complementation and determined that the complementing gene was open reading frame YOR046C, in a region close to *RAT1* and adjacent to *STD1*.

To prove that *YOR046C* was the gene defective in the *rat8-1* mutant strain, and not a suppressor of the *rat8-1* mutation, we determined that a single mutation was responsible for both temperature-sensitive growth and the mRNA export block, and that this mutation was located very close to a *LEU2* marker inserted adjacent to the *RAT1* locus (see Materials and methods). This indicates that *RAT8* is identical to *DBP5*, which encodes a putative DEAD-box RNA helicase. Part of this gene was identified earlier in a search for DEAD-box RNA helicases that used a PCR-based strategy, and it was designated *CA5* (Chang *et al.*, 1990). No analyses of the function of CA5 were carried out in that study. It has subsequently been given the name *DBP5* for DEAD-box protein 5. A BLAST search of the *S.cerevisiae* genome database indicates that of the more than two dozen proteins in yeast that belong to the DEAD-box RNA helicase family, Dbp5p/Rat8p is most closely related to Tif1p and Tif2p (Altschul *et al.*, 1990, 1997). Tif1p and Tif2p are eIF4A, a highly conserved protein found in all eukaryotic organisms. A key feature of all DEAD-box RNA helicases is the presence of seven characteristic motifs separated one from another by conserved distances (Schmid and Linder, 1992). These motifs are all present in Dpb5p/Rat8p and are bold-faced in Figure 2. Dbp5p/Rat8p differs from Tif1p and Tif2p by having an unrelated N-terminal region of ~100 amino acids and in having a six amino acid insertion at a novel location within its conserved helicase core between the sixth and seventh conserved motifs. Dbp5p/Rat8p is the only DEAD-box protein in *S.cerevisiae* that contains this six amino acid insertion, but database analyses indicate that the *Schizosaccharomyces pombe*, *Xenopus laevis*, *Dictyostelium*, *Drosophila*, mouse and human genomes each contain a protein very closely related to Dbp5p/Rat8p, and carrying a related six amino acid insertion at precisely the same position. An alignment of some of these putative homologs with Dbp5p and Tif1p/eIF4A is shown in Figure 2. All of these proteins from other species are more closely related to Dbp5p/Rat8p than Tif1p/eIF4A.

Preparation and analysis of additional temperature-sensitive alleles of DBP5/RAT8

Genetic analyses indicated that *DBP5/RAT8* is an essential gene in yeast. To gain further insight into the role of Dbp5p/Rat8p, and because the *rat8-1* strain grew so poorly even at 23°C, we prepared additional temperature-sensitive alleles of *DBP5/RAT8* using a PCR-based mutagenesis strategy. We obtained five temperature-sensitive strains

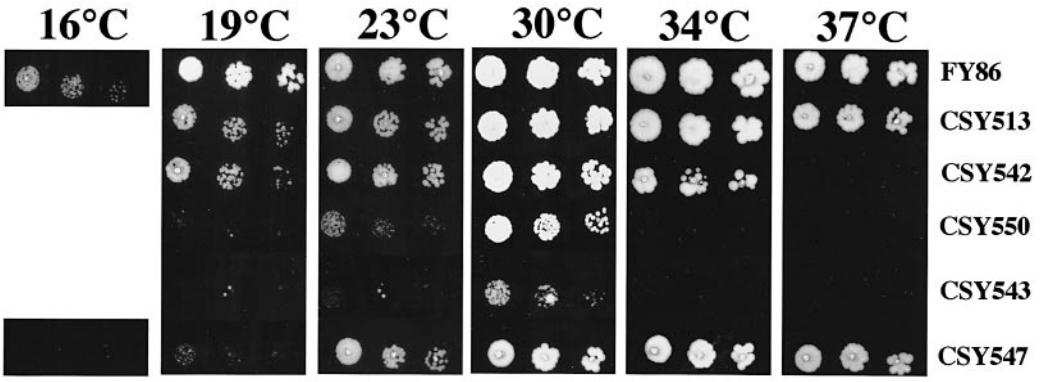

Fig. 3. Growth properties of mutant strains at several temperatures. Serial dilutions of wild-type and mutant yeast strains were plated on YPD plates and incubated for 3 days (23–37°C) or 4 days (16 or 19°C). The strains examined were FY86 (wild-type), CSY513 (disrupted for *rat8*, and containing wild-type *RAT8* on a *URA3/CEN* plasmid), CSY542 (disrupted for *rat8* and carrying *rat8-2* on a *LEU2/CEN* plasmid), CSY550 (carrying *rat8-2* integrated into the yeast genome at the site of the *RAT8* gene; only the *rat8-2* allele is present in this strain), CSY543 (disrupted for *rat8* and carrying the *rat8-3* allele on a *LEU2/CEN* plasmid) and CSY547 (disrupted for *rat8* and carrying the *rat8-7* allele on a *LEU2/CEN* plasmid).

that grew well at 23°C but failed to grow at 37°C, and one which grew well at 30°C but failed to grow at 16°C. These new heat-sensitive alleles are called *rat8-2* to *rat8-6*, and the cold-sensitive allele is called *rat8-7*. Mutants *rat8-2*, *rat8-3* and *rat8-7* were selected for further study. The *rat8-2* allele was integrated back into the genome at the *RAT8* locus and the resulting strain was included in all subsequent experiments, including genetic analyses (see below).

The growth properties of several mutant strains were compared by plating serial dilutions of cells on YPD plates and monitoring the formation of colonies following incubation at several different temperatures. These data are shown in Figure 3. Wild-type cells (FY86 and CSY513) grew well at all temperatures. The *rat8-2* allele (strains CSY542 and CSY550) permitted cells to grow as well as wild-type at 30°C, but growth was slower than wild-type at 23°C, especially for the strain carrying a single integrated copy of the *rat8-2* allele (CSY550). Cells carrying the integrated *rat8-2* allele did not form visible colonies after 4 days at 19°C or 3 days at 34°C, while the strain carrying *rat8-2* on a *CEN* plasmid (CSY542) grew nearly as well as wild-type at 19°C and 34°C. The CSY543 strain, carrying the *rat8-3* allele, grew best at 30°C, but showed very little growth at any other temperatures, although some growth was seen at 23°C following further incubation. The cold-sensitive CSY547 strain (carrying the *rat8-7* allele) grew as well as wild-type at all temperatures from 23°C to 37°C, showed very slow growth at 19°C, and did not grow at 16°C.

Plasmids containing these mutant alleles were sequenced. Each mutant contained a single amino acid change. *rat8-2* contains a Leu→Pro change at amino acid 267; *rat8-3* contains a Ile→Asn change at amino acid 385; while the cold-sensitive *rat8-7* allele contains a Pro→His change at amino acid 170, which is located within the second DEAD-box helicase canonical motif, P(S/T)RELA. Because the N-terminus of Dbp5p/Rat8p is unrelated to sequences in other yeast DEAD-box proteins (or in any other yeast proteins), we constructed a mutant lacking the DNA encoding amino acids 9–79 of Dbp5p/Rat8p and determined that this region of Dbp5p/Rat8p was completely dispensable (data not shown).

We next examined the mRNA export properties of representative strains. As shown in Figure 4, cells carrying the *rat8-2* and *rat8-3* alleles (strains CSY542 and CSY543, respectively) showed limited nuclear accumulation of poly(A)$^+$ RNA when grown at 23°C (Figure 4, panels E and I). Poly(A)$^+$ RNA accumulated rapidly in all nuclei within 20 min of a shift to 37°C (Figure 4, panels G and K). The onset of the defect in the cold-sensitive strain (CSY547) was slower. These cells showed little or no nuclear accumulation of poly(A)$^+$ RNA when grown continuously at 30°C (Figure 4, panel M), and a modest increase in the nuclear signal after 20 min at 16°C (data not shown); all cells showed strong nuclear accumulation after a 1 h shift to 16°C (Figure 4, panel O).

Liang *et al.* (1996) previously described Mtr4p as a DEAD-box family member involved in mRNA export, and localized this protein to the nucleus, including the nucleolus. We obtained from them their strain carrying the *mtr4-1* allele and compared it with the *rat8-2* allele using *in situ* hybridization analysis. Figure 5 shows that *mtr4-1* cells have a minimal defect in RNA export. A minority of the cells show an increased nuclear signal for poly(A)$^+$ RNA following a shift to 37°C for 30 min (Figure 5, panel E), and the percentage of cells showing accumulation increased to nearly 100% after 3 h at 37°C (Figure 5, panel F). However, note that there was no diminution in the cytoplasmic signal for poly(A)$^+$ RNA in *mtr4-1* cells, even after 3 h. For comparison, note that all cells carrying the *rat8-2* allele showed strong nuclear accumulation within 30 min (Figure 5, panel H), and a very low cytoplasmic signal after 30 min or 3 h at 37°C (Figure 5, panel I). This implies that Dbp5p/Rat8p plays a direct role in mRNA export.

Dbp5p/Rat8p is located both at the nuclear rim and in the cytoplasm

We generated a polyclonal antiserum to the N-terminal portion of Dbp5p/Rat8p. To minimize the possibility that our antiserum would cross-react with any of the large number of yeast DEAD-box RNA helicase family members, we chose amino acids 8–105 because this region shows the greatest divergence from other yeast DEAD-box proteins. A GST fusion protein was prepared, expressed in *Escherichia coli*, and isolated by affinity chromatography on glutathione–Sepharose. The antiserum was sub-

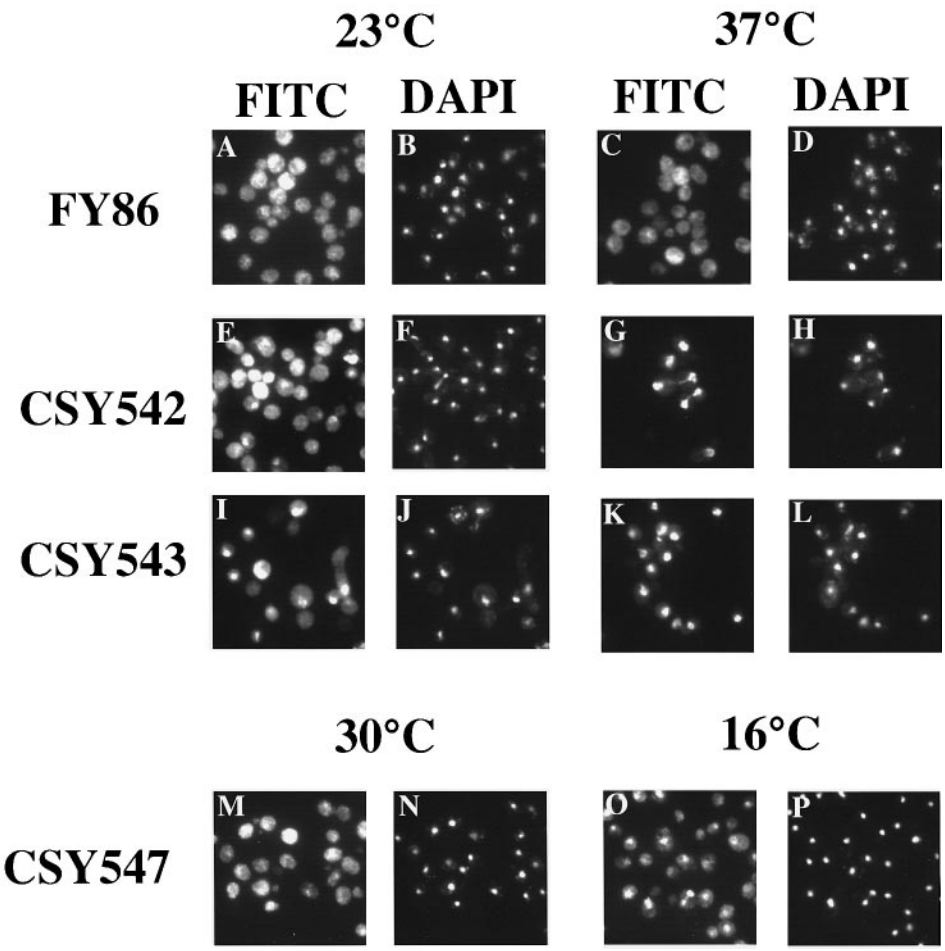

Fig. 4. PCR-generated heat- or cold-sensitive *rat8* strains show a block in poly(A)$^+$ RNA export. Strains CSY542, CSY543 and CSY547 contain plasmid-borne *rat8* mutant alleles (*rat8-2*, *rat8-3* and *rat8-7*, respectively) in a *rat8* null background. They were grown to mid-log phase at 23°C and subjected to a 20 min incubation at the non-permissive temperature of 37°C (CSY542 and CSY543) or 1 h incubation at the non-permissive temperature of 16°C (CSY547) before fixation and *in situ* hybridization using a digoxigenin-tagged oligo $(dT)_{50}$ probe for localization of poly(A)$^+$ RNA. Panels A, C, E, G, I, K, M and O show the fluorescence signal after the cells were probed with FITC-conjugated anti-digoxigenin antibody. The remaining panels show DAPI staining of the same fields of cells and indicate the positions of the nuclei.

sequently affinity purified. A Western blot using this antiserum is shown in Figure 6A. Lane 1 shows Dbp5p/Rat8p in wild-type cells. Lane 2 shows a myc-epitope-tagged form of Dbp5p/Rat8p in cells disrupted for *DBP5/RAT8* and carrying the myc-tagged *DBP5/RAT8* on a *CEN* plasmid. Lane 3 shows the same myc-tagged Dbp5p/Rat8p, but carried on a 2μ vector. This higher-level expression of Dbp5p/Rat8p had no deleterious effect on cell growth (data not shown).

We also examined Dbp5p/Rat8p in each of the mutant strains (Figure 6B) grown at 23°C or shifted to 37°C for 1 h (strains CSY542 and CSY543 harboring the *rat8-2* and *rat8-3* alleles, respectively) or shifted to 16°C for 1 h (strain CSY547, harboring the *rat8-7* allele). Cells carrying the *rat8-3* allele appeared to have slightly higher levels of Dbp5p/Rat8p at both 23 and 37°C than did wild-type cells or cells carrying either of the other two alleles. Some apparent breakdown products can also be seen when $Dbp5p_{myc}/Rat8p_{myc}$ was overexpressed (Figure 6A, lane 3) or in the *rat8-3* strain (CSY543; Figure 6B, lanes 5 and 6). However, none of the mutations caused Dbp5p/Rat8p to become thermolabile. Note that a lighter band can be seen above the Dbp5p/Rat8p band in each lane. This band is also present in cells expressing only the myc-tagged form of Dbp5p/Rat8p (Figure 6 A, lane 2), indicating that it is not Dbp5p/Rat8p, but a cross-reacting polypeptide.

We took several approaches to localize Dbp5p/Rat8p within yeast cells. We prepared an epitope-tagged form of Dbp5p/Rat8p and localized the tagged protein using both the affinity-purified polyclonal antibody to the N-terminus of Dbp5p/Rat8p and the 9E10 anti-myc monoclonal antibody. We also localized wild-type Dbp5p/Rat8p in wild-type cells using the affinity-purified antibody. Results of immunofluorescence experiments are shown in Figure 7. Using either the N-terminal polyclonal antibody or the anti-myc antibody, we observed a diffuse though punctate staining of the cytoplasm as well as a clear concentration of signal very close to the nuclear rim.

We next prepared a fusion of GFP to Dbp5p/Rat8p. In this construct, GFP was inserted at the same place within the N-terminal region as the triple-myc epitope tag. As was the case with the myc-tagged construct, GFP–Dbp5p/Rat8p completely covered the disruption of *DBP5/RAT8*. We examined the location of GFP–Dbp5p/Rat8p in wild-type yeast cells (Figure 8). In contrast to the results obtained by indirect immunofluorescence, the cytoplasmic staining was considerably less punctate and

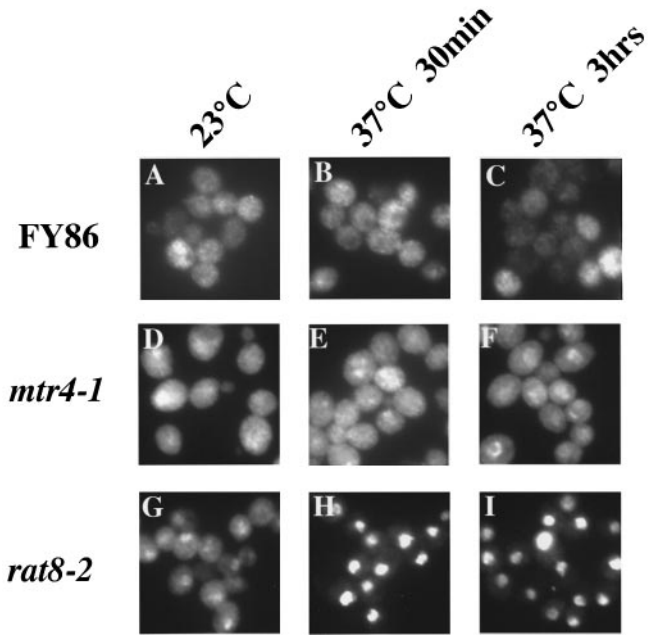

Fig. 5. Comparison of another DEAD-box helicase mutant, *mtr4-1*, with *rat8-2* by *in situ* hybridization analysis. *mtr4-1 cells* showed a slight increase in nuclear signal for poly(A)$^+$ RNA, but no decrease in cytoplasmic staining when shifted to the non-permissive temperature for 20 min or 3 h (panels E and F, respectively). However, in cells carrying the *rat8-2* allele and shifted to the non-permissive temperature, a very rapid and strong nuclear accumulation of poly(A)$^+$ RNA was observed, as well as very faint cytoplasmic staining (panels H and I).

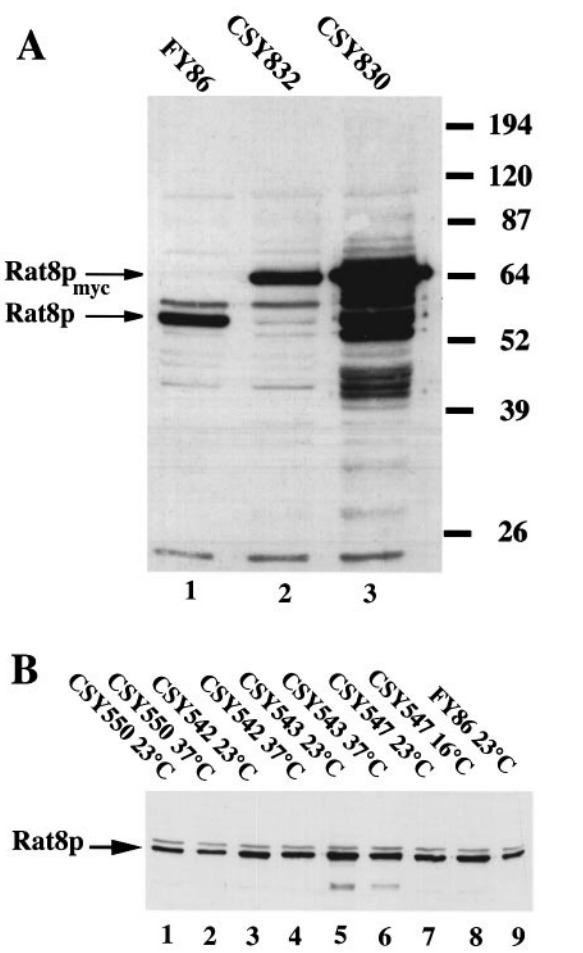

Fig. 6. Western analysis of Rat8p in various wild-type and mutant strains. (**A**) A wild-type strain (FY86) and strains CSY832 and CSY830, harboring Rat8p$_{myc}$ on *CEN* and 2μ plasmids, respectively, were grown to mid-log phase. Extracts were prepared and equal amounts from each strain were separated by SDS–PAGE, transferred to nitrocellulose and probed with an affinity-purified antibody generated against the N-terminal portion of Rat8p. The numbers on the right indicate the positions of molecular weight markers (in kDa). (**B**) Strains harboring heat- or cold-sensitive mutant alleles of *RAT8* were grown at 23°C to mid-log phase and incubated at the appropriate non-permissive temperatures for 1 h. Extracts were prepared and their protein concentrations determined; equal amounts of protein were analyzed.

more uniform (Figure 8A and B), suggesting that the punctate signal seen in Figure 7C and E may reflect, at least in part, fixation artifacts. However, the most significant finding using GFP–Dbp5p/Rat8p was the clear staining surrounding the nucleus (Figure 8A). In each cell, the dark area that is not surrounded by the GFP signal is the vacuole. The same results were obtained when the gene encoding GFP-tagged Dbp5p/Rat8p was examined in a strain containing a disruption of *DBP5/RAT8* (data not shown).

We then examined the localization of GFP–Dbp5p/Rat8p in cells whose NPCs are clustered because they carry a mutant allele affecting the nucleoporin Rat2p/Nup120p (Heath *et al.*, 1995). In Figure 8B, it can be seen that the signal for GFP–Dbp5p/Rat8p close to the nucleus no longer surrounds the nucleus but is clustered into a small portion of the nuclear circumference. We also performed two-color indirect immunofluorescence in *rat2-1* cells to compare the location of Dbp5p/Rat8p and a nucleoporin, Rat7p/Nup159p, using confocal microscopy. As expected, Rat7p/Nup159p was detected exclusively at the nuclear rim, and its staining pattern was largely identical to the portion of the Dbp5p/Rat8p staining pattern that is nearest the nucleus (Figure 8, compare C and D). The data indicate that the portion of Dbp5p/Rat8p closest to the nucleus is co-localized with nucleoporins. We conclude that a portion of Dbp5p/Rat8p is associated with nuclear pore complexes.

Mutation of DBP5/RAT8 does not cause defects in translation initiation or RNA splicing

Because Dbp5p/Rat8p is very closely related by sequence to RNA helicases involved in translation initiation, and because a substantial part of the protein is cytoplasmic, we examined the effect of mutations of *DBP5/RAT8* on protein synthesis. Yeast strains carrying mutant alleles of *DBP5/RAT8* were compared with wild-type cells and cells carrying the temperature-sensitive *prt1-1* allele (Evans *et al.*, 1995). Prt1p is one of the subunits of translation initiation factor 3 (eIF3) (Moldave and McLaughlin, 1988). Cells were grown at 23°C, and subsequently shifted to 37°C for 5 min prior to a 10 min labeling period with [^{35}S]methionine plus [^{35}S]cysteine. Extracts were prepared and amounts from the same number of cells loaded onto an SDS–polyacrylamide gel (Figure 9). While there was a very rapid cessation of protein synthesis in the *prt1-1* strain, consistent with published results (Evans *et al.*, 1995), incorporation of radiolabel into proteins continued in *dbp5/rat8* mutant strains to the same extent as in wild-type cells. We conclude that Dbp5p/Rat8p does not participate directly in protein synthesis.

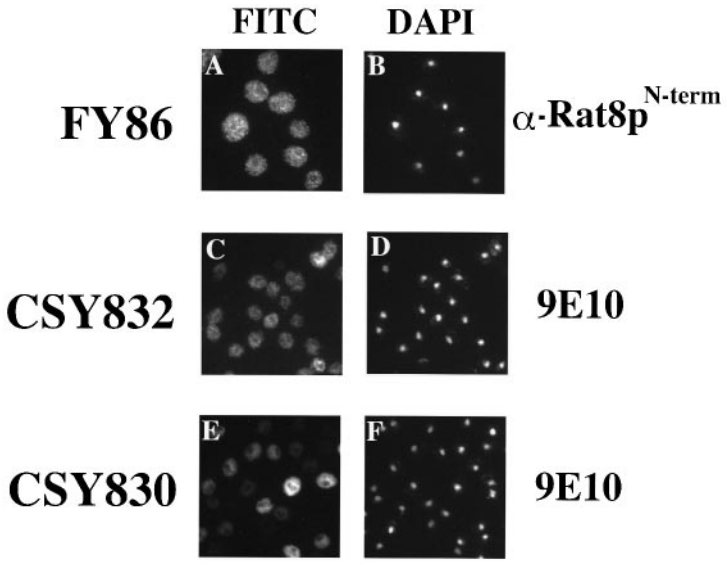

Fig. 7. Indirect immunofluorescence localizes Dbp5/Rat8p to the nuclear rim and cytoplasm. Similar staining patterns were obtained in wild-type cells using the polyclonal antibody to the N-terminus of Rat8p (panel A) or the 9E10 monoclonal antibody in the strain producing only myc-tagged Rat8p (panels C and E). The remaining panels show DAPI staining of the same fields of cells to identify the positions of the nuclei.

We also examined cells carrying *dbp5/rat8* mutant alleles for defects in splicing since several members of the DEAD-box family of putative RNA helicases have been shown to be required for pre-mRNA splicing. We saw no accumulation of unspliced U3 snoRNA in the ts strains following a 1 h shift to 37°C, while a severe splicing defect was seen in cells carrying a temperature-sensitive mutation in *prp16* (data not shown). In addition, our *in situ* hybridization results (Figure 4) show a dramatic decrease in the cytoplasmic signal for poly(A)$^+$ RNA following a shift to 37°C, suggesting that mutation of Rat8p/Dbp5p had no or very little effect on the normal turnover of cytoplasmic mRNA.

Synthetic lethal analyses demonstrate a genetic link between Dbp5p/Rat8p and other proteins involved in mRNA export

To gain additional insight into the function of Dbp5p/Rat8p, we determined whether the *rat8-2* mutant allele was synthetically lethal with mutant alleles affecting other genes whose products are known to be involved in mRNA export as well as several genes whose products are involved in other aspects of RNA metabolism. The results are presented in Figure 10. The data obtained indicate that *rat8-2* is synthetically lethal with mutant alleles affecting three nucleoporins known to be required for efficient mRNA export, Rat7p/Nup159p, Rat9p/Nup85p and Nup145p (Gorsch *et al.*, 1995; Kraemer *et al.*, 1995; Goldstein *et al.*, 1996; Siniossoglou *et al.*, 1996; Dockendorff *et al.*, 1997; Emtage *et al.*, 1997; Teixeira *et al.*, 1997). We also observed synthetic lethality between the *rat8-2* allele and temperature-sensitive alleles of *rna1*, the GTPase-activating protein for Gsp1p (Hopper *et al.*, 1990; Amberg *et al.*, 1992) and *rss1/gle1* (Del Priore *et al.*, 1996; Murphy and Wente, 1996). These data indicate a strong genetic interaction between *RAT8* and a subset of genes whose products play important roles in mRNA export. In addition, we observed a weak synthetic interaction with a strain disrupted for the gene encoding the non-essential nucleoporin Rip1p/Nup42p and carrying the *rat8-2* allele; although this strain could grow at 23°C, it failed to grow at 30°C, whereas strains either harboring the *rat8-2* mutation or disrupted for *RIP1* grew well at 30°C. We saw no synthetic lethality between *rat8-2* and mutant alleles of several other genes whose products are involved in RNA export, including *GSP1*, *PRP20* and *NPL3*. We also observed no synthetic lethality between *rat8-2* and temperature-sensitive alleles of *RPA190*, encoding a subunit of RNA polymerase I; *RPB1*, encoding a subunit of RNA polymerase II; *PRT1*, encoding a subunit of translation initiation factor eIF3; or *HMT1*, encoding a non-essential arginine methyltransferase whose substrates include the abundant yeast hnRNP protein Npl3p (Henry and Silver, 1996), an important mediator of RNA export (Lee *et al.*, 1996).

Based on the rapidly occurring mRNA nuclear accumulation phenotype, the association of a fraction of Dbp5p/Rat8p with NPCs, and the genetic interactions between Dbp5p/Rat8p and proteins known to be involved in nucleocytoplasmic transport, our data provide strong support for a direct role of Dbp5p/Rat8p in RNA export.

Discussion

In a screen for yeast genes whose products are required for efficient export of mRNA from the nucleus to the cytoplasm, we have identified Dbp5p/Rat8p, a member of the DEAD-box RNA helicase family of proteins. This screen was based on the accumulation of poly(A)$^+$ RNA in nuclei of mutant yeast cells shifted to the non-permissive temperature of 37°C. We believe that Dbp5p/Rat8p plays an important role in RNA export for the following reasons: (i) while yeast strains carrying temperature-sensitive alleles of *dbp5/rat8* showed little accumulation of poly(A)$^+$ RNA under permissive growth conditions, poly(A)$^+$ RNA accumulated rapidly and synchronously in all cells shifted to the non-permissive temperature (16°C for the cold-sensitive strain and 37°C for the heat-sensitive

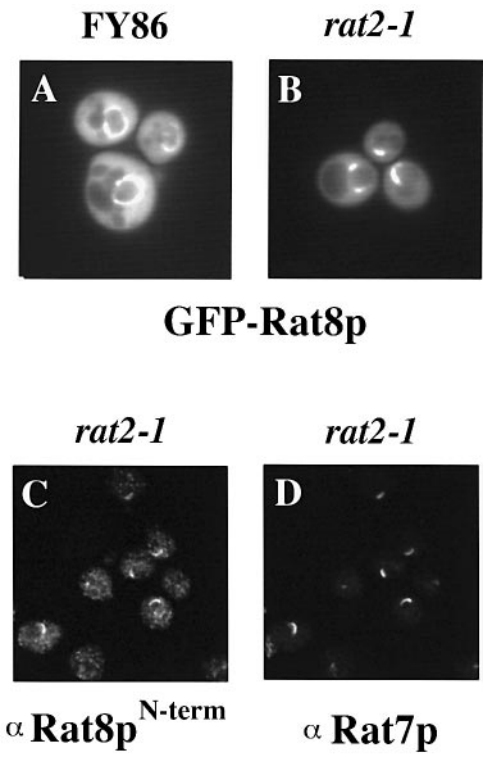

Fig. 8. Localization of GFP–Dbp5/Rat8p in living yeast cells. The staining pattern is similar to that seen by indirect immunofluorescence (Figure 7), except that the cytoplasmic signal is more diffuse. (**A**) Wild-type cells. (**B**) *rat2-1/nup120-1* cells. (**C** and **D**) The same *rat2-1* cells double-stained with the polyclonal antibody to the N-terminal portion of Rat8p (C) and a polyclonal antibody to Rat7p (D). An FITC-conjugated secondary antibody was used to detect the location of Dbp5p/Rat8p and a Texas Red-conjugated second antibody was used to detect the location of Rat7p/Nup159p. The images in (C) and (D) were acquired using filters which permitted completely separate observation of the signals from the FITC-coupled and the Texas Red-coupled second antibodies. Control experiments confirmed that no signal for FITC was detected in the Texas Red channel and no signal for Texas Red in the FITC channel (data not shown). In addition, controls were also performed in which both secondary antibodies and one primary antibody were used. When the primary antibody to Dbp5p/Rat8p was not included, no signal was obtained in the FITC channel (data not shown). Similarly, when the primary antibody to Rat7p/Nup159p was omitted, no signal was observed in the Texas Red channel (data not shown). Note that the nuclear signal seen with the anti-Rat8p antibody coincides with that seen with the anti-Rat7p antibody. (C) and (D) were obtained using a Bio-Rad M1000 confocal microscope.

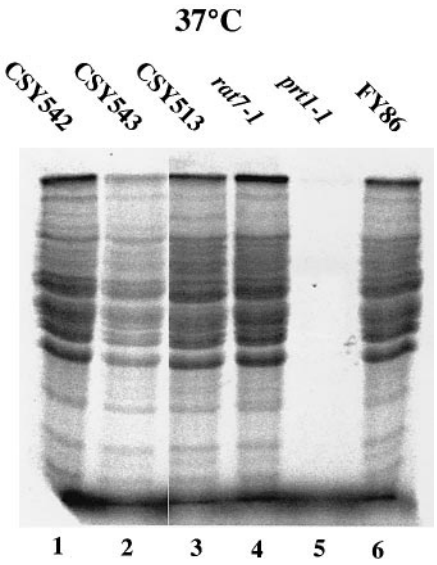

Fig. 9. *rat8* strains do not show a translation defect. Cultures of *rat8* mutant strains, wild-type strains, and strains harboring temperature-sensitive mutations in *RAT7* or *PRT1* were shifted to 37° for 5 min prior to addition of [^{35}S]Met/Cys. Following an additional 10-min incubation, extracts were prepared and equal amounts analyzed by SDS–PAGE. Prt1p is a component of translation initiation factor eIF3.

	Synthetic interaction with *rat8-2*	No synthetic lethality with *rat8-2*
Nucleoporins	*RAT7/NUP159* *RAT9/NUP85* *RAT10/NUP145* *RIP1/NUP42*	
Transport factors	*RSS1/GLE1* *RNA1*	*GSP1* *PRP20* *NPL3*
Other		*RPA190* *RPB1* *PRT1* *HMT1*

Fig. 10. The *rat8-2* allele shows synthetic interactions with mutant alleles of several genes encoding proteins important for RNA export.

strains); (ii) the signal for cytoplasmic poly(A)$^+$ RNA declined rapidly after a shift to non-permissive conditions, indicating that the pre-existing pool of cytoplasmic mRNA was being turned over and was not being replaced by newly exported mRNA; (iii) a significant fraction of Dbp5p/Rat8p was associated with nuclear pore complexes. GFP–Dbp5p/Rat8p was detected in a ring surrounding the nucleus as well as in the cytoplasm. In addition, in strains where NPCs are clustered due to a mutant nucleoporin (e.g. *rat2-1*), that portion of the signal for Dbp5p/Rat8p nearest the nucleus overlapped the clustered signal for the nucleoporin Rat7p/Nup159p; and (iv) genetic tests demonstrated that mutations of *DBP5/RAT8* were synthetically lethal with mutations affecting Rna1p, the GTPase-activating protein for Gsp1p/Ran, and with mutant alleles affecting three nucleoporins that play roles in RNA export but do not appear to be involved in nuclear protein import or nuclear pore complex biogenesis. Synthetic lethality was also observed with mutant alleles of *RSS1/GLE1*. Rss1p/Gle1p contains a region that can function as a nuclear export signal (Murphy and Wente, 1996) and is also located in the cytoplasm and near the nuclear rim (Del Priore *et al.*, 1996; Murphy and Wente, 1996). Temperature-sensitive alleles of *RSS1/GLE1* show rapidly occurring defects in export of poly(A)$^+$ RNA and synthetic lethality with several genes whose products are known to be involved in RNA export (Del Priore *et al.*, 1996; Murphy and Wente, 1996). Synthetic interactions were also observed between the *rat8-2* allele and a strain disrupted for *RIP1/NUP42*. Rip1p/Nup42p is not essential for growth at 23 or 37°C, but is required for export of heat-shock mRNAs following stress (Saavedra *et al.*, 1997; Stutz *et al.*, 1997). We also determined that protein transport, both into and out of the nucleus, was unaffected by mutations in *DBP5/RAT8* (C.A.Snay-Hodge and C.N.Cole, unpublished results).

An enzyme required for RNA export?

Very few enzymes involved in RNA export have been identified. Nuclear protein import and the export of proteins and most classes of RNA require the small GTPase, Gsp1p (Melchior *et al.*, 1993; Moore and Blobel, 1993; Schlenstedt *et al.*, 1995), and the activities of its GTPase-activating protein, Rna1p (Shiokawa and Pogo, 1974; Amberg *et al.*, 1992; Forrester *et al.*, 1992) and its GTP/GDP exchange factor, Prp20p (Forrester *et al.*, 1992; Amberg *et al.*, 1993). However, it appears that export of tRNA does not require hydrolysis of GTP by Ran (Izaurralde *et al.*, 1997b) or the action of its exchange factor, RCC1 (the metazoan homolog of Prp20p) (Chang *et al.*, 1995). Although nuclear protein export requires Ran, recent studies using a mutant of Ran unable to hydrolyze GTP suggest that protein export does not require GTP hydrolysis (Richards *et al.*, 1997). Other genes whose products have been shown to play a role in RNA export include several nucleoporins (for example, see Gorsch *et al.*, 1995; Heath *et al.*, 1995; Li *et al.*, 1995; Goldstein *et al.*, 1996) as well as Npl3p (Lee *et al.*, 1996) and Hrp1p (Henry and Silver, 1996; Kessler *et al.*, 1997), two proteins that bind poly(A)$^+$ RNA in the nucleus and accompany it to the cytoplasm before they dissociate from the RNA and shuttle back into the nucleus. RNA export also requires the participation of receptors including Xpo1p/Crm1p (Fornerod *et al.*, 1997; Stade *et al.*, 1997) that recognize nuclear export signals and are related to the protein import receptor Kap95p/Rsl1p. Other factors important for RNA export have been identified, including the NES-containing proteins Rss1p/Gle1p (Del Priore *et al.*, 1996; Murphy and Wente, 1996) and Mex67p (Segref *et al.*, 1997). The roles of Rss1p/Gle1p and Mex67p are unknown, but neither of these proteins resembles proteins of known function, so it is uncertain whether either possesses any enzymatic activity.

Liang *et al.* (1996) identified Mtr4p as a putative RNA helicase involved in RNA export. Only limited nuclear accumulation of poly(A)$^+$ RNA was seen in *mtr4-1* cells following a shift to 37°C for periods as long as 3 h, and little or no diminution of the cytoplasmic signal for poly(A)$^+$ RNA was observed. This suggests that poly(A)$^+$ mRNA continued to be exported after *mtr4-1* cells were shifted to 37°C. Possibly, the *mtr4-1* allele is a weak allele; other alleles might show a stronger mRNA export block. Depletion of Mtr4p by using a glucose-repressible *GAL* promoter showed stronger nuclear accumulation of poly(A)$^+$ RNA 20 h after depletion was initiated (Liang *et al.*, 1996). Recent data suggest that Mtr4p is essential for pre-rRNA processing (de la Cruz *et al.*, 1998).

Only a limited number of DEAD-box proteins have been shown to possess helicase activity *in vitro*, and we have not assayed purified Dbp5p/Rat8p for its ability to unwind RNA. However, it is very closely related to eIF4A (Tif1p/Tif2p in yeast), which is known to have RNA helicase activity (Pause and Sonenberg, 1992; Pause *et al.*, 1993). It is generally assumed that canonical DEAD-box proteins are likely to function as RNA helicases *in vivo* and we think it highly likely that Dbp5p/Rat8p does indeed possess RNA unwinding activity. The helicase activity of eIF4A *in vitro* is dramatically stimulated by the presence of eIF4B (Goyer *et al.*, 1993), a protein that binds eIF4A and also contains RNA recognition motifs. Because of its small size, Dbp5p/Rat8p is likely to have binding partners to provide specificity or otherwise modulate its activity, and we are currently searching for such proteins using both genetic and biochemical strategies.

What kinds of functions might Dbp5p/Rat8p provide for RNA export?

RNA helicases are widely distributed in cells and have been shown to be important for many steps of RNA metabolism, including RNA processing, translation and turnover (for reviews see Schmid and Linder, 1992; Fuller-Pace, 1994). It would be surprising if RNA helicases were not involved in RNA export, since export probably requires that ribonucleoproteins (RNPs) and large proteins be restructured in order to fit through the 25 nm channel of the NPC. The RNA molecules that are exported range in size from 100 to several thousand nucleotides. Ribosomal subunits contain several proteins and large RNA molecules and are known to have complicated secondary structures. Many mRNA molecules are also likely folded into complex structures. Studies of the export of the very large Balbiani ring granules in *Chironomus tentans* showed that these large circular RNPs were dramatically restructured prior to their passage through NPCs (Mehlin *et al.*, 1995; Visa *et al.*, 1996). Thus, there is a strong expectation that RNA helicases will be involved in RNA export.

One might expect that RNA helicases involved in RNA export would be required within the nucleus. In several recent studies (Grimm *et al.*, 1997a,b; Panté *et al.*, 1997) it was proposed that RNA molecules are transiently retained within the nucleoplasm and need to be actively released in order to be exported—a process that could involve ATP hydrolysis and helicase-mediated restructuring. A recent study reported that small colloidal gold particles microinjected into *Xenopus* oocyte nuclei could diffuse into the central channel of the NPC before encountering a barrier or gate within the NPC near the central plane of the nuclear envelope (Feldherr and Akin, 1997). In contrast, large microinjected colloidal gold particles had difficulty moving into the nuclear basket and the central channel of the NPC unless they were coated with nuclear export signals. This suggests that the export of large RNP particles might be facilitated by helicases acting at the nuclear basket as well as within the central channel. It is possible that Dbp5p/Rat8p is a shuttling protein; inspection of its sequence reveals several stretches that are leucine-rich and which could possibly function as nuclear export signals. If it is a shuttling protein, it could function within the nucleoplasm close to the NPC, within the central channel of the NPC, and in late steps of RNA export as the RNP particle emerged from the NPC. Alternatively, proteins other than Dbp5p/Rat8p but with similar activity might function within the nucleus and at the nuclear basket, with Dbp5p/Rat8p performing its functions on the cytoplasmic side of the NPC and within the cytoplasm.

In addition to restructuring RNA molecules, a DEAD-box protein could play a role in removing shuttling proteins from exported ribonucleoprotein particles to allow the hnRNP proteins to return to the nucleus. These proteins, which include Npl3p in *S.cerevisiae* and a large group of metazoan hnRNP proteins (e.g. A1), bind to pre-mRNA

molecules in the nucleus and participate in RNA processing reactions; many of them accompany the RNA to the cytoplasm. Releasing these proteins and rearranging the RNP complexes could well be an energy-requiring process; for example, the DEAD-box RNA helicase Prp22p releases the mature transcript and its RNP proteins from the spliceosome in an ATP-dependent reaction (Company *et al.*, 1991; Ono *et al.*, 1994; Ohno and Shimura, 1996). If Dbp5p/Rat8p was normally involved in removing shuttling proteins, there might be a cytoplasmic accumulation of Npl3p in *rat8* mutant strains. We have examined the location of Npl3p in *rat8* mutant cells and found that it is located primarily in the nucleus, as in wild-type cells (data not shown). However, Lee *et al.* (1996) showed that Npl3p remained in the nucleus and did not shuttle when RNA export was prevented, making it unlikely that mislocalization of Npl3p could occur in mutant strains defective for RNA export. Perhaps the export of messenger ribonucleoprotein (mRNP) is coupled with removal of Npl3p from the mRNA in the cytoplasm such that mRNA export ceases when Npl3p (and other proteins) cannot be removed from exported mRNP particles.

Significant cytoplasmic localization of Dbp5p/Rat8p was observed using both indirect immunofluorescence and GFP-tagged Dbp5p/Rat8p, in addition to its clear association with the nuclear envelope and NPCs. This suggests that there is a substantial pool of Dbp5p/Rat8p which is not NPC-associated. This large pool of Dbp5p/Rat8p could insure its presence at the pore as needed. However, we think it quite possible that Dbp5p/Rat8p associates with RNA during export and, after performing an ATP-dependent rearrangement, could remain associated with the resulting mRNP particle until the translation initiation complex eIF4F (which includes the helicase eIF4A/eIF4B) binds to the RNA and displaces Dbp5p/Rat8p. When we determined that some Dbp5p/Rat8p is cytoplasmic, we assayed for a primary translation defect in *rat8* mutant strains. Compared with strains mutant for known translation factors, however, *rat8* strains at the non-permissive temperatures were not deficient in translation, indicating that the export defect is not an indirect consequence of a defect in translation. A recent report on the DEAD-box helicase Fal1p further supports the notion that great similarity to eIF4A does not indicate a role in translation. Fal1p was identified as the yeast protein closest in sequence to eIF4A but it is a nuclear protein involved in 40S ribosomal subunit biogenesis (Kressler *et al.*, 1997).

A conserved and ancient putative RNA helicase

DEAD-box proteins represent a large and diverse group of proteins found in all organisms. They range in size from <500 to >1500 amino acids. The most thoroughly studied RNA helicase is the protein synthesis initiation factor eIF4A. eIF4A is very highly conserved; all eukaryotic organisms contain proteins that play the same role in protein synthesis initiation as human eIF4A and show a very high degree of identity to human and yeast eIF4A. Database analyses suggest the possibility that Dbp5p/Rat8p is the founding member of another broadly distributed and highly conserved family of DEAD-box proteins. While Dbp5p/Rat8p is more closely related to Tif1p/Tif2p (yeast eIF4A) than to any other yeast helicases, it is highly divergent in its N-terminal region, which we have shown to be dispensable, and in having a six amino acid insertion, relative to all other yeast DEAD-box family proteins, between the sixth and seventh highly conserved helicase motifs. Interestingly, a single putative RNA helicase containing a related insertion at the identical location exists in several other organisms, including *S.pombe*, *Drosophila melanogaster*, *X.laevis* (M.L.King, personal communication), *Mus musculus* (Gee and Conboy, 1994) and *Dicytostelium discoideum.* There is also a *Caenorhabditis elegans* protein that includes sequences related to the six-amino acid insertion, though the insertion in the *C.elegans* protein is considerably longer than six amino acids. The human EST database contains partial cDNAs for a protein that contains this insertion and that is more closely related to yeast Dbp5p/Rat8p than to yeast Tif1p/Tif2p/eIF4A.

There is a high degree of conservation among factors and receptors previously identified as required for nuclear transport. Nuclear pore complexes are also very closely related among species, particularly in their 8-fold symmetry, overall shape and the size of their channels, and many nucleoporins are closely related in yeast and metazoans. This makes it likely that other factors critical for efficient RNA export will also be highly conserved among eukaryotic organisms. Dbp5p/Rat8p and related proteins in other organisms may play a role in RNA export as ancient and fundamental as the role played in protein synthesis initiation by eIF4A.

Materials and methods

Yeast strains and genetic methods

The strains and plasmids used in this study are listed in Tables I and II. Growth conditions and general genetic methods have been described previously (Gorsch *et al.*, 1995).

Cloning and sequencing of RAT8

The generation and screening of temperature-sensitive *rat* mutants has been described (Amberg *et al.*, 1992). The *RAT8* gene was cloned by complementation of the temperature-sensitive growth phenotype of the *rat8-1* strain. Sequence analysis of the complementing fragment identified ORF YOR046C, which is located in a region close to *RAT1* and adjacent to *STD1*. In order to ascertain that the rescuing gene represented the wild-type form of the gene mutant in *rat8-1*, we mapped the mutant allele genetically by crossing the *rat8-1* strain with a strain carrying a *LEU2* marker adjacent to the *RAT1* gene. The patterns of segregation of the *rat8-1* allele and the *LEU2* marker indicated that *RAT8* was located ~7.7 cM from *RAT1* (YOR046C's location is consistent with this position).

Disruption of DBP5/RAT8

The *DBP5/RAT8* gene was deleted by first amplifying the *HIS3* gene using oligonucleotide primers that included sequence complementary to the flanking regions of *DBP5/RAT8*; the upstream primer was 5′-AGCAACCAAGAACATCTATAAAATTACTTACCACCTTAGATCG-GAGGCCTCCTCTAGTACACTC-3′ and the downstream primer was 5′-GATTAAAGCTTTTACGTATTTTGAGGTATTATGTACTGAATTC-TAGCGCGCCTCGTTCAGAATG-3′. The PCR product was gel-purified and transformed into ACY1 and ACY2, two wild-type diploid strains homozygous for the *his3-Δ200* mutation, and plated onto SC-His. His^+ colonies were picked and checked by colony PCR for the replacement of *DBP5/RAT8* by the *HIS3* gene. The PCR contained 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 100 μg/ml gelatin, dATP, dCTP, dGTP and dTTP at 0.2 mM each, 2 U Taq DNA polymerase (Boehringer Mannheim) and 1 μM primers. One primer was complementary to an internal region of *HIS3* (5′-GCCTCATCCAAAGGCGC-3′) and the other to a region just upstream of the start codon of *DBP5/RAT8* (5′-CTTACCACCTTAGATCGGA-3′). A small amount of each colony was transferred to the PCR mix, which was then subjected to a 35-cycle

Table I. Yeast strains used in this study

Strain name	Genotype	Source
ACY1	*MATa/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/his3Δ200 trp1Δ63/TRP1*	Anita Corbett (Emory Univ.)
ACY2	*MATa/MATα ura3-52/ura3-52 leu2Δ1/leu2Δ1 his3Δ200/his3Δ200 trp1Δ63/trp1Δ63*	Anita Corbett (Emory Univ.)
AG157.206A	*MATa ura3-52 leu2-3 ts157 rat8-1*	Goldstein (1995)
AG195.206A	*MATα ura3-52 leu2-1 his3-200 ts195 rat9-1*	Goldstein (1995)
CSY512	*MATα trp1Δ63 leu2Δ1 ura3-52 his3Δ200 Rat8::HIS3* pRat8.31 (*YCplac33 Rat8*)	this study
CSY513	*MATa trp1Δ63 leu2Δ1 ura3-52 his3Δ200 rat8::HIS3* pRat8.31 (YCplac33 *RAT8*)	this study
CSY542	*MATa trp1Δ63 leu2Δ1 ura3-52 his3Δ200 rat8::HIS3* pCS542 (YCplac111 *rat8-2*)	this study
CSY543	*MATa trp1Δ63 leu2Δ1 ura3-52 his3Δ200 rat8::HIS3* pCS543 (YCplac111 *rat8-3*)	this study
CSY547	*MATa trp1Δ63 leu2Δ1 ura3-52 his3Δ200 rat8::HIS3* pCS547 (YCplac111 *rat8-7*)	this study
CSY550	*MATa leu2Δ1 trp1Δ63 ura3-52 rat8-2*	this study
CSY560	*MATa leu2Δ1 trp1Δ63 ura3-52 rat8-2* pRat8.31(YCplac33 *RAT8*)	this study
CSY561	*MATα MATa leu2Δ1 trp1Δ63 ura3-52 rat8-2* pRat8.31(YCplac33 *RAT8*)	this study
CSY570	*MATα his3Δ200 ura3-52 leu2Δ1* pCS835 (YCplac111 $RAT8^{GFP}$)	this study
CSY571	*MATα rat2-1 his3Δ200 ura3-52 leu2Δ1* pCS835 (YCplac111 $RAT8^{GFP}$)	this study
CSY830	*MATa trp1Δ63 leu2Δ1 ura3-52 his3Δ200 rat8::HIS3* pCS830 (YEplac181 $RAT8^{myc}$)	this study
CSY832	*MATa trp1Δ63 leu2Δ1 ura3-52 his3Δ200 rat8::HIS3* pCS830 (YCplac111 $RAT8^{myc}$)	this study
CSY835	*MATa trp1Δ63 leu2Δ1 ura3-52 his3Δ200 rat8::HIS3* pCS835 (YCplac111 $RAT8^{GFP}$)	this study
CCY1	*MATα ura3 leu2 his3 prp20-1*	P.Silver (Harvard Med. School)
F294	*MATa prt1-1 ade1 leu2-3112 ura3-52*	Chuang *et al*. (1997)
FY23	*MATa ura3-52 trp1Δ63 leu2Δ1*	Winston *et al*. (1995)
FY86	*MATα his3Δ200 ura3-52 leu2Δ1*	Winston *et al*. (1995)
LGY101	*MATα rat7-1 his3Δ200 ura3-52 leu2Δ1*	Gorsch *et al*. (1995)
PSY1026	*MATα npl3-17 ura3-52 leu2Δ1 ade2-101 ade8 his3Δ200 lys1-1 can1-100*	Lee *et al*. (1996)
nsp1-13A	*MATa ade2-1 can1-100 his3Δ200 leu2-3 lys1-1*	Nehrbass *et al*. (1993)
NOY259	*MATα trp1-Δ1 his4Δ400 leu2-3112 ura3-52 rpa190-1*	Oakes *et al*. (1993)
NOY 267	*MATα trp1-Δ1 his4Δ400 leu2-3112 ura3-52 rpa190-5*	Oakes *et al*. (1993)
TDY109	*MATα nup145-10 ura3 leu2 trp1 ade2 ade3*	Dockendorff *et al*. (1997)
OHY068	*MATα ura3-52 his3Δ200 leu2-3 trp1Δ63 gsp1::HIS3, gsp2::HIS3* pDW36 (*gsp1-1 TRP1*)	C.Hammell (Dartmouth Medical School)
ts382	*MATa rna11–1 ade1 ade2 gal1 his7 lys2 tyr1 ura1*	*Hartwell Laboratory*
VDPY130	*MATα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 rss1::HIS3* (YCpLac111 *rss1-16*)	this study
YSL401(T188)	*MATa mtr4-1 ura3-52 lys2-801 ade2-1 his3Δ200 pep4::HIS3 prp1-Δ1.6 RGal+*	Chuang *et al*. (1997)

Table II. Plasmids used in this study

pRat8.1	original complementing clone *LEU2/CEN* (Hieter Library)
pRat8.26	6.5 kb *Xba*I fragment from pRat8.1 subcloned into YCplac33
pRat8.31	3.5 kb *Stu*I fragment from pRat8.26 subcloned into YCplac33
pRat8.32	triple myc epitope cloned into *Eag*I site within pRat8.31
pCS831	3.5 kb *Stu*I fragment from pRat8.1 subcloned into YCplac111
pCS832	2.8 kb *Pst*I fragment from pRat8.32 subcloned into YCplac111
pCS830	2.8 kb *Pst*I fragment from pCS832 subcloned into YEplac181
pCS835	GFP–S65T subcloned into *Eag*I site within pCS831
pCS82T	443 bp GST–Rat8p N-terminal fusion subcloned into *Bam*HI and *Eco*RI sites of pGEX2T
pCS542	Leu→Pro mutation at amino acid 267 of Dbp5p/Rat8p in YCplac111 (pCS831)
pCS543	Ile→Asn mutation at amino acid 385 of Dbp5p/Rat8p in YCplac111 (pCS831)
pCS547	Pro→His mutation at amino acid 170 of Dbp5p/Rat8p in YCplac111 (pCS831)

amplification (94°C, 1.5 min; 50°C, 2.0 min; 72°C, 3 min). The products were analyzed electrophoretically for the presence of a 600 bp fragment indicating successful replacement of *DBP5/RAT8* by *HIS3*. To test whether *DBP5/RAT8* is essential, the His^+ diploids were transformed with pRat8-31 (a *URA3/CEN* plasmid containing *DBP5/RAT8*). Ura^+His^+ colonies were sporulated and tetrads tested for their ability to grow on 5-FOA. In all cases, the tetrads segregated 2:2 for growth on 5-FOA plates, indicating that *DBP5/RAT8* is essential.

Generation of temperature-sensitive alleles of DBP5/RAT8

A PCR-based mutagenesis procedure was used to generate temperature-sensitive mutants of *DBP5/RAT8*, as previously described (Cadwell and Joyce, 1992; Lee *et al*., 1996). A portion of the *DBP5/RAT8* ORF was amplified using primers complementary to a region 148 bp upstream of the start codon and a region 91 bp downstream of the stop codon (5′-CTAGTCTAGACGCGGTATTTACCCGTATGC-3′ and 5′-TCCCCCC-GGGGGGACCCATTGATGTCG-3′, respectively). The mutagenic PCR buffer contained: 80 ng of template pCS831 (a *LEU2/CEN* plasmid containing the *DBP5/RAT8* ORF), 50 mM KCl, 10 mM Tris–HCl pH 8.3, 7 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.2 mM dGTP, 0.2 mM dATP, 1 mM dCTP, 1 mM dTTP, 30 pmol of each primer, and 5 U Taq DNA polymerase. The amplification reaction consisted of 30 cycles, as follows: 94°C, 1 min; 45°C, 1 min, and 72°C, 1 min. To create a fragment suitable for recombination with the PCR products, most of the *DBP5/RAT8* ORF was removed from pCS831 by partial digestion with *Eco*RI followed by digestion with *Eag*I, yielding a linearized plasmid containing short regions of overlap with the PCR products. This linear fragment was co-transformed with the PCR products into strain CSY513, followed by plating onto SC-Leu to select for strains in which the linear plasmid fragment and the mutagenized PCR product recombined to yield a *LEU2/CEN* plasmid. Colonies were then replica-plated onto 5-FOA to select for loss of wild-type *DBP5/RAT8*. Colonies growing on 5-FOA were replica-plated to SC-Leu and plates were incubated at 23 and 37°C (to identify heat-sensitive strains) or at 30°C and 16°C (to identify cold-sensitive strains). Plasmid DNA was isolated from Ura^-Leu^+ colonies that showed the desired temperature-sensitive growth properties (Rose and Broach, 1990) and retransformed into strain CSY513 to confirm that the growth phenotypes were properties of the mutant *DBP5/RAT8* plasmids. The nature of each mutation was determined by DNA sequencing.

Replacement of DBP5/RAT8 with a dbp5/rat8 mutant allele

The mutant *dbp5/rat8* allele present in strain CSY542 was integrated into the yeast genome at the *DBP5/RAT8* locus to yield strain CSY550 by the pop-in/pop-out replacement method (Rothstein, 1991). Briefly, the mutant *dbp5/rat8* ORF from strain CSY542 was subcloned into YIplac211. The resulting plasmid was linearized with either *Eag*I or *Age*I and transformed into wild-type strains FY86 and FY23. The transformants were selected on SC-Ura plates and then restreaked onto 5-FOA plates, to select for replacement of the wild-type gene by the mutant allele. Colonies that grew on 5-FOA were then streaked onto duplicate YPD plates and incubated at 23 and 37°C. The presence of the mutation in strains that failed to grow at 37°C was confirmed by sequencing PCR products made directly from the colonies (see above).

Tagging of Dbp5/Rat8p with the myc-epitope and with green fluorescent protein

DNA fragments encoding either GFP or three copies of the epitope recognized by the anti-myc monoclonal antibody 9E10 were generated by PCR and inserted separately into *DBP5/RAT8* at the unique *Eag*I site 24 bp downstream of the start codon, as previously described (Goldstein, 1995). The primers used for insertion of the 'myc' epitope were 5′-AGACCGGCCGCTAGAGGTGAACAAAG-3′ and 5′-GTCTCGGCC-GATCCGTTCAAGTCTTC-3′. The primers used for insertion of GFP were 5′-GATCCGGCCGTCATGAGTAAAGGAGAAG-3′ and 5′-GAT-CGCGGCCGGGCCAGCACCAGCACC-3′.

Immunofluorescence and in situ hybridization

Indirect immunofluorescence was performed as previously described (Gorsch *et al.*, 1995). Dbp5/Rat8p$_{myc}$ was visualized with monoclonal antibody 9E10, using undiluted tissue culture fluid used for growing the 9E10 hybridoma cell line. An affinity-purified anti-Dbp5/Rat8p antibody (see below) was used at a dilution of 1:10 000. An antibody raised against the FG-repeat region of Rat7p/Nup159p was used at a dilution of 1:3000. The secondary antibodies included FITC- and Texas Red-labeled horse anti-mouse and FITC-labeled goat anti-rabbit antisera (Vector Laboratories), all diluted 1:250. The *in situ* hybridization assay described previously (Amberg *et al.*, 1992; Gorsch *et al.*, 1995; Saavedra *et al.*, 1996) was used to localize poly(A)$^+$ RNA.

Tests for synthetic lethality

Yeast strains of both *MATa* and *MAT*α mating types (CSY560 and CSY561, respectively) carrying the *rat8-2* allele at the *DBP5/RAT8* locus and also carrying wild-type *DBP5/RAT8* on pRAT8-31 (see above) were mated with various mutant strains of interest. The resulting diploids were sporulated and tetrads dissected; haploids were tested for the presence of appropriate auxotrophic markers. In addition, they were tested for their ability to grow on 5-FOA in the absence of the wild-type *DBP5/RAT8* gene. For 10 four-spore tetrads analyzed following sporulation of a strain heterozygous for the *rat8-2* and *rat7-1* mutations, the ratio of those yielding four, three or two haploids able to grow on 5-FOA was 0:7:3. Similarly, the ratio for the 10 four-spore tetrads obtained from a diploid heterozygous for the *rat8-2* and *rat9-1* mutations was 0:6:4; the ratio for the nine four-spore tetrads obtained by sporulating a diploid heterozygous for the *rat8-2* and *nup145-10* mutations was 1:7:1; and the ratio for the eight tetrads obtained from a diploid heterozygous for the *rat8-2* allele, a plasmid-borne *rss1/gle1* mutation, and a disruption of the *RSS1/GLE1* gene was 1:6:1. In cases where four haploids grew on 5-FOA, all four were temperature-sensitive. In all cases where two haploids were able to grow on 5-FOA, neither was temperature-sensitive, and when three haploids could grow on 5-FOA, only one was not temperature-sensitive. All other markers segregated as expected. The other tested heterozygous strains carried *rat8-2* and one of the following mutant alleles: *gsp1-1*, *hmt1*Δ, *rpa190-1*, *rpa190-5*, *rpb1-1*, *prt1-1*, *npl3-17*, *prp20-1* or *rip1*Δ. In all of these cases, all four haploid strains resulting from four-spore tetrads were able to grow at 23°C on plates containing 5-FOA. However, haploid strains which carried both the *rat8-2* and *rip1*Δ mutations were unable to grow at temperatures of 30°C or greater. To test for synthetic lethality between *rat8* alleles and *rna1-1*, a strain disrupted for *RAT8*, carrying wild-type *RAT8* on a *URA3/CEN* plasmid, and carrying the *rna1-1* allele was transformed with a *LEU2/CEN* plasmid carrying ts or cs mutant alleles of *rat8*. Following selection and growth on SC-Leu-Ura plates, colonies were plated to 5-FOA. An inability to grow following loss of wild-type *RAT8* was taken as evidence of synthetic lethality.

Generation of polyclonal antibodies to Dbp5/Rat8p and Western analyses

An antigen for raising antibodies against Dbp5/Rat8p was made as a fusion protein between GST and the N-terminal portion of Dbp5/Rat8p (amino acids 8–105). Briefly, a PCR fragment was synthesized using primers (5′-CGCGGATCCCCGGCCGATTTACTAGCT-3′ and 5′-GCG-AATTCTTGTTAGCATCGTTAAAGAAAAGGC-3′, respectively) that are complementary to the appropriate upstream and downstream portions of the coding sequence for Dbp5/Rat8p and that added to the ends of the PCR product one *Bam*HI and one *Eco*RI site. The conditions used for PCR were the same as described above for the colony PCR. The PCR product was cloned into pGEX-2T (Pharmacia, Inc.) which had been digested in its polylinker region with *Bam*HI and *Eco*RI. After transformation of the resulting plasmid into protease-deficient strain BL21(DE3), fusion protein was prepared essentially as described by Moazed and Johnson (1996), except that the cells were grown in 2XTY with carbenicillin (100 μg/ml) and the protein was induced with IPTG at 0.5 mM for 2 h at 37°C. The fusion protein was eluted from the glutathione–Sepharose beads (Sigma) with 10 mM glutathione and dialyzed against 20 mM HEPES–KOH pH 7.6, 350 mM NaCl, 20% glycerol. This antigen was used to prepare antisera in rabbits (Cocalico, Inc.). To affinity purify antibodies specific for Dbp5/Rat8p, the GST–N-terminal fusion protein was bound to Affi-Gel 10 (Bio-Rad), followed by binding and elution of the antibody, according to the manufacturer's directions. For Western analysis, protein extracts were prepared from cultures in mid-log phase by using the TCA/glass bead procedure (Stutz *et al.*, 1997) and analyzed on 10% polyacrylamide–SDS gels. Dbp5p/Rat8p was visualized using the affinity-purified antibody described above (diluted 1:10 000) and ECL (Amersham, Inc.).

Assay for translation efficiency

Cultures were grown at the permissive temperature (30°C for the cold-sensitive strain and 23°C for all other strains) to mid-log phase; 1.5 OD of cells were then resuspended in 0.5 ml YPD pre-equilibrated at the permissive or non-permissive temperature (16°C for cold-sensitive strain and 37°C for all other strains). After a 5 min preincubation at the same temperature, 10 μCi [^{35}S]Translabel ([^{35}S]methionine plus [^{35}S]cysteine; ICN, Inc.) were added to each culture, followed by a 10 min incubation. The cells were collected by brief centrifugation and TCA/glass bead extracts prepared as described (Stutz *et al.*, 1997). Equal volumes of extracts (representing the same number of cells) were analyzed by SDS–PAGE and the labeled proteins visualized on a PhosphorImager (Molecular Dynamics).

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