

Synthetic Genetic Array Analysis in *Saccharomyces cerevisiae* Provides Evidence for an Interaction Between *RAT8/DBP5* and Genes Encoding P-Body Components

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ABSTRACT

Coordination of the multiple steps of mRNA biogenesis helps to ensure proper regulation of gene expression. The *Saccharomyces cerevisiae* DEAD-box protein Rat8p/Dbp5p is an essential mRNA export factor that functions at the nuclear pore complex (NPC) where it is thought to remodel mRNA/protein complexes during mRNA export. Rat8p also functions in translation termination and has been implicated in functioning during early transcription. We conducted a synthetic genetic array analysis (SGA) using a strain harboring the temperature-sensitive *rat8-2* allele. Although *RAT8* had been shown to interact genetically with >15 other genes, we identified >40 additional genes whose disruption in a *rat8-2* background causes synthetic lethality or dramatically reduced growth. Included were five that encode components of P-bodies, sites of cytoplasmic mRNA turnover and storage. Wild-type Rat8p localizes to NPCs and diffusely throughout the cell but *rat8-2p* localized to cytoplasmic granules at nonpermissive temperature that are distinct from P-bodies. In some genetic backgrounds, these granules also contain poly(A)-binding protein, Pab1p, and additional mRNA export factors. Although these foci are distinct from P-bodies, the two merge under heat-stress conditions. We suggest that these granules reflect defective mRNP remodeling during mRNA export and during cytoplasmic mRNA metabolism.

GENE expression requires several steps that are integrated with one another, beginning with chromatin remodeling, transcription, and pre-mRNA processing, followed by nuclear export, and ending with translation and mRNA turnover (for reviews, see STUTZ and IZARRALDE 2003; RODRIGUEZ *et al.* 2004; COLE and SCARCELLI 2006). The coordination of these events allows for accurate and efficient production of proteins in eukaryotic cells. The coupling of these events is mediated primarily by proteins that interact with mRNAs at different stages of the gene expression pathway. These mRNA/protein complexes are called messenger ribonucleoprotein particles (mRNPs). The association of a particular set of proteins with an mRNA at a given time reflects the step in the gene expression pathway through which an mRNP complex is currently progressing. Deposition of a specific protein on an mRNP may signal the completion of one step. The presence of some

proteins in mRNPs may also inhibit subsequent downstream events of gene expression from occurring prematurely, thus providing coordination.

One important protein involved in the coordination of gene expression events is the essential DEAD-box RNA helicase Rat8p/Dbp5p. DEAD-box helicases function in all steps of RNA metabolism, and several have the ability to unwind short, double-stranded stretches of RNA or remove bound proteins from RNA (for reviews, see CORDIN *et al.* 2006; JANKOWSKY and BOWERS 2006). We identified Rat8p/Dbp5p in a genetic screen designed to isolate temperature-sensitive yeast mutants defective in mRNA export (SNAY-HODGE *et al.* 1998). Rat8p binds to the cytoplasmic fibrils of the nuclear pore complex (NPC), where it interacts directly with two nucleoporins, Rat7p/Nup159p and Gle1p (HODGE *et al.* 1999; STRAHM *et al.* 1999; WEIRICH *et al.* 2004). This positions it to function in the remodeling of mRNPs that is thought to occur as mRNPs pass through NPCs. Rat8p/Dbp5p has also been shown to shuttle in and out of the nucleus (HODGE *et al.* 1999). Rat8p/Dbp5p has RNA-dependent, ATPase activity *in vitro* that is stimulated by Gle1p and the polyphosphoinositide IP₆ (ALCAZAR-ROMAN *et al.* 2006; WEIRICH *et al.* 2006). This indicates that the activity of Rat8p/Dbp5p is spatially regulated and that activation at the nuclear periphery is

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important for mRNP remodeling via removal of bound proteins from mRNP complexes. This would prepare the emerging mRNP for translation and allow recycling of mRNA-binding proteins that accompanied the mRNA during passage through the NPC. These proteins are known to reenter the nucleus to participate in additional rounds of export. Recently, the interaction of Rat8p with Gle1p and IP₆ was shown to enable Rat8p to remove Nab2 from mRNA *in vitro* in an ADP-dependent manner (TRAN *et al.* 2007). Nab2 is one of the mRNA-binding proteins that associates with mRNAs in the nucleus, is important for pre-mRNA processing, and normally leaves the nucleus as part of mRNPs (GREEN *et al.* 2002).

Aside from its function at the cytoplasmic fibrils of NPCs, Rat8p/Dbp5p plays other roles in mRNA metabolism. The *Chironomus tentans* homolog of Rat8p/Dbp5p (Ct-DBP5) binds to Balbiani ring mRNPs co-transcriptionally and was observed on mRNPs from the time of synthesis through translocation through the nuclear pore complex as well as in the cytoplasm as the mRNA associated with ribosomes. This demonstrates that Rat8p/Dbp5p association with mRNA is not restricted to NPC cytoplasmic filaments (ZHAO *et al.* 2002). Rat8p/Dbp5p can be detected diffusely in the cytoplasm and is found in polysomes. Furthermore, it interacts with translation termination factors Sup45p (eukaryotic release factor 1, eRF1) and Sup35p (eukaryotic release factor 3, eRF3) and is required for efficient stop codon recognition as well as for recruitment of Sup35p into termination complexes (GROSS *et al.* 2007). In addition, genetic and physical interactions between Rat8p/Dbp5p and proteins functioning in transcription initiation suggest a nuclear function for Rat8p/Dbp5p (ESTRUCH and COLE 2003).

Previous studies by ourselves (SNAY-HODGE *et al.* 1998; HODGE *et al.* 1999; ESTRUCH and COLE 2003; ESTRUCH *et al.* 2005; GROSS *et al.* 2007) and others (STRAHM *et al.* 1999; MILLER *et al.* 2004; ALCAZAR-ROMAN *et al.* 2006; WEIRICH *et al.* 2006) have revealed extensive genetic interactions between *RAT8* and other genes. Earlier screens identified both suppressors of *rat8-2* and genes that are synthetically lethal (SL) with *rat8-2*. Because many of these were identified only once in these screens, it is clear that the screens were not saturated. In an effort to extend our understanding of Rat8p/Dbp5p, we performed a synthetic genetic array (SGA) analysis (TONG *et al.* 2004) and mated a yeast strain harboring the *rat8-2* allele with a collection of >4000 strains, each disrupted at one nonessential genetic locus.

Here, we report on the outcome of the SGA screen. The screen identified a large number of genes whose disruption was not previously known to be SL with *rat8-2*. These include genes whose products are involved in chromatin remodeling, transcription, mRNA metabolism, and mRNA transport. Particularly noteworthy was finding that *rat8-2* is SL with disruptions of several

genes encoding nonessential components of P-bodies. P-bodies are discrete cytoplasmic foci that were originally characterized as centers for RNA degradation (SHETH and PARKER 2003). Recently, P-bodies have been shown to be sites of mRNA storage as well (BRENGUES *et al.* 2005; TEIXEIRA *et al.* 2005).

The strong genetic interactions between *rat8-2* and mutations affecting proteins involved in mRNA turnover led us to examine the distribution of wild-type and mutant *rat8-2p* in cells lacking a P-body component, and we observed that, at nonpermissive temperatures, *rat8-2p* mislocalizes to cytoplasmic foci that are distinct from P-bodies. Like P-bodies, these foci did not appear if cytoplasmic mRNA was sequestered in polysomes due to the action of cycloheximide, suggesting that these foci also contain mRNA. Several other mRNA export factors, including Mtr2p, Mex67p, and Pab1p, were found in these foci in certain genetic backgrounds. We call these foci RNA export granules (REGs) and suggest that their appearance in some mRNA export mutants reflects a failure to complete remodeling of mRNPs during export. Consistent with this, some mRNAs are degraded more slowly in *rat8-2* cells (HILLERIN and PARKER 2001), which could be due to the continued abnormal association of mRNA export factors with mRNPs, perhaps slowing their entry into P-bodies. REGs and P-bodies show only limited overlap, and this varies depending on which P-body components are examined. However, following heat stress, there is complete overlap of REGs and P-bodies.

MATERIALS AND METHODS

Yeast strains and plasmids: The strains and plasmids used in this study are listed in supplemental Tables 1 and 2, respectively. All strains were grown and media were prepared using standard methods (SAMBROOK *et al.* 1989). Wild-type strains used in this study are the FY23 (*MATa leu2Δ 1 ura3-52 trp1Δ63*) and FY86 (*MATα leu2Δ1 ura3-52 his3Δ200*) derivatives from S288c (WINSTON *et al.* 1995). The strains CHy2000 and PBy1 were created by transformation of the diploid strain SGAY9 with a mixture of PCR products produced using overlapping primers that amplified either the *rat8-2* (CHy2000) or the *mex67-5* (PBy1) allele and the nourseothricin resistance (*nat^r*) gene. The diploids were sporulated and tetrads were dissected. Identification of haploid spores harboring the respective mutations was determined by sequencing following genomic PCR, as well as scoring for cosegregation of resistance to nourseothricin, temperature sensitivity, and a defect in mRNA export. CHy2000 was backcrossed twice to strains yRP1730 (*XRN1-GFP*), yRP1724 (*DHH1-GFP*), and yRP1727 (*DCP2-GFP*) (gifts of Roy Parker) and scored for temperature sensitivity and growth on media lacking leucine. Strains JSy036, JSy041, and JSy040 were derived from individual colonies that were temperature sensitive, while JSy034 was not temperature sensitive. For creation of strain CSy1110, PBy1 was backcrossed to FY23 twice and then crossed to CSy550 (*rat8-2*, FY23 background), and a Ts⁻ spore resulting from a nonparental ditype tetrad was selected.

To generate plasmid pJS3, pCS837 (YCplac111-*RAT8-GFP*) was digested with *PacI* and *AsdI* and the excised *PacI* and *AsdI* fragment was replaced with a fragment encoding the mCherry variant of RFP. This fragment was produced from pRSET-B-

mCherry (SHANER *et al.* 2004) using primers designed to yield *PacI* and *AsdI* ends. This resulted in pJS2 (p*RAT8-RFP*). To create pJS3, the *rat8-2* mutation (P267L) was introduced by digesting pJS2 with *BlnI* and *AgeI*. This removed 700 bp of the *RAT8* ORF. This was replaced with the corresponding *BlnI/AgeI* fragment from pCS542 (YCplac111-*rat8-2*). pCS542-GFP was made by linearizing pCS542 with *EagI* and by ligating in a PCR fragment of DNA coding for GFP with in-frame *EagI* ends. The red mCherry protein is referred to as RFP (red fluorescent protein) throughout.

SGA analysis: SGA analysis was performed via robotics essentially as described (DANIEL *et al.* 2006). Random spore analysis was used to determine if synthetic lethals identified robotically were actually synthetic lethals. Briefly, diploids that were heterozygous for the *rat8-2* mutation and disruption of a nonessential putative interactor were sporulated, spun down, and resuspended in 250 μ g/ml zymolyase. Cultures were rotated for 1 hr at 30°. Spores were disrupted by addition of glass beads and vortexing. Cells were then plated onto canavanine-supplemented plates with either G418 or nourseothricin or with both drugs. Because the genes encoding resistance to each drug are not linked, half of the spores are expected to yield cells resistant to one drug and one quarter of the spores to yield cells resistant to both drugs. Cases where few or no haploids grew on plates containing both drugs were scored as synthetically lethal. Cases where haploids grew considerably more slowly on plates with both drugs than on plates with only one or neither drug were scored as synthetically sick.

Microscopy of live yeast cells: All fluorescent microscopy was performed using live cells grown in SCD media to an OD₆₀₀ of 0.3–0.5. When cells contained GFP- and RFP-tagged proteins, images were obtained for both colors automatically within <1 sec of each other. For temperature shifts, cells were transferred for 20 min (unless otherwise stated) to the indicated temperature (42° for heat shock) and then visualized and photographed immediately (within 4 min). For cycloheximide treatment, cycloheximide was added to a final concentration of 100 μ g/ml. Images were processed using Adobe Photoshop 7.0 (Adobe Systems). The images in Figures 2A, 3B, 7, and supplemental Figure 1 were acquired using a Nikon TE2000-E microscope fitted with a \times 100 Plan Apochromat oil objective (NA 1.4) and an Orca-ER CCD camera (Hamamatsu); images were acquired with Phylum Live Software, version 3.5.1 (Improvision) and processed by using Openlab (Improvision) and Photoshop. GFP and RFP (mCherry; SHANER *et al.* 2004) were visualized by using an X-cite 120 UV lamp and Chroma filter sets. The images in Figure 5 were acquired using a compound wide-field microscope (Olympus model BX51) equipped with a Plan Apochromat \times 60 objective (NA 1.4) and a Sencam QE CCD camera (Cooke); images were processed with IP lab software (Scanalytics). GFP was visualized with a 100-W mercury arc lamp and an Endow GFP filter set (Chroma). All other images were acquired using a confocal microscope (Leica, model TCS SP UV) fitted with a \times 100 Plan Apochromat oil objective (NA 1.4); images were processed with Leica LCS Software. GFP was visualized using an argon laser (488 nm) and RFP was visualized using a krypton laser (568 nm).

RESULTS

SGA analysis of the *rat8-2* allele: Previous work from our laboratory and many others has demonstrated the value of synthetic lethal analyses for understanding the roles that specific *Saccharomyces cerevisiae* gene products

play in mRNA export as well as identifying novel interactions between mRNA export factors and proteins that function in other processes (DEL PRIORE *et al.* 1996; MURPHY *et al.* 1996; STRASSER and HURT 2001; STRASSER *et al.* 2002; GROSS *et al.* 2007). One focus of our work is the DEAD-box protein and mRNA export factor Rat8p/Dbp5p. Previously, multiple types of genetic screens (synthetic lethality, dosage suppression, extragenic suppression; SNAY-HODGE *et al.* 1998; HODGE *et al.* 1999; ESTRUCH and COLE 2003) were employed to learn more about Rat8p/Dbp5p and identified more than a dozen genes that, when mutant, are synthetically lethal with the *rat8-2* Ts⁻ mutation. To extend our genetic analyses further, we performed an SGA analysis using the well-studied *rat8-2* allele (SNAY-HODGE *et al.* 1998).

We replaced the wild-type *RAT8/DBP5* gene in the SGA query strain with *rat8-2* and an adjacent drug resistance marker (nourseothricin resistance). Using the previously described SGA methodology (TONG *et al.* 2001; DANIEL *et al.* 2006), we systematically crossed the query strain to >4000 yeast strains, each harboring a disruption of a nonessential gene (nonessential genes were replaced with a gene encoding kanamycin resistance), and isolated diploids (resistant to both nourseothricin and kanamycin), induced sporulation, and selected at 30° (permissive for *rat8-2*) for haploids resistant to both kanamycin and nourseothricin, and therefore harboring both the *rat8-2* mutation and a deletion of one nonessential gene. Plates were examined visually to identify strains that failed to grow (synthetic lethality) or grew poorly (synthetic sickness) on plates containing both drugs.

The robotic screen was performed twice, followed by a round of screening by hand. After examining data from these analyses, as well as considering gene ontology, 100 potential *rat8-2* synthetic lethals were chosen for further study (supplemental Table 3). A random spore analysis was performed by mating the *rat8-2* strain and the selected knockout strains followed by sporulation of heterozygous diploid strains. The strains were chosen for random spore analysis primarily on the basis of their known or suspected link to nucleic acid metabolism or because of a strong synthetic lethal interaction in both runs of the SGA screen. Resulting haploids were examined on plates containing neither drug, only kanamycin, only nourseothricin, or both drugs. The genes that tested positive for a synthetic interaction with *rat8-2* by using random sporulation analysis (poor or no growth on plates with both drugs) are displayed in Figure 1.

Synthetic lethality or growth defects were seen between *rat8-2* genes whose products are involved in diverse steps of gene expression, extending all the way from chromatin remodeling through pre-mRNA processing and mRNA export to translation and cytoplasmic mRNA turnover. As expected, the synthetic interactions detected included several previously reported for *rat8-2* (SNAY-HODGE *et al.* 1998; LEI *et al.* 2003; MILLER *et al.* 2004): disruptions of *SAC3*, *NUP42*, *NUP133*, and *IPK1*

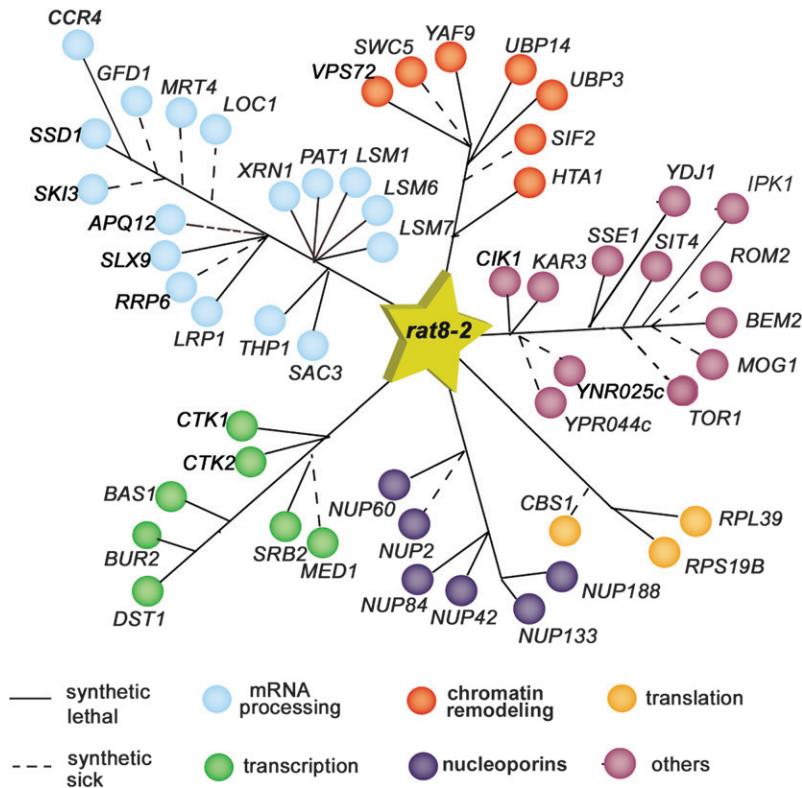


FIGURE 1.—Genetic interaction web displaying the SGA analysis of the *rat8-2* allele. Deletion strains are grouped according to gene ontology.

were identified in this screen. The nucleoporins identified are located at sites throughout the pore, including the cytoplasmic filaments (Nup42p), the central pore framework (Nup84p, Nup133p, Nup188p), and the nuclear basket (Nup2p, Nup60p).

Particularly striking was the discovery that *rat8-2* was synthetically lethal with disruptions of most of the nonessential genes encoding a component of P-bodies (*LSM1*, *LSM6*, *LSM7*, *PAT1*, and *XRN1*). P-bodies are sites where 5′–3′ mRNA degradation takes place in the cytoplasm and are also sites of mRNA storage. They contain the decapping enzyme Dcp1p/Dcp2p, the 5′–3′ exoribonuclease Xrn1p, the DEAD-box protein Dhh1p, the seven Lsm proteins (Lsm1p–Lsm7p), Edc3p, Scd5p, Sbp1p, and Pat1p (PARKER and SHETH 2007). We mated *rat8-2* with *dhh1Δ* and found that the two mutations were not synthetically lethal (data not shown). Previously, another allele of *RAT8* was shown to be synthetically lethal with a disruption of *DH11*, which encodes a DEAD-box protein (TSENG-ROGENSKI *et al.* 2003).

The *rat8-2* protein mislocalizes to cytoplasmic foci that are distinct from P-bodies: Several temperature-sensitive alleles of *RAT8* have been previously characterized (SNAY-HODGE *et al.* 1998; TSENG *et al.* 1998). We determined that protein thermolability was not the cause of temperature sensitivity of the *rat8-2* strain (SNAY-HODGE *et al.* 1998). To determine whether localization of *rat8-2p* and its interaction with NPCs were different from that of wild-type Rat8p/Dbp5p, we constructed a GFP-tagged form of *rat8-2p*. *rat8-2p*-GFP

rescued the *rat8* null mutation at 30° but not at 37° (data not shown, as is also the case with untagged *rat8-2p*). Live cells expressing *rat8-2p*-GFP were compared, both before and after a shift to 37°, to identically treated cells expressing wild-type Rat8p-GFP. As reported previously, wild-type Rat8p-GFP displayed a punctate nuclear rim localization and a diffuse cytoplasmic signal at both 23° and after a 20-min shift to 37° (Figure 2A). A faint nuclear rim localization for *rat8-2p*-GFP was observed at 23°, but it was much less distinct than that of wild-type Rat8p/Dbp5p and the cytoplasmic signal was stronger. After a 20-min shift to 37°, the nuclear rim signal could no longer be detected, and *rat8-2p*-GFP became concentrated in cytoplasmic granules. These granules were detected within 5 min of the shift and became brighter during the following 15–30 min (data not shown). *rat8-2p*-GFP also accumulated in foci following a shift to 34° (Figure 2A), where *rat8-2p* retains sufficient functionality that cells can grow slowly (HODGE *et al.* 1999; ESTRUCH and COLE 2003). Interestingly, cytoplasmic *rat8-2p*-GFP granules were detected even when wild-type Rat8p was also present (supplemental Figure 1).

Because of the localization of *rat8-2p* in cytoplasmic granules and the synthetic interactions between *rat8-2* and disruption of genes encoding P-body components, we investigated whether there was overlap between P-bodies and the *rat8-2p* granules. Because P-bodies are quite small in exponentially growing cells, cells were grown to an A_{600} of 8, which leads to enlarged P-bodies (BREGUES and PARKER 2007). We saw no overlap

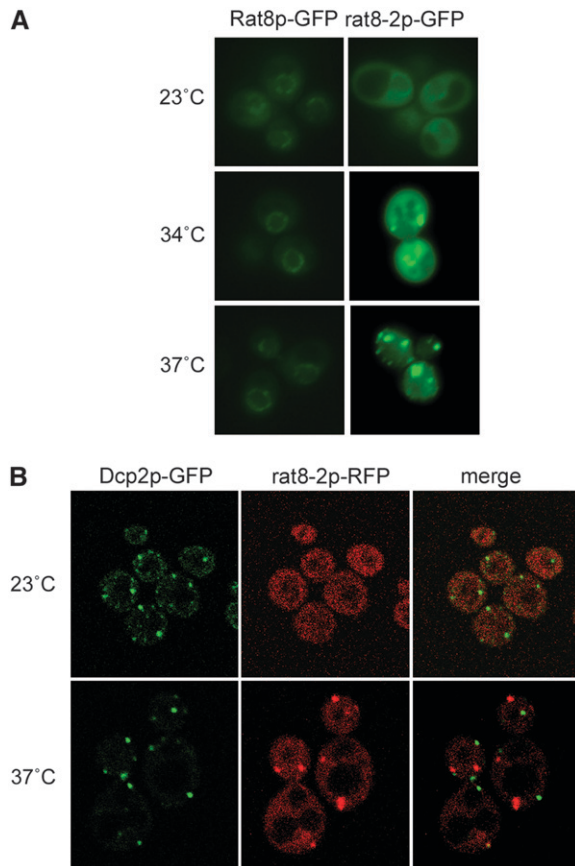


FIGURE 2.—The mutant mRNA export factor rat8-2p mislocalizes to cytoplasmic granules distinct from P-bodies at the nonpermissive temperature. (A) CSy513 (*Rat8::His*) cells harboring either pCS835 (*pGFP-RAT8*) or pCS542-GFP (*pGFP-rat8-2*) were visualized at room temperature or after a shift to 37° for 20 min. (B) JSy040 (*DCP2-GFP/rat8-2*) cells were transformed with pJS3 (*prat8-2-RFP*) and visualized after a 20-min shift to 37°.

between rat8-2p-RFP and Dcp2p-GFP (Figure 2B) and very limited overlap between rat8-2p-RFP and Xrn1p-GFP (supplemental Figure 2A). A very similar result was obtained when cells were examined while in exponential growth (supplemental Figure 2B), although in this experiment, a small minority of rat8-2p-RFP foci overlapped with Dcp2p foci, while most rat8-2p foci and P-bodies did not overlap.

Additional mRNA export factors are found in cytoplasmic granules: We wondered whether other RNA export factors were present in *rat8-2* granules or in cells that contained mutant alleles of genes encoding other mRNA export factors. The mRNA export receptor Mex67p is normally detected at the nuclear periphery where it binds to components of the NPC (SEGREF *et al.* 1997). It was reported previously that the protein encoded by the *mex67-5* Ts⁻ allele mislocalizes to cytoplasmic foci after a shift to the nonpermissive temperature (37°), both when *MEX67* is mutated (*e.g.*, *mex67-5*) and when the gene encoding its binding partner, Mtr2p, is mutant (SANTOS-ROSA *et al.* 1998). To determine if the

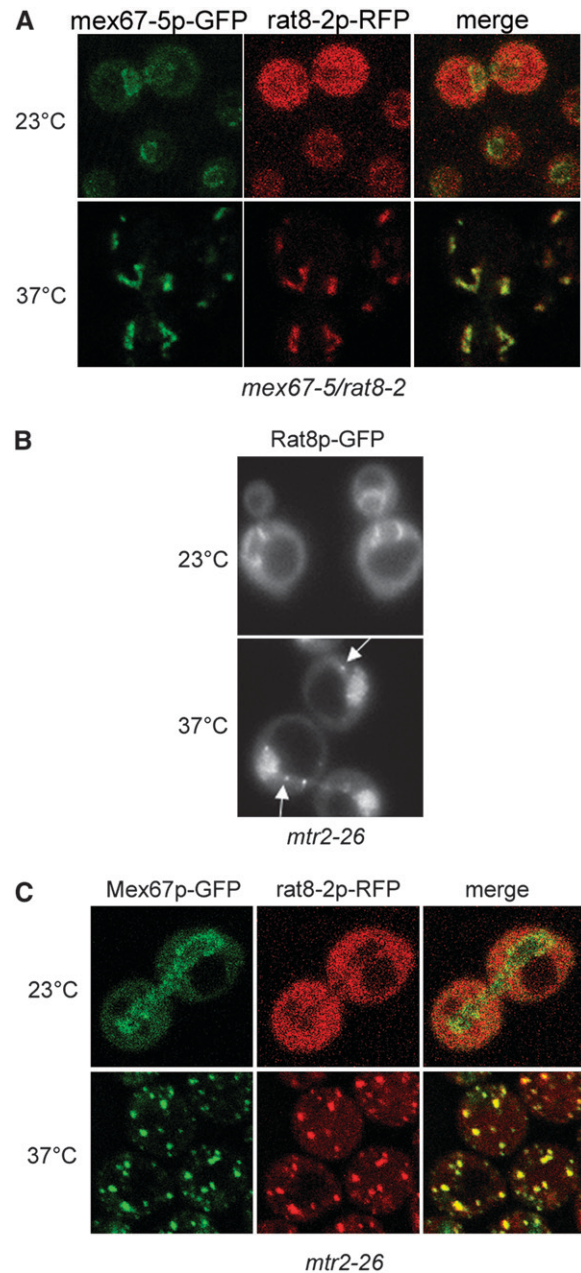


FIGURE 3.—The mutant mRNA export factor rat8-2p colocalizes with Mex67 under certain genetic conditions. (A) CSy1110 cells were transformed with pJS3 (*prat8-2-RFP*) and *p mex67-5-GFP* and visualized prior to or after a 20-min shift to 37°. (B) *mtr2-26* cells were transformed with pCS835 (*pGFP-RAT8*) and visualized at room temperature or after a 20-min shift to 37°. (C) *mtr2-26* cells were transformed with both *pMEX67-GFP* and pJS3 (*prat8-2-RFP*) and visualized before and after a 20-min shift to 37°.

mex67-5p granules were the same as *rat8-2p* granules, plasmids expressing *rat8-2p-RFP* and *mex67-5p-GFP* were introduced into a *mex67-5/rat8-2* double-mutant strain. Interestingly, the double mutant grows as well as either single-mutant strain. We observed extensive colocalization of *rat8-2p-RFP* and *mex67-5p-GFP* in cytoplasmic granules following a shift to 37° (Figure 3A).

Since mutation of *MTR2* has a profound effect on the localization of wild-type Mex67p, we tested for perturbations in the location of wild-type Rat8p-GFP in the *mtr2-26* mutant, both before and after a shift to the nonpermissive temperature. As seen in Figure 3B, Rat8p-GFP was detected primarily at the nuclear periphery at the permissive temperature but became predominantly nuclear following a shift to 37°. This is not surprising, since mislocalization of Rat8p to the nucleus also occurs in a *mex67-5* strain (HODGE *et al.* 1999). This indicates that Rat8p becomes predominantly nuclear when either *mex67* or *mtr2* are mutated, conditions that cause Mex67p to mislocalize to cytoplasmic granules. However, some wild-type Rat8p-GFP could be detected in bright foci in *mtr2-26* cells (arrows in Figure 3B). However, these foci were seen in only a fraction of cells, while all cells had many bright foci containing Mex67p (Figure 3C). Furthermore, the Rat8p-GFP foci were found very close to the nucleus and may be associated with it. Therefore, the Rat8p-GFP foci in *mtr2-26* cells are probably not REGs. We conclude that wild-type Mex67p and mutant *mex67-5p* localize to cytoplasmic granules in certain mutant yeast strains.

We also transformed *mtr2-26* cells with plasmids encoding Mex67p-GFP and *rat8-2p*-RFP and shifted the cells to the nonpermissive temperature. All granules that contained *rat8-2p*-RFP also contained wild-type Mex67p-GFP, although a fraction of Mex67p-GFP was still at the nuclear periphery (Figure 3C). However, redistribution of *rat8-2p* to granules did not depend on Mex67p also localizing to granules, since granules containing *rat8-2p* were seen even when Mex67p and Mtr2p were wild type and properly localized (Figures 2A; see also Figure 4, top). Similarly, localization of *mex67-5p* to granules did not depend on the presence of *rat8-2p* since *mex67-5p* localized to granules even when *RAT8* was wild type and Rat8p was correctly localized (Figure 4, top, and see SEGREF *et al.* 1997). Thus, these proteins show independent localization to granules and when both localize to granules in the same cell, they colocalize. Because these granules contain mRNA export factors (mutant, and sometimes wild-type, factors, depending on the genotype of the cells), we have designated them RNA export granules, or REGs.

Like P-bodies, granules that contain *rat8-2* are not seen if cytoplasmic mRNA is sequestered in polysomes: It has been shown that P-bodies contain mRNA and do not form if mRNA is sequestered in inactive polysomes (SHETH and PARKER 2003). To determine whether the *rat8-2p* and *mex67-5p* granules share this property with P-bodies, we treated cells with inhibitors of translation. Cycloheximide blocks ribosomal translocation and prevents release of mRNA from polysomes. Cells expressing either *rat8-2p*-GFP or *mex67-5p*-GFP were treated with cycloheximide for 20 min prior to, simultaneously with, or after a 20-min shift to 37° (Figure 4, bottom). When cycloheximide was added following the

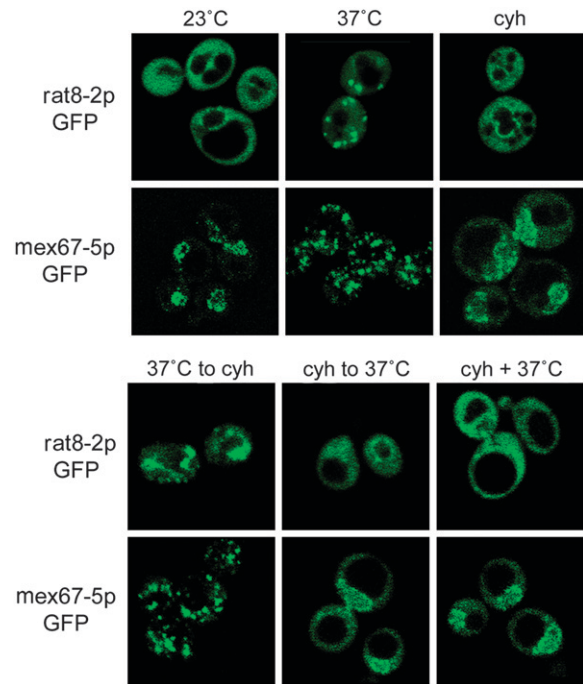


FIGURE 4.—Formation of the granules that contain mRNA export factors requires nonpolysomal mRNA. CSy513 (*rat8::His*) harboring either pCS835 (*pRAT8-GFP*) or pCS542 (*prat8-2-GFP*) and CSy956 (*MEX67Δ*, *p mex67-5-GFP*) cells were visualized at room temperature after a 20-min temperature shift to 37°, 20 min after treatment with cycloheximide, or after a simultaneous temperature shift and cycloheximide treatment (“cyh + 37°”). In addition, cells that had been exposed to cycloheximide for 20 min were then shifted to 37° for 20 min (“cyh to 37°”); cells that had been shifted to 37° for 20 min were then treated with cycloheximide and kept at 37° for an additional 20 min (“37° to cyh”).

shift, *rat8-2p*-GFP and *mex67-5p*-GFP remained in cytoplasmic granules. However, when cells were treated with cycloheximide before or simultaneously with the shift to 37°, these proteins did not redistribute to granules (Figure 4). We conclude that formation of these granules depends on the availability of nonpolysomal cytoplasmic mRNA.

Effect of *rat8-2* on the localization of P-body proteins and the effect of absence of P-body proteins on the distribution of Rat8p: To further characterize the relationship between P-body components and Rat8p/Dbp5p, we sought to determine whether or not mutation of *RAT8* would have an effect on P-body size or number. We compared the distribution of Xrn1p-GFP in wild-type and *rat8-2* cells at 23° and following a shift to 37° (Figure 5). In wild-type cells at both temperatures, Xrn1p-GFP was distributed diffusely throughout the cytoplasm with very small foci of Xrn1p-GFP detected against a bright background. In contrast, in *rat8-2* cells shifted to 37°, Xrn1p foci were considerably more distinct, due primarily to a reduction in the intensity of the diffuse cytoplasmic signal. Even at 23°, the diffuse cytoplasmic signal for Xrn1p-GFP was lower in *rat8-2*

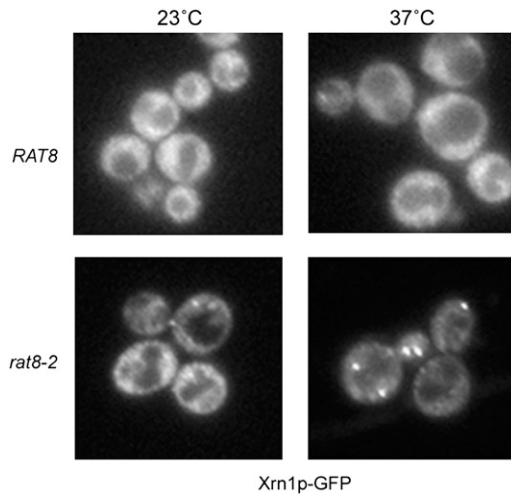


FIGURE 5.—Effect of the *rat8-2* mutation on the localization of Xrn1p-GFP. JSy034 (*XRN1-GFP*) or JSy036 (*XRN1-GFP, rat8-2*) cells were visualized at room temperature and after a 20-min shift to 37°.

cells than in wild-type cells and, in some cells, P-bodies were more easily seen. Similar analyses were performed using GFP fusions to other P-body proteins, and little difference was seen when comparing wild-type and *rat8-2* cells (data not shown).

Pab1p localizes to REGs in a *mex67-5*-dependent manner: Pab1p shuttles between nucleus and cytoplasm but is more abundant in the cytoplasm. We reported previously (DUNN *et al.* 2005) that Pab1p accumulates in nuclei in several strains with defects in mRNA export following a shift to 37°, including *xpo1-1*, *mal1-1*, and *prp20-1*. Rat8p/Dbp5p also accumulates in the nuclei of these strains. Only in *mex67-5* cells does Rat8p/Dbp5p but not Pab1p accumulate in nuclei. We investigated the localization of Pab1p-GFP in double-mutant *mex67-5/rat8-2* cells expressing *rat8-2p-RFP*. Following a shift to 37°, Pab1p-GFP and *rat8-2p-RFP* colocalized to REGs (Figure 6A). While most *rat8-2p-RFP* was in granules at 37°, a substantial fraction of Pab1p-GFP remained uniformly distributed in the cytoplasm. Remarkably, all granules containing *rat8-2p-RFP* also contained Pab1p-GFP. We found that Pab1p-GFP was also present in granules in *mex67-5* and *rat8-2/mex67-5* cells shifted to 37° (Figure 6B), but did not localize to granules in *MEX67/rat8-2* cells or in *MEX67/mex67-5* cells, even though *mex67-5p* is still found in granules at 37° when wild-type Mex67p was also present (Figure 6C). Thus, the absence of wild-type Mex67p, but not of wild-type Rat8p/Dbp5p, was required for localization of Pab1p to REGs.

P-bodies and REGs overlap during stress conditions: Previously, it was shown that Pab1p could be found in P-bodies under the stress conditions of glucose deprivation and growth to stationary phase (BRENGUES and PARKER 2007), although the signal for Pab1p-GFP in foci following glucose deprivation was only moderate. We wondered how stress would affect the localization of

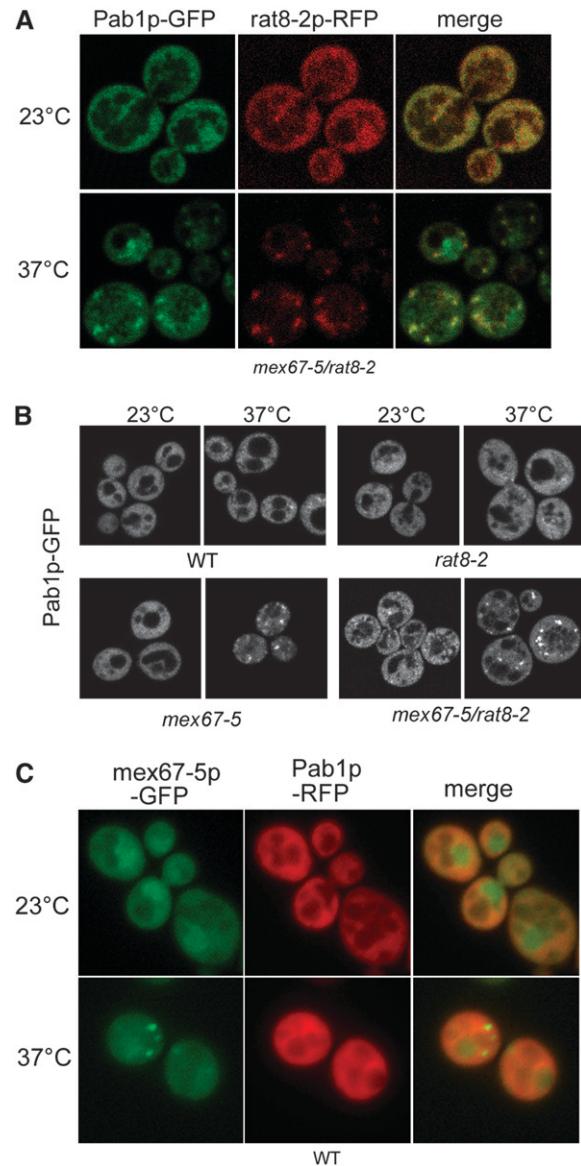


FIGURE 6.—(A) CSy1110 (*mex67-5/rat8-2*) cells were transformed with pCMH035 (*pPABI-GFP*) and pJS3 (*prat8-2-RFP*). Cells were visualized before and after a 20-min shift to 37°. (B) Cells were transformed with pCMH035 (*pPABI-GFP*) and visualized either at 23° or after a 20-min shift to 37°. (C) Wild-type cells containing *pPABI-RFP* and *pmex67-5-GFP* were visualized at 23° and after a 20-min shift to 37°.

proteins found in REGs and analyzed the distribution of *rat8-2p-RFP*, Xrn1p-GFP, and Dcp2p-GFP under heat-stress conditions by shifting cells to 42° for 20 min. As shown in Figure 7A, heat stress caused the two types of cytoplasmic foci to merge. This suggests that there may be some functional overlap between P-bodies and REGs.

A shift of cells to 37° prior to shifting to 42° induces thermotolerance in yeast cells, allowing continuation of some processes that normally cease following heat shock (*e.g.*, splicing) (YOST and LINDQUIST 1991). We wondered whether or not incubating cells at 37° (a temperature where P-bodies and REGs do not overlap)

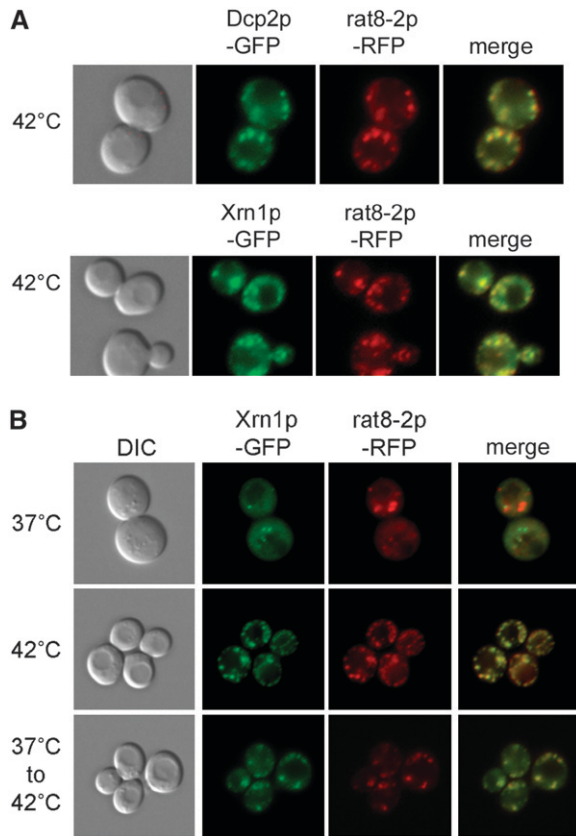


FIGURE 7.—Under stress conditions, REGs and P-bodies colocalize. (A) JSy040 (*DCP2-GFP/rat8-2*) and JSy036 (*XRN1-GFP/rat8-2*) were transformed with pJS3 (*prat8-2-RFP*) and visualized 20 min after a shift to 42°. (B) JSy036 cells were transformed with pJS3 and were shifted to either 37° or 42° for 20 min and visualized. Cells that had been shifted to 37° were subsequently shifted to 42° for 20 min and visualized again (37°–42°). DIC, differential interference contrast microscopy.

prior to shifting to 42° would prohibit the two from colocalizing. As shown in Figure 7B, prior incubation at 37° did not prevent the two types of foci from merging following a shift to 42°. However, under these conditions, there was a modest reduction in the number of foci containing Xrn1p and rat8-2p.

DISCUSSION

SGA analysis of the *rat8-2* mutation: SGA screens have proven to be a powerful tool for gaining insight into and information about yeast gene products. In the two independent robotic screens performed with *rat8-2*, we identified 217 and 294 genes with possible synthetic interactions with *rat8-2*. A total of 108 strains were found in both screens. We chose 100 for random spore analysis, on the basis of both the results from the robotic screens and gene ontology, focusing on genes whose products function or may function in gene expression. Random spore analyses confirmed synthetic interactions between *rat8-2* and 52 of these. These can be

clustered into several subgroups on the basis of function (see Figure 1). The average nonessential gene has ~30 synthetic genetic interactions (TONG *et al.* 2004). The fact that the *rat8-2* mutation has a much higher number likely reflects that *DBP5/RAT8* is an essential gene and that Rat8p functions not only in mRNA export but also in additional aspects of mRNA metabolism (ESTRUCH and COLE 2003; GROSS *et al.* 2007).

The *rat8-2* mutation is synthetically lethal with several genes functioning in chromatin modification and transcription. A recent report identified a robust network of synthetic genetic interactions among genes involved in chromatin remodeling and/or transcriptional elongation (KROGAN *et al.* 2003). In addition, that study also found interactions between chromatin-remodeling genes and genes coding for proteins that participate in other aspects of RNA metabolism, including *CCR4*, encoding an mRNA deadenylase; *NUP60*, encoding a nucleoporin; and both *SAC3* and *THP1*, encoding proteins that associate with NPCs and connect mRNA biogenesis and export. The finding that *rat8-2* was also synthetically lethal with *sac3Δ*, *thp1Δ*, *ccr4Δ*, and *nup60Δ* fits with Rat8p being among the proteins involved in linking diverse steps of mRNA metabolism.

Other groups have examined the collection of strains lacking a nonessential gene for defects in mRNA metabolism. HIERONYMUS *et al.* (2004) screened the entire knockout collection using *in situ* hybridization to determine whether there were additional nonessential genes beyond the many already identified that might be important for mRNA export. They identified only four genes, *lhp1Δ*, *rrp6Δ*, *slx9Δ*, and *apq12Δ*, and all four were identified in our SGA screen. Roy Parker's group screened the same strain collection for defects in mRNA poly(A) tail length (BAKER *et al.* 2004) and identified *apq12Δ*. Our analysis indicates that Apq12p function is important for proper nuclear pore biogenesis (SCARCELLI *et al.* 2007). Therefore, *apq12Δ* likely affects mRNA metabolism indirectly. Additional studies will be required to understand the mechanistic basis for many of the *rat8-2* genetic interactions that we detected.

Multiple types of mRNA-containing granules are found in yeast cells: Cytoplasmic mRNA-containing granules are present in both yeast and metazoan cells. Both contain P-bodies, sites where 5'–3' mRNA degradation takes place (for review, see PARKER and SHETH 2007). Treatment of metazoan cells with heat or other stresses leads to the formation of stress granules (SGs), which are distinct from, although often adjacent to, P-bodies (KEDERSHA *et al.* 2005). SGs are thought primarily to be sites where mRNAs are stored during stress, when the pattern of protein synthesis shifts to produce high levels of stress response proteins (for review, see ANDERSON and KEDERSHA 2006). SGs have not been identified in yeast.

The studies presented here show that, in certain genetic backgrounds, mutant, and sometimes wild-type,

mRNA export factors, Rat8p and Mex67p, as well as Pab1p, can be found in granules (*e.g.*, REGs) that are distinct from P-bodies. Although we have not shown directly that REGs contain mRNA, this is highly likely because REGs, like P-bodies (SHETH and PARKER 2003), do not form if mRNAs are sequestered in polysomes due to cycloheximide treatment (Figure 3A) whereas treatment with another protein synthesis inhibitor, puromycin, which leads to release of mRNA from polysomes, does not prevent formation of REGs (Figure 3B) or P-bodies (J. J. SCARCELLI and C. N. COLE, unpublished results). When yeast cells are heat-shocked, the components of REGs and P-bodies colocalize in granules (Figure 7).

What are REGs? REGs differ from P-bodies in several ways. First, the conditions under which each can be seen and the way in which each responds to changes in growth conditions are distinct. P-bodies increase in number when a nonessential P-body component is missing and intermediates in the turnover process accumulate, indicating that P-body function is compromised (BOUVERET *et al.* 2000). P-bodies also increase when cells reach stationary phase (BRENGUES and PARKER 2007), a condition where translation slows and more mRNAs may need to be stored or degraded. mRNA export factors are not found in P-bodies and P-body proteins are absent from or present at a very low level in REGs (Figure 2B).

Pab1p is difficult to detect in P-bodies when cells are growing exponentially and is present at a very low level in P-bodies in cells deprived of glucose and at an increased level in cells grown to stationary phase (BRENGUES and PARKER 2007). In contrast, in *mex67-5* cells, a strong signal for Pab1p-GFP is detected in REGs. Growth to stationary phase also leads to enlarged and more numerous P-bodies, but this treatment had no effect on REGs (Figure 2B). The fact that the number of P-bodies is normally very low suggests that turnover is usually efficient enough that few P-bodies are present in exponentially growing wild-type cells, and the few that are detected during normal growth may be different from those that appear when there is a defect in 5'-3' mRNA turnover. For example, P-bodies in normal cells may contain RNAs that are unusually difficult to degrade.

In contrast to P-bodies, REGs are not detected in wild-type cells. The dramatic appearance of large numbers of REGs in response to mutations affecting Rat8p/Dbp5p, Mex67p, and Mtr2p suggests that some step of cytoplasmic mRNA metabolism is compromised by mutation of these genes and that this process normally occurs with sufficient efficiency that REGs are not seen. Because mRNA export slows dramatically or ceases in these strains within minutes of a shift to nonpermissive temperature and REGs appear very rapidly, we think it is likely that mRNPs that become components of REGs were exported prior to the temperature shift, even though slow export of some mRNA appears to occur

in *rat8-2* and *mex67-5* strains at nonpermissive temperature (HILLERIN and PARKER 2001).

HILLERIN and PARKER (2001) showed that some mRNAs become more stable when *rat8-2* and *mex67-5* cells are shifted to nonpermissive temperature. We suggest that this increased mRNA stability occurs because of a defect in remodeling of mRNPs during export slows the movement of mRNPs through various cytoplasmic compartments and eventually to P-bodies. The finding that the *rat8-2* mutation had little effect on the number or size of P-bodies suggests that the mRNAs in REGs are readily degraded once they are able to interact with P-body proteins.

The observation that mutant *rat8-2p* and *mex67-5p* enter REGs even when wild-type Rat8p or Mex67p, respectively, are present, suggests that, in cells containing both mutant and wild-type protein, both interact with mRNA. Because these mutations are recessive yet REGs still form, the presence of the wild-type proteins is not sufficient to overcome the conditions that lead *rat8-2p* and *mex67-5p* to enter REGs, yet the formation of REGs does not prevent growth and mRNA export from occurring at wild-type rates.

An intriguing finding is that Pab1p is found in REGs in *mex67-5* cells but not in cells that express both wild-type and mutant Mex67p, nor in *rat8-2* cells. We reported earlier that Rat8p/Dbp5p accumulates in nuclei in many strains that are defective for nuclear transport, including *xpo1-1*, *rna1-1*, *prp20-1*, and *mex67-5* (HODGE *et al.* 1999). Pab1p also accumulates in nuclei in each of these strains except *mex67-5*. This suggests that Mex67p plays a role in nuclear import of Pab1p. This could be direct or indirect, but one possibility is that Mex67p interacts with Pab1p bound to mRNA, releasing it from this association and permitting it to be reimported into the nucleus for loading onto new mRNAs. Although Pab1p is thought to be able to rebind mRNA in the cytoplasm, perhaps it cannot rebind to mRNAs sequestered in REGs.

BRENGUES and PARKER (2007) described an mRNP complex that can appear in P-bodies following glucose deprivation or growth to stationary phase. These mRNPs contained polyadenylated mRNA, Pab1p, eIF4E (Cdc33p), and eIF4G2 (Tif4632p), although other translation initiation components analyzed were absent. We observed that glucose deprivation and growth to stationary phase do not lead to the appearance of REGs. Although REGs are likely to contain polyadenylated RNA and, in *mex67-5* cells, Pab1p, we did not find eIF4E/Cdc33p in REGs (J. J. SCARCELLI and C. N. COLE, unpublished results) and did not test whether eIF4G2 was present. We suspect that under the conditions used by BRENGUES and PARKER (2007), mutant mRNA export factors do not remain associated with mRNAs released from polysomes, even though *rat8-2p* is present in polysomes (GROSS *et al.* 2007). In contrast, at 37°, and to a lesser extent at 34°, *rat8-2p* and *mex67-5p* appear

unable to terminate their association with mRNA, and REGs form when these mRNAs are released from polysomes. HOYLE *et al.* (2007) also observed a class of mRNP-containing granules distinct from P-bodies that formed after glucose deprivation and contained, in addition to mRNA, eIF4E, eIF4G1, and Pab1p.

Under heat-shock conditions, the cellular response leads to formation of granules that contain not only P-body components but also mRNA export factors and Pab1p. These granules may be a yeast form of SGs, even though P-body proteins are not found in mammalian SGs but are often adjacent to them. Additional studies will be required to better define the role and composition of the granules that form following heat shock.

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