Genetic Interactions of Yeast Eukaryotic Translation Initiation Factor 5A (eIF5A) Reveal Connections to Poly(A)-Binding Protein and Protein Kinase C Signaling

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ABSTRACT

The highly conserved eukaryotic translation initiation factor eIF5A has been proposed to have various roles in the cell, from translation to mRNA decay to nuclear protein export. To further our understanding of this essential protein, three temperature-sensitive alleles of the yeast *TIF51A* gene have been characterized. Two mutant eIF5A proteins contain mutations in a proline residue at the junction between the two eIF5A domains and the third, strongest allele encodes a protein with a single mutation in each domain, both of which are required for the growth defect. The stronger *tif51A* alleles cause defects in degradation of short-lived mRNAs, supporting a role for this protein in mRNA decay. A multicopy suppressor screen revealed six genes, the overexpression of which allows growth of a *tif51A-1* strain at high temperature; these genes include *PAB1*, *PKC1*, and *PKC1* regulators *WSC1*, *WSC2*, and *WSC3*. Further results suggest that eIF5A may also be involved in ribosomal synthesis and the *WSC/PKC1* signaling pathway for cell wall integrity or related processes.

REGULATION of the fate of cytoplasmic mRNA involves a delicate balance between its recruitment to ribosomes for translation and its degradation by nucleolytic enzymes. As researchers have studied separately the factors that are important for translation and those that are required for mRNA turnover, it has become clear that these two processes are intimately linked. eIF5A is an enigmatic protein that has been implicated in several steps of RNA metabolism including both translation and mRNA degradation.

eIF5A is a highly conserved protein encoded in the genomes of eukaryotes and archaebacteria (Chen and Liu 1997). Yeast and mammalian eIF5A proteins are 63% identical, suggesting the importance of this protein in basic cellular processes (Schnier *et al.* 1991). Originally purified from ribosomes of rabbit reticulocyte lysates (Kemper *et al.* 1976), eIF5A was described as a translation initiation factor due to its ability to stimulate the synthesis of methionyl-puromycin *in vitro* (Benne and Hershey 1978; Park *et al.* 1993). However, depletion of this factor in yeast caused only a small (30%) reduction in the protein synthesis rate (Kang and Hershey 1994). These results have been used to argue against eIF5A being an initiation factor for general protein synthesis.

Alternatively, it was suggested that eIF5A could be involved in the translation of a specific subset of mRNAs, for example, those involved in the cell cycle progression (G1/S transition; Park *et al.* 1993, 1997). Expression of eIF5A has also been correlated with cell proliferation: an increase in G1-arrested cells is observed after depletion of this factor in yeast (Kang and Hershey 1994). Conversely, eIF5A expression is induced in activated human T lymphocytes (Bevec *et al.* 1994).

The yeast *Saccharomyces cerevisiae* contains two 90% identical genes encoding eIF5A, *TIF51A* (*HYP2*), and *TIF51B* (*HYP1*) (Schnier *et al.* 1991), and each of these proteins undergoes two post-translational modifications. The N-terminal acetylated serine residue of eIF5A is phosphorylated (Kang *et al.* 1993; Klier *et al.* 1993), and mutational analysis revealed that unphosphorylated eIF5A is sufficient to support growth of a *tif51A*Δ haploid strain (Klier *et al.* 1993), demonstrating that phosphorylation is not essential for eIF5A function *in vivo*. The second modification is hypusination of a specific lysine (Chen and Liu 1997; Park *et al.* 1997). Hypusination of eIF5A is essential in yeast: strains in which hypusination is blocked, by mutation of the target lysine (K51R) or deletion of the deoxyhypusine synthase gene, are inviable (Schnier *et al.* 1991; Sasaki *et al.* 1996; Park *et al.* 1998). Similarly, inhibitors of hypusination block the proliferation of different mammalian cell lines (Han-

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TABLE 1

Yeast strains used in this study

Strain	Genotype	Source
W303a	*MAT***a** *ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100*	A. Tzagoloff
W303α	*MAT*α *ade2-1 trp1*Δ*63 ura3-1 leu2-3,112 his3-11,15 can1-100*	A. Tzagoloff
FY23	*MAT***a** *ura3-52 trp1*Δ*63 leu2*Δ*1*	F. Winston
DL376	*MAT***a** *trp1-1 ura3-52 leu2-3,112 his4 can1*^{r} *pkc1*Δ*::LEU2*	D. Levin
xpo1-1	*MAT***a** *ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 xpo1::LEU2 + pKW457 (xpo1-1 HIS3)*	K. Weis
PSY8	*MAT***a** *ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 cyt1::HIS3 tif51A-1*	A. Chiang
PSY338	*MAT*α *ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 cyt1::HIS3 tif51A-2*	A. Chiang
PSY341	*MAT***a** *ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100 cyt1::HIS3 tif51A-3*	A. Chiang
PSY1241	*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11 can1-100 TIF51A::URA3*	This study
PSY1242	*MAT*α *ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 cyt1::HIS3 tif51A-1*	This study
PSY1243	*MAT*α *ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 cyt1::HIS3 tif51A-2*	This study
PSY1244	*MAT*α *ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 cyt1::HIS3 tif51A-3*	This study
PSY1245	*MAT*α *ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 tif51A-1*	This study
PSY1248	*MAT***a** *ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 tif51A-2*	This study
PSY1249	*MAT***a** *ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 tif51A-3*	This study
PSY1250	*MAT***a**/α *ura3-52/- leu2*Δ*1/- his3*Δ*200/- trp1*Δ*63/+*	This study
PSY1251	*MAT*α *ura3-52 leu2*Δ*1 his3*Δ*200 tif51a::HIS3* [pPS1592]	This study
PSY1252	*MAT***a** *ade2-1 trp1-1 ura3-1 his3-11,15 leu2-3,112 can1-100 cyt1::HIS3 tif51A-1*	This study

auske-Abel *et al.* 1994; Park *et al.* 1994; Chen *et al.* 1996; Shi *et al.* 1996a). eIF5A is the only eukaryotic protein known to be hypusinated.

Although eIF5A has been suggested to participate in the nucleocytoplasmic trafficking of the HIV-1 Rev protein/RRE complex (Ruhl *et al.* 1993; Bevec *et al.* 1996; Bevec and Hauber 1997; Liu *et al.* 1997; Hofmann *et al.* 2001), other studies do not agree on such a role for eIF5A (Henderson and Percipalle 1997; Shi *et al.* 1997; Lipowsky *et al.* 2000). Localization studies also do not support the involvement of this protein in the nuclear transport of macromolecules. In mammalian cells, eIF5A is mainly cytoplasmic with a fraction associated with the endoplasmic reticulum (ER) membrane through ribosomes (Shi *et al.* 1996b). Its cytoplasmic localization, combined with its interaction with the ribosomal protein L5 (Schatz *et al.* 1998), strengthens the connection between eIF5A and translation.

Recently it was shown that a yeast mutant harboring a temperature-sensitive allele of *TIF51*A exhibits a defect in mRNA decay, accumulating uncapped mRNAs at the restrictive temperature. In addition, this strain shows an ~30% decrease in protein synthesis at high temperature (Zuk and Jacobson 1998). These results suggest that eIF5A may be involved in mRNA degradation as well as translation.

Here we have characterized three novel alleles of *TIF51A* and have used them to address the proposed functions of eIF5A. We also present data linking eIF5A with both poly(A)-binding protein and protein kinase C. The isolation of *PAB1* and *PKC1* as multicopy suppressors of a temperature-sensitive allele of *TIF51A*, *tif51A-1*, suggests important connections between these three proteins and their roles in RNA metabolism, including translation, mRNA decay, and ribosome biogenesis.

MATERIALS AND METHODS

Yeast strains, growth conditions, and plasmids: *S. cerevisiae* strains and their genotypes used in this work are listed in Table 1. Procedures for cell growth and genetic manipulations were according to standard protocols (Rose *et al.* 1990). Plasmids used in this work were obtained as described below and are listed in Table 2. Cloning by PCR was performed with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) or Vent DNA polymerase (New England Biolabs, Beverly, MA) and oligonucleotides listed in Table 3.

Construction of a marked *TIF51A* allele and linkage assays: To mark *TIF51A* with the *URA3* gene, a 1.4-kb *Pst*I fragment containing *TIF51A* was removed from the plasmid pPS1483 and ligated into the *Nsi*I site of the vector YIp5, generating the plasmid pPS1591. This plasmid was linearized using *Hpa*I, which cleaves within the *TIF51A* gene, and used to transform a W303a/α diploid strain. After selection on uracil dropout plates, transformed diploids were sporulated. Genomic DNA from the selected diploids and their haploid progeny was digested with *Pst*I and subjected to Southern blot analysis using the 1.4-kb *Pst*I fragment as a probe. One of the haploid cells that contained the *URA3* gene integrated next to *TIF51A* was chosen for linkage assay (PSY1241). To examine linkage of the *tif51A* temperature-sensitive (ts) alleles and *TIF51A*, the *tif51A* mutants (PSY1242–PSY1244) were initially crossed to this strain containing a *TIF51A*-marked allele (PSY1241). The diploids resulting from these crosses were sporulated and subjected to tetrad analysis.

Amplification and sequencing of the *tif51A* temperature-sensitive alleles: Genomic DNA was prepared from the *tif51A* temperature-sensitive strains (PSY1245, PSY1248, and PSY1249). The *TIF51A* gene was amplified by PCR using Taq DNA polymerase (Perkin-Elmer, Norwalk, CT) and the primers NPL2-A and NPL2-B. PCR products were purified and se-

TABLE 2

Plasmids used in this study

Plasmid	Features	Source
pGEX-4T-1	Bacterial expression vector for GST fusion proteins	Pharmacia
pRS315	Yeast LEU2 CEN expression vector	Sikorski and Hieter (1989)
pRS316	Yeast URA3 CEN expression vector	Sikorski and Hieter (1989)
pRS426	Yeast URA3 2μ expression vector	Christianson *et al.* (1992)
pPS1483	1.4-kb PstI fragment with *TIF51A* in YCpLacIII LEU2 CEN	R. Zitomer
pPS1494	Yeast Gal GFP-Rev URA3 2μ	Taura *et al.* (1998)
YIp5	URA3 targeted integration vector	Struhl *et al.* (1979)
pCGF-1a	Yeast Gal GFP URA3 2μ expression vector	Kahana and Silver (1996)
pPS1591	*TIF51A Pst*I fragment in YIp5	This study
pPS1592	*TIF51A* in pRS316	This study
pPS1593	*tif51A-1* in pRS315	This study
pPS1594	*tif51A-2* in pRS315	This study
pPS1595	*tif51A-3* in pRS315	This study
pPS1596	*TIF51A* in pGEX-4T-1	This study
pPS1597	*TIF51A* in pCGF-1a	This study
pPS1598	*TIF51A* in pRS426	This study
pPS1599	*YOR137C* in pRS426	This study
pPS1600	*FspI-Not*I fragment containing *PKC1* in pRS426	This study
pPS1601	*PAB1* in pRS426	This study
pPS1602	*WSC1* in pRS426	This study
pPS1603	*WSC2* in pRS426	This study
pPS1604	*WSC3* in pRS426	This study
pPS2440	*PKC1::HA* in pPS1600	This study
pPS2441	*pkc1-K853R::HA* in pPS1600	This study

quenced using the same primers at the Dana-Farber Cancer Institute Molecular Biology Core Facility.

Cloning *TIF51A* and the *tif51A* temperature-sensitive alleles: *TIF51A* was cloned from genomic DNA purified from a wild-type strain (FY23) and the ts alleles from the *tif51A* ts strains (PSY1245, PSY1248, and PSY1249). PCR reactions were performed using primers NPL2-C and NPL2-D, which contain a *Bam*HI site. PCR products were digested with *Bam*HI and gel purified. The fragment containing wild-type *TIF51A* was cloned into the *Bam*HI site of pRS316, generating the plasmid pPS1592. For the mutated alleles, PCR fragments were transferred into pRS315, resulting in plasmids pPS1593 (*tif51A-1*), pPS1594 (*tif51A-2*), and pPS1595 (*tif51A-3*). All constructs were confirmed by sequencing.

Disruption of *TIF51A*: A PCR strategy (Baudin *et al.* 1993) was used to disrupt *TIF51A* in a wild-type diploid (PSY1250). Briefly, a DNA fragment containing the *HIS3* gene flanked by the 5′ and 3′ sequences of *TIF51A* was obtained by PCR using primers NPL2-G and NPL2-H. The PCR product was purified and transformed into PSY1250. Transformants were selected on histidine dropout plates and screened by PCR. A diploid strain harboring a disrupted copy of *TIF51A* was selected, transformed with pPS1592, sporulated, and subjected to tetrad analysis. The *tif51A*::*HIS3* disruption was confirmed by PCR and Southern blot. One strain carrying the *tif51A::HIS3* disruption (PSY1251) was chosen to confirm the function of the plasmid-borne mutated alleles.

Anti-eIF5A Western blotting: To produce anti-eIF5A antisera, a plasmid to express GST-eIF5A in *Escherichia coli* (pPS1596) was constructed as follows. The *TIF51A* gene was amplified by PCR from genomic DNA of a wild-type strain (FY23) using primers TIF51A-1 and TIF51A-2. The PCR product was digested with *Bam*HI and *Ava*I, gel purified, and ligated into pGEX-4T-1 (Pharmacia, Piscataway, NJ) that had been digested with *Bam*HI and *Sal*I. GST-eIF5A was bacterially expressed from this plasmid, the fusion protein purified, and eIF5A released from GST-eIF5A by thrombin (Sigma, St. Louis) cleavage essentially as described (Henry and Silver 1996). A polyclonal anti-eIF5A rabbit antiserum was raised at Covance Research Products using this recombinant eIF5A as immunogen.

To determine eIF5A levels in *tif51A* mutant strains, cells were grown to midlog phase at 25°, shifted to 37° or left at 25° for 3 hr, and then lysed in radio-immune precipitation buffer (150 mm NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mm Tris-HCl, pH 8.0) with 1 mm phenylmethylsulfonyl fluoride as previously described (McBride *et al.* 2000). Total protein (5 μg) was resolved by SDS-PAGE, transferred to nitrocellulose, and eIF5A levels were detected by immunoblotting with a 1:10,000 dilution of anti-eIF5A antiserum and enhanced chemiluminescence detection (Amersham, Buckinghamshire, UK).

Localization of eIF5A: Indirect immunofluorescence was used to localize endogenous eIF5A using the polyclonal anti-eIF5A at a dilution of 1:1000. This experiment was performed essentially as described (Lee *et al.* 1996). Localization of eIF5A in wild-type (FY23) and *xpo1-1* (Stade *et al.* 1997) cells was determined following a 1-hr shift to 37°.

To localize eIF5A in living cells, a plasmid that expressed a fusion between green fluorescent protein (GFP) and eIF5A was transformed into a wild-type strain (FY23). This plasmid, pPS1597, was generated by PCR amplification of *TIF51A* from FY23 genomic DNA using primers NPL2-L and NPL2-M. The PCR product was digested with *Bam*HI and *Hin*dIII and ligated into the pCGF-1a vector (Kahana and Silver 1996), placing *GFP-TIF51A* under the control of a galactose-inducible promoter. GFP fusion proteins were visualized after 1–3 hr of induction as described (Lee *et al.* 1996).

TABLE 3

Oligonucleotides used in this study

Oligo	Sequence (5′-3′)[a]	Site
NPL2-A	GCCAATTACTCATAGACTCC	
NPL2-B	GCGAAGAGTACATGATGTGA	
NPL2-C	CGCGGATCCTGTATTCCGTAGCGTTATATCG	*Bam*HI
NPL2-D	CGCGGATCCGAATTACACTGCCTGCATAAGG	*Bam*HI
NPL2-G	*ACTCATAGACTCCCAAACACACACAAATACCAACTCATATATACA* GGCCTCCTCTAGTACACTC	
NPL2-H	*TTTTTTCTTTTTTCATTTATATCCCATGCCATGATGTTAACCGGT* GCGCGCCTCGTTCAGAATG	
NPL2-L	CGCGGATCCATGTCTGACGAAGAACATACC	*Bam*HI
NPL2-M	CCCAAGCTTAGCCGGTAGATATGCGC	*Hin*dIII
TIF51A-1	CGCGGATCCATGTCTGACGAAGAACATACCCCGCTCGAG	*Bam*HI TIF51A-2
	CCGGTAGATATGCGC	*Xho*I/*Ava*I
137-E	CGCGGATCCCGGACATAAATTAGACTCTAAGG	*Bam*HI
137-F	CGCGGATCCGTATCTACTCCAAACTTCTATGG	*Bam*HI
PAB1-C	CGCGGATCCAAGAGGTCATACTGTATGAAGCC	*Bam*HI
PAB1-D	CGCGGATCCAGTATTTTCCTGGAACCTGTTGG	*Bam*HI
WSC1-A	CGCGGATCCACTACTTTCGTAGAATTCCTTCC	*Bam*HI
WSC1-B	CGCGGATCCCTATGTAACTAAGTTAAGGTTGG	*Bam*HI
WSC2-A	CGCGGATCCATCTAGCACTTCTCCCAGAAGTGC	*Bam*HI
WSC2-B	CGCGGATCCTGATGGTGATTTGAGACAGACC	*Bam*HI
WSC3-A	CGCGGATCCAGCCGATTCGTTAGTAGGAATGC	*Bam*HI
WSC3-B	CGCGGATCCGTACCACAAACATCATTAGAAGCTGC	*Bam*HI

[a] Introduced restriction sites are underlined and italics indicate *TIF51A* sequences.

mRNA stability assay: Exponentially growing yeast cultures were shifted from 25° to 37°. At various times, samples were collected and quickly frozen in a dry ice-ethanol bath. Total RNA was isolated from yeast cells by a modified hot phenol method (Oliveira and McCarthy 1995). Yeast strains were incubated for 4 hr at 37°, when 20 μg/ml thiolutin was added and samples were taken at 0, 5, 10, 15, 20, and 30 min after addition of thiolutin. RNAs were separated by electrophoresis on 1.3% agarose gels, after denaturing with glyoxal. RNA gels were subjected to Northern blotting using Hybond nylon membranes (Amersham). Membranes were probed with a ^{32}P-labeled oligonucleotide complementary to the 25S rRNA and a random primer-labeled DNA fragment corresponding to the *HHT2* gene. Blots were analyzed by using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

High-copy suppressor screen: A *URA3*/2μ genomic yeast library (Connelly and Hieter 1996) was transformed into a *tif51A-1* strain (PSY1252). Approximately 140,000 transformants were selected by plating on uracil dropout plates, incubating at 25° overnight, and shifting plates to 36° for 3–4 days. Plasmids were rescued from temperature-resistant clones and retransformed into PSY1252 to show plasmid linkage. The genomic segment present in each of the selected clones was determined by sequencing the ends with T3 and T7 primers and using these sequences to search the *S. cerevisiae* genome database.

High-copy suppressor genes were characterized by subcloning different segments of the original clone into pRS426 (*URA3*/2μ) and testing in PSY1252. The minimal suppressing subclone containing the *PKC1* open reading frame (ORF) is pPS1600. Also, *TIF51A* and the five other high-copy suppressor genes were cloned into pRS426 using the following primers containing a *Bam*HI site: NPL2-C and NPL2-D to clone *TIF51A* (pPS1598); 137-E and 137-F to clone YOR137C (pPS1599); PAB1-C and PAB1-D to clone *PAB1* (pPS1601); WSC1-A and WSC1-B to clone *WSC1* (pPS1602); WSC2-A and WSC2-B to clone *WSC2* (pPS1603); and WSC3-A and WSC3-B to clone *WSC3* (pPS1604). In addition, hemagglutinin (HA)-tagged forms of wild-type and K853R mutant *PKC1* were subcloned into pPS1600 by digestion of pGAL[PKC1::HA] and pGAL[pkc1-K853R::HA] (Watanabe *et al.* 1994) with *Msc*I and *Sph*I and insertion of the 2.4-kb fragment into *Msc*I/*Sph*I-digested pPS1600, resulting in pPS2440 and pPS2441, respectively.

RESULTS

Characterization of three new temperature-sensitive alleles of *TIF51A*: In a screen for conditional mutants defective in nuclear protein localization in the yeast *S. cerevisiae*, several complementation groups were obtained (Sadler *et al.* 1989). Five temperature-sensitive mutants were classified in a complementation group initially named *npl2* (*n*uclear *p*rotein *l*ocalization 2). However, these mutants did not show a general defect in nuclear protein import or export or mRNA export. They did show defects in localization of the large ribosomal subunit (Stage-Zimmermann *et al.* 2000).

Using one of the original mutants (PSY8) and a yeast genomic library, *TIF51A* was cloned by complementation of the temperature-sensitive phenotype (Chiang 1993). Here, three of these mutants (PSY8, PSY338, and PSY341) were further characterized. These strains were first backcrossed three times to the parental W303 strains to generate a number of new strains containing the same mutated alleles of *TIF51A*. To determine whether the temperature-sensitive mutations were linked to the *TIF51A* locus, *npl2* strains (PSY1242–PSY1244)

were crossed to a strain containing the *URA3* gene inserted next to *TIF51A* (PSY1241). The diploids that resulted from these crosses were sporulated and subjected to tetrad analysis. If the mutations present in the *npl2* strains were linked to *TIF51A*, the temperature-sensitive phenotype of the mutants would never cosegregate with the *URA3* gene. For *npl2-1*, 25 complete asci were analyzed and no temperature-sensitive Ura+ spore was observed. The same result was obtained with *npl2-2* and *npl2-3*, after analyzing 15 and 17 complete asci, respectively. These results demonstrate that *npl2-1*, *npl2-2*, and *npl2-3* are linked to *TIF51A*. Therefore, the *npl2* mutants presented here contain temperature-sensitive alleles of the gene encoding eIF5A and are henceforth referred to as *tif51A-1*, *tif51A-2*, and *tif51A-3*.

These three *TIF51A* temperature-sensitive alleles were cloned by PCR into pRS315 (*CEN/LEU2*) using genomic DNA obtained from the *tif51A* strains. To test whether the cloned mutant alleles of *TIF51A* were functional, we generated a strain in which *TIF51A* was disrupted. Sporulation of a heterozygous *TIF51A/tif51AΔ::HIS3* diploid revealed that *TIF51A* is an essential gene since only two viable his$^-$ spores were obtained in 24 tetrads analyzed. This result agreed with Wohl *et al.* (1993) and with the isolation of temperature-sensitive alleles of *TIF51A*. In contrast, another report showed that cells lacking *TIF51A* grow slowly and become inviable only when *TIF51B* is also disrupted (Schnier *et al.* 1991). This discrepancy may be due to strain background differences.

The three *TIF51A* alleles demonstrate varying degrees of temperature sensitivity upon growth at 37° on rich media, YEPD (Figure 1A). While the allele *tif51A-2* displays a moderate reduction in growth at the nonpermissive temperature, alleles *tif51A-1* and *tif51A-3* display more pronounced growth defects. To determine the time of onset of the growth defect in the *tif51A* mutant strains, midlog phase cultures were monitored at different times after shift to the nonpermissive temperature (Figure 1B). Although *tif51A* mutant strains showed slightly slower growth than the wild-type strain at 25° (left), they showed a significant decrease in growth rate by 2–3 hr after shift to 37° (right). The discrepancy in the relative growth defect between *tif51A-1* and *tif51A-2* on plates *vs.* liquid may reflect a growth defect that is overcome after longer incubation times.

To define the mutations in the *TIF51A* gene present in the *tif51A* temperature-sensitive mutants, genomic DNA was isolated from the *tif51A* strains and the *TIF51A* gene was amplified by PCR. The 0.7-kb fragment containing *TIF51A* was purified and then sequenced. These mutations were confirmed by sequencing the cloned alleles in pPS1593, -1594, and -1595. A single mutation of the same residue was detected for the *tif51A-1* and *tif51A-2* alleles. The proline at position 83 was changed to serine (P83S) or leucine (P83L), respectively. Two mutations were mapped in the *tif51A-3* allele, a cysteine-to-tyrosine change at position 39 (C39Y) and a glycine-to-aspartic acid change at position 118 (G118D).

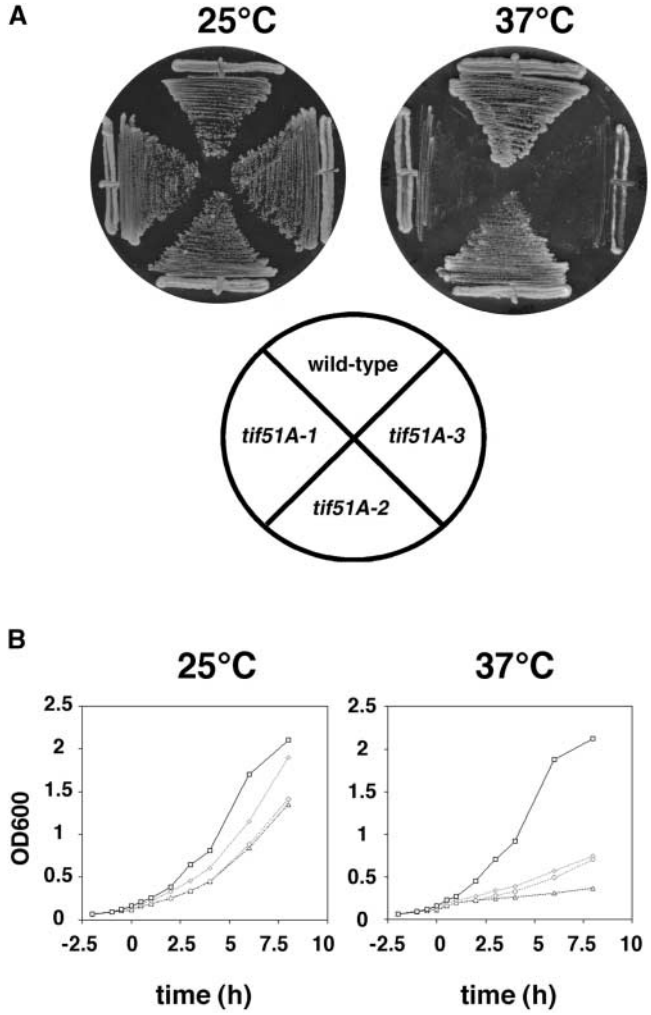

Figure 1.—Temperature sensitivity of *tif51A* alleles. (A) Temperature sensitivity of *tif51A* mutant strains: Wild-type (W303), *tif51A-1* (PSY1245), *tif51A-2* (PSY1248), and *tif51A-3* (PSY1249) strains were streaked to rich plates (YEPD) and grown at either 25° or 37° for 3–4 days. (B) Strains W303, PSY1245 (*tif51A-1*), PSY1248 (*tif51A-2*), and PSY1249 (*tif51A-3*) were grown at 25° in rich medium (YEPD) to midlog phase (2.5 hr). The cultures were then split between 25° and 37° and growth was monitored for 8 hr by spectrophotometry ($OD_{600\ nm}$). (□) Wild type, (◇) *tif51A-1*, (○) *tif51A-2*, (△) *tif51A-3*.

Two crystal structures of archaebacterial eIF5A proteins have been solved (Kim *et al.* 1998; Peat *et al.* 1998) and the positions of these mutations in the *Methanococcus jannaschii* eIF5A structure (Kim *et al.* 1998) are shown (Figure 2). The proline mutated in tif51A-1 and tif51A-2 proteins is at the junction between the two domains of eIF5A. In contrast, the mutations in tif51A-3, both of which are required for temperature sensitivity (data not shown), are found on separate domains of eIF5A. C39Y is found in a β-sheet 12 residues N-terminal to the hypusinated lysine that is critical for eIF5A function; G118D

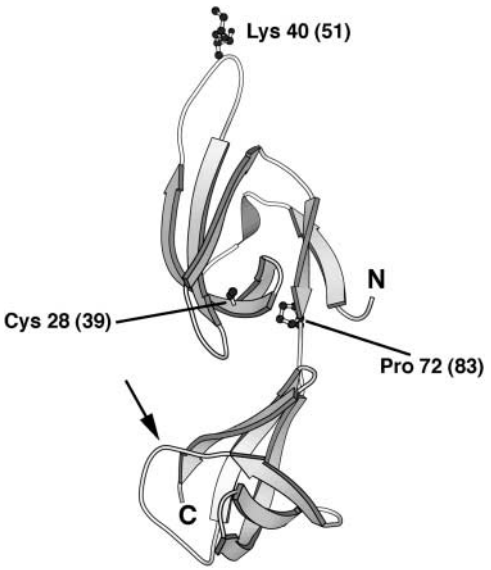

FIGURE 2.—Lesions in *tif51A* temperature-sensitive alleles. The locations of mutated residues in the eIF5A structure are shown on the archaebacterial eIF5A crystal structure (KIM *et al.* 1998; yeast eIF5A residue numbers are shown in parentheses). Tif51A-1 and tif51A-2 have mutations at Pro83 and tif51A-3 has mutations at Cys39 and Gly118 in the C-terminal domain loop (arrow). The essential hypusinated lysine is also shown.

is located within a loop in the C-terminal domain. Thus both domains of eIF5A appear to be important for its function.

To determine eIF5A protein levels in the *tif51A* mutant strains, a rabbit polyclonal antiserum against eIF5A was produced using recombinant yeast eIF5A expressed in *E. coli*. This antiserum was used to detect eIF5A by Western blot in cell lysates prepared from wild-type and temperature-sensitive *TIF51A* strains grown at the permissive and nonpermissive temperature (Figure 3). At 25°, eIF5A of the expected molecular mass (~20 kD) was detected in the wild-type (WT) and in the *tif51A-1* and *tif51A-2* strains. In the case of *tif51A-3*, eIF5A migrates slightly more slowly, due to the G118D mutation in eIF5A in the *tif51A-3* strain (data not shown). After a 3-hr shift to 37°, levels of mutant eIF5A proteins are significantly lower than that of the wild-type protein,

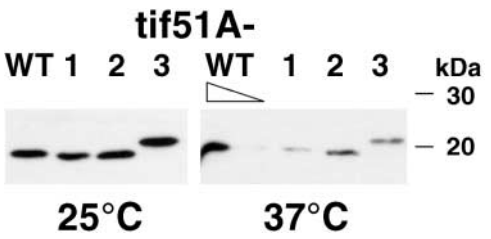

FIGURE 3.—Expression of mutant eIF5A proteins. Samples from cultures in Figure 1B were collected at 3 hr postshift, cells were lysed, and 5 μg (or 1 μg for the lower dilution of the wild-type culture at 37°) of total protein was analyzed by Western blotting with an anti-eIF5A antiserum.

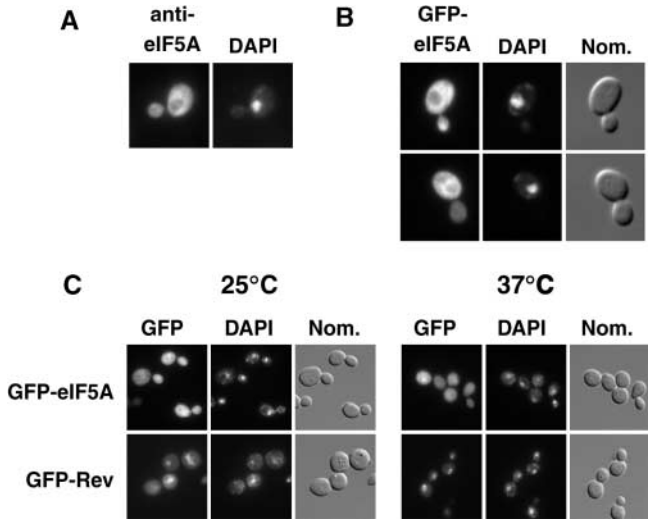

FIGURE 4.—Localization of wild-type eIF5A. (A) Endogenous eIF5A localization in fixed cells. Wild-type cells (FY23) were grown at 25° to midlog phase and fixed by incubation with formaldehyde. Cells were placed on polylysine-coated slides and, after permeabilization, incubated first with a rabbit anti-eIF5A polyclonal serum and then with an anti-rabbit fluorescein-conjugated secondary antibody. Localization of eIF5A was then visualized by fluorescence microscopy. (B) GFP-eIF5A localization in living cells. A functional GFP-eIF5A fusion protein was expressed in wild-type yeast cells (FY23) under the control of a galactose promoter. Production of the fusion protein was induced for 1–2 hr at 25° prior to visualization by fluorescence microscopy. (C) Localization of eIF5A and Rev in *xpo1-1* cells. *Xpo1-1* cells (PSY1105; STADE *et al.* 1997) were transformed with 2μ *URA3* plasmids containing either GFP-eIF5a (pPS1597) or GFP-Rev (pPS1494) under the control of the inducible GAL promoter. Cells were grown in 2% raffinose until midlog phase, induced with 2% galactose for 3–5 hr, shifted to glucose, incubated at 25° or 37° for 1 hr, and fixed, and eIF-5A and REV localization was determined by fluorescence microscopy.

suggesting that these are loss-of-function alleles. Indeed, after 12 hr at 37°, eIF5A mutant protein is no longer detectable by Western blotting (data not shown).

Localization of eIF5A in the yeast *S. cerevisiae*: eIF5A has been proposed to be an export factor for the human immunodeficiency virus (HIV) Rev protein, by virtue of acting as an adapter to the transporter exportin/CRM1 (RUHL *et al.* 1993; BEVEC *et al.* 1996; BEVEC and HAUBER 1997; HOFMANN *et al.* 2001). Therefore, we wished to test whether the localization of yeast eIF5A was consistent with this hypothesis, and, if so, whether *tif51A* mutant alleles affected nuclear export. eIF5A localization was performed initially by indirect immunofluorescence, using formaldehyde-fixed wild-type cells and anti-eIF5A antiserum (Figure 4A). eIF5A is localized mainly in the cytoplasm with a concentration in the perinuclear region. A comparison of the eIF5A localization to 4′,6-diamidino-2-phenylindole (DAPI) staining of nuclei reveals that only minor amounts of eIF5A are found in the nucleus.

To ascertain whether this localization reflected eIF5A

distribution in live cells, *TIF5A* was inserted downstream of a galactose-inducible green fluorescent protein gene. This plasmid produces functional eIF5A in that it restores the ability of *tif51A-1* to grow at the restrictive temperature after galactose induction (data not shown). This plasmid was transformed into a wild-type strain and after 1–2 hr induction with galactose, GFP-eIF5A localization was determined by fluorescence microscopy (Figure 4B). As expected, GFP-eIF5A was detected predominantly in the cytoplasm with a stronger signal in the perinuclear region (Figure 4B, left). These results conclusively show that eIF5A is a cytoplasmic protein and accumulates in the perinuclear region.

If eIF5A were to facilitate HIV Rev export by binding both Rev and the Xpo1/Crm1 exporter, export of eIF5A itself would be expected to be blocked by mutations in the export machinery. To test if eIF5A is exported by Xpo1/Crm1, its localization was monitored in *xpo1-1* cells, which are temperature sensitive for export by this pathway. *Xpo1-1* cells carrying *GFP-eIF5A* or *GFP-Rev* on 2μ *URA3* plasmids under the galactose-inducible promoter were grown to midlog phase and then induced with galactose for 3 hr, shifted to 37° for 1 hr, fixed, and then visualized by fluorescence microscopy (Figure 4C). GFP-eIF5A localization was predominantly cytoplasmic in *xpo1-1* cells at 37°. Comparison with DAPI staining shows no marked increase in nuclear localization of eIF5A, which would be expected if eIF5A shuttled in an Xpo1-dependent manner, suggesting that eIF5A is not exported by Xpo1 in yeast. In contrast, GFP-Rev showed a marked accumulation of fluorescent signal in nuclei of *xpo1-1* cells after 1 hr at the nonpermissive temperature (Figure 4C).

To further test the dependence of Rev export upon eIF5A, GFP-Rev localization was examined in strains containing the *TIF51A* temperature-sensitive alleles. After a 3-hr shift to the nonpermissive temperature, GFP-Rev displayed cytoplasmic localization similar to that seen in the wild-type control (Figure 5). The slight increase in signal in the mutant alleles after temperature shift does not coincide with the nucleus as determined by DAPI staining (Figure 5). These data are consistent with a recent study in mammalian cells that revealed that exportin 4, rather than CRM1, exports eIF5A (Lipowsky *et al.* 2000). Since no yeast homolog of exportin 4 exists, eIF5A localization was tested in a battery of strains with mutations in putative transport proteins. None of these strains showed a nuclear accumulation of eIF5A (data not shown), suggesting either that yeast eIF5A does not shuttle between the nucleus and the cytoplasm or that its exporter has no homology to known transport proteins. These data, in combination with the finding that Rev export is not blocked in *tif51A* mutant strains, argue against the hypothesis that Rev export by Xpo1/CRM is mediated by eIF5A.

mRNA decay defect of *tif51A* alleles: eIF5A has also been proposed to play a role in mRNA decay (Zuk and Jacobson 1998). To test whether the *tif51A* alleles described above affect mRNA stability at the nonpermissive temperature, wild-type or mutant strains were grown for 4 hr at 37°, transcription was inhibited, and samples were taken at several times post-transcription (Figure 6). Northern blot analysis with a probe against the moderately stable *HHT2* mRNA shows a significant decrease in the levels of these RNAs in wild-type cells over the course of the assay as compared to the 25S rRNA loading control (Figure 6, TIF51A). However, in *tif51A-1* and

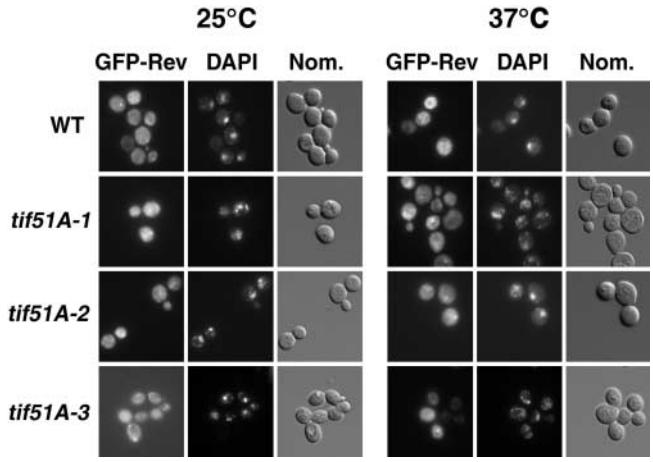

Figure 5.—Localization of GFP-Rev in *tif51A* mutant strains. Wild-type (W303), *tif51A-1* (PSY1245), *tif51A-2* (PSY1248), and *tif51A-3* (PSY1249) strains were transformed with a 2μ *URA3* plasmid containing GFP-Rev (pPS1494) under the control of the inducible GAL promoter. Cells were prepared as in Figure 4C with a temperature shift to 37° for 3 hr and visualized by fluorescence microscopy.

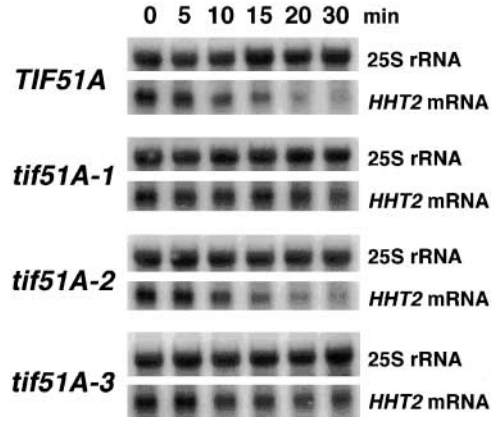

Figure 6.—mRNA decay defect in *tif51A* temperature-sensitive alleles. Northern blot analysis of *HHT2* mRNA. Following incubation at 37° for 4 hr, thiolutin was added to cultures to the final concentration of 20 μg/ml and cell samples were collected at the times indicated. Total RNA was extracted and analyzed by Northern blotting using probes complementary to the 25S rRNA and to the *HHT2* mRNA. Quantitation of the 25S rRNA served as an internal control to visualize the amount of total RNA loaded in each lane. Blots were analyzed and bands quantitated by using a phosphorimager.

TABLE 4

Multicopy suppressors of the temperature-sensitive mutant *tif51A-1*

Clone	No. of isolates	Chromosome	Suppressor
1	8	XV	*YOR137C* (*SIA1*)
2	2	II	*PKC1*
3	2	XIV	*WSC2*
4	1	V	*PAB1*
5	1	XV	*WSC1/SLG1/HCS77*
6	1	XV	*WSC3*

tif51A-3 strains the *HHT2* RNA is stabilized, with levels slightly higher than those in wild-type cells at 15–30 min post-inhibition (Figure 6). The *HHT2* RNA level in *tif51A-2* cells is similar to that in wild-type cells. Similar results were obtained by probing the *PAB1* transcript (data not shown). Cells submitted to heat shock for a shorter period of time (2 hr at 37°) did not display significant stabilization of *CYH2*, *PAB1*, and *HHT2* mRNAs (data not shown).

Suppression of the temperature-sensitive phenotype of *tif51A-1*: To expand our understanding of the function of eIF5A, we wished to identify other genes that encode proteins that work similarly or together with eIF5A, and therefore we performed a high-copy suppressor screen. A yeast genomic library was transformed into the *tif51A-1* strain and transformants that could grow at 36° were selected. Twenty-three plasmids that still suppressed temperature sensitivity after retransformation into *tif51A-1* were characterized by DNA sequencing. Six clones, representing 15 of these plasmids, were selected and used to define the ORF that suppresses the temperature-sensitive phenotype of *tif51A-1*. These clones are presented in Table 4. One clone contained the entire *PKC1* gene, which was cloned twice in this screen, without flanking ORFs. Subcloning revealed the other suppressing ORFs to be the following: *YOR137C*, *WSC2*, *WSC1*, *WSC3*, and *PAB1*. To eliminate partial flanking ORFs, these five genes were also cloned by PCR and the constructs tested for suppression of the temperature-sensitive phenotype of *tif51A-1* strain. Figure 7 shows that the ability of *tif51A-1* to grow at 36° was restored by expression of these genes in high copy. In addition, all *tif51A-1* suppressors were allele specific, as they were unable to rescue growth of *tif51A-3* at high temperatures (data not shown). The plate phenotype of *tif51A-2* at 36° was too weak to test suppression in this manner.

To test whether the two most-well-studied suppressor proteins, poly(A)-binding protein (Pab1) and protein kinase C (Pkc1), acted by increasing the levels of mutant eIF5A protein, mutant strains bearing the multicopy *PAB1* and *PKC1* plasmids were grown at 25° or 37° for 3 hr and eIF5A levels were monitored by Western blotting

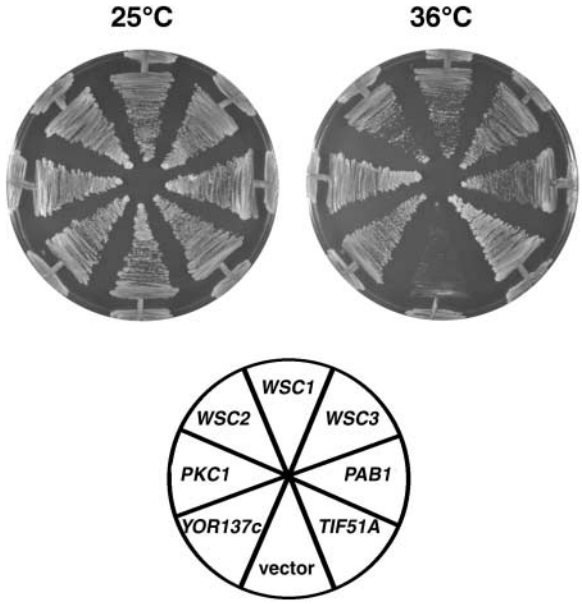

FIGURE 7.—High-copy suppressors of *tif51A-1* temperature sensitivity. *tif51A-1* strain PSY1252 was transformed with a 2μ *URA3* library of plasmids and transformants were screened for restoration of growth at 36°. Candidates that still suppressed after plasmid rescue and retransformation into PSY1252 were restreaked to plates lacking uracil at 25° and 36°. Wild-type *TIF51A* and the 2μ *URA3* vector, positive and negative controls, are also shown.

(Figure 8). Overexpression of *PAB1* and *PKC1* did not have a significant effect on steady-state levels of eIF5A at either permissive or nonpermissive temperatures. The slight increase in eIF5A-3 levels in the presence of excess Pkc1 was not reproducible.

Connections between *PKC1* signaling and eIF5A: To examine the importance of *PKC1* signaling in *tif51A* mutant strains, we tested whether a kinase-inactive form of *PKC1* could suppress the *tif51A-1* temperature sensitivity. Strain PSY1245 was transformed with high-copy

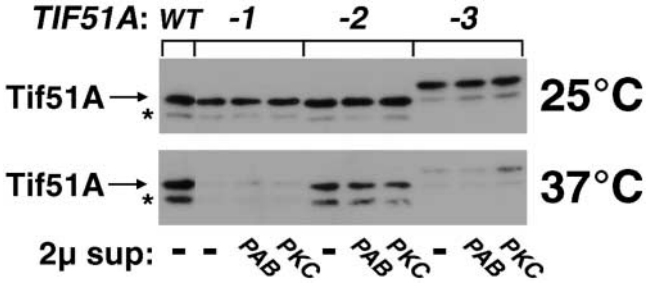

FIGURE 8.—eIF5A protein levels in the presence of high-copy *PAB1* and *PKC1*. W303 bearing high-copy vectors pRS426 and PSY1245 (*tif51A-1*); PSY1248 (*tif51A-2*); and PSY1249 (*tif-51A-3*) bearing pRS426, pPS1600 (*PKC1*), or pPS1601(*PAB1*) were grown at 25° to midlog phase and then shifted to 37° or left at 25° for 3 hr. Cells were lysed and 5 μg total protein was analyzed by anti-eIF5A Western blotting as in Figure 3. The asterisk denotes a common degradation product of eIF5A (KANG *et al.* 1993).

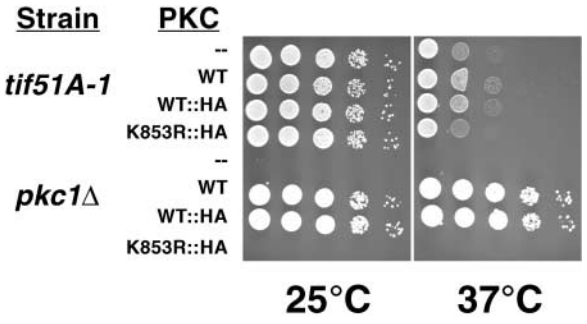

FIGURE 9.—PKC kinase activity is required for suppression of *tif51A-1* temperature sensitivity. Dilutions of PSY1245 (*tif51A-1*) and DL376 (*pkc1*Δ) strains bearing plasmids pRS426 (−), pPS1600 (WT), pPS2440 (WT::HA), and pPS2441 (K853R::HA) were grown for 2 days on plates lacking uracil and sorbitol at 25° and 37°.

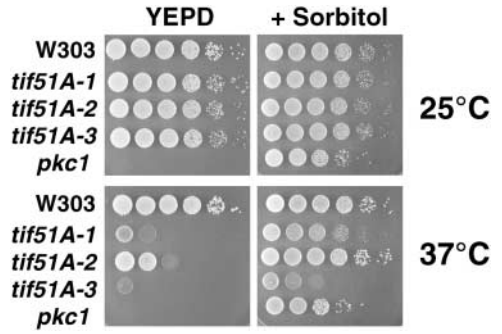

FIGURE 10.—Temperature sensitivity of *tif51A* mutant strains is partially suppressed by sorbitol. Dilutions of wild-type (W303), *tif51A-1* (PSY1245), *tif51A-2* (PSY1248), *tif51A-3* (PSY1249), and *pkc1*Δ (DL376; LEVIN and BARTLETT-HEUBUSCH 1992) strains were grown for 2 days at 25° or 37° on YEPD plates with or without 1 M sorbitol as an osmotic stabilizer.

plasmids encoding wild-type Pkc1, HA-tagged wild-type Pkc1, or HA-tagged Pkc1 with a lysine-to-arginine change in the active site (K853R). Serial dilutions of each transformed strain were then plated at 25° or 37° (Figure 9). Whereas the ability of *PKC1* to suppress *tif51A-1* temperature sensitivity was not impaired by the addition of an HA tag (WT *vs.* WT::HA), the active site mutation (K853R::HA) eliminated suppression. Growth of the *pkc1*Δ strain, which requires PKC activity, is shown as a control (WATANABE *et al.* 1994). Therefore, kinase activity of *PKC1* is required both for signaling and for growth of the *tif51A-1* strain, indicating a functional link between eIF5A and *PKC1* signal transduction.

Although *PKC1* is involved in multiple signaling pathways in yeast, the additional isolation of three *WSC* genes as high-copy suppressors of *tif51A-1* suggested a connection between eIF5A and a specific pathway involved in stress response and maintenance of cell wall integrity (for review see HEINISCH *et al.* 1999). Deletion of *PKC1* or *WSC1* results in cell wall defects and cell lysis on media lacking an osmotic stabilizer (PARAVICINI *et al.* 1992; VERNA *et al.* 1997). To determine whether the temperature sensitivity of *tif51A* mutant strains might reflect a defect in cell wall integrity, dilutions of each strain were grown at 37° on rich media without or with 1 M sorbitol to lend osmotic stability (Figure 10). The addition of sorbitol significantly increased the ability of *tif51A-1* and *tif51A-3* strains to grow at 37° and completely suppressed the growth defect of *tif51A-2*. Growth of a strain lacking *PKC1*, which requires an osmotic stabilizer at all temperatures, is shown as a control (LEVIN and BARTLETT-HEUBUSCH 1992). These results suggest that the ability of *PKC1* and *WSC* genes to suppress the temperature sensitivity of *tif51A-1* is due to their roles in maintaining cell wall integrity.

DISCUSSION

In spite of two decades of research on eIF5A, the role of this ubiquitous protein remains mysterious. In this report, three temperature-sensitive alleles of *TIF51A* were characterized (*tif51A-1*, *tif51A-2*, and *tif51A-3*). While mRNA decay experiments indicated that the stronger mutations led to stabilization of mRNAs, localization studies of wild-type eIF5A suggested that this protein is unlikely to be involved in HIV Rev export. The *tif51A-1* allele was used to contribute further to the search for a function for this highly conserved protein. Screening of a 2μ plasmid library containing genomic DNA uncovered six genes that allowed growth of *tif51A-1* strains at high temperature. Further experiments supported the importance of the PKC/WSC signaling pathway for cell wall integrity in *tif51A-1* mutant cells.

In fluorescence microscopic experiments, eIF5A was detected primarily in the cytoplasm with a concentration in the perinuclear region (Figure 4). This result agrees with the subcellular localization of mammalian eIF5A (SHI *et al.* 1996b, 1997). RUHL *et al.* reported that a substantial fraction of eIF5A was also present in the nucleus and suggested that eIF5A was a shuttling protein involved in HIV Rev export (RUHL *et al.* 1993; BEVEC *et al.* 1996; BEVEC and HAUBER 1997). However, in yeast, eIF5A is not mislocalized to the nucleus in an *xpo1-1* strain, a temperature-sensitive mutant of the export receptor for Rev and other proteins that contain leucine-rich nuclear export signals (NESs), nor does eIF5A itself have a canonical NES (STADE *et al.* 1997, Figure 4). Indeed, exportin 4, rather than CRM1, was recently found to mediate eIF5A export in mammalian cells (LIPOWSKY *et al.* 2000). Thus, our data from *S. cerevisiae* support the cytoplasmic localization of eIF5A, perhaps associated with the ER/ribosomes as suggested by its concentration in the perinuclear region. Furthermore, our data do not support the hypothesis of eIF5A being involved in the export of NES-containing proteins through the export receptor Xpo1p/Crm1p.

The identification of conditional *tif51A* alleles in a screen for mislocalization of a nuclear localization se-

quence (NLS)-reporter protein initially would seem to suggest that eIF5A is crucial for nuclear import (Sadler *et al.* 1989; Chiang 1993). The reporter protein in this screen contained an SV40 NLS fused to cytochrome c_1 (Cyt1). Thus, in a strain with a null allele of *CYT1*, growth on glycerol media requires the mislocalization of the normally nuclear NLS-cytochrome c protein to mitochondria. The *npl2/tif51A* complementation group of mutant strains grew on glycerol plates at permissive temperature; however, the NLS reporter proteins still localized to the nucleus as detected by immunofluorescence microscopy (Chiang 1993). In addition, strains bearing the mutant *tif51A* alleles did not mislocalize the nuclear protein Npl3p, a cytoplasmic NLS-NES-GFP fusion protein, mRNA, or the mutant eIF5A proteins themselves (data not shown). These contradictory results suggest that the mechanism by which mutations in *TIF51A* allow growth on a nonfermentable carbon source does not involve redirecting the majority of cytochrome c_1 to mitochondria. These mutations may alter the translation or decay rate of the CYT1 mRNA and thereby increase the production of the fusion protein, perhaps allowing a minor fraction to reach the mitochondria to allow growth.

A high-copy suppressor screen with the *tif51A-1* allele revealed six genes that could suppress the temperature sensitivity of this mutant. These suppressors are *PAB1*, *YOR137C*, *PKC1*, *WSC1*, *WSC2*, and *WSC3*. The most frequent suppressor in the screen, *YOR137C* [named here suppressor of eIF5A (*SIA1*)], encodes a protein with a peptide signal for membrane localization and was recently associated with activation of the yeast plasma membrane H^+-ATPase by glucose (de la Fuente *et al.* 1997). Although the fluorescence pattern of a GFP-Sif51A fusion protein, cytoplasmic and concentrated in the perinuclear region, is similar to that of eIF5A (data not shown), it is not clear how overexpression of Sif51Ap could suppress *tif51A-1* temperature sensitivity.

Another high-copy suppressor of *tif51A-1*, *PAB1* has been shown to be involved in multiple aspects of RNA metabolism, including 3′-end processing, translation, and mRNA decay. The yeast gene, *PAB1*, is essential but the lethality caused by deletion of *PAB1* can be suppressed by mutations in a number of other genes, including genes that encode components of the translational and mRNA degradation machinery (Sachs and Davis 1989, 1990; Caponigro and Parker 1995; Boeck *et al.* 1998; Bonnerot *et al.* 2000). Pab1 participates in the length control of poly(A) tails of messenger RNAs in the yeast nucleus (Amrani *et al.* 1997; Kessler *et al.* 1997; Minvielle-Sebastia *et al.* 1997), and the poly(A) tail enhances the translation of mRNAs (Jacobson and Favreau 1983; Galili *et al.* 1988; Munroe and Jacobson 1990). This translational enhancement is mediated by the binding of Pab1p to initiation factor eIF-4G, which is part of the mRNA cap-binding complex eIF-4F (Tarun *et al.* 1997). The intimate connections between translation and mRNA decay are underscored by the roles of these proteins in both processes: binding of Pab1p stabilizes mRNA in a translation-dependent manner (Coller *et al.* 1998) and mutations in eIF-4F complex genes as well as in *PAB1* result in destabilization of mRNA (Schwartz and Parker 1999; Brown *et al.* 2000).

Like Pab1, eIF5A has been implicated in both translation and mRNA decay. Although eIF5A was first thought to be a translation initiation factor (Benne and Hershey 1978; Park *et al.* 1993), a temperature-sensitive mutant of *TIF51A* (ts1159) decreases the decay rate of several mRNAs tested and results in a twofold accumulation of uncapped *URA5* mRNA (Zuk and Jacobson 1998). This mutant also displays slowed translation, as was shown in the eIF5A depletion studies (Kang and Hershey 1994; Zuk and Jacobson 1998). Here we have shown that *tif51A-1* and *tif51A-3* alleles result in mRNA decay defects at the nonpermissive temperature (Figure 5), although the rate of mRNA stabilization seen in the mutants *tif51A-1* and *tif51A-3* is less drastic than that observed in the strain *ts1159* (Zuk and Jacobson 1998). No effect on mRNA decay was observed in the mutant strain *tif51A-2*. These results support the idea that the effects on mRNA decay are allele specific. Significant mRNA stabilization is not detected until after 4 hr of heat shock, suggesting the possibility that secondary effects are being measured. Furthermore, these results suggest that the suppression of *tif51A-1* temperature sensitivity by overexpression of *PAB1* may reflect complementary roles of eIF-5A and Pab1 in mRNA decay.

The high-copy suppressor focused on in this work, *PKC1*, is the yeast homolog of the metazoan *PKC* gene and encodes a regulator of one of the four mitogen-activated protein-kinase cascades characterized so far in *S. cerevisiae* (Posas *et al.* 1998). Pkc1-signaling pathways can be activated by hypotonic and heat shock and they are involved with cell cycle control and cell wall biosynthesis (Levin *et al.* 1990; Madden *et al.* 1997; Posas *et al.* 1998). Remarkably, three other suppressors isolated simultaneously are upstream regulators of the cell wall integrity signaling pathway: *WSC1* (*SLG1* or *HCS77*), *WSC2*, and *WSC3* (Gray *et al.* 1997; Verna *et al.* 1997; Jacoby *et al.* 1998).

Although PKC signaling has not been shown to have a direct role in mRNA translation or decay, recent studies have revealed the importance of *PKC/WSC* signaling in the regulation of ribosome synthesis (Nierras and Warner 1999; Li *et al.* 2000). Ribosomal RNA and ribosomal protein mRNA transcription are inhibited during the stress response caused by a secretion block and this downregulation requires *PKC1* and the *WSC* genes. Several results also point to the possibility that eIF5A may have a role in regulating ribosome synthesis. At high temperature two of the mutant strains characterized in this study, *tif51A-1* and *tif51A-2* (referred to as *npl2-1* and *npl2-2*), were found to mislocalize the large ribosomal

subunit protein L11 fused to GFP in 10–40% of cells, reflecting a defect in either ribosome assembly or export (Stage-Zimmermann *et al.* 2000). In permeabilized cell assays, purified eIF5A was shown to accumulate in nucleoli, the site of ribosome synthesis (Lipowsky *et al.* 2000). The initial identification of eIF5A as a ribosome-associated factor (Kemper *et al.* 1976; Benne and Hershey 1978) and the 30% decrease in translation found in cells depleted of eIF5A (Kang and Hershey 1994) may reflect the involvement of eIF5A in ribosome synthesis, rather than a direct role in translation.

The ability of *tif51A-1* temperature sensitivity to be partially suppressed either by overexpression of *PKC* or *WSC* genes (Figure 6) or by the addition of sorbitol (Figure 9) suggests that eIF5A may act either in the PKC pathway for cell integrity or in a parallel pathway. eIF5A is phosphorylated *in vivo*, but the kinase responsible for this nonessential modification has not been identified. *PKC1* overexpression may suppress *tif51A-1* by increasing phosphorylation of the mutant protein. Alternatively, *PKC1* could phosphorylate a protein that interacts with eIF5A and that is important for its function. One candidate eIF5A-interacting protein is deoxyhypusine synthase, the essential enzyme that catalyzes the first step in the lysine-to-hypusine modification (Kang *et al.* 1995; Sasaki *et al.* 1996). This protein has been shown to be phosphorylated specifically by PKC *in vitro* (Kang and Chung 1999) and contains three potential target S/T residues with basic residues on both sides. It is also intriguing to note that Pkc1p is an upstream regulator of the SBF transcription factor, which is a regulator of the G1-to-S phase (Madden *et al.* 1997), and that eIF5A has been implicated in the translation of mRNAs involved in the same cell cycle transition (Hanauske-Abel *et al.* 1995). The exact mechanism of PKC suppression will be the focus of future investigation.

The isolation of *PAB1*, *PKC1*, and the *WSC* genes as high-copy suppressors of *tif51A-1* points to different roles for eIF5A in RNA metabolism from mRNA decay to ribosome synthesis. The allele specificity of suppression suggests that eIF5A may have more than one important role in the cell, leading to functional differences between the alleles. It is interesting to note that *PKC1* was identified in a Pab1p two-hybrid screen (Mangus *et al.* 1998). The genetic interactions of these genes with *TIF51A* suggest that these three proteins are intimately associated, but further experiments will be necessary to clarify these interactions. Thus, although the precise function of eIF5A remains an open question, the data presented in this report suggest that eIF5A acts in multiple steps of RNA metabolism. Future research will help define the exact roles played by these proteins in post-transcriptional regulation.

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