Novel Characteristics of the Biological Properties of the Yeast Saccharomyces cerevisiae Eukaryotic Initiation Factor 2A*

Eukaryotic initiation factor 2A (eIF2A) has been shown to direct binding of the initiator methionyl-tRNA (Met-tRNA<sub>i</sub>) to 40 S ribosomal subunits in a codon-dependent manner, in contrast to eIF2, which requires GTP but not the AUG codon to bind initiator tRNA to 40 S subunits. We show here that yeast eIF2A genetically interacts with initiation factor eIF4E, suggesting that both proteins function in the same pathway. The double eIF2A/eIF4E-ts mutant strain displays a severe slow growth phenotype, which correlated with the accumulation of 85% of the double mutant cells arrested at the G2/M border. These cells also exhibited a disorganized actin cytoskeleton and elevated actin levels, suggesting that eIF2A might be involved in controlling the expression of genes involved in morphogenic processes. Further insights into eIF2A function were gained from the studies of eIF2A distribution in ribosomal fractions obtained from either an eIF5BΔ (fun12Δ) strain or an eIF3b-ts (prt1-ts) strain. It was found that the binding of eIF2A to 40 and 80 S ribosomes was not impaired in either strain. We also found that eIF2A functions as a suppressor of Ure2p internal ribosome entry site-mediated translation in yeast cells. The regulation of expression from the URE2 internal ribosome entry site appears to be through the levels of eIF2A protein, which has been found to be inherently unstable with a half-life of ~17 min. It was hypothesized that this instability allows for translational control through the level of eIF2A protein in yeast cells.

Initiation of protein synthesis in eukaryotes is a complex process requiring numerous accessory proteins called initiation factors. At least 12 different initiation factors have been identified, comprising over 30 polypeptide chains (1). The function of many of these factors have been established in detail (1, 2); however, the precise role of some of them, their mechanism of action, and the particular step in the initiation process at which these factors function still remains obscure. It should be noted that recent studies have pointed to the key role of translational control (which is mainly exerted at the initiation step of protein synthesis) in regulating gene expression during development, differentiation, cell cycle progression, cell growth, apoptosis, and stress (3–5). Studies of the responses of a large variety of cell systems to different physiological stimuli have shown that protein synthesis can be modulated by both changes in the state of phosphorylation of initiation factors and changes in the levels of these factors in the cell. These effects allow rapid modification of the overall rate of translation as well as post-transcriptional regulation of gene expression due to changes in the relative selection of different mRNA species utilizing different mechanisms of translation initiation. In view of these observations, it is of importance to establish the molecular mechanisms by which changes in the levels or activities of various initiation factors could affect cell fate.

Eukaryotic initiation factor 2A (eIF2A<sub>1</sub>) is a 65-kDa protein that was first identified in the early 1970s on the basis of its stimulation of initiator methionyl-tRNA (Met-tRNA<sub>i</sub>) binding to 40 S ribosomal subunits, its participation in methionyl puromycin synthesis, and its ability to stimulate poly(U)-directed polyphenylalanine synthesis at low [Mg<sup>2+</sup>] (6). eIF2A was initially believed to be the functional homologue of prokaryotic IF2, since IF2 catalyzes biochemically similar reactions (6, 7). Subsequent identification of a multisubunit factor eIF2 (8–10) showed that this factor and not eIF2A is primarily responsible for the delivery of Met-tRNA<sub>i</sub> to 40 S subunits in eukaryotes. Whereas both eIF2 and eIF2A function similarly in model assays, the order of events differs between the two (Fig. 1). eIF2A binds Met-tRNA<sub>i</sub> to 40 S subunits in a codon-dependent manner, whereas eIF2 binds Met-tRNA<sub>i</sub> to 40 S subunits in a GTP-dependent manner (7, 11–13). Recently, the yeast homologue of mammalian eIF2A was identified, and yeast strains were obtained that lacked the gene for eIF2A (14). The eIF2A<sub>Δ</sub> strains were viable and showed no apparent phenotype (although this strain sporulated with about one-third the efficiency of the wild type (14)), suggesting that the eIF2A does not function in major (key) steps in the initiation process but, perhaps, could act at some late steps or be involved in minor alternative initiation events such as reinitiation, internal initiation, or non-AUG initiation. Of these alternatives, it does not appear that eIF2A influences reinitiation, at least as tested using the GCN4 reporter system in yeast cells (14). Genetic interaction of the eIF2A<sub>Δ</sub> and eIF5B was also observed, suggest-

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Yeast cultures were grown as indicated using either synthetic media containing 0.67% Difco yeast nitrogen base, 1% ammonium sulfate, 2% glucose (or galactose) and supplemented with the appropriate amino acids or complete (YPD) medium (17). Transformation was performed using the lithium acetate method (18). For the polysome expression analysis of HA-tagged eIF2A, yeast were grown in complete medium with 2% galactose.

Plasmids—The pTB328_y_2A shuttle vector bearing yeast eIF2A (fused to an HA epitope) under control of the GAL promoter has been previously described (14). The YCplac111 (CEN, LEU2) vector bearing yeast eIF2A under its own promoter was produced by BamHI-PstI subcloning, using the PCR-amplified (3339 bp) yeast genomic DNA fragment (with 5’-AAGCGGATCCCTATTATCTGATATACGTGTCATTTTTTC-3′ as the upstream and 5’-AAAAACTGACAGCACTGTTTCATTTCTCAGTATATATACCC-3′ as the downstream primer) into the YCplac111 vector. This fragment contains 843 bp upstream of the eIF2A AUG start codon. The pRS316_y_2A (CEN, URA3) plasmid was constructed as follows. Yeast eIF2A genomic DNA fragment (3809 bp) was PCR-amplified with 5′-TCTGTTCAATTAAATCCTGTTAGG-3′ as an upstream primer and 5′-TTGATGAGACGTGTTGTG-3′ as a downstream primer, and cloned into KpnI-SalI-digested pRS316 vector. The pTB328_h_2A vector bearing the cDNA copy of human eIF2A under control of GAL promoter was produced as follows. cDNA encoding human eIF2A (1,156 bp; cDNA Consortium, Clontech; clone ID 3688407) was amplified with 5′-AAAACGGGATCCATGCGGATCCCTACCACGC-3′ as the upstream and 5′-AAAACTGACAGCACTGTTTCATTTCTCAGTATATATACCC-3′ as the downstream primer), digested with BamHI and PsiI, and cloned into pTB328 (CEN, LEU2, GAL1 promoter, HA tag) vector. The p281 plasmid containing lacZ under GAL promoter has been described previously (19). The pDA2-eIF4E/HA tag) vector containing HA-tagged eIF4E under the control of GAL promoter (UR2 as an auxotrophic marker) was kindly provided by Dr. Nahum Sonenberg (McGill University). The p281-4-URE2 and p281-4-URE2_CT vectors have also been previously described (20).

Northern Blot Analysis—Northern blotting was performed generally as described (21) with slight modifications following the general procedures described previously (20). For detection of Ure2_lacZ mRNAs, the Prodigium fractionation 3-kb lacZ fragment (with 5′-GCTTCTACAGTTTGCACTACCATATTATAGTCTGGACATTTTG-3′ as the downstream primer) was amplified with 5′-GCCCGCATATGTTCAATTAAATCCTGTTAGG-3′ as the upstream primer and 5′-TTGATGAGACGTGTTGTG-3′ as a downstream primer, and cloned into KpnI-SalI-digested pRS316 vector.

Fractionation of Ribosomes—Fractionation of ribosomes was performed at 4°C except where indicated. Yeast cells from 50 ml of log phase culture were pelleted, treated for 1 min with 10 μl/ml cycloheximide (Calbiochem), and resuspended. Lysates were made by glass bead cell disruption (3–5 cycles, 1 min each), with intermittent cooling on ice, in buffer that contained 100 mM KCl, 2 mM magnesium acetate, 20 mM HEPES-KOH, pH 7.4, 14.4 mM β-mercaptoethanol, 100 μg/ml cycloheximide. Cell debris was removed by centrifugation at 7000 rpm for 8 min. Ribosomes were resolved in 10–25% sucrose gradients containing 100 mM KCl, 5 mM MgCl2, 20 mM HEPES-KOH, pH 7.4, and 2 mM dithiothreitol (Beckman SW28 rotor, 20,000 rpm, 19.5 h). Gradients were collected with continuous monitoring at 254 nm using an ISCO UA-5 absorbance detector and 1840 gradient collector. Proteins collected from sucrose gradient fractions were precipitated with 10% trichloroacetic acid and resolved in 10% Laemmli SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon (Millipore Corp.) membranes.

Western Blotting—Western blotting was performed following standard procedures (22). For studying eIF2A time course degradation experiments, yeast extracts were obtained by glass bead disruption of the yeast cells mixed with protein loading buffer. After vigorous vortexing, the beads and cell debris were removed by centrifugation at 14,000 rpm for 5 min, and Western blots were decorated with various monoclonal anti-HA tag antibodies (Cell Signaling, Inc.) or custom made rabbit anti-eIF2A antibodies followed by incubation with either goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated antibodies. The antibodies for actin and phosphoglycerate kinase were obtained from...
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Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The blots were then detected using an ECL™ kit (Amersham Biosciences).

Expression and Purification of a Recombinant eIF2A Protein Fragment—eIF2A cDNA fragment (comprising residues Gly39–Glu420) was amplified by PCR using 5′-AACGGGATCCGGTCCATGTTGGATA-ACGGTCTATTAAC-3′ as an upstream primer and 5′-TCCGGAATT-CCCTTCTAAACTAAAGAGCCTGATACATGC-3′ as a downstream primer, digested with BamHI and EcoRI, and cloned into pMW-127 vector (23, 24) as a fusion to the gene encoding staphylococcal nuclease bearing on its N-terminal end a His6 tag. The plasmid was transformed into BL21-CodonPlus (DE3)-RIL (Stratagene) cells. Cells were grown at 37 °C to an A660 of 0.6, induced with 1 mM isopropyl-β-D-thiogalactoside, harvested by centrifugation after 3 h of induction, and lysed by sonication in 20 mM Tris/HCl, pH 8.0, buffer, containing complete EDTA-free protease inhibitor mixture tablets (Roche Applied Science). The recombinant protein was found to be insoluble and was further used from the inclusion bodies under denaturing conditions (6 M urea, 20 mM Tris/HCl, pH 8.0, 0.5 M NaCl, 10 mM imidazole, 1 mM β-mercaptoethanol) on a Ni2+-nitrilotriacetic acid beads following standard procedures described elsewhere. After the Ni2+-nitrilotriacetic acid column purification, the protein was found to be 95–97% pure and was then dialyzed against 10 mM Tris/HCl buffer, pH 7.5, containing 0.2% SDS, 1 mM EDTA, and 10 mM β-mercaptoethanol and concentrated by 15-centrifugal filter device (Millipore Corp.) and further used for immunization of rabbits. Rabbit polyclonal antibodies were produced by United States Biological, Inc.

Actin Phalloidin Staining—Yeast strains were grown in YEPD medium for 16 h in log phase by continual dilution at 30 °C. Fixation was performed by adding formaldehyde and Triton X-100 to a final concentration of 4 and 0.5%, respectively, and incubated for 30 min at room temperature. Yeast pellets were resuspended in PBS buffer plus 0.5 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Cells were washed once with PBS prior to phalloidin addition to a final concentration of 0.6 μM in PBS and incubated for 60 min at room temperature in the dark. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting medium (90% glycerol, 0.1% PBS, 92 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast pellets were resuspended in PBS buffer plus 0.5 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting medium (90% glycerol, 0.1% PBS, 92 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast pellets were resuspended in PBS buffer plus 0.5 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting medium (90% glycerol, 0.1% PBS, 92 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting medium (90% glycerol, 0.1% PBS, 92 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting medium (90% glycerol, 0.1% PBS, 92 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting medium (90% glycerol, 0.1% PBS, 92 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting medium (90% glycerol, 0.1% PBS, 92 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting medium (90% glycerol, 0.1% PBS, 92 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature. Yeast samples were subsequently washed three times with PBS and resuspended in 1× mounting medium (90% glycerol, 0.1% PBS, 92 mM MgCl2 and 4% formaldehyde for further fixation for 90 min at room temperature.

RESULTS

The eIF2A null mutant has been shown to have no apparent phenotype in a variety of systematic tests assaying growth, metabolism, cell cycle progression, cytoskeleton and mitochondrial morphology, mating, stability of an artificial minichromosome, rate of spontaneous mutation, etc. (14, 28, 29), although it was found to possess reduced sporulation efficiency (14).

We have, however, previously shown that the double AΔΔBΔ mutant is “synthetically sick.” This suggested a genetic interaction between eIF2A and eIF5B (14). To further investigate the cellular functions of eIF2A in Saccharomyces cerevisiae, we decided to look for other initiation factors that could possibly genetically interact with eIF2A. Such genetic relationships, if they exist, suggest a functional interaction between the corresponding gene products. However, disruption of the vast majority of the initiation factors in yeast is known to be lethal. Thus, we decided to take advantage of combining the eIF2A mutation with some of the yeast temperature-sensitive mutants carrying ts mutations in the respective initiation factors. As a first step in this line of reasoning, the eIF2A gene (open reading frame YGR054W) was disrupted in the eIF4E-ts strain 4-2 using the kanMX disruption module and the resulting eIF4E-ts strain 4-2/2A (MATa, ade2-1, leu2-3,112, his3-11,15, trp1-1, ura3, ygr054w::KanMX, cdc33::LEU2 <cdc33-4-2; TRP1, ARS CEN>) was obtained.

The eIF4E-ts strain with a Disrupted Copy of the eIF2A Gene Is Synthetically Sick and Displays an Altered Phenotype—eIF4E-ts strain 4-2 has two point mutations in the eIF4E gene, which confer a temperature-sensitive phenotype, resulting in a rapid shutoff of protein synthesis and cell growth at the nonpermissive temperature (37 °C) (15). However, at permissive temperatures (28–30 °C), this strain displays growth rates that are only slightly reduced (15).

Surprisingly, the double eIF4E-ts and eIF2A mutant strain revealed a severe slow growth phenotype (Fig. 2A) when compared with the parental strains. The doubling time for this strain at 28 °C in liquid YPD glucose medium was found to be 4–4.2 h, whereas for the eIF4E-ts strain, it is about 2.5 h. This severe slow growth phenotype can be reverted by reintroducing the eIF2A gene on a CEN plasmid (Fig. 2A). Mutations in the eIF4E (CDC33) gene are known to arrest yeast cells at random points in the cell cycle (30, 31). To determine whether the eIF4E-ts/eIF2AΔ null mutant also presents a defect in cell cycle progression, we analyzed all strains by flow cytometry. At 30 °C, the eIF4E-ts 4-2 strain showed an increased cell population in G2 (73% total) (Fig. 2B). This effect was further exaggerated to more than 82% in eIF4E-ts/eIF2AΔ null mutant cells (Fig. 2B), perhaps contributing to the synthetic slow growth phenotype observed (Fig. 2A).

The 4-2/2A strain also displayed an altered phenotype (cell morphology) in comparison with ancestor eIF4E-ts strains 4-2 and WT CWO4 (Fig. 2C). On average, the 4-2/2A cells were larger in size and more rounded in shape, representing almost perfect circles (Fig. 2C). The establishment of cell morphology is universal during the development of both uni- and multicellular organisms (32). Wild type yeast cells have a relatively simple ellipsoidal shape; however, a number of mutants with elongated, round, small, large, pointed, clumped, and other shapes have been reported (33). In general, yeast cell growth and morphogenesis is a complex process, which is tightly linked to the cell cycle (34). The organization of the actin cytoskeleton plays a critical role in this morphogenic process (35). It is therefore possible that in the double eIF4E-ts and eIF2A mutant strain, the expression and/or organization of the actin cytoskeleton is affected. To check the latter, the actin cytoskeleton from the different yeast strains was stained using rhodamine phalloidin, and the samples were analyzed by fluorescence microscopy (Fig. 2D). The parental strains CWO4 and BY4741 revealed similar actin organization in the eIF4E-ts strain was somewhat altered with a partial loss of actin cables and depolarization. In contrast to CWO4 and eIF4E-ts strains, cytoskeletal organization, and polarization in the eIF4E-ts and eIF2A double mutant strain was severely affected, with almost complete disruption of the actin...
cytoskeleton resulting in a total loss of the actin cables and almost complete depolarization (Fig. 2D). It should be noted, however, that no change in actin cytoskeleton organization was observed in the single eIF2A/H9004 mutant strain (data not shown).

To determine whether actin expression was altered at the protein level, cell extracts were prepared and Western blots for actin were performed. To our surprise, it was found that actin levels were not constant in all samples. Strains carrying the eIF4E-ts mutation presented an increase in actin protein when compared with its wild type counterpart (30% increase), and the double eIF4E-ts and eIF2A mutant strain presented even greater changes with more than a 50% increase in actin levels (Fig. 2E). Thus, eIF2A might have a critical function in yeast in controlling (under certain specific conditions) the expression of the genes involved in cytoskeleton formation and assembly and this could ultimately lead to a change of cell morphology.

The observed genetic interaction of eIF2A and eIF4E suggests that both proteins function in the same pathway, namely initiation of protein synthesis. However, we have previously shown that eIF2A does not affect cap-dependent initiation or reinitiation in yeast as monitored using various GCN4 LacZ fusion constructs (kindly provided by Dr. Thomas Dever, National Institutes of Health) (14).

It was, however, still possible that eIF2A might affect internal initiation. To study the effect of eIF2A on IRES-mediated expression in yeast, the Ure2p-lacZ reporter system was used (20). In this construct, the URE2 IRES (URE2 open reading frame amino acids 3–353) was inserted in frame with lacZ and...
placed behind a stable hairpin structure (>−30 kcal/mol). This hairpin structure reduces cap-dependent lacZ expression almost completely (20, 36). Two constructs were produced; they carried at position 94 either ATG-Met (internal initiation start) or CCT-Leu (ATG was mutated to CCT in order to abolish internal initiation, used as a negative control) (20). Although there was almost no expression from the CCT-Leu construct, there was a significant increase in the level of lacZ-galactosidase expressed in an eIF4E-ts strain at the nonpermissive temperature (37 °C) compared with expression in wild type cells, indicating that the expression from the URE2 IRES is eIF4E- and cap-independent (Fig. 3) (20).

Expression from the URE2 IRES Is Up-regulated in Yeast Cells Lacking eIF2A—Surprisingly, it was found that lacZ expression driven by the URE2 IRES was dramatically enhanced in the eIF2A null strain in comparison with the WT strain having the same genetic background and that the observed increase in the activity was even greater than that measured for the eIF4E-ts strain at the nonpermissive temperature (Fig. 3A). At the same time as it was noted before (14), cap-dependent initiation in the eIF2A strain was not significantly affected, although a slight increase in its level was observed (Fig. 3A) when using a construct encoding lacZ mRNA with a 5'-untranslated region derived from the GAL1 gene (19). Surprisingly, we also found that the activity of URE2 IRES was not up-regulated in the double eIF4E-ts and eIF2A mutant strain at the permissive temperature and was only slightly elevated at the nonpermissive temperature (Fig. 3A), suggesting that expression of some other genes required for efficient URE2 IRES utilization may have also been affected in this strain. It
should be noted that no reduction of URE2_lacZ mRNA levels in the double 4-2/2A mutant strain in comparison with the elf4e-ts strain 4-2 (at both permissive and nonpermissive temperatures) was observed that could account for the observed differences in URE2 IRES activity (Fig. 3B). Also, the level of cap-dependent initiation and the extent of its inhibition at the nonpermissive temperature in 4-2/2A strain was found to be very similar to that of elf4e-ts strain 4-2 (Fig. 3A). In both cases an ~5-fold reduction in the expression of lacZ reporter driven by cap-dependent initiation was observed. Since elf2A was also found to genetically interact with GIM3, GIM5, and PAC10 genes (members of the prefoldin complex) (37), we also tested whether mutations in these genes could affect the activity of the URE2 IRES. No apparent change in URE2 IRES activity was observed when comparing isogenic GIM3, GIM5, and PAC10 null mutants and the WT BY4741 strain (Table I, Fig. 3C). It was concluded that up-regulation of URE2 IRES in elf2A null strain could primarily be related to the activity of elf2A protein.

Expression of elf2A Down-regulates URE2 IRES Activity in Yeast Cells—To further address the possibility that elf2A functions as a suppressor of Ure2p IRES-mediated translation initiation, the yeast elf2A gene was reintroduced into the elf2A knockout yeast strain expressing the Ure2p-IRES fusion construct. elf2A was expressed either from its own promoter in a YCplac111 plasmid (Fig. 4A) or as an HA-tagged elf2A expressed from the GAL promoter in the pBT328 plasmid. In both cases, down-regulation of URE2 IRES activity was observed (Fig. 4A); however, overexpression of elf2A from the GAL promoter down-regulated URE2 IRES activity more efficiently. The elf2A knockout strain transformed with either the pBT328 or YCplac111 plasmids was used as a control, and no down-regulation of URE2 IRES activity was observed in the latter cases. Also tested was whether human elf2A (which is 28% identical and 58% similar to the yeast protein in amino acid sequence) could substitute for its yeast homologue in vivo. It was found that human HA-tagged elf2A could also suppress the expression from the URE2 IRES element, but it functions about 80% as well as yeast HA-tagged elf2A in repressing the expression of lacZ (Fig. 4A). Both yeast and human proteins were found to be associated with 40 and 80 S ribosomes; however, association of the human protein with 80 S ribosomes was significantly reduced (Fig. 4B), and in contrast to the yeast protein, the human homologue was found predominantly associated with 40 S ribosomes. We anticipate that the reduced association of human elf2A with 80 S ribosomes could account for its reduced ability to suppress expression from the URE2 IRES element (by comparison with the yeast protein).

It should be noted that no apparent change in URE2_lacZ mRNA levels was observed when comparing WT yeast, yeast elf2A knockout, and yeast elf2A knockout strains transformed with either yeast or human elf2A that could account for the observed difference in lacZ activity (Fig. 4C). Thus, it was concluded that elf2A functions as a negative regulator of Ure2p IRES-mediated expression. Interestingly, only a 2-fold reduction of URE2 IRES activity was observed in the WT strain when overexpressing HA-tagged yeast elf2A. This could indicate that under the experimental conditions used, elf2A is present in wild-type yeast cells at sufficiently high concentrations such that an increase in elf2A levels does not further reduce expression from the Ure2p IRES element.

Association of elf2A with 40 and 80 S Is Not Impaired in the FUN12Δ (elf5BΔ) Strain—We have previously shown the genetic interaction between elf2A and elf5B (14). elf5B was also known to interact with elf2A in the process of 80 S complex formation in a reconstituted system when using AUG codon (7). We decided to check whether the presence of elf5B affected the binding of elf2A to either 40 or 80 S ribosome in vivo in yeast cells. The HA-tagged elf2A was transformed into an isogenic WT strain BY4741 (MATa, his3-1, leu2-0, met15-0, ura3-0) and elf5BΔ (MATa, his3-1, leu2-0, met15-0, ura3-0, fun12-2::KanMX) and we found that similar amounts of HA-tagged elf2A were present in the 40 and 80 S ribosomes of the WT and elf5BΔ strains (Fig. 5A). It was concluded that the absence of elf5B does not affect binding of elf2A to 40 and 80 S ribosomes. If, as we hypothesized, suppression of IRES activity through elf2A protein in yeast cells occurs at the level of 40 S/80 S ribosomes, one would then expect that there will be no change in URE2 IRES activity in elf5BΔ cells. Indeed, as expected, we observed no difference in lacZ expression driven by URE2 IRES in elf2A cells in comparison with WT yeast cells (Fig. 3A).

elf2A Remains Bound to 40 and 80 S Subunits in Extracts of prt1-t Cells Incubated at the Nonpermissive Temperature—The prt1-t mutation replaces Ser518 with Phe in the Prt1p (elf3b) subunit of yeast elf3 and confers a ts phenotype. Incubation of prt1-t cells at the nonpermissive (37°C) temperature produces a run-off of polysomes and accumulation of 80 S monosomes (38). We asked whether incubating prt1-t cells at the nonpermissive temperature would change the amount of elf2A associated with 40 S/80 S subunits. We found that elf2A remained bound to 40 and 80 S subunits in the prt1-t cells at the nonpermissive temperature and that the amount of elf2A bound to 80 S ribosomes increased slightly with the accumulation of 80 monosomes at the nonpermissive temperature (Fig. 5B, right).

elf2A Is an Inherently Unstable Protein—From the Stanford yeast genome expression connection data base (available on the World Wide Web at genome-www.stanford.edu/yeast_stress/), it is known that the mRNA levels for elf2A are highest under optimal growth conditions and decrease 2–8-fold under a wide variety of stress conditions, including heat shock, nitrogen depletion, amino acid starvation, diauxic shift, and stationary phase among others. We hypothesize that the decrease in elf2A mRNA levels would be accompanied by a decrease in
eIF2A protein levels. To address this question, we monitored the levels of HA-tagged eIF2A (which is under the control of the GAL promoter) by immunoblotting with anti-HA antibodies and compared them with the level of HA-tagged eIF4E (placed also under the control of the GAL promoter). A vector lacking the eIF2A gene was used as a control. β-Galactosidase activity (relative units) was determined after a 20-h induction. B, Western blot analysis of eIF2A, strain transformed with an HA-tagged eIF2A clones. Extracts from eIF2AΔ yeast strains carrying plasmids encoding either yeast or human HA-tagged eIF2A were subjected to sucrose gradient (10–50%) centrifugation. Fractions from the gradients were collected and resolved by SDS-PAGE. Subsequently, proteins were transferred to Immobilon membranes and probed with antibodies to the HA epitope. C, Northern blot analysis of Ure2-lacZ mRNA expressed in WT strain, eIF2AΔ strain, eIF2AΔ strain transformed with yeast HA-tagged eIF2A, or eIF2AΔ strain transformed with human HA-tagged eIF2A. For each lane, 25 μg of total yeast RNA was separated on a denaturing agarose gel, transferred onto a BrightStar™Plus Nylon membrane (Ambion), and hybridized to a 32P-labeled DNA lacZ probe. rRNA loading control is shown below the Northern blot.

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**FIG. 4.** eIF2A acts as a suppressor of the Ure2p IRES element in yeast cells. A, cells expressing the Ure2-lacZ reporter constructs under the control of the GAL1/10 promoter were co-transformed with vectors expressing either HA-tagged yeast eIF2A or HA-tagged human eIF2A from their respective GAL promoters. A vector lacking the eIF2A gene was used as a control. β-Galactosidase activity (relative units) was determined after a 20-h induction. B, Western blot analysis of eIF2A, strain transformed with an HA-tagged eIF2A clones. Extracts from eIF2AΔ yeast strains carrying plasmids encoding either yeast or human HA-tagged eIF2A were subjected to sucrose gradient (10–50%) centrifugation. Fractions from the gradients were collected and resolved by SDS-PAGE. Subsequently, proteins were transferred to Immobilon membranes and probed with antibodies to the HA epitope. C, Northern blot analysis of Ure2-lacZ mRNA expressed in WT strain, eIF2AΔ strain, eIF2AΔ strain transformed with yeast HA-tagged eIF2A, or eIF2AΔ strain transformed with human HA-tagged eIF2A. For each lane, 25 μg of total yeast RNA was separated on a denaturing agarose gel, transferred onto a BrightStar™Plus Nylon membrane (Ambion), and hybridized to a 32P-labeled DNA lacZ probe. rRNA loading control is shown below the Northern blot.

The lack of a drop in signal in the case of the HA-tagged eIF4E reflects, in part, a lag in the growth curve for the yeast in their adaptation to growth on glucose (i.e. there is no apparent change in A500 until roughly 2 h following the switch to glucose (Fig. 6B)). With more extended periods of time, there is the expected decrease in HA-tagged eIF4E concomitant with the increase in cell number (i.e. a dilution effect; data not shown). In contrast to HA-tagged eIF2A, a rapid drop-off in the signal for HA-tagged eIF2A was observed, suggesting that HA-tagged eIF2A is an inherently unstable protein. We used an HA epitope tag for detection, since the monoclonal antibodies against HA epitope are extremely sensitive, allowing one to detect very low levels of the tagged protein. One caveat to this experiment is that the observed instability of HA-tagged eIF2A could be apparent and might result from the rapid cleavage of the HA epitope. To verify our conclusion, we also used a polyclonal antibody derived against the central core fragment (Gly59–Glu420) of the recombinant eIF2A and measured the half-life of the HA-tagged eIF2A after repression of the GAL1 promoter by glucose. A similar decay of the eIF2A signal was observed (Fig. 6F).

Interestingly, a strong proline, glutamic acid, serine, threonine (PEST) motif (41, 42) of 24 amino acids (KSSETSPDST-...
the monoclonal antibodies to the HA epitope.
proteins were transferred to Immobilon membranes and probed with
dient was precipitated with cold 10% trichloroacetic acid, resuspended
sucrose gradient (10–25%) centrifugation. Each fraction from the gra-
medium at 28 °C and treated for 15 min at 37 °C were subjected to
eIF2A under control of a galactose-inducible promoter) grown in YPD
(H1676) cells (carrying the same plasmid encoding the wild type HA-
strains transformed with an HA-tagged eIF2A clone. eIF2A has now appeared in several genetic screens (14, 37).
main targets for translational control (51–55). In general, their
factors eIF4E, eIF4G, eIF2, and eIF2B have proven to be the
affecting their integrity or protein levels). So far, initiation
products are responsible for rapid degradation of proteins containing this motif, and a PEST score of more than 5 denotes
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Martin Rechsteiner in 1986. It is widely believed that PEST
webs/pestfind.html) originally developed by Scott Rogers and
World Wide Web at www.hgmp.mrc.ac.uk/Software/EMBOSS/
calculated using the PEST-FIND program (available on the
PAPSAPSTNAPTNNK) exists between positions 559 and 584
of the eIF2A amino acid sequence with a PEST score of 16.12
calculated using the PEST-FIND program (available on the
World Wide Web at www.hgmp.mrc.ac.uk/Software/EMBOSS/Apps/pestfind.html) originally developed by Scott Rogers and
Martin Rechsteiner in 1986. It is widely believed that PEST
sequences are responsible for rapid degradation of proteins containing this motif, and a PEST score of more than 5 denotes
a very strong proteolytic degradation signal (41, 42). eIF2A is also found to be among 1075 proteins identified as yeast ubiquitin conjugates (43). Ubiquitinated proteins are well known to be substrates for rapid turnover (44). This was not the case for eIF4E or any of the three subunits of initiation factor eIF2 (43). It should be noted that no significant difference in the levels of the two mRNAs was observed as determined by Northern blotting, and both mRNAs were almost completely degraded after 20 min following the switch from galactose to glucose (Fig. 4E).

DISCUSSION

Genetic analyses have proven to be a powerful tool for elucidating the biological function of proteins in S. cerevisiae, and eIF2A has now appeared in several genetic screens (14, 37). First, it was shown to genetically interact with eIF5B (14). This suggests that both proteins function in the same pathway (14). Although our experiments with FUN12A strain did not support the idea of a direct physical interaction of these two proteins in vivo during binding to 40 S/80 S ribosomes, they did not exclude the possibility that this interaction could be through some other protein or the ribosome either. Indeed, genetically interacting genes do encode proteins often found in the same complex, however, global analyses have shown that only 30 of 4039 genetically interacting gene pairs in S. cerevisiae encode physically interacting proteins (37). Very recently, eIF2A was also found to genetically interact with the members of the prefoldin complex, GIM3, GIM5, and PAC10 (37). The double knockouts of eIF2A/GIM3, eIF2A/GIM5, and eIF2A/PAC10 mutants display a severe slow growth phenotype (37). Members of the GIM family in yeast are important for the folding of tubulin and actin (45). Prefoldin binds ribosome-associated actin chains after synthesis of the first ~145 amino acids and remains bound to the actin polypeptide until its posttranslational delivery to the cytosolic chaperonin (46). Members of the prefoldin complex in contrast to eIF2A are predominantly associated with polysomes and not with 40 S/80 S ribosomes (46). The rapid assembly and disassembly of actin and tubulin filaments at specific subcellular locations provides the mechanistic basis for various dynamic activities such as segregation of chromosomes, change of cell shape/morphology, translocation of intracellular organelles, and others (47). Although none of the above mentioned genes have an obvious direct relationship with the translation process, this observation provides additional evidence that eIF2A might have a critical function in yeast in controlling (under certain specific conditions) the expression of the genes involved in cytoskeleton formation and assembly, and this could finally lead to a change of cell morphology. Our findings that the eIF4E-ts/eIF2A strain displays altered actin organization when compared with the parental strains as well as an affected morphogenetic processes supports this suggestion. The significant increase in actin levels observed in the eIF4E-ts/eIF2AΔ strain may be the cause of a slow growth phenotype. Remarkably, overexpression of actin is lethal in yeast; however, the reason for this sensitivity is not well understood (48, 49). It is unclear which gene expression is affected in the 4-22A strain that subsequently causes the observed changes in cell shape, actin disorganization, and actin levels. However, the genetic interaction of eIF4E and eIF2A reported here clearly shows that both proteins function in the same pathway.

It should be noted that translational control of gene expression has become the focus of many studies during the last 10 years. Recent studies have led to an increase in our understanding of how the balance between different initiation mechanisms (namely cap-dependent initiation, leaky scanning, reinitiation, and internal initiation) might influence cell fate (3–5, 50). This can be accomplished through the mechanisms that target specific initiation factors (altering their activity, affecting their integrity or protein levels). So far, initiation factors eIF4E, eIF4G, eIF2, and eIF2B have proven to be the main targets for translational control (51–55). In general, their inactivation is triggered by stress and their activation by growth proliferation signals. A part of the overall effect of stress is the decrease in cap-dependent translation through a reduction of eIF4F activity. At the same time, however, many mRNAs continue to be translated or become more efficiently translated under these conditions (56). These mRNAs are the most competitive, cap-dependently translated mRNAs or those mRNAs that are internally initiated (IRES containing).

In the past few years, IRES elements have been detected in an increasing number of cellular mRNAs from various species.
(57, 58). Remarkably, most of these IRES elements initiate translation of proteins that protect cells from stress or at least help them to cope with transient stress conditions (57, 58). The exact molecular mechanisms that redirect the ribosome from the m7G cap structure to the IRES elements under such conditions are unknown. One simple possibility is that the inhibition of cap-dependent translation through sequestration of eIF4E by the eIF4E binding protein frees other initiation fac-

FIG. 6. Yeast eIF2A is unstable protein. A, eIF2AΔ yeast cells co-transformed with HA-tagged eIF2A and HA-tagged eIF4E expression plasmids were grown overnight (18 h) on galactose YP-rich medium to allow for the expression of both HA-tagged eIF2A and HA-tagged eIF4E from their respective GAL promoters. Yeast cells co-transformed with ancestor plasmids that do not contain either eIF2A or eIF4E genes were used as control (lane C). At time 0, the cells were washed with water and then resuspended in YP medium containing glucose. Aliquots (50 μl) of the cells were taken at the indicated times. The yeast were pelleted and then dissolved in 200 μl of 20 mM HEPES/KOH buffer (pH 7.4) containing 100 mM KCl, 2 mM MgAc, 14.4 mM β-mercaptoethanol, 100 μg/ml cycloheximide. The cells were lysed by subsequent cycles of freezing in liquid nitrogen and thawing at 37 °C, and insoluble material was pelleted by centrifugation. Approximately equal amounts of protein from the supernatants were subjected to SDS Tris/Tricine 11.5% acrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and probed with anti-HA antibodies (Santa Cruz Biotechnology). B, yeast cell growth (A600) following the shift from galactose to glucose. C, the relative ratio of HA-tagged eIF4E and HA-tagged eIF2A as determined densitometrically from A. D, time course of HA-tagged eIF4E and HA-tagged eIF2A degradation in eIF2AΔ yeast cells. The quantitative data from C are plotted as log (relative intensity) versus time in order to estimate the protein half-life of HA-tagged eIF4E and HA-tagged eIF2A. Under the experimental conditions, no loss of HA-tagged eIF4E was observed, whereas HA-tagged eIF2A disappeared with a half-life of 17–18 min. E, Northern blot analysis of mRNA for HA-tagged eIF2A and HA-tagged eIF4E expressed in the eIF2AΔ strain. Total RNA was isolated from eIF2AΔ yeast strain harboring plasmids expressing HA-tagged eIF2A and HA-tagged eIF4E at 0, 10, 20, and 30 min following the switch from galactose to glucose. For each lane, 15 μg of total yeast RNA was separated on a denaturing agarose gel, transferred onto BrightStart-Plus nylon membrane (Ambion), and hybridized to a 32P-labeled DNA HA probes. The half-life of both mRNAs was less than 10 min. F, Western blot analysis of the eIF2AΔ strain transformed with an HA-tagged eIF2A clones. Custom made antibodies derived against a central core fragment (Gly59–Glu420) of recombinant eIF2A were used. Samples were prepared as in A. Protein loading control is shown to the right of the Western blot.
tors for the IRES-mediated processes. Few of the IRES containing mRNAs require the cap-binding factor eIF4E or intact eIF4G (i.e. missing the eIF4E-binding domain in the N terminus) for their translation (57) and, under conditions of reduced global translation, mRNAs containing IRES elements generally become more competitive for ribosome binding.

We have also shown here that eIF2A functions as a suppressor of IRES-mediated translation in yeast S. cerevisiae at least as tested using the Ure2p IRES reporter system. The mechanistic basis for this increased induction of Ure2p IRES expression in eIF2AΔ cells over what is seen in the WT yeast is unclear. Two possibilities exist. First, eIF2A might act directly by slowing the rate of IRES-containing mRNA binding to 40 S subunits or by slowing the steps between the 48 S complex and the elongating 80 S ribosome. In this regard, the finding of eIF2A bound to 40 and 80 S ribosomes would support this suggestion (14). Second, eIF2A might function indirectly by affecting the synthesis of some protein that directly influences IRES-mediated expression. Given that mammalian eIF2A was previously shown to not stimulate expression from globin mRNA (7), it is possible that eIF2A specifically binds and affects the translation of only a subset of mRNAs such as IRES-containing mRNAs. If one assumes a mechanism for eIF2A function based upon its similarity in biochemical properties to those of IF2 (binding of an aminoacyl-tRNA to ribosomes in a codon-dependent manner), it would appear likely that eIF2A actively participates in the initiation process. The finding of eIF2A in 48 and 80 S complexes (Fig. 4 and data in Ref. 14), and the fact that it marginally affects cap-dependent initiation supports this suggestion. We hypothesize that the release of eIF2A from 48 and 80 S complexes is much slower than the release of eIF2, and thus, although eIF2A is functioning in a positive, synthetic direction, the fact that it is acting so much more slowly than eIF2 results in “apparent suppression,” causing a delay in the transition of the 80 S ribosome to the elongation cycle. This suggestion is supported by an earlier observation on AUG-directed methionyl-puromycin synthesis using salt-washed ribosomes and purified rabbit eIF2 and eIF2A (7).

Concordant with this hypothesis is the observation in yeast that human eIF2A can only partially suppress Ure2p IRES activity and was found to be less abundant in the 80 S ribosomes (Fig. 4B). At the same time, no change in URE2 IRES activity was observed in eIF5BΔ cells, and the binding of the yeast eIF2A to 40 and 80 S ribosomes was not impaired either. The accumulation of eIF2A in 80 S complexes in prt1Δ cells, and the binding of the human eIF2A to 40 and 80 S ribosomes was not impaired either. This suggests that the free 80 S ribosomes (7) that eIF2A specifically binds and affects the translation of only a subset of mRNAs such as IRES-containing mRNAs. If one assumes a mechanism for eIF2A function based upon its similarity in biochemical properties to those of IF2 (binding of an aminoacyl-tRNA to ribosomes in a codon-dependent manner), it would appear likely that eIF2A actively participates in the initiation process. The finding of eIF2A in 48 and 80 S complexes (Fig. 4 and data in Ref. 14), and the fact that it marginally affects cap-dependent initiation supports this suggestion. We hypothesize that the release of eIF2A from 48 and 80 S complexes is much slower than the release of eIF2, and thus, although eIF2A is functioning in a positive, synthetic direction, the fact that it is acting so much more slowly than eIF2 results in “apparent suppression,” causing a delay in the transition of the 80 S ribosome to the elongation cycle. This suggestion is supported by an earlier observation on AUG-directed methionyl-puromycin synthesis using salt-washed ribosomes and purified rabbit eIF2 and eIF2A (7).

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