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Effect of Initiation Factor eIF-5A Depletion on Protein Synthesis and Proliferation of *Saccharomyces cerevisiae**

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Eukaryotic translation initiation factor eIF-5A (formerly eIF-4D) is thought to function in protein synthesis by promoting synthesis of the first peptide bond because it stimulates methionyl-puromycin formation *in vitro*. eIF-5A is encoded by two genes (*TIF51A* and *TIF51B*) in *Saccharomyces cerevisiae*; the protein and its hypusine modification are essential for cell viability. To analyze the factor's function *in vivo*, we expressed from the repressible *GAL* promoter a functional but unstable eIF-5A fusion protein (R-eIF-5A) with an NH_2-terminal arginine which is subject to rapid turnover through the NH_2-terminal end rule proteolytic pathway. When the conditional mutant strain is shifted from galactose to glucose medium, the rapid disappearance of R-eIF-5A protein occurs within one generation, causing an immediate inhibition of cell growth. However, eIF-5A-depleted cells synthesize protein at about 70% of the wild type rate and exhibit only a slight change in polysome profiles reflecting a subtle defect in a late step of translation initiation. These results suggest that the activity of eIF-5A may not be absolutely essential for general protein synthesis. Rather, eIF-5A may be selectively required for translation of certain mRNAs and/or may be involved in some other aspect of cell metabolism.

Eukaryotic translation initiation factor eIF-5A,[1] formerly called eIF-4D, is a small (16–18 kDa) abundant protein that is highly conserved in eukaryotes (1, 2). eIF-5A originally was isolated from the ribosomal high salt wash fraction of rabbit reticulocytes based on its activity in stimulating the synthesis of methionyl-puromycin (3, 4). This *in vitro* assay involves prior formation of an 80 S initiation complex containing methionyl-$tRNA_i$ and subsequently the reaction with puromycin which mimics synthesis of the first peptide bond (4, 5). Since eIF-5A is not required for 80 S initiation complex formation, it appears to be involved solely in the step where the first peptide bond is formed. Unfortunately, the methionyl-puromycin assay is the only means available for measuring the activity of eIF-5A *in vitro*. Furthermore, rather high concentrations of eIF-5A are required, well in excess of the amounts of methionyl-puromycin formed. As a result, there is concern whether or not eIF-5A truly functions in the initiation phase of protein synthesis. Clearly, the precise role of eIF-5A in translation *in vivo* has not yet been defined.

eIF-5A is the only known cellular protein that undergoes an unusual post-translational modification on a specific lysine residue to form hypusine (N^{ϵ}-(4-amino-2-hydroxybutyl)lysine) (6). The unique hypusine modification in mammalian cells occurs by a two-step pathway that involves the attachment of an aminobutyl group from spermidine to the ϵ-amino group of lysine 50, followed by hydroxylation on the number 2 carbon of the butyl group to form hypusine (7). The lysine residue is modified immediately after the synthesis of the protein, and hypusine is not altered or removed until the protein is degraded (8, 9). Hypusine in eIF-5A is found in all eukaryotic species investigated (2), suggesting an important role for the hypusinated protein in cellular metabolism. Furthermore, the hypusine modification is essential for eIF-5A activity in stimulating methionyl-puromycin synthesis in the *in vitro* reaction (10, 11).

To better study the structure and function of eIF-5A in eukaryotic cells, we have cloned two yeast genes encoding eIF-5A, *TIF51A* and *TIF51B*, using the mammalian eIF-5A cDNA as a probe (12). The two yeast genes encode proteins which share 90% amino acid sequence identity and are about 63% identical to human eIF-5A. Expression of at least one of the two yeast eIF-5A genes is required for cell viability, and the hypusine modification of the protein is vital for cell growth in *Saccharomyces cerevisiae*. Purified yeast eIF-5A protein stimulates methionyl-puromycin formation in the mammalian *in vitro* assay system (12), and the mammalian cDNA substitutes for the *TIF51* genes in yeast, demonstrating that human and yeast eIF-5A are not only conserved at the sequence level but are functionally interchangeable *in vivo* (13). Interestingly, the two yeast genes are members of a duplicated gene cluster (14), where sets of homologous genes, such as the *TIF51* genes and the *CYC* genes, are regulated by oxygen at the transcriptional level (15). Yeast cells grown aerobically express only mRNA transcribed from *TIF51A*, whereas cells grown anaerobically express only the *TIF51B* gene (16). However, growth analyses of strains constructed to express only *TIF51A* or *TIF51B* under the *GAL1,10* promoter showed that either gene product supports cell growth identically under either aerobic or anaerobic conditions, demonstrating that the two gene products are functionally indistinguishable (13). Besides hypusination, yeast eIF-5A undergoes another post-translational modification, partial phosphorylation on a serine residue, which generates two hypusinated isoelectric variants, eIF-5Aa and eIF-5Ab (17). Both isoelectric forms were purified separately and shown to possess identical activity in the methionyl-puromycin assay. Thus, hypusination is an essential modification for the activity of eIF-5A both *in vivo* and *in vitro*, but phosphorylation does not have an observable effect, at least in the *in vitro* assay (17). The physiological significance and site(s) of phosphorylation in eIF-5A are not yet known.

To investigate whether or not eIF-5A is truly involved in protein synthesis *in vivo*, we set out to deprive cells of the protein and characterize the effects on protein synthesis and on

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[1] The abbreviations used are: eIF, eukaryotic initiation factor; kb, kilobase pair(s); Ub, ubiquitin; PAGE, polyacrylamide gel electrophoresis.

intermediates in the initiation pathway. We constructed a *TIF51A* conditional expression system using a genetic cassette containing the repressible *GAL* promoter system in combination with protein-destabilizing elements which has been developed as an effective way to rapidly deplete a protein of interest (18). When expression of the altered *TIF51A* gene fused to the protein destabilizing elements is turned off by shifting the culture from galactose to glucose medium, the pre-existing destabilized eIF-5A fusion protein is eliminated from the cells within a single generation. Thus the effect of rapid depletion of eIF-5A on cell proliferation and protein synthesis could be analyzed.

MATERIALS AND METHODS

Strains and Growth Conditions—Escherichia coli strain MC1066 (F⁻ *ΔlacX74 hsdR rpsL galU galK trpC9830 leuB6 pyrF::Tn5*) (19) was the host for plasmid amplication and was grown at 37 °C in LB medium (0.5% yeast extract, 1% bactotryptone, 1% sodium chloride) supplemented with 100 μg/ml ampicillin to propagate plasmids, if required. *S. cerevisiae* strain W303-1A (MAT**a** *leu2–3, 112 his3–11, 15 ade2–1 ura3–1 trp1–1 can1–100*) and HHY13 (MATα *leu2–3, 112 his3–11, 15 ade2–1 ura3–1 trp1–1 can1–100* (pBM-TIF51A)) are described in a previous paper (13). Strain HHY132 (MATα *leu2–3, 112 his3–11, 15 ade2–1 ura3–1 trp1–1 can1–100* (pHSTC-TIF51A)) is a derivative of strain HHY13, obtained by transforming strain HHY13 with the *TRP1* plasmid, pHSTC-TIF51A, which contains the *TIF51A* gene, and then streaking the Trp⁺ transformants on 5-fluoro-orotic acid plates to select cells that have lost the *URA3* plasmid, pBM-TIF51A. The plasmid pHSTC was constructed by subcloning a 1.45-kb *Eco*RI fragment from plasmid YRp7 containing the yeast *ARS1/TRP1* sequence (20) into the *Eco*RI site of the bacterial vector pGEM-1 (Promega), followed by inserting a 1.6-kb blunt-ended *Sal*I fragment from plasmid p10-2-3 containing the *CEN11* sequence (obtained from M. Holland, University of California, Davis) into the *Pvu*II site of the pGEM-1 derivative. Then a 1.3-kb *Bam*HI-*Sph*I fragment containing the *TIF51A* gene, which was generated from pJA2 (12) was subcloned into the *Bam*HI and *Sph*I sites of pHSTC to generate pHSTC-TIF51A. The strains UBHY-M (MATα *leu2–3, 112 his3–11, 15 ade2–1 ura3–1 trp1–1 can1–100* (YCpUB-M5A)), and UBHY-R (MATα *leu2–3, 112 his3–11, 15 ade2–1 ura3–1 trp1–1 can1–100* (YCpUB-R5A)) are derivatives of strain HHY132 and express eIF-5A fusion proteins with protein destabilizing elements (see below). YP medium and minimal medium supplemented with the relevant amino acids, bases, and 2% glucose or 2% galactose were prepared as described (21). Yeast cells were grown at 30 °C and monitored by measuring the optical density at 600 nm in a Beckman spectrophotometer.

Construction of Plasmids for Expressing Variant Forms of the TIF51A Gene—The plasmid pGem-flu, which contains the *Bam*HI-*Xba*I fragment encoding a *lacI*-flu segment (18), was used to make an in-frame fusion of the *TIF51A* coding region to the *lacI*-flu segment. An *Xba*I-*Pst*I fragment containing the entire *TIF51A* coding sequence was generated by digestion of a 1.0-kb DNA fragment which was amplified from the plasmid pJA2 (12) by the polymerase chain reaction by using the 26-base synthetic DNA (5′-GGGGAATTCT AGA ATG TCT GA CGA AG-3′), which has an *Xba*I site (underlined) just 5′ to the initiator AUG of *TIF51A*, and the reverse sequencing primer (5′-GAAACAGCTATGAC-CATGATTACG-3′) for pUC19. This *Xba*I/*Pst*I fragment was subcloned into the *Xba*I and *Pst*I sites in the multicloning site of pGem-flu to generate an in-frame fusion of the *lacI*-flu segment to the *TIF51A* coding region. The resulting plasmid pGem-flu-TIF51A was digested with *Hin*dIII and then treated with Klenow fragment to make blunt ends. The linearized and blunt-ended plasmid was digested with *Bam*HI to generate a 1.1-kb *Bam*HI/blunt-ended fragment containing the *lacI*-flu-*TIF51A* fragment. To make an in-frame fusion with the *UBI4* gene under the control of the *GAL* promoter, the *lacI*-flu-*TIF51A* fragment was ligated with the *Bam*HI/*Pvu*II fragment of pUB23 which contains: Amp^r-2 micron origin-*URA3*-upstream activation sequence of the *GAL* promoter (UAS_{Gal})-ubiquitin (Ub)-Xaa (22). The resulting plasmids were named pUB-Xaa-5A, where Xaa represents either methionine or arginine. To change the pUB-Xaa-5A plasmid to a centromeric plasmid, a 2.9-kb *Eco*RI/FspI fragment containing the *URA3* gene and the Ub-*lacI*-flu-*TIF51A* fusion gene under the *GAL* promoter were obtained from the pUB-Xaa-5A plasmid and ligated with a 5.4-kb *Eco*RI/*Sma*I fragment containing Amp^r, *ARS1*, and the *CEN4* elements of YCp50 (23) to generate YCpUB-Xaa-5A (Fig. 1). The plasmid was introduced into the strain HHY132 which has the disrupted chromosomal copies of *TIF51A* and *TIF51B*, but harbors the plasmid pHSTC-TIF51A containing the wild type *TIF51A* gene and *TRP1* as a selectable marker. The transformants of HHY132 having the two plasmids, pHSTC-TIF51A and YCpUB-Xaa-5A, were grown for 20 generations in galactose containing complete minimal media but lacking uracil (SG-Ura) to allow the loss of the pHSTC-TIF51A plasmid by segregation. Then the cells were plated on SG-Ura plates and replica-plated into SG-Trp (lacking tryptophan) and SG-Ura (lacking uracil) plates to screen for colonies that cannot grow on SG-Trp due to the loss of the pHSTC-TIF51A plasmid but can grow on SG-Ura due to the presence of the YCpUB-Xaa-5A plasmid. The strain expressing the M-eIF-5A fusion protein (containing methionine at the NH_2 terminus of the mature protein) was designated as UBHY-M, and the strain expressing the R-eIF-5A protein (arginine NH_2 terminus) was designated as UBHY-R (Fig. 1).

Measurement of Protein Stability by Pulse-Chase Labeling and Immunoprecipitation—The W303-1A and UBHY-R strains were grown in methionine-labeling medium containing 2% galactose, 0.67% yeast nitrogen base without amino acids, uracil, adenine, and amino acids, except methionine, until the culture reached mid-exponential growth phase (OD_{600} = 0.25). Cultures (25 ml) were harvested, and cells were resuspended in 300 μl of the same medium. [^{35}S]Methionine (100 μCi; 1000 Ci/mmol) was added and the cells were incubated at 30 °C for 5 min. The cells were pelleted and resuspended in 0.6 ml of the labeling medium containing 0.5 mg/ml of cycloheximide and 0.2 mg/ml nonradioactive methionine. The cultures were incubated at 30 °C, and 100-μl aliquots were removed at various times. The aliquots were added to 700 μl of buffer A (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM Na-HEPES, pH 7.5, 0.5 mg/ml bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, 1 mM dithiothreitol). After adding 0.5 ml of glass beads, cells were disrupted by vortexing, and the lysate was centrifuged for 15 min at 12,000 × *g* at 4 °C. The amount of [^{35}S]methionine incorporated into protein was determined by hot trichloroacetic acid precipitation. Equal amounts radioactivity were used for immunoprecipitations with the polyclonal antibody against yeast eIF-5A as described elsewhere (17). Immunoprecipitates were subjected to 15% SDS-PAGE and radioactivity in eIF-5A bands was assessed by autoradiography and densitometric scanning.

Measurement of Protein Synthesis Rates—Precultures of strains W303-1A or UBHY-R were inoculated into methionine-labeling medium (see above) containing 2% galactose and grown to early log phase. Cultures were harvested, and cells were resuspended in the same labeling medium except that it contained 2% glucose instead of galactose in order to deplete eIF-5A protein in the UBHY-R strain. At various times after the shift to glucose-containing medium, cultures were harvested and cells (corresponding to OD_{600} = 1) were resuspended in 300 μl of 2% glucose-containing labeling medium. [^{35}S]Methionine was added, and cells were incubated at 30 °C for 5 min. To stop incorporation of [^{35}S]methionine, 1 ml of labeling medium containing cycloheximide (0.5 ml/ml) and unlabeled methionine (1.2 mg/ml) was added to the culture, followed by quick-freezing in liquid nitrogen. Cells were lysed in NaOH/β-mercaptoethanol, and proteins were precipitated with hot trichloroacetic acid (24). The pellet was washed with acetone, dissolved in 200 μl of 1% SDS solution, and heated immediately at 95 °C for 5 min. The incorporation of [^{35}S]methionine into total protein was determined by counting radioactivity in the SDS extract. The protein concentration of the SDS extract was analyzed by the micro-BCA protein assay reagent kit (Pierce Chemical Co.) as described by the manufacturer. The rate of protein synthesis is expressed as counts/min of [^{35}S]methionine incorporated into 1 μg of trichloroacetic acid-precipitable protein/min.

Polysome Profile Analysis—Cultures grown to mid-exponential phase in YP medium containing 2% galactose were shifted to YP medium containing 2% glucose. At various times after the shift to glucose medium, 100 μg/ml cycloheximide was added to the culture, followed by quick-cooling by swirling the culture on ice. The cells were collected by centrifugation and washed with buffer B (10 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) plus 100 μg/ml cycloheximide. The cell pellets were broken by vortexing with glass beads, and cell lysates were clarified by centrifugation at 10,000 × *g* for 5 min at 4 °C. The extracts corresponding to A_{260} = 10 were layered onto 12.5-ml sucrose density gradients (7–47% in Buffer A) prepared in a Gradient Master 105 (Biocomp) apparatus. The gradients were centrifuged in a Beckman SW40 rotor at 40,000 rpm for 150 min at 4 °C and fractionated in an ISCO density gradient fractionator.

RESULTS

Construction of a TIF51 Conditional System—An effective way to deplete a protein in yeast is to place its gene under the control of a repressible promoter such as the *GAL1,10* pro-

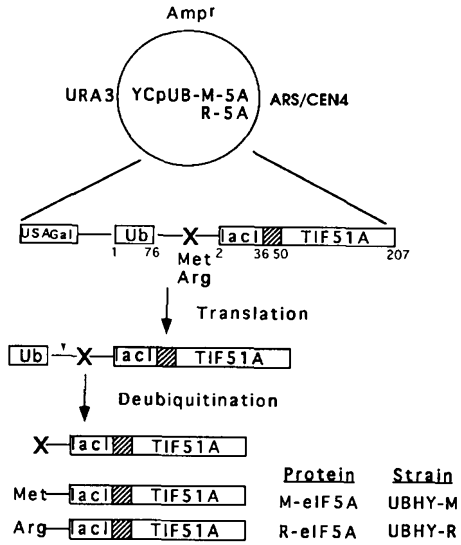

FIG. 1. **Schematic view of the plasmid constructs.** The plasmid carries *URA3*-UAS$_{Gal}$-Ub-Xaa region from pUB-23 (27) and the Amp^r-*ARS1-CEN11* fragment from YCp50 (23). Abbreviations used are: *UAS$_{Gal}$*, the upstream activation sequence of the *GAL* promoter (25); *Ub*, ubiquitin; *Xaa*, the codon for either arginine or methionine; *Amp^r*, ampicillin-resistant gene; *ARS*, autonomous replication sequence; *lacI*, a *Bam*HI-*Hin*fI fragment encoding amino acid residues 318–346 of the *lac* repressor. The *shaded box* represents an epitope from the influenza virus hemagglutinin protein fused to the *lacI* fragment (18). The *small arrowhead* indicates the site of de-ubiquitination cleavage in the nascent fusion protein.

moter, grow the cells in galactose medium, then shift to a medium containing glucose which strongly represses the *GAL* promoter (25). Using the *GAL* promoter, we first constructed the haploid strain HHY13 which has the chromosomal copies of both *TIF51A* and *TIF51B* disrupted, but harbors a centromeric plasmid containing the *TIF51A* coding region under the *GAL1* promoter (13). The steady state concentration of *TIF51A* mRNA in the wild type strain W303-1A and that of the hybrid *TIF51A* mRNA from the *GAL* promoter in strain HHY13 are very similar in the exponential growth phase. Likewise, HHY13 expresses almost the same level of eIF-5A protein as do wild type cells when analyzed by Western immunoblotting (data not shown). When a culture of strain HHY13 is shifted from galactose medium to glucose medium, transcription of *TIF51A* from the *GAL* promoter is turned off. Since eIF-5A is a very stable protein, the level drops essentially only by dilution due to cell division and cell growth continues normally for up to four to five generations before declining. We observed that eIF-5A must be reduced about 20-fold in cells of strain HHY13 before a growth rate phenotype is detectable, based on calculations from the number of cell divisions and Western blot analysis which is sufficiently sensitive to detect a few nanograms of eIF-5A protein (data not shown). Even though strain HHY13 shows the conditional phenotype dependent on carbon source, the long delay in the depletion of eIF-5A protein complicates analyses of the depleted cells, since many secondary effects may be occurring after five generations.

In order to deplete eIF-5A protein more rapidly, we constructed another conditional system using the *GAL* promoter together with protein destabilizing elements (26) which are fused in-frame to the coding region of the *TIF51A* gene. The vector system containing protein destabilizing elements (18) comprises: a ubiquitin gene at the 5′ end of the gene, followed by a codon for either Met or Arg; an N terminal extension derived from a *lacI*-flu fragment; and finally the coding region of *TIF51A* (Fig. 1). The purpose of ubiquitin-protein fusions is to generate otherwise identical proteins except bearing different NH_2-terminal residues. When such a fusion gene is expressed in cells, ubiquitin is cleaved from the fusion protein by the ubiquitin-specific isopeptidase (26) leaving either methionine or arginine as the NH_2-terminal amino acid residue. The NH_2-terminal end rule proteolytic pathway degrades proteins at variable rates according to their NH_2-terminal amino acid (26). Methionine is known as a stabilizing amino acid whereas arginine is a severely destabilizing amino acid. In addition, the *lacI* segment included in the construction of the fusion protein serves as a second determinant of the NH_2-terminal end rule degradation signal (27).

The Ub-Xaa-*lacI-TIF51A* fusion gene was placed behind the *GAL1,10* promoter in a *URA3* centromeric vector (Fig. 1) as described under "Material and Methods." This generates either plasmid YCpUB-M-5A for expression of the eIF-5A fusion protein whose mature NH_2 terminus is methionine or plasmid YCpUB-R-5A for the eIF-5A protein having arginine as the NH_2 terminus. Strains UBHY-M and UBHY-R were constructed (see "Material and Methods") that carry the respective plasmids as the sole source of the *TIF51* gene. Western immunoblot analyses of these cells grown in galactose medium show only de-ubiquitinated fusion proteins of the expected size, 26 kDa, but not the wild type eIF-5A protein of 20 kDa (Fig. 2). The ability to grow on galactose medium demonstrates that the eIF-5A fusion proteins function sufficiently well to complement the *TIF51* null mutations on the chromosomes. The two strains do not grow in glucose-containing medium (Fig. 4*C*), but growth is restored by transforming the strains with the plasmid pHSTC-TIF51A, which expresses wild type eIF-5A from its own promoter. This indicates that the lack of growth on glucose-containing medium is caused by depletion of eIF-5A.

Physiological Characterization of the Conditional System—To examine the conditional expression and the steady state level of eIF-5A protein, exponential cultures of strains UBHY-M and UBHY-R were shifted from galactose to glucose medium and cell lysates were prepared at various times thereafter. Western immunoblot analysis shows that the level of the M-eIF-5A fusion protein decreases steadily over a period of 6 h (Fig. 2*B*), whereas eIF-5A from the nonrepressed wild type gene in W303-1A remains constant (*A*). In contrast, strain UBHY-R shows a rapid disappearance of R-eIF-5A within 1 h after the shift to the glucose-containing medium (Fig. 2*C*). This result demonstrates that the R-eIF-5A protein, which has Arg as the NH_2 terminus, is so unstable that repression of transcription from the *GAL* promoter leads to a rapid depletion of R-eIF-5A protein. To demonstrate directly that the rapid disappearance of the R-eIF-5A protein is due to protein destabilization, pulse-chase labeling followed by immunoprecipitation was carried out with strains W303-1A and UBHY-R as described under "Material and Methods." Fig. 3 shows that unmodified eIF-5A expressed in the wild type cell is stable over a 60-min chase period (*A*), which is consistent with the greater than 24-h half-life of wild type eIF-5A protein reported previously (2). In contrast, the half-life of R-eIF-5A is about 10 min (Fig. 3*B*), which is a little longer than the 2-min half-life of Arg-β-galactosidase reported by Bachmair *et al.* (22). These results clearly show that R-eIF-5A, whose mature NH_2 terminus is the destabilizing residue arginine, is subject to rapid turnover through the NH_2-terminal end rule proteolytic pathway.

To investigate the effect of eIF-5A depletion on cell growth and viability, growth was monitored by measuring OD_{600} of liquid cultures and viability was analyzed by plating cells onto galactose medium throughout the growth period of strains UBHY-M and UBHY-R. The strains expressing the eIF-5A fusion proteins grow well with doubling times of 2.0 and 2.2 h, comparable with 1.9 h for the wild type strain W303-1A in

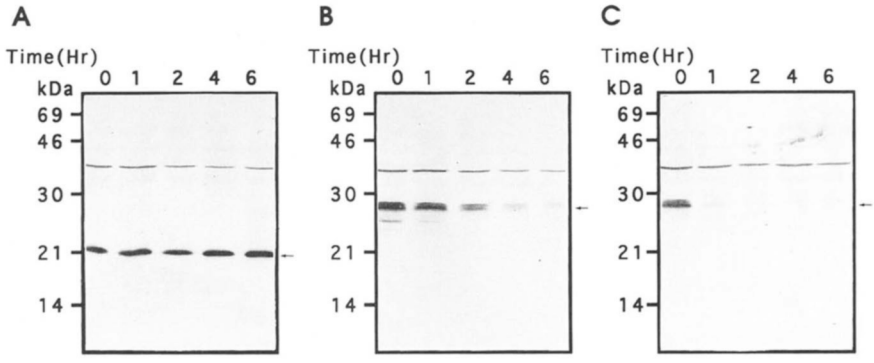

FIG. 2. **Western immunoblot analysis of eIF-5A protein in cell lysates.** S-30 cellular fractions were prepared from strain W303-1A (*A*), UBHY-M (*B*), and UBHY-R (*C*) as described under "Material and Methods" at various times after the shift from galactose to glucose medium. Total S-30 protein (20 μg) was fractionated by 15% SDS-PAGE (36), transferred to Immobilon membranes, and treated with affinity-purified anti-eIF-5A antibody as described (17). The figure shows a photograph of the blot after color development. The wild type eIF-5A in *A* migrates as a ~20-kDa protein, and the de-ubiquitinated eIF-5A fusion protein (*B* and *C*) migrates as a ~26-kDa protein (identified by *arrows* on the *right* of each panel). The ~38-kDa band is an unknown protein which cross-reacts with some batches of affinity-purified eIF-5A antibody; it serves as an internal standard. Molecular mass markers are indicated in kilodaltons on the *left*.

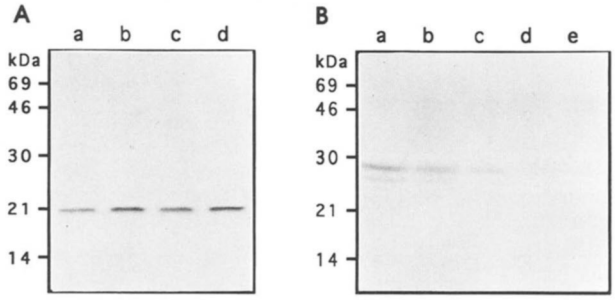

FIG. 3. **Pulse-chase experiment to determine the half-life of the R-eIF-5A protein.** *A*, pulse-chase analysis of wild type eIF-5A protein. After a 5-min pulse labeling, cells were analyzed immediately (*lane a*) and after a chase of 10 min (*lane b*), 30 min (*lane c*), and 60 min (*lane d*) in the presence of cycloheximide. eIF-5A was immunoprecipitated from cell lysates, and the precipitates were fractionated by SDS-PAGE as described under "Materials and Methods." *B*, pulse-chase analysis of the R-eIF-5A fusion protein. After a 5-min pulse labeling, cells were analyzed immediately (*lane a*) and after a chase of 5 min (*lane b*), 10 min (*lane c*), 30 min (*lane d*), and 60 min (*lane e*) in the presence of cycloheximide, as described for *A*. The figure shows a photograph of the autoradiogram of the gel. The sizes of molecular mass markers are indicated in kilodaltons.

galactose-containing medium (Fig. 4*A*). When the conditional strains are shifted from galactose to glucose medium, the UBHY-M strain grows normally for up to four generations, then starts to decrease in rate as observed previously for the HHY13 strain (Fig. 4*B*). In contrast, the growth rate of the UBHY-R strain begins to decrease after just one generation, consistent with the observation that the R-eIF-5A fusion protein is much less stable and thus is depleted more quickly. The cells gradually cease their growth and almost completely arrest after approximately three doublings (~16 h in glucose medium) as determined by OD_{600} (Fig. 4*B*). However, whereas the OD_{600} of the UBHY-R strain steadily increases to a moderate extent in glucose medium, the number of viable cells stops increasing immediately after one generation (data not shown). After 16 h of culture in glucose medium, cell viability is reduced to 30% as calculated by dividing the number of viable cells by the number of counted cells. Microscopic examination reveals that cells begin to increase in volume as eIF-5A is depleted. Most cells arrest as large single cells or as cells having a large bud that is almost the same size as the mother cell (after 16 h). At this time, the population of small cells and cells having a small bud is very low compared with wild type cells. Therefore, the discrepancy between the OD_{600} values and the number of viable cells seems partly due to the increase of cell volume and partly due to the defect in cell division. In the glucose-containing plate, the conditional strains do not form any detectable colonies at all (Fig. 4*C*). These observations suggest that the function of eIF-5A is required for initiation of cell proliferation, either directly or indirectly.

Analyses of Protein Synthesis Parameters upon Depletion of eIF-5A—Having established that the R-eIF-5A protein is rapidly depleted after transfer to glucose-containing medium, we used the UBHY-R strain to investigate the effect of the depletion of eIF-5A on protein synthesis. The rate of UBHY-R protein synthesis was measured with 5-min pulses of [^{35}S]methionine over the first 5 h of culture in glucose medium as described under "Material and Methods." After 1–2 h the cells "grow" for several more hours at about 40% the rate of wild type as detected by OD_{600}, but apparently have ceased cell division as analyzed by cell viability. We chose to analyze the early stage of the culture after the shift to glucose medium in order to avoid indirect effects of prolonged depletion of eIF-5A on protein synthesis, which may be caused by the decreased growth rate or by other factors. Surprisingly, the rate of protein synthesis is about 60–70% of that of wild type cells from 1 to 5 h (Fig. 5). Continuation of protein synthesis well after essentially complete depletion of eIF-5A suggests that eIF-5A is not required for global protein synthesis.

To investigate whether the slight decrease in the incorporation of [^{35}S]methionine into protein comes from a small defect in the protein synthesis machinery, polysome profiles were obtained from eIF-5A-depleted and nondepleted cells to determine whether or not the initiation phase might be affected. When initiation of translation is slowed or blocked, ribosomes finish translating mRNA but do not efficiently reinitiate the translation process, leading to a reduction in the size of polysomes (number of ribosomes per mRNA) and the accumulation of 80 S ribosomes. Exponential cultures of strains W303-1A (control) and UBHY-R (R-eIF-5A) were shifted to glucose medium and extracts were prepared and fractionated by sucrose density gradient centrifugation as described under "Material and Methods." The polysomes obtained from either strain grown in galactose medium show almost the same profile (Fig. 6, *left panels*). After 4-h growth in glucose medium, the polysome profile from the control cells is essentially unchanged (Fig. 6*A*, *right panel*), whereas that from the eIF-5A-depleted cells shows only slight differences (Fig. 6*B*, *right panel*). Substantial amounts of intact polysomes are seen in these UBHY-R cells, although a slight shift toward smaller polysomes is detected. In addition, a small increase in the 60 S peak and the presence of "half-mers" are observed for monosomes and small polysomes that are not seen in control cells. Half-mers are

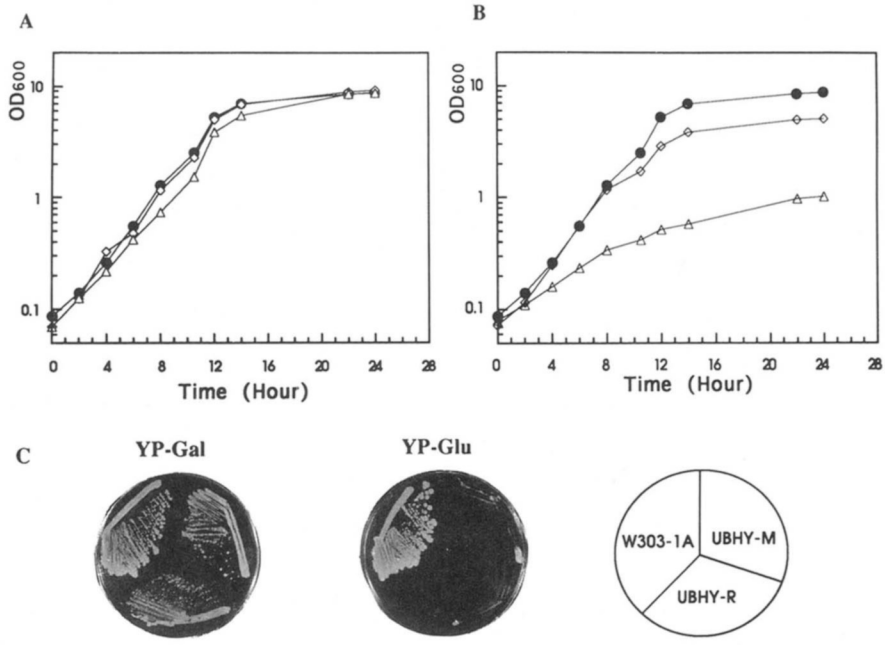

FIG. 4. **Effect of eIF-5A depletion on cell growth.** Exponential cultures of strain W303-1A (●), UBHY-M (◇), and UBHY-R (△) grown in YP media containing galactose were diluted to an OD_{600} = ~0.06–0.08 in YP media containing either galactose (*A*) or glucose (*B*). Growth was monitored by measuring optical density at 600 nm. *C*, the yeast strains W303-1A, UBHY-M, and UBHY-R were streaked on YP plates containing either galactose (*YP-Gal*) or glucose (*YP-Glu*) and grown at 30 °C for 3 days. The figure shows a photograph of the plates; a template indicating the locations of the streaked strains is shown on the *right*.

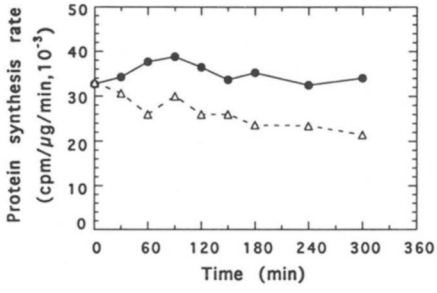

FIG. 5. **Protein synthesis rates in cells after a shift from galactose to glucose medium.** W303-1A (●) and UBHY-R (△) cells grown to early exponential phase in galactose-containing methionine-labeling medium were inoculated into glucose-containing labeling medium. At the times indicated, cells were taken from each culture and subjected to 5-min pulse labeling and analysis as described under "Material and Methods." The rate of [^{35}S]methionine incorporation into protein (counts/min/μg of protein/min) was calculated by dividing the counts/min of the hot trichloroacetic acid precipitate by the μg of protein in the sample and by the time of pulse labeling (5 min).

thought to be polysomes with an extra 40 S ribosomal subunit attached, presumably in the scanning mode or bound to the initiator AUG awaiting junction with the 60 S ribosomal subunit. These observations are consistent with a small effect of eIF-5A on a late step of initiation, leading to the slight decrease of the protein synthesis rate, but rule out that eIF-5A is essential for the initiation process in general protein synthesis.

DISCUSSION

eIF-5A is unique in that it is the only known cellular protein that undergoes the hypusine modification by spermidine at one of its lysine residues. Polyamines, and specifically spermidine, have been implicated in a wide variety of physiological effects, including cellular proliferation and differentiation. It has been reported that polyamines affect nucleic acid and protein synthesis both *in vivo* and *in vitro* (28). It is possible that eIF-5A is the intermediator through which polyamines exert such effects. Based on its activity in the methionyl-puromycin assay, a model system that mimics the initiation phase of eukaryotic translation, eIF-5A has been thought to be involved in the initiation step of protein synthesis. Since eIF-5A stimulates methionyl-puromycin synthesis after formation of the 80 S initiation complex (4), eIF-5A may be required specifically for formation of the first peptide bond. We have speculated that the positive charge on the non-acylated Met-$tRNA_i$ may not be favorable for tight binding in the donor site of the peptidyl transferase center of the 80 S initiation complex, and thus eIF-5A may provide stabilization for the binding and positioning of Met-$tRNA_i$ in the 80 S initiation complex (29). Demonstration of the involvement of eIF-5A in a reconstituted *in vitro* protein synthesis system with natural mRNA has been largely unsuccessful. It has been reported that eIF-5A slightly lowers the Mg^{2+} concentration for optimal protein synthesis, but does not affect the level of translation (30).

As a result of the facts cited above, there has been concern about the role of eIF-5A in the methionyl-puromycin assay. Even though stimulation of up to 3–6-fold has been obtained in the mammalian assay system, it is troubling that quite large amounts of protein are required to obtain such stimulation. Nearly 40–50 pmol of homogenous eIF-5A protein are added to obtain 0.2–0.3 pmol of methionyl-puromycin synthesis in the assay (12). Therefore there is doubt that eIF-5A is truly an initiation factor, as opposed to a protein that artifactually stimulates the formation of methionyl-puromycin. Thus it is important to test the possibility that while eIF-5A is an important yeast protein required for yeast growth and viability, it may not be a translation factor required for protein synthesis.

As an approach to investigate the role of eIF-5A in protein synthesis *in vivo*, we tried to deplete eIF-5A in cells and analyze the effect of eIF-5A depletion on protein synthesis. Depletion of a protein of interest has the advantage that it leads to the complete loss of function of the protein, in contrast to temperature-sensitive mutants that may still retain partial activity, especially if the protein has more than one function. For example, analysis of cell-free extracts from eIF-4A-depleted cells demonstrated that eIF-4A is truly involved in translation (31). Using protein destabilizing elements and the *GAL* promoter, we constructed a strain that shows rapid disappearance of eIF-5A protein within one generation in glucose medium. Analyses of protein synthesis parameters such as protein synthesis rate and polysome profiles upon rapid eIF-5A depletion show that cells support protein synthesis well, even when cell division is severely inhibited. A similar observation has been also made with strain HHY151, a null strain complemented by the human eIF-5A cDNA (13). This strain expresses human eIF-5A protein from the *GAL* promoter at such a low level that

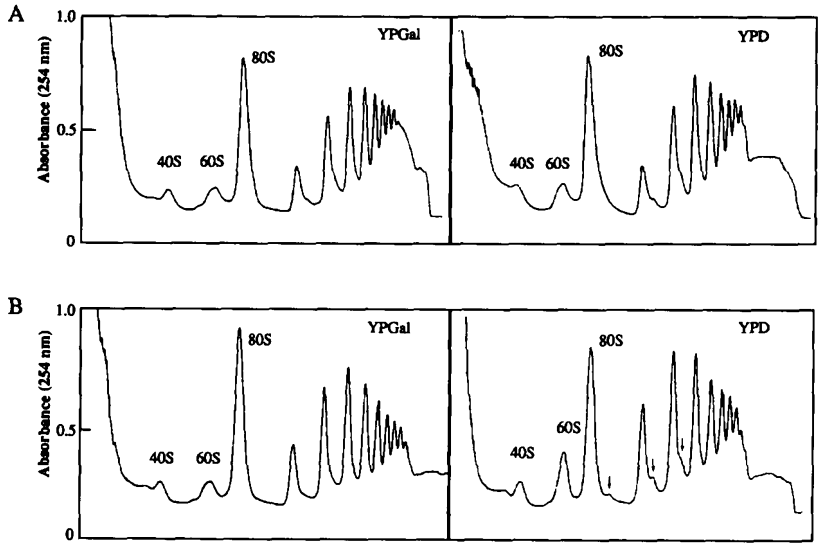

FIG. 6. **Polysome analyses of wild type and eIF-5A-depleted cells.** W303-1A (*A*) and UBHY-R (*B*) cells grown in galactose medium (*YPGal, left*) and those grown for 4 h after a shift to glucose medium (*YPD, right*) were harvested, lysed, and 10 $A_{260\ units}$ from each extract were fractionated by centrifugation on 7–47% sucrose gradients as described under "Materials and Methods." Gradients were collected from the top by using an ISCO density gradient fractionator and were scanned at A_{254} with an ISCO UA-5 detector. Sedimentation is from *left* to *right*. The *small arrows* in *B*, *right*, identify possible half-mers as discussed in the text.

it is barely detected by radiolabeling in galactose medium, yet the strain grows at an appreciable rate. The doubling time of strain HHY151 is ~2.5-fold greater than that of the wild type strain W303-1A, but we do not see any significant differences in the former's polysome profiles except for a very slight diminution of large polysomes.[2] The lack of a clear correlation between protein synthesis activity and growth rate in eIF-5A-depleted cells strongly argues against the idea that eIF-5A is required for general protein synthesis. It rather suggests that eIF-5A may be involved in some other aspect of cellular metabolism. But based on the observation that a slight decrease in protein synthesis occurs upon eIF-5A depletion, it still remains possible that eIF-5A is required for translation of a small set of mRNAs. We also cannot rule out that very low (undetectable) levels of eIF-5A generated by leaky transcription of the *GAL* promoter in glucose are sufficient to promote translation. This seems unlikely, however, since eIF-5A is an abundant protein in both yeast and mammalian cells.

Although eIF-5A was originally isolated from the ribosomal high salt wash (3), it is a very abundant protein in the cytoplasm, unlike most of the other initiation factors (1). It is largely present unbound to ribosomes, and only a small fraction of the protein is in the ribosomal high salt fraction. Such abundance of eIF-5A in the cytoplasm may imply multiple functions, like another abundant translation factor, eEF-1α (32). Recently the *TIF51A* gene was isolated as an multicopy suppressor of a defective RNA polymerase II (RPO21-mCTD51) in which a portion of the mouse COOH-terminal domain was placed at the carboxyl terminus of the largest subunit of yeast RNA polymerase II. The mutant strain shows cold sensitivity and an Ino$^-$ phenotype.[3] In another case, *TIF51A* was isolated as a multicopy suppresser of a *fus3* null strain, which shows defects in its ability to arrest in G1 phase in the presence of mating factor treatment.[4] However, it is not yet known how overexpression of eIF-5A suppresses these mutations. It is enigmatic that overexpression of eIF-5A suppresses such mutant phenotypes, when it already is an abundant protein in the nonsuppressed cells. The overproduction of eIF-5A in wild type cells does not change the growth phenotype of either HeLa cells (33) or yeast,[5] suggesting that it is not limiting.

On the other hand, the observation that eIF-5A depletion leads eventually to enlarged cells in G1 arrest is intriguing in that certain metal chelation inhibitors of deoxyhypusine hydroxylase, which is involved in one of the steps of hypusination, exhibit antiproliferative defects by arresting mammalian cells at the G1/S boundary of the cell cycle (34). Based on these observations, it was speculated that eIF-5A acts as an initiation factor selective for a subset of mRNAs encoding proteins that have a critical function in the initiation of DNA replication (35). To test the requirement of eIF-5A for a small class of mRNAs, we are in the process of evaluating the relative rates of synthesis of individual proteins in eIF-5A-depleted and nondepleted yeast cells. Work also is in progress to investigate possible other functions of eIF-5A by using the R-eIF-5A construct where eIF-5A can be depleted rapidly.

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[2] H. G. Schwelberger and H. A. Kang, unpublished results.
[3] H. Y. Rienhoff, personal communication.
[4] B. Satterberg and E. A. Elion, personal communication.
[5] H. A. Kang and J. W. B. Hershey, unpublished data.

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