Eukaryotic translation initiation factor 5 (eIF5),\(^1\) in conjunction with GTP and other initiation factors, plays an essential role in initiation of protein synthesis (for reviews, see Refs. 1–5). \(\textit{In vitro}\) studies using purified initiation factors have shown that the overall initiation reaction proceeds with the initial binding of the initiator Met-tRNA\(\text{f}\) as the Met-tRNA\(\text{f}\)-eIF2-GTP ternary complex to a 40 S ribosomal subunit followed by the positioning of the 40 S preinitiation complex (40 S-eIF3-Met-tRNA\(\text{f}\)-eIF2-GTP) at the initiation AUG codon of the mRNA to form the 40 S initiation complex (40 S-eIF3-mRNA-Met-tRNA\(\text{f}\)-eIF2-GTP). The initiation factor eIF5 then interacts with the 40 S initiation complex to mediate the hydrolysis of ribosome-bound GTP. Hydrolysis of GTP causes the release of eIF2-GDP (and P\(_i\)) from the 40 S ribosomal initiation complex which is essential for the subsequent joining of the 60 S ribosomal subunit to the 40 S complex to form a functional 80 S initiation complex (80 S-mRNA-Met-tRNA\(\text{f}\)) that is active in peptidyl transfer (6–9).

eIF5 was purified originally from rabbit reticulocyte lysates on the basis of its ability to stimulate \(\textit{in vitro}\) translation of globin mRNA in a partially reconstituted system (10–12). The purified protein was shown to be essential for the joining of 60 S ribosomal subunits to the 40 S initiation complex to form the 80 S initiation complex (7, 13, 14). In these initial studies the apparent molecular weight of the purified protein varied between 125,000 (10) and 150,000–168,000 (11, 12). In our laboratory, however, using an assay that measured directly the formation of an 80 S initiation complex by the joining of 60 S ribosomal subunits to a preformed AUG-dependent 40 S initiation complex, we purified eIF5 both from mammalian cells (15–18) as well as from the yeast \(\textit{Saccharomyces cerevisiae}\) (19). The purified mammalian and yeast proteins were shown to be monomers that migrated on SDS gels with an apparent molecular weight of about 58,000 and 54,000, respectively (15–19). More recently, we have cloned and expressed the mammalian cDNA encoding functional eIF5 of calculated \(M_r = 48,926\) (20–22). The \(\textit{S. cerevisiae}\) gene encoding eIF5, designated TIF5, has also been characterized and shown to be a single copy essential gene that encodes a protein of 45,346 daltons (23). The purified mammalian protein has also been used to characterize \(\textit{in vitro}\) the eIF5-mediated subunit joining reaction using AUG-dependent 40 S initiation complex as a substrate (8, 9, 17, 18, 24). However, the involvement of either the 49-kDa mammalian eIF5 or the 45-kDa yeast protein in translation of natural mRNAs \(\textit{in vivo}\) or \(\textit{in vitro}\) has not yet been defined. This is relevant in view of previous observations (1–5) that proteins, \(e.g.\) eIF5A (25), isolated on the basis of their ability to stimulate partial reactions in the initiation pathway, were later shown not to be involved in translation of mRNAs \(\textit{in vivo}\).

In this paper, we have used the \(\textit{S. cerevisiae}\) system to further characterize the function of eIF5 in protein synthesis \(\textit{in vivo}\). We have constructed a conditional eIF5 expression system in which functional eIF5 was expressed from a transcription unit consisting of a ubiquitin gene cassette which acts as a protein destabilizing genetic element fused to the NH\(_2\)-terminus of the open reading frame of the yeast TIF5 gene under the transcriptional control of \(\textit{GAL10}\) promoter. Similar expression systems were previously used by Park \textit{et al.} (26) and Kang and Hershey (25) as an effective way to rapidly deplete yeast cells of protein of interest. The effect of depletion of eIF5 on cell growth as well as protein synthesis \(\textit{in vivo}\) and \(\textit{in vitro}\) were then analyzed. The results of these experiments show that eIF5 plays an essential role in translation of mRNAs \(\textit{in vivo}\) and \(\textit{in vitro}\). Additionally, the rat cDNA encoding functional eIF5 was expressed in yeast cells lacking TIF5. We demonstrate that the...
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rat protein can functionally substitute for the homologous yeast protein in vivo.

**EXPERIMENTAL PROCEDURES**

**Media, Growth Conditions, and DNA Manipulations**—Yeast strains were grown at 30 °C in either YPD medium (1% (w/v) yeast extract, 2% (w/v) Bacto-peptone, and 2% (w/v) dextrose) or in YPGal medium where 2% (w/v) galactose (galactose-free) replaced dextrose as the carbon source. Where indicated, haploid yeast cells were also grown in either synthetic complete medium containing galactose as the sole carbon source, designated SGal medium (2% galactose, 0.67% Bacto-yeast nitrogen base without amino acids, 0.2% amino acid mixture) or in SD medium where 2% dextrose replaced galactose as the carbon source. For in vivo [35S]methionine incorporation, the 0.2% amino acid mixture in either the SGal or the SD media did not contain methionine. These methionine-lacking media were designated SGal-Met and SD-Met, respectively. Yeast transformations and tetrad analysis were performed as described by Rose et al. (27). Methods for plasmid and genomic DNA preparations, restriction enzyme digestion, DNA ligation, cloning, and bacterial transformation were according to standard protocols (28).

**Construction of Plasmids for Conditional Expression of Yeast eIF5 or Mammalian eIF5**—A 2.5-kb yeast genomic fragment containing the entire coding region for yeast eIF5 (23) was separately cloned into the XbaI/HindIII sites of centromeric plasmids pRS316 (URA3-based) (29) and pRS315 (LEU2-based) (29) to yield pRS316-TIF5 and pRS315-TIF5, respectively. In these newly constructed plasmids the expression of yeast eIF5 is under the transcriptional control of its endogenous promoter present in the inserted 2.8-kb fragment. For expression of yeast and mammalian eIF5 under the control of galactose-inducible GAL1 promoter, we first isolated a 0.6-kb BamHI/EcoRI fragment containing GAL1-GAL10 promoter from plasmid pBM272 (30), and cloned it at the same sites of pRS315 to generate a new recombinant vector, pTM100. For cloning of the yeast eIF5 open reading frame into pTM100 vector, two primers were synthesized bearing the NH2-terminal and COOH-terminal ends of yeast eIF5 coding sequences (23) that were flanked by BamHI and EcoRI restriction sites: N1, 5′-GCGGATCCATGTCTATTAATATTTGTAG-3′ and N2, 5′-CGAATTCTCTCTGCTGTCATC-3′. These primers were used in high fidelity-polymerase chain reaction using Pyrococcus DNA polymerase I (Stratagene) and the polymerase chain reaction product was digested with BamHI and EcoRI and cloned into the same sites of pGEM-TIF5+ vector to yield pGEM-TIF5+. DNA sequencing of the eIF5 coding region in the recombinant plasmid indicated error-free DNA synthesis. The 1.2-kb TIF5 coding region was then amplified from pGEM-TIF5+ by digestion with BamHI and XbaI and cloned into the same sites of pTM100 vector to generate the new recombinant plasmid pTM100-TIF5. For expression of mammalian eIF5 under GAL1 promoter, the recombinant rat eIF5 expression plasmid, pET5(TIF5), (20, 22), was digested with EcoRI, blunt-ended by treatment with klenow DNA polymerase, in the presence of 4 mM DTT, and then cut with 96 units of NdeI to generate the 1.34-kb fragment that contained the entire coding sequence of rat eIF5 cDNA, starting 42 base pairs upstream of the initiating ATG codon and 8 base pairs after the termination codon. This fragment was then cloned into the NotI (blunt-ended)/XbaI sites of pTM100 vector to yield the new recombinant plasmid pTM100-EIF5. We also constructed a plasmid for conditional expression of a rapidly-degradable form of yeast eIF5 fusion protein (see Fig. 1). For this purpose, the coding region of TIF5 was first fused in-frame to a lacI-flu segment present in the plasmid pGEM-flu (26) as follows. The vector pGEM-TIF5 was digested with BamHI and SpI and the 1.235-kb BamHI (blunt-ended)/SpI fragment containing the entire TIF5 coding region was cloned into the PsI (blunt-ended)/SpI site of the vector pGEM-flu to yield pGEM-flu-TIF5. This new vector was then cut with BamHI and SpI and the resulting 1.34-kb lacI-flu/TIF5 fragment was fused in-frame to the ubiquitin gene, UBAI (26) under the control of the GAL10 promoter as follows. The plasmid pUB23 which contains the UBAI gene was cut with EcoRI and BamHI and the resulting EcoRI/BamHI fragment that encodes URA3 upstream activation sequence of the GAL promoter (UAASgal-ubiquitin-(Ub)-X (where X is the truncine-otid portion of eIF5)) was ligated with the corresponding BamHI/SpI fragment that encodes the lacI-flu/TIF5 gene derived from the pGEM-flu vector. The resulting EcoRI/SpI fragment was then cloned into the EcoRI/SpI site of the centromeric HIS3 vector pSE362 (26) to generate pUB-TIFS5 (Fig. 1). Expression of yeast eIF5 from the GAL10 promoter in this plasmid initially generates a NH2-terminal ubiquitylated eIF5 fusion protein, which is rapidly processed in yeast cells by a deubiquitination enzyme to yield free ubiquitin plus an eIF5 fusion protein having arginine (R) as the NH2-terminal amino acid (see Fig. 1).

**Construction of Haploid Yeast Strains for Expression of Yeast and Mammalian eIF5 Proteins**—The haploid yeast strain TMY101 (see Table I), containing the chromosomal copy of the TIF5 gene disrupted with TRP1 marker gene and harboring the plasmid pRS316-TIF5 that expresses yeast eIF5 from its own promoter, was crossed to the diploid strain W303(a/a) following the one-step gene disruption method of Rothstein (31) as described for a similar disruption procedure with URA3 gene by Chakravarti and Maitra (23). The newly generated diploid strain (trf5::TRP1/TIF5) was then transformed with plasmid pRS316-TIF5 and Ura- transformants were selected. One of the transformants was then sporulated at 30 °C, and the resulting tetrads were dissected and spores were germinated on YPD plates followed by selection on SD-Trp-Ura plates. Only spores containing the trf5::TRP1 gene and harboring the plasmid, pRS316-TIF5 will germinate to yield Trp- Ura- colonies. This haploid strain, named TMY101 (Table I), was the host strain used for all plasmid shuffling experiments described in this study. The genotype of the TIF5 locus on the chromosome and on the plasmid of TMY101 was confirmed by Southern blot analysis (23) using relevant DNA restriction fragments as probes. For construction of haploid yeast strains for conditional expression of ubiquitinated eIF5 fusion protein, the strain TMY101 was transformed with LEU2-plasmid pRS315-TIF5, and Trp- Ura- colonies were selected on SD plates and replica-plated onto another SD plate which contained uracil and thiamine to select the URA3 plasmid. The yeast strain recovered from the 5-FOA plate, designated TMY205, has the disrupted chromosomal copy of TIF5 but now harboring pRS315-TIF5 as the complementing plasmid. This strain was then transformed with pUB-TIFS5 that contains the TIF5 gene fused to the ubiquitin gene cassette (see Fig. 1). The resulting Leu' His' Ura- transformants were then selected and grown in SGAHis-Ura media to promote the loss of the LEU2-plasmid, pRS315-TIF5 by segregation. The newly generated yeast strain, which has the disrupted chromosomal copy of the TIF5 gene, but harboring only the plasmid, pUB-TIFS5, was selected on appropriate SGAi media. This strain, designated TMY201R, expresses eIF5 fusion protein (arginine at the NH2 terminus of the mature protein (Fig. 1).

**Cell Line Profile Analysis**—The haploid yeast strains W303n or TMY201R were grown in YP medium containing 2% galactose (YPGal) to early logarithmic phase. The cells were harvested and 3 A260 units of cells were resuspended in 100 ml of YPD medium supplemented with 0.4 mg/ml adenine sulfate and grown for about 10 h at 30 °C. Following addition of cycloheximide (50 μg/ml), cultures were rapidly chilled in an ice-water bath and centrifuged. The cells were washed twice with LHB buffer (10 mM Tris-acetate, pH 7.5, 150 mM NaCl, 30 mM MgCl2, and 50 μg/ml cycloheximide). The washed cells were then suspended in 1.2 ml of the LHB buffer and lysed by vortexing with an equal volume of glass beads. After adding an additional 0.5 ml of the same buffer, the cell lysates were clarified by centrifugation first at 12,000 × g for 10 min, followed by recentrifugation at 17,600 × g for another 10 min. Equivalent amounts of A250 absorbing material (approximately 10 A250 units in 300 μl) were layered on 11 ml of 7–47% (w/v) sucrose gradients in either a low salt buffer (10 mM Tris acetate, pH 7.0, 12 mM MgCl2, 50 mM NaCl) or in a high salt buffer (10 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 800 mM KC1) and centrifuged at 40,000 rpm for 2.5 h at 4 °C in a Beckman SW41 rotor. The gradients were fractionated in an ISCO density gradient fractionator and the absorbance profile at 254 nm was analyzed in an ISCO UA-5 absorbance monitor.

**Cell-free Translation**—The strain TMY201R was grown in YPGal supplemented with adenine sulfate (0.4 mg/ml) overnight at 30 °C (A250 = 1.0). Cells were harvested and suspended in YPD medium containing adenine sulfate (0.4 mg/ml) such that the initial absorbance at 600 nm was about 0.03 and grown at 30 °C for about 18 h (about 2 generations in YPD medium) when growth was nearly completely arrested. Cells were then harvested and cell-free translation extracts were prepared and mRNA-dependent cell-free translation was performed using [35S]methionine as the labeled amino acid as described by Hussain and Liebowitz (33).

**Other Materials and Methods**—Total RNA of S. cerevisiae was isolated as described by Rose et al. (27) while poly(A) RNA was isolated from total yeast RNA by chromatography on oligo(dT)-cellulose columns (Pharmacia LKB Biotech Inc.) using the protocol of the manufacturer. Luciferase mRNA was purchased from Promega and capped at the 5′ end with vaccinia guanylylmethyltransferase as described (34). The vaccinia vaccine was a kind gift of Dr. Stewart Shuman of Sloan-Kettering Cancer Institute, New York. Purified eIF5 from rabbit reticulocyte lysates as well as recombinant rat eIF5 were isolated as described (18, 22). Yeast eIF5 was purified from S. cerevisiae strain.
BJ926, as described by Chakravarti et al. (19). The purified yeast eIF5 preparation exhibited two polypeptide bands of 54 and 56 kDa upon SDS-polyacrylamide gel electrophoresis. Each of the two polypeptides was found to contain eIF5 activity and they were immunologically related to each other (19). The preparation of specific rabbit antisera against purified rabbit and yeast eIF5 and their affinity purification were as described (17, 19). The procedure used for preparation of yeast cell lysates for immunoblot analysis was adapted from that described by Sachs and Davis (35).

RESULTS

Construction and Expression of a Conditional Mutant Form of eIF5 in S. cerevisiae—To understand the function of eIF5 in the yeast S. cerevisiae, we depleted yeast cells of endogenous eIF5 and measured the effects of such depletion on cell growth as well as protein synthesis in vivo and in vitro. We initially constructed a haploid yeast strain in which the chromosomal copy of the TIF5 gene was inactivated by disruption with TRP1 marker gene and the essential eIF5 function was provided by maintenance of a centromeric plasmid that contained the open reading frame of the TIF5 gene under the transcriptional control of the GAL1 promoter. This yeast strain, designated TMY201 (Table I), as expected, grew on YPGal plates but did not form detectable colonies on YPD plates (data not shown). To deplete yeast cells of eIF5, TMY201 cells were grown in galactose-containing medium to early exponential growth phase and then transferred to glucose medium to turn off transcription of the TIF5 gene from the GAL1 promoter and thus, in essence turning off synthesis of new eIF5 protein in cells. Surprisingly, we observed that under these conditions, cell growth continued normally for five to six generations before declining. Immunoblot analysis of cell lysates showed that while the level of eIF5 progressively decreased with time following transfer from galactose to glucose medium, a significant level of eIF5 was still detectable in cell lysates even after six generations of growth (data not shown). Presumably, the rate of degradation of pre-existing eIF5 protein was not rapid enough to deplete yeast cells of detectable levels of eIF5 within one or two generations. In view of the observation (9, 17) that eIF5 acts catalytically in initiation of protein synthesis in vitro, the presence of very low levels of the protein in yeast cells may be sufficient to sustain slow cell growth for many generations even in the absence of new eIF5 synthesis. Furthermore, because depletion of eIF5 in TMY201 cells required several generations of growth under repressing conditions, we were concerned of the possibility that any secondary effects might be induced under these conditions that could complicate interpretation of results obtained with such eIF5-depleted cells.

To further lower the cellular level of eIF5 and to ensure more rapid depletion of eIF5 from yeast cells following transfer to repressing conditions, we constructed a haploid yeast strain TMY201R as described under “Experimental Procedures” in which the chromosomal copy of the TI5 gene was inactivated with TRP1 marker gene and the strain was kept viable by maintenance of the URA3-centromeric plasmid that harbors a conditional eIF5 expression system in which eIF5 was expressed from a genetic cassette designed to destabilize the eIF5 protein. In such a system, constructed by utilizing a strategy first used by Park et al. (26) and later by Kang and Hershey (25), eIF5 was expressed from a transcription unit consisting of a ubiquitin gene cassette (Ub-X-lacI-TIF5) which acts as a protein destabilizing element fused to the NH2 terminus of the open reading frame of the TIF5 gene under the transcriptional control of GAL10 promoter (Fig. 1). The eIF5 fusion protein synthesized from this construct contains ubiquitin at the NH2 terminus followed by a 31-amino acid segment of the lacI repressor which acts as a recognition element for ubiquitin-dependent protein degradation by NH2-terminal recognition pathway (36, 37). Deubiquitination of this eIF5 fusion protein exposes arginine (R) as the NH2 terminus amino acid (see Fig. 1) and should lead to rapid degradation of the protein by the N-end rule (36, 37). As expected the strain TMY201R grew well on plates containing galactose as the carbon source while it did not form detectable colonies on plates containing glucose as the sole source of carbon (data not shown). When growth of the strain TMY201R was monitored in liquid cultures containing galactose as the carbon source, it grew with doubling time of 3.4 h comparable with 3.2 h for the wild-type W303a strain (Fig. 2, panel A). However, when the exponentially growing cultures of these strains were shifted from galactose to glucose-containing medium to repress transcription of the TIF5 gene from the GAL10 promoter, the strain W303a, in which eIF5 expression occurred from its own promoter, as expected, grew well with a doubling time of about 2 h (Fig. 2, panel B). In contrast, the growth rate of TMY201R, in which expression of eIF5 is under the control of GAL10 promoter, began to decrease after about one generation and the growth rate was drastically reduced in about two generations (17 h in glucose medium) (Fig. 2, panel B).

The level of eIF5 protein in cell lysates following the shift from galactose to glucose medium was also analyzed using affinity-purified anti-yeast eIF5 antibodies as probes. Fig. 2 (lower panel) shows that the level of eIF5 in cell lysates prepared from non-repressed W303a cells remained constant during the growth period. In contrast, there was a rapid disappearance of R-eIF5 fusion protein in strain TMY201R. After about 17 h there was no detectable level of R-eIF5 fusion protein (Fig. 2, lower panel). These results demonstrate that in strain TMY201R, which synthesizes eIF5 fusion protein having Arg as the NH2 terminus, a more rapid depletion of eIF5 can be achieved following repression of transcription of the TIF5 gene cassette from the GAL10 promoter, and this depletion of eIF5...
The plasmid contains correlation between the rate of protein synthesis over the growth period. Fig. 3 shows that there was a clear protein synthesis. After about 17–18 h (between 7.5 and 10 h), there was a marked decrease in the rate of protein synthesis remained fairly constant. However, because of the presence of pre-existing eIF5 in cells, the rate of protein synthesis was decreased even further. The decrease in protein synthesis was due to the presence of eIF5 in cells, as TMY201R cells continued to grow exponentially following the shift from galactose to glucose medium, as TMY201R cells growing in galactose medium (Fig. 4, panel B) or TMY201R cells growing in galactose medium (Fig. 4, panel D). When equivalent amounts of cell extracts were also analyzed in sucrose gradients containing 0.8 M KCl, the majority of the accumulated monoribosomes in eIF5-depleted TMY201R cells were found to be dissociated into subunits (Fig. 4, panel F), indicating that they are neither bound to mRNA nor arise due to nonspecific RNase activity in extracts. The observed decrease in polyribosome content with an increase in the amount of free 80 S ribosomes and 60 S ribosomal subunits when eIF5 was depleted, indicates a block in translation initiation, and not in the elongation or termination of protein synthesis.

**eIF5-dependent Cell-free Yeast Translation System**—We used eIF5-depleted TMY201R cells to prepare a yeast cell-free translation system to demonstrate the requirement of eIF5 for translation of mRNAs in vivo (Fig. 5). Cell-free translation extracts prepared (33) from exponentially growing cultures of wild-type W303α cells in glucose medium was highly active in translation of total yeast poly(A)⁺ RNA without any exogenously added protein factors (Fig. 5, panel A). In contrast, when similar cell-free extracts were prepared from TMY201R cells that were initially grown in galactose-containing medium and then shifted to glucose medium and grown until the growth of cells virtually stopped (about 18–20 h) due to depletion of eIF5, they were rather inactive in translation of both total yeast poly(A)⁺ RNA (Fig. 5, panel B) as well as luciferase mRNA (Fig. 6). Translation could be restored in these lysates
by the addition of purified yeast eIF5 (Figs. 5B and 6). In the absence of mRNA, addition of eIF5 had virtually no effect (Fig. 5B and Fig. 6). Recombinant rat eIF5 was also able to substitute for yeast eIF5 in restoring translation in such eIF5-depleted cell-free extracts (Fig. 5B). However, the rat protein was about 50% as effective as an equimolar amount of yeast eIF5 in restoring translation in eIF5-depleted translation extracts (Fig. 5, panel B, compare stimulation of translation by 10 ng of each protein factor). In contrast to strong dependence of eIF5 for translation of mRNAs in these cell extracts, extracts prepared from wild-type yeast cells which were highly active in translation of poly(A)+ RNA, was not stimulated by exogenously added eIF5 (Fig. 5A), indicating that wild-type yeast extracts contained saturating levels of eIF5.

**Mammalian eIF5 Can Functionally Substitute for the Homologous Yeast Protein in the Yeast S. cerevisiae**—Results presented above showed that recombinant rat eIF5 can substitute for yeast eIF5 in supporting protein synthesis in *in vitro* yeast translation system. We employed the plasmid shuffling technique to determine if the wild-type mammalian (rat) eIF5 protein can functionally substitute for the corresponding yeast protein in *vivo*. For this purpose, we constructed the haploid *S. cerevisiae* strain TMY101 as described under “Experimental Procedures” and Table 1. The strain TMY101 carries inactive *TIF5* disrupted with the *TRP1* marker gene and is kept viable by maintenance of a centromeric *URA3* plasmid, pRS316-TIF5 that contains the yeast wild-type *TIF5* gene under the transcriptional control of its own promoter. For expression of rat eIF5 in TMY101, we cloned the open reading frame of rat eIF5 cDNA under the control of yeast galactose-inducible *GAL1* promoter in the vector, pTM100 that contains LEU2 selectable marker to yield the plasmid, pTM100-TIF5 (see “Experimental Procedures”). As controls, we constructed two additional plasmids, pTM100-TIF5 and pRS315-TIF5, in which the expression of yeast eIF5 is under control of the *GAL1* promoter and endogenous yeast eIF5 promoter, respectively (“Experimental Procedures”). The host strain TMY101 was transformed individually with each of the three recombinant eIF5 expression plasmids and the parental vector, pRS315. Trp” Ura” Leu” transformants were selected on SGal plates (Fig. 7A, left panel) and replica-plated onto another similar plate which also contained uracil and 5-fluoro-orotic acid (5-FOA) to promote the loss of the original *URA3* plasmid, pRS316-TIF5. Fig. 7 (panel A) shows that cells transformed with control plasmids, pRS315-TIF5 and pTM100-TIF5 (that express yeast eIF5 from endogenous eIF5 promoter and *GAL1* promoter, respectively), grew on 5-FOA plates, as expected. Cells transformed with pTM100-EIF5 that express rat eIF5 from *GAL1* promoter were also able to lose the *URA3* plasmid, pRS316-
TIF5, and could thus grow on 5-FOA plates. In contrast, cells transformed with the vector pRS315 failed to grow, as expected. These results show that mammalian eIF5 when expressed from its own promoter in the plasmid pRS315-TIF5 failed to grow on plates containing glucose as the sole carbon source (Fig. 7, panel B). Final confirmation that the growth of the yeast strain harboring the plasmid pTM100-EIF5 (strain TMY202) is due solely to expression of rat eIF5 that came from immunoblot analysis using monospecific anti-rabbit and anti-yeast eIF5 antibodies as probes (Fig. 7, panel C). These antibodies are highly specific for their own antigen and do not cross-react either with heterologous eIF5 or any other yeast protein (Fig. 7, lanes a and b, upper and lower panels). When lysates of strain TMY202 which express rat eIF5 from the GAL1 promoter in plasmid pTM100-EIF5 were subjected to immunoblot analysis, a strong immunoreactive polypeptide was observed only with anti-rabbit eIF5 antibodies (Fig. 7, upper panel, lane f). Anti-yeast eIF5-antibodies did not recognize any polypeptide in this cell lysate (Fig. 7, lower panel, lane f). As expected, lysates of strain TMY201 harboring plasmid pTM100-TIF5 and wild-type strain W303a reacted strongly only with anti-yeast eIF5 antibodies yielding an immunoreactive polypeptide band corresponding to the molecular mass of yeast eIF5 (56 and 54 kDa doublets) (lanes e and c of Fig. 7C, lower panel). Anti-mammalian eIF5 antibodies did not recognize any polypeptide in these cell lysates (lanes e and c of Fig. 7C, upper panel). However, a yeast strain carrying null mutations in the chromosomal copy of TIF5 and harboring both plasmids, pRS316-TIF5 and pTM100-EIF5, expressed both mammalian and yeast eIF5 (lane d of Fig. 7C, upper and lower panels). These results firmly establish that yeast cells without intact endogenous eIF5 loci but expressing mammalian eIF5 can maintain cell growth and viability. Thus, mammalian eIF5 expressed from an extra-chromosomally replicating plasmid can functionally substitute for the homologous yeast protein in vivo although such cells did not grow as rapidly as either the wild-type W303a cells or the strain TMY201 overexpressing yeast eIF5 from the GALI promoter (Fig. 7, panel D), i.e. doubling time of 4.3 h versus 2.2 and 2.7 h, respectively. It should be noted that comparison of the steady state level of eIF5 by immunoblotting of cell lysates...
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Addition of 45-kDa purified yeast eIF5 was able to restore translation of mRNAs in eIF5-depleted cells showing greatly diminished polysomes. Data presented in this paper clearly show that as yeast cells were depleted of eIF5, the rate of protein synthesis in these cells was inhibited in parallel. Analysis of the polysome profiles of eIF5-depleted cells showed greatly diminished polyribosomes with the simultaneous increase in 80 S ribosomes (Fig. 4), which suggests that eIF5 plays an essential role in the initiation phase of protein synthesis. Furthermore, lysates of cells depleted of eIF5 were inactive in translation of mRNAs in vitro. Addition of 45-kDa purified yeast eIF5 was able to restore translation in these cell lysates. These results show that the TIF5 gene product, a protein of 45,346 daltons, is a translation factor involved in initiation of protein synthesis. Furthermore, in agreement with the 39% identity and 60% homology in amino acid sequence between mammalian and yeast eIF5 (21), we observed that rat eIF5 can functionally substitute for yeast eIF5 in restoring in vitro translation of mRNAs in eIF5-depleted yeast cell-free translation extracts (Fig. 5). In accord-

FIG. 7. Growth of haploid yeast transformants expressing yeast or rat eIF5 from the GAL1 promoter. A, haploid yeast strain TMY101 harboring the URA3 plasmid pRS316-TIF5 was transformed separately with different recombinant eIF5 expression plasmids as indicated. Transformants were patched onto SGal plates lacking tryptophan, leucine, and uracil (SGal-Trp-Leu-Ura) and then replica-plated onto both SGal-tryptophan-Ura-LEU plates (left panel) and SGal plates containing 5-FOA and uracil (SGal-Trp-Leu-Ura+5-FOA) (right panel). Cells were allowed to grow on these plates for 3 and 5 days, respectively. B, haploid yeast cells recovered from 5-FOA plates each containing different eIF5 expression plasmids, pTM100-TIF5 (strain TMY201), pTM100-EIF5 (strain TMY202), and pRS315-TIF5 (strain TMY203) were grown on a SGal-Trp-Leu plate (left panel), or on a SD-Trp-Leu plate (right panel) at 30 °C for 3 days. C, immunoblot analysis of eIF5 in lysates of yeast cells expressing either yeast or rat eIF5 or both from the recombinant plasmids. Yeast cells harboring different recombinant eIF5 expression plasmids were grown to mid-logarithmic phase in synthetic medium containing 2% galactose as the sole source of carbon. Cell lysates were prepared as described under “Experimental Procedures” and analyzed by Western blotting using either anti-rabbit eIF5 antibodies (upper panel) or anti-yeast eIF5 antibodies (bottom panel). Lanes a and b, purified recombinant rat eIF5, and purified yeast eIF5, respectively; lane c, extracts from W303a cells; lanes d-f, extracts from tif5::TRP1 yeast cells expressing plasmids as follows: lane d, pRS316-TIF5 and pTM100-EIF5; lane e, pTM100-TIF5; lane f, pTM100-EIF5. D, growth curves of yeast strains expressing either yeast eIF5 or mammalian eIF5 from GAL1 promoter. The indicated yeast strains were grown in synthetic media containing 2% galactose as the only carbon source and the growth was monitored by measuring absorbance at 600 nm in a Beckman spectrophotometer. The growth curve of wild-type parental yeast strain W303a is also shown.

showed that while the molar concentration of eIF5 in strain TMY201 in which yeast eIF5 was expressed from the GAL1 promoter was nearly 4-fold higher relative to wild-type W303a cells, the concentration of rat eIF5 expressed from the GAL1 promoter in strain TMY202 was similar to that of the W303a strain (data not shown).

DISCUSSION

eIF5 was isolated in our laboratory both from mammalian cell extracts (15–18) as well as from the yeast S. cerevisiae (19) based on an in vitro assay that measured the ability of the factor to mediate the joining of 60 S ribosomal subunits to a

preformed AUG-dependent 40 S initiation complex (40 S-AUG-Met-tRNA2-eIF2-GTP) to from the functional 80 S initiation complex. Studies with the purified factor as well as subsequent cloning and characterization of the mammalian cDNA and yeast gene encoding eIF5 (20–23) have led to the demonstration that both mammalian and yeast proteins are monomers of calculated Mr 48,926 and 45,346, respectively. For reasons summarized below, it is, however, important to show that the 45–49-kDa eIF5, purified on the basis of in vitro partial initiation reaction assay, is indeed involved in the translation of natural mRNAs in vivo and in vitro. First, prior to our work, eIF5 was isolated from mammalian cells in several laboratories (11–14) based on its ability to stimulate globin mRNA-directed protein synthesis in a partially reconstituted translation system. The purified factor was reported to be a monomer of about 150,000–160,000 kDa (10–14) and shown to be specifically required for the joining of the 60 S ribosomal subunits to the 40 S initiation complex formed with natural mRNA to form the 80 S initiation complex. Second, there are reports in literature of several protein factors that were isolated from rabbit reticulocyte lysates based on the in vitro activity of each protein in stimulating a partial reaction in the initiation pathway (for review, see Refs. 1–5). Although these protein factors were called translation initiation factors, the role of many of these proteins, e.g. eIF5A (formerly called eIF4D), eIF6, CoeIF2A, CoeIF2B, in initiation of protein synthesis in vivo is far from clear. For example, initiation factor eIF5A, a 16–18-kDa highly conserved protein in eukaryotes (38, 40, 41), was isolated from ribosomal salt-wash proteins of rabbit reticulocytes based on its activity to stimulate the formation of methionyl puromycin by interaction of Met-tRNA and bound to the 80 S initiation complex with puromycin (12, 41, 42). Based on the activity of this protein in the above in vitro assay, eIF5A was thought to be a translation initiation factor required for the synthesis of the first peptide bond following 80 S initiation complex formation. However, subsequent cloning of the S. cerevisiae genes encoding eIF5A (43, 44) and studies on protein synthesis in vivo in eIF5A-depleted yeast cells (25) showed that while eIF5A is an important protein required for yeast cell growth and viability, it may not be a translation factor required for global protein synthesis in yeast cells. These observations lend strong support to the view that the requirement of a protein isolated on the basis of an in vitro partial reaction assay must be demonstrated for protein synthesis in vivo before it can be regarded as a translation factor.

In this paper, we have used the S. cerevisiae system to investigate the function of the 45-kDa yeast eIF5 in protein synthesis in vivo and in vitro. We have used a conditional eIF5 expression system to rapidly deplete eIF5 from yeast cells. Data presented in this paper clearly show that as yeast cells were depleted of eIF5, the rate of protein synthesis in these cells was inhibited in parallel. Analysis of the polysome profiles of eIF5-depleted cells showed greatly diminished polyribosomes with the simultaneous increase in 80 S ribosomes (Fig. 4), which suggests that eIF5 plays an essential role in the initiation phase of protein synthesis. Furthermore, lysates of cells depleted of eIF5 were inactive in translation of mRNAs in vitro. Addition of 45-kDa purified yeast eIF5 was able to restore translation in these cell lysates. These results show that the TIF5 gene product, a protein of 45,346 daltons, is a translation factor involved in initiation of protein synthesis. Furthermore, in agreement with the 39% identity and 60% homology in amino acid sequence between mammalian and yeast eIF5 (21), we observed that rat eIF5 can functionally substitute for yeast eIF5 in restoring in vitro translation of mRNAs in eIF5-depleted yeast cell-free translation extracts (Fig. 5). In accord-
Yeast eIF5 and Mammalian eIF5

ance with these results, we also observed that in ΔTIF5 yeast cells in which no functional eIF5 was synthesized due to disruption of the essential chromosomal copy of the TIF5 gene, expression of either rat or S. cerevisiae eIF5 from centromeric plasmids under the transcriptional control of the GAL1 promoter can support growth and viability of yeast cells. It is to be noted, however, that the growth rate of ΔTIF5 yeast cells expressing mammalian eIF5 from the GAL1 promoter is about 40–50% of that exhibited by either the wild-type W303α strain or by a yeast strain expressing yeast eIF5 from the GAL1 promoter. This difference in growth rate between the two eIF5 expression systems presumably reflects subtle evolutionary differences between the components of the translation machinery of the unicellular yeast and the multicellular mammalian systems. We should also point out that among many translation initiation factors that have been tested for the ability of their cDNAs to functionally substitute for the corresponding yeast gene in yeast cells, only eIF4A has not been found to complement (for review, see Ref. 5). These observations lend strong support to the view that the basic mechanism of initiation of translation is highly conserved from the unicellular yeast and the multicellular mammalian systems. Finally, availability of both a cell-free yeast translation system dependent on exogenous eIF5 for efficient in vitro translation of mRNAs and a ΔTIF5 yeast strain in which functional eIF5 is produced in vivo from the TIF5 gene under the control of GAL promoter should be powerful tools to analyze structure-function studies of eIF5 in vivo and in vitro.

Acknowledgments—We are grateful to Dr. Eun-Chung Park, Massachusetts General Hospital, for the kind gift of plasmids pGEM-flu, pUB23, and pSE362 used in this study. We are also indebted to Dr. Philip Hieter, Johns Hopkins University School of Medicine, Baltimore, MD, for providing us with the yeast plasmids, pRS315 and pRS316, and Dr. Michael J. Leibowitz, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, for help in preparation of yeast cell-free translation extracts. We thank Dr. Stewart Shuman, Sloan-Kettering Cancer Institute, NY, and Dr. Jayanta Chaudhuri of this institution for critically reading the manuscript.

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