Translation Initiation Factor 5A and Its Hypusine Modification Are Essential for Cell Viability in the Yeast Saccharomyces cerevisiae

JOACHIM SCHNIER,
† HUBERT G. SCHWELBERGER, ZELJKA SMIT-MCBRIDE, HYUN AH KANG,
AND JOHN W. B. HERSHEY*

Department of Biological Chemistry, School of Medicine, University of California, Davis, California 95616

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Translation initiation factor eIF-5A (previously named eIF-4D) is a highly conserved protein that promotes formation of the first peptide bond. One of its lysine residues is modified by spermidine to form hypusine, a posttranslational modification unique to eIF-5A. To elucidate the function of eIF-5A and determine the role of its hypusine modification, the cDNA encoding human eIF-5A was used as a probe to identify and clone the corresponding genes from the yeast Saccharomyces cerevisiae. Two genes named TIFS1A and TIFS1B were cloned and sequenced. The two yeast proteins are closely related, sharing 90% sequence identity, and each is ca. 63% identical to the human protein. The purified protein expressed from the TIFS1A gene substitutes for HeLa eIF-5A in the mammalian methionyl-puromycin synthesis assay. Strains lacking the A form of eIF-5A, constructed by disruption of TIFS1A with LEU2, grow slowly, whereas strains lacking the B form, in which HIS3 was used to disrupt TIFS1B, show no growth rate phenotype. However, strains with both TIFS1A and TIFS1B disrupted are not viable, indicating that eIF-5A is essential for cell growth in yeast cells. Northern (RNA) blot analysis shows two mRNA species, a larger mRNA (0.9 kb) transcribed from TIFS1A and a smaller mRNA (0.8 kb) encoded by TIFS1B. Under the aerobic growth conditions of this study, the 0.8-kb TIFS1B transcript is not detected in the wild-type strain and is expressed only when TIFS1A is disrupted. The TIFS1A gene was altered by site-directed mutagenesis at the site of hypusination by changing the Lys codon to that for Arg, thereby producing a stable protein that retains the positive charge but is not modified to the hypusine derivative. The plasmid shuffle technique was used to replace the wild-type gene with the mutant form, resulting in failure of the yeast cells to grow. This result indicates that hypusine very likely is required for the vital in vivo function of eIF-5A and suggests a precise, essential role for the polyamine spermidine in cell metabolism.

Eukaryotic initiation factor 5A (eIF-5A; previously named eIF-4D) (initiation factors are named according to the revised nomenclature recommended by the International Union of Biochemistry [37]) is one of a number of protein factors that stimulate the initiation phase of protein synthesis (29). The purified protein from mammalian cells is small (16 to 18 kDa) and acidic (pI = 5.4) and is one of the most abundant of the initiation factors (2, 21). eIF-5A is distinguished by possession of a unique residue, hypusine [N°-(4-amino-2-hydroxybutyl)-lysine], formed posttranslationally by transfer of a butylamine group from spermidine to a specific lysine followed by a hydroxylation reaction (9, 33). The eIF-5A protein and its hypusine modification are highly conserved from yeasts to humans (12), suggesting an important role in protein synthesis, cellular metabolism, or both. eIF-5A appears to function in protein synthesis by promoting formation of the first peptide bond, a reaction usually studied in vitro by a model reaction, the synthesis of methionyl-puromycin (3, 28). No other functional assay for eIF-5A is available. Thus, the precise functional roles of eIF-5A and its hypusine modification are yet to be elucidated.

We have cloned a human cDNA encoding eIF-5A in order to better study the role of hypusine and the factor in protein synthesis (40). From the cDNA sequence and the sequence of a hypusine-containing peptide (34), we identified the site of hypusination as Lys-50. Expression of the cDNA in Escherichia coli results in a precursor form, now named ec-eIF-5A(Lys), which lacks the hypusine modification (41). Purified ec-eIF-5A(Lys) fails to stimulate methionyl-puromycin synthesis in vitro (41). However, when the precursor is modified by in vitro conversion of Lys-50 to deoxyhypusine, the resulting protein, named eIF-5A(Dhp), becomes active in the methionyl-puromycin assay (35). These results, together with those reported by Park (32), strongly suggest that the hypusine (or deoxyhypusine) modification is an essential element in the function of eIF-5A in protein synthesis, at least as measured by in vitro reactions.

Because of uncertainties in interpreting in vitro assays in general, we sought evidence for a role in protein synthesis for eIF-5A and hypusine in intact mammalian cells. The eIF-5A cDNA was overexpressed in transiently transfected COS-1 cells, but no effect on protein synthesis was detected, indicating that eIF-5A is not limiting in such cells (41). A mutant form of the cDNA was constructed, resulting in the substitution of Arg for Lys-50, the site of hypusination. Expression of the mutant protein in COS-1 cells also caused no change in the rate of protein synthesis. Possible explanations are that the mutant form of eIF-5A may fail to compete with the high levels of endogenous eIF-5A, that it may be active (unlikely, since the Arg mutant expressed in E. coli is not active in vitro), or that eIF-5A itself may play no essential role in the cell. Clearly, transfection of mammalian cells has not been a useful approach to studying the function of eIF-5A.

To better evaluate eIF-5A's function in intact eukaryotic cells, we have turned to studies of the yeast Saccharomyces cerevisiae. We report here the cloning of two genes whose products share homology with mammalian eIF-5A and show...
that purified yeast eIF-5A stimulates the formation of methionyl-puromycin in the heterologous mammalian assay system. At least one functional gene is required for cell viability and growth. Furthermore, a mutation expressing Arg in place of Lys at the site of hypusination renders yeast eIF-5A inactive, thereby demonstrating that the modification by spermidine very likely is essential for the factor’s activity in vivo.

**MATERIALS AND METHODS**

**Sources and manipulations of yeast strains.** The genotypes and sources of *S. cerevisiae* strains used or constructed in this work are described in Table 1. The diploid JS10 was made by mating S173-6B with S173-6BMF9. Construction of strains carrying disrupted eIF-5A genes and/or plasmids expressing eIF-5A are described below. Culture media such as YEPD, YEPG, and minimal SD were prepared as described previously (38). Minimal SD medium was supplemented with the relevant amino acids. For sporulation, cells were grown on a YEPD plate and then streaked on 1% potassium acetate and incubated for 3 days. All experiments were carried out at 30°C. Transformations in yeast cells were carried out according to Ito et al. (19). Growth rates were determined by measuring turbidity at 650 nm in a Beckman spectrophotometer. Labeling experiments were carried out in minimal SD medium. Cells in 2 ml of culture medium were labeled with 20 μCi of [35S]methionine (333 Ci/mmol; New England Nuclear) for 10 min at mid-log phase (A650 = 0.3). For the detection of hypusine, cells from a 2-ml culture were labeled with 10 μCi of either [35S]spermidine (90 Ci/mol; Research Products International) or [3H]spermidine (15 Ci/ mmol; New England Nuclear) for two to three generations during mid-log phase.

**Cloning and sequencing of the TIF51A and TIF51B genes.** For the cloning of TIF51A, chromosomal DNA was prepared from strain S173-6B as described previously (38). The DNA was digested with BamHI to completion and separated by 0.8% agarose gel electrophoresis. Southern blot analysis with a 32P-labeled 0.5-kb EcoRI-PstI fragment of human eIF-5A cDNA (40) produced a strong signal at 8 kb. DNA of ca. 8 kb was electroeluted and ligated into the BamHI site of the bacterial plasmid pACYC184 previously treated with calf intestinal alkaline phosphatase. About 200 individual clones

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**TABLE 1. Yeast strains and plasmids**

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<th>Strains or plasmid</th>
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* ORI, origin of replication.
were stabbed and scraped in groups of 10 from the agar plates. Plasmid DNA was prepared from each group and spotted onto a nitrocellulose filter. The filter was subjected to hybridization with the labeled human CDNA probe. One pool gave a positive signal, and the individual clones were then probed similarly to identify the single clone that hybridized. A 1.4-kb Psrl fragment that hybridized to the probe was subcloned into the Psrl site of pUC19 to yield pJS1A. 

TIFS1B was cloned in a similar fashion by digesting chromosomal DNA from strain S173-6B with HindIII. A 13-kb fragment was cloned into plasmid pACYC184; among 650 clones, 1 positive clone was identified by using the TIFS1A gene as a probe. A 3.5-kb EcoRI fragment containing the gene was subcloned in a Bluescript (Stratagene) derivative (in which the SalI site in the polylinker had been destroyed by digesting with HindIII and XhoI, filling in with Klenow fragment, and ligating) to yield pJSB1. 

Gene replacements and disruptions. Plasmid pJS2A was constructed for the disruption of TIFS1A as follows. To destroy the SalI site of pUC19, plasmid DNA was digested with Sall and the 5' ends were filled in with Klenow fragment. After religation and transformation, the pUC19 derivative was digested with Psrl and the 1.4-kb Psrl fragment containing the TIFS1A gene was inserted to obtain plasmid pJS2A. This plasmid was digested with Sall and HpaI, and the linearized 3.7-kb plasmid lacking the 0.4-kb Sall-HpaI fragment from the coding region of the TIFS1A gene was isolated. The 2-kb Sall-HpaI fragment from plasmid YEp13 containing the intact yeast LEU2 gene was inserted into the linearized plasmid pJS2A. The resulting plasmid pJS3A was digested with Psrl, and a 3.0-kb Psrl fragment carrying the disrupted TIFS1A gene and ca. 0.5 kb of flanking sequences on either side was isolated. Strain JS10 was transformed with the 3.0-kb fragment, and Leu+ transformants were selected. Disruption of the TIFS1A genes was confirmed by Southern blot analysis of genomic DNA probed either with the 1.4-kb Psrl fragment from pJSB1 or with the 0.4-kb Sall-HpaI fragment that had been removed from the coding region of TIFS1A. 

The TIFS1B gene was disrupted as follows. Plasmid pJSB1 (see above) was digested with Sall (which cuts in the N-terminal third of the TIFS1B coding region) and partly filled with Klenow fragment, dCTP, and dTTP. The HIS3 gene was excised from pLAC15 by digestion with BamHI, and the ends were partly filled in with Klenow fragment, dGTP, and dATP. The 2-nucleotide overhangs of the 1.8-kb HIS3 fragment and those of pJSB1 are now complementary, so that ligation and transformation yielded pJSB2 with the HIS3 gene inserted into TIFS1B. pJSB2 was digested with EcoRI to yield a 5.3-kb fragment with TIFS1B flanking sequences that was used to transform JS10. His+ transformants were selected, and disruption of the TIFS1B gene was confirmed by Southern blot analyses. 

To construct a strain with both TIFS1A and TIFS1B disrupted, JSDB3 (a/a tifs1A::LEU2/tifs1A::LEU2) was constructed with the 5.3-kb linear DNA fragment carrying TIFS1B disrupted by HIS3. The resulting diploid, JSDB2A, was transformed with YCp50-5A constructed by digestion of YCp50 with HindIII and BamHI and ligation with the 1.4-kb HindIII-BamHI fragment from pJS1A that carries TIFS1A. Cells were sporulated and tested for mitotic viability on 5-fluoro-orotic acid (5-FOA) plates. 

Substitution of TIFS1A with tifs1A(K51R). The haploid strain JSDB3 [YCp50-5A] was transformed with either YRp7-5A or YRp7-5A(K51R), and Trp+ Ura+ cells were selected. Transformants were streaked on 5-FOA plates (5 to score for cells that can lose the YCp50-5A plasmid. YRp7-5A was constructed by opening pJS2A (a pUC19 derivative carrying TIFS1A) with EcoRI and inserting an EcoRI fragment from YRp7 that carries TRP1 and ARS1. To construct YRp7-5A(K51R), the TIFS1A gene was mutated at the codon for Lys-51 by using the polymerase chain reaction method (16, 17). A 0.9-kb fragment of DNA from pJS2A was amplified with the reverse sequencing primer (Stratagene) and a 44-base synthetic DNA, 5'-GGG-GTC-GAC-ATG- TCC-CTC-CTT-AAG-AGT-AGA-CAC-GGT-CAC- GC-3'. The 44-mer spans the SalI site at its 5' end and converts the Lys-51 codon to AGA (underlined) for Arg. The amplified DNA was cut with Sall and HindIII (from the pUC19 polylinker) and subcloned into the corresponding sites of M13mp19, and the insert was sequenced from the SalI site to the HpaI site to confirm that the only sequence change was the AGA codon. M13 replicative-form DNA was cut with Sall and HpaI, and the fragment carrying the mutation was inserted into the HpaI site of YRp7-5A to yield YRp7-5A(K51R). Confirmation that the plasmid carries the K51R mutation was made by sequencing plasmid DNA minipreparations by using the primer 5'-ATGTTT GACGAAGAACA-3', which hybridizes to a region ca. 75 bp upstream of codon 51. 

To demonstrate that the mutant eIF-5A protein, eIF-5A(K51R), is stable and not hypusinised, plasmids that overexpress the wild-type and mutant proteins were constructed. YEp352T-SA and YEp352T-SA(K51R) were constructed by inserting the 1.4-kb Psrl fragment encoding the eIF-5A proteins from YRp7-5A and YRp7-5A(K51R), respectively, into the Psrl site of the high-copy-number plasmid YEp352 (15). Then the 0.52-kb Ndel-StuI fragment (blunt ended) containing a major part of the URA3 gene was replaced with the 0.85-kb EcoRI-BglII fragment (blunt ended) of YRp7 containing TRP1. In both constructs, the TIFS1A alleles as well as the TRP1 marker genes were inserted in the same orientation in relation to the vector backbone. For overexpression of the proteins, strain S173-6B was transformed with each of the plasmids. 

Nucleotide sequence accession numbers. The sequences of TIFS1A and TIFS1B have been assigned the GenBank accession numbers M63541 and M63542, respectively.

RESULTS

Cloning of a yeast eIF-5A gene. A number of genes encoding yeast protein synthesis initiation factors have been cloned and sequenced. The derived amino acid sequences for eIF-2α, eIF-2β, and eIF-4A share 58% (8), 42% (10), and 65% (23) identity with their corresponding mammalian factors, respectively, indicating considerable conservation of structure for this class of proteins. eIF-5A itself is highly conserved (12), especially in the region where hypusine is located. We therefore proposed to employ the human cDNA encoding eIF-5A as a hybridization probe to clone the corresponding yeast gene. The potential feasibility of the approach was supported by the detection of only a few discrete bands when labeled cDNA was used to probe a Southern blot of BamHI-restricted yeast genomic DNA at low stringency. A single prominent 8-kb band remained after washes of increasing stringency (results not shown). An 8-kb BamHI fragment ligated into pACYC184 was cloned in E. coli as described in Materials and Methods. From this clone, a 1.4-kb Psrl fragment that hybridizes to the mammalian cDNA was subcloned into the Psrl site of pUC19 to yield pJS1A.
FIG. 1. Sequences of the eIF-5A genes and proteins. DNA sequences are shown for the coding and flanking regions of TIFS1A and TIFS1B that have been determined on both strands as described in Materials and Methods. The two sequences are aligned by matching their initiator codons, whose A is numbered +1. The derived amino acid sequence of TIFS1A [labeled eIF-5A (yeast A)] is shown; for TIFS1B [labeled eIF-5A (yeast B)] and for the human protein [labeled eIF-5A (human)], only residues that differ from those in TIFS1A are shown. The lysine residue that is modified to hypusine is enclosed in a box. The tRNA<sub>Sec</sub> gene at the distal end of the TIFS1A sequence is underlined.

The sequence of the 1.4-kb insert in pJS1A was determined for both strands and is reported in Fig. 1. An open reading frame (ORF) was detected that codes for a 157-amino-acid protein (17, 103 Da) with homology to mammalian eIF-5A. The yeast and human proteins are similar throughout their lengths, sharing 63% sequence identity and especially high conservation in the vicinity of the Lys hypusine site, which is residue 51 in the yeast protein. The similar size and sequence of the yeast protein strongly suggest that we have identified a gene for eIF-5A in S. cerevisiae, which we named TIFS1A. (Yeast genes encoding translation initiation factors have been named in numerous ways, depending in part on how they were selected or identified. We prefer the generic name TIF for translation initiation factor, as applied to the genes encoding eIF-4A, but suggest that numbers used to indicate the factor's precise name [37] whenever possible. Thus, the genes for eIF-5A become TIFS1, the 5 and 1 corresponding to the 5A in the factor's name. Uppercase letters [e.g., A and B] are then applied in cases of more than one gene, as has been done for the duplicated genes for ribosomal proteins.) Further evaluation of the sequence is provided below.

Disruption of TIFS1A with LEU2. To determine whether yeast eIF-5A is essential for cell viability and growth, we sought to construct a null mutant strain lacking the gene (see Materials and Methods for details). A plasmid, pJS3, was constructed that contained a 73% deletion of the TIFS1A coding region and an insertion of the LEU2 gene (Fig. 2A). A 3.0-kb linear F<sub>2</sub>II fragment containing LEU2 and the TIFS1A flanking sequences was prepared from pJS3 and used to transform the diploid yeast strain JS10 (see Table 1 for yeast strain genotypes). Stable Leu<sup>+</sup> transformants were selected, and the disruption of one of the TIFS1A genes in the diploid genome was confirmed by Southern blot analysis (results not shown). Independent isolates were sporulated, and tetrads were dissected. Surprisingly, all spores germinated and grew, although each tetrad gave rise to two fast-growing and two slow-growing haploid cell lines (results not shown).

The slower-growing haploid cells were Leu<sup>+</sup>, whereas the faster-growing cells were Leu<sup>-</sup>. Disruption of TIFS1A by
LEU2 in one of the slower-growing haploids, named JSDA1, was confirmed by Southern blot analyses of PstI-cleaved DNA. We noted that some of the haploid cell lines, after a delay, grew significantly faster than the others, exhibiting 60% of the growth rate of the parental strain, S173-6B. A likely explanation of this observation will be provided later. A diploid strain, JSDA3, homozygous for tif51A::LEU2, was constructed by mating JSDA1 and JSDA2 (a similar haploid of opposite mating type). The slower-growth phenotype for TIF51A-disrupted haploid and diploid strains also was observed when they were grown on nonfermentable carbon sources such as ethanol or glycerol which were tested by streaking cells on agar plates. No significant difference between the null and parental strains was detected in mating ability, sporulation efficiency, or recovery after limiting a required amino acid (histidine) or shifting from 2 to 0.02% glucose for 24 h to arrest the cells in the G1 phase. Furthermore, the disrupted strains were stable when grown several generations in YEPD with no loss of the LEU2 marker, indicating stable insertion of the plasmid DNA into a chromosome. We conclude that TIF51A is not essential for cell viability but that it is required for maximal rates of growth under the conditions of this study.

If TIF51A is the sole gene encoding eIF-5A in yeast cells, the constructed null strains should lack the eIF-5A protein entirely. Neither pure yeast eIF-5A nor specific antisera were available to help us to identify the protein in crude cell lysates. Instead, we attempted to demonstrate the disappearance of eIF-5A by failure of the cell to label the protein with [35S]methionine. Analysis of lysate protein from the wild-type strain (JS10) by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3A) shows two radiolabeled bands of mass 18 and 20 kDa. Two hypusine-containing proteins of differing molecular weight had been observed previously in yeast cells (32a), but...
there is no explanation for their differences in mass. When the TIFS1A-disrupted strain JSDA3 was analyzed (Fig. 3B), the lower band was not detected and the intensity of the upper band was greatly reduced. The result indicates that TIFS1A encodes the protein that is labeled by spermidine. Since an eIF-5A-like protein is present at a low concentration in the tifs1A::LEU2/tifs1A::LEU2 cells, one or more additional genes encoding eIF-5A may occur in yeast cells. Further evidence for the presence of eIF-5A in JSDA3 was obtained by identifying eIF-5A in the pattern of proteins produced by high-resolution two-dimensional isoelectric focusing (IEF)/SDS-PAGE analyses. Strain JS10, exhibits three [35S]methionine-labeled spots (Fig. 3C; labeled a, b, and c) that are relatively more intense than those in strain JSDA3 (Fig. 3D). The same three spots increase in intensity (results not shown) in gel patterns from cells carrying the eIF-5A-overproducing plasmid YRp7-5A (described below) and therefore are good candidates for yeast eIF-5A proteins. The identifications were confirmed by analyzing cells labeled with [14C]spermidine. In the parental strain (Fig. 3E), radiolabel was found only in positions corresponding to the proteins labeled a, b, and c. In the null strain (Fig. 3F), the intensities of spots a and b are greatly reduced and spot c is not detectable. The results together indicate that TIFS1A encodes the greater part of all three spermidine-labeled proteins and suggest that a second gene encoding eIF-5A exists which is expressed more weakly than TIFS1A. How the three labeled proteins differ structurally from one another is not known, but presumably the differences are not caused simply by the spermidine modification.

**Cloning and disruption of TIFS1B.** To pursue the idea of a second TIFS1 gene, we probed Southern blots of restricted genomic DNA at low stringency with labeled TIFS1A DNA (a 0.9-kb PstI-HpaI fragment; Fig. 2) instead of with the mammalian cDNA. DNA restricted with HindIII showed a strong hybridizing band at 5 kb corresponding to TIFS1A and a much weaker band at 12 kb. DNA from the 12-kb region was eluted, a library was constructed, and a hybridization-positive clone was isolated essentially as described for the cDNA of TIFS1A (see Material and Methods for details). A 3.5-kb EcoRI fragment was subcloned into a Bluescript-derived plasmid that had its SalI site destroyed, to yield pJSB1. A portion of the insert was sequenced on both strands (Fig. 1). An ORF was detected which codes for a protein that is essentially identical in size (157 residues; 17,120 Da) and very strongly resembles in sequence the product of the TIFS1A gene (Fig. 1). The two yeast proteins share 90% amino acid identity, and most of the differing residues are physically similar. The lysine target of hypusination and surrounding region are especially well conserved. In addition, the new gene product shares 62% sequence identity with human eIF-5A. We conclude that the isolated gene also encodes an eIF-5A protein and have named the gene TIFS1B. TIFS1A and TIFS1B possess 91% identical nucleotides in the coding region but are unrelated in their 5' and 3' noncoding regions.

The TIFS1B gene was disrupted as described in Materials and Methods by cutting the structural gene with Sall and inserting a 1.8-kb fragment carrying the HIS3 gene (Fig. 2B). A linear 5.3-kb EcoRI DNA fragment containing the disrupted gene and 3.5 kb of flanking sequences was purified and used to transform the diploid strain JS10. His+ transformants were selected, and disruption of one of the TIFS1B genes was confirmed by Southern blot analyses (results not shown). The heterozygous TIFS1B/tifs1B::HIS3 strain was sporulated, and tetrads were dissected and analyzed. Each tetrad yielded four viable spores that germinated and grew at wild-type rates, whereas the His+ and His− phenotypes segregated 2:2. One of the His+ haploids, named JSDB1, was analyzed by Southern blotting to confirm disruption of TIFS1B. We conclude that disruption of TIFS1B does not affect the growth of yeast strains under these conditions, presumably because of the presence of the stronger-expressing TIFS1A gene.

**Yeast eIF-5A stimulates methionyl-puromycin formation in vitro.** To obtain more compelling evidence that the TIFS1 genes encode the mammalian equivalent of eIF-5A, we purified the yeast protein and tested its activity in the mammalian methionyl-puromycin synthesis assay for eIF-5A (41). Yeast eIF-5A was purified from strain S173-6B[YEp352-5A], which overexpresses TIFS1A about 10-fold (see the legend to Fig. 4). The essentially homogeneous protein (stained gel insert in Fig. 4) stimulates the in vitro assay with a specific activity of about half that of human eIF-5A (Fig. 4). This finding demonstrates that the product of the TIFS1A gene is functionally related to mammalian eIF-5A and is highly conserved in structure since it is
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FIG. 5. Northern blot hybridization analyses. Total yeast RNA was prepared from strains S173-6B (lanes 1 and 4), JSDB2 (lanes 2 and 5), and JSDB1 (lanes 3 and 6); equal amounts of RNA (20 μg) were applied to each lane of a formaldehyde–1.75% agarose gel, electrophoresed, and then blotted onto HyBond-N (Amersham) (26). The Northern blot was probed with either the 1.4-kb FstI fragment of pJSA1 carrying the coding region of TIFSJA (A) or the 1.4-kb Smal-Dral fragment of pJSBI that includes the coding region of TIFSJB (B), each labeled to comparable specific activity. The Northern blots were exposed to Kodak X-omat film for 8 h.

capable of acting in an assay system composed only of mammalian components.

Northern (RNA) blot analyses of eIF-5A mRNAs. To characterize the mRNAs encoding eIF-5A, Northern blot analyses of total yeast RNA were carried out (Fig. 5). When probed with TIFSJA DNA (Fig. 5A), the parental strain S173-6B (lane 1) and the tifsJB::HIS3 null strain JSDB1 (lane 3) generate a strong band at ca. 0.9 kb, whereas the tifsJA::LEU2 null strain JSDB2 (lane 2) generates a very weak, slightly smaller band at ca. 0.8 kb not detected in the other strains. Similar results are obtained when the same RNA preparations are probed with TIFSJB DNA (Fig. 5B) except that the 0.9-kb bands are less intense and the 0.8-kb band is more intense. The slower-migrating band in Fig. 5B, lane 3, is thought to be a larger transcript made from both the disrupted TIFSJB gene and the HIS3 gene, although this has not been proven. The results indicate that the larger 0.9-kb RNA is transcribed from TIFSJA and the smaller 0.8-kb RNA is transcribed from TIFSJB. The efficiency of hybridization to the two RNA transcripts varies with the probe. We estimate that the TIFSJA transcript is ca. 5- to 10-fold more abundant than the TIFSJB transcript. This roughly equals the difference in apparent rates of eIF-5A synthesis deduced from [35S]spermidine labeling when parental and TIFSJA-disrupted strains are compared (Fig. 3A and B).

eIF-5A is essential for cell growth. To demonstrate the phenotype of a null mutant completely lacking eIF-5A, we constructed diploid strains which upon sporulation would generate spores with null alleles in both TIFSJA and TIFSJB. We mated the haploid strains JSDA1 (MATα tifsJA::LEU2) and JSDB2 (MATα tifsJB::HIS3) to produce the diploid JSDA1B, which is Leu" His". Upon sporulation and tetrad analysis of 20 ascii, Leu" and His" phenotypes were scored, but no spores were both Leu" and His". By assigning to nonviable spores the presence of both disrupted alleles, the pattern of tetrad phenotypes was 2 parental diploids, 5 non-parental ditypes, and 13 tetraploids, indicating that the two eIF-5A genes are unlinked. The failure to detect Leu" His" spores suggests that lack of eIF-5A either is lethal or prevents germination.

To determine whether eIF-5A is required for cell growth, the diploid strain JSDA3 (tifsJA::LEU2/tifsJA::LEU2) was transformed with the 5.3-kb EcoRI fragment carrying tifsJB::HIS3. His" transformants were selected, and those which had a disruption of a single TIFSJB gene were identified by Southern blots analyses (results not shown) to yield JSDB2. The transformants sporulate poorly, giving a very low number of asci, making tetrad analysis impractical. Then JSDB2 was transformed with a YCP50 derivative carrying a functional TIFSJA gene, YCP50-5A (Fig. 3C). Ura" transformants (named JSDA2[YCP50-5A]) were selected, and these were sporulated. The cells produce asci with a frequency comparable to that of the parental strain JS10. Dissection of tetrads resulted in the identification of spores that were Leu" and His", indicative of haploids in which the chromosomal genes for TIFSJA and TIFSJB are disrupted. A number of dissected spore colonies were patched onto plates with minimal media lacking histidine and leucine. Half of the cells grew (Fig. 6A), indicating they are His" (i.e., carry both TIFSJA disruptions), whereas half did not grow, presumably because they are His" (with an intact TIFSJB gene). When the same colonies were replica patched on a 5-FOA plate (Fig. 6B), which selects for the loss of URA3, the His" cells grew, indicating that they could lose the URA3-TIFSJA plasmid. However, the His" cells did not grow on 5-FOA, indicating that they are not able to lose the URA3-TIFSJA plasmid. Thus, the YCP50-5A plasmid is essential for growth of the double-null haploids; at least one copy of a TIFSJA gene is required for cell viability.

The hypusine modification is essential for cell growth. We next asked whether the hypusine modification of eIF-5A is
required for cell growth. Possession of a double-null haploid strain requiring expression of eIF-5A from a plasmid enables us to test mutant forms of the gene by using the plasmid shuffle technique. A mutation of TIF51A was constructed in which the codon for Lys-51, the site of hypusination, was altered to that for Arg, thereby providing a protein, eIF-5A(K5,R), which cannot be modified by spermidine yet continues to carry a positive charge at that position. The mutated gene, called tif51A(K5,R), and the wild-type gene TIF51A each were combined with a portion of YRP7 DNA to yield YRP7-5A(K5,R) and YRP7-5A, respectively, as described in Materials and Methods. The plasmids were transformed into strain JSDB3[YCP50-5A], and Trp+ transformants were selected. Thirty transformants carrying YRP7-5A and 60 carrying YRP7-5A(K5,R) were streaked onto supplemented SD plates to score for Trp+ and Ura+ phenotypes. As shown in Fig. 6C and D for representative transformants called JSDB3[YCP50-5A]YRP7-5A and JSDB3[YCP50-5A]YRP7-5A(K5,R), all transformants grew on both selection media. When the transformants were streaked onto plates supplemented with 5-FOA, all 30 of the wild-type YRP7-5A transformants grew, indicating that many cells in each streak had lost the YCP50-5A plasmid and that the TIF51A gene in the YRP7-derived plasmid expresses functional eIF-5A (Fig. 6E). In contrast, none of the 60 mutant YRP7-5A(K5,R) transformants grew on 5-FOA (as shown for one of these in Fig. 5B). This finding suggests that the eIF-5A(K5,R) mutant form is inactive for a function required for cell growth.

To show that the mutant protein eIF-5A(K5,R) actually is synthesized and is stable in yeast cells, cells overexpressing tif51A(K5,R) were analyzed. Since the YRP-derived plasmids are unstable and give only about two- to threefold overexpression, the accumulation of mutant protein is difficult to demonstrate unambiguously. We therefore excised the DNAs encoding the wild-type and mutant proteins from YRP7-5A and YRP7-5A(K5,R) and inserted these DNA fragments into the high-copy-number plasmid YEps3527 (as described in Materials and Methods) to yield YEps3527-5A and YEps3527-5A(K5,R), respectively. The plasmids were tested in the plasmid shuffle experiment as described above, and results comparable to those shown in Fig. 6C to E were obtained (results not shown). Following their transformation into strain S173-6B, the cells generate a more intense 20-kDa protein band (Fig. 7A, lanes 2 and 3) compared with the control (lane 1; vector alone) when analyzed by SDS-PAGE. The migration position of the bands corresponds to that of yeast eIF-5A, indicating that both forms of eIF-5A protein accumulate. To determine whether the mutant protein is modified by spermidine, the cells were labeled with [3H] spermidine for three generations, and proteins were analyzed by SDS-PAGE and autoradiography (Fig. 7B). Overexpression of the wild-type protein (lane 2) but not the K5,R mutant form (lane 3) results in enhanced spermidine labeling of the 20-kDa protein. The results indicate that eIF-5A(K5,R) is indeed synthesized, is stable in yeast cells, and is not modified to hypusine. It is therefore highly likely that the mutant protein’s inability to support growth derives from the failure to undergo the hypusine modification reactions.

**DISCUSSION**

The process of initiation of protein synthesis is promoted by numerous soluble initiation factors that transiently associate with ribosomes. The mechanism of action of the initiation factors has been elucidated primarily by in vitro studies with purified mammalian cell components (29). Because translation is so complex, it is especially desirable to study the process both genetically and biochemically, yet genetic approaches for mammalian cells are cumbersome and have not been used extensively. Recent studies of protein synthesis in the yeast *S. cerevisiae* have been carried out, and genes have been cloned for eIF-2α (8), eIF-2β (10), eIF-4A (23), and eIF-4E (1). We report here the cloning of two genes, named TIF51A and TIF51B, that encode proteins homologous to mammalian eIF-5A (40). The identity of these gene products as homologs of eIF-5A is based on the facts that they share ca. 63% amino acid sequence identity with human eIF-5A, they are uniquely modified by spermidine, and the purified yeast protein substitutes for HeLa eIF-5A in the in vitro mammalian methionyl-puromycin synthesis assay. The simultaneous disruption of both genes results in the failure of yeast cells to grow, thereby indicating that yeast eIF-5A is essential for cell viability. This result is especially important, since the major evidence for a functional role for eIF-5A is its stimulation of a nonphysiological reaction, the synthesis of methionyl-puromycin. However, it has not yet been demonstrated that the essential function of eIF-5A in *S. cerevisiae* involves the translation pathway.

Two genes encoding eIF-5A also have been identified in a number of different yeast strains (37a). Duplicate genes for translational components appears to be a frequent occurrence, having been observed for the eIF-4A (23) and numerous ribosomal proteins (47). It is interesting to note that both TIF51 genes are followed by genes that express serine tRNA1, the major serine tRNA in yeast cells (42). The two serine tRNA1 genes that are linked to TIF51A and TIF51B possess identical sequences in their coding regions but differ in their flanking regions. The serine tRNA1 gene detected next to TIF51A represents a new member of the yeast serine tRNA1 family (31).

While this report was in preparation, Mehta and coworkers (27) reported the sequence of a gene named *ANB1* which
is homologous to human eIF-5A cDNA (40). ANB1 is equivalent to TIF51B; of the 745 bp of DNA sequence reported in Fig. 1, only two T's at positions −203 and +93 differ from the sequence of ANB1, and the derived protein sequences are identical. ANB1 (and thus TIF51B) maps adjacent to the 5′ side of CYC1 on chromosome X. Hybridization of Northern blots with DNA probes from the TIR region produces two bands, named tr1 (0.9 kb) and tr2 (0.8 kb) (25). The tr2 transcript is encoded by ANB1 (TIF51B), whereas the tr1 transcript is produced from a gene mapping elsewhere in the genome. Our Northern blot analyses (Fig. 4) indicate that tr1 is encoded by TIF51A. The expression of tr1 and tr2 (and therefore TIF51A and TIF51B, respectively) is regulated by both oxygen and heme, which stimulate the synthesis of tr1 and repress the other gene (24). In contrast, anaerobic conditions or a heml mutation (causing a lack of porphyrins) stimulate tr2 synthesis. Why the genes for eIF-5A are reciprocally regulated by oxygen and heme is not known. It is tempting to postulate that the two protein forms may influence the translation of specific mRNAs differently. However, we observe no obvious differences in the intensities of radiolabeled protein spots (other than eIF-5A proteins) in autoradiograms of IEF/SDS-PAGE from wild-type cells (predominantly the eIF-5A A form) and the tif51A::LEU2 strain (containing only the eIF-5A B form). Furthermore, the strictly complementary nature of the two genes, such that either one alone supports growth at nearly wild-type rates, argues that their respective roles in gene expression must be very similar. Since no TIF51B mRNA (tr2) accumulates in wild-type strains under aerobic conditions (24; Fig. 4B), it is surprising that the tif51A::LEU2 null strain expresses significant amounts of TIF51B mRNA and grows. The synthesis of the B form of eIF-5A in strains lacking the A form suggests that the A form may repress expression of TIF51B. Alternatively, the tif51A null strains may be altered in the TIF51B allele such that repression by oxygen is reduced. However, no change in the Southern blot pattern of the TIF51B region was detected in these strains, ruling out large rearrangements of the chromosome.

The cis-acting DNA sequences involved in regulation of the expression of the eIF-5A genes have not been studied in this work. Mehta et al. (27) demonstrated three major sites of transcription initiation for ANB1 (TIF51B) and identified an upstream TATA box as well as a HOMOL1 consensus sequence frequently found in genes expressing ribosomal proteins (47). Analysis of the TIF51A sequence identifies two putative GFI-1 DNA-binding sequences (11) at −181 to −169 (one mismatch of the consensus sequence) and at −203 to −215 (opposite DNA strand; two mismatches), but we have not attempted further characterization of the transcription signals. At the translation level, both genes initiate protein synthesis at the first AUG in the ORF. The initiator regions of both genes strongly resemble the consensus sequence for S. cerevisiae AA(A/T)AATGCTT (7), each differing at only a single position at positions −1 and −2, respectively. Codon usage is highly biased; of the 157 codons in each gene, TIF51A uses 143 and TIF51B uses 135 that are most favored in highly expressed proteins in yeast cells (4). We also note that in the TIF51A sequence, there is a −1 ORF in the complementary DNA strand that extends from +639 backwards to −69. The ORF contains 236 codons and an in-frame AUG at +507, and it completely overlaps the TIF51A coding region. Curiously, a similar ORF also is found in the −1 reading frame of the complementary DNA strand of TIF51B, extending from +534 (using the published sequence of the ANB1 downstream untranslated region [27]) to −42, spanning 192 codons. However, in the latter case, there are no in-frame AUG codons in the sequence until the last third of the ORF (at +174). The function and expression of these putative overlapping genes have not been pursued further. However, Northern blot analyses do not detect additional transcripts, which means that these genes are very poorly if at all expressed or that they transcribe mRNAs precisely matching the TIF51A gene products.

A major purpose of cloning the genes encoding eIF-5A was to test whether the hypusine modification is required for eIF-5A function. We constructed a strain in which the chromosomal genes for TIF51A and TIF51B were disrupted and eIF-5A was expressed from the TIF51A gene on a plasmid. A mutant form of TIF51A was constructed in which the site of hypusination, Lys-51, was altered to Arg, thereby preventing the modification but preserving a positive charge at that position. The mutant gene, tif51A(K5,R), is expressed and the mutant protein accumulates in cells, indicating that it is stable and not degraded rapidly. Furthermore, the mutant protein is not modified by spermidine, as expected. However, eIF-5A(K5,R) does not complement the eIF-5A− defect of wild-type cells, and thus the mutant protein is not functional. This indicates that the hypusine modification is very likely required for the essential in vivo function of eIF-5A and is consistent with our demonstration that eIF-5A lacking hypusine is inactive in vitro in the methionyl-puromycin synthesis assay but is active following modification by spermidine to deoxyhypusine (13, 39). The roles of spermidine and other polyamines have been studied extensively (44). Polyamines are known to bind to nucleic acids, e.g., to specific sites in tRNA (36), and to affect the process of protein synthesis in vitro (18, 20, 46) and in vivo (45). Yet no precise functions for these small molecules have been identified. Our results indicate that an essential role for spermidine is to participate in the unique and necessary hypusine modification of eIF-5A. Experiments are in progress to determine how the hypusine modification may contribute to the essential function of eIF-5A in yeast cells.

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