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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 *TIF51A* and *TIF51B* 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 *TIF51A* 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 *TIF51A* with *LEU2*, grow slowly, whereas strains lacking the B form, in which *HIS3* was used to disrupt *TIF51B*, show no growth rate phenotype. However, strains with both *TIF51A* and *TIF51B* 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 *TIF51A* and a smaller mRNA (0.8 kb) encoded by *TIF51B*. Under the aerobic growth conditions of this study, the 0.8-kb *TIF51B* transcript is not detected in the wild-type strain and is expressed only when *TIF51A* is disrupted. The *TIF51A* 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^{ϵ}-(4-amino-2-hydroxybutyl)-lysine], formed posttranslationally by transfer of a butylamino 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 ec-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

* Corresponding author.
† Present address: Department of Pathology, School of Medicine, University of California San Diego, La Jolla, CA 92093.

TABLE 1. Yeast strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
S173-6B	*MATa leu2-3,112 ura3-52 trp1-289 his3-1*	M. Holland
S173-6BMF9	*MATα leu2-3,112 ura3-52 trp1-289 his3-1*	M. Holland
JS10	*MATa/MATα leu2-3,112/leu2-3,112 ura3-52/ura3-52 trp1-289/trp1-289 his3-1/his3-1*	This work
JSDA1	*MATa ura3 trp1 his3 leu2 tif51A::LEU2*	This work
JSDA2	*MATα ura3 trp1 his3 leu2 tif51A::LEU2*	This work
JSDA3	*MATa/MATα ura3/ura3 trp1/trp1 his3/his3 leu2/leu2 tif51A::LEU2/tif51A::LEU2*	This work
JSDB1	*MATa leu2 ura3 trp1 his3 tif51B::HIS3*	This work
JSDB2	*MATα leu2 ura3 trp1 his3 tif51B::HIS3*	This work
JSDB3	*MATa/MATα leu2/leu2 ura3/ura3 trp1/trp1 his3/his3 tif51B::HIS3/tif51B::HIS3*	This work
JSDAB1	*MATa/MATα leu2/leu2 ura3/ura3 trp1/trp1 his3/his3 TIF51A/tif51A::LEU2 TIF51B/tif51B::HIS3*	This work
JSDAB2	*MATa/MATα leu2/leu2 ura3/ura3 trp1/trp1 his3/his3 tif51A::LEU2/tif51A::LEU2 TIF51B/tif51B::HIS3*	This work
JSDAB2[YCp50-5A]	*MATa/MATα leu2/leu2 trp1/trp1 ura3/ura3 his3/his3 tif51A::LEU2/tif51A::LEU2 TIF51B/tif51B::HIS3* [YCp50-5A]	This work
JSDAB3[YCp50-5A]	*MATα leu2 ura3 trp1 his3 tif51A::LEU2 tif51B::HIS3* [YCp50-5A]	This work
JSDAB3[YCp50-5A]-[YRp7-5A (K_{51}R)]	*MATα leu2 ura3 trp1 his3 tif51A::LEU2 tif51B::HIS3* [YCp50-5A][YRp7-5A(K_{51}R)]	This work
S173-6B[YEp352-5A]	*MATa leu2 ura3 his3 trp1* [YEp352-5A]	This work
S173-6B[YEp352T-5A]	*MATa leu2 ura3 his3 trp1* [YEp352T-5A]	This work
S173-6B[YEp352T-5A(K_{51}R)]	*MATa leu2 ura3 his3 trp1* [YEp352T-5A(K_{51}R)]	This work
Plasmids		
YCp50	*CEN4 ARS1 URA3* Ap^r Tc^r	14
YCp50-5A	*CEN ARS1 URA3 TIF51A* Ap^r	This work
YRp7	*ARS1 TRP1* Ap^r Tc^r	43
YRp7-5A	*ARS1 TRP1 TIF51A* Ap^r	This work
YRp7-5A(K_{51}R)	*ARS1 TRP1 tif51A(K_{51}R)* Ap^r	This work
YEp13	2μm ORI[a] *LEU2* Ap^r Tc^r	6
pLAC-15	*CEN4 ARS1 HIS3* Ap^r	M. Holland
YEp352	2μm ORI *URA3* Ap^r	15
YEp352-5A	2μm ORI *URA3* Ap^r *TIF51A*	This work
YEp352T	2μm ORI *TRP1* Ap^r	This work
YEp352T-5A	2μm ORI *TRP1* Ap^r *TIF51A*	This work
YEp352T-5A(K_{51}R)	2μm ORI *TRP1* Ap^r *tif51A(K_{51}R)*	This work

[a] ORI, origin of replication.

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 [^{35}S]methionine (333 Ci/mmol; New England Nuclear) for 10 min at mid-log phase (A_{650} = 0.3). For the detection of hypusine, cells from a 2-ml culture were labeled with 10 μCi of either [^{14}C]spermidine (90 Ci/mol; Research Products International) or [^{3}H]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 *Bam*HI to completion and separated by 0.8% agarose gel electrophoresis. Southern blot analysis with a ^{32}P-labeled 0.5-kb *Eco*RI-*Pst*I 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 *Bam*HI site of the bacterial plasmid pACYC184 previously treated with calf intestinal alkaline phosphatase. About 200 individual clones

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 *Pst*I fragment that hybridized to the probe was subcloned into the *Pst*I site of pUC19 to yield pJSA1.

TIF51B was cloned in a similar fashion by digesting chromosomal DNA from strain S173-6B with *Hin*dIII. A 13-kb fragment was cloned into plasmid pACYC184; among 650 clones, 1 positive clone was identified by using the *TIF51A* gene as a probe. A 3.5-kb *Eco*RI fragment containing the gene was subcloned in a Bluescript (Stratagene) derivative (in which the *Sal*I site in the polylinker had been destroyed by digesting with *Hin*dIII and *Xho*I, filling in with Klenow fragment, and ligating) to yield pJSB1.

Gene replacements and disruptions. Plasmid pJSA2 was constructed for the disruption of *TIF51A* as follows. To destroy the *Sal*I site of pUC19, plasmid DNA was digested with *Sal*I and the 5′ ends were filled in with Klenow fragment. After religation and transformation, the pUC19 derivative was digested with *Pst*I and the 1.4-kb *Pst*I fragment containing the *TIF51A* gene was inserted to obtain plasmid pJSA2. This plasmid was digested with *Sal*I and *Hpa*I, and the linearized 3.7-kb plasmid lacking the 0.4-kb *Sal*I-*Hpa*I fragment from the coding region of the *TIF51A* gene was isolated. The 2-kb *Sal*I-*Hpa*I fragment from plasmid YEp13 containing the intact yeast *LEU2* gene was inserted into the linearized plasmid pJSA2. The resulting plasmid pJSA3 was digested with *Pst*I, and a 3.0-kb *Pst*I fragment carrying the disrupted *TIF51A* 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 *TIF51A* genes was confirmed by Southern blot analysis of genomic DNA probed either with the 1.4-kb *Pst*I fragment from pJSA1 or with the 0.4-kb *Sal*I-*Hpa*I fragment that had been removed from the coding region of *TIF51A*.

The *TIF51B* gene was disrupted as follows. Plasmid pJSB1 (see above) was digested with *Sal*I (which cuts in the N-terminal third of the *TIF51B* coding region) and partly filled with Klenow fragment, dCTP, and dTTP. The *HIS3* gene was excised from pLAC-15 by digestion with *Bam*HI, 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 *TIF51B*. pJSB2 was digested with *Eco*RI to yield a 5.3-kb fragment with *TIF51B* flanking sequences that was used to transform JS10. His$^+$ transformants were selected, and disruption of the *TIF51B* gene was confirmed by Southern blot analyses.

To construct a strain with both *TIF51A* and *TIF51B* disrupted, JSDA3 (**a**/α *tif51A*::*LEU2*/*tif51A*::*LEU2*) was transformed with the 5.3-kb linear DNA fragment carrying *TIF51B* disrupted by *HIS3*. The resulting diploid, JSDAB2, was transformed with YCp50-5A constructed by digestion of YCp50 with *Hin*dIII and *Bam*HI and ligation with the 1.4-kb *Hin*dIII-*Bam*HI fragment from pJSA2 that carries *TIF51A*. Cells were sporulated and tested for mitotic viability on 5-fluoro-orotic acid (5-FOA) plates.

Substitution of *TIF51A* with *tif51A*($K_{51}R$). The haploid strain JSDAB3[YCp50-5A] was transformed with either YRp7-5A or YRp7-5A($K_{51}R$), 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 pJSA2 (a pUC19 derivative carrying *TIF51A*) with *Eco*RI and inserting an *Eco*RI fragment from YRp7 that carries *TRP1* and *ARS1*. To construct YRp7-5A($K_{51}R$), the *TIF51A* 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 pJSA2 was amplified with the reverse sequencing primer (Stratagene) and a 44-base synthetic DNA, 5′-GGG-GTC-GAC-ATG-TCC-ACT-TCT-AAG-ACT-GGT-AGA-CAC-GGT-CAC-GC-3′. The 44-mer spans the *Sal*I site at its 5′ end and converts the Lys-51 codon to AGA (underlined) for Arg. The amplified DNA was cut with *Sal*I and *Hin*dIII (from the pUC19 polylinker) and subcloned into the corresponding sites of M13mp19, and the insert was sequenced from the *Sal*I site to the *Hpa*I site to confirm that the only sequence change was the AGA codon. M13 replicative-form DNA was cut with *Sal*I and *Hpa*I, and the fragment carrying the mutation was inserted into the *Sal*I-*Hpa*I sites of YRp7-5A to yield YRp7-5A($K_{51}R$). Confirmation that the plasmid carries the $K_{51}R$ mutation was made by sequencing plasmid DNA minipreparations by using the primer 5′-ATGTCT GACGAAGAACA-3′, which hybridizes to a region ca. 75 bp upstream of codon 51.

To demonstrate that the mutant eIF-5A protein, eIF-5A($K_{51}R$), is stable and not hypusinated, plasmids that overexpress the wild-type and mutant proteins were constructed. YEp352T-5A and YEp352T-5A($K_{51}R$) were constructed by inserting the 1.4-kb *Pst*I fragment encoding the eIF-5A proteins from YRp7-5A and YRp7-5A($K_{51}R$), respectively, into the *Pst*I site of the high-copy-number plasmid YEp352 (15). Then the 0.52-kb *Nde*I-*Stu*I fragment (blunt ended) containing a major part of the *URA3* gene was replaced with the 0.85-kb *Eco*RI-*Bgl*II fragment (blunt ended) of YRp7 containing *TRP1*. In both constructs, the *TIF51A* 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 *TIF51A* and *TIF51B* 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 *Bam*HI-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 *Bam*HI fragment ligated into pACYC184 was cloned in *E. coli* as described in Materials and Methods. From this clone, a 1.4-kb *Pst*I fragment that hybridizes to the mammalian cDNA was subcloned into the *Pst*I site of pUC19 to yield pJSA1.

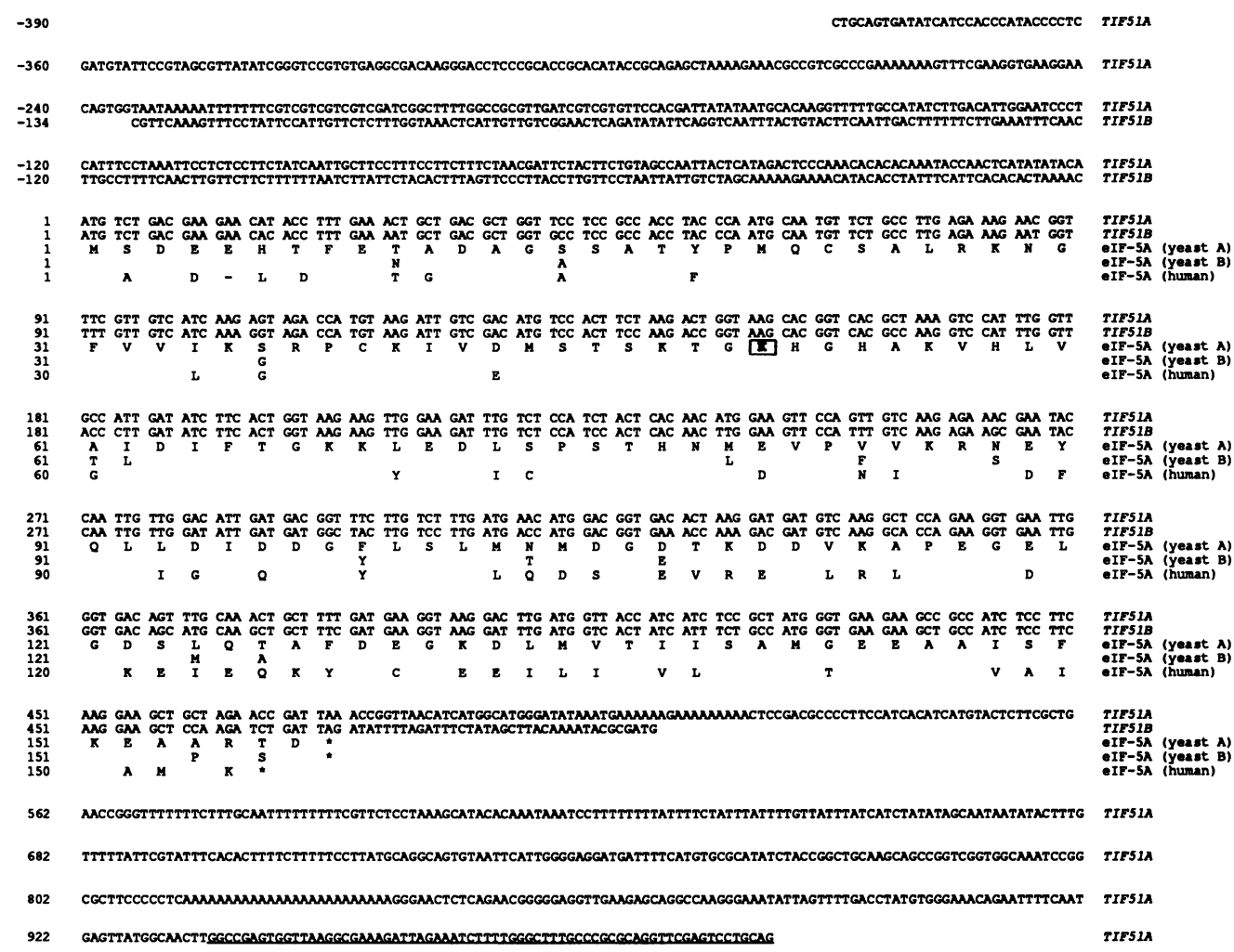

FIG. 1. Sequences of the eIF-5A genes and proteins. DNA sequences are shown for the coding and flanking regions of *TIF51A* and *TIF51B* that have been determined on both strands as described in Materials and Methods. The two sequences are aligned by matching their initiator codons, whose A is numbered +1. The derived amino acid sequence of *TIF51A* [labeled eIF-5A (yeast A)] is shown; for *TIF51B* [labeled eIF-5A (yeast B)] and for the human protein [labeled eIF-5A (human)], only residues that differ from those in *TIF51A* are shown. The lysine residue that is modified to hypusine is enclosed in a box. The $tRNA_2^{Ser}$ gene at the distal end of the *TIF51A* sequence is underlined.

The sequence of the 1.4-kb insert in pJSA1 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 hypusination 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 *TIF51A*. (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 be used to indicate the factor's precise name [37] whenever possible. Thus, the genes for eIF-5A become *TIF51*, 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 *TIF51A* 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, pJSA3, was constructed that contained a 73% deletion of the *TIF51A* coding region and an insertion of the *LEU2* gene (Fig. 2A). A 3.0-kb linear *Pst*I fragment containing *LEU2* and the *TIF51A* flanking sequences was prepared from pJSA3 and used to transform the diploid yeast strain JS10 (see Table 1 for yeast strain genotypes). Stable Leu^+ transformants were selected, and the disruption of one of the *TIF51A* 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^+, whereas the faster-growing cells were Leu^-. Disruption of *TIF51A* by

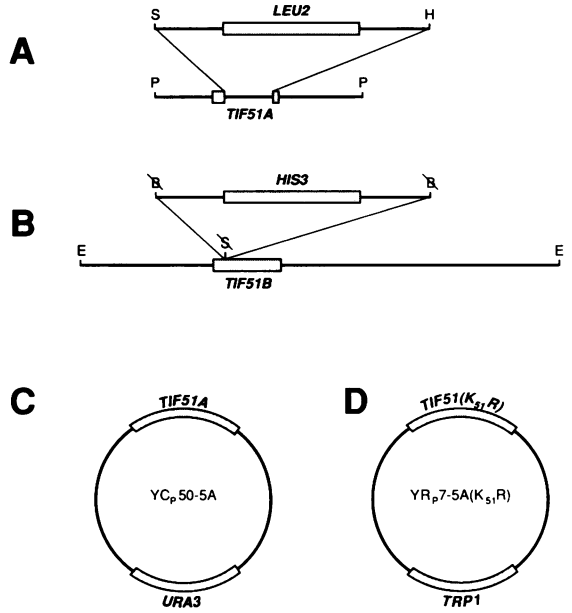

FIG. 2. Gene disruption constructs and expression vectors. Construction of the DNAs used to disrupt the *TIF51A* and *TIF51B* genes is described in Materials and Methods. (A) The *TIF51A* gene partially deleted and disrupted by insertion of the *LEU2* gene; (B) the *TIF51B* gene disrupted by insertion of the *HIS3* gene; (C) YCp50-5A; (D) YRp7-5A. Open rectangles refer to gene coding regions and are labeled appropriately, as are relevant restriction sites: S, *Sal*I; H, *Hpa*I; P, *Pst*I; E, *Eco*RI; and B, *Bam*HI. Panels A and B are drawn approximately to scale, whereas the plasmids in panels C and D are not.

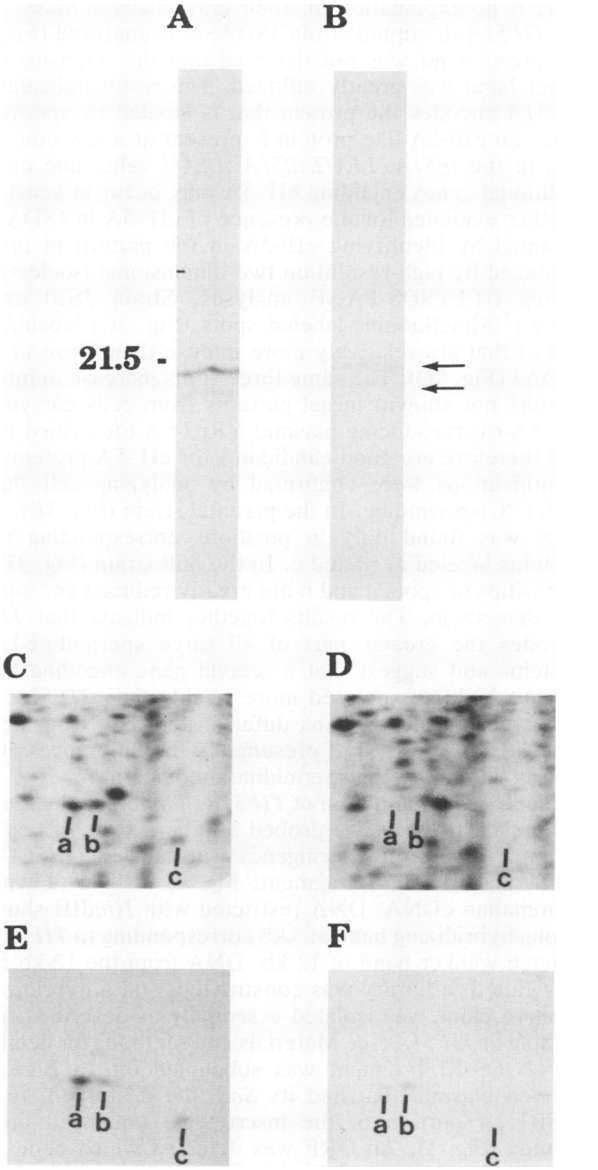

FIG. 3. Gel electrophoretic analysis of eIF-5A forms. Total protein in lysates of parental strain JS10 (A, C, and E) or the *TIF51A*-disrupted cell line JSDA3 (B, D, and F) were subjected to one-dimensional PAGE (A and B) or two-dimensional IEF/SDS-PAGE (C to F) as described by Laemmli (22) or O'Farrell (30), respectively. Prior to analysis, the cells analyzed in panels A, B, E, and F were grown in 2 ml of minimal SD medium containing 10 μCi of [^{14}C]spermidine (90 Ci/mol) for two generations (A_{650} of 0.1 to 0.4). Cells in panels C and D were labeled in 2 ml of minimal SD medium with 20 μCi of [^{35}S]methionine (333 Ci/mmol; New England Nuclear) for 10 min at an A_{650} of 0.3. The figure shows the autoradiograms on Kodak X-Omat film which were exposed overnight (C and D), for 3 days (A and B), or for 3 weeks (E and F). In the two-dimensional gels, only identical portions of the gel near the eIF-5A proteins are shown.

LEU2 in one of the slower-growing haploids, named JSDA1, was confirmed by Southern blot analyses of *Pst*I-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 we used 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 G_1 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 [^{14}C]spermidine. 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 *TIF51A*-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 *TIF51A* encodes the protein that is labeled by spermidine. Since an eIF-5A-like protein is present at a low concentration in the *tif51A*::*LEU2*/*tif51A*::*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 [^{35}S]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 eIF-5A proteins. The identifications were confirmed by analyzing cells labeled with [^{14}C]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 *TIF51A* 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 *TIF51A*. 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 *TIF51B*. To pursue the idea of a second *TIF51* gene, we probed Southern blots of restricted genomic DNA at low stringency with labeled *TIF51A* DNA (a 0.9-kb *Pst*I-*Hpa*I fragment; Fig. 2) instead of with the mammalian cDNA. DNA restricted with *Hin*dIII showed a strong hybridizing band at 5 kb corresponding to *TIF51A* 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 cloning of *TIF51A* (see Materials and Methods for details). A 3.5-kb *Eco*RI fragment was subcloned into a Bluescript-derived plasmid that had its *Sal*I 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 *TIF51A* 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 *TIF51B*. *TIF51A* and *TIF51B* possess 91% identical nucleotides in the coding region but are unrelated in their 5′ and 3′ noncoding regions.

The *TIF51B* gene was disrupted as described in Materials and Methods by cutting the structural gene with *Sal*I and inserting a 1.8-kb fragment carrying the *HIS3* gene (Fig. 2B). A linear 5.3-kb *Eco*RI 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 *TIF51B* genes was confirmed by Southern blot analyses (results not shown). The heterozygous *TIF51B*/*tif51B*::*HIS3* strain was sporulated, and tetrads were dissected and analyzed. Each

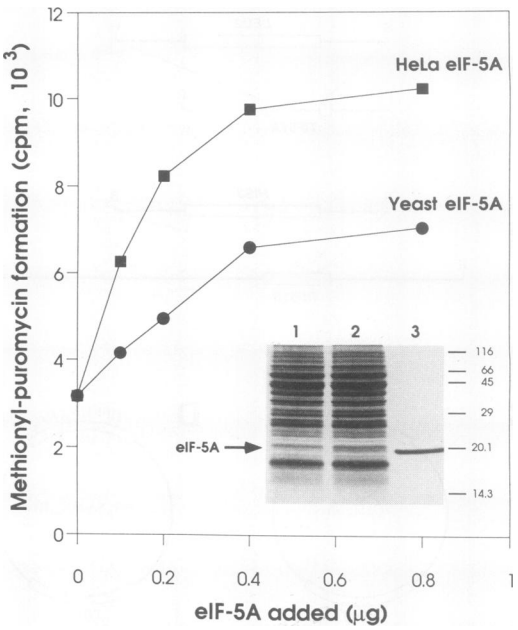

FIG. 4. Activity of yeast eIF-5A in vitro. Yeast eIF-5A was purified from the overproducing strain S173-6B[YEp352-5A], which overproduces eIF-5A about 10-fold from the *TIF51A* gene. A postribosomal supernatant fraction of the cell lysate was chromatographed consecutively on Mono S and Mono Q columns of the FPLC system (Pharmacia) essentially as described for the mammalian protein synthesized in *E. coli* (41). Details of the procedures will be presented elsewhere. The insert shows 15% SDS-PAGE analyses of Coomassie-stained proteins in the postmitochondrial lysates of control cells (lane 1) and overexpressing cells (lane 2) and the protein in the Mono Q peak fraction (lane 3). Purified HeLa and yeast eIF-5A were tested at the indicated amounts for stimulation of methionyl-puromycin synthesis in a 30-μl assay mixture containing 20 mM Tris-HCl (pH 7.6), 2 mM magnesium acetate, 70 mM KCl, 10 mM 2-mercaptoethanol, 1.6 pmol of [^{3}H]Met-tRNA (specific activity, 4.45×10^4 cpm/pmol), 0.8 mM GTP, 1 mM puromycin, 33 μM ApUpG, 0.06 and 0.15 A_{260} units of 40S and 60S ribosomal subunits from rat liver, respectively, 0.9 μg of HeLa eIF-2, 3.12 μg of HeLa eIF-3, 0.64 μg of rabbit reticulocyte eIF-5, and 0.27 μg of HeLa eIF-1A. Reaction mixtures were incubated and analyzed as described previously (41).

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 *TIF51B*. We conclude that disruption of *TIF51B* does not affect the growth of yeast strains under these conditions, presumably because of the presence of the stronger-expressing *TIF51A* gene.

Yeast eIF-5A stimulates methionyl-puromycin formation in vitro. To obtain more compelling evidence that the *TIF51* 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 *TIF51A* 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 *TIF51A* gene is functionally related to mammalian eIF-5A and is highly conserved in structure since it is

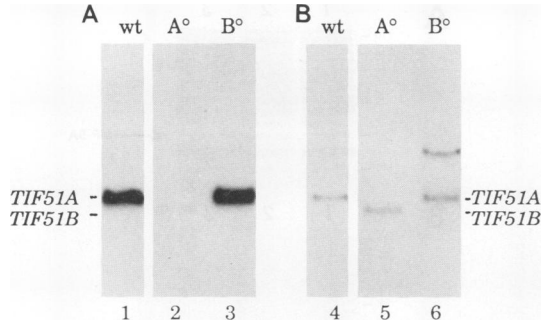

FIG. 5. Northern blot hybridization analyses. Total yeast RNA was prepared from strains S173-6B (lanes 1 and 4), JSDA2 (lanes 2 and 5), and JSDB1 (lanes 3 and 6); equal amounts of RNA (20 μg) were applied to each lane of a formaldehyde–1.75% agarose gel, electrophoresed, and then blotted onto HyBond-N (Amersham) (26). The Northern blot was probed with either the 1.4-kb *Pst*I fragment of pJSA1 carrying the coding region of *TIF51A* (A) or the 1.4-kb *Sma*I-*Dra*I fragment of pJSB1 that includes the coding region of *TIF51B* (B), each labeled to comparable specific activity. The Northern blots were exposed to Kodak X-Omat film for 8 h.

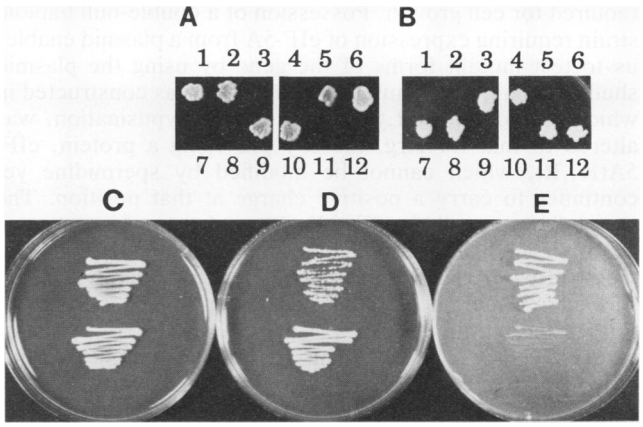

FIG. 6. Growth analyses of strains lacking eIF-5A or hypusine. (A and B) Analysis of haploids lacking a *TIF51* gene. Strain JSDAB2[YCp50-5A] was sporulated, and haploids were grown on minimal SD plates supplemented with histidine and tryptophan. A random sample of 12 haploids was selected and replica patched onto minimal SD plates containing either tryptophan and uracil (A) or tryptophan, uracil, histidine, and 5-FOA (B). Patched cells were grown at 30° for 2 days (A) or 1 week (B) and then photographed. (C to E) Analysis of haploid strains with wild-type and mutant *TIF51A*. Haploid strains JSDAB3[YCp50-5A][YRp7-5A] (upper streaks) and JSDAB3[YCp50-5A][YRp7-5A(K_{51}R)] (lower streaks) were constructed by transformation of JSDAB3[YCp50-5A] with the YRp7 plasmid derivatives as described in the text and were selected by growth on minimal SD plates supplemented with all amino acids except tryptophan. The figure shows one of each type of transformant streaked onto minimal SD plates supplemented with all amino acids but no uracil (C), uracil and all amino acids except tryptophan (D), and all amino acids, uracil, and 5-FOA (E). The plates were incubated at 30°C for 2 days (C and D) or 7 days (E) and photographed.

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 *TIF51A* DNA (Fig. 5A), the parental strain S173-6B (lane 1) and the *tif51B*::*HIS3* null strain JSDB1 (lane 3) generate a strong band at ca. 0.9 kb, whereas the *tif51A*::*LEU2* null strain JSDA2 (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 *TIF51B* 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 *TIF51B* gene and the *HIS3* gene, although this has not been proven. The results indicate that the larger 0.9-kb RNA is transcribed from *TIF51A* and the smaller 0.8-kb RNA is transcribed from *TIF51B*. The efficiency of hybridization to the two RNA transcripts varies with the probe. We estimate that the *TIF51A* transcript is ca. 5- to 10-fold more abundant than the *TIF51B* transcript. This roughly equals the difference in apparent rates of eIF-5A synthesis deduced from [^{14}C]spermidine labeling when parental and *TIF51A*-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 *TIF51A* and *TIF51B*. We mated the haploid strains JSDA1 (*MAT***a** *tif51A*::*LEU2*) and JSDB2 (*MAT*α *tif51B*::*HIS3*) to produce the diploid JSDAB1, which is Leu$^+$ His$^+$. Upon sporulation and tetrad analysis of 20 asci, 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 ditype, 5 nonparental ditype, and 13 tetratype, 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 (*tif51A*::*LEU2*/*tif51A*::*LEU2*) was transformed with the 5.3-kb *Eco*RI fragment carrying *tif51B*::*HIS3*. His$^+$ transformants were selected, and those which had a disruption of a single *TIF51B* gene were identified by Southern blot analyses (results not shown) to yield JSDAB2. The transformants sporulate poorly, giving a very low number of asci, making tetrad analysis impractical. Then JSDAB2 was transformed with a YCp50 derivative carrying a functional *TIF51A* gene, YCp50-5A (Fig. 3C). Ura$^+$ transformants (named JSDAB2[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 *TIF51A* and *TIF51B* 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 *TIF51* disruptions), whereas half did not grow, presumably because they are His$^-$ (with an intact *TIF51B* 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* plasmid. However, the His$^+$ cells did not grow on 5-FOA, indicating that they are not able to lose the *URA3*-*TIF51A* plasmid. Thus, the YCp50-5A plasmid is essential for growth of the double-null haploids; at least one copy of a *TIF51* 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(K_{51}R), which cannot be modified by spermidine yet continues to carry a positive charge at that position. The mutated gene, called *tif51A*($K_{51}R$), and the wild-type gene *TIF51A* each were combined with a portion of YRp7 DNA to yield YRp7-5A(K_{51}R) and YRp7-5A, respectively, as described in Materials and Methods. The plasmids were transformed into strain JSDAB3[YCp50-5A], and Trp^+ transformants were selected. Thirty transformants carrying YRp7-5A and 60 carrying YRp7-5A(K_{51}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 JSDAB3[YCp50-5A][YRp7-5A] and JSDAB3[YCp50-5A][YRp7-5A(K_{51}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(K_{51}R) transformants grew on 5-FOA (as shown for one of these in Fig. 5B). This finding suggests that the eIF-5A(K_{51}R) mutant form is inactive for a function required for cell growth.

To show that the mutant protein eIF-5A(K_{51}R) actually is synthesized and is stable in yeast cells, cells overexpressing *tif51A*($K_{51}R$) 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(K_{51}R) and inserted these DNA fragments into the high-copy-number plasmid YEp352T (as described in Materials and Methods) to yield YEp352T-5A and YEp352T-5A(K_{51}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 [^{3}H] 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 K_{51}R mutant form (lane 3) results in enhanced spermidine labeling of the 20-kDa protein. The results indicate that eIF-5A(K_{51}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.

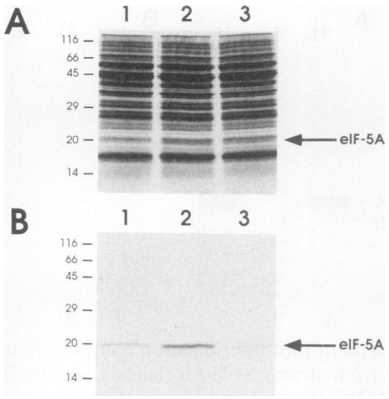

FIG. 7. Evidence that mutant eIF-5A(K_{51}R) is stable and not hypusinated. Total protein of postmitochondrial supernatant fractions of cell lysates from the control strain S173-6B[YEp352T] (lane 1), S173-6B[YEp352T-5A] (lane 2), and S173-6B[YEp352T-5A(K_{51}R)] (lane 3) were subjected to SDS-PAGE on a 15% polyacrylamide gel as described previously (22). Prior to analysis, cells were grown in 2 ml of SD medium containing 10 μCi of [^{3}H]spermidine as described in Materials and Methods. (A) Coomassie-stained gel; (B) autoradiogram of a duplicate gel exposed to Kodak X-Omat film for 3 days. The migration positions of molecular weight markers are shown on the left in kilodaltons; the migration position of eIF-5A is indicated by an arrow.

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 $tRNA_2$, the major serine tRNA in yeast cells (42). The two serine $tRNA_2$ genes that are linked to *TIF51A* and *TIF51B* possess identical sequences in their coding regions but differ in their flanking regions. The serine $tRNA_2$ gene detected next to *TIF51A* represents a new member of the yeast serine $tRNA_2$ 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 *ANB1* 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 *hem1* 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 GF1-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* A(A/T)AATGTCT (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 either that the genes are very poorly if at all expressed or that they transcribe mRNAs precisely matching the *TIF51* 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*($K_{51}R$), 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, *tif51A*($K_{51}R$) does not replace the-wild type gene; 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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