Eukaryotic Translation Initiation Factor 5 from *Saccharomyces cerevisiae*

CLONING, CHARACTERIZATION, AND EXPRESSION OF THE GENE ENCODING THE 45,346-Da PROTEIN*

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Eukaryotic translation initiation factor 5 (eIF-5),1 in conjunction with other initiation factors and GTP, plays an essential role in the initiation of protein synthesis in eukaryotic cells (Maitra et al., 1982; Kozak, 1983; Hershey, 1991; Merrick, 1992). Following ribosomal scanning of mRNA and formation of the 40 S initiation complex (40 S·mRNA·Met-tRNAf), the bound eIF-2 and the guanine nucleotide must be released from the 40 S initiation complex prior to the joining of a 60 S ribosomal subunit to the 40 S complex (40 S·mRNA·Met-tRNAf) to form the elongation-competent 80 S initiation complex (80 S·mRNA·Met-tRNAf). The release of eIF-2 and the guanine nucleotide is accomplished by the interaction of the 40 S initiation complex with eIF-5, which catalyzes the quantitative hydrolysis of ribosome-bound GTP (Trachsel and Staehelin, 1978; Peterson et al., 1979; Raychaudhuri et al., 1985b; Chakrabarti and Maitra, 1991). The eIF-2·GDP and P, formed in the reaction lack affinity for the 40 S ribosomal complex (40 S·mRNA·Met-tRNAf), and are released. The subsequent joining of the 60 S ribosomal subunit to the 40 S·mRNA·Met-tRNAf complex to form the 80 S initiation complex (80 S·mRNA·Met-tRNAf) does not require the participation of eIF-5 (Chakrabarti and Maitra, 1991). However, since the hydrolysis of GTP and release of the 40 S·GDP is not a prerequisite for subsequent joining of 60 S ribosomal subunits to the 40 S·mRNA·Met-tRNAf complex, eIF-5 was conveniently assayed by its ability to catalyze the joining of 60 S ribosomal subunits to the 40 S complex, eIF-5 was conveniently assayed by its ability to catalyze the joining of 60 S ribosomal subunits to the 40 S·mRNA·Met-tRNAf complex.

The nucleotide sequence(s) reported in this paper have been submitted to the GenBank™/EMBL Data Bank with accession number(s) L10524.

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1 The abbreviations used are: eIF-2 and eIF-5, eukaryotic (translation) initiation factors 2 and 5, respectively; IPTG, isopropyl-β-D-thiogalactopyranoside; GMP-PCP, 5′-guanylyl methylenediphosphonate; bp, base pair(s); kb, kilobase pair(s); CAPS, 3-(cyclohexylamino)propanesulfonic acid; Tricine, N-tris(hydroxymethyl) methylglycine; CHEF, contour-clamped homogeneous electric field.

tRNA, eIF-2·GTP), the bound eIF-2 and the guanine nucleotide must be released from the 40 S initiation complex prior to the joining of a 60 S ribosomal subunit to the 40 S complex (40 S·mRNA·Met-tRNAf) to form the elongation-competent 80 S initiation complex (80 S·mRNA·Met-tRNAf). The release of eIF-2 and the guanine nucleotide is accomplished by the interaction of the 40 S initiation complex with eIF-5, which catalyzes the quantitative hydrolysis of ribosome-bound GTP (Trachsel and Staehelin, 1978; Peterson et al., 1979; Raychaudhuri et al., 1985b; Chakrabarti and Maitra, 1991). The eIF-2·GDP and P, formed in the reaction lack affinity for the 40 S ribosomal complex (40 S·mRNA·Met-tRNAf), and are released. The subsequent joining of the 60 S ribosomal subunit to the 40 S·mRNA·Met-tRNAf complex to form the 80 S initiation complex (80 S·mRNA·Met-tRNAf) does not require the participation of eIF-5 (Chakrabarti and Maitra, 1991). However, since the hydrolysis of GTP and release of the 40 S·GDP is not a prerequisite for subsequent joining of 60 S ribosomal subunits to the 40 S·mRNA·Met-tRNAf complex, eIF-5 was conveniently assayed by its ability to catalyze the joining of 60 S ribosomal subunits to the 40 S·mRNA·Met-tRNAf complex.
contains an activity that can functionally substitute for mammalian eIF-5 in catalyzing the formation of an 80 S initiation complex from a preformed 40 S initiation complex. This activity, designated yeast eIF-5, has recently been purified and characterized biochemically as well as immunochemically (Chakravarti et al., 1993). It has been demonstrated that purified yeast eIF-5 bound to CNBr elution columns as a protein of about 45–50 kDa, exhibits two polypeptide bands of apparent molecular mass of 54 and 56 kDa in denaturing gel electrophoresis (Chakravarti et al., 1993). Each of the two polypeptides individually has been found to contain eIF-5 activity, and they are immunologically related to each other (Chakravarti et al., 1993).

The availability of monospecific rabbit antibodies specific for yeast eIF-5 has now allowed us to carry out immunoscreening of an αgt11 yeast genomic expression library to isolate the gene for yeast eIF-5. In this paper, we describe the cloning and characterization of the yeast gene TIF5 (translation initiation factor 5) that encodes eIF-5. Expression of the catalytically active eIF-5 protein in *Eschericia coli* confirms that the gene identified by immunoscreening is indeed the structural gene of yeast eIF-5. The TIF5 gene encodes an intron-free, single large open reading frame of 405 amino acid residues, predicting a protein of 45,546 daltons. This value is in close agreement with the apparent molecular weight of eIF-5 isolated from yeast cells. Analysis of the predicted amino acid sequences of the yeast eIF-5 protein reveals structural motifs characteristic of proteins of the GTPase superfamily (Bourne et al., 1991). Furthermore, inactivation of one copy of the gene in a diploid strain shows that TIF5 has an essential function in mitotic growth of yeast cells.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Genetic Methods**—Standard genetic techniques and media used for these studies were as described by Sherman et al. (1986) and Rose et al. (1989). αgt11 phage was grown in *E. coli* strain Y1090, and lysogens were made in strain Y109B (Young and Davis, 1983). The *S. cerevisiae* diploid strain B3926 (MATαα, trp1/+, his/p, prel-126/prel-126; prel-407/prel-407, pep3-3/prep3-3, prel-1122/prel-1122, can1/ran1, gal2/gal2) obtained from the Yeast Genetic Stock Center (Berkeley, CA) is protease-deficient and was used to isolate eIF-5. The gene disruption experiment was carried out in the homzygous diploid strain, W303 (MATαMATα, ade2-1, leu2-3, -112, his3-11, -15, trpl-1, ura3-1, can1-100). This strain and its haploid derivatives were kind gifts of Dr. R. Rothstein and were made available to us by Dr. Jonathan Warner of this institution. Electrophoretic karyotyping and chromosomal mapping were carried out in the yeast strain YPH149 (MATα, ura3-52, his3-200, ade2-1, his7, trpl-1 (CF/URA3 + RAD2 distal) (CF/TRPI + RAD2 proximal)) (a kind gift of Dr. Philip Hieter, Johns Hopkins University School of Medicine).

**tRNA, Ribosomes, and Protein Factors**—The preparation of *α*-labeled rabbit liver initater Met-tRNA, ribosomal subunits from *Artemia salina* eggs, purified eIF-2, and eIF-5 from rabbit reticulocyte lysates was described by Chakraborti and Maizel (1991). Yeast eIF-5 was purified from *S. cerevisiae* strain B3926 as described by Chakravarti et al. (1993). Yeast eIF-5 is a monomeric protein that eluted in the homzygous diploid strain, W303 (MATαMATα, ade2-1, leu2-3, -112, his3-11, -15, trpl-1, ura3-1, can1-100). This strain and its haploid derivatives were kind gifts of Dr. R. Rothstein and were made available to us by Dr. Jonathan Warner of this institution. Electro- phoretic karyotyping and chromosomal mapping were carried out in the yeast strain YPH149 (MATα, ura3-52, his3-200, ade2-1, his7, trpl-1 (CF/URA3 + RAD2 distal) (CF/TRPI + RAD2 proximal)) (a kind gift of Dr. Philip Hieter, Johns Hopkins University School of Medicine).

**Cyanochrome B550 Digestion and Amino Acid Sequence Analysis**—Digestion of eIF-5 with CNBr was performed as described by Charbonneau (1989). Purified eIF-5 protein (50 μg) was digested with trichloroacetic acid and resuspended in 200 μl of 70% formic acid containing 200 μg of CNBr. After incubation in the dark under nitrogen at room temperature for 24 h, the sample was subjected to repeated lyophilization to remove unreacted reagents. The resulting peptide mixture was resolved by electrophoresis in Tricine-SDS-polyacrylamide gradient gel (17% acrylamide and 4.5% cross-linker piperazine diacrylamide to 14% acrylamide and 3% piperazine diacrylamide) as described by Schägger and von Jagow (1987). The separated peptides were electrophotoretherically transferred to ProBlott™ membrane using 10 mM CAPS, pH 11.0, 10% methanol, and 0.4 mM sodium thioglycollate as the transfer buffer. The membrane was washed extensively with water, stained with 0.1% Coomassie Brilliant Blue, and destained in developing an applied Biomass model 477A protein Sequence to determine their amino acid sequence.

**Immunochemical Techniques and Immunoscreening of Yeast Genomic Library**—The preparation and characterization of polyclonal antisera raised in rabbits against purified yeast eIF-5 were described by Chakravarti et al. (1993). Polyclonal anti-yeast eIF-5 antibodies were affinity-purified using purified yeast eIF-5 (either the 56- or the 54-kDa polypeptide), blotted onto aminopropylthieter paper by an adaptation of the procedure of Olmsted (1981) as described by Chakravarti et al. (1993). Affinity-purified anti-yeast eIF-5 antibodies were specific for the yeast eIF-5 antigen, since analysis of total proteins derived from yeast cells lysed directly into denaturing buffers containing 5% SDS showed only two immunoreactive polypeptide bands corresponding to the purified yeast eIF-5 (apparent M, = 54,000 and 56,000) (Chakravarti et al., 1993). Furthermore, these anti-eIF-5 antibodies did not cross-react with either mammalian eIF-5 (Chakravarti et al., 1993) or with yeast eIF-2 (data not shown). The procedure for immunoblotting yeast eIF-5 was similar to that used in this laboratory for mammalian eIF-5 (Ghosh et al., 1989). A *λ*gt11 genomic library of *S. cerevisiae* (2180, ATCC 26109), prepared by Clonetech, was a gift from Dr. Jonathan Warner of this Institution, and was screened using affinity-purified anti-yeast eIF-5 antibodies essentially as described by Snyder et al. (1987). A 1.100 to 1.300 dilution of the affinity-purified anti-yeast-eIF-5 antibodies was routinely used for immunochecmical detection as well as for immunoscreening of the αgt11 library. A goat anti-rabbit IgG coupled to alkaline phosphatase was used as the secondary antibody to detect the binding of the primary antibody to the immunoblots. The formation of immuno complexes on the blots were ascertained by placing the blots in the color development solution (1 ml of 1.5% nitro blue tetrazolium and 1 ml of 3% 5-bromo-4-chloro-3-indolyl phosphate dissolved in 100 ml of 0.1 M Tris-HCl, pH 9.5, and 0.5 mM MgCl₂) until the desired intensity of immunoreactive plaques was achieved. The putative positive phase plaques were plaque-purified by several rounds of re-screening.

**Preparation of Cell Lysates from Recombinant αgt11 Lysogens—** *E. coli* Y1089 was infected with recombinant λ-phage clones at a multiplicity of infection of 10. The infected cells were spread onto LB/ ampicillin plates (Sambrook et al., 1989) and grown at 30°C overnight. Lysogenic colonies were detected by their temperature sensitivity. A 0.1% 75% glycerol culture was grown in the presence of 150 μg/ml ampicillin, 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride, and then suspended in 1 ml of the same buffer. Cell lysis was achieved by rapid freeze and thaw followed by brief sonication. The resulting extract was centrifuged in an Eppendorf microcentrifuge and the supernatant dialyzed for 24 h at 4°C in the presence of 20 mM Tris-HCl, pH 7.5, 0.1 M KCl, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride. The dialyzed material was centrifuged to remove any insoluble debris and stored in small aliquots at −155°C until assayed for eIF-5 activity.

**Subcloning and DNA Sequencing—** Standard techniques described by Sambrook et al. (1989) were employed to isolate plasmid and phasmid DNA, as well as for the generation of subclones for sequencing. The EcoRI fragments from XC14 and XC16, clones were separately subcloned into pGEM7Zf(+) vector (Promega). The recombinant plasmid was digested with EcoRI and Smal and subjected to exonuclease digestion and treatment to generate fragments. Functional deletions that were subsequently cloned and sequenced by the dideoxy chain termination (Sanger et al., 1977) method using a United States Biochemical Corp. sequencing kit. After one strand had been sequenced, a series of appropriate 1-mer oligonucleotides were synthesized and used to sequence the complementary DNA strand. DNA sequences from overlapping clones were determined, compared, and
checked using the GCG-Wisconsin program.

Primer Extension Mapping of the TIF5 Transcription Initiation Sites—TIF5 transcription initiation sites were mapped using the oligonucleotide-directed primer extension method of Sambrook et al. (1989). A 5'-32P-labeled oligonucleotide (approximately 50-mer) polyphosphorylated total RNA isolated from the yeast strain W303-2a was used as template RNA, while 20 ng of the 5'-32P-labeled deoxyoligonucleotide 5'-AAAAACGAGCTTTGATACCCGTCTTCTA-3', which is complementary to nucleotides +110 to +80 of TIF5 gene, was used as the primer. Following reverse transcriptase reaction with 20 units of avian myeloblastosis virus reverse transcriptase (BRL) and 40 units of RNAsin, the DNA-RNA hybrids formed were isolated, treated with RNase A, then analyzed on a 6% sequencing gel as described by Ju et al. (1990).

TIF5 Gene Disruption—The one-step gene disruption method of Rothstein (1983) was used to construct the null allele of the TIF5 gene. For this purpose the 2.192-kb yeast DNA insert present in the XC14 clone was subcloned into pGEM7Zf(+) vector. The recombinant plasmid was digested with BsmI and PstI to remove a 285-bp fragment which is epithelial RNA of 18S. The plasmid was then used to transform E. coli XL1-blue (Stratagene) cells. The recombinant plasmid DNA (containing a 3.067-kb EcoRI-EcoRI fragment) was isolated and digested with EcoRI. The DNA was purified by extraction with phenol and phenol-chloroform, followed by ethanol precipitation. The purified DNA containing the 3.067-kb EcoRI-EcoRI fragment was dissolved in 10 µl of TE (10 mM Tris-Cl, pH 7.5 + 1 mM EDTA), mixed with 40 µg of carrier DNA, and used to transform W303-2a (a/a) cells. The transformants were selected on SD-URA media. The disruption of the genomic TIF5 gene in the transformants was verified by Southern blot analysis of total DNA using a 5'-32P-labeled 740-bp HhaI fragment of the TIF5 gene as a probe. Spores obtained from one such transformant were subjected to tetrad analysis as described by Rothstein (1983).

Other Methods—Chromosome-length DNA molecules, total and poly(A)^+ RNA of S. cerevisiae were isolated as described by Rose et al. (1989). Northern and Southern blot analyses were performed as described by Sambrook et al. (1989). Yeast transformation and tetrad analysis were performed as described by Rose et al. (1989).

RESULTS

Molecular Cloning of the Yeast TIF5 Gene—To isolate the yeast gene encoding eIF-5, we carried out immunoscreening of a λgt11 yeast genomic DNA expression library using affinity-purified polyclonal anti-eIF-5 antibodies as probes. Three positive clones were obtained by screening a total of approximately 10^8 recombinant phages. Each positive clone was plaque-purified to homogeneity and shown to encode protein that reacted strongly with anti-eIF-5 antisera but not with preimmune sera (data not shown). These clones were designated λC14, λC16, and λC17. An EcoRI digest of DNA isolated from each clone followed by agarose gel electrophoresis revealed the presence of a 2.2-kb DNA insert in λC14, a 2.8-kb DNA insert in λC16, and a 3.5-kb DNA insert in λC17 (data not shown).

To identify the polypeptides produced by these clones, immunological cross-reactivity between clone-specified polypeptides and yeast eIF-5 was monitored by analyzing whether polypeptides encoded by cloned sequences could affinity-purify antibodies that reacted with yeast eIF-5 (epitope selection). For this purpose, total proteins obtained from E. coli cells infected separately with λC14, λC16, and λC17 phage clones and subsequently induced with IPTG were immobilized on separate nitrocellulose membrane filters. Each filter was then incubated with anti-eIF-5 antisera. After washing the filters to remove antibodies that were not specific for clone-specified polypeptides, the bound antibodies were eluted with 4 M guanidine HCl and then used for immunoblot analysis of purified yeast eIF-5 as well as total yeast cell lysates. As shown in Fig. 1, when protein blots of purified yeast eIF-5 (lanes b–d) or of total yeast cell lysates (lanes e–g) were probed with each set of affinity-purified antibodies, two major immunoreactive polypeptide bands were observed. The apparent molecular weights of these polypeptides were similar to those observed when isolated yeast eIF-5 (lane a) was probed with affinity-purified anti-eIF-5 antibodies. Several minor but lower molecular weight immunoreactive polypeptide bands observed in the immunoblots of purified yeast eIF-5 preparations presumably represented proteolytic degradation products of yeast eIF-5.

Expression of Active eIF-5 in E. coli—The nature of the yeast polypeptide antigen encoded by each of the three positive clones was analyzed by infecting E. coli with each λ clone, followed by inducing the synthesis of the polypeptide encoded by cloned sequences by IPTG. When the proteins produced in each induced E. coli lysogenic culture were subjected to polyacrylamide gel electrophoresis, followed by immunoblotting with anti-eIF-5 antibodies, a major immunoreactive protein band of 54 kDa and a minor band of 56 kDa were clearly detected in all three extracts examined (Fig. 2, lanes b–d). The size of these polypeptide bands were similar to those observed when eIF-5 isolated from yeast was probed with anti-eIF-5 antibodies (Fig. 2, lane a). E. coli cells infected with a λ clone that did not give positive signals in the initial immunoscreening procedure did not yield immunoreactive protein bands (Fig. 2, lane a). Interestingly, in all three crude E. coli lysates examined, an additional immunoreactive polypeptide (of 38 kDa) was also observed (Fig. 2, lanes b–d). This polypeptide, which was not present in purified yeast eIF-5 preparations (Fig. 2, lane a), may arise by limited proteolysis of either or both 54- and 56-kDa polypeptides by proteases present in crude E. coli extracts. Alternatively, this polypeptide may have been synthesized by bacterial ribosomes by initiation from an internal AUG codon located on the yeast gene. At any rate, these results demonstrate that all three positive clones contained yeast genes that code for the same polypeptide whose molecular size is similar to that of eIF-5 isolated from yeast cells.

Additional evidence that each of the positive recombinant clones contained the structural gene encoding eIF-5 was obtained by assaying the crude extracts prepared from IPTG-induced E. coli lysogens (Fig. 3). When a preformed 40 S

![Fig. 1. Identification of recombinant clones by epitope selection.](image-url)
were grown and induced with IPTG, as described under “Experimental Procedures.” Approximately 0.75 ml of each infected culture was centrifuged and the cells suspended in 12.5 μl of water. To this suspension, 12.5 μl of a denaturing gel loading buffer (4% SDS, 100 mM Tris-HCl, pH 6.8, 0.2 mM dithiothreitol, 20% glycerol, and 0.2% bromphenol blue) was added. The suspension was heated at 90°C for 10 min and electrophoresed in a 10% SDS-polyacrylamide gel. The proteins were transferred to Immobilon-P (Millipore) membrane filters and probed with an 1:100 dilution of affinity-purified anti-eIF-5 antibodies, as described under “Experimental Procedures.”

Fig. 2. Immunoblot analysis of cell lysates prepared from IPTG-induced recombinant λ lysogens. Recombinant λ lysogens were grown and induced with IPTG, as described under “Experimental Procedures.” Approximately 0.75 ml of each infected culture was centrifuged and the cells suspended in 12.5 μl of water. To this suspension, 12.5 μl of a denaturing gel loading buffer (4% SDS, 100 mM Tris-HCl, pH 6.8, 0.2 mM dithiothreitol, 20% glycerol, and 0.2% bromphenol blue) was added. The suspension was heated at 90°C for 10 min and electrophoresed in a 10% SDS-polyacrylamide gel. The proteins were transferred to Immobilon-P (Millipore) membrane filters and probed with an 1:100 dilution of affinity-purified anti-eIF-5 antibodies, as described under “Experimental Procedures.” Lane a, cell extracts prepared from non-immunoreactive λgt11 lysogenic cells; lane b, λC14 lysogenic extracts; lane c, λC16 lysogenic extracts; lane d, λC17 lysogenic extracts; lane e, partially purified yeast eIF-5 preparation.

initiation complex containing bound [35S]Met-tRNAe (Fig. 3, panel D) was incubated with 60 S ribosomal subunits and an aliquot of crude extract prepared from λC14-, λC16-, or λC17-infected E. coli cells, formation of the 80 S initiation complex readily occurred (Fig. 3, panel A). Formation of the 80 S initiation complex was dependent on the presence of λC14-, λC16-, or λC17-infected E. coli extracts (Fig. 3, compare panel A with panel C). In contrast, extracts prepared from IPTG-induced uninfected E. coli cells were inactive in promoting 80 S initiation complex formation (Fig. 3, panel B). Extracts prepared from cells lysogenic for a non-immunoreactive λ clone were also inactive in the subunit joining reaction (data not shown). The amount of 80 S initiation complex formed was proportional to the amount of λC14 or λC16 lysogenic cell extracts added to the initiation reaction mixture (Fig. 4). Similar results were obtained with the λC17 cell extracts (data not shown). Furthermore, when the 40 S initiation complex was formed using the non-hydrolyzable analogue of GTP, (GMP-PCP) instead of GTP, neither purified yeast eIF-5 nor any of the λ lysogenic cell extracts was catalytic in the formation of 80 S initiation complex (Fig. 5). These results are in keeping with the observations made previously that both mammalian eIF-5 as well as yeast eIF-5 catalyze the hydrolysis of GTP bound to the 40 S initiation complex (Raychaudhuri et al., 1965b; Chakravarti et al., 1993). Hydrolysis of GTP is essential for the joining of 60 S ribosomal subunits to the 40 S complex to form the 80 S initiation complex. At any rate, these results clearly demonstrated that λC14, λC16, and λC17 clones each contained the structural gene encoding yeast eIF-5 and that the protein synthesized in E. coli was catalytically active in promoting the formation of an 80 S initiation complex from a preformed 40 S initiation complex. Additionally, since the polypeptides encoded by each clone were similar in size to that of purified yeast eIF-5, it is likely that each λ clone contained a functionally oriented open reading frame encoding yeast eIF-5 and that the open reading frame was translated using its own translation initiation codon but not as a fusion protein with β-galactosidase.

Fig. 3. Expression of active eIF-5 in E. coli lysogens. Induced lysogen extracts were prepared as described under “Experimental Procedures.” eIF-5 activity in each extract was measured by its ability to promote the joining of a 60 S ribosomal subunit to a preformed 40 S initiation complex to form an 80 S initiation complex, as described by Chakravarti et al. (1993). Reaction mixtures (45 μl each) containing 20 mM Tris-HCl, pH 7.5, 0.1 mM KCl, 5 mM 2-mercaptoethanol, 20 μg of bovine serum albumin, 0.5 mM GTP, 0.5 A260 unit of rabbit liver tRNA containing 9 pmol of [35S]Met-tRNAe (20,000 cpm/pmol), and 1 μg of purified rabbit reticulocyte eIF-2 were incubated at 37°C for 3 min to form the [35S]Met-tRNAe-eIF-2-GTP ternary complex. Subsequently, each reaction mixture was adjusted to 5 mM MgCl2 (final concentration) then supplemented with 0.5 A360 unit of 40 S ribosomal subunits and 0.1 A360 unit of the AUG codon, and the mixtures were incubated for an additional 4 min at 37°C to form the 40 S initiation complex (40 S-AUG-[35S]Met-tRNAe-eIF-2-GTP). The reaction mixtures were then chilled in an ice bath and 0.8 A360 unit of 60 S ribosomal subunits and 90 μg of crude cell extract (source of eIF-5) were added and incubation was continued for an additional 5 min at 37°C. Each reaction mixture was then chilled in an ice bath and layered onto a 5-ml linear 5-25% (w/v) sucrose gradient containing 20 mM Tris-HCl, pH 7.5, 0.1 mM KCl, 5 mM MgCl2, and 5 mM 2-mercaptoethanol. After centrifugation for 82 min at 45,000 rpm at 2°C in a Beckman 50.1 rotor, 0.25-ml fractions were collected from the bottom of the tube, and 32P radioactivity was determined by counting in Aquasol (Du Pont-New England Nuclear) in a liquid scintillation spectrometer. Panel A represents three separate complete reaction mixtures as follows: •, λC14 cell extracts; □, λC16 cell extracts; ▲, λC17 cell extracts. Panel B represents complete system with induced uninfected E. coli extract. Panel C represents cell extract omitted. Panel D represents cell extract and 60 S ribosomal subunits omitted.

This conclusion is supported by (a) our inability to detect any immunoreactive polypeptide bands with a molecular weight higher than that of yeast eIF-5 (Fig. 2) and (b) the sequence data presented in the following section that the reading frame
of β-galactosidase is different from the open reading frame of yeast eIF-5. It has been well documented (Snyder et al., 1987) that when many yeast genes cloned in λgt11 vectors are expressed in E. coli, the reading frame of the yeast gene is translated using its own translation initiation codon but not as a fusion protein with β-galactosidase.

**Sequence Analysis of the Yeast TIF5 Gene**—Since all three positive clones contain the yeast gene that codes for eIF-5, we initially chose the λC14 clone containing the smallest yeast DNA insert (approximately 2.2 kb) for sequencing. The complete nucleotide sequence of the λC14 insert is presented in Fig. 6 (nucleotide positions −139 to +2053). We also characterized the λC16 clone that had a larger insert (approximately 2.8 kb). Restriction enzyme mapping and sequencing of the ends of the DNA inserts indicated that the λC16 clone contained the entire 2.192-kb yeast DNA insert present in the λC14 clone plus an additional 0.6-kb DNA sequence at one end, upstream of nucleotide position −139 of the λC14 insert presented in Fig. 6. This entire additional region was also sequenced completely on both strands and is presented in Fig. 6 (nucleotide positions −734 to −138).

The nucleotide sequence of the TIF5 gene presented in Fig. 6 lacks the conserved invariant sequence TACTAAC as well as the conserved 5' and 3' sequences present in all intron-containing yeast genes transcribed by RNA polymerase II (Langford et al., 1984). This suggests that the TIF5 gene has no intron and that the gene sequence can therefore be directly examined for an open reading frame. Analysis of the nucleotide sequence presented in Fig. 6 revealed an uninterrupted long open reading frame starting with a first in-frame ATG codon at position +1 and terminating with a TAG codon at position +1216. The open reading frame is preceded by an in-frame translation termination codon TAA at position −24, indicating that the ATG at +1, which satisfies the consensus rule for yeast translational start sites (Cigan and Donahue, 1987), is probably the true initiating ATG codon for yeast eIF-5. The open reading frame encodes a polypeptide of 405 amino acids with a predicted molecular mass of 45,346 daltons. This value is fairly close to the experimentally determined size of purified yeast eIF-5 (Chakravarti et al., 1993). To confirm that the open reading frame encodes yeast eIF-5, we determined the partial amino acid sequences of three peptides generated by digestion of purified yeast eIF-5 with cyanogen bromide (see “Experimental Procedures”). The NH2-terminal sequences of these three peptides (using the single-letter amino acid code) were: (a) PPIQAXVXGRG, (b) GXIERFXGLEHKXLI, and (c) RFGTKSXXKKVFKEV, where residue X indicates that no positive identification could be made. All three sequenced peptides were present in the predicted translated sequence of eIF-5 (Fig. 6), thereby confirming that the 2.192-kb fragment was indeed derived from the yeast gene TIF5 encoding eIF-5.

A closer examination of the open reading frame indicates a second in-frame ATG codon at position +52, which also satisfies the consensus rule for yeast translational start sites (Cigan and Donahue, 1987). The existence of two possible translational start sites for yeast eIF-5 suggests that TIF5 may encode two proteins with eIF-5 activity, a shorter one of 388 amino acids corresponding to a molecular mass of 43,500 daltons, and a longer one with 17 additional amino acids at the amino-terminal end corresponding to a molecular mass of 45,346 daltons. Such a possibility may explain why SDS-polyacrylamide gel electrophoresis of purified yeast eIF-5, isolated from yeast cells, exhibits two polypeptide bands differing in size by about 1,500-2,000 daltons (Chakravarti et al., 1993) (see also Fig. 2, panel e).

Analysis of the derived amino acid sequence of yeast eIF-5 revealed a high content of glutamic acid residues (39 residues; 9.6%) as well as aspartic acid residues (35 residues; 8.6%). eIF-5 is an acidic protein with a calculated pI of 4.82. eIF-5 is also relatively rich in lysine residues (37 residues; 9.16%). The stretch of 5 lysine residues (152-156) in yeast eIF-5 may be involved in the interaction of eIF-5 with mRNA, ribosomal
RNA, or initiator tRNA present in the 40 S initiation complex. Finally, comparison of the predicted amino acid sequence of yeast eIF-5 with protein sequences in the GenBank or EMBL data bank indicated no significant amino acid sequence homology to other known proteins. However, a portion of the antisense sequence of yeast eIF-5 exhibits about 60% homology to a 1017-bp rat brain cDNA clone of unknown function. This cDNA clone was isolated on the basis of its ability to hybridize with a 82-nucleotide-long brain-specific RNA, termed "identifier sequence" (Milner et al., 1984). The significance of this homology is not clear at present.

Characterization of eIF-5 mRNA by Northern Blot and Primer Extension Analysis—The size of the eIF-5 transcript(s) synthesized in yeast cells as well as the transcription initiation site(s) of eIF-5 mRNA were determined. Fig. 7
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Fig. 7. Analysis of eIF-5 mRNA. A, Northern blot analysis. Total RNA was isolated from haploid yeast cells, while poly(A)+ RNA was isolated by oligo(T)-cellulose column chromatography of total RNA as described under "Experimental Procedures." Poly(A)+ RNA (4 µg) (lane a) and total RNA (24 µg) (lane b) were electrophoresed in 15% agarose gel containing 6% formaldehyde. The separated RNA species were transferred onto a Nytran membrane and hybridized with a 32P-labeled 740-bp HhaI fragment of TIF5 (Fig. 6). The positions of a set of RNA size markers obtained from BRL, run in a parallel lane in the same gel, are indicated. B, primer extension analysis of eIF-5 mRNA. Total yeast RNA (50 µg) and a 32P-labeled oligonucleotide complementary to nucleotides +110 to +80 of TIF5 were used for primer extension reaction by reverse transcriptase, as described under "Experimental Procedures." The products were analyzed on a 6% denaturing sequencing gel (lane P). Molecular weight sequencing markers were run in parallel lanes using a M13 mp18 DNA template and M13 DNA universal primer (lanes GATC). The arrow shows the major product of primer extension reaction at nucleotide −253 of Fig. 6, indicated by an arrowhead. It is to be noted that in the absence of primer, no 32P-labeled cDNA product was observed (data not shown).

(panel A) shows that when the 740-bp HhaI restriction fragment derived from TIF5 (see Fig. 6, panel B) was radiolabeled with 32P and used as a probe for blot hybridization of electrophoretically separated yeast total and poly(A)+RNA, a single intense 32P-labeled band of mRNA corresponding to about 1.75 kb was observed. This mRNA size is sufficient to code for a protein of 45,346 daltons.

To map the transcription initiation site for eIF-5 mRNA, we carried out a reverse transcription reaction using yeast total RNA as a template and an oligonucleotide (complementary to nucleotide sequence +110 to +80 of TIF5, see Fig. 6) as a primer. The size of the cDNA synthesized under these conditions was determined by comparison with a DNA sequencing reaction run on the same gel using the appropriate oligonucleotide primer (Fig. 7, panel B). A 363-nucleotide-long cDNA was found to be the major reaction product under these conditions, indicating that the majority of the 5′ end of eIF-5 mRNA initiated at nucleotide position −253 (Figs. 6 and 7). However, in addition to this major product, a number of minor extended cDNA products were also observed indicating heterogeneity in the 5′ ends of eIF-5 mRNA. Many yeast genes including the gene for the translation initiation factor eIF-4E have multiple transcription initiation sites located downstream from the TATA elements resulting in the heterogeneity in the transcription start sites (Altmann et al., 1987; Guarente, 1987; Struhl, 1989). It should be noted that as a control, yeast poly(A)+ RNA was also reverse-transcribed using a primer, termed JW233, corresponding to the REB1 gene of the S. cerevisiae (Ju et al., 1990), and analyzed in same gel in a parallel lane (not shown). Under the conditions of our reverse transcription assay, a 366-nucleotide-long cDNA product was obtained as described by Ju et al. (1990). These results clearly show that the 5′ ends of eIF-5 mRNA map upstream of both in-frame methionine (ATG) codons.

Analysis of the transcriptional regulatory signals present at the 5′- and 3′-flanking sequences of the open reading frame revealed that the major transcription initiation site at −253 is preceded by TATA sequences at positions −320 and −283. Downstream from the translational termination codon TAG at position +1216, the sequence TAAG...TAGT...TTT was found starting at position +1273. A similar sequence has been proposed by Zaret and Sherman (1982) to be the consensus sequence for transcription termination of yeast genes by RNA polymerase II. The 3′-untranslated region also contains the sequence AATAAA starting at position +1438. Such a sequence has been implicated as a signal for poly(A) addition to many RNA polymerase II transcripts (Proudfoot, 1991).

From the positions of the transcription start and termination/poly(A) addition signals, an mRNA size of about 1.7 kb is predicted, which is in good agreement with the value obtained by the Northern blot analysis presented in Fig. 7.

TIF5 Is a Single Copy Gene Located on Chromosome XVI—The copy number of TIF5 was determined by Southern analysis. For this purpose, total genomic DNA isolated from haploid strain W303-a was digested separately with BamHI, EcoRI, and HindIII and then subjected to Southern analysis using the 740-bp-long HhaI fragment of TIF5 as a probe (Fig. 8, panel A). The appearance of a single 32P-labeled band with all three restriction digests indicates that either a single gene or, less likely, very closely linked genes encode eIF-5.

The chromosomal location of TIF5 gene was determined using contour-clamped homogeneous electric field (CHEF) gel electrophoresis (Gerring et al., 1991). After separating the chromosomal DNAs by CHEF, DNA blots were probed with the 32P-labeled 740-bp HhaI fragment of TIF5. As shown in Fig. 8B, when the yeast strain YPH149 was used as the source of DNA, 17 distinct bands were detected (lane EB) and TIF5 mapped to the band corresponding to chromosome XVI (lane AR). This assignment was confirmed by hybridizing the TIF5 probe to an ordered collection of λ vector-cloned yeast DNA fragments, generously provided by Drs. J. Warner, L. Riles, and M. Olson. A detailed analysis (data not shown) indicated that TIF5 maps on the right arm of chromosome XVI between HTS1 (histidinyl tRNA synthetase) and MAK3 (maintenance of killer) genetic markers.

TIF5 Gene Is Essential for Cell Growth—To determine whether TIF5 is essential for cell viability, we constructed a null allele of the TIF5 gene as shown schematically in Fig. 9A and described under "TIF5 Gene Disruption." A 285-bp BsmI/PstI fragment was deleted from the TIF5 gene present in the pGEM7zf (+) plasmid and replaced by a 1.16-kb HindIII fragment containing the yeast URA3 gene that served as a selectable marker. The resulting construct was digested with EcoRI and used to transform ura3 homozygous diploid yeast strain, W303;MATa/MATa), according to the single-step disruption protocol of Rothstein (1983). Stable Ura+ transformants were isolated, and the site of integration of the transforming DNA was determined by Southern analysis of DNA isolated from several transformants (Fig. 9, panel B). As expected, under these conditions, disruption of the TIF5 locus of only one chromosome by homologous recombination was achieved. One of the transformants was then sporulated...
The separated chromosomal DNAs were then transferred to a Nytran membrane filter and probed with a 32P-labeled HhaI fragment (Fig. 6) as a probe. Following hybridization, the blot was washed under increasingly stringent conditions (Sambrook et al., 1989) with the final wash in 0.1 × SSC and 0.1% SDS at 68°C. Panel B, electrophoretic karyotyping and mapping of TIF5. Chromosome-size yeast DNA (strain YPH149) was isolated in the form of DNA-agarose plugs as described by Rose et al. (1989). Separation of chromosomal DNAs was achieved in a 1% agarose CHEF gel in 0.5 × TBE buffer system as described by Gerring et al. (1991). Electrophoresis was performed initially at 200 V at 10°C for 15 h with 60-s pulse frequency, followed by 200 V at 10°C for 9 h with 90-s pulse frequency. Following completion of electrophoresis, the gel was stained with EtBr, destained, and then photographed. The separated chromosomal DNAs were then transferred to a Nytran membrane filter and probed with a 32P-labeled 740-bp HhaI fragment of TIF5 (Fig. 6). Lanes EB, ethidium bromide-stained gel. Lanes AR, autoradiogram of the blot probed with a 32P-labeled HhaI fragment.

at 25°C, and the resulting tetrads were dissected and spores were germinated at 30°C in a rich (YPD) medium. Only two of the four spores from each ascus were viable even after prolonged growth for 5 days (Fig. 9, panel C). Furthermore, all the viable spores yielded Ura+ colonies (data not shown). Since the URA3 gene is physically linked to the disrupted TIF5 gene in the transforming DNA, these results suggest that the lethal mutation is genetically linked to the disrupted chromosomal copy of the TIF5 gene. Taken together, these results indicate that TIF5 is a single copy gene that is essential for mitotic growth of yeast cells.

**DISCUSSION**

Several lines of evidence presented in this paper and summarized below indicate that we have cloned the complete structural gene encoding yeast eIF-5 that we have designated TIF5 (translation initiation factor 5). First, we demonstrated immunological cross-reaction of affinity-purified antibodies to peptides synthesized from the recombinant Agt11 clones with yeast eIF-5. Subsequently, it was shown that catalytically active eIF-5 was synthesized in E. coli cells containing the appropriate recombinant Agt11 clone. Western blot analysis of the bacterially produced eIF-5 using affinity-purified anti-yeast eIF-5 antibodies as probes showed that both the 54- and 56-kDa polypeptides were synthesized in E. coli. Finally, nucleotide sequence analysis of the cloned yeast gene revealed the presence of a long uninterrupted open reading frame that could encode a 405-amino acid-long polypeptide with a calculated molecular mass of 45,346 daltons. This value is close to the native molecular weight of purified eIF-5 isolated from
yeast cells. Furthermore, the amino acid sequence predicted from the open reading frame contained the partial NH2-terminal amino acid sequences of three CNBr peptides generated from purified yeast eIF-5. Interestingly, this open reading frame is preceded by an in-frame translation termination codon TAA at nucleotide position -24, confirming that the open reading frame is an independent reading frame that can code for eIF-5 and not part of a larger polypeptide. Thus, yeast eIF-5, as is its mammalian counterpart, is a monomeric protein that migrate on SDS gels with an apparent molecular mass of 54-56 kDa (Chakravarti et al., 1993). These results therefore provide additional evidence that earlier reports from other laboratories (Schreier et al., 1977; Benne et al., 1978; Merrick, 1992) that mammalian eIF-5 is a protein of 125-168 kDa probably reflected the presence of proteins unrelated to eIF-5 activity.

Although the molecular cloning data indicate that eIF-5 isolated from yeast and mammalian cells are similar in size, analysis of purified preparations of yeast eIF-5 by SDS-polyacrylamide gel electrophoresis showed only a single polypeptide band of 58 kDa. Although the yeast strain used was of the eIF-5 was deficient in a major protease activity and several protease inhibitors were added to buffers used during the isolation of yeast eIF-5, the possibility that the 54 kDa form was generated by a specific proteolytic cleavage of the message form could not be excluded. However, eIF-5 expressed from TIF5 in E. coli also exhibited one major and one minor immunoreactive polypeptide band with molecular mass of 54 kDa and 56 kDa, respectively (Fig. 2). The nucleotide sequence of TIF5 gene revealed the presence of an in-frame downstream AUG codon at position +52. Both ATGs (at +1 and at +52) occur in a sequence context that is a good match for the consensus (5'-A^A^AUAUCUC-3') associated with translational initiators in yeast (Cigan and Donahue, 1987). Initiation of translation from these two ATG codons would generate two proteins, one having 17 additional amino acid residues at the amino terminus than the other. A similar difference in size (1,846 daltons) was observed between the two forms of eIF-5 obtained either from yeast cell lysates or by expression of TIF5 in E. coli. These observations suggest that the two forms of eIF-5 may be produced by alternative usage of the two ATG codons at the 5' end of eIF-5 mRNA. We have also detected a third strongly immunoreactive polypeptide band with an apparent size of 38 kDa when λ lysogens carrying the yeast TIF5 gene were expressed in E. coli and the expressed proteins were analyzed by Western blotting (Fig. 2). However, this polypeptide band was not observed when eIF-5 isolated from yeast cell lysates was analyzed by Western blot. It is likely that the 38-kDa polypeptide was synthesized by bacterial ribosomes from an in-frame third AUG codon at nucleotide position +388, which is preceded by a strong "Shine-Dalgarno" ribosome binding sequence located at nucleotide positions +378 to +382. This could explain why the bacterially expressed yeast eIF-5 protein preparation contained the 38-kDa polypeptide while eIF-5 preparations isolated from yeast cells did not contain it.

It is not immediately apparent why yeast cells synthesize two forms of eIF-5. It is, however, well established that there exist a number of single copy nuclear genes of S. cerevisiae that code for functionally analogous proteins (isozymes), that are present in different sub-cellular compartments. A few examples of such yeast genes are MOD5 (Gillman et al., 1991), HTS1 (Natsoulis et al., 1986), LEU4 (Beltzer et al., 1988), TRM1 (Ellis et al., 1987), and SUC2 (Carlson and Botstein, 1982). Recent studies carried out in the above laboratories have demonstrated that all these genes, with the exception of MOD5, yield multiple mRNAs by initiating transcription at sites located upstream of, as well as between, the two potential translation initiation sites. Such a mechanism of transcription initiation produces two sets of messages, a long one with two in-frame AUGs and a short one containing only the downstream AUG. The longer message codes for a protein with an amino-terminal extension that contains the signal for targeting the protein to another cellular compartment, e.g. mitochondria, whereas the shorter message produces a shorter cytoplasmic protein lacking the amino-terminal signal sequence. In contrast to these genes, the MOD5 gene of S. cerevisiae, which codes for proteins involved in tRNA modification (Gillman et al., 1991), produces mRNAs that are all initiated upstream of the open reading frame of the gene. The MOD5 mRNAs contain two closely spaced in-frame translation initiation sites; initiation of translation from the first AUG codon produces the longer form of the protein that is targeted to mitochondria, whereas translation from the second in-frame AUG codon produces the shorter form of the protein that remains in the cytoplasm.

It is clear that if indeed the two forms of yeast eIF-5 are produced by alternative AUG selection, the situation will be similar to the translation of MOD5 mRNA. Because both the AUGs present in the open reading frame of the eIF-5 gene are in the proper sequence context, this raises the interesting question of whether both AUGs are actually used in the synthesis of the two forms of eIF-5 and, if so, whether the longer form of the protein with an amino-terminal extension is sorted to different subcellular compartments. A genetic approach (e.g. mutations of initiation codons, deletion and insertion mutagenesis at sites preceding and following each initiation codon) will be necessary to resolve this issue.

Examination of the putative amino acid sequence of yeast eIF-5 protein reveals regions with sequence similarities to proteins belonging to the GTPase superfamily (Bourne et al., 1991). Members of the GTPase superfamily include tralation initiation and elongation factors, IF-2, EF-Tu, EF-G, EF-1α, the α-subunit of G proteins, Ha-Ras, yeast proteins CDC42 and SEC4, and a large number of other GTP-binding proteins and GTPases. These proteins have characteristic sequence motifs in four distinct domains, designated G-1 through G-4, as shown in Table I. Such sequence similarities between the yeast eIF-5 and the proteins of the GTPase superfamily

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| Table I |

| Sequence homology of yeast eIF-5 with proteins of the GTPase superfamily |

Comparison of the sequences of yeast eIF-5 with the conserved sequence motifs in the GTPase superfamily (Bourne et al., 1991). The single-letter amino acid code is used. The numbers preceding the sequence motifs in eIF-5 represent the position of the first amino acid in each binding motif.

<table>
<thead>
<tr>
<th>Motifs</th>
<th>G-1</th>
<th>G-2</th>
<th>G-3</th>
<th>G-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consensus</td>
<td>G(X)XGK(S/T)</td>
<td>D-(X)-T</td>
<td>DXXG</td>
<td>(N/T)(K/Q)XD</td>
</tr>
<tr>
<td>Yeast eIF-5</td>
<td>r2GFRNGKKT34</td>
<td>pGpD-Xp-T3p7</td>
<td>p2GpDGTG106</td>
<td>p2GpTQLD140</td>
</tr>
</tbody>
</table>
superfamily (Table I) correlate well with the known GTP hydrolysis function of eIF-5 in the initiation of protein synthesis.

It is now well established that regulation of gene expression in eukaryotes occurs at the level of translation initiation (Hershey, 1991). The eIF-5-dependent 80 S initiation complex formation may be an important step in this overall regulatory process. In this connection, it should be mentioned that a large number of yeast mutants has recently been isolated that are defective in translation control of GCN4 biosynthesis (Foiani et al., 1991; Hinnebusch and Liebman, 1991). Some of these mutants, designated gcd mutants, have been shown to be defective in initiation factor genes. It would be particularly interesting to investigate whether some of these gcd mutations that lead to defects in ribosomal subunits joining (Hinnebusch and Liebman, 1991) map in the eIF-5 gene. The availability of such yeast strains defective in TIF5 should allow us to examine in more detail the function of eIF-5 in the regulation of initiation of protein synthesis.

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REFERENCES