Characterization of the p33 Subunit of Eukaryotic Translation Initiation Factor-3 from Saccharomyces cerevisiae*

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Eukaryotic translation initiation factor-3 (eIF3) is a large multisubunit complex that binds to the 40 S ribosomal subunit and promotes the binding of methionyl-tRNA, and mRNA. The molecular mechanism by which eIF3 exerts these functions is incompletely understood. We report here the cloning and characterization of TIF35, the Saccharomyces cerevisiae gene encoding the p33 subunit of eIF3. p33 is an essential protein of 30,501 Da that is required in vivo for initiation of protein synthesis. Glucose repression of TIF35 expressed from a GAL1 promoter results in depletion of both the p33 and p93 subunits. Expression of histidine-tagged p33 in yeast in combination with Ni\(^{2+}\) affinity chromatography allows the isolation of a complex containing the p135, p110, p90, p39, and p33 subunits of eIF3. The p33 subunit binds both mRNA and rRNA fragments due to an RNA recognition motif near its C terminus. Deletion of the C-terminal 71 amino acid residues causes loss of RNA binding, but expression of the truncated form as the sole source of p33 nevertheless supports the slow growth of yeast. These results indicate that the p33 subunit of eIF3 plays an important role in the initiation phase of protein synthesis and that its RNA-binding domain is required for optimal activity.

There are five major steps involved in initiation of eukaryotic protein synthesis: dissociation of ribosomes into 40 S and 60 S subunits, binding of Met-tRNA\(_\text{i}\), to the 40 S ribosomal subunit; binding of mRNA to the 40 S preinitiation complex; scanning and initiation codon recognition, and the joining of the 60 S subunit to the 40 S initiation complex (1, 2). The initiation phase is promoted by at least 10 soluble proteins known as eukaryotic initiation factors (eIFs).\(^1\) eIF3 is the largest and most complex initiation factor, comprising 10 or more subunits in mammalian cells (3) and up to eight subunits in yeast (4, 5). Several functions have been assigned to eIF3 in the translation initiation pathway in mammalian cells (1). It promotes dissociation of 80 S ribosomes into 40 S and 60 S subunits and stabilizes Met-tRNA\(_\text{i}\), binding to the 40 S ribosomal subunit.

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\(^{1}\) The abbreviations used are: eIFs, eukaryotic initiation factors; RRM, RNA recognition motif; PCR, polymerase chain reaction; kb, kilobase pair; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; RNP, ribonucleoprotein.

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and its role in protein synthesis were not addressed in the reports. We describe here the cloning of TIF35, characterization of the function of p33 in protein synthesis, and analysis of its RNA recognition motif (RRM). This completes the detailed characterization of the genes encoding eight elf3 subunits in yeast.

MATERIALS AND METHODS

Strains and Media—Escherichia coli strain XL1-Blue was used for plasmid propagation. The various Saccharomyces cerevisiae strains used in this work are based on strain W303-1A (MATa, leu2-3,112 his3-11,152 trp1-1 can1-100) or its isogenic diploid, W303-1B. Yeast cells were grown at 30 °C inYP or synthetic (S) medium supplemented with the relevant amino acids and 2% glucose (YPD or SD) or 2% galactose (YPG or SG) as described previously (21); growth was monitored by measuring absorbance at 600 nm (A600). Sporulation was carried out at room temperature on plates containing 0.3% potassium acetate, 0.02% raffinose, and 10 μg/ml each amino acid. Tetrad dissections and DNA transformations were carried out by standard procedures (22).

Cloning and Disruption of TIF35—The yeast gene encoding elf3-p33 was tentatively identified as a homolog of the gene for mammalian elf3-p44. The mouse elf3-p44 sequence (34) was used to conduct a TblastN search of the entire yeast genome sequence, and a putative protein was identified whose amino acid sequence exhibits 35% identity to mouse elf3-p44. The coding sequence, together with flanking regions, was amplified from total yeast genomic DNA by PCR. The upstream (5′-CTCTTACACGTTCGAAAAATCCACATT-3′) and downstream (5′-GCTTAATGTTGTTGGCCTTTATATGGCCC-3′) primers generate a single 2.1-kb DNA fragment. The fragment was gel-purified and subcloned into pNoTA (5 Prime, Inc., Boulder, CO) to create pNo-TIF35. Sequencing confirmed that the 2.1-kb insert contains an 825-base-pair open reading frame (ORF) with 607 and 674 base pairs of DNA flanking the 5′- and 3′-ends, respectively.

To disrupt the TIF35 gene, pNo-TIF35 was digested with BsaAI and BamHI to remove 91% of the TIF35 coding region, and a 1.7-kb BamHI DNA fragment containing HIS3 was inserted to generate pNoTA-ti55:HIS3. The upstream and downstream cloning primers described above were used to generate a 3.1-kb Ti55:HIS3 PCR fragment, which was transformed into the diploid yeast strain W303 to create a one-step gene deletion/disruption (23). One of the stable His+ transformants (PH33D-7) was selected, and the disruption of one of the TIF35 genes was confirmed by Southern blot analysis (data not shown).

Plasmid Constructions—pRS16-TIF35 was constructed by digesting pNo-TIF35 with BamHI and subcloning the resulting fragment into BamHI-cleaved pRS316 (American Type Culture Collection). p415Gal1-NH33 is a CEN4 LEU2 plasmid that allows expression of N-terminal His+ tagged proteins (26). p415Gal1-NH33 and p415Gals-NH33 were digested with AluI and SalI, respectively (24). p415Gals-NH33 was digested with BamHI and NcoI sites (underlined); the bases corresponding to the initiation codon of wild-type TIF35 are in boldface) and 5′-CCGGTCGACCTGGCACTATCTGGCATCTA-3′ (tagged with SalI and XhoI sites (underlined); the bases corresponding to the stop codon are in boldface). The resulting 0.9-kb DNA fragment was subcloned into pNoTA and sequenced, yielding pNo-NH33 (NH represents N terminus). The 0.9-kb fragment was subcloned into the BamHI/SalI sites of p415Gal1 and p415GalS, respectively (24). p415p33A (kindly provided by M.-H. Verlaic, University of California, San Francisco) (14) is identical to p415Gal1-NH33, except that the encoded p33 lacks the C-terminal 71 amino acids.

To express a recombinant form of His-tagged p33 in E. coli, pet-NH33 was constructed by inserting the NcoI/XhoI fragment from pNo-NH33 into the corresponding sites in pet28c (Novagen). To generate pet-NH33A33C, p415p33A33C was digested with BamHI and SalI, and the 0.7-kb fragment was subcloned into pet28c digested with BamHI/SalI. Construction of Plasmid Strains—Strains PH33, PH33L, and PH33-—Strains PH33 and PH33L was transformed with p415Gals-NH33, p415Gal1-NH33, or p415p33A33C, and transformants were selected on SD-His-Leu plates. The resulting transformants were sporulated, and their ascii were dissected. Two, three, and four viable spores were obtained on YPG plates and were streaked on SG-His-Leu plates to identify TIF35-disrupted cells carrying p415Gals-NH33, p415Gals-NH33, and p415p33A33C. The corresponding haploid strains were named PH33S, PH33H, and PH33L, respectively.

Antibodies—Rabbit polyclonal antibodies against yeast elf3 has been described previously (5). Rabbit anti-p33 antibody was a gift from A. G. Hinnenbush (National Institutes of Health), and affinity-purified rabbit anti-p33 antibody (14) was kindly provided by M.-H. Verlaic. To obtain anti-p33 antisera, rabbit antibodies were raised against purified His6-p33 (Babco). For affinity-purified anti-recombinant p33 antibodies, His6-p33 was overexpressed from pET-NH33 in E. coli BL21(DE3), affinity-purified by Ni2+ affinity chromatography (Novagen), and fractionated by SDS-PAGE, followed by transfer to a polyvinylidene difluoride membrane (Millipore Corp.). The anti-p33 antisera was incubated with a piece of the membrane containing His6-p33, and antibodies bound to His6-p33 were eluted with 0.2 ml of low-pH buffer (0.2 M glycine HCl and 1 mM EGTA, pH 2.5). The eluate was quickly neutralized with 0.2 ml of 100 mM Tris-HCl, pH 8.8, diluted with 1 volume of blotto (0.5% (w/v) nonfat dry milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.075% (v/v) Tween 20); and stored frozen at −80 °C. A second batch of affinity-purified anti-p33 antibodies was prepared similarly, but from the anti-elf3 antisera, and was used as indicated in the figure legends. Anti-p135 and anti-p110 antibodies were affinity-purified against recombinant His6-tagged p135 and p110 as described elsewhere (24).

Analysis of Polyome Profiles—Strains PH33S and W303-1A were grown in YPG medium at 30 °C to early log phase and shifted into YPD medium. Five, nine, and twelve hours after the shift to glucose, cycloheximide was added to a final concentration of 100 μM, followed by quick cooling of the cultures on ice. The cells were harvested by centrifugation and washed with buffer A (10 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM MgCl2, and 1 mM dithiothreitol) plus 100 μM cycloheximide. Cells were broken by vortexing with glass beads in lysis buffer (20 mM HEPEs, pH 7.5, 5 mM MgCl2, 150 mM KCl, 5% (v/v) glycerol, and 1× Complete™ protease inhibitors (Boehringer Mannheim), and cell lysates were clarified by centrifugation at 20,000 × g for 10 min at 4 °C. Aliquots (10 μg amounts) from each extract were fractionated on 15–45% sucrose gradients in buffer A by centrifugation at 38,000 rpm in a Beckman SW 40 rotor for 2.25 h at 4 °C. The gradients were analyzed by upward displacement, and A260 profiles were obtained with a density gradient fractionator and UV monitor (Isoch Model 185).

Measurement of Protein Synthesis Rates—Strains W303-1A and PH33S were grown overnight in SG complete minus methionine media. Cells were harvested, washed in water, and resuspended in the same medium complete minus methionine or SD complete minus methionine medium at a density of 0.05 A600. During incubation at 30 °C, cells corresponding to 1 A600 unit were withdrawn at the indicated time points, harvested, washed in buffer A, and resuspended in 300 μl of the same medium containing 100 μM of [35S]methionine (1× 106 cpm/ml). The cells were incubated at 30 °C for 5 min, followed by addition of 1 ml of a “stop buffer” containing 1.2 mg/ml nonradioactive methionine and 0.1 mg/ml cycloheximide. Cells in Stop buffer were kept on ice. Total cell pellet was washed with water and sonicated with cell beads, and proteins from the cleared lysate were precipitated with 10% trichloroacetic acid. The pellet was washed with acetone and dissolved in 200 μl of 1% SDS. The incorporation of [35S]methionine into total protein was determined by counting radioactivity in an aliquot of the SDS extract. The protein concentration of the SDS extract was determined by the micro-BCA protein assay reagent kit (Pierce) as described by the manufacturer. The rate of protein synthesis is expressed as cpm × min−1 × μg of protein−1.

Immolized Metal Affinity Chromatography—Cells harvested at an A600 of 0.9–1 were disrupted with glass beads in lysis buffer by eight 30-s pulses in a Bead Beater (BioSpec Products, Inc.). The lysate was centrifuged for 15 min at 12,000 × g at 4 °C, and the supernatant was centrifuged at 65,000 rpm in a Beckman TL100 rotor for 80 min at 4 °C. The ribosomal pellet was suspended in 500 mM KCl in lysis buffer and centrifuged as described above. The resulting ribosomal salt wash enriched in elf3 was incubated in batch for 1 h at 4 °C with 0.8 ml of His-bind™ resin (Novagen) equilibrated with binding buffer (20 mM Tris-HCl, pH 7.9, 10% (v/v) glycerol, 30 mM imidazole, and 500 mM NaCl). After pouring the resin into a column, unbound proteins were washed with 10 ml of wash buffer. The elf3 fraction was eluted with the same buffer containing 500 mM imidazole. Eluted fractions were analyzed by SDS-PAGE and Western immunoblotting.

Northwestern Blot Analysis—Plasmid pRIB-S1 (kindly provided by J. Warner, Albert Einstein University) carrying a copy of the yeast rDNA gene was used as template to amplify two fragments of yeast 18 S rDNA overlapping at the unique Scn1 site. rDNA nucleotides 1–1248 were

3 http://genome-www.stanford.edu/Saccharomyces/.
amplified with primers 5'-CCCTCGGATCTCAGTCAGGACGCGCAG-3' (introducing an XhoI site (underlined) upstream of the first rDNA nucleotide) and 5'-CCCGAATTCGAGCTCTCAATCTGT-3' (introducing a EcoRI site downstream of the SacI site in the rDNA). Membranes were then incubated for 20 min with 200,000 tRNA, and 200 units of ribonuclease inhibitor (Amersham Pharmacia Biotech). The 32P-labeled pSP18S-fl. pSP18S-fl and pSP18S-39 were cloned into pSP73 digested with EcoRI to generate pSP185S-39. pSP185S-39 was created by ligating the XhoIClal fragment from pNo18S-39 into XhoIClal-digested pSP73. Ligating a SacClIaI fragment from pNo18S-39 into the SacClIaI sites of pSP18S-39 resulted in pSP18S-fl and pSP18S-39 were ligandized with Clal and transcribed with T7 RNA polymerase to yield rRNA-(1–1800) and rRNA-(1243–1800), respectively. pSP18S-39 was digested with EcoRI and BstRI generated rRNA-(1–1248) and rRNA-(1248–1248), respectively. To synthesize rRNA-(263–1248), pSP18S-39 was digested with XhoI and BstBI, blunt-ended with Klenow DNA polymerase, religated, and digested with EcoRI prior to transcription. All T7 transcripts carry the sequence 5'-GGGAGCCGGCCGCCAG at the 5'-end of the rRNAs, corresponding to the pSP73 sequence following the transcription start site. The sequence was carried out in the presence of 50 μCi of [α-32P]UTP (800 Ci/mmol) with the T7/SP6 transcription MAXIscript kit (Ambion Inc.) according to the manufacturer’s recommendations. Unincorporated nucleotides were removed using MicroSpin S-200 HR columns (Amersham Pharmacia Biotech). The 32P-labeled 5.5-globin mRNA was prepared as described previously (25). Isotopically labeled transcripts were then analyzed on denaturing 4% (for long transcripts) or 5% (for shorter transcripts) polyacrylamide gels.

For Northern RNA binding experiments, purified His8-p33 (3 μg), His8-p33ΔC (3 μg), and yeast lysate (10 μg) were subjected to SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. The membranes were treated for 20 min with binding buffer containing 20 μmol HEPES-KOH, pH 7.5, 2 μmol Mg(OAc)2, 75 μmol KAc, 1 μmol EDTA, 1 μmol diithiothreitol, 0.2% (w/v) CHAPS, 1 mg/ml E. coli tRNA, and 200 units of ribonuclease inhibitor (Amersham Pharmacia Biotech). The membranes were then incubated for 20 min with 200,000 cpm/ml of the 32P-labeled 18 S rRNA or 39-globin transcripts in 8 ml of binding buffer. The blots were washed three times for 5 min each with binding buffer and subjected to autoradiography.

RESULTS

Cloning and Characterization of TIF35—When this work was initiated, the gene encoding the 33-kDa subunit of eIF3 had not been identified. An attempt to obtain partial amino acid sequences from tryptic digests of p33 from purified yeast eIF3 was not successful. However, at this time, partial amino acid sequence information was being developed in the laboratory (34) for one of the last subunits of mammalian eIF3 to be characterized, namely eIF3-p44. The mouse p44 amino acid sequence was used to search the yeast data base as described under “Materials and Methods,” and an ORF encoding a putative homolog of mammalian eIF3-p44 was identified. Since the yeast ORF appeared to encode a 30.5-kDa protein, we considered it to be a good candidate for the gene for yeast eIF3-p33, which we named TIF35. Evidence reported below and elsewhere (18) shows that the protein product of TIF35 is a 33-kDa protein that is present in a complex with other known eIF3 subunits, confirming that the gene encodes the p33 subunit. A 2.1-kb fragment of DNA containing TIF35 was amplified by PCR from yeast genomic DNA and cloned into the pNOTA vector to yield pNo-TIF35 as described under “Materials and Methods.” The 2.1-kb DNA contained ORF of 825 bp that encodes for a protein of 274 amino acid residues with a calculated mass of 30,501 Da. The sequence context of the first AUG in the ORF, AUAAUG (the initiation codon is underlined), resembles the yeast consensus context, A(A/U)AAUG (26). This AUG is preceded by an in-frame UAG termination codon, whereas the next in-frame AUG is found far downstream at codon 117. Thus, the first AUG very likely serves as the initiation codon. Hybridization of 32P-labeled DNA probes (derived from the coding region) to a single band of genomic DNA individually digested with four different restriction enzymes suggested the presence of a single gene locus (data not shown).

Sequence comparisons revealed amino acid sequence identities/similarities of 35.8/46.9, 33.1/42.5, 33.3/43.1, and 33.6/47.3% when yeast eIF3-p33 was compared with the corresponding homologous proteins from Schizosaccharomyces pombe (GenBank/EMBL accession number AB011882, human (U96074), mouse (AA109090, AA270800, and W18370), and Caenorhabditis elegans (Z50044; protein F22B5.2), respectively. When restricting the sequence comparison to the C-terminal 93-amino acid region of p33 that contains the RRM, identity/similarity values range between 43.7/56.3% for S. pombe and 36.4/47.7% for C. elegans. Therefore, eIF3-p33 appears to be present and moderately conserved in essentially all eukaryotic cells. In contrast, no homolog is found in Archaea sequences.

TIF35 Is an Essential Gene—To examine whether or not TIF35 is an essential gene, we constructed a diploid strain, PH33D-7, in which one of the TIF35 genes is nearly entirely deleted and is replaced by HIS3, as described under “Materials and Methods.” Tetrad analysis of PH33D-7 revealed that only two of the four spores in each of 30 asci formed colonies on rich medium (YPD), even after a long incubation at 30°C (data not shown). All viable spores were unable to grow on SD-His medium, suggesting that the phenotype of tif35::HIS3 is lethal. The segregation pattern of the tetrad spores (2:2:2:2) and the fact that no His+ segregants were found indicate that TIF35 is necessary for germination and/or cell viability.

To confirm that the lethal phenotype is due to disruption of TIF35, plasmid pRS316-TIF35, which expresses TIF35 from its own promoter on a centromeric plasmid carrying a URA3 marker gene, was constructed and transformed into PH33D-7. Ura+ transformants were selected; two were sporulated; and the resulting asc i were dissected. Two, three, or four viable spore colonies per ascus were obtained (data not shown). Only one spore from the three viable tetrads or two from the four viable tetrads grew on SD-His plates, and all His+ spore colonies were also Ura+. Thus, spores containing the tif35::HIS3 allele must harbor the URA3 plasmid, which carries TIF35. The results show that TIF35 is the only gene affected and that the disruption is complemented by the cloned gene.

p33 Is Present in a Complex with Other eIF3 Subunits—To obtain further evidence that TIF35 encodes a subunit of eIF3, we fused six histidine residues to the N terminus of p33 to create His8-p33. Strain PHL33, expressing His8-p33 as the sole source of this subunit, grows at the wild-type rate, indicating that the histidine tag is not deleterious to p33 function. Ribosomal salt wash fractions were prepared from strains expressing the wild-type (strain W303-1A) and histidine-tagged (strain PHL33) forms of p33, and the preparations were fractionated on Ni2+-affinity columns as described under “Materials and Methods.” Bound proteins were eluted with 500 mM imidazole and analyzed by SDS-PAGE and Western immunoblotting (Fig. 1). When the blot was analyzed with antiseraum to eIF3, no p33 or other eIF3 subunit was detected in the eluted fraction prepared from the strain expressing the wild-type form of p33 (W303-1A). However, with histidine-tagged p33 (strain PHL33), His8-p33 and numerous other eIF3 subunits were detected. Bands with mobilities corresponding to p135, p110, p90, p39, and p33 were readily identified. To confirm that these bands correspond to true eIF3 subunits, the blot was probed with antibodies affinity-purified against recombinant p135,
p110, p39, and p33 and with an antiserum highly specific for Prt1p (p90). Each of these proteins was found bound to the Ni\textsuperscript{2+} affinity column only when p33 was histidine-tagged. We do not have antisera to the Nip1p (p93), Gcd10p (p62), and Sui1p (p16) subunits of eIF3, so we could not determine whether or not these proteins were present as well. However, it is apparent that p33 is present in complexes that contain most if not all of the putative eight subunits of eIF3.

Effect of TIF35 Depletion on Cell Growth and eIF3 Subunit Levels—To investigate the function of eIF3-p33 in vivo, we depleted yeast cells of endogenous p33 and measured the effect of such depletion on cell growth and eIF3 subunit levels. As described under "Materials and Methods," we placed the TIF35 ORF under the glucose-repressible GAL1 promoter, an attenuated form of the GAL1 promoter (24). The strain carrying this construct as the sole source of TIF35, called PHS33, was grown in galactose-containing medium to early exponential growth phase and then transferred to glucose medium to turn off transcription of TIF35 from the GAL1 promoter. PHS33 in liquid cultures containing galactose as the carbon source grew with a doubling time of 90 min, comparable to that of the parental strain, W303-1A (Fig. 2B). When shifted to glucose-containing medium, strain W303-1A (in which p33 expression occurs from its own promoter) grew with a doubling time of ~90 min (Fig. 2A). In contrast, the growth rate of PHS33 began to decrease after about four generations (6 h), and the apparent growth rate was drastically reduced after 12 h (Fig. 2A). The very slow rate of growth seen thereafter may be due to extremely low levels of expression of TIF35 resulting from incomplete repression of transcription by glucose. The near-cessation of growth is consistent with an essential role for eIF3-p33.

The levels of p33 and other eIF3 subunits in lysates prepared from W303-1A and PHS33 cells were determined at 5, 9, and 12 h after shift to glucose medium. Equal amounts of whole cell lysates were subjected to SDS-PAGE and Western immunoblotting with anti-eIF3 antibodies (Fig. 3, upper panel). The level of p33 in cell lysates prepared from the W303-1A strain remained constant up to 12 h (lane 8). In contrast, the level of p33 in strain PHS33 was greatly diminished after 9 h (lane 3), and the protein was nearly undetectable at 12 h (lane 4). The experiments were performed at least five times, each time resulting in a depletion of only p33 and p39. The results suggest an important role of
p33 in stabilizing the p39 subunit in the cell. The observation is consistent with our earlier results (14) and the results of Asano et al. (19) that demonstrate that p39 interacts with p33 in the yeast two-hybrid assay and that overexpression of p33 suppresses the slow growth phenotype of tif34-ts mutants. These observations support the view that p33 is physically associated with p39 in the eIF3 complex.

p33 Depletion Inhibits Protein Synthesis in Vivo—The observation that TIF35 is an essential gene that encodes the p33 subunit of eIF3 suggests that p33 is required for initiation of translation. To test this idea, we first analyzed the effect of p33 depletion on protein synthesis in vivo. The rate of protein synthesis in strain PHS33 was measured by pulse-labeling cells for 5 min at various times following the shift from galactose- to glucose-containing medium (Fig. 4). The data demonstrate that ribosomes run off mRNAs following depletion of p33. Such behavior is indicative of a severe decrease in the rate of translation initiation.

Fig. 4. Inhibition of protein synthesis in p33-depleted cells. W303-1A cells grown in YPD medium (○), PHS33 cells grown in YPG medium (△), and PHS33 cells shifted from YPG to YPD medium (□) were subjected to a 5-min pulse labeling at the indicated times as described under “Materials and Methods.” The rate of [35S]methionine incorporation into protein was calculated as cpm × min⁻¹ × μg of protein⁻¹.

Methods.” PHS33 cells harvested 5 h after the shift to glucose showed large polysomes (Fig. 5A), consistent with little or no inhibition of protein synthesis rates at this time (Fig. 4). The gradient profiles from p33-depleted PHS33 cells analyzed at 9 and 12 h (Fig. 5, B and C) showed a marked reduction in the amount of large polysomes, with a proportionate increase in the amount of 80 S ribosomes when compared with parental W303-1A cells (Fig. 5D). The percentage of ribosomes remaining in polysomes in the p33-depleted cells was ~10–15% compared with 65% in non-depleted W303-1A cells, consistent with the ~6-fold inhibition of protein synthesis observed at those times (Fig. 4). The data demonstrate that ribosomes run off mRNAs following depletion of p33. Such behavior is indicative of a severe decrease in the rate of translation initiation.

eIF3-p33 Is An RNA-binding Subunit—One function of the eIF3 complex is to promote the binding of mRNA to the 40 S ribosomal subunit (27, 28). Of the eight subunits of yeast eIF3, three contain RNA-binding motifs in their sequences: p90, p62, and p33. p62 (Gcd10p) binds RNA strongly when assayed by Northernwestern analysis (29); however, RNA binding to these subunits was not detected by Northernwestern blotting (10, 21). To assess the RNA-binding ability of p33, purified His₆-p33 was subjected to Northernwestern analysis with radiolabeled 18 S rRNA (Fig. 6B, lane 2) or β-globin mRNA (Fig. 6C, lane 5). The protein bound either type of RNA, suggesting a lack of specificity (but see “Discussion” below). The possibility that p33 binds a specific region of the 18 S rRNA was examined by Northernwestern blotting with 32P-labeled frag-
ments of 18S rRNA (Fig. 6, A and C). All fragments bound, again suggesting little or no specificity. The role of the RRM in RNA binding was examined by testing purified recombinant His<sub>6</sub>-p33. No binding to 18S rRNA was detected (Fig. 6B, lane 3). The results demonstrate that p33 has nonspecific RNA-binding activity and that its C-terminal RRM is essential for the RNA-binding function.

The C-terminal RNA-binding Domain of p33 Is Not Essential for Cell Growth—To assess whether or not the RRM of p33 is essential, the C-terminal 71 amino acid residues were deleted, thereby removing the RNP-1 element and most of the RRM (14). p415p33ΔC, which expresses the C-terminal truncated p33 (p33ΔC) from a GAL1 promoter, was transformed into strain PH33D-7, and transformants were sporulated and analyzed by tetrad dissection. From each ascus, two fast growing haploid cells plus none, one, or two very slow growing cells were obtained in galactose-containing medium (Fig. 7A). The slow growing haploid cells were His<sup>−</sup>, indicative of carrying the disrupted tif35::HIS3 gene on a chromosome, and Leu<sup>−</sup>, due to the presence of p415p33ΔC (Fig. 7B). One of the haploid slow growing colonies was selected and called PH133. Strain PH133 did not grow on glucose-containing medium, consistent with the plasmid being the only source of TIF35 expression. It exhibited a doubling time of 6 h in galactose-containing medium, thereby suggesting that the RRM is not absolutely essential when p33ΔC is expressed from a GAL1 promoter. The slow growth phenotype could be caused either by a reduced specific activity or by a reduced level of p33ΔC. To distinguish between these possibilities, the cellular levels of p33ΔC and p33 were measured by Western immunoblotting of lysates derived from strain PH133 and the parental strain, W303-1A (Fig. 7C). The band corresponding to full-length p33 was absent in PH133, and instead a strong band with the expected apparent molecular mass of 25 kDa was seen that was absent in W303-1A. The intensity of the p33ΔC band in PH133 is comparable to that of p33 in W303-1A, indicating that p33ΔC is stable and accumulates to wild-type levels. We conclude that the slow growth phenotype is caused by an appreciable loss of p33 activity due to the removal of its RRM.

DISCUSSION

Yeast eIF3 was purified in this laboratory based on its activity in an eIF3-dependent mammalian assay for the synthesis of methionyl-tRNA
transcripts were prepared with T7 RNA polymerase as described under “Materials and Methods.” The numbers on the right describe the range of nucleotides in the transcript. A, upper panel: Northwestern blotting of His<sub>6</sub>-p33 and His<sub>6</sub>-p33ΔC. His<sub>6</sub>-p33 and His<sub>6</sub>-p33ΔC were produced in E. coli BL21(DE3) and purified as described under “Materials and Methods.” Purified recombinant (rc) proteins (2–5 μg) and 20 μg of yeast lysate were subjected to 7.5% SDS-PAGE, followed by blotting onto a polyvinylidene difluoride membrane and probing with [32P]-labeled 18S rRNA (1–1800) as described under “Materials and Methods.” Lower panel: Coomassie Blue staining of recombinant His<sub>6</sub>-p33 and His<sub>6</sub>-p33ΔC in the blot above. The migration positions of full-length and truncated recombinant tagged p33 proteins are shown; only a portion of the blot is shown. C, Northwestern blotting of His<sub>6</sub>-p33 (2–3 μg) with truncated forms of 18S rRNA and β-globin mRNA. Only the portions of the blots containing tagged p33 are shown.

The cloning of TIF35 was based initially on its homology (33% sequence identity and 43% similarity) to mouse or human eIF3-p44. Further evidence that the putative cloned gene actually encodes the p33 subunit of eIF3 follows. 1) TIF35 encodes a protein with a calculated mass of 30.5 kDa, close to the apparent mass of the 33-kDa subunit of eIF3. It should be noted, however, that the apparent mass of this protein depends on the gel system used, and values range from 32 to 36 kDa. 2) Antibodies raised against the product of TIF35 expressed in E. coli react with the p33 subunit in purified eIF3 preparations. 3) By using the yeast two-hybrid system and co-immunoprecipitations, p33 was shown to interact with a previously characterized subunit of eIF3, namely p39 (14, 19). 4) When p33 tagged with His<sub>6</sub> is expressed in yeast as the sole source of p33,
a complex is isolated by Ni²⁺ affinity chromatography that contains numerous other subunits of eIF3, namely p135, p110, p90, and p39. 5) The TIF35 gene product was identified by mass spectroscopy in a complex with p110, p93, His₆-p90, and p39, which was isolated by Ni²⁺ affinity chromatography (18). 6) Depletion of p33 results in lowered levels of p39. 7) Finally, overexpression of p33 from TIF35 suppresses the temperature-sensitive phenotype of mutant forms of p39 (14, 19). These findings provide convincing evidence that p33 expressed from TIF35 is a subunit of yeast eIF3.

Deletion/disruption of TIF35 is lethal and, together with depletion experiments, shows that p33 is required for cell growth. Depletion also causes an inhibition of the initiation phase of protein synthesis as deduced from polysome profiles. This is consistent with p33 being a subunit of eIF3, and comparable effects on protein synthesis are seen when other eIF3 subunits are depleted or inactivated. However, a precise role for p33 in initiation cannot be determined from these experiments. Because p39 is depleted along with p33, the loss of eIF3 function could be due to the lack of p39, rather than p33 itself. The availability of cloned TIF35 should expedite the isolation of mutant forms of p33 that may shed light on its function.

One of the possible functions of p33 is to bind RNA. Possible targets are the 18 S rRNA in the 40 S ribosomal subunit, mRNA, and Met-tRNAᵢ residing in the ternary complex with eIF2 and GTP. Northwestern blotting was used to examine binding to radiolabeled 18 S rRNA and β-globin mRNA. Because p33 binds to all fragments of 18 S rRNA and to mRNA, it appears that the RNA-binding activity is nonspecific for both sequence and structure. The human homolog, eIF3-p44, also binds RNA nonspecifically (34). An important caveat is that there could be significant differences in RNA-binding affinities between the different RNA probes that are not detected by the method employed. Deletion of most of the C-terminal RRM results in loss of RNA binding as determined by Northwestern blotting. It is surprising, however, that the C-truncated protein, p33ΔC, nevertheless supports growth (albeit slow) of yeast as the sole source of the p33 subunit. Therefore, the RRM does not perform an essential function, but clearly is required for optimal activity of the protein.

For further insight into the RNA-binding properties of yeast eIF3-p33, a comparison was made of the RRM sequences of four other p33 homologs derived from S. pombe, C. elegans, Homo sapiens, and Mus musculus (Fig. 8). The RRM regions of p33 from these species share sequence identities/similarities with the RRM of S. cerevisiae p33 of 41/63, 32/53, 39/55, and 40/56%, respectively. In particular, each of the four putative β-sheet structures appears well conserved, suggesting common functions. The RRMs of the five p33 homologs also are similar to the...
RRM in the spliceosomal protein U1A (Fig. 8). Structural data for a U1A-RNA interaction are available from crystallographic (30) and NMR (31) studies. The U1A RRM contains a four-stranded b-sheet that is stabilized by two a-helices. The singlestranded loop of the RNA binds to the surface formed by the b-sheets, interacting mainly with b-sheets 1 and 3. Most amino acids required to form the hydrophobic core of the U1A RRM (Leu-17, Leu-26, Phe-34, Phe-59, and Ala-68) are also conserved in p33 and its homologs (residues denoted by solid squares in the figure). Half of the residues implicated in U1A binding to RNA also are conserved in p33 (solid circles), whereas half are not (open circles), especially those in b-sheet 4. A striking difference corresponds to Tyr-13 in b-sheet 1 of U1A, which is a basic amino acid residue in all of the p33 homologs. In U1A, Tyr-13 stacks with a C base in the RNA, whereas in p33, this interaction presumably is replaced by ionic interactions. Although b-sheets 2 and 4 differ in sequence from the corresponding regions in U1A, b-sheet 2 in particular contains numerous basic residues that may contribute to RNA binding. The sequence similarities and differences between U1A and the p33 homologs suggest that slightly different modes of RNA binding may be involved that may contribute to different binding specificities.

Work from several laboratories has identified eight possible subunits in yeast eIF3: p135, p110, p93, p90, p62, p39, p33, and p16. Evidence for the presence of p135 and p110 in eIF3 will be published elsewhere.2 The p33 subunit, encoded by NIP1, has been seen in a complex with p110, p90, p39, and p33 obtained by Ni2+ affinity chromatography with His6-tagged p90 (18). As reported elsewhere, p90, p62, p39, and p16 are present in the various immunoprecipitates of eIF3 formed by antibodies to these proteins (5, 8, 10, 11). The sum of the evidence therefore indicates that a complex of eight subunits exists in yeast.

We show here that His6-tagged p33 allows the isolation of a complex in which p135, p110, p90, and p39 are positively identified with affinity-purified antibodies. Lacking antibodies to p135 and p33, as shown by two-hybrid analyses (14, 19). Work is in progress to further elucidate the structure of eIF3 by employing a number of methods that detect protein-protein interactions. The characterization of TIF35 contributes to these studies. We anticipate that further detailed structural studies of eIF3 will help to elucidate the function of this key initiation factor.

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