A 110-Kilodalton Subunit of Translation Initiation Factor eIF3 and an Associated 135-kilodalton Protein Are Encoded by the Saccharomyces cerevisiae TIF32 and TIF31 Genes*

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Translation initiation factor eIF3 is a multisubunit protein complex required for initiation of protein biosynthesis in eukaryotic cells. The complex promotes ribosome dissociation, the binding of the initiator methionyl-tRNA to the 40 S ribosomal subunit, and mRNA recruitment to the ribosome. In the yeast Saccharomyces cerevisiae eIF3 comprises up to 8 subunits. Using partial peptide sequences generated from proteins in purified eIF3, we cloned the TIF31 and TIF32 genes encoding 135- (p135) and 110-kDa (p110) proteins. Deletion/disruption of TIF31 results in no change in growth rate, whereas deletion of TIF32 is lethal. Depletion of p110 causes a severe reduction in cell growth and protein synthesis rates as well as runoff of ribosomes from polysomes, indicative of inhibition of the initiation phase. In addition, p110 depletion leads to p90 co-depletion, whereas other eIF3 subunit levels are not affected. Immunoprecipitation or nickel affinity chromatography where other eIF3 subunit levels are not affected. Immunoprecipitation or nickel affinity chromatography where other eIF3 subunit levels are not affected. Immunoprecipitation or nickel affinity chromatography where other eIF3 subunit levels are not affected. Immunoprecipitation or nickel affinity chromatography where other eIF3 subunit levels are not affected.

The initiation phase of protein synthesis in eukaryotes is promoted by 10 or more proteins called eukaryotic initiation factors (eIFs)‡ (for reviews, see Refs. 1 and 2). The largest and most complex of these, eIF3, is a 600-kDa factor with 8 or more subunit proteins. Based on in vitro biochemical studies of the mammalian system, eIF3 is implicated in a large number of reactions in the initiation pathway. eIF3 alone among the initiation factors binds stably to 40 S ribosomal subunits (3). The factor promotes the dissociation of 80 S ribosomes into 40 S and 60 S subunits, affects the stability of the ternary complex comprising methionyl-tRNA-eIF2-GTP in the absence of ribosomes but in the presence of mRNA, and stabilizes methionyl-tRNA binding to 40 S subunits (1). It also is absolutely required for mRNA binding to ribosomes, where eIF3 already bound to the 40 S ribosome interacts with a region of eIF4G, a component of the mRNA m7G-cap binding complex, eIF4F (4). Thus eIF3 acts as a bridge between the 40 S ribosome and the mRNA-eIF4F complex. It is apparent that eIF3 plays a central role in initiation by interacting with numerous other translational components.

To better understand the function of eIF3, the cDNAs encoding 11 human eIF3 subunits have been cloned and characterized: p170 (5), p116 (6), p110 (7), p66 (8), p48 (9), p47 (8), p44 (10), p40 (8), p36 (7), p35 (10), and p28.‡ Knowledge of the primary sequences of these proteins sheds light on their possible functions. Three of the human subunits contain RNA-binding motifs (p116, p66, and p44), and the gene for p48 (Int-6) is a frequent site of integration by the mouse mammary tumor virus, possibly implicating p48 in the regulation of eIF3 activity (9). Thus, the cloned cDNAs provide insights into subunit functions and tools for the study of the structure of eIF3 and its interactions with other translational components.

To elucidate the function of eIF3 during the initiation phase, we turned our attention to the budding yeast, Saccharomyces cerevisiae, where genetic approaches and gene replacement strategies are well developed. The other initiation factors from mammalian and yeast cells are quite strongly conserved, ranging from 17 to 72% sequence identity (1). Thus information obtained about yeast eIF3 may be applicable to understanding the structure/function of human eIF3. A yeast homolog of mammalian eIF3 was isolated on the basis of its activity in an in vitro methionyl-puromycin synthesis assay constructed with purified mammalian components of initiation (11). The yeast eIF3 preparation contains proteins with masses of 135, 90, 62, 39, 33, 29, 21, and 16 kDa. The genes encoding p90 (PRT1), p62 (GCD10), and p16 (SUI1) had been identified previously, and were subsequently shown to encode eIF3 subunits (11–13). The genes encoding the p39 (TIF34) and p33 (TIF35) subunits have been cloned and characterized more recently (14, 15). The p29 subunit was shown to be a degradation product of p39 (14), whereas a protein of 93 kDa has been identified as an eIF3 subunit (p93) encoded by NIP1 (16, 17). Nip1p (p93) is not present in our preparation described above, apparently due to proteolysis during purification. In this report, we describe the cloning of TIF31 encoding p135 and show that the 21-kDa protein is derived from a 110-kDa subunit encoded by TIF32.

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1 The abbreviations used are: eIF, eukaryotic initiation factor; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; IMAC, immobilized metal affinity chromatography; kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction.

2 G. L. Mayeou and J. W. B. Hershey, unpublished results.

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TIF31 and TIF32 Encode Yeast eIF3-p135 and -110

| Strain          | Genotype                                      | Source/reference |
|-----------------|-----------------------------------------------|------------------|
| W303            | MATα/MATα leu2–3,112/leu2–3,112 his3–11,15/his3–11,15 ade2–1/ade2–1 trp1–1/trp1–1 can1–100/can1–100 ura3–1/ura3–1 | Ref. 43          |
| W303–1A         | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | Ref. 43          |
| YAS538          | prb1Δ prb1C1 ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 pep4–4 HIS3 | A. Sachs         |
| HV110–2         | MATα/prb1Δ prb1C1 ade2–1 his3–11,15 leu2–3,112 trp1–1 ura3–1 | This work        |
| HV110–13        | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |
| HV110–24        | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |
| HV110–29        | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |
| HV110–33        | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |
| HV110–42        | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |
| PH533           | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | Ref. 15          |
| PH1–39a         | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |
| PH135D-8        | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |
| PH135H-B        | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |
| PH1–135         | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |
| PH1–414         | MATα leu2–3,112 his3–11,15 ade2–1 trp1–1 can1–100 ura3–1 | This work        |

Thus, 8 proteins associated with yeast eIF3 have been cloned and characterized: p135, p110, p93, p90, p62, p39, p33, and p16. Interactions of p135, p110, and eIF4B with other eIF3 subunits are also defined. The report thereby completes the detailed characterization of yeast genes encoding known eIF3 components and employs the genes in studies designed to define the subunit composition and structure of eIF3.

**MATERIALS AND METHODS**

*Strains, Cell Growth, and General Procedures*—The strains of *S. cerevisiae* employed in this study are listed in Table I. Yeast cells were grown at 30 °C in yeast-peptone (YP) medium or in synthetic complete (SC) medium lacking the indicated amino acids and were supplemented with 2% glucose (D) or galactose (G) (18). Cell growth was monitored as optical density at 600 nm (OD600). Yeast cells were sporulated on 2% agar plates with 0.3% potassium acetate, 0.02% raffinose, and 10 mg/ml of each amino acid as sporulation medium, and tetrad analyses were carried out as described (19). Yeast chromosomal DNA and RNA were prepared and transformations performed according to Rose et al. (18). *Escherichia coli* XL-1 blue was used for all plasmid cloning procedures and the recombinant *Trx*-p110 fusion protein was expressed in *E. coli* AD494(DE3) (Novagen). Plasmid preparations, recombinant DNA techniques, and Northern and Southern blotting were performed as described (20, 21).

Cloning and Chromosomal Disruption of TIF31 and TIF32—eIF3 was purified as described previously (11) and was fractionated by SDS-PAGE. Internal peptide sequences for the p135 and p21 subunits were determined in the Protein Structure Laboratory (University of California, Davis) as described (22). The 5 peptide sequences from p135 and the two from p21 are reported in Table II and were used to perform a TblastN search of the GenBank database.

The five p135 peptide sequences match 51 out of 55 residues with a putative protein encoded by cosmid clone c8270 (American Type Cell Culture). The 7.2-kb Ape I and Xho I fragment (GenBank accession number AF004911) was ligated into the same sites of pBluescript KSII (Stratagene) to yield pKS(Cos)TIF31. For disruption of the open reading frame (ORF), tentatively called TIF31 (for translation initiation factor 3, 1st subunit), the gene was PCR-amplified from total yeast genomic DNA to generate a 4.6-kb DNA fragment that includes 519- and 257-bp flanking the 5′ and 3′ ends of the ORF. The PCR product was cloned into the *Sma*I site of pBluescript KSII to yield pKS-TIF31. pKS-TIF31 was digested with *Bsr*BI and *Bgl*II to remove 98% of the TIF31 coding region and a 1.7-kb *Bam*HI DNA fragment containing the HIS3 gene was inserted (see Fig. 1). The resulting plasmid, named pKS-TIF31::HIS3, was digested with *Sac*I and *Asel*I to release a 2.5-kb DNA fragment containing the *TIF31::HIS3* allele. The fragment was transformed into the diploid yeast strain W303 to create a one-step gene deletion/disruption (23), confirmed by Southern blot analysis, to yield PH135D-8. PH135D-8 was sporulated, tetrads were dissected, and a His− haploid spore colony containing *tj31::HIS3* was selected and named PH135H-B.

For the cloning of the p110 gene (*TIF32*), the two p21 peptide sequences (Table II) matched two regions of a putative 110-kDa protein derived from the yeast genome data base (ORF YBR079c). To obtain TIF32 DNA, yeast genomic DNA (200 μg) was digested with *Nhe*I and *Pst*I, subcloned into pBluescript KS (Stratagene) and transformed into *E. coli* XL1-blue. Colonies containing the desired plasmid were identified by hybridization with a labeled 3.9-kb PCR product amplified from genomic DNA. One of the plasmids carrying TIF32 was named pHV110-1 and the sequence of the 4565-bp insert was determined and submitted to GenBank under accession number AF004912. A *Sac*I/Asgp 178I fragment from pHV110-1 carrying the putative TIF32 gene was subcloned into the same sites of the centromeric URA3 plasmid pRS316 (24) to generate pHV110-2.

To disrupt a chromosomal TIF32 gene by a one-step gene replacement, the 3.9-kb PCR product described above, which includes 557 and
462 bp of DNA flanking the 5’ and 3’ ends of the TIF32 coding region, respectively, was inserted into the Small site of pbScript KS’ to generate pKSp110. The TIF32 coding region was deleted by digestion with Styl and BspMI (see Fig. 1) and the 1.7-kb BamHI fragment carrying the HIS3 gene was ligated into form pHV110-9. The TIF31-HIS3 allelic replacement of pHV110-3 with AclI and PacI and the resulting 2.3-kb DNA fragment was transformed into the diploid yeast strain W303. One of the His- transformants was selected and named HV110-D20/D. The correct disruption of one of the TIF32 genes was confirmed by Southern blot analysis (not shown).

Construction of Plasmids Expressing TIF31—p414GaINH135, a CEN plasmid which allows expression of the N-terminal (His)6-tagged p135 under a GAL1 promoter, was constructed as follows. The p135 coding sequence (3.8 kb) was PCR amplified from pKSCos-TIF31 and subcloned into pNOTA (5 Prime — 3 Prime Inc., Boulder, CO) to generate pNo-NH135F. A BamHI fragment from pNo-NH135F was subcloned into the BamHI site of p414Ga1L (25) to generate p414Ga1L-NH135. Transformation of PH135H-B with this plasmid yielded PH1-135. An N-terminal-(His)6-tagged 151-amino acid fragment of p135 was PCR amplified and cloned into pET28c to yield pET-NH135. Affinity purification of eIF3—The resulting His tag on eIF3 was determined (27) and adjusted with buffer C350 containing 350 mM KCl) to 2 mg/ml. Lysates (500 μl of Protein A/G-agarose beads loaded with anti-(His)6 antibody (CLONTECH) or anti-c-Myc antibody 9E10 (Santa Cruz) were incubated with 1 μg of probe, but 1 μg of Protein A/G-agarose beads loaded with anti-(His) antibody (CLONTECH) or anti-c-Myc antibody 9E10 (Santa Cruz Biotechnology). Beads were washed 3 times with 500 μl of buffer C350 and resuspended in SDS-PAGE sample buffer.

RESULTS

Identification and Cloning of TIF31 and TIF32 Encoding eIF3—To complete the identification of genes encoding possible subunits of eIF3, we report the cloning and characterization of TIF31 and TIF32. Each of these genes was cloned by obtaining partial peptide sequences derived from the 135- and 21-kDa proteins present in our preparations of eIF3. The sequences reported in Table II were used to search yeast data bases, an open reading frame was identified that encodes each set of peptides, and the genes were cloned as described under “Materials and Methods.” pKSCos-TIF31 carries an insert of a 3834-bp coding region (assuming the first AUG as the initiator codon), with 1752 and 1638 bp of DNA flanking the 5’ and 3’ regions, respectively. An in-frame stop codon exists 21 nucleotides upstream of the first AUG, ruling out initiation sites upstream. However, AUGs found 62 and 88 codons downstream of the first AUG possibly may serve as initiator codons. The absence of a strict consensus sequence surrounding the initiator AUG in yeast (28) does not allow a firm prediction of which AUG is actually used. The hypothetical p135 product of TIF31 translated from the first AUG contains 1277 amino acid residues with a calculated mass of 145,165 Da and a pI of 6.0. A search of data bases indicates that p135 has no mammalian homolog (but see “Discussion” below).

For p110, plasmid pHV110-1 was constructed as described under “Materials and Methods” which contains a 2892 bp ORF with 920 and 748 bp of 5’- and 3’-flanking DNA, respectively. The first AUG is preceded by an in-frame stop signal 16 codons upstream, whereas the second in-frame AUG is 169 codons downstream. TIF32 mRNA translated from the first AUG generates a protein with 965 amino acid residues, a mass of 110,329 Da and a calculated isoelectric point of 6.1. The two peptide sequences from the 21-kDa protein are derived from a stable C-terminal fragment of p110. Apparently, the p110 subunit is readily degraded in yeast strains derived from W303, and a fragment of p110 is detected in eIF3 prepared by classical protein fractionation techniques (11). Northern blot hybridizations (not shown) with probes derived from either the N- or C-terminal coding regions of TIF32 show a single 3.2-kb band indicative of only one size class of mRNAs. Further evidence that TIF31 and TIF32 encode the p135 and p110 proteins in eIF3 is provided below and is summarized under “Discussion.”

The p110 amino acid sequence has a high content of charged residues (33.5%) evenly distributed over the entire protein. Data base searches identify homologous proteins from human,
mouse, Caenorhabditis elegans, and Nicotiana tabacum with amino acid sequence identity/similarity values around 26/38%.

The mammalian homologs encode the p170 subunit of eIF3. An alignment of these sequences has been published elsewhere (5).

The p110 Subunit Is Essential for Cell Growth, but p135 Is Not.—To explore the physiological role of p135 and p110 in eIF3, we attempted to construct haploid strains lacking these proteins. Individually, most of the coding region of one of the TIF31 or TIF32 genes in the diploid strain W303 was deleted and disrupted by insertion of HIS3 as described under “Materials and Methods.” The gray vertical bar at the 3'-end of TIF32 and TIF31 identifies the coding regions not deleted. PCR primers used to generate the 3.9-kb DNA fragment are indicated by arrows in the TIF32 scheme.

FIG. 1. Disruption of TIF32 and TIF31. The figure shows schematic descriptions of the TIF32 (3.9 kb, upper portion) and TIF31 (4.6 kb, lower portion) regions, with the ORFs shown as stippled rectangles translated from left to right. Restriction enzyme sites mentioned in the text also are indicated. The inner dotted lines mark the DNA deleted and replaced by HIS3 (shown as an open rectangle), whereas the outer dotted lines depict the region excised (shown below the gene) and used for transformation of the diploid W303 as described under “Materials and Methods.” The gray vertical bar at the 3'-end of TIF32 and TIF31 identifies the coding regions not deleted. PCR primers used to generate the 3.9-kb DNA fragment are shown by arrows in the TIF32 scheme.

Depletion of p110 Inhibits Cell Growth and Protein Synthesis.—The cellular level of eIF3-p110 was reduced by placing TIF32 under control of glucose-repressible promoters. pHPp110EX1 and pHPp110EX1-S were constructed to express TIF32 from the strong GAL1 promoter and a weaker GAL1 promoter as described under “Materials and Methods.” Each plasmid was transformed into the diploid strain HV110-20/D, followed by tetrad dissection, to yield haploid strains HV110-24 and HV110-29, respectively, that express TIF32 only from the plasmid. Both haploid strains grow rapidly (100 min doubling times) in YPG liquid medium and no overexpression of p110 is detected by Western immunoblotting (results not shown), even though these expression vectors normally produce higher levels of protein. This suggests that p110 is rapidly degraded when not incorporated into the eIF3 complex. The absence of intact p110 in our purified eIF3 preparations (11) also indicates that p110 is an unstable protein readily susceptible to degradation. Because no difference was detected in either growth rate or p110 expression level for strains HV110-24 and HV110-29 (results not shown), all subsequent experiments were carried out with HV110-29.

To determine the effects of p110 depletion, we compared the growth rates of strains HV110-29 and HV110-13 (expressing TIF32 under control of its own promoter) in both galactose and glucose media (Fig. 3A). Although both strains grow equally well in galactose medium, only HV110-29 shifted to glucose grows progressively more slowly, exhibiting a doubling time of...
rates by pulse labeling with [35S]methionine. At the indicated time non-depleted level (Fig. 3 after 10 h of depletion the rate has dropped to about 20% of the initial reduction of the rate of protein synthesis is seen by 4 h, and thionine pulse labeling as described previously (15). A substantial reduction of the rate of protein synthesis rates.

Protein synthesis rates are expressed as counts/min of incorporated methionine × µg⁻¹ protein min⁻¹.

220 min after 3 h and cessation of growth by about 20–25 h (after 4 generations). The depletion results are consistent with the requirement of p110 for cell division.

Depletion of an eIF3 subunit is expected to result in reduced protein synthesis. The rate of protein synthesis in strain HV110-29 after the shift to glucose was measured by [35S]methionine pulse labeling as described previously (15). A substantial reduction of the rate of protein synthesis is seen by 4 h, and after 10 h of depletion the rate has dropped to about 20% of the non-depleted level (Fig. 3B, solid circles). Little or no inhibition of protein synthesis is seen in strain HV110-13 in glucose (closed squares). This result suggests that p110 depletion does not occur; the slight decrease in protein synthesis rates at later times likely is due to their approach to stationary growth. The continued slow growth and low-level protein synthesis with HV110-29 after 12 h in glucose suggest either that p110 is not absolutely essential, or that low levels are sufficient to provide some eIF3 function. Maintenance of low p110 levels may be due to incomplete glucose suppression of TIF32 expression.

**Effects of p110 Depletion on Polysome Profiles and eIF3 Subunit Composition**—To determine the phase of protein synthesis that is inhibited upon p110 depletion, we analyzed the distribution of ribosomes on mRNAs. Reducing the rate of translation initiation without changing the rate of elongation leads to a reduction of polysome size (number of ribosomes per mRNA) and an increase in free 80 S ribosomes. When HV110-29 cells are shifted to glucose for 2 h, a time when protein synthesis already is inhibited over 2-fold (Fig. 3B), a reduction of polysome size and an increase in 80 S ribosomes are observed compared with cells grown in galactose for the same time (Fig. 4). After 6 h the polysome size is severely reduced and after 12 h polysomes are hardly detectable. When the same cells are grown in galactose, little or no reduction of polysome size is apparent after 6 h. The more pronounced effect on polysome size after 12 h again may be due to exit from the exponential growth phase (see Fig. 3A). The results demonstrate that the reduced protein synthesis rate observed after depletion of p110 (Fig. 3B) is due to decreased efficiency of translation initiation, indicating that eIF3-p110 plays an important role in the initiation pathway.

To demonstrate that p110 actually is depleted from the HV110-29 cells after the shift to glucose, we analyzed the levels of p110 and some other eIF3 subunits in crude cell lysates prepared at various times after the carbon source shift. eIF3 subunit levels in equal amounts of cell lysates were determined by Western blotting with specific antibodies as described under “Materials and Methods.” As shown in Fig. 5A, an immunoreactive protein of 110 kDa is indeed reduced over the time course of glucose repression of TIF32 expression. This is seen with blots subjected to treatment with a crude anti-eIF3 antiserum (upper panel), and also with antibodies affinity purified from the anti-eIF3 antiserum with recombinant p110 protein. Specific detection of a 110-kDa protein in undepleted cells with the affinity-purified antibodies demonstrates that TIF32 indeed encodes a 110-kDa protein.

The crude anti-eIF3 antiserum also detects the p33 subunit, whose level does not change following p110 depletion. The p39 and p90 subunits are not readily detected in lysates by the antiserum to eIF3, but analyses with affinity-purified anti-p39
p110 Is a Integral Part of the eIF3 Complex—Further evidence that p110 is in fact a subunit of yeast eIF3 was obtained by immunoprecipitation of the eIF3 complex. Strain HV110-33 was constructed which overexpresses an N-terminal (His)_6-tagged p110 protein as the sole source of p110. Since the growth rate of the strain is normal, we conclude that the (His)_6 tag is not detrimental to the function of the protein. Strains expressing (His)_6-tagged p33 and p135, and Myc-tagged p90, were subjected to protein labeling with [35S]methionine and immunoprecipitation with (His)_6-specific or anti-Myc antibodies as described under “Materials and Methods.” To control for nonspecific immunoprecipitation, strain W303–1A, which contains no (His)_6- or Myc-tagged protein, was treated similarly. A number of radiolabeled protein bands are seen in the autoradiogram of the tagged immunoprecipitates that also are seen above the gel lanes of eIF3 subunits from lysates derived from strains W303–1A (control), HV110-33 (expressing (His)_6-tagged p110), and PH1-135 ((His)_6-tagged p135). Coimmunoprecipitation was carried out with anti-(His)_6 antibody (CLONTECH) for lanes 1, 2, 4, and 5 and anti-c-Myc antibody (Santa Cruz Biotechnology) for lane 3 as described under “Materials and Methods.” The immunoprecipitates were fractionated by SDS-PAGE, and an autoradiogram of the gel is shown. Migration positions of molecular mass markers are shown on the left and eIF3 subunits are identified on the right. A putative p110 degradation product is labeled p110*, the 95-kDa band is labeled with a dot, and nonspecifically bound proteins are denoted by the symbol u. Panel B, coimmunoprecipitations with or without anti-(His)_6 antibodies (labeled above the gel lanes) of eIF3 subunits from lysates derived from strains W303–1A (control), HV110-33 (expressing (His)_6-p110), and PH333 (expressing (His)_6-p33). Proteins were coimmunoprecipitated as described under “Materials and Methods,” separated by SDS-PAGE, and detected with anti-eIF3 serum (upper panel) or affinity-purified antibodies against individual eIF3 subunits as indicated. Migration positions of molecular mass markers and eIF3 subunits are indicated on the left and right, respectively. The positions of full-length p110 are indicated by dots.

antibodies and an antiserum specific for p90 show that p39 levels remain constant, whereas p90 levels decrease. Quantitation of the Western blot signals (Fig. 5B) reveals that the p110 level drops following the shift to glucose and by 12 h is only 10% of the normal concentration. The p90 level decreases similarly. The quantitations also confirm that p39 and p33 levels, as well as that of eIF5A which is not associated with eIF3, do not significantly change over the time course of the experiment. Whether p110 depletion regulates the synthesis of p90 or makes p90 more accessible to degradation remains to be determined. The observed co-depletion of p90 and p110 suggests an interaction between the two subunits. Since p90 is a proven component of eIF3, the finding supports the view that p110 is associated with the eIF3 complex.

Fig. 5. Quantitation of eIF3 subunits in p110-depleted cells. Panel A, lysates (30 μg protein) from HV110-29 cells shifted to galactose (left 2 lanes) or glucose (right 8 lanes) for the times in hours indicated above the lanes were analyzed by SDS-PAGE and immunoblotting with anti-eIF3 antibodies. Lysate protein concentrations were determined by the BCA assay (Pierce). Loading of equal amounts of protein was confirmed by Coomassie Blue staining of parallel gels (not shown) and by probing with anti-eIF5A. Blots were probed with anti-eIF3 antiserum (top panel) or with affinity-purified antibodies against the indicated subunits (lower panels) as described in the legend to Fig. 2. Molecular weight standards are indicated to the left of the upper panel and eIF3 subunits are identified on the right. Panel B. The signal intensities for the individual subunits shown in panel A were quantitated with a GS-505 Imager System (Bio-Rad). Signal intensities for p110 were constructed which overexpresses an N-terminal (His)_6-tagged p110 protein as the sole source of p110. Since the

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Panel A, lysates (30 μg protein) from HV110-29 cells shifted to galactose (left 2 lanes) or glucose (right 8 lanes) for the times in hours indicated above the lanes were analyzed by SDS-PAGE and immunoblotting with anti-eIF3 antibodies. Lysate protein concentrations were determined by the BCA assay (Pierce). Loading of equal amounts of protein was confirmed by Coomassie Blue staining of parallel gels (not shown) and by probing with anti-eIF5A. Blots were probed with anti-eIF3 antiserum (top panel) or with affinity-purified antibodies against the indicated subunits (lower panels) as described in the legend to Fig. 2. Molecular weight standards are indicated to the left of the upper panel and eIF3 subunits are identified on the right. Panel B. The signal intensities for the individual subunits shown in panel A were quantitated with a GS-505 Imager System (Bio-Rad). Signal intensities for p110 were constructed which overexpresses an N-terminal (His)_6-tagged p110 protein as the sole source of p110. Since the
have masses of 110 and 105 kDa, the former corresponding to (His)6-tagged p110 and the latter probably being its partial degradation product (labeled p110* in Fig. 6A). Additionally, proteins of 90, 39, and 33 kDa are coimmunoprecipitated in the HV110-33 strain but not in strain W303-1A, except that the 39-kDa protein is precipitated in both strains (see “Discussion” below). A similar pattern of labeled immunoprecipitated proteins is seen with strain PHS33 containing (His)6-tagged p33 and strain PH1-39 containing Myc-tagged p39 (Fig. 6A). Interestingly, PH1-39 produces an additional weak band of ~95 kDa (labeled with a dot) which might correspond to the p93 (Nip1p) subunit of eIF3. The results indicate that p110 is found in complexes with the 90, 39, and 33 kDa proteins. Since all of these proteins correspond in size to subunits of eIF3, the results imply that p110 is a part of the eIF3 complex. It is noteworthy that in Fig. 6A, the bands corresponding to the (His)6- and Myc-tagged p33 and p39 subunits are very intense, indicating substantial accumulation of these proteins in the cells, whereas that for (His)6-tagged p110 is not intense, consistent with an innate lability of p110.

To confirm the identity of the immunoprecipitated proteins detected by autoradiography, nonradiolabeled lysates from strains W303-1A, HV110-33, and PHS33 were subjected to immunoprecipitation with the anti-(His)6 antibodies as described above. Following SDS-PAGE fractionation of the proteins in the immunoprecipitates, we used anti-eIF3 antisera (Fig. 6B, upper panel) as well as highly specific antibodies (lower panels) to p110, p90, p39, and p33 to detect these eIF3 subunits in the immunoprecipitates. The p110, p90, and p33 subunits are not detected when the immunoprecipitating antibody is omitted or in the precipitate from W303-1A. However, they are present in the anti-(His)6 precipitates from strains HV110-33 and PHS33 when analyzed by anti-eIF3 and the specific antibodies. Detection of the prominent 105-kDa band (just below the faint p110 band labeled by dots) from strain HV110-33 with the anti-p110 antibodies indicates that this protein is very likely a (His)6-p110 degradation product. In the case of p90, its absence in the W303-1A precipitate is obscured by a defect in the blot with the highly specific antibody; however, it clearly is not present in the anti-eIF3 blot or in the labeled precipitate analyzed in panel A. Unexpected is the apparent precipitation of p39 from W303-1A by the anti-(His)6 antibodies seen in panel A and confirmed with the anti-p39 antibodies (panel B). Noteworthy is the fact that the p39 subunit from W303-1A also co-purifies by IMAC (see Fig. 8 below). Although this might suggest that p39 contains an oligo-histidine tract, no such tract longer than two residues is present in the protein (14). We are not able to explain the presence of p39 in the W303-1A precipitates. In summary, the coimmunoprecipitation experiments demonstrate that p110 is part of a multisubunit complex containing at least two of the known subunits of eIF3, namely p33 and p90.

A second approach to obtaining eIF3 complexes rapidly is to employ IMAC. The eIF3 complex was purified on a HIS-Bind™ resin by utilizing the N-terminal (His)6-tagged p110 from strain HV110-33, as described under “Materials and Methods.” Fractions from each elution step were subjected to SDS-PAGE and Western immunoblotting with the anti-eIF3 antisera (Fig. 7A). The majority of eIF3 elutes with 250 mM imidazole. Because the 250 mM imidazole fraction contains numerous proteins as detected by Coomassie staining (result not shown), it was subjected to size exclusion chromatography on a Superdex 200 column as described under “Materials and Methods.” Aliquots of various fractions were subjected to SDS-PAGE, and eIF3 was detected with the anti-eIF3 antisera (Fig. 7B, upper panel) and by Coomassie staining (middle panel). The strongest signals with the antibody are seen in fractions 11–13. Coomassie staining also shows the presence of 33, 39, 90, and 110 kDa proteins in fractions 11–13. A complex of identical subunit composition was obtained from strain HV110-42, which expresses p110 with a C-terminal (His)6 tag (results not shown). The proteins in fractions 11–13 elute with an apparent molecular mass of 530 kDa (Fig. 7B, lower panel), consistent with the size of the eIF3 complex.

p135 Also May Be Present in a Complex with Other eIF3 Subunits—Further evidence that TIF31 encodes a protein associated with eIF3 was obtained by constructing strain PH1-135 which expresses (His)6-tagged p135 as the sole source of this protein from a GAL1 promoter. Ribosomal salt wash fractions were prepared from strain PH1-135 and from strain PH1-414 which expresses wild type p135 (untagged) from its own promoter. The preparations were subjected to IMAC and analyzed by SDS-PAGE and Western immunoblotting as described under “Materials and Methods.” When the blots are analyzed with antisera to eIF3, a number of bands corresponding to eIF3 subunits (135, 110, 90, 39, and 33 kDa) are detected with strain PH1-135 which are not present with PH1-414 (results not shown). To demonstrate that these bands correspond to eIF3 subunits, the blot was probed with antibodies affinity purified against recombinant p33, p110, p90, and p33, and with an antisera specific to p90 (Fig. 8). Each of these proteins is found in the eluate fractions from the (His)6-tagged p135 strain, PH1-135, but barely (e.g. p39) or not at all in the eluate fractions from the non-tagged strain PH1-414 (labeled Wt in the figure). The faint p110 immunoreactive band with PH1-135 suggests either partial degradation during purification or dissociation from the eIF3 complex under the conditions used. However, an association of p135 with the eIF3 complex is not always seen, as immunoprecipitation of (His)6-p135 (Fig. 6A, lane 5) does not bring down other eIF3 subunits. The relationship of p135 to eIF3 is discussed below.

Interactions of p110 or p135 with Other eIF3 Subunits and Initiation Factors—To obtain further evidence for the presence of p135 and p110 in the eIF3 complex, and to generate information leading to a structural model of eIF3, we attempted to define subunit-subunit interactions by two methods: far Western blotting and two-hybrid analyses. Because depletion of p110 leads to depletion of p90 as well, it seemed likely that these two proteins interact directly in the eIF3 complex. We therefore asked whether p110 might bind to p90 directly, rather than through other subunits in the eIF3 complex. As probes for the far Western blot analyses, p110 and p90 were synthesized in vitro in reticulocyte lysates in the presence of [35S]methionine as described under “Materials and Methods.” Full-length p110 and p90, as well as numerous smaller proteins likely due to degradation during the translation incubation, are seen when analyzed by SDS-PAGE and autoradiography (Fig. 9A). The radiolabeled p110 and p90 proteins were used to probe eIF3 subunits fractionated by SDS-PAGE. The eIF3 complexes were purified from strain HV110-33 by IMAC and Superdex chromatography as described above (Fig. 7B). Radiolabeled p110 binds only to p90 (Fig. 9A, lane 2), whereas radiolabeled p90 binds most strongly to p110 (lane 1), confirming the interaction. p90 also binds to another protein of ~95 kDa, possibly a degradation product of p110, and less strongly to p39. Interactions of p90 with p39 and p110 also have been detected by two-hybrid analyses and by glutathione S-transferase pull-down experiments (29, 30).

Possible interactions of p135 with other eIF3 subunits were sought by far Western blotting. Radiolabeled p135 binds to recombinant p33 (Fig. 9C, lane 3), but not to recombinant p39 or p62 under these conditions (results not shown). To further
substantiate this interaction, the two-hybrid system was employed as described in the legend to Table III. A positive interaction is seen between p135 and p33 (Table III), but not with p93 or p39 although the fusion proteins are expressed as detected by Western immunoblotting (not shown). Thus a p135-p33 interaction is confirmed.

Because mammalian eIF4B has been shown by far Western blotting to bind to the p170 subunit of human eIF3 (31), we asked whether or not yeast eIF4B might bind to the yeast homolog of human p170, namely p110. Radiolabeled eIF4B (Fig. 9A) was used to probe the eIF3 complex that contains (His)_6-p110, but no binding was detected (Fig. 9B, lane 3). Instead, labeled eIF4B binds to recombinant p33 (Fig. 9C, lane 1), but not to recombinant p39, p93, or p110 (results not shown). The binding interaction also is seen in a two-hybrid analysis (Table III). The eIF4B-p33 interaction appears to depend on the C-terminal 71 amino acid residues of p33 which contain the RNA recognition motif (29), as the mutant form lacking this region fails to bind to eIF4B (Fig. 9C, lane 2). The result suggests that eIF4B binds to the C-terminal region of p33. We cannot rule out the possibility, however, that the C-terminal deletion of p33 causes misfolding of other parts of the protein and thereby its loss of eIF4B binding activity.

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![FIG. 7. Affinity purification of eIF3 by IMAC. Panel A, analysis of different fractions during eIF3 purification on a HIS-Bind™ resin. Proteins were analyzed by SDS-PAGE and immunoblotting with anti-eIF3 antiserum. The gel contains the indicated subcellular fractions (lanes 1–3) as follows: S-30 and S-100 (30 µg of protein); high salt wash (15 µg). After the flow-through (fourth lane) was collected, proteins were eluted from the IMAC column as described under “Materials and Methods” with 5 mM (lanes 5–8), 30 mM (lanes 9–10), and 250 mM (lanes 11–13) imidazole, and 10-µl aliquots were analyzed (fraction numbers are shown above each gel lane). Panel B, size exclusion chromatography of eIF3 from the IMAC column. Proteins eluted with 250 mM imidazole (fractions 1 and 2) were subjected to fractionation on a Superdex 200 column as described under “Materials and Methods.” Aliquots (10 µl) of column fractions were analyzed by SDS-PAGE and proteins were detected with anti-eIF3 antiserum (upper panel), or 100-µl aliquots of each fraction were precipitated with trichloroacetic acid, separated by SDS-PAGE, and proteins were stained with Coomassie Blue (middle panel). Column fractions are labeled above the upper panel. The lower panel shows the column absorption profile at 280 nm, with fraction numbers indicated below. Elution positions of molecular mass markers are indicated by arrows: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa).](image-url)

![FIG. 8. Analysis of eIF3 subunits associated with (His)_6-tagged p135. Equal amounts of protein in the high salt wash fractions (Load) prepared from strain PH1-114 (labeled Wt, expressing untagged p135) and PH1-135 (expressing (His)_6-p135) were fractionated by IMAC as described (15). Unbound (wash, with 10 mM imidazole) and bound (eluate, with 250 mM imidazole) fractions were analyzed by 7.5% SDS-PAGE and immunoblotting with the affinity-purified antibodies indicated on the right. The figure shows relevant portions of the immuno-blots developed by nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate color reagents.](image-url)
DISCUSSION

We report here the cloning and analysis of TIF31 and TIF32 encoding p135 and p110, the largest and second largest proteins associated with eIF3. The conclusion that TIF31 encodes the 135-kDa protein found in eIF3 preparations is based on 4 major lines of evidence. 1) The encoded protein contains regions that match 51 out of 55 residues of 5 sequenced peptides derived from the 135-kDa protein in purified eIF3. 2) The calculated molecular mass of the protein encoded by TIF31 (145,165 Da) is consistent with the apparent mass of 135 kDa determined by SDS-PAGE. 3) Antibodies in an antiserum raised against purified eIF3 recognize a polypeptide of 135 kDa in crude extracts prepared from TIF21-expressing cells but not in extracts of cells lacking the TIF31 gene. Affinity-purified antibodies raised against recombinant p135 give the same result (data not shown). 4) When (His)_6-tagged p135 is expressed in cells where the chromosomal copy of TIF31 is disrupted, the protein that is immunoreactive with anti-p135 antibodies possesses an apparent mass of 135 kDa. We conclude that the cloned TIF31 gene encodes the 135-kDa protein found in our eIF3 preparations.

The role of p135 in yeast cells is not known. TIF31 deletion/disruption displays no obvious phenotype when cells are grown at temperatures lower or higher than 30 °C (data not shown) and thus is not an essential gene. Furthermore, analysis of polysome profiles of strain PH135H-B carrying the disrupted gene reveals no defect in protein synthesis (results not shown). It is noteworthy that p135 is not related to any subunit of mammalian eIF3, but is 27% identical and 50% similar to a 150-kDa protein encoded in the Dictyostelium genome. Recently, Zhu et al. (32) isolated the Dictyostelium gene and named it cluA. Disruption of cluA impairs cytokinesis and results in the clustering of mitochondria near the cell center. While this manuscript was in preparation, Fields et al. (33) cloned the S. cerevisiae homologue of Dictyostelium cluA and named it CLU1. CLU1 and TIF31 are identical. Consistent with the results reported here, deletion of CLU1 from S. cerevisiae does not affect cell viability even when cells are exposed to stress conditions such as heat and osmotic shock. However, disruption does result in the clustering of mitochondria as was observed in Dictyostelium. It is difficult to envision a mechanism whereby eIF3 directly affects cytokinesis and the clustering of mitochondria. The results suggest instead that p135 has a role in cells other than, or in addition to, initiation of protein synthesis. Nevertheless, there is strong evidence that p135 can associate with eIF3; it is present in highly fractionated eIF3 preparations; it co-purifies with other eIF3 subunits by IMAC when either p135 (Fig. 8) or p33 (15) is (His)_6-tagged; and it binds specifically to p33 as determined by far Western blotting and two-hybrid analyses. In effect, the precise physiological function of p135 and why it is sometimes found associated with the eIF3 complex are unclear.

Evidence that TIF32 encodes the p110 subunit of eIF3 also is strong. 1) The sequence of two internal peptides from p21 match precisely the C-terminal region of the putative product of TIF32. Although no 110-kDa protein is detected in our initial preparations of eIF3 (11), the 21-kDa protein in such preparations is derived from the p110 subunit through partial prote-
TIF31 and TIF32 Encode Yeast eIF3-p135 and -110

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ylation, likely during the purification process. 2) TIF32 encodes a putative protein of 110 kDa which is present in eIF3 preparations prepared rapidly by IMAC. 3) Antibodies affinity purified from anti-eIF3 with recombinant p110 expressed from TIF32 recognize a 110-kDa protein in eIF3 rapidly purified by IMAC. Taken together with other experiments discussed below, the data clearly establish that the 110-kDa protein in eIF3 is encoded by TIF32.

In contrast to p135, the p110 subunit plays an important role in protein synthesis. The protein is essential for growth of yeast, and depletion results in slowed growth and an inhibition of protein synthesis. Polysome profile analyses of p110-depleted cells indicate that p110 is required for efficient initiation, consistent with its being a subunit of eIF3. Similar effects are observed when other eIF3 subunits are depleted (14, 15).

Considerable additional evidence has been generated to demonstrate that p110 is a component of the eIF3 complex. Coimmunoprecipitates from lysates containing epitope-tagged p110 contain p33 and p90 previously characterized as eIF3 subunits. In addition, precipitates from lysates with other tagged eIF3 subunits contain p110. The p110 subunit interacts with p90 as shown by far Western blotting experiments. Depletion of p110 causes degradation of p90 as well, consistent with a direct interaction within the eIF3 complex. Furthermore, the p110 subunit is homologous to the p170 subunit of mammalian eIF3 (5), thus supporting the notion that it is present in yeast eIF3. In work published while this manuscript was in preparation, the p110 subunit was identified by mass spectroscopic analysis of preparations of eIF3 obtained from cells expressing (His)6-tagged p90 (16) and was shown to interact with p90 by glutathione S-transferase pull-down experiments (30).

The experiments above establish that p110 is an integral part of the eIF3 complex and that it plays an important role in the initiation phase of protein synthesis. A gene identical to TIF32, called RPG1, was cloned independently through a screen for yeast proteins that cross-react with antibodies to mammalian microtubule-associated protein 2 (34). The protein product of RPG1 was not characterized then, but analysis of a temperature-sensitive rpg1-1 allele demonstrated that cells shifted to nonpermissive temperature arrest in the G1 phase (34). A similar phenotype was observed for mutant alleles of PRT1 (encoding p90) (35) and TIF34 (encoding p39) (29). These data suggest that a properly functioning eIF3, presumably providing efficient translation initiation and protein synthesis, is required for progression through START and the cell cycle (36, 37). In a recent publication (38), RPG1 was further characterized by employing the temperature-sensitive mutant to show that initiation of protein synthesis is diminished. Their results and the results reported here are entirely compatible and support and extend each other.

The composition of yeast eIF3 has been controversial, as different preparations appear to contain different subunits with different apparent masses (11, 39). (His)6-tagging of eIF3 subunits followed by IMAC has shed light on the composition of eIF3. When (His)6-tagged p90 is employed, a complex is isolated that contains p33, p39, p90, p93, and p110 as well as eIF5 (16). This may represent a core complex of eIF3, as it possesses activity in vitro. In the IMAC and immunoprecipitation experiments reported here, tagged p33, p39, or p110 lead to copurification of the same 5 subunits, except for p93 which is known to be unstable in strains derived from W303 (17) and presumably is degraded in the lysates examined. It is noteworthy that only the 5 core subunits have homologs in mammalian eIF3 (see below).

The results presented above indicate that p135 also may associate with the eIF3 complex, albeit with lower affinity than the core subunits. Since p16 and p62 are co-immunoprecipitated with antibodies specific for other eIF3 subunits (12, 13), these proteins may be present in eIF3 as well, although p62 is not required for eIF3 activity in vitro (16). We lack suitable antibodies to p16, p62, and p93, and thus are not able to determine unambiguously whether or not they are present in our (His)6-tagged preparations. Taking into account all of the evidence available, we believe that yeast eIF3 may contain up to 8 subunits, although the p16, p62, and p135 subunits appear not to be as firmly associated with the complex as the core subunits. The more loosely associated subunits may not play essential roles in all aspects of eIF3 function.

The 5 core subunits in yeast eIF3 are homologous with 5 proteins identified as subunits of mammalian eIF3: yeast p110 is homologous with mammalian p170 (29% identity); p93, with p110 (31%); p90, with p116 (31%); p39, with p36 (46%); and p33 with p44 (33%). Thus yeast and mammalian eIF3 subunit structures are conserved, although more weakly than most of the other initiation factors (1). This conservation is reflected in the ability of yeast eIF3 to substitute for mammalian eIF3 in an in vitro initiation assay (11). On the other hand, there is considerable structural diversity in that the less tightly bound yeast subunits are not related to mammalian eIF3 subunits (although yeast p16 is homologous with mammalian eIF1). Since the overall eIF3 structures appear to be related, interactions of eIF3 with other translational components might be conserved as well. One such interaction concerns mammalian eIF4B, which binds to the p170 subunit of eIF3 (31). The interaction involves the DRYG-rich motif in the central region of eIF4B as demonstrated by far Western blotting. It has been postulated that the C-terminal repeat region of p170 also may be involved in this interaction (5). Our attempts to detect a yeast eIF4B interaction with yeast p110, the homolog of mammalian p170, were not successful. Close examination of the yeast and mammalian eIF4B sequences (40, 41) indicates that the two proteins are poorly conserved (17% identity), especially in the DRYG region implicated in the interaction with eIF3. Furthermore, yeast p110 differs considerably from the mammalian p170 subunit. The N-terminal third of yeast p110 shares 35% sequence identity with eIF3-p170, but the C-terminal two-thirds is less well conserved (20% identity) and the C-terminal repeat region of human p170 is lacking entirely in yeast p110. Our two-hybrid and far Western blotting results indicate that yeast eIF4B interacts instead with the p33 subunit of eIF3. Thus the interaction of yeast eIF4B with eIF3 may differ from that occurring in mammalian cells. Along similar lines, mammalian eIF4G binds to eIF3, but no such interaction has been demonstrated in yeast although numerous attempts have been made to do so. Further work is required to determine the three-dimensional structure of eIF3 in both yeast and mammals and to define the subunits responsible for its interaction with other translational components. Until then, caution must be exercised when extrapolating results from one system to the other.

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