Conservation and Diversity of Eukaryotic Translation Initiation Factor eIF3*

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The largest of the mammalian translation initiation factors, eIF3, consists of at least eight subunits ranging in mass from 35 to 170 kDa. eIF3 binds to the 40 S ribosome in an early step of translation initiation and promotes the binding of methionyl-tRNAi and mRNA. We report the cloning and characterization of human cDNAs encoding two of its subunits, p110 and p36. It was found that the second slowest band during polyacrylamide gel electrophoresis of eIF3 subunits in sodium dodecyl sulfate contains two proteins: p110 and p116. Analysis of the cloned cDNA encoding p110 indicates that its amino acid sequence is 31% identical to that of the yeast protein, Nip1. The p116 cDNA was cloned and characterized as a human homolog of yeast Prt1, as described elsewhere (Méthot, N., Rom, E., Olsen, H., and Sonenberg, N. (1997) J. Biol. Chem. 272, 1110–1116). p36 is a WD40 repeat protein, which is 46% identical to the p39 subunit of yeast eIF3 and is identical to TRIP-1, a phosphorylation substrate of the TGF-β type II receptor. The p116, p110, and p36 subunits localize on 40 S ribosomes in cells active in translation and co-immunoprecipitate with affinity-purified antibodies against the p170 subunit, showing that these proteins are integral components of eIF3. Although p36 and p116 have homologous protein subunits in yeast eIF3, the p110 homolog, Nip1, is not detected in yeast eIF3 preparations. The results indicate both conservation and diversity in eIF3 between yeast and humans.

The initiation phase of eukaryotic protein synthesis is rate-limiting for most mRNAs and is frequently the site of translational control (1). The pathway of initiation involves dissociation of the 80 S ribosome into 40 S and 60 S ribosomal subunits, the binding of the initiator Met-tRNAi and mRNA to the 40 S ribosomal subunit, and subsequent junction of the 60 S ribosomal subunit to form an 80 S initiation complex. The reactions are promoted by at least 10 proteins called eukaryotic initiation factors (eIFs), which have been purified and characterized biochemically (see Ref. 2 for a recent review). The largest of these, eIF3, is a complex of eight or more polypeptides and plays a central role in the initiation pathway. eIF3 binds to 40 S ribosomal subunits in the absence of other translational components and helps maintain 40 S and 60 S ribosomal subunits in a dissociated state. It interacts with the ternary complex of eIF2-GTP-Met-tRNAi, and prevents its destabilization caused by RNA (3). eIF3 stabilizes Met-tRNAi binding on 40 S ribosomal subunits and is absolutely required for the binding of mRNA to 40 S and 80 S ribosomes when evaluated in vitro with purified translational components (4, 5). Furthermore, eIF3 associated with 40 S subunits may be directly involved in the initial stages of mRNA binding. The eIF4G subunit of the cap-binding complex, eIF4F, binds to eIF3 (6), as does eIF4B, a factor involved in RNA helicase activity (7).

A detailed understanding of the molecular events of initiation of protein synthesis benefits from a knowledge of the primary sequences of the initiation factors. Of the ~25 polypeptides comprising the initiation factors, cDNAs encoding all of them have been cloned and sequenced except for the subunits of eIF3 and the eIF6 polypeptide (reviewed in Ref. 2). Purified eIF3 has been characterized as a complex of eight nonidentical subunits, called p170, p115, p66, p47, p44, p40, p36, and p35 (4, 8, 9). The subunits co-purify during numerous fractionation procedures, although the p35 subunit appears to dissociate partially during nondenaturing gel electrophoresis (8). The p66 subunit is a strong RNA-binding protein (10), but the functions of the other subunits are not known.

Insight into the function of eIF3 comes from studies of the corresponding initiation factor in yeast. Yeast eIF3 has been purified on the basis of its stimulation of methionyl-puromycin synthesis in an assay composed of mammalian components that requires the formation of 80 S initiation complexes (11). The active yeast eIF3 preparation contains eight major polypeptides, called p135, p90, p62, p39, p33, p29, p21, and p16. That the yeast factor can replace mammalian eIF3 in the methionyl-puromycin synthesis assay indicates strong conservation of function.

The availability of purified yeast eIF3 led to the identification of genes encoding three of its subunits and characterization of their mutant forms has shed light on the function of the factor. p90, p62, and p16 are encoded by PRT1 (11), GCD10 (12), and SUI1 (13), respectively. A conditional lethal mutation in PRT1 was isolated (14), which shows a defect in Met-tRNAi binding to ribosomes (15). GCD10 was isolated as a gene whose mutations constitutively derepress the expression of GGN4 in rich medium (16). This gcd phenotype for p62 is consistent with the proposed role of eIF3 in stabilizing Met-tRNAi binding to 40 S subunits. Gcd10/p62 also is an RNA-binding protein and therefore might correspond to mammalian p66 (12). Mutant forms of SUI1 were identified from a genetic screen for proteins that affect initiator codon recognition and allow initiation at a UUG codon (17). SUI1 encodes p16, which implicates eIF3.
along with eIF2, in initiation codon recognition. However, p16/Sui1 exists both in the eIF3 complex and as a free form (13). Since a mammalian homolog of Sui1 corresponds to the poorly characterized initiation factor eIF1 (18), which is not thought to be a component of mammalian eIF3, p16/Sui1 may perform more than one function in yeast. A fourth gene, TIF34, encoding the p39 subunit, has been cloned and characterized recently \(^2\) and appears to be required for the integrity of the entire eIF3 complex. Work is in progress to clone the remaining genes for yeast eIF3 subunits.

We report here the cloning and characterization of cDNAs encoding two of the human eIF3 subunits, namely p36 and p110. The eIF3-p36 subunit is homologous to the yeast p39 subunit. eIF3-p110 is one of two proteins found in the previously described p115 band and is related to the yeast protein, Nip1 (20). The other protein in the p115 band, eIF3-p116, is a homolog of yeast Prt1 (36). We discuss the conservation and divergence of eIF3 subunit structures between mammals and yeast.

**EXPERIMENTAL PROCEDURES**

**Materials**—eIF3 was prepared from human HeLa cells essentially as described previously (21), except that the otherwise exchange chromatography employed FPLC (Pharmacia Biotech Inc.) Mono Q and Mono S columns. The polyclonal antiserum against rabbit reticulocyte eIF3 was prepared in a goat and was characterized previously (22).

**Affinity Purification of Antibodies**—HeLa eIF3 (100 µg) was subjected to electrophoresis in a 7.5% polyacrylamide gel containing SDS (23) and transferred to a nitrocellulose membrane (AB 084, Schleicher & Schuell). The membrane was stained with Ponceau S to locate eIF3 proteins and then washed with water. After incubation at room temperature for 3 h with 5 µl of Blotto (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.075% Tween 20, 0.5% dried milk) containing 200 µl of crude goat anti-eIF3 polyclonal antiserum, the membrane was washed with TST (0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.075% Tween 20), and portions that contained eIF3 subunits were excised. Antibodies bound to the excised membrane pieces containing the p170 and p115 bands were eluted with a low pH buffer (2 M glycerin, 1 mg EGTA, pH 2.5) for 30 min. The resulting affinity-purified anti-p170 and anti-p115 antibodies were neutralized by addition of 1 x Tris-HCl, pH 8.8, diluted with one volume of Blotto and stored frozen at -80°C.

For affinity purification with recombinant eIF3-p116 (hPrt1), eIF3-p110 and eIF3-p36, 1–3 mg of Escherichia coli lysate expressing their respective cDNA vectors (see below) was fractionated by SDS-PAGE. The bands containing the recombinant proteins were excised and antibodies in the crude goat anti-eIF3 antisera were affinity-purified as described above. In the case of the anti-p116 antibody, 50 µg of GST-p110 fusion protein immobilized on a nitrocellulose membrane chip was included in the incubation to remove anti-p110 antibodies whose high titer otherwise would seriously contaminate the anti-p116 antibody preparation.

**Screening the Human HeLa cDNA Library**—About 1 × 10\(^9\) bacteriophages from a human HeLa cDNA library in λgt11 (Clontech) were grown on a lawn of E. coli Y1090/150-mm plate. Twenty plates were screened according to the method described in Ref. 24 with affinity-purified anti-p115 antibody. Bound antibodies were detected by incubating the filters with rabbit anti-goat IgG antibodies conjugated with alkaline phosphatase (Sigma), followed by treatment with nitro blue tetrazolium as recommended by the manufacturer. Putative positive plaques were picked and rescreened until purified. Thirty immunopositive plaques were obtained. The sizes of the inserts were determined by the polymerase chain reaction (PCR) with λgt11 primers, FW 5'-GG-TGGCAGACCTCTGGAGCCCC-3' and RV 5'-TGTGACCCAGACCC-AACGTGATG-3', which flank the EcoRI cloning site, and restriction sites were mapped. Two phases containing the longest related inserts (~3.0 kb), called 11a and 19a, were selected and their DNAs were prepared as described (25). Since the EcoRI sites in the phase DNAs were lost, the 3' sites flanking the inserts were used instead, and 3.3-kb SphI fragments were subcloned into the Asp718I site of pTZ19R (26). The resulting plasmids are called pTZp110–11a and pTZp110–19a, respectively. The 5'-ends of the cDNA inserts were sequenced and clone 11a was found to be 68 bp longer at the 5'-end. For sequencing clone 11a on both strands, deletions were constructed from pTZp110–11a with restriction enzymes NdeI, BamHI and SphI, which cleave at 0.3, 1.7, and 1.8 kb from the 5'-end, respectively, and with PsI, which cleaves both at 2.0 and 2.6 kb, and the remaining portions were sequenced with eight custom-made primers.

**Determination of the Amino Acid Sequences of eIF3-p36 Fragments**—The human eIF3-p36 DNA was digested with restriction enzymes EcoRI and NdeI, which cleave at 2.0 and 2.6 kb, and the remaining portions were sequenced with eight custom-made primers. Determination of the Amino Acid Sequences of eIF3-p36 Fragments and Identification and Sequencing of eIF3-p36 DNA—eIF3 (100 µg) purified from rabbit reticulocytes was applied to a standard SDS gel (15% acrylamide, 0.2% bisacrylamide), and individual subunits were resolved by electrophoresis at 30 milliamperes for 3 h. The subunit bands were excised and transferred to Immobilon-P (Millipore) in 10 mM sodium phosphate, pH 12, 10% methanol for a period of 1 h at 60 V. The membrane was stained with Coomassie Brilliant Blue R-250 to identify protein bands, then fully destained in 50% methanol, 10% acetic acid. The membrane was rinsed several times with distilled water, and individual bands containing the p36 subunit were excised. The excised strips were subjected to automated amino acid sequencing with an Applied Biosystems Inc. model 477A protein microsequencer with an on-line phenylthiohydantoin analyzer in the Molecular Biology Core Laboratory at Case Western Reserve University. Internal peptide sequences were obtained by trypsin digestion of the protein in the p36 band, high performance liquid chromatography purification, and sequencing essentially as described (27).

Sequences in the expressed sequence tag (EST) database that match the NH\(_2\)-terminal amino acid sequence were sought in the National Center for Biomedical Information data base by using the GCG BLAST program. One of the identified ESTs, clone obb04, was kindly provided by Genethon, Evry, France. Clone obb04 from a human infant brain cDNA library contains a 1.4-kb insert cloned into the HindIII (5') and NolI (3') sites of lamdf BA, derived from pEMBL (28). Lamdf BA has an EcoRI site next to the NorI site and the annealing sites for standard M13 primers in the regions flanking these cloning sites. For dideoxynucleotide DNA sequencing, deletions in the insert were constructed by digestion with BamHI or HindIII, which cleave the insert at 0.4 and 0.7 kb from the 5'-end, respectively. The remaining portions of both strands were sequenced with three custom-made primers.

**Northern Blot Analysis**—Total RNA was isolated (29) from HeLa cells, which were grown exponentially to a density of 5 × 10\(^5\) cells/ml in spinner flasks. Poly(A)+ RNA was isolated from total RNA, and Northern blotting was conducted as described previously (30) with radioactive probes derived from pTZp110–11a (PCR-amplified with the M13 forward and reverse primers) and obb04 (1.4-kb HindIII-EcoRI fragment). Hybridizing bands were visualized by autoradiography.

**Expression of Recombinant p110 and p36 in Escherichia coli**—A 2.9-kb NdeI-EcoRI fragment of pTZp110–11a (the EcoRI site is located 3' to the insert in the multiple cloning site of pTZ19R), which lacks 314 bp including the initiator AUG at the 5'-end, was subcloned into the corresponding sites of pT7–7 (31). The resulting plasmid, pTZp110–NdeI, was introduced into E. coli strain DH5α carrying pGFP–1 and expression was heat-induced as described (31). The size of the protein produced was shorter than that of the full-length p110 target, since initiation likely occurs at the internal AUG codon (Met-130, preceded by a good Shine-Dalgarno sequence) because the AUG in the NdeI site is out-of-frame. To construct pGEXp110 for expression of the GST-p110 fusion protein, the 5'-terminal half of the p110 coding region was amplified from pTZp110–11a with primers 5'-CCCGAATTCATATGTTGGCTTGTTCACC-3' (tagged by EcoRI and NdeI sites; the bases corresponding to the initiator codon are underlined) and 5'-TTGCAACCAGACC-AACGTGATG-3', which flank the EcoRI cloning site, and restriction sites were mapped. The two phases containing the longest related inserts (~3.8 kb), called 11a and 19a, were selected and their DNAs were prepared as described (25). Since the EcoRI sites in the phase DNAs were lost, the 3' sites flanking the inserts were used instead, and 3.3-kb SphI fragments were subcloned into the Asp718I site of pTZ19R (26). The resulting plasmids are called pTZp110–11a and pTZp110–19a, respectively. The 5'-ends of the cDNA inserts were sequenced and

\(^a\) Naranda, T., Kinumawa, M., MacMillan, S. E., and Hershey, J. W. B. (1997) Mol. Cell. Biol. 17, in press.
of p110, cells were harvested early at 10 min after induction because the fusion protein is unstable.

In Vitro Transcription/Translation with cDNAs—The 1.6-kb NdeI-BamHI fragment from pTZp110N was used to replace the 1.4-kb NdeI-BamHI fragment of pT7p110N(NdeI) to generate plasmid pT7p110. Plasmid pT7p110 was constructed by subcloning the 1.4-kb HindIII-EcoRI fragment of bo04 into pTZ19R. pT7p110 and pTzp36 were transcribed and translated in vitro with the T7 Tnt-Coupled reticulocyte lysate system (Promega) and \(^{35}\)S-methionine. Reaction mixtures were incubated at 30 °C for 90 min, and an aliquot of the mixture was fractionated by SDS-PAGE. Gels were stained, dried, and exposed to x-ray film at −80 °C for 16–32 h.

Two-dimensional IEF/SDS-PAGE—Two-dimensional gel analyses followed procedures described previously (33), with minor modifications. Briefly, 2 μg of HeLa eIF3 was subjected to isoelectric focusing in the first dimension gel (4% acrylamide, 0.1% piperazinediacrylamide (Bio-Rad), 1.5% CHAPS (Boehringer Mannheim), 1.5% Nonidet P-40 (Fierce), 4% amylpectins, 0.8 μm urea) at 3500 V-h with 25 μm histidine as the cathode buffer and 25 μm phosphoric acid as the anode buffer. The amphotericities were two pHs 5–7 and one pH 3–10 (Serva). The second dimension employed a 7.5% polyacrylamide gel in SDS (23).

Polysome Profile Analyses—Cos-1 cells freshly fed with serum were lysed with buffer containing 20 mM HEPES-KOH, pH 7.5, 100 mM KCl, 10 mM magnesium chloride, 1 mM dithiothreitol, 0.25% Nonidet P-40, 1 μg/ml leupeptin, 2 μg/ml pepstatin, and 10 μg/ml cycloheximide with 10 strokes of a narrow gauge syringe. The lysate was clarified by centrifugation at 10,000 rpm for 5 min.

RESULTS

Cloning and Characterization of eIF3-p110 cDNA—A goat antiserum raised against rabbit eIF3 recognizes eight subunits in the multiprotein complex (22). The strongest antigen gives rise to a broad band or doublet at about 115 kDa when HeLa eIF3 is analyzed by SDS-PAGE and immunoblotting (34) (see also Fig. 2A, lane 4). Antibodies recognizing the p115 protein(s) were affinity-purified and used to screen a library containing HeLa cDNAs as described under “Experimental Procedures.” Among the 2 × 10^6 plaques screened, 13 positives were identified and plaque-purified. Insert sizes were determined by PCR analysis with primers that flank the cloning site in the phage. The two largest inserts, called 11a and 19a, were subcloned into pT7Z19B and sequenced with strands described under “Experimental Procedures.” Clone 11a, called pTZp110, contains an insert of 2945 bp whereas clone 19a is somewhat shorter, with 2877 bp. Clone 11a was named pTZp110 rather than pTZp115, because the p115 band from which the antibodies were purified contains two proteins with apparent masses of 116 and 110 kDa, as explained below.

The DNA sequence in pTZp110 contains an open reading frame of 2739 bp that encodes a protein of 913 amino acid residues with a calculated mass of 105,277 Da and a pl of 5.41. The sequence context surrounding the first AUG, GCCAUGU, compares favorably to the consensus sequence for moderately strong initiator AUGs (35); furthermore, the AUG is preceded by an in-frame TGA termination codon in the 5’-UTR. Thus, this AUG very likely serves as the initiator codon, as discussed below. The amino acid sequence derived from the open reading frame is shown in Fig. 1 (labeled human p110); the DNA sequence is not shown but is deposited in the GenBank™ data base with accession number U46025. The 5’-UTR in the cDNA insert contains 49 bp, whereas the 3’-UTR has 89 bp and ends with a string of 48 A residues preceded by an ACTAAA sequence, which may possibly serve as the polyadenylation signal.

Northern blot analysis of poly(A)+ RNA from HeLa cells generates a single band of 3.0 kb (results not shown), suggesting that pTZp110 contains a nearly full-length cDNA.

The sequence of the cDNA insert was compared with other known sequences by searching the EST data base. The cDNA was found to match identical or near-identical (>95%) partial DNA sequences in ESTs from numerous human tissues, as shown in Table I. The 72 matches found in all tissues represent about 0.02% of the total EST entries examined. Three independent human ESTs (H27714, H27960, and D81864) overlapped and match the 5’-end of the insert in pTZp110, ruling out the possibility that foreign DNA fused to the NH2-terminal coding region of the 11a cDNA during construction of the library. The frequency and wide distribution of ESTs matching pTZp110 indicate that p110 is likely expressed in all cells as a moderately abundant protein.

The Insert in pTZp110 Encodes the p110 Subunit of eIF3—A COOH-terminal portion of the cDNA insert in pTZp110 carrying about 85% of the coding region was subcloned into a bacterial expression vector and introduced into E. coli as described under “Experimental Procedures.” Shortly after heat induction, a 90-kDa protein was overexpressed but then disappeared at later times (data not shown). By lysing the cells at 10 min following induction, the 90-kDa protein was readily detectable upon SDS-PAGE and was used to affinity-purify antibodies from a crude goat anti-eIF3 antiserum. The resulting affinity-purified antibodies generate a single band with a mobility corresponding to a 110-kDa protein when either a HeLa lysate or purified human eIF3 is analyzed by Western blotting (Fig. 2A). Further evidence that pTZp110 encodes a subunit of eIF3 was obtained by in vitro transcription of the entire cDNA insert coupled with translation in a rabbit reticulocyte lysate as described under “Experimental Procedures.” The resulting 35S-labeled proteins were subjected to analysis by SDS-PAGE and autoradiography as shown in Fig. 2B. A single major band of 110 kDa was detected. Since the size of the translation product and the protein detected in HeLa lysates by the affinity-purified antibodies is the same, this supports the view that the cDNA contains the entire coding region for a 110-kDa subunit of eIF3 (named eIF3-p110). Further evidence that the eIF3-p110 cDNA encodes a subunit of eIF3 is provided below.

eIF3-p110 Is Homologous to the Yeast Protein Nip1—A search of EST sequences that are related to eIF3-p110 reveals homologs in rat, mouse, Caenorhabditis elegans, and Arabidopsis thaliana. The amino acid sequences derived from the rat and mouse ESTs (rat, H31971, H34067, H34777, H34840; mouse, Z62929) show 97–100% identity to the corresponding regions of eIF3-p110, whereas those from C. elegans (D36264, D36431, D36784, D36806, D37362, and D37595) show 38–58% identity and from A. thaliana (T13976 and T88395), 48 and 60% identity. The eIF3-p110 sequence is 30.9% identical and 44.1% similar to the yeast Nip1 sequence (20; see Fig. 1), but is not related to Prt1, which is reported to be the second largest
subunit in the yeast eIF3 preparation (11) and therefore was presumed to be the homolog of the mammalian protein in the p115 band. No obvious peptide motif was found for either the mammalian or yeast proteins by the GCG MOTIFS program. However, it is noteworthy that the NH₂-terminal third of both Nip1 and eIF3-p110 is hydrophilic and is also rich in serine, as shown by bold letters in the sequence shown in Fig. 1. Nip1 was originally identified as a protein involved in nuclear import (20), but it may also play a role in the initiation phase of protein synthesis since a conditional mutant strain altered in \textit{NIP1} exhibits reduced polysomes and increased 80 S ribosomes when shifted to a nonpermissive temperature.

**eIF3-p116 Is the Human Homolog of Yeast Prt1**

While analyzing the components of HeLa eIF3, it was discovered that SDS-PAGE with a lower (7.5%) concentration of acrylamide than the usual 10% separates the p115 band into two components with apparent masses of 110 and 116 kDa (Fig. 2, lane 1). Two components in the 115-kDa region of the gel were recognized earlier (22) but were thought to be due to limited proteolysis of the protein in the slower migrating band. The affinity-purified antibodies prepared against the recombinant p110 fragment described above recognize only the p110 band and not the p116 band (lane 2). During the course of this work, N. Me\'thot and N. Sonenberg (McGill University) identified human sequences in the data base of Human Genome Science Inc. that are homologous to yeast \textit{PRT1} and proceeded to clone a human cDNA encoding a Prt1 homolog (hPrt1). They constructed a fusion of GST DNA with the major part of the hPrt1 cDNA coding region and expressed a 120-kDa protein in \textit{E. coli} (36). Using the \textit{E. coli} lysate kindly provided by N. Me\’thot and N. Sonenberg, we affinity-purified antibodies from the goat.

### Table I

| Tissue                  | p110  | p36  |
|-------------------------|-------|------|
| A Adipose tissue        | 2     | 1    |
| B Brain (infant)        | 14    | 9    |
| C Brain (adult)         | 11    | 2    |
| D Breast                | 7     | 1    |
| E Heart                 | 1     | 2    |
| F Hippocampus           | 1     | 2    |
| G Melanocyte            | 1     | 3    |
| H Lung                  | 6     | 6    |
| I Placenta              | 3     | 4    |
| J Synovial membrane     | 2     | 2    |
| K Testis                | 1     | 4    |
| L White blood cells     | 1     | 9    |
| M Others                | 22    | 11   |

* GenBank accession numbers for p110 ESTs: A. T29990; B. F08530, H08137, H08138, H16749, R29027, A34067, T30592, T08143, T09008, T25570, Z11055, Z12461, H21211, H25461, H40299, H44909, H50415, H50416, R18517, T31326, T31327, T33434, T33435; D. H25091, H26388, H27714, H29760, H42551, H43030, R15569; F. M78803; G. N20146; H. T16253, T39747, T36691, T29830, T94738, T47939, I. D58773, D58904, R26885, R27117; J. T35728, T35901, K. T30244, T32428, T32529, T34560, L. T41223, M. D25679 (colon mucosa), T31531, T31658, T31684, T31721 (embryo), N29061 (epidermal keratinocyte), D54554, D55023, T60560 (fetal brain), H27993, H29794, H87994, R39451, R83150, T81525, T90534 (fetal liver spleen), R95472 (ocular ciliary body), T35740 (ovary), Z17695 (skeletal muscle), T57333 (HM1 cells), T27501 (WATM1 cells).

* GenBank accession numbers for p36 ESTs: A. T30831; B. F08234, F08629, R14471, R59837, R61400, T26406, F04308, Z42307, Z43588, Z44802; C. T31325, D. R53192; E. T32683; F. M76301, N25173; G. H9689, H97009, N26173, H29764, T30244, T32428, T34454, T35503, T35590, T35563, I. T81939, R24486, R28478, R78618; J. T35743, T35814; K. T18990; L. T33981, T34033, T34160, T34231, T34280, T34659, T33926, T33928, M. T34038, T34428 (endothelial cells), T36020 (eye), T32529 (gall bladder), T32299 (kidney), T47680, T47646 (liver), T34348 (prostate gland), H41100 (retina), T22920 (skin), T31617, T32644 (thymus gland).

Aspartate, and glutamate, as shown by bold letters in the sequence shown in Fig. 1. Nip1 was originally identified as a protein involved in nuclear import (20), but it may also play a role in the initiation phase of translation due to its potential role in the initiation phase of translation and synthesis since a conditional mutant strain altered in \textit{NIP1} exhibits reduced polysomes and increased 80 S ribosomes when shifted to a nonpermissive temperature.

**eIF3-p116 Is the Human Homolog of Yeast Prt1**—While analyzing the components of eIF3, it was discovered that SDS-PAGE with a lower (7.5%) concentration of acrylamide than the usual 10% separates the p115 band into two components with apparent masses of 110 and 116 kDa (Fig. 2, lane 1). Two components in the 115-kDa region of the gel were recognized earlier (22) but were thought to be due to limited proteolysis of the protein in the slower migrating band. The affinity-purified antibodies prepared against the recombinant p110 fragment described above recognize only the p110 band and not the p116 band (lane 2). During the course of this work, N. Méthot and N. Sonenberg (McGill University) identified human sequences in the data base of Human Genome Science Inc. that are homologous to yeast \textit{PRT1} and proceeded to clone a human cDNA encoding a \textit{Prp1} homolog (\textit{Prp1}). They constructed a fusion of GST DNA with the major part of the \textit{Prp1} cDNA coding region and expressed a 120-kDa protein in \textit{E. coli} (36). Using the \textit{E. coli} lysate kindly provided by N. Méthot and N. Sonenberg, we affinity-purified antibodies from the goat.

\* D. Goldfarb, personal communication.
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anti-eIF3 antiserum that bound to the 120-kDa fusion protein. The affinity-purified antibodies recognize the 116-kDa band in eIF3 (Fig. 2C, lanes 3 and 4) and react most strongly with a ca. 116-kDa protein in HeLa lysates (lane 5). Because the titer of antibodies that recognize p116 is quite low, a high concentration of the affinity-purified antibody preparation is needed and cross-reaction with other proteins in the lysate is apparent. We conclude that both p110 (the homolog of Nip1) and p116 (hPrt1) are present in HeLa eIF3.

To further characterize and differentiate the p116 and p110 subunits of eIF3, we conducted two-dimensional IEF/SDS-PAGE analyses of purified eIF3. eIF3 proteins in the 115-kDa region of such gels are known to focus into two spots or streaks at about pI = 5.2 and 6.5 (37, 34). When purified HeLa eIF3 was fractionated by the two-dimensional gel system, followed by immunoblotting with antibodies affinity-purified against recombinant p110 and p116, it was observed that the anti-p110 antibodies recognize a spot with a pI of 5.8, whereas the anti-p116 antibodies recognize two streaky spots with pIs of 4.8 to 5.0 (Fig. 3). Thus p116 is more acidic than p110, consistent with their calculated pIs, and exhibits two isoelectric variants that might be due to phosphorylation (see “Discussion”).

Cloning and Characterization of a cDNA Encoding eIF3-p36—The titer of antibodies against the p36 subunit in the goat anti-eIF3 antiserum appears quite low (Fig. 2A, lane 4). Rather than using antibodies to screen for p36 cDNA in an expression library, we relied on partial amino acid sequencing of the p36 subunit from rabbit reticulocyte eIF3 to clone the cDNA. NH2-terminal sequencing was carried out as described under “Experimental Procedures.” The initiator codon is identified unambiguously by the NH2-terminal sequence and lies in a favorable context.

Fig. 2. Western immunoblot analyses and in vitro synthesis of human eIF3 subunits. Panel A, purified eIF3 (200 ng; lanes 1 and 3–5) and HeLa cell extracts (20 μg; lanes 2 and 6) were fractionated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to Coomassie Blue staining (lane 3) or immunoblotting with affinity-purified anti-recombinant p110 antibodies (lanes 1 and 2), crude anti-eIF3 antiserum (lane 4), and affinity-purified anti-recombinant p36 antibodies (lanes 5 and 6). The migration positions of molecular mass markers and eIF3 subunits are indicated. The p115 band comprises both p110 and p116, as explained in the text. Panel B, expression products of plasmids pT7p110 (lane 1) and pT7p36 (lane 2), carrying p110 and p36 cDNAs behind the T7 promoter, respectively, were expressed in the T7-coupled transcription/translation system (Promega) as described under “Experimental Procedures.” The panel shows a autoradiogram of the 35S-labeled translation products fractionated by 10% SDS-PAGE. Purified HeLa eIF3 was subjected to electrophoresis in parallel lanes and the migration positions of the subunits are indicated. Panel C, HeLa eIF3 (lane 1, 1 μg; lanes 2 and 3, 200 ng) was fractionated by 7.5% SDS-PAGE and electrophoresis was continued to allow run-off of proteins smaller than about 70 kDa and thereby to enhance the separation of the p115 band into two protein species. Lane 1 was excised for Coomasie Blue staining, whereas lanes 2 and 3 were subjected to immunoblotting with affinity-purified anti-recombinant-p110 and anti-recombinant-p116 antibodies, respectively, as described under “Experimental Procedures.” In lanes 4 and 5, purified eIF3 (200 ng) and HeLa lysate (20 μg) were fractionated by 7.5% SDS-PAGE as described in panel A and immunoblotted with affinity-purified anti-recombinant-p116 antibodies. eIF3 subunit bands are identified to the right of the gel lanes.
post-translational modification: a single search of the sequence identifies the following possible sites for modification: the COOH-terminal region of the proteins. Although the eIF3 subunits co-purify and all elements (Fig. 4A) and compared to the consensus sequence (Fig. 4B) identical in the folding of p36, and are discussed in greater detail below. A search of the data base also identifies a large number of ESTs derived from many different human tissues that match to the eIF3-p36 sequence (Table I). This indicates that the p36 cDNA is expressed as a housekeeping gene, and the frequency of appearance of the ESTs suggests a moderately abundant protein. The p36 subunit also shares 45.8% identity and 58.8% similarity with a 39-kDa protein in yeast, which was recently identified as the p39 subunit of yeast eIF3.2 Therelatednessof the p36 subunit also shows 36 to 71% identity to eIF3-p36. In addition, one EST each from rice (accession numbers Z46547, and Z46548) whose translation products show 36 to 50% identity to eIF3-p36 and yeast eIF3-p39, are aligned with the WD repeat consensus sequences (38). Residues matching the consensus sequence are shown in bold. The putative positions of the four strands of B sheets (41) are shown by arrows. The positions of loops that do not conform to the consensus are defined with triangles and numbers of residues. The consensus sequence is defined with the following symbols: \( ^{\text{\*}} \), for hydrophobic amino acids; \( ^{\text{\#}} \), for D; \( ^{\text{\*}} \), for W, F, or Y; \( ^{\text{\*}} \), for K, R, N, or D. The amino acid sequence of eIF3-p36 was analyzed further. To demonstrate that the cDNA in pTZp36 encodes the p36 subunit of eIF3, antibodies specific for p36 were prepared as described under "Experimental Procedures." Briefly, the coding region of the pTZp36 insert was subcloned into a derivative of pGEX-2T and the resulting GST-p36 fusion protein was expressed in E. coli. Antibodies in the goat anti-eIF3 antiserum retained when lysine follows the methionine (Huang et al., 1987). The theoretical isoelectric point of the protein is 5.28, although two-dimensional IEF/SDS-PAGE analysis (Fig. 3) indicates a somewhat more acidic protein (pl = 5.2). A ProSite search of the sequence identifies the following possible sites for post-translational modification: a single N-glycosylation site, eight protein kinase C and four casein kinase II phosphorylation sites, five N-myristoylation sites, and an ATP/GTP-binding motif A site. From information currently in hand, it is likely that none of these ProSite-identified post-translational modification sites is of biological importance. Comparison of the eIF3-p36 sequence with other proteins in the data base shows a match to the \( ^{\text{\*}} \)-transducin family, and to the eIF3-p36 sequence (Table I). This indicates that the p36 cDNA is expressed as a housekeeping gene, and the frequency of appearance of the ESTs suggests a moderately abundant protein. The p36 subunit also shares 45.8% identity and 58.8% similarity with a 39-kDa protein in yeast, which was recently identified as the p39 subunit of yeast eIF3.2 Therelatednessof the mammalian and yeast proteins extends throughout their entire length (Fig. 4A) and is apparent in their WD repeat elements (Fig. 4B). Besides the human ESTs listed in Table I, the p36 cDNA sequence is homologous with 12 ESTs from A. thaliana (accession numbers H36544, H76382, T13850, T34639, T42527, T43638, T46040, T75627, Z27523, Z29843, Z46547, and Z46548) whose translation products show 36 to 71% identity to eIF3-p36. In addition, one EST each from rice and Zea mays (D23414 and T23353) exhibits 66% identity in the COOH-terminal region of the proteins. The p116, p110, and p36 Subunits of eIF3 Are Part of the Same Complex—Although the eIF3 subunits co-purify and all
Further support for p116, p110, and p36 being subunits of eIF3 comes from co-fractionation by FPLC with a Mono S (Pharmacia) ion exchange column. Each of the three affinity-purified antibodies reacts with its cognate protein in the same region where the other eIF3 subunits elute (results not shown). Finally, eIF3 complexes were immunoprecipitated with anti-eIF3 antiserum and with antibodies affinity-purified against the p170 subunit (Fig. 6). Four major immunoreactive eIF3 subunit bands (p170, p115, p47, and p35) are detected by SDS-PAGE and Western blotting with anti-eIF3 in the anti-eIF3 precipitate (panel A, lane 2), but not when a preimmune serum was used (lane 3). When the anti-p170 precipitate was analyzed (lane 4), bands corresponding to p170, p115, and p47 are detected, but not the p35 band. Apparently, p35 either readily dissociates upon immunoprecipitation with anti-p170 antibodies or is not a true component of eIF3. The same immunoprecipitates were then tested for the presence of p116, p110, and p36. Analysis by Western blotting with antibodies affinity-purified against recombinant p116, p110, and p36 (panel B) each detects its respective cognate subunit in the anti-eIF3 and anti-p170 precipitates, but not in the “precipitates” generated by preimmune or no antibodies. The results demonstrate that the three subunits are present in complexes with p170. Attempts to precipitate the eIF3 complex with affinity-purified anti-p110 antibodies were not successful (panel C, lane 4). Because the antibody immunoprecipitates the GST-p110 fusion protein and its partial degradation products (lane 5), its failure to immunoprecipitate eIF3 implies that the p110 epitopes are masked or altered when the subunit is incorporated in the eIF3 complex.

**DISCUSSION**

We have cloned and characterized cDNAs encoding human eIF3-p110 and eIF3-p36. The following facts establish the authenticity of the coding regions. The calculated masses of the proteins encoded by the two cDNAs, 105.4 and 36.5 kDa, are consistent with their assigned masses measured by SDS-PAGE. Transcription in vitro of the cDNAs and subsequent translation of the transcripts in reticulocyte lysates result in radiolabeled protein products that migrate in SDS-PAGE at precisely the same positions as the corresponding subunits in highly purified eIF3 or in crude HeLa cell lysates (Fig. 2B). Antibodies from a crude goat antiserum made against purified rabbit eIF3 that were affinity-purified against recombinant p110 and p36 overexpressed in E. coli specifically recognize the corresponding proteins in purified eIF3 (Fig. 2A). Furthermore, for the eIF3-p36 cDNA, an NH₂-terminal and two internal partial amino acid sequences obtained from the 36-kDa subunit of rabbit eIF3 match the deduced amino acid sequence.

The initiator AUG codon for each cDNA also has been identified unambiguously. For p110, a possible upstream AUG is ruled out by the presence of an in-frame UAG codon 18 nucleotides upstream of the initiator AUG. The sequence for the initiator AUG and NH₂-terminal coding region was obtained and confirmed by analysis of three independent EST cDNAs. For p36, the NH₂-terminal peptide sequence establishes the initiator AUG assignment.

During the characterization of purified eIF3 preparations, we noticed that the p115 band can be resolved by SDS-PAGE into two distinct bands of similar intensity when stained with Coomassie Blue (Fig. 2C). Protein in the faster migrating band is named the p110 subunit whose cDNA is cloned here. This band is recognized by the affinity-purified antibodies prepared with recombinant p110. Protein in the slower migrating band is named the p116 subunit (or hPrt1), which reacts with antibodies affinity-purified against a recombinant protein fragment expressed from a cDNA that is homologous to yeast PRT1.
Fig. 6. Co-immunoprecipitation of eIF3 subunits. Panel A, HeLa eIF3 purified by Mono Q column chromatography was incubated with no antibody (lane 1), crude anti-eIF3 antiserum (lane 2), its preimmune serum (lane 3), or affinity-purified anti-p170 antibody (lane 4). The immune complexes were isolated with Gamma Bind G Sepharose beads (Pharmacia) and analyzed by 10% SDS-PAGE and Western immunoblotting with anti-eIF3 antisera as described under "Experimental Procedures." The migration positions of eIF3 subunits are indicated on the upper panel, recombinant p110 (middle panel), and recombinant p36 (lower panel). Panel B, GST-p110 immunoprecipitated with the same antibodies as in panel A, but the immunoblots were analyzed with affinity-purified antibodies to recombinant p116 (upper panel), recombinant p110 (middle panel), and recombinant p36 (lower panel). Panel C, lanes 1–4 contain eIF3 immunoprecipitated with the same antibodies as in panel A; lane 5 contains the GST-p110 protein purified as described under "Experimental Procedures" and precipitated with affinity-purified antibodies against recombinant p110. The Western blot of the immunoprecipitates was performed with anti-eIF3 antiserum. The migration positions of eIF3 subunits are indicated on the left; that of GST-p110, on the right. The appearance of faster moving bands in lane 5 likely is due to partial proteolysis of the GST-p110 fusion protein.

(kindly provided by N. Méthot and N. Sonenberg). The two eIF3 subunits also are resolved by IEF/SDS-PAGE according to their isoelectric points, with p110 being more basic (Fig. 3). Although two similar proteins were resolved previously by IEF/SDS-PAGE, the more acidic protein was labeled "p115" and the more basic protein was apparently incorrectly believed to be a partial degradation product of the p170 subunit (34).

A number of lines of evidence indicates that p116, p110, and p36 are integral parts of the eIF3 complex. All three proteins are found in the 40 S region of sucrose gradients following sedimentation analysis of HeLa cell lysates. Each co-elutes with the other eIF3 subunits during ion exchange chromatography and are found in highly purified eIF3 preparations. Affinity-purified antibodies specific for eIF3-p170 precipitate p116, p110, and p36, thereby demonstrating that these proteins are present in complexes that contain p170. However, the data collected to date do not prove that a single type of eIF3 complex exists with all four proteins present in the same complex. It remains possible that there are different eIF3 complexes where, for example, either p110 or p116 is present, but not both, even though the apparent stoichiometry of p170, p116, and p110 is 1:1:1 (see Fig. 2C, lane 1). Attempts to demonstrate the simultaneous presence of p116 and p110 by immunoprecipitation with affinity-purified anti-110 antibodies were not successful, as these antibodies do not precipitate any of the eIF3 subunits, although they precipitate recombinant p110 from an E. coli lysate (Fig. 6).

Mammalian and yeast eIF3 are functionally conserved, inasmuch as the yeast complex functions nearly as well as mammalian eIF3 in an in vitro assay for methionyl-puromycin synthetase with purified ribosomes and initiation factors (11). It is therefore not surprising that the human p116 subunit is homologous with the yeast p90 subunit, also called Prt1. In addition, the human p36 subunit shares 46% sequence identity with yeast eIF3-p39, although the human cDNA does not produce an active protein in yeast. Although the human p110 subunit is homologous with a yeast protein called Nip1, Nip1 is not found in highly fractionated preparations of yeast eIF3. Either Nip1 dissociates from yeast eIF3 during fractionation, or the human and yeast eIF3 complexes differ in this respect. Another difference concerns the p16 (Su1) subunit of yeast eIF3, which is homologous to the mammalian eIF3, although eIF1 is not found in preparations of mammalian eIF3. It is surprising that the apparent subunit composition of eIF3 from mammals and yeast differs in these respects, given the strong conservation of most of the other initiation factors (2). Future efforts to obtain eIF3 complexes through tagging of individual subunit proteins, rather than by classical purification methods, may result in a better determination of the composition of eIF3 from yeast and mammalian cells.

It is noteworthy that eIF3-p36 and the homologous yeast subunit, p39, are members of the WD repeat family of proteins, as this has implications for their function in eIF3. The structures of prototypical WD repeat proteins, the β-subunit of G proteins, have been solved (39–41). The last three β-strands of the COOH-terminal WD repeat make a continuous β-sheet with the first β-strand of the NH2-terminal repeat, giving the protein a circular structure called a β propeller. eIF3-p36 contains at least five WD repeat elements (Fig. 4B) and in principle should be capable of forming a β propeller. WD repeat proteins are thought to interact with other proteins. For example, G, has been implicated in promoting the assembly of macromolecular effector complexes (42). One may speculate that the p36 subunit acts as a core subunit to which other eIF3 subunits bind. In support of this notion, the yeast p39 subunit of eIF3 appears to be essential for maintaining the integrity of the complex, as deletion of p39 results in diminished levels of all of the eIF3 subunits.2

The primary structure of eIF3-p36 is identical to a protein called TRIP-1 (19). TRIP-1 cDNA transfection of mammalian cells does not result in accumulation of the protein to levels much higher than those found in untransfected cells, a result reminiscent of that with the yeast homolog, eIF3-p39.2 TRIP-1 was identified in a yeast two-hybrid selection as a protein that associates with the TGF-β type II receptor. The association was also demonstrated by coimmunoprecipitation of the tagged proteins. Since TRIP-1 is phosphorylated by the activated receptor, the results suggest that eIF3 is a phosphorylation target of the TGF-β type II receptor. However, the antibodies that precipitate TRIP-1 or TGF-β type II do not coprecipitate other proteins such as the other subunits of eIF3 (19). It is therefore possible that the entire eIF3 complex does not associate with the receptor. Alternatively, the presence of the tag on TRIP-1 may have prevented the protein’s assembly into eIF3 com-

5. T. Naranda, unpublished results.
plexes. A third possibility is that eIF3-p36 may play a functional role as a free protein apart from eIF3, as appears to occur for the yeast eIF3 subunit, p16/Suc1 (13). Finally, it is possible that the eIF3 complex is the true target of the TGF-β type II receptor, but its association with eIF3 complexes is not sufficiently stable to persist during the immunoprecipitations. Further work is required to better elucidate the function of eIF3-p36 and its unexpected association with a membrane receptor.

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Conservation and Diversity of eIF3