Characterization of cDNAs Encoding the p44 and p35 Subunits of Human Translation Initiation Factor eIF3*

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Eukaryotic translation initiation factor 3 (eIF3) is a large multisubunit complex that plays a central role in the initiation of translation. It binds to 40 S ribosomal subunits resulting in dissociation of 80 S ribosomes, stabilizes initiator methionyl-tRNA binding to 40 S subunits, and is required for mRNA binding. eIF3 has an aggregate molecular mass of ~600 kDa and comprises at least 10 subunits. The cDNAs encoding eight of the subunits have been cloned previously (p170, p116, p110, p66, p48, p47, p40, and p36). Here we report the cloning and characterization of human cDNAs encoding two more subunits of human eIF3, namely eIF3-p44 and eIF3-p35. These proteins are immunoprecipitated by affinity-purified anti-eIF3-p170 antibodies, indicating they are components of the eIF3 complex. Far Western analysis shows that eIF3-p44 interacts strongly and specifically with the eIF3-p170 subunit, and weakly with p110/p116, p106, p40, and itself. eIF3-p44 contains an RNA recognition motif near its C terminus. Northwestern blotting shows that eIF3-p44 binds 18 S rRNA and β-globin mRNA. Possession of cloned cDNAs encoding all 10 subunits of eIF3 provides the tools necessary to elucidate the functions of the individual subunits and the structure of the eIF3 complex.

Translational control plays an important role in the regulation of gene expression in eukaryotes. The initiation phase of translation is one of the points at which changes in the rate of protein synthesis occur (1). Initiation of translation begins with dissociation of 80 S ribosomes into 40 and 60 S subunits. The 40 S subunit then binds a ternary complex consisting of eukaryotic initiation factor 2 (eIF2), GTP, and methionyl-tRNA (Met-tRNA). This 40 S preinitiation complex recognizes the m7G-capped 5' end of a mRNA, binds to the mRNA, and scans toward the 3' end until it forms a stable complex at the first AUG initiation codon. Subsequently, the 60 S subunit joins to form the 80 S initiation complex.

The variations in the initiation pathway are promoted by 11 or more soluble proteins called eIFs (2, 3). One of these, eIF3, plays a central role in the process. It binds to 40 S ribosomal subunits, thereby preventing 60 S association and promoting dissociation of 80 S ribosomes (4). It stabilizes Met-tRNA, binding to 40 S subunits and contributes to mRNA binding (5, 6) through its interaction with the eIF4F subunit of the mRNA m7G-cap binding protein complex, eIF4F (7) and with eIF4B (8). eIF3 also may be involved in the recognition of the initiation codon (9) and the GTPase activity of eIF2 activated by eIF5 (10). Therefore, elucidating the structure and function of eIF3 is essential for understanding the pathway and regulation of initiation.

eIF3 is the largest of the eukaryotic translation initiation factors, with an apparent mass of about 600 kDa and a shape resembling a flat triangular prism (11). Initial characterizations of mammalian eIF3 were based on biochemical methods applied to purified preparations from rabbit reticulocytes and HeLa cells (12–14). eIF3 also has been purified from the budding yeast, Saccharomyces cerevisiae (15, 16), as well as from numerous other species. The human factor has 10 or more different subunits named according to their apparent masses as determined by SDS-PAGE: p170, p116, p110, p66, p48, p47, p44, p40, p36, and p35. More recently, the cDNAs encoding eight of the subunits of human eIF3 have been cloned and sequenced: p170 (17), p116 (18), p110 and p36 (19) p48 (20), and p66, p47, and p40 (21). Similarly, the genes for the eight subunits of yeast eIF3 have been identified (22). The cloning of the cDNAs or genes encoding eIF3 subunits provides structural information about the factor and enables researchers to develop tools to better characterize its structure/function. In this paper, we describe the cloning and characterization of the cDNAs encoding eIF3-p44 and eIF3-p35, provide evidence that these subunits are part of the eIF3 complex, and show that the p44 subunit, which contains an RNA recognition motif (RRM), binds RNA. This report completes descriptions of the cloning of cDNAs encoding the 10 eIF3 subunits, thereby providing a firm base for further structural work on this important initiation factor.

EXPERIMENTAL PROCEDURES

Cloning of p44 and p35—eIF3 was purified from HeLa S3 cells essentially as described (14) and was subjected to SDS-PAGE. Bands corresponding to the p44 and p35 subunits were excised and digested with Lys-C protease in the gel. Following high performance liquid chromatography fractionation, N-terminal sequences of internal peptides were obtained by automated Edman degradation in the Protein Structure Laboratory (University of California, Davis). The sequences (shown by black overlines in Figs. 1 and 4) were used to search for matching sequences in the National Center for Biotechnology Information EST (expressed sequence tag) data base using the TBLASTN program (23). Mouse ESTs matching p44 peptide sequences were found and used to identify overlapping EST clones (GenBank accession numbers

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1 The abbreviations used are: eIF, eukaryotic initiation factor; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; UTR, untranslated region; EST, expressed sequence tag; bp, base pairs; KB, kilobase pairs; RACE, rapid amplification of cDNA ends; PVDF, polyvinylidene difluoride; RRM, RNA recognition motif; CHAPS, 3-(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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AA109090, AA270800, and W18370, and a hypothetical cDNA for mouse eIF3-p44 was assembled. The deduced amino acid sequence was used in a TBLASTN search to identify matching human EST clones. The longest clone (identification no. 293583) was purchased from ATCC (Rockville, MD) as a 1,237-bp insert in pT7T3D-PAC and sequenced. The sequence matching, namely Z25214 (clone identification no. HSBA6A022). By using the EST data base with the C-proximal peptide (Fig. 4) identified a human clone with 12 of 15 amino acid matches, namely Z25214 (clone identification no. HSBA6A022). For searching the EST data base with the C-proximal peptide (Fig. 4) identified a human clone with 12 of 15 amino acid matches, namely Z25214 (clone identification no. HSBA6A022).  

Northern Blotting—Total RNA was isolated from exponentially growing HeLa S3 cells using the RNAeasy Midi total RNA isolation kit (Qiagen, Santa Clarita, CA). RNA was subjected to 1.2% agarose gel electrophoresis (24) and blotted onto a nitrocellulose membrane using a 1-h downward capillary blot procedure (25). Probes corresponding to the coding regions of eIF3-p44 (bp 420–829) and eIF3-p35 (bp 307–824) were amplified by PCR-amplified full-length DNAs were digested (p44, primer 3; p35, primer 8) and subcloned into pET28c (Novagen, Madison, WI) to generate the tagged protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside.  

Western Immunoblotting—HeLa S3 cells were lysed in 20 mM HEPES/KOH (pH 7.5), 500 mM KCl, 1.3 mM magnesium acetate, 1% Triton X-100, and 0.5% SDS, hybridized overnight (hybridization buffer plus 10% dextran sulfate), washed repeatedly with Protein Binding Buffer (PBB containing 5% bovine serum albumin at 4 °C, and probed overnight at 4 °C with the radiolabeled reticulocyte lysates in 10 ml of PBB containing 3% bovine serum albumin. Blots were washed eight times for 10 min each with 10 ml of 0.1% Nonidet P-40 in PBB (28), dried, and exposed to film.  

Characterization of eIF3-p44 and eIF3-p35

| Primers used in cloning and analysis of eIF3-p44 and p35 | Primer sequence |
|----------------------------------------------------------|-----------------|
| Primer number | Primer sequence |
| 1 | GCCGTCATGCTTCCCGCCAGCAGTT |
| 2 | CCGGAATTCCTCATGCTACTGGGGACTTTG |
| 3 | CCGGAATTCCTGAGCCGCGCGCGCGCGCGC |
| 4 | CCGGAATTCCTATGCGCGCGCGCGCGCGC |
| 5 | CCAGAATTCCTGGGAGGGAGGT |
| 6 | TACCTCTCCACCAATGATTG |
| 7 | CAGAGCATACATGAGGTTCAGT |
| 8 | CCGGTACCTATGCTACTGGGGACTTTG |

TABLE I

| Primers used in cloning and analysis of eIF3-p44 and p35 | Primer sequence |
|----------------------------------------------------------|-----------------|
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| 3 | CCGGAATTCCTGAGCCGCGCGCGCGCGCGC |
| 4 | CCGGAATTCCTATGCGCGCGCGCGCGCGC |
| 5 | CCAGAATTCCTGGGAGGGAGGT |
| 6 | TACCTCTCCACCAATGATTG |
| 7 | CAGAGCATACATGAGGTTCAGT |
| 8 | CCGGTACCTATGCTACTGGGGACTTTG |
buffer (20 mM HEPES-KOH, 75 mM KOAc, 2 mM Mg(OAc)$_2$, 1 mM EDTA, 0.2% (w/v) CHAPS (pH 7.5)) plus 1 mg/ml yeast tRNA for 20 min. The blot was then hybridized in binding buffer plus yeast tRNA and RNase inhibitor with the $^{32}$P-labeled 18 S rRNA or $\beta$-globin mRNA (10$^7$ cpm) for 30 min, washed three times for 5 min each in binding buffer, and subjected to autoradiography, and subsequently to staining or Western immunoblotting.

### RESULTS

#### Cloning and Characterization of Human eIF3-p44 cDNA

To clone the cDNA encoding the p44 subunit of eIF3, a preparation of purified HeLa eIF3 was fractionated by SDS-PAGE and the band corresponding to eIF3-p44 was excised and used to obtain partial peptide sequences. As described in detail under “Experimental Procedures,” the peptide sequences were used to search the EST data base at the National Center for Biotechnology Information, and finally, three overlapping human ESTs encoding one of the partial peptides were identified. The longest of these, clone 293583, was sequenced but lacked DNA encoding the N-terminal 100 amino acids, as concluded from comparison to the mouse protein sequence deduced from overlapping ESTs. The missing 5'-region of the cDNA was obtained by the 5'-RACE procedure with a human pancreas cDNA library to yield DNA that codes for the N-terminal region and the putative initiator codon. DNA encoding the entire eIF3-p44 was constructed in pNoTA to yield pNo44–7, and the nucleotide sequence was deposited in the GenBank data base.

The cloned cDNA insert in pNo44–7 comprises 1,115 bp with 963-bp open reading frame that encodes a putative 35,694 Da protein of 320 amino acids (Fig. 1). The two peptide sequences mentioned above are found within the p44 sequence (identified with black overlines in Fig. 1). The 5'-UTR of the cDNA contains 9 bp, whereas the 3'-UTR contains 96 bp plus another 47 A residues that are preceded by the sequence AATAA 20 bp upstream. This sequence presumably serves as the polyadenylation signal. Northern blot analysis of HeLa total RNA identifies a major RNA species migrating at 1.4 kb (Fig. 2A), indicating that the cloned cDNA is not full-length, lacking a substantial portion likely at the 5' end. Nevertheless, the putative AUG initiation codon (residues 10–12) is very likely correctly identified, as the next in-frame AUG codon is far downstream, corresponding to amino acid residue 128. In addition, a nearly identical mouse cDNA (GenBank accession no. AA109090) corresponding to the 5'-end of eIF3-p44 contains an in-frame termination codon 4 codons upstream from the putative initiator AUG.

#### Comparison of the human eIF3-p44 amino acid sequence with those of other species.

The amino acid sequence of human eIF3-p44 has been aligned with its yeast homolog, yeIF3-p33 (GenBank accession no. AF004913), its mouse homolog (accession nos. AA109090, AA270800, and W18370), the homolog in S. pombe (accession no. AB011823), and the homolog in C. elegans (accession no. Z50044). The alignment was conducted with the Clustal X program and modified using Boxshade. Residues identical in three or more of the five species are shown with a black background, whereas three or more similar residues are shown with a gray background. The black bars over the sequence identify the peptides sequenced; the light gray bars correspond to the RNP1 and RNP2 motifs.

[FIG. 1. Comparison of the human eIF3-p44 amino acid sequence with those of other species.](#)

Cloning and Characterization of Human eIF3-p44 cDNA—To clone the cDNA encoding the p44 subunit of eIF3, a preparation of purified HeLa eIF3 was fractionated by SDS-PAGE and the band corresponding to eIF3-p44 was excised and used to obtain partial peptide sequences. As described in detail under “Experimental Procedures,” the peptide sequences were used to search the EST data base at the National Center for Biotechnology Information, and finally, three overlapping human ESTs encoding one of the partial peptides were identified. The longest of these, clone 293583, was sequenced but lacked DNA encoding the N-terminal 100 amino acids, as concluded from comparison to the mouse protein sequence deduced from overlapping ESTs. The missing 5'-region of the cDNA was obtained by the 5'-RACE procedure with a human pancreas cDNA library to yield DNA that codes for the N-terminal region and the putative initiator codon. DNA encoding the entire eIF3-p44 was constructed in pNoTA to yield pNo44–7, and the nucleotide sequence was deposited in the GenBank data base.

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Comparison of the human eIF3-p44 amino acid sequence with translated overlapping mouse EST sequences, using the GAP program (GCG, Madison, WI), indicates that the mouse and human proteins share 97% amino acid sequence identity (Fig. 1). The human eIF3-p44 amino acid sequence is also homologous to the eIF3-p33 subunit from the yeast S. cerevisiae, sharing 33% sequence identity and 42% similarity. As seen from Fig. 1, eIF3-p44 shares a fair amount of sequence.

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**FIG. 1.** Comparison of the human eIF3-p44 amino acid sequence with those of other species. The amino acid sequence of human eIF3-p44 has been aligned with its yeast homolog, yeIF3-p33 (GenBank accession no. AF004913), its mouse homolog (accession nos. AA109090, AA270800, and W18370), the homolog in S. pombe (accession no. AB011823), and the homolog in C. elegans (accession no. Z50044). The alignment was conducted with the Clustal X program and modified using Boxshade. Residues identical in three or more of the five species are shown with a black background, whereas three or more similar residues are shown with a gray background. The black bars over the sequence identify the peptides sequenced; the light gray bars correspond to the RNP1 and RNP2 motifs.
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identity and similarity with its homologs in Schizosaccharomyces pombe and Caenorhabditis elegans, where highly conserved regions are potentially important for function or structure. A PROSITE search identifies several potential phosphorylation sites and a consensus RRM (30) in the C-terminal quarter of the protein. The RNPI and RNP2 motifs are located between residues 280–287 and 241–246, respectively, and are identified with gray lines above them in Fig. 1.

Two experimental approaches were used to demonstrate that the cloned cDNA encodes the p44 subunit of eIF3. The coding region of pNo44–7 was inserted into pET28c (an E. coli expression vector) such that the final product contains an N-terminal 6-histidine tag. As described under “Experimental Procedures,” Ni2+ affinity chromatography. The resulting recombinant tagged p44 subunit was used to affinity-purify anti-p44 antibodies present in crude goat serum containing anti-eIF3 antibodies. Immunoblot analysis of purified eIF3 and HeLa lysate with anti-eIF3 serum and with affinity-purified anti-p170 antibodies is shown in Fig. 5 (lanes 8 and 9, which contain eIF3 and HeLa lysate, respectively). As shown in Fig. 5A (lane 3), the affinity-purified anti-p170 antibodies clearly immunoprecipitate p170, p116, p110, p47, p40, and maybe p35. Because the titers of antibodies to p44 is low in the crude anti-eIF3 serum, detection of p44 was problematic. Therefore, aliquots of the immunoprecipitated sample were subjected to Western blotting and incubation with affinity-purified antibodies to p44 and p35. As shown in Fig. 5B (lane 4) and C (lane 2), affinity-purified p170 antibodies co-immunoprecipitate p44 and p35, respectively, from HeLa lysates. These results indicate that p44 and p35 are part of the complex or can bind other eIF3 subunits in vitro.

eIF3 complexes were immunoprecipitated from a HeLa cell lysate with anti-eIF3 serum and with affinity-purified anti-p170 antibodies. The high specificity of the affinity-purified anti-p170 antibodies is shown in Fig. 5 (lanes 8 and 9, which contain eIF3 and HeLa lysate, respectively). As shown in Fig. 5A (lane 3), the affinity-purified anti-p170 antibodies clearly immunoprecipitate p170, p116, p110, p47, p40, and maybe p35. Because the titers of antibodies to p44 is low in the crude anti-eIF3 serum, detection of p44 was problematic. Therefore, aliquots of the immunoprecipitated sample were subjected to Western blotting and incubation with affinity-purified antibodies to p44 and p35. As shown in Fig. 5B (lane 4) and C (lane 2), affinity-purified p170 antibodies co-immunoprecipitate p44 and p35, respectively, from HeLa lysates. These results indicate that p44 and p35 are part of the eIF3 complex. The presence of p35 in both the eluate and supernatant fractions suggests that p35 partially dissociates from the eIF3 complex under the stringent assay conditions.

A demonstration that a putative subunit interacts directly with an authentic eIF3 subunit supports the view that it may
Comparison of the human eIF3-p35 amino acid sequence with its homolog in yeast.

The amino acid sequence of human eIF3-p35 is aligned with its yeast homolog (SwissProt identification no. Q05775) by procedures described in the legend to Fig. 1. Identical and similar residues are shown with black and gray backgrounds, respectively. The black bars indicate peptides used for BLAST searches.

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be a part of the eIF3 complex. Far Western analysis was performed as described under “Experimental Procedures” to look for possible direct protein-protein interactions of p44 and p35 with eIF3 subunits. Radiolabeled p44 and p35 probes were prepared in vitro translation and were incubated with blots containing eIF3 subunits fractionated by SDS-PAGE. As shown in Fig. 6A (lane 2), recombinant 35S-labeled p44 binds most strongly to the p170 subunit, less so to p116/110 and p66, and most weakly to itself (p44) and p40. Comparable analysis of a HeLa lysate shows that 35S-labeled p44 binds specifically and most strongly to the p170 subunit, less so to p66, and most weakly to itself (p44) and p40. Black staining (lane 1, 3.5 µg) and immunoblot analysis (lane 2, 0.5 µg) of purified eIF3 with goat antiserum to eIF3, fortified with affinity-purified antibodies to eIF3-p170, -p66, and -p44 (Fig. 7C, lanes 2 and 4). The probe binds to both 18 S rRNA and other eIF3 subunits and initiation factors have been shown to bind RNA, we wished to determine whether or not p44 binds RNA in vitro. To accomplish this aim, we employed Northern blot analysis. rc-eIF3-p66, rc-eIF3-p44, and purified eIF3 were subjected to SDS-PAGE, transferred to a PVDF membrane, and incubated with 32P-labeled 18 S rRNA (31) and was therefore regarded as a positive control. Proteins in purified eIF3 corresponding to p170, p66, and p44 bind to both 18 S rRNA and β-globin mRNA (Figs. 7, A and B, lane 1). Recombinant p66 and p44 also bind to either of the radiolabeled probes (lanes 2 and 3). The identities of the three RNA-binding subunits seen with purified eIF3 are established by immunoblotting the membrane used in panel A with affinity-purified antibodies to eIF3-p170, -p66, and -p44 (Fig. 7C). Proteins in the gel that bind RNA also react with the appropriate antibodies. Recombinant p44 appears to bind the 18 S
ribosomal RNA ~4 times more strongly than recombinant p66 (as calculated using a PhosphorImager (STORM 860; Molecular Dynamics, Sunnyvale, CA)), whereas rc-p44 and rc-p66 bind β-globin mRNA with equal intensity, comparable to what is seen when eIF3 subunits are probed (lane 1). It is noteworthy that p170 binds both RNAs, since the p170 sequence contains no discernible RNA-binding motif. The RNA binding activities appear to be specific because other proteins on the membrane do not bind to the RNA probe. However, one cannot determine absolute affinities with the Northwestern blotting procedure because this method may yield similar extents of binding even when binding affinities differ substantially.

**DISCUSSION**

We have cloned and characterized cDNAs encoding human eIF3-p44 and eIF3-p35. The following facts indicate that the cloned cDNAs indeed encode the two subunits of human eIF3. In *vitro* transcription and translation of the cDNAs in reticulocyte lysates result in radiolabeled protein products that comigrate in SDS-PAGE with the corresponding subunits in a purified preparation of eIF3. Antibodies from a crude anti-eIF3 goat antiserum that were affinity-purified with recombinant p44 and p35 overexpressed in *E. coli* specifically recognize the cognate protein in purified eIF3 and in HeLa lysates (Fig. 3B). In addition, partial peptide sequences for p44 and p35 match regions in the amino acid sequences deduced from the cloned cDNAs.

Two different approaches were used to prove that p44 and p35 are part of the eIF3 complex. First, co-immunoprecipitation of eIF3 subunits with affinity-purified antibodies to the p170 subunit was performed. The results show that both p44 and p35 are co-immunoprecipitated with the p170 antibodies.

Far Western analyses were then used to demonstrate that p44 and p35 interact with other eIF3 subunits.35S-Labeled p44 binds strongly and specifically not only to the p170 subunit in purified eIF3, but also to a 170-kDa protein in a HeLa lysate. The labeled probe also appears to bind unresolved p116/p110, p66, and more weakly to itself and to p40 (Fig. 6A). The weak interactions were confirmed with purified recombinant p44 and p40. Further support for these findings comes from preliminary yeast two-hybrid data, which show human eIF3-p44 interacts
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Fig. 6. eIF3-p44 interacts directly with a restricted set of eIF3 subunits. A, Far Western blots of purified eIF3 (5 μg; lanes 2, 4, and 6) and HeLa lysate (60 μg; lanes 3, 5, and 7) were incubated with reticulocyte lysates containing [35S]methionine-labeled eIF3-p44 (lanes 2 and 3), p35 (lanes 4 and 5), or unprogrammed lysate (lanes 6 and 7) as described under “Experimental Procedures.” Lane 1 contains 3.5 μg of purified eIF3 stained with Amido Black. B, Far Western blot of eIF3 (lane 2), re-eIF3-p40 (lane 3), and re-eIF3-p44 (lane 4) probed with [35S]methionine-labeled eIF3-p44. Lane 1 contains 3.5 μg of purified eIF3 stained with Amido Black. C, Far Western blot of eIF3 (lane 1) and Novex markers (lane 2) probed with [35S]methionine-labeled green fluorescent protein (GFP).

with p170, p116, p110, p44, and p40.2 Unfortunately, Far Western analysis is not a useful tool for studying proteins interacting with p35, because the protein is unusually sticky and binds to nearly everything on the membrane, even at high stringency. A Prosit search with eIF3-p44 identifies several potential protein kinase C and casein kinase II phosphorylation sites, myristoylation sites, and glycosylation sites (not shown). An RRM consensus sequence (30) close to the C terminus of the protein also is apparent. Northwestern analysis shows that p44 binds 18 S rRNA and this activity is destroyed when the RRM is deleted.3 Although yeast eIF3 functionally replaces human eIF3 in a methionyl-ppuromycin synthesis assay (15), p44 does not complement a p33 yeast knock-out.4 Similar failures to complement have been reported for the human p170 (32) and human p36 (33) subunits, suggesting that there is considerable divergence in the overall structures of yeast and human eIF3. Yeast eIF3-p33 has been shown to interact with both the p39 (homolog of human p36) and p90 (homolog of human p116) subunits (34, 35), whereas here we show a strong interaction of human p44 with p170 (homolog of yeast p110), and none with p36.4 The interaction of p44 with p116 seen by both the Far Western and two-hybrid analyses appears to be conserved, however. A precise determination of the composition and subunit stoichiometry of both human and yeast eIF3 remains to be made. The identification of subunit-subunit interactions, some of which are defined above, contributes to elucidating the structure of eIF3. Further such experiments are in progress and exploit the availability of the cloned cDNAs reported here.

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