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Cloning and characterization of the p42 subunit of mammalian translation initiation factor 3 (eIF3): demonstration that eIF3 interacts with eIF5 in mammalian cells

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ABSTRACT

Eukaryotic translation initiation factor 3 (eIF3) is a large multisubunit protein complex that plays an essential role in the binding of the initiator methionyl-tRNA and mRNA to the 40S ribosomal subunit to form the 40S initiation complex. cDNAs encoding all the subunits of mammalian eIF3 except the p42 subunit have been cloned in several laboratories. Here we report the cloning and characterization of a human cDNA encoding the p42 subunit of mammalian eIF3. The open reading frame of the cDNA, which encodes a protein of 320 amino acids (calculated M_r 35 614) has been expressed in *Escherichia coli* and the recombinant protein has been purified to homogeneity. The purified protein binds RNA in agreement with the presence of a putative RNA binding motif in the deduced amino acid sequence. The protein shows 33% identity and 53% similarity with the Tif35p subunit (YDR 429C) of yeast eIF3. Transfection experiments demonstrated that polyhistidine-tagged p42 protein, transiently expressed in human U20S cells, was incorporated into endogenous eIF3. Furthermore, eIF3 isolated from transfected cell lysates contains bound eIF5 indicating that a specific physical interaction between eIF5 and eIF3 may play an important role in the function of eIF5 during translation initiation in eukaryotic cells.

INTRODUCTION

Eukaryotic translation initiation factor 3 (eIF3) is a complex protein that plays an essential role in the initial binding of the initiator Met-$tRNA_f$ to the 40S ribosomal subunit, and also in the subsequent binding and scanning of the mRNA by the 40S preinitiation complex (40S·eIF3·Met-$tRNA_f$·eIF2·GTP), leading to the recognition of the initiation AUG codon to form the 40S initiation complex (40S·eIF3·mRNA·Met-$tRNA_f$·eIF2·GTP) (1–3). There is considerable uncertainty regarding the subunit composition of both mammalian and yeast eIF3. The factor has been isolated in several laboratories from a variety of eukaryotic sources based on an assay that measured its ability to stimulate mRNA translation in a protein synthesizing system using partially purified proteins (4–11). Mammalian eIF3, purified in this way, was reported to consist of nine major polypeptides, p170, p115, p110, p66, p47, p44, p40, p36 and p35 (12). In our laboratory we have purified eIF3 from rabbit reticulocyte lysates using an assay that directly measured the ability of eIF3 to bind to 40S ribosomal subunits and stimulate the transfer of Met-$tRNA_f$ to a 40S·eIF3 complex to form the 40S preinitiation complex (13). Our purified eIF3 preparation consisted of six major polypeptides of molecular masses 110, 67, 42, 40, 36 and 35 kDa but lacked the 170 kDa polypeptide reported by others (5,7,12) to be a constituent of mammalian eIF3. We have shown that p170 dissociates from mammalian eIF3 during the later stages of purification, and is not required for eIF3-mediated transfer of Met-$tRNA_f$ to 40S ribosomal subunits (13).

In the case of yeast eIF3, the protein purified on the basis of AUG-dependent methionyl-puromycin synthesis, was shown to consist of eight major polypeptides (14). Purified eIF3 was shown to stimulate methionyl puromycin synthesis ~3.5-fold. In contrast, when yeast eIF3 was purified based on a direct assay for the presence of Prt1p, a known subunit of yeast eIF3, it was observed that Prt1p co-purified with only four other polypeptides (15,16). This five-subunit complex was shown to stimulate the transfer of Met-$tRNA_f$ to 40S ribosomal subunits nearly 10-fold (15,16).

It appears clear that the true subunit composition of eIF3 cannot be resolved until the functional protein is reconstituted from separated subunits. Of the nine subunits comprising mammalian eIF3, cDNAs encoding all of them have been cloned in several laboratories (13,17–20) except for the p42 subunit of our mammalian eIF3 preparation. [This subunit is equivalent to the subunit p44 by Hershey's group (12).] Here we report the molecular cloning and characterization of a human cDNA encoding the p42 subunit of mammalian eIF3. The open reading frame (ORF) of the cDNA has been expressed in *Escherichia coli* and the purified recombinant protein has been shown to bind to

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RNA. When expressed in mammalian cells, p42 became incorporated into cellular eIF3. Furthermore, a major fraction of cellular eIF5 was found to be associated with eIF3. The implications of this association between eIF3 and eIF5 in the overall function of eIF5 in translation initiation are discussed.

MATERIALS AND METHODS

Initiation factors and antibodies

Initiation factor eIF3 was isolated from rabbit reticulocyte lysates as we described recently (13). It consists of six major polypeptide bands of apparent molecular masses 110, 67, 42, 40, 36 and 35 kDa but lacks the 170 kDa polypeptide reported by others (5–8) to be a constituent of the eIF3 complex. Total IgY antibodies specific for mammalian eIF3 subunits were isolated from egg yolks of laying hens immunized with purified rabbit reticulocyte eIF3 and subsequently purified by polyethylene glycol 8000 fractionation as described (13). IgY antibodies specific for p42 were subsequently isolated by affinity purification using purified recombinant p42 protein blotted onto aminophenyl-thioether paper (Schleicher and Schuell) following the procedure described by Ghosh *et al*. (21). Immunoblot analysis of eIF3 and p42 were carried out using appropriate purified chicken antibodies as probes. Rabbit anti-chicken IgY coupled to alkaline phosphatase was used to detect the binding of the primary antibody to eIF3 polypeptide(s) in the blots.

Protein sequencing

Approximately 1 mg of purified eIF3 was electrophoresed on an SDS–polyacrylamide gel (15% gel) and the separated subunits were then electrotransferred onto a nitrocellulose membrane. The blot was stained with Ponceau S (Sigma), and the portion of the membrane corresponding to the p42 subunit was excised and washed with water to remove excess stain. *In situ* protein digestion with trypsin, HPLC purification of tryptic peptides, and sequencing of several well-resolved peptides were performed at the Protein Microsequencing Facility of Sloan Kettering Cancer Research Institute, New York.

cDNA cloning, sequencing and other nucleic acid methods

To clone the cDNA for the 42 kDa subunit of mammalian eIF3, a HeLa cell λZapII cDNA library (Stratagene) was screened using as a probe a 385 bp polymerase chain reaction (PCR)-amplified cDNA as described by Sambrook *et al*. (22). This cDNA probe was synthesized by a reverse transcription (RT)–PCR reaction of total HeLa cell RNA using a Life Technologies, Inc. Kit. The primer sequences used were derived from a mouse EST clone (W29786) that encoded the two tryptic peptides of p42 subunit of rabbit reticulocyte eIF3. The cDNA inserts present in several homogeneous positive clones were isolated by *in vivo* excision as recombinant pSK-plasmids using filamentous helper phage. DNA was sequenced from both ends by the dideoxy chain termination method (22) using US Biochemical Sequencing Kit and a series of appropriate 17mer deoxyoligonucleotide primers. The DNA sequencing was also verified by automated sequencing using ABI PRISM™ AmpliTaq FS dye and Rhodanine terminator cycle sequencing kits. A ^{32}P-labeled luciferase RNA transcript was synthesized by *in vitro* transcription of linearized pGEM-luciferase cDNA (Promega) with T7 RNA polymerase in the presence of [α-^{32}P]UTP as the labeled ribonucleoside triphosphate (1600 c.p.m./pmol), according to manufacturer's specifications. A similar method was used to synthesize ^{32}P-labeled lacZ transcript. It should be noted that these *in vitro* transcripts lacked either the 5′-cap or the 3′-poly(A) tail.

Expression of p42 in *E.coli* and purification of the recombinant p42 protein

The ORF of the p42 cDNA in pGEM7Z(+) vector was amplified by PCR with *Pyrococcus* DNA polymerase (Stratagene) and two primer sequences as follows. N-terminus, 5′-dGGAAGATCTGCC-ATATGCCTACTGGAGACTTT-3′ having a *Bgl*II/*Nde*I overhang; C-terminus, an SP6 primer. The PCR product was sequenced to ensure error-free DNA synthesis, digested with *Nde*I and *Eco*RI and cloned into the same sites of pET-5a plasmid (Novagen) to yield the recombinant pET-5a-p42 expression plasmid. The recombinant plasmid was transformed into *E.coli* BL21 (DE3) cells, and the expression of the plasmid-encoded p42 protein was then induced by the addition of 1 mM isopropyl β-D-thiogalacto-pyranoside (IPTG) to an exponentially growing bacterial culture (3 l). The cells were harvested by centrifugation at 3 h post-induction. The frozen cells (5.6 g) were suspended in 17 ml of 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 30 mM KCl, 10 mM 2-mercaptoethanol and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), treated with 200 µg of lysozyme for 30 min at 4°C, and then disrupted by sonication. After the cell debris was removed by centrifugation at 15 000 *g* for 10 min, the supernatant was treated with 15 µg of pancreatic DNase, incubated at 0°C for 30 min, and then centrifuged at 48 000 r.p.m. for 150 min at 4°C in a Beckman 50 Ti rotor. The post-ribosomal supernatant (10 ml containing 80 mg protein) was adjusted to 0.28 M KCl by the addition of 4 M KCl and then loaded onto an 8 ml bed volume of a DEAE-cellulose column equilibrated in 50 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA and 10% glycerol (buffer A) + 0.28 M KCl. The unadsorbed proteins were washed from the DEAE-cellulose column with buffer A + 0.28 M KCl and then treated with solid ammonium sulfate to 70% saturation. The precipitated proteins were dissolved in 8 ml of buffer A + 70 mM KCl containing 0.5 mM PMSF and then dialyzed against a large excess of the same buffer for ~10 h. The dialyzed protein fraction was applied to a 9 ml bed volume of a column of DEAE-Sephacel equilibrated in buffer A + 150 mM KCl. The column was washed with this buffer. Under these conditions, the p42 protein did not bind to DEAE-Sephacel and emerged in the flow-through protein fraction. This protein fraction was then applied to a 5 ml bed volume of a phosphocellulose column equilibrated in buffer A + 150 mM KCl. After washing the column with ~40 ml of the same buffer, the bound p42 protein was eluted from the phosphocellulose column with buffer A + 300 mM KCl. The fraction containing p42 was pooled, diluted with an equal volume of buffer A and then applied to a 1 ml bed volume FPLC-Mono Q column (Pharmacia BioTech) equilibrated in buffer A +150 mM KCl. After washing the column with this buffer, the bound proteins were eluted with a linear gradient (1 ml/min) of 20 ml total volume from buffer A + 150 mM KCl to buffer A + 1 M KCl. Fractions of 0.5 ml were collected and assayed for p42 by western blotting. Fractions containing p42 (eluting at ~310 mM KCl) were diluted with an equal volume of buffer A and applied to a 0.5 ml bed volume of a poly(U) Sepharose column equilibrated in buffer A + 150 mM KCl. After

washing the column with the same buffer, a linear gradient of 4 ml total volume from buffer A + 150 mM KCl to buffer A + 0.8 M KCl was then applied. Fractions of 150 µl were collected and assayed for p42 by western blotting. Fractions containing p42 were pooled and stored at 0°C. The yield of homogeneous recombinant p42 was ~385 µg.

Cell culture and expression of p42-Myc-His in U20S cells

The human U2OS cell lines, obtained from ATCC, were kindly provided by Peter Guida and Dr Liang Zhu of this institution. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in a 7% CO_2 atmosphere.

The coding sequence of the p42-cDNA cloned in pGEM7Z vector was amplified by PCR with *Pyrococcus* DNA polymerase and two primer sequences as follows: N-terminus, 5′-dCCGCTC-GAGATGTCTGTCAATGTCAACCGC-3′ having a *Xho*I over-hang; C-terminus, 5′-dCCATCGATGAATTCAATGGCATCA-ATATCGATGTCGT-3′ having *Cla*I/*Eco*RI overhang. The PCR product was sequenced to ensure error-free DNA synthesis, digested with *Xho*I and *Eco*RI, and cloned into the same sites of pcDNA3.1(+)/*Myc*-His B plasmid (Invitrogen) to generate the recombinant pcDNA3.1(+)/*Myc*-HisB-p42 expression plasmid. U2OS cells were transfected with this expression plasmid by the calcium phosphate coprecipitation technique (23). Briefly, cells were seeded 12 h before transfection at 5×10^6/10 cm diameter plate. Transfections were carried out in four such plates with 20 µg of purified expression plasmid per plate. Another set of four plates were transfected with 20 µg of an empty expression vector, pcDNA3.1(+)Myc-HisB plasmid. Twelve hours post-transfection, the transfection medium was replaced by DMEM plus 10% fetal bovine serum. Cells were harvested 42 h post-transfection. The harvested cells were lysed by three cycles of freezing and thawing in a buffer containing 20 mM sodium phosphate, pH 7.4, 0.5 M NaCl and 1% Triton-X (Buffer B). The lysates were centrifuged at 10 000 *g* for 10 min and the supernatant was loaded onto a 0.5 ml bed volume of a Ni-NTA-agarose column (Qiagen) which was previously equilibrated with buffer B. The column was washed sequentially with (a) 5 ml of buffer B and (b) 5 ml of buffer B containing 25 mM potassium imidazole. The expressed p42-Myc-His protein was then eluted from the column using buffer B containing 250 mM potassium imidazole.

RESULTS

Cloning and characterization of the cDNA encoding the p42 subunit of translation initiation factor eIF3

The partial amino acid sequence of two peptides derived from the trypsin-digested p42 subunit of mammalian eIF3 (Fig. 1) was used to search the non-redundant database of the GenBank EST Division. A 385 bp mouse embryo cDNA clone (W29786) was found to encode both these tryptic peptides. This cDNA insert was amplified by RT–PCR of total HeLa cell RNA as described in Materials and Methods. The PCR product was sequenced confirming its identity with the EST sequence, and the ^{32}P-labeled PCR product was then used as a probe to screen a HeLa cell λZapII cDNA library yielding several positive clones. The largest cDNA insert (1.040 kb) present in one of the clones (clone a of Fig. 1A) was sequenced completely on both strands. Analysis of the nucleotide sequence (nucleotides +41 to +1080 of Fig. 1A) of

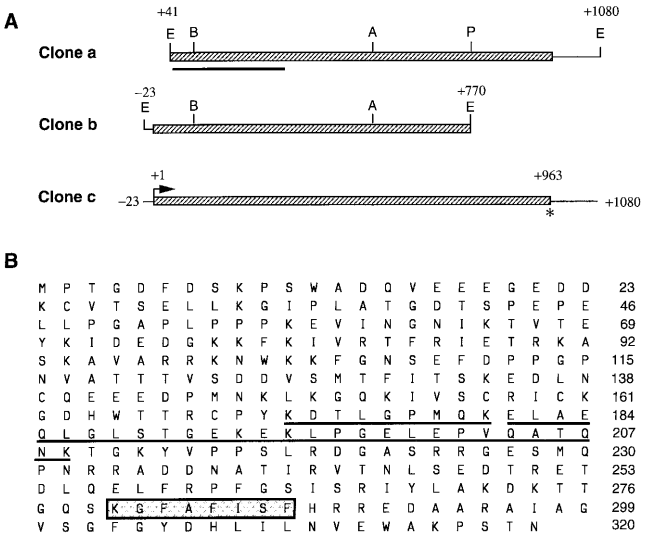

Figure 1. Characterization of the cDNA encoding the p42 subunit of mammalian eIF3. (**A**) Alignment of the cDNA clones used for sequencing and generation of a cDNA clone encoding the full-length p42 protein. Clone a, the 1040 bp partial HeLa cDNA clone; clone b, the 793 bp partial human skeletal muscle cDNA clone. The indicated *Bam*HI site in clones a and b was utilized to ligate the 121 bp *Eco*RI/*Bam*HI fragment (the 5′ end) of clone b to the 982 bp *Bam*HI/*Eco*RI fragment (the 3′ end) of clone a to generate the 1.103 kb cDNA (clone c) that encodes the full-length eIF3-p42 protein. The arrow in clone c represents the proposed start ATG codon (numbered +1) that was found in-frame with the partial amino acid sequences of two tryptic peptides of eIF3-p42. There is no other ATG in the cloned 5′ untranslated region. The translation stop codon TAA at nucleotide position +961 is marked by an asterisk. The black bar represents the HeLa cell cDNA fragment of clone a used as a probe for screening a λgt 10 skeletal muscle library as well as for northern analysis in Figure 2. Restriction sites are indicated. B, *Bam*HI; E, *Eco*RI, P, *Pst*I; A, *Apa*I. (**B**) The predicted amino acid sequence of full-length eIF3-p42 is shown with the partial amino acid sequences of two tryptic peptides of eIF3-p42 underlined. The putative RNP region is boxed.

this cDNA revealed that the fragment encodes both the p42 peptide sequences and the termination codon TAA in the same reading frame. The potential termination codon is followed by a poly(A) addition signal AATAAA and a poly(A) tail indicating that the cDNA encodes the complete C-terminus of the protein. Northern blot analysis of total poly(A) + RNA derived from different human tissues, using as a probe a ^{32}P-labeled 235 bp PCR product derived from the 5′ end of the 1.040 kb cDNA fragment of clone a (Fig. 1A, black bar), showed one major transcript of 1.5 kb in human heart and skeletal muscle tissues while in other tissues examined, the size of the transcript was ~2.2 kb (Fig. 2).

To obtain the complete cDNA, we rescreened a human skeletal muscle cDNA library (CLONETECH) using as a probe the above 235 bp PCR product derived from the 5′ end of the 1.04 kb cDNA of clone a. Several independent positive clones were identified. The largest cDNA insert (793 bp) in one clone (clone b of Fig. 1) contained the nucleotide sequence –23 to +770 (Fig. 1A). Since clones a and b contained overlapping p42 cDNA inserts, the presence of a unique *Bam*HI site at position +98 in both clones was utilized to construct the 1.103 kb p42 cDNA (–23 to +1080) in pGEM7Zf (+) vector (clone c). The nucleotide sequence of this cDNA has been deposited in the GenBank (accession no. AF020833). Block, Vornlocher and Hershey have also deposited

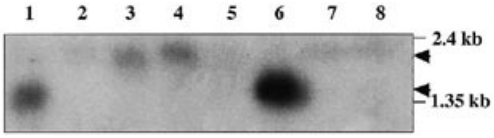

Figure 2. Northern blot analysis. A human multiple-tissue northern blot (Clontech, Palo Alto, CA) containing 2 µg of electrophoretically separated poly(A)+ RNA isolated from human organs was hybridized to a ^{32}P-labeled 235 bp *Bam*HI/*Eco*RI fragment of clone a (Fig. 1A, black bar). Conditions for hybridization and washing of the blot were according to standard protocols (22). The blot was analyzed by autoradiography. Lanes 1–8 represent poly(A)+ RNA isolated from human heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis, respectively. The blot also contained a set of RNA size markers, of which the positions of 1.35 and 2.4 kb RNA are indicated. The arrowheads indicate the positions of two size classes of p42 RNA transcripts.

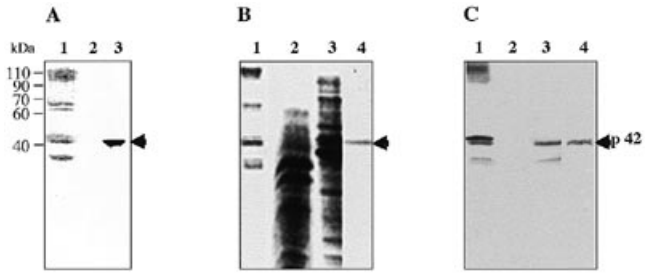

Figure 3. Expression of the eIF3-p42 cDNA clone. (**A**) SDS–PAGE (15%) of [^{35}S]methionine-labeled translation products in a rabbit reticulocyte transcription–translation system (TNT system, Promega). Lanes 2 and 3 are, respectively, *in vitro* translation in the presence of parental vector, pGEM7Zf(+) and recombinant vector pGEM7Zf(+) containing cloned p42 cDNA (clone c) under the transcriptional control of a T7 RNA polymerase promoter. Lane 1 denotes the Coomassie Blue-stained purified rabbit reticulocyte eIF3. The dried gel was analyzed by autoradiography. (**B**) Expression of eIF3-p42 in *E.coli*. Cell-free extracts, prepared from IPTG-induced cultures of *E.coli* BL21 (DE3) cells harboring either the parental plasmid pET-5a (lane 2) or the recombinant plasmid pET-5a-p42 containing the p42 coding region under the transcriptional control of T7 RNA polymerase promoter (lane 3), and purified recombinant p42 (lane 4) were electrophoresed in SDS–15% polyacrylamide gel followed by Coomassie Blue staining. Lane 1 shows Coomassie Blue-stained separated polypeptide subunits of purified eIF3. (**C**) Immunoblot analysis of bacterially expressed p42. Cell-free extracts of IPTG-induced cultures of *E.coli* BL21 (DE3) cells harboring either the parental plasmid pET-5a (lane 2) or the pET-p42 recombinant expression plasmid (lane 3), were subjected to western blot analysis using affinity purified anti-p42 antibodies as probes. Purified recombinant p42 (lane 4) and purified mammalian eIF3 (lane 1) were also analyzed by western blot. A set of molecular weight markers were run in a separate lane of each gel (not shown). The position of p42 is indicated by arrowhead in each panel.

the nucleotide sequence of a human cDNA encoding this eIF3 subunit which has been designated as p44 subunit by these investigators (accession no. U96074).

Analysis of the nucleotide sequence of the 1.103 kb cDNA in clone c showed that ATG codon numbered +1 is the first ATG codon at the 5′ end of the cDNA and is presumed to be the initiating AUG codon for p42 mRNA. Based on this presumption, the ORF of this cDNA encodes a protein of 320 amino acids with a predicted molecular mass of 35 614 (Fig. 1B). The sequence context surrounding this first ATG, GCGATGC, compares favorably with the consensus sequence for moderately strong initiation AUGs (24). However, this ATG codon in this cDNA is not preceded by an in-frame translational stop codon. Furthermore, the size of the cDNA is somewhat smaller than the size of the p42 mRNA as determined by northern analysis, indicating that we did not clone the complete nucleotide sequence preceding the first ATG codon of the 1.103 kb cDNA. We therefore further characterized the cDNA to show that it indeed encodes the full-length p42 subunit of mammalian eIF3 as follows.

First, the 1.103 kb cDNA present in pGEM7Zf(+) vector under the transcriptional control of a T7 RNA polymerase promoter was translated in a rabbit reticulocyte coupled transcription/translation system. Analysis of the ^{35}S-labeled translation products by SDS–PAGE showed that the major polypeptide synthesized migrated with the same mobility as the p42 subunit of purified rabbit reticulocyte eIF3 (Fig. 3A, compare lanes 1 and 3). Second, the putative ORF of the 1.103 kb p42-cDNA (nucleotides +1 to + 963 of Fig. 1) was expressed in *E.coli* (Materials and Methods) and the cell lysates were analyzed by SDS–PAGE. A polypeptide of ~42 kDa that migrated with the same mobility as the p42 subunit of purified mammalian eIF3 was observed (Fig. 3B, compare lanes 1 and 3). This polypeptide, which reacted strongly with affinity-purified chicken anti-p42 antibodies (Fig. 3C, lane 3), was not observed in *E.coli* cells containing the parental non-recombinant vector (Fig. 3B, lane 2 and C, lane 2). Finally, we examined whether expression of p42 in mammalian cells led to the incorporation of the expressed protein into the endogenous eIF3 protein complex. For this purpose, we transiently overexpressed p42 tagged with Myc and $(His)_6$ at the C-terminus in human osteosarcoma U2OS cells and the cell lysates were subjected to affinity chromatography in a Ni^{2+}-NTA affinity column initially equilibrated in a buffer containing 25 mM potassium imidazole. We observed that under these conditions, a major fraction of the expressed p42 protein was specifically retained on the column and could be eluted with 0.25 M imidazole buffer as judged by western blot analysis using anti-Myc antibodies as probes (data not shown). When these protein fractions were analyzed by western blot using chicken anti-eIF3 antibodies, we observed that a major fraction of eIF3 polypeptides were also retained on the Ni^{2+}-NTA column in 25 mM potassium imidazole buffer and coeluted with His_6-Myc-p42 in the 0.25 M imidazole eluate (Fig. 4A, compare lanes 6 and 7). In contrast, when U2OS cells were transfected with empty vectors, and the cell lysates were treated the same way, endogenous eIF3 polypeptides were not retained in the column, but emerged in the flow-through fraction (Fig. 4A, lane 3). The 0.25 M imidazole eluate did not contain any eIF3 polypeptides (Fig. 4A, lane 4). Taken together, these results indicate that we have cloned the complete coding sequence of the p42 subunit of mammalian eIF3.

Initiation factor eIF5 interacts with eIF3 in mammalian cells

Since expression of polyhistidine-tagged p42 in mammalian cells allowed specific retention of endogenous eIF3 on a Ni^{2+}-NTA agarose column, we tested whether any other initiation factors were bound to eIF3. In particular, we wanted to determine whether initiation factor eIF5, whose function in translation initiation is to specifically interact with the 40S initiation complex (40S·eIF3·mRNA·Met-tRNA$_i$·eIF2·GTP) to mediate the hydrolysis of bound GTP (25), associates with eIF3 *in vivo*. For this purpose, cell extracts prepared from U2OS cells expressing His-tagged p42 were subjected to Ni^{2+}-NTA agarose chromatography and both the column flow-through fraction containing unretarded proteins and 0.25 M imidazole eluate (containing eIF3) were assayed for eIF5 by western blotting using anti-eIF5 antibodies.

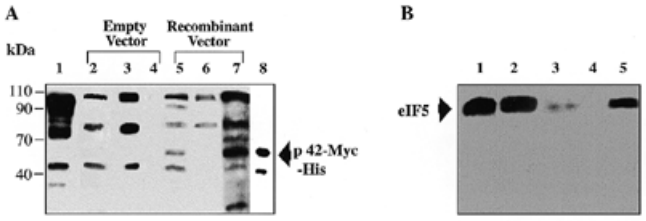

Figure 4. Expression of p42 in mammalian cells. (**A**) p42 associates with the eIF3 subunits *in vivo*. Human U20S cells were transiently transfected with either a p42 expression plasmid, pc DNA 3.1 (+)*Myc*-His B (recombinant vector) or the control vector not containing the p42-ORF (empty vector). Harvested cells were lysed, and proteins in cell lysates were subjected to Ni^{2+}-NTA-agarose column chromatography to purify the His-tagged p42 protein as described in Materials and Methods. For each cell lysate as indicated in the figure, the original lysate as well as the unretarded flow through fraction and the 0.25 M imidazole buffer eluate from the Ni^{2+}-column were subjected to SDS–15% PAGE followed by electrophoretic transfer to a PVDF membrane. In lane 1, purified eIF3 was electrophoresed to detect the position of eIF3 polypeptides. Lanes 2–4 represent protein fractions derived from cells in which the empty vector was expressed as follows: lane 2, crude lysate prior to Ni^{2+}-NTA chromatography; lane 3, Ni^{2+}-NTA column flow-through fraction; lane 4, 0.25 M imidazole eluate. Lanes 5–8 represent protein fractions derived from cells in which p42 was expressed as follows: lane 5, crude lysate prior to Ni^{2+}-NTA chromatography; lane 6, Ni^{2+}-NTA column flow-through fraction; lanes 7 and 8, 0.25 M imidazole eluate. The PVDF membrane was probed with 1:2000 dilution of total chicken anti-mammalian eIF3 antibodies (lanes 1–7) to detect eIF3 polypeptides, while lane 8 blotted separately was probed with anti-*Myc* antisera to detect the position of His and Myc-tagged p42 polypeptide. It should be noted that two additional polypeptide bands of ~100 and 48 kDa were also observed in lysates of cells expressing p42, compared with cells harboring empty vector (compare lanes 2 and 5). The band at p48 corresponds to His6- Myc tagged p42 while the band at 100 kDa presumably arose due to limited proteolysis of p110. (**B**) eIF5 associates with eIF3 in mammalian cells. The protein fractions obtained from the Ni^{2+}-NTA column chromatography described above were subjected to western blot analysis using affinity-purified anti-eIF5 antibodies (21) as probes. Lane 1, purified recombinant human eIF5; lanes 2 and 3, unretarded flow-through protein fractions from the Ni^{2+}-NTA column obtained from empty vector and p42 expression plasmid-transfected cell extracts, respectively; lanes 4 and 5, 0.25 M imidazole buffer eluates from the Ni^{2+}-NTA column obtained from empty vector and p42 expression plasmid-transfected cell extracts, respectively.

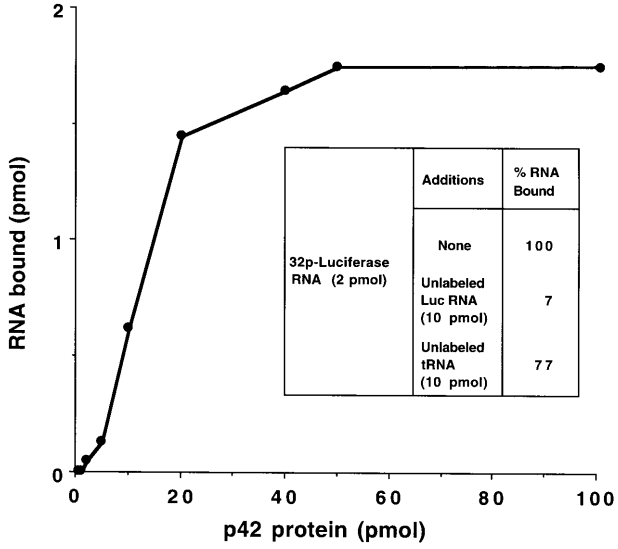

Figure 5. Binding of mRNA to eIF3-p42. Reaction mixtures (25 µl) contained 20 mM Tris–HCl, pH 7.5, 100 mM KCl, 5 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 2 pmol of T7-derived ^{32}P-labeled luciferase RNA transcript (167 000 c.p.m./pmol of RNA chain) and recombinant p42 protein as indicated. Following incubation at 30°C for 5 min, each reaction mixture was filtered through nitrocellulose membrane filters that were pre-soaked in the reaction buffer containing 1 mM sodium pyrophosphate. The filters were washed with ~4 ml of the reaction buffer, dried and assayed for radioactivity in a liquid scintillation spectrometer. Control values (<0.01 pmol) of [^{32}P]RNA bound to filters in reaction mixtures containing no p42 were subtracted to calculate RNA binding activity of p42. Insert table, reactions were carried out and analyzed as described above except that unlabeled luciferase RNA or tRNA was also added as indicated. Reactions were initiated by the addition of 20 pmol p42. The amount of ^{32}P-labeled luciferase RNA bound in the absence of competitor RNA was taken as 100% RNA bound.

As shown in Figure 4B, a major fraction (>90%) of endogenous eIF5, like eIF3, was retained on the Ni^{2+}-NTA column and coeluted with eIF3 from the column with 0.25 M imidazole treatment (Fig. 4B, compare lanes 3 and 5). In contrast, when similar experiments were carried out using cell extracts prepared from empty vector-transfected U2OS cells, endogenous eIF5, like eIF3, was not retained in the column and was detected in the unretarded column flow-through protein fraction (Fig. 4B, lane 2). The 0.25 M imidazole eluate did not contain eIF5 (Fig. 4B, lane 4). These results indicate that eIF5 associates with eIF3 in mammalian cells *in vivo*. Similar interaction between yeast eIF5 and yeast eIF3 was also observed by Phan *et al.* (16) when His-tagged Prt1p, a bona fide subunit of yeast eIF3, was expressed in yeast cells.

Interaction of recombinant p42 with RNA

To characterize recombinant p42, we purified the protein from the soluble fraction of extracts of *E.coli* expressing human p42 protein as described in Materials and Methods. p42 was monitored at different purification steps by SDS–PAGE followed by Coomassie Blue staining as well as immunoblot analysis of protein fractions by using affinity-purified polyclonal anti-p42 antibodies (data not shown). The final p42 fraction was >90% pure (Fig. 3B, lane 4). Purified p42 bound *in vitro* synthesized luciferase mRNA transcript synthesized from a T7 RNA polymerase promoter (Fig. 5). The protein also bound T7 promoter-derived lacZ transcript, a prokaryotic mRNA (data not shown). Under the same experimental conditions, the protein did not bind [^{32}P]tRNA (data not shown). The binding of ^{32}P-labeled luciferase transcript was nearly completely inhibited (>90%) by the addition of a 5-fold molar excess of unlabeled luciferase transcript. In contrast, addition of a 5-fold molar excess of unlabeled tRNA caused only ~23% inhibition of binding of luciferase RNA (Fig. 5, table). It should be noted that RNA transcripts used in these binding studies did not contain either a 5′-cap or a 3′poly(A) tail (Materials and Methods). These results, therefore, show that p42 binds unstructrued RNA in accord with the presence of an RNP motif in p42 (see Fig. 1B where the RNP motif is boxed).

DISCUSSION

Several lines of evidence presented in this paper indicate that we have cloned the human cDNA encoding one full-length p42 subunit of mammalian eIF3. The coding region of the cDNA has been expressed in *E.coli* and the recombinant p42 protein has been purified to near homogeneity. The purified recombinant protein (calculated M_r 35 614), which migrated with the same

mobility in SDS–PAGE as the p42 subunit of purified mammalian eIF3 (apparent M_r 42 000), binds a T7-derived luciferase mRNA transcript as well as a prokaryotic mRNA transcript but did not bind ^{32}P-labeled tRNA. These results are in accord with the presence of RNP motifs in p42 (Fig. 1). Among the other subunits of mammalian eIF3 only p110, the mammalian homolog of yeast Prt1p (26–28), has RNP motifs (13,18). However, p110 was unable to bind a radiolabeled RNA probe as measured by UV photocrosslinking and northwestern assays (18). In contrast, the p66 subunit of mammalian eIF3 which does not contain any obvious RNA-binding motif was shown to bind radioactive β-globin RNA in northwestern analysis (20). It is likely that the binding of eIF3 to mRNA (29,30) may occur via the p42 and the p66 subunits.

Comparison of the predicted amino acid sequence of mammalian p42 with the protein sequences in the yeast genomic database revealed that p42 has 33% identity and 53% similarity with the Tif35p (YDR429C) subunit of yeast eIF3 (Fig. 6). This subunit has been shown to be one of the five subunits of the yeast eIF3 core complex (16) and is also a constituent of the eight-subunit yeast eIF3 purified by Naranda *et al.* (14). The yeast Tif35p subunit also contains the RNA binding motif, RGLAFVTF (Fig. 6). Further analysis of the predicted amino acid sequence of mammalian eIF3-p42 showed that it is an acidic protein with a calculated pI of 5.9 and contains multiple potential phosphorylation sites for protein kinase C and casein kinase II. It remains to be seen whether eIF3-p42, like many other translation initiation factors, is phosphorylated *in vitro* and *in vivo* and whether phosphorylation of this subunit is involved in the regulation of eIF3 activity.

The subunit composition of eIF3 is variable depending on both the assay system used and the nature of the purification steps employed in different laboratories to purify the protein. Since eIF3 performs a variety of essential functions at different steps of the initiation pathway, the possibility exists that eIF3 may be a protein complex consisting of a core of a small number of subunits with one or more additional polypeptides able to associate with this core as required for discrete partial reactions in the initiation pathway. With the completion of the cloning and characterization of all the known subunits of mammalian eIF3, it will now be possible to express each of these subunits individually and in a variety of combinations in mammalian expression vectors, and isolate these different forms of eIF3 and examine each of them in all the partial reactions of the translation initiation pathway.

Finally, an important outcome of the present study is our finding that when eIF3 was isolated from extracts of mammalian cells by Ni^{2+}-NTA affinity chromatography (directed against polyhistidine-tagged p42 that was incorporated into the eIF3 complex), a major fraction of endogenous eIF5 was associated with eIF3 (Fig. 4B). The significance of this specific interaction between eIF3 and eIF5, which has also been observed in yeast cells by Phan *et al.* (16), is not immediately apparent. However, it is important to note that eIF5 functions in the translation initiation pathway after the 40S subunit containing bound eIF3 and Met-tRNA$_f$·eIF2·GTP ternary complex is positioned at the AUG codon of mRNA to form the 40S initiation complex (40S·eIF3·mRNA·Met-tRNA$_f$·eIF2·GTP) (1–3). It has been demonstrated that eIF5 interacts with the 40S initiation complex to mediate the hydrolysis of bound GTP (25). Hydrolysis of GTP causes the release of both eIF2 (as an eIF2·GDP complex) and eIF3 from the 40S subunit, an event that is essential for the subsequent joining of the 60S ribosomal subunit to the 40S complex to form a functional 80S initiation complex (30–33). However, eIF5, by itself, neither binds nor hydrolyzes either free GTP or GTP bound in a Met-tRNA$_f$·eIF2·GTP ternary complex in the absence of 40S ribosomal subunits (25,33). These observations suggest the possibility that the protein–protein interactions between eIF5 and the 40S subunit-bound eIF2 and eIF3 may be critical for the hydrolysis of GTP bound to the 40S initiation complex. In agreement with this hypothesis, a direct interaction between eIF2 and eIF5 (both in the mammalian and the yeast systems) was previously demonstrated in this laboratory (34,35). In the present study, we now show a specific complex formation between mammalian eIF3 and eIF5. Future experiments will focus on the significance of these protein–protein interactions in the mechanism and regulation of GTP hydrolysis mediated by eIF5 during translation initiation in eukaryotic cells.

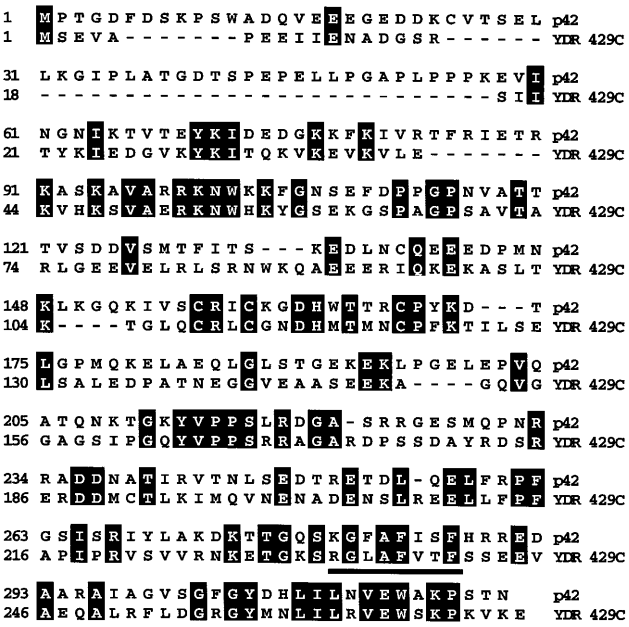

Figure 6. Alignment of the predicted amino acid sequences of human eIF3-p42 and its putative yeast *Saccharomyces cerevisiae* homolog, Tif35p (YDR 429C), a subunit of yeast eIF3. The deduced amino acid sequence of human eIF3-p42 was used to search the yeast genomic database by using the BLAST Search Program to identify the yeast homolog of human eIF3-p42. The yeast protein identified was YDR 429C/Tif35p, a subunit of yeast eIF3 (GenBank accession no. AF004913). The amino acid sequences of human eIF3-p42 and the yeast protein Tif35p are shown and are aligned for maximum sequence identity. The conserved residues are highlighted. The putative RNA binding motif in each sequence is underlined.

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NOTE ADDED IN PROOF

While this manuscript was under review, a paper was published [Block,K.L., Vornlocher,H.-P. and Hershey,J.W.B. (1998) *J. Biol. Chem.*, **273**, 31901–31908] that described the cloning of the p44 subunit of human eIF3. This subunit is equivalent to the p42 subunit described in this paper.