A cDNA encoding human eukaryotic initiation factor (eIF) 4H was subeloned into a bacterial expression plasmid for purification of recombinant protein. Recombinant human eIF4H (heIF4H) was purified to greater than 95% homogeneity and shown to have similar physical characteristics to eIF4H purified from rabbit reticulocyte lysate as described previously. Functional studies have revealed that recombinant heIF4H functions identically to rabbit eIF4H in stimulating protein synthesis, and the ATP hydrolysis and helicase activities of eIF4A. More detailed enzymatic studies revealed that eIF4H increases the affinity of eIF4A for RNA by 2-fold, but has no effect on the binding of ATP by eIF4A. eIF4H stimulates the helicase activity of eIF4A at least 4-fold, and it is postulated that this stimulation occurs through increasing the processivity of eIF4A. Northern blot analysis shows that eIF4H is expressed ubiquitously in human tissues, and displays different levels of expression in given tissues relative to eIF4B. Secondary structure analysis of heIF4H by circular dichroism suggest that eIF4H has a mostly β-sheet structure, which appears similar to other RNA recognition motif-containing proteins. Finally, it is suggested that eIF4H functions in translation initiation through protein-protein interactions that possibly stabilize conformational changes that occur in eIF4A during RNA binding, ATP hydrolysis, and RNA duplex unwinding.

The initiation of protein synthesis in eukaryotes is a complex process involving almost a dozen initiation factors that work in combination to bring the mRNA, the initiating methionyl-tRNA (Met-tRNA),1 and the 40 S ribosomal subunit together into a 48 S translation complex. This 48 S complex can then associate with the 60 S ribosomal subunit to complete the initiation phase of protein synthesis (for recent reviews on translation initiation, see Refs. 1–3). Eukaryotic initiation factor 4A, eIF4B, and eIF4F specifically interact with the mRNA and facilitate its binding to the 43 S ribosome complex (40 S subunit-eIF2-GTP-Met-tRNA). eIF4H has been identified as a new protein translation initiation factor that enhances the activities of initiation factors eIF4A, eIF4B, and eIF4F in various protein synthesis assays (4).

Previously, it was reported that eIF4H is not an abundant protein, and that ~1 mg can be purified from 6 liters of rabbit reticulocyte lysate. It was also reported that the human eIF4H protein is encoded by the human cDNA sequence KIAA0038 (GenBank™ accession number D26068) (4, 8, 9). Therefore, this human cDNA sequence was obtained for expression and purification of recombinant human eIF4H protein (heIF4H) from a bacterial system. Here, methodology is presented for easily obtaining large amounts of very pure heIF4H that allow for further biochemical and kinetic characterization of eIF4H. Results from studies described herein show that recombinant heIF4H has physical properties similar to those found in eIF4H purified from rabbit reticulocytes, with activities identical to those previously reported for rabbit eIF4H (4). The availability of large quantities of active and pure protein made it feasible to examine more closely the functional properties of heIF4H in translation. In addition, by using recombinant heIF4H in the studies described below, it was possible to begin examining the differences between eIF4B and eIF4H, which are known to be similar in both activity and amino acid sequence (human eIF4H is 39% identical and 62% similar to human eIF4B). From these studies, it may be possible to develop a model of how eIF4H functions during the initiation of protein synthesis.

**EXPERIMENTAL PROCEDURES**

**Materials**

Reagents were purchased from the following suppliers: restriction endonucleases, T4 DNA ligase, shrimp alkaline phosphatase, Tag DNA polymerase, PCR-grade NTPs, ampicillin, and IPTG from Roche Molecular Biochemicals; T4 polynucleotide kinase from New England Bio-labs; DNA oligonucleotides from Molecular Biology Core Laboratory, Case Western Reserve University; RNA-10 through RNA-15 oligonucleotides from Cybersyn; Prime-a-Gene labeling kit from Promega; human multiple tissue blot from CLONTECH; Megashortscript transcription kit from Ambion; rabbit reticulocyte lysate from Green Hectares, Oregon, WI; Superdex-75 FPLC chromatography resin from Amersham Pharmacia Biotech; CM-cellulose (type 52), DEAE-cellulose (type 52),...
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and phosphocellulose (type P-11) from Whatman Inc.; IEF and high molecular weight SDS-PAGE standards from Bio-Rad; pH 3.5–10 amphotolites from Amersham Pharmacia Biotech; pH 7–9 and pH 8–10 amphotolites from Bio-Rad; nitrocellulose filters (type HA) from Millipore Corp.; [3H]cysteine, [32P]CTP, and [γ-32P]ATP from NEN Life Science Products; and ATP, GTP, bovine serum albumin, phosphoenolpyruvate, and pyruvate kinase from Sigma.

Methods

Subcloning of Recombinant Human eIF4H—A clone of hIF4H cDNA was obtained as a generous gift from Dr. Nobuo Nomura at the Kazusa DNA Research Institute in Japan. This was provided in the pBluescript SK+ vector as an insert between the EcoRV and NotI sites oriented to allow for transcription of the sense strand from the T7 promoter (named pBS4H). DNA mini-preps were performed using the alkaline lysis technique (10). PCR primers were designed to engineer a unique NdeI restriction site at the AUG start codon for hIF4H (4H1: 5'-TGAT-GAGCGGATATGGCGGATTCC-3', NdeI site underlined), and a unique SpeI site at 15 nucleotides 3' of the UGA stop codon (4H2: 5'-CCCCAGC-CACGACTGCTCCACCA-3', SpeI site underlined) for subcloning into the pET-17b expression vector. The PCR product and pET-17b plasmid were digested with NdeI and SpeI. The pET-17b fragment was gel purified and recovered by electrophoresis, treated with shrimp alkaline phosphatase, and then ligated to the digested PCR fragment. The ligation reaction was transformed directly into NM522 competent cells, and selected by ampicillin resistance. Restriction digestion and DNA sequencing confirmed the presence of the hIF4H coding region within the plasmid, which was named pET4H. Subsequently, pET4H was transfected into bacterial strain BL21(DE3) for expression of recombinant human eIF4H upon IPTG induction.

DNA Sequencing—DNA sequencing was performed by either the Molecular Biology Core Laboratory at Case Western Reserve University, or the Molecular Biology Core Laboratory at the Cleveland Clinic Foundation.

Purification of Initiation Factors from Rabbit Reticulocyte Lysate—Purification of eIF4A from rabbit reticulocytes was described previously (4). Purification of other eukaryotic initiation factors has been described previously (11, 12).

Expression and Purification of Recombinant Human eIF4H from Bacteria—One liter of BL21(DE3)/pET4H culture was induced at mid-log phase with 50 μM IPTG for 4 h. Bacteria were pelleted, washed once in cold 25 mM Tris (pH 8.0), 10 mM EDTA, and then resuspended in cold standard buffer (SB = 20 mM Tris (pH 7.5), 1 mM DTT, 0.1 mM EDTA) with 25% glycerol and protein inhibitors (0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2.2 mg/mL aprotinin). Bacteria were lysed by sonication, and insoluble material was removed by centrifugation for 1 h at 50,000 × g.

Purification of recombinant hIF4H follows the standard procedures used to purify rabbit eIF4H (4) with some modifications and additions as outlined below. All steps in the purification of hIF4H were performed at 4 °C. Soluble protein was applied by batch wash sequentially to DEAE-cellulose and CM-cellulose, both equilibrated in SB + 50 mM KCl + 25% glycerol. Unabsorbed protein was collected by filtering the resin using a small column, and then washing with an equal volume of SB + 50 mM KCl + 25% glycerol. The protein that did not bind to either DEAE- or CM-cellulose (flow-through) was then applied to a column containing phosphocellulose equilibrated in SB + 50 mM KCl + 25% glycerol. The column was washed with at least 1 column volume of equilibrating buffer at −0.5 ml/min collecting 10-min fractions. A 50–500 ml KCl gradient (50 ml KCl/column volume) in SB + 25% glycerol was applied at −0.5 ml/min collecting fractions every 10 min and monitoring absorbance at 280 nm. Fractions were analyzed on a 15% SDS-polyacrylamide gel, and those containing eIF4H were pooled and dialyzed against a saturated (NH4)2SO4 solution with 20 mM Tris (pH 7.5) overnight to precipitate the protein. Precipitated protein was pelleted, resuspended in a small volume of SB + 500 mM KCl + 25% glycerol, and dialyzed overnight against the same. The sample was then applied to a Superdex-75 FPLC column and developed with SB + 500 mM KCl + 25% glycerol with 0.1 mM EDTA collecting 0.5-ml fractions and monitoring absorbance at 280 nm. Fractions were analyzed on 15% SDS-polyacrylamide gels, and those containing eIF4H were pooled and used in an ATPase assay both with and without eIF4A. Fractions that were free of containing Escherichia coli proteins with ATPase activity, that stimulated the RNA-dependent ATPase activity of eIF4A, and with the highest concentration of eIF4H were pooled and dialyzed against SB + 100 mM KCl + 25% glycerol. Protein concentration was determined by Bradford assay (eIF4H has no tryptophan and gives a poor UV reading at 280 nm), and stored at the liquid nitrogen vapor temperature.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)—SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (13). Separating gels used were 15% acrylamide-bisacrylamide (ratio acrylamide: bisacrylamide, 58:7).

Liquid Chromatography Mass Spectrometry (LC/MS)—Samples of rabbit and recombinant eIF4H were analyzed by LC/MS at the Cleveland Mass Spectrometry Facility, Cleveland State University. Approximately 50 pmol of sample (20–50 μl) was injected onto a 2.1 mm × 25 cm C-18 reverse phase HPLC column (Vydac) coupled to a Micro- mass Quadrupole mass spectrometer. Peaks from the HPLC were analyzed by mass spectrometry and molecular weights determined.

Two-dimensional Isoelectric Focusing/Sodium Dodecyl Sulfate (Two-dimensional IEF/SDS)-Polyacrylamide Gel Electrophoresis—Purified eIF4H was analyzed by two-dimensional IEF/SDS electrophoresis as described previously (4). Where noted, samples were dephosphorylated by treatment with shrimp alkaline phosphatase for 2.5 h at 37 °C.

Globin Synthesis Assay—Human and rabbit eIF4H were analyzed in a reconstituted rabbit reticulocyte lysate system lacking eIF4F, eIF4E, and eIF4H, by measuring the incorporation of [3H]cysteine into newly synthesized globin as described previously (4, 11, 14). Briefly, 50-μl reactions contained 20 mM HEPES (pH 7.5), 80 mM KCl, 1 mM DTT, 3 mM Mg(CH3CO2)2, purified elongation factors, aminoacyl-tRNA synthetases, ribosomes, globin mRNA, tRNA, 80 μM amino acids (minus leucine), 0.3 μCi of [3H]cysteine (specific activity 320 μCi/μmol), 1 mM ATP, 0.2 mM GTP, 0.34 unit of pyruvate kinase, and 3 mM phosphoenolpyruvate. The remaining initiation factors (eIF1A, eIF2, eIF4A, eIF5A) were added to the assay in partially purified form as fractions from phosphocellulose chromatography, except for eIF3, which was added in its purified form. After a 20-min incubation at 37 °C, the incorporation of [3H]cysteine into hot trichloroacetic acid-precipitable protein was measured by scintillation spectrometry. The amount of human eIF4H and rabbit eIF4H used was 1.5 μg.

RNA-dependent ATP Hydrolysis Assay—Combinations of the initiation factors eIF4A, eIF4F, eIF4E, and eIF4H were used to investigate RNA-dependent ATP hydrolysis by measuring the release of 32P, from [γ-32P]ATP as described previously (4, 15). Briefly, 20-μl reactions contained 15 mM HEPES (pH 7.5), 80 mM KCl, 2.5 mM Mg(CH3CO2)2, 1 mM DTT, 0–400 μM [γ-32P]ATP (specific activity of 2,000–4,000 cpm/pmol), 0–100 μM poly(A) (concentration determined using 100-mer units), and various concentrations and combinations of initiation factors. Reactions were incubated at 37 °C for 15 min, and 32P release was quantitated by precipitation of initiation factors eIF4A, eIF4E, eIF4F, and eIF4H added are shown in the legend of Table I. The amounts of eIF4A and eIF4F used to determine kinetic constants were 1.25 μg each.

Helicase Assay—The ability of eIF4H to stimulate the helicase activity of eIF4A was performed as described in detail in Ref. 7. Duplexes consisted of a 50-nucleotide RNA (RNA-1) made by in vitro transcription of an RNA oligonucleotide that is 10–15 bases in length (RNA-10 through 15), which are 5’-end labeled with 32P (using [γ-32P]ATP and T4 polynucleotide kinase). Preparation and sequences of RNA duplexes, as well as their stabilities are described in Ref. 7. In general, 20-μl reactions contained 20 mM HEPES (pH 7.5), 70 mM KCl, 2 mM DTT, 1 mM Mg(CH3CO2)2, 0.5 mM ATP, 2.0–4.0 μM eIF4A, and 0–4 μM eIF4H. Reactions were incubated at 35 °C for 0–15 min, and terminated by adding 5 μl of stop solution (50% glycerol, 2% SDS, 20 mM EDTA, 0.01% bromphenol blue, and 0.01% xylene cyanol). Reactions were analyzed on a 12% native polyacrylamide gel (19:1, acrylamide:bisacrylamide) (pre-electrophoresed at 4 °C for 20–30 min) for 1.5 h at 200 V, 4 °C. Gels were then scanned directly using an Ambis RADIONYALYSIS scanner for 50–60 min. Results were quantitated as described previously (7). Curves in Fig. 4B were fit using a single phase exponential equation as described in Ref. 7, and the curve in Fig. 4C was fit using a one-site binding equation, both using the GraphPad graphing software (Fitz).

Northern Blot—Probes were made from cDNA fragments of human eIF4H, human eIF4F, and β-actin by the random primer method using a SciScan 5000 densitometer (U. S. Biochemical Corp.) and OS-
RESULTS

Subcloning and Expression of Human eIF4H

The sequence of the cDNA encoding human eIF4H (heIF4H) and the aligned protein sequence, is shown in Fig. 1. This cDNA was subcloned into an expression vector to facilitate purification of large quantities of recombinant protein from bacteria. PCR primers 4H1 and 4H2 (shown in Fig. 1) were used to amplify the coding sequence for heIF4H with a unique NdeI restriction site engineered at the AUG start codon, as well as a unique SpeI site 18 base pairs from the UGA stop codon at the 3' end of the coding sequence. This PCR product was then subcloned into the pET-17b expression vector between the NdeI and SpeI sites, and this plasmid was named pET4H. Restriction digestion and DNA sequencing confirmed the presence and integrity of the heIF4H coding sequence. Fig. 2 shows the levels of recombinant heIF4H expressed using 50 μM IPTG.
and the molecular mass of standards (in kDa) are shown to the left.

Polyacrylamide gel and stained with Coomassie Brilliant Blue. Purification of recombinant heIF4H. Samples were analyzed on a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue.

FIG. 2. Expression and purification of heIF4H. A, expression of recombinant human eIF4H upon induction with IPTG. Samples were analyzed on a 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, molecular mass marker; lane 2, 1 μg of rabbit eIF4H; lane 3, 10 μl of total protein from an uninduced BL21(DE3) culture; lane 4, 10 μl of total protein after induction with 50 μM IPTG; lane 5, 10 μl of soluble protein from high speed centrifugation; lane 6, protein from the insoluble fraction. Migrations of heIF4H and the reference protein, “X,” are designated by arrows at the right of the gel, and the molecular mass of standards (in kDa) are shown to the left. B, purification of recombinant heIF4H. Samples were analyzed on a 15% polyacrylamide gel and stained with Coomassie Brilliant Blue. Lane 1, molecular mass marker; lane 2, 1 μg of rabbit eIF4H; lane 3, 10 μl of soluble protein from high speed centrifugation; lane 4, 10 μl of flow-through from DEAE and CM-cellulose columns; lane 5, 10 μl of flow-through from phosphocellulose column; lane 6, 10 μl of pool from phosphocellulose column; lane 7, 3 μg of heIF4H from Superdex-75 FPLC column. An arrow at the right of the gel designates migration of heIF4H, and molecular mass of standards (in kDa) are shown to the left.

(lane 4), and the amounts in the soluble and insoluble fractions (lanes 5 and 6, respectively). As can be seen, high quantities of heIF4H are expressed (compare lanes 3 and 4), and a significant portion of the heIF4H was in the soluble fraction (at least 75%). This was determined by comparing the ratios of protein “X” to heIF4H in the total protein fraction, soluble fraction, and insoluble fraction.

Purification of Recombinant Human eIF4H

To purify recombinant heIF4H from E. coli, techniques that were successful in purifying eIF4H from rabbit reticulocytes (4) were employed. As was done with rabbit eIF4H, all buffers used during the purification contained 25% glycerol to stabilize the protein. A detailed protocol for purification of heIF4H is described under “Methods.” Soluble heIF4H was obtained upon clarification of the cell lysate at 50,000 × g (Fig. 2B, lane 3), applied to DEAE- and CM-cellulose columns by batch wash in 50 mM KCl, and heIF4H was recovered in the flow-through (Fig. 2B, lane 4). This fraction was now enriched for heIF4H (compare lanes 3 and 4), and the majority of nucleic acids that bind tightly to DEAE-cellulose at low salt concentrations have been removed (data not shown). heIF4H from the DEAE- and CM-cellulose columns was then applied to a phosphocellulose column in 50 mM KCl and eluted using a 50–500 mM KCl gradient. Fig. 2B, lane 5, shows that the flow-through from phosphocellulose chromatography contained most of the contaminating E. coli proteins, while the majority of heIF4H was found to elute between 200 and 225 mM KCl (pool from gradient shown in lane 6). At this point the heIF4H preparation was ~70–80% pure, yet contained high amounts of contaminating RNA-dependent and -independent ATP hydrolysis activity (data not shown), and therefore would not be useful in some functional studies. Previous studies suggested that eIF4H by itself has no ATPase activity (4). To further purify heIF4H, it was applied to a Superdex-75 FPLC column in 500 mM KCl, and column fractions containing heIF4H were analyzed in an ATP hydrolysis assay. The final pool was made from fractions that were most concentrated for heIF4H, were free of contaminating ATPase activity, and stimulated the RNA-dependent ATPase activity of eIF4A. A sample from this pool is shown in Fig. 2B, lane 7. After this purification step, heIF4H was >95% pure, and was used in physical analysis and functional activity assays as described below.

Physical Characterization of heIF4H Relative to Rabbit eIF4H

Determination of Molecular Weight—Recombinant human eIF4H, when compared with the rabbit protein by SDS-PAGE, has a similar apparent molecular weight (refer to Fig. 2). The native molecular weight of heIF4H was determined empirically by size exclusion chromatography using the Superdex-75 FPLC column. Profiles from purifications of both rabbit and human eIF4H showed that both proteins eluted at the same position from this column (data not shown).

To determine their actual molecular weight, samples of both rabbit and recombinant human eIF4H were analyzed by LC/MS. Mass spectrometry analysis of the main reverse phase HPLC peak showed that the heIF4H preparation contained two species (data not shown). The major species of heIF4H has a molecular mass of 25,072 Da, and the minor species has one of 24,340. The molecular mass of human eIF4H calculated from its amino acid sequence is 25,200 Da. Removal of the initiating formylated methionine (fMet) after protein synthesis would yield a molecular mass of 25,069 Da, which is essentially identical to that of the major species determined by LC/MS within error (±2–4 Da). This result confirms, along with the complete DNA sequencing of the coding region in pET4H, that the expressed heIF4H protein has the expected amino acid sequence. The minor species seen in the heIF4H sample (24,340 Da) was most likely one of the contaminating E. coli proteins seen in Fig. 2B, lane 7, which may have co-eluted by HPLC with heIF4H.

Analysis of rabbit eIF4H by LC/MS revealed three species within the major HPLC peak with the following molecular masses: 25,166 Da (major species), 25,277 Da, and 25,387 Da (minor species) (data not shown). The calculated molecular mass of human eIF4H, as stated above, is 25,200 Da, yet it is known that the N terminus is blocked in the rabbit protein (4) so that the first initiating methionine is most likely removed and the new N-terminal amino acid subsequently acetylated (16–18). When the molecular mass is recalculated for the N-terminal blocked human eIF4H, the resulting size is 25,111 Da. However, all of the molecular masses determined by LC/MS for rabbit eIF4H were slightly higher than that calculated, and the
differences between the three species were 110–111 Da. The increase in molecular mass of the rabbit protein relative to that calculated for the human eIF4H sequence (55 Da) was most likely due to minor differences in amino acid sequence. The differences between the three species in the rabbit eIF4H preparation may be due to addition or removal of an amino acid(s) (average amino acid ~120 Da), or post-translational modification(s) of the protein.

**Determination of Isoelectric Point**—The isoelectric point for heIF4H was determined using two-dimensional IEF/SDS-polyacrylamide gel electrophoresis and results are shown in Fig. 3A. The heIF4H migrated on two-dimensional IEF/SDS gels as two distinct spots with the same apparent molecular weight, but separate pl values. The major band has a pl of ~8.4 and the minor ~8.2. The calculated pl of heIF4H with removal of the N-terminal fMet (as determined by mass spectrometry above) is 8.0. The minor band seen in the heIF4H preparation may be due to random loss of an amide nitrogen, which would decrease the protein’s pl, but not significantly affect its molecular weight. The migration of heIF4H on two-dimensional IEF/SDS gels was similar to that seen for rabbit eIF4H shown in Fig. 3B. Rabbit eIF4H migrated as two or more distinct bands, with the major band migrating at a pl of ~8.7, and a minor band migrating at an apparently higher molecular weight and lower pl close to 8.5. These results are similar to what was previously reported for rabbit eIF4H (4). It is possible that the slight discrepancies between rabbit and recombinant human eIF4H seen by two-dimensional IEF/SDS/PAGE are due to differences in amino acid sequence or protein modification, as is also suggested by the mass spectrometry data (see above).

Results from mass spectrometry analysis show that there are indeed multiple species within the rabbit eIF4H preparation. The major species seen by LC/MS had the lower molecular mass (25,166 Da), and may have been represented by the major band seen on two-dimensional IEF/SDS gels. The minor band migrating at the higher molecular weight and more acidic pl on two-dimensional IEF/SDS gels may have represented one of the minor species of rabbit eIF4H seen by LC/MS (25,277 or 25,387 Da), which could be a post-translationally modified form of eIF4H. A phosphorylation would increase the molecular mass of eIF4H by 85 Da and decrease the pl by ~0.2 pl units. To determine if rabbit eIF4H is phosphorylated, the protein was treated with alkaline phosphatase and reanalyzed by two-dimensional IEF/SDS/PAGE. Results of this experiment showed that there was no difference in the migration of bands between the untreated and phosphatase-treated samples (data not shown). Therefore rabbit eIF4H does not appear to be phosphorylated, but the possibility that it is modified in some other way cannot be ruled out at this time.
eIF4H has on the $K_{act}$ is minimal. It was found previously that eIF4B did not change the $K_{s}$ of eIF4A for ATP (80 μM), but did increase the $V_{max}$ by 2-fold when using 16 μM poly(A) (19). Here, the $K_{s}$ and $V_{max}$ values of eIF4A for ATP using 100 μM poly(A) were determined to be 286 μM and 187 fmol/s, respectively, and the addition of heIF4H had no effect on either the $K_{s}$ (261 μM) or $V_{max}$ (181 fmol/s) (data not shown). Differences between the $K_{s}$ and $V_{max}$ of eIF4A for ATP reported in this and previous studies will be discussed later.

Helicase Assay—Recently, it was reported that rabbit eIF4H stimulates the helicase activity of eIF4A (7). To determine if recombinant heIF4H is similar to rabbit eIF4H, both were analyzed in a RNA helicase assay using 2.0 nM RNA-1/RNA-12 duplex as substrate (described under “Methods” and Ref. 7). Fig. 4A shows a schematic representation of the helicase assay showing the RNA duplex being converted to single-stranded RNA monomers by incubation with eIF4A, ATP, and + eIF4H. Results of these experiments are shown in Fig. 4B. When 0.2 μM eIF4H was used, both rabbit and recombinant proteins stimulated the helicase activity of 0.4 μM eIF4A by a factor of 2 when measuring total amount of duplex unwound, and by a factor of 3.5 when measuring the initial rate of the reaction (femt mole of duplex unwound per min). In addition, heIF4H proved to be as active as rabbit eIF4H in this assay, and displayed less contaminating helicase activity than the rabbit protein. To determine the optimal ratio of eIF4H relative to eIF4A, 0–0.4 μM heIF4H was titrated into reactions containing 0.2 μM eIF4A and 2 nM RNA-1/RNA-12 duplex substrate. Fig. 4C shows that half-maximal unwinding was achieved when 0.11 μM heIF4H was used. Therefore, under the conditions used in this assay, maximal stimulation of eIF4A’s activity occurs when eIF4A and heIF4H are present in equimolar amounts.

To determine how eIF4H affects the helicase activity of eIF4A, the initial rates of unwinding were measured in the helicase assay using RNA duplexes of varying length and stability and equimolar amounts of eIF4A and eIF4H (0.2 μM). Fig. 5A shows the determination of initial rates using 0.4 μM eIF4A alone, while Fig. 5B shows the initial rates of 0.2 μM eIF4A with 0.2 μM heIF4H added. The addition of heIF4H increased the initial rate of unwinding by eIF4A for all duplexes analyzed (see below). Fig. 5C shows that there was a linear relationship between the ln(initial rates of unwinding) and the $ΔG$ values of the RNA duplexes for both eIF4A only and the eIF4A + heIF4H combination. The slope of the line obtained for eIF4A equals 0.31, which is similar to that reported earlier using 0.4 μM eIF4A (7). When heIF4H was added in equimolar amounts to eIF4A (0.2 μM each), the slope of the line changed to 0.19. The significance of this change will be discussed below.

It should be noted that the initial rates for eIF4A alone were determined using 0.4 μM protein to allow for comparison with previously published results, while those measured for the eIF4A + heIF4H combination used half as much eIF4A (0.2 μM). This was necessary since the helicase activity using 0.4 μM of both initiation factors was too fast for accurate measurement of the initial rate of unwinding of the less stable substrates (data not shown). Therefore, the initial rates for the eIF4A + heIF4H combination (0.2 μM each) should be compared with those obtained using 0.2 μM eIF4A. Since previous studies have shown that there is a linear relationship between the concentration of eIF4A and the initial rate of duplex unwinding (7), the slope of the line shown in Fig. 5C will be unchanged at 0.2 μM eIF4A, only shifted to lower ln(initial rate) values at the same $ΔG$ values. The calculated values for 0.2 μM eIF4A are shown in Fig. 5C as a calculated curve (dashed line). Therefore, when comparing the value calculated for 0.2 μM eIF4A alone to that obtained experimentally for eIF4A + heIF4H (0.2 μM each) using the RNA-1/RNA-10 duplex, heIF4H would increase the initial rate of eIF4A-dependent duplex unwinding by a factor of 2. Therefore, heIF4H does increase the initial rate of unwinding by eIF4A for all duplexes analyzed, and by examining the lines shown in Fig. 5C, the fold stimulation by heIF4H increases with the stability of the duplexes.

Expression of eIF4H mRNA in Human Tissue

Several translation initiation factors have been shown to be expressed ubiquitously in human tissues (20–22), and it is expected that all translation factors are expressed in all tissue types. To determine if eIF4H is also expressed ubiquitously in human tissues, a radiolabeled probe was created from the cDNA sequence of human eIF4H to hybridize to a human multiple tissue blot. Fig. 6A shows that eIF4H was expressed in all tissues analyzed. This confirms results from previous studies performed by Nomura et al. (8), who stated that HUMORFU_1 (heIF4H) was present in all human tissues and cell lines they examined. Also, the mRNA for human eIF4H was found to be approximately the same size as the reported length for the human cDNA, HUMORFU_1 (2,477 nucleotides) (8).

Because eIF4H and eIF4B both stimulate the ATPase and helicase activities of eIF4A, the question arises why are there two translation initiation factors with related functions. eIF4H and eIF4B may function differently in the translation of divergent mRNAs, in other non-standard initiation events (i.e. internal initiation, reinitiation, etc.), during development, or in particular tissues. To begin answering these questions, the expression of eIF4H relative to eIF4B was compared on the human multiple tissue blot to determine if there is tissue-specific expression of either mRNA. A cDNA probe for eIF4B was created and used to examine the same blot as eIF4H (Fig. 6A). eIF4B was also expressed ubiquitously in all tissue types, and the size of eIF4B mRNA was determined to be approximately the reported length for human eIF4B cDNA (3,877 nucleotides) (5). Fig. 6B shows a quantitative comparison of eIF4B and eIF4H expression in these tissues using β-actin (blot shown in Fig. 6A) as a control. It should be noted that this experiment does not indicate if there are equal amounts of

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**Table I**

| Factor | No eIF4H added | Rabbit eIF4H | Recombinant heIF4H |
|--------|---------------|--------------|-------------------|
| None (base line) | 1.8          | 8.9          | 2.8               |
| eIF4A  | 11.7          | 25.9         | 14.6              |
| eIF4A + eIF4B | 23.0        | 33.5         | 21.4              |
| eIF4A + eIF4F | 21.8        | 50.0         | 30.3              |
| eIF4A + eIF4B + eIF4F | 133.2 | 180.9 | 144.9 |

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2 N. Sonenberg, personal communication.
eIF4H and eIF4B mRNA in a given tissue, only their relative ratios. When the expression of a given mRNA in each tissue was standardized to that in liver on the same blot, it can be seen that eIF4B and eIF4H were expressed differently in several of the tissues examined. eIF4B expression was approximately 1.7-fold higher than eIF4H in pancreas, 3.7-fold higher in skeletal muscle, and 4-fold higher in kidney (this latter value may be slightly high due to background signal on the blot). Conversely, eIF4H was expressed approximately 2-fold higher than eIF4B in brain, and about 1.4-fold higher in placenta. It should be noted that the signal for the 2,000-nucleotide β-actin mRNA in skeletal muscle was obscured by the very intense signal from the muscle-specific 1,800-nucleotide β-actin mRNA (seen in heart and skeletal muscle), and therefore accurate quantitation of β-actin mRNA cannot be performed for this tissue. These results show that there was a difference between the expression of eIF4B and eIF4H mRNA in several tissues,
and may suggest tissue-specific functions of the factors with varying mRNAs.

**Analysis of Secondary Structure Using Circular Dichroism**

With milligram quantities of highly pure and active heIF4H, it was now possible to perform preliminary structural analysis of the protein. The heIF4H preparation used in these studies was the same preparation described in all enzymatic assays described above. Examination of the amino acid sequence of human eIF4H using the PHD structural prediction program suggested that eIF4H is an α-β protein (4). To assess the accuracy of this prediction, determination of heIF4H’s secondary structure was performed using circular dichroism and a representative spectrum is shown in Fig. 7A. Deconvolution of the heIF4H spectrum revealed that the protein had a high content of β-sheet and random coil (−50 and 45%, respectively), and very little α-helix (−1%) or turn (−4%). Denaturation studies using urea are seen in Fig. 7B with the change in molar ellipticity at 212 nm (β-sheet) shown as a function of urea concentration. Secondary structure was lost upon treatment with urea, proving that heIF4H was indeed structured, and the inflection point of the denaturation curve was at 5.8 M urea.

In this study, the cDNA for human eIF4H (HUMORFU_1) (4, 8, 9) was overexpressed in E. coli, and the recombinant protein, heIF4H, was purified without the use of an affinity tag to greater than 95% homogeneity using standard chromatography procedures. The recombinant protein was shown to have many of the same physical characteristics as rabbit eIF4H, and slight differences suggested there are minimal discrepancies in the amino acid sequence of human and rabbit eIF4H. Results obtained for the recombinant heIF4H in the globin synthesis and ATPase assays proved that the recombinant heIF4H has activities similar to rabbit eIF4H shown both in these and previous studies (4). When rabbit eIF4H and heIF4H were compared in the helicase assay, it was also found that both proteins had the same activities. In conclusion, all of the assays indicated that the rabbit and the recombinant human eIF4H had identical activity, confirming that the cDNA for HUMORFU_1 is indeed eIF4H, and supporting the conclusion that eIF4H is a translation initiation factor (4). In addition, heIF4H purified from E. coli was free of other contaminating eukaryotic translation initiation factors, had less contaminating ATP hydrolysis and helicase activities than the rabbit eIF4H preparation used, and therefore was the better candidate for more detailed studies.

Since heIF4H was functionally identical to rabbit eIF4H, it was used in the ATP hydrolysis assay to determine how eIF4H affects the ability of eIF4A to bind RNA and utilize ATP. It was found that heIF4H decreased the Km of eIF4A for poly(A) by a factor of 2, which suggests that eIF4H has only a mild effect on the affinity of eIF4A for RNA. This is also supported by previous studies that report eIF4H does not enhance the ATP-dependent binding of RNA by eIF4A in nitrocellulose filter binding assays (4). When heIF4H was added to similar experiments that measured the Km, and Vmax of eIF4A for ATP, it had no effect on either the Km or Vmax of eIF4A for ATP.
The $K_m$ of eIF4A for ATP measured in these studies, 290 $\mu$M, was different than that reported previously in similar assays (80 $\mu$M) (19). This can be explained by the fact that previous experiments were performed using subsaturating levels of poly(A) (16 $\mu$M, equal to the $K_{act}$ of eIF4A), while those performed here used saturating amounts (100 $\mu$M). The $K_{act}$ value obtained here was similar to that obtained by Lorsch et al. (23) of 330 $\mu$M under similar conditions. This would also explain why previous studies showed eIF4B to increase the $V_{max}$ by 2-fold (19), since experiments including eIF4B were done using saturating amounts of poly(A) ($K_{act}$ of eIF4A decreases to 60 $\mu$M in presence of eIF4B) and those with eIF4A only were not.

Despite the minimal effect seen by heIF4H in the ATPase assays, heIF4H appeared to enhance the RNA helicase activity of eIF4A to a much greater degree. To better understand how heIF4H enhanced this activity, the initial rate of unwinding by eIF4A with and without heIF4H was measured using different RNA duplexes, and the $\ln$(initial rate of unwinding) for each of these was then plotted against the $\Delta G$ values of the RNA duplexes. Results showed that heIF4H increased the initial rate of unwinding by eIF4A for all duplexes, and that the slope of the line in Fig. 5C ($\ln$(initial rate of unwinding) versus $\Delta G$) decreased upon addition of heIF4H. By examining how the addition of heIF4H affected the slope of the line in this experiment, it may be possible to determine the mechanism by which eIF4H stimulates the activity of eIF4A.

If the addition of eIF4H increased only the affinity of eIF4A for the RNA substrate, this would increase the number of active eIF4A-duplex complexes and would be analogous to increasing the concentration of eIF4A. It would then be expected that the resulting line from the $\ln$(initial rate of unwinding) versus $\Delta G$ plot would be parallel to the eIF4A only line, but with higher initial rate values at each $\Delta G$ value (refer to Fig. 5C comparing 0.2 $\mu$M eIF4A calculated to 0.4 $\mu$M experimental). This was not seen when heIF4H was added to the reaction. Therefore, the increase in helicase activity by eIF4H is not merely due to an increase in the affinity of eIF4A for substrate, which is consistent with the results from ATPase studies (heIF4H decreases $K_{act}$ only 2-fold). If the addition of eIF4H increases the activity of eIF4A by making it more processive (hydrolyze more than one ATP and/or actively unwind more RNA base pairs per RNA binding event), the initial rate would be expected to increase at all $\Delta G$ values, but the linear relationship might be different. Fig. 5C shows that the addition of heIF4H lead to a decrease in the slope of the line by allowing eIF4A to efficiently unwind more stable substrates, and unwind more RNA base pairs in the RNA duplex before dissociating. This is also seen in the ability of heIF4H to increase the maximum amount of duplex unwound over the course of the reaction (refer to Fig. 4B). These results all suggest that eIF4H increases the processivity of eIF4A.

Previous studies have shown that eIF4A dissociates from its substrate faster than it can hydrolyze ATP, implying that the protein cannot function by itself as a processive helicase (23). Related studies have shown that eIF4A goes through a cycle of conformational changes upon substrate binding, ATP hydrolysis, and product dissociation (24), and that these conformational changes are believed to be the actual helicase "motor" of eIF4A that causes unwinding of the RNA duplexes. This mechanism has also been suggested for the NS3 RNA helicase from hepatitis C virus (25). Further support for this mechanism in eIF4A comes from recent crystallographic studies of the ATPase domain of eIF4A, which was shown to have structure nearly identical to the corresponding domain of NS3 (26). Since eIF4H has only a mild effect on the affinity of eIF4A for mRNA, and does not increase the catalytic step of ATP hydrolysis, it is possible that the ability of eIF4H to enhance the helicase activity of eIF4A may be through a stabilization of one or more of the conformational changes necessary for eIF4A to unwind RNA duplexes. This would increase the processivity of eIF4A and allow for more unwinding by eIF4A before product dissociation.

Although eIF4H and eIF4B have similar functions in stimulating translation initiation, one can notice significant differences in their mechanisms from results of this and previous studies. First, eIF4B functions as a dimer while eIF4H functions as a monomer. Second, eIF4B has a greater ability to stimulate the ATPase activity of eIF4A than eIF4H. Using equimolar amounts, the eIF4B dimer stimulates the ATPase activity of eIF4A almost 5 times more than eIF4H (4). Third, eIF4B has been shown to greatly increase the affinity of eIF4A for RNA (11, 19, 27), while eIF4H has only a mild effect on the affinity. This third difference could be due to the fact that eIF4B contains a second arginine-rich RNA-binding domain in addition to the RRMs in its C terminus that is essential for RNA binding of eIF4B (6, 28). This additional RNA-binding domain, which is not present in eIF4H, may play a role in the increased affinity of eIF4A for RNA in the presence of eIF4B. Fourth, results from Northern blots show that eIF4B and eIF4H mRNA are expressed at different levels in varying tissue types. Most interesting is the fact that expression of eIF4B mRNA is elevated relative to eIF4H mRNA in one tissue type, while the converse is true in another tissue type. Quantitation of protein levels within these same tissues would need to be performed to confirm these results. The significance of these differences is not apparent at this time, but it does suggest that there is tissue-specific expression of eIF4B and eIF4H, and that the two initiation factors may have different effects on varying mRNAs.

With large quantities of highly pure heIF4H, it was possible for the first time to perform structural analysis experiments. In this report, the secondary structure of heIF4H was analyzed using circular dichroism (CD), and compared with what was predicted from the amino acid sequence. Previous computer analysis of the protein sequence using the PHD structural prediction program predicted eIF4H to be an $\alpha$-$\beta$ protein (4) with 22.4% $\alpha$-helix and 7.0% $\beta$-sheet. Eleven other secondary structure prediction programs gave differing results, and all 12 programs together provide predictions ranging from 9.2 to 31.6% $\alpha$-helix and 5.7–38.6% $\beta$-sheet. Actual analysis of heIF4H showed that the protein is composed mostly of $\beta$-sheet and random coil (50 and 45%, respectively), which is quite different than what was predicted by all programs used.

It is known that there is an RRm in eIF4H (4) that delineates approximately 36% of the amino acid sequence of heIF4H. When the structures of other RRm-containing proteins were examined, it was found that RRms typically have a $\beta\alpha\beta$-$\beta\alpha\beta$ structure with the RNP-2 and RNP-1 domains found in structurally adjacent $\beta$-strands ($\beta_1$ and $\beta_3$, respectively) (29–34). Two of the programs used (GOR I and SOPM) predicted a $\beta\alpha\beta$-$\beta\alpha\beta$ structure in the RRm of eIF4H, and all other programs predicted $\beta$-sheet and $\alpha$-helix in at least three of the six corresponding sections, suggesting that the RRm of eIF4H may exhibit typical secondary and tertiary structure. Two classes of RRms have been described where one class has been shown to contain the canonical $\beta\alpha\beta$-$\beta\alpha\beta$ structure and interacts well with RNA, while the second class contains less $\alpha$-helix and does not bind RNA (33, 35). Since eIF4H binds weakly to RNA (4) and has a low content of $\alpha$-helix (as determined by CD) the RRm of eIF4H may belong to this second class of RRms.

Although heIF4H has a high amount of random coil, this does not suggest that the protein is unstructured, as is confirmed by denaturation studies. It might be possible that part
of eIF4H is unstructured when not complexed with other initiation factors or RNA. This may explain why eIF4H is unstable when stored in solutions containing less than 25% glycerol (4). Other proteins are known to be partially unstructured until they interact with other proteins or ligands (36–43). An example is also found in eukaryotic protein translation where the eIF4E-binding proteins, 4E-BP1 and 4E-BP2, are completely unstructured in solution by themselves, but can inhibit translation by binding to eIF4E, which induces structural changes in the 4E-BPs (44, 45). Since eIF4H has activity only upon interacting with other translation initiation factors and mRNA, further structural studies may reveal changes in eIF4H protein conformation upon interaction with these other components.

In conclusion, this study provides a method for purifying large amounts of active human eIF4H from E. coli, and begins to describe how eIF4H functions in the initiation of translation through more detailed enzymatic analysis. Studies have shown that eIF4H works in translation initiation during the steps of mRNA recognition and utilization by stimulating the ATPase and helicase activities of eIF4A, and enhancing the translational activities of the other initiation factors eIF4B and eIF4F. eIF4H increases the affinity of eIF4A for poly(A) by 2-fold, yet has no effect on the affinity of eIF4A for ATP, and results suggest that eIF4H stimulates the helicase activity of eIF4A by making it a more processive helicase. From helicase studies it was also determined that eIF4H and eIF4A function optimally under these conditions when both proteins are present in a 1:1 molar ratio, and one might extrapolate that this could also be true in vivo. Since its affinity for RNA is weak, eIF4H most likely functions in translation initiation through protein-protein interactions with the other eIF4 initiation factors. These interactions might stabilize the conformation of eIF4H, as well as conformational changes in eIF4A that occur upon RNA binding and ATP hydrolysis that may promote RNA helicase activity.

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