A new protein with translational activity has been identified on the basis of its ability to stimulate translation in an in vitro globin synthesis assay deficient in eukaryotic initiation factor (eIF) 4B and eIF4F. This protein has been purified to greater than 80% homogeneity from rabbit reticulocyte lysate and has been given the name eIF4H. eIF4H was shown to stimulate the in vitro activities of eIF4B and eIF4F in globin synthesis, as well as the in vitro RNA-dependent ATPase activities of eIF4A, eIF4B, and eIF4F. Three tryptic fragments of eIF4H yielded amino acid sequences that were 100% identical to a human sequence found in the GeneBank™ that codes for a previously uncharacterized protein (HUMORFU_1). The calculated molecular weight of the protein encoded by this sequence, its predicted cyanogen bromide fragmentation, and calculated isoelectric point are all consistent with those determined experimentally for eIF4H. Also, the presence of an RNA recognition motif within HUMORFU_1 suggests that eIF4H may interact with mRNA. We conclude that this newly characterized protein, eIF4H, functions to stimulate the initiation of protein synthesis at the level of mRNA utilization, and is encoded by the gene for HUMORFU_1.

Protein synthesis is the process by which an mRNA sequence is translated into protein on ribosomes using aminoacyl-tRNAs as substrates. In eukaryotes, this process involves many accessory proteins, which are transiently associated with the ribosome and function either in the initiation, elongation, or termination of protein synthesis. The steps involving delivery of the mRNA to the ribosome and positioning of the first initiating methionyl-tRNA (Met-tRNAi) at the AUG start codon is termed initiation. This process involves at least 11 protein initiation factors (for a recent review on translation, see Merrick and Hershey (1)).

The first step in the initiation of the majority of mRNAs is the specific recognition of the 7-methylguanosine (m7G) cap of the mRNA by the multisubunit complex eukaryotic initiation factor (eIF) 4F. eIF4F is composed of three protein subunits, eIF4E (25 kDa), eIF4A (46 kDa), and eIF4G (150 kDa). The interaction of eIF4F with the m7G cap is facilitated specifically through the eIF4E subunit (2). Following binding of the eIF4F complex to the 5' end of mRNA, the initiation factors eIF4A (46 kDa) and eIF4B dimer (160 kDa) interact with the mRNA and facilitate “melting” of mRNA secondary structure through the helicase activity of eIF4A (3). This mechanism facilitates binding of the 40 S ribosomal subunit to the 5' end of the mRNA and may also provide the motor for the ATP-dependent process of scanning.

The other major step in the initiation of protein synthesis involves binding of the first aminoacyl-tRNA, Met-tRNAi, to the 40 S ribosomal subunit. This is facilitated by the translation factors eIF1A, eIF2, and eIF3. The Met-tRNAi forms a ternary complex (3°) with eIF2 and GTP (3° = eIF2-GTP-Met-tRNAi) that will then bind to the 40 S ribosomal subunit to form the 43 S preinitiation complex (40 S-eIF1A-eIF3-3°) (1). The 43 S complex binds to unfolded mRNA near the m7G cap, which may involve an interaction of the complexed eIF3 with the eIF4G subunit of eIF4F (4). The 43 S complex “scans” down the mRNA until the initiator AUG codon is recognized by the Met-tRNAi (1). Once the ribosome is in the correct position, eIF5 promotes the hydrolysis of the GTP in the 3° complex to GDP (1, 5, 6), causing the release of the 40 S-associated initiation factors and leaving a 40 S-Met-tRNAi-mRNA complex. Subsequently, the 60 S ribosomal subunit joins to form 80 S ribosomes on the mRNA with the Met-tRNAi positioned in the P site of the ribosome (1). In this state, the ribosome is ready to begin the elongation phase of protein synthesis.

Many of the factors involved in the initiation of eukaryotic protein synthesis as described above have been purified and characterized, although there is still an enormous amount of information not yet known about the details of initiation. The last eukaryotic initiation factor to be biochemically identified and characterized was eIF4F (7). Subsequently, several attempts have been made to identify novel initiation factors by genetic studies (8–10). These studies have yielded several proteins characterized as essential for protein synthesis of uniquely selected mRNA. Several of these were found to code

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† Current address: National Institutes of Health, Bethesda, MD 20892.

‡ To whom correspondence should be addressed: Dept. of Biochemistry, Case Western Reserve University, 10900 Euclid Ave., Cleveland, OH 44106-4935. Tel.: 216-368-3578; Fax: 216-368-3419; E-mail: wcm2@po.cwru.edu.

§ The abbreviations used are: Met-tRNAi, initiator methionyl-tRNA; m7G, 7-methylguanosine; eIF, eukaryotic initiation factor; eEF, eukaryotic elongation factor; 3°, ternary complex; HPLC, high pressure liquid chromatography; IEF, isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; NEPHGE, nonequilibrium pH gradient electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; SB, standard buffer; RRM, RNA recognition motif.
for known translation factors (10), while some were found to also have additional primary functions (8). Here we report the biochemical purification and characterization of a new protein from rabbit reticulocyte lysate that stimulates translation. This protein’s identity was determined by amino acid sequencing and it was defined as a unique translation factor with a sequence identical to a protein found in the data base of undescribed function. In the following article, the activities and characteristics of this protein, which has been named eIF4H, are described.

**EXPERIMENTAL PROCEDURES**

**Materials**

Reagents were purchased from the following suppliers: rabbit reticulocyte lysate from Green Hectors, Oregon, WI; Sephadex G-75, Sephadex G-75 superfine, and Ultrogel AcA-34 chromatography resins from Amersham Pharmacia Biotech; CM-cellulose (type 52), DEAE-cellulose (type 52), and phosphocellulose (type P-11) from Whatman; SynChropak RP-P-100 C-18 reverse phase HPLC column from SynChrom Incorporated; isoelectric focusing (IEF) and high molecular weight SDS-polyacrylamide gel electrophoresis (PAGE) standards from Bio-Rad; high and low molecular weight rainbow-labeled SDS-PAGE protein standards from Amersham Pharmacia Biotech; nonequilibrium pH gradient electrophoresis (NEPHGE) protein standards from Life Technologies, Inc.; polyvinylidene difluoride membrane (Immobilon-P) and nitrocellulose filters (type HA) from Millipore Corporation; pH 3.5–10 ampholytes from Amersham Pharmacia Biotech; pH 7–9 and pH 8–10 ampholytes from Bio-Rad; [14C]leucine and [32P]ATP from NEN Life Science Products; and trypsin, phosphoamidovvpyruvate, and pyruvate kinase from Sigma.

**Methods**

**Purification of eIF4H from Rabbit Reticulocyte Lysate**—Purification of eIF4H follows the standard procedures used to purify protein translation initiation factors (7, 11) with some modifications and additions as outlined below. All steps in the purification of eIF4H were performed at 4 °C. Rabbit reticulocyte lysate was centrifuged at 100,000 × g for 4 h, and the supernatant, containing initiation factors (7, 11) with some modifications and additions, was further separated within a 5–15% (w/v) sucrose gradient containing 500 mM KCl at 100,000 × g for 4 h, and the supernatant, containing initiation factors (7, 11) with some modifications and additions, was further separated within a 5–15% (w/v) sucrose gradient containing 500 mM KCl at 100,000 × g for 4 h, and the supernatant, containing initiation factors (7, 11) with some modifications and additions, was further separated within a 5–15% (w/v) sucrose gradient containing 500 mM KCl at 100,000 × g for 4 h, and the supernatant, containing initiation factors (7, 11) with some modifications and additions, was further separated within a 5–15% (w/v) sucrose gradient containing 500 mM KCl at 100,000 × g for 4 h, and the supernatant, containing initiation factors (7, 11) with some modifications and additions, was further 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**RESULTS**

**Purification of a New Translation Factor, eIF4H**—During the purification of eIF4B and eIF4F from rabbit reticulocyte lysate, an additional protein possessing translational activity was identified on the basis of its ability to stimulate protein translation in an *in vitro* globin synthesis assay deficient in eIF4B and eIF4F (Fig. 1). This new activity was found to elute from an Ultrogel AcA-34 gel filtration column later than eIF4F and eIF4B. This factor has been named eIF4H based on its various activities associated with mRNA utilization in protein translation, as will be described in this article. During further rounds of purification, eIF4H’s stimulatory activity was found to be unstable in the standard buffers containing 10% glycerol, which may explain why the factor was not identified earlier.

Subsequent steps employed buffers containing 25% glycerol, which have been used previously in the purification of eukaryotic elongation factor (eEF) 1A to maintain its stability and activity (18). Purification of eIF4H by its activity, as described under “Methods,” resulted in a greater than 80% pure preparation containing a polypeptide band with an apparent molecular weight of 25 kDa by SDS-polyacrylamide gel electrophoresis (Fig. 2A), and a native molecular mass of 36.75 kDa as determined by gel filtration chromatography (Fig. 2B). This discrepancy in size may indicate that the native protein has a nonglobular, asymmetric conformation, therefore affecting its mobility on the column.

To determine whether or not eIF4H is a unique translation factor, it was compared by SDS-polyacrylamide gel electrophoresis (Fig. 2A) to other previously characterized low molecular mass (<40 kDa) initiation factor peptides. eIF4H was found to migrate differently than the other factors shown: eIF1A (17 kDa), eIF4E (25 kDa subunit of eIF4F), and the α (35 kDa), β (36 kDa), and γ (40 kDa) subunits of the eIF3 complex. eIF4H was also found to differ from eIF6 (25 kDa) by its interactions with DEAE-cellulose and phosphocellulose: eIF4H bound to phosphocellulose and not to DEAE-cellulose, while eIF6 has been reported to not bind to phosphocellulose but does bind to DEAE-cellulose (19, 20).

**Translational Activities of eIF4H**—Purified eIF4H was found to barely stimulate protein synthesis (18% increase over background), if at all, in a globin synthesis assay deficient in eIF4B, eIF4F, and eIF4H (Table I). This low activity was different than that which had initially been seen during purification of eIF4H on the AcA-34 column (230% increase over background, Fig. 1). These fractions, though, may have been contaminated with small amounts of eIF4B, which then contributed to the higher levels of activity seen. When assayed at saturating levels, eIF4F by itself stimulated globin synthesis slightly better than eIF4H (Table I), while eIF4F stimulated synthesis by 3-fold over background. The combination of eIF4B + eIF4F increased activity by an additional 2-fold over that seen with eIF4B alone, and the combination of all three factors, eIF4B + eIF4F + eIF4H, led to an additional 30% increase in globin synthesis over that seen with eIF4B + eIF4F only.

Although eIF4H appears to stimulate protein synthesis only mildly in Table I, Fig. 3A shows that eIF4H dramatically stimulates (300–400% increase) globin synthesis when titrated into...
reactions containing subsaturating levels of eIF4B (0.3 μg). This significant stimulation of activity confirms that eIF4H is not eIF4B, and may also explain the higher activity seen for eIF4H-containing fractions during purification if these contained small amounts of eIF4B. Fig. 3B shows a similar titration curve of eIF4B with an amount of eIF4H that stimulates eIF4B’s activity well (0.7 μg). In this titration experiment, it can be seen that there is little stimulation of activity at saturating levels of eIF4B (>2.5 μg), which is similar to the lower stimulation seen in Table I using saturating levels of eIF4B and eIF4F. The dramatic stimulation seen at subsaturating levels of eIF4B suggests that eIF4H does function as a stimulatory factor in protein synthesis and that it may function in the same steps in initiation as eIF4B and eIF4F; however, eIF4H is not a cap-binding protein due to its inability to bind to am7GTP-Sepharose column (data not shown).

To further identify eIF4H’s function in translation, this protein was examined in an RNA-dependent ATPase assay (Table II). This model initiation assay measures indirectly the interactions of eIF4A, eIF4B, and eIF4F, with mRNA and ATP. Previously, eIF4A and eIF4F have been shown to possess ATPase activity in the presence of RNA, which is stimulated by eIF4B, and the activity is maximal when all three of these factors are present (15). Assays were performed using 2 μg of eIF4A and subsaturating levels of eIF4B and eIF4F to stimulate eIF4A’s RNA-dependent ATP hydrolysis activity (Table II, column 3). As expected, eIF4A displayed limited RNA-dependent ATP hydrolysis activity (50% increase in the activity seen without poly(A) added) that was stimulated by the addition of eIF4B (3.5-fold increase) and maximal when all three factors (eIF4A, eIF4B, and eIF4F) were present (13.8-fold increase over eIF4A alone).

When eIF4H was analyzed in the assay, we found that it had minimal ATPase activity either alone or in the presence of mRNA (Table II, row 1). Yet when eIF4H was added to ATPase assays containing various combinations of the initiation factors eIF4A, eIF4B, and eIF4F, the RNA-dependent ATPase activities of all combinations of these initiation factors were increased (Table II, last column). These results are consistent...
with the hypothesis that eIF4H stimulates the activities of the initiation factors eIF4A, eIF4B, and eIF4F during the steps involving mRNA binding and utilization in the initiation of protein synthesis.

As with the globin synthesis assay, titration curves of eIF4F and eIF4B in the RNA-dependent ATPase assays were performed to investigate more closely the stimulation by eIF4H. Fig. 4A shows the titration of eIF4B into reactions containing subsaturating levels of eIF4A and eIF4F with and without eIF4H. As with the globin synthesis assay, eIF4H enhances eIF4F's stimulatory activity markedly (3-fold) at subsaturating levels of eIF4B (≤0.6 μg). This increase in eIF4B's ATPase stimulatory activity is similar to that which was seen for protein synthesis in Fig. 3B. Fig. 4B shows a titration curve of eIF4F into reactions containing subsaturating levels of eIF4A and eIF4B with and without eIF4H. Unlike the results seen for the eIF4B titration in Fig. 4A, eIF4H stimulates eIF4F's ATPase activity less dramatically at low concentrations. At low levels of eIF4F (≤1 μg) there is very little increase in ATP hydrolysis by the addition of eIF4H.

**Amino Acid Sequence of eIF4H**—To further characterize eIF4H, and to confirm that it is truly a unique translation factor, the protein was subjected to amino acid sequencing. Amino-terminal sequencing of eIF4H resulted in no detectable sequence, suggesting that the amino terminus is blocked. Therefore, eIF4H was digested with trypsin, fragments were separated by reverse phase HPLC, and peptides were subjected to sequencing by Edman degradation. Because eIF4H was the main component of this preparation (greater than 80%), major peaks seen by HPLC analysis represent eIF4H peptide fragments. Several peaks yielded amino acid sequences, and these are shown in Fig. 5A.

When these sequences were checked against the protein data base using a BLAST enhanced alignment utility, they were found to match the amino acid sequence for the human open reading frame, HUMORFU_1, coded by the gene KIAA0038 (GeneBank™ accession no. D26068) (21, 22) with 100% homology for those HPLC fractions that yielded only one sequence (fractions 106, 156, and 170) (Fig. 5B). The amino acid sequence of fraction 64 matched two separate peptides with similar sequences, and fractions 156 and 170 contained overlapping sequences. This overlap was most likely due to a minor contamination of trypsin with chymotrypsin.

**Predicted Physical Characteristics of HUMORFU_1**—The amino acid sequence of HUMORFU_1 was further analyzed using various sequence analysis programs. The calculated molecular mass of HUMORFU_1 was 25.2 kDa, which is similar to that seen for rabbit eIF4H, and its amino acid composition was found to be similar to that obtained for eIF4H by amino acid hydrolysis (data not shown). Cyanogen bromide digestion of HUMORFU_1 was predicted to result in three fragments, an amino-terminal fragment of 17.3 kDa, and two additional fragments equalling 1.6 and 6.1 kDa each. Cyanogen bromide digestion of rabbit eIF4H yielded bands with molecular masses of ~18 kDa and ~7 kDa (Fig. 6). The 17.3- and 6.1-kDa predicted fragments coincide with what was seen experimentally with the hypothesis that eIF4H stimulates the activities of the initiation factors eIF4A, eIF4B, and eIF4F during the steps involving mRNA binding and utilization in the initiation of protein synthesis.

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As with the globin synthesis assay, titration curves of eIF4F and eIF4B in the RNA-dependent ATPase assays were performed to investigate more closely the stimulation by eIF4H. Fig. 4A shows the titration of eIF4B into reactions containing subsaturating levels of eIF4A and eIF4F with and without eIF4H. As with the globin synthesis assay, eIF4H enhances eIF4F's stimulatory activity markedly (3-fold) at subsaturating levels of eIF4B (≤0.6 μg). This increase in eIF4B's ATPase stimulatory activity is similar to that which was seen for protein synthesis in Fig. 3B. Fig. 4B shows a titration curve of eIF4F into reactions containing subsaturating levels of eIF4A and eIF4B with and without eIF4H. Unlike the results seen for the eIF4B titration in Fig. 4A, eIF4H stimulates eIF4F's ATPase activity less dramatically at low concentrations. At low levels of eIF4F (≤1 μg) there is very little increase in ATP hydrolysis by the addition of eIF4H.

| Initiation factors | No poly(A), no eIF4H | No poly(A) + 1.5 μg eIF4H | Poly(A) + 1.5 μg eIF4H |
|--------------------|-----------------------|--------------------------|-----------------------|
| None               | 0.0                   | 8.0                      | 2.1                   |
| eIF4A              | 7.8                   | 18.1                     | 11.7                  |
| eIF4B              | 1.0                   | 15.4                     | 11.1                  |
| eIF4F              | 0.4                   | 6.6                      | 4.3                   |
| eIF4A + eIF4B      | 10.6                  | 16.4                     | 40.6                  |
| eIF4A + eIF4F      | 8.7                   | 11.6                     | 40.9                  |
| eIF4B + eIF4F      | 5.9                   | 9.0                      | 32.9                  |
| eIF4A + eIF4B + eIF4F | 14.3               | 17.8                     | 161.0                 |

**TABLE II**

Effect of eIF4H on RNA-dependent ATP hydrolysis activity

Activities shown in picomoles of $^{32}$P, released in 15 min. A background of 24.6 pmol of $^{32}$P, released in the absence of protein and mRNA was subtracted from each value. Initiation factors were added to the reaction in the following amounts: eIF4A = 2 μg, eIF4B = 1 μg, eIF4F = 1 μg, and eIF4H = 1.5 μg. Where indicated, reactions contain 0.01 A$_{260}$ units of poly(A).

![Fig. 4](http://www.jbc.org/)

![Fig. 5](http://www.jbc.org/)

![Fig. 6](http://www.jbc.org/)
that eIF4H is at best present in less than one copy per 10
comparable staining. Based upon this analysis, it would appear
18% polyacrylamide SDS gel.
eIF4H loaded onto the gel in Fig. 7
B
), an identical gel containing both eIF4H
along with ribosomal proteins prepared from sucrose-cushion
possibility, eIF4H was analyzed by two-dimensional NEPHGE
ated primarily with the initiation process. To rule out this
translation factors from the polysomes, there is a possibility
by nitrocellulose filter binding assays (data not shown).
modification of the protein, which has yet to be characterized. The
protein may represent some basic post-translational mod-
which might have occurred during purification. Another expla-
for the more basic pl seen for eIF4H is that this form of
ated form of eIF4H may be a result of some limited proteolysis
C-terminal glutamic acid from the HUMORFU_1 sequence
a C-terminally truncated protein, since removal of a single
weight causing it to migrate with the dye front. Attempts to
m on the gel. This was most likely due to its small molecular
eIF4H, while the 1.6-kDa predicted fragment was not visual-
onized on the gel. This was most likely due to its small molecular
ancing causing it to migrate with the dye front. Attempts to
sequence the 18-kDa peptide fragment were unsuccessful, sug-
gesting that it is amino-terminally blocked. This coincides with
the 17.3-kDa predicted fragment of HUMORFU_1 being the
amino-terminal peptide fragment. The 7-kDa band from eIF4H
cyanogen bromide digestion did not transfer well to a poly-
vinyldiene difluoride membrane and therefore was not
The isoelectric point of eIF4H was determined by two-dimen-
sional IEF/SDS-polyacrylamide gel electrophoresis (Fig. 7A).
eIF4H was seen to migrate as two distinct forms, one with a pl
of ~7.8 and another more predominate form at ~8.5. The calculated
pl of the HUMORFU_1 sequence is 7.79. The later, more basic form seen in the eIF4H preparation may represent
a C-terminally truncated protein, since removal of a single
C-terminal glutamic acid from the HUMORFU_1 sequence
would result in a calculated pl of 8.6. This C-terminally trunc-
atd form of eIF4H may be a result of some limited proteolysis
which might have occurred during purification. Another expla-
ation for the more basic pl seen for eIF4H is that this form of
the protein may represent some basic post-translational mod-
ification of the protein, which has yet to be characterized. The
sequence for HUMORFU_1 contains a putative RNA binding
region RNP-1 sequence between residues 82 and 89 (KGF-
CYVEF), which is consistent with preliminary studies that
show eIF4H to bind weakly to mRNA by UV cross-linking and
by nitrocellulose filter binding assays (data not shown).
Because of the high ionic strength used to separate the translation factors from the polysomes, there is a possibility that
eIF4H might be a loosely bound ribosomal protein associ-
ated primarily with the initiation process. To rule out this
possibility, eIF4H was analyzed by two-dimensional NEPHGE
along with ribosomal proteins prepared from sucrose-cushion
polysomes. When compared with a gel displaying only polye-
somal proteins (Fig. 7B), an identical gel containing both eIF4H
plus polysomal proteins (Fig. 7C) showed that eIF4H migrated
as a distinct band separate from the characteristically basic
ribosomal proteins. Most importantly, eIF4H was undetectable
in the gel containing only polysomal proteins. It was estimated
that each ribosomal protein is present on the gel in approxi-
mately 0.5–1-µg quantities. This is equivalent to the amount of
eIF4H loaded onto the gel in Fig. 7C and shows that they have comparable staining. Based upon this analysis, it would appear
that eIF4H is at best present in less than one copy per 10
ribosomes.
eIF4H did show a higher level of stimulation in the globin
synthesis assay (300–400% increase), as well as in the ATPase
assay (200% increase), when it was measured using subsatu-
rating levels of eIF4B (Figs. 3B and 4A, respectively). The
dramatic stimulation seen at subsaturating levels of eIF4B in
Fig. 3B show that the activities of eIF4H and eIF4B in the
globin synthesis assay are not merely additive, and we can
conclude that eIF4H does function as a stimulatory factor in
protein synthesis. A similar increase in eIF4B's ATPase stim-
ulatory activity was observed in Fig. 3A, and suggests that eIF4H functions the same in the two different assays. Conversely, in Fig. 4B we see that eIF4H does not enhance eIF4F's activity at low concentrations, but
that their RNA-dependent ATPase activities are additive. It is
possible that eIF4H acts as a stimulatory protein which func-
tions primarily to augment eIF4F's activities, or it may func-
tion when levels of eIF4B are limiting.
Initial mRNA-binding studies suggest that there is at least a
weak association of eIF4H with mRNA. This is also substanti-
ated by eIF4H's interaction with phosphocellulose requiring an
ionic strength of approximately 225 mM for elution. Yet, eIF4H
does not enhance the ATP-dependent RNA-binding of eIF4A,
DISCUSSION
In this report, we have described a novel translational activ-
ity that stimulates protein synthesis in a reconstituted reticu-
locyte lysate system. This activity has been purified to near
homogeneity from rabbit reticulocyte lysate and was identified
as a protein of 25-kDa molecular mass. This protein has been
shown to stimulate the translational activities of eIF4B and
eIF4F in an in vitro globin synthesis assay, as well as stimulate
the RNA-dependent ATP hydrolysis activities of the initiation
factors eIF4A, eIF4B, and eIF4F. Amino acid sequencing has
identified this new protein as being coded by the gene
KIAA0038 for the human open reading frame, HUMORFU_1, a
previously uncharacterized protein whose predicted physical
characteristics are identical to those seen experimentally for
the purified rabbit protein.
Stimulation of globin synthesis by this newly purified pro-
tein may occur at any step during protein synthesis (initiation
or elongation), but the stimulation of the ATPase activities
of eIF4A, eIF4B and eIF4F strongly indicates that it functions
during the same steps in initiation of protein synthesis involv-
ing mRNA utilization that these eIF4 factors do. Historically,
the initiation factors that interact with mRNA and involve
subsequent binding of the mRNA to the 43 S preinitiation
complex are placed in the eIF4 group. Therefore, we have
named this new translation factor eIF4H, and believe that it
most likely interacts with one or more of the previously char-
erized eIF4 proteins to stimulate their activities.
When analyzed in in vitro translation assays, eIF4H stimu-
lated the globin synthesis activities of eIF4B + eIF4F by 30%
(Table I), while it stimulated the ATPase activities of eIF4A +
eIF4B and eIF4A + eIF4B + eIF4F by 130% and 71%, respec-
tively (Table II). The limited stimulation of eIF4B and eIF4F
activities by eIF4H in the globin synthesis assay relative to the
stimulation of eIF4A, eIF4B, and eIF4F activities in the
ATPase assay is most likely due to the fact that subsaturating
levels of proteins were used in the ATPase assay, while satu-
brating levels of the initiation factors were used in the globin
synthesis assay. Because eIF4H stimulated globin synthesis in
the presence of saturating levels of both eIF4B and eIF4F
(Table I), and eIF4H on its own had very little activity, it would
appear that eIF4H's stimulatory activity is not due to contam-
nation with either eIF4B or eIF4F and that eIF4H is a unique
and real protein synthesis stimulatory factor.

eIF4B, and eIF4F in preliminary assays (data not shown), but does stimulate the RNA-dependent ATP hydrolysis activity of these factors (Table II). This apparent discrepancy may be due to the differences in reaction conditions between the two assays, or that the eIF4H-mRNA complex is not stable enough to fully stimulate the mRNA-binding assay. Another possibility may be that eIF4H's stimulatory activity is exhibited not through an enhancement of binding affinities ($K_m$) of the factors for mRNA, but through the enhancement of the factors' catalytic activities ($V_{max}$) in ATP hydrolysis. In the case of eIF4B, it has been shown that the addition of this factor increases both the $V_{max}$ of ATP hydrolysis by eIF4A, as well as (and more dramatically) the $K_{act}$ (similar to $K_m$) of eIF4A for mRNA (23). When all three initiation factors (eIF4A + eIF4B + eIF4F) were present in ATPase reactions, an additional increase in the $V_{max}$ was also seen (23). The exact effect of eIF4B and eIF4F on eIF4A's ATPase activity when all factors are present in the assay is quite complicated, and suggests that the kinetics of this reaction are not simply defined. The addition of eIF4H may add yet another level of complexity to our understanding of this reaction. Furthermore, it is possible that eIF4H may react more strongly with the initiation factors than with mRNA, and therefore exhibit its stimulatory activity through these interactions and not necessarily through stimulation of RNA binding per se.

eIF4H has been shown to be separate from other initiation factors characterized so far by SDS-PAGE (Fig. 2A), as well as through differential interactions with various chromatographic resins during purification. To demonstrate that eIF4H is a nonribosomal protein, we utilized two-dimensional polyacrylamide electrophoresis to show that eIF4H has a differential mobility relative to other ribosomal proteins (Fig. 7, B and C). Also, if eIF4H were indeed a ribosomal protein, it should be present in levels equimolar to the other ribosomal proteins, yet eIF4H is not visible in a gel containing only polysomal proteins. These data prove that eIF4H is a nonribosomal protein, as well as support its identity as a distinct initiation factor.

eIF4H has also been identified as a unique translation factor by amino acid sequencing and therefore does not represent a truncated form of any other initiation factor or previously characterized ribosomal protein. Several peptide sequences from eIF4H were found to match identically to portions within the sequence encoded by the gene for the human open reading frame, HUMORFU_1. We believe that the sequence for HUMORFU_1 is identical to that for rabbit eIF4H due to the precedence for mammalian translation factors to all be ≥93% identical to each other (24).

The amino terminus of eIF4H was shown to be blocked due to the inability to sequence either the amino terminus of the intact protein or the 17-kDa band seen during cyanogen bromide digestion that represents the amino-terminal fragment by comparison to the predicted fragmentation of HUMORFU_1. Blockage of the amino terminus is a common feature seen for most of the eukaryotic protein translation factors sequenced so far (25). This process involves the cleavage of the amino-terminal methionine by aminopeptidase (26) and subsequent blockage of the new amino terminus by the addition of an acetyl group to this residue by amino-terminal acetylase (27, 28). The presence of either an alanine, glycine, serine, or threonine as the second amino acid in the protein promotes this process (26). The second amino acid in the sequence for HUMORFU_1 is an alanine, suggesting that this protein would be amino-terminally blocked in eukaryotes.

Other physical properties of eIF4H determined experimentally (molecular weight, isoelectric point, and amino acid composition) are consistent with those predicted for HUMORFU_1, supporting the identity of eIF4H as the protein encoded by the gene for HUMORFU_1. An analysis of the amino acid sequence of HUMORFU_1 using PROSITE identified several possible phosphorylation and myristylation sites, and one amidation site within the protein sequence, but as yet there is no indication that any of these post-translational modifications occur. One of these possible modifications, or another which has yet to be identified, may be responsible for the more basic pl seen experimentally for eIF4H (8.5) than was predicted (7.79) (Fig. 7A). Alternatively, eIF4H may have been proteolytically cleaved at the C terminus during purification resulting in an increase in pl (see “Results”). Analysis of the amino acid sequence of HUMORFU_1 using the PHD structural prediction program (at EMBL-Heidelberg) predicted its structure to be an α-β protein.

Searches for eIF4H homologs in the various data bases identified several proteins with a significant degree of homology. The sequence for HUMORFU_1 was found to have a 39%
and binding along with the basic region between residues 367 and 423 (32). This RRM region is not essential for RNA binding of eIF4B, but is required for the maximal stimulation of the eIF4A helicase activity (32). Since this RRM region in eIF4B is 45% identical to a segment of nearly identical length in eIF4H, it may suggest that eIF4H can also stimulate the helicase activity of eIF4A as it did the ATPase activity. This hypothesis has not been specifically addressed here in this article, but will be investigated in future studies. In addition, this similar RRM region within Tif3/Stm1 was also found to be important for its function since site-directed mutagenesis within the RNP domains eliminated the protein’s suppressor activity (30). This finding adds to the possible importance of this RRM region within eIF4H for its translational activity.

It is possible that eIF4H may facilitate the ability of some of the other initiation factors to overcome negative regulation, such as inhibition of eIF4E by 4E-BP1 (34, 35); or even enhance their positive regulation, such as the enhancement of eIF4A’s activities by eIF4B (1, 23). Closer examination of the titration curves in Figs. 3 and 4 show that eIF4H can greatly stimulate the activities at limiting amounts of eIF4B, and suggests that eIF4H serves as a positive regulator of protein synthesis at the level of initiation. eIF4H may also have an effect on the translation of different messages, where it may facilitate the translation of inefficient messages, or even affect internal initiation. The fact that eIF4H appears to stimulate the ATP-dependent process of mRNA binding to ribosomes offers the possibility that it may influence the competition between different mRNAs for translation.

It is estimated that ~1 mg of eIF4H can be purified from 6 liters of rabbit reticulocyte lysate. This is ~5- to 10-fold less than the milligram amounts of eIF4B or eIF4F and ~100-fold less than the mg amounts of eIF3 which can be obtained from the same volume of starting material (these are based on purification and may not reflect actual relative levels intercellularly). Low levels of eIF4H are also seen relative to ribosomal proteins during analysis of polysomal proteins by two-dimensional gel electrophoresis (Fig. 7, B and C). These limited amounts of eIF4H relative to other initiation factors also support the hypothesis that eIF4H plays a role as a stimulatory or regulatory factor in translation. It is known that the relative concentration of translation factors in different tissues in mammalian systems varies significantly, but at this point there is little experimental data evaluating the protein levels of eIF4H relative to other mRNA binding factors (eIF4A, eIF4B, or eIF4F) in discrete tissues, or its effect on differential translation of mRNAs in these tissue extracts.

This report has provided evidence for a new eukaryotic initiation factor, eIF4H, which stimulates protein translation. This new factor has been the first to be biochemically identified within the past 14 years since the identification of eIF4F. Current research on eIF4H involves studies of this new factor’s activities and interactions with other components involved in the initiation of protein synthesis in eukaryotes. These studies will help determine relative affinities of eIF4H for other protein factors as well as mRNA, and may help identify where in the process of mRNA binding and utilization eIF4H functions. Future studies will also analyze possible post-translational modifications of eIF4H and any effect these may have on activity. In addition, eIF4H’s concentration within various tissues will be measured and it will be determined whether this influences competition for mRNA translation.

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REFERENCES

1. Merrick, W. C., and Hershey, J. W. (1996) Translational Control, Chapter 2, pp. 31–69, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
2. Carberry, S. E., Friedland, D. E., Rhoads, R. E., and Goss, D. J. (1992) Biochemistry 31, 1427–1432
3. Rozen, F., Edery, I., Meirovitch, K., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1990) Mol. Cell. Biol. 10, 1134–1144
4. Joshi, B., Yan, R., and Rhoads, R. E. (1994) J. Biol. Chem. 269, 2048–2055
5. Trachsel, H., Erni, B., Schreier, M. H., and Staehelin, T. (1977) J. Mol. Biol. 116, 755–767
6. Benne, R., and Hershey, J. W. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 3005–3009
7. Carvalho, J. F., Carvalho, M. D., and Merrick, W. C. (1984) Arch. Biochem. Biophys. 234, 591–602
8. Valenzuela, D. M., Chaudhuri, A., and Maitra, U. (1982) J. Biol. Chem. 257, 7712–7719
9. Raychaudhuri, P., Stringer, E. A., Valenzuela, D. M., and Maitra, U. (1984) J. Biol. Chem. 259, 11830–11835
10. Nomura, N., Miyajima, N., Sazuka, T., Tanaka, A., Kawarabayashi, Y., Sato, S., Nagase, T., Seki, N., Ishikawa, K., and Tabata, S. (1994) DNA Res. 1, 27–35
11. Nomura, N., Miyajima, N., Sazuka, T., Tanaka, A., Kawarabayashi, Y., Sato, S., Nagase, T., Seki, N., Ishikawa, K., and Tabata, S. (1994) DNA Res. 1, 47–56
12. Abramson, R. D. (1987) Messenger RNA-specific Eukaryotic Initiation Factors. Ph.D. thesis, Case Western Reserve University
13. Merrick, W. C., Dever, T. E., Kinzy, T. G., Conroy, S. C., Cavalli, J., and Owens, C. L. (1990) Biochim. Biophys. Acta 1050, 235–240
14. Merrick, W. C., Dever, T. E. (1990) Characterization of Eukaryotic Protein Synthesis Factors. Ph.D. thesis, Case Western Reserve University
15. Flinta, C., Persson, B., Jornvall, H., and von Heijne, G. (1986) Eur. J. Biochem. 154, 193–196
16. Huang, S., Elliott, R. C., Liu, P. S., Koduri, R. K., Weickmann, J. L., Lee, J. H., Blair, L. C., Ghosh-Dastidar, P., Bradshaw, R. A., Bryan, K. M., Einarson, B., Kendall, R. L., Kolacz, K. H., and Saito, K. (1987) Biochemistry 26, 8242–8246
17. Persson, B., Flinta, C., von Heijne, G., and Jornvall, H. (1985) Eur. J. Biochem. 152, 523–527
18. Altmann, M., Wittmer, B., Methot, N., Sonenberg, N., and Trachsel, H. (1995) EMBO J. 14, 3820–3827
19. Coppeolcchia, R., Buser, P., Stutz, A., and Linder, P. (1993) EMBO J. 12, 4005–4011
20. Schmidt, S., Hofmann, K., and Simonis, V. (1997) Nucleic Acids Res. 25, 3433–3439
21. Methot, N., Pause, A., Hershey, J. W., and Sonenberg, N. (1994) Mol. Cell. Biol. 14, 2307–2316
22. Bandziulis, R. J., Swanson, M. S., and Dreyfuss, G. (1989) Genes Dev. 3, 431–437
23. Pause, A., Belsham, G. J., Gingras, A. C., Denwe, O., Lin, T. A., Lawrence, J. C., Jr., and Sonenberg, N. (1994) Nature 371, 762–767
24. Lin, T. A., Kong, X., Haystead, T. A., Pause, A., Belsham, G., Sonenberg, N., and Lawrence, J. C., Jr. (1994) Science 266, 653–656
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EUKARYOTIC INITIATION FACTOR 4H
Nancy J. Richter-Cook, Thomas E. Dever, Jack O. Hensold and William C. Merrick

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