

A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5' cap-binding protein, eIF4E

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The eukaryotic translation initiation factor 4E (eIF4E) plays an important role in the control of cell growth. eIF4E binds to the mRNA 5' cap structure m⁷GpppN (where N is any nucleotide), and promotes ribosome binding to the mRNA in the cytoplasm. However, a fraction of eIF4E localizes to the nucleus. Here we describe the cloning and functional characterization of a new eIF4E-binding protein, referred to as 4E-T (eIF4E-Transporter). We demonstrate that 4E-T is a nucleocytoplasmic shuttling protein that contains an eIF4E-binding site, one bipartite nuclear localization signal and two leucine-rich nuclear export signals. eIF4E forms a complex with the importin $\alpha\beta$ heterodimer only in the presence of 4E-T. Overexpression of wild-type 4E-T, but not of a mutant defective for eIF4E binding, causes the nuclear accumulation of HA-eIF4E in cells treated with leptomycin B. Taken together, these results demonstrate that the novel nucleocytoplasmic shuttling protein 4E-T mediates the nuclear import of eIF4E via the importin $\alpha\beta$ pathway by a piggy-back mechanism.

Keywords: cap-binding protein/eIF4E localization/initiation factor/shuttling protein/transport

Introduction

Regulation of translation plays an important role in the control of gene expression. In eukaryotes, translation regulation occurs primarily at the initiation step, which is rate limiting under most circumstances (reviewed in Mathews *et al.*, 1996). Translation initiation is facilitated by the mRNA 5' cap structure, m⁷GpppN, which is present on all nuclear encoded mRNAs (Shatkin, 1976). The stimulatory effect of the cap on translation is mediated by eukaryotic translation initiation factor 4E (eIF4E), which directly contacts the cap structure (Sonenberg *et al.*, 1979; Marcotrigiano *et al.*, 1997). eIF4E exists either as a monomer or as a subunit in a complex called eIF4F. eIF4F is a heterotrimeric complex composed of eIF4E, eIF4A and eIF4G, where eIF4A is an ATP-dependent RNA

helicase (Gingras *et al.*, 1999). eIF4G is a modular scaffolding protein, which binds to eIF4E, eIF4A and the ribosome-associated initiation factor, eIF3, thus bridging the mRNA and the ribosome (Lamphear *et al.*, 1995; Imataka and Sonenberg, 1997; reviewed in Gingras *et al.*, 1999). There are two functional homologs of eIF4G, eIF4GI and eIF4GII, which function interchangeably as part of the eIF4F complex (Gradi *et al.*, 1998).

eIF4E is a major target for translational control. It is the least abundant of the initiation factors (Duncan *et al.*, 1987) and is considered to be the limiting factor for cap-dependent translation initiation (Mader and Sonenberg, 1995). eIF4E overexpression in NIH 3T3 cells causes malignant transformation (Lazaris-Karatzas *et al.*, 1990), whereas depletion of eIF4E by antisense RNA partially reverses the transformed phenotype of *ras*-overexpressing cells (Rinker-Schaeffer *et al.*, 1993). eIF4E is phosphorylated on Ser209 by the serine/threonine kinase Mnk1 (Waskiewicz *et al.*, 1997; Pyronnet *et al.*, 1999). Phosphorylation of eIF4E enhances its affinity for the cap structure (Minich *et al.*, 1994), and increased eIF4E phosphorylation correlates with elevated translation rates in response to extracellular stimuli (Gingras *et al.*, 1999). Cell stimulation also induces the phosphorylation and subsequent dissociation of eIF4E-binding proteins (4E-BPs) from eIF4E (Pause *et al.*, 1994). The 4E-BP family of translation repressors comprises three members (Pause *et al.*, 1994; Poulin *et al.*, 1998) that compete with eIF4G for the interaction with eIF4E, and thus inhibit translation by preventing the assembly of the eIF4F complex (Haghighat *et al.*, 1995).

Cell fractionation and immunofluorescence analysis demonstrated that between 12 and 33% of total eIF4E is localized to the nucleus of mammalian cells (Lejbkowitz *et al.*, 1992). Electron microscope studies showed that eIF4E is also partially nuclear in *Saccharomyces cerevisiae* (Lang *et al.*, 1994). Nuclear eIF4E co-localizes with splicing factors in speckles, suggesting that eIF4E may also play a role in pre-mRNA maturation or another function in the nucleus (Dostie *et al.*, 2000). However, nothing is known about the nuclear import or export mechanism of eIF4E. Nucleocytoplasmic transport of macromolecules occurs through the nuclear pore complex (NPC) and is usually an energy-dependent, signal-mediated process (reviewed in Mattaj and Englmeier, 1998; Nakielny and Dreyfuss, 1999). Various types of nuclear localization signals (NLSs), nuclear export signals (NESs) and their receptors have been described. The best characterized NLSs are those of SV40 large T and nucleoplasmin, which consist of either a single or a bipartite cluster of basic amino acids, respectively (Kalderon *et al.*, 1984; Robbins *et al.*, 1991). Basic NLSs are recognized in the cytoplasm by importin α , an adaptor protein that is associated with the receptor protein,

A

ATG GAT AGG AGA AGT ATG GGT GAA ACA GAA AGT GGA GAT GCT TTC CTT GAC CTG AAG AAG 112
 CCT CTT ACC TCC AAA TGC CCC CAT CGC TAT ACA AAA GAA GAA CTC TTG GAT ATA AAA GAA 172
 F F A P D F H A E L Y P A S Q R E S P V 260
 CTC CCC CAT TCC AAA CAG AGG CTT TCA TGC CTT TCT GAA AAA TAT GAC AGT GAT GGT GTC 232
 L P H S K Q R P S C L S E K Y D S D G V 60
 TGG GAC CCT GAG AAG TGG CAT GCC TCT CTC TAC CCA GCT TCA GGG CGG AGC TCA CCA GAT 292
 W S P P D F H A E L Y P A S Q R E S P V 260
 GAA AGT CTG AAG AAA GAG TTG GAT ACA GAC CGG CCT TCC GTG CGC AGG ATA GTA GAT 352
 E S L K K E L D T D R P S L V R R I V D 100
 CCA CGA GAG COT GTG AAA GAA GAT TTA GAT GTT TTT ACC CCT CAG AGA CGG AGC 412
 F R E R V K E D D L D V V P Q R R S 120
 TTT GGA GGC GGC TGC CAC GTG ACA GCC GCT GTT AGC TCC CGG CGC TCA GGA AGT CCA TTA 472
 I S A R T T F E K D H R L S D K D L R D L 180
 GAG AAA GAT AGT GAT GGG CTT COT CTG CTT GGT GGA COT AGG ATT GGC AGT GGG AGG ATA 532
 E K D S D G L R L R L G R R I G S G R I 160
 ATC TCT GCC CGG ACC TTT GAG AAG GAT CAC COT CTT AGC GAT AAG GAC CTG CGG GAC TTG 592
 I S A R T T F E K D H R L S D K D L R D L 180
 AGA GAC AGA GAC CGA GAG AGG GAC TTC AAG GAC AAG COT TTC AGG AGA GAG TTT GGA GAT 652
 R D R D R R E R D F K D K R F R R E R G D 200
 AGT AAG COT GTC TTT GGT GAG COT GAA AAT GAT TCT TAC ACA GAA GAA GAA CCA GAG 712
 I S A R T T F E K D H R L S D K D L R D L 180
 TGG TTC TCT GCT GGA CCC ACA AGT CAG TCT GAA ACC ATC GAA CTG ACT GGC TTT GAT GAT 772
 M F S A G P T S A S E T I E L T G F D D 240
 AAG ATA CTA GAA GAA GAT CAC AAA GGG AGA AAA AGA ACA AGG CGA CGG ACA GCC TCT GTG 832
 K I L E E D H R K R R T R R R A S V 260
 AAG GAA GGT ATA GTA TGC TGC AAT GGA GGA GTG GCC GAA GAG GAT GAA GTG GAG GTC ATC 892
 K E G I V E C N G G V A E E D R V E V I 280
 CTT GCA CAG GAG CCT GGC GGT GAT CAG GAA GTG CCA AGG GAT GCT GTC TTG CCT GAG CAG 952
 L A Q E P A A D Q E V P R D A V L P E Q 300
 TCC CCA GGA GAC TTT GAC TTT AAT GAG TTC TTT AAC CTT GAT AAG GTG CCA TGC TTG GCT 1012
 S P S R S G S R S R S S L G S T P H E 360
 TCG ATG ATA GAA GAT GTT TTG GGA GAA GGG TCA GTC TCT GCC AGT CGG TTC AGT AGG TTG 1072
 S M I E D V L G E A V G S A S R F S R W 340
 TTC TCT AAG CGG AGC AGA TCA GGA AGC CGA TCC AGC AGT CTT GGG TCA ACA CCA CAT GAA 1132
 F S H P S R S G S R S S S L G S T P H E 360
 GAG CTA GAG AGA CTT GCA GGT GAG CAA GCC ATC CTC TCT CCT GGA CAG AAG CAG TCC GGG 1192
 E L E R L L A G L E Q A I L S P G Q N S G 380
 AAT TAC TTT CCT ATA CCA TTG GAA GAC CAT GCT GAA AAT AAA GTG GAT ATT TTA GAA 1252
 N Y F A F I P L E D H A E N K V D I L R 400
 ATG CTA CAG AAA GCC AAA GTG GAT TTG AAA CCT CTT CTT TCC AGC CTT TCT GCA AAT AAA 1312
 M L Q Q K A K V D L K P L L S S L S A N K 420
 GAA AAA CTT AAA GAA AGC TCA CAT TCA GGG GTT GTG CTT TCA GTG GAG GAG GTA GAA GAA 1372
 E K L K E S S H S G V V L S V E V E A 440
 GGT CTG AAG GGC TTT AAG CTT GAC CAG CAA ATG AAG AAT TCA ACT CCT TTT ATG GCA GAA 1432
 G L K K G G D Q Q V K H S T P F M A E 460
 CAC CTA GAA GAG ACC TTG AGT GGC GTA ACC AAT CCA CCA CTG AAG AAA GAC GGA GAC 1492
 H L E E T L A S A V T N N R A G T G L K K D G D 480
 ATG ACT GCG TTC AAC AAG CTA GTG ACA ATG AAG ACA AGT GGG ACT TTG COT TCT CAG 1552
 M T A F H I V S T M K A S G T L P S Q 500
 CCC AAA GTC AGC CGA AAC CTT GAA AGC CAT TTG ATC TCC CCT GCT GAG ATT CCA GGC CAG 1612
 P K V S R N L E S H L M S P A E I P G Q 520

CCT GTC CCT AAG AAC ATC CTG CAG GAA CTT CTG GGT CAA CCA GTT CAG AGA OCT GCT TCT 1672
 P V P P K N I L Q E L L G Q P V Q R P A S 540
 TCC AAT CTT CTG AGT GGC CTT ATG GGG AGC TTG GAG CTT ACA ACA TCT TTA CTG GGC CAA 1732
 F F A P D F H A E L Y P A S Q R E S P V 260
 AGA GCA CCC TCT CCT CCT CTG TGC TCA CAG GTG TTT CAA ACT CGA GCA GCC TCA GCT GAC TAC 1792
 R A P S P P L S Q V F Q T R A A S A D Y 580
 CTT CGC CCA AGA ATA CCA TCA CCA ATT GGT TTC ACA CCA GGA CCA CAG CAG CTA CTC GGA 1852
 L R P R I G S P I G P Q Q L L G 600
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 D P F Q G M R R K P M S P T A Q H S Q L 620
 GAG TTG CAA CAG CCA GCT TTA GAA GGG CTG GCT TTG CCA CAT GAC CTT GGT GTA CAG GCA 1972
E L Q Q Q A A L E G L A L P H D L A V Q A 640
 GCA AAC TTC TAC CAG COT GGT TTT GGC AAA CCA CAG GTG GAC AGA ACC AGA GAT GGA TTC 2032
 A H P Y Q P G F G K C P Q V D E T R D G 660
 AGA AAC AGG CAA CAG CGA GTG ACC AAG TCA CCA CCA CCG GTG CAT CGA GGG AAT TCC TCT 2092
 R N R Q Q R V T K S P A P V H R G N S S 680
 TCC CTT GCC CCT GCT GCC TCC ATC ACA AGC ATG CTT TCT CCT TCC TTT ACC ACC TCA 2152
 S P A P A A S I T S H L S P S F T P T S 700
 GTG ATT COT AAG ATG TAC GAG AGC AAA GAG AAA AGG AAG GAG GAG CCA TCT GGA AAA 2212
 V I R K M Y E S K E K S K E P A S G K 720
 GCA GCT CTT GGT GAC AGT AAA GAG GAT ACT CAG AAG GCC AGT GAA GAA AAC CTC CTG TCA 2272
 A A L G D S K E D T Q K A S E H N L L S 740
 TCC AGC TCT GTA CCC AGT GCC GAT CGA GAC TCT TCT CCC ACT ACA AAT TCC AAA CTG TCA 2332
 S S S V P S A D R D S S P T N S K L S 760
 GCA TTA CAG AGG TCT TCG TGT TCC ACC CCA CTG TCC CAG GCC AAC CGT TAC ACC AAA GAA 2392
 A L Q R S S S S S T P L S Q A N R Y Y T K E 780
 CAA GAT TAT GCA CTT AAA CGA ACT GGG AGA AAA ACA CCC ACT TTG CCA TCC CCA GTT CCT 2452
 Q D V R H P K A T G R K T P T L A S P V D 800
 ACA ACA CTT TTT CCT GGC CTT GTC CAA GTT CCT CTT GTC CCA CAT GTC CCT ATG GPT 2512
 T T P P L R P H P G L V Q V P L V P H V 820
 AGG COT CCT CCA CAG CTT CAC CCA GGG TTG GTA CAG AGG ATG CTG GCC CAG GCA GAT CAT 2572
 R P A H Q L H P G L V Q R M L A Q G V H 840
 CCA CAG CAT CTT CCA ACT TTG CTC CAA ACT GGT GTC CTT CTT GGT ACC GAC TTG AGT 2632
 P Q H L P S L L Q T G V L P G M D L S 860
 CAT TTA CAG GGA ATA TCT GGC CCC ATC CTG GGT CAG CCC TTT TAC COT TTA COT GCT 2692
 H L Q Q I S G P I L G Q P F Y P L F A A 880
 AGT CAC COT CTT TTA AAC CTT CST CTT GGA ACA COT CTG CAT CTG GCA ATG GTG CAA CAG 2752
 S H P L L N P R P F G T P L H L A M V Q Q 900
 CAG CTA CAG CGC TCA GTT CTT CAT CTT CCA GGC TCT GGT TCC CAT GCA GCA GCT GTC AGC 2812
 Q L Q R S V L H F P G S G S H A A A V S 920
 GTT CAG ACA ACC CTT CAG AAC GTG CCC AGC CGG TCA GGC CTG CCC CAC ATG CAC TCC CAG 2872
 V Q T P Q N V P S R S G T P H M H S Q 940
 CTG GAG CAT CGC CCC AGC CAG AGG AGC AGC TCT CCT CTG GGC CTT GGC AAA TGG TTT GGC 2932
 L E H R P S Q R S S S P V G L A K M F G 960
 TCA GAT GTG CTA CAG CAA CCC CTG CCC ATG CCG GCC AAA GTT ATC AGT GTA GAT GAA 2992
 S D V L Q Q F L F S A K V I S V D E 980
 TTG GAA TAC CGA CAG TGAGCAGGGCAGGCAGCTCAACTAAGCCCGACCTGTGGTGGCAGCTGGGGCGACCC 3066
 L E Y R Q
 TGCTCATCTCGGGTTGGTTATGGGCTTTTACTTTGGAGCACTCTGTGTGAAGCTGTTGGTGAACCCATGCATCTG 3145
 GTGTGGTCGGCATATGATGGAGAGGATCTTAAACAGTCGAGTGGAGGTGATGCTGTGCAATACAGGAGGCAAAATGPT 3224
 GTCAATCCCGGAATAGGCTCTCTTTTGTAGATATGTGAATGAAGGTGTCTCTCCACCCAGAGGGGCACTCA 3303
 AGGGTTCTGGGAAATAAAGTAGACCCCTATGTACAGACCTGTGTATAAACAACCTTTGTATATACATATAGGATA 3382
 CTTTTTGAACATACAGCTGTACATAAAAGTAGCTATATTAGTAGGCCCTGTGTCAACAGTTTGGATTTTTTTCAC 3461
 TTGTACATTTGGGATTTCTCTTTGGTTTATTAATAATGCATATGCTAAGTGTGTGAATG 3520

B

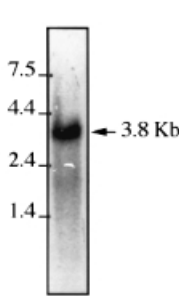


Fig. 1. 4E-T cDNA. (A) Nucleotide and amino acid sequence of human 4E-T. The first methionine is circled. The stop codon and polyadenylation signal are underlined and the eIF4E-binding site is boxed. The nuclear localization signal and nuclear export signals are highlighted with gray and black boxes, respectively. The 4E-T sequence has been deposited in the DDBJ/EMBL/GenBank database (accession No. AF240775). (B) Northern blotting analysis of 4E-T. HeLa poly(A)⁺ RNA (5 μg) was probed with a 4E-T 5' end probe as described in Materials and methods.

importin β. Interaction of the import cargo with the importin αβ complex triggers docking of the heterotrimeric complex to the NPC via importin β (Adam and Adam, 1994; Gorlich *et al.*, 1995). The best described nuclear export signal is the leucine-rich NES, which was first identified in the human immunodeficiency type 1 (HIV-1) Rev protein (Fischer *et al.*, 1995), and in the cellular protein kinase A inhibitor, PKI (Wen *et al.*, 1995). Leucine-rich NESs are recognized directly by the nuclear export receptor CRM1/exportin 1 (Fornerod *et al.*, 1997; Stade *et al.*, 1997).

Translocation of transport cargoes through the NPC requires, in addition to receptors, several other proteins including the GTPase Ran/TC4 and p10/NTF2 (Mattaj and Englmeier, 1998). Ran/TC4 cycles between a GDP-bound and GTP-bound state, with Ran-GDP being predominantly cytoplasmic and Ran-GTP nuclear. Ran-GTP interacts directly with importins and exportins, and promotes directionality of transport by triggering the disassembly

and assembly of import and export complexes, respectively (Gorlich *et al.*, 1996; reviewed in Moore, 1998). When complexed with Ran-GTP, importin β releases the import cargo, whereas its association with the export receptor CRM1/exportin 1 is required for the formation of a stable export cargo–receptor complex (reviewed in Mattaj and Englmeier, 1998; Nakiely and Dreyfuss, 1999).

Here we report the cloning and functional characterization of a new eIF4E-binding protein termed 4E-T (eIF4E-Transporter). We show that 4E-T is a novel nucleocytoplasmic shuttling protein that targets eIF4E for nuclear import.

Results

Cloning of 4E-T cDNA

Two partial human 4E-T cDNA clones were isolated from a placental cDNA expression library by far-western

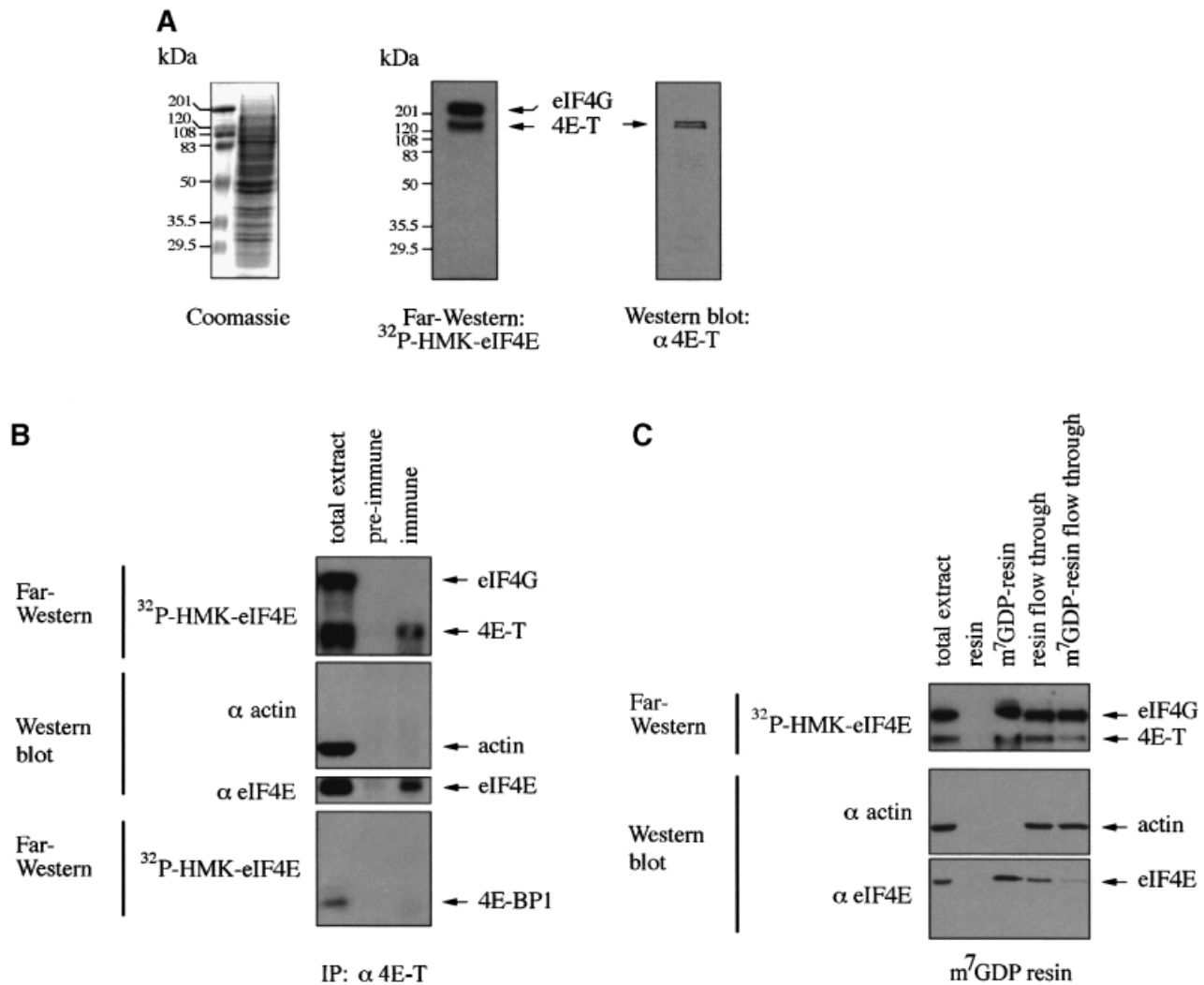


Fig. 2. Interaction of 4E-T with eIF4E. (A) eIF4E interacts with 4E-T as determined by far-western. HeLa cell extract (100 μ g) was resolved by SDS-PAGE and stained with Coomassie Blue R-250 (left), or transferred to nitrocellulose and probed by far-western with a [32 P]HMK-eIF4E probe (middle) or by western blotting with anti-4E-T antibody (right). (B) eIF4E specifically co-immunoprecipitates with 4E-T. HeLa cell extract (1 mg) was incubated with either pre-immune sera or anti-4E-T antibody as described in Materials and methods. Total extract [5% of input (50 μ g)] and total immunoprecipitates from 1 mg of extract were resolved by SDS-PAGE, and analyzed by far-western with a [32 P]HMK-eIF4E probe or by immunoblotting with anti-actin or anti-eIF4E monoclonal antibodies. (C) 4E-T interacts with eIF4E on a cap affinity resin. HeLa cell extract (1 mg) was incubated with resin or m^7 GDP resin, and processed as described in Materials and methods. Total extract [5% of input (50 μ g)], resin, m^7 GDP resin-bound material from 1 mg of extract and flow through (5%) were resolved by SDS-PAGE, and analyzed by far-western with a [32 P]HMK-eIF4E probe or by immunoblotting with anti-actin or anti-eIF4E monoclonal antibodies.

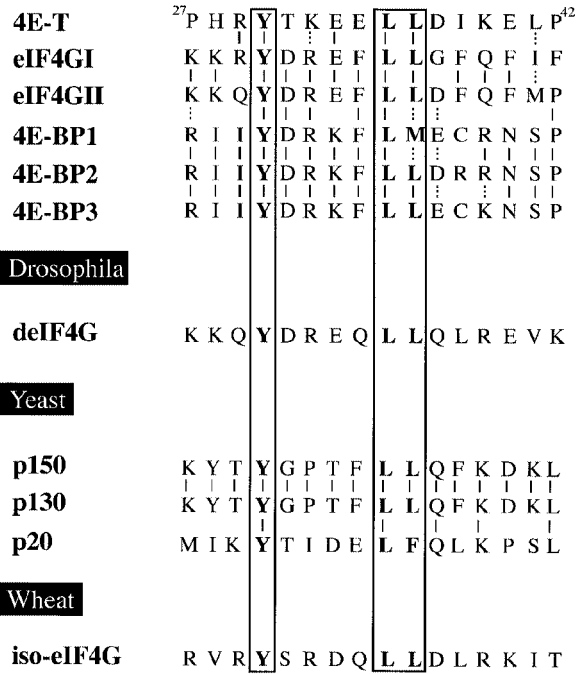
screening using a [32 P]HMK-eIF4E probe. To obtain a full-length cDNA, the longest (1.5 kb) cDNA was used to screen a human fetal brain cDNA library. Two overlapping partial clones were ligated to form a contiguous cDNA of 3520 bp (Figure 1A). Northern blotting analysis, using probes from three different regions, revealed one prominent mRNA of \sim 3.8 kb (Figure 1B and data not shown). A similar sized RNA was also detected in all tissues tested including heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (data not shown). 4E-T cDNA encodes a 5'-untranslated region (UTR) of 52 nucleotides, an open reading frame (ORF) of 2958 nucleotides and a 3'-UTR of 513 nucleotides. The ORF encodes a protein of 985 amino acids with a predicted molecular mass of 108.2 kDa. Because expression of the cDNA in HeLa cells generates a protein that co-migrates with the

endogenous protein (see below; Figure 3), it is most probable that the first AUG is the authentic initiation codon, inasmuch as the length of the cDNA closely resembles that of the mRNA. Based on the results described below, we have named the protein encoded by this cDNA 4E-T.

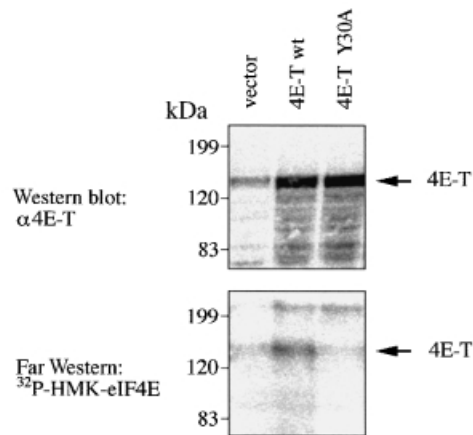
4E-T interacts with eIF4E in vitro and in vivo

To study the expression of 4E-T in cells, two different antisera against a glutathione S-transferase (GST)-4E-T N-terminal fusion protein were raised in rabbits, and antibodies from one antiserum were affinity purified. Both crude antisera and purified antibodies recognized a protein migrating at \sim 140 kDa, which is higher than the predicted mass of 108 kDa in HeLa cells (Figure 2A, right) and in other cells, including NIH 3T3, COS-7, CV-1 and 293

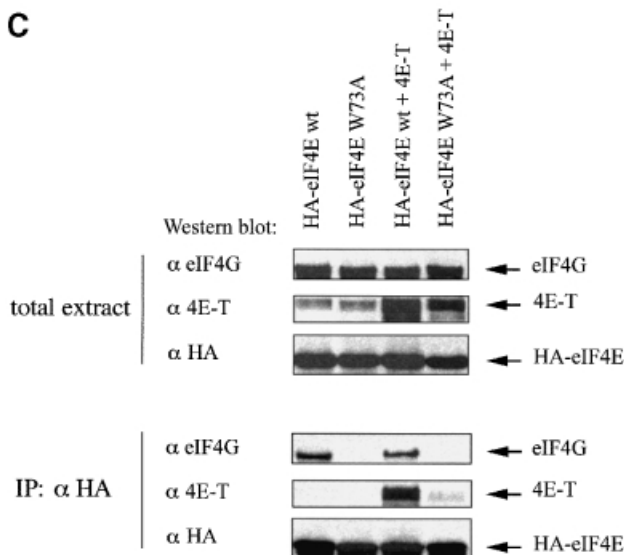
A Human



B



C



(data not shown). An ~140 kDa polypeptide was also recognized by a [³²P]HMK-eIF4E probe in a far-western assay (Figure 2A, middle). As expected (Mader *et al.*, 1995), eIF4E also bound to eIF4G in this assay. The 4E-T protein expressed from the 4E-T cDNA in HeLa cells also migrates aberrantly on SDS-PAGE and co-migrates with endogenous 4E-T (see below; Figure 3B and C). The slower mobility of 4E-T may be due to phosphorylation (see Discussion) or to its high proline content, as previously suggested for proline-rich proteins (See and Jakowski, 1989).

To determine whether 4E-T interacts with eIF4E *in vivo*, a co-immunoprecipitation experiment was performed with HeLa cell extracts. eIF4E specifically co-immunoprecipitated with 4E-T (Figure 2B). eIF4G and 4E-BP1 did not co-immunoprecipitate with 4E-T, suggesting that when complexed with 4E-T, eIF4E can no longer interact with eIF4G or 4E-BP1. Furthermore, 4E-T co-precipitated with eIF4E from HeLa extracts using an m⁷GDP resin (Figure 2C). Actin, tested as a negative control, did not co-immunoprecipitate with eIF4E, and was not retained on the m⁷GDP resin (Figure 2B and C). Taken together, these results demonstrate that 4E-T interacts with eIF4E both *in vitro* and *in vivo*.

4E-T and eIF4E interact through conserved binding sites

Interaction of the eIF4Gs and 4E-BPs with eIF4E is mutually exclusive (Haghighat *et al.*, 1995) and occurs through the conserved eIF4E recognition motif Tyr-X-X-X-X-Leu-φ (where φ is a hydrophobic amino acid and X is any amino acid; Mader *et al.*, 1995). Mutation of either Tyr or Leu-φ to alanine residues abolishes the binding of eIF4GI and 4E-BP1 to eIF4E (Mader *et al.*, 1995). There is one putative eIF4E-binding site (Tyr-Thr-Lys-Glu-Glu-Leu-Leu) in the N-terminus of 4E-T (Figure 3A). To demonstrate that the putative eIF4E-binding site of 4E-T mediates its interaction with eIF4E, wild-type 4E-T (4E-T wt) or 4E-T Y30A mutant proteins were expressed in HeLa cells using the vaccinia virus system (Poulin *et al.*, 1998). Expression of transfected 4E-T was assessed by immunoblotting with purified anti-4E-T antibody (Figure 3B, top), and the interaction with eIF4E was analyzed by far-western using a [³²P]HMK-eIF4E probe.

Fig. 3. 4E-T and eIF4E interact through conserved binding sites.

(A) Amino acid sequence alignment of the eIF4E-binding site of 4E-T with those found in human eIF4GI (AF012088), human eIF4GII (AF012072), human 4E-BP1 (L36055), human 4E-BP2 (L36056), human 4E-BP3 (AF038869), *Drosophila* eIF4G (AF030155), *S.cerevisiae* eIF4GI (p39935), *S.cerevisiae* eIF4GII (p39936), *S.cerevisiae* p20 (X15731) and wheat iso-eIF4G (M95747). Residues which are critical for eIF4E binding are boxed. Solid and dashed lines represent identical and conserved residues, respectively. (B) 4E-T associates with eIF4E through a conserved motif. Untagged 4E-T wt and Y30A mutant were overexpressed in HeLa cells using the vaccinia virus system. Top: immunoblotting with anti-4E-T antibody. Bottom: far-western with [³²P]HMK-eIF4E. (C) eIF4E binds to 4E-T and eIF4G through a shared sequence. HeLa cells were infected with vTF7-3 and transiently transfected with the plasmids indicated. Total extract [25% of input (50 μg)] was analyzed by western blotting with the indicated antibodies. Immunoprecipitation (from 200 μg of extract) of HA-eIF4E with anti-HA 12CA5 was performed as described in Materials and methods, and analyzed by western blotting with the antibodies indicated.

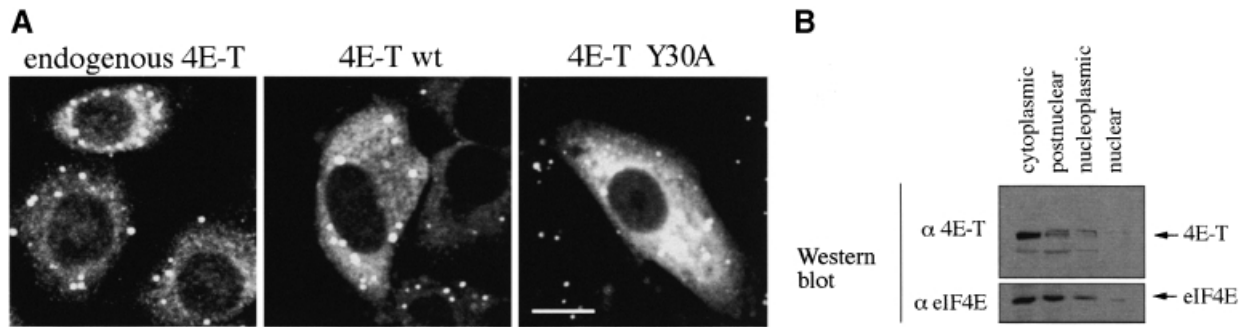


Fig. 4. 4E-T localizes predominantly to the cytoplasm. **(A)** Indirect immunofluorescence analysis with anti-4E-T. Localization of endogenous 4E-T in HeLa cells (left). HeLa cells were transiently transfected with either 4E-T wt (middle) or Y30A (right). At 36 h post-transfection, cells were fixed and immunostained with anti-4E-T antibody as described in Materials and methods. Bar, 10 μ m. **(B)** Cell fractionation. HeLa cells were fractionated as described in Materials and methods, and an equal cell volume from each fraction was resolved by SDS-PAGE. Fractions were analyzed by western blotting with anti-4E-T (top) or anti-eIF4E monoclonal antibody (bottom).

4E-T wt, but not the Y30A mutant, associated with eIF4E, indicating that 4E-T interacts with eIF4E through the conserved eIF4E recognition motif (Figure 3B, bottom).

A phylogenetically conserved Trp73 is located on the convex dorsal surface of eIF4E (Marcotrigiano *et al.*, 1997), and interacts with the invariant Leu and ϕ residues of the eIF4E-binding motif of eIF4G and 4E-BP1 (Marcotrigiano *et al.*, 1999). The eIF4E W73A mutant protein does not interact with eIF4GI *in vivo* or *in vitro* (Pyronnet *et al.*, 1999). To determine whether eIF4E interacts with 4E-T through the conserved Trp73, HeLa cells were infected with the recombinant vaccinia virus ν TF7-3 (Fuerst *et al.*, 1986), and then transfected with either hemagglutinin (HA)-eIF4E wt or the W73A mutant, alone or together with 4E-T wt. 4E-T co-immunoprecipitated with eIF4E wt, but not with the W73A mutant (Figure 3C). Interaction of HA-eIF4E wt with endogenous 4E-T was not detectable in this assay, presumably because of the small amount of extract used. Taken together, these results demonstrate that eIF4E and 4E-T interact through a conserved binding site.

4E-T contains one functional bipartite NLS

Sequence analysis of 4E-T revealed a putative bipartite NLS (Robbins *et al.*, 1991; Figure 5A; see also Figure 1) in its N-terminus that could be important for its physiological function. Consequently, the cellular localization of 4E-T was examined. 4E-T localizes predominantly to the cytoplasm in HeLa cells as demonstrated by indirect immunofluorescence with anti-4E-T (Figure 4A, left). Overexpressed untagged 4E-T wt and Y30A mutant also localize to the cytoplasm in HeLa cells (Figure 4A, middle, right; cells overexpressing 4E-T were identified by their stronger fluorescence intensity as compared with the endogenous signal) and COS-7 cells (data not shown). Similarly, the localization of HA-tagged 4E-T wt and Y30A mutant is cytoplasmic (Figures 5, 6 and 7). However, in a small percentage of cells (~5%), endogenous and transfected 4E-T is found in the nucleus (data not shown). HeLa cell fractionation shows that 4E-T is predominantly in the cytoplasmic and post-nuclear fraction. eIF4E is present in somewhat higher amounts in the nucleoplasmic fraction than was demonstrated earlier (Figure 4B; Lejbkovicz *et al.*, 1992).

Our results show that although 4E-T contains a putative NLS, it is predominantly cytoplasmic. It is possible that the NLS is functional, but that other segments in 4E-T could cause its cytoplasmic localization. To determine whether the putative NLS motif is functional, several HA-4E-T deletion mutants were constructed and analyzed by indirect immunofluorescence (Figure 5B and D). While the full-length HA-4E-T localized to the cytoplasm (Figure 5D, panel a), the HA-4E-T 1–359 fragment, which contains the putative NLS, localized to the nucleus (panel b). However, in contrast, longer fragments of 4E-T (1–504) localized to the cytoplasm in a manner similar to the full-length protein (panel c). The exclusive nuclear localization of 4E-T (1–359) is not the result of free diffusion because of its low molecular weight, inasmuch as a deletion of the putative NLS (HA-4E-T 1–359 Δ NLS) results in an even distribution of this fragment between the nucleus and the cytoplasm (panel d). Mutation of Y30A did not affect the nuclear targeting of HA-4E-T 1–359 (panel e), indicating that the interaction with eIF4E is not required for nuclear import of 4E-T.

To establish further that the putative NLS of 4E-T is functional, we examined its ability to confer nuclear import on a heterologous cytoplasmic protein. The 4E-T 1–359 fragment was fused to myc-tagged chicken muscle pyruvate kinase (myc-PK; Siomi and Dreyfuss, 1995; Figure 5C and E). The 4E-T fragment 1–359, but not 4E-T 1–359 Δ NLS, caused myc-PK to accumulate in the nucleus (Figure 5E, panels a, b and c). Furthermore, mutation of the first two arginines in the NLS motif in 4E-T 1–359 (R195N/R196S) prevented the import of myc-PK into the nucleus (panel d). This demonstrates the presence of a bipartite NLS in the 4E-T 1–359 fragment. Y30A mutation did not affect the nuclear localization of myc-PK-4E-T 1–359 (panel e). These results indicate that 4E-T contains one functional bipartite NLS, and demonstrate that nuclear import of 4E-T does not require interaction with eIF4E.

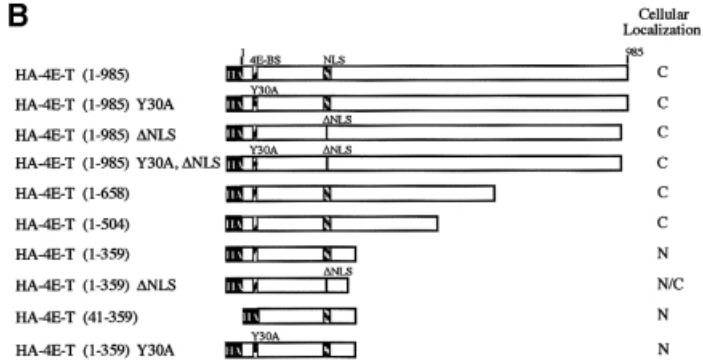
4E-T contains two functional leucine-rich NESs

One possible explanation for the predominant cytoplasmic localization of 4E-T could be that its nuclear import is regulated, either by phosphorylation, as was shown for example for NF-AT4 (Zhu *et al.*, 1998), or through its interaction with cytoplasmic proteins, as was demonstrated for NF- κ B (Henkel *et al.*, 1992). It is also possible

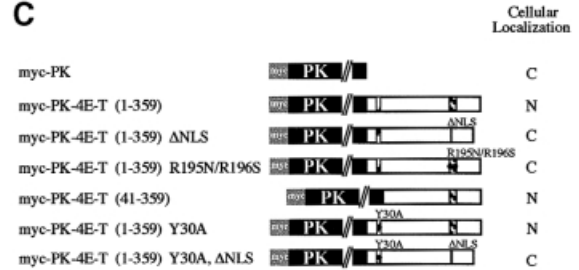
A

bipartite NLS consensus: $(K/R)_2 X_{10} (K/R)_{3/5}$ 4E-T bipartite NLS: $\text{RREFGDSKR} \text{VFGERRR} \text{N}$

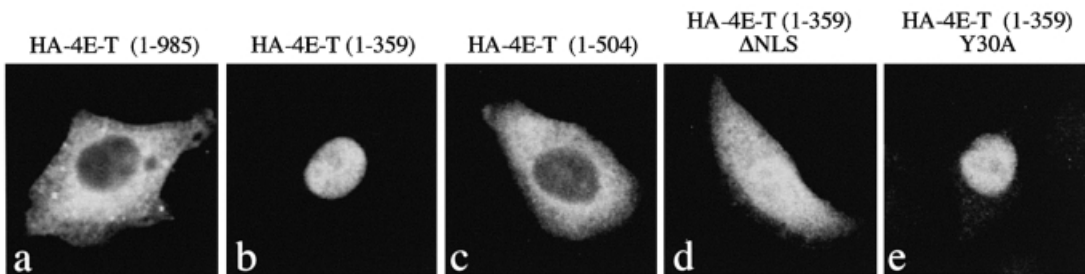
B



C



D



E

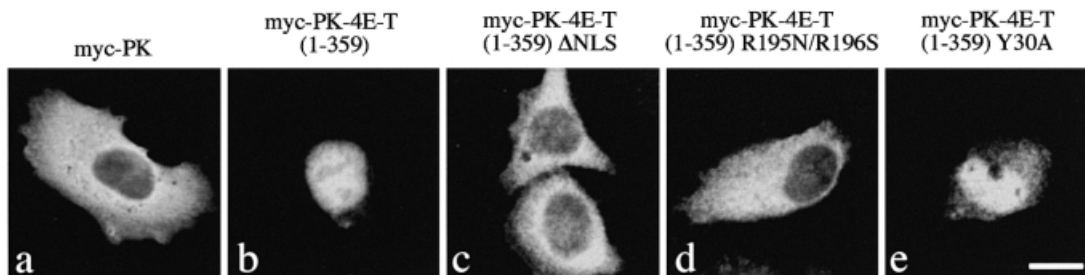


Fig. 5. 4E-T contains a functional bipartite NLS. (A) Putative bipartite NLS of 4E-T. Invariant residues are underlined. (B) and (C) Schematic maps of HA-4E-T and myc-PK-4E-T expression constructs used for NLS mapping. HeLa cells were transfected with either HA-4E-T or myc-PK-4E-T expression constructs. Cells were fixed at 36 h post-transfection and immunostained with either anti-HA 12CA5 (D) or anti-myc 9E10 antibody (E). The transfected construct is indicated above each panel. Bar, 10 μ m.

that the NLS of 4E-T is always active, but that 4E-T shuttles between the nucleus and the cytoplasm. To determine whether 4E-T is a shuttling protein, HeLa cells were treated with the nuclear export inhibitor leptomycin B (LMB). LMB is a *Streptomyces* metabolite that inhibits export of leucine-rich NES-containing proteins by interacting with the export receptor CRM1/exportin 1, and preventing its association with the NES substrate (Kudo *et al.*, 1998). Endogenous and transfected HA-4E-T wt, but not HA-4E-T Δ NLS, accumulated in the nucleus upon LMB treatment (Figure 6). LMB also caused

the nuclear accumulation of HA-4E-T Y30A, confirming that association with eIF4E is not required for nuclear import of 4E-T. This result shows that 4E-T is a nucleocytoplasmic shuttling protein and suggests that 4E-T contains a leucine-rich NES.

There are two putative NESs in the middle region of 4E-T (Figure 7A). To determine whether these motifs are functional, HA-4E-T deletion mutants were constructed (Figure 7D), transfected into HeLa cells and analyzed by indirect immunofluorescence. 4E-T 321–658, which contains both putative NESs, is predominantly cytoplasmic

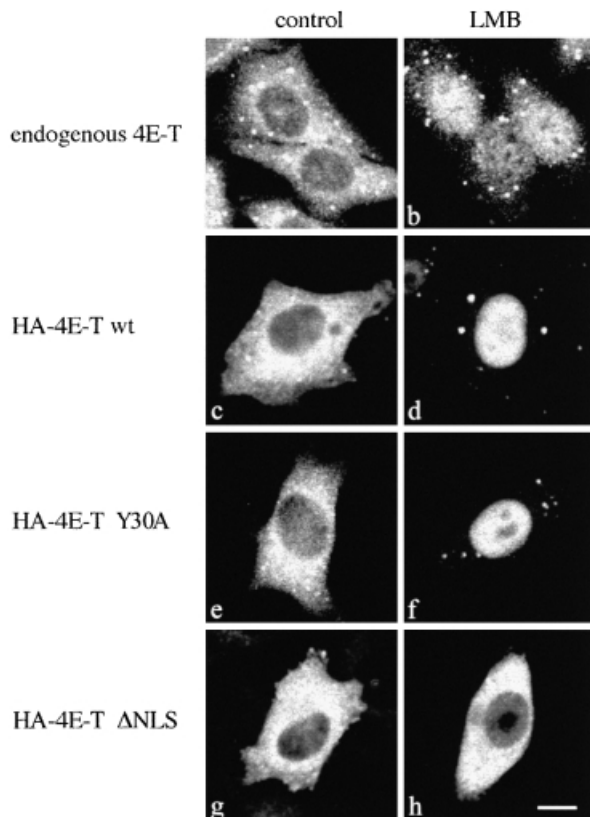


Fig. 6. 4E-T is a shuttling protein. HeLa cells were incubated with medium in the absence (A) or the presence of leptomycin B (LMB) (B) for 5 h prior to fixation and immunostaining with anti-4E-T as described in Materials and methods. (C–H) HeLa cells were transfected with either HA-4E-T wt, Y30A or Δ NLS expression constructs. At 36 h post-transfection, the medium was replaced with medium either lacking (control) or containing LMB. Cells were processed as described above, except that anti-HA 12CA5 antibody was used for immunoblotting. Bar, 10 μ m.

(Figure 7B, panel c), whereas HA-4E-T 1–359 is nuclear (panel b). HA-4E-T 659–985 is distributed evenly between the cytoplasm and the nucleus (panel d). These data show that nuclear export activity is present only in the middle region of 4E-T. To determine which of the putative NES motifs is functional, deletion and point mutants of 4E-T 321–658 were fused to the nuclear myc-tagged nucleoplasmin core protein (myc-NPC; Michael *et al.*, 1997), and examined for their ability to confer export activity on NPC (Figure 7E). Fusion of 4E-T fragments 321–658, 321–504 and 505–658 to the NPC conferred on it nuclear export (Figure 7C), indicating that 4E-T contains at least two independent functional NESs. Deletion of NES1 in 4E-T 321–504 or NES2 in 4E-T 505–658 abolished the nuclear export activity of these fragments (Figure 7E). This result confirms that both NESs are functional, and indicates that there are no additional NESs in the 4E-T 321–658 fragment. Point mutations of all the invariant residues in NES1 and NES2 (4E-T NES1;NES2 mutations) also abolished nuclear export of 4E-T 1–985 and 4E-T 321–658 (Figure 7B and C). Y30A mutation or deletion of the eIF4E-binding site did not affect nuclear export of 4E-T (Figures 6 and 7D). These results show that 4E-T contains two separate and independent functional NESs, and

indicate that nuclear export of 4E-T does not require binding to eIF4E.

4E-T mediates the nuclear import of eIF4E

Although eIF4E is sufficiently small to diffuse passively through the NPC, the nucleocytoplasmic transport of most low molecular weight proteins and that of all known RNAs is believed to be an active, signal-mediated process (Mattaj and Englmeier, 1998). Also, since eIF4E is complexed with either eIF4Gs or 4E-BPs in the cytoplasm, it is very likely that eIF4E requires an active process for its import into the nucleus. To determine whether 4E-T mediates the nuclear import of eIF4E, we first examined the ability of eIF4E to form a quaternary complex with 4E-T, importin α and importin β . Purified recombinant proteins were pre-incubated before the addition of glutathione–Sepharose resin to precipitate the complex via GST–importin β . 4E-T associated with importin β only in the presence of importin α (Figure 8, compare lanes 5 and 6), and eIF4E interacted with importin α and importin β only in the presence 4E-T (compare lanes 7 and 8). A lower amount of importin α was precipitated with GST–importin β when 4E-T was present. We found that this was dependent on the order in which the recombinant proteins were added to the reaction mixture. When importin α was pre-incubated with importin β , this effect was not observed, but noticeably less eIF4E was pulled-down in the quaternary complex. This effect may be due to some extent to improper folding of the recombinant proteins or may reflect the physiological order in which the complex is formed in the cell. This assay shows that the interaction of eIF4E with the importin $\alpha\beta$ complex requires 4E-T, and that interaction of eIF4E and importin α with 4E-T is therefore not mutually exclusive. Thus, although nuclear import of 4E-T does not require an interaction with eIF4E, it is plausible that 4E-T mediates the nuclear import of eIF4E by a piggy-back mechanism (Turpin *et al.*, 1999).

To demonstrate that 4E-T mediates the nuclear import of eIF4E *in vivo*, HeLa cells were co-transfected with HA-eIF4E together with 4E-T wt or 4E-T Y30A, the mutant defective in eIF4E binding (see Figure 3B). The localization of the proteins was analyzed by double-label immunofluorescence using anti-HA and anti-4E-T antibodies. HA-eIF4E was shown previously to localize partially to the nucleus (Lejbkiewicz *et al.*, 1992), while in another report, it localized to the cytoplasm (Waskiewicz *et al.*, 1999). We found that this discrepancy is most probably due to different eIF4E expression levels in the two studies. Transfection with low amounts of DNA results in cytoplasmic accumulation, while transfection with high amounts results in a predominant nuclear accumulation of HA-eIF4E (data not shown). Similarly, the localization of a green fluorescent protein (GFP)–Cdc25 fusion protein was also shown to be dependent on the expression levels (Kumagai and Dunphy, 1999). The cytoplasmic retention of eIF4E, which is expressed at low levels, occurs presumably because of its interaction with cytoplasmic binding partners such as the eIF4Gs and the 4E-BPs. To demonstrate the effect of 4E-T on eIF4E nuclear transport, HA-eIF4E was expressed at low levels, where it localizes predominantly to the cytoplasm (Figure 9A and B, left). Expression of 4E-T caused the

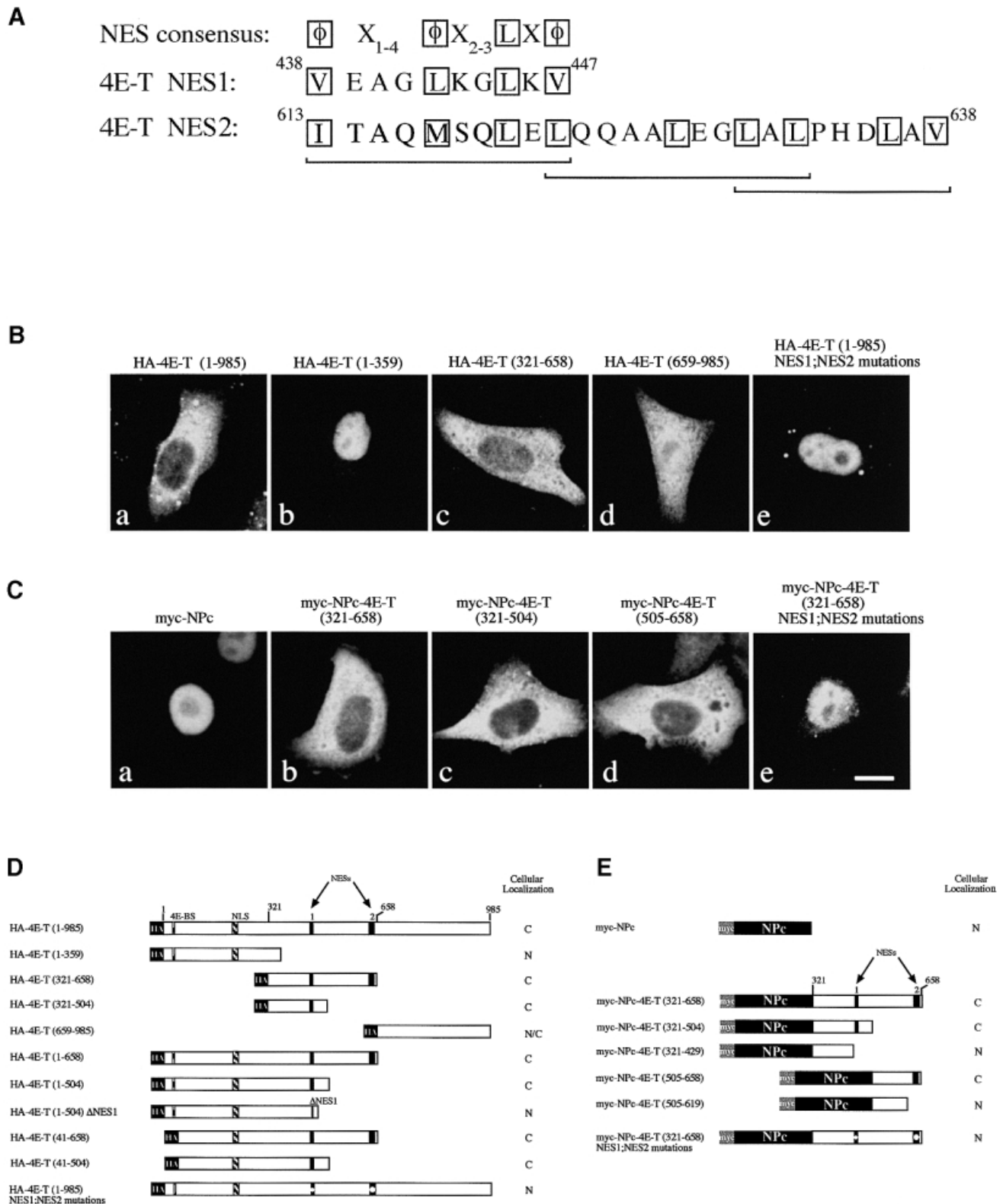


Fig. 7. 4E-T contains two functional NESs. (A) Putative NESs of 4E-T. Invariant residues are boxed. HeLa cells were transfected with either HA-4E-T (B) or myc-NPc-4E-T expression constructs (C). Cells were fixed at 36 h post-transfection and immunostained with either anti-HA 12CA5 (B) or anti-myc 9E10 antibody (C). Transfected construct is indicated above each panel. (D) and (E) Schematic maps of HA-4E-T and myc-NPc-4E-T expression constructs used for NES mapping. Bar, 10 μ m.

nuclear accumulation of HA-eIF4E in cells treated with LMB (Figure 9A). However, in sharp contrast, expression of 4E-T Y30A, which accumulated in the nucleus in the

presence of LMB, failed to cause the import of eIF4E into the nucleus. That the localization of HA-eIF4E was not affected by 4E-T Y30A substantiates the idea that 4E-T is

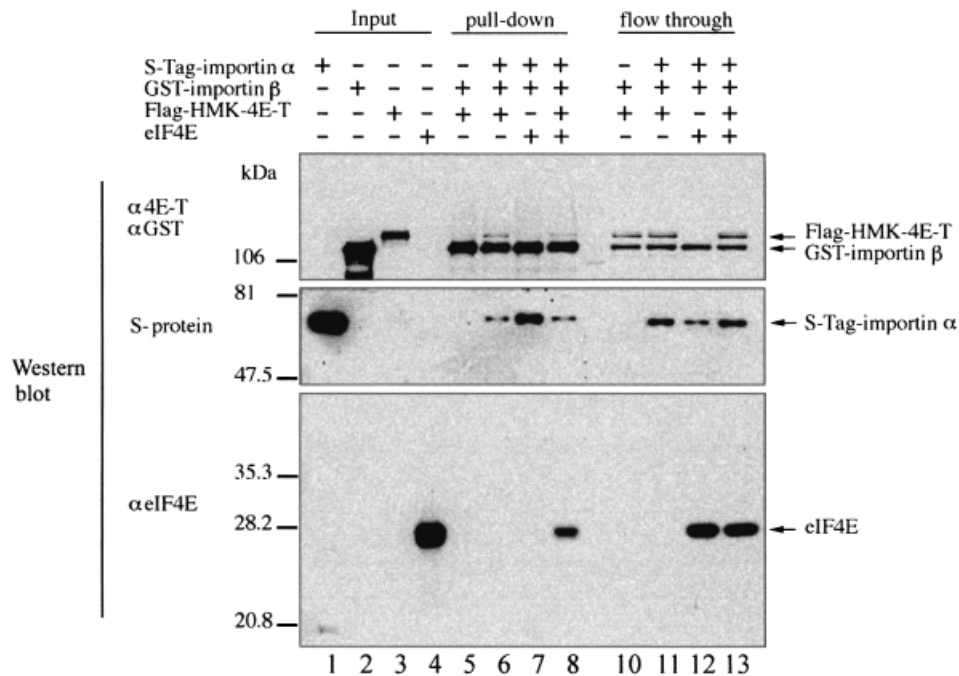


Fig. 8. eIF4E forms a complex with importin α and β only in the presence of 4E-T. The purified proteins indicated were incubated with glutathione–Sepharose beads as described in Materials and methods. Precipitates were analyzed by western blotting with anti-4E-T, anti-GST, S-protein (Novagen) and anti-eIF4E (No. 5853) as indicated. Input represents 25% of total protein used for pull-down; flow through represents 10% of unbound material.

required for the nuclear import of eIF4E. Taken together, our results show that 4E-T mediates the nuclear import of eIF4E by a piggy-back mechanism.

Discussion

Here we provide evidence that the nuclear import of murine eIF4E occurs via the importin $\alpha\beta$ pathway by a piggy-back mechanism, which requires a novel nucleocytoplasmic shuttling protein, 4E-T. Several other proteins lacking a nuclear import signal rely on the use of the NLS of binding partners for import into the nucleus. For example, the interleukin (IL)-5 α - and β -receptor subunits require IL-5, adenovirus E1B 55-kDa requires the adenovirus E4 34-kDa, and Cdk2 requires cyclin E for nuclear import (Goodrum *et al.*, 1996; Jans *et al.*, 1997; Moore *et al.*, 1999). Although I κ B α is sufficiently small to diffuse passively through the NPC, a recent report suggests that it is also imported by a piggy-back mechanism through its association with an ankyrin repeat-binding protein (Turpin *et al.*, 1999). The nuclear import of several small polypeptides such as histone H1 (Jakel *et al.*, 1999) and ribosomal proteins (Jakel and Gorlich, 1998) is carrier-mediated, whereas rad24 (Lopez-Girona *et al.*, 1999) and U1 snRNP-specific protein C (Klein Gunnewiek *et al.*, 1997) localize to the nucleus by passive diffusion and subsequent retention in the nucleus.

Why does eIF4E require a carrier for nuclear import? Since eIF4E, which is the most limiting initiation factor (Duncan *et al.*, 1987), interacts with several more abundant cytoplasmic proteins, it is probable that in the absence of 4E-T, eIF4E would be retained in the cytoplasm. This is in good agreement with our results showing that overexpression of 4E-T wt, but not the Y30A mutant, causes

the nuclear accumulation of HA-eIF4E in cells treated with LMB (Figure 9). Also, the observations that eIF4E, which immunoprecipitates with 4E-T, is not associated with eIF4Gs or 4E-BPs (Figure 2B), and that eIF4E and 4E-T interact through conserved binding domains (Figure 3), demonstrate that the interaction of 4E-T, the eIF4Gs and the 4E-BPs with eIF4E is mutually exclusive. Indeed, eIF4G is not co-precipitated with the eIF4E–4E-T complex (Figure 2B). Consistent with this, overexpression of 4E-T wt, but not 4E-T Y30A, strongly inhibits cap-dependent translation of a reporter luciferase construct (J.Dostie, unpublished observations). Thus, the use of 4E-T in mediating the nuclear import of eIF4E provides an efficient mechanism specifically to recruit eIF4E to the import machinery by competing with eIF4E-binding proteins. Direct binding of eIF4E to importin α as an alternative means to import eIF4E to the nucleus would not be effective, since importin α is a general adaptor protein, which localizes primarily to the nucleus and at the nuclear membrane, and thus would not be an effective competitor with eIF4Gs and 4E-BPs for binding to eIF4E.

A database search identified 4E-T orthologs in mouse and rat but did not identify any putative 4E-T homolog in *S.cerevisiae*. The *Drosophila* cup protein (Keyes and Spradling, 1997) and CG11097 gene product (DDBJ/EMBL/GenBank accession No. AAF59398.1), and the *Caenorhabditis elegans* clone F26F3 (DDBJ/EMBL/GenBank accession No. S43585), share limited homology with a short segment of 4E-T (amino acids 201–261). It is possible that in yeast and less complex eukaryotes, eIF4E uses a different adaptor protein that shares little homology with 4E-T. It is also possible that in *S.cerevisiae* there is more free eIF4E and that, consequently, eIF4E localizes to the nucleus by passive diffusion.

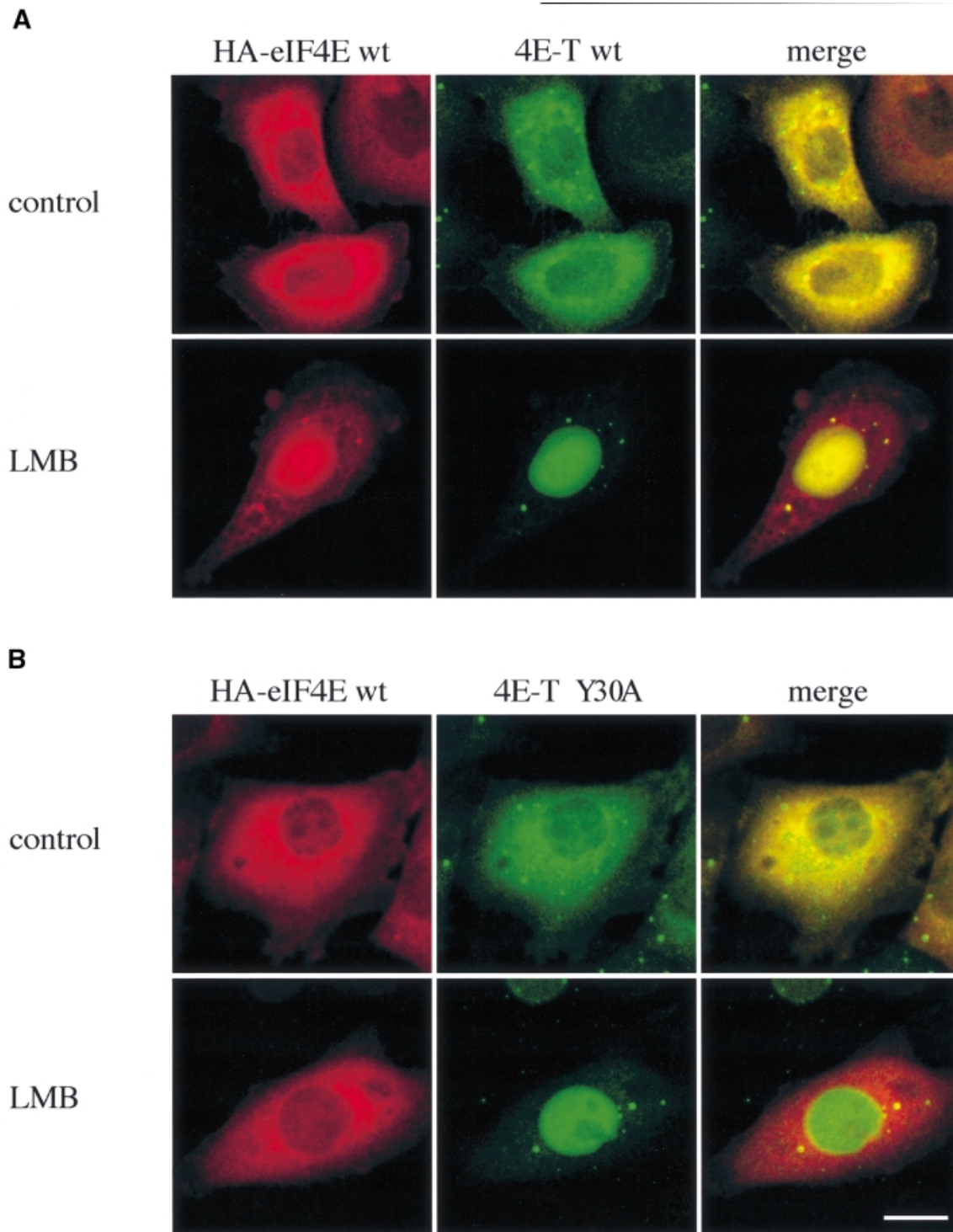


Fig. 9. 4E-T imports HA-eIF4E into the nucleus. HeLa cells were co-transfected with either HA-eIF4E and 4E-T wt (A), or HA-eIF4E and 4E-T Y30A (B). At 36 h post-transfection, the medium was replaced with fresh medium (control) or medium containing leptomycin B (LMB), and cells were incubated for 5 h prior to fixation. The localization of HA-eIF4E was determined by indirect immunofluorescence with anti-HA 12CA5 and Texas red-conjugated anti-mouse IgG (left), and that of 4E-T with anti-4E-T and fluorescein-conjugated anti-rabbit IgG (middle). The co-localization of HA-eIF4E and 4E-T appears yellow (right). Bar, 10 μ m.

The use of 4E-T might provide a means for the control of eIF4E nuclear import through the regulation of 4E-T. Since 4E-T shuttles between the nucleus and the cytoplasm, either the nuclear import or export of 4E-T could be modulated. Phosphorylation affects the nuclear import and/or export of numerous proteins. For example,

nuclear import of nucleoplasmin (Vancurova *et al.*, 1995) and c-rel (Gilmore and Temin, 1988; Mosialos *et al.*, 1991) is enhanced by phosphorylation. Also, phosphorylation of Jak family members by STAT kinases (Leonard and O'Shea, 1998) triggers their nuclear import, whereas dephosphorylation of NF-AT4 by calcineurin is required

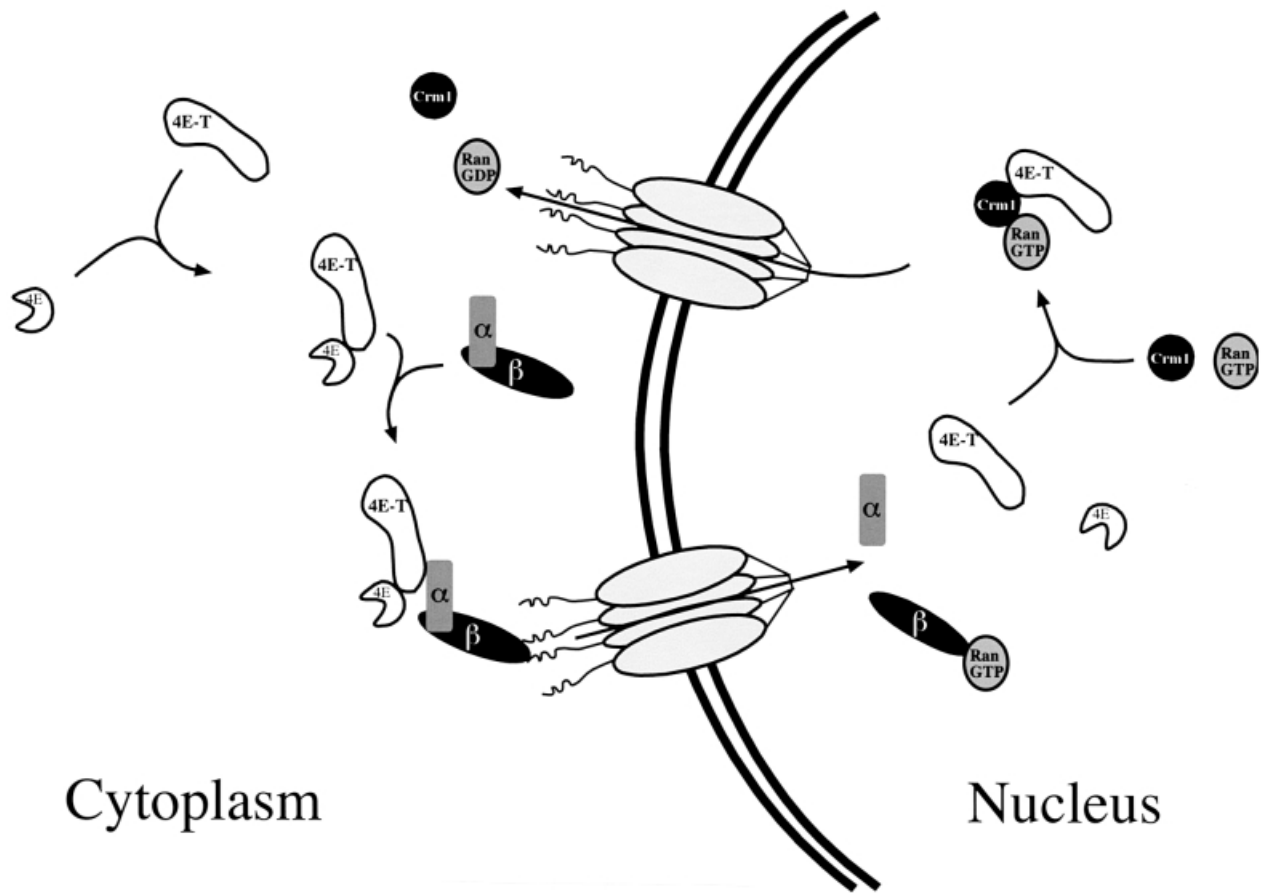


Fig. 10. 4E-T mediates the nuclear import of eIF4E. See the text for details.

for nuclear entry (Zhu *et al.*, 1998). Additionally, nuclear export of MK2 (Engel *et al.*, 1998) is activated by phosphorylation, whereas phosphorylation of cyclin B1 (Li *et al.*, 1997a) inhibits its nuclear export. Since 4E-T is a phosphoprotein (J.Dostie, unpublished observations), the nuclear localization of 4E-T and, consequently, that of eIF4E may also be regulated by phosphorylation. eIF4E contains a putative NES (amino acids 126–137 in human eIF4E), but it is not known whether it is functional. Further studies with the heterokaryon assay using eIF4E mutants will be required in order to determine the nuclear export mechanism of eIF4E.

The results presented here support the conclusion that eIF4E is imported into the nucleus via the importin $\alpha\beta$ pathway by a piggy-back mechanism through its association with 4E-T (see model in Figure 10). Upon nuclear entry, eIF4E and 4E-T would dissociate from importin α as a result of the interaction of Ran-GTP with importin β . This process probably occurs immediately and rapidly following the transport, because 4E-T resides most of the time in the cytoplasm. Although the relative affinity of 4E-T for eIF4E in the presence or absence of importin α is not known, it is possible that dissociation of 4E-T from importin α also results in the release of eIF4E. 4E-T and eIF4E may dissociate because of a decrease in affinity or because of their interaction with nuclear proteins. Following the release of eIF4E, 4E-T shuttles back to the cytoplasm via the CRM1/exportin 1 pathway. Since

nuclear import or export of 4E-T does not require interaction with eIF4E, it is possible that 4E-T also functions as an import or export adaptor for other proteins. Therefore, the identification of 4E-T-binding proteins is of interest.

The nuclear function of eIF4E is not known. The cap is involved in several aspects of RNA metabolism in the nucleus, including 3' end mRNA processing (Hart *et al.*, 1985), pre-mRNA splicing (Konarska *et al.*, 1984; Izaurralde *et al.*, 1994) and nuclear export of RNA polymerase II transcripts (Hamm and Mattaj, 1990; Jarmolowski *et al.*, 1994). Several nuclear cap-binding proteins mediate the nuclear function of the cap structure (Patzelt *et al.*, 1983; Rozen and Sonenberg, 1987; Izaurralde *et al.*, 1992). For example, the nuclear cap-binding complex (CBC), which is composed of CBP20 and CBP80, functions in pre-mRNA splicing (Izaurralde *et al.*, 1994; Lewis *et al.*, 1996) and mRNA 3' end processing (Flaherty *et al.*, 1997). CBC is also required for U snRNA export (Izaurralde *et al.*, 1995), and although it does not seem to facilitate mRNA export dramatically, a study on the *Chironomus tentans* Balbiani ring mRNA export demonstrates that CBC binds co-transcriptionally to the cap and accompanies the ribonucleoprotein particle during nuclear export (Visa *et al.*, 1996).

We have shown recently that nuclear eIF4E co-localizes with splicing factors in speckles (Dostie *et al.*, 2000). The pattern of eIF4E nuclear localization is modulated by

inhibitors of RNA polymerase II, and is regulated by the dual specificity kinase Clk/Sty, suggesting that, in addition to its role in translation, eIF4E may also be involved in nuclear mRNA metabolism. Another intriguing, but more remote possibility is that eIF4E is involved in mRNA surveillance by nuclear translation to target mRNAs that contain premature nonsense codons for degradation (Li *et al.*, 1997b).

Materials and methods

Isolation of 4E-T cDNA clones and DNA sequence analysis

Partial 4E-T cDNA clones were isolated from a λ gt11 human placenta cDNA library (kindly provided by M.Park, McGill University) following far-western screening using a 32 P-labeled eIF4E probe as previously described (Pause *et al.*, 1994). An 809 bp DNA fragment was generated by PCR using a partial cDNA clone as template and the following primers: 5'-GCCTTCATGCCTTCTG-3' and 5'-GGCACCTTATCAA-GGTTAAAG-3'. The amplified DNA was 32 P-labeled by random priming using [α - 32 P]dCTP, random hexamers and the Klenow fragment of DNA polymerase (Feinberg and Vogelstein, 1984). A λ Uni-ZAP-XR human fetal brain cDNA library (kindly provided by G.Rouleau, Université de Montréal) was screened with this probe to obtain a longer cDNA clone. Phages (1×10^6) displayed on duplicate sets of filters (Hybond-N⁺; Amersham) were screened. Isolation of the positive clones was performed as described in the Stratagene ZAP Express instruction manual. Oligonucleotides used for sequencing were derived from either pBluescript or from the 4E-T DNA sequence. Nucleotide sequence was determined from both strands using the dideoxy chain termination method (Sanger *et al.*, 1977) and the T7 polymerase sequencing kit (Amersham Pharmacia Biotech). Regions of compression were re-sequenced using 7-deaza-dGTP.

Northern blotting

Total RNA from HeLa cells was isolated using Trizol (Life Technology, Inc.) and purified twice in batch mode with oligo(dT) beads (Amersham Pharmacia Biotech). Poly(A)⁺ RNA (5 μ g) was fractionated by electrophoresis on a 1% agarose/formaldehyde gel, transferred to a Hybond-N⁺ membrane and cross-linked to the membrane using UV light (Stratalinker). The membrane was pre-hybridized in 5 \times SSPE (0.9 M sodium chloride, 50 mM sodium phosphate, 5 mM EDTA pH 7.7), 5 \times Denhardt's solution, 50% formamide, 1% SDS, 10% dextran sulfate, 100 μ g/ml denatured salmon sperm for 4 h at 42°C. The probe was generated as described above, and hybridization was performed in the same buffer containing the 4E-T probe at 1×10^6 c.p.m./ml for 16 h at 42°C. The membrane was washed to a final stringency of 0.1% SSPE, 0.1% SDS at 68°C, and exposed against a Kodak XAR film for 48 h with intensifying screens.

Antibodies

A GST-4E-T fusion protein comprising amino acids 1-471 of 4E-T was purified on glutathione-Sepharose according to the manufacturer's instructions (Amersham Pharmacia Biotech). Two New Zealand White rabbits were immunized with GST-4E-T as previously described (Gradi *et al.*, 1998). Anti-GST- and anti-4E-T-specific antibodies were purified from the resulting serum by affinity chromatography using GST and GST-4E-T (1-471) columns. Anti-eIF4E no. 5853 (Frederickson *et al.*, 1991), and anti-eIF4G1 C (Imataka *et al.*, 1997) were described previously. Anti-eIF4A and anti-myc 9E10 antibody were kindly provided by H.Trachsel (University of Berne) and M.Tremblay (McGill University), respectively. Monoclonal anti-eIF4E and anti-actin antibodies were purchased from Transduction Laboratories. The anti-HA 12CA5 was purchased from BabCo. Peroxidase-coupled anti-mouse and anti-rabbit IgG were purchased from Amersham Corp. Texas red- and fluorescein-coupled secondary antibodies were purchased from Molecular Probes.

Cell culture, cell extracts and western blotting

Cells were plated on 100 mm dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). At 80% density, the cells were washed twice in phosphate-buffered saline (PBS), scraped and pelleted by slow centrifugation. Cell pellets were resuspended in either buffer B [50 mM Tris-HCl pH 7.4, 150 mM potassium chloride, 20% glycerol, 1 mM EDTA, 1 mM

dithiothreitol (DTT), 0.2% Tween-20 and CompleteTM (Boehringer Mannheim)] or RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate and CompleteTM) and incubated for 30 min at 4°C. Alternatively, cells were resuspended in buffer A (20 mM Tris-HCl pH 7.5, 100 mM potassium chloride, 2 mM EDTA, 2 mM DTT and CompleteTM) and lysed by three freeze-thaw cycles. Cell debris was removed by centrifugation at 10 000 g for 10 min at 4°C, and protein concentration was determined with the Bio-Rad assay. Polypeptides were resolved by SDS-PAGE and transferred onto 0.45 μ m nitrocellulose membranes. Membranes were blocked for 16 h at 4°C with 5% skim milk in PBS containing 0.2% Tween-20 (PBST). Primary antibodies were incubated for 2 h at room temperature followed by four washes of 15 min in PBST. Membranes were incubated with either peroxidase- or 125 I-coupled secondary antibodies for 30 min at room temperature and washed four times for 15 min in PBST. Detection of peroxidase-coupled secondary antibodies was performed with Enhanced-ECL (Amersham Corp.).

Far-western analysis and Coomassie Blue staining

Partially purified mouse Flag-HMK-eIF4E fusion protein (2.5 μ g) was 32 P-labeled with heart muscle kinase (Sigma) as described (Blancar and Rutter, 1992). Membranes were incubated with the probe as previously described (Methot *et al.*, 1997). HeLa cell extract (100 μ g) was resolved by SDS-PAGE, incubated for 30 min at room temperature in Coomassie Blue staining solution [50% methanol, 0.05% Coomassie Brilliant Blue R-250 (Bio-Rad), 10% acetic acid], and unbound dye was removed with destaining solution (5% methanol, 7% acetic acid).

Vaccinia virus infection, immunoprecipitation and m⁷GDP resin precipitation

HeLa cells (70% confluency) were infected with recombinant vaccinia virus vTF7-3 for 1 h at 37°C (Fuerst *et al.*, 1986). Cells were washed with serum-free medium and transfected with plasmid DNA (5 μ g) using Lipofectin (Life Technologies, Inc.) according to the manufacturer's recommendations. Cell extract (200 μ g) prepared with buffer B as described above was incubated with 2 μ g of anti-HA 12CA5 antibody for 2 h at 4°C. Protein G-Sepharose (50 μ l of a 50% slurry) was added and the mixture was incubated for an additional hour at 4°C (Amersham Corp.). Immunoprecipitates were collected by slow centrifugation (3000 g), washed three times with buffer B, boiled for 6 min in Laemmli buffer and resolved by SDS-PAGE. For co-immunoprecipitation of total cell extract, 1 mg of HeLa extract in buffer B was incubated with 1 μ g of anti-4E-T and processed as described above. For m⁷GDP resin precipitation, 1 mg of HeLa extract in buffer A was incubated with 25 μ l of m⁷GDP resin (Edery *et al.*, 1988) for 2 h at 4°C. The resin was washed three times with 1 ml of buffer A, boiled for 6 min in Laemmli buffer and resolved by SDS-PAGE.

Cell fractionation

HeLa cell fractionation into cytoplasmic, post-nuclear, nucleoplasmic and nuclear fractions was performed as previously described (Lejbkowitz *et al.*, 1992) with minor modifications. Briefly, cells (5×10^6) were washed twice with ice-cold PBS, scraped and pelleted by slow centrifugation. Cell pellets were washed with isotonic buffer 1 [10 mM HEPES-KOH pH 7.4, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF) and CompleteTM], resuspended in 1 ml of buffer 2 (10 mM HEPES-KOH pH 7.4, 250 mM sucrose, 2 mM EDTA, 0.1 mM DTT, 1 mM PMSF and CompleteTM) and disrupted by 50 strokes with a pestle B homogenizer. Lysates were centrifuged at 1000 g for 10 min at 4°C. This step was repeated twice, and the supernatant was regarded as the cytoplasmic fraction. Nuclei were resuspended in 1 ml of buffer 3 (10 mM HEPES-KOH pH 7.4, 250 mM sucrose, 2 mM EDTA, 0.1 mM DTT, 2 mM magnesium chloride, 0.1% Triton X-114, 1 mM PMSF and CompleteTM), incubated on ice for 15 min and centrifuged at 1000 g for 10 min at 4°C. The supernatant was collected and regarded as the post-nuclear fraction. Nuclei were resuspended in 0.5 ml of buffer 4 (10 mM HEPES-KOH pH 7.4, 250 mM sucrose, 2 mM EDTA, 0.1 mM DTT, 0.5% NP-40, 50 mM sodium chloride, 1 mM PMSF and CompleteTM), incubated on ice for 30 min and centrifuged at 1000 g for 10 min at 4°C. This step was repeated twice, and the combined supernatants were regarded as the nucleoplasmic fraction. The NP-40-extracted nuclear pellet was resuspended in 0.2 ml of buffer 5 (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM sodium chloride, 1.5 mM magnesium chloride, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF and CompleteTM), incubated on ice for 20 min and centrifuged at 14 000 g for 2 min at 4°C. The supernatant was collected and regarded as the nuclear fraction.

Construction of plasmids

pcDNA3-4E-T was constructed by cloning the full-length 4E-T cDNA into pcDNA3 (Invitrogen). HA-4E-T was generated by subcloning the coding region of 4E-T from pcDNA3-4E-T into PACTAG2 (kindly provided by M.Tremblay, McGill University). All 4E-T mutants were constructed by PCR using either pcDNA3-4E-T or HA-4E-T as template. The plasmids myc-PK and myc-NPc (kindly provided by G.Dreyfuss, University of Pennsylvania School of Medicine) were used to generate myc-PK- and myc-NPc-4E-T constructs. To subclone 4E-T into myc-PK, primers were designed to include a *KpnI* site and a *NotI* site in the forward and reverse oligonucleotide, respectively (Siomi and Dreyfuss, 1995). To generate myc-NPc-4E-T constructs, primers were designed to include an *EcoRI* site and an *XhoI* site in the forward and reverse oligonucleotide, respectively (Michael et al., 1997). HA-eIF4E wt and HA-eIF4E W73A were described previously (Pyronnet et al., 1999). For the purification of baculovirus-expressed recombinant Flag-HMK-4E-T, the coding region of 4E-T was subcloned into the baculovirus transfer vector pVL1392-FlagHMK (Pharming) (Haghighat et al., 1996).

Transient transfections and immunofluorescence

HeLa cells (50% confluency) were transfected with 10 µg of plasmid DNA by the calcium phosphate transfection method (Graham and Van Der Eb, 1973). After 24 h, cells were trypsinized and plated at 2×10^4 per chamber on Lab-Tek chamber slides (Nunc). At 36 h post-transfection, cells were fixed for 30 min with 4% formaldehyde in PBS and permeabilized for 30 min with 4% formaldehyde/0.2% Tween-20 in PBS at room temperature. Cells were rehydrated briefly with 0.2% Tween-20 in PBS before blocking for 16 h in a solution containing 50% FBS, 6% skim milk, 3% bovine serum albumin (BSA), 0.2% Tween-20 and 0.02% sodium azide. Cells were incubated with primary antibodies for 2 h at room temperature and washed extensively with 0.2% Tween-20/PBS before and after incubation with secondary antibodies for 30 min at room temperature. Cells were mounted in 30% glycerol in PBS and analyzed with a Kontron IBAS confocal imaging system. Images were taken from a $63\times$ objective of a Zeiss LSM 410 microscope. For drug treatment, LMB (kindly provided by M.Yoshida, University of Tokyo) was added to the media at a final concentration of 2 ng/ml, and incubated for the time period indicated in the figure legends.

Pull-down assay

S-Tag-importin α and GST-importin β were purified as previously described (Chi et al., 1996). Baculovirus Flag-HMK-4E-T was purified from *Spodoptera frugiperda* (Sf9) cells as previously described (Haghighat et al., 1995), except that cells were disrupted by a Dounce homogenizer (50 strokes, pestle B) in transport buffer (20 mM HEPES-KOH pH 7.0, 150 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, 0.1% casamino acids, 0.1% Tween-20 and Complete™) and purification was performed in transport buffer. Purification of untagged eIF4E was performed as previously described (Edery et al., 1988), except that the protein was eluted in transport buffer. Approximately 3 µg of S-Tag-importin α , 375 ng of GST-importin β , 1 µg of Flag-HMK-4E-T and 1 µg of eIF4E were used in the assay. Proteins were mixed and pre-incubated on ice for 30 min in a final volume of 30 µl. Transport buffer (250 µl) and 15 µl of glutathione-Sepharose resin (Amersham Pharmacia Biotech) were then added to the samples. The samples were incubated for 40 min at room temperature end-over-end and washed three times with transport buffer. The precipitates were eluted in sample buffer, resolved by SDS-PAGE and analyzed by western blotting.

Nucleotide sequence accession number

The nucleotide and protein sequence of 4E-T have been submitted to the DDBJ/EMBL/GenBank database under accession No. AF240775.

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