Journal of Cellular Biochemistry 86:590–600 (2002)

# Subcellular Localization of the Hypusine-Containing Eukaryotic Initiation Factor 5A by Immunofluorescent Staining and Green Fluorescent Protein Tagging

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**Abstract** Eukaryotic initiation factor 5A (eIF-5A) is the only protein in nature that contains hypusine, an unusual amino acid residue formed posttranslationally by deoxyhypusine synthase and deoxyhypusine hydroxylase. Although the eIF-5A gene is essential for cell survival and proliferation, the precise function and localization of eIF-5A remain unclear. In this study, we have determined the subcellular distribution of eIF-5A by indirect immunofluorescent staining and by direct visualization of green fluorescent protein tagged eIF-5A (GFP-eIF5A). Immunofluorescent staining of the formaldehyde-fixed cells showed that eIF-5A was present in both the nucleus and cytoplasm. Only the nuclear eIF-5A was resistant to Triton extraction. Direct visualization of GFP tagged eIF-5A in living cells revealed the same whole-cell distribution pattern. However, a fusion of an additional pyruvate kinase (PK) moiety into GFP-eIF-5A precluded the nuclear localization of GFP-PK-eIF-5A fusion protein. Fusion of the GFP-PK tag with three different domains of eIF-5A also failed to reveal any nuclear localization of the fusion proteins, suggesting the absence of receptor-mediated nuclear import. Using interspecies heterokaryon fusion assay, we could detect the nuclear export of GFP-Rev, but not of GFP-eIF-5A. The whole-cell distribution pattern of eIF-5A was recalcitrant to the treatments that included energy depletion, heat shock, and inhibition of transcription, translation, polyamine synthesis, or CRM1-dependent nuclear export. Collectively, our data indicate that eIF-5A gains nuclear entry via passive diffusion, but it does not undergo active nucleocytoplasmic shuttling. J. Cell. Biochem. 86: 590–600, 2002. 

**Key words:** eIF-5A; hypusine; GFP-tagging; subcellular localisation

Eukaryotic initiation factor 5A (eIF-5A) is the only protein in nature that contains a hypusine residue. This unusual amino acid formed posttranslationally through the action of deoxyhypusine synthase and deoxyhypusine hydroxylase [review by Park et al., 1993, 1997; Chen and Liu, 1997; Chen and Jao, 1999]. Disruption of either eIF-5A or deoxyhypusine synthase gene in yeast leads to a lethal phenotype [Schnier et al., 1991; Sasaki et al., 1996]. Inhibition of deoxyhypusine synthase activity in mammalian cells causes growth arrest [Jakus et al., 1993; Park et al., 1994], cell death [Tome et al., 1997], or tumor differentiation [Chen et al., 1996]. In addition, hypusine formation exhibits a striking attenuation in senescent cells [Chen and Chen, 1997a], but a marked increase in virally-transformed cells [Chen and Chen, 1997b].

Although, eIF-5A is essential for cell survival and proliferation, its physiological function is unclear. Due to the lack of a clear correlation with general protein synthesis, eIF-5A may not be a bona fide translation initiation factor [Kang and Hershey, 1994]. The suggestion that eIF-5A may serve as a target protein for Rev or Rex [Ruhl et al., 1993; Katahira et al., 1995] has been questioned because a direct protein interaction cannot be established in vitro [Henderson and Percipalle, 1997; Mattaj and Englmeier, 1998; Lipowsky et al., 2000]. Based on the finding that eIF-5A can bind to RRE

Grant sponsor: National Cancer Institute, NIH (United States Public Health Services); Grant number: RO1 CA49695.

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Received 23 January 2002; Accepted 30 April 2002
DOI 10.1002/jcb.10235

and U6 and that eIF-5A recognizes synthetic RNA in a sequence-dependent manner, we proposed that eIF-5A may function as an RNA-binding protein [Liu et al., 1997; Xu and Chen, 2001]. The finding that eIF-5A complements the defect in mRNA decay phenotypes of a temperature-sensitive yeast mutant [Zuk and Jacobson, 1998] indirectly suggests that eIF-5A may be involved in the mRNA metabolism.

The intracellular localization of eIF-5A has been previously studied, but with conflicting results [Ruhl et al., 1993; Shi et al., 1996]. Since the information of protein localization can provided important clues on protein function, we decided to examine the subcellular distribution of eIF-5A not only by in vitro immunofluorescent staining, but also by direct visualization of eIF-5A using the GFP-tagging technique. GFP is an autofluorescent protein derived from the jellyfish *Aequorea victoria* and GFP tagging has been widely used as a marker for protein targeting and localization in a living cell [Tsien, 1998]. Moreover, fusion proteins of GFP with truncated eIF-5A allowed us to determine possible presence of domains for nuclear import and export.

## MATERIALS AND METHODS

### Materials

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Polyclonal antibody against recombinant human eIF-5A was raised in chicken and affinity-purified by recombinant eIF-5A proteins. The purified antibody does not cross-react with other cellular proteins [Chen and Jao, 1999]. Rabbit anti-GFP antibody was purchased from Clontech (Palo Alto, CA). Anti-calnexin monoclonal antibody was purchased from StressGen (Victoria, BC). Anti-human CRM1 antiserum was kindly provided by Minoru Yoshida [Kudo et al., 1998].

### Cell Culture

Human epithelial carcinoma cell (HeLa), COS-7 (green monkey kidney cell), and NIH3T3 (mouse embryonic fibroblast) were cultured in Dulbecco's modified Eagles's medium (DMEM) containing with 10% fetal bovine serum (FBS) at 37°C. For transient transfection, cells grown on glass coverslips in 35-mm dishes were transfected with 1-μg plasmid DNA using GenePORTER™ transfection reagent (Gene Therapy Systems, San Diego, CA).

### Molecular Cloning

Human eIF-5A cDNA was cloned into *Hin*dIII-cut pEGFP-C1 (Clontech) to generate pGFP-5A. GFP-pyruvate kinase (GPK) tag was constructed as follows: the cDNA corresponding to chicken muscle PK from codon 17 to the carboxyl terminal end was PCR-amplified from the pMyc-PK plasmid [Siomi and Dreyfuss, 1995]. The amplified PK cDNA fragment was then cloned between *Ssp*BI and *Xho*I sites of pEGFP-C1 and pGFP-5A to generate pGPK and pGPK-5A, respectively. For the construction of GFP- or GPK-tagged eIF-5A deletion mutants, PCR-generated cDNAs corresponding to amino acid sequences 1–83, 84–154, and 41–120 of human eIF-5A were substituted for the full-length eIF-5A cDNA in pGPK-5A to create pGPK-5A(1–83), pGPK-5A(84–154), and pGPK-5A(41–120), respectively. To construct pGPK-5A (K50R), the lysine residue at codon 50 in the human eIF-5A gene of pGPK-5A was mutated to arginine using Transformer™ Site-Directed Mutagenesis Kit (Clontech). The pGPK-NLS was generated by replacing the *Hin*dIII-Myc-PK-*Eco*RI fragment in pMyc-PK-NLS [Michael et al., 1995] with *Nhe*I-GPK-*Eco*RI fragment obtained from pGPK. The *Eco* RI ends were ligated first and then the *Hin*dIII and *Nhe*I ends were blunted by T4 DNA polymerase followed with ligation. To create pGPK-5A-NLS, the primers 5′-CCAAAAGCTTAATGGCAGATGACTTGGACTTCGAG-3′ (sense) and 5′-TTAAAGCTTCCTTTTGCCATGGCCTTGATTGC-3′ (antisense) were used to amplify the human eIF-5A cDNA by PCR. After digestion, the eIF-5A cDNA was cloned into the *Hin*dIII site of pGPK-NLS to generate pGPK-5A-NLS. To generate pGPK-Rev-NLS and pGPK-M10-NLS, the Rev and Rev M10 cDNA fragments were amplified from pCsRevsg25GFP and pCs RevM10BLsg25 [Afonina et al., 1998]. After digestion, the corresponding fragment was cloned into the *Eco*RI site of pGPK-NLS to generate pGPK-Rev-NLS and pGPK-M10-NLS, respectively.

### Immunostaining and Fluorescence Microscopy

Cells were fixed either with methanol or 4% paraformaldehyde in PHEM buffer (60 mM PIPES, pH 6.9, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$) for 15 min and permeabilized with 0.5% Triton X-100 for 10 min. For detergent extraction prior to fixation, the cells were

incubated first with 0.2% of Triton X-100 in PHEM buffer for 1 min at room temperature before fixation. The cells were then incubated with the blocking solution containing 10% bovine serum albumin (BSA), 0.1% Tween-20, and 0.02% sodium azide in PHEM buffer for 30 min. The cells were probed with anti-eIF-5A (1:20 dilution), anti-calnexin (1:200 dilution), anti-CRM1 (1:200 dilution), or anti-GFP (1:100 dilution) antibodies for 1 h at room temperature, followed by washing with the blocking solution. The treated cells were incubated with appropriate secondary antibodies (1:100 dilution) coupled with fluorescein isothiocyanate (FITC) or Cy3 fluorophores for 30 min. The cells were then washed in PHEM buffer extensively, mounted in PHEM buffer containing 10% glycerol, 2.5% 1,4-diazabicyclo-[2.2.2] octane (DABCO), and analyzed with an Olympus BH-2 fluorescence microscope equipped with a digital camera (MDS120, Kodak). To observe GFP fusion proteins in living cells, cells were seeded onto glass coverslips and maintained at 37°C with full humidity and 5% $CO_2$. The coverslip was removed from the incubator and mounted upside-down onto a hanging-drop slide containing DMEM about 24–48 h post-transfection for visualization. The cells were observed immediately after the mounting under the fluorescence microscope at 25°C.

### Interspecies Heterokaryon Assay

The interspecies heterokaryon assay was performed as described [Pinol-Roma and Dreyfuss, 1992]. Briefly, 24 h after transfection, the transfected HeLa cells were seeded with a fivefold excess of untransfected mouse NIH3T3 cells. The following day, cells on the coverslip were washed with DMEM and then inverted onto a drop of polyethylene glycol 3350 (50% w/w in Earl's balanced salt solution) for 2 min. The coverslip was rinsed three times with DMEM and returned to heterokaryon growth medium (DMEM, 10% FBS, 20% water, 100 μg/ml cycloheximide, and 1% penicillin and streptomycin) for 3 h before fixation for fluorescence microscopy. To block new protein synthesis, the culture was incubated for 3 h in the presence of 50 μg/ml cycloheximide and 30 min in the presence of 100 μg/ml cycloheximide before fusion. All solutions were pre-warmed to 37°C. In order to distinguish the 3T3 mouse nuclei from the HeLa nuclei, cells were stained with propidium iodide (PI, 0.5 μg/ml) and observed by the rhodamine filter set. Mouse nuclei display a speckled pattern after PI staining. In addition, they are less transparent than human nuclei after PI staining.

### Western Blot Analysis

Transfected COS-7 cells growth in 35-mm dishes were trypsinized, washed in PBS (50 mM sodium phosphate, pH 7.5, and 150 mM NaCl), and lyzed in 500 μl of lysis buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, and a panel of protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN). Cell lysates were clarified by centrifugation at 14,000*g* for 10 min. The supernatant was subjected to SDS–PAGE and Western blot analysis using antibodies against eIF-5A or GFP (1:1,000 dilution). The proteins were detected by enhanced chemical luminescence method (Amersham Pharmacia Biotech, Piscataway, NJ).

## RESULTS

### Indirect Immunofluorescent Staining of Endogenous eIF-5A

The immunofluorescent staining of eIF-5A in cells was performed under two fixation conditions, one with formaldehyde and the other with methanol. As shown in Figure 1A, the fluorescent signal in cells fixed with formaldehyde displayed a whole-cell distribution pattern and appeared to be more prominent at the site that resembles the structure termed annulate lamellae. In contrast, Figure 1C shows that the fluorescent signal was apparent only in the cytoplasm of cells fixed with methanol, although with an intense staining near the perinuclear region. As a control, Figure 1E shows that no signal was observed when the pre-absorbed antibody was used for staining. The corresponding phase-contrast images of cells were included to indicate the integrity of cells after fixation (Fig. 1B,D,F). This result suggests that the controversy about the eIF-5A localization in the literature [Ruhl et al., 1993; Shi et al., 1996] could be due to different fixation protocols used. Although, formaldehyde and methanol are both commonly used in immunofluorescent staining, they can produce different staining patterns in some cases. For example, the nuclear presence of plakophilin [Mertens et al., 1996] and feline immunodeficiency virus, Vif [Chatterji et al., 2000] can be demonstrated only by

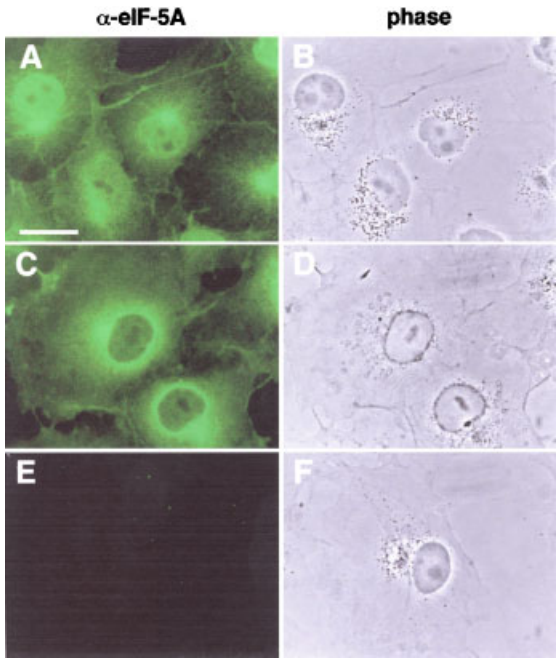


**Fig. 1.** Subcellular localization of eIF-5A by indirect immunofluorescence with two different fixation protocols. Immunofluorescence (left-hand panel) and corresponding phase-contrast images (right-hand panel) of COS-7 cells fixed with 4% paraformaldehyde (**A** and **B**; **E** and **F**) or methanol (**C** and **D**) are shown. Fixed cells were treated with the purified anti-eIF-5A antibody (1:20). FITC-conjugated rabbit anti-chicken immunoglobulin (IgG) was used as the secondary antibody to visualize the protein. As a control, fixed cells were also probed with the anti-eIF-5A antibody that was pre-absorbed with recombinant human eIF-5A proteins (E and F). Bar: 25 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

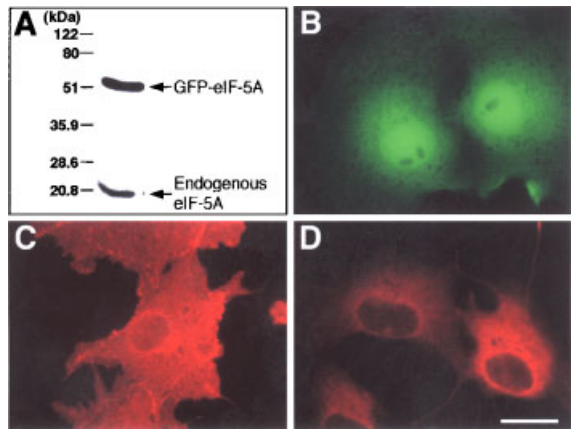


**Fig. 2.** Expression and localization of GFP-eIF-5A in transiently transfected COS-7 cells. (**A**) Western blot analysis of GFP-eIF-5A in the transfected cells. (**B**) Direct visualization of GFP-eIF-5A. (**C**) Indirect immunofluorescent staining of GFP-eIF-5A in the transiently transfected cells (fixed with 4% paraformaldehyde). (**D**) Indirect immunofluorescent staining of the same cells in methanol. Bar: 25 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

formaldehyde fixation, but not by methanol fixation. Formaldehyde fixation is a more gentle method in preserving cellular structure because of the formation of cross-linked networks of biopolymers [Solomon and Varshavsky, 1985]. In contrast, methanol fixation tends to collapse the nuclear structure, which may randomize the availability of the epitope to the antibody or lead to leaking of certain nuclear proteins [Schimenti and Jacobberger, 1992; Chatterji et al., 2000].

### Direct Visualization of GFP-Tagged eIF-5A in Living Cells

To further confirm the whole-cell distribution pattern of eIF-5A that we observed in the formaldehyde-fixed cells, we took advantage of the approach of GFP-tagging, which allows direct observation of tagged protein in vivo. Figure 2A shows the expression level of green fluorescent protein tagged eIF-5A (GFP-eIF-5A) ($Mr = 45$ kDa) in the transiently transfected COS-7 cells as compared to that of endogenous eIF-5A. Using yeast plasmid shuffling assay, we have shown that GFP-eIF-5A fusion protein is fully functional (Chatterjee and Chen, unpublished data). Figure 2B shows that the GFP-eIF-5A in the living cells exhibited a whole-cell distribution pattern. We then compared the pattern with that obtained by immunostaining using anti-GFP antibody. While GFP-eIF-5A displayed a whole-cell distribution in the formaldehyde-fixed cells (Fig. 2C), it was apparent only in the cytoplasm in the methanol-fixed cells (Fig. 2D). This result clearly indicated that formaldehyde fixation produced a staining pattern most closely resembles that obtained by in vivo GFP-tagging. We, therefore, concluded that the whole-cell distribution pattern probably represents more closely to the true localization of eIF-5A in living cells.

### Does eIF-5A Co-Localize With Calnexin or CRM1?

Earlier studies have suggested that eIF-5A co-localizes either with calnexin, a resident protein of endoplasmic reticulum [Shi et al., 1996] or CRM1, a general nuclear export receptor [Rosorius et al., 1999]. Since protein localization revealed by immunofluorescent staining can be influenced by fixation protocols, we decided to re-examine the possible co-localization

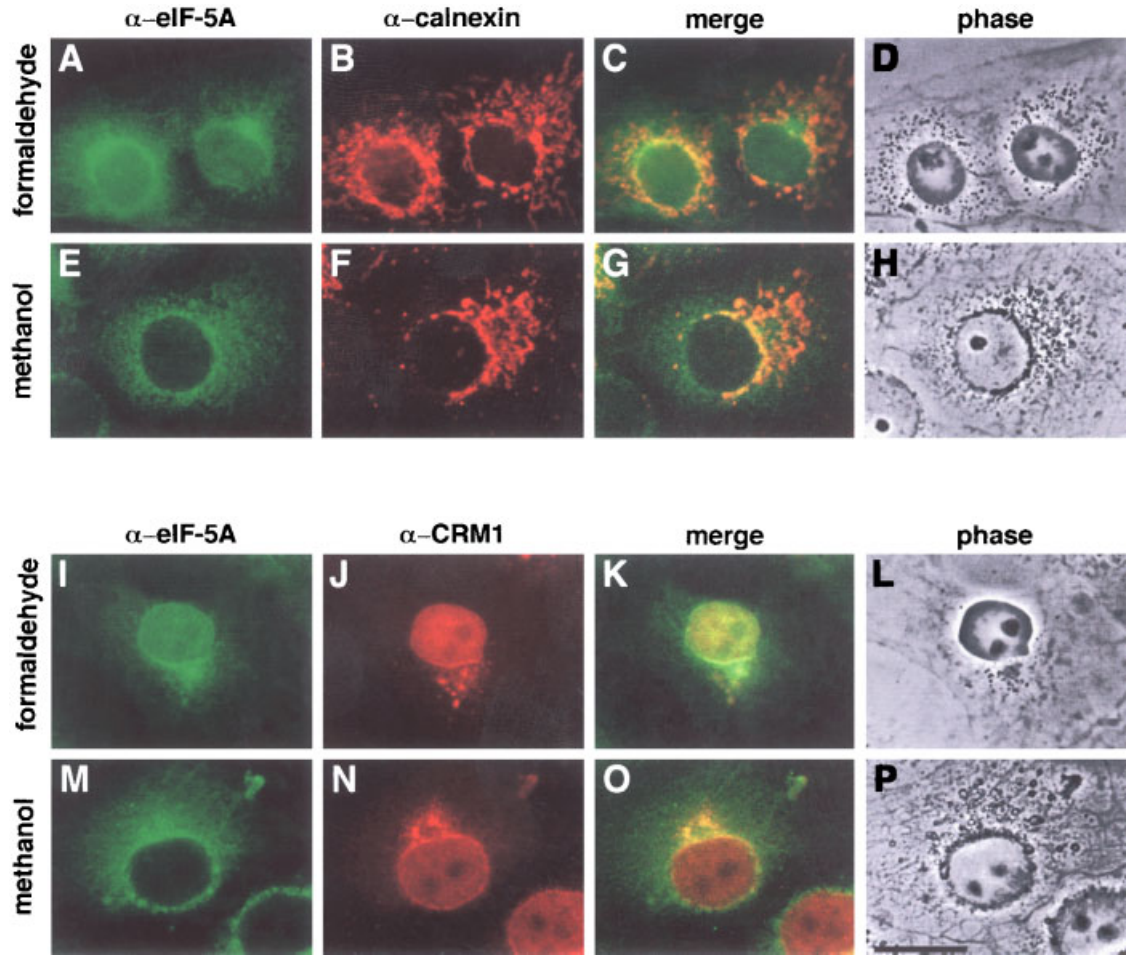


**Fig. 3.** Staining of eIF-5A and calnexin (top panels) or eIF-5A and CRM1 (bottom panels) by double immunofluorescence microscopy. COS-7 cells were fixed with formaldehyde or methanol and then stained with anti-eIF-5A and anti-calnexin antibodies (top panel) or anti-eIF-5A and anti-CRM1 antibodies (bottom panel). FITC-fluorescence images revealed the distribution of eIF-5A (**A**, **E**, **I**, and **M**). Cy3-fluorescence images revealed the distribution of calnexin (**B** and **F**) or CRM1 (**J** and **N**). Double immunofluorescence images revealed the merged images of eIF-5A and calnexin (**C** and **G**) or eIF-5A and CRM1 (**K** and **O**). Phase contrast images of the corresponding fixed cells are shown in (**D**), (**H**), (**L**), and (**P**). Bar: 25 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of eIF-5A with calnexin and CRM1. The top panels of Figure 3 show that both formaldehyde and methanol fixation revealed the same cytoplasmic localization of calnexin with the characteristic punctate staining pattern (Fig. 3B,F). This staining pattern of calnexin was clearly different from that of eIF-5A (Fig. 3A,E). There appeared to be certain overlap of calnexin and eIF-5A in the merged images (Fig. 3C,G). This partial overlap is likely to be fortuitous due to the whole-cell distribution nature of eIF-5A. However, other biochemical approach is needed to determine whether there is any functional association of eIF-5A with ER or other structures such as microtubule organization center (MTOC).

The bottom panels of Figure 3 show that CRM1 was found to be in the nucleus of both the formaldehyde- and methanol-fixed cells, (Fig. 3J,N). Double immunofluorescent staining shows a substantial overlap of eIF-5A and CRM1 signals in the nuclear area of the formaldehyde-fixed cells (Fig. 3K), and very little overlap was observed in the methanol-fixed cells (Fig. 3O). In the formaldehyde-fixed cells, the signals of both eIF-5A and CRM1 were prominent at the site corresponding to annulate lamellae. Although, the functional role of annulate lamellae is unclear [Meier et al., 1995], the presence of CRM1 in annulate lamellae has been demonstrated [Fornerod et al., 1997]. These results suggest that eIF-5A does not co-localize with

calnexin, but may co-localize with CRM1 in the nucleus.

### How Does eIF-5A Gain Nuclear Entry?

The existence of eIF-5A in the nucleus poses the question on how eIF-5A gains nuclear entry. Because of the relatively small size, either eIF-5A or GFP-eIF-5A can enter the nucleus through passive diffusion. Since globular proteins with size larger than 60 kDa cannot diffuse across the nuclear pore barrier [Ohno et al., 1998], the fusion of PK moiety to a small polypeptide has been used to minimize the concern of passive diffusion [Siomi and Dreyfuss, 1995]. Thus, to distinguish the receptor-mediated import from passive diffusion, we have constructed GPK-tagged eIF-5A chimera proteins and examined their localization in intact cells. Figure 4A shows that the expression level of GPK-eIF-5A in the log phase cells as compared to that of the endogenous eIF-5A. Figure 4B shows that GPK-eIF-5A, with a size of about 98 kDa, was localized exclusively in the cytoplasm in the intact cells. This result suggests strongly that eIF-5A may gain nuclear entry via passive diffusion.

### Does eIF-5A Contain Nuclear Localization Signal (NLS)?

If indeed eIF-5A enters the nucleus via diffusion process, it would imply that eIF-5A does not contain a functional NLS. Alternatively, the NLS may be dormant due to masking and thus non-functional. To differentiate these possibilities, we have constructed chimeras with GPK tag fused to the N-terminal domain (amino acids 1–83), C-terminal domain (amino acids 84–154), and the central region (amino acids 41–120) of eIF-5A. Figure 5A shows the expression levels of these different chimera proteins in the transfected cells. Although, GPK-eIF-5A(1–83) protein cannot be recognized by our anti-eIF-5A antibody, its expression was confirmed by Western blot using anti-GFP antibody (Fig. 5A). Although all three chimera proteins were expressed, none of them was detected in the nucleus, suggesting a lack of NLS in any of these regions (Fig. 5B–D). Furthermore, we found that mutation of hypusine site did not affect the subcellular distribution of GPK-eIF-5A (Fig. 5E), suggesting that the hypusine residue is not involved in the nuclear import. We do not think that GPK tag itself could affect the import activity of eIF-5A, since addition of a small NLS segment derived from hnRNP K to the GPK-eIF-5A led to nuclear import (see below). Collectively, these results suggests that receptor-mediated nuclear import activity of eIF-5A was either absent or very

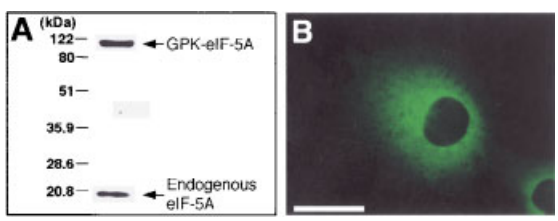


**Fig. 4.** Expression and localization of GPK-eIF-5A in transiently transfected COS-7 cells. Visualization was carried out at room temperature. (**A**) Western blot analysis of GPK-eIF-5A in the transfected cells as detected by anti-eIF-5A antibody. (**B**) Direct visualization of GPK-eIF-5A in COS-7 cells. Bar: 25 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

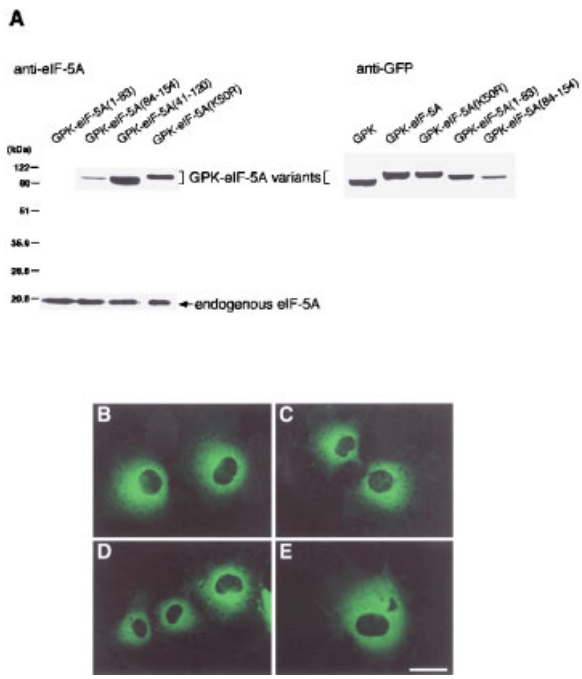


**Fig. 5.** Expression and subcellular localization of GPK-tagged eIF-5A domain chimera proteins in living cells. (**A**) Western blot analysis of various GPK-eIF-5A fusion proteins using anti-eIF-5A or anti-GFP antibody. Note that GPK-eIF-5A(1–83) could not be detected by the anti-eIF-5A antibody, since the epitopes of this antibody are located to the C-terminal portion of eIF-5A. However, it can be detected by anti-GFP antibody. (**B**) Direct visualization of GPK-eIF-5A (aa 1–83). (**C**) Direct visualization of GPK-eIF-5A (aa 41–120). (**D**) Direct visualization of GPK-eIF-5A (aa 84–154). (E) Direct visualization of GPK-eIF-5A (KSOR). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

weak and that the nuclear localization of endogenous eIF-5A was most likely due to passive diffusion.

### Nuclear Export Activity of eIF-5A

The human eIF-5A contains a leucine-rich region at the C-terminal domain (amino acids 89–102), with a sequence similar to the nuclear export signal (NES) in HIV-1 Rev and Influenza A NS1 [Liu et al., 1997]. Thus, it is still possible that eIF-5A may contain a functional NES and thus can engage nucleocytoplasmic trafficking. To determine whether this is the case, we performed an interspecies heterokaryon assay. We first used Rev and Rev mutant M10, whose NES is mutated, to demonstrate the feasibility of the assay. HeLa cells were first transfected with an expression plasmid encoding GPK-Rev-NLS fusion protein (i.e., GPK-Rev fused at its carboxyl terminus to a classic NLS of hnRNP K) or GPK-M10-NLS. The transfected human cells were then fused with mouse NIH 3T3 cells to form heterokaryons. The heterokaryons were fixed and the nucleocytoplasmic transport properties of the GPK fusion proteins were examined under the fluorescence microscope. Figure 6 shows that GPK-Rev-NLS shuttled between the heterologous nuclei (Fig. 6A,B), whereas the NES-mutated GPK-M10-NLS protein was retained in the human nuclei (Fig. 6D,E). Under the same condition, the GPK-eIF5A-NLS fusion protein was retained in the human nuclei and did not exhibit any nuclear export (Fig. 6G,H). This result suggested that eIF-5A does not contain a functional NES for active nucleocytoplasmic shuttling.

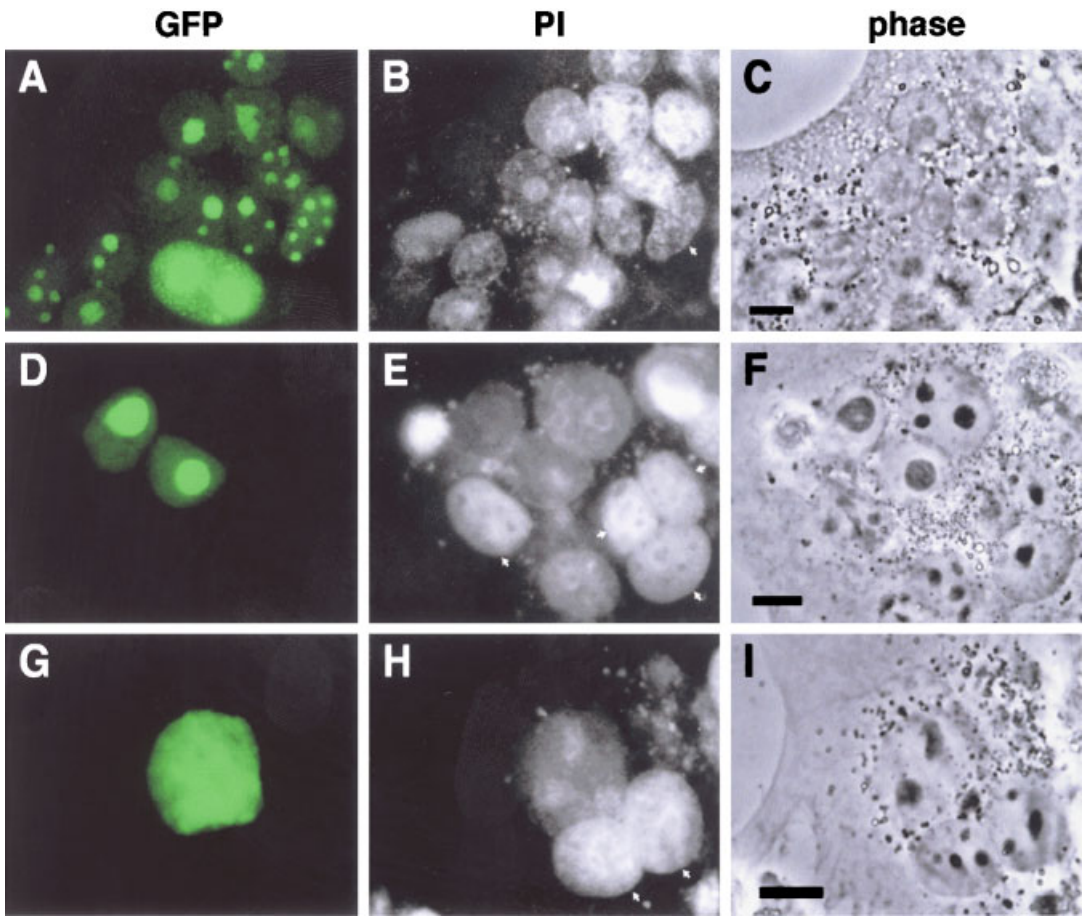


**Fig. 6.** Heterokaryon analysis of the nuclear export of eIF-5A. HeLa cells were transfected with GPK-Rev-NLS, GPK-M10-NLS, or GPK-5A-NLS and then fused with mouse 3T3 cells as described under Materials and Methods. (**A**), (**D**), and (**G**) are the intrinsic GFP signals of GPK-Rev-NLS, GPK-M10-NLS, and GPK-5A-NLS, respectively. (**B**), (**E**), and (**H**) are the PI staining of the same cells in (A), (D), and (G), respectively. (**C**), (**F**), and (**I**) are the phase-contrast images in (A), (D), and (G), respectively. Arrows denote the mouse nuclei. Bar: 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

### Nuclear eIF-5A is Resistant to Detergent Extraction

Treatment of cells with non-ionic detergent before fixation has been employed to distinguish two populations of antigens, one soluble and the other presumably associated with certain cytoskeletal structures [Bravo and MacDonald-Bravo, 1987]. We have adopted this method to determine whether the cytoplasmic and the nuclear presence of eIF-5A can also be distinguished by Triton X-100 extraction. Figure 7 shows that while Triton X-100 removed the eIF-5A signal in the cytoplasm, the eIF-5A signal in nucleus remained strong (Fig. 7A). This is to be contrasted with GFP, which also exhibited a whole-cell distribution pattern after transfection (data not shown). The GFP signal, both in the nucleus and cytoplasm, was completely eliminated by Triton X-100 treatment (Fig. 7C). These results suggest that the cytoplasmic eIF-5A may be soluble, whereas the nuclear eIF-5A may be associated with certain structural components in the nucleus.

### Subcellular Distribution of eIF-5A Under Different Conditions

Changes in the subcellular distribution of a target protein under different pharmacological or physiological conditions can yield clues on protein function. We have examined whether the eIF-5A localization may be perturbed by treatments, such as heat shock, energy depletion, cytoskeleton disruption, polyamine depletion, and inhibition of protein synthesis, transcription, or CRM1-dependent nuclear export. The results were summarized in Table I, which indicated that none of the treatments significantly altered the subcellular localization of endogenous eIF-5A or transfected GFP-eIF-5A. Thus, the global distribution of eIF-5A inside the cell appeared to be recalcitrant to many conditions that severely alter the metabolic and physiological state. Nevertheless, we still cannot exclude the possibility that our detection method based on immunostaining or GFP-tagging may not be sensitive enough to detect a small fraction of eIF-5A pool that undergoes changes in distribution.

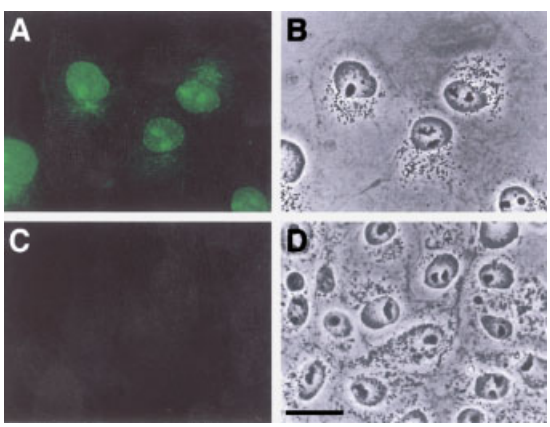


**Fig. 7.** Effect of detergent extraction on the immunostaining patterns of eIF-5A and GFP in the COS-7 cells. COS-7 cells were treated with 0.2% Triton X-100 before fixation as described under Materials and Methods. (**A**) Immunofluorescent staining of the Triton-extracted COS-7 cells using anti-eIF-5A antibody. (**B**) Phase-contrast image in (A). (**C**) Immunofluorescent staining of the Triton-extracted GFP-transfected COS-7 cells using anti-GFP antibody. (**D**) Phase-contrast image in (C). Bar: 25 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

## DISCUSSION

In the present study, we have employed both the in vitro and in vivo approach and demonstrated that eIF-5A is present in the cytoplasm and nucleus (Figs. 1 and 2). The use of GFP- and GPK-tagging for direct visualization of marked eIF-5A in living cells provided the in vivo data suggesting that eIF-5A enters the nucleus via passive diffusion (Fig. 2 vs. 4). The absence of detectable NLS activity in various GPK-tagged chimeras (Fig. 5) substantiates the notion that passive diffusion is the mechanism for eIF-5A to gain nuclear entry. Furthermore, heterokaryon assay results indicate that eIF-5A does not undergo any appreciable nucleocytoplasmic shuttling (Fig. 6).

The whole-cell distribution pattern of eIF-5A is consistent with the notion that eIF-5A may function as an RNA-binding protein. It is known that many RNA-binding proteins are distributed in the cytoplasm and nucleus [Shen et al., 2000]. The lack of any appreciable nuclear signal in the methanol-fixed cells could be attributed to either the randomization of the available epitope or the leaking. Similar findings have been reported for other nuclear proteins after methanol fixation [Schimenti and Jacobberger, 1992; Chatterji et al., 2000].

That eIF-5A may function as an RNA-binding protein is supported by the following evidence: (i) motif analysis revealed that it has a bimodular structure similar to RNA-binding proteins such as Rev and NS-1 [Liu et al., 1997]; (ii) two X-ray diffraction studies of the archae

**TABLE I. Effects of Various Pharmacological and Physical Treatments on the Subcellular Distribution of eIF-5A in COS-7 Cells**

| Effect of treatments | Conditions of treatments[a] | Localization[b] |
|---|---|---|
| Inhibition of polyamine biosynthesis | 5 mM α-difluoromethylornithine (DFMO)<br>5 μM methylglyoxal *bis*(guanyl-hydrazone) (MGBG)<br>40 μM $N^1$-guanyl-1,7-diaminoheptane (GC7)<br>Incubated for 48 h at 37°C | N, C |
| Depletion of energy source | 10 mM deoxyglucose<br>20 μM CCCP<br>10 mM sodium azide<br>Incubated in dialyzed serum-containing medium for 3 h at 4°C | N, C |
| Inhibition of CRM1-dependent nuclear export | 100 nM leptomycin B for 2 h at 37°C<br>200 nM leptomycin B for 9 h at 37°C | N, C |
| Inhibition of protein synthesis | 50 μg/ml puromycin for 16 h at 37°C<br>100 μg/ml cycloheximide for 4 h at 37°C | N, C |
| Inhibition of RNA synthesis | 5 μg/ml actinomycin D for 4 h at 37°C | N, C |
| Disruption of cytoskeleton | 5 μg/ml cytochalasin B<br>5 μg/ml cytochalasin D<br>5 μg/ml colchicine<br>Incubated for 2 h at 37°C | N, C |
| Heat stress | Incubated at 44°C for 3 h | N, C |

[a]Cells at logarithmic growth were treated under various conditions as listed and then processed for determining the localization of eIF-5A.
[b]Subcellular distribution of eIF-5A was determined by either in vitro indirect immunofluorescent staining in all cases or direct visualization of GFP-tagged eIF-5A in the cases of inhibition of nuclear export and protein synthesis. N, present in the nucleus; C, present in the cytoplasm.

eIF-5A precursor show that it is composed of two domains, one containing many positively charged residues and the other is similar to cold shock domain of CspA, linked by a flexible hinge [Kim et al., 1998; Peat et al., 1998]. Both the positively-charged hypusine and the cold shock domain have the potential to interact with nucleic acids; (iii) we have shown that eIF-5A is capable of binding to RNA in vitro and that the binding requires either deoxyhypusine or hypusine [Liu et al., 1997]; and (iv) more recently, we have demonstrated by SELEX method that eIF-5A recognizes RNA in a sequence-dependent manner [Xu and Chen, 2001]. The notion that eIF-5A may function as an RNA binding protein is also consistent with the finding that an expression of *TIF51A* gene, which encodes eIF-5A, complements the impaired mRNA decay phenotypes of a yeast mutant [Zuk and Jacobson, 1998].

Although, eIF-5A is termed as initiation factor, the evidence is still weak. In fact, it fails to show any significant effect on the translation with physiological mRNA in vitro [Kemper et al., 1976; Benne and Hershey, 1978]. Further, depletion of eIF-5A in a conditional yeast mutant only inhibits the protein synthesis by 30% [Kang and Hershey, 1994]. It is still possible that eIF-5A may be required for translation of selective mRNAs [Kang and Hershey, 1994]. If this is the case, eIF-5A may interact with a small class of mRNA in vivo. In this regard, it is interesting to note that the consensus sequence identified by SELEX is present in over 400 human EST sequences [Xu and Chen, 2001]. It is quite possible that some of these sequences will serve as the physiological targets of eIF-5A.

Although, eIF-5A has been suggested to target HIV-Rev and to mediate its nuclear export [Ruhl et al., 1993], no direct binding between eIF-5A and Rev could be demonstrated so far [Henderson and Percipalle, 1997; Lipowsky et al., 2000]. On the other hand, two recent studies have demonstrated the interaction of eIF-5A with the nuclear export receptor, CRM1 [Rosorius et al., 1999] and exportin4 [Lipowsky et al., 2000]. It remains to be investigated, however, whether these interactions may be related to the binding of eIF-5A to RNA. Nevertheless, both findings are consistent with the nuclear localization of eIF-5A as shown in the present study.

The presence of eIF-5A in the nucleus poses the following questions: (i) what is the physiological function of the nuclear eIF-5A? (ii) is the function of nuclear eIF-5A different from that of the cytoplasmic eIF-5A? and (iii) how the level of nuclear eIF-5A is regulated? The possible interaction of eIF-5A with nuclear exporter such as CRM1 [Rosorius et al., 1999] or exportin4 [Lipowsky et al., 2000] would implicate a role for eIF-5A in nuclear transport. However, despite the high binding affinity between exportin4

and eIF-5A, we could not detect any appreciable nucleocytoplasmic shuttling of eIF-5A by the heterokaryon assay (Fig. 6C). In contrast, we have no problem to demonstrate the nuclear export of Rev in the same assay (Fig. 6A,B). The nuclear export of eIF-5A was previously demonstrated in vitro by either microinjection or cell permeabilization using exogenously added eIF-5A [Rosorius et al., 1999; Lipowsky et al., 2000]. Since both methods could result in very high concentration of eIF-5A in the nucleus, we suspect that in our study, the nuclear level of GFP-eIF-5A may not be high enough to trigger receptor-mediated export. Alternatively, it is possible that the degree of receptor-mediated nuclear export, if present, is too small to be detected by the assay method. Although, the specificity and high affinity of eIF-5A-exportin4 interaction suggests that this binding has to be functional, exportin4 is absent in the yeast *Saccahromyces cerevisiae* [Lipowsky et al., 2000]. However, we noted that the concentration of eIF-5A in HeLa cell is about 10-fold higher than that in the yeast (data not shown). In light of this, it will be of interest to know whether exportin4 is evolved to endow eIF-5A with expanded functions or simply to prevent any untimely accumulation of eIF-5A in the nucleus. Our finding that nuclear eIF-5A is resistant to detergent extraction (Fig. 7) suggests that it may interact with some structural components in the nucleus. This would also suggest that the chemical potential of the nuclear eIF-5A could be significantly lower than that of the cytoplasmic eIF-5A. If this is the case, it will make sense to have some mechanism in place to prevent the accumulation of nuclear eIF-5A.

In conclusion, the whole-cell distribution pattern of eIF-5A is consistent with the notion that eIF-5A functions as an RNA binding protein [Liu et al., 1997; Xu and Chen, 2001]. In addition, our study revealed: (i) eIF-5A enters nucleus via passive diffusion; (ii) there is an apparent lack of detectable nucleocytoplasmic shuttling; (iii) the nuclear eIF-5A is resistant to non-ionic detergent extraction; and (iv) the subcellular distribution of eIF-5A is recalcitrant to various pharmacological treatments (Table I). Future research on the function of eIF-5A should accommodate these findings. However, the nuclear presence of eIF-5A will have to be reconciled with the apparent dilemma that archaea have no nuclei, although clearly eIF-5A is essential for the survival of archaea [Jansson et al., 2000].

## ACKNOWLEDGMENTS

We are grateful to Dr. Gideon Dreyfuss, University of Pennsylvania for pcDNA3-myc-PK and Myc-PK-NLS; Dr. George N. Pavlakis, NCI-Frederick Cancer Research and Development Center for pCsRevsg25GFP and pCs RevM10BLsg25; Dr. Barbara Wolff, Norvatis Research Institute, for leptomycin B. The work was supported in part by United States Public Health Services Grant RO1 CA49695 awarded by the National Cancer Institute, NIH.

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