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Differential expression of eIF5A-1 and eIF5A-2 in human cancer cells

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Abstract

Eukaryotic translation initiation factor 5A (eIF5A) is the only cellular protein that contains the unusual amino acid hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine]. Vertebrates carry two genes that encode two eIF5A isoforms, *eIF5A-1* and *eIF5A-2*, which, in humans, are 84% identical. eIF5A-1 mRNA (1.3 kb) and protein (18 kDa) are constitutively expressed in human cells. In contrast, expression of eIF5A-2 mRNA (0.7–5.6 kb) and eIF5A-2 protein (20 kDa) varies widely. Whereas eIF5A-2 mRNA was demonstrable in most cells, eIF5A-2 protein was detectable only in the colorectal and ovarian cancer-derived cell lines SW-480 and UACC-1598, which showed high overexpression of eIF5A-2 mRNA. Multiple forms of eIF5A-2 mRNA (5.6, 3.8, 1.6 and 0.7 kb) were identified as the products of one gene with various lengths of 3′-UTR, resulting from the use of different polyadenylation (AAUAAA) signals. The eIF5A-1 and eIF5A-2 precursor proteins were modified comparably in UACC-1598 cells and both were similarly stable. When eIF5A-1 and eIF5A-2 coding sequences were expressed from mammalian vectors in 293T cells, eIF5A-2 precursor was synthesized at a level comparable to that of eIF5A-1 precursor, indicating that the elements causing inefficient translation of eIF5A-2 mRNA reside outside of the open reading frame. On sucrose gradient separation of cytoplasmic RNA, only a small portion of total eIF5A-2 mRNA was associated with the polysomal fraction, compared with a much larger portion of eIF5A-1 mRNA in the polysomes. These findings suggest that the failure to detect eIF5A-2 protein even in eIF5A-2 mRNA positive cells is, at least in part, due to inefficient translation.

Keywords

alternative polyadenylation; eIF5A; hypusine; post-translational modification; translation

Translational control of gene expression is vital in the regulation of cell proliferation [1]. Eukaryotic initiation factor 5A (eIF5A) stimulates methionyl-puromycin synthesis, a model

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assay for the first ribosomal peptidyl transfer reaction [2]. Although the true physiological activity of this factor has yet to be elucidated, it has been proposed to be a mRNA-specific initiation factor [3,4] and to be involved in transport of HIV mRNAs [5] and mRNA turnover [6]. This protein is unique in that it contains the polyamine-derived amino acid hypusine [N^{ε}-(4-amino-2-hydroxybutyl)lysine], which is essential for its activity [7]. Hypusine synthesis occurs only in this protein in two post-translational modification steps. First, deoxyhypusine synthase (DHS; EC 2.5.1.46) catalyzes NAD-dependent transfer of the aminobutyl moiety of the polyamine spermidine to the ε-amino group of a single conserved lysine residue (Lys50 in the human eIF5A precursor) to form deoxyhypusine [N^{ε}-(4-aminobutyl)lysine] [8]. In the second step, deoxyhypusine hydroxylase (EC 1.14.99.29) converts the deoxyhypusine residue into hypusine [9] to complete eIF5A activation.

eIF5A is essential for sustained proliferation of mammalian cells [7,10–14], yeast [15–17] and an eIF5A homolog, aIF5A, for archaea [18]. Disruption of both of the eIF5A genes (*TIF51A* and *TIF51B*) in the yeast *Saccharomyces cerevisiae*, or substitution with a mutated eIF5A gene (Lys50Arg) causes growth arrest. Likewise, inactivation of the single DHS gene, or substitution with a gene encoding an enzymatically inactive protein, also leads to cessation of yeast growth [19,20] Furthermore, inhibitors of DHS, such as N^1-guanyl-1,7-diaminoheptane, and of deoxyhypusine hydroxylase, such as ciclopirox, exert strong antiproliferative effects in mammalian cells, including various human cancer cell lines, and cause arrest of cell cycle progression [21–25]. These findings indicate that eIF5A is intimately involved in cell proliferation, and may therefore contribute to malignant cell transformation.

Two or more eIF5A genes have been identified in many eukaryotic organisms from fungi to humans (Z. A. Jenkins, P. G. Hååg, M. H. Park and H. E. Johansson, unpublished work) [26]. However, in mammalian cells, only one hypusine-containing protein, corresponding to eIF5A-1, is readily detected by radiolabeling with [^{3}H]spermidine. Only in the chicken embryo has the simultaneous expression of two protein isoforms been reported [27,28]. In addition to the EIF5A1 gene [29,30], a second human eIF5A gene, EIF5A2, has been identified, which encodes an isoform with 84% identity with eIF5A-1 [26]. Weak expression of EIF5A2 was seen in various human tissues (multiple-tissue expression array) and high levels of expression only in parts of the brain and testis. In contrast, eIF5A-1 mRNA and its cognate protein are expressed in all human cells and tissues examined. We have identified the eIF5A-2 protein as a bona fide product of EIF5A2 [31] and shown its expression in an ovarian cancer cell line, UACC-1598, and a colorectal adenocarcinoma cell line, SW-480, that overexpresses eIF5A-2 mRNA. Furthermore, we demonstrated that eIF5A-2 precursor also undergoes hypusine modification and that it shares functional similarity with eIF5A-1 in supporting the growth of the yeast strain in which tif51A and tif51B (encoding yeast eIF5A) were disrupted. Overexpression of eIF5A-2 mRNA has also been observed in ovarian cancer tissues and cell lines, including UACC-1598, and EIF5A2 has been suggested as a candidate oncogene associated with ovarian cancers, which display frequent amplification of 3q26 [32]. More recently, Guan *et al.* [33] reported evidence that substantiates the oncogenic role of eIF5A-2 in ovarian cancers, including transformation of NIH3T3 cells and human liver cell line LO2 by overexpression of eIF5A-2, as assessed by

anchorage-independent growth and by tumorigenicity in nude mice. Although the two human eIF5A isoforms share functional similarity in supporting yeast growth, they probably exert specialized activities in certain tissues and cancer cells. In this study, we investigated the differential expression of eIF5A-1 and eIF5A-2 mRNA in various human cancer cells and identified multiple eIF5A-2 mRNA species as products of alternative polyadenylation. We also demonstrate that poor translatability of eIF5A-2 mRNAs contributes to the lack, or low expression, of eIF5A-2 protein.

Results

Expression of eIF5A-1 and eIF5A-2 proteins in normal human cells and cancer cell lines

As eIF5A is the only cellular protein in which hypusine is synthesized, it is readily detected as the exclusively radiolabeled protein after culture of cells in the presence of [^{3}H]spermidine. Until recently, only one protein band, namely eIF5A-1, had been detected in human cells by this method. The discovery of a gene encoding a functional second isoform of eIF5A was unexpected and raised the question of why this isoform, eIF5A-2, had gone unobserved. As the EIF5A2 gene has been implicated in ovarian cancers, we screened a panel of human cancer cell lines for the expression of eIF5A-1 and eIF5A-2 proteins. Radiolabeled eIF5A-1 (newly synthesized and hypusine modified) was consistently detected in a panel of 15 human cancer cell lines and two normal human cells (Fig. 1A). Western blot analysis with eIF5A antibody (NIH353), which recognizes the hypusine-modified eIF5A-1 but not the unmodified precursor [34], also revealed comparable steady-state concentrations of eIF5A-1 in all the cancer cells and human umbilical vein endothelial cells (Fig. 1B). The only exception was normal human epidermal keratinocytes, which showed low steady-state concentration, as well as low synthesis rate. In contrast with eIF5A-1, eIF5A-2, which migrates slightly more slowly than eIF5A-1, was detected only in the ovarian cancer cell line UACC-1598 (strong labeling) and the colorectal cancer line SW-480 (weak labeling) by labeling with [^{3}H]spermidine. Likewise, a strong signal for eIF5A-2 was detected in UACC-1598 by immunoblotting using eIF5A-2 antibody (Fig. 1B bottom panel). Faint bands immunoreactive to eIF5A-2 antibody were also detectable in SW-480, Caki and SNB19. Although eIF5A-2 antibody recognizes predominantly eIF5A-2 (eIF5A-2 precursor as well as the modified protein), faint signals were also visible at the position of eIF5A-1 (Fig. 1B), suggesting a weak cross-reactivity with eIF5A-1. These findings suggest that, except for UACC-1598, expression of eIF5A-2 protein is extremely low in normal and a wide array of human cancer cells and that overexpression of eIF5A-2 is not generally associated with human cancers.

Northern blot analysis of eIF5A-1, eIF5A-2 and DHS mRNA expression

In an effort to understand the basis of the differential expression of eIF5A isoforms in the various human cells, we carried out northern blot analysis of eIF5A-1, eIF5A-2 and DHS expression. The eIF5A-1 mRNA was expressed in all cells as one band ($\approx$ 1.3 kb) and there were no significant differences in its level in various cell lines (Fig. 2), in accordance with data on the protein concentration (Fig. 1). In contrast with eIF5A-1 mRNA, eIF5A-2 mRNA showed multiple bands (0.7–5.6 kb), with one at 3.8 kb being the major form; the total levels of eIF5A-2 mRNAs varied widely from low to high (SW-480) and to an extremely high

expression in UACC-1598. The total concentration of eIF5A-2 mRNA in UACC1598 was > 10-fold higher than in other cell lines (data not shown). Five eIF5A-2 hybridizing bands (5.6, 3.8, 2.0, 1.6 and 0.7 kb) were clearly visible not only in UACC1598 cells but also in other cancer cell lines, including SW-480, HS578T, Colo 205, Caki, SNB19, Lox IMV1, K562. Thus, the occurrence of multiple eIF5A-2 mRNAs is not specific to the genetically modified cell line UACC-1598 (with gene amplification of the 3q26 locus), but seems to be a general phenomenon in normal cells (not shown) as well as in various cancer cell lines. Interestingly, DHS mRNA (1.4 kb) also showed quite variable expression and was highly expressed in K562, MCF7, PC3, COLO205, OVCAR4 and HS578T. Although the significance of DHS overexpression in certain cancer cell lines is unknown, the DHS gene was reported to be one of a signature set of amplified genes in cancer metastases [35]. The expression of the three genes encoding eIF5A-1, eIF5A-2 and DHS does not seem to be co-ordinately regulated, as no correlations were observed in their mRNA levels.

Identification of multiple eIF5A-2 mRNA forms and their stabilities

To verify that the multiple bands shown in Fig. 2 are indeed eIF5A-2 mRNAs, we undertook to characterize the different forms of eIF5A-2 mRNA. All six bands detected in the total RNA (Fig. 3A, lane 2) were also present in the RNA purified from an oligo-dT resin (Fig. 3A, lane 1), indicating that they are all poly(A) RNAs. The EIF5A2 gene contains putative polyadenylation signals (AATAAA) at multiple loci in the 3′-UTRs of the fifth exon; the four AATAAA sites are 588, 1460, 3608 and 5511 bases from the 5′-terminal of 5′-UTR (Fig. 3B). Polyadenylation at the four predicted sites, 638 (A), 1483 (B), 3627 (C) and 5534 (D) bases from the 5′-terminal would yield four mRNA species 50–200 bases larger after addition of poly(A) tails. To test this possibility and, if true, to identify which of the AAUAAA signals was used for different eIF5A-2 mRNAs, we constructed a set of ^{32}P-labeled eIF5A-2 probes (Pr1–Pr5, Fig. 3B). Probe 1 corresponds to the ORF of eIF5A-2 and the second probe includes the 5′-UTR and 5′-terminal portion of the ORF. Probes 3–5 were designed to hybridize with sequences in the 3′-UTR immediately preceding each AAUAAA signal site (Table 1 and Fig. 3B). The Northern blot showed that probes 1 and 2 recognize all the eIF5A-2 mRNA species, suggesting that they share the same 5′-UTR and ORF. On the other hand, selective hybridization was observed with probes 3–5, each probe recognizing those larger than the size of the probe location. For example, probe 3 hybridized to bands higher than 1.6 kb, probe 4 only to those higher than 3.8 kb, and probe 5 mainly to the 5.6-kb band. These findings indicate that the 5.6-kb mRNA arose from polyadenylation at site D, the 3.8-kb form at site C, the 1.6-kb form at site B, and the 0.7-kb form at site A. They confirm that these four mRNA bands are the products of one gene derived from alternative polyadenylations at different AAUAAA signals in the 3′-UTR. The two bands at 4.5 kb and 2.0 kb could not be clearly identified here. The 4.5-kb form was hybridizable to all the probes including probe 5, but it is shorter than 5.6 kb. The 2.0-kb form contains sites A and B, but may have a longer poly(A) tail than the 1.6-kb form. The 2.0-kb and 4.5-kb forms may be variants generated by alternative splicing or unknown recombination events.

To compare the stability of eIF5A-1 mRNA and the different eIF5A-2 mRNA species, UACC-1598 cells were cultured in the presence or absence of 5 μg·mL^{-1} actinomycin D, and total RNA isolated after 1, 2 and 4 h was analyzed. Two identical panels of membrane

containing ≈ 10 μg total RNA from each time point were hybridized with labeled eIF5A-1 or eIF5A-2 probes (Pr1) followed by a control glyceraldehyde dehydrogenase (GAPDH) probe (Fig. 3C). eIF5A-1 mRNA was fairly stable up to 4 h of actinomycin treatment, comparable to that of GAPDH. The stability of eIF5A-2 mRNA varied with the size. Whereas the short 0.7-kb form was quite stable during the actinomycin treatment, the largest band (5.6 kb) disappeared almost completely. The other bands (3.8, 2.0 and 1.8 kb) displayed intermediate stability. The stability appeared to be inversely related to the length of 3′-UTR of eIF5A-2 mRNAs, suggesting that elements in the 3′-UTR of eIF5A-2 mRNA contribute to the instability.

Hypusine synthesis in eIF5A-1 and eIF5A-2 precursors and the stability of eIF5A-1 and eIF5A-2 proteins in UACC-1598 cells

We have shown previously that recombinant eIF5A-2 (precursor protein) acts as a substrate for DHS *in vitro* [31], although the K_m for the eIF5A-2 precursor was higher than that for the eIF5A-1 precursor. The failure to detect the eIF5A-2 isoform protein by radiolabeling with [^{3}H]spermidine in most human cells may be due to inefficient modification of the eIF5A-2 precursor. Therefore, we determined the concentrations of eIF5A-1 and eIF5A-2 precursors in UACC-1598 cells, untreated or treated with GC7, a potent inhibitor of DHS. When UACC-1598 cells were incubated with [^{3}H]spermidine for 24 h, almost equal labeling of eIF5A-1 and eIF5A-2 was observed, indicating that the two eIF5A precursors are synthesized and modified at similar rates in the control cells (Fig. 4A). In the presence of GC7 at 2–20 μM, labeling of the two eIF5A isoforms was inhibited in a parallel manner, with almost complete inhibition at > 10 μM (Fig. 4A). We also estimated the concentrations of unmodified eIF5A precursors using cellular proteins from these cells as substrates for DHS *in vitro*. Both of the eIF5A-1 and eIF5A-2 precursor isoforms were detectable in untreated and GC7-treated cells. A significant increase in the two precursor isoforms occurred on inhibition of cellular DHS by GC7 (5–20 μM; Fig. 4B). The amounts of accumulated eIF5A precursor isoforms are similar, judging from the labeling intensity, an indication that there is no selective modification of one isoform and that the two precursors are similarly stable. These findings suggest that the eIF5A-1 and eIF5A-2 precursors are synthesized at a similar level, and the modification of the two isoforms occurs with comparable efficiency in UACC-1598 cells.

Next, we examined the stability of the two eIF5A isoforms. UACC-1598 cells were first cultured in the presence of [^{3}H]spermidine for 24 h to label eIF5A-1 and eIF5A-2. The cells were then washed and incubated in fresh medium free of [^{3}H]spermidine. As intracellular [^{3}H]spermidine is not readily removed by washing the cells, the turnover of prelabeled eIF5A was examined in the presence of GC7 (10 μM) to inhibit new synthesis of hypusine. When equal amounts of cellular proteins were analyzed after incubation for 8 and 24 h in the presence of GC7, the labeling intensity of the two isoforms decreased over the time period, and no difference in the decay rate was observed between the two isoforms (Fig. 4C). Thus, the eIF5A-1 and eIF5A-2 isoforms seem to be similarly stable in UACC-1598 cells.

Overexpression of eIF5A-1 and eIF5A-2 precursors and their modification on transfection of 293T cells

To compare the translation efficiency of the eIF5A-1 and eIF5A-2 ORFs, we overexpressed eIF5A-1 and eIF5A-2 cDNA alone or together with DHS in the human embryonic kidney cell line, HEK293T. The ORFs were inserted into the mammalian expression vectors pCEFL and p3XFLAGCMV7.1. Expression from the latter vector yields FLAG-tagged fusion proteins that are larger and migrate at higher apparent molecular mass than the natural eIF5A-1 (18 kDa) and eIF5A-2 (20 kDa) proteins. Expression and modification of eIF5A precursors were studied by western blotting (Fig. 5A) and metabolic [^{3}H]spermidine labeling (Fig. 5B). eIF5A-1 (as the precursor protein) or its FLAG-tagged counterpart was overexpressed on transfection of 293T cells with a eIF5A-1 expression vector alone or on cotransfection with DHS expression vector (Fig. 5A, top panel). eIF5A-2 (the precursor protein), which is not normally expressed in 293T cells, and the FLAG-tagged counterpart were similarly overexpressed on transfection (Fig. 5A, second panel). However, the overexpressed eIF5A-1 or eIF5A-2 precursor proteins as shown by the western blotting were not modified in 293T cells, unless cells were cotransfected with DHS vector. Little increase in the labeling was observed in cells transfected with each eIF5A expression vector alone on labeling with [^{3}H]spermidine (Fig. 5B, lanes 2–5). Only on coexpression with DHS was a marked increase in the labeling of eIF5A-1, eIF5A-2 or their FLAG-tagged proteins observed (Fig. 5B, lanes 7–10). This finding indicates that the normal concentration of endogenous DHS in 293T cells is too low to modify the induced excess of precursor proteins and that coexpression of the enzyme was needed to modify all the overexpressed eIF5A precursor isoforms. Expression of precursor proteins (Fig. 5A) and their modification on induced DHS expression (Fig. 5B) seemed equally efficient for eIF5A-1 and eIF5A-2, as judged from the signals achieved with both techniques. Then we analyzed the radioactive components of the labeled proteins after acid hydrolysis. Whereas the radioactivity was mostly in [^{3}H]hypusine for the labeled proteins in lanes 2–5 (low labeling), most of the radioactivity ($\approx$ 80%) in the cotransfected samples (high labeling in lanes 7–10) was in [^{3}H]deoxyhypusine. This finding indicates that 293T cells do not contain enough deoxyhypusine hydroxylase activity to convert all the eIF5A intermediate (deoxyhypusine form) overproduced in cotransfected cells (Fig. 5B, lanes 7–10) into the hypusine form. That is, the endogenous activity of the second enzyme limits the rate of hydroxylation, resulting in the accumulation of deoxyhypusine-containing eIF5A intermediates. Thus, overexpression of either eIF5A isoform seems to overwhelm the modification system, DHS (lanes 2–5) and deoxyhypusine hydroxylase, when the first modification enzyme is coexpressed (lanes 7–10). The lack of co-ordinated upregulation of the two enzymes suggests a lack of coupling of the synthesis of the eIF5A precursor protein and its modification enzymes. Furthermore, the results from the transfection experiments show that ORFs of eIF5A-1 and eIF5A-2 are translated and modified with comparable efficiency in 293T cells. Therefore, the negative elements causing poor translation of eIF5A-2 mRNAs must reside outside of the ORF, in the 5′-UTR or 3-′UTR of the natural mRNAs.

Polysome profiles of eIF5A-1 and eIF5A-2 mRNAs in UACC-1598 cells

To test if eIF5A-1 and eIF5A-2 mRNAs are differentially translated in cells, we analyzed their association with polysomes. Exponentially growing UACC-1598 cells were treated

with cycloheximide, which arrests elongating ribosomes while keeping mRNA–ribosome complexes intact [36], and separated cytoplasmic polysomes by sucrose density gradient centrifugation. To compare the polysomal profile of eIF5A-1 and eIF5A-2 mRNAs, the same membrane blotted with the RNA of fractions from sucrose density gradient centrifugation was hybridized sequentially with ^{32}P-labeled eIF5A-2, eIF5A-1 and GAPDH probes (Fig. 6A–C). GAPDH mRNA was distributed mainly in the polysomal fractions (Fig. 6C, fractions 9–14, with peaks in fractions 10 and 11) and served as a control indicator of sucrose gradient separation based on the sizes. The Northern blot analysis revealed that the majority of eIF5A-1 mRNAs are associated with monosome and polysomes, with barely detectable amounts found in the mRNP fraction close to the top of the gradient (Fig. 6B). In contrast, almost all of each of the eIF5A-2 mRNA forms were associated with monosomes (Fig. 6A), with very little associated with larger polysomes (Fig. 6A). Although association of the five major mRNA bands (5.6, 3.8, 2.0, 1.8 and 0.7 kb) with the monosomes suggests that they are all potentially translatable forms, the actual amounts in the polysomal fractions were extremely low for reasons unknown at present. The experiment also shows that the 4.5-kb eIF5A-2 hybridizing band from total RNA is absent from the cytoplasmic RNA fraction and probably represents partially spliced pre-mRNA. The poor association of eIF5A-2 mRNAs with polysomes may be an important factor contributing to inefficient translation of eIF5A-2 mRNA, compared with eIF5A-1 mRNA, in UACC-1598 and other human cells.

Discussion

We investigated the differential expression of the two known isoforms of eIF5A. Unlike eIF5A-1, which is ubiquitous in human and mammalian cells, the recently discovered second human isoform, eIF5A-2, was detected only in cells that are overexpressing the encoding gene, although all cells examined express eIF5A-2 mRNA. Equal amounts of both isoforms were found in UACC-1598 cells. Given the high amplification of EIF5A2 as double minutes and high expression of eIF5A2 mRNA in these cells, the concentration of eIF5A-2 protein seems surprisingly low. The protein is barely detectable in SW-480, a cell line that shows modest overexpression of eIF5A-2 mRNA. The extent of modification of the precursors of the two isoforms to the deoxyhypusine-containing or hypusine-containing form was similar in UACC-1598 cells, and there was little difference in the stabilities between the two eIF5A isoforms. Thus low expression of eIF5A-2 protein may be due to transcriptional repression, mRNA instability, and inefficient translation. Our results show that inefficient translation of eIF5A-2 mRNA definitely contributes to the low expression of eIF5A-2 protein.

The ORFs of the two eIF5A isoforms were expressed equally well in 293T cells on transfection with eIF5A vector alone or in combination with DHS vector, indicating that the eIF5A-2 ORF can be translated as efficiently as the eIF5A-1 ORF. Thus, the negative *cis* elements that retard the translation of eIF5A-2 mRNAs may reside in the 5′-UTR or 3′-UTR. It is curious that multiple forms of eIF5A-2 mRNA are capable of forming monosomes, but not efficiently elongating polysomes. Although monosomal association and mRNA stalling were observed for both eIF5A isoforms, the extent was much greater for eIF5A-2 mRNAs than eIF5A-1 mRNA. This is probably the critical reason for the inefficient translation of eIF5A-2 mRNA. Negative *cis* element(s) in the 5′-UTR, 3′-UTR and putative *trans*-acting

cellular factors may cause monosome stalling and poor translation of eIF5A-2 mRNAs. Considering that all eIF5A-2 mRNAs are stalled, the *cis*-acting element(s) probably reside in the 5′-UTR or within the short (48 nucleotide) stretch common to all eIF5A-2 mRNAs. Future experiments will be needed to determine the influence of eIF5A mRNA UTRs on translation.

The results in Fig. 5 reveal an intrinsic problem associated with overexpression of eIF5A by transfection in mammalian cells. The endogenous activity of DHS and/or deoxyhypusine hydroxylase appears to limit the overproduction of the mature eIF5A proteins. It is clear that the vast majority of the eIF5A-1 or eIF5A-2 precursor proteins overproduced by transfection were not modified to the active hypusine form after 24 h of transfection. Even when DHS is coexpressed with eIF5A precursors, the deoxyhypusine-containing eIF5A intermediate accumulates but not the hypusine-containing form, indicating that the normal cellular level of deoxyhypusine hydroxylase is not sufficient for modification of the excess eIF5A intermediate produced. Therefore, transfection studies with eIF5A expression vectors, such as those by Li and colleagues [37] and Guan and colleagues [33], should be re-evaluated by assessing the actual changes in the concentrations of eIF5A precursor or the fully modified protein to determine the true cause of the effects observed. Recent cloning of deoxyhypusine hydroxylase cDNA has enabled us to overexpress hypusine-containing (biologically active) eIF5A-1 or eIF5A-2 isoforms [38] and will permit assessment of the effects of overexpression of mature eIF5A proteins.

This is the first report of the occurrence of multiple forms of eIF5A-2 mRNA in various human cancer cell lines. Previous reports of eIF5A-2 mRNA overexpression in ovarian cancer tissues and cell lines, including UACC-1598, failed to recognize the multiple eIF5A-2 mRNAs and evaluated the gene expression on the basis of one band of eIF5A-2 mRNA. By Northern blot analysis, we have identified the multiple forms as the products of alternative polyadenylation at the four sites following the AAUAAA signals [A–D, located in the 3′-UTR (Fig. 3) at 588, 1460, 3608 and 5511 bases, respectively, from the 5′-terminus]. Polyadenylation at the four sites (638, 1483, 3627 and 5534 bases from the 5′-terminus) would give rise to four mRNAs ≈ 0.7, 1.6, 3.8 and 5.6 kb long. These four eIF5A-2 mRNAs are identified in Fig. 3 (solid arrowheads). It is not clear how the two other forms (4.5 kb and 2.0 kb, gray arrowheads) were generated. The 4.5-kb RNA, present in the total RNA, but not in the cytoplasmic RNA pool (Fig. 6), may be a partially spliced nuclear premRNA variant that does not enter the cytoplasm. The structural relation of the two bands at 1.6 and 2.0 kb are not clear. They are both hybridizable with probes 1–3. The presence of the 2.0-kb band suggests an AAUAAA hexanucleotide at or close to site B and sequences surrounding the polyadenylation site that together support very efficient poly(A) tail extension. However, no poly(A) signal, conserved or degenerate, can be discerned between 1483 (B) and 3627 (C) bases. It is possible that the 2.0-kb mRNA is specific to cancer cells and to UACC-1598 cells in particular. Curiously, Guan and colleagues [32] reported a 2.2-kb eIF5A-2 cDNA (GenBank accession number AF262027) from an ovarian cancer cell line. The Guan cDNA is a chimera between the first 1410 nucleotides of eIF5A-2 mRNA and the mRNA encoding RAD23B (normal mRNA; GenBank accession number NM_002874) which may have arisen through recombination at the gene level at Alu sites on

chromosomes 3 and 9 or during the construction of the cDNA library. This abnormal cDNA lacks the polyadenylation signal at 1460–1465 and instead uses the unconventional UAUAAA polyadenylation signal of the RAD23B pre-mRNA.

The existence of multiple 3′-UTRs in eIF5A-2 mRNAs is probably biologically relevant as the 3′-UTR and the length of the poly(A) tail are known to play important roles in the translational regulation of gene expression. Comparison with other mammals reveals that at least three poly adenylation sites are conserved in mouse, rat, bull, horse and pig, suggestive of a conserved function for the 3′-UTRs. In other studies, differential expression of the eIF5A-2 mRNA isoforms in normal tissue has been detected in both human and mouse (Z. A. Jenkins, P. G. Hååg, M. H. Park and H. E. Johansson, unpublished work). When the relative intensities of these multiple bands were compared in different cancer cells and also in mouse or human tissues (unpublished results), we observed variations in the ratios of these forms. For example, whereas the 3.8-kb form is the major one in most cells, the short 0.7-kb form is the major eIF5A-2 mRNA in human and mouse testis. These findings suggest that the polyadenylation of eIF5A-2 mRNA is regulated in a cell-specific or tissue-specific manner depending on the contents of *trans*-acting factors binding at the polyadenylation sites. It may also depend on the physiology of cells. Numerous genes have been reported to use alternative polyadenylation signals to generate multiple mRNAs that encode identical proteins, including mammalian translation initiation factors eIF5 [39] and eIF4E [40]. Regulated polyadenylation depends on the interaction of RNA-binding proteins at specific sites close to the cleavage site in the 3′-UTR of the pre-mRNA. The best example comes from the snRNP protein U1A which autoregulates its polyadenylation by binding to its pre-mRNA and directly interacts with and inhibits the poly(A) polymerase [41–43]. The second example involves the switch from cell-attached to secreted IgM by B-cells [44,45]. The significance of the alternative polyadenylation in the regulation of eIF5A-2 expression is not yet clear. However, the 3′-UTR and poly(A) tails are implicated in the mRNA stability and translation efficiency and may be regulated during embryonic development and differentiation. The long 3′-UTR of eIF5A-2 mRNAs (especially the long ones, 3.8 kb and 5.5kb) may contribute to their instability and poor translation. Future work will be needed to determine the mechanism(s) whereby polyadenylation sites in the 3′-UTR of eIF5A-2 mRNAs are selected and whether altered expression of eIF5A-2 mRNA and hypusine biosynthetic enzymes contribute to tumorigenesis and metastasis.

Experimental procedures

Materials

Human umbilical vein endothelial cells, endothelial cell media bullet kits including EBM-2 and supplements (Single-Quots), normal human epidermal keratinocytes, and keratinocyte growth media KGM Bullet Kit were purchased from Cambrex (Walkersville, MD, USA). The human ovarian cancer cell line UACC-1598 was obtained from the University of Arizona Cancer Center (Tucson, AZ, USA). The human embryonic kidney cell line 293T, the colorectal adenocarcinoma line SW-480, the acute myeloid leukemia line HL-60, the breast adenocarcinoma line MCF7, the prostate adenocarcinoma line PC-3, and the cervix adenocarcinoma line HeLa were from ATCC. The renal clear cell carcinoma line Caki-1, the

chronic myelogenous leukemia line K562, the epithelial breast cancer line HS578T, the malignant melanoma line Lox IMV1, the ovarian adenocarcinoma line Ovcar-3, the ovarian carcinoma line Ovcar-4, the glioblastoma line SNB19, the squamous lung carcinoma line H226, and the colorectal adenocarcinoma line Colo-205 were kindly provided by the Developmental Therapeutics Program, NCI, NIH (Frederick, MD, USA).

Lipofectamine Plus reagent was purchased from Life Technologies (Rockville, MD, USA), [*Terminal methylenes*-1,8-^{3}H]spermidine·3HCl from DuPont/New England Nuclear (Boston, MA, USA), Amplify from Amersham Pharmacia Biotech (Piscataway, NJ, USA) and ProSTAR HF Single-Tube RT-PCR System from Stratagene (La Jolla, CA, USA). Mouse monoclonal antibody against human eIF5A-1 fragment (amino acids 58–154) was purchased from BD Biosciences (Palo Alto, CA, USA), rabbit polyclonal antibody against eIF5A-2 precursor from NOVUS (Littleton, CO, USA), and goat anti-actin IgG from Santa Cruz Biotech Inc (Santa Cruz, CA, USA). Peroxidase-linked donkey anti-rabbit IgG and ECL Plus chemiluminescence reagents were from Amersham. Renaissance Western blot chemiluminescence reagents were from NEN Life Sciences (Boston, MA, USA). The vector p3XFLAG-CMV-7.1, actinomycin D and protease inhibitor cocktail were from Sigma (St Louis, MO, USA).

N^{1}-Guanyl-1,7-diaminoheptane (GC7) was synthesized as reported [46]. Polyclonal antibody against rabbit eIF5A (NIH353) specific for the hypusine-containing eIF5A-1 was raised against human red blood cell eIF5A-1 (hypusine-containing protein) [47]. Recombinant human eIF5A-1, eIF5A-2 precursor proteins and human DHS were purified as described [31,48–50].

Northern blot analysis

Total cellular RNA was isolated from exponentially growing cells by using Trizol Reagent from Life Technologies, and poly(A) RNA by using Oligotex Direct mRNA kit. Approximately 10 μg total RNA or 0.03–0.33 μg poly(A) RNA was separated on 1% agarose gels and transferred to nitrocellulose membranes for hybridization with ^{32}P-labeled probes. The probes used were generated by PCR using primer pairs (Table 1): 363-bp probe (a portion of the ORF) for eIF5A-1, 490-bp probe (probe 1 for the entire eIF5A-2 ORF) for eIF5A-2 and 700-bp probe (a portion of the ORF) for DHS. For identification of multiple species of eIF5A-2 mRNA, additional probes, probes 2 (5′-UTR and 5′-end of ORF), 3, 4 and 5 (different locations in the 3′-UTR) as indicated in Fig. 3 and a GAPDH probe were made by RT-PCR using UACC-1598 total RNA as template and the primer pairs (Table 1) employing the Single-Tube RT-PCR kit (Stratagene).

Construction of mammalian expression vectors encoding human eIF5A-1, eIF5A-2 and DHS

The ORFs of human eIF5A-1, eIF5A-2 and DHS were amplified by PCR using the primer pairs shown in Table 1 and using pET11a vectors encoding each protein as template [31,48,49]. The following program [(95 °C, 10 min) + (95 °C, 0.5 min; 56 °C, 0.5 min; 72 °C, 2.0 min) × 35 + (72 °C, 7 min)] was used for DNA amplification on a Perkin–Elmer GeneAmp 9700 (Perkin–Elmer, Boston, MA, USA). Insertion of amplified DNA into the

*Hin*dIII and *Bam*HI sites of pCEFL yielded the mammalian expression vector, pCEFL-heIF5A-1, pCEFL-heIF5A-2 and pCEFL-DHS. A separate set of primers was used for PCR with the same template for expression of FLAG-tagged proteins (Table 1). In this case PCR-amplified ORFs were inserted into *Eco*RI and *Sal*I sites of the p3XFLAGCMV-7.1 vector.

Radiolabeling of eIF5A isoforms by incubation with [^{3}H]spermidine

Human umbilical vein endothelial cells were grown in supplemented EBM-2 and normal human epidermal keratinocytes in supplemented KGM. All the human cancer cells were grown in RPMI 1640 containing 10% fetal bovine serum. Exponentially growing cells were incubated with [^{3}H]spermidine (5 μCi·mL^{-1}) for 24 h. Cells were harvested, and cellular proteins were precipitated with 10% trichloroacetic acid containing 1 mM unlabeled polyamines, and washed three times to remove free [^{3}H]spermidine. Half of the trichloroacetic acid precipitate was hydrolyzed in 6 M HCl, and radiolabeled deoxyhypusine and hypusine were quantitated after ion-exchange chromatographic separation [24]; the other half was used for SDS/PAGE, and radiolabeled proteins were visualized by fluorography using Amplify.

Detection of eIF5A isoforms by Western blotting

Exponentially growing cells (100-mm dishes) were lysed in 0.5 mL lysis buffer [20 mM Hepes, pH 7.5, containing 10 mM EGTA, 2.5 mM $MgCl_2$, 1% Nonidet P40, 1 mM dithiothreitol and protease inhibitor cocktail (Sigma)]. After incubation on ice for 30 min, the lysates were centrifuged. About 50 μg supernatant protein was used for SDS/PAGE. Proteins transferred on to a nitrocellulose membrane were subjected to immunodetection by using primary antibodies against human eIF5A-1, human eIF5A-2 or human DHS, followed by the secondary antibody and detection using Renaissance Western blot or ECL Plus chemiluminescence reagents.

Detection of eIF5A precursors by DHS assay

Accumulation of eIF5A precursors in UACC-1598 cells or 293T cells was detected by using cellular proteins as substrate in the *in vitro* DHS assay. One dish (100 mm) of cells was harvested, and the cell pellet was lysed by sonication. After removal of debris by centrifugation at 10 000 ***g***, the supernatant was treated with ammonium sulfate (40–75% saturation). The precipitated protein was dissolved in 0.1 mL NaCl/P_i containing protease inhibitors, and dialyzed against the same buffer for 4 h, at 4 °C. The resulting protein fraction was used as substrate for human DHS. A typical 50-μL reaction mixture contained 0.1 M glycine/NaOH buffer, pH 9.5, 1 mM dithiothreitol, 100 μg BSA, 1 mM NAD, 10 μCi [^{3}H]spermidine (10 μM), 0.1 μg human enzyme, and 100 μg cellular proteins or 3 μg purified eIF5A-1 precursor as indicated. After incubation at 37 °C for 2 h, labeled proteins were precipitated with 10% trichloroacetic acid containing unlabeled polyamines (1 mM each putrescine, spermidine and spermine) and washed thoroughly to remove free radioactive spermidine. One half of the sample was used for SDS/PAGE and the other half for acid hydrolysis in 6 M HCl and determination of radioactive deoxyhypusine.

Polysome analysis of eIF5A-1 and eIF5A-2 mRNAs

UACC-1598 cells were grown to 70% confluency in 100-mm dishes and given fresh medium 2.5 h before treatment with cycloheximide (added to the medium to a concentration of 0.1 $mg \cdot mL^{-1}$) for 10 min at 37 °C. The cells were next carefully washed twice with 5 mL ice-cold NaCl/P_i containing 0.1 $mg \cdot mL^{-1}$ cycloheximide, and the dish placed on ice. The cells were lysed directly in the dish after addition of 600 μL polysome extraction buffer (10 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 100 mM KCl, 0.5% Nonidet P40, 0.1 $mg \cdot mL^{-1}$ cycloheximide, 0.2 $mg \cdot mL^{-1}$ heparin, 1 : 100 diluted protease inhibitor cocktail and 20 $U \cdot mL^{-1}$ RNasin). Extracts were transferred to 1.9-mL Microfuge tubes and incubated on ice for 12 min with occasional mixing. The nuclei and debris were removed by centrifugation at 12 000 ***g*** for 10 min in a Microfuge. Supernatants were recovered, and 300-μL aliquots were layered on to 11 mL 10–50% sucrose gradients in extraction buffer lacking Nonidet P40. The gradients were sedimented at 150 000 ***g*** for 120 min in a Beckman SW41 rotor (Beckman Coulter, Fullerton, CA, USA) at 4 °C. Fractions (800 μL) were collected from the top of each gradient. A_{260} and A_{280} were measured to verify the intactness of the polysomes. RNA was extracted from 100 μL of the cytoplasmic extract and from the full volume of each gradient fraction with two-third volumes of Trizol and coprecipitated with 30 μg glycogen and propan-2-ol. Ethanol-washed pellets were resuspended in denaturing formamide RNA loading buffer and two-thirds of each sample processed as above for Northern blotting. The polysome gradients were repeated twice, and a representative experiment is shown.

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Abbreviations

DHS	deoxyhypusine synthase
eIF5A	eukaryotic initiation factor 5A, eIF5A is defined as a fully modified protein containing hypusine
eIF5A(Lys)	unmodified eIF5A termed eIF5A precursor
eIF5A(Dhp)	eIF5A intermediate containing deoxyhypusine
EIF5A1 and EIF5A2	the human genes encoding eIF5A-1 and eIF5A-2, respectively
GAPDH	glyceraldehyde dehydrogenase
heIF5A	human eIF5A
yeIF5A	yeast eIF5A

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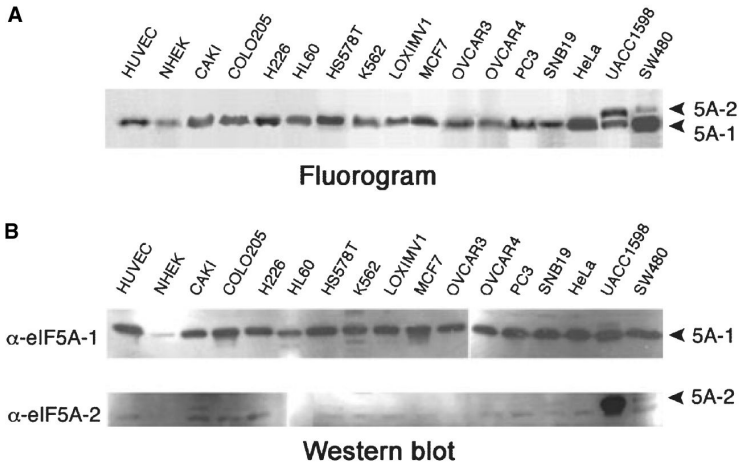

Fig. 1.

Expression of eIF5A-1 and eIF5A-2 proteins in normal human cells and a panel of human cancer cell lines. (A) Proteins (≈ 150 μg) from exponentially growing cells incubated with 5 μCi·mL^{-1} [^{3}H]spermidine for 20 h were separated by SDS/PAGE, and labeled proteins detected by fluorography. (B) Proteins (≈ 50 μg) from exponentially growing cells were separated by SDS/PAGE, and processed for western blotting with eIF5A-1 antibody, NIH353 (top panel) and eIF5A-2 antibody (NOVUS) (bottom panel) as described in Experimental procedures.

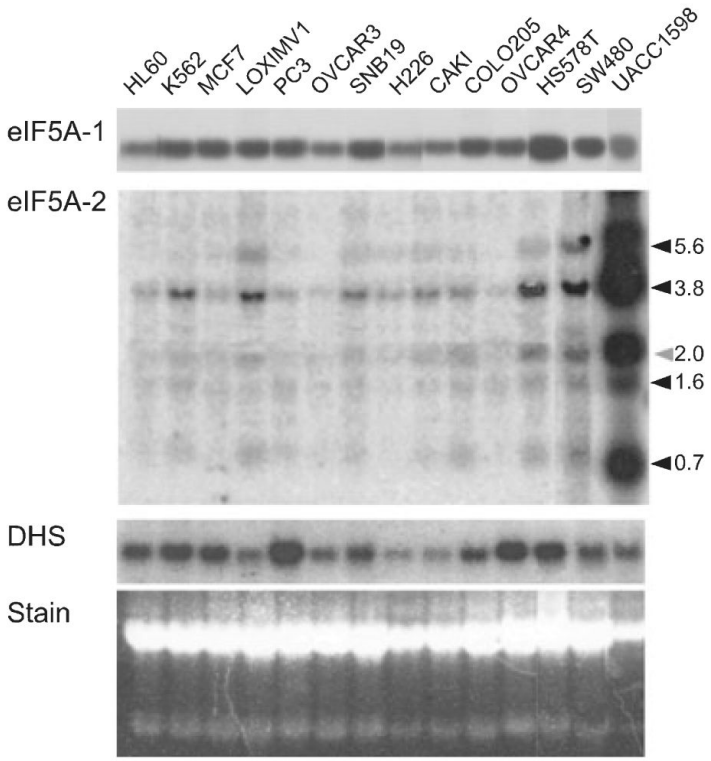

Fig. 2.

Northern blot analysis of eIF5A-1, eIF5A-2 and DHS mRNA expression in various human cells. Approximately 10 μg total RNA from exponentially growing cells were used for northern blots except for UACC-1598, for which 5 μg total RNA was used, as the eIF5A-2 signal was too strong with 10 μg.

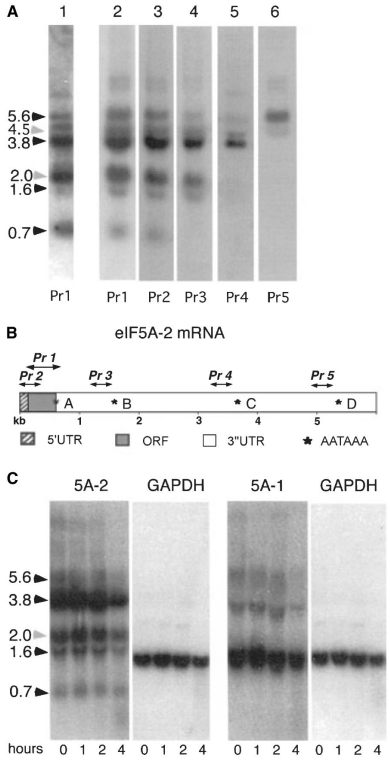

Fig. 3.

Identification of multiple species of eIF5A-2 mRNA and their stability. (A) Northern blots using UACC-1598 RNA (lane 1, 0.3 μg polyA RNA; lanes 2–6, 10 μg total RNA) and different ^{32}P-labeled eIF5A-2 probes. (B) The positions of polyadenylation signals, AATAAA, 588–593 (A), 1460–1465 (B), 3608–3613 (C) and 5511–5516 (D) and the positions of probes Pr1–Pr5 are indicated. (C) Stability of eIF5A-1 and eIF5A-2 mRNAs compared with that of GAPDH mRNA. Total RNA was isolated from UACC-1598 cells treated with 5 μg·mL^{-1} actinomycin D for 0, 1, 2 and 4 h. Approximately 10 μg total RNA was used for each time point, and two identical gels were run for blotting and hybridization with eIF5A-1 probe (left) or with eIF5A-2 probe Pr1. Subsequently, each membrane was stripped and reprobed with ^{32}P-labeled GAPDH probe.

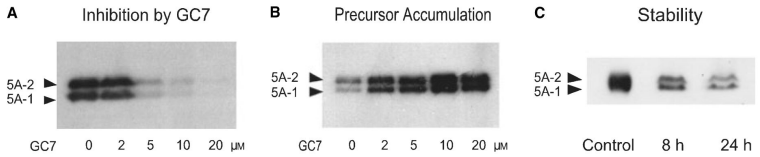

Fig. 4.

Inhibition of hypusine synthesis in eIF5A-1 and eIF5A-2, accumulation of eIF5A precursors on treatment with GC7, and stability of eIF5A isoforms in UACC-1598 cells. (A) Cells were cultured with 5 $\mu Ci \cdot mL^{-1}$ [^{3}H]spermidine without or with 2, 5, 20, or 20 μM GC7 for 20 h. Cellular proteins (150 μg) were separated by SDS/PAGE (fluorogram). (B) Cells were treated without or with 2, 5, 10 or 20 μM GC7 for 20 h. Cellular proteins were used as substrates for DHS *in vitro* using [^{3}H]spermidine as described in Experimental Procedures, and labeled proteins analyzed (fluorogram). (C) Cells were cultured with [^{3}H]spermidine (5 $\mu Ci \cdot mL^{-1}$) for 24 h, washed and incubated in medium free of [^{3}H]spermidine and with 10 μM GC7. Cells were harvested at 0, 8 and 24 h after a medium change, and cellular proteins analyzed by SDS/PAGE (fluorogram).

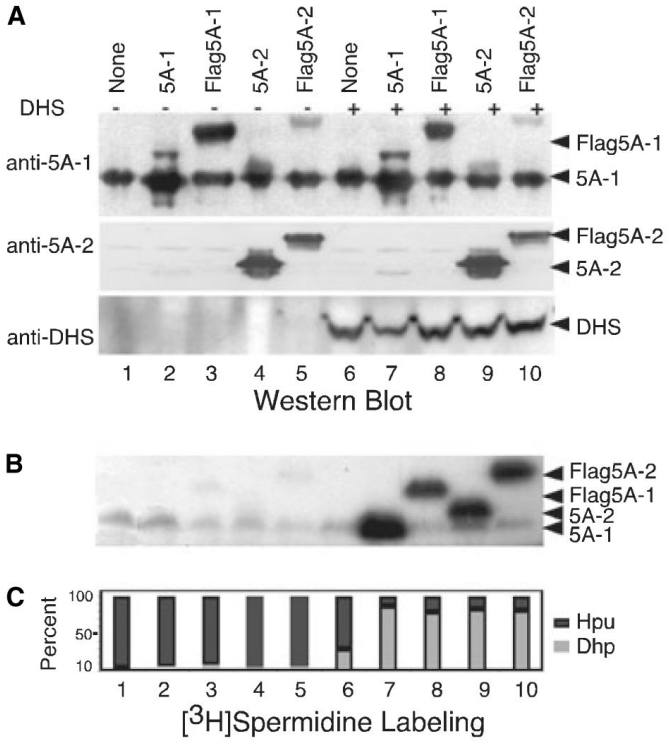

Fig. 5.

Expression of eIF5A-1 and eIF5A-2 isoforms and their precursors, and DHS in 293T cells on transfection with mammalian expression vectors. (A) Steady-state concentration of eIF5A-1 and eIF5A-2 isoforms in control and transfected 293T cells. At 20 h after transfection, cells were harvested and cellular proteins were processed for western blotting using a monoclonal mouse antibody (BD Sciences) against eIF5A-1 and polyclonal rabbit antibodies against eIF5A-2 (NOVUS) and DHS. DHS protein concentration is too low to be visible in the cells not transfected with DHS vector (lanes 1–5). However, there is sufficient enzyme activity to modify endogenously expressed eIF5A precursor (lane 1). (B) Newly synthesized and modified eIF5A isoforms detected by [^{3}H]spermidine labeling. Cells were treated as in (A) except that [^{3}H]spermidine (5 μCi·mL^{-1}) was added after change to a regular medium. Cells were harvested after 20 h, and half of cellular proteins analyzed by SDS/PAGE (fluorogram). (C) The other half of the labeled proteins in (B) were precipitated with trichloroacetic acid and, after thorough washing, hydrolyzed in 6 M HCl. Labeled components were analyzed by ion-exchange chromatography. The percentages of radioactivity in hypusine and deoxyhypusine for each sample are indicated as bar graphs. Hpu, hypusine; Dhp, deoxyhypusine.

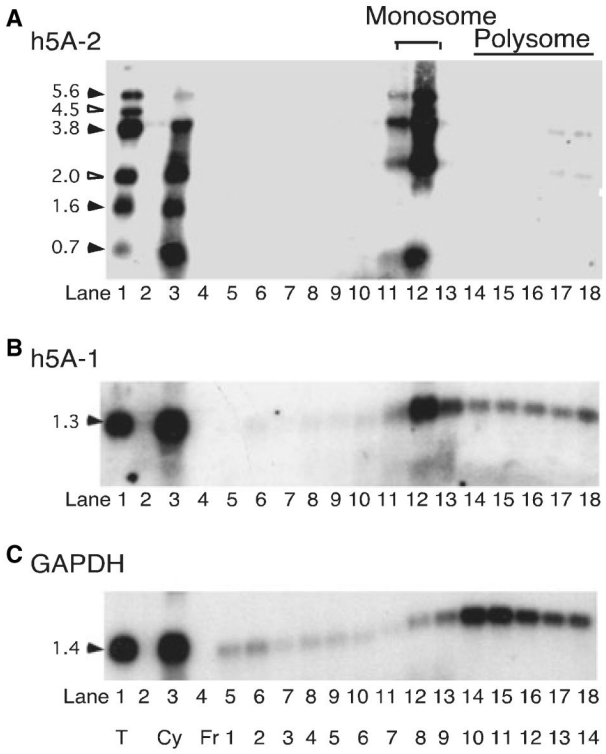

Fig. 6.

Distribution of eIF5A-mRNAs on polysomes. Cytoplasmic RNAs from exponentially growing UACC-1598 cells were separated by sucrose density gradient centrifugation as described in Experimental procedures, and RNA was separated on a 1% agarose gel. After blotting, the membrane was hybridized sequentially with ^{32}P-labeled eIF5A-2 probe (Pr1) (A), eIF5A-1 probe (B) and GAPDH probe (C). The membrane was stripped free of each ^{32}P-labeled probe before hybridization with the next one. Lane 1, 3 μg total UACC-1598 RNA; lane 2, 3 μg Ambion RNA size marker; lane 3, cytoplasmic RNA from UACC-1598 cells; lanes 4–18, RNA from 800-μL fractions (Fr1–Fr14) taken from top to bottom of a 11.5-mL sucrose gradient.

Table 1

Oligonucleotides used for PCR.

Name		Sequence	cDNA amplified
For plasmid in pCEFL			
PC11-F		cttccagtatgctaagcttgccaccATG GCAGAT GACTTGGACTTCGAG	eIF5A-1
PC12-R		cttccagtctggatccTTA TTT TGCCATGGCCTTGAT TGC	
PC13-F		cttccagtatgctaagcttgccaccATG GCAGAC GAAATTGAT TTCACTACTGG	eIF5A-2
PC4-R		cttccagtctggatccTTA TTT GCAGGGTTTTATGGCTACAGCATATTC	
PC14-F		cttccagtatgctaagcttgccaccATG GAA GGT TCCCTGGAACGGGAG	hDHS
PC15-R		cttccagtagctggatccTCAGTCCTCGTTCTTCTCATG AT GAAGG	
For plasmid construction in p3xFLAGCMV7.1			
CH27-F		ctaccagtatcgaattcaATGGCAGATGACTTGGACTTCGAGACA	eIF5A-1
CH9-R		ctaccagtagcgtcgactTTATTTTGCCATGGCCTTGATTGCAAC	
J26-F		gctaccagtagcgaattcaATGGCAGACGAAATTGATTTCACTAC	eIF5A-2
J27-R		ctacccagtagcgtcgacTTATTTGCAGGGTTTTATGGCTACAGC	
CH30-F		ctaccagtatcgaattcaATGGAAGGTTCCCTGGAACGGGAGGCG	hDHS
CH10-R		ctaccagtagcgtcgacTCAGTCCTCGTTCTTCTCATGCATGAA	
For northern probes			
EK35-F		ATGCAGTGCTCAGCATTAGCTAAGAATGGC	eIF5A-1
EK36-R		GGCAGACAGCACCGTGATCAGGATCTC	
EK37-F		ATCCGTGAGACCATTCGCTACCTTGTG	hDHS
EK38-R		TTGCCCCAGGAGACAGCCTCGTCTGG	
MP42-4		ATGGGGAAGGTGAAGGTCGGAGTC	GAPDH
MP43		CATGCCAGTGAGCTTCCCGTTCAG	
Pr1	PC3-F	cttccagtatgctcatATGGCAGACGAAATTGATTTCACTACTGGAG	eIF5A-2 ORF
	PC4-R	cttccagtctggatccTTATTTGCAGGGTTTTATGGCTACAGCATATTC	
Pr2	PC26-F	agacgaaaggcgctctttgccagc	eIF5A-2 5′UTR + ORF
	PC5-R	GAGCACTGCATAGGGTAAGTGC	
Pr3	PC18-F	cttgatgtaaccttatatggtggt	eIF5A-2 3′UTR
	PC19-R	tatcatgtaaagcagatcactccc	
Pr4	PC20-F	gatatcattgtacagtgataagcc	eIF5A-2 3′UTR
	PC21-R	agggaaacagagtcaagagcttgg	
Pr5	PC22-F	cgaacagcttgtcacacatcatag	eIF5A-2 3′UTR
	PC23-R	acaattagtaatacccatactacg	