Regulation of gene expression for translation initiation factor eIF-2α: importance of the 3′ untranslated region

Suzanne MIYAMOTO*, John A. CHIORINI, Elena URCELAY and Brian SAFER

Molecular Hematology Branch, Section on Protein and RNA Biosynthesis, NHLBI, Bld. 10, Room 7D18, 10 Center Dr MSC 1654, Bethesda, MD 20892-1654, U.S.A.

Gene expression of the α-subunit of eukaryotic initiation factor-2 (eIF-2α), involves transcriptional and post-transcriptional mechanisms. eIF-2α is a single-copy gene expressing two mRNAs, 1.6 and 4.2 kb in size. Cloning and sequencing of the cDNA for the 4.2 kb mRNA revealed that it is the result of alternative polyadenylation site selection. Four polyadenylation sites were identified within the 3′ untranslated region (UTR) of eIF-2α, only two of which are normally utilized in human and mouse tissues. A functional role for the extended 3′ UTR was assessed by comparing the translatability and stability of the 1.6 and 4.2 kb mRNAs. Both the 1.6 and 4.2 kb transcripts could be translated *in vitro* and were identified *in vivo* as being distributed on large polyribosomes. This indicates that both mRNAs are efficiently translated. Stability studies showed that in activated T-cells the 4.2 kb mRNA was more stable than the 1.6 kb mRNA. Polyadenylation site selection and mRNA stability differ for the two mRNAs of eIF-2α. These activities might be modulated by sequence elements contained within the untranslated regions of the eIF-2α gene.

INTRODUCTION

One of the key factors involved in the initiation of protein synthesis is eukaryotic initiation factor 2 (eIF-2). During the process of initiation, eIF-2 catalyses binding of the initiator $tRNA^{Met}$ and GTP to the 40 S ribosomal subunit. This pre-initiation complex then binds mRNA, followed by joining of the 60 S ribosomal subunit to form the active 80 S ribosome. eIF-2 is a heterotrimer containing 38 kDa (α), 52 kDa (β), and 33 kDa (γ) subunits. We have been particularly interested in studying the gene expression of the α-subunit (eIF-2α). This subunit has been shown to have a regulatory role in protein synthesis [1–3]. Phosphorylation of this subunit on Ser-51 leads to inhibition of protein synthesis by interfering with the activity of eIF-2B, the guanine nucleotide exchange factor [2]. eIF-2α is a single-copy gene with only one protein product [4]. During T-cell activation (the first 24 h), eIF-2α mRNA levels increase more than 50-fold, with a subsequent increase in protein levels [5]. The transcriptional rate of eIF-2α, however, increases only slightly and does not account for the large increase in eIF-2α mRNA levels [5,6]. These previous studies consistently showed induction of two mRNAs reacting with the eIF-2α cDNA probe [5,6]. One hybridizing band, 1.6 kb in size, corresponded to the previously described mRNA for eIF-2α [7]. A second, considerably larger, 4.2 kb hybridizing band was also strongly induced in activated T-cells [5,6]. The 4.2 kb mRNA was determined as not being nuclear precursor RNA, because it could be identified in cytoplasmic $poly(A)^+$ RNA [5].

It is important to study the significance of multiple mRNA species encoded by a single gene to understand fully its expression and post-transcriptional regulation. The presence of multiple mRNAs could indicate utilization of different transcription start sites or different RNA processing (alternative splicing or alternative polyadenylation). A number of genes express multiple mRNAs, the best characterized of which is the immunoglobulin μ heavy chain gene [8–10]. Multiple mRNA species have also shown to be differentially regulated by events such as development, infection and differentiation [11,12], giving credibility to the importance of their existence.

We wanted to determine the mechanism by which the two mRNAs for eIF-2α are generated, and the functional significance of the two forms. To accomplish this it was necessary to clone and sequence the cDNA for the 4.2 kb mRNA. Cloning and sequencing of the 4.2 kb cDNA revealed that it contained the same 5′ upstream and coding sequences as the 1.6 kb mRNA, but differed from it in having a 2.5 kb downstream extension of the 3′ untranslated region (UTR). To assess the functional role of the extended 3′ UTR, stability studies *in vivo*, and studies *in vitro* comparing the translatability of the two mRNAs, were conducted. Understanding the functional consequences of the extended 3′ UTR of the 4.2 kb mRNA will improve our understanding of its importance and potential contribution to eIF-2α gene expression.

MATERIALS AND METHODS

Isolation and induction of T-cells

Enriched T lymphocytes were prepared from peripheral blood mononuclear cells, obtained from normal volunteers as previously described [5]. Whole blood was fractionated by continuous counterflow elutriation [13]. T lymphocytes were further enriched by Ficoll (BioWhittaker) gradient centrifugation and passage over nylon wool columns (Robbins Scientific). Quiescent G_0 T-cells were activated by incubation with 0.25 μM ionomycin (Sigma) and 10 ng/ml phorbol 12-myristate 13-acetate (PMA;

Abbreviations used: ActD, actinomycin D; CHX, cycloheximide; eIF-2α, eukaryotic initiation factor, α-subunit; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte/macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; poly(A) site, polyadenylation site; RACE, rapid amplification of cDNA ends; UTR, untranslated region.

* To whom correspondence should be addressed.

The nucleotide sequence data reported will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U26032.

Sigma) in 10 % (v/v) fetal bovine serum (Hyclone), RPMI 1640 with 25 mM Hepes (Gibco/BRL), 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin (Gibco/BRL).

Cloning of the 4.2 kb mRNA

The cDNA for the 4.2 kb mRNA was isolated from three commercially available libraries: (1) oligo(dT) and a random primed 5′ stretch gt11 library from PMA-treated T-cell lymphoma Hut 78 cell line (Clontech), (2) oligo(dT)-primed gt11 library from phytohaemagglutinin-activated T-cells (Clontech), and (3) oligo(dT)-primed gt11 library prepared from Jurkat T-cells (in Lambda ZAP II vectors, Stratagene). The final 4.2 kb cDNA was constructed from three clones and subcloned into Stratagene's Bluescript $(SK)^+$ vector. Sequencing was performed either manually (Sequenase, USB) or by automated means (Prism PCR sequencing, Applied Biosystems).

Confirmation of the ends of the 1.6 and 4.2 kb mRNAs was accomplished by using the 3′ rapid amplification of cDNA ends (RACE) procedure (BRL). Total or $poly(A)^+$ RNA isolated from activated T-cells was used and two rounds of PCR cycling were performed with nested primers corresponding to less than 1000 bp from the anticipated end of the message. The resulting PCR product was subcloned with the TA cloning kit (Invitrogen) and sequenced.

Preparation of RNA and Northern blot analysis

Total RNA was purified from induced cells with RNAzol B, as described by the manufacturer (Tel-Test, Inc., Friendswood, TX, U.S.A.). Northern blot analysis was performed as previously described [5]. RNA (10 μg) was fractionated by denaturing formaldehyde agarose gel electrophoresis, transferred to Nytran (S&S) and cross-linked by UV irradiation (Stratagene). These blots were hybridized with random-primed probes, washed in accordance with the procedure of Church and Gilbert [14] and exposed to XAR film (Kodak). Results were quantified by laser densitometry (Molecular Dynamics Personal Densitometer).

Transcription and translation of cDNAs *in vitro*

The 1.6 and 4.2 kb cDNAs were transcribed into capped RNA (Epicenter Ampliscribe T3 kit). Each cDNA was linearized with *Eco*RI and purified before transcription. The size of each transcript was confirmed by electrophoresis on denaturing formaldehyde agarose gels. The 1.6 and 4.2 kb transcripts were translated by using micrococcal nuclease-treated rabbit reticulocyte lysates (Promega). Translations were monitored by incorporation of [^{35}S]methionine into protein, which was analysed by SDS gel electrophoresis [15]. Results were quantified by laser densitometry (Molecular Dynamic Personal Densitometer).

Sucrose-density-gradient centrifugation

Washed Jurkat cells were lysed with 10 mM Hepes buffer, pH 7.9, containing 40 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 5 % (v/v) glycerol and 0.2 % Nonidet P40, with 8 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1 mM PMSF and 100 μg/ml cycloheximide (Sigma), incubated at 4 °C for 15 min with rotation, and centrifuged at 25000 ***g*** for 10 min at 4°C (Beckman SS34 rotor). Aliquots of the cytoplasmic supernatant were layered on a 10–40 % continuous sucrose density gradient containing 20 mM Tris buffer, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol, and centrifuged at 360000 ***g*** at 4°C for 90 min (Beckman SW41). The gradients were fractionated with an ISCO gradient fractionator and 0.5 ml fractions collected with a Gilson fraction collector. Each sample was treated with 5 μl of 20 % (w/v) SDS and 5 μl of 20 mg/ml proteinase K, then incubated for 15 min at 37 °C. Samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated by ethanol. Samples were subjected to formaldehyde agarose gel electrophoresis, transferred to Nytran membrane, baked and probed with α-^{32}P-labelled random primed cDNA for eIF-2α.

Stability studies

Stability of the 1.6 and 4.2 kb mRNAs was monitored by Northern blot analysis after activation of purified T-cells with 0.25μM ionomycin and 10 ng/ml PMA. After mitogenic activation, actinomycin D (ActD; 5 μg/ml; Sigma) or cycloheximide (CHX; 10 μg/ml) was added. Cells were harvested every hour over a 4 h period and RNA was isolated. Northern blot analysis was performed on 10 μg samples of RNA with α-^{32}P-labelled eIF-2α cDNA as a probe.

RESULTS

Both mRNAs for eIF-2α increase during T-cell activation

eIF-2 is a rate-limiting factor of translation, catalysing binding of GTP and the initiator $tRNA_i^{met}$ to the 40 S ribosomal subunit. The α-subunit of eIF-2 is encoded by a single-copy gene, but previously two distinct eIF-2α mRNAs, 1.6 and 4.2 kb, were observed in G_0 T-cells activated by treatment with phytohaemagglutinin plus the phorbol ester PMA [5], αCD3 plus PMA or ionomycin plus PMA [5,6]. Although the presence of the two mRNAs had been noted in these studies, quantification of only the 1.6 kb one was performed. We therefore repeated activation of enriched peripheral-blood G_0 T-cells by treatment with 0.25 μM ionomycin and 10 ng/ml PMA to measure the expression of both mRNAs (Figure 1). Levels of both mRNAs (detectable as early as 0.5–1 h) increased with time. Interestingly, the rate at which each mRNA increased was different. The increase was greater for the 1.6 kb mRNA than for the 4.2 kb mRNA (Table 1). By 8 h the 1.6 kb mRNA increased 11.5-fold, whereas the 4.2 kb mRNA increased only 4-fold. As a result there was an increase in the ratio of the 1.6 to 4.2 kb mRNA from 1:1 to 3:1 during early activation, and within 24 h of activation the ratio increased to 10:1. Therefore the potential contribution of the 4.2 kb mRNA to eIF-2α gene expression could be significant in G_0 T-cells when the ratio of the two mRNAs is 1:1.

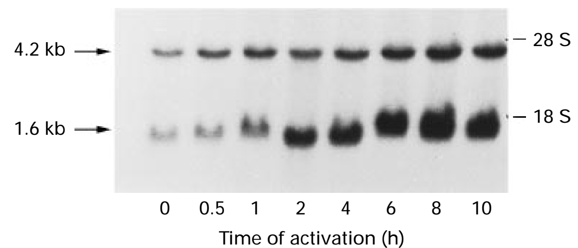

Figure 1 Northern blot analysis of the 1.6 and 4.2 kb mRNAs of eIF-2α during T-cell activation

Total RNA (10 μg) from T-cells activated with 0.25 μM ionomycin and 10 ng/ml PMA was fractionated on a formaldehyde agarose gel, transferred to Nytran membrane and hybridized with a ^{32}P-labelled eIF-2α cDNA probe.

Table 1 Comparison by densitometry of readings for the 1.6 and 4.2 kb mRNAs of eIF-2α

Results are calculated as fold increase over the $t = 0$ baseline The data include results from two overlapping time-point experiments. Ranges where shown are standard deviations.

Time of activation (h)	1.6 kb mRNA	4.2 kb mRNA
0	1.00	1.00
0.5	1.29	1.64
1.0	2.43	2.24
2.0	4.18 ± 1.16	1.82 ± 0.28
4.0	5.37 ± 0.74	2.77 ± 0.28
6.0	8.42	3.38
8.0	10.64 ± 0.29	4.53 ± 0.26
10.0	8.19	3.72
12.0	12.35	8.21
18.0	16.14	7.58
24.0	24.19	10.15

Analysis of sequences within the 3′ UTR of the 4.2 kb mRNA for eIF-2α

To elucidate the significance of the 4.2 kb mRNA, the cDNA for this mRNA was cloned from cDNA libraries prepared from activated T-cells or T-cell lines (see the Materials and methods section). Analysis of 4.2 kb eIF-2α cDNA sequences revealed that the 5′ UTR and coding sequences were identical with those of the cDNA previously reported for the 1.6 kb mRNA [7]. Only one large open reading frame was identified within each cDNA, which corresponded to the coding sequence for eIF-2α. We therefore determined that the difference between the 1.6 and 4.2 kb mRNAs was due to an extended 3′ UTR.

Four consensus polyadenylation sites (AAUAAA) were identified within the 3′ UTR of the 4.2 kb mRNA corresponding to 1487, 1614, 1663 and 4135 nt (Figure 2). The second and third polyadenylation sites were identified in close proximity (within 200 nt) to the first site. The fourth site was located 2.5 kb downstream of the first three sites. Determination of the poly(A) site used for the 1.6 and 4.2 kb mRNAs was performed by using the 3′ RACE procedure (see the Materials and methods section). This procedure used oligonucleotide primers targeted to specific gene sequences located upstream of the poly(A) sites. Poly(A)$^+$ RNA from activated T-cells was first reverse-transcribed by using oligo(dT) primers and then the 3′ ends of both the 1.6 and 4.2 kb cDNAs were amplified with the gene-specific primer and universal oligo(dT) primer by PCR. PCR products were cloned and sequenced. Results from the sequencing confirmed that the 3′ ends of the 1.6 and 4.2 kb mRNA corresponded to utilization of the first and last polyadenylation sites (results not shown). The two middle poly(A) sites were not used in activated T-cells.

Eleven AUUUA sequences were found within the 3′ UTR of the 4.2 kb cDNA. AUUUA-rich elements have been identified in the 3′ UTR of granulocyte/macrophage colony-stimulating factor (GM-CSF) as sequences that contribute to mRNA destabilization [16]. In eIF-2α, nine of these sequences occur in the 2.5 kb extension of the 4.2 kb 3′ UTR, whereas only two were found in the 3′ UTR of the 1.6 kb mRNA. Unlike the tandem multiple copies of AUUUA sequences found in GM-CSF, c-*fos* and interferon-γ [16–18], AUUUA sequences in the 3′ UTR of eIF-2α were widely spaced.

A long tract of poly(A) followed by a 200 bp region and then an equally long tract of poly(T) flanked by direct repeats of GCCTGGG were also found within the 3′ UTR of the 4.2 kb cDNA (Figure 2). Long stretches of poly(T) tracts flanked by

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TAA ctttgtgggaaacagagtccaatttaaggaacacagagcagcgcttcctggctgtaaatcctagacttgaaagttttccagta   1171
ttgaaaacttcaaagctgaatatttttatttctaagtatttaaatgttctaacagatcagaacatgaaatgccctcctaaatgt   1261
cagctgttgtcacacagtagctccaacactttgagcattttaagggagtggcctcatttcactagagacaaatctttaagaata   1341
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ggggaggtccaagcatttgtacttggcaggaatgctggcaatagcttgtgccagattactgtataaacttgaaagtAATAAAaat   4143
gttctttaaaagcaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
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Figure 2 3′ UTR sequence of the cDNA for eIF-2α 4.2 kb mRNA

The sequence starts with the termination sequence UAA (indicated in capital letters) for the coding region. Non-coding sequences are in lower-case letters except where indicated. Polyadenylation sites are in bold, double underlined and capital letters. AUUUA sequences are underlined. Poly(A) and poly(T) tracts are italicized. Repeat sequences flanking the poly(T) tract are indicated by capital italicized letters that are underlined.

direct repeats have been observed in the unusually long 3′ UTRs of hormone and steroid receptor mRNAs [19]. It has been postulated that direct repeats flanking poly(T) regions represent vestiges of prior retrotransposon activity within the gene [20]. We have no evidence, however, at present that these sequences are the result of prior retrotransposon activity. This generally GC-rich region also contained direct repeats of the 11 bp sequence, AAATCTTGAAT.

Presence of multiple mRNAs for eIF-2α in human and mouse tissues

Northern blot analysis of poly(A)$^+$ RNA isolated from human tissues revealed that both the 1.6 kb and the 4.2 kb mRNAs of eIF-2α were expressed in all tissues examined, i.e. heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Figure 3A). Measurement by laser densitometer scanning of the Northern blot showed that the ratio of 1.6 kb mRNA to 4.2 kb mRNA varied between different tissues. Ratios of 1.6 to 4.2 kb mRNAs ranged from almost 1:1 in brain and skeletal muscle to 10:1 in placenta, liver and pancreas. Hybridization of the human eIF-2α cDNA probe to a mouse multiple-tissue blot also showed the presence of both 1.6 and 4.2 kb mRNAs (Figure 3B). The amount of 4.2 kb mRNA in the mouse, however, seemed to be considerably less than that observed in the human tissues. Similarly to the human tissue blot, higher levels of the 4.2 kb mRNA were observed in mouse skeletal muscle and lung tissue and lower levels were observed in liver and heart. Interestingly, in mouse testes there was an additional 1.7 kb band hybridizing to the eIF-2α cDNA, seen more clearly in a shorter exposure (Figure 3B, lane 9). Direct comparison by Northern blot analysis

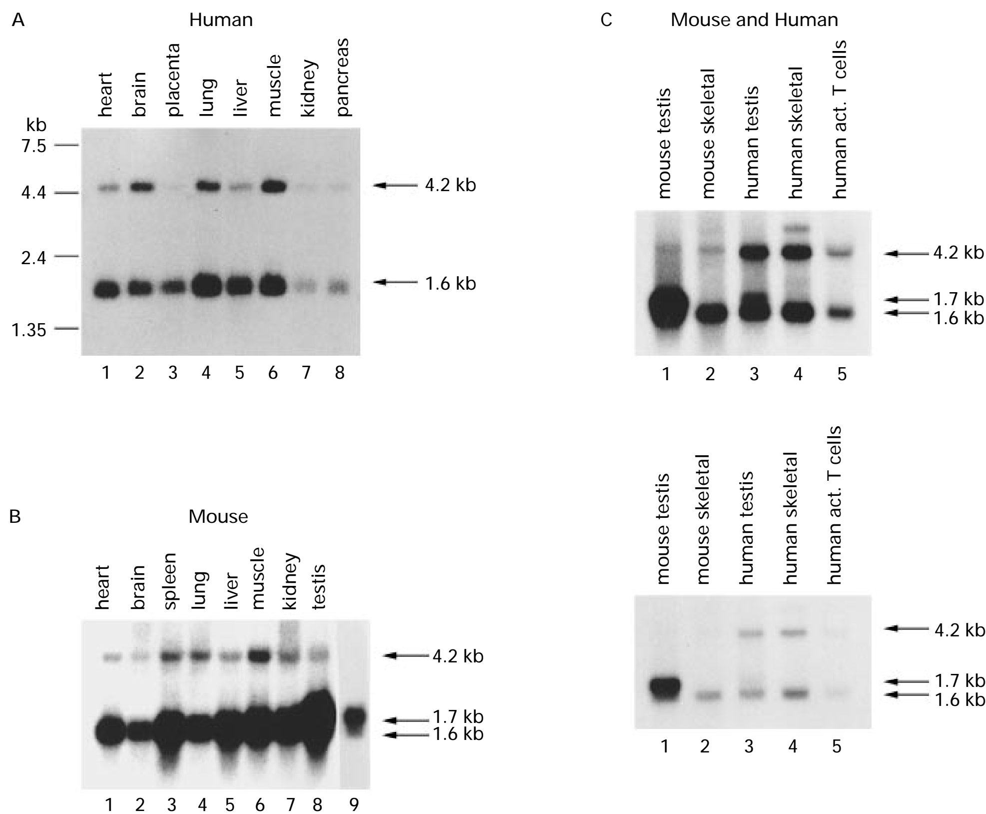


Figure 3 Northern blot analysis of a human multiple-tissue blot

(**A**) Multiple-tissue blot containing poly(A)$^+$ RNA from human heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), muscle (lane 6), kidney (lane 7) and pancreas (lane 8), probed with ^{32}P-labelled eIF-2α cDNA. The 1.6 and 4.2 kb mRNA are indicated by arrows. (**B**) Multiple-tissue blot containing poly(A)$^+$ RNA from mouse heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), muscle (lane 6), kidney (lane 7) and testis (lane 8); a shorter exposure of testis sample is shown in lane 9. (**C**) Multiple-tissue blot using poly(A)$^+$ RNA (1 μg per lane) from mouse testis (lane 1), mouse skeletal muscle (lane 2), human testis (lane 3), human skeletal muscle (lane 4) and 10 μg of total RNA from activated (act.) T-cells (lane 5). The lower panel is a shorter exposure of the same blot.

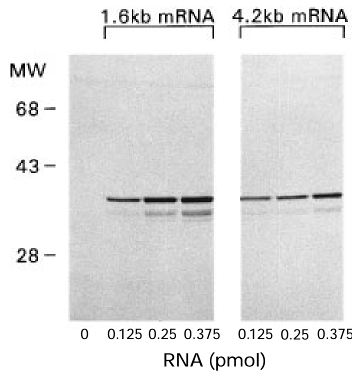
with an eIF-2α cDNA probe of poly(A)$^+$ RNA from mouse and human testes and skeletal muscle (Figure 3C) reproduced the results shown previously and also showed that there was a 1.7 kb band present in human testis tissue. The human tissues examined contained considerably higher ratios of the 4.2 kb mRNA than the mouse tissues. These results demonstrate that expression of the 1.6 and 4.2 kb mRNAs is conserved from mouse to human and that utilization of a third site is also conserved between mouse and human, but only found in testis tissue. The 1.7 kb band was confirmed as an eIF-2α isoform by 3′ RACE with primers targeted to eIF-2α sequences (results not shown). Because there are four potential poly(A) sites, with the second and third poly(A) sites located 127–176 nt downstream of the first poly(A) site, it is quite possible that the 1.7 kb mRNA is the result of utilization of either the second or third site. We also observed another band approx. 5–5.5 kb in size, in human and weakly in mouse skeletal muscle RNA. The nature of this eIF-2α isoform is unknown and must await further sequencing of the eIF-2α gene. Although the basis for the variability in the ratio of the 1.6 to 4.2 kb mRNAs between tissues remains to be determined, the patterns of poly(A) site utilization in mouse parallels that of human, i.e. larger amounts of 4.2 kb mRNA were found in the same tissue from mouse or human origin. These results indicate that RNA processing, i.e. cleavage, termination and poly-adenylation, plays an important role in the generation of different levels of eIF-2α mRNAs in a tissue-specific manner.

Translation of the 1.6 and 4.2 kb mRNAs *in vitro*

Translation of the 1.6 and 4.2 kb mRNAs *in vitro* by rabbit reticulocyte lysate was compared. Capped transcripts from each cDNA were synthesized *in vitro* by using the bacterial T_3 promoter and bacterial RNA polymerase. Equal molar quantities were then translated into protein by using [^{35}S]methionine and rabbit reticulocyte lysate. Only one product, corresponding to the 36 kDa α-subunit of eIF-2, was synthesized by either the 1.6 or 4.2 kb transcript (Figure 4A). Slightly more (2–3-fold) eIF-2α was synthesized by the 1.6 kb transcript than by the 4.2 kb transcript (Figure 4B). Rates of protein synthesis were compared for the amount of mRNAs and they were found to be linear for the time course of the assay. The ratio of the linear rate of the 1.6 to 4.2 kb mRNAs was calculated as 2.4 (results not shown).

Both mRNAs are located on polyribosomes

Although both the 1.6 and 4.2 kb mRNAs could be translated into proteins *in vitro*, we still had not demonstrated that both mRNAs could be translated *in vivo*. One way of demonstrating translatability of the mRNAs *in vivo* is to determine the position of the mRNAs on polyribosomes. Jurkat cells (a lymphocytic cell line often used to study activation of T-cells) were lysed, fractionated into cytosolic and nuclear fractions and the cytosol was analysed on 15–40 % continuous sucrose density gradients



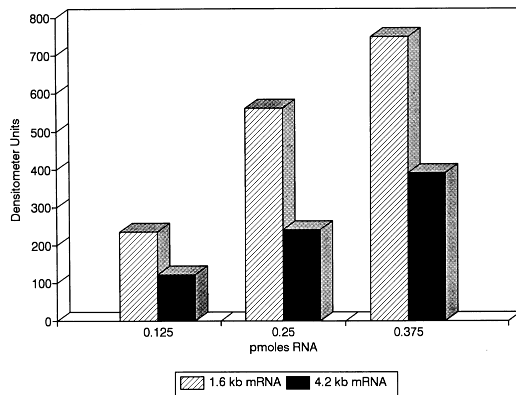


Figure 4 Translation *in vitro* of capped 1.6 and 4.2 kb transcripts generated from their respective cDNAs

(**A**) SDS gel electrophoresis of the protein products after translation with rabbit reticulocyte lysate. Molecular masses (MW; in kDa) are indicated on the left. (**B**) Quantification of eIF-2α by densitometry.

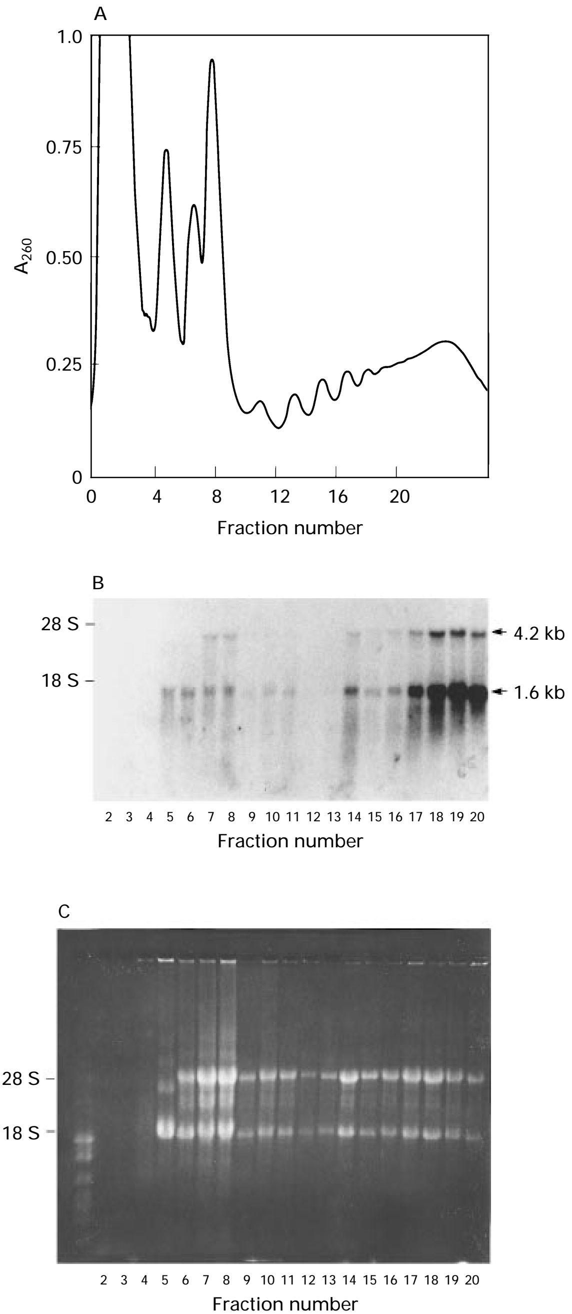


Figure 5 Northern blot analysis of fractions from sucrose density gradients of Jurkat cell lysates

Jurkat cells were lysed with 0.2% Nonidet P40 and cytosols fractionated on 10–40% continuous sucrose density gradients. (**A**) A_{260} of sucrose-density-gradient fractions. (**B**) Northern blot analysis of gradient fractions. (**C**) Ethidium bromide staining of fractions.

(see the Materials and methods section). The A_{260} profile of the gradient is shown in Figure 5(A). 40 S and 60 S ribosomal subunits were detected in fractions 5 and 6, and 7 respectively, with 80 S ribosomes present in fractions 8 and 9. Polyribosomes were distributed in fractions 13–20. RNA extracted from each fraction was electrophoresed on formaldehyde agarose gels, transferred to nylon membranes and probed with cDNA for eIF-2α. Northern blot analysis of these fractions with eIF-2α cDNA (Figure 5B) showed that the 1.6 and 4.2 kb mRNAs were associated with polyribosomes in fractions 14–20 (Figure 5B). The majority of each mRNA was, however, located near the bottom of the gradient, in fractions 17–20, corresponding to large polyribosomes. Ethidium bromide staining of the gel showed that the RNA was reasonably intact and identified the 18 S and 28 S rRNA species (Figure 5C). Treatment of cytosols with EDTA before sucrose-density-gradient centrifugation resulted in shifting of both mRNAs and ribosomes to the top of the gradient (results not shown). This indicates that both mRNAs are specifically associated with large polyribosomes and are not co-sedimenting as large ribonuclear particles. Association with large polyribosomes suggests that both mRNAs were being actively and efficiently translated. Sucrose-density-gradient analysis of cytoplasm isolated from activated human primary T lymphocytes showed a similar distribution of eIF-2α 1.6 and 4.2 kb mRNAs on large polyribosomes (results not shown).

The 4.2 kb mRNA is more stable than the 1.6 kb mRNA

Because alternative poly(A) site utilization generated at least two mRNAs, the effect on stability and translatability was investi-

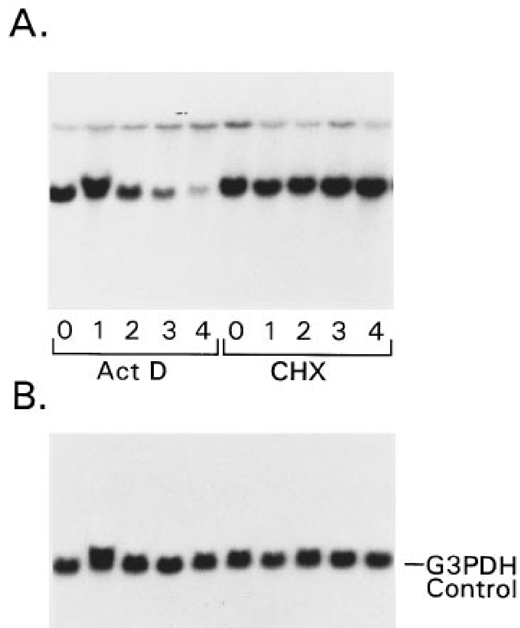


Figure 6 Stability studies of the 1.6 and 4.2 kb mRNAs of eIF-2α

Purified T-cells were activated with ionomycin and PMA for 6 h. They were then treated with either ActD or CHX and samples removed at 0, 1, 2, 3 and 4 h afterwards. RNA was extracted from each sample and analysed by Northern blot, with eIF-2α cDNA as probe (**A**). The blot was stripped and rehybridized with the control probe G3PDH to verify equal loading of RNA (**B**). (The experiment with and without ActD or CHX was repeated once at 6 h of activation and twice at 3 h of activation.)

gated as a means of establishing a functional role for the extended 3′ UTR of the 4.2 kb mRNA. Studies of the stability of the 1.6 and 4.2 kb mRNAs were conducted in mitogen-activated T-cells by Northern blot analysis. Because the levels of the two mRNAs increased differentially during T-cell activation, experiments were designed to determine whether the degradation rates during induction of the two mRNAs differed with T-cell activation. Initially, purified G_0 T-cells were activated for 6 h with ionomycin plus PMA. Decay was monitored by Northern blot analysis of RNA extracted from cells 1, 2, 3 and 4 h after the addition of ActD (Figure 6). The protein synthesis inhibitor CHX was added to assess the importance of protein synthesis for stabilization of the mRNAs. Results of a representative study showed degradation of the 1.6 kb mRNA, but little or no degradation of the 4.2 kb mRNA. This blot was also probed with cDNA for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) as a control for equal loading of RNA. These results also show that mRNA for G3PDH was relatively stable after treatment with ActD. The addition of CHX stabilized the 1.6 kb mRNA, but did not seem to affect the 4.2 kb mRNA or the G3PDH control. CHX might inhibit the synthesis of a labile protein required for degradation of the 1.6 kb mRNA, or it might indicate that translation itself, i.e. association of the mRNA with ribosomes, is important for degradation of the mRNA. It is clear from these experiments that the 1.6 kb mRNA is more sensitive to degradation than the 4.2 kb mRNA.

We wanted to see if degradation rates for the 1.6 kb mRNA or the 4.2 kb mRNA changed with activation of T-cells. To accomplish this, degradation of eIF-2α mRNAs with increasing times of T-cell activation was examined (Figure 7). Time points used were 0, 1, 3 and 6 h of activation before treatment with ActD. Degradation rates were calculated from results from Northern Blot analysis of the time points, scanned by laser densitometry, normalized to G3PDH levels, averaged for time points and plotted as a percentage of the first time point ($t = 0$ of ActD treatment). Rates of degradation for the 1.6 kb mRNA are similar for 1, 3 and 6 h of activation. There was little on no degradation of the 4.2 kb mRNA. Table 2 contains calculated $t_{1/2}$ values for each time point, which range from 2.71 h to 2.8 h

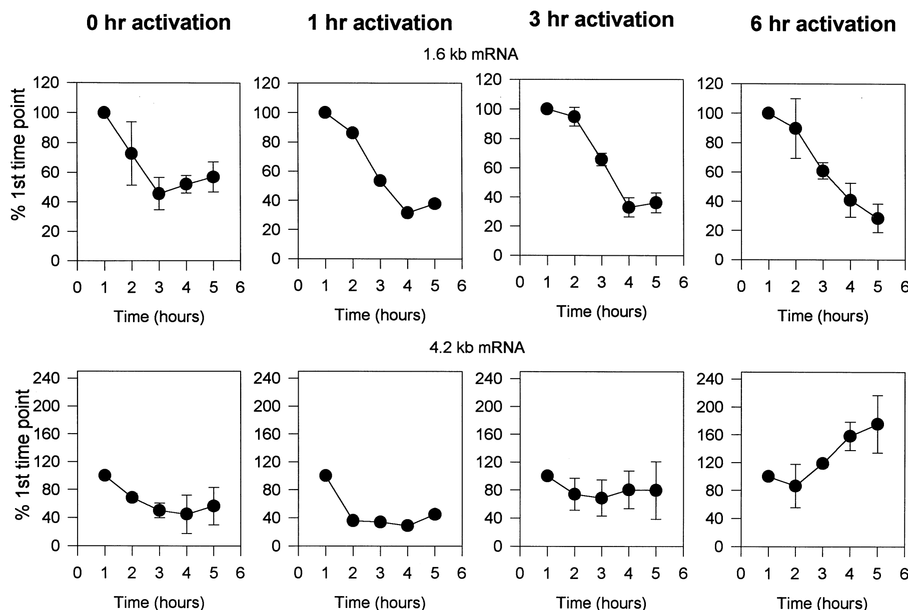


Figure 7 Time course of stabilization/degradation of the 1.6 and 4.2 kb mRNAs of eIF-2α during T-cell activation

Northern blot analysis of purified T-cells that had been activated for 0, 1, 3 or 6 h before addition of ActD, after which samples were taken at 0, 1, 2, 3 and 4 h. Results of densitometer scans of the blots were normalized to the control signals for G3PDH, calculated as a percentage of the first time point ($t = 0$) and then averaged for each period of activation. Results are plotted either as a single point (1 h of activation) or as the mean $\pm$ S.D. for two or three experiments (0, 3 and 6 h).

Table 2 Degradation of the 1.6 kb mRNA of eIF-2α during T-cell activation

Ranges where shown are means ± S.D.

Time of activation (h)	$t_{1/2}$ (h)	Number of experiments
0	3.92 ± 0.54	2
1	2.8	1
3	2.71 ± 0.63	3
6	2.73 ± 0.76	3

for 1, 3 and 6 h of activation. It therefore seems that the rate of degradation for the 1.6 kb mRNA did not change significantly with activation. Little degradation was observed for the 4.2 kb mRNA during those same periods of activation and ActD treatment. The amount of degradation of the mRNAs may be sensitive to the concentration of ActD, but it is clear from experiments conducted that the 1.6 kb mRNA is more sensitive to degradation than the 4.2 kb mRNA at the concentration of ActD used, regardless of the extent of activation.

DISCUSSION

The major conclusion that can be drawn from this work is that the gene for eIF-2α produces multiple forms of eIF-2α mRNAs with different metabolic profiles. The two major forms accumulate differently, in a tissue-specific manner that is evolutionarily conserved from mouse to man. Both forms are translated equally well. As a result of cloning the 4.2 kb mRNA for eIF-2α, we have identified potential regulatory functions within the 3′ UTR: polyadenylation site selection is one and mRNA stability is another. Four potential poly(A) sites were identified within the 3′ UTR of the 4.2 kb cDNA. All four sites are within one contiguous exon, as demonstrated by PCR probing of human genomic DNA, with oligonucleotides targeted to regions within the 3′ UTR (results not shown). Therefore differential forms of eIF-2α mRNA result from alternative poly(A) site use and not alternative splicing. Northern blot analysis of a variety of human and mouse tissues shows that two sites are used predominantly, corresponding to the first and fourth poly(A) sites. The third site may be used, but only in mouse and human testes. Synthesis of these various forms of eIF-2α mRNA seems to be regulated in a tissue-specific manner.

Poly(A) site selection involves 3′ end processing of mRNA, which includes transcription termination, cleavage and polyadenylation. RNA *Pol*II transcripts are synthesized up to 4000 nt beyond the poly(A) site before termination [21,22]. Termination is dependent on an intact poly(A) site [21–23], and it is not known what other sequence and/or structural elements may also be required [21,24]. For cleavage and polyadenylation, poly(A) site use normally requires the consensus AATAAA sequence and is influenced by a downstream GU-rich region [25–28], upstream *cis* elements [29] and secondary structure [30]. In genes containing multiple tandem poly(A) sites, the upstream site (proximal to the promoter) is selected preferentially [31]. Preferential use of the first poly(A) site for eIF-2α is consistent with this mechanism. The 147 nt region between the first and second sites is GU-rich. Three sites are located within 300 nt of each other, with the second and third sites separated by only 49 nt. Space and distance between poly(A) sites [32], as well as availability of factors involved in *Pol*II transcription, termination, cleavage and polyadenylation of RNA [31,33], are important contributing parameters in poly(A) site utilization. Limited amounts of specific cleavage and polyadenylation factor(s) could favour read-through of the upstream poly(A) sites, and continuation of transcription until the last poly(A) site of the eIF-2α gene. We are in the process of isolating the complete genomic clone for eIF-2α. This will enable us to examine the downstream region from the last poly(A) site for the GU-rich region and other sequence/structural elements responsible for the use of the last poly(A) site in the 4.2 kb mRNA.

Transcripts generated from both the 1.6 and 4.2 kb cDNAs were translated *in vitro* with rabbit reticulocyte lysate into a single 36 kDa protein. Transcripts from the 1.6 kb cDNA were translated more efficiently (2–3-fold) than those from the 4.2 kb cDNA. This may indicate that the additional 2.5 kb 3′ UTR slightly represses translation of the 4.2 kb mRNA *in vitro*. These results must be interpreted with caution, however, because they do not necessarily reflect the true physiological state of the 1.6 and 4.2 kb mRNAs. Another means of assessing translation of mRNAs *in vivo* was to demonstrate that both the 1.6 and 4.2 kb mRNAs were associated with large polyribosomes. Ribosome density and association with large polyribosomes has been shown to be a good indicator of translational efficiency [34]. Recent evidence suggests that the 5′ UTR is responsible for the translational status of mRNAs [35]. As both isoforms of eIF-2α have the same 5′ UTRs and coding region, ribosomal loading and translation would be predicted to be similar. Treatment with EDTA caused both mRNAs to shift to the top of the sucrose density gradient, demonstrating that both mRNAs were indeed associated with large polyribosomes and were actively being translated.

The 4.2 kb mRNA is more stable than the 1.6 kb mRNA in activated T-cells. Although 11 AUUUA sequences [16], which have been identified as destabilization sequences in GM-CSF [16], c-*fos* [17] and interferon-β [18] mRNAs, are identified within the 3′ UTR of the eIF-2α 4.2 kb mRNA, these sequences are widely spaced (36–800 nt) with no overlapping sequences. The optimal sequence UUAUUUAU [36,37] was not found within the 3′ UTR of the 4.2 kb mRNA. The AUUUA would therefore not be predicted to have a destabilizing effect on the 4.2 kb mRNA. Because the 4.2 kb mRNA is actually more stable than the 1.6 kb mRNA, it is possible that either the length of the transcript or unidentified stabilizing elements contribute to its increased stability. Stability of mRNA does not necessarily correlate with length [38,39]. It is therefore possible that sequence and/or structural elements is responsible for the greater stability of the 4.2 kb eIF-2α mRNA. Although a number of RNA elements responsible for destabilizing mRNA have been well characterized [40], stabilizing elements are only recently being identified. Such elements are found in the 3′ UTR of α-globin [41]. We are in the process of determining whether stabilizing elements exist within the 3′ UTR of eIF-2α.

Conservation of poly(A) site use for the 4.2 kb mRNA from mouse to man is strong evidence of its importance for gene expression of eIF-2α. In quiescent T-cells, the 4.2 kb mRNA is found in a 1:1 ratio with the 1.6 kb mRNA. Because of the greater lability of the shorter mRNA, the 4.2 kb mRNA must contribute significantly to the expression of eIF-2α in these cells. We postulate that synthesis of the 4.2 kb mRNA in cells is dependent upon the availability of factors necessary for RNA processing, i.e. cleavage/polyadenylation/termination of RNA transcripts. These factors may be in limited supply in quiescent, non-proliferating cells.

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