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Cloning and expression of eukaryotic initiation factor 4B cDNA: sequence determination identifies a common RNA recognition motif

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Eukaryotic protein synthesis initiation factor 4B (eIF-4B) is an 80 000 dalton polypeptide which is essential for the binding of mRNA to ribosomes. A highly purified preparation of eIF-4B from HeLa cells was subjected to enzymatic cleavage and amino-terminal amino acid sequence analysis. Degenerate oligonucleotide probes were used to isolate a 3851 bp cDNA encoding eIF-4B from a human cDNA library. The DNA encodes a protein comprising 611 residues with a mass of 69 843 daltons. The amino-terminal domain of eIF-4B contains a consensus RNA binding domain present in a number of other RNA binding proteins. Expression of eIF-4B in transfected COS-1 cells yielded a polypeptide which reacted with anti-eIF-4B antiserum and comigrated with purified eIF-4B. Expression of eIF-4B in COS-1 cells resulted in a general inhibition of translation, possibly due to a 50-fold eIF-4B overproduction.

Key words: cap binding protein/RNA binding protein/ translation

Introduction

The mechanism by which ribosomes bind to the 5′ end of the eukaryotic mRNAs has been extensively studied *in vitro* with purified components. These studies have identified several proteins which bind the 7-methyl guanosine (m^7G) cap structure at the 5′ end of mRNAs and melt RNA secondary structure (for review see Sonenberg, 1988). Eukaryotic initiation factor 4B (eIF-4B) functions in close association with eIF-4F and eIF-4A. All three factors are required for the binding of mRNA to ribosomes (Grifo *et al.*, 1984). The individual roles for these initiation factors in mRNA binding are not known. eIF-4B is a single polypeptide of ~80 000 daltons (Trachsel *et al.*, 1977; Benne and Hershey, 1978) which may function as a dimer of identical subunits (Abramson *et al.*, 1988). eIF-4B binds near the 5′-terminal cap of mRNA (Edery *et al.*, 1983; Milburn *et al.*, 1988) in the presence of eIF-4F and ATP. eIF-4B may have a preference for binding the AUG translation initiation codon (Butler and Clark, 1984; Goss *et al.*, 1987). eIF-4B promotes the ATPase activity (Grifo *et al.*, 1984; Abramson *et al.*, 1987) and the ATP-dependent RNA unwinding activities of both eIF-4A and eIF-4F (Ray *et al.*, 1985; Rozen *et al.*, 1990). eIF-4B has also been found to stimulate the release and rebinding (recycling) reaction of eIF-4F with the mRNA cap (Ray *et al.*, 1986). Although eIF-4B stimulates many of the activities of eIF-4A and eIF-4F, no specific unique function has been attributed to eIF-4B. In all of the assays characterizing eIF-4B activity, large amounts of eIF-4B protein are utilized, resulting in some uncertainty that the 80 kd eIF-4B polypeptide in these preparations is the active component. To enhance our understanding of the function of eIF-4B, we have used purified eIF-4B to isolate and express a cDNA clone encoding human eIF-4B.

Results

Cloning cDNAs encoding eIF-4B

cDNA sequences encoding eIF-4B were isolated by screening a human cDNA library with oligonucleotides based on partial protein sequences. Although antibodies specific for eIF-4B were available (Meyer *et al.*, 1982), attempts at screening λgt11 expression libraries were unsuccessful. The inability to generate amino-terminal sequence data from intact eIF-4B by Edman degradation suggested that the amino terminus was blocked. Therefore, internal protein sequences were obtained from four chymotryptic peptide fragments derived from highly purified HeLa cell eIF-4B. The HPLC elution profile of the chymotryptic digest and amino acid sequences obtained are shown in Figure 1. Peptides 1 and 4 generated double signals at several residues, suggesting the presence of two similar peptides.

A 32 base non-degenerate oligonucleotide probe ('guessmer') and two overlapping 17 base degenerate oligonucleotide probes were synthesized based on the amino acid sequence of peptide 2. Labeled probes were used to screen a human fetal liver cDNA library in λ Charon 21A as described in Materials and methods. A positive hybridizing recombinant phage was purified and sequence analysis confirmed that the recombinant phage encoded a portion of eIF-4B. To obtain full-length cDNA inserts, two *Eco*RI fragments from the recombinant λ Charon 21A phage which encode peptides 1 and 2 were used to screen a human osteosarcoma cDNA library in λ ZAP. Four positively hybridizing phage were isolated harboring inserts of 1.9, 2.9, 3.0 and 3.9 kb which were subcloned into Bluescript plasmids. All clones were subjected to terminal DNA sequence analysis, restriction endonuclease mapping, and hybridization to oligonucleotide probes. The 2.9 and 3.0 kb clones were found to be incomplete reverse transcription products of the longer 3.9 kb mRNA encoding eIF-4B and thus lack 5′ sequences present within the 3.9 kb clone. The eIF-4B insert from the 3.9 kb clone was sequenced on both strands to obtain a sequence of 3840 bases up to the poly(A) tract (Figure 2). The sequence contains an open reading frame (ORF) of 611 codons beginning near the 5′ terminus.

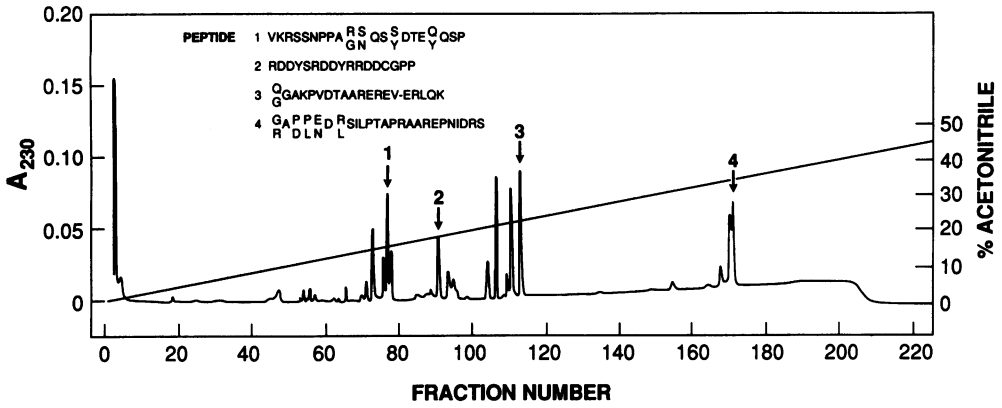

Fig. 1. Fractionation of eIF-4B chymotryptic peptides by reverse phase HPLC. Chymotryptic peptides from eIF-4B were separated using a Vydac C-4 column as described in Materials and methods. Individual peaks were subjected to gas-phase microsequencing and the resulting amino acid sequences are shown. For positions where double sequences were obtained, both residues are indicated.

The following results indicate that the 1.9 kb insert was derived from an mRNA which codes for the same protein as the 3.9 kb insert, but is shortened at its 3′ end (Figure 3A). Its nucleotide sequence over 350 bp is identical at the 5′ end to that of the 3.9 kb DNA, except that it is shorter by 7 bp. Its sequence at the 3′ end matches a 350 bp internal sequence, but diverges at bp 1965 in the 3.9 kb sequence (Figure 3A). Finally, the DNA sequence encoding peptide 1, where a mixture of two amino acid sequences was found, is identical to that in the 3.9 kb clone. It is therefore highly likely that the 1.9 and 3.9 kb cDNAs are derived from mRNAs transcribed from the same gene. However, at present we cannot rule out the existence of another mRNA derived from an eIF-4B related gene which encodes the secondary amino acid sequences shown in Figure 1.

Two sizes of mRNA encode eIF-4B in vivo

Northern blot analysis using an internal 1.8 kb *Eco*RI fragment (Figure 3A) derived from the 3.9 kb clone confirmed the presence of two distinct size classes of mRNA, with approximate sizes of 4.4 and 2.3 kb in total RNA from human adenovirus transformed 293 cells (Figure 4, lane 1) and from poly(A)$^+$ RNA isolated from colon carcinoma cells (lane 2) and human osteosarcoma cells (lane 3). In 293 cells and in the colon carcinoma cells the ratio of 4.4 kb to 2.3 kb mRNA is ~3:1, whereas in the osteosarcoma cells the two mRNAs are present in equal amounts. Northern blot hybridization with a probe derived from the 3′ untranslated region of the 3.9 kb clone detected only the larger of the two eIF-4B specific mRNAs (data not shown).

The two eIF-4B clones with insert sizes of 3.9 kb and 1.9 kb are consistent with the size of the two eIF-4B mRNAs detected by Northern blot analysis. The 3.9 kb clone possesses a consensus polyadenylation signal AATAAA (residues 3822–3827) 25 bp upstream from a polyadenylate tract. The shorter 1.9 kb clone is probably derived from the 2.3 kb mRNA since it also contains a hexanucleotide AATAAA (residues 1942–1947) 22 bp upstream from a poly(A) tract. Downstream of the point where the two clones diverge, there is a T rich sequence (residues 2008–2019) in the 3.9 kb clone (Figure 3A). The AATAAA and downstream T rich sequence are hallmarks for polyadenylation (Birnstiel *et al.*, 1985). The Northern blot analysis and DNA sequence analysis from these two clones indicates that two structural mRNAs encode eIF-4B which differ by alternate utilization of two polyadenylation signals within the 3′ untranslated region.

Genomic Southern blots of human DNA digested with several restriction enzymes produced complex patterns of 8–15 bands when hybridized with nick-translated eIF-4B cDNA sequences. These results suggest that multiple genes or pseudogenes may exist in the human genome or that many introns are present within one gene. The cloning of the eIF-4B gene(s) will be necessary to define the precise number and structure and to determine the full-length 5′ untranslated region of the mRNA.

Analysis of the eIF-4B coding sequence

The 3.9 kb sequence contains a single large ORF which extends from the 5′ terminus to nucleotide 1852 (Figure 2). The deduced amino acid sequence encodes all four peptide sequences identified (underlined in Figure 2), as well as numerous rabbit eIF-4B tryptic peptides (William C.Merrick, personal communication, data not shown). The amino acid composition as determined by standard acid hydrolysis is in agreement with that of the deduced polypeptide (data not shown). The AUG at nucleotides 20–22, tentatively identified as the translation initiation site (see below), possesses a consensus nucleotide sequence for a favorable initiation codon, with an A at position −3 and a G at position +4 (Kozak, 1987).

Analysis of the derived amino acid sequence for the eIF-4B suggests the presence of three domains which are depicted in Figure 3B. The amino-terminal domain of ~150 residues contains a consensus RNA binding site (RNP-CS) which has been identified in a number of RNA binding proteins including snRNA, pre-RNA and other mRNA binding proteins for which sequence information is available (Table I) (for recent reviews see Bandziulis *et al.*, 1989; Mattaj, 1989). This sequence contains a consensus hexapeptide sequence 33 amino acids upstream from a conserved octapeptide sequence. The hexapeptide sequence is less well conserved than the octapeptide sequence although its position relative to the octapeptide sequence and the general character of its amino acid content are conserved. Although eIF-4B is acidic overall (pI = 5.8), the most conserved portion of

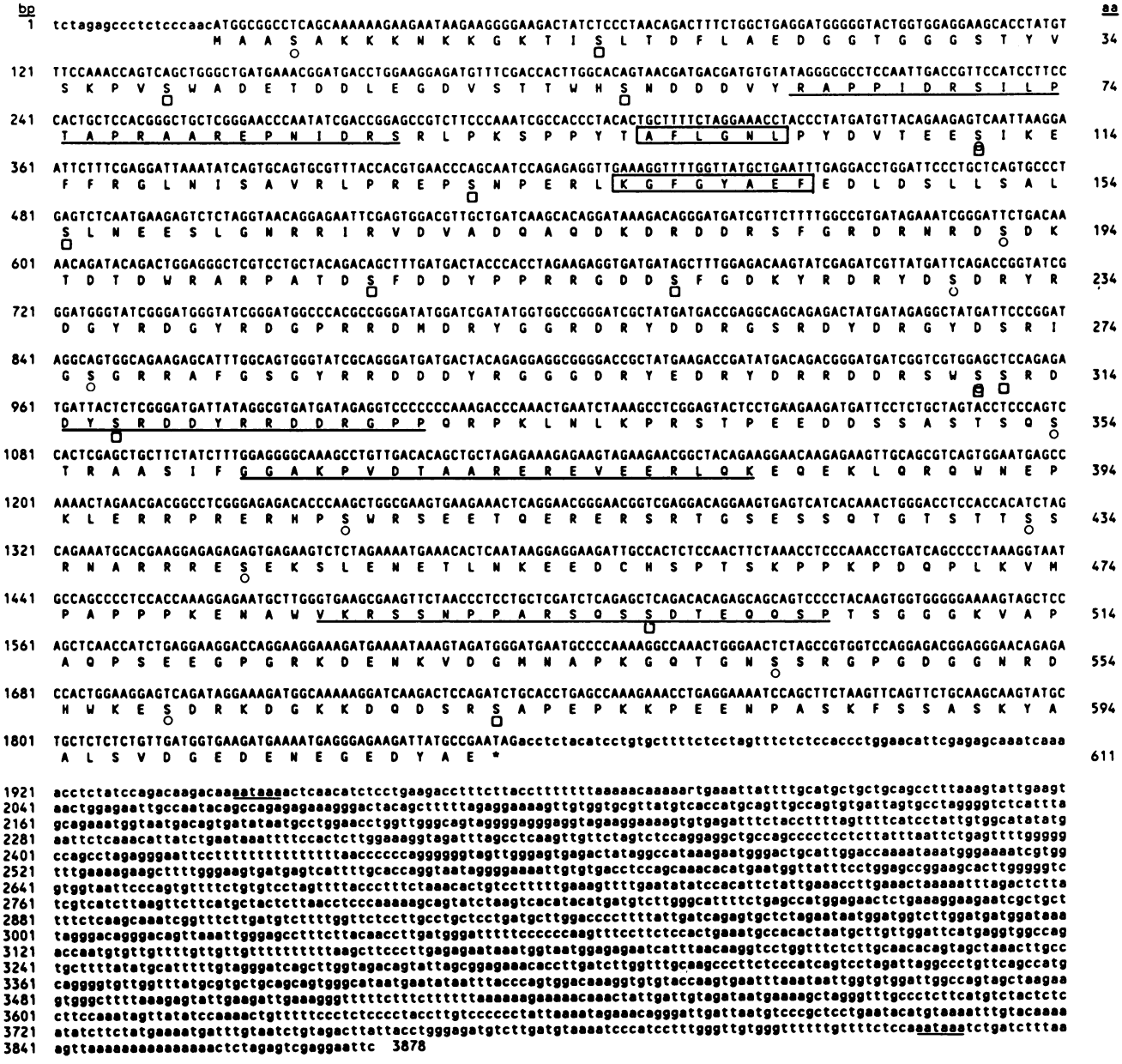

Fig. 2. Sequence of the 3.9 kb cDNA clone encoding eIF-4B. The eIF-4B cDNA sequence of the coding strand is shown with the deduced amino acid sequence of eIF-4B below. The octamer and hexamer of the proposed RNA binding domain are identified by boxes. The four underlined peptide sequences correspond to the sequences determined from direct protein sequencing (Figure 1). The two polyadenylation signals in the 3′ untranslated region are underlined. The location of potential protein kinase C (Woodget *et al.*, 1986) and casein kinase II (Kuenzel *et al.*, 1987) serine phosphorylation sites determined from the Prosite sequence analysis program (Intelligenetics) are shown by an open circle (protein kinase C) or an open box (casein kinase II) below the putative phosphorylated serine.

the RNA binding region (residues 100−150) contains six basic and three acidic amino acids. This is the most basic region of the protein.

A second domain (residues 164−356) consists of a very high proportion (52%) of charged amino acids: 50 basic and 50 acidic amino acids. The region contains numerous copies of the dipeptide aspartic acid—arginine, frequently adjacent to glycine or tyrosine residues (DRYG rich). The highly polar region may be non-structured (Figure 3B), and may account for the aberrant migration of eIF-4B on SDS−PAGE. The factor has an apparent mol. wt of 80 000 even though the cDNA encodes a protein with a mol. wt of 69 843. U1 70K snRNP has a similar highly polar region which has been demonstrated to retard mobility upon SDS−PAGE (Query *et al.*, 1989).

The carboxy-terminal domain of eIF-4B (residues 360−611) also consists of an unusually large number of polar residues (Figure 3B), namely glutamic acid (13%), arginine (9%), lysine (8%), proline (10%) and serine (14%). Multiple serine residues within eIF-4B are covalently modified by phosphorylation and may be involved in translational control (Hershey, 1990). Computer searches for protein kinase C (Woodgett *et al.*, 1986) and casein kinase II (Kuenzel *et al.*, 1987) serine phosphorylation sites identified multiple potential phosphorylation sites within eIF-4B (identified in Figure 2). The La RNA binding protein

which is an RNA binding protein (Chambers *et al.*, 1988) that exhibits nucleic acid dependent ATPase and unwinding activity (Bachmann *et al.*, 1990) is also extensively modified by phosphorylation. All phosphorylated serine and threonine residues within the La RNA binding protein are located in the carboxy-terminal domain of the protein (Chambers *et al.*, 1988).

Expression of eIF-4B in COS-1 cells

The ORF encoding eIF-4B contained within a *Pst*I fragment was introduced into the mammalian cell expression vector pMT2 (Kaufman *et al.*, 1989) for transient DNA transfection into COS-1 monkey kidney cells. At 44 h post-transfection, cell lysates were prepared and analyzed by SDS−PAGE and immunoblotting procedures using a goat anti-human eIF-4B antiserum. This analysis detects an 80 kd polypeptide species which is overrepresented in the transfected cells (Figure 5A, lane 3) compared with cells that received vector alone (lane 4) or did not receive DNA (lane 5). This 80 kd species comigrates with human HeLa cell purified eIF-4B (compare lanes 1, 2 and 3). By comparison of the band intensities obtained from titrating the amount of pMT-4B-transfected cell lysate loaded onto the gel, it is possible to estimate the amount of eIF-4B overproduction to be ~50-fold. These results demonstrate that pMT-4B encodes an 80 kd polypeptide which reacts with the anti-eIF-4B antiserum, comigrates with purified human eIF-4B, and is highly overexpressed in transfected cells.

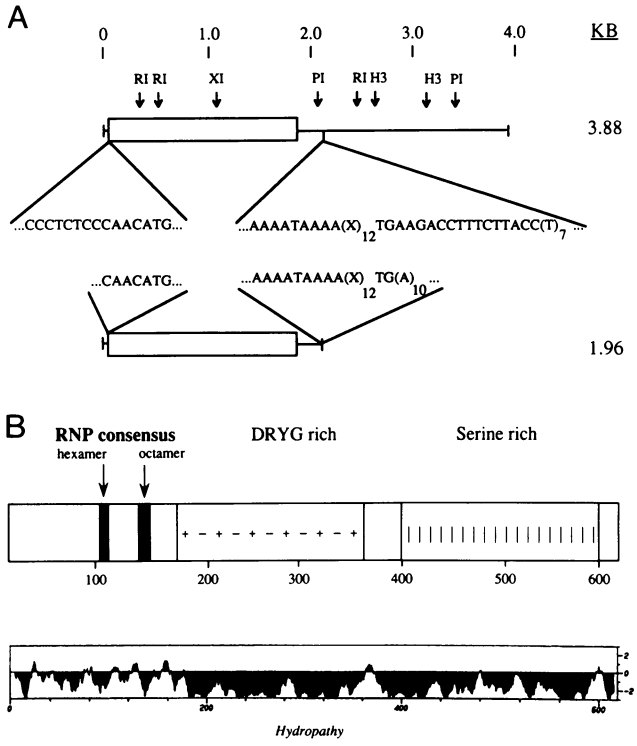

Fig. 3. Representation of the mRNA structure and protein domains of eIF-4B. (**A**) mRNA structure of eIF-4B. The 1.9 and 3.9 kb cDNA clones encoding eIF-4B are depicted. The DNA sequences presented are where the DNA sequence of the two clones diverge. Restriction sites indicated are *Eco*RI (RI), *Pst*I (PI), *Hin*dIII (H3) and *Xho*I (XI). (**B**) Domains and hydrophobicity of eIF-4B. The RNA binding domain (RNP-CS) is shown with the position of the octapeptide and hexapeptide conserved sequences. Also shown are the highly charged region (DRYG rich) and the serine rich region. The hydropathy of eIF-4B was determined as described by Devereux *et al.* (1984).

Table I. RNA binding protein homology

Protein		Domain	RNA Binding Domain	
mRNP			RNP 2	RNP 1
			*	*
eIF-4B	human	1	TFLGNL .PYDVTEESIKEFFRGLN.ISAVRLPREPSNPERL	KGFGYAEF
PABA	yeast	1	LYVGDL .EPSVSKAHLYDIFSPI.GSVSSIRVCRDAITKTS.	LGYAYVNF
PABA	human	1	LYVGDL .HPDVTKAMLYEKFSPA.GPILSIRVCRDMITRRS.	LGYAYVHF
PABA	yeast	2	IFIKNL .HPDIDNKALYDTFSV.FGDILSSKIATDEN.GKS.	KGFGFVHF
PABA	human	2	IFIKNL .DKSIDNKALYDTFSA.FGNILSCKVVCDEN.G.S.	KGYGFVHF
PABA	yeast	3	LYVKNI NS.ETTDEQFQELFAK.FGPIVSASLEKDAD.GKL.	KGFGFVXY
PABA	human	3	FTNVYI .KNFGEDMDDERLKDL.FGPALSVKVMTDES.GKS.	KGFGFVSF
PABA	yeast	4	LFVKNL .DDSVDDEKLEEEFAP.YGTITSAKVMRTEN.GKS.	KGFGFVCF
PABA	human	4	LYVKNL .DDGIDDERLRKEFSP.FGTITSAKVM.MEG.GRS.	KGFGFVCF
snRNP				
U1A	human	-	IYINNL NEKIKKDE.LKKSLYAIFSQFGQILDILVSRSLKM.	RGQAFVIF
U1B	human	-	IYINNM NDKIKKEE.LKRSLYALFSQFGHVVDIVALKTMKM.	RGQAFVIF
U1 70K	human	-	LFVARV N.YDTTESKLRREFEV.YGPIKRIHMVYSKRSGKP.	RGYAFIEY
pre-rRNP				
Nucleolin		1	LFIGNL N.PNKSVAELKVAISEPFAKNDLA.VVDVRT.GTN.	RKFGYVDF
hamster		2	LLAKNL .SFNITEDELKEVFEDAL.EIRLVSQD.....GKS.	KGIAYIEF
		3	LVLSNL .SYSATEETLQEVFEKATF.IKVPQNQQ....GKS.	KGYAFIEF
		4	LFVKGL .SEDTTEETLKESFEG...SVRARIVTDRET.GSS.	KGFGFVDF
SSB1	yeast	-	IFIGNV .AHECTEDDLKQLFVEEFGDEVSVEIPIKE..SAN.	RGMAFVTF
hn-RNP				
A1	rat	1	LFIGGL .SFETTDESLRSHFEQ.WGTLTDCVVMRDPNTKRS.	RGFGFVTY
A1	drosphila	1	LFIGGL .DYRTTDENLKAHFEK.WGNIVDVVVMKDPRTKRS.	RGFGFITY
A1	rat	2	IFVGGI .KEDTEEHHLRDYFEQ.YGKIEVIEIMTDRGSGKK.	RGFAFVTF
A1	drosphila	2	LFVGAL .KDDHDEQSIRDYFQH.FGNIVDINIVIDKETGKK.	RGFAFVEF
C1	human	-	VFIGNL NTLVVKKSDVEAIFSK.YGKIV.....GCSVH.	KGFAFVQY
C1	xenopus	-	VFIGNL NTLVVKKTDVEAIFSK.YGKIV.....GCSVH.	KGFAFVQF
other				
La	human	-	VYIKGF PTDATLDD.IKE.WLEDKGQVLNIQMRRTLH..KAF	KGSIFVVF
Ro	human	-	LFALAI .CSQCSDISTKQA...AFKAVSEVCRIPT.....	HLVTFIQF
			LFVGNL E L F FG I K	KGFGFVXF
			IYIKG D Y R	R YA Y

The amino acid sequence homologies between the RNP-CS of eIF-4B are shown in comparison with sequences from the other RNP-CS containing proteins. The table shows only the peptide sequences between the highly conserved octamer (RNP-1) and hexamer (RNP-2) sequences; however, homologies of a lesser degree do extend beyond the region depicted. Sequence alignments were taken from Bandziulis *et al.* (1989). The location of phenylalanine residues within A1 hnRNP protein that crosslink RNA are shown by * (Merrill *et al.*, 1988). Not shown are the RNP-CS containing proteins that have been implied to be important in *Drosophila* development and have been reviewed recently (Rebagliati, 1989). Sequences were obtained as follows: hnRNP A1 from rat (Cobianchi *et al.*, 1986), human (Swanson *et al.*, 1987), *Xenopus* (Preugschat and Wold, 1988) and *Drosophila* (Haynes *et al.*, 1987); human hnRNP E(UP2) (Lahiri and Thomas, 1986); SSB1 from yeast (Jong *et al.*, 1987); polyadenylate binding protein (PABP) from human (Grange *et al.*, 1987), yeast (Adam *et al.*, 1986; Sachs *et al.*, 1986); human La (Chambers *et al.*, 1988), human Ro (Deutscher *et al.*, 1988); human snRNP A and B (Sillekens *et al.*, 1987); human snRNP U1 70K (Theissen *et al.*, 1986), hamster pre-rRNP nucleolin (Lapeyre *et al.*, 1987).

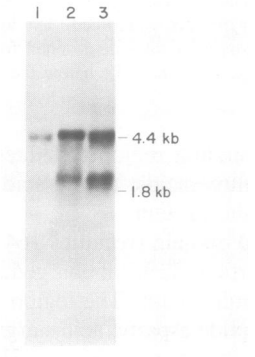

Fig. 4. Northern blot hybridizion analysis of eIF-4B mRNA. Northern blot analysis of RNA was performed with an eIF-4B cDNA probe as described in Materials and methods. Arrows indicate the migration of 18S and 28S rRNA. **Lane 1**, 10 μg of total 293 cell RNA; **lane 2**, 2.6 μg of poly(A)$^+$ RNA from colon carcinoma (LS-174) cells; **lane 3**, 2.6 μg of poly(A)$^+$ RNA from osteosarcoma (U2-OS) cells.

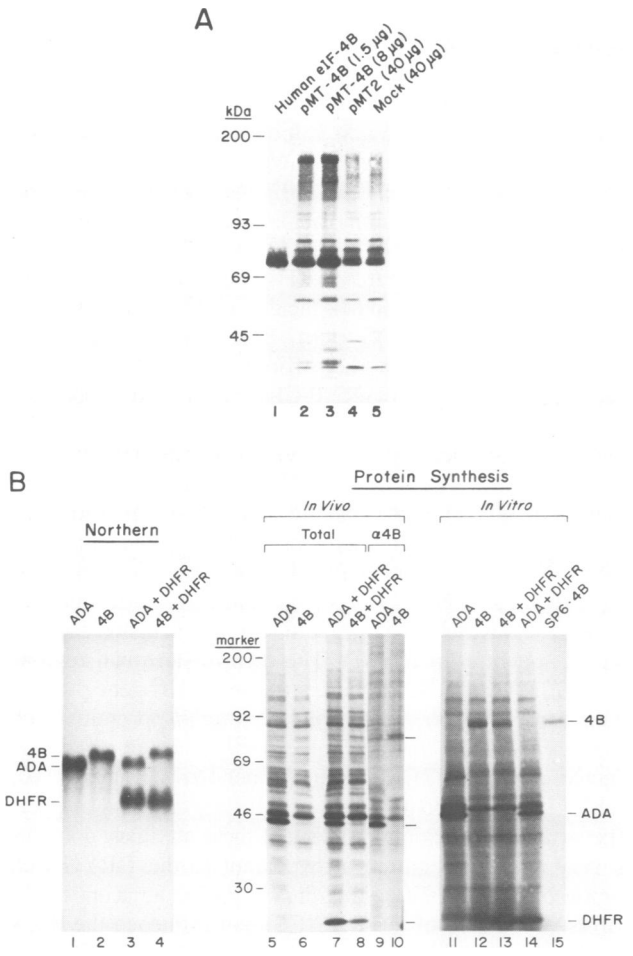

Fig. 5. Expression of eIF-4B in COS-1 cells. **Panel A**. The eIF-4B cDNA was introduced into the expression vector pMT-2 as described in Materials and methods. pMT-2 contains the SV40 enhancer and origin of replication for SV40 T antigen dependent replication in COS-1 cells. The transcription unit within pMT-2 utilizes the adenovirus major late promoter for transcription initiation, contains a small intron within the 5′ untranslated region, a dihydrofolate reductase coding region within the 3′ untranslated region and a polyadenylation signal derived from the SV40 early transcript (Kaufman *et al.*, 1989). COS-1 cells were transfected with the eIF-4B expression plasmid pMT-4B or the vector pMT-2. At 44 h post-transfection cells were harvested as described in Materials and methods for analysis by SDS−PAGE and Western immunoblotting procedures. Forty μg of protein from cells that did not receive DNA (**lane 5**) or that received pMT-2 vector alone (**lane 4**) was applied to polyacrylamide gel. To ensure linearity of the signal for eIF-4B, 8 μg (**lane 3**) and 1.5 μg (**lane 2**) of the pMT-4B COS-1 cell extract was added to 32 μg and 38.5 μg, respectively, of cell extract from COS-1 cells that did not receive DNA. **Lane 1** represents 320 ng of purified human eIF-4B. Mol. wt markers are indicated at the right. **Panel B**. COS-1 cells were transfected with either pMT-4B (4B) or pMT2-ADA (ADA) in the presence (**lanes 3, 4, 7, 8, 13** and **14**) or absence (**lanes 1, 2, 5, 6, 9−12**) of the DHFR expression plasmid pMT-2 (DHFR). At 48 h post-transfection, cells were pulse labeled with [^{35}S]methionine and cell extracts were prepared and analyzed by SDS−PAGE. Cell extracts were also immunoprecipitated with an anti-eIF-4B antiserum and the immunoprecipitates were analyzed by SDS−PAGE (**lanes 9** and **10**). RNA was isolated from parallel transfected plates and analyzed by Northern blot hybridization (**lanes 1−4**) to a DHFR probe which detects transcripts expressed from all vectors: pMT-2 and the two dicistronic vectors pMT-4B and pMT2-ADA. RNA was also analyzed by *in vitro* translation in reticulocyte lysates (**lanes 11−14**). Also shown are products from *in vitro* translation of SP6 derived eIF-4B mRNA (**lane 15**). Migration of DHFR, ADA and eIF-4B mRNA and polypeptides is indicated.

The synthesis of eIF-4B was monitored in transfected COS-1 cells by pulse labeling with [^{35}S]methionine at 48 h post-transfection, a time at which the steady state of the expressed eIF-4B has accumulated to high level. Cell extracts were prepared for analysis by SDS−PAGE and fluorography. A band migrating in the 80 kd region of the gel is faintly visible in cells which received pMT-4B (Figure 5B, lane 6). By comparison, COS-1 cells transfected with a control vector containing an adenosine deaminase (ADA) cDNA yielded a predominant band of ADA migrating at 45 kd (Figure 5B, lane 5). The ADA band intensity is ~25-fold greater than the eIF-4B band intensity. For a more sensitive detection of eIF-4B expression, the cell extracts were immunoprecipitated with a goat anti-human eIF-4B antibody. In extracts of cells transfected with pMT-4B a polypeptide migrating at 80 kd was detected which is not observed in control extracts from transfected cells (Figure 5B, compare lanes 9 and 10). These results show that at this 48 h time point, eIF-4B synthesis was very much reduced compared wtih the ADA control.

To identify reasons for the low eIF-4B synthesis, total cellular RNA was prepared from the transfected COS-1 cells and analyzed by Northern blot hybridization and *in vitro* translation. Northern blot hybridization identified an abundant mRNA migrating at the appropriate size expected for an mRNA transcribed from the expression plasmid. Its abundance is 2-fold less than that observed from the control plasmid expressing ADA (Figure 5B, lanes 1 and 2). *In vitro* translation of the COS-1 cell derived RNA in a reticulocyte lysate identified an abundant 80 kd polypeptide specific to the RNA from the pMT-4B transfected cells (Figure 5B, lane 12), which comigrated with an eIF-4B translation product derived from an SP6 transcribed RNA (Figure 5B, lane 15). Both translated eIF-4B polypeptides were immunoprecipitated by anti-human eIF-4B antibody (not shown). The amount of [^{35}S]methionine incorporation *in vitro* into eIF-4B was 5-fold less than incorporation into ADA (Figure 5B, compare lanes 11 and 12). The reduced incorporation into eIF-4B is attributed to a 2-fold lower level of eIF-4B mRNA in the transfected cells and differences in methionine content between ADA (Met = 9) and eIF-4B (Met = 3). These results demonstrate that both mRNAs are translated with roughly equal efficiencies *in vitro* and that eIF-4B exhibits a reduced efficiency of translation *in vivo* compared with ADA.

Whether the inefficient translation of eIF-4B was unique to that mRNA in the transfected cells was studied by a cotransfection experiment. The eIF-4B expression vector or the control ADA expression vector were each cotransfected with another marker gene encoding murine DHFR (pMT2) into COS-1 cells. At 48 h post-transfection cells were pulse labeled with [^{35}S]methionine and cell extracts were prepared and analyzed by SDS−PAGE. Analysis of *in vivo* labeled proteins shows that ADA and DHFR synthesis can readily be detected in the total cell lysates of control ADA transfected cells (Figure 5B, lane 7). In cells transfected with pMT-4B and pMT2, DHFR expression was reduced 4- to 5-fold and eIF-4B was not detectable above background (Figure 5B, lane 8). Quantification of RNA in these co-transfected cells by Northern blot analysis, by hybridization to a DHFR probe which detects all three mRNA species, demonstrates equal amounts of DHFR mRNAs in the two samples and similar amounts of eIF-4B and ADA mRNAs (Figure 5B, lanes 3 and 4). *In vitro* translation of isolated

RNA shows equal amounts of DHFR synthesis (lanes 13 and 14). Whereas eIF-4B is not detected by *in vivo* pulse labeling (lane 8), *in vitro* translation of the COS-1 cell RNA produces readily detectable synthesis of eIF-4B (lane 13). Quantification of these results shows that both eIF-4B and DHFR translation are reduced 4- to 5-fold in the eIF-4B cotransfected cells compared with the translation of DHFR in control ADA cotransfected cells or *in vitro* translation of RNA isolated from the eIF-4B cotransfected cells. These results suggest that accumulation of eIF-4B at this 48 h time point reduces the translation efficiency of the cell.

Discussion

Two cDNA clones encoding eukaryotic initiation factor 4B were isolated and characterized, and appear to be derived from two size classes of eIF-4B mRNA which were detected by Northern blot analysis of several human cell lines. Several findings support the view that the entire coding region of eIF-4B mRNA is represented in this reading frame. First, the ORF encodes all four eIF-4B peptide sequences (Figure 2) as well as numerous rabbit eIF-4B tryptic peptides (William C.Merrick, personal communication). Second, the AUG at nucleotides 20–22 possesses a consensus nucleotide sequence for a favorable translation initiation codon, with an A at position −3 and a G at position +4 (Kozak, 1987). Third, the amino acid composition of eIF-4B as determined by standard acid hydrolysis is in agreement with that of the predicted polypeptide (data not shown). Finally, the expression of the cDNA generates a polypeptide which comigrates with purified eIF-4B upon SDS–PAGE analysis and which is immunoprecipitated with anti-eIF-4B antiserum. This apparent equality of protein sizes strongly suggests that the AUG codon at nucleotides 20–22 is used for initiation of eIF-4B synthesis. However, until additional sequences upstream of the sequenced cDNA sequences are determined, it is not possible to rule out initiation from an AUG on eIF-4B mRNA upstream from the 5′ end of the cDNA isolated.

eIF-4B plays a crucial role in promoting the binding of ribosomes to mRNA, although its precise mode of action is not understood. The elucidation of the primary structure for eIF-4B facilitates our understanding of how eIF-4B may exert its action. One striking structural feature identified for eIF-4B is the presence of a consensus RNA binding site (RNP-CS) which is found in several different types of RNA binding proteins from phylogenetically diverse organisms. This binding domain has been observed in many proteins involved with RNA processing; however, this is the first example of the RNP-CS present in a translation initiation factor. The consensus octapeptide within the RNP-CS contains three aromatic residues that might be expected to interact with RNA by intercalating between bases whereas the nearby positively charged residues may facilitate binding by countering negative charges on the nucleic acid phosphate backbone as proposed by Prigodich *et al.* (1986). The RNA binding domain from rat A1 nuclear RNP crosslinks RNA via two phenylalanine residues; one in the conserved hexamer and the other in the conserved octamer (Table I) (Merrill *et al.*, 1988). eIF-4B binds RNA by UV induced crosslinking analysis (Pelletier and Sonenberg, 1985). Fluorescence spectroscopy measurements of RNA binding to eIF-4B show that the AUG triplet most effectively competes with poly(A) binding (Goss *et al.*, 1987). These data suggest that eIF-4B may exhibit preferential binding to a specific RNA sequence. The RNP-CS domains of poly(A) binding protein and U1 RNP have been shown to bind single-stranded nucleic acids *in vitro* (Sachs *et al.*, 1987; Query *et al.*, 1989), although there is little data to suggest that the RNP-CS domain may exhibit sequence specific RNA binding (Bandziulis *et al.*, 1989; Mattaj, 1989). Expression of specific mutants of eIF-4B will enable us to identify the functionally important amino acids mediating RNA binding for eIF-4B.

Another unusual feature of eIF-4B is a region consisting of a high content of aspartic acid, arginine, tyrosine and glycine residues (DRYG rich). Since intact hnRNP A1 exhibits a cooperative higher affinity RNA binding than a fragment consisting of the RNP-CS domain itself (Cobianchi *et al.*, 1988), it is thought that other portions of hnRNP A1 contribute significantly to the overall binding energy. The carboxy-terminal region of hnRNP A1 contains a very high content of glycine and synthetic peptides of this sequence content exhibit poly(A) binding properties (Cobianchi *et al.*, 1988). Nucleolin, another member of the class of proteins that contain an RNP-CS, has a domain carboxy-terminal to the RNP-CS which is very rich in glutamic acid and aspartic acid (Lapeyre *et al.*, 1987). The carboxy-terminal regions of the 70K U1 snRNP protein, a murine MHC gene product (Levi-Strauss *et al.*, 1988), and *Drosophila* proteins such as *suppressor of white apricot* [*su(w^a)* (Chou *et al.*, 1987; Zachar *et al.*, 1987)] and transformer [*tra*, (Boggs *et al.*, 1987)] contain repetitions of arg-glu, arg-asp and arg-ser. The structurally flexible property of these auxiliary domains is very similar in amino acid content to the DRYG rich region of eIF-4B. These auxiliary domains adjacent to the carboxy-terminus of the RNP-CS may influence the RNA binding by interacting with other proteins and/or RNAs.

The abundance of eIF-4B in HeLa cells has been estimated to be ~0.3 molecules/ribosome (Duncan and Hershey, 1983). To determine if eIF-4B is potentially a limiting factor in regulating translation, we studied the effect of eIF-4B overexpression. Fifty-fold overexpression of the cloned eIF-4B cDNA resulted in a 5-fold reduction in translation of both the eIF-4B mRNA and a DHFR mRNA derived from a cotransfected plasmid. Thus, the overexpression of eIF-4B inhibited translation in transfected cells. Further studies are required to demonstrate that the expressed eIF-4B is functional and to identify the reason(s) for the reduced translation. It is interesting to speculate that eIF-4B as a single molecular or homodimer binds simultaneously with both mRNA and the 40S ribosomal subunit, but that upon overexpression these sites are saturated with separate eIF-4B molecules. Alternatively, overexpression of the eIF-4B may alter the phosphorylation status of eIF-4B by saturating activities of a protein kinase. Experiments are presently in progress to determine how the overexpression of eIF-4B inhibits translation.

Materials and methods

Materials

Reagents were purchased from the following suppliers: HPLC grade trifluoroacetic acid and acetonitrile, from Pierce Chemical Co.; protein sequencing grade chymotrypsin, from Calbiochem; λ ZAP, from Stratagene. Anti-eIF-4B was raised in goats immunized with human eIF-4B (Meyer *et al.*, 1982). All other chemicals were reagent grade.

Purification and sequencing of eIF-4B peptides

eIF-4B was purified from HeLa cells through the sucrose density gradient step as described (Brown-Luedi *et al.*, 1982). Gradient fractions containing

eIF-4B were pooled and protein was precipitated with ammonium sulfate, resuspended and dialyzed against 100 mM KCl in buffer H [20 mM HEPES, pH 7.5, 7 mM β-mercaptoethanol, 0.2 mM EDTA, 10% glycerol and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)]. Two batches of 105 mg protein each were fractionated by FPLC on a Mono Q (10/10) column eluted with a linear gradient of 100–600 mM KCl in buffer H. eIF-4B eluted at 270–320 mM KCl and pooled fractions (9 mg protein) were diluted to 75 mM KCl with buffer H. Further fractionation was achieved with a Mono S column eluted with a 75–425 mM KCl gradient in buffer H. eIF-4B eluted at 110–140 mM KCl and was >98% pure based on SDS–PAGE and Coomassie blue staining.

eIF-4B (200 μg; 2.5 nmol) was treated with 0.1 μg chymotrypsin for 90 min at 37°C. The resulting peptides were separated by reverse phase HPLC on a Vydac C-4 column by eluting with a linear 0–45% acetonitrile gradient in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Fractions containing well separated peptides were pooled and lyophilized. Amino acid sequences were determined by gas phase microsequencing as described (Hewick *et al.*, 1981).

Cloning procedures

A 32 base oligonucleotide complementary to the mRNA encoding the last 11 amino acids of peptide 2 (Figure 1) was synthesized to contain nucleotides at degenerate positions based on the empirical rules of Lathe (1985). This 'guessmer', probe 1, was labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP to 8 × 10^6 c.p.m./pmol and used to screen a λ Charon 21A human fetal liver cDNA library (Toole *et al.*, 1984). 250 000 recombinants were screened at 12 000 plaques/150 mm plate by hybridizing to duplicate nitrocellulose filters at 45°C for 24 h with 1 × 10^6 c.p.m./ml probe in 5 × SSC (1 × = 150 mM sodium chloride, 15 mM sodium citrate), 5 × Denhardt's reagent (Denhardt, 1966), 0.1% SDS and 100 μg/ml of sonicated salmon sperm DNA. Filters were washed with 5 × SSC, 0.1% SDS at 45°C and exposed to film. Plaques positive on both filters were picked for secondary screening by the phage amplification procedure (Woo, 1979) with two degenerate 17mer oligonucleotide probes. Probes were based on overlapping portions of the amino acid sequence from peptide 2: DDYSRD [probe 2: GA(C/T)GA(C/T)TA(C/T)TCNCGNGA] and YSRDDY [probe 3: TA(C/T)TCNCGNGA(C/T)GA(C/T)TA]. Each probe was hybridized at 50°C for 24 h in 3 M tetramethylammonium chloride (TMAC) (Wood *et al.*, 1985), 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, 5 × Denhardt's reagent, 0.6% SDS and 100 μg/ml salmon sperm DNA. Filters were washed in 3 M TMAC, 50 mM Tris–HCl, pH 8.0, at 50°C for 3 h and exposed to film. A recombinant phage that hybridized to both probes 2 and 3 was plaque purified. Phage DNA was isolated, digested with *Sau*3A, and the fragments were subcloned into M13 vectors and transformed into *Escherichia coli* TG1. Phage hybridizing with both probes 2 and 3 were isolated and their inserts were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). One of the insert sequences yielded a deduced amino acid sequence that matched the 17 amino acid peptide 2 (Figure 1) except that the Cys residue of the peptide was Arg in the DNA. Excision of the insert with *Eco*RI yielded three fragments of 0.2, 0.5 and 1.8 kb, apparently due to incomplete methylation of insert cDNA when the Charon 21A library was constructed (Toole *et al.*, 1984). The entire 0.5 kb *Eco*RI fragment and parts of the 1.8 kb fragment were sequenced and were found to encode peptides 1 and 2, respectively (Figure 1).

A full-length clone was isolated by screening an oligo(dT) primed human osteosarcoma (U2-OS) cell cDNA library constructed in λ ZAP (Stratagene) with the 0.5 and 1.8 kb *Eco*RI fragments as probes. 175 000 λ ZAP recombinants were hybridized at 68°C for 18 h in 5 × SSC, 5 × Denhardt's reagent, 0.1% SDS, 100 μg/ml sonicated salmon sperm DNA and 1 × 10^6 c.p.m./ml nick-translated probe. The phage from four of the 24 positive plaques observed were purified and characterized.

DNA sequencing

The 3.9 kb eIF-4B cDNA clone was digested with *Sma*I and a *Kpn*I linker was ligated to the 5′ end. The DNA was digested with *Kpn*I to isolate a 3.9 kb fragment which was subcloned into pGEM-3 for DNA sequencing. The DNA sequence of the 3.9 kb insert was determined for one strand using a Bal31 nuclease deletion series protocol (Poncz *et al.*, 1982) with subsequent subcloning into appropriate M13 vectors (Messing and Vieira, 1982). DNA sequence on the opposite strand was determined by synthesizing 17mer oligonucleotide primers for sequencing directly the 3.9 kb insert in M13. DNA was sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977).

Blot hybridizations

Total RNA was extracted from human 293 cells (Chirgwin *et al.*, 1979). RNA from human osteosarcoma (U2-OS) and colon carcinoma (LS-174) cells was extracted and poly(A)$^+$ RNA isolated by oligo(dT) cellulose chromatography as described (Toole *et al.*, 1984). The RNA was fractionated by electrophoresis on a 1% agarose formaldehyde gel and transferred to nitrocellulose (Derman *et al.*, 1981). The filters were hybridized in 2 × SSC, 5 × Denhardt's reagent, 0.1% SDS, 100 μg/ml sonicated salmon sperm DNA with nick-translated 1.8 kb *Eco*RI fragment of eIF-4B cDNA. After 15 h at 68°C the filters were washed in 0.2 × SSC, 0.1% SDS for 2 h at 68°C and exposed to X-ray film with intensifying screens at −70°C for 18 h or longer.

In vitro translation

SP6 transcription reactions using pGEM-4B and reticulocyte lysate translation reactions (Promega Biotech) were performed as described by the supplier. Translation reactions were immunoprecipitated with a goat anti-human eIF-4B antiserum (Meyer *et al.*, 1982) using rabbit anti-goat antiserum and protein A–Sepharose for immunoadsorption. Aliquots from the translation reactions were electrophoresed on an SDS–10% polyacrylamide gel before and after immunoprecipitation.

Expression of eIF-4B in transfected COS-1 cells

The eIF-4B coding region was excised from pGEM3-4B by digestion with *Pst*I. This liberates a 2.0 kb fragment which contains the pGEM3 polylinker sequences (*Pst*I site to the *Kpn*I site) at the 5′ end and 170 bases of 3′ non-coding sequences from the eIF-4B cDNA extending to the *Pst*I site at residue 2020 within the 3′ end. The *Pst*I fragment was cloned into the *Pst*I site of the expression vector pMT2 (Kaufman *et al.*, 1989) to generate pMT-4B harboring the eIF-4B sequence in the correct orientation for transcription from the adenovirus major late promoter. DNA was transfected into COS-1 cells by the DEAE–dextran method (Kaufman *et al.*, 1989). Under these conditions 15–20% of the cells express the transfected DNA (Kaufman *et al.*, 1989).

Steady state analysis of eIF-4B levels was performed by Western immunoblotting procedures. At 44 h post-transfection, cells were harvested by lysis in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% NP-40, 1% SDS and 1 mM PMSF. 40 μg of protein was applied to an 8% SDS–polyacrylamide gel. After electrophoresis the proteins were electroblotted onto nitrocellulose and the filter was then incubated in blocking buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.075% Tween 20 and 0.005% sodium azide) with addition of 1% skimmed milk for 1 h at room temperature. The filter was then incubated overnight in blocking buffer with goat anti-human eIF-4B (1:400 dilution). The filter was washed four times in blocking buffer and then incubated with 1:7500 dilution of rabbit anti-goat alkaline phosphatase conjugate (Sigma Chemical Co., St Louis, MO) into blocking buffer for 1 h. The filter was then washed three times in blocking buffer and then once more in 100 mM Tris–HCl, pH 8.0, 100 mM NaCl and 5 mM $MgCl_2$. Bands were developed in the same solution by addition of NBT and BCIP reagents as recommended by the supplier (Protoblot System, Promega, Madison, WI).

For measurement of protein synthesis, at 48 h post-transfection cells were pulse labeled for 20 min with [^{35}S]methionine (New England Nuclear Corp., MA) and total cell lysates were prepared by lysis in radioimmunoprecipitation buffer (RIPA, Kaufman and Sharp, 1982). Immunoprecipitation of the cell extracts was performed with eIF-4B antibody as described above and aliquots of the total cell extract and immunoprecipitates were electrophoresed on SDS–10% polyacrylamide gels (Laemmli, 1970). Quantification of band intensity was performed by visual comparison of autoradiograms of different exposure times.

From parallel plates of transfected COS-1 cells, total cellular RNA was isolated at 48 h post-transfection as described (Chirgwin *et al.*, 1979). RNA was analyzed by Northern blot hybridization (Derman *et al.*, 1981) to either eIF-4B or DHFR specific ^{32}P-labeled probes synthesized using [α-^{32}P]dCTP (Amersham), random primers and Klenow fragment of DNA polymerase I as described by the supplier (Pharmacia LKB Biotech). Total RNA was translated in rabbit reticulocyte lysates as described above.

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