CLONING AND SEQUENCING OF A cDNA

ENCODING eIF-2Bε

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Translational control is exerted predominantly at the initiation step in eukaryotic cells. Regulatory control in these cells is mediated by specific alterations in the activities of key initiation factors (1, 2, 3, 4). The best characterized regulatory process involve the three subunits (α = 38 kDa, β = 36 kDa, γ = 56 kDa) of eukaryotic initiation factor-2 (eIF-2). eIF-2 is the initiation factor involved in the formation of a ternary complex that includes met-tRNA$_i$, eIF-2 and GTP (5, 6). This ternary complex facilitates the positioning of the met-tRNA$_i$ on the 40S ribosomal subunit to form the 43S initiation complex (7, 8). Once formed, the 43S initiation complex binds to eukaryotic mRNA complexes containing eIF-4F, eIF-4A, and eIF-4B in a ATP dependent reaction and scans to the first AUG sequence to form the 48S initiation complex. eIF-5 catalyzes the hydrolysis of eIF-2 bound GTP, causing the release of eIFs from the 48S initiation complex and the binding of the 48S initiation complex to the 60S ribosomal subunit to form the 80S initiation complex. The formation of this 80S initiation complex completes eukaryotic translational initiation (9, 10). Ternary complex formation is believed to be the committed step in eukaryotic translational initiation, so regulation of its formation is expected to

play a key role in the overall control of protein synthesis. Such appears to be the case and central to this control is the phosphorylation of the α-subunit of eIF-2 by specific kinases (4, 11).

This regulation has been most extensively studied in the reticulocyte lysate system, which contains two kinases specific for the α-subunit of eIF-2 (eIF-2α). The heme-regulated eIF-2α kinase (HRI) becomes activated in response to hemin deficiency, addition of oxidized glutathione, heavy metals, and heat shock (11, 12, 13, 14). The double-stranded RNA activated inhibitor (DAI) is activated by low concentrations of double-stranded RNA (15, 16). Activation of either of these kinases results in the phosphorylation of eIF-2α, followed by distintegration of polysomes and termination of protein synthesis. Termination of protein synthesis is caused by the inability of the lysate to regenerate functional eIF-2.

Upon joining of the 48S initiation complex with the 60S ribosomal subunit to form the 80S initiation complex, GTP is hydrolysed with subsequent release of eIF-2 in a binary complex with GDP (10, 17). eIF-2/GDP is nonfunctional in translational initiation, so the reutilization of eIF-2 in subsequent rounds of initiation requires replacement of bound GDP with GTP. At physiological concentration of Mg^{2+}, eIF-2 has a 400 fold higher affinity for GDP than GTP and the GDP is bound so tightly that it effectively does not dissociate from eIF-2 (18). The exchange of GTP for GDP thus requires the participation of another initiation factor.

The factor with this exchange activity is the five-subunit (α = 26 kDa, β = 40 kDa, γ = 58 kDa, δ = 68 kDa, ε = 82 kDa) eukaryotic initiation factor-2B (eIF-2B),

also known as reversing factor (RF) or guanine nucleotide exchange factor (GEF) (19, 20, 21). The termination of protein synthesis upon phosphorylation of eIF-2α is due to the loss of eIF-2B activity. The loss of eIF-2B exchange activity most likely is the result of formation of a stable nonfunctional complex of eIF-2B with phosphorylated eIF-2 (22). Since eIF-2 is about 15 fold more abundant than eIF-2B in reticulocyte lysate, formation of this nonfunctional complex results in sequesteration of the low abundance eIF-2B and a termination of protein synthesis. Although regulation of protein synthesis has been extensively documented in reticulocyte lysates, analogous studies with nonerythoid cells indicates that this may be a general mechanism by which eIF-2 phosphorylation (via alteration of eIF-2B activity) regulates protein synthesis in all eukaryotic cells.

While a regulatory role for eIF-2B in protein synthesis has been established, its reaction mechanism, mode of interaction with eIF-2 and the specific roles of its subunits remains largely unknown. Indeed the cellular concentration of eIF-2B, upon which the current hypothesis of the control of protein synthesis by eIF-2 phosphorylation is based, remains to be conclusively established. In addition, the role of the phosphorylation of the 82 kDa subunit of eIF-2B (eIF-2Bε) in the control of eIF-2B activity remains to be determined. In order to begin to dissect the structure and function of eIF-2B, the present study is designed to clone and sequence the complementary DNA (cDNA) to its 82 kDa subunit. Information obtained from the cDNA sequence will provide the amino acid sequence of this subunit, thus providing a means of generating antibodies against it. The availability of such

antibodies will enable the characterization of the cellular concentration of eIF-2B and the role of phosphorylation of eIF-2Bε in the control of eIF-2B activity.

CHAPTER II

LITERATURE REVIEW

Properties of eIF-2B

eIF-2B is a nonribosomal associated initiation factor that plays a key regulatory role in eukaryotic protein synthesis. It has been purified from different sources to apparent homogenity either as complex with eIF-2 or as free eIF-2B, as judged by SDS polacryamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue or silver staining (19, 23, 24, 25, 26). From all sources, eIF-2B preparations show similar subunit composition. Slight variations in the molecular weight of the subunits of eIF-2B are observed in some cases. All five subunits appear to be present in equal molar amounts, although the 58 kDa subunit appears to be less abundant. The difference in the stoichemistry of the 58 kDa subunit has been attributed to its susceptibilty to proteolysis. Purified eIF-2B has an apparent molecular weight of approximately 250 kDa as determined by its sedimentation rate during centrifigation in sucrose density gradient. eIF-2B forms a tight complex of about 450 kDa with eIF-2 that is dissociable by high salt concentration (19). Although nonribosomal associated, eIF-2B has been shown to interact transciently with the 60S ribosomal subunit. It has been proposed that eIF-2B mediates the

guanine nucleotide exchange reaction with eIF-2 on this ribosomal subunit (21, 22). eIF-2B has also been shown to be a phosphoprotein, phosporylated on its 82 and 67 kDa subunits. Phosphorylation on the 82 kDa subunit is mediated by casein kinase II (27) while the 67 kDa subunit is phosphorylated by a mechanism which is predicted to involve autophosphorylation (6). Using photoaffinity labeling with 8-azidoadenosine-5-phosphate Dholakia, et al. (28), have demonstrated that the 58 and 67 kDa subunits of eIF-2B binds ATP. The binding of ATP to these subunits appears consistent with autophosphorylation. This proposition, however, remains speculative and requires more detailed analysis. In addition to labeling with 8-azido ATP, eIF-2B is also labeled with 8-azidoguanine-5-phosphate on its 40 kDa subunit indicating that it may also be a guanine nucleotide binding protein. At high concentrations, eIF-2B also binds 2 moles of NADPH per molecule (23). The interaction of eIF-2B with NADPH is stablized by the presence of DTT and glycerol, but the full implication of this association with respect to its activity in vivo has not been demonstrated.

Regulation of eIF-2B Activity

The primary means by which eIF-2B activity is regulated is by phosphorylation of eIF-2α (11). This phosphorylation results in the formation of a tight complex with eIF-2B that sediments with a sedimentation coefficient of 15S on glycerol gradient centrifugation (22). GDP is freely dissociable from this complex, but the eIF-2B in this complex is unavailable to catalyze the exchange reaction, because it

cannot freely dissociate from phosphorylated eIF-2. The k_i of eIF-2(αP) for eIF-2B is approximately 0.29 nm, so this inhibition is essentially irreversible and eIF-2B is effectively sequestered (41). eIF-2B can be released from this complex with consequent restoration of protein synthesis by phosphatase treatment, but it is unclear which cellular phosphatase may catalyse the reaction (22).

Since the sequesteration of eIF-2B in a complex with eIF-2(αP) and subsequent inhibition of its activity results in termination of protein synthesis, the inhibition of protein synthesis observed when eIF-2 is phosphorylated is only physiologically relevant, if the cellular concentration of eIF-2 exceeds that of eIF-2B. Indeed in reticulocyte lysates, eIF-2 is estimated to be 15 fold more abundant than eIF-2B and the phosphorylation of only 30% of the eIF-2 results in almost a 90% reduction in protein synthesis (20, 29, 30). In Ehrlich ascites tumor cells, the ratio of eIF-2 to eIF-2B is approximately two to one and phosphorylation of 50% of eIF-2 results in a considerable reduction in protein synthesis (31). In T lympocytes the levels of eIF-2B and eIF-2(αP) are also similar, but upon activation by mitogens, the observed increase in protein synthesis coincides with an increase in the synthesis/activity of eIF-2B with no corresponding increase in phosphorylation of eIF-2 (34). Thus, although the cellular concentrations of eIF-2B in other cell types has yet to be determined, it seems likely that differences in the relative abundance of eIF-2 and eIF-2B may be common to all cells.

As a major regulatory protein in protein synthesis, the activity of eIF-2B is also regulated by cellular nutrients, redox state and growth requirements of the cell. The

first indication of the regulatory influence of a cellular nutrient came from the work of Ersnt, et al (12). Using gel filtered reticulocyte lysates, they demonstrated that glucose-6-phosphate prevents or reverses the inhibition of protein synthesis due to activation of HRI by hemin deficiency and oxidized glutathione. Glucose-6-phosphate (G-6-P) is also required as a stimulatory factor for initiation, an effect which is independent of its role as a metabolite. This effect of G-6-P has been proposed to be exerted on the activity of eIF-2B (35). It is postulated that G-6-P may act as an effector that directly stimulates eIF-2B activity. This may account for the observed ability of insulin to stimulate eIF-2B activity in rat fast twitch fibre (36). In these cells, insulin deficiency does not lead to an increase in eIF-2α phosphorylation (71), but the activity of eIF-2B is reduced up to 80%. It is possible that during insulin deficiency, the limited availability of G-6-P leads to a reduction in eIF-2B activity.

G-6-P may also have indirect effects on eIF-2B activity, since it is required for NADPH production. Wabha et al (23) have recently demonstrated that eIF-2B binds NADPH and that this association stimulates eIF-2B activity. Consistent with a role for NADPH in eIF-2B activation is the observed increase in eIF-2B activity upon fertilization of sea urchin eggs; this process is accompanied by an increase in the concentration of NADPH (33). Although the exact role of NADPH in regulating eIF-2B activity is not known, it has been suggested that it may be required for the maintenance of its structural integrity, since eIF-2B preparations retain their activity until subjected to repeated freezing and thawing or prolonged storage (25).

The activity of eIF-2B is also modulated by the availability of amino acids. In reticulocyte lysate, amino acid deficiency leads to a 50% reduction in the activity of eIF-2B, but no change in eIF-2α phosphorylation is observed (37). The corresponding reduction in protein synthesis is due to a decrease in polypeptide chain elongation. In nonerythoid cells, however, amino acid deficiency leads to the inhibition of protein synthesis initiation via increased phosphorylation of eIF-2α and a consequent reduction in eIF-2B activity. Chinese Hamster ovary cells with a temperature sensitive leucyl-tRNA synthetase activity mimic the effect of amino acid deficiency at non permissive temperatures (39, 40). It is possible that the mutation in leucyl-tRNA synthetase leads to accumulation of uncharged $tRNA_{leu}$, which may activate a kinase that phosphorylates eIF-2α. The inhibition of protein synthesis initiation that results from amino acid deficiency would then be analogous to GCN4 regulation in yeast where accumulation of uncharged $tRNA_{his}$ has been proposed to be the signal which activates the GCN2 kinase (52).

Polyamines also regulate the activity of eIF-2B (37). Their depletion in reticulocyte lysate, by gel filtration results in a 35% reduction in the activity of eIF-2B and a corresponding 70% reduction in the rate of protein synthesis. The reduction in protein synthesis is due to inhibition of initiation of protein synthesis, resulting from a reduction in the activity of eIF-2B. The precise mechanism by which polyamines mediate their effect on eIF-2B activity is unclear. One possible explanation is that polyamines regulate casein kinase II (42) and this in turn alters the phosphorylation state of eIF-2B. Dholakia and Wahba (27) have demonstrated

in a recent study that phosphorylation of eIF-2Bε by casein kinase II results in a 2.5 fold increase in eIF-2B activity, and that dephosphorylation of the subunit by treatment with alkaline phosphatase results in a five fold reduction of its activity. The five-fold reduction in eIF-2B activity on dephosphorylation does not, however, account for the 35% reduction in the activity of eIF-2B upon depletion of polyamines.

Mechanism of eIF-2B Catalyzed Guanine Nucleotide Exchange Reaction

While the role of eIF-2B in the control of protein synthesis in eukaryotic cells is generally agreed on, the mechanism of the reaction it catalyzes remains controversial. eIF-2B catalyzes the two substrate reaction represented below, so its reaction is

eIF-2/GDP + GTP --------> eIF/GTP + GDP

expected to follow either sequential or a substituted (ping pong) enzyme mechanism. Both mechanisms have been proposed for this reaction by different groups. Using measurements of the fluroscence anisotropy effect of eIF-2B's interaction with dansylated eIF-2, and a filtration assay which measures the binding of radiolabeled GDP or GTP to eIF-2, Goss *et. al.* (43) proposed a substituted mechanism for the eIF-2B catalyzed reaction. In their propsed mechanism, eIF-2B binds to the binary complex eIF-2/GDP to form eIF-2/GDP/eIF-2B. GTP then displaces eIF-2B and GDP from this ternary complex with the formation of eIF-2/GTP. In another study, Rowland et al, (41) used results of the kinetic analysis of the eIF-2B reaction to also

propose a ping pong mechanism. In their proposed mechanism, the binding of eIF-2B to eIF/GDP causes the displacement of the bound GDP to form a eIF-2/eIF-2B complex. GTP then displaces free eIF-2B from this complex regenerating the functional eIF-2. This mechanism differs slightly from the mechanism of Ochoa *et. al.* (44), who proposed that following the formation of the eIF-2/eIF-2B/GTP complex, met-tRNA$_i$ binds to form a quaternary complex. Free eIF-2B is then released upon binding of the quaternary complex to the 40S rRNA. Recently, Dholakia *et. al.* (45) have demonstrated that eIF-2B is a G-binding protein, and this finding has lead them to propose a sequential mechanism for the eIF-2B catalyzed reaction. In the sequential model, the substates eIF-2/GDP and GTP bind to eIF-2B to form a quaternary complex. The exchange reaction by implication then takes place in this complex with the release of the products eIF-2/GTP and GDP. Their study did not demonstrate however whether the eIF-2B exchange reaction followed an ordered or a random sequential mechanism.

eIF-2B in Yeast

The development of a cell free translation system from reticulocyte lysates has facilitated the detailed study of protein synthesis in mammalian cells. Recently, an in vitro translation system has been developed from the yeast Saccharomyces cerevisae (46). The availability of this system, coupled with the ability to perform genetic manipulation is beginning to yield information on the regulation of protein synthesis in lower eukaryotes (47). Similar to that found in the mammalian system,

protein synthesis in yeast is also regulated at the initiation step. Much of the supporting information comes from the study of the control of amino acid biosynthesis in yeast (48). In yeast, amino acid deficiency leads to the activation of several genes encoding the enzymes required for amino acid biosynthesis (49). The activation of these genes is mediated by the transcription activator GCN4. When yeast cells have adequate supply of amino acid the synthesis of GCN4 is repressed, but amino acid starvation results in a derepression of GCN4 synthesis.

Regulation of GCN4 is exerted at the translational level. Studies in yeast strains with defects in GCN4 regulation indicate that this control is mediated at the initiation step by modulation of the interactions between some initiation factors and regulatory elements in the 5′ leader sequence of the GCN4 mRNA (48, 50, 51, 55). Similar to the regulation of translation in mammalian system, eIF-2 appears to play a major role in the regulation of GCN4 expression and yeast protein synthesis in general. Genetic studies have indicated that the α and β subunits of yeast eIF-2, are negative regulators of GCN4. In addition, GCN2 a positive regulator of GCN4, has been shown to be a protein kinase, specific for eIF-2α. The GCN2 protein has extensive sequence similarity to mammalian DAI and HRI (72). The N-terminal domain of GCN2 has significant homology to his-tRNA aminoacyl synthetase. It has been proposed that amino acid starvation leads to accumulation of uncharged $tRNA_{his}$, which binds to GCN2 and activates its kinase activity. Based on these findings, Dever *et. al.* (54), have proposed that the derepression of GCN4 upon amino acid starvation results from increased phosphorylation of eIF-2α by GCN2.

The increase in phosphorylation is believed to impair the assembly of functional initiating ribosomes, which leads to a reduced level of reinitiation at upstream AUG codons in the regulatory leader sequence, thus facilitating initiation at the proper GCN4 AUG start site.

In mammalian cells, phosphorylation of eIF-2α impairs the exchange reaction catalyzed by eIF-2B, so eIF-2B is expected to play a role in this eIF-2 mediated control of GCN4 expression and the overall regulation of yeast protein synthesis. Although the yeast equivalent of eIF-2B has not been isolated, available information indicates that yeast GCD genes and GCN3 may code for the subunits of eIF-2B. The GCD genes are negative regulators of GCN4 expression and deletion of any of the genes leads to unconditional lethality in yeast, suggesting an essential role (56, 57). The essential role of the GCD gene products appears to involve their requirement for translational initiation, since temperature sensitive mutants of these genes show a depletion of the 43S initiation complex and polyribsome disintegration, two conditions characteristic of defects in translational initiation (58).

Biochemical studies also support a role for these genes in protein synthesis initiation and indicate that their gene products may be the yeast analog of eIF-2B. Cigan *et. al.* (59) haved shown that antibodies directed against GCD1 or GCD2 co-immunoprecipitate GCD1, GCD2 and GCN3 suggesting that the three proteins are associated with each other. Furthermore, these proteins coelute from a Sephacryl S-300, and much like the mammalian eIF-2/eIF-2B complex, they co-sediment with

eIF-2 as a high molecular weight complex of approximately 600 kDa during sucrose density gradient centrifugation. eIF-2 can be dissassociated from the complex in the presence of high salt, but GCD1, GCD2, and GCN3 still co-sediment together, further indicating that they are part of the same complex.

Other Functions of eIF-2B

Although the exchange activity of eIF-2B is its most extensively studied function, available data suggests that eIF-2B may have additional functions. eIF-2B has been shown to bind ATP, however, the binding of this nucleotide has not been found to have any functional effect on the exchange activity of eIF-2B. Thus, in addition to a potential regulatory role, this nucleotide may be involved in an unidentified function of eIF-2B. In a recent study, Van Heugter *et. al.* (60), have shown that eIF-2/eIF-2B binds to mRNA cap via the β and α subunits of eIF-2. The efficiency of this interaction is greatly reduced in the absence of eIF-2B, suggesting that it plays a role in the cap binding. Moreover, an unidentified 40 kDa protein also crosslinked to the cap, this protein is probably the β subunit of eIF-2B, since eIF-2 does not have a subunit of this molecular weight. Further studies on the fate of this protein in the absence of eIF-2B in the crosslinking reaction will be required to determine if eIF-2B plays a direct role in cap binding of the complex.

CHAPTER III

MATERIALS AND METHODS

Materials

The synthetic oligodeoxyribonucleotide primers used for sequencing were synthesized by Oligos *et. al.* and National Bioscience. Sequencing reactions were performed with reagents in the Sequenase sequencing kit from United States Biochemical Corporation (USB, Cleveland Ohio). Sequencing gels were cast with USB ultrapure reagents. The radioactive materials [α^{32}P] CTP, [α^{35}S] ATP (sequencing grade), and ^{35}S-methionine were purchased from Dupont/New England Nuclear. The n-(4-azido-2-nitrophenyl)-n-(3-biotinyl-aminopropyl)-n-methyl-1, 3-propanediamine (photobiotin) used for labeling the probe for nonradioactive hybridization was from Sigma. The photogene nucleic acid detection kit and biotinylated lambda DNA (Hind III fragments) were from GIBCOBRL (Gaithersburg, MD). Nitrocellulose membranes (150mm and 100mm circles) used for antibody screening and Western blotting were obtained from Millipore. Photogene Nylon membrane used for Northern blotting and nucleotide screening were purchased from GIBCOBRL. The restriction enzyme PpuMI was obtained from United States Biochemicals and all other enzymes were from GIBCOBRL or

Promega. The affinity purified alkaline phosphatase linked Rabbit anti-chicken IgG was purchased from USB. Rabbit reticulocytes used for RNA isolation was from Green Hectares (Oregon, WI). The nuclease free reticulocyte lysate used for in vitro translation was purchased from Ambion Inc (Austin, TX). The *E. coli* strain XL-1-Blue was from Stratagene (LaJolla, California). All other reagents were molecular biology reagent grade.

Methods

Isolation of the cDNA Encoding eIF-2Bε

The cDNA encoding eIF-2Bε was isolated by screening a rabbit reticulocyte lysate λ-ZAP library, supplied by Dr. J. J. Chen, (M. I. T) with a polyclonal antibody raised against all the five subunits of eIF-2B. 337,500 recombinant phage were grown on a lawn of XL-1-Blue for 3.5 hours at 42°C at a density of 37,500 pfu per 150 mm plate. Plates were overlayed with 120 mm nitrocelluse filters previously saturated with 10 mM IPTG and incubation was continued for an additional 4 hours at 37°C. Because the rabbit anti-chicken IgG cross reacts with a 26 kDa protein in reticulocyte lysates, a second filter was overlayed on the plates and incubated for an additional 3.5 hours. Next nitrocelluse filters were incubated with a blocking solution containing 10% calf fetal serum in TBS (50 mM Tris/HCl (pH 7.5), 150 mM NaCl) for two hours. Following incubation with the blocking solution, one set of the filters was incubated overnight at 4°C with a 100 fold

dilution of the eIF-2B antibody preabsorbed with *E. coli*/phage extract. The filters were washed with TBST (TBS, 0.05% Tween 20) three times at room temperature. A replicate set of filters were left overnight in the blocking solution and then washed three times with TBST. Next the filters were incubated with a 4000 fold dilution of alkaline phosphatase-linked Rabbit anti-chicken IgG (15 ml/filter) for one hour at room temperature. Positive clones were detected by incubation with 10 μg NBT and 167 μg BCIP in a 30 ml buffer containing 100 mM Tris/HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$ at room temperature for approximately 5 minutes. Clones with positive signals on both filters were discarded as false positives. One positive phage (λ-pA1) was isolated and plaque purified by sequential screening at low phage density. The corresponding cDNA of the positive clone was subcloned into pBluescript in vivo by infecting XL-1-Blue with λ-pA1 and the helper phage R408, followed by overnight incubation on an ampicilin LB agar plate at 37°C as described in Stratagene manual. The resulting plasmid with the cDNA insert (pA1) was subjected to restriction endonuclease and nucleotide sequence analysis.

Because the putative nucleotide sequence of pA1 lacked an immediate upstream AUG sequence,(see following section for sequence analysis) a 1068 bp probe (P1068) was amplified using PCR from the insert and used to rescreen the λ-ZAP reticulocyte library. As with antibody screening, recombinant phage were grown on a lawn of XL-1-Blue overnight at 37°C. The plates were incubated at 4°C for two hours and overlayed with duplicate nylon filters. Bound phage DNA were denatured and cross linked to the nylon filters using Stratagene stratalinker. The filters were

hybridized to P1068 at high stingency in a hybridization buffer containing 50 ng/ml of biotinylated P1068, 50% formamide, 900 mM NaCl, 60 mM NaH_2PO_4 (pH 7.4), 6 mM EDTA, 0.1% ficoll, 0.1% polyvinylpymolidone, 0.1% BSA, 1% SDS, 100 μg sheared denatured salmon sperm DNA, and 10% dextran sulfate overnight at 42°C. Following hybridization, duplicate filters were washed twice at 65°C with 5 x SSC; (150 mM NaCl, 15 mM sodium citrate pH 7.0) containing 0.5% SDS for 15 min and once at 60°C with 0.1 x SSC containing 1% SDS for 30 min. The filters were then incubated with alkaline phosphatase-linked streptavidin (1.0 μg/ml) for 15 mins at room temperature and positive clones were detected by incubation of the filters with 4-methoxy-4-(3-phosphatephenyl) spiro [1,2-dioxoethane-3, 3-adamantene] at room temperature for three hours and followed by autoradiography. Clones giving positive signals on both filters were isolated, and the corresponding cDNA subcloned into pBluescript and subjected to restriction endonuclease restriction analysis and DNA sequence analysis.

PCR Amplification, Biotinylation and Radiolabeling of P1068

Two primers (P6 sense strand and P10 anti-sense strand) spanning 1068 bp of pA1 were synthesized and used to amplify P1068 sequences. The amplified sequence extended from nucleotide 931 to 2000 on the sense strand of pA1. 1 ng of pA1 was amplified with 2.5 U of ampliTaq DNA polymerase and 20 μM of each primers in a 100 μl reaction volume. The reaction conditions were 94°C for two minutes, 55°C for one minute, 72°C for three minutes. This cycle was repeated 40

times followed by a 10 minute final extension at 72 °C. The amplified product was purified by phenol/chloroform extraction followed by ethanol precipitation. For biotinylation, 6 μg of purified P1068 and 25 μg photobiotin were photolyzed with a 500 volt white light source in 50 μl reaction volume on ice for 20 minutes. The unreacted photobiotin was removed by chromotography on Bio-gel P60. P1068 was radiolabeled to a specific activity of 6.2 x 10^8 μCi/μg by random priming with Klenow. 50 ng of heat denatured P1068 was incubated for three hours at room temperature with 2.5U Klenow in a 25 μl reaction buffer containing 100 μCi [α^{32}p] CTP, 10 mg BSA, 50 μmM ATP, GTP, and TTP. Following the incubation, unreacted radionucleotide was removed by spin column chromatography on Sephadex G-50.

Expression of pA1 in *E.coli*

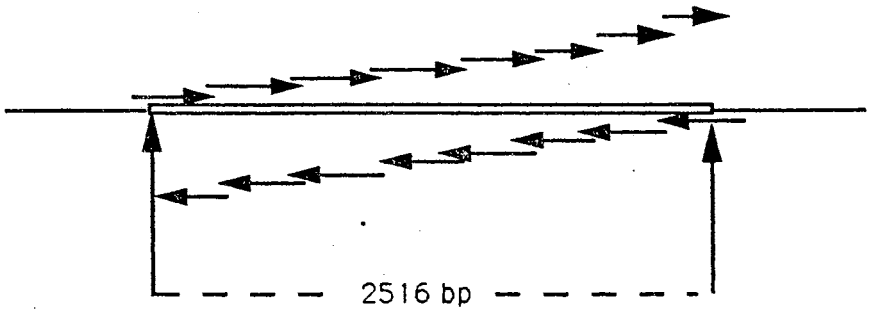
XL-1-Blue harboring pA1, was grown to mid log phase (A_{600} of approximately 0.20) in 30 ml LB broth at 37 °C and expression of the fusion protein was induced by addition of IPTG to a final concentration of 10 mM. Incubation was continued until the absorption at 600 nm for the cells was 1.0 OD/ml. The cells were pelleted by centrifugation at 6000g for 15 minutes, and resuspended in 7.5 ml of lysis buffer (50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1 mM PMFS, 10% sucrose, and 1 mg/ml lysozyme) and incubated on ice for 10 minutes. Triton X-100 was added to a final concentration of 0.1% and incubation was continued for an additional 10 minutes. Following incubation with Triton X-100, cell debris was removed by centrifugation

at 12,000 rpm for 90 minutes. 30 µl of the clarified supernatant was electrophoresed on a 10% SDS-PAGE gel, stained with Coomassie blue staining, or immunoblotted.

Sequence Analysis of pA1 cDNA

The nucleotide sequence of the isolated cDNA was determined by the dideoxynucleotide chain termination method (73). Double stranded DNA was sequenced completely using the sequenase kit (USB) and overlapping oligonucleotide primers made from sequences complementary to previously sequenced regions. The sequencing strategy is summarized in Figure 1. The first 200 nucleotides 5′ of the clone was very GC rich, so to remove sequence compression and band artifacts, this region was sequenced using double stranded DNA and dITP, with addition of 2 U of terminal deoxynucleotidyl transferase (TdT) to the sequence reactions after the dideoxy nucleotide termination (ddNTP) step, as described by Fawcett *et. al.* (61). Addition of TdT extends sequences not terminated by incorporation of ddNTP, thus eliminating the band artifacts resulting from their presence on sequencing gels. As an additional check, this region was also sequenced from single stranded DNA. The nucleotide sequence of the pA1 cDNA was analyzed using the Macvector sequence analysis programs (IBI). Sequence homology of the pA1 cDNA and putative amino acids sequence to other protein was determined by searching GenBank using the Fasta program of Pearson and Lipman (62) and the NBRF data bank, respectively.

Figure 1. Sequencing Strategy of pA1 cDNA.
Oligo nucleotide primers (18 to 22 nucleotides long) were synthesized and used as primers in the sequencing reaction. Overlapping sequence were identified using the computer program DNA strider.


2516 bp

In Vitro Transcription and Translation of pA1 cDNA

pA1 DNA was purified by double banding on cesium chloride gradient centrifugation. Following purification, 10 μg of pA1 was extracted with phenol/$CHCl_3$ and linearized with 20U of EcoRV, followed by ethanol precipitation in the presence of 1M NH_4OAc. For the transcription reaction 3 μg of linearized pA1 was incubated with 50 U T_3 RNA polymerase in a 50 μl reaction buffer containing 40 mM Tris/HCl (pH 7.5), 50 mM NaCl, 8 mM MgCl, 2 mM spermidine, 0.6 mM r(UTP, CTP, ATP), 0.18 mM rGTP, 30 mM DTT, 40U RNasin and 0.36 mM 5′ 7meGppp 5′G for one hour at 37°C. Uncapped mRNA was transcribed in the absence of 5′-7meGppp 5′G. After one hour, an additional 30 U of T_3 RNA polymerase was added and the reaction was incubated at 37°C for an additional 30 minutes. pA1-230 was transcribed with T_7 RNA polymerase and the DNA template was removed by treatment of the reaction with 15 U of RNase free DNase and 2 mM CaCl for 30 minutes at 37°C. Following the DNase treatment, the reaction volume was brought to 100 μl by addition of RNase free dH_2O and phenol/chloroform extracted two times. The synthesized capped RNA was then ethanol precipitated and resuspended in 5 μl RNase-free dH_2O. The RNA was translated with 15 μl of Ambion nuclease treated reticulocyte lysate in the presence of 50 μCi [^{35}S] Methionine. To determine the time course of the translation reaction, 5 μl aliquots were removed from the reactions at 15, 30, and 60 minutes. The translation products were analyzed by electrophoresis through 10% SDS

polyacramide gels followed by western blotting and autoradiography. For western blots, the SDS-PAGE seperated proteins were transfered to a nitrocellulose paper with a Poly blot at 2.5 mA/cm^2 for 40 min. After the transfer, the nitrocellulose paper was incubated in a blocking solution containing 10% calf serum in TBS for one hour and incubated overnight with a 100 fold dilution of the eIF-2B antibody. Following this incubation, detection of immunoreactive polypeptides were essentially the same as those used for immuno screening of the cDNA library.

Characterization of the mRNA Encoding eIF-2Bε

Poly (A^+) RNA was isolated from rabbit reticulocytes by a slight modification of the method of Badley *et. al.* (63). 5 ml of a buffer containing 400 mM NaCl, 400 mM Tris/HCl (pH 7.5), 3.0 mM $MgCl_2$, 4% SDS and 400 μg Proteinase K was added to 5 ml of rabbit reticulocyte lysate, followed by incubation of the lysate at 45 °C for two hours with intermittent agitation. The lysate was then added directly to oligo (dT) cellulose previously equilibrated with a buffer containing 10 mM Tris/HCl (pH 7.5) and 500 mM NaCl. The poly (A^+) RNA was bound by incubating the mixture at room temperature with agitation for 20 minutes. The oligo (dT) cellulose was washed with the binding buffer until no absorbance was detected at 260 nm. Poly (A^+) RNA was then eluted from the oligo (dT) cellulose with 10 mM Tris/HCl (pH 7.5), 1 mM EDTA and 0.1% SDS (elution buffer). The RNA was precipitated by the addition of 2.5 volume 95% ethanol. The precipitated mRNA was resuspended in 20 μl of the elution buffer.

The isolated poly (A^+) RNA preparations were treated with glyoxal at 65°C for one hour and fractionated on a 1% agarose gel. After electropherorsis, the fractionated RNA was transferred to a nylon membrane by capillary transfer for 24 hours and hybridized to radiolabeled P1068 under conditions identical to those described for hybridization of biotinylated P1068. Following hybridization, the membrane was washed four times for 15 minutes each at 55°C with 2xSSC, 1% SDS; two times with 1xSSC, 1% SDS at 55°C for 15 minutes each; two times with 0.5x SSC, 1% SSC at 55°C for 15 minutes each; and a final wash with 0.5x SSC at room temperature. The membrane was then dried and exposed to Kodak XAR-5 film at 70°C for one week.

Deletion Analysis of the 5′ end of pA1 cDNA

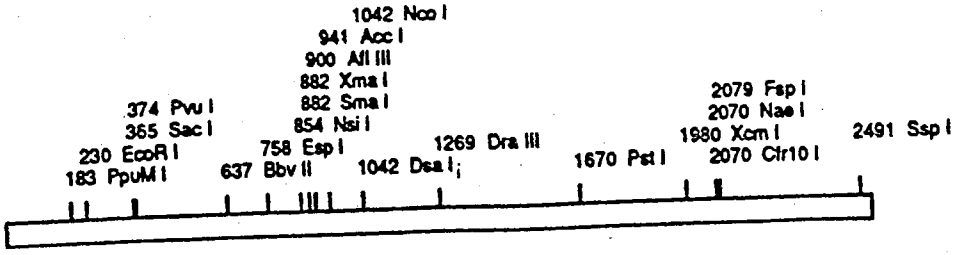
To help determine the initiating codon for the isolated cDNA, three deletions were made into pA1 with unique restriction at the 5′ terminal of pA1 to generate the following plasmids; pA1-172, pA1-230 and pA1-369 (figure 2). pA1-172 was prepared by digesting 5 μg of cesium chloride gradient purified pA1 with 10 U of BamHI at 37°C for 90 minutes, followed by purification by phenol/chloroform extraction and ethanol precipitation. 1 μg of the BamHI linearized pA1 was then digested with 2 U of Ppum1. The ends of the resulting DNA were filled in by the addition of 1 U of Klenow and 0.2 mM NTP with a further incubation at 37°C for 30 minutes. Intramolecular recircularization of the DNA was carried out by the addition of 1 U of T_7 ligase in a reaction mixture containing 10 mM

Figure 2. 5′ Deletion Analysis of pA1 cDNA.

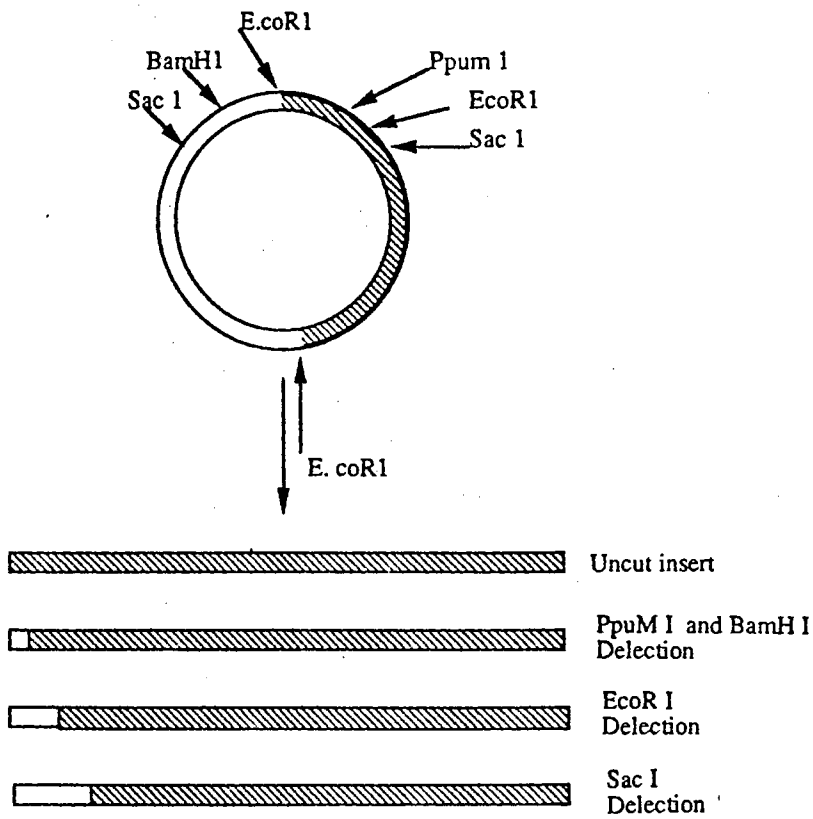
a; Partial Restriction Map of pA1 showing unique restriction enzyme sites and their relative positions on pA1. These sites include the restriction enzymes used for the deletion analysis.

b; Deletion with BamHI on pBS and pPum1 on pA1 removes 183 nucleotides from the 5′ terminus of pA1 to generate the plasmid pA1-172. The plasmid pA1-230 was generated by digestion of pA1 with EcoRI, which removes 230 nucleotides from the 5′ terminus of pA1. Restriction with SacI removes 365 nucleotides from pA1 generating the plasmid pA1-369. Because all deletions were made using unique restriction sites on pBS multiple cloning site and pA1, the T3 and T_7 promotor were not destroyed, allowing <u>in vitro</u> synthesis of mRNA using the respective polymerase.

a


1042 Nco I
941 Acc I
900 Afl III
882 Xma I
882 Sma I
854 Nsi I
758 Esp I
1042 Dsa I
1269 Dra III
374 Pvu I
365 Sac I
230 EcoR I
183 PpuM I
637 Bbv II
1670 Pst I
2079 Fsp I
2070 Nae I
1980 Xcm I
2070 Clr10 I
2491 Ssp I

b


E.coR1
BamH1
Sac 1
Ppum 1
EcoR1
Sac 1
E. coR1
Uncut insert
PpuM I and BamH I Delection
EcoR I Delection
Sac I Delection

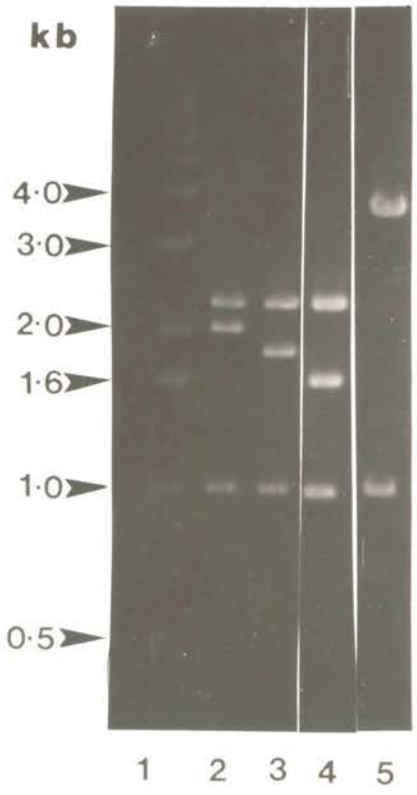
hexaaminecobalt, 25 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT and 400 mM KCl in a total reaction volume of 50 μl for one hour at room temperature. 10 μl of the recircularization reaction mixture was used to transform component XL-1-Blue cells as described by Hannaha (63). Transformed cells were plated on a LB agar plate supplemented with X-gal and 50 μg ampicilin (amp) and grown overnight at 37°C. Clear colonys from this plate were restreaked on amp supplemented LB plates and incubated overnight at 37°C. pA1-172 was isolated from bacteria culture grown from a single colony on this plate using the alkaline denaturation method described in USB sequencing support manual.

For pA1-369, 1 μg of pA1 was digested with 2 U of Sac1 and purified by phenol/chloroform extraction and ethanol precipitation. After purification, the experimental procedures used to generate pA1-369 were essentially the same as those used for pA1-172 except that end filling with Klenow was not performed.

Restriction of pA1 with EcoRI frees it from the vector. So to obtain pA1-230, 10 μg of EcoR1 digested pA1 was electrophoresed on a 1.2% low melting point agarose. The band corresponding to the 2328 bp fragment was then extracted from the agarose gel using USBioclean kit. The extracted DNA was then subcloned into pBluescript generating pA1-230. The plasmid isolation was the same as that described for pA1-172.

To verify the extent of the deletions, each of the constructed plasmids was restricted with PvuI and the reaction mixture electrophoresed on a 1.2% agarose gel (figure 3).

Figure 3. Restriction Analysis of pA1, pA1-172, pA1-230, and pA1-369.
0.3 μg of each of the plasmid were digested with 1 U of Pvu1 and electrophoresed through a 1.2% agorose gel. Lanes: 1, 1 kb ladder DNA; 2, pA1; 3, pA1-172; 4, pA1-230; 5, pA1-369.


kb
4·0
3·0
2·0
1·6
1·0
0·5
1
2
3
4
5

Glycerol Gradient of In Vitro Translated eIF-2Bε

5 μl of the translated product for pA1 was diluted with one half volume of a buffer containing 20 mM Tris/HCl (pH 7.5), 1 mM DDT and 0.2 mM EDTA and separated on a linear 15% to 50% glycerol gradient by centrifugation at 45,000xg for 18 hours. 200 μl fractions were collected. Fractionated proteins were precipated with 300 mM NaOAc, 5 μg BSA and 2 volume of 95% ethanol at -20°C overnight. Precipitated protein were collected by centrifugation at 12,000g for 15 minutes and redissolved in 50 μl of Laemmilli buffer (65). The resuspended proteins were denatured by boiling for one minute and electrophoresed through a 10% SDS polyacrylamide gel. After electrophoresis, the proteins were transfered to a PVF membrane as described previously. Proteins were visualized by autoradiography with Kodak XAR-5 film at room temperature.

CHAPTER IV

RESULTS

Isolation and Analysis of the Nucleotide Sequence of the cDNA Encoding eIF-2Bε

The cDNA encoding eIF-2Bε was isolated by antibody screening of a rabbit reticulocyte lysate library in λ-ZAP (Stratagene). Of the 337,500 recombinants screened, only a single immunopositive clone was isolated. To determine the approximate size and confirm the immunoreactivity of the encoded protein, the isolated clone was subsequently subcloned into pBluescript by in vivo excision and expressed in *E.coli*. In *E.coli*, the recombinant protein is expressed as a fusion protein with 34 amino acids of β-galactosidase under the control of the lac repressor (66). Upon induction with IPTG, a new protein with a molecular weight of approximately 88 kDa was observed to be present in cell extracts analyzed on a 10% SDS polyacryamide gel (figure 4). Western blot of the gel with antibody directed against eIF-2B showed two bands, one corresponding to the induced fusion protein (figure 4) and the other comigrating with the 82 kDa subunit of purified nonrecombinant eIF-2B (figure 5). The expressed protein did not react with preimmune antiserum indicating that it is specific to the eIF-2B antibody.

Figure 4. Expression of pA1 in *E.coli.*

Following induction of the synthesis of fusion protein with IPTG, the *E.coli* cells were lysed and 30 μl of the soluble protein supernatant NAS electrophoresed through a 10% SDS polyacryamide gel. The proteins were then visualized by Coomassie blue staining. Lanes: 1, IPTG induces expression from pBS; 2, IPTG induced expression of pA1 (-- shows the new protein); 3, uninduced expression of pA1; 4, purified eIF-2B (* shows eIF-2Bε); 5, molecular weight standard.

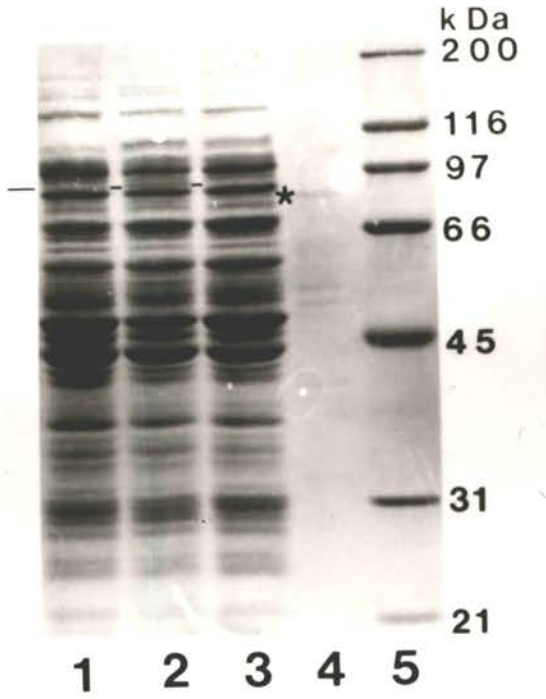
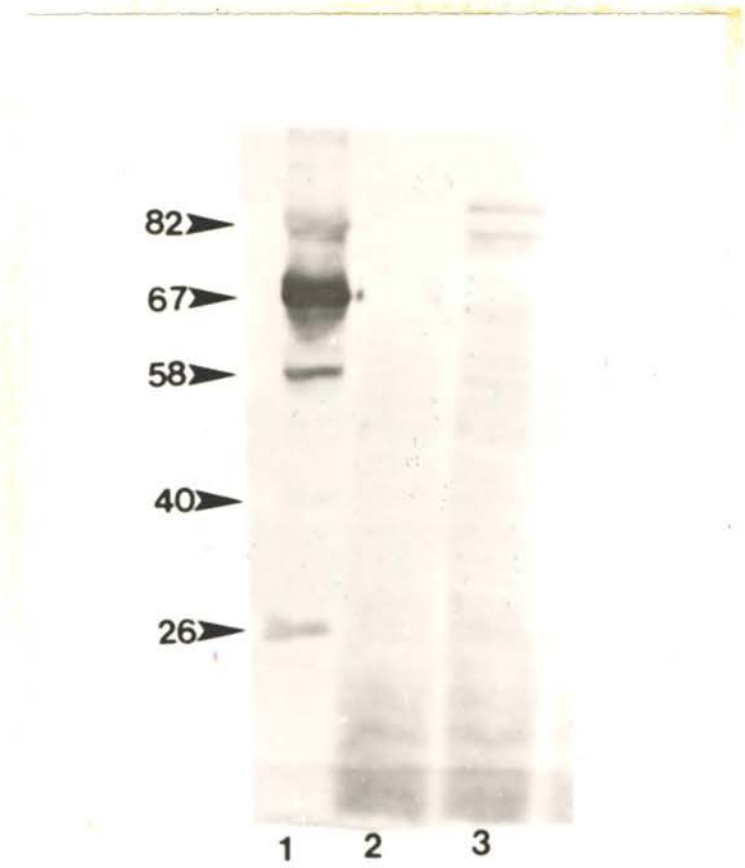

k Da
200
116
97
66
45
31
21
1
2
3
4
5

Figure 5. Western Blot Analysis of the Protein Expressed from pA1 in *E.coli*. Protein samples from *E.coli* expressing pA1 were electrophoresed through a 10% SDS polyacramide gel, transfered to nitrocellulose membrane and probed with a 1:100 dilution of a polyclonal antibody directed against all subunits of eIF-2B. Lanes: 1, purified eIF-2B; 2, protein sample from the IPTG induction of pBS expression in *E.coli*; 3, proteins from the IPTG induced expression of pA1 in *E.coli*.


82
67
58
40
26
1
2
3

After digestion of pA1 with EcoRI, DNA fragments were separated by electrophoresis through agarose gels. Three bands with sizes of approximately 2900, 2200, and 230 bp (figure 6, lane 2) were observed upon staining the gels with ethidium bromide. Since the upper 2900 bp band corresponds to the vector sequence, the cDNA insert contains one internal EcoRI site and is approximately 2500 bp in size. The average molecular weight of an amino acid is 110 Da, therefore, approximately 2240 bases of coding sequence is required to produce a polypeptide of 82 kDa protein. Therefore, the 2500 bp insert of pA1 cDNA contains sufficient DNA sequence to encode the 82 kDa subunit of eIF-2B.

Nucleotide sequencing of cDNA revealed the size of the pA1 cDNA is 2516 nucleotides, consistent with the size of the cDNA estimated by agarose gel electrophoresis. Analysis of the pA1 cDNA sequence using Macvector sequence analysis program revealed an opening reading frame extending from the second nucleotide to nucleotide 2152. The open reading frame is followed by 408 nucleotides of untranslated 3′ sequence that lacks a consensus polyadenlylation signal and a poly-A tail. The first 120 nucleotides of the pA1 cDNA is extremely GC rich with a G+C content between 70% - 90% (figure 7a & 7b). The pA1 cDNA sequence lacks an initiating AUG codon immediately downstream from the 5′ terminus. The first AUG codon is found at nucleotide 550 which would code for a protein with a predicted molecular weight of 65 kDa. Therefore, one possibility is that the cDNA is not full length. Another possibility is that the cDNA does encode

Figure 6. EcoRI Restriction Analysis of pA1, pA2, and pA3.
0.3 μg of the respective plasmids wre digested with 1 U of EcoRI and electrophoresed through a 1.2% agarose gel. The bands were then visualized by ethidium bromide staining. Lanes: 1, 1 kb DNA ladder; 2, pA1; 3, pA2; 4, pA3.

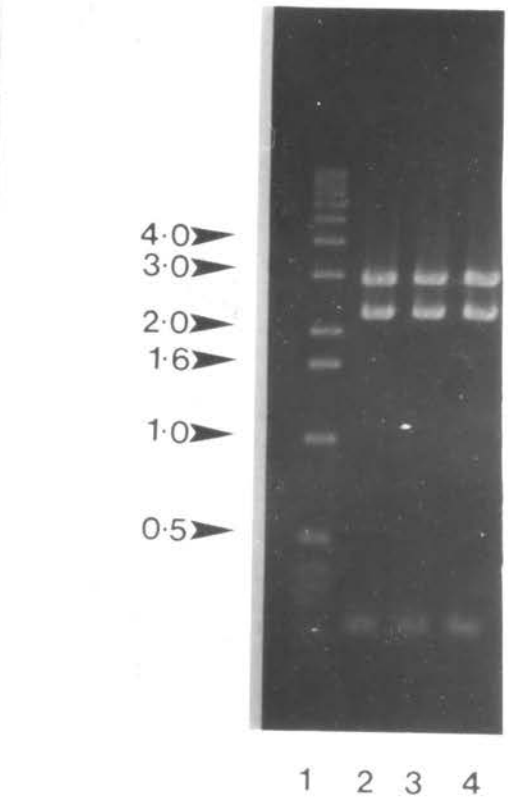
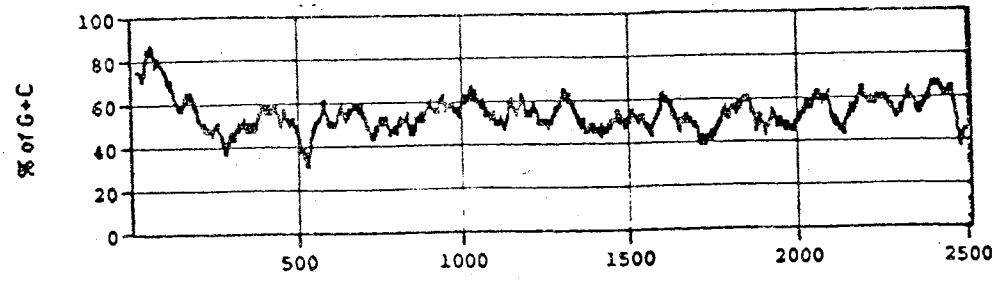

4·0
3·0
2·0
1·6
1·0
0·5
1 2 3 4

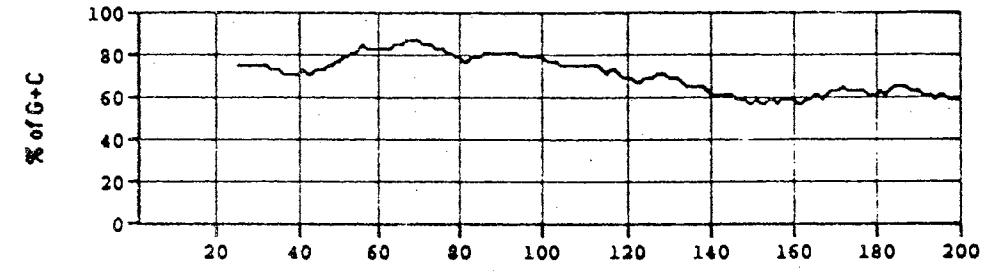
Figure 7. Percent G+C Content of the cDNA for eIF-2Bε.

a. The percent of G+C content of the entire nucleotide sequence of the cDNA encoding eIF-2Bε was analyzed using the percent G+C analysis program on Macvector.
b. Percent G+C content of only the first 200 nucleotide of the cDNA for eIF-2Bε.

a


% of G+C
100
80
60
40
20
0
500
1000
1500
2000
2500

b


% of G+C
100
80
60
40
20
0
20
40
60
80
100
120
140
160
180
200

full-length, but the observed band at 82 kDa is due to anomolus migration on SDS gels or due to post-trranslational modification of the proteins.

In order to distinguish between these alternatives, an attempt was made to isolate a longer cDNA by rescreening the reticulocyte lysate library with P1068, a nucleotide probe generated from the sequences of the immunopositive clone. Of the 150,00 recombinant phages screened, two additional positive clones were isolated. Plasmid DNAs were recovered by in vivo excision and the cDNA inserts were analyzed by EcoR1 digestion followed by electrophoresis through a 1% agarose gel. Both plasmids (pA2 and pA3) were found to have fragments of similar size to those generated from pA1 (figure 6). Terminal DNA sequence analysis with T_3 primer showed that all three have identical terminal sequences. Therefore, pA1, pA2, and pA3 are identical clones.

Analysis of the Deduced Amino Acid Sequences of eIF-2Bε

The nucleotide sequence and the deduced amino acid sequence of pA1 cDNA are shown in figure 8. The longest open reading frame begins at nucleotide 2, however, the GTG codon at nucleotide position 23 has been tentatively identified as the initiating codon (see next section for explanation). The predicted protein is 711 amino acid residues in length and has an estimated molecular weight of 79,381 kDa, consistent with the observed mobility of eIF-2Bε on 10% SDS-polyacrylamide gels. The amino acid composition of the derived sequence of the predicted protein is shown in table I. The percent composition of the amino acids in the protein is

consistent with those expected for vertebrate proteins except for a 50% elevation in the number of glutamate residues. This increase in glutamate residues gives the predicted protein an acidic pI of 4.9. Although, the percent composition of glycine falls within the the expected value, the predicted protein has an unusual cluster of glycine at its N-terminus.

Microsequence analysis of SDS-PAGE purified eIF-2Bε was performed, following partial hydrolysis with V8 protease (Shaun N. Thomas, unpublished results). The two peptide sequences H_2N-N V LLGSFNVIGSN-CO_2 and H_2N-XXVIXLXXPXA-CO_2 were obtained. The predicted amino acid sequence of pA1 cDNA contained the sequence H_2N-NVLLGSGTVIGS N-CO_2 (underlined in figure 8), this sequence differed at only two positions from that obtained from microsequencing of one of the V8 fragments. On the V8 fragment, residue 7 and 8 are phenylalamine and alanine instead of glycine and threonine observed in the predicted amino acid sequence. The amino acid sequence H_2N-GSVISLHPPEA-CO_2 was also present in the predicted amino acid of pA1 cDNA. Although, the underlined amino acid residues in the predicted sequence (H_2N-GSVISLHPPEA-CO_2) were not conclusively determined by microsequencing, this sequence like the previous sequence is preceded by an acidic residue in the pA1 sequence; acidic residues mark the cut site for V8 protease. Moreover, the spacing between the identified and the unidentified residues in the V8 fragment sequence are an exact match to the predicted sequence. The presence of these peptide sequences in the

Figure 8. Nucleotide and Deduced Amino Acid Sequences of eIF-2Bε.
The deduced amino acid sequence of eIF-2Bε is given below the nucleotide sequence. Translation is postulated to begin from the GTG codon begining at nt 23. The open reading frame then extends from this codon to the termination codon TGA beginning at nt 2158. This corresponds to a protein of 711 amino acids to give a protein with a calculated molecular weight of 79 kDa. The underlined amino acids represent those corresponding to the sequence obtained from the peptide sequencing of V8 fragments of eIF-2Bε. The ATG codons beginning at nt 530 or 536 and 854 represent possible translation of initiation codons for the 65 and 58 kDa proteins observed on SDS polyacrylamide electrophoresis gel of the translation product of this cDNA. -- indicates possible phosphorylation site for casein kinase II.

```
          10          20          30          40          50
           *           *           *           *           *
GCGGC CGCCG CCGCC TGGTG CA GTG TCT GAT CGG GCC AAC AAG CGC GGC GGG GGG
                          Val Ser Asp Arg Ala Asn Lys Arg Gly Gly Gly>

     60          70          80          90          100
      *           *           *           *           *
CCA GGA GGC GGT GGC GGC GGC GGG GGA GCC CGA GGT GCG GAG GAG GAG TCT CCG
Pro Gly Gly Gly Gly Gly Gly Gly Gly Ala Arg Gly Ala Glu Glu Glu Ser Pro>

110         120         130         140         150         160
 *           *           *           *           *           *
CCG CCC CTC CAG GCG GTC CTG GTG GCC GAT AGC TTC AAC CGC CGT TTC TTC CCC
Pro Pro Leu Gln Ala Val Leu Val Ala Asp Ser Phe Asn Arg Arg Phe Phe Pro>

       170         180         190         200         210
        *           *           *           *           *
ATC TCC AAG GAC CAG CCT CGG GTC CTC CTG CCT CTG GCC AAT GTG GCG CTA ATT
Ile Ser Lys Asp Gln Pro Arg Val Leu Leu Pro Leu Ala Asn Val Ala Leu Ile>

  220         230         240         250         260         270
   *           *           *           *           *           *
GAC TAC ACT CTG GAA TTC CTG ACT GCC ACA GGT GTA CAG GAG ACC TTT GTG TTT
Asp Tyr Thr Leu Glu Phe Leu Thr Ala Thr Gly Val Gln Glu Thr Phe Val Phe>

        280         290         300         310         320
         *           *           *           *           *
TGT TGC TGG AAG GCT GCT CAG ATC AAA GAA CAT TTG CAA AAA TCC AAG TGG TGC
Cys Cys Trp Lys Ala Ala Gln Ile Lys Glu His Leu Gln Lys Ser Lys Trp Cys>

    330         340         350         360         370
     *           *           *           *           *
CGC CCA ACA TCC CTC AAC GTG GTT CGG ATA ATT ACA TCA GAG CTC TAC CGA TCG
Arg Pro Thr Ser Leu Asn Val Val Arg Ile Ile Thr Ser Glu Leu Tyr Arg Ser>

380         390         400         410         420         430
 *           *           *           *           *           *
CTG GGA GAT GTC CTC CGT GAT GTT GAC GCC AAA GCC CTG GTG CGC TCT GAC TTC
Leu Gly Asp Val Leu Arg Asp Val Asp Ala Lys  Ala Leu Val Arg Ser Asp Phe>

       440         450         460         470         480
        *           *           *           *           *
CTC CTG GTG TAC GGA GAT GTC GTC TCC AAC ATC AAT GTC ACC AGG GCC CTC GAG
Leu Leu Val Tyr Gly Asp Val Val Ser Asn Ile Asn Val Thr Arg Ala Leu Glu>

490         500         510         520         530         540
 *           *           *           *           *           *
GAA CAT AGG TTA AGG CGG AAG CTA GAA AAA AAT GTG TCT GTG ATG ACA ATG ATC
Glu His Arg Leu Arg Arg Lys Leu Glu Lys Asn Val Ser Val Met Thr Met Ile>

        550         560         570         580         590
         *           *           *           *           *
TTC AAA GAA TCA TCC CCC AGC CAC CCG ACT CGT TGC CAT GAG GAC AAT GTG GTA
Phe Lys Glu Ser Ser Pro Ser His Pro Thr Arg Cys His Glu Asp Asn Val Val>

    600         610         620         630         640
     *           *           *           *           *
GTG GCT GTG GAT AGT GCC ACA AAC CGG ATT CTC CAC TTC CAG AAG ACC CAG GGC
Val Ala Val Asp Ser Ala Thr Asn Arg Ile Leu His Phe Gln Lys Thr Gln Gly>
```

```
650          660          670          680          690          700
 *            *            *            *            *            *
CTC CGA CGT TTC TCT TTT CCT CTG AGC CTG TTC CAA GGC AGT GGC GCT GGC GTG
Leu Arg Arg Phe Ser Phe Pro Leu Ser Leu Phe Gln Gly Ser Gly Ala Gly Val>

      710          720          730          740          750
       *            *            *            *            *
GAG ATT CGG TAT GAC TTA TTG GAT TGT CAT ATC AGC ATC TGC TCC CCT CAG GTG
Glu Ile Arg Tyr Asp Leu Leu Asp Cys His Ile Ser Ile Cys Ser Pro Gln Val>

760          770          780          790          800          810
 *            *            *            *            *            *
GCT CAG CTC TTC ACA GAC AAC TTT GAC TAC CAG ACT CGA GAT GAC TTT GTG CGA
Ala Gln Leu Phe Thr Asp Asn Phe Asp Tyr Gln Thr Arg Asp Asp Phe Val Arg>

        820          830          840          850          860
         *            *            *            *            *
GGC CTG TTA GTG AAC GAG GAG ATC CTA GGG AAC CAG ATC CAC ATG CAT GTA ACA
Gly Leu Leu Val Asn Glu Glu Ile Leu Gly Asn Gln Ile His Met His Val Thr>
              870          880          890          900          91
    *            *            *            *            *
ACT AGG GAA TAC GGT GCC CGG GTC TCC AAC CTG CAC ATG TAC TCT GCT GTC TGT
Thr Arg Glu Tyr Gly Ala Arg Val Ser Asn Leu His Met Tyr Ser Ala Val Cys>

920          930          940          950          960          970
 *            *            *            *            *            *
GCC GAT GTC ATC CGC CGA TGG GTC TAC CCT CTC ACC CCA GAG GCA AAC TTC ACT
Ala Asp Val Ile Arg Arg Trp Val Tyr Pro Leu Thr Pro Glu Ala Asn Phe Thr>

      980          990         1000         1010         1020
       *            *            *            *            *
GAC AGC ACC GCT CAG AGC TGC ACT CAT TCC CGG CAC AAC ATC TAC CGA GGG CCC
Asp Ser Thr Ala Gln Ser Cys Thr His Ser Arg His Asn Ile Tyr Arg Gly Pro>

1030         1040         1050         1060         1070         1080
 *            *            *            *            *            *
GAG GTC AGC TTG GGC CAT GGC AGC ATC CTG GAG GAG AAT GTA CTC CTG GGC TCT
Glu Val Ser Leu Gly His Gly Ser Ile Leu Glu Glu Asn Val Leu Leu Gly Ser>

       1090         1100         1110         1120         1130
        *            *            *            *            *
GGC ACT GTC ATT GGC AGC AAC TGC TCT ATC ACC AAC AGT GTC ATT GGT CCT GGC
Gly Thr Val Ile Gly Ser Asn Cys Ser Ile Thr Asn Ser Val Ile Gly Pro Gly>

  1140         1150         1160         1170         1180
    *            *            *            *            *
TGC TGC ATT GGT GAT AAC GTG GTG CTG GAC CGG GCC TAC CTG TGG AAA GGT GTT
Cys Cys Ile Gly Asp Asn Val Val Leu Asp Arg Ala Tyr Leu Trp Lys Gly Val>

1190         1200         1210         1220         1230         1240
 *            *            *            *            *            *
CAA GTG GCT TCT GGG GCG CAG ATC CAT CAG TCT CTG CTC TGT GAC CAT GCT GAG
Gln Val Ala Ser Gly Ala Gln Ile His Gln Ser Leu Leu Cys Asp His Ala Glu>
```

```
     1250         1260         1270         1280         1290
      *            *            *            *            *
GTC AAG GAA CAA GTG ACA CTG AAG CCA CAC TGT GTC CTT ACT TCC CAG GTG GTA
Val Lys Glu Gln Val Thr Leu Lys Pro His Cys Val Leu Thr Ser Gln Val Val>

1300         1310         1320         1330         1340         1350
  *            *            *            *            *            *
GTG GGC CCC AAC ATC ACG CTG CCT GAG GGC TCG GTC ATC TCT TTG CAC CCT CCA
Val Gly Pro Asn Ile Thr Leu Pro Glu Gly Ser Val Ile Ser Leu His Pro Pro>

       1360         1370         1380         1390         1400
        *            *            *            *            *
GAT GCA GAA GAA GAT GAG GAT GAT GGC CAG TTC AGT GAT GAT TCT GGG GTC AAC
Asp Ala Glu Glu Asp Glu Asp Asp Gly Gln Phe Ser Asp Asp Ser Gly Val Asn>

   1410         1420         1430         1440         1450
    *            *            *            *            *
CAA GCA AAG GAG AAA GCG AAG CTG AAA GGT TAC AAT CCA GCG GAA GTA GGA GTT
Gln Ala Lys Glu Lys Ala Lys Leu Lys Gly Tyr Asn Pro Ala Glu Val Gly Val>

1460         1470         1480         1490         1500         1510
  *            *            *            *            *            *
GCA GGC AAA GGC TAC CTC TGG AAA GCT GCT GAC ATG AAC ACG GAG AAA GAG GAA
Ala Gly Lys Gly Tyr Leu Trp Lys Ala Ala Asp Met Asn Thr Glu Lys Glu Glu>

     1520         1530         1540         1550         1560
      *            *            *            *            *
GAA CTG CGG CAG AGT CTG TGG GGA CTC ACA ATC AAT GAG GAA GAG GAG AGT GAG
Glu Leu Arg Gln Ser Leu Trp Gly Leu Thr Ile Asn Glu Glu Glu Glu Ser Glu>

1570         1580         1590         1600         1610         1620
  *            *            *            *            *            *
ACT GAA AGT GAG CGA AGC ATG GAT TCT GAG GAG CTG GAC AGC CGA GCA GGC TCC
Thr Glu Ser Glu Arg Ser Met Asp Ser Glu Glu Leu Asp Ser Arg Ala Gly Ser>

       1630         1640         1650         1660         1670
        *            *            *            *            *
CCC CAG TTG GAT GAC ATC AAA GTG TTC CAG AAT GAA GTC CTG GGA ACA CTG CAG
Pro Gln Leu Asp Asp Ile Lys Val Phe Gln Asn Glu Val Leu Gly Thr Leu Gln>

   1680         1690         1700         1710         1720
    *            *            *            *            *
CGG GGC AAA GAA GAG AGC ATT TCT TGT GAC AAT CTC ATC CTG GAG ATC AAC TCT
Arg Gly Lys Glu Glu Ser Ile Ser Cys Asp Asn Leu Ile Leu Glu Ile Asn Ser>

1730         1740         1750         1760         1770         1780
  *            *            *            *            *            *
CTC AAG TAT GCC TAC AAC ATA AGC CTA AAG GAG GTG ATG CAG GTA CTG AGC CAT
Leu Lys Tyr Ala Tyr Asn Ile Ser Leu Lys Glu Val Met Gln Val Leu Ser His>

     1790         1800         1810         1820         1830
      *            *            *            *            *
GTG GTC CTG GAG TTC CCA CTG CAA CAA ATG GAT TCC CCG CTG GAG GCA AAC CGC
Val Val Leu Glu Phe Pro Leu Gln Gln Met Asp Ser Pro Leu Glu Ala Asn Arg>
```

```
1840          1850          1860          1870          1880          1890
 *             *             *             *             *             *
TAC TGT GCC CTG CTG CTT CCT TTG CTC AAG GCC TGG AGC CCT GTT TTT AGG AAC
Tyr Cys Ala Leu Leu Leu Pro Leu Leu Lys Ala Trp Ser Pro Val Phe Arg Asn>

        1900          1910          1920          1930          1940
          *             *             *             *             *
TAC ATA AAG CGT GCA GCC GAC CAT TTG GAA GCA TTG GCA GCC ATT GAG GAG TTC
Tyr Ile Lys Arg Ala Ala Asp His Leu Glu Ala Leu Ala Ala Ile Glu Glu Phe>

    1950          1960          1970          1980          1990
      *             *             *             *             *
TTC CTG GAG CAT GAA GCT CTT GGT ACT TGC ATA GCC AAG GTA CTG ATG GGT TTC
Phe Leu Glu His Glu Ala Leu Gly Thr Cys Ile Ala Lys Val Leu Met Gly Phe>

2000          2010          2020          2030          2040          2050
 *             *             *             *             *             *
TAC CAG CTA GAG ATC CTG GCT GAG GAG ACG ATC CTG AGC TGG TTC GGC CAA AGG
Tyr Gln Leu Glu Ile Leu Ala Glu Glu Thr Ile Leu Ser Trp Phe Gly Gln Arg>

      2060          2070          2080          2090          2100
        *             *             *             *             *
GAT GTA ACG GAC AAG GGC CGG CAG CTG CGC AAG AAC CAG CAG CTA CAG AGG TTC
Asp Val Thr Asp Lys Gly Arg Gln Leu Arg Lys Asn Gln Gln Leu Gln Arg Phe>

2110          2120          2130          2140          2150          2160
 *             *             *             *             *             *
ATC CAG TGG CTA AAA GAG GCA GAA GAG GAG TCA TCT GAA GAT GAC TGA AGTCA
Ile Gln Trp Leu Lys Glu Ala Glu Glu Glu Ser Ser Glu Asp Asp ***>

        2170          2180          2190          2200          2210          2220
          *             *             *             *             *             *
CATGC TGCCC GCTCC TGGGG CTCTG AGTCA TTGTC CTCCT GGTTC CTGGG CCAGG ACAAG

        2230          2240          2250          2260          2270          2280
          *             *             *             *             *             *
TGCGG AGCTG GTTGT GGAAG GATGA GTGAC CACCG TCCCG TGACC CAAGG AGCAG AGGCT

        2290          2300          2310          2320          2330          2340
          *             *             *             *             *             *
GGAAC TACAG TATTC ACACT GCCAG CAACC ATGTG CCTCC CGTCC CAACT GGGGC ACTGG

        2350          2360          2370          2380          2390          2400
          *             *             *             *             *             *
GATGA GGGAA GTCAG ACTGG AACAA AGCAT CTGCC TAGGG AGGAG CTGGG CAGGC CTGCG

        2410          2420          2430          2440          2450          2460
          *             *             *             *             *             *
GTTGG AGGAA GGCCA GAGGA ACCTG TGTGC TCGGG CTCTC CCTCA GGGAA CAGCA GAGAG

        2470          2480          2490          2500          2510
          *             *             *             *             *
CAGCT GGCCT CTCTG CTGCT TGTAT TTGTT AATAT TAAAA AAGAG AGGCG GCCGC G
```

Table 1: Amino Acid Composition of the 82 kDa Subunit of eIF-2B

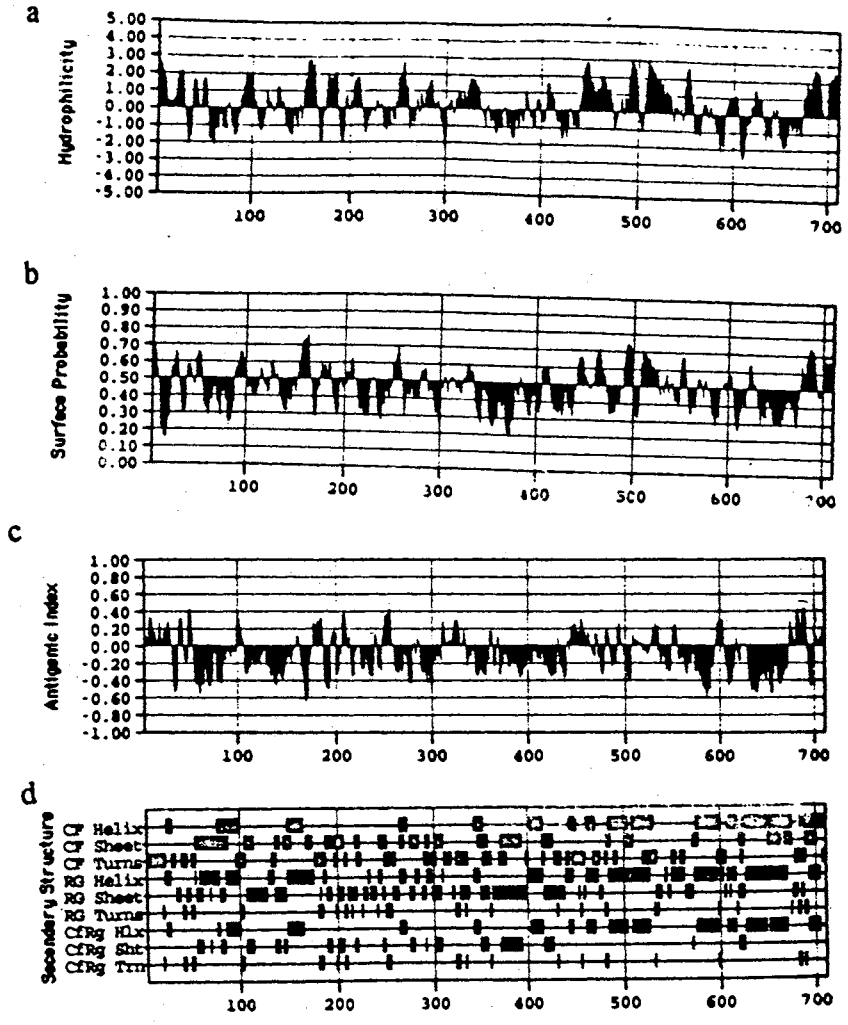
Amino acid	No.present	Percent
Ala	46	6.47
Val	58	8.16
Leu	76	10.69
Ile	35	4.92
Pro	27	3.80
Met	9	1.27
Phe	25	3.52
Trp	9	1.27
Gly	49	6.89
Ser	55	7.74
Thr	32	4.50
Cys	16	2.25
Tyr	17	2.39
Asn	31	4.36
Gln	34	4.78
Asp	40	5.63
Glu	61	8.58
Lys	31	4.36
Arg	40	5.63
His	19	2.67

sequence of amino acids predicted in the open reading from pA1 cDNA confirms that the isolated cDNA encodes eIF-2Bε.

The native eIF-2Bε subunit is believed to be a substrate for phosphorylation by casein kinase II, a serine/threonine kinase which phosphorylates the consenus sequence Ser/Thr XX Glu/Asp. The deduced amino acid sequence of eIF-2Bε contains several putative phosphorylation sites, at positions Thr-281, Ser 343, Thr 493, Thr 507, Ser 514, and Ser 706. The sequence Ser-514 Glu Thr Glu is the same as the consensus sequence phosphorylated by casein kinase II in fibrogen (65).

A hydrophilicity plot generated for the derived eIF-2Bε sequence predicts that eIF-2Bε is a hydrophilic protein (figure 9a). The antigenic index and surface probability of the deduced sequence were also obtained to identify peptides of eIF-2Bε that would have a high probability of generating an immune response in a host (figure 9b). The overall antigenicity index of eIF-2Bε is low, however, synthetic peptides corresponding to the residues spanning amino acids 250 to 258 and 690 to 700 could be useful for antibody production, since their surface probabilities are high. The secondary structure profile of the derived sequence indicated that eIF-2Bε has a complex secondary structure with no predominant secondary structure pattern (figure 9d).

Figure 9. Structural Analysis of the Amino Acid Sequence of eIF-2Bε.
a. Hydrophility plot of eIF-2Bε was performed with a window of 8.
b. Surface probability of eIF-2Bε.
c. Antigenic index of eIF-2Bε.
d. Secondary structure analysis of eIF-2Bε.


a
Hydrophilicity
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00
100
200
300
400
500
600
700
b
Surface Probability
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100
200
300
400
500
600
700
c
Antigenic Index
1.00
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
100
200
300
400
500
600
700
d
Secondary Structure
Helix
Sheet
Turns
RG Helix
RG Sheet
RG Turns
CfRg Hlx
CfRg Sht
CfRg Trn
100
200
300
400
500
600
700

In Vitro Transcription and Translation of eIF-2Bε cDNA

Since pA1 cDNA (eIF-2Bε cDNA) is expressed as fusion protein that contains 34 amino acids of β-galacosidase in *E.coli*, it is more difficult to deduce the molecular weight of the protein encoded by the cDNA. To verify that the eIF-2Bε cDNA encodes full length eIF-2Bε and to determine the apparent molecular weight of the encoded protein without interference from the 34 amino acids of β-galacosidase, eIF-2Bε cDNA was transcribed in vitro. The capped mRNA was translated in nuclease treated reticulocyte lysate in the presence of [^{35}S] methionine and the translation products were separated by SDS/PAGE and transfered to PVDF membranes. The autoradiograph of the Western blot revealed multiple protein bands (figure 10). Three of the protein bands comigrated with the 82, 65, and 58 kDa subunits of endogenous eIF-2B detected by western blotting using the chicken anti-eIF-2B antiserum. A fourth protein of approximate molecular weight of 72 kDa was also observed. A time course of the translation of the transcribed mRNA indicates that the 72 kDa, 65 and 58 kDa products are not produced by proteolytic digestion of the 82 kDa protein (figure 11). The 82 kDa protein is the largest protein translated and its presence indicates that the isolated cDNA encodes full length eIF-2B. Therefore, the most likely explanation is that the 65 and 58 kDa proteins are due to initiation at internal AUG codons located at nucleotide 550 and 854 respectively. The 72 kDa protein is also most likely the result of internal initiation

Figure 10. In Vitro Transcription and Translation of eIF-2Bε cDNA.
3 μg of EcoRV linearized pA1 was transcribed and the resulting mRNA translated in nuclease treated reticulocyte lysate. Autoradiography of western blot of the translation products separated by SDS-PAGE, gave multiple protein bands, three of which correspond to eIF-2B subunits. Lanes: 1, western blot of endogenous eIF-2B; 2, autoradiography of the translation product of eIF-2Bε cDNA.

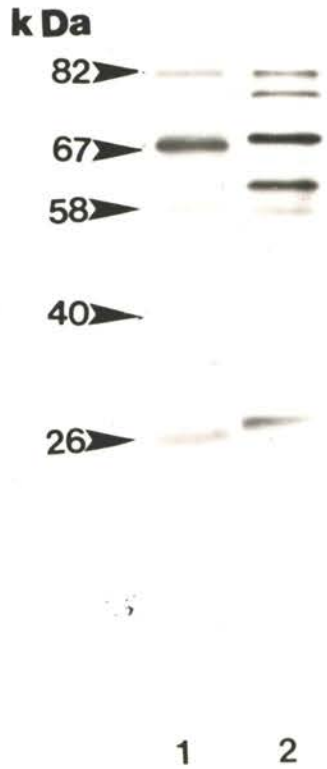
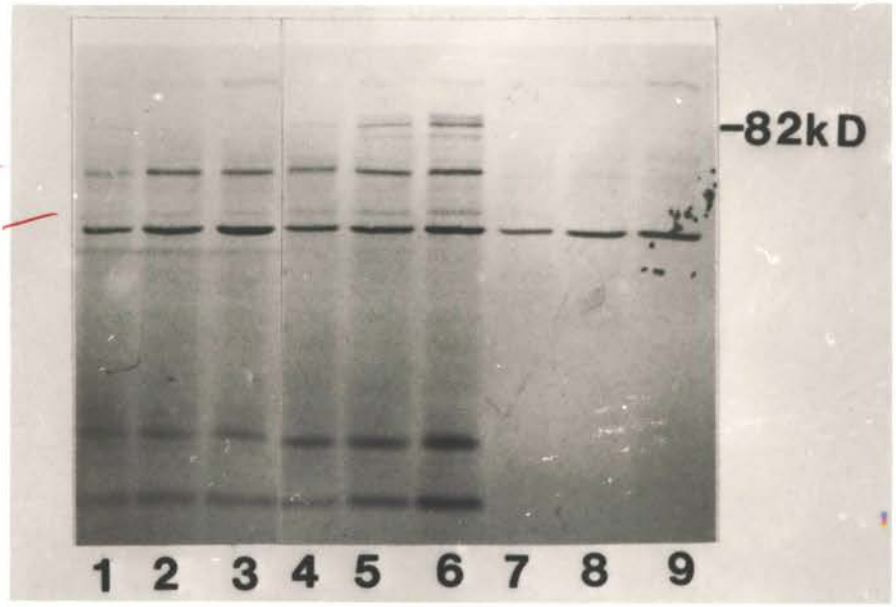

k Da
82
67
58
40
26
1
2

Figure 11. Time Course of eIF-2Bε cDNA In Vitro Translation.
3 μg of EcoRV linearized pA1 was transcribed in the presence and absence of the cap analog. The resulting capped and uncapped mRNA were translated in nuclease treated reticulocyte lysate. Aliquots were removed from the translation reactions containing uncapped mRNA (lanes 1-3), capped mRNA (lanes 4-6), or no mRNA (lanes 7-9) at 15, 30, and 60 minutes, and analyzed. Lanes: 1, 4, and 7, aliquots taken at 15 minutes; lanes 2, 5, and 8, aliquots taken at 30 min; lanes 3, 6, and 9, aliquots taken at 60 minutes.

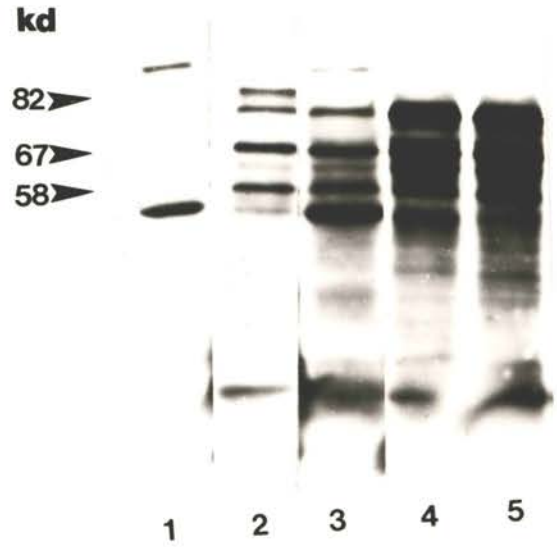

-82kD
1 2 3 4 5 6 7 8 9

from non AUG codon upstream of these AUG codons. The predominance of the 72 kDa, 65 kDa, and 58 kDa proteins in translation products of uncapped mRNA transcribed from eIF-2Bε cDNA appear to support the explanation that they are produced due to internal initiation. The presence of the 65 and 58 kDa bands also suggests that the longer 82 kDa translation product is generated from a non AUG initiation codon located upstream of the AUG codons at nucleotides 550 and 854.

To determine the location of the putative non AUG start codon, three deletions 5′ to the first AUG codon were generated by digesting with pPumI and BamHI (pA1-172), EcoR1 (pA1-230) and SacI (pA1-369). The truncated mRNA generated from pA1-172 lacked the codons GTG at nucleotide 23, CTG at nucleotide 128, and GTG at nucleotide 131, which are the likely non AUG codon used for initiation in eukaryotic cells (69). pA1-230 and pA1-369 also lacked the potential initiation codon GTG located at nucleotide 206 and 344, respectively. The truncated mRNAs generated from these deletions were translated in nuclease-treated reticulocyte lysates and the translation products were separated on a SDS/PAGE gel. Analysis of the translation products of each deletion indicated that all three deletions abolish the expression of the 82 kDa protein (figure 12 lane 3). In all cases however, the 72 kDa, 65 kDa and 58 kDa protein continued to be expressed. This suggests that the 72 kDa protein translation is initiated from a non AUG codon after nucleotide 369. Furthermore, since the expression of 82 kDa protein is abolished in the pA1-172 construct, this suggests that its initiating codon is within the first 172 nucleotide of the cDNAs. The most likely codons to be used within this region includes the GTG

Figure 12. In Vitro Translation of mRNA Transcribed from pA1-172, pA1-230, and pA1-369.

Truncated mRNA transcribed from pA1-172, pA1-230, and pA1-369 were translated in nuclease treated reticulocyte lysate in the presence of [^{35}S] methionine. The translation products were electrophoresized through a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Protein bands were visualized by autoradiography. Lanes: 1, negative control (-RNA); 2, pA1 translation; 3, pA1-172 translation; 4, pA1-230 translation; 5, pA1-365 translation. The high molecular weight band on lanes 1 and 3 are absent in lanes 2, 4, and 5 because of difference in exposure time.


kd
82
67
58
1
2
3
4
5

codon beginning at nucleotide 23, CTG beginning at nucleotide 128 and GTG beginning at nucleotide 131.

Similarity Between the Amino Acid Sequence of eIF-2Bε and Yeast GCD6

The deduced amino acid sequence of eIF-2Bε was compared with protein sequences in NBRF protein databank. The predicted amino acid sequence of eIF-2Bε showed a low homology with yeast GDC1 and an unidentified open reading frame in *E. coli*. The homology with yeast GCD1 (homology score 149) may be significant since this protein is postulated to be a subunit of eIF-2B in yeast. However, when the sequence of eIF-2Bε was compared with yeast GCD6 (provided by J. Bushman & A. Hinnebusch, NIH), eIF-2Bε was found to be 30% identical to GCD6 (figure 13) with the quality score for eIF-2Bε aligned with GCD6 being over 34 standard deviations greater than the mean score of randomized sequences. Comparison of eIF-2Bε and GCD6 sequences revealed four regions with significant homology (figure 14). The highest degree of similarity was found in N-terminal regions which span amino acid 9 through 177 in eIF-2Bε and 4 to 172 in GCD6. (figures 10, 15). Within this region, 55% of the amino acids are either identical or conservative substitutions. Strong similarity was also observed in region 2 which includes residues 221 to 283 of eIF-2Bε and residues 220 to 283 of GCD6 (Figures 14, 15) where 51% of the amino acids are either identical or are conservative substitutions. A threonine residue at position 281 is conserved in both eIF-2Bε and GCD6 and forms part of a concensus casein kinase II phosphorylation site.

Figure 13. Alignment of the Amino Acids of eIF-2Bε and GCD6.
The alignment of eIF-2Bε amino acid sequence with that of GCD6 was performed with best fit program. In this alignment, lines indicate identical amino acid substitutions, colons indicate conservative substitutions with similarity scores greater than 1.0 and periods represent similarity scores between 0.5 to 1.0.

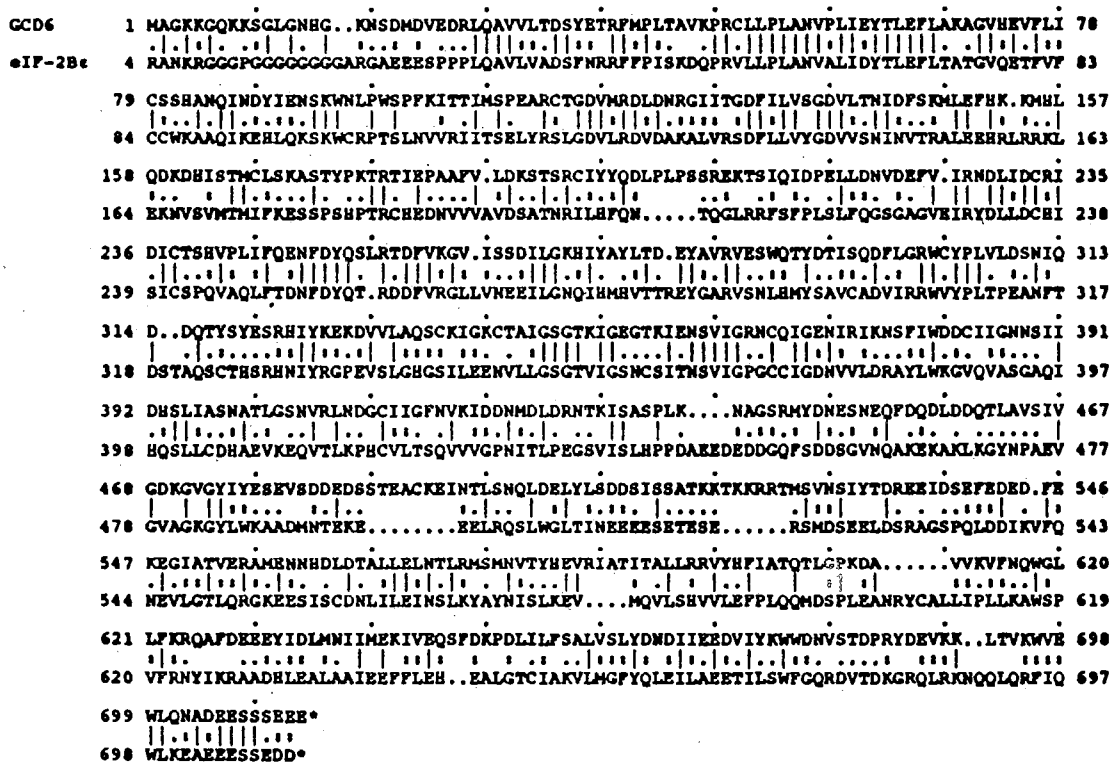
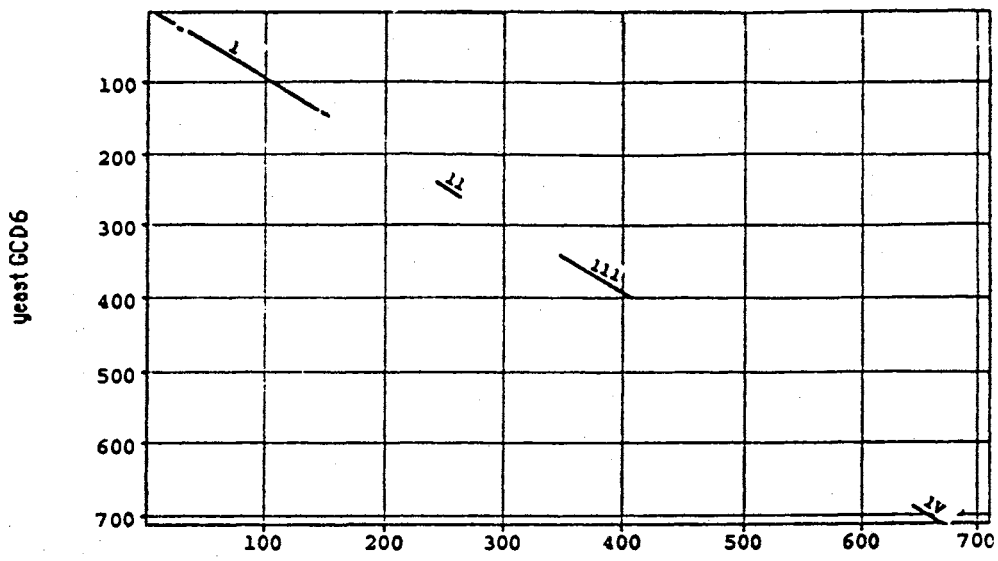

GCD6
eIF-2Bε

Figure 14. Dot-matrix Comparison of eIF-2Bε and GCD1.
Dot-matrix comparison analysis of the two proteins were performed with a window of 60 and stingency of 15. Four regions of sequence similarity between the two protein were apparent. Regions 1, 2, and 3 had over 50% amino acid similarity between the two proteins, while region 4 showed a low percentage similarity of 21%. The strongest sequence similarity was observed at the N-terminal of the two proteins.


yeast GCD6
100
200
300
400
500
600
700
100
200
300
400
500
600
700
I
II
III
IV
eIF-2Bε.

Cysteine-237 is also conserved on both sequences in this region. The third region of sequence similarity extends from amino acid 322 to 428 of eIF-2Bε and 316 to 423 of GCD6 (figure 14 and 15). 46% of the amino acids within the region are either identical or are conservative substitutions. A fourth region of identity was found between residues 639 to 669 of eIF-2Bε and 681 to 715 of yeast GCD6. However, this region is only 21% similar (figure 14, 15).

In addition to the strong sequence similarity in Region 2 and 3, both protein sequences have three cysteine residues that are conserved. In region 2, cysteine-232 is conserved at the same position on both proteins. The other conserved cysteines are present in region 3 at positions 372 and 418 in eIF-2Bε, and at positions 367 and 413 in yeast GCD6. The second amino acid following these conserved cysteines is either a leucine or isoleucine in all cases. Similar internal repeats containing cysteines have also been observed in yeast GCD1, suggesting that they may play a significant role in the secondary structure of these proteins.

Glycerol Gradient Analysis of In Vitro Translated eIF-2Bε

To provide additional proof that the pA1 cDNA encoded protein is the authentic eIF-2Bε, the translation products of the cDNA were subjected to glycerol gradient centrifugation to determine if their sedimentation properties are identical to subunits of authentic eIF-2B. Following centrifugation, fractions were collected and subjected to SDS-PAGE and autoradiography. Three protein bands of approximately

Figure 15. Amino Acid Sequence of the Regions with Similarity Between eIF-2Bε and GCD6.

Line 1 corresponds to GCD6 sequences and line 2 corresponds to eIF-2Bε sequences.

Region 1 is comprised of amino acid residues 9 to 177 of eIF-2Bε and 4 to 172 of GCD6;

Region 2 includes amino acid residues 220 to 283 of eIF-2Bε and residues 220 to 283 of yeast GCD6;

Region 3 shows similarity between amino acids 322 to 428 of eIF-2Bε and 316 to 423 of yeast GCD6;

Region 4 is toward the C-terminal of both proteins and includes amino acids 639 to 669 of eIF-2Bε and 681 to 715 of GCD6.

↓ conserved cysteines and threonine.

Region 1

```
1     kk Gqkks GlGnh gknsd mdved rLQAV vltDS yetRF mPlta vkPRc LLPLA NVpLI>
      vv ^-vv- ^v^-v -^--^ v^--- -^^^^ ^^-^^ ^--^^ -^^-- v-^^v ^^^^^ ^^-^^
2     GG GPGGG GGGGG ARGAE EESPP PLQAV LVADS FNRRF FPISK DQPRV LLPLA NVALI

1     eYTLE FLaka GVhEv FliCs shAnQ Indyi enSKW nlPwS pfkit tImSp eaRct>
      ^^^^^ ^^--- ^^^^- ^^-^- v-^-^ ^-^-^ ^-^^^ vv^v^ vvv^- -^-^- vv^-v
2     DYTLE FLTAT GVQET FVFCC WKAAQ IKEHL QKSKW CRPTS LNVVR IITSE LYRSL


1      GDVmR DlDnr giitg DFiLV sGDVl tNIdf skmLE fHkkm hlqdK dhist mclsK aS>
       ^^^^^ ^^^-^ -^^-- ^^^^^ v^^^^ -^^^- -^-^^ v^^v- ^vv^^ ^v--- -v^v^ -^
2      GDVLR DVDTK ALVRS DFLLV YGDVV SNINV TRALE EHRLR RKLEK NVSVM TMIFK ES
```

Region 11

```
                          ↓    ↓                                                ↓
1      dnvd efvIR nDLiD CrIdI CtshV pliFq eNFDY Qslrt dfvkg vissd ILGkh Iyayl Tde>
       ---- --v^^ v^^^^ ^^^-^ ^--^^ -v^^- ^^^^^ ^-v-- v-vvv ^^--^ ^^^-^ ^---^ ^--
2      GSGA GVEIR YDLLD CHISl CSPQV AQLFT DNFDY QTRDD FVRGL LVNEE ILGNQ IHMHV TTR
```

Region 111

```
                                                                  ↓
1      Qtys yesrh IYkek dVvLa qscki gkcta iGSGT kIGeg tkIeN SVIGr nCqIG eNiri>
       ^--- ---^^ ^^^-- ^^-^- ^--v^ --v-v ^^^^^ v^^-- v-^-^ ^^^^- -^v^^ ^^^v^
2      QSCT HSRHN IYRGP EVSLG HGSIL EENVL LGSGT VIGSN CSITN SVIGP GCCIG DNVVL
                                                  ↓
1      knsfi Wddci ignns iIdhS Liasn Atlgs nVrLn dgCii gfnVk idd>
       ---^^ ^--vv ^---- v^-^^ ^^v-^ ^-^v- -^-^- -v^^^ -v-^v ^--
2      DRAYL WKGVQ VASGA QIHQS LLCDH AEVKE QVTLK PHCVL TSQVV VGP
```

Region 1V

```
1      vstd pryde vkklT vkwve wlqna deEss sEEe*>
       ---^ vv-^- v-vv^ vvvvv v^-vv ^v^-v -^^-v
2      AIEE FFLEH EALGT CIAKV LMGFY QLEIL AEETI
```

82, 65, and 58 kDa of eIF-2B and a fourth of 72 kDa were observed in fractions with the same sedimentation properties as those expected for purified eIF-2/eIF-2B complex (figure 16). This result suggests that in vitro translated eIF-2Bε is incorporated into endogenous eIF-2/eIF-2B complex. In addition, the continued presence of the 65 and 58 kDa polypeptides indicate that they might be authentic eIF-2B subunits or that the N-terminal amino acids of eIF-2Bε are not required for the incorporation of eIF-2Bε into the eIF-2B complex. At present the peptide sequences of these subunits are unavailable for comparison.

Characterization of eIF-2Bε mRNA

In order to determine if the pA1 (eIF-2Bε) cDNA is full length we performed Northern blot analysis of mRNA isolated from rabbit reticulocyte lysates. When reticulocyte lysate poly (A^+) RNA was probed with radiolabeled P1068, a single mRNA of approximate 2.8 kb was observed (figure 17) which is very close in size to the 2510 kb insert. The approximately 300 base pairs difference in length between the observed message and the cDNA can be accounted for in part by the absence of a poly (A^+) tail on isolated cDNA. In addition, it is possible that the 5′ terminus lacks some 5′ non-coding sequence. However, the size of the eIF-2Bε mRNA is very close in size to the cDNA pA1 insert and suggests that the pA1 clone contains the complete coding region of eIF-2Bε gene.

Figure 16. Glycerol Gradient Analysis of the Products of the In vitro Translation of eIF-2Bε cDNA.

In vitro translated pA1 was fractionated on a 15% to 50% glycerol gradient and protein fractions collected. Proteins precipitated from these fractions were redissolved in sample buffer, electrophoresed on a 10% SDS gel and transferred to PVDF membrane. Autoradiography showed incorporation of in vitro translated eIF-2Bε into endogenous eIF-2/eIF-2B complex. Lanes 15 and 16 shows the incorporation of eIF-2Bε, the 67 and 58 kDa subunit of eIF-2B into this complex.

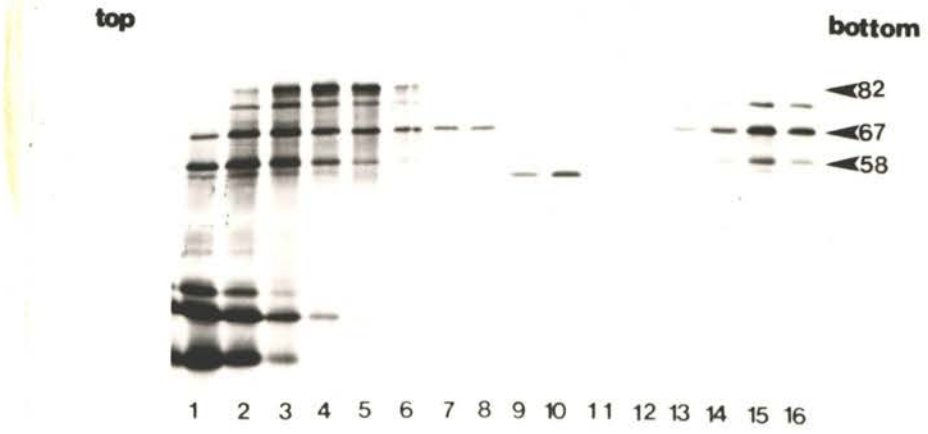
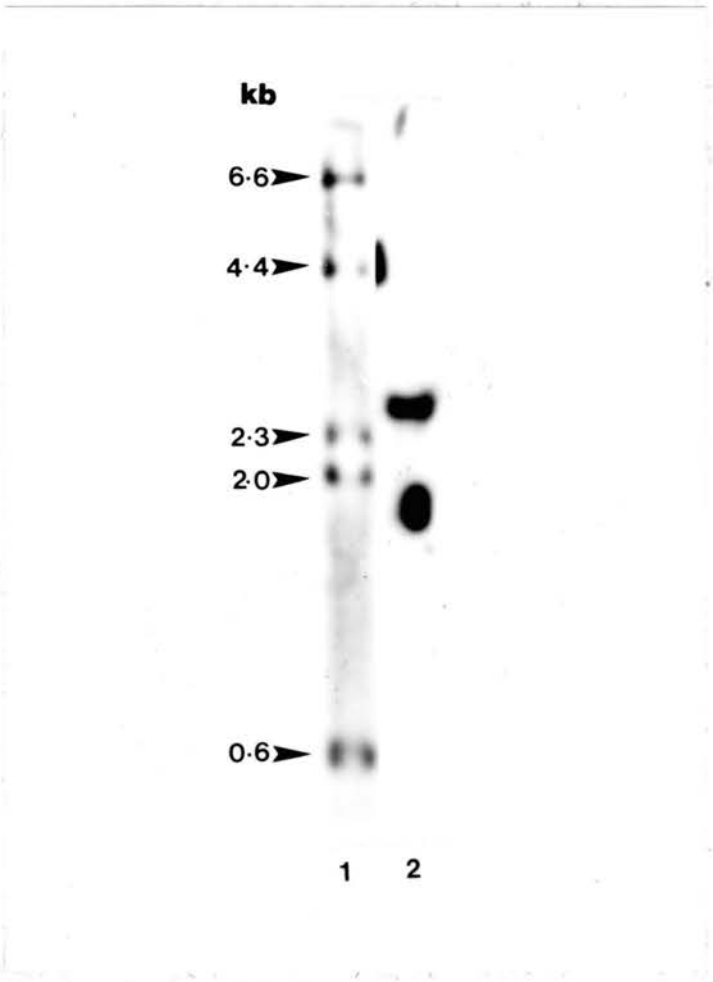

top
bottom
82
67
58
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 17. Northern Blot Analysis of eIF-2Bε mRNA.
10 μg of glyoxated poly (A^+) RNA from rabbit reticulocyte lysate was electrophoresed on a 1.2% agarose gel. The fractionated RNA were transferred to a nylon membrane and probed with radiolabelled P1068. Lanes: 1, Biotinylated DNA standard; 2, Poly(A^+) RNA from rabbit recticulocyte lysate.


kb
6·6
4·4
2·3
2·0
0·6
1
2

CHAPTER V

DISCUSSION

We have isolated and characterized a 2516 bp cDNA encoding the 82 kDa subunit of rabbit reticulocyte lysate eIF-2B. The identification of eIF-2Bε was based on the following criteria. First, expression of pA1 cDNA in *E.coli* results in production of a 88 kDa fusion protein that specifically cross reacts with a chicken anti-eIF-2B antiserum. Second, the open reading frame of pA1 cDNA contains two peptide sequences obtained from amino acid microsequencing of V8 fragments of SDS-PAGE purified eIF-2Bε. Third, the deduced amino acid sequence has strong homology with yeast GCD6, a protein proposed to be the eIF-2Bε analog in yeast (73).

The isolated cDNA encodes full-length eIF-2Bε. This conclusion is based on the following. (i) The open reading frame of isolated cDNA extends from nt 23 to nt 2158 and encodes protein with a calculated molecular weight of 79 kDa. When the possible mobility change induced by phosphorylation of eIF-2Bε is taken into account, the 79 kDa molecular weight is close to the 82 kDa observed for non recombinant eIF-2Bε on SDS polyacryamide gels. (ii) Northern blot analysis of mRNA corresponding to eIF-2Bε cDNA indicates that both are almost the same size.

(iii) Upon in vitro translation in nuclease treated reticulocyte lysate of mRNA transcribed in vitro from pA1 cDNA, one of the translation products comigrated with the 82 kDa subunit of endogenous eIF-2B. (4) The 82 kDa polypeptide is no longer expressed from mRNA transcribed in vitro from cDNAs containing deletion from either the first 172 nt or nt 230 and 369 at the 5'end of eIF-2B cDNA. The inability to express eIF-2Bε from any of the three truncated mRNA shows that eIF-2Bε translation must be initiated from a non AUG codon since the first AUG codon is located at nucleotide 550. Further, the similarity between eIF-2Bε and GCD6 is highest at the N-terminal of both proteins which is part of the open reading frame that precedes the first AUG. The amino acid residues between 30 to 49 residues on eIF-2Bε has one of the highest similarity indicating that GTG and CTG beginning at nt 128 and nt 131 precedes the initiation codon. Therefore, the GTG codon at nucleotide position 23 is the most likely candidate for initiation.

In most mammalian mRNAs studied to date, non AUG codons are used for initiation in conjunction with the AUG codon (69). eIF-2Bε is unique in this regard in that its translation from this cDNA appears to be initiated exclusively from a non AUG codon. However, the possibility exists that there might be an AUG codon in frame with the eIF-2Bε translational reading frame upstream of the proposed initiation codon that is not present in the pA1 cDNA.

The eIF-2Bε cDNA contains several characteristics that is associated with genes whose translation are initiated from non AUG codons. The 5′ terminus is extremely GC rich with the region of highest GC content closely following the proposed

initiating codon. In genes that lack an AUG codon, GC-rich sequences have been proposed to facilitate formation of secondary structure which may slow down ribsome scanning and result in initiation at non AUG codons. However, translation initiation at non AUG codons is generally inefficient and we found that the expression of the 82 kDa product from the transcribed mRNA in nuclease treated lysate was generally less efficient than the expression of the lower molecular weight products that presumably initiate at internal AUGs.

The high GC content of the 5′ sequences in the eIF-2Bε cDNA also suggests that the expression of eIF-2Bε may be highly regulated as would be expected of a protein involved in translation initiation. Other mammalian mRNAs that have been found to contain GC rich 5′ termini encode proteins that play key roles in cell growth (69). Because GC rich 5′ termini modulate expression of proteins by forming secondary structures that impede translation, the lower translational efficiency results in low levels of these proteins, which is often the case for proteins whose expression is tightly regulated. Consistent with an essential role in cell growth for eIF-2B, deletion of any of the GCD genes in yeast results in cells that are non viable.

The primary amino acid sequence of eIF-2Bε showed some distinct features. One of these features is the cluster of 11 glycines at its N-terminus. Eight of these glycines are tandemly repeated. Glycine rich domains have been implicated in poly (A^+) tail binding, raising the possibility that eIF-2Bε may interact with the poly (A^+) tail of mRNA. Van Heugten *et. al.* (60), have recently shown that eIF-2/eIF-2B

complex interacts with the cap structures of mRNAs. Secondary structure analysis of the deduced eIF-2Bε sequence also indicates that the glycine rich region is highly flexible. This flexibilty may be necessary for its role in eIF-2B activity/function. Of interest is the identification of nine of these glycines as possible myristoylation sites. Although they are all internal sequences, proteolytic processing may leave any of them as the N-terminal residue, which would be capable of being myristoylated. Thus, the N-terminal block observed for the eIF-2Bε subunit may possibly be myristate.

Another feature of the eIF-2Bε sequence is the presence of six potential casein kinase II sites. The most notable site is located at ser-514, which contains identical sequence to the site phosphorylated by casein kinase in fibrogen. Other potential casein kinase II phosphorylation sites include Thr-493 and Thr-507, which both contain consensus sequences followed by two or more acidic residues. Similar clusters of acidic residues at the C-terminal side of casein kinase II phosphorylation sites have been shown to increase the likelyhood of phosphorylation (65). In addition, these residues are in a region with one of the highest surface probabilities, indicating that they should be accessible to kinase. Ser-706 is of particular interest because this potential casein kinase II phosphorylation sequence is conserved in both eIF-2Bε and yeast GCD6. This observation suggests that this amino acid residue may be a phosphorylation site, if the regulation of eIF-2B activity through the phosphorylation of its 82 kDa subunit is conserved evolutionarily. Phosphopeptide

mapping, specific mutations and characterization of the mutants will be necessary to determine the exact residues phosphorylated.

Comparison of the sequence of eIF-2Bε with yeast GCD6 revealed strong similarity between both proteins. The 30% overall identity between the proteins was the highest among any of the putative subunits of yeast eIF-2B suggesting that both proteins may share a common function in eIF-2B. The shared function may be mediated via their N-terminal since this region showed the strongest sequence similarity. Another region of interest includes the pocket of amino acids with conserved cystines followed by leucine or valine at the +2 positions. Similar patterns are observed in GCD1 and GCD7, which are both postulated to be subunits of yeast eIF-2B. Perhaps these cystines are required for folding/assembly of eIF-2B.

The expression of the 72 kDa, 65 and 58 kDa polypeptides from mRNA transcribed from the eIF-2Bε cDNA was not cap dependent, suggesting that their expression may have no in vivo significance. Although, they comigrated with the γ and δ subunits of eIF-2B, these eIF-2B subunits are unlikely to be encoded by the same mRNA. In yeast, the five genes postulated to encode the subunits of yeast eIF-2B have been cloned and sequenced, making it unlikely that a single gene will encode three eIF-2B subunits in mammalian cells (72). Moreover, the 65 kDa and 58 kDa subunit of eIF-2B are both proposed to bind ATP, but 82 kDa does not. Since the expression of 72, 65, and 58 kDa polypeptides in vitro is cap independent, we suggest that they are due to internal initiation from the eIF-2Bε DNA. The observed incorporation of the 72, 65 and 58 kDa polypetide into endogenous eIF-2B

complex suggests that the amino acid sequences lacking in these polypeptides are not necessary for incorporation of eIF-2Bε into eIF-2B complex.

Future Studies

The isolation and characterization of eIF-2Bε cDNA provides the first primary structure of any of the subunits of eIF-2B. This will facilitate the characterization of the function of eIF-2B. To facilitate characterization of eIF-2B, accurate knowledge of the initiating codon for eIF-2Bε cDNA is necessary. This can be accomplished by:

1. Specific mutation of the postulated initiation codon and analysis of the effect of the mutation on eIF-2Bε expression.
2. Isolation and analysis of the genomic DNA for eIF-2Bε, to obtain its full 5′ non coding sequence and verify that there is no upstream AUG in frame with its translational reading frame. In addition, information will be obtained on possible upstream sequences that may be important for the binding of transcription factors that regulate eIF-2Bε transcription.

The determination of the residues present in eIF-2Bε that are phosphorylated by casein kinase II is of great interest. This will require microsequencing of radiolabeled peptide fragments obtained from eIF-2Bε phosphorylated in the eIF-2B complex in vivo and by casein kinase II in vitro. The comparison of the sequences of these fragments with the deduced sequence of eIF-2Bε could enable the

determination of the phosphorylated residues and whether the site phosphorylated by casein kinase II in vitro is also phosphorylated in vivo. Determining the phosphorylated residues would enable specific mutation(s) to be designed that would facilitate determining if phosphorylation actually has a regulatory effect in vivo.

Expression of eIF-2Bε would make larger quantities of the protein available for the production of antibodies specific for this subunit. The antibody currently available is limited in its usefullness since it recognizes only denatured eIF-2B. Production of antibody to eIF-2B is expected to solve this problem and facilitate the quantitation of eIF-2B in vivo.

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