MOLECULAR CLONING AND SEQUENCE ANALYSIS OF HUMAN EUKARYOTIC INITIATION FACTOR 2B (eIF-2B) 82-kDa SUBUNIT PARTIAL cDNA

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ATP	Adenosine Triphosphate
cDNA	complementary DNA
DNA	Deoxyribonucleic Acid
dsI (DAI)	double-stranded RNA activated Inhibitor
EtBr	Ethidium Bromide
GDP	Guanosine Diphosphate
GEF	Guanine nucleotide Exchange Factor
GTP	Guanosine Triphosphate
HRI	Heme Regulated Inhibitor
IPTG	Isopropyl-β-D-Thiogalactopyranoside
kb	kilo-base pairs
kDa	kiloDalton
mRNA	messenger RNA
PCR	Polymerase Chain Reaction
pfu	plaque forming unit
RF	Reversing Factor
RNA	Ribonucleic Acid
tRNA	transfer RNA
UV	UltraViolet
X-gal	5-Bromo-4-Chloro-3-Indolyl-β-D-Galactoside

CHAPTER I

INTRODUCTION

Protein synthesis is an integral part of the pathway of gene expression and makes important contributions to modulation of the expression of specific genes (1, 2). Being a highly energy demanding process, rates of global protein synthesis must be controlled in order to be in accord with the overall metabolic activity of the cell (3). The dominant mechanism of control of global protein synthesis is phosphorylation/dephosphorylation of translational components, primarily initiation and elongation factors (4, 45). It is generally believed that the rate limiting step of protein synthesis under most physiological conditions is the initiation phase (5, 6). To date, studies on the phosphorylation of the α subunit of the eukaryotic initiation factor 2 (eIF-2) constitute one of the best characterized regulatory mechanisms known. In order to understand how translation is regulated at this level, knowledge of the mechanism of protein synthesis is required and is thus briefly described below (7).

In eukaryotic cells, the translation of mRNA occurs in the cytoplasm. It can be divided into three phases, namely initiation, elongation and termination, which are promoted by the action of a group of protein factors known as initiation factors (eIFs), elongation factors (eEFs) and a release factor (eRF, also termination factor), respectively. In the first step of initiation, free eukaryotic 80S ribosomes are in equilibrium with their 40S and 60S subunits. eIF-3 and eIF-4C shift the equilibrium towards ribosome dissociation by binding to the 40S subunit and thus inhibiting the joining of the 60S subunit (8). The resulting structure is the 43S ribosomal complex. eIF-6 is thought to act similarly by reacting with 60S rather than 40S subunits (9). In the next step, eIF-2 binds

GTP and the initiator tRNA, Met-tRNA$_i$ (also Met-tRNA$_f$), in a ternary complex (10). Ternary complex formation is the committed step of initiation. The resulting complex can then bind to the 43S ribosomal complex to form the 43S preinitiation complex (11).

On the other hand, mRNA forms a complex with eIF-4A, eIF-4B and eIF-4F in an ATP-dependent reaction. eIF-4F is believed to be the 5'-cap binding protein, while eIF-4A and eIF-4B are ATP-dependent helicases which are thought to play a role in melting mRNA secondary structures (12, 13). The 48S preinitiation complex is formed upon binding of the mRNA complex to the 43S preinitiation complex near the 5'-cap structure. The 40S ribosomal subunit subsequently scans down the mRNA to select the proper start site. The anticodon of the Met-tRNA$_i$ probably participates in the scanning and recognition of the AUG start codon. The joining of the 48S preinitiation complex and the 60S subunit is catalyzed by eIF-5 which has a ribosome-dependent GTPase activity. The joining reaction is accompanied by the release of the eIF-3, eIF-4C and eIF-2·GDP binary complex (14). The formation of the resulting 80S initiation complex completes the initiation phase of translation. However, the released eIF-2·GDP binary complex is nonfunctional in translational initiation. To reutilize eIF-2 in the subsequent rounds of initiation, the bound GDP must be substituted by GTP (15). At physiological concentration of Mg^{2+} (>1 mM), the binding affinity of eIF-2 for GDP is approximately 400-fold greater than that for GTP (K_d GDP ~ 5.0×10^{-9} M versus K_d GTP ~ 1.6×10^{-6} M) (16), and eIF-2·GDP is so stable that the bound GDP dissociates at an unusually slow rate ($t_{1/2}$ > 30 minutes at 30°C) (17). This difference in affinity and the slow dissociation rate of the eIF-2·GDP complex under physiological conditions makes it necessary to have another initiation factor, eIF-2B (also referred to as "RF", or "GEF") to catalyze the GDP-GTP exchange reaction for eIF-2 recycling.

One apparent exception to this general scheme is wheat germ extracts (44), where eIF-2 has been found to have four rather than three subunits and its affinity for GDP is only 10 times higher than that for GTP (K_d GTP ~ 1.5×10^{-6} M), suggesting that at a

sufficiently high [GTP]/[GDP] ratio, the guanine nucleotide exchange reaction in wheat germ cells may take place without other specific factors such as eIF-2B.

Phosphorylation of the α-subunit of the eIF-2 is believed to be a general pathway in controlling global protein synthesis. Extensive research has been done in the rabbit reticulocyte lysate *in vitro* translation system. Phosphorylation of eIF-2α has no effect on the binding affinity of eIF-2 for GDP (18), nor does it affect spontaneous dissociation of GDP from eIF-2 (19). Rather, eIF-2(αP)·GDP binds tightly to eIF-2B to form a complex whose GDP is readily exchangeable yet the eIF-2B in the resulting eIF-2(αP)·eIF-2B complex is essentially nondissociable and thus nonfunctional (19). Since eIF-2B is present in lysates at a much lower concentration than eIF-2 (20), phosphorylation of only 20-40% of the eIF-2α present is sufficient to bind all the available eIF-2B in a nonfunctional complex. This will effectively impede eIF-2 recycling and shut off global protein synthesis.

The single phosphorylation site on the eIF-2α has been identified as residue Ser-51 (21). Two kinases are specific for eIF-2α in reticulocyte lysate, the heme-regulated eIF-2α kinase (HRI) and the double-stranded RNA activated inhibitor (DAI or dsI). HRI is activated in response to hemin deficiency, addition of oxidized glutathione, heavy metals, and heat shock (7, 22-24), while DAI is sensitive to low levels of dsRNA (25, 26). Activation of either of these two kinases results in the phosphorylation of the same Ser-51 residue of eIF-2α, followed by an abrupt decline in protein synthesis rate and disaggregation of polyribosomes. On the other hand, a protein phosphatase (classified as type-2A) (27) for eIF-2(αP) exhibits broad specificity (28). Also temporal correlations of phosphorylation and activity have been made, a change in factor activity has been demonstrated *in vitro* (29), and *in vivo* evidence has been generated for a role in translational control (30). All of these provide a convincing although possibly incomplete explanation for translational repression by HRI or DAI.

Elucidation of the mechanisms controlling the rate of protein synthesis in other cells has lagged somewhat behind the studies with reticulocytes. However, it is becoming clear

that regulation at the level of eIF-2 function is by no means unique to the reticulocyte. Kinases and phosphatases acting on eIF-2α have been identified in a wide variety of cells and tissues (31-35); eIF-2B preparations with very similar activity and subunit structure to the reticulocyte factor have been isolated from Ehrlich ascites cells (36) and rat liver (37) and a protein fraction with eIF-2B activity has been obtained from neuroblastoma cells (38). It is probably true that altering eIF-2B activity via eIF-2α phosphorylation is a general mechanism of controlling global protein synthesis used by many, if not all, eukaryotic cells.

Although a regulatory role for eIF-2B in global protein synthesis has been implicated, the story is possibly incomplete. Substantial variation of the eIF-2B/eIF-2 ratio in different cell types and different levels of eIF-2α phosphorylation in different cell types have been observed, raising the possibility that a component limiting in one cell type or physiological state may not be limiting in another. If this is true, the reason for this difference needs to be found. Also, the subcellular distribution and concentration of eIF-2B and eIF-2 remains unclear. The central assumption that eIF-2B is far less abundant than eIF-2 can only be stated confidently after these issues are resolved. For the protein itself, its reaction mechanism, the exact nature of its interaction with both phosphorylated and dephosphorylated eIF-2, its interaction with possible cofactors or allosteric regulators (22, 41), the specific role of its five subunits, and the possible physiological role of phosphorylation on its 82-kDa subunit (eIF-2Bε) remain to be determined. This study seeks to understand the structure and function of human eIF-2B by cloning and sequencing the complementary DNA (cDNA) of its 82-kDa ε subunit.

CHAPTER II

LITERATURE REVIEW

Properties of eIF-2B

The eukaryotic initiation factor 2B (eIF-2B, also referred to in the literature as RF, or GEF) is a nonribosomal associated multipolypeptide factor consisting of 5 asymmetric subunits with approximate molecular weights of 82, 65, 55, 40, and 32 kDa respectively (18, 19, 36, 39, 40). Purified from both post ribosomal supernatants and ribosomal salt washes, eIF-2B has been isolated in either a free form or complexed in a 1:1 stoichiometry with eIF-2 (18, 19, 36, 39-41). In all cases, eIF-2B shows similar subunit composition, although slight variations in the molecular weights of the individual subunits have been observed in different labs. All five subunits appear to exist in equal molar amounts. This conclusion is further supported by the agreement between its calculated molecular weight of 274 kDa and its apparent molecular weight of about 250 kDa determined by sucrose gradient centrifugation (40). The purified eIF-2·eIF-2B complex has an apparent molecular weight of 450 kDa and a calculated molecular weight of 418 kDa and this complex can be dissociated at high salt concentrations (40). The reason for the slight disagreement between 450 kDa and 418 kDa is unclear although impurities and/or true missing structural components have been proposed to account for the difference (40).

At least two subunits of eIF-2B can be phosphorylated. The 82 kDa ε subunit can be phosphorylated by casein kinase II *in vitro* in the presence of ATP (42). The extent of phosphorylation is 0.55 mole of phosphate per mole of eIF-2B, and this results in a 2.3 fold increase in eIF-2B activity. Alkaline phosphatase treatment will reduce the activity of this protein by a factor by 5. These observations suggest that eIF-2B phosphorylation

contributes to the control of eIF-2 recycling, although it is not known if eIF-2Bε subunit phosphorylation is regulated *in vivo*. The 65 kDa δ subunit can also be phosphorylated. This has been suggested to be autophosphorylation by an intrinsic kinase activity of eIF-2B. The functional significance of this phosphorylation is not known (18).

Photochemical labeling with the 8-azidopurine analogs reveals that the 40 kDa β subunit of eIF-2B binds GTP, and that the 55 kDa γ and the 65 kDa δ subunits bind ATP·Mg^{2+} and $NADP^+$ interferes with these bindings. Spectrofluorometric analysis of eIF-2B suggests that NADPH is bound to eIF-2B in a 2:1 ratio (41). The binding of NADPH can be enhanced by glycerol, dithiothreitol and limited dialysis. The findings that NADH or NADPH have no effect on eIF-2B activity, while NAD^+ or $NADP^+$ inhibit the GTP-dependent nucleotide exchange reaction, raise the possibility that eIF-2B activity can be regulated by the redox state of the cell. However, further *in vivo* studies are required to make this issue clear.

eIF-2B is generally believed to exist in the cytoplasm and is defined as a nonribosomal associated initiation factor. But recent observations have indicated that eIF-2·GDP binds tightly to the 60S ribosomal subunit. The dissociation of eIF-2·GDP complex from 60S subunit is promoted by eIF-2B (46), suggesting eIF-2B may also interact transiently with 60S subunit. In fact, the inability of eIF-2B to dissociate eIF-2(αP)·GDP from 60S subunit has led to the proposal that inhibition of protein synthesis due to eIF-2α phosphorylation is mediated, at least in part, by this mechanism (47). However, a different view is held by other authors who doubt the role of 60S ribosomal subunits as the direct carrier of eIF-2·GDP in the release and recycling of eIF-2 in initiation reactions (48). So the biological function of the association of eIF-2 or eIF-2·eIF-2B with 60S ribosomal subunits remains unclear.

Proposed Catalytic Mechanisms of eIF-2B

The precise mechanism by which eIF-2B catalyzes the guanine nucleotide exchange reaction is not clearly understood. Various models have been proposed (65-74), yet the exact sequence of events involved during the exchange reaction has not been clearly established. The overall reaction catalyzed by eIF-2B may be written as

$$\text{eIF-2}\cdot\text{GDP} + \text{GTP} \leftrightarrow \text{eIF-2}\cdot\text{GTP} + \text{GDP}$$

The most common model for a two-substrate -two-product reaction may be either a sequential or a ping pong mechanism, with the sequential model as being far the more common one.

Ochoa (65) proposed that the eIF-2B catalyzed reaction follows a ping pong mechanism in which eIF-2B binds eIF-2·GDP to form a ternary complex. Then GDP is released after which GTP is bound. eIF-2B then releases eIF-2·GTP which in turn forms a ternary complex with Met-tRNA$_i$.

Another ping pong mechanism suggested by Safer (69) is quite similar, but differs in that the functioning eIF-2B exists inside a ternary complex including eIF-2 and GTP/GDP. The eIF-2·GDP complex displaces eIF-2·GTP bound to eIF-2B, to give eIF-2·GDP·eIF-2B. GTP then replaces the GDP inside this complex to regenerate the active eIF-2·GTP·eIF-2B ternary complex. The eIF-2·GTP that is released is used to form eIF-2·GTP·Met-tRNA$_i$.

The ping pong model of Salimans *et al* (67) is the same as that of Ochoa, except after the addition of GTP to the eIF-2·eIF-2B complex. In this model, Met-tRNA$_i$ subsequently joins to form a quaternary complex. Upon binding of the 40S ribosomal subunit, eIF-2B is released while eIF-2·GTP·Met-tRNA$_i$ stays on the ribosome.

Enzyme kinetic data from Henshaw's lab (71) support a ping pong mechanism as the mode of eIF-2B function. This mechanism proposes the existence of a eIF-2B·eIF-2·GDP complex and excludes the possibility of two guanine nuceotide binding sites on eIF-2.

A sequential model has been proposed by Goss (68) *et al.*, in which both eIF-2·GDP and GTP must be bound by eIF-2B before any product is released. The product, eIF-2·GTP is quickly used to form eIF-2·GTP·Met-tRNA$_i$ ternary complex. Recently, Dholakia *et al.* (72) have found that eIF-2B is a GTP binding protein. Based on this observation and other data, a sequential model has been proposed for the guanine nucleotide exchange reaction. However, further information concerning substrate addition order (random or ordered) is not available.

Each of these models has been proposed on the basis of experimental data, yet no study has been definitive. The uncertainty may be due to several factors: the purity of the eIF-2B preparations; the instability of eIF-2B (66, 71); and the possible existence of more complicated mechanisms. Also, the activity of eIF-2B can be regulated by a variety of effectors (see below) which might somehow affect the reaction mechanism. Furthermore, the role of eIF-2B in protein synthesis is likely to be more complex than simply to increase the rate of GDP release. As reported by other investigators (46), eIF-2B may affect the binding of eIF-2 to 40S subunits and it may also have additional functions while complexed with ribosomal subunits.

Interestingly, analogous reaction mechanisms have been found in other systems. Investigators favoring the ping pong model point out that bacterial elongation factor EF-Ts displaces GDP from EF-Tu·GDP complex (76) by a mechanism similar to that which they propose, while those supporting the sequential model argue that the eukaryotic receptor G-protein system (75) may be considered analogous to the eIF-2B catalyzed reaction.

Regulation of eIF-2B Activity

The guanine nucleotide exchange activity of eIF-2B is mainly regulated by phosphorylation of the α-subunit of eIF-2 (7). Upon phosphorylation, eIF-2(αP)·GDP forms a tight complex with eIF-2B which is not readily dissociable (19). Since eIF-2B is believed to be much less abundant than eIF-2, phosphorylation of only a part of the eIF-2α

present is sufficient to abolish all available eIF-2B activity. Although originally studied in the rabbit reticulocyte lysate system, this mechanism is believed to be a general pathway in controlling global protein synthesis in many, if not all, eukaryotic cells. However, exceptions to this mechanism may exist. In the wheat germ system, eIF-2 has been isolated as a four (rather than a three) subunit factor. The 40 kDa β- and 42 kDa γ-subunits, but not the 37 kDa α-subunit can be readily phosphorylated by the dsRNA activated kinase (DAI) from rabbit reticulocytes (44). Furthermore, the affinity of eIF-2 for GDP is only 10 times that for GTP, making it possible that the GDP-GTP exchange at high [GTP]/[GDP] ratios may not require eIF-2B. These observations suggest that eIF-2B activity in wheat germ may not be regulated by phosphorylation of eIF-2 subunits. Another exception might arise from the controversial role played by 60S ribosomal subunit mediated interaction between eIF-2·GDP and eIF-2B (47, 48). The possible existence of other pathways for controlling global protein synthesis via eIF-2α phosphorylation may account for the substantial variations in the eIF-2/eIF-2B ratio observed and the different levels of eIF-2α phosphorylation in different eukaryotic cells (20, 31, 49). Clearly, there is still a great deal to learn concerning this.

Two kinases specific for eIF-2α and one phosphatase with broad specificity have been implicated in regulating the phosphorylation status of eIF-2α. HRI is active in response to hemin deficiency, addition of oxidized glutathione, heavy metals, methylene blue and heat shock, while DAI is activated by low levels of dsRNA. Both activated kinases phosphorylate the same Ser-51 residue on eIF-2α. Inhibition of eIF-2α phosphatase activity, while eIF-2α kinase activity remains unchanged, has also been reported to affect eIF-2B activity via changing the status of eIF-2α phosphorylation in rat livers deprived of single essential amino acids (50). In other studies, vasopressin (51) and mobilization of sequestered Ca^{2+} in the endoplasmic reticulum in rat liver (52), and a temperature sensitive mutation in Leu-tRNA synthetase in Chinese Hamster Ovary cells (53) have been demonstrated to cause changes in eIF-2α phosphorylation status. However,

the specific signal transduction pathways involved need to be identified. Since kinases and phosphatases acting on eIF-2α have been identified in a wide variety of cells and tissues (31-35), regulation of eIF-2B activity via regulating eIF-2α phosphorylation status through eIF-2α kinases and phosphatases is likely a general mechanism adopted by many other eukaryotic cells, although it is not yet clear if other kinases or phosphatases are also involved.

eIF-2B activity may also be regulated by various other factors without changing eIF-2α phosphorylation status. Glucose-6-phosphate (Glc-6-P) has been demonstrated to be required for maintaining active states of translation (22), but its mechanism of action is currently unsettled. Jackson *et al* (54) concluded that Glc-6-P has a dual role: it is required to provide a suitable reducing system; and, in addition, sugar phosphate has some unknown activating role on chain initiation. The reducing system is required to maintain HRI in an inactive form (54). In addition, Gross *et al* (56) demonstrate that Glc-6-P regulates, perhaps directly, the activity of eIF-2B in a manner that is independent of eIF-2α phosphorylation. If this is true, it may also account for the observed ability of insulin to stimulate eIF-2B activity in rat fast twitch fiber (57). Given the central position of Glc-6-P in glycolysis and the pentose phosphate pathway, the effect of Glc-6-P on eIF-2B activity might well integrate the rate of global protein synthesis with carbohydrate metabolism in the cell.

A variety of evidence suggests that redox potential may affect protein synthesis activity by influencing eIF-2B activity without changing of eIF-2α phosphorylation status. Regeneration of NADPH is known to be required for protein synthesis (22), and NADPH has been suggested to be an allosteric regulator of eIF-2B activity (41). However, other evidence suggests that NADPH itself may not be responsible for increased eIF-2B activity (58). In *in vitro* studies, the exchange of eIF-2-bound GDP is markedly inhibited by NAD^+ and $NADP^+$ and restored by NADPH (41). But eIF-2B activity in this system is dependent on the presence of Glc-6-P (56). In another study, where eIF-2α phosphorylation status

was not related to the eIF-2B activity, inhibition of protein synthesis in mixed fiber muscles of diabetic rats has been proposed to involve a change in the phosphorylation status of eIF-2Bε subunit or a change in the NADPH/$NADP^+$ ratio (59). However, the mechanism by which redox potential and NADPH affect eIF-2B activity is still unclear.

Met-$tRNA_i$ is also required for efficient recycling of eIF-2·GDP to eIF-2·GTP under physiological conditions (60). It is believed that Met-$tRNA_i$ stabilizes the exchange reaction product eIF-2·GTP by forming a Met-$tRNA_i$·eIF-2·GTP ternary complex.

Polyamines also appear to regulate eIF-2B activity (61). Although the precise mechanism of action is unclear, it has been proposed that polyamines exert their effect by regulating casein kinase II (62) which in turn activates eIF-2B (42).

In reticulocyte lysate, amino acid deficiency may lead to reduction of eIF-2B activity while no change in eIF-2α phosphorylation is observed (61). The reduction of eIF-2B activity is insufficient, by itself, to slow the rate of protein synthesis. The translational inhibition is exerted on polypeptide chain elongation, not initiation, suggesting that amino acid deficiency regulates eIF-2B activity by a different mechanism. This seems to have changed in nonerythroid cells. A study on a temperature-sensitive $tRNA^{Leu}$ synthetase mutant of Chinese Hamster Ovary cell shows that reduction in eIF-2B activity is associated with an increased level of eIF-2(αP) (62). However, recharging $tRNA^{Leu}$ *in vivo* at non-permissive temperature failed to reverse the inhibition of eIF-2 function, indicating that tRNA charging *per se* is not involved in the regulatory mechanism. In addition, aging has been shown to decrease eIF-2B activity in rats. No clear mechanism is available for this effect (64).

Given their important roles in controlling global protein synthesis, it is not surprising to see that so many factors can exert effects on eIF-2 and eIF-2B, either directly or indirectly. Such is in accord with the notion that protein synthesis must be controlled and integrated into the overall metabolic activity of the cell in order to respond properly to many

different stimuli, including environmental stresses, viral infection, and developmental signals.

Yeast eIF-2B Analog

Much of the information concerning protein synthesis regulation in yeast comes from the study of the control of amino acid biosynthesis. In response to amino acid starvation, the budding yeast *Saccharomyces cerevisiae* increases expression of the GCN4 protein, which in turn activates transcription of more than 30 genes encoding enzymes involved in amino acid biosynthesis (general amino acid control). Such increased expression of *GCN4* is primarily regulated at the level of translation initiation (77). Four upstream open reading frames (uORFs) in the leader of *GCN4* mRNA couple *GCN4* translation to the level of eIF-2 activity (78). Under non-starvation conditions, it appears that ribosomes scanning from the 5' end of *GCN4* mRNA translate the first ORF and reinitiate at one of the three remaining uORFs instead of at the *GCN4* start codon. Under starvation conditions, the GCN2 eIF-2α kinase becomes activated and phosphorylates yeast eIF-2α. In the presence of low levels of functional eIF-2·GTP·Met-tRNA$_i$ ternary complexes, ribosomes scanning downstream from uORF1 skip uORF2 to -4 and reinitiate further downstream at the *GCN4* start codon (79). Thus, a decrease in the eIF-2 activity (and thus the efficiency of reinitiation) leads to an increase in *GCN4* translation.

In higher eukaryotes, exchange of GDP for GTP is catalyzed by eIF-2B, a five subunit protein. A high molecular weight "GCD complex" associated with eIF-2 that contains GCD1, GCD2 and GCN3 was postulated to be eIF-2B in *S. cerevisiae* (80). Recently, two new essential genes, GCD6 and GCD7, have been identified in *S. cerevesiae* (81). Although direct biochemical experiments will be required to demonstrate an association of GCD6 and GCD7 with other GCD factors, many observations strongly suggest that GCD6 and GCD7 are additional subunits of the GCD complex. The most convincing evidence is the discovery that GCD6 is 30% identical in sequence to the 82 kDa

subunit of rabbit eIF-2B, with the greatest similarity occurring at their amino termini. The quality score for GCD6 aligned with eIF-2Bε was 36 standard deviations above the mean score for the randomized sequences, indicating a very high level of statistical significance for the sequence similarity between GCD6 and eIF-2Bε.

In higher eukaryotes, phosphorylation of eIF-2α plays an important role in controlling global protein synthesis according to the sequestration model (7). Dever *et al* (79) found that the yeast protein kinase GCN2 stimulates *GCN4* translation in amino acid starved cells by phosphorylating eIF-2α on Ser-51 residue. This evidence together with the finding that GCN2 is homologous to rabbit reticulocyte HRI and human DAI (82) strongly suggest that GCN2 plays a role in global regulation of protein synthesis in yeast by controlling phosphorylation status of yeast eIF-2 in response to signals such as amino acid starvation.

The observation that the budding yeast *S. cerevisiae* has a mechanism for regulating global protein synthesis which is quite similar to that of many higher eukaryotes, suggests that this mechanism is likely to be of vital importance since it is conserved from yeast to human.

CHAPTER III

MATERIALS AND METHODS

Materials

All restriction endonucleases were purchased from United State Biochemical (USB), Bethesda Research Laboratory (GIBCO BRL), or Promega; T4 DNA ligase, Exonuclease III, and S1 Nuclease were from Promega; the BIOPRIME™ DNA Labeling System was purchased from GIBCO BRL; the pA1 plasmid containing the rabbit eIF-2Bε cDNA was obtained from this lab (Dr. Agatha Asuru); the human histiocytic lymphoma (U-937) cell cDNA library constructed in λgt10 was a gift kindly provided by Dr. N. Shaun B. Thomas (University College London); the bacterial strain *E. coli* C600*hfl* (for lambda phage) was from Promega and MAX EFFICIENCY DH5α™ Competent Cells were from BRL; the 150 mm and 82 mm nylon membrane circles were from MagnaGraph (Micron Separations Inc.) and BRL, respectively; the streptavidin-alkaline phosphatase (SA-AP) conjugate was bought from BRL; the 5' RACE kit for rapid amplification of cDNA 5' Ends was purchased from GIBCO BRL; the human gene specific primer 1 (hGSP1) was synthesized by Oklahoma State University Core Facility; the nested human gene specific primer 2 (hGSP2) was synthesized by National Biosciences Inc. (NBI); the human HeLa cell total RNA was from Clontech; the GeneAmp^R PCR reagent kit with AmpliTaq^R DNA polymerase was bought from Perkin Elmer Cetus Corp.; chemicals for color detection (NBT, Nitroblue Tetrazolium Chloride; BCIP, 5-Bromo-Chloro-3-Indolylphosphate) were from Sigma; Sequenase^R Version 2.0 DNA sequencing Kit was purchased from USB and dATPαS[^{35}S] was from New England Nuclear Research products of DuPont; ultrapure sequencing reagents (urea, acrylamide and bis-acrylamide)

were bought from USB. All other chemicals and reagents are from Sigma, USB, EM Science (of EM Industries, Inc.) or Fisher Scientific.

Media prepared: LB medium is 1% (w/w) Bactotryptone, 1% (w/w) NaCl, and 0.5% (w/w) yeast extract; LM agar is 1% (w/w) Bactotryptone, 0.5% (w/w) yeast extract, 10 mM NaCl, 10 mM $MgSO_4$, and 1.5% agar; and TAXI plates are 5 μg/ml Thiamine, 20 μg/ml ampicillin, 33 μg/ml X-gal, and 8.1 μg/ml IPTG in LM agar.

20 x SSC is 3.0 M NaCl and 0.3 M sodium citrate, pH 7.0.

Methods

Nonradioactive (Biotin-14-dCTP) Labeling of DNA Probes

The 2.5 kb rabbit reticulocyte eIF-2Bε cDNA was digested out as a *Bam*HI-*Hin*dIII (USB) fragment from about 10 μg of CsCl-EtBr gradient purified pA1 plasmid which contains the cDNA inserted into the *Eco*RI site of the pBluescript vector. The digestion product was separated on a 0.8% agarose gel. The 2.5 kb cDNA insert was excised from the gel, and the DNA was then recovered by the freeze-squeeze method as described (83). Using the BIOPRIME™ DNA labeling system (GIBCO BRL), this 2.5 kb insert was then non-radioactively labeled with biotin-14-dCTP via the random priming method according to manufacturer's suggestion.

Screening the Human λgt10 cDNA Library

About 500,000 phages from a human histiocytic lymphoma (U-937) cell cDNA library constructed in λgt10 were plated out on C600*hfl* (Promega) lawns at a density of about 50,000 pfu per 150 mm plate (84). The plates were kept at 37°C for 9-16 hours until the plaques just touched. After chilling the plates at 4°C for 2 hours, plaques from each plate were transferred to a pair of nylon membranes (MagnaGraph) as previously described (84). Each membrane was then washed successively with 0.5 N NaOH containing 1.5 M

NaCl; 0.5 M Tris·HCl (pH 8.0) containing 1.5 M NaCl; and 2 x SSC (84), respectively. The plaque DNA was then cross-linked to the membranes by UV cross-linking. The prehybridization (4 hours) and hybridization (overnight, >12 hours) were carried out in the presence of 50% formamide at 42°C according to the protocol provided by PHOTOGENE (GIBCO BRL) non-radioactive nucleic acid detection system. Binding the streptavidin-alkaline phosphatase conjugate (GIBCO BRL) was carried out according to PHOTOGENE protocol and detection of biotin-labeled probe DNA-target DNA hybrids was done according to a protocol from the Nonradioactive DNA Labeling and Detection Kit from Boehringer-Mannheim.

Positive plaques were picked out from the original plates as described (84). Secondary screening was done essentially the same way as the primary screening, except that plaque density was reduced to below 500 pfu per 80 mm plate. Well resolved plaques were then isolated and stored individually.

Dot Blot Analysis of Positive Lambda Plaques

Each positive lambda isolate was grown as described (84) in a small-scale liquid culture until lysis occured. Then 5 µl of supernatant from each lysate was spotted on a nylon filter. This filter was then treated exactly the same as in the "library screening" section. For positive control, 800 pg of pA1 plasmid was also spotted. A randomly selected λgt10 phage was also included as negative control.

Subcloning into Sequencing Vector pBluescript SK(+)

Isolation of lambda phages was performed as described (84). About 1 µg of each phage DNA was digested completely by *Eco*RI. The digestion products were separated on a 0.8% agarose gel. Only the phage with the largest insert was chosen to be subcloned.

Since the largest insert had an internal *Eco*RI site, partial digestion was performed at 37°C for 20 minutes with 1 µg of λ DNA being digested by 20 units of *Eco*RI (USB).

The digestion product was separated on a 0.8% agarose gel, and the DNA band corresponding to the intact insert was excised from the gel. The insert DNA was recovered (83) and subsequently ligated into the pBluescript vector as described (84).

Transformants were screened on TAXI plates. Several white colonies bearing inserts were randomly isolated and their plasmid DNAs were further characterized by restriction endonuclease (*Eco*RI, *Bam*HI and *Hin*dIII) digestion.

Southern Blotting to Check Insert Identity

Lambda DNA and plasmid DNA were digested by appropriate restriction endonucleases (*Eco*RI, *Bam*HI or *Hin*dIII). The digestion products were separated on a 0.8% agarose gel. After staining by EtBr and photography, the gel was treated once in 0.25 M HCl for 15 minutes for depurination, twice in 0.5 N NaOH containing 1.5 M NaCl for 20 minutes to denature the DNA, and twice in 1.0 M Tris·HCl containing 1.5 M NaCl for 20 minutes to neutralize the gel. The DNA was then transferred onto a nylon membrane as described in PHOTOGENE protocol. Hybridization with biotin-14-dCTP labeled cDNA probe, binding of SA-AP conjugates, and color detection reaction were performed exactly the same way as that in the "library screening" section.

Generating Unidirectional Deletions to Facilitate Sequencing

To facilitate fast sequencing of relatively large inserts with one (or two) primer(s), a series of unidirectional deletions (Exo III library) were generated according to "Protocols and Applications Guide" (2nd Edition) from Promega. The recombinant pBluescript containing insert was first linearized by *Kpn*I (GIBCO BRL) and *Eco*RV (USB) and then extracted with TE-saturated phenol/chloroform. The DNA was then precipitated and redissolved in Exo III buffer (66 mM Tris·HCl, pH 8.0; 0.66 mM $MgCl_2$). Exo III deletion at 37°C, ligation and transformation procedures were exactly the same as described in the protocol with each sample being taken out at a 30 second interval.

The progressive deletion process was checked on a 0.8% agarose gel as recommended. The transformants selected from different LB plates were further characterized by isolating plasmid DNA and restriction endonuclease digestion. This process can be continued until a series of plasmids with satisfactory (200-300 bp shorter) lengths are obtained. The same procedure was used to generate another Exo III library from another recombinant pBluescript plasmid bearing the same insert but with a different orientation. The same restriction endonucleases (*Kpn*I and *Eco*RV) were used in this case.

Sequencing

Nucleic acid sequencing using dideoxy method was carried out with a Sequenase^R Version 2.0 DNA Sequencing Kit from U. S. Biochemical Corp. (Cleveland, Ohio) and dATPαS[^{35}S] (NEN-DuPont) as recommended by manufacturer. Sequences for both strands were obtained by this method from the two Exo III libraries described above.

5' RACE (Rapid Amplification of 5' cDNA End)

In an attempt to get the missing 5' end of the human cDNA, a combined approach of reverse transcription and PCR called 5'-RACE (Rapid Amplification of 5'-cDNA Ends) was employed by using the 5' RACE Kit bought from GIBCO BRL. The whole procedure was performed according to the manufacturer's recommendation. Briefly, available sequence information downstream of the internal *Eco*RI site was used to synthesize a human gene specific primer (hGSP1). After priming the first strand cDNA synthesis from 1 μg of human HeLa cell total RNA (Clontech) with this hGSP1, RNA templates were digested away with RNaseH. The cDNA purified by GLASSMAX^R DNA Isolation Spin Cartridge was then tailed with dCTP by Terminal deoxynucleotidyl Transferase (TdT) and served as the PCR template for an anchor primer (with a complementary poly(dG) tail) and a nested gene specific primer 2 (hGSP2). PCR reaction conditions were 94°C for 45 seconds, 57°C for 25 seconds, and 72°C for 2 minutes. This cycle was repeated 35 times

followed by 8 minutes final extention at 72°C. PCR products were analyzed on a 0.8% agarose gel. Southern blotting of the gel and subsequent hybridization with eIF-2Bε specific probes and color development were the same as those in the "Southern Blotting" section.

Data Analysis

Sequencing data were analyzed by MacVector™ 4.0 software on a Macintosh computer. Genetics Computer Group (GCG) sequence analysis software package based on the University of Oklahoma VAX computer was employed for mRNA secondary structure prediction and the multisequence alignment.

CHAPTER IV

RESULTS AND DISCUSSION

Screening the Human λgt10 cDNA Library

Twenty-three positive plaques were isolated from the primary screening from which 13 well resolved single positive plaques were obtained during the secondary screening. Dot blot analysis on a nylon membrane circle indicated that they were all positive by showing strong color development. The negative control (a randomly selected lambda phage from the human cDNA library) did not have any color development, while the positive control (800 pg of pA1 plasmid) showed strong color development (Fig. 1). Therefore, the 13 individually isolated recombinant λgt10 phages all bore inserts which were somewhat similar to the rabbit reticulocyte eIF-2Bε cDNA. The different color intensities probably reflect different DNA amounts or different degrees of insert sequence similarity.

Characterization of Inserts

The human λgt10 cDNA library was constructed so that foreign DNA is inserted into a unique *Eco*RI site on this recombinant vector. This *Eco*RI site cuts the 43.34 kb lambda DNA into a 32.71 kb left arm and a 10.63 kb right arm. All 13 recombinant phage isolates were digested by *Eco*RI to completion. The digestion products were separated on 0.8% agarose gels (Fig. 2A and 2B). One isolate showed two insert bands whose approximate sizes were 0.5 kb and 2.3 kb, respectively. Nine isolates had 2.3 kb insert each. Inserts for the other two isolates were around 1.6 kb, although slight

Figure 1. Dot blotting assay of 13 positive phage plaques.

From upper left corner to lower right corner, the spotted 16 samples are: phage isolates 1-21-1, 1-31-1, 1-41, 1-51, 2-11, 2-21, 3-11, 3-21, 3-31, 3-41, 4-11, 6-31, 7-11, 9-11, negative control (a randomly selected recombinant lambda phage), and positive control (800 pg pA1 plasmid), respectively. For each phage isolate, 5 μl of supernatant from small scale phage growth lysate was spotted on the nylon membrane as described in the text. The phage isolate 1-21-1 was later shown to have contained the largest 2.8 kb insert.

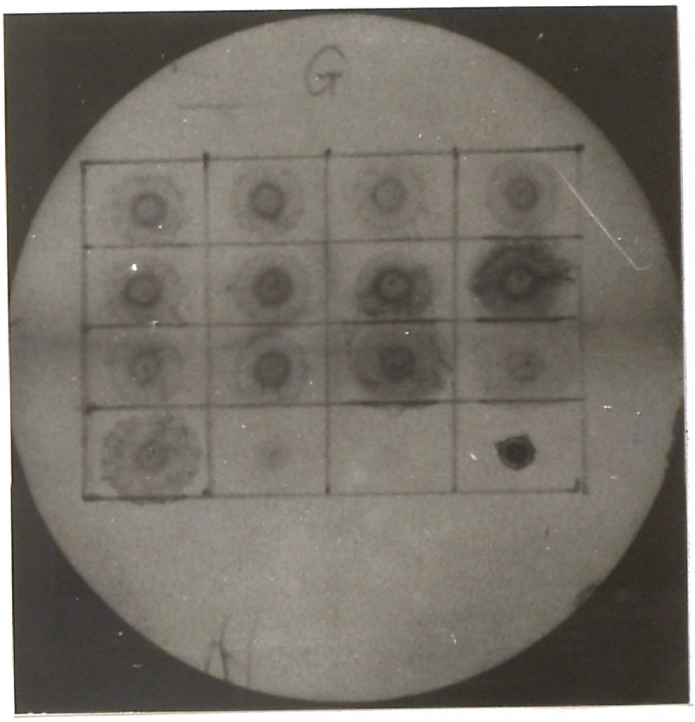
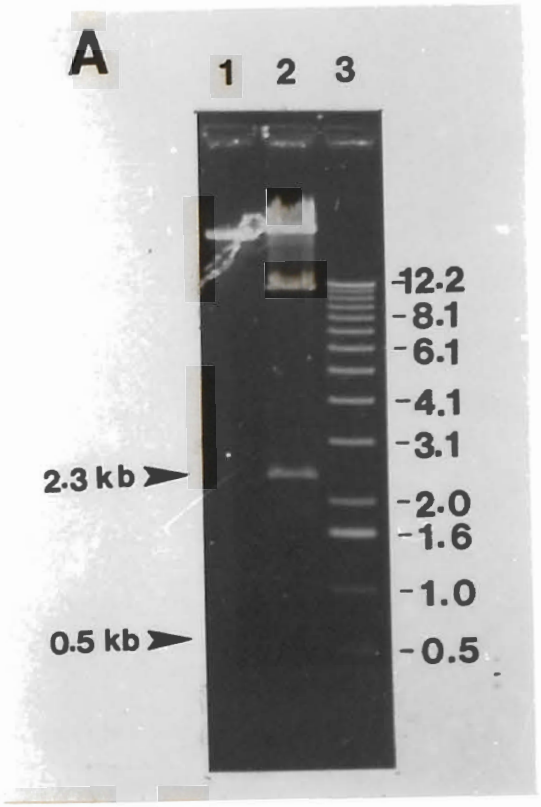
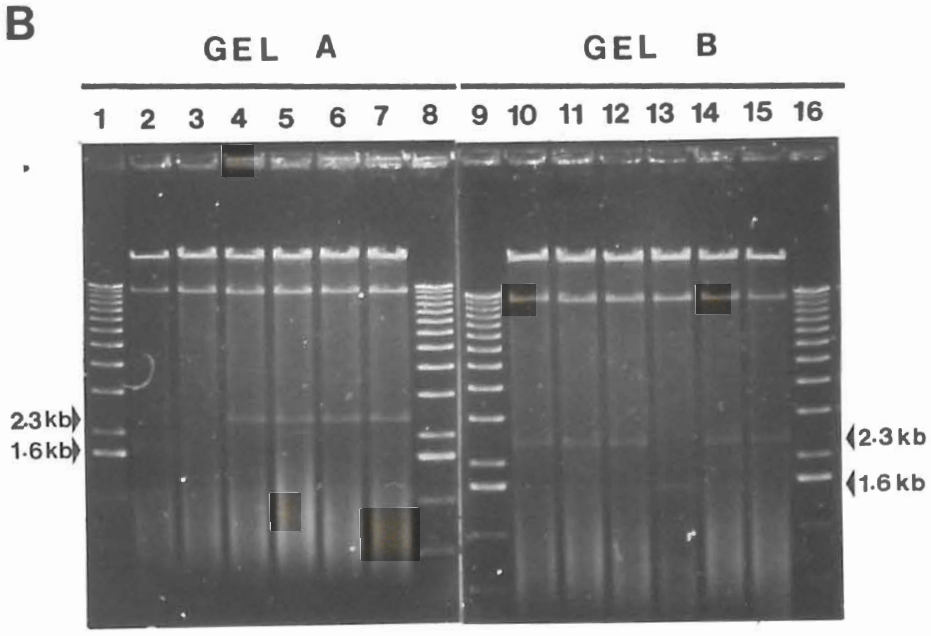

G

Figure 2. *Eco*RI digestion of the 13 positive lambda phage isolates.

(A) *Eco*RI digestion of phage 1-21-1. Lane 1, undigested 50 kb re
binant phage DNA. Lane 2, *Eco*RI digested 1-21-1. A 0.5 kb insert ba
visible in the original film. Lane 3, 1 kb DNA ladder (GIBCO B
(B) *Eco*RI digestion of the other 12 positive phage isolates. Lanes 1, 8,
are 1 kb DNA ladders (BRL). Lanes 2-7 and 10-15 are phage isolates
6-31, 4-11, 3-41, 3-31, 3-21, 3-11, 2-21, 2-11, 1-51, 1-41, and 1-
respectively.


A
1 2 3
12.2
8.1
6.1
4.1
3.1
2.3 kb
2.0
1.6
1.0
0.5 kb
0.5


B
GEL A
GEL B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
2.3 kb
1.6 kb
2.3 kb
1.6 kb

differences in length exist. The digestion product of one isolate did not appear to have a detectable insert band.

Restriction enzyme analysis indicated that the largest insert was about 2.8 kb, and had an internal *Eco*RI site that cuts the insert into a 0.5 kb and 2.3 kb fragments. The 2.3 kb fragment is the same as the inserts of the other 9 isolates. It was not clear what the two isolates with 1.6 kb inserts were, but it was probable that they were truncated versions of the 2.3 kb insert. The isolate which did not contain an insert was odd, since λgt10 phage without an insert are difficult to grow on the C600*hfl* host strain.

Subcloning into the Sequencing Vector

Since the phage isolate with the largest insert had an internal *Eco*RI site, partial digestion by *Eco*RI was employed to retrieve the intact insert from the lambda vector. Digestion at 37°C for 20 minutes with 20 units of *Eco*RI (USB) seemed to give the highest yield of the intact insert from 1 μg of λ DNA (Fig. 3). The DNA bands corresponding to the intact insert were then excised from the gel. The DNA was recovered by freeze-squeeze method (83) and ligated into pBluescript SK(+) vector at its *Eco*RI site.

Transformation using MAX EFFICIENCY DH5α™ Competent Cells (BRL) was highly efficient (>1000 colonies formed with about 1 ng input DNA) and about 1/4 of the colonies were white on the TAXI plates. Ten white colonies were picked (84). Of the eight plasmids whose DNAs were isolated, all contained the expected 2.8 kb insert, since *Eco*RI digestion of the plasmids yielded 0.5 kb and 2.3 kb insert bands in addition to the 3.0 kb vector band (Fig. 4). Two plasmids, designated pBRFA and pBRFI, were further characterized by restriction endonuclease digestion (*Hin*dIII, etc.) and were confirmed to have the same insert with different orientations (Fig. 5).

Figure 3. Partial *Eco*RI digestion of recombinant lambda phage isolate 1-21-1.

Lanes 1 to 7 are different loads of the same digestion reaction. Lane 8
1 kb DNA ladder (BRL). A total of 1 μg of phage DNA was subjectec
unit of *Eco*RI (USB) partial digestion at 37°C for 20 minutes. The
2.8 kb intact insert band was subsequently recovered from the g
ligated into pBluescript SK(+) vector.

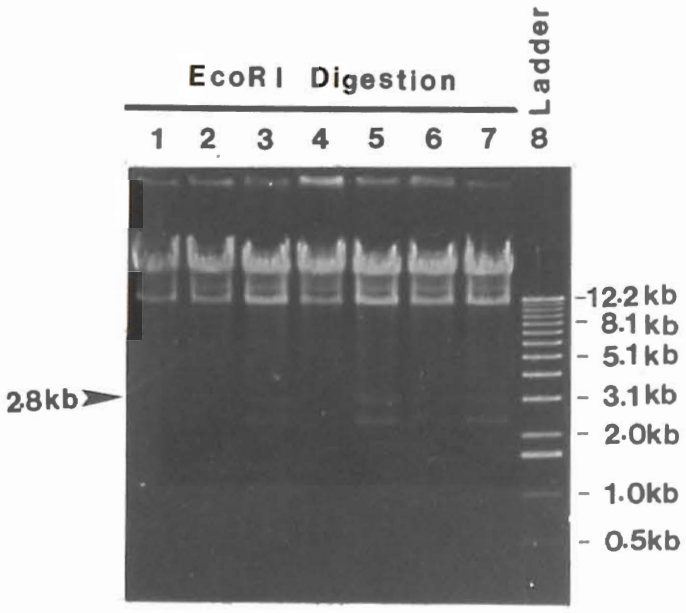

EcoR I Digestion
Ladder
1 2 3 4 5 6 7 8
12.2 kb
8.1 kb
5.1 kb
3.1 kb
2.0 kb
1.0 kb
0.5 kb
2.8kb

Figure 4. Characterization of 10 randomly selected white transformants from TAXI by *Eco*RI digestion.

Lanes 1, 9, 15, and 23 are supercoiled pBluescript SK(+) plasmid Lanes 6, 14, 20, and 28 are 1 kb DNA ladders (BRL). Lanes 2, 4, 12, 16, 18, 21, 24, and 26 are supercoiled forms of recombinant pl DNAs prepared from transformants A, B, C, D, E, F, G, H, I, a respectively. Their corresponding *Eco*RI digestion products are in lane 8, 11, 13, 17, 19, 23, 25, and 27, respectively. No DNAs seem present in lanes corresponding to samples G and H probably beca mistake occured during the plasmid isolation step. All other samples c the expected 2.8 kb insert since *Eco*RI digestion yielded two insert (0.5 kb and 2.3 kb) in each case. Two plasmids, pBRFA and p (from A and I respectively), were chosen for further characteriz sc: supercoiled DNA; M: 1 kb ladder.

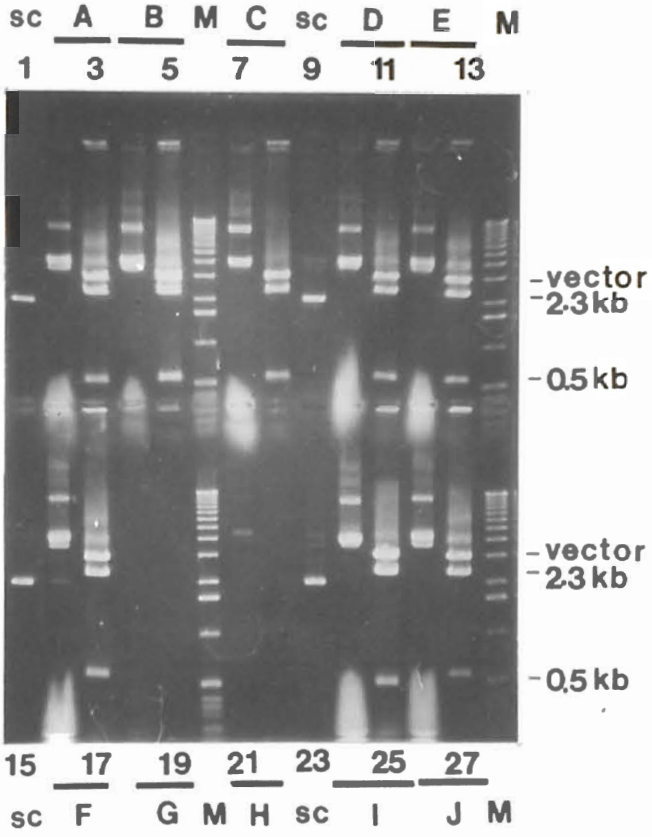
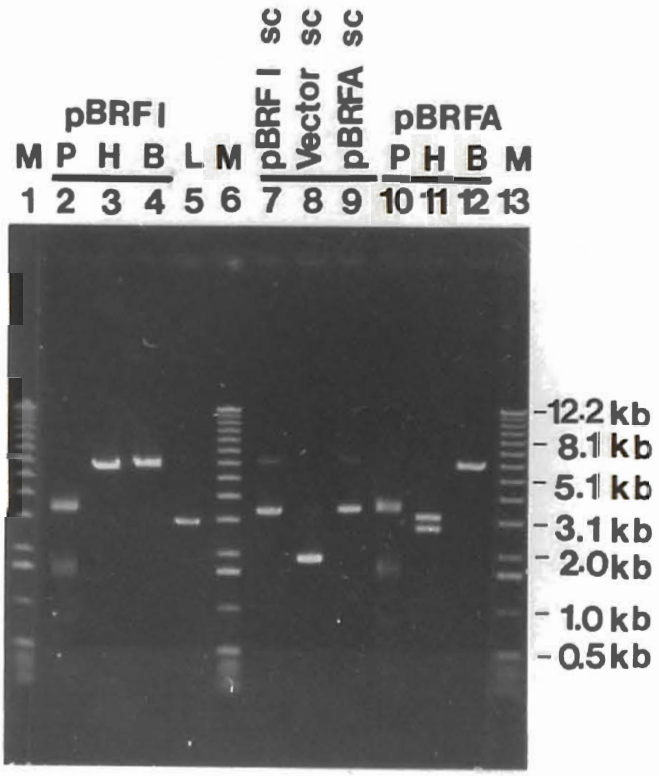

sc A B M C sc D E M
1 3 5 7 9 11 13
vector
2.3kb
0.5kb
vector
2.3kb
0.5kb
15 17 19 21 23 25 27
sc F G M H sc I J M

Figure 5. Characterization of pBRFA and pBRFI by restriction endonuclease diges

Lanes 1, 6, and 13 are 1 kb DNA ladders (BRL). Lane 2, *Pst*I digestion of pBRFI. Lane 3, *Hin*dIII (USB) digestion of pBRFI. I *Bam*HI (USB) digestion of pBRFI. Lane 5, *Eco*RI linearized pBlu SK(+) vector DNA. lane 7, supercoiled DNA of pBRFI. Lane 8, supe DNA of pBluescript SK(+). Lane 9, Supercoiled DNA of pBRFA. L *Pst*I (BRL) digestion of pBRFA. Lane 11, *Hin*dIII digestion of p Lane 12, *Bam*HI digestion of pBRFA. Note that *Hin*dIII digesti pBRFA and pBRFI gave different band patterns, indicating di orientations of insert in these two plasmids. M: 1 kb ladder; P: *P* *Hin*dIII; B: *Bam*HI; sc: supercoiled DNA.


pBRFI
pBRFA
M P H B L M pBRF I sc Vector sc pBRFA sc P H B M
1 2 3 4 5 6 7 8 9 10 11 12 13
12.2 kb
8.1 kb
5.1 kb
3.1 kb
2.0kb
1.0 kb
0.5kb

Southern Blotting

To further confirm that pBRFA and pBRFI contain the target cDNA inserts, Southern blotting was performed. The lambda phage from which the 2.8 kb insert was isolated, and plasmids pBRFA and pBRFI were digested with restriction endonucleases as indicated (Fig. 6A). Southern blotting (Fig. 6B) indicated that all bands (except one, see below) related to the insert fragment hybridized strongly with the eIF-2Bε cDNA specific probe, implying sequence homology. But quite unexpectedly, the 0.5 kb fragment derived from the insert did not hybridize with the probe. The reason is explained below. In addition, the pUC plasmid related vector bands (including pBluescript SK(+) and the vector used in the commercial product 1 kb DNA ladder from BRL) unexpectedly hybridized strongly with the eIF-2Bε specific probe. It is unlikely that the probe prepared is nonspecific (so that it detects all DNA bands) because of the following reasons:

1) The probe does not hybridize with λgt10 arms. The hybridization band near the right arm is the incomplete digestion product of λgt10 DNA which contains the 2.3 kb cDNA fragment. The 10.63 kb right arm does not hybridize with the probe at all. The broad band at the 32.71 kb left arm position is likely due to the presence of incomplete digestion product.

2) The probe does not hybridize with the 1 kb DNA ladder which is derived from the yeast 2μ plasmid.

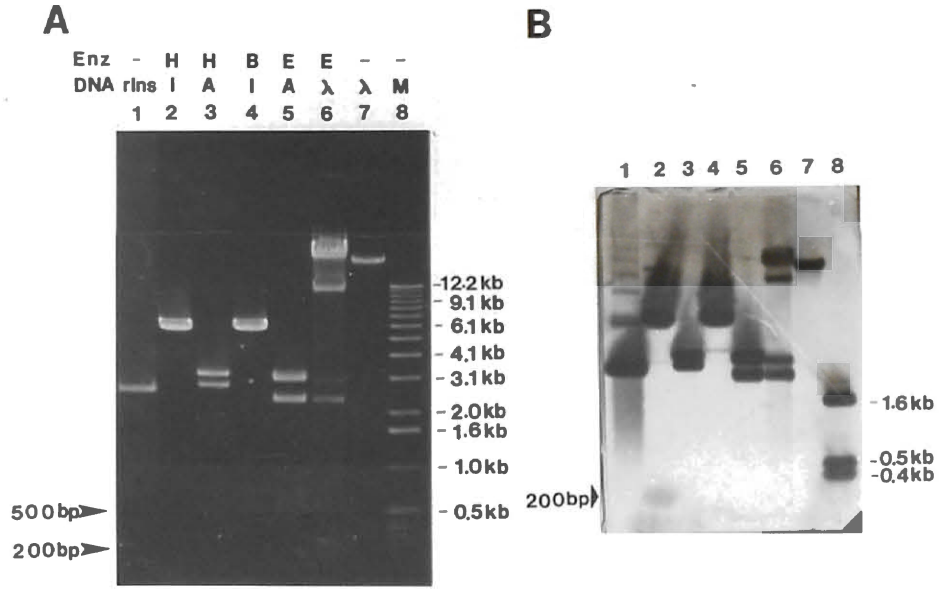
3) The probe does not hybridize with other numerous foreign insert cDNAs in the λgt10 library, since this probe was used to isolate 13 individual lambda plaques from the cDNA library, which all seem to contain the same insert DNA.

4) The probe does not hybridize with the 0.5 kb insert band derived from the intact 2.8 kb insert. Given the sensitivity of this method which can detect homologous DNA at levels of less than 1 pg, all DNA bands detectable in EtBr stained gel (>10 ng) should

Figure 6. Southern blotting to check the identity of the 2.8 kb insert.

(A) Lane1, the 2.5 kb rabbit eIF-2Bε cDNA insert isolated from pA
*Bam*HI - *Hin*dIII fragment. Lane 2, *Hin*dIII digestion of pBRFI. La
*Hin*dIII digestion of pBRFA. Lane 4, *Bam*HI digestion of pBRFI. La
*Eco*RI digestion of pBRFA. Lane 6, *Eco*RI digestion of phage is
1-21-1. Lane 7, phage isolate 1-21-1. Lane 8, 1 kb DNA Ladder (I

(B) Southern blotting result of (A). Rabbit eIF-2Bε cDNA specific p
were used for hybridization. All lanes are in the same order as in (A). (
bands were present in the *Eco*RI digestion of pBRFA and lambda
(lanes 5 and 6), but no corresponding hybridization bands apeared
Southern blotting result.


A
Enz - H H B E E - -
DNA rlns I A I A λ λ M
1 2 3 4 5 6 7 8
12.2kb
9.1 kb
6.1 kb
4.1 kb
3.1 kb
2.0kb
1.6kb
1.0kb
0.5kb
500bp
200bp
B
1 2 3 4 5 6 7 8
1.6kb
0.5kb
0.4kb
200bp

hybridize strongly with the probe if they are homologous. In fact, it is this result that gave us the first hint that the 0.5 kb insert might not be related to human eIF-2Bε cDNA (see "Sequence and Data Analysis" part below).

5) The reasons cited above do not rule out the possible existence of minor amounts of contaminating vector DNA in the gel purified 2.5 kb rabbit eIF-2Bε cDNA preparation used to generate the probe. While this contamination could be hard to avoid when trying to excise the 2.5 kb insert band which migrates close to the 3.0 kb vector band, it should be fairly easy to avoid when excising the 5' end 230 bp *Eco*RI fragment of rabbit eIF-2Bε cDNA. Yet even this DNA preparation gave a probe that hybridized strongly with those pUC related vectors (data not shown).

6) A eukaryotic probe derived from yeast 2μ plasmid has similarly been found to cross-hybridize with prokaryotic DNA vector pBR322 (85). This had been attributed to the possible sequence similarity between the probe and the vector, although it was later shown by computer search that no extensive identities exist and that the longest perfect identity was only 13 base pairs (86).

Generating Unidirectional Deletions to Facilitate Sequencing

Preliminary sequencing data revealed that one end of the 2.8 kb insert had a long stretch of poly(A) sequence (>100 bp) which was impossible for the Sequenase^R Version 2.0 T7 DNA Polymerase (USB)to pass through. To overcome this problem, a series of unidirectional deletions were made from both the pBRFA and pBRFI plasmid. The deletion products were monitored by withdrawing ongoing reactions at different times. The electrophoresis of the reaction products is shown in Fig. 7A. Because the reaction rate can not be predicted accurately, the reaction was repeated for a specific time period in order to get proper deletion products. Because of the heterogeneity of the reaction, plasmid transformants from the same plate do not necessarily have the same size. Abnormal plasmids may also appear. So after the general characterization of various deletions by

plasmid isolation and enzyme digestion (Fig. 7B and 7C), additional rounds of plasmid isolation and enzyme digestion were required to obtain the expected deletion products. Finally, two series of unidirectional deletions were made with 200-350 bp insert length differences between two successive plasmids.

Sequencing and Data Analysis

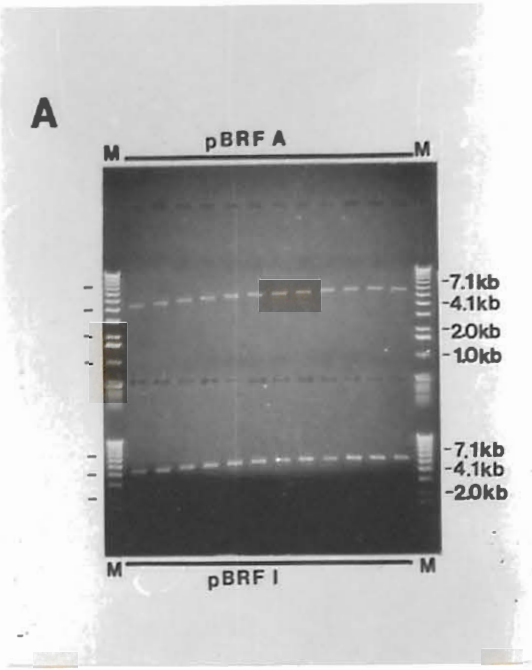
Sequencing reactions were performed using the Sequenase^{R} Version 2.0 DNA sequencing kits from U. S. Biochemical Corp. (Cleveland, Ohio). Overlapping pieces of DNA sequences were used to reconstruct the complete sequence using the MacVector™ 4.0 program in a Macintosh computer. Sequences for both strand were obtained and they agreed with each other (Fig. 8).

Sequencing data confirm that the 0.5 kb *Eco*RI fragment is totally unrelated to the eIF-2Bε cDNA. It was probable that this fragment was ligated to the 2.3 kb partial cDNA of human eIF-2Bε cDNA merely by chance during the construction of the library. This is supported by the fact that human eIF-2Bε cDNA is nearly 90% identical to its rabbit reticulocyte counterpart up to the internal *Eco*RI site. But when the sequence goes further upstream beyond this site, an additional poly(A) sequence appears, followed by a sequence which shows virtually no homology with the 230 bp rabbit eIF-2Bε cDNA 5' end. This is unlikely to be the case if the 0.5 kb fragment is part of the human eIF-2Bε cDNA, since the most conserved region between the rabbit eIF-2Bε cDNA and its proposed yeast counterpart *GCD6* cDNA resides at the 5' end (81).

Starting from the internal *Eco*RI site, the partial human eIF-2Bε cDNA contains a single large open reading frame which starts at base 301 and ends at base 1926. This open reading frame will encode a peptide of 541 amino acid residues. If the average molecular weight of an amino acid residue is taken to be 110 Da, the molecular weight of this protein is less than 60 kDa, far less than the reported 82 kDa value. Furthermore, no in-frame stop codons have been observed in the upstream 300 bases, so it is quite certain that

Figure 7. Generation of the unidirectional deletions (Exo III library).

(A) Monitoring the progressive Exonuclease III digestion reaction. S
were withdrawn at 30 second intervals. The left and right most
are 1 kb DNA ladders (BRL). Upper half, pBRFA; lower half, p
(B) *Bam*HI digestion of Exo III libraries for pBRFA and pBRFI. Upp
Lane M, 1 kb DNA ladder (BRL). Lane 13, *Bam*HI digestion of pl
Lanes 1-12, *Bam*HI digestion of pBRFA Exo III library. Lower hal
M, 1 kb DNA ladder. Lane 13, *Bam*HI digestion of pBRFI. Lane
*Bam*HI digestion of pBRFI Exo III library. (C) *Hin*dIII digestion of
libraries for pBRFA and pBRFI. Lane assignments were the same as
Note that some individual members of the library are abnormal and th
to be replaced by other proper ones. (see text)


A
M
pBRF A
M
7.1kb
4.1kb
2.0kb
1.0kb
7.1kb
4.1kb
2.0kb
M
pBRF I
M

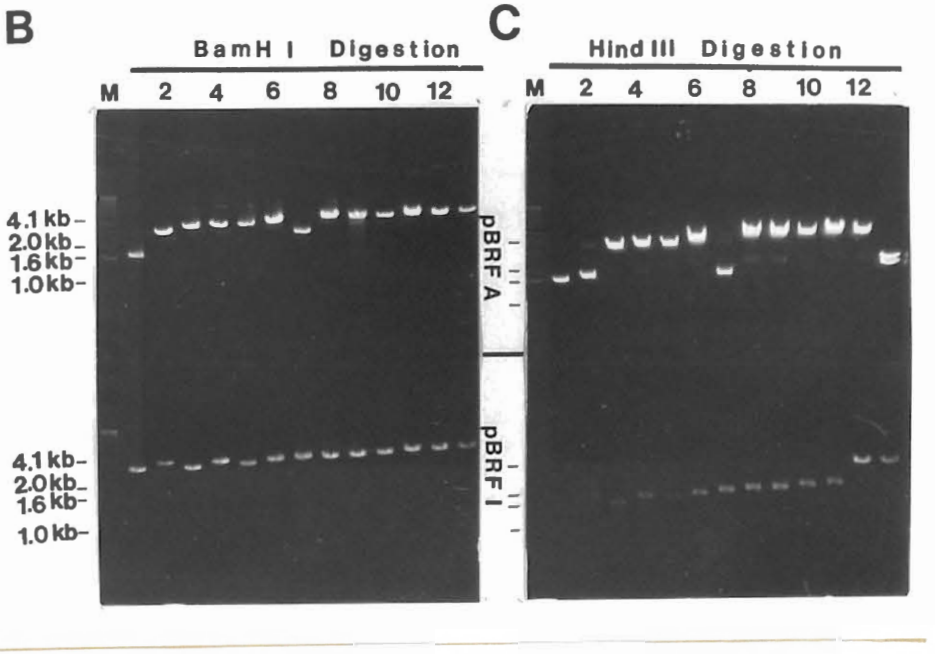

B
BamH I Digestion
M 2 4 6 8 10 12
4.1 kb
2.0 kb
1.6 kb
1.0 kb
pBRF A
pBRF I
4.1 kb
2.0 kb
1.6 kb
1.0 kb
C
Hind III Digestion
M 2 4 6 8 10 12

Figure 8. Nucleotide and Amino Acid Sequences of Human eIF-2Bε Subunit.

The *Eco*RI site and the first in-frame ATG start codon are underline
ATTAAA sequence which is very close to the consensus sec
(AATAAA) for adding poly(A) tail is in bold letter. Amino aci
represented by one-letter-code (singlet).

```
GAATTCCTGACTGCCACAGGTGTACAGGAAACATTTGTCTTTTGTTGCTGGAAAGCTGCTCAAATCAAAGAACATTTA    78
 E  F  L  T  A  T  G  V  Q  E  T  F  V  F  C  C  W  K  A  A  Q  I  K  E  H  L
CTGAAGTCAAAGTGGTGCCGCCCTACATCTCTCAATGTGGTTCGAATAATTACATCAGAGCTCTATCGATCACTGGGA   156
 L  K  S  K  W  C  R  P  T  S  L  N  V  V  R  I  I  T  S  E  L  Y  R  S  L  G
GATGTCCTCCGTGATGTTGATGCCAAGGCTTTGGTGCGCTCTGACTTTCTTCTGGTGTATGGGGATGTCATCTCAAAC   234
 D  V  L  R  D  V  D  A  K  A  L  V  R  S  D  F  L  L  V  Y  G  D  V  I  S  N
ATCAATATCACCAGAGCCCTTGAGGAACACAGGTTGAGACGGAAGCTAGAAAAAAAATGTTTCTGTGATGACGATGATC   312
 I  N  I  T  R  A  L  E  E  H  R  L  R  R  K  L  E  K  N  V  S  V  M  T  M  I
TTCAAGGAGTCATCCCCCAGCCACCCAACTCGTTGCCACGAAGACAATGTGGTAGTGGCTGTGGATAGTACCACAAAC   390
 F  K  E  S  S  P  S  H  P  T  R  C  H  E  D  N  V  V  V  A  V  D  S  T  T  N
AGGGTTCTCCATTTTCAGAAGACCCAGGGTCTCCGGCGTTTTGCATTTCCTCTGAGCCTGTTTCAGGGCAGTAGTGAT   468
 R  V  L  H  F  Q  K  T  Q  G  L  R  R  F  A  F  P  L  S  L  F  Q  G  S  S  D
GGAGTGGAGGTTCGATATGATTTACTGGATTGTCATATCAGCATCTGTTCTCCTCAGGTGGCACAACTCTTTACAGAC   546
 G  V  E  V  R  Y  D  L  L  D  C  H  I  S  I  C  S  P  Q  V  A  Q  L  F  T  D
AACTTTGACTACCAAACTCGAGATGACTTTGTGCGAGGTCTCTTAGTGAATGAGGAGATCCTAGGGAACCAGATCCAC   624
 N  F  D  Y  Q  T  R  D  D  F  V  R  G  L  L  V  N  E  E  I  L  G  N  Q  I  H
ATGCACGTAACAGCTAAGGAATATGGTGCCCGTGTCTCCAACCTACACATGTACTCAGCTGTCTGTGCTGACGTCATC   702
 M  H  V  T  A  K  E  Y  G  A  R  V  S  N  L  H  M  Y  S  A  V  C  A  D  V  I
CGCCGATGGGTCTACCCTCTCACCCCAGAGGCGAACTTCACTGACAGCACCACCCAGAGCTGCACTCATTCCCGGCAC   780
 R  R  W  V  Y  P  L  T  P  E  A  N  F  T  D  S  T  T  Q  S  C  T  H  S  R  H
AACATCTACCGAGGGCCTGAGGTCAGCCTGGGCCATGGCAGCATCCTAGAGGAAAATGTGCTCCTGGGCTCTGGCACT   858
 N  I  Y  R  G  P  E  V  S  L  G  H  G  S  I  L  E  E  N  V  L  L  G  S  G  T
GTCATTGGCAGCAATTGCTTTATCACCAACAGTGTCATTGGCCCCGGCTGCCACATTGGTGATAACGTGGTGCTGGAC   936
 V  I  G  S  N  C  F  I  T  N  S  V  I  G  P  G  C  H  I  G  D  N  V  V  L  D
CAGACCTACCTGTGGCAGGGTGTTCGAGTGGCGGCTGGAGCACAGATCCATCAGTCTCTGCTTTGTGACAATGCTGAG  1014
 Q  T  Y  L  W  Q  G  V  R  V  A  A  G  A  Q  I  H  Q  S  L  L  C  D  N  A  E
GTCAAGGAACGAGTGACACTGAAACCACGCTCTGTCCTCACTTCCCAGGTGGTCGTGGGCCCAAATATCACGCTGCCT  1092
 V  K  E  R  V  T  L  K  P  R  S  V  L  T  S  Q  V  V  V  G  P  N  I  T  L  P
GAGGGCTCGGTGATCTCTTTGCACCCTCCAGATGCAGAGGAAGATGAAGATGATGGCGAGTTCAGTGATGATTCTGGG  1170
 E  G  S  V  I  S  L  H  P  P  D  A  E  E  D  E  D  D  G  E  F  S  D  D  S  G
GCTGACCAAGAAAAGGACAAAGTGAAGATGAAAGGTTACAATCCAGCAGAAGTAGGAGCTGCTGGCAAGGGCTACCTC  1248
 A  D  Q  E  K  D  K  V  K  M  K  G  Y  N  P  A  E  V  G  A  A  G  K  G  Y  L
TGGAAAGCTGCAGGCATGAACATGGAGGAAGAGGAGGAACTGCAGCAGAATCTGTGGGGACTCAAGATCAACATGGAA  1326
 W  K  A  A  G  M  N  M  E  E  E  E  E  L  Q  Q  N  L  W  G  L  K  I  N  M  E
GAAGAGAGTGAAAGTGAAAGTGAGCAAAGTATGGATTCTGAGGAGCCGGACAGCCGGGGAGGCTCCCCTCAGATGGAT  1404
 E  E  S  E  S  E  S  E  Q  S  M  D  S  E  E  P  D  S  R  G  G  S  P  Q  M  D
GACATCAAAGTGTTCCAGAATGAAGTTTTAGGAACACTACAGCGGGGCAAAGAGGAGAACATTTCTTGTGACAATCTC  1482
 D  I  K  V  F  Q  N  E  V  L  G  T  L  Q  R  G  K  E  E  N  I  S  C  D  N  L
GTCCTGGAAATCAACTCTCTCAAGTATGCCTATAACGTAAGTCTAAAGGAGGTGATGCAGGTACTGAGCCACGTGGTC  1560
 V  L  E  I  N  S  L  K  Y  A  Y  N  V  S  L  K  E  V  M  Q  V  L  S  H  V  V
CTGGAGTTCCCCCTGCAACAGATGGATTCCCCGCTTGACTCAAGCCGCTACTGTGCCCTGCTGCTTCCTCTGCTAAAG  1638
 L  E  F  P  L  Q  Q  M  D  S  P  L  D  S  S  R  Y  C  A  L  L  L  P  L  L  K
GCCTGGAGCCCTGTTTTTAGGAACTACATAAAGCGCGCAGCCGACCATTTGGAAGCGTTAGCAGCCATTGAGGACTTC  1716
 A  W  S  P  V  F  R  N  Y  I  K  R  A  A  D  H  L  E  A  L  A  A  I  E  D  F
TTCCTAGAGCATGAAGCTCTTGGTATTTCCATGGCCAAGGTACTGATGGCTTTCTACCAGCTGGAGATCCTGGCTGAG  1794
 F  L  E  H  E  A  L  G  I  S  M  A  K  V  L  M  A  F  Y  Q  L  E  I  L  A  E
GAAACAATTCTGAGCTGGTTCAGCCAAAGAGATACAACTGACAAGGGCCAGCAGTTGCGCAAGAATCAACAGCTGCAG  1872
 E  T  I  L  S  W  F  S  Q  R  D  T  T  D  K  G  Q  Q  L  R  K  N  Q  Q  L  Q
AGGTTCATCCAGTGGCTAAAAGAGGCAGAAGAGGAGTCATCTGAAGATGACTGAAGTCACACTGCCTGCTCCTTTGGG  1950
 R  F  I  Q  W  L  K  E  A  E  E  E  S  S  E  D  D  *

TGTGATTGAGTGCCCTCCTGGCTCCTGGGCTGGGACAAGTGAGGAACTAGCTGCAGAGGGATGAGTGACCACCATCCA  2028
GGCTGAGACTGAAAGGAGCAGAGGCTGGAACTACAGTATTCTTTCCCCTGCTAGCAACCATGTGCCTCCCATCCTGAC  2106
TGTGGAGTTGGGATGTGGAAGTGGGGCTGGAACAAAGCTTCTGCCTAGGGAGGAGCTAAGCAGGCCCGGCAGTTGGAG  2184
GAAGGCCAGAGGAACAGCTTTGTGCTCCGGCTTTCCCTCAGGGAACAGCAGAGAGCAGTTGGCTCTTTCTGCTGCTTG  2262
TATATGTTAATATTAAAAGAGAGTGGTGTAAAAAAAAAAAAAAAAAAAA
```

sequence information at the 5' end of the intact mRNA for eIF-2Bε (including part of the coding region and the 5' end untranslated region) has been lost. The loss of the 5' end is most likely due to insufficient methylation of the internal *Eco*RI sites while constructing the cDNA library. Since the cDNAs were size selected before ligation into the λgt10 vectors and none of the 13 individually selected recombinant phages contained the full length eIF-2Bε cDNA, rescreening the same cDNA library to search for that missing 5' end is unlikely to be worthwhile. Either other approaches or rescreening other better human cDNA libraries might be employed to get the full length cDNA.

Alignment of three sequences, human and rabbit eIF-2Bε proteins together with their yeast counterpart GCD6 sequence, was done by using the Genetics Computer Group (GCG) sequence analysis software package (87) (Fig. 9). The consensus sequence of the three proteins showed that the N-terminus was more conserved. Also, downstream from the internal *Eco*RI site, human and rabbit cDNA show strong sequence similarity (about 90% identity, data not shown). Only the 3'-untranslated region has several insertions or deletions which are not observed within the coding region. These data further support the notion that these three homologous sequences encode eIF-2Bε subunit.

5' RACE (Rapid Amplification of 5' cDNA End)

Attempts to amplify the 5' end of the eIF-2Bε mRNA using a 5'-RACE kit from BRL have not been successful. A single 340 bp hybridization band was detected after southern blotting the PCR product (Fig. 10). The two "x" arrows in Fig. 10B and 10C seem to point to the same band, but careful examination revealed that the "x" in Fig. 10B actually corresponded to the white marks just above the "x" in Fig. 10C. The hybridization band in Fig. 10C was real. However, its corresponding DNA band in the gel can not be seen, indicating that the PCR product of interest is present at such a low level that it will be difficult to clone. Even if it were cloned, it still may not give us a full length cDNA. This problem probably arises from the fact that the 5' end of the mRNA is very GC-rich; the

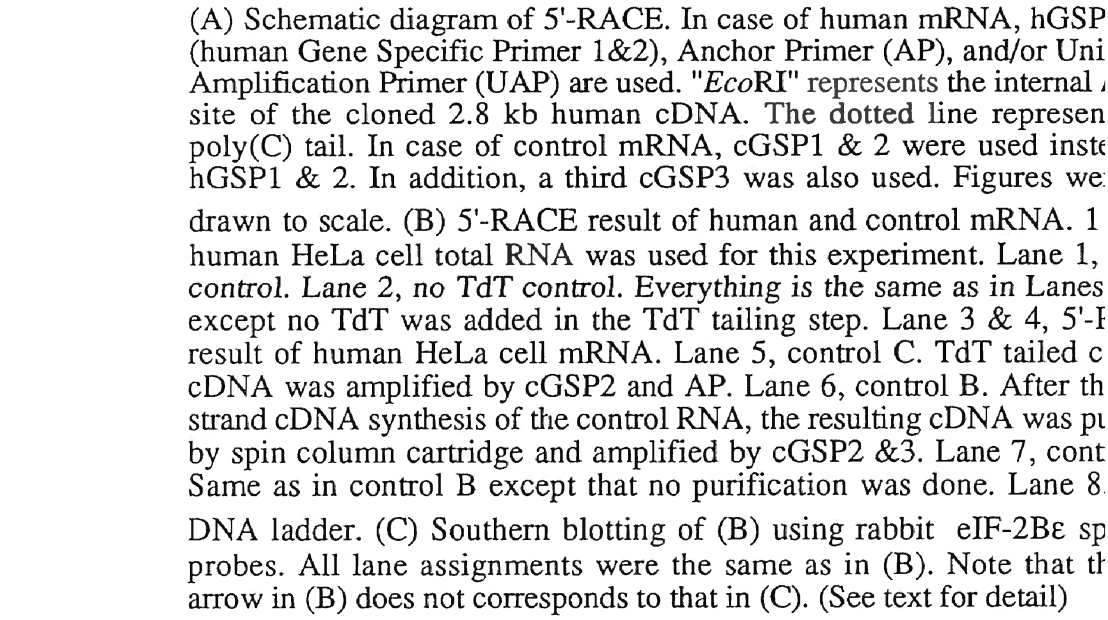
first 150 bases at the 5' end of the cloned rabbit cDNA is > 80% GC. To investigate this issue further, a computer program of the GCG package was used to predict the potential mRNA secondary structures within the first 230 bases of the rabbit eIF-2Bε sequence. The results (Fig. 11) show that a set of very stable secondary structures potentially exists within this region. For the first very GC-rich 100 bases, a 13 bp double helix structure exists with a free energy of -43.6 Kcal/mol at 37°C and -2.4 Kcal/mol at 100°C! As a control, the average free energy for a 100 base RNA sequence is just around -20 Kcal/mol and virtually all structures melt out at about 75°C. Since the SUPERSCRIPT™ reverse transcriptase provided with the BRL kit is only active at temperatures below 45°C, the stable mRNA secondary structures present at that temperature may prevent the enzyme from reverse transcribing through this region. However, this problem might be alleviated by using a reverse transcriptase (Retrotherm™ RT DNA Polymerase from Epicentre Technologies, Madison, Wisconsin) which functions at 70°C or above.

Figure 9. Three-way Alignment of Human, Rabbit and Yeast eIF-2Bε Proteins.

The three-way alignment was generated by using progressive pa
alignment. Numbers are arbitrary units for blocks, periods represen
inserted in the sequence, and asterisks occur at the C terminus o
sequence. a consensus sequence is shown below the yeast sequence;
case letters indicate that three sequences are identical at that positio
lower case letters indicate that two of the three sequences are identical.

```
          1                                                                   70
Prn-Hum   .......... .......... .......... .......... .......... .......... ..........
Prn-Rab   ...RPPPPGA VSDRANKRGG GPGGGGGGGG ARGAEEESPP PLQAVLVADS FNRRFFPISK DQPRVLLPLA
Prn-Yst   .......... .....MAGKK GQKKSGLGNH GKNSDMDVED RLQAVVLTDS YETRFMPLTA VKPRCLLPLA
Consens   .......... .......... g....g.g.. .......... .lqav...ds ...rf.p... ..pr.llpla
         71                                                                  140
Prn-Hum   .........E FLTATGVQET FVFCCWKAAQ IKEHLLKSKW CRPTSLNVVR IITSELYRSL GDVLRDVDAK
Prn-Rab   NVALIDYTLE FLTATGVQET FVFCCWKAAQ IKEHLQKSKW CRPTSLNVVR IITSELYRSL GDVLRDVDTK
Prn-Yst   NVPLIEYTLE FLAKAGVHEV FLICSSHANQ INDYIENSKW NLPWSPFKIT TIMSPEARCT GDVMRDLDNR
Consens   nv.li.ytlE FLtatGVqEt FvfCcwkAaQ Ikehl.kSKW crPtSlnvvr iItSelyRsl GDVlRDvD.k
         141                                                                 210
Prn-Hum   ALVRSDFLLV YGDVISNINI TRALEEHRLR RKLEKNVSVM TMIFKESSPS HPTRCHEDNV VVAVDSTTNR
Prn-Rab   ALVRSDFLLV YGDVVSNINV TRALEEHRLR RKLEKNVSVM TMIFKESSPS HPTRCHEDNV VVAVDSATNR
Prn-Yst   GIITGDFILV SGDVLTNIDF SKMLEFHK.K MHLQDKDHIS TMCLSKASTY PKTRTIEPAA FV.LDKSTSR
Consens   alvrsDFlLV yGDV.sNIn. traLEeHrlr rkLeknvsvm TMifkesSps hpTRchEdnv vVavDs.TnR
         211                                                                 280
Prn-Hum   VLHFQK.... .TQGLRRFAF PLSLFQGSSD GVEVRYDLLD CHISICSPQV AQLFTDNFDY QT.RDDFVRG
Prn-Rab   ILHFQK.... .TQGLRRFSF PLSLFQGSGA GVEIRYDLLD CHISICSPQV AQLFTDNFDY QT.RDDFVRG
Prn-Yst   CIYYQDLPLP SSREKTSIQI DPELLDNVDE FV.IRNDLID CRIDICTSHV PLIFQENFDY QSLRTDFVKG
Consens    lhfQk.... .tqglrrf.f plsLfqgs.. gVeiRyDLlD ChIsICspqV aqlFtdNFDY Qt.RdDFVrG
         281                                                                 350
Prn-Hum   LLVNEEILGN QIHMHVTAKE YGARVSNLHM YSAVCADVIR RWVYPLTPEA NFTDSTTQSC THSRHNIYRG
Prn-Rab   LLVNEEILGN QIHMHVTTRE YGARVSNLHM YSAVCADVIR RWVYPLTPEA NFTDSTAQSC THSRHNIYRG
Prn-Yst   .VISSDILGK HIYAYLTD.E YAVRVESWQT YDTISQDFLG RWCYPLVLDS NIQD..DQTY SYESRHIYKE
Consens   llvneeILGn qIhmhvT..E YgaRVsnlhm YsavcaDvir RWvYPLtpea NftDst.Qsc thsrhnIYrg
         351                                                                 420
Prn-Hum   PEVSLGHGSI LEENVLLGSG TVIGSNCFIT NSVIGPGCHI GDNVVLDQTY LWQGVRVAAG AQIHQSLLCD
Prn-Rab   PEVSLGHGSI LEENVLLGSG TVIGSNCSIT NSVIGPGCCI GDNVVLDRAY LWKGVQVASG AQIHQSLLCD
Prn-Yst   KDVVLAQSCK IGKCTAIGSG TKIGEGTKIE NSVIGRNCQI GENIRIKNSF IWDDCIIGNN SIIDHSLIAS
Consens   peVsLghgsi leenvllGSG TvIGsnc.It NSVIGpgC.I GdNvvld..y lW.gv.va.g aqIhqSLlcd
         421                                                                 490
Prn-Hum   NAEVKERVTL KPRSVLTSQV VVGPNITLPE GSVISLHPPD AEEDEDDGEF SDDSGADQEK DKVKMKGYNP
Prn-Rab   HAEVKEQVTL KPHCVLTSQV VVGPNITLPE GSVISLHPPD AEEDEDDGQF SDDSGVNQAK EKAKLKGYNP
Prn-Yst   NATLGSNVRL NDGCIIGFNV KIDDNMDLDR NTKISASP.. ..LKNAGSRM YDNESNEQFD QDLDDQTLAV
Consens   nAevke.VtL kp.cvltsqV vvgpNitLpe gsvISlhPpd aeededdg.f sDdsg..Q.k .k.k.kgynp
         491                                                                 560
Prn-Hum   AEVGAAGKGY LWKAAGMNME EE........ EELQQNLWGL KINMEEESES ESE......Q SMDSEEPDSR
Prn-Rab   AEVGVAGKGY LWKAADMNTE KE........ EELRQSLWGL TINEEEESET ESE......R SMDSEELDSR
Prn-Yst   SIVGDKGVGY IYESEVSDDE DSSTEACKEI NTLSNQLDEL YLSDDSISSA TKKTKKRRTM SVNSIYTDRE
Consens   aeVG.aGkGY lwkaa.mn.E .e........ eeL.q.LwgL .in.eeeSe. ese....... SmdSee.Dsr
         561                                                                 630
Prn-Hum   GGSPQMDDIK VFQNEVLGTL QRGKEENISC DNLVLEINSL KYAYNVSLKE V....MQVLS HVVLEFPLQQ
Prn-Rab   AGSPQLDDIK VFQNEVLGTL QRGKEESISC DNLILEINSL KYAYNISLKE V....MQVLS HVVLEFPLQQ
Prn-Yst   EIDSEFED.E DFEKEGIATV ERAMENNHDL DTALLELNTL RMSMNVTYHE VRIATITALL RRVYHFIATQ
Consens   .gspq.dDik vFqnEvlgTl qRgkEenisc Dnl.LEiNsL kyayNvslkE V....mqvLs hvVleFplqQ
         631                                                                 700
Prn-Hum   MDSPLDSSRY CALLLPLLKA WSPVFRNYIK RAADHLEALA AIEDFFLEH. .EALGISMAK VLMAFYQLEI
Prn-Rab   MDSPLEANRY CALLLPLLKA WSPVFRNYIK RAADHLEALA AIEEFFLEH. .EALGTCIAK VLMGFYQLEI
Prn-Yst   TLGPKDA... ...VVKVFNQ WGLLFKRQAF DEEEYIDLMN IIMEKIVEQS FDKPDLILFS ALVSLYDNDI
Consens   mdsPlda.ry calllpllka WspvFrnyik raadhleala aIeefflEh. .ealg...ak vLm.fYqleI
         701                                                         747
Prn-Hum   LAEETILSWF SQRDTTDKGQ QLRKNQQLQR FIQWLKEAEE ESSEDD*
Prn-Rab   LAEETILSWF GQRDVTDKGR QLRKNQQLQR FIQWLKEAEE ESSEDD*
Prn-Yst   IEEDVIYKWW DNVSTDPRYD EVKK..LTVK WVEWLQNADE ESSSEEE
Consens   laEetIlsWf .qrdttdkg. qlrKnqqlqr fiqWLkeAeE ESSedd*
```

Figure 10. 5' RACE Result



(A) Schematic diagram of 5'-RACE. In case of human mRNA, hGSP (human Gene Specific Primer 1&2), Anchor Primer (AP), and/or Uni Amplification Primer (UAP) are used. "*Eco*RI" represents the internal site of the cloned 2.8 kb human cDNA. The dotted line represen poly(C) tail. In case of control mRNA, cGSP1 & 2 were used inste hGSP1 & 2. In addition, a third cGSP3 was also used. Figures we drawn to scale. (B) 5'-RACE result of human and control mRNA. 1 human HeLa cell total RNA was used for this experiment. Lane 1, control. Lane 2, no TdT control. Everything is the same as in Lanes except no TdT was added in the TdT tailing step. Lane 3 & 4, 5'-F result of human HeLa cell mRNA. Lane 5, control C. TdT tailed c cDNA was amplified by cGSP2 and AP. Lane 6, control B. After th strand cDNA synthesis of the control RNA, the resulting cDNA was pu by spin column cartridge and amplified by cGSP2 &3. Lane 7, cont Same as in control B except that no purification was done. Lane 8 DNA ladder. (C) Southern blotting of (B) using rabbit eIF-2Bε sp probes. All lane assignments were the same as in (B). Note that th arrow in (B) does not corresponds to that in (C). (See text for detail)

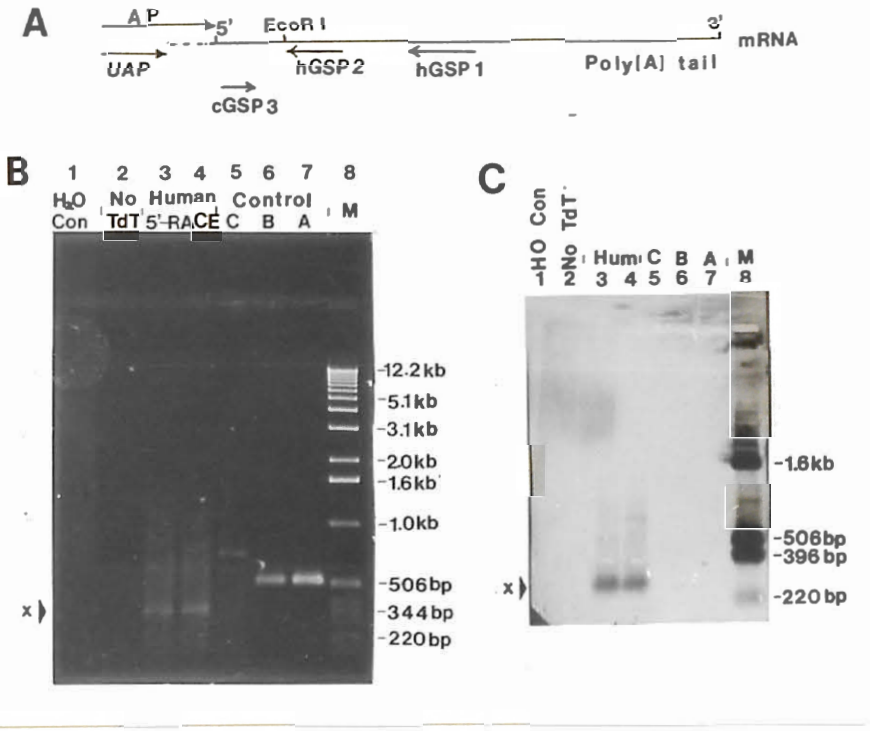

A
AP
5'
EcoR I
3'
mRNA
UAP
hGSP2
hGSP1
Poly[A] tail
cGSP3
B
1 2 3 4 5 6 7 8
H2O Con
No TdT
Human 5'-RACE
Control C B A
M
12.2kb
5.1kb
3.1kb
2.0kb
1.6kb
1.0kb
506bp
344bp
220bp
x
C
H2O Con
No TdT
Hum
C B A M
1 2 3 4 5 6 7 8
1.6kb
506bp
396bp
220bp
x

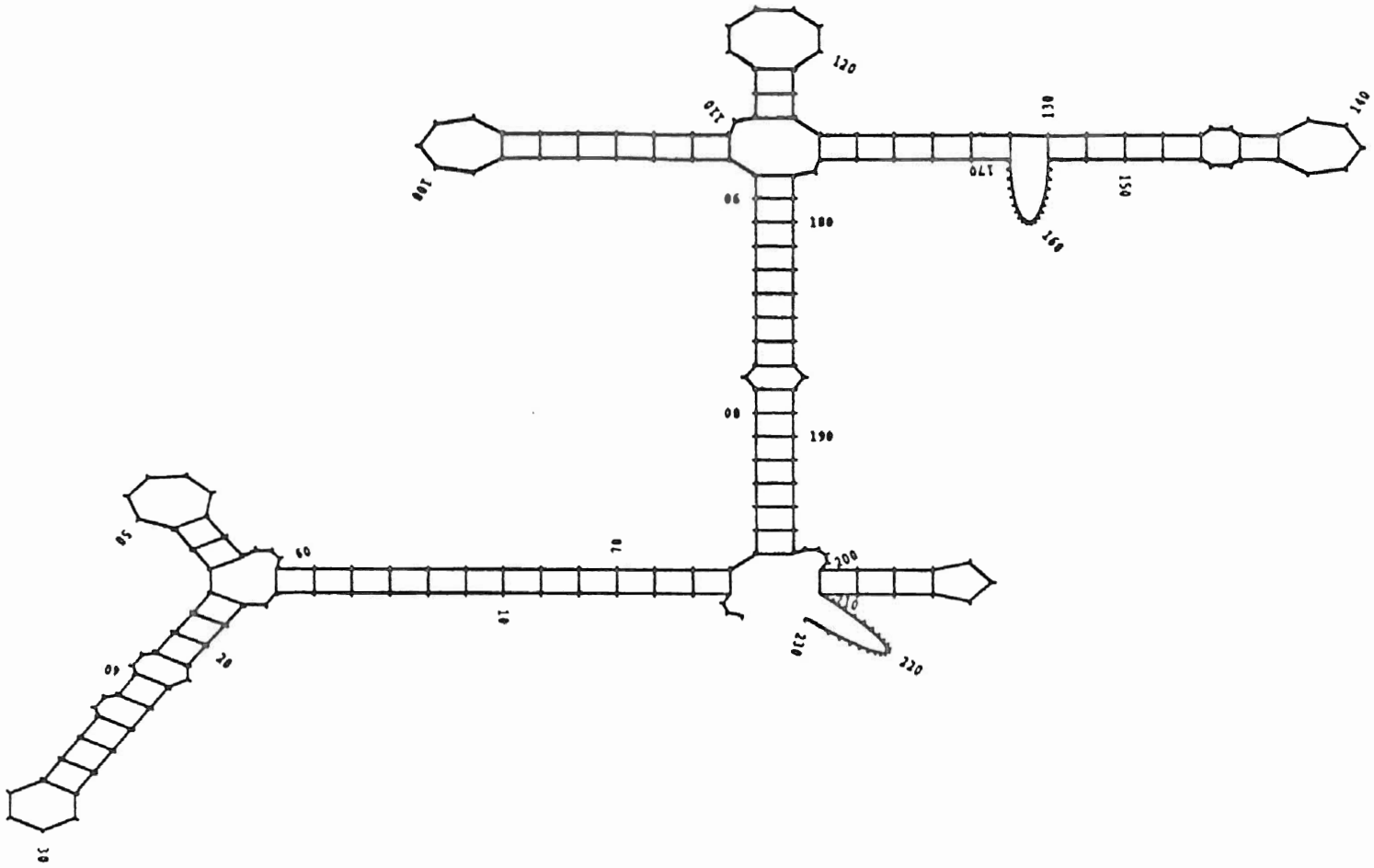
Figure 11. Potential 5' End mRNA Secondary Structures of Rabbit eIF-2Bε

The 5'-end 230 bp of rabbit eIF-2Bε mRNA was plotted by us
MFOLD program of the GCG package. The folding temperature wa
The free energy unit was Kcal/mole.

Squiggle plot of: [.Mfold]82-1_230bp.Mfold;1 April 29, 1993 20:58

(Linear) MFOLD of: 82.Gcg T: 37.0 Check: 4937 from: 1 to: 230 April 28, 1993 22:16

Length: 230 Energy: -93.8



CHAPTER V

CONCLUSIONS AND FUTURE STUDIES

Conclusions

A 2312 bp partial cDNA encoding human eIF-2Bε (82 kDa) subunit has been cloned and analyzed. Sequence analysis shows that the human partial cDNA and its rabbit counterpart share significant sequence homology; approximately 90% identity for both nucleotide sequences and protein sequences have been observed. For the aligned 641 amino acid residues, only 55 changes have been observed. Among them, 38 are conservative substitutions. Thus the overall similarity between human and rabbit peptide sequence is above 97%.

Several lines of evidence from *in vitro* studies indicate that the rabbit cDNA encodes eIF-2Bε: (1) the cDNA codes for an isopropylthio-galactoside-inducible 86 kDa fusion protein that reacts with the anti-eIF-2B antiserum on immunoblots; (2) the cDNA contains a single long ORF whose predicted amino acid sequence encompasses the sequence of a peptide isolated after partial hydrolysis of the purified eIF-2Bε subunit; (3) translation in reticulocyte lysate of mRNA transcribed *in vitro* from the cDNA yields a ^{35}S-labeled protein that comigrates with endogenous eIF-2Bε (88). Because of the significant sequence homology observed, it is almost certain that the cloned partial human cDNA encodes human eIF-2Bε subunit. However, it is still not certain whether translation of rabbit eIF-2Bε mRNA starts at a non-AUG (GUG in this case) start codon or at an upstream AUG codon which has not yet been cloned. mRNA secondary structure prediction reveals that potential stable secondary structures exist at the 5' end of the mRNA. The unusual structure might play a role in the translation initiation if GUG is used

as the start codon. Preliminary results from this lab seem to favor this assumption (90). Because the human cDNA is highly homologous to the rabbit cDNA, it is very likely that these secondary structures also exist in human mRNA. Whether human cDNA uses GUG as start codon needs to be determined.

Future Studies

Theoretically, at least two approaches can be employed to obtain the lost 5' end coding region. One is to screen the appropriate genomic library which will not only give the 5' end coding region but also the 5'-untranslated region. Once obtained, it may help to resolve the dispute whether translation of eIF-2Bε mRNA is initiated through a normal AUG codon or a non-AUG codon (in this case, a GUG codon) and help to identify the N-terminus of the mature peptide. Studies on the regulation of the gene expression will be possible if the upstream untranslated region and promoter region are available (For example, antisense RNA can be used to suppress eIF-2Bε expression if the 5' end sequence is obtained).

In addition, the 5'-RACE (Rapid Amplification of 5'-cDNA Ends) procedure can be modified to overcome the problems encountered. A reverse transcriptase (Retrotherm™ RT DNA Polymerase from Epicentre Technologies, Madison, Wisconsin) which functions at 70°C or above can substitute the SuperScript reverse transcriptase so that the reverse transcription can be carried out at a temperature high enough to melt most secondary structures.

Site-directed mutagenesis can be performed by PCR to change the first postulated non-AUG start codon (GUG) to AUG. This mutant cDNA can be used in an *in vitro* transcription and translation system to check if the expression level of the full length translation product is increased; previous work in this lab reported that an 80 kDa *in vitro* translation product of the rabbit eIF-2Bε cDNA did exist, although at a low level.

Regulation of translation initiation factor gene expression during human T cell activation has been reported for eIF-2α, -4E, and -4A (89). Regulation of eIF-2B expression may also occur. In preliminary studies, western blot analysis indicated that the eIF-2Bε levels increased upon mitogen stimulation of T cells. eIF-2Bε mRNA levels are currently being semi-quantitated by the RT-PCR method since the available sequence information allows us to synthesize eIF-2Bε specific primers.

Casein kinase II has been shown to be able to phosphorylate eIF-2Bε subunit in vitro (42). It would also be of interest to see if this also happens *in vivo* and if the phosphorylation sites in both cases are the same. If these are true, then what is the possible signal transduction pathway that leads to the phosphorylation of eIF-2Bε and how is it regulated? The cloning of this eIF-2Bε cDNA will greatly facilitate such kind of investigation.

The cloned eIF-2Bε cDNA can be used to generate large amounts of proteins for monoclonal antibody production. The availability of these antibodies may help to determine the eIF-2B/eIF-2 ratio *in vivo*, which is the primary value upon which the sequestrated model for global protein synthesis regulation is based.

Partial peptide sequences for other eIF-2B subunits are available. These will be used to synthesize degenerate primers for PCR. The resulting gene fragment will be used to clone cDNAs encoding other eIF-2B subunits. Once available, their specific functions and mutual interactions can be studied in a greater detail.

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