1997


Zelpha Elizabeth Floyd
Louisiana State University and Agricultural & Mechanical College

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CHARACTERIZATION OF BEAN EUKARYOTIC TRANSLATION INITIATION FACTOR 5: CLONING, SEQUENCING, AND STRUCTURE-FUNCTION STUDIES

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Department of Biochemistry

by

Zelpha Elizabeth Floyd
B.S.N., University of Alabama in Birmingham, 1977
August 1997

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Dedication

This dissertation is dedicated to my husband, Steven Barker. Steve has always supported my decision to return to school and he has been confident that I would reach my goal, even when I was not. He is the sturdiest and truest of partners and his unwavering belief in me has been essential over the past few years. I would not have finished graduate school without him.
Acknowledgments

I am indebted to quite a few people who have helped me navigate graduate school. My graduate advisory committee, Drs. Patrick DiMario, Ding Shih, and Gary Winston have consistently supported my research efforts and I am grateful for all of their contributions. I would also like to thank Dr. Simon Chang for his encouragement; it has meant a lot to me. My graduate advisor, Dr. Sue Bartlett, expects all of her graduate students to challenge themselves and her enthusiasm for biochemistry and willingness to embark on unexpected paths has provided me with an exceptional learning experience. I am grateful to Dr. Bartlett for all of her advise and support.

There are others who have helped me complete graduate school by sharing the results of their efforts with me. The bean seed cDNA expression library was obtained from Dr. Norimoto Murai; Dr. Karen Leach generously shared her anti-hsp 56 antibody; Dr. Andrew Bent provided me with a detailed protocol for vacuum infiltration of Arabidopsis thaliana, and Dr. Jay Means shared his expertise with me in performing the ICP-MS zinc analysis.

My lab mates, past and present: Michael Bracey, Hee Jin Kim, Carmen Dessauer, Wei Liu, Katrina Ramonell, Pilar Tovar, Linda Shaffer, and Xiaochun Xi. I have been fortunate to work with each of them. They have taught me a lot about doing research and I will always value their friendship.

Finally, I would like to thank my parents. I am indebted to them for many things; among those is having shared with me the joy of reading, a sense of curiosity, and the pleasures of solving puzzles. And a sense of humor. On the occasion of my parents’
fortieth anniversary, I asked my father what was his advise for a long and happy marriage. He laughed and replied, "perseverance". It turns out to be good advise not only for marriage, but for graduate school as well.
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Abstract

Translation initiation is emerging as one of the important mechanisms involved in regulation of gene expression and our understanding of this process in eukaryotes has greatly increased over the past twenty years. Research in translation initiation in plants has lagged behind efforts in the mammalian system and a number of the initiation factors have not been characterized. Eukaryotic initiation factor 5 (eIF5), the protein mediating hydrolysis of the eIF2-bound GTP and the subsequent formation of the 80S ribosome, is among the least characterized initiation factor in plants. This dissertation describes the cloning of the cDNA encoding eIF5 from a bean seed cDNA expression library as well as the sequencing of the cDNA, and the purification of the recombinant protein.

Bean eIF5 shares 37-39% identity and 59-60% similarity with rat and yeast eIF5, respectively, with the majority of the conserved residues at the amino-terminal region of each of these proteins. The conserved elements of the carboxy-terminal region of eIF5 include two highly conserved tryptophans and an acidic tail. While eIF5 is considered to function as a GTPase, the G1 and G2 domains at the amino-terminus are the only strictly conserved GTPase domains found in all of the eIF5s and the G1 domain is atypical of the ras-type GTPase superfamily.

It is shown that eIF5 is a C2C2-type zinc-finger protein, binding zinc in a 1:1 molar ratio. Substitution of two of the zinc-binding cysteines with alanine reveals that the zinc ion is an important structural element of eIF5 but it remains unclear what role the zinc-finger may play in eIF5's ribosomal function. However, eIF5 does interact with nucleic acids and this interaction requires the native form of eIF5. In particular, eIF5 will
interact with rRNA in an interaction that requires the presence of the carboxy-terminal region in order to occur with full avidity.
Chapter 1

Literature Review

In 1955, Paul Zamecnik and coworkers determined that $^{14}$C-labeled leucine and valine were transiently associated with subcellular structures in microsomal fractions that had been termed "ribonucleoprotein" particles (Littlefield et al., 1955). The bulk of the protein in the microsomal preparations was labeled more slowly. This experiment cemented the idea that the newly described ribonucleoprotein particles or ribosomes (Zamecnik, 1969) were the sites of protein synthesis in the cell. Ribosome-mediated protein synthesis is now understood to be one of the central processes involved in the flow of genetic information from DNA to RNA to proteins.

The translation of genetic information occurs in three distinct steps at the ribosome: initiation, elongation, and termination (Voet and Voet, 1995). While each of these steps are clearly important, most of the mechanisms involved in regulation of translation are known to have their effect at the point of translation initiation (Pain, 1996). The process of translation initiation is very similar in all eukaryotes (Browning, 1996) and can also be divided into three steps. The first step is concerned with positioning the initiator tRNA at the 40S ribosomal subunit to form a 43S preinitiation complex. This complex then binds to the messenger RNA (mRNA) and the mRNA is "scanned" until the correct starting codon is found in the second step. Initiation of protein synthesis is completed when the 60S ribosome joins the 43S initiation complex and elongation of the polypeptide begins (Merrick, 1992; Pain, 1996). These three...
Figure 1.1 Overview of translation initiation. Adapted from Merrick, 1992 and Pain, 1996.
Table 1.1 Comparison of mammalian and plant translation initiation factors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Mammals</th>
<th>Plants</th>
<th>mass (kDa)</th>
<th>function</th>
<th>mass (kDa)</th>
<th>function</th>
</tr>
</thead>
<tbody>
<tr>
<td>elF1A (elF4C)</td>
<td>delay reassociation of 40S and 60S subunits</td>
<td>unknown</td>
<td>16.5</td>
<td>forms initiation complex with GTP and tRNA$_{\text{Met}}$</td>
<td>12.7</td>
<td>forms initiation complex with GTP and tRNA$_{\text{Met}}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\alpha$ 36.2</td>
<td></td>
<td>$\alpha$ 42.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\beta$ 38.4</td>
<td></td>
<td>$\beta$ 38.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$\gamma$ 51.8</td>
<td></td>
<td>$\gamma$ 50.0</td>
</tr>
<tr>
<td>elF2</td>
<td>interacts with elF2 as a GDT-GTP exchange factor</td>
<td>contains five subunits, in yeast, Mr = 290 kDa</td>
<td>unknown</td>
<td>has not been isolated from plants</td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>elF3</td>
<td>delay reassociation of 40S and 60S subunits; binds mRNA to initiation complex</td>
<td>eight subunits, Mr 500-750 kDa</td>
<td>binds mRNA to the initiation complex</td>
<td>ten subunits, sediments as 15S particle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>elF4A</td>
<td>ATP-dependent RNA unwinding</td>
<td>46.4</td>
<td>ATP-dependent RNA unwinding</td>
<td>46.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>elF4B</td>
<td>cofactor for the helicase activity of elF4A</td>
<td>69</td>
<td>stimulates helicase activity of elF4A (not required)</td>
<td>59.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>elF4E</td>
<td>$\text{m}^7\text{G}$ cap-binding protein</td>
<td>25.1</td>
<td>$\text{m}^7\text{G}$ cap-binding protein</td>
<td>26.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>elFiso4E</td>
<td>not found</td>
<td></td>
<td></td>
<td>$\text{m}^7\text{G}$ cap-binding protein</td>
<td>28.0</td>
<td></td>
</tr>
<tr>
<td>elF4G</td>
<td>align elF4E and 40S ribosome</td>
<td>154</td>
<td>ATP-dependent unwinding of mRNA</td>
<td>220</td>
<td></td>
<td></td>
</tr>
<tr>
<td>elFiso4G</td>
<td>not found</td>
<td></td>
<td></td>
<td>ATP-dependent unwinding of mRNA</td>
<td>86.0</td>
<td></td>
</tr>
<tr>
<td>elF5</td>
<td>mediate hydrolysis of elF2-bound GTP; subunit joining</td>
<td>49.0</td>
<td>activity not yet determined in plants</td>
<td>48.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>elF5A</td>
<td>positioning of tRNA$_{\text{Met}}$</td>
<td>18.0</td>
<td>$^c$positioning of tRNA$_{\text{Met}}$</td>
<td>18.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>elF6</td>
<td>antireassociation factor, binds 60S subunit</td>
<td>25</td>
<td>antireassociation factor</td>
<td>23.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* adapted from Pain, 1996; † adapted from Browning, 1996; ‡Mehta et al., 1994
steps require the participation of over ten different soluble initiation factors and many of these eukaryotic translation initiation factors have been characterized. An overview of translation initiation is shown in Figure 1.1 and a comparison of mammalian and plant initiation factors is given in Table 1.1.

The eukaryotic translation initiation factors have been shown to be involved in positioning the initiator tRNA at the 40S ribosome; binding the 5' cap structure of the mRNA; mediating the scanning of the mRNA; promoting the dissociation or slowing the reassociation of the 40S and 60S ribosomal subunits and in the joining of the two ribosomal subunits to form the 80S ribosome (Kozak, 1983; Pain, 1996). One of the best characterized of these initiation factors is eIF2. This protein is composed of three subunits and is a GTP-binding protein. The eIF2-GTP binary complex functions to recruit the initiator tRNA and to carry that tRNA to the 40S ribosome as part of a ternary complex of eIF2 - GTP - tRNA$_{\text{Met}}$. Once this complex is in place, the mRNA is scanned until the start site is chosen. At this point, the eIF2-bound GTP is hydrolyzed and the 80S ribosome is formed. The hydrolysis of the GTP is a pivotal step in the process of translation initiation and another translation initiation factor, eIF5, is required at this juncture (Merrick, 1992; Pain, 1996).

The characterization of eIF5 has lagged behind that of the other translation initiation factors due to the lack of abundance of eIF5 in the cell coupled with the realization that eIF5 is the most catalytically active initiation factor (Merrick, 1992). These two factors misled earlier investigators to focus on a high molecular weight contaminant associated with eIF5 activity and call into question the conclusions that have been reached regarding eIF5 based on these early experiments. It was first isolated in
1975 as a single polypeptide chain with a molecular weight of 125 kDa on the basis of an assay that measured translation of globin mRNA (Merrick et al., 1975). The purified protein was termed IF-M2A and was also reported to have ribosome-dependent GTPase activity in the absence of any other initiation factor. At about the same time, Schreier and Staehelin (1973) isolated a protein having equivalent activity which they termed IF-E5. Further studies involved in examining the mechanism of translation initiation supported the molecular weight determination of 125-150 kD for the protein, which was termed eIF5 (Schreier et al., 1977, Benne and Hershey, 1978; Brown-Luedi et al., 1982).

While these studies generally supported the notion that eIF5 had ribosome-dependent GTPase activity, other assays of eIF5 activity were also being used. In particular, it was determined that eIF5 was required for the joining of the 60S subunit to the 40S initiation complex containing the template mRNA and initiator tRNA although eIF5 was not required for the binding of the initiator tRNA to the 40S ribosomal subunit (Nombela et al., 1975). A role for eIF5 in subunit joining was consistent with its ribosome-dependent GTP hydrolysis activity since it had been shown that GTP hydrolysis was required for subunit joining (Benne and Hershey, 1978).

Experiments carried out since 1978 have served to further clarify the purpose of eIF5 in the process of translation initiation. It is now understood that the 43S initiation complex is fully assembled before eIF5 interacts with the complex (Peterson et al., 1978; Raychaudhuri et al., 1985). The ternary initiation complex can be formed in the presence of a nonhydrolyzable form of GTP (Peterson et al., 1978) but this form of GTP will not support the formation of the 80S ribosome nor allow for the release of the
eIF2-GDP complex from the 40S subunit. Hydrolysis of the eIF2-bound GTP is clearly mediated by eIF5 and this step is required for the formation of the 80S ribosome. However, subunit joining can be demonstrated in experiments using a purified intermediary initiation complex of eIF2-GDP and 60S ribosomal subunits without the addition of eIF5. Moreover, eIF5 is not associated with the intermediate complex nor can eIF5 be found associated with either the 60S or 80S ribosome (Chakrabarti and Maitra, 1991). In addition, P_i and the eIF2-GDP complex are released from the ribosome prior to the joining of the 60S subunit to the 40S subunit (Raychaudhuri et al., 1985; Raychaudhuri and Maitra, 1986). Taken together, these results indicate that the sole function of eIF5 in translation initiation is to mediate hydrolysis of the GTP bound to the initiation complex. Since GTP hydrolysis is an obligatory step immediately preceding subunit joining, eIF5 is required for formation of the 80S ribosome.

However, the designation of eIF5 as a GTPase is problematic. In a series of carefully controlled experiments, Chakrabarti and Maitra (1991) examined hydrolysis of the ribosome-bound GTP. The series of experiments shows that there is a basal level of GTP hydrolysis when the 40S initiation complex alone is present. This result is the same regardless of what factor is added to the assay if the AUG codon is absent. This indicates a basal level of GTP hydrolysis that occurs when eIF2, GTP, and the initiator tRNA are present on the 40S subunit, but eIF5 is absent. When eIF5 is added to the initiation complex, GTP hydrolysis reaches its maximum level, a level that is not increased by the addition of the 60S subunit. However, eIF5 does not hydrolyze GTP in the absence of the components required to form the 43S initiation complex (Raychaudhuri et al., 1985). Although this result could support consideration of eIF5 as
a GTPase Activating Protein (Boguski and McCormick, 1993), eIF5 is still regarded as a GTPase. Indeed, Maitra notes that his results differ from the earlier finding by Merrick (Merrick et al., 1975) that eIF5 (termed IF-M2A) could act as a GTPase in the absence of any other initiation factor.

The discrepancy between the two results has been resolved by Maitra's purification of eIF5 and use of the isolated initiation complex to assay the activity of eIF5. Using a series of purification steps, eIF5 can be isolated from various sources as a single polypeptide having a molecular mass of 45-62 kD (Raychaudhuri et al., 1985; Raychaudhuri et al., 1987; Das et al., 1993; Chakravarti and Maitra, 1993). The first report of the lower molecular weight eIF5 (Raychaudhuri et al., 1985) also serves to illustrate the catalytic ability of eIF5; one pmol eIF5 is able to promote the formation of nearly 150-200 pmol 80S ribosome under in vitro conditions. So, as little as 45-60ng of eIF5 is sufficient to saturate the in vitro assays of eIF5 activity. This level of catalytic activity, coupled with the low abundance of eIF5 in the cell, makes the purification of eIF5 a difficult task and earlier methods of purification may have been copurifying other proteins along with eIF5. This can account for the appearance of a high molecular weight polypeptide along with eIF5 activity and the finding that eIF5 acts as a GTPase in the absence of any other initiation factor.

In 1993, Maitra’s group reported the cloning, sequencing, and expression of eIF5 from both rat and Saccharomyces cerevisiae (Das et al., 1993; Chakravarti and Maitra, 1993). This has been followed by the cloning and sequencing of the human eIF5 cDNA in 1996 by the same laboratory (Kausik et al., 1996). These results have settled the
question of the molecular weight of eIF5 and have opened the door to a more complete examination of the structure and function of eIF5.

The cDNA corresponding to the rat eIF5 encodes a protein having a predicted molecular weight of 48,926; the protein migrates on SDS-gel electrophoresis at 58,000 daltons. The *Saccharomyces cerevisiae* of eIF5 is encoded by an open reading frame predicting a molecular weight of 45,346 and the human eIF5 cDNA encodes a protein having a molecular weight of 47,410.

Each of the purified recombinant eIF5s is catalytically active when assayed for promoting formation of the 80S ribosome. In fact, the specific activity of rat eIF5 is determined to be 40,000 units per mg (1 unit of eIF5 is defined to be that amount of protein required to promote the formation of 1 pmol 80S ribosome), corresponding to almost 2000 units per nmol eIF5 (Das *et al*., 1993). This is higher than the value initially reported for eIF5 and serves to reinforce the idea that eIF5 is catalytically active in vivo in very small amounts. In addition, the recombinant eIF5s are able to mediate the hydrolysis of ribosome-bound GTP as shown for the rabbit reticulocyte purified eIF5. It was also shown, using knockout mutations, that the yeast eIF5 gene is essential for cell growth (Chakravarti and Maitra, 1993).

A comparison of the deduced amino acid sequences of rat and yeast eIF5 reveals 60% homology and 39% identity between the proteins with the majority of the conserved amino acids at the amino terminal region of each protein. Maitra has noted that eIF5 contains regions of homology with the GTPase superfamily, with characteristic sequence motifs in four of the five domains identified for GTPases (Bourne *et al*., 1991). Two of these domains, G1 and G2, are at the highly conserved N-terminus of eIF5 and
are identified as being involved in forming bonds with the α- and β- phosphates of either GTP or GDP in the case of the G1 domain or interacting with the Mg²⁺ ion coordinated to the β- and γ- phosphates for the G2 domain. The remaining two domains are involved in binding to the Mg²⁺ and forming a hydrogen bond with the γ- phosphate of GTP for the G3 domain and forming hydrogen bonds with the groups on the guanine ring and stabilizing the nucleotide binding site in the case of G4. None of the eIF5s have a region of homology with the remaining GTPase domain, G5. This domain interacts with the guanine nucleotide and may be the only region with direct contact with GTP. However, it cannot be clearly identified in all of the subfamilies of the GTPase superfamily. The designation of eIF5 as a GTPase is further complicated by the G1 domain as it is found in eIF5. The consensus domain for the phosphate binding loop is GXXGXGK(S/T), but this is represented in eIF5 as GXGXGXK(S/T). As Maitra notes, “insertion of an extra amino acid into the consensus loop sequence suggests that eIF5, by itself, is not functional as a GTPase” (Das et al., 1993).

There are additional regions of interest in eIF5, including potential phosphorylation sites. Analysis of the amino acid sequence indicates that eIF5 contains potential phosphorylation sites for casein kinase II, protein kinase C, p34cdc2/cyclin B kinase and protein kinase A (Das et al., 1993). Other translation initiation factors are known to be phosphorylated both in vitro and in vivo and the modification has been shown to be involved in regulation of translation initiation in the case of the cap-binding complex (eIF4F = eIF4A, eIF4E, and eIF4G) and eIF2 (Pain, 1996). The phosphorylation level of eIF4E is increased under conditions in which the level of protein synthesis is increased (Merrick, 1992; Pain, 1996) and the pathways thought to be
involved in phosphorylation of eIF4E include the MAP kinase (Flynn and Proud, 1996) and the p70S6K pathways (Mader et al., 1995; Zanchin and McCarthy, 1995). Phosphorylation of eIF2 has been well studied and Ser51 of the eIF2α subunit is known to be phosphorylated by three different kinases: the heme-controlled repressor, the double-stranded RNA protein kinase (PKR) (Proud, 1995), and GCN2, a kinase activated by amino acid starvation (Hinnebusch, 1994). In each case, phosphorylation of eIF2α interferes with the ability of the GDP-GTP exchange factor, eIF2B, to remove the eIF2-bound GTP, thus blocking the formation of the eIF2-GTP-tRNA<sub>Met</sub> initiation complex (Siekierka et al., 1984). Because of eIF5’s position in translation initiation and its relative low abundance, post-translational modification of the protein could have significance in the regulation of translation. It has been shown that eIF5 immunoprecipitated from rabbit reticulocytes is phosphorylated in vitro at serine residues (Chevesich et al., 1993). The phosphopeptide maps found using rabbit reticulocyte lysate in in vitro assays correspond to the phosphopeptide maps obtained when using casein kinase II in vitro (Ghosh et al., 1989; Chevesich et al., 1993). In each case, there were two major phosphopeptides and two minor phosphopeptides, corresponding to multiple serine phosphorylations. However, there are different phosphopeptides identified when rat pituitary GH<sub>3</sub> cells are used to examine phosphorylation in vivo (Chevesich et al., 1993). In this instance, although there are two major phosphopeptides and one minor phosphopeptide corresponding to serine residues, the phosphorylated serines comigrate with only one of the rabbit reticulocyte lysate phosphorylated serines by two-dimensional mapping. While eIF5 can clearly be phosphorylated in vitro and in vivo, it is unclear what role phosphorylation may play in the function of eIF5.
Phosphorylation of eIF5 with casein kinase II does not alter the catalytic activity of purified eIF5 in the standard \textit{in vitro} assays designed to measure either promotion of subunit joining or mediation of GTP hydrolysis (Chakrabarti and Maitra, 1991). In fact, Maitra has now used phosphorylation of eIF5 as a tool to increase the sensitivity of detection of eIF5 in some assay conditions (Chaudhuri \textit{et al.}, 1994). In this regard, phosphorylation of purified recombinant eIF5 does not prohibit interaction of eIF5 with eIF2 in an \textit{in vitro} experiment (Chaudhuri \textit{et al.}, 1994). However, phosphorylation of eIF5 could still play a regulatory role \textit{in vivo}. Maitra’s group has noted that eIF5 remains bound to other cellular proteins through several purification steps (Chevesich \textit{et al.}, 1993) and earlier attempts to purify eIF5 consistently isolated a copurifying 125-150 kD protein that was mistaken for eIF5 since the assays were measuring eIF5 activity (Benne and Hershey, 1978). It is possible that (de)phosphorylated eIF5 could have a higher affinity for a cellular protein that could sequester it from the translational apparatus. Alternatively, phosphorylation could alter the half-life of eIF5.

Experiments to examine the interaction of eIF5 with eIF2 are now possible because of the availability of the recombinant form of eIF5 and these experiments have demonstrated that eIF5 forms a specific complex with eIF2 (Chaudhuri \textit{et al.}, 1994). In partially purified preparations from rabbit reticulocytes and yeast, eIF5 remains bound to another protein and will sediment as a 160 kD particle in glycerol gradient centrifugations (Chakravarti \textit{et al.}, 1993). This second protein can be identified as eIF2. Using recombinant eIF5, Maitra has shown that eIF5 will interact with purified eIF2 using gel filtration chromatography and glycerol gradient centrifugation. Using the same experiments, eIF5 will not form a complex with either eIF3 or eIF2B. This is important
since eIF2 does interact with eIF2B and both eIF3 and eIF2B are present at the 40S initiation complex prior to subunit joining (Siekierka et al., 1984; Chakrabarti and Maitra, 1992). Interestingly, the authors noted that, according to unpublished data, prior incubation of eIF2 with GTP or GDP does not have an effect on the interaction of eIF5 with eIF2. This indicates that eIF5 and eIF2 form a stable complex in the absence of bound nucleotide, an observation with potential relevance to the issue of regulation of the interaction of eIF5 and eIF2 at the ribosome.

Maitra reasons that the protein-protein interactions of eIF5 and eIF2 are crucial for the hydrolysis of GTP bound to the initiation complex. He compares the situation in eukaryotes to that in prokaryotic systems. In prokaryotes, IF2 is solely responsible for binding of the initiator tRNA and GTP to the 30S ribosome and for the hydrolysis of GTP after the addition of the 50S ribosome (Maitra, 1982). So, in the eukaryotic system the function of one protein is now shared by two separate proteins, indicating an increased level of complexity in the mediation of GTP hydrolysis and subunit joining in the eukaryotes (Chaudhuri et al., 1994).

This increased level of complexity in control of translation initiation may also be reflected in regulation of the expression of eIF5 itself. The rat and human eIF5 genes each contain two small open reading frames in the 5' untranslated region and this is a feature found in many genes encoding proteins involved in regulatory functions (Geballe and Morris, 1994). A particularly interesting example of this is found in the regulation of expression of GCN4, a transcription factor that regulates the expression of genes encoding proteins involved in de novo amino acid synthesis. Expression of GCN4 under well-fed and starvation conditions is regulated at the translational level under the control
of four short open reading frames in the 5' UTR of GCN4 and the phosphorylation of eIF2α (Hinnebusch, 1994). Under well-fed conditions, eIF2α is dephosphorylated and there are plenty of eIF2-GTP-tRNAInitiation complexes to bind the multiple 5'ORFs of GCN4, some of which inhibit reinitiation, precluding the expression of GCN4. Under starvation conditions, the phosphorylated eIF2α inhibits formation of initiation complexes and recognition of the 5'ORFs that block scanning is suppressed in the presence of limiting amounts of the initiation complex (Hinnebusch, 1994; Pain, 1996). In this way, the expression of GCN4 is increased at the translational level under conditions of stress. Multiple 5' ORFs for initiation factors, including eIF5, may also provide an additional level of translational response to physiological stresses using the same interplay of the 5' ORFs.

Also in the higher eukaryotes, northern blot analysis has shown that multiple mRNAs encode eIF5 (Das et al., 1993 and Si et al., 1996). Primer extension analysis maps transcription initiation to a single site, ruling out different 5'untranslated regions as the source of the multiple mRNAs. Instead, the various mRNAs differ only in the length of the 3' untranslated region and the varying lengths are attributed to selection of different polyadenylation signals at the 3' untranslated region of the transcript (Si et al., 1996). The significance of generating multiple mRNAs using alternative polyadenylation signals has not been established. However, it is known that at least one other translation initiation factor, eIF4E, is included in a large number of mammalian genes that use various polyadenylation signals to produce different mRNAs encoding the same protein (Jaramillo et al., 1991). In addition, it is becoming increasingly clear that the 3'untranslated region and the length of the poly(A) tail play important roles in regulating
gene expression (Wahle and Keller, 1996). The biological significance of the differences in the 3’untranslated region of eIF5 is currently being investigated (Si et al., 1996).

While the information regarding eIF5 has increased dramatically since it was first described in the 1970s, some basic questions about the protein have not been addressed. One of the central questions is whether eIF5 interacts with the ribosome itself. While it is possible that eIF5’s role in translation initiation could be carried out while interacting only with eIF2, it seems to be a general characteristic of initiation factors to interact with nucleic acids (Sonenberg and Shatkin, 1978; Chaudhuri et al., 1981). There are sequence motifs in eIF5 that are associated with nucleic acid binding and an interaction of eIF5 with any of the three types of RNA found at the ribosome would be plausible. The most prominent motif of interest is a putative zinc-binding domain that is found at the N-terminus of eIF5 and is strictly conserved in all of the eIF5s sequenced to date. Clarification of the nucleic acid binding capabilities of eIF5 will potentially contribute to an understanding of the regulation of translation initiation.

All of the research completed to date on eIF5 has centered on characterizing the protein from a group of eukaryotic species that has not included plants. Currently, eIF5 has only been partially purified from wheat germ extract (Lax et al., 1986) and has not been characterized. Any discussion of universal features of eIF5 will have to include characterization of these features in plants as well as animals.

This dissertation discusses the initial characterization of eIF5 from Phaseolus vulgaris (L.), the common bean. It describes the isolation and sequencing of the cDNA encoding eIF5, the purification of the protein, and the generation of transgenic Arabidopsis in order to study the phosphorylation of eIF5. In addition, the dissertation
addresses whether eIF5 is a zinc binding protein and demonstrates that eIF5 does interact with ribosomal RNA. Herein is presented the first discussion of structure-function relationships in eIF5 in plants and it is hoped that these results will further the understanding of translation initiation in plants and in eukaryotes in general.
Chapter 2

Identification of eIF5 in Phaseolus vulgaris (L.):
Screening, Sequencing, Expression, Purification, and Characterization of eIF5 as a Zinc-binding Protein

Introduction

Translation initiation in all eukaryotes requires the participation of over 10 different soluble initiation factors that can be dissociated from the ribosome using high salt conditions (Pain, 1996; Browning, 1996). Each of these initiation factors has a specific role to play in the assembly of the 80S ribosome and all of the factors must be removed from the ribosome prior to the commencement of translation elongation.

Release of these factors from the 40S initiation complex depends on the hydrolysis of the eIF2-bound GTP and eIF5 is responsible for mediating this reaction. After the GTP is hydrolyzed, the 40S and 60S subunits are joined to form the active 80S ribosome. Elucidation of how subunit joining occurs has been greatly facilitated by the cloning and sequencing of eIF5 from yeast and mammals.

Eukaryotic initiation factor 5 is a monomeric protein having a molecular weight ranging from 45 kDa to 50 kDa as deduced from conceptual translation of the cDNA’s from yeast and mammals (Das et al., 1993; Chakravarti and Maitra, 1993). Examination of the primary sequence of eIF5 reveals regions of the protein having homology with the GTPase superfamily (Bourne et al., 1991). This is consistent with eIF5’s role in mediating GTP hydrolysis but it remains unclear whether eIF5 is itself a GTPase (Das et al., 1993). Other regions of interest in the primary sequence are the putative zinc finger domain (Klug and Rhodes, 1987) at the conserved N-terminus of eIF5 and a motif at the
C-terminus that contains highly conserved tryptophan residues and was speculated to be involved in protein-protein interactions (Koonin, 1995).

Using recombinant eIF5, Maitra’s group has shown that eIF5 associates with eIF2 in a highly specific interaction (Chaudhuri et al., 1994). It is plausible that the two initiation factors interact at the surface of the 40S ribosome after the ternary complex of eIF2-GTP-tRNA\textsubscript{Met} has been positioned at the initiator codon (Pain, 1996).

While the structure-function relationships of eIF5 are being investigated for the yeast and mammalian eIF5, nothing is known about eIF5 from plants. The protein has been partially purified from a wheat germ extract but has not been characterized (Lax et al., 1986). An understanding of the structure-function relationships in plant eIF5 will be important in understanding the regulation of translation in plants in response to developmental cues or to environmental stresses (Browning, 1996). In addition, characterization of eIF5 from plants will allow for a more complete understanding of translation initiation in all eukaryotes.

This chapter discusses the isolation of a cDNA encoding eIF5 in the common bean, Phaseolus vulgaris (L.), and the derived protein sequence is compared with that of eIF5 from rat and yeast. A strategy for overexpressing and purifying the recombinant protein is described. Finally, the characterization of eIF5 as a zinc-binding protein is also presented.

Materials and Methods

Screening a bean seed cDNA expression library. Phaseolus vulgaris (L.) (common bean) seeds were harvested and processed as total seed, embryo, or cotyledon to assay for immunoreactivity with anti-hsp 56 antibody (gift from Dr. Karen Leach, Upjohn
Laboratories) in an effort to identify hsp 56, a prolyl isomerase, in bean tissue. The tissue was ground with a mortar and pestle after freezing the tissue using liquid N\textsubscript{2}. An extraction buffer containing 20 mM Tris-Ac, pH 7.6, 90 mM KCl, 5 mM MgCl\textsubscript{2}, and 1 mM DTT was added to the ground tissue and the mixture was incubated on ice for 30 minutes with occasional mixing. The extract was subjected to centrifugation at 14,474 x g using an SA600 rotor for 15 minutes and the supernatant was analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) (Laemmli, 1970) followed by electrophoretic transfer to nitrocellulose for western blot analysis. Rabbit anti-hsp56 (UPJ56, Pharmacia and Upjohn) was used as the primary antibody and goat anti-rabbit IgG conjugated to horse radish peroxidase (Sigma Co) was used as the secondary antibody. Cross-reacting proteins were detected by chemiluminescence using a detection solution containing 200 mM Na\textsubscript{2}CO\textsubscript{3}, pH 8.8, 10 mM imidazole, 5 mM Luminol, and 5 mM para-iodophenol.

The bean cDNA expression library in λZAPII was prepared by Dr. Yasushi Kawagoe in Dr. Norimoto Murai’s laboratory (Kawagoe, 1994). The cDNA library was phage titered to confirm a concentration of 1.1x10\textsuperscript{11} pfu per mL. Infection of \textit{E. coli} XL1Blue cells, plating of cells, and induction of expression was done according to Maniatis \textit{et al.}, (1982). Two hundred thousand plaques (50,000 plaques/plate) were screened using the hsp56 antibody after optimizing the screening protocol. Anti-carbonic anhydrase antibody was used as a positive control and uninfected XL1Blue cells as a negative control. After plating the infected cells, the plates were incubated at 42 °C for 2-3 hours prior to induction of β-galactosidase fusions with 10 mM Isopropylthiolgalactoside (IPTG)-impregnated nitrocellulose filters. Before the first
IPTG filter was placed on the plate, a nitrocellulose filter was briefly placed on the plates and removed in order to reduce the background at the detection step. The plates were then incubated with the first IPTG filter for 2 hours followed by incubation with a second filter for an additional 3 hours. At the end of each incubation, the filter was briefly rinsed in Tris-buffered saline (TBS) and placed in 0.1 M acetic acid for 20 minutes with gentle rocking at room temperature. This was followed by a neutralization step consisting of a second room temperature incubation with TBS for 10 minutes. The filters were then placed on a glass support and covered with an acetate sheet for overnight storage at 4 °C. The filters were blocked with a solution of 5% nonfat dry milk in TBS for 30 minutes prior to incubation with a 1:500 dilution of the anti-hsp56 antibody for 2 hours at room temperature. This was followed by an incubation with the secondary antibody and detection using chemiluminescence as previously described. Positive plaques were isolated and subjected to a secondary and tertiary screen.

The phagmid containing the cDNA giving a positive signal was excised from the λZAPII vector using the λZAPII in vivo excision protocol as described in the Stratagene protocol manual. The resulting cDNA was obtained as an insert in Bluescript SK (Stratagene). Expression of the encoded protein was assayed by induction of 3 mL XL1Blue cells by addition of IPTG to a final concentration of 10 mM after growth at 37 °C to an A600 = 0.6. The cells were grown for 2 hours after induction and were pelleted and resuspended in 0.1 M Na2CO3, 0.1M dithiothreitol (DTT) (0.1 M Na2CO3/DTT) prior to sonication. The sonicated cells were subjected to centrifugation and the supernatant was analyzed by SDS-PAGE followed by Coomassie staining and western blotting. A negative control of E. coli XL1Blue cells only was used.
Western blotting was accomplished as previously described using anti-hsp56 as the primary antibody and goat anti-rabbit nonspecific IgG conjugated to horse radish peroxidase as the secondary antibody.

**Sequencing the cDNA encoding eIF5.** The size of the cDNA was determined by restriction enzyme analysis. All DNA manipulations were carried out according to Maniatis *et al.*, (1982). All restriction enzymes were purchased from New England Biolabs (NEB). The Bluescript SK plasmid containing the cDNA was isolated and digested with *EcoR* I and *Xho* I followed by separation of the fragments on a 1.2% agarose gel. Bluescript KS alone digested with the same enzymes was used as a negative control. Additional restriction mapping of the cDNA was done using *BamH* I, *Pst* I, *Hind* III, *Nco* I, *Kpn* I, and *EcoR* V (Figure 2.1).

All sequencing reactions were carried out using double-stranded DNA purified by precipitation in polyethylene glycol (PEG). Dideoxy sequencing reactions were performed according to standard protocols (United States Biochemical) with modifications (Fawcett and Bartlett, 1990). The cDNA was sequenced using a combination of subcloned fragments of the full length cDNA and oligo-priming. All fragments of the cDNA were subcloned into a derivative of Bluescript KS containing a *Nco* I site in the polylinker region and transformed into XL1Blue cells. Restriction sites used in creating the eIF5 cDNA fragments were *EcoR* I, *Apo* I, *Pst* I, *Dra* I, *Sma* I, and *BamH* I. The internal primers were:

5' GAGCTTGGAGCCCAATCAA 3'

5' CCAGCTGAAATGTGCTGCTT 3'
Figure 2.1 Strategy for sequencing the cDNA encoding eIF5. The full-length cDNA is indicated by the solid bar and the hatched bar indicates restriction sites used in sequencing eIF5. The arrows represent sequencing direction and length of sequenced fragment, showing overlaps. E, EcoR I; Ap, Apo I; P, Pst I; B, BamH I; D, Dra I; *, oligomer primers; TAA, stop codon.
Translation of the DNA sequence, amino acid sequence homology searches and alignments were accomplished using GCG and the National Center for Biotechnology Information’s Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) network service.

Expression of bean eIF5. The full length cDNA encoding eIF5 was subcloned into the pAlter vector (Promega) for generation of a Nco I site at the initiator AUG and a Xho I site at the 3' end of eIF5. These sites were needed in order to clone eIF5 into the pET28b vector (Novagen), generating a C-terminal hexaHis-tagged protein. The Nco I and Xho I sites were created by site-directed mutagenesis using the Altered Sites Mutagenesis System (Promega). Bluescript-eIF5 was digested with Xba I and Kpn I and the insert was ligated into pAlter digested with the same restriction enzymes. This placed eIF5 in pAlter in the 3’ to 5’ orientation behind the lac promoter. The pAlter-eIF5 plasmid was transformed into E. coli JM109 cells and single-stranded DNA was prepared according to the manufacturer’s protocol (Promega). The mutagenic oligonucleotide for generation of a Nco I site (eIF5NcoI) at the initiator ATG was:

5' TAAAGCCATGGTGAGTTTCAG 3'

The mutagenic oligonucleotide for generation of a Xho I (eIF5NcoLYhol) site at the 3’ end of eIF5 was:

5'AATGATCACACGCTCGAGTTCATCGATTTCAG 3'

Generation of the Xho I site at the 3’ end added a leucine as the final amino acid and eliminated the stop codon. Mutagenesis was carried out according to the manufacturer’s protocol (Promega). Briefly, double-stranded DNA was synthesized using an ampicillin repair oligomer and the mutagenic oligomer as primers for T4 DNA Polymerase. The
mutagenesis reaction was transformed into *E. coli* BMH 71-18 mut S cells and the resulting plasmid DNA was miniprepped and transformed into *E. coli* JM109. Both mutations were confirmed by dideoxy sequencing (Fawcett and Bartlett, 1990) using a modified Sequenase Version 2.0 (United States Biochemical).

The pAlter-eIF5NcoXhoI was then digested with *Nco I* and *Xho I* and the insert was ligated into pET28b digested with the same enzymes. This created a construct that would express eIF5 with a C-terminal hexaHis tag. This construct was transformed into *E. coli* DH5α cells and the presence of the insert was confirmed by restriction analysis of the plasmid DNA using *Nco I* and *Xho I*. The pET-eIF5(N/X) was then transformed into *E. coli* BL21(DE3) cells (Novagen) for expression of the protein. The presence of pET-eIF5His was confirmed by restriction analysis and the cells containing the pET-eIF5His construct were stored as 8% glycerol stocks at -70 °C.

Expression of eIF5His was examined by induction of 3 mL of cells grown to an $A_{600} = 0.6$. A series of inductions was carried out to determine the optimal induction time for obtaining soluble eIF5His. The BL21(DE3) cells were first grown at 37 °C for 6 hours starting from the glycerol stock. These cells were either used directly or stored overnight at 4 °C. An aliquot of 100 μL cells per tube was then added to a set of overnight tubes, each containing 3 mL fresh LB media and 30 μg per mL kanamycin. These cells were grown until the $A_{600}=0.6$ and induced by addition of IPTG to a final concentration of 1 mM IPTG. Uninduced pET-eIF5His was used as the negative control and β-galactosidase induction from pET28b was used as the induction control in accordance with the technical manual (Novagen). Induction time points from 30 minutes to 3 hours were used and the cells were harvested and pelleted prior to sonication into
0.1 M Na₂CO₃/0.1 M DTT. After sonication, the soluble and membrane fractions were collected by centrifugation and the level of induced expression of eIF5His in both fractions was evaluated by SDS-PAGE and Coomassie staining.

**Purification of eIF5His.** The overexpressed eIF5His was first purified from induced BL21(DE3) cells by chromatography on a Ni²⁺ column according the Novagen technical manual. The cells were grown at 37 °C to A₆₀₀ = 0.6 and were induced with 1 mM IPTG for 1 hour. The cells were centrifuged and suspended in one-tenth volume (based on media volume) Ni²⁺ binding buffer (20 mM Tris-Cl, pH 7.9, 5 mM imidazole, 0.5M NaCl, 2 mM PMSF, and 25 μg/mL leupeptin). The cells were sonicated and the supernatant was obtained by centrifugation at 14,474 x g for 10 minutes using a SA600 rotor. The crude extract was then loaded onto the Ni²⁺ column. Each fraction eluting from the Ni²⁺ column was analyzed by SDS-PAGE and the amount of purified eIF5His recovered was determined using the Bradford assay. A 1:1 ratio of bovine serum albumin and lysozyme was used as the standard.

Purification of eIF5His by classical chromatography was carried out using a crude extract obtained from cells grown at 30 °C in LB media containing 0.5 mM ZnCl₂. The cells were grown until the A₆₀₀ = 0.6 and were then induced with the 1 mM IPTG and grown for another hour. The cells were harvested as described earlier and then sonicated into one-twentieth the media volume of a buffer containing 0.1 M Tris-Cl, pH 8.8, 25 mM NaCl, 10% glycerol, 1 mM EDTA, 2 mM PMSF, and 25 μg/mL leupeptin. The theoretical pI of eIF5His was determined to be 7.8 using the method of Bjellqvist et al., (1993). This value was used to determine the pH of the buffer.
required in the diethylaminoethyl (DEAE) chromatography to ensure eIF5 would bind the column.

The DEAE column was equilibrated with the sonication buffer and the eluate pH was determined to be 8.8 prior to adding the crude extract. The crude extract was loaded onto the DEAE column with a bed volume of 50 mL per 50 mL sonication buffer. The DEAE column was developed with 1 column volume of starting buffer followed by 300 mL of a linear NaCl gradient from 25 mM NaCl to 200 mM NaCl. All glassware used for fraction collection was siliconized. The fractions containing eIF5 eluted at 100 mM - 125 mM NaCl. These fractions were pooled and concentrated by (NH₄)₂SO₄ precipitation prior to dialyzing into 20 mM potassium phosphate, pH 6.8, 0.1 mM EDTA and, 10% glycerol. The dialyzed sample was loaded onto a 10 mL hydroxylapatite (HAP) column. The column was rinsed with one column volume of the starting buffer and developed using a 10X column volume linear gradient of 20 mM potassium phosphate to 500 mM potassium phosphate. The fractions containing eIF5 eluted at 400 mM potassium phosphate. Those fractions were pooled and precipitated with ultrapure (NH₄)₂SO₄ (ICN). The concentrated sample was dialyzed into 0.1 M Tris-Cl, pH 8.0, 250 mM NaCl, and 10% glycerol. Protein concentration was determined using a theoretical extinction coefficient calculated according to Gill and von Hippel, (1989) at (http://expasy.hcuge.ch/cgi-bin/protparam). The validity of this calculation was checked by comparing the value obtained for EcoR I and comparing this value to the experimentally determined extinction coefficient. The values agreed to within 10%. For measurement of protein concentration, samples were diluted into 6.0 M urea, 20 mM potassium phosphate, pH 6.8. The purity of the eIF5 preparation was checked using
Coomassie staining after SDS-PAGE. Purified eIF5 was stored at -70 °C in 0.1 M Tris-Cl, pH 8.0, 250 mM NaCl, and 10% glycerol.

**Site-Directed Mutagenesis of pETeIF5.** The C101AC104A eIF5 mutant was created using the Altered Sites System (Promega; Figure 2.2). To generate a template for mutagenesis, eIF5 from pETeIF5His was digested with Nco I and Xho I and the insert was ligated into pBluescript Nco I digested with the same enzymes. This construct was then digested with Kpn I and Hind III and this insert was ligated into pAlter digested with the same enzymes. This placed eIF5 in pAlter in the 3' to 5' direction behind the lac promoter. Single-stranded DNA was isolated according to the manufacturer’s protocol (Promega). This pAlter eIF5 was transformed into *E. coli* JM109. The mutagenic oligomer for generation of the C101AC104A double mutant was:

\[
5'\text{CAGTTTCAGGATTGCGGGCACATAGCTGGACATAT}3'
\]

In addition to the cysteine to alanine mutagenic sites noted in bold italics, the oligomer contained a silent mutation (G) that introduced a unique *NgoM* I site, which is underlined. The mutagenesis was done according to the Promega protocol as described for the expression of pETeIF5His. The mutation was analyzed by restriction digestion with *NgoM* I and confirmed by dideoxy sequencing. The pAlter eIF5 C101AC104A mutant was digested with Nco I and Xho I and the insert was ligated into pET28b digested with the same enzymes. This results in expression of eIF5 containing a C-terminal hexaHis tag. The construct was transformed into *E. coli* DH5α cells and the presence of the insert was confirmed by restriction analysis of the plasmid DNA using Nco I, Xho I, and *NgoM* I. The pETC101AC104A was transformed into *E. coli* BL21(DE3) cells (Novagen) for expression of the protein. The presence of the construct
Figure 2.2 Site-directed mutagenesis of eIF5 to obtain eIF5C101AC104A. C101AC104A was constructed as shown. The potential zinc-binding residues, cysteine\textsuperscript{101} and cysteine\textsuperscript{104}, were substituted with alanine in order to abolish the zinc-binding capacity of eIF5.
was again confirmed by restriction analysis and *E. coli* BL21(DE3) cells containing the plasmid were stored as 8% glycerol stocks at -70°C.

**Expression and Purification of C101AC104A.** Expression of pETC101AC104A was optimized and assessed as previously described for pETeIF5. Wild-type eIF5His was used as a control to gauge the expression level of C101AC104A and expression was confirmed by western blotting using anti-hsp 56 as the primary antibody.

An *E. coli* extract containing the eIF5 C101AC104A mutant protein was obtained as follows. The cells were grown to $A_{600} = 0.6$ at 30°C in LB media containing 0.5 mM ZnCl$_2$, induced with 1 mM IPTG and grown for an additional 2 hours. The cells were harvested as described for the wild-type eIF5. Purification of C101AC104A was attempted using Ni$^{2+}$ chromatography as well as classical chromatography including ammonium sulfate precipitation along with DEAE, HAP, or gel filtration chromatography. The mutant proved to be unstable and sufficient quantities of full-length C101AC104A for elemental analysis or later RNA binding assays were not obtained. In light of this, all experiments involving C101AC104A were carried out using C101AC104A crude extract and the amount of mutant used in each experiment relative to wild-type eIF5 was standardized by SDS-PAGE.

**Zinc Content Analysis Using ICP-MS.** Wild-type eIF5 was purified as described in Chapter 2 using classical chromatography. Five hundred microliters of the purified protein was loaded onto a 1.5 mL Chelex (BioRad) column and adventitious zinc was removed by eluting the protein into 15 mL orange-capped plastic tubes. The plastic tubes had been preincubated with two changes of 0.5 M EDTA. The concentration of eIF5 was determined by removing an aliquot from each tube and using the theoretical
extinction coefficient of 36,400 M⁻¹ cm⁻¹ at A₂₈₀ (in 6.0 M urea, 20 mM PO₄, pH 6.8). Polyethylene gloves (Baxter) were worn while processing the samples for zinc analysis. Control samples included a buffer blank of 0.1 M Tris-Cl, pH 8.0, 0.1 M NaCl and 10% glycerol and a protein blank of recombinant carboxyltransferase kindly provided by Dr. Grover Waldrop. The carboxyltransferase contained an N-terminal hexaHis tag and had been purified by affinity chromatography on a Ni²⁺ column. In addition, the carboxyltransferase has no metal requirements for activity, indicating that zinc is not bound to the protein (Polakis et al., 1974). Adventitious zinc was removed from the control samples exactly as described for eIF5.

Zinc analysis of the buffer blank, eIF5, and carboxyltransferase was conducted by Dr. Jay Means, School of Veterinary Medicine, Louisiana State University using a Fisons ICP-MS (inductively coupled plasma mass spectrometer). The instrument was tuned using reference standards and the instrument background was determined. A standard curve was generated using serial dilutions of certified reference standards for zinc and other metals.

Data analysis was done by subtracting the value of zinc present in the buffer and protein blank from the value obtained for eIF5 and converting the resulting μg per mL zinc into nmol per mL of zinc. The nmol eIF5 per mL was calculated from the concentration and molecular mass of eIF5 and the ratio of nmol per mL zinc: nmol per mL eIF5 was determined.

Crude extracts were used for zinc analysis of the C101AC104A mutant since this construct could not be purified to homogeneity. Cultures of pET28b only, pETeIF5, and pETC101AC104A were grown under identical conditions: 50 mL LB with 0.5 mM
ZnCl₂ at 30°C to an A₆₀₀ = 0.6, induced with 1 mM IPTG and grown for 1 hour after induction.

The C101AC104A mutant does not express at the same level as the wild-type eIF5 and the amount of total protein submitted for ICP-MS analysis of each construct was calibrated so that roughly equal amounts of eIF5 and C101AC104A were analyzed for zinc content. The amount of each crude extract was calibrated using SDS-PAGE and the amount of pET28b crude extract used as a control for each construct was also determined by SDS-PAGE. Adventitious zinc was removed from these samples as described for the purified eIF5 with the following modifications. Two mL of pET28b and pETeIF5 crude extracts were each diluted into a total volume of 5.0 mL and loaded onto a 1.5 mL Chelex column. Three mL of pET28b and pETC101AC104A crude extracts were each diluted into a total volume of 5.0 mL and loaded onto a 1.5 mL Chelex column. Adventitious zinc was also removed from a control sample of sonication buffer and all of the samples were collected in EDTA-treated orange-capped plastic tubes. An aliquot was removed from each tube for protein concentration determination using the Bradford assay and a 1:1 ratio of bovine serum albumin:lysozyme as the standard. ICP-MS analysis was carried out as previously described. The zinc content of the paired pET28b control extract was subtracted from the zinc content of pETeIF5 or pETC101AC104A and the amount of zinc associated with expression of either construct was reported qualitatively as percent change.

Results and Discussion

The hsp-56 antibody was found to cross react with a protein of approximately 50 kD from total bean extract and the antibody was used to screen a bean seed cDNA
Figure 2.3 Anti-hsp56 cross-reacts with a protein from bean extract at about 50 kD. (A) Total wheat germ extract (lane 1), total bean extract (lane 2), and a 10-fold increase in concentration of bean extract (lane 3) were obtained from ground tissue, separated by SDS-PAGE and stained with Coomassie Blue. Molecular weight markers (M) are shown at the left. (B) Western blotting of total wheat germ extract (lane 1), total bean extract (lane 2), and a 10-fold concentration of total bean extract (lane 3) using anti-hsp56 as the primary antibody.
Figure 2.4 Expression of proteins obtained from the tertiary screen of the bean seed cDNA expression library. The phagmid containing the cDNA giving a positive response on the tertiary screen was excised from the λZAPII vector and the encoded protein was expressed in XL1Blue cells by induction with 10 mM IPTG. Four positive plaques were analyzed, 2A-1, 2A-2, 2C-1, and 2C-2, and the in vivo excision was carried out using either K07 or R01 helper phage. (A) Lanes 1-4 correspond to excision using K07 and lanes 5-8 correspond to excision using R01; 2A-1 (lane 1,5); 2A-2 (lane 2,6); 2C-1 (lane 3,7); 2C-2 (lane 4,8). XL1Blue cells alone (Neg) were used as a negative control. Proteins were separated by SDS-PAGE and stained with Coomassie Blue. Molecular weight markers are shown at the left (M). (B) Western blotting of (A) using anti-hsp56 as the primary antibody.
expression library (Figure 2.3). While screening the bean seed cDNA expression library using the antibody against hsp56, a cDNA encoding eIF5 was obtained (Figure 2.4). This cDNA is considered to be a false positive since eIF5 has only a small region of homology with the prolyl isomerases or other heat shock proteins. The region of homology is at the C-terminal portion of the encoded protein and it may overlap one of the two antigenic determinants that are recognized by the anti-hsp56 antibody (Ruff et al., 1992; Lebeau et al., 1992). However, the sequencing and characterization of the cDNA was pursued and this is the first cDNA encoding eIF5 to be reported in plants.

The isolated cDNA is 1690 base pairs and the longest open reading frame encodes a protein containing 443 amino acids with a predicted molecular weight of 49,100 (Figure 2.5). There are two potential polyadenylation sites at the 3’ untranslated region of the cDNA. The first, AATAAA, occurs at base 1508 and the second, AATAAAA, begins at base 1650. The translation start site occurs at base 89 and the AUG codon context is AACAUGG, corresponding to the consensus initiation start site of A/G NNAUGG (Kozak, 1983; Futterer and Hohn, 1996). The two potential polyadenylation signals in the 3’ untranslated region (3’ UTR) are also found in the cDNA encoding mammalian eIF5 (Si et al., 1996) and the multiple mRNAs encoding mammalian eIF5 can be correlated with alternative polyadenylation site selection. Given the emerging understanding of the role of the 3’ UTR and the polyA tail in regulating gene expression (Jackson and Standart, 1990; Pain, 1996), the multiple polyadenylation sites in the 3’ UTR of bean eIF5 are an interesting feature of the cDNA.
Figure 2.5 cDNA sequence and the deduced amino acid sequence of bean eIF5.
The identity of the protein encoded by the cDNA was determined by homology with other eIF5s as a result of submitting the sequence to a BLAST homology search. The bean eIF5 has 59% homology and 37% identity with rat eIF5. There is 60% homology and 39% identity between bean eIF5 and yeast eIF5, indicating a highly conserved protein throughout eukaryotes (Figure 2.6). The N-termini of the eIF5s contain the majority of the conserved residues. Examination of the sequence of bean eIF5 reveals three of five GTPase domains found in both rat and yeast eIF5 are also present in bean eIF5. The G1 region of bean eIF5 is GRGNGIKT corresponding to the consensus sequence of G-R/K-GNGIKT for all eIF5s. The G2 region of DX₈₋₁₀₉ and the G3 domain can be identified at 2₄₆DDDG₂₄₃. The G4 domain is not present in bean eIF5. The domain is described as consisting of four apolar or hydrophobic amino acids followed by (N/T)(K/Q)XD (Bourne et al., 1991) and has been identified in rat and yeast eIF5 as 4₁₉NKDD₄₂₂ and 2₄₆TQLD₂₄₉, respectively. It is interesting to note that sequences of three other cDNAs encoding eIF5 have been sequenced since we reported the sequence of bean eIF5; one of these is a cDNA encoding eIF5 in maize (Genbank X99517) and the other two are the human (genbank U49436) and Schizosaccharomyces pombe (Genbank Q09689) eIF5 cDNAs. The maize eIF5, like bean eIF5, contains the G1, G2, and G3 GTPase domains, but the G4 domain is not present. Both the human and fission yeast eIF5 have all four of the GTPase domains that are easily identified in the fusion yeast and rat eIF5s. However, the G4 domain, although present in these eIF5s, does not contain the same residues among the eIF5s, nor is the location of this domain conserved across the eIF5s. The residues of
Figure 2.6 Amino acid sequence homology of bean eIF5 with rat and *Saccharomyces cerevisiae* eIF5. The yeast and rat eIF5 amino acid sequences were obtained from the Swiss-Prot data bank and the homology was determined using the University of Wisconsin GCG program (Altschul *et al.*, 1990). The putative zinc-finger is indicated in the block; the GTPase domains (G1,G2,G3,G4) are underlined and the C-terminal conserved tryptophans and acidic tail are noted by overlining the residues, superfamily (Bourne *et al.*, 1991).
this domain are usually highly conserved among related members of the GTPase superfamily (Bourne et al., 1991). None of the protein sequences contain the G5 domain, but this is a domain that cannot be clearly identified in all GTPases (Bourne et al., 1991). The significance of the absence of the G4 domains in plants is not clear. However, there are examples of other bona fide GTPases that do not have the typical GTPase domains as defined by (Bourne et al., 1991) based on the p21ras family.

An example of this is β-tubulin, which has none of the typical GTPase domains (Sage et al., 1995). It has been suggested that $\text{DAKN}_{298}$ of β-tubulin is functionally equivalent to the NKAD domain of the GTPase superfamily (Sternlicht et al., 1987). This odd reversal of consensus sequences has precedent; there have been other sequences that have been shown to be functional in the forward or reverse direction (Chiang and Dice, 1988; Becker and Roth, 1992). For example, it is intriguing that both plant eIF5s have this reversed motif but site-directed mutagenesis in this region of β-tubulin did not alter the nucleotide binding properties of the protein (Sage et al., 1995).

The situation for β-tubulin may be instructive in the case of eIF5. While it is clear that β-tubulin is a GTPase, the protein is presently described as an atypical GTPase because of the lack of GTPase domains that are found in the ras family of GTPases (Sage et al., 1995). There are other examples of atypical GTPases: proteins that are well established as GTPases but contain only some or none of the known GTPase domains (deBoer et al., 1992; Takeuchi et al., 1992; Nakaoka et al., 1994). It is possible that eIF5 belongs to this group of “atypical” GTPases.

Another possibility is that eIF5 functions as a GTP Activating Protein (GAP) and eIF2 with the bound GTP is the GTPase. While eIF5 has no homology with most of the
GAPs currently identified (Boguski and McCormick, 1993), there is similarity to the ADP-ribosylation factor-directed (ARF-directed) GTPase activating protein, including a N-terminal putative zinc-binding domain (Makler et al., 1995; Cukierman et al., 1995).

This putative zinc-binding domain is completely conserved in all eIF5s and is part of the highly conserved N-terminal portion of eIF5. The conserved residues are Cys101, Cys104, Cys124, and Cys127, making this region a putative Cys2Cys2-type zinc finger with a consensus sequence of Cys-X2-Cys-X18-19-Cys-X2-Cys. There is a strictly conserved sequence in the intervening region: AsnProGluThr(Glu/Asp) and the last X2 residues are LysAla in all eukaryotes other than plants. These two residues are AlaAla in both of the plant eIF5s. This type of zinc finger domain is found in glucocorticoid receptor family proteins (Schwabe and Klug, 1994); however, there is always more than one zinc finger present in these proteins. This potential zinc binding domain is like the zinc binding domain found in the ARF1 GAP protein described in (Cukierman et al., 1995) and that zinc finger is required for GTPase activation.

ICP-MS offers the opportunity to examine the zinc content of eIF5 with sensitivity in the pmol range and analysis of the purified wild-type eIF5 (Figure 2.7) indicates that there is an average of 0.83 mol zinc: mol eIF5, essentially a 1:1 molar ratio (Table 2.1). This ratio is consistent with binding of the zinc in the putative single zinc finger domain at the N-terminus of eIF5. In order to abolish the zinc binding capability without greatly altering the steric properties of the potential zinc ligands, cysteine101 and cysteine104 were mutated to alanines. Alanine is similar in size to cysteines but it is a nonpolar amino acid that will not interact with metals nor will it allow for disulfide bond formation in the absence of zinc. The first two potential zinc binding
Figure 2.7 Purification of recombinant bean eIF5. The expression of eIF5 in *E. coli* BL21(DE3) cells was induced by the addition of IPTG and the crude extract was obtained as described in chapter two. The amount of eIF5 containing the hexaHis tag was relatively low, necessitating purification of eIF5 by classical approaches. (A) Affinity purification of eIF5His by Ni\(^{2+}\) chromatography. Total *E. coli* crude extract (crude), Ni\(^{2+}\) column flow-through (FT), and eIF5His eluted from the Ni\(^{2+}\) column (elute) were analyzed by SDS-PAGE followed by staining with Coomassie Blue. (B) The classical approach to the purification of eIF5 included isolation of the *E. coli* crude extract (crude) followed by ion exchange chromatography (DEAE) and hydroxylapatite chromatography (HAP). The purification scheme was analyzed by SDS-PAGE followed by staining with Coomassie Blue. Molecular weight markers are shown at the left (M) in (A) and (B).
Table 2.1  ICP-MS analysis of zinc content of eIF5His

<table>
<thead>
<tr>
<th>Sample</th>
<th>nmol protein</th>
<th>Zn ng/mL</th>
<th>mol Zn/mol protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>buffer blank</td>
<td>blank</td>
<td>10.4</td>
<td>N/A</td>
</tr>
<tr>
<td>carboxyltransferase-His¹</td>
<td>2.7</td>
<td>12.1</td>
<td>0.07</td>
</tr>
<tr>
<td>eIF5His² A</td>
<td>3.2</td>
<td>184.0</td>
<td>0.91</td>
</tr>
<tr>
<td>eIF5His B</td>
<td>3.3</td>
<td>189.2</td>
<td>0.79</td>
</tr>
<tr>
<td>eIF5His C</td>
<td>3.7</td>
<td>168.2</td>
<td>0.80</td>
</tr>
</tbody>
</table>

¹MW 130,000 daltons  ²MW 48,971 daltons
amino acids were chosen rather than cysteine\textsuperscript{124} and cysteine\textsuperscript{127} because the placement of alanine in those positions would produce a segment containing four contiguous alanines followed by a glycine, phenylalanine, and valine. This combination would have a greater potential to introduce large changes in secondary structure than the introduction of the alanines into a stretch of amino acids with the resulting sequence: alanine, tryosine, glycine, alanine, asparagine, proline, glutamate (Cantor and Schimmel, 1980).

Two of the four cysteines were substituted because earlier studies of the UvrA protein indicated that if only one cysteine is replaced in a C\textsubscript{2}C\textsubscript{2}-type zinc finger, biological activity can be unchanged (Wang and Grossman, 1993). This indicates that any three cysteine residues of the zinc finger motif could bind the zinc with sufficient affinity to have no effect on the structure of the motif. However, if one of the cysteines is replaced by a bulky, aromatic amino acid, biological activity is lost (Wang et al., 1994). Presumably, this is due to the introduction of steric hindrance in the zinc-finger domain and disruption of the secondary structure required for activity. Rather than introduce a bulky amino acid at one of the cysteines, two of the four cysteines were substituted with sterically similar residues, eliminating the possibility of zinc binding without introducing additional structural considerations. In addition, substitution of the two closely spaced cysteines rather than mutating a single cysteine eliminates the possibility that any nearby cysteine could substitute in liganding the zinc ion.

Since the eIF5C101AC104A mutant could not be purified to homogeneity, the construct was analyzed crude protein extracts for zinc content by ICP-MS. Extracts expressing the C101AC104A showed no increase in zinc content when compared to the background control, while a crude extract from cells expressing the wild-type eIF5
showed a 31% increase over background, corresponding to an increase of 7.5 mol Zn per mL (Table 2.2).

Taken together, these results indicate that eIF5 is a zinc finger protein of the \( \text{C}_2\text{C}_2 \)-type. The inability to achieve high-level expression of eIF5C101AC104A or to purify it suggests that the zinc finger is an important structural component of eIF5. This is consistent with what is known about the role of zinc in stabilizing the domain structure found in zinc finger proteins (Freedman et al., 1988; Coleman, 1992). It is notable that several other reports of mutagenesis of a zinc-finger domain do not suggest that loss of zinc binding leads to instability of the protein (Severne et al., 1988; Wang et al., 1994; Zang et al., 1995), although mutant proteins were not purified in most of these cases.

The difference between eIF5 and these other proteins is that the mutagenesis studies have generally been carried out on proteins containing multiple zinc-finger domains with the mutagenesis involving only one of the zinc-fingers. There is only one such domain in eIF5 and any destabilization of the protein structure associated with the loss of the zinc-finger will be fully realized.

Other regions of interest in eIF5 are found in the C-terminal region of the protein. One is the conserved tryptophans that are found at Trp\(^{408}\) and Trp\(^{432}\) in bean eIF5. A stretch of highly acidic amino acids precedes Trp\(^{408}\) and follows Trp\(^{432}\). All other eIF5s contain these tryptophans except eIF5 from \textit{Saccharomyces cerevisiae}, which has phenylalanine at the first position instead of tryptophan. It has been postulated that this domain could be involved in interacting with eIF2 (Koonin, 1995) and the domain does resemble the WW domain that has been identified as interacting...
Table 2.2 ICP-MS analysis of zinc content of eIF5 and C101AC104A crude extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crude Extract mg/mL</th>
<th>Zn Content nmol/mL</th>
<th>% Change Zn Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET28b control (eIF5)</td>
<td>0.34</td>
<td>23.6</td>
<td>30.1 (increase)</td>
</tr>
<tr>
<td>pETeIF5</td>
<td>0.71</td>
<td>30.9</td>
<td></td>
</tr>
<tr>
<td>pET28b control (C101AC104A)</td>
<td>0.84</td>
<td>42.8</td>
<td>8.9 (decrease)</td>
</tr>
<tr>
<td>pETC101AC104A</td>
<td>1.36</td>
<td>39.0</td>
<td></td>
</tr>
</tbody>
</table>
with polyproline regions of other proteins in competition with SH3 domains (Macias et al., 1996). This polyproline domain, PPxY, can be found in the α subunit of all eIF2s for which sequences are available as PP(R/L)Y with the exception of the yeast eIF2α, which has the sequence APLY. These conserved tryptophans in eIF5 also resemble a region that is found in the DNA-binding domains of telomere binding proteins (Konig and Rhodes, 1997). Finally, the ARF1 GAP protein contains nine tryptophans in the C-terminal region with some of the tryptophans resembling the WW domain, further increasing the similarity between ARF1 GAP and eIF5. It would seem that this region of eIF5 could be implicated in either protein-protein interactions or protein-nucleic acid interactions.

Many translation initiation factors are regulated by phosphorylation, a modification having a profound effect on the rate of protein synthesis. Because of the importance of phosphorylation in the regulation of cellular events, the last areas of interest are the potential phosphorylation sites. Bean eIF5 has motifs that could serve as recognition sites for a variety of kinases including protein kinase C, protein kinase A, casein kinase II and p34cdc2/cyclin B protein kinase. *In vitro* and *in vivo* phosphorylation has been demonstrated for mammalian eIF5 (Chevesich et al., 1993) although the significance of this post-translational modified has not been appreciated. Nonetheless, as Pain (1996) indicates, subunit joining represents a potentially important control point in translation and the observation that eIF5 is a phosphoprotein is “provocative”.

The data presented here show that eIF5 is a zinc-finger protein of the C2C2-type and that zinc ion is an important structural element in the secondary structure of eIF5. The bean eIF5 described here has been purified to homogeneity, taking advantage of the
ability to overexpress the protein in *E. coli* along with the ability of the protein to interact with hydroxylapatite at an acidic pH, typical of nucleic acid binding proteins. This will allow one to begin to answer some of the questions raised by sequence comparisons. Is this eIF5 a phosphoprotein? Does eIF5 interact with nucleic acids; in particular, ribosomal RNA? These questions are addressed in the following chapters.
Chapter 3

Phosphorylation of Bean eIF5

Introduction

Translation is now understood to be as important as transcription in the regulation of gene expression in eukaryotic organisms (Norbury and Nurse, 1992). Alterations in the overall rate of protein synthesis are required for progression through the cell cycle and changes in rates of synthesis of specific proteins are involved in the cellular response to stress and developmental tasks. Results of studies to date suggest that regulation of eukaryotic translation involves primarily the influence of structural components of the mRNA itself and the phosphorylation of specific initiation factors. However, there is evidence that elongation is also a target of regulation (Hinnebusch, 1994; Pain, 1996; Brown and Schreiber, 1996).

Translation initiation is the rate limiting step in protein synthesis and the initiation factors known to be regulated by phosphorylation are eIF4E and eIF2. Eukaryotic initiation factor 4E is part of the cap-binding complex and recruits the mRNA to the ribosome by recognizing the 5’ m7GpppN cap on the mRNA (Merrick, 1992; Pain, 1996). This represents a key control point in translation initiation and the activity of eIF4E is modulated by altered levels of expression of mRNA encoding the protein as well as by phosphorylation of the protein. The correlation between eIF4E phosphorylation and increased rates of translation have been well-characterized, and it has been shown that phosphorylated eIF4E has a higher affinity for the mRNA cap than nonphosphorylated eIF4E (Minich et al., 1994). Although eIF4E was considered to be the rate-limiting factor in initiation, it has now been shown that eIF4E is present in higher
concentrations than previously realized and it partitions between a ribosome-associated form that is highly phosphorylated and a soluble, less phosphorylated form (Rau et al., 1996). This suggests that phosphorylation is involved in the partitioning of eIF4E between an active, ribosome-associated form and an inactive, soluble form. Multiple pathways are thought to regulate the phosphorylation of eIF4E, including the p70S6K pathway, a mitogen-activated pathway leading to the ribosomal S6 protein kinase (Frederickson and Sonenberg, 1993; Zanchin and McCarthy, 1995).

Eukaryotic initiation factor 2 (eIF2) is a complex of three proteins that brings the initiator tRNA to the ribosome as the ternary complex, eIF2-tRNAiM^Met-GTP (Merrick, 1992; Pain, 1996) and regulation of eIF2 by phosphorylation has been well documented. The primary phosphorylation target on eIF2 is Ser\(^{51}\) of the alpha subunit; phosphorylation of this residue inhibits the interaction of eIF2 with eIF2B, an interaction needed to recycle eIF2 between rounds of translation initiation (Proud, 1995; Dever et al., 1995; Pain, 1996). Hence, phosphorylation of eIF2 results in a decrease in the formation of the ternary initiation complex and a corresponding decrease in the initiation of protein synthesis.

While the phosphorylation of eIF2 has been extensively studied in mammals and yeast, the first evidence of eIF2 phosphorylation in plants has only recently been reported (Langland et al., 1996). It was demonstrated that Ser\(^{51}\) of eIF2\(\alpha\) from wheat germ is phosphorylated by the plant encoded double-stranded RNA-dependent protein kinase and that this modification inhibits protein synthesis in wheat germ. This is the first clear evidence that plant protein synthesis is regulated by phosphorylation.
Very little is known about the phosphorylation status of the remaining initiation factors. Browning (Gallie et al., 1997) has shown, using isoelectric point determinations, that developmentally induced changes can be correlated with changes in the phosphorylation status of eIF2, eIF4B, and eIF4E from wheat germ. In the case of eIF2, the protein is phosphorylated when isolated from embryonic tissue but is either dephosphorylated or hypophosphorylated when isolated from leaf tissue. This suggests that eIF2 is inactive in the embryo and is shifted to an active state during germination. In contrast, eIF4B is dephosphorylated when isolated from embryos and is phosphorylated in wheat leaf extract. Three different isoforms of eIF4E were isolated, corresponding to the embryonic tissue, three-day-old seedlings, and mature leaves; indicating developmental regulation of eIF4E associated with changing phosphorylation states. The study did not examine the phosphorylation state of any other initiation factors, including eIF5.

While nothing is known about whether plant eIF5 is phosphorylated, mammalian eIF5 has been shown to be a phosphoprotein in vivo (Ghosh et al., 1989; Chevesich et al., 1993). Casein kinase II (CKII) phosphorylates the mammalian eIF5 in vitro, but it is not clear whether CKII phosphorylates the protein in vivo. Phosphorylation by CKII has not been shown to affect the activity of eIF5 in vitro nor does it appear to alter the interaction between eIF5 and eIF2 in vitro (Chaudhuri et al., 1994). However, the assay of eIF5 and eIF2 interaction was carried out using the two proteins in a 1:2 molar ratio of eIF5 to eIF2, corresponding to an excess of eIF5 when compared to the 1:8 molar ratio of eIF5 to eIF2 used in subunit joining assays or the 1:100 molar ratio of eIF5 to GTP used in GTP hydrolysis assays (Das et al., 1993; Chaudhuri et al., 1994). Clearly,
eIF5 was in excess when assayed for interaction with eIF2 and any influence of phosphorylation may be masked under these conditions. Nonetheless, eIF5 is well positioned at a potential regulatory step in translation initiation and phosphorylation states that do not affect *in vitro* assays may have a role in regulation of the *in vivo* activity of eIF5. It has been established that eIF5 is a very low abundance protein functioning in assays of subunit joining at nanomolar concentrations (Chevesich *et al.*, 1993). In addition, subunit joining is the final step of translation initiation and occurs at a slower rate than the binding of the mRNA to the 40S ribosomal subunit (Anthony and Merrick, 1992). It is a reasonable assumption that this slow step might ensure the correct assembly of the necessary components of the initiation complex prior to beginning elongation. Finally, bean eIF5 contains five consensus targets for CKII and one sequence which is similar to the target of p34cdc2/cyclin B kinase. Phosphorylation of eIF5 by either of these kinases offers a route to regulate subunit joining.

This chapter discusses the generation of transgenic *Arabidopsis thaliana* (L.) plants in order to study the *in vivo* phosphorylation status of bean eIF5. Evidence that bean eIF5 is a phosphorylated *in vitro* is also presented.

**Materials and Methods**

**Generation of Transgenic *Arabidopsis thaliana* (L.) (ecotype WS).** All manipulations of DNA were performed according to Maniatis *et al.*, (1982). The binary vector, pBI101M9#10 (pBI101 from Clontech) was provided by Hee Jin Kim. This vector contains a truncated promoter for carbonic anhydrase (CA) 5' to the gene encoding β-glucuronidase (GUS) along with part of the polylinker regions from pSP65 (Promega) and pBluescript KS (Stratagene). The truncated CA promoter is 268 bases in length and
is the smallest segment of the promoter that will support expression of the GUS gene product (Kim, 1997). The remainder of the plasmid section enclosed by the left and right border repeats from the *Agrobacterium* Ti plasmid contains the nopaline synthase (NOS) gene promoter and polyadenylation signal sequence (NOS-Ter) flanking the gene encoding neomycin phosphotransferase II (NPTII) with the NOS-Ter also located 3' to the GUS gene. The strategy for inserting eIF5His into this segment of pBI101M9#10 at the *Sal* I and *Sst I/Sac I* sites involved first generating a *Sac I* site in pETeIF5His. This was accomplished by digesting pETeIF5His at the *Bsp I* site followed by 3' end filling using the exo- Klenow fragment of DNA polymerase I (Ambion). The DNA was then ligated to *Sac I* linkers; digested with *Sac I*, and religated.

To create an initiator ATG codon in the correct reading frame, the pETeIF5His was digested with *Nco I* followed by digestion with Mung Bean Nuclease to remove the 5' extension. The pBI101M9#10 was digested with *Sal I* followed by digestion with Mung Bean Nuclease to remove the 5' extension. Each of these vectors was then digested with *Sac I* and the 1.3 Kb insert from pETeIF5His containing eIF5His and 167bp of the vector 3' to eIF5His was ligated into pBI101M9#10 at the blunt/Sac I sites to obtain pBI101eIF5His (Figure 3.1). *E. coli* DH5α cells were used in all transformations.

The sequence of pBI101eIF5His was confirmed by restriction analysis and dideoxy sequencing. The *Dra I/Pst I* fragment of pBI101eIF5His was subcloned into a derivative of pBluescript KS (BlueNco) containing an *Nco I* site in the polylinker. The *Dra I/Pst I* fragment containing eIF5 from pBI101eIF5His was subcloned into
Figure 3.1 pBI101eIF5His vector for production of transgenic *Arabidopsis*. The bean eIF5 cDNA was placed under the control of a truncated carbonic anhydrase (CA) promoter in order to obtain low level expression of bean eIF5 limited to the green tissues. NOS, Nopaline Synthase gene; NOS-pro, NOS promoter; NPTII, Neomycin Phosphotransferase II; NOS-ter, NOS terminator; CA-pro, truncated carbonic anhydrase promoter; bean eIF5His, bean eIF5His cDNA from pETeIF5His; RB and LB, right and left border of *agrobacterium* Ti plasmid.
BlueNcoI digested with EcoRV and PstI. Dideoxy sequencing was carried out as described earlier.

The correct construct was transformed into *Agrobacterium tumefaciens* GV3101 pMP90 (Koncz and Schell, 1986; gift from Andrew Bent) made competent by a standard CaCl₂ method (Walkerpeach and Velten, 1992). After transformation, the bacteria were incubated for 4 hours at 28°C in Luria Broth (LB) with 50 μg/mL gentamycin to maintain the Ti-derived pMP90 helper plasmid present in the *Agrobacterium* and then plated on LB with gentamycin (50 μg/mL) and kanamycin (30 μg/mL) to select for pMp90 and pBI101eIF5His and incubated for 48 hours at 28°C. Colonies were selected and grown in LB plus gentamycin and kanamycin for 48 hours at 28°C. Digestion of medium-scale DNA preparations with *Pst* I (from 25 mL culture) confirmed the presence of pBIeIF5His. The controls were DNA from untransformed *Agrobacterium*, pBI101, and pBIeIF5His. Glycerol stocks (15%) were made from each culture harboring the plasmid and these were stored at -70°C, both individually and as a mixture of all of them. The mixed stock was used for transformation into *Arabidopsis*.

*Arabidopsis thaliana* (L.), ecotype Wassilevskija (WS) was used to generate transgenic plants by vacuum infiltration (Bechtold *et al.*, 1993; Bent *et al.*, 1994). Seeds were vernalized by placing ca. 15-20 mg seeds in 10 mL H₂O in a 15 mL orange-capped tube and rocking for 2 days at 4°C. The seeds were then spread over soil in a 3.5” pot; a nylon screen was placed over the seeds and secured with a rubber band. The pots were placed in continuous light at 25°C and the plants were thinned to 15-20 plants/pot approximately two weeks after the appearance of the cotyledons. Plants were watered daily using a wash bottle and fertilized with the addition of a dilute solution of
MiracleGro™ (Stern's MiracleGro), 1-2 times/week. The plants were then grown two more weeks until emerging bolts were evident. The bolts were pruned and the plants continued to grow until the flowers began to appear after an additional 7 days. At this point, the plants were ready for infiltration (Figure 3.2). Each pot was well-watered in the morning prior to infiltration.

*Agrobacterium* containing pBlieF5His was grown in 3 mL LB culture with antibiotic selection (gentamycin 50 μg/mL and kanamycin 30 μg/mL) for 8 hours and then 1.5 mL was transferred to 12.5 mL LB with antibiotic selection. This was grown for 16 hours and then added to 200 mL LB with selection. The 200 mL culture was grown for 24 hours, until the A₆₀ₒ was >2.0. All cultures were grown at 28°C. At this point, the cultures were centrifuged at 4,229 x g (GSA rotor) for 10 minutes at room temperature and the cell pellet was resuspended in a volume of infiltration media (see appendix) that was 3 times the culture volume. Vac-In-Stuff (Lehle Seeds), a surfactant, was added to the solution at 0.03% v/v. All solutions were kept at room temperature.

The *Agrobacterium* solution was added to a 600 mL beaker (ca. 400 mL solution) that was placed in a vacuum dessicator. A pot containing the plants was inverted into the solution, making sure that aerial portions, including the rosette, were completely submerged in the solution. A vacuum was established using a water trap aspirator and was maintained until bubbles formed on the leaf and stem surfaces. In general, the vacuum was held for 8 minutes. At the end of 8 minutes, the vacuum was quickly released and the pot was placed on its side in a tray. The 400 mL solution was used for infiltration of three pots and the solution was then changed.
Figure 3.2 *Arabidopsis thaliana*, ecotype Wassilevskija (WS). The *Arabidopsis* plants were grown in pots with screens in place to facilitate transformation with *Agrobacterium* as described in chapter Three and the Appendix.
The infiltrated plants were returned to the growth chamber. Pots were placed on their sides on a tray which was loosely covered with plastic wrap to maintain humidity. After twenty-four hours, the plastic wrap was removed and the pots were placed upright.

The plants were grown at 25°C under continuous light until seeds were harvested. Mature seeds were collected daily and stored in the growth chamber until all of the seeds had been harvested. After the seeds were completely dry, they were surface sterilized by washing in 100% ethanol (1 minute), 25% bleach and 0.01% tween 20 (5 minutes, only Clorox® bleach should be used at this step) followed by three rinses in sterile water. Sterile seeds were sown on selection media (see Appendix) at a density of about 1000 seeds per 150 mm Petri plate. The seeds (T₀) were spread on the selection plates using 5 mL of 0.1% agarose per 1000 seeds and allowed to stand until the ‘top agarose’ was set. The plates were then placed in the dark at 4°C for 48 hours prior to putting them under continuous light in the growth chamber. The transgenic seedlings (T₁) were identified after about 7 days by the formation of dark green secondary leaves and a root system that extended into the selection media. In contrast, the wild-type (untransformed) seedlings were small, white, and lacked any secondary leaves (Figure 3.3). The transgenic plants were moved to soil after 7-10 days on the selection plates. The pots containing the transplants were loosely covered with plastic wrap for 48 hours to maintain high humidity and the plants were grown under continuous light until the seeds were harvested. This process was repeated for two subsequent generations, yielding the T₂ and T₃ generations of transgenic plants. At the T₂ generation, the ratio of the number of transgenic seedlings to the total number of seedlings was calculated in
Figure 3.3 Selection plates used in screening for eIF5 transgenic Arabidopsis. Selection of the T₀ generation is shown on the left. The majority of the seedlings are yellowing and lacking any secondary leaves while a few of the seedlings are dark green and are beginning to produce secondary leaves, characteristic of transgenic seedlings. The arrow indicates a transgenic seedling. The homozygous T₃ generation of eIF5 transgenic plants is shown on the right. All of the seedlings are dark green and have produced secondary leaves, indicating resistance to kanamycin.
order to determine which plants were homozygotes. The homozygous seeds were collected, completely dried, and stored at -20°C.

**Analysis of transgenic *Arabidopsis***. Confirmation of the presence of bean eIF5 in the transgenic *Arabidopsis* plants was accomplished at the DNA level by genomic Southern blotting. Both wild-type and the C-1-B negative control plants were analyzed along with the T₃ eIF5His transgenic plants. The genomic DNA was obtained according to the Puregene protocol (Gentra) with the exception that 2 M NaCl was added to the 10 mM Tris-Cl, pH 8.0 with 1 mM EDTA (TE) buffer on the first resuspension of the DNA pellet. This allowed for more complete removal of the cell wall carbohydrates during the final DNA precipitation. The final DNA pellet was resuspended in TE overnight at 4°C and the concentration of DNA was determined by UV absorbance. Equal amounts of DNA were loaded onto a 0.8% agarose gel and subjected to electrophoresis at 40V for 6 hours and 10V for 14 hours. The agarose gel was processed for unblotting (Wallace and Miyada, 1987). The DNA probe was the 330 bp *Pst I/Sac I* fragment of pETeIF5His that included the 3' end of the eIF5 transgenic insert and part of the pET vector. The probe was labeled to a specific activity of 2x10⁹ cpm/μg by random priming using exo‘Klenow and the manufacturer’s protocol (Ambion). The gel was incubated with the probe overnight at 55°C with constant gentle rocking, rinsed and placed with film for 48 hours.

Any phenotypic differences in the T₂ and T₃ generations were noted by comparing the eIF5 transgenic plants with the C-1-B negative control transgenic plant containing GUS instead of eIF5His (gift from Hee Jin Kim). The plants were transferred...
from the selection plates to soil and were grown at least two weeks before phenotypic differences became apparent.

Leaves were collected for western blot analysis from the T\textsubscript{2} generation after the seedlings had been transferred to soil and had grown for an additional 4-5 days. The T\textsubscript{3} generation leaf samples were collected from seedlings grown directly on soil after vernalization at 4°C x two-four days. Additional leaf samples were collected from the T\textsubscript{3} plants that had been grown in long day (16 hour) light conditions rather than continuous light and that had been harvested at various stages of growth after appearance of the cotyledons (5 days, 7 days, or 2 weeks). The leaves were rinsed with water and then ground after freezing with liquid N\textsubscript{2}. The ground leaves were placed in a 1.5 mL Eppendorf tube and 1.0 mL of extraction buffer was added (0.1 M Tris-Cl, pH 7.5, 25 mM NaCl, 5 mM EDTA, 1 mM ZnCl\textsubscript{2}, 0.1% (w/v) ascorbate, 1 mM DTT, 5% PVPP w/v, 25 μg/mL leupeptin, 2 mM PMSF, 0.1 mM E-64). The ground leaves were incubated on ice for 30 minutes and centrifuged at 13,200 rpm in a microcentrifuge for 10 minutes. The supernatant was removed and analyzed by SDS-PAGE followed by electrophoretic transfer to nitrocellulose. Western blotting was performed as previously described using anti-hsp56 as the primary antibody. A parallel set of samples was subjected to SDS-PAGE and Coomassie stained for comparison with the western blot. The C-1-B transgenic plant served as a negative control.

\textit{In vitro} Phosphorylation. Recombinant eIF5His was purified as described in Chapter 2. The C-terminal truncation of eIF5 was constructed by site-directed mutagenesis using the Altered Sites Kit from Promega. The eIF5\textsubscript{NcoI} in pSelect was used to create eIF5\textsubscript{Δ408-443} by inserting a \textit{Xho I} coinciding with the codon for Trp\textsuperscript{408}. The mutagenic
primer was: 5'CCCTTTTGATACTCGAGAACTATGGCC 3'. This resulted in an eIF5 truncated at the C-terminus and removed the conserved tryptophans along with the acidic carboxy-terminal tail.

The C-terminal truncation was partially purified by chromatography on a Ni$^{2+}$ column. Minor contaminating proteins were revealed after the eluate from the Ni$^{2+}$ column was subjected to SDS-PAGE and Coomassie staining. Protein concentration was determined by Bradford assay (BioRad) using a 1:1 ratio of bovine serum albumin and lysozyme as the standard. The proportion of eIF5Δ408-443 in the eluate was estimated from the Coomassie stained gel. The concentration of eIF5Δ408-443 was estimated to be 0.1 mg per mL based on these comparisons. The partially purified eIF5Δ408-443 was stored in 0.1 M Tris-Cl, pH 8.0, 0.1 M NaCl and 10% glycerol at -70°C.

In vitro phosphorylation was assayed according to the protocol of New England Biolabs for casein kinase II and p34cdc2/cyclin B kinase. Each kinase was used at a concentration of 20 units per 30 μL, an amount that was experimentally determined. ATP was supplemented with γ-32P-ATP to 100 μCi per μmol ATP in each reaction. Kinase reaction buffers (CKII: 20 mM Tris-Cl, pH 7.5, 50 mM KCl, 10 mM MgCl$_2$, 100 μM ATP and p34cdc2/cyclin B kinase: 50 mM Tris-Cl, pH 7.5, 10 mM MgCl$_2$, 2 mM DTT, 1 mM EGTA, 0.01% Brij 35, 100 μM ATP) were supplemented with 1 mM Na$_3$VO$_4$ and 10 mM Na$_2$MoO$_4$ as phosphatase inhibitors. Control reactions contained all components of the assay except the kinases. Assays contained 60 nM or 600 nM eIF5 and approximately 30 nM or 300 nM eIF5Δ408-443. The kinase reactions were incubated at 30 °C for 45 minutes and were stopped by the addition of 10 μL.
SDS-PAGE loading solution (30% sucrose, 0.1% Bromphenol Blue (BPB), 5% SDS, 100 mM DTT) and heating to 65 °C for 15 minutes. The kinase reactions were subjected to SDS-PAGE followed by collection of the data as a phosphorimage and analysis of the image using a Molecular Dynamics Storm Phosphorimager. The amount of phosphate incorporated into the proteins was determined by excising the band from the dried gel and counting the $^{32}\text{P}$ by liquid scintillation (Beckman). Sections of the gel removed from the lane containing the molecular weight markers and from each of the control reactions served as controls for background. Two additional sample sets were subjected to SDS-PAGE and the proteins were detected by either Coomassie or silver staining. These samples were prepared in the same manner as the kinase assay samples except that no $\gamma^{32}\text{P}$-ATP was added. The molar ratio of incorporated phosphate to the full length eIF5 and eIF5Δ408-443 was calculated using these data.

Results and Discussion

In an effort to examine the \textit{in vivo} phosphorylation status of bean eIF5, a transgenic line of \textit{Arabidopsis} containing bean eIF5 was constructed using the vacuum infiltration method of Bechtold \textit{et al.}, (1993) as modified by Bent \textit{et al.}, (1994). A truncated carbonic anhydrase promoter was used to control expression of the cDNA due to the concern that the high level of expression of a protein which is normally of low-abundance would have deleterious effects. The truncated CA promoter is a weak, leaf-specific promoter and so any deleterious effects of overexpression would be minimized and confined to green tissues.
Figure 3.4 Confirmation of bean eIF5 in the T₃ generation of eIF5 transgenic plants by genomic Southern blotting. Genomic DNA was purified from the T₃ transgenic plant line, 3K, as described in Chapter Three. The blot was probed with a fragment of the pETeIF5His vector containing the 3' end of the eIF5 cDNA and part of the pET28b vector. Wild-type Arabidopsis (lane 1), the transgenic control, C-1-B (lane 2), and four separate plants from the eIF5 transgenic line, 3K (lane 3-6) were probed with the ³²P-labeled cDNA probe.
Figure 3.5 Confirmation of expression of bean eIF5 in the T2 generation of transgenic Arabidopsis. Leaf extracts were obtained as described in Chapter Three and subjected to SDS-PAGE followed by western blot analysis using anti-hsp56 as the primary antibody. (A) A control of purified eIF5 (lane 1) was analyzed along with total leaf extracts of C-1-B, the negative control transgenic plant (lane 2), transgenic plant 3N (lane 3) and transgenic plant 3K (lane 4). 3N and 3K were from the same transgenic line. Molecular weight markers (M) are shown at the left. (B) Western blot of the SDS-PAGE indicating the expression of bean eIF5 in transgenic plants 3N and 3K. Lane assignments are identical to (A).
The presence of the DNA encoding bean eIF5 was confirmed in the T3 generation (homozygotes) of plants by genomic Southern blotting (Figure 3.4) and the bean eIF5 protein can be detected in the T2 generation of plants by western analysis (Figure 3.5). However, efforts to consistently detect eIF5 at the protein level in the homozygous plants proved unsuccessful. This lack of eIF5 expression in the homozygotes is unrelated to the light cycles or the age of the plants, indicating that the dampening of protein detection in the homozygotes is not due to harvesting the plants at inopportune developmental stages. In addition, the T2 generation of eIF5 transgenic plants exhibited phenotypic changes that are absent in the homozygous plants.

Phenotypic changes were noted when the eIF5 transgenic plants were compared to the C-1-B negative control plants as each were grown side-by-side starting from plate selection and progressing to growth on soil (Figure 3.6). The transgenic control plants began to bolt earlier than the transgenic plants containing eIF5 and were moving into senescence while the eIF5 plants lagged behind in a prolonged vegetative phase. An explanation for this observation is difficult to formulate without further experiments. It is possible that the overexpression of eIF5 has an effect on the rate of protein synthesis and this alteration in protein synthesis is reflected in the phenotypic changes. It is also possible that growth on the selection media altered the phenotype of the eIF5 transgenic plants, although this is unlikely given the phenotype of the negative control. A third possibility is the eIF5 gene insertion interrupted a coding region involved in the transition to senescence in Arabidopsis. This is considered unlikely since the phenotype does not persist in the homozygous plants. A final possibility is that the phenotype was lost as a result of vernalization, the period of exposure to cold temperatures that induces
Figure 3.6 The $T_2$ generation of eIF5 transgenic plants are phenotypically different from the C-1-B transgenic control plants grown under the same conditions. The eIF5 transgenic plants and the C-1-B control transgenic plants were grown as described in Chapter Three and the phenotypic differences were apparent in 3-4 weeks after transfer to soil from the selection plates. The plants on the left (eIF5 transgenic plants) remain in a prolonged vegetative state while the plants on the right (C-1-B transgenic control plants) have progressed to seed production and senescence.
germination in seeds in the laboratory or flowering in plants requiring a winter season for proper growth cycles. The phenomenon of phenotype suppression after vernalization has been described in Arabidopsis for a mutant of FCA, a gene encoding a protein that participates in controlling flowering time (Koomneef, M. et al., 1991; MacKnight et al., 1997). This fca allele, one of the late flowering mutants from Arabidopsis thaliana, ecotype Landsberg erecta, is particularly affected by vernalization. Rather than flowering very late, the mutant will flower at the same time as wild-type Arabidopsis after prolonged vernalization. The phenotypic differences we observed for the eIF5 transgenic plants occurred after the plants had been transferred directly from selection plates to soil, bypassing the vernalization step that was used in every other instance. It is intriguing to note that the FCA gene product is a RNA-binding protein containing two RNA-binding domains and is thought to likely function as a posttranscriptional regulator of gene expression (MacKnight et al., 1997).

The lack of phenotypic changes in the homozygous plants correlates with the loss of adequate detection of the protein by western blotting. The remaining possibility to consider is suppression of the expression of bean eIF5 in the homozygous plants. ‘Coexpression suppression’ has been reported for homologous genes (Napoli et al., 1990; van der Krol et al., 1990) but has not been reported for heterologous genes as is found in our eIF5 transgenic plants. However, this is an hypothesis that can easily be tested in the eIF5 transgenic plants in further experiments using western analysis of the T2 and T3 generations grown simultaneously.

Purified eIF5 (as described in Chapter 2) and eIF5Δ408-443 (Figure 3.7) were used in the in vitro phosphorylation experiments. It was observed that casein kinase II
Figure 3.7 Site-directed mutagenesis of eIF5 to obtain eIF5Δ408-443 and purification of eIF5Δ408-443. (A) eIF5Δ408-443 was constructed as shown and (B) affinity-purified by Ni²⁺ chromatography. Total E. coli crude extract (lane 1) and the Ni²⁺ column eluate (lane 2) were analyzed using SDS-PAGE followed by staining with Coomassie Blue. Molecular weight markers (M) are shown at the left.
Figure 3.8 Full-length eIF5 and eIF5Δ408-443 are phosphorylated by casein kinase II. The purified proteins were used in *in vitro* casein kinase II assays as described in Chapter Three. (A) The kinase reactions were analyzed by SDS-PAGE followed by silver-staining. Control reactions for eIF5 (lane 1) and eIF5Δ408-443 (lane 2) were assayed with the eIF5 (lane 3) and eIF5Δ408-443 (lane 4) kinase reactions. (B) Autoradiography of the SDS-PAGE of the kinase reactions. The incorporation of phosphate was analyzed by phosphorimaging the dried gel followed by the excision of the labeled bands and obtaining $^{32}$P counts by liquid scintillation.
(CKII) phosphorylates full-length eIF5 and the truncated eIF5Δ408-443 in vitro (Figure 3.8). The molar ratio of incorporated phosphate to eIF5 was calculated to be 7.3 and the ratio for eIF5Δ408-443 was 5.2, indicating the C-terminal truncation may be phosphorylated by casein kinase II to a lesser extent than the full-length eIF5. However, that observation is inconclusive for several reasons. The molar ratio for incorporated phosphate to wild-type eIF5 and eIF5Δ408-443 appears high based on the number of clearly identifiable CKII consensus sites at Ser^{209}, Thr^{244}, Thr^{268}, Ser^{438}, and Ser^{440}. The unexpectedly high number may be due to experimental error introduced by pipetting or miscalculation of protein concentrations. Another consideration is the additional sites, Ser^{11}, Thr^{109}, Ser^{120}, Ser^{207}, Thr^{269} and Ser^{364}, that contain a serine or threonine near acidic residues. These sites may also have been phosphorylated under the assay conditions.

In addition, the two proteins were not present in equal concentrations in the assay and the concentration determined for the partially purified eIF5Δ408-443 is not equivalent to the concentration of eIF5Δ408-443 due to the presence of the contaminating proteins. A conservative estimate places the concentration of eIF5Δ408-443 as one-half the concentration of the full-length eIF5 as judged by silver staining. This would suggest that region of eIF5 deleted in eIF5Δ408-443 does contain two sites for phosphorylation by casein kinase II; however, further experiments are needed to adequately address this point. This is an important issue in considering whether eIF5 is involved in the regulation of translation initiation since the C-terminal region of eIF5 has domains that are potentially involved in either protein-protein or protein-nucleic acid interactions (Koonin, 1995; Maciase et al., 1996; Konig and Rhodes, 1997).
Nonetheless, it has been shown that bean eIF5 is an *in vitro* substrate for casein kinase II. This is consistent with the results found in the mammalian version of eIF5, but it remains unclear what significance this post-translational modification may have *in vivo*.

Casein kinase II is a serine-threonine kinase that recognizes either serine or threonine positioned within clusters of acidic amino acids (Pearson and Kemp, 1991; Marshak and Carroll, 1991); a motif that appears in bean eIF5 at 290Ser-Asp-Glu-Asp, 244Thr-Ser-Leu-Asp, 268Thr-Thr-Asp-Glu, 438Ser-Glu-Ser-Asp, and 440Ser-Asp-Glu-Glu. Three of the five sites, 268Thr-Thr-Asp-Glu, 438Ser-Glu-Ser-Asp, and 440Ser-Asp-Glu-Glu, are conserved in all eIF5s. Casein kinase II is found in all eukaryotic cells (Edelman *et al.*, 1987) and its activity is detected in the cytosol as well as the nucleus with *in vitro* substrates identified from both regions (Picton *et al.*, 1982; Luscher *et al.*, 1989; Luscher *et al.*, 1990). Many of the cytosolic substrates identified for casein kinase II are translation initiation factors, including eIF5, eIF4E, and eIF2 (Ghosh *et al.*, 1989; Zanchin and McCarthy, 1995; Tuazon *et al.*, 1980; Gil *et al.*, 1996). However, as mentioned earlier, the *in vivo* significance of phosphorylation of these proteins by CKII is not known since, in most cases, the assays for CKII phosphorylation are *in vitro* (Yu *et al.*, 1991). Even so, there continue to be intriguing observations about casein kinase II. One is that casein kinase II and p34<sup>cdc2</sup>/cyclin B kinase share a number of substrates including p53 (Bischoff *et al.*, 1990; Meek *et al.*, 1990) and nucleolin (Schneider and Issinger, 1988; Belenguer *et al.*, 1990). Another is that casein kinase II does phosphorylate p34<sup>cdc2</sup>/cyclin B *in vivo* (Russo *et al.*, 1992) at the M-G1 phase transition. These observations suggest that casein kinase II phosphorylation may be involved in regulation of cellular events.
The consensus recognition motif for p34\textsuperscript{cdc2}/cyclin B kinase is Ser/Thr-Pro-X-Arg/Lys (Kennelly and Krebs, 1991) and bean eIF5 contains a similar sequence at Arg-Arg\textsubscript{215}Ser-Pro-Thr-His-Lys\textsubscript{219}. This sequence is also present in eIF5 from maize as Ser-Ser-Pro-Thr-Arg\textsubscript{226} but it cannot be found in any other eIF5. This suggests a potentially unique regulatory site in plants. However, phosphorylation by p34\textsuperscript{cdc2}/cyclin B was not significantly above background under the assay conditions used in this experiment. This would argue against a unique site, although it is possible that the p34\textsuperscript{cdc2}/cyclin B kinase used in this experiment did not recognize the plant eIF5 as a substrate.
Chapter 4
Examination of eIF5-Nucleic Acid Interactions

Introduction

Initiation of protein synthesis in eukaryotic cells is a process involving the coordinated recruitment of the initiator tRNA and a messenger RNA to the 40S ribosomal subunit followed by binding of the 60S ribosomal subunit to form the 80S ribosome. This process requires the participation of over ten different soluble translation initiation factors that are associated with the ribosome. The majority of these factors have been shown to interact with one of the forms of RNA that are present at the ribosome. Most prominent among the initiation factors that are known to interact with RNA are the proteins that are involved in recruiting the mRNA to the 40S ribosomal subunit. A well characterized example of these proteins is eIF4E, the initiation factor that recognizes the m7 GpppN cap at the 5' end of the mRNA via an interaction that requires the highly conserved tryptophan residues found in eIF4E (Altmann et al., 1988).

Other initiation factors use different strategies for interacting with nucleic acids. The helicase activity of eIF4A is ATP-dependent and requires the presence of an arginine-rich region that is found at the C-terminal of the polypeptide (Merrick, 1992) while the factor that stimulates the helicase activity, eIF4B, uses a N-terminal RNA recognition motif (RRM) along with an arginine-rich region to facilitate RNA binding (Naranda et al., 1994). The RRM is described below. The arginine-rich motif (ARM), defined only as a preponderance of arginines, is thought to function as a probe of the RNA surface. The arginine residues of the ARM domain increase nonspecific binding of
RNA, facilitating high affinity RNA binding by localizing the protein at the RNA surface (Tan et al., 1993).

A second initiation factor containing a RRM domain is eIF4G, the initiation factor responsible for connecting and orienting the eIF4E associated mRNA with the 40S initiation complex (Lamphear et al., 1995). The RRM domain is at the C-terminal region of eIF4G and this region is required for interacting with the ribosome. However, the ribosomal interaction of eIF4G could also be mediated by its interaction with eIF3, an association that also requires the C-terminal region of eIF4G (Lamphear et al., 1995). The third initiation factor known to contain an RRM domain is eIF3, the largest of the initiation factors. The N-terminal domain of yeast PRT1, a subunit of eIF3, contains the RRM domain and this region is probably involved in ribosome binding (Evans et al., 1995). Another subunit of yeast eIF3, p62, has also been shown to be involved in binding eIF3 to mRNA (Naranda et al., 1994; Danaie et al., 1995) but this subunit does not contain a RRM domain. At present, it is not clear which motifs mediate the interaction of eIF3 with the 40S ribosome and mRNA.

RNA recognition motifs (RRM), also known as the ribonucleoprotein motif (RNP) domain, are the most common RNA-binding motifs (Burd and Dreyfuss, 1994). This domain occurs in over 200 RNA-binding proteins and is identified by two short sequences, RNP1 and RNP2, and other, mostly hydrophobic, amino acids distributed along the motif (Nagai et al., 1995). The consensus sequences for the RNP2 and RNP1 domains, -I/L/V-Y/F-I/L/V-X-X-I/L/V- and -R/K-G-Y/F-A/G-Y/F-V-X-Y/F, respectively, contain hydrophobic and aromatic amino acids, with one basic amino acid. These domains form the two central β strands of the RNP domain (Nagai et al., 1990).
and the structure of the RNP domain is essentially unchanged when RNA is bound to a RNP domain protein. This leaves the RNA at the surface of the RNP domain protein and available to interact with other ligands, which could include RNA-binding proteins or other nucleic acids (Gorlach et al., 1990).

Other RNA-binding domains that have been identified include the RGG domain, the hnRNP K homology (KH) motif, the double-stranded RNA-binding motif (DSRM) (Burd and Dreyfuss, 1994), and the tudor domain (Ponting, 1997). The RGG domain usually occurs in RNA-binding proteins that also have other types of RNA-binding domains and is defined by the presence of a variable number of closely spaced Arg-Gly-Gly repeats (Kiledjian and Dreyfuss, 1992). The KH motif was first identified in the human hnRNP K protein and is composed of invariant glycines interspersed with highly conserved hydrophobic amino acids (Siomi et al., 1993). The double-stranded RNA-binding motif (DSRM) is a loosely defined region of approximately seventy amino acids that contains both lysine and arginine along with hydrophobic amino acids, highly conserved aromatic residues and three invariant glycines (St. Johnston et al., 1992; Green et al., 1992). Many of the proteins having this domain, such as Staufen, which is involved in Drosophila anterior/posterior axis formation and the DAI kinase, involved in viral infection responses, are involved in regulation of translation (Burd and Dreyfuss, 1994). However, none of the translation initiation factors have been identified as having the DSRM domain. The tudor domain has only recently been described and it is found in a group of proteins involved in Drosophila embryogenesis as well as other proteins that are known to interact with RNA (Ponting, 1997). The domain is presently defined by a
series of small hydrophobic amino acids associated with a highly conserved aspartate and tryptophan although a physiological role for this domain has not been established.

The remaining nucleic acid binding domain found in a small number of RNA binding proteins is the zinc finger domain characterized by Cys-X_{2,5}-Cys-X_{4,12}-Cys/His-X_{2,4}Cys/His, also known as the zinc finger-knuckle motif. The best known example of this motif mediating RNA binding is TFIIB, where the motif appears nine times and is responsible for the interaction of TFIIB with both the 5S rRNA gene and 5S rRNA (Theunissen, 1992). The glucocorticoid receptors contain multiple zinc fingers of the C_{2}C_{2} type (Severne et al., 1988) and the C_{2}C_{2} zinc-finger motif is represented in the translation initiation factors eIF2 and eIF5 as a single putative zinc finger.

Eukaryotic initiation factor 2 interacts specifically with the initiator tRNA (Merrick, 1992; Pain, 1996). Although the binding site for tRNA\textsuperscript{\text{Met}} has not been definitively located, studies indicate that the C-terminal region of eIF2\text{\textbeta} is involved, along with the N-terminal region of eIF2\gamma (Gaspar et al., 1994). The C-terminal region of eIF2\text{\textbeta} contains the putative zinc finger domain of the type, Cys-X_{2}-Cys-X_{17,19}-Cys-X_{2}-Cys. This region has also been implicated in interactions of eIF2 with mRNA using cross-linking studies and filter-binding assays (Flynn et al., 1994). While the physiological significance of an interaction between eIF2 and mRNA is not clear, it is known that eIF2\text{\textbeta} plays a role in start site selection (Dasso et al., 1990) and that mutations in the yeast eIF2\text{\textbeta} gene affecting start site selection map to amino acids in the zinc finger region (Castilho-Valavicius et al., 1992). However, Merrick (1992) claims that "active" eIF2 does not contain zinc, although no data supporting this contention have been presented.
The single zinc finger domain in eIF5 is found at the N-terminal region and is represented as Cys-X$_2$–Cys–X$_{18-19}$–Cys–Lys/Ala–Ala–Cys. The intervening region of 18-19 amino acids contains a stretch of amino acids including the sequence, Asn-Pro-Asp-Thr-Asp/Glu-Ile/Leu. Finally, the C-terminus of eIF5 contains conserved tryptophans that are similar to the telomeric recognition domain found in the *Saccharomyces cerevisiae* RAP1 protein (Konig and Rhodes, 1997), raising the possibility that these conserved tryptophans in eIF5 could be involved in nucleic acid interactions at the ribosome.

The nucleic acid binding properties of eIF5 have not been examined since 1978 when Sonenberg (1978) reported that eIF5, along with other translation initiation factors, would bind to reovirus capped mRNA. These experiments were carried out in the context of examining the role of the newly discovered mRNA 5'-m$^7$GpppN cap structure (Shafritz *et al.*, 1976) in mRNA recognition by initiation factors. However, it is now clear that Sonenberg and coworkers were not focusing on eIF5 in their experiments, but a 150 kD polypeptide that copurified with eIF5 and was thought at the time to be eIF5 (Benne *et al.*, 1978b). Recent research has established unequivocally that eIF5 is a monomer of molecular mass 45-62 kD (Raychaudhuri *et al.*, 1985; Das *et al.*, 1993; Chakravarti and Maitra, 1993; Floyd and Bartlett, 1995). Hence, the nucleic acid binding properties of eIF5 remain an open question.

Experiments described in this chapter clearly demonstrate that eIF5 binds RNA. The rRNA-binding interaction of eIF5 requires elements of the secondary structure, potentially including structural components of the C-terminal region of eIF5.
Materials and Methods

Site-Directed Mutagenesis of eIF5. The eIF5 C101AC104A mutation has been described in Chapter Two and the eIF5Δ408-443 mutation has been described in Chapter Three.

Southwestern Blotting. Southwestern blotting was done using eIF5 partially purified by DEAE chromatography and eIF5 purified by Ni²⁺ chromatography. A nuclear extract from Xenopus oocytes (gift from Dr. Patrick DiMario) served as a positive control since multiple proteins from this extract will bind nucleic acids under the assay conditions. The samples were resolved by SDS-PAGE followed by electrophoretic transfer to nitrocellulose. A second set of samples were Coomassie-stained after separation by SDS-PAGE. The nitrocellulose was blocked by incubating for an hour at room temperature in 10 mM HEPES, pH 8.0, 25 mM NaCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 5% nonfat dry milk (blocking buffer). The nonspecific DNA probe was a cDNA encoding ubiquitin which was excised from pAW1.1 (gift from Michael Bracey) by digesting the plasmid with Not I and EcoR I. The insert was purified by electrophoresis in low-melting agarose. Radiolabeled DNA was prepared by random priming according to the manufacturer’s protocol (Ambion) except the labeled DNA was not boiled before incubating with the blot. The specific activity of the probe was 1.50 X 10⁹ cpm per µg. The probe was diluted to 1.5 X 10⁶ cpm per mL in binding buffer (10 mM HEPES, pH 8.0, 50 mM NaCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT and 0.25% nonfat dry milk) and incubated with the blot overnight at 37°C with agitation. After incubation, the nitrocellulose was rinsed with the binding buffer until there was no further decrease in the background radiation and was placed with X-ray film (Kodak) for 24 hours.
**Southwestern Dot Blotting.** Increasing amounts of eIF5 (25 μg-500 μg aliquoted from 5 μg/μL purified eIF5) and 250 μg of BSA as a negative control were blotted onto nitrocellulose that had been pre-rinsed with 1X Tris-buffered saline (10 mM Tris-Cl, pH 7.4 with 0.9% NaCl). The nitrocellulose was then blocked by incubation with the blocking buffer without DTT added (blocking buffer B) at 4°C for 1 hour. The nonspecific DNA probe was prepared as described above. The probe was incubated with the nitrocellulose at 1.4 X 10^4 cpm per mL overnight at 37°C in binding buffer without DTT added (binding buffer B). After incubation, the nitrocellulose was rinsed extensively in binding buffer B followed by rinsing with binding buffer B + 0.05% Triton X-100 and then binding buffer B + 0.5 M NaCl in order to reduce the background counts to a minimum. The nitrocellulose was placed with X-ray film (Kodak) for 15 minutes.

**rRNA Binding Assay.** Ribosomal RNA was isolated from wheat germ 80S ribosomes. Wheat germ (General Mills) was ground in 4 batches of 1.5gm per batch using a mortar and pestle after freezing the wheat germ in liquid N2. The ground wheat germ was placed in a 15 mL Corex™ tube and incubated with 10 mL of 50 mM Tris-Cl, pH 7.50, 10 mM MgCl2, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.25 M sucrose (ribosome buffer A) on ice for 15 minutes. The solution was transferred to ultracentrifuge tubes containing 1 mL 10% sucrose in ribosome buffer A and subjected to centrifugation at 100,000 x g in a Ti 70 rotor (Beckman). The supernatant was discarded and the ribosomal pellet was resuspended in 10 mL 50 mM Tris-Cl, pH 7.50, 5 mM MgCl2, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.25 M sucrose (ribosome buffer B) by allowing the mixture to stand overnight at 4°C. The resuspended ribosomes
were centrifuged at 32,566 x g for 30 minutes in an SA600 rotor and the resulting supernatant was repelleted by centrifugation at 100,000 x g for 2 hours through a 1 mL solution of 10% sucrose in ribosome buffer B. The final ribosome pellet was resuspended overnight in 1 mL ribosome buffer B at 4°C and stored at -70°C.

All solutions used to isolate rRNA from the ribosomes were made using water treated with 0.1% (v/v) diethylpyrocarbonate (DEPC). All glassware was cleaned and baked at 240°C for 4 hours and all plastics were autoclaved prior to use. The rRNA (and any accompanying mRNA) was isolated from the ribosomes by extraction with an equal volume of phenol/chloroform (24:23:1 phenol:chloroform: isoamyl alcohol), pH 8.0 until the interface was clear. The aqueous phase was transferred to a second tube and one tenth volume of 3 M NaAc, pH 7.9 was added. The RNA was precipitated by adding two volumes of 100% ethanol and incubating on ice for 1 hour. Small RNA molecules, including tRNA, will remain in the supernatant under these conditions (Maniatis et al., 1982; Robyt and White, 1987). The RNA was centrifuged in a microfuge at 13,200 rpm for 15 minutes and the RNA pellet was resuspended in 0.5 mL TE per tube. The RNA concentration was determined by UV absorbance at 260 nm and the purity of the RNA preparation was determined by the A$_{260}$/A$_{280}$ ratio.

The CF11 cellulose was placed in a beaker and autoclaved. The rRNA was added to a slurry of 3 gm CF11 cellulose (Whatman) and 200 mL 100% ethanol (Fedoroff and Zinder, 1971; Nygard et al., 1980). The slurry was stirred for 1.5 hours under (ca.1-2 feet) UV lights, followed by UV irradiation of the solution using the Stratalinker™ (Stratagene) for 30 seconds. The slurry was rinsed with 0.8 M KCl using a Buchner funnel to remove the unbound RNA and the percent bound RNA was
determined from the concentration of RNA in the flow-through. Eighty-four percent of the rRNA was bound to the cellulose. The resulting rRNA-cellulose was rinsed extensively with ethanol and stored under 100% ethanol at 4°C.

Crude extracts of eIF5, eIF5A408-443, and C101AC104A were prepared from 250 mL cultures as previously described, except the cells were sonicated into 20 mL of a starting buffer consisting of 50 mM Tris-Cl, pH 8.0, 50 mM KCl, 10% glycerol, 25 μg/mL leupeptin, and 2 mM PMSF. The relative amount of each construct was calibrated by SDS-PAGE so that an approximately equal concentration of each construct was added to a 3 mL rRNA matrix (Figure 4.1). Each rRNA column had been washed with 20 column volumes of the starting buffer. The flow-through from each crude extract was collected and reloaded onto the column twice. The third flow through was retained for further analysis and the matrices were then subjected to a step-wise gradient of starting buffer as follows: 15 mL at 100 mM KCl, 15 mL at 500 mM KCl and 15 mL at 800 mM KCl. The matrices were then rinsed with the starting buffer followed by a final rinse of 100% ethanol for storage at 4°C. Each eluate was precipitated with 10% trichloroacetic acid overnight at 4°C. The precipitated proteins were collected by centrifugation at 7,996 x g for 15 minutes in an HB-4 swinging bucket rotor. The pellets were rinsed with 100% acetone and recentrifuged before being allowed to air-dry. The dried pellets were resuspended in 0.1 M Na2CO3/0.1 M DTT and SDS-PAGE loading buffer (5% SDS, 30% sucrose, 0.1% BPB) before being subjected to SDS-PAGE. One set of samples was stained with Coomassie Blue and the other set was transferred electrophoretically to nitrocellulose for western blot analysis. Anti-hsp56 antibody (UPJ 56) was used as the primary antibody and goat anti-rabbit IgG conjugated to horse radish...
Figure 4.1 Approximately equal amounts of eIF5, C101AC104A, and eIF5Δ408-443 cross-react with anti-hsp56 with varying intensities. As a control for the rRNA-cellulose binding experiments, amounts of wild-type eIF5 along with the C101AC104A and eIF5Δ408-443 mutants expressed in BL21(DE3) cells after IPTG induction were calibrated using SDS-PAGE followed by western blotting. (A) Pre-and post induction crude extracts of each construct were separated by SDS-PAGE and stained with Coomassie Blue. Pre-and post induction eIF5 crude extract, (lanes 1 and 2); pre and post induction C101AC104A crude extract, (lanes 3 and 4); pre and post induction eIF5Δ408-443 crude extract, (lanes 5 and 6). Molecular weight markers(M) are shown at the left. (B) Western blot of the SDS-PAGE from (A). The lane assignment is identical to (A). Anti-hsp56 was used as the primary antibody and the decreased cross-reactivity with C101AC104A is apparent.
peroxidase was used as the secondary antibody. Cross reacting proteins were detected by chemiluminescence as previously described.

Results and Discussion

Since eIF5 functions at the ribosome, it is reasonable to examine the interaction of eIF5 with nucleic acids. As noted earlier, no studies of the nucleic acid binding properties of bona fide eIF5 have been undertaken. It has been shown, using gel filtration assays, that eIF5 can interact with eIF2 when both of these proteins are present in a purified form (Chaudhuri et al., 1994). Clearly, this protein-protein interaction can occur in the absence of any other element of the ribosome. In contrast, reconstitution of eIF5's function using in vitro assays requires the 40S ribosome along with the AUG codon, eIF2, and GTP. These functional assays measure either GTP hydrolysis or ribosomal subunit joining and do not directly address how eIF5 interacts with this system.

The results clearly show that purified eIF5 does interact with nucleic acids and that this interaction requires the native structure of eIF5. Southwestern blotting of eIF5 that had been subjected to SDS-PAGE showed no binding of nucleic acid (Figure 4.2). However, when the southwestern blot was carried out as a dot blot using native eIF5, the protein clearly bound nucleic acid (Figure 4.3). This indicates that the native structure of eIF5 is required for this interaction, arguing for a role for the zinc-finger domain rather that features of the primary sequence such as the arginine-rich region, \texttt{Arg-Arg-Ala-Glu-Lys-Glu-Arg}, found in bean eIF5. Bovine serum albumin, a protein found to bind zinc nonspecifically (Grondin et al., 1996) did not interact with the nucleic
Figure 4.2 Bean eIF5 will not bind nucleic acids under denaturing conditions. Recombinant bean eIF5 was purified as described in Chapter Two and southwestern blotting was carried out using eIF5 transferred to nitrocellulose from SDS-PAGE. (A) A positive control of *Xenopus* nuclear extract (POS) was separated by SDS-PAGE along with eIF5 partially purified by DEAE chromatography (lane 1, 10μL; lane 2, 15μL of DEAE pooled fractions), and Ni²⁺-affinity purified eIF5 (lane 3). Molecular weight markers (M) are labeled at the left. (B) Southwestern blotting of the SDS-PAGE gel shown in (A). The lane assignments are identical to (A). The southwestern blot was carried out using ^32^P-labeled nonspecific DNA as described in Chapter Three.
Figure 4.3 Bean eIF5 will interact with nucleic acids when the native structure of eIF5 is maintained. Recombinant eIF5 was purified as described in Chapter Two. Southwestern blotting was carried out as a dot blot, allowing for the retention of any secondary structure of eIF5 required for nucleic acid interactions. eIF5 was dot blotted in increasing amounts: 25 µg (lanes 1 and 2), 50µg (lane 3), 250 µg (lane 4), and 500 µg (lane 5) and 250 µg bovine serum albumin was used as a negative control (BSA). Lane 6 is blank. $^{32}$P-labeled nonspecific DNA was used as a probe.
acid, ruling out nonspecific structural elements associated with the zinc as responsible for nucleic acid binding by eIF5.

Using rRNA affinity chromatography, it has also been shown that eIF5 interacts with rRNA (Figures 4.4 and 4.5). When approximately equal amounts of wild-type eIF5, C101AC104A, and eIF5Δ408-443 are added to the rRNA column as crude extracts, the wild-type eIF5 interacted with the rRNA, but was completely removed from the rRNA matrix with a 0.1 M KCl wash. In comparison, eIF2 and eIF3 eluted from rRNA-cellulose at 0.25-0.32 M KCl (Nygard et al., 1980; Nygard and Westermann, 1982). Eukaryotic initiation factor 3 is essential for the interaction of the initiation complex with the 40S ribosome and for keeping the two subunits apart while the initiation complex is assembled at the 40S subunit while eIF2 is responsible for delivering the initiator tRNA to the 40S subunit and remains associated with the ribosome until eIF5 mediates GTP hydrolysis and subsequent subunit joining. These functions imply stronger interactions with the ribosome than would be found with eIF5 and, in fact, eIF5 has not been isolated associated with either the 40S or 60S ribosomal subunits. The relative strength of eIF5's interaction with the rRNA-cellulose is consistent with its transient interaction with the 40S ribosomal initiation complex when compared to the more prolonged interactions of eIF2 and eIF3 at the ribosome.

The C101AC104A mutant is not retained on the rRNA column under the conditions of the assay. The simplest explanation for this result is that the zinc-finger is involved in the rRNA interaction of eIF5, but this may not be completely accurate. The C101AC104A mutant is unstable and exhibits a greater tendency to adhere to surfaces.
Figure 4.4 SDS-PAGE of wild-type eIF5, C101AC104A, and eIF5Δ408-443 fractions from an rRNA column. IPTG-induced crude extracts of eIF5, C101AC104A, and eIF5Δ408-443 were calibrated for loading onto the rRNA column as described in Chapter Four. Pre-column crude extracts are shown in lanes 1, 5, and 9. The crude extract that did not interact with the rRNA matrix is shown in lanes 2, 6, and 10. The protein eluting from the rRNA matrix with 0.1 M KCl is shown in lanes 3, 7, and 11 and the protein eluting from the matrix with 0.5 M KCl is shown in lanes 4, 8, and 12. Molecular weight markers (M) are indicated at the left.
Figure 4.5 Wild-type eIF5 interacts with rRNA and the C-terminal region of eIF5 is required for eIF5 to bind rRNA with full avidity. (A) Western blot of the SDS-PAGE shown in Figure 4.4 with identical lane assignments. Anti-hsp56 was used as the primary antibody. Wild-type eIF5 is clearly shown to bind the rRNA matrix with this exposure, but the decreased cross-reactivity of C101AC104A with anti-hsp56 precludes interpretation of lanes 5-8 at this exposure time. (B) Prolonged exposure confirms that wild-type eIF5 interacts with the rRNA and that C101AC104A does not interact with the rRNA matrix. This may be due to the overall structural changes in the protein associated with loss of the zinc ion. eIF5Δ408-443 interacts with the rRNA matrix with decreased avidity, indicating that the C-terminal region of eIF5 is involved in nucleic acid binding.
than the wild-type eIF5, suggesting that it is folded incorrectly. This indicates alterations in the secondary structure of the protein associated with loss of the zinc ion. It is possible that the failure to interact with the rRNA-cellulose simply reflects the lack of appropriate structure rather than a specific role of the zinc-finger in RNA binding. This result is consistent with the earlier finding that the native structure of eIF5 is required for nonspecific nucleic acid interactions when measured by southwestern blotting.

However, the zinc-finger domain is clearly not the only consideration in the interaction of eIF5 with rRNA. The eIF5Δ408-443 mutant interacts with the rRNA-cellulose with much less avidity than wild-type eIF5, indicating that part of the truncated C-terminus is involved in the nucleic acid interaction. The truncated region includes the conserved tryptophans as well as the acidic amino acids at the C-terminal end. Similar domains are involved in both protein-protein (Macias et al., 1996) and protein-nucleic acid interactions (Konig and Rhodes, 1997). This region of eIF5 has homology with the C-terminal region of eIF4γ (a subunit of eIF4G) and eIF2βε (subunit of eIF2B). Both eIF5 and eIF2 are known to interact with eIF2. Koonin (1995) has proposed that eIF4γ could also interact with eIF2 at the 40S initiation complex and hypothesized that the C-terminal region of each of these proteins could mediate its interaction with eIF2. However, the ability of wild-type eIF5 to bind rRNA in the absence of other proteins, combined with the decrease in the avidity of rRNA binding by eIF5Δ408-443 support a role for the C-terminal region in binding nucleic acid. Since eIF4γ, eIF2βε, and eIF5 all could bind rRNA, it is hypothesized that the C-termini of all of these proteins mediates rRNA binding. Nonetheless, it is possible that some portion of the C-terminal region could also be involved in the interaction of eIF5 with eIF2.
Chapter 5
Conclusions and Future Directions

Eukaryotic initiation factor 5 was first described in the 1970s in research involving mammalian translation initiation and was characterized as a 150 kD ribosome-dependent GTPase (Merrick et al., 1975) that was required for 40S and 60S subunit joining (Nombela et al., 1975). Studies carried out since the initial characterization of eIF5 have confirmed that eIF5 plays a central role in translation: ribosomal subunit joining cannot occur until the eIF2-bound GTP is hydrolyzed and eIF5 mediates hydrolysis of the GTP (Benne and Hershey, 1978; Chakrabarti and Maitra, 1991). However, acquisition of cDNAs encoding eIF5 in yeast and mammals and the subsequent expression of the encoded protein have shown that eIF5 functions as a monomer of 45-62 kD, rather than 150 kD (Das et al., 1993; Chakravarti and Maitra, 1993).

This dissertation describes the first report of a cDNA encoding eIF5 from plants. While translation initiation has been studied extensively in other eukaryotes, information about plant protein synthesis has lagged behind. Very little is known about some of the initiation factors in plants and eIF5 is among those that previously had only been partially purified (Browning, 1996).

The cDNA corresponding to eIF5 from Phaseolus vulgaris (L.) encodes a protein with a predicted molecular weight of 49,100. The primary sequence of eIF5 from bean shares 59% homology and 37% identity with the sequence of eIF5 from rat while there is 60% homology and 39% identity with eIF5 from yeast, indicating that the
protein is highly conserved throughout all eukaryotes. Bean eIF5 contains three of the five GTPase domains that have been identified for the ras-protein-like GTPase superfamily (Bourne et al., 1991) with the G1 domain being strictly conserved among all eIF5s. However, it remains unclear that eIF5 actually functions as a GTPase and the data indicating that there is a basal level of GTP hydrolysis when the 40S initiation complex alone is present leaves open the possibility that eIF5 could act as a GTPase-activating-protein. The increase in GTP hydrolysis associated with the addition of eIF5 to the 40S initiation complex is consistent with a GTPase-activating protein. In that regard, eIF5 has some features similar to the GTPase-activating-protein, adenosine diphosphate ribosylation factor1-GAP (ARF1-GAP). ARF1 is a small GTP-binding protein that acts as a regulator of protein sorting at the Golgi (Cukierman et al., 1995).

The most notable similarity involves the N-terminal single zinc-finger of the C2C2-type found in both eIF5 and ARF1-GAP. The GAP activity of ARF1-GAP has been localized to the N-terminal region of the protein, encompassing the zinc-finger domain. This indicates that the ARF1-GAP zinc-finger is involved in protein-protein interactions. Whether the zinc finger of eIF5 is involved in the interaction of eIF5 with eIF2 remains to be seen. But the question presents an opportunity to study the basis of the interaction between eIF5 and eIF2 as well as begin to address the question of whether eIF5 functions as a GTPase or a GTPase activating protein (GAP).

This dissertation research indicates the presence of a zinc-finger of the C2C2-type in eIF5 but characterization of the role of the zinc-finger in either protein-protein or protein-nucleic acid interactions will require further experiments. It is difficult to determine if the failure of the C101AC104A mutant to interact with rRNA is due to a
global change in the protein structure associated with the loss of the zinc ion or to the specific loss of the zinc-finger domain. Regardless, eIF5 is a RNA-binding protein and this interaction depends on the presence of the zinc-finger domain and future experiments may be aimed at understanding how the zinc-finger domain influences rRNA binding. An interesting feature of the zinc-finger is the stretch of strictly conserved residues in the loop region of the domain. This group of amino acids, Asn-Pro-Glu-Thr-Glu/Asp-Ile/Leu, would be well-situated to make specific contact with the ribosome and could serve to help correctly position eIF5 at the 40S initiation complex. Experiments involving site-directed mutagenesis of the group of residues while leaving the zinc binding residues in place could serve as a probe of the specificity of the eIF5-rRNA interactions.

Another region of eIF5 that is clearly needed for eIF5-rRNA interactions is the C-terminal portion of the protein. This is perhaps the more intriguing result of the rRNA-binding experiment. The experiment revealed that removal of the C-terminal amino acids markedly affects the ability of eIF5 to interact with the RNA, even though the zinc-finger is present in the eIF5Δ408-443 construct. This suggests that eIF5 contacts the ribosome with the C-terminal region potentially serving as a probe to detect the presence of the eIF2-GTP-tRNA\textsubscript{Met} complex at the 40S ribosome. This would require the C-terminus of eIF5 to contain both a nucleic acid and a protein binding site and it has already been noted (Koonin, 1995) that the C-terminal region of eIF5 contains two conserved motifs: the acidic residues at the C-terminal tail and the conserved tryptophans near the C-terminal tail. Koonin has hypothesized that these two motifs are involved in binding to eIF2 since the same motifs can be found in eIF2B and eIF4γ.
(subunit of eIF4G), both of which also interact with eIF2 (Pain, 1996). The N-terminal region of eIF2β contains a basic region along with the C-terminal zinc-finger domain (Flynn et al., 1994) making this subunit of eIF2 a potential target of the acidic C-terminal tail of eIF5. The conserved tryptophans may be analogous to the conserved tryptophans of eIF4E that are essential for mRNA binding in eIF4E (Altmann et al., 1988) or may represent a new domain as hypothesized by Koonin (1995).

Since a low concentration of salt removes eIF5 from the rRNA-cellulose column, it is reasonable to hypothesize that eIF5 binds the ribosome with a relatively low affinity. If the initiation complex containing eIF2 is assembled at the ribosome, eIF5 may sense the complex through an interaction involving its C-terminal tail, which could be free at the surface of eIF5 and the ribosome. When eIF5 interacts with eIF2, a conformational change would decrease the affinity of the C-terminal region for the ribosome and, in turn, greatly decrease eIF5’s overall affinity for the ribosome (fig.5.1). This model would imply that eIF5 acts as a sensor of the assembly of the initiation complex, an idea that is consistent with eIF5’s function in translation initiation. Although it has been shown that eIF5 and eIF2 will interact in vitro without the presence of the ribosome (Chaudhuri et al., 1994), the model suggests that eIF5 and eIF2 would not interact in vivo unless eIF2 is bound to the ribosome. The requirement for the presence of the 40S ribosome would ensure that eIF5 and eIF2 interact only when eIF2 is part of the initiation complex and could function in the regulation of protein synthesis.

Since eIF5 is centrally located in the process of translation and is present in very low abundance, it presents a good target for regulation. Mammalian eIF5 is known to be phosphorylated in vitro and in vivo, although the physiological significance is not yet
Figure 5.1 Hypothetical model of the interactions of eIF5 at the 40S ribosome.
known. In light of this, the in vitro phosphorylation results indicating two CKII phosphorylation sites at the C-terminal region of eIF5 become more interesting, since this region would be an ideal target for regulation. Although it has been shown that phosphorylation of eIF5 does not affect its interaction with eIF2 in vitro at the molar ratios used in the experiments (Chaudhuri et al., 1994), in vivo phosphorylation could serve to partition eIF5 into active and inactive states much like the situation for eIF4E. Future research may be directed at understanding the importance of phosphorylation in the C-terminal region of eIF5. It is possible that modification of eIF5 in this region could play a role in regulating any potential interactions with eIF2 or with rRNA. Investigation of the role of phosphorylation of the acidic tail will need to involve site-directed mutagenesis of the phosphorylation sites in in vivo experiments using transgenic Arabidopsis. Other approaches could involve examining what cytosolic proteins interact with eIF5 as the C-terminal region is altered and if these interactions affect the activity of eIF5. In addition, further examination of the rRNA binding properties of this region may reveal a role for phosphorylation in this interaction.

The experimentally testable predictions of the model for eIF5's interactions at the ribosome are that (1) the C-terminal acidic tail of eIF5 is responsible for the interaction of eIF5 with eIF2, possibly at eIF2β; (2) the conserved tryptophans at the C-terminus of eIF5 are involved in rRNA binding; (3) the zinc-finger region of eIF5 could interact specifically with the 40S ribosome; either in protein-protein or protein-nucleic acid interactions (4) the removal of the conserved tryptophans at the C-terminal of eIF5 will decrease the affinity of eIF5 for the ribosome; and (5) removal of the C-terminal of eIF5 will disrupt 80S ribosome formation in in vitro assays.
Translation initiation is now understood to be crucial to the regulation of gene expression and while translation initiation has been well studied in mammalian systems, relatively little is known about the process in higher plants. In either system, eIF5 has not been a major focus of study although the protein is centrally located in the translation initiation scheme. Further work involving eIF5 will offer an exciting opportunity to delve into the mechanisms of one of the most essential processes in the cell, protein synthesis.


Electrophoresis 14, 1023-1031.


Journal 11, 3289-3295.


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Microbiological Reviews 56, 291-315.


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Appendix

Forwarded message

Date: Mon, 2 Oct 1995 10:12:58 -0500
From: Andrew Bent <abent@ux1.cso.uiuc.edu>
To: Zelpha Elizab Floyd <zfloyd@chris1.chem.lsu.edu>
Subject: Arabidopsis transformation

Dear Beth,

Brian Staskawicz told me you are interested in Arabidopsis transformation. Here is our version of the Agrobacterium vacuum infiltration protocol for use with Arabidopsis. The citation for this protocol is:


It would also be good to cite the inventors of this approach, Bechtold et al.:


Transformation of Arabidopsis by Vacuum Infiltration

This protocol is based on the work of Nicole Bechtold, Jeff Ellis and Georges Pelletier. My modifications were incorporated to streamline their procedure. The most significant changes eliminate the need to uproot and re-plant infiltrated plants.

- Andrew Bent

Plant Growth:
1. Grow plants of the appropriate genotype to a stage at which bolts are just emerging.

It works well to grow 12-15 plants in a 3.5" pot. Mound the soil up above the top of the pot, and after planting cover the soil with nylon window screen and fasten with a rubber band. Plants grow through the screen and when pot is inverted for infiltration less dirt falls out. Plants can be grown in continuous light or in short days (10 hr.) for the first four weeks to get larger plants and a greater seed yield. Transfer to long days to induce bolting.
Success may also depend on frequent fertilization and strong light intensity. You want to start with healthy plants!

2. Clip off emerging bolts to encourage growth of multiple secondary bolts.

Infiltration will be done 4 to 8 days after clipping, when there are many bolts that are 1-5 cm long (and some longer, but only a few dozen open flowers).

Vacuum Infiltration:
3. Grow a large liquid culture of Agrobacterium carrying the appropriate construct.

Start a 25 ml overnight (LB + antibiotics) two to three days ahead of time. Add this culture to 400ml of LB + antibiotic the day before infiltration. My experiments were done using A. tumefaciens GV3101pMP90 (C. Koncz and J. Schell, 1986, Mol. Gen. Genet. 204:383-396).

4. Harvest cells by centrifugation (5K 10min. in GSA rotor, preferably at room temp.) and resuspend in 3 volumes infiltration medium (OD600 approx. 0.8).

Harvest cells at an OD600 of >2.0. A 400 ml culture will give enough cells for infiltration of at least six pots.

5. Add Agrobacterium (in infiltration medium) to a dish or beaker and invert plants (pot, soil, and all) into liquid solution. Be sure bolts and entire rosettes are submerged.

A one liter beaker filled with >200 ml of solution fits well with our 3.5" pots. Bacterial solution can be extended by reusing for at least one additional pot.

6. Place beaker into bell jar. Draw a vacuum until bubbles form on leaf and stem surface and the solution bubbles, then release vacuum very rapidly.

The necessary time and vacuum pressure will vary lab-to-lab. Practice on a few dispensable plants first. Good infiltration is visibly apparent as uniformly darkened, water-soaked tissue. Treatments that close stomates (such as recent transfer from humid to dry air) will make infiltration more difficult. Be sure to have good traps in your vacuum system or you will quickly saturate the pump oil.

7. Remove plants from beaker, lay them on their side into a plastic flat and cover with plastic wrap or a dome to maintain humidity. The next day, uncover plants and set upright.

8. Grow approximately four weeks, keeping bolts from each pot together and separated from neighboring pots.

9. When siliques on plants are very dry, harvest seed (all seed from one
Selection of Putative Transformants:
Kanamycin selection protocol: (Note that Basta selection is much less labor intensive - but your present binary vector system is more likely to encode antibiotic resistance.)


Plastic 150 x 15 mm petri dishes are convenient.

11. Sterilize seed.

A variety of sterilization protocols are appropriate.
I place seed in 15 ml plastic orange cap tubes and then treat:
1 minute in ethanol or isopropanol
5 minutes in 50% Bleach/50% water/0.05% Tween.
3 rinses with sterile water.

It is advisable to add one or two control seeds from a known transformed plant onto a marked location on at least a few of the selection plates.
Sterilize these seed also.

12. Plate seed by resuspending in sterile, room temperature 0.1% agarose and spreading onto selection plates. Dry plates in laminar flow hood until seed no longer flows when plate is tipped.

Use one ml agarose for every 500-1000 seed.
Plate 2000 to 4000 seed per 150 x 15 mm plate. Higher densities can make antibiotic selection less effective.

13. Vernalize plates for two nights in cold room. Move plates to growth chamber.

14. After about 7 days, transformants should be clearly identifiable as dark green plants with healthy green secondary leaves and roots that extend over and into the selective medium.

15. Transplant plantlets to soil, grow, and collect seed.

Transplanting success is improved by breaking up agar around root prior to pulling, by removing any adhering chunks of agar from root before planting, by saturation of soil with water after transplanting, and by growing plants under a dome (for high humidity) for the first day or two. If you break the root, put plantlet onto a new selection plate for a few days before transplanting.

Infiltration Medium:
1/2 X Murashige & Skoog salts
1 X B5 vitamins
5.0% Sucrose
.044 uM Benzylamino Purine (10 ul per liter of a 1 mg/ml stock in DMSO)
Selection Plates:
1/2 X Murashige & Skoog salts
0.8% Agar
Autoclave, cool, then add:
1 X B5 vitamins
Antibiotic (such as Km 50 ug/ml)

Good Luck!

- Andrew Bent
Vita

Zelpha Elizabeth (Beth) Floyd attended the University of Alabama at Birmingham and graduated in December, 1977 with a bachelor’s degree in nursing. She worked in various clinical settings before returning to graduate school at Louisiana State University in Baton Rouge, Louisiana in 1990. Ms. Floyd will graduate with a doctor of philosophy degree in biochemistry from Louisiana State University in August, 1997.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Zelpha Elizabeth Floyd

Major Field: Biochemistry

Title of Dissertation: Characterization of Bean Eukaryotic Translation Initiation Factor 5: Cloning, Sequencing, and Structure-Function Studies

Approved:

[Signatures and names]

EXAMINING COMMITTEE:

[Signatures and names]

Date of Examination:

June 26, 1997