EUKARYOTIC INITIATION FACTOR 4F (elF4F) ENHANCES HIGH AFFINITY BINDING OF 40S RIBOSOMAL SUBUNIT TO TOBACCO ETCH VIRUS (TEV) INTERNAL RIBOSOME ENTRY SITE (IRES)

by

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Abstract

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Many viruses and some eukaryotic mRNAs employ a cap-independent pathway in which an RNA element called the internal ribosome entry site (IRES), drives preinitiation complex formation by positioning the ribosome on the message, either at or just upstream of the start site. We have studied binding of wheat germ 40S subunit of wheat germ ribosome to wild type PK1 RNA, which shows 100 % translation activity. To explore the specificity of the IRES RNA•40S interaction, we also measured the affinity for IRES RNA mutants, S2-3 RNA and to S1-3 RNA that have been previously characterized in terms of in vitro translation initiation activity (1). The level of expression from each mutant is calculated relative to the corresponding wild-type sequence, which is set at 100%.S2-3 RNA shows about 30% translation activity while S1-3 RNA shows about 7% translation activity.

While ribosome binding to eukaryotic mRNAs requires the participation of eukaryotic initiation factors eIF4A, eIF4B, and eIF4F and the hydrolysis of ATP, our

results suggest that the 40S ribosomal subunit binds directly to the PK1 RNA with dissociation constant (Kd) of 67 nM. While 40S ribosome bound to S2-3 RNA with Kd of 138 nM; significant binding to S1-3 RNA was not observed. Direct binding measurements of both wild type and mutant TEV IRES RNAs suggest that TEV message is driven through a high affinity TEV RNA•40S interaction, involving a novel strategy of ribosome recruitment distinct from eukaryotic cap-dependent mechanisms.

Fluorescence anisotropy data showed that eIF4F increased 40S Ribosome binding affinity to PK1 RNA by approximately 2.6 fold. To determine the effects of initiation factor mediated RNA unwinding in cap independent initiation, we determined RNA binding activity of elF4A, elF4B and 40S Ribosome with TEV RNAs. The fluorescence anisotropy data revealed that in the presence of eIF4A, eIF4B, and hydrolysis of ATP, binding affinity of 40S ribosome to PK1 RNA increased 4 fold; giving Kd of 17 nM. Under the same conditions, while binding affinity of 40S ribosome to S2-3 RNA increased 2 fold, there was no significant change in S1-3 RNA binding to 40S.These data correlate well with the observed translational data and provide more detailed information on the translational strategy of tobacco etch virus.

This thesis is dedicated to M. Fethullah Gulen

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LIST OF ABBREVIATIONS

elF: Eukaryotic Initiation Factor

TEV: Tobacco Etch Virus

IRES: Internal Ribosome Entry Site

UTR: Untranslated Region

EMCV: Encephalomyocarditis Virus

FMDV: Foot and Mouth Disease Virus

HCV: Hepatitis C Virus

CrPV: Cricket Paralysis virus

CV: Coxsackievirus

HRV: Human Rhinovirus

PV: Poliovirus

HAV: Hepatitis A Virus

nt: Nucleotide

LB: Luria-Bertani Broth

IPTG: Isopropyl β-D-1-thiogalactopyranoside

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

PMSF: Phenylmethanesulphonylfluoride

DTT: Dithiothreitol

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Kd: Dissociation constant

1.0 Introduction

Translation is the process where ribosome synthesizes protein using the mRNA transcript. Translation of an mRNA into its cognate protein requires three sequential steps of initiation, elongation and termination (2). In the initiation step of protein synthesis, initiator $tRNA_i^{Met}$, 40S, and 60S ribosomal subunits assemble at the initiation codon of an mRNA to form an 80S initiation complex (3). The translation elongation cycle adds one amino acid at a time to a growing polypeptide chain according to the sequence of codons found in the mRNA. Termination of protein synthesis in eukaryotes is governed by three stop codons, UAA, UAG or UGA of mRNA by releasing the newly synthesized polypeptide from peptidyl-tRNA which takes place on the ribosome (4-6).

Initiation is typically rate-limiting and therefore an objective for regulation. Translation initiation is composed of three steps. The first step is the formation of a 43S complex which contains eukaryotic initiation factors eIF2, eIF3, and initiator Met-$tRNA_i$ bound to the 40S subunit (3, 7). In the second step that is rate limiting; 43S complex must bind to an mRNA and locate the initiation codon to form the 48S preinitiation complex. The last step is the addition of the large ribosomal subunit, 60S, to generate a translational-competent ribosome, 80S, which is capable to continue with translation initiation (2). The second step occurs in two ways according to the place where the 40S ribosomal subunit binds. One principal pathway is the attachment of the small ribosomal subunit, 40S, to the 5'-terminal cap structure (m^7GpppN, where N is any nucleotide), so called cap-dependent translation initiation.

The second way is the cap-independent translation initiation in which the small ribosomal subunit is recognized by internal ribosome entry site (IRES) sequence in the 5'-UTR.

1.1 Cap Dependent Translation Initiation

In eukaryotes, protein synthesis starts by a mechanism which relies on the 5'-terminal cap structure m^7GpppN, where N is any nucleotide, 3' poly(A) tail and numerous initiation factor proteins (8). The cap serves as a docking point for a cap-binding protein complex that can mediate recruitment of the small subunit of the ribosome to the 5' end of the mRNA. Under normal conditions, translation initiation in the eukaryotes follows a 'ribosome scanning model'. According to this model, initiation of cap dependent translation starts in the nucleus where capped mRNA unites with translation initiation factor 4E (elF4E), the small subunit of elF4F (9). The association of elF4E-mRNA complex with elF4G takes place in the cytoplasm. The secondary and tertiary structures in the 5' end of the mRNA are unwound with the assistance of elF4A, elF4B and hydrolysis of ATP to promote ribosome binding (2). Afterwards, 43S initiation complex which is composed of the small ribosomal subunit (40S), the initiator transfer RNA carrying the amino acid methionine (Met-$tRNA^{Met}$), and some initiation factors (elF2, elF3, elF1 and elF1A) binds to the cap structure at the 5' end of the mRNA and scans towards to the 3' end of the mRNA until the initiation codon is encountered followed by association with the large ribosomal subunit (60S)

which culminates with an 80S ribosome located on the mRNA to start protein synthesis.

Previously, the binary and ternary interactions among many proteins, m7G cap analogue, and poly-(A) was examined using fluorescence spectroscopy (10). The binding affinity of the cap-protein complex for PABP and the effects of PABP on the cap-binding affinity of eIF-4F and eIF-(iso)4F was studied. It was concluded that the cap-binding affinity of both proteins was enhanced around 40-fold by PABP (10). In addition, a comparison study of two subunits of elF(iso)4F , elF(iso)4E (p28) and elF(iso)4G (p86), was performed. The small subunit, p28, was found to be responsible for cap recognition. On the other hand, p86 increases the interaction with capped oligonucleotides and apparently participated in protein-protein interactions (11). Recently, elF4B was demonstrated to enhance the binding affinity of elF4F for the cap structure and in the presence of poly(A), the energy barrier was reduced and the binding rate was increased (12).

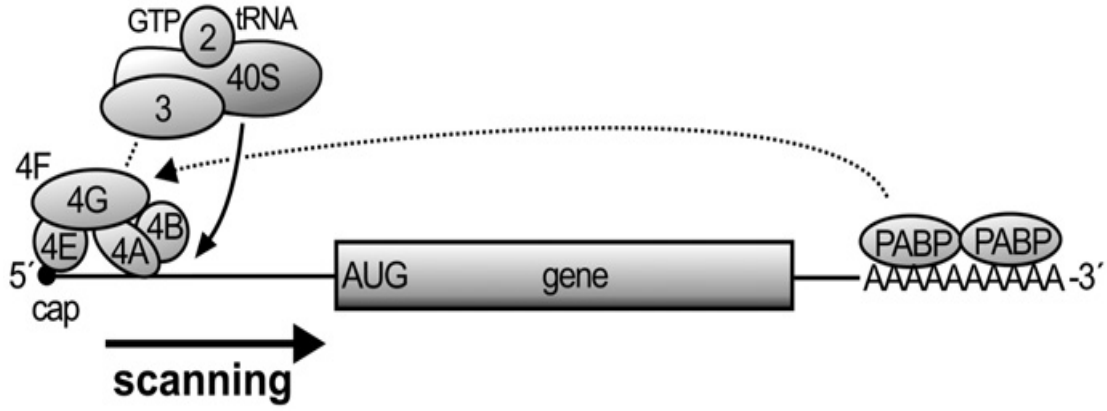


Figure 1. Cap-dependent translation initiation, the heterotrimeric initiation factor eIF4F binds with its eIF4E subunit to the cap, and the eIF4G subunit mediates the recruitment of the small ribosomal subunit to the 5′-end of the mRNA by interaction with the ribosome-bound eIF3.

Niepmann et al 2009

1.2 Cap-Independent Translation Initiation

The canonical cap-dependent mechanism is responsible for protein synthesis on the majority of eukaryotic mRNAs (8). The viral RNA molecule has no opportunity to be capped by the nuclear mRNA processing machinery because the virus totally replicates in the cytoplasm. However, upon infection, the viral RNAs are efficiently translated by the host translational machinery, even though the initiation codons are deeply inserted within internal and structured regions of the message. In addition, picornavirus replication can be very quick and effective that a single foot and mouth disease virus (FMDV) can infect a host cell and cultivate more than 100,000 progeny viruses and destroy the cell in a few hours (13).In 1988, the mystery of how translation initiation takes place on this human pathogen's RNA was resolved, when it was discovered that both poliovirus (PV) and encephalomyocarditis virus (EMCV) RNA utilize a cap-independent internal-translation initiation mechanism (14,15). This mechanism does not rely on the cap structure, instead it is driven by special RNA sequences, internal ribosome entry sites (IRES), which are present in the untranslated region (UTR). Briefly, in cap-independent translation initiation mechanism, cap-dependent translation initiation is shut down and the 40S ribosomal subunit is recruited, positioned, and activated by IRES sequences (16). However, the source of the IRES RNA and the requirements for the other initiation factors can differ dramatically (17). In addition, some cellular mRNAs, for instance translation initiation factors, transcription factors, oncogenes, growth factors, homeotic genes, and survival proteins carry IRES sequences which allow them to be translated under stress conditions, e.g.

hypoxia, apoptosis, amino acid starvation, and irradiation or when they need to escape translation repression during mitosis (18).

IRES-dependent translation initiation completely relies on the structural unity of the IRES. It was discovered that small deletions, insertions or even replacement of a single nucleotide can critically increase or decrease IRES activity (19, 20). Generally, there are no consensus similarities between individual IRESs. Encephalomyocarditis virus (EMCV) has been indicated to require almost all the initiation factors as the cap-dependent initiation mechanism except for elF4E for the formation of 48S complex (3). On the other hand, biochemical reconstruction of the initiation process on the cricket paralysis virus (CrPV) and hepatitis C virus (HCV) IRESs demonstrated that they do not require any initiation factor for 40S/IRES complex formation (21, 22). Another supplemental support for the concept of IRES-mediated translation initiation resulted from studies conducted to investigate the mechanism of inhibiting translation of capped cellular mRNAs. During infection, the viral proteases 2A of PV, coxsackievirus (CV), human rhinovirus (HRV) or the leader protease of FMDV cleave an amino-terminal fragment of elF4G which has the binding site for elF4E and a carboxyl-terminal fragment which include elF3 and elF4A binding sites (23,24). In contrast, hepatitis A virus (HAV) does not impair elF4G because the activity of HAV requires full length elF4G and elF4E (25). Moreover, the EMCV 2A protein binds to 40S ribosomal subunit and damages its interaction with capped mRNA but keeps it suitable for EMCV translation (26).

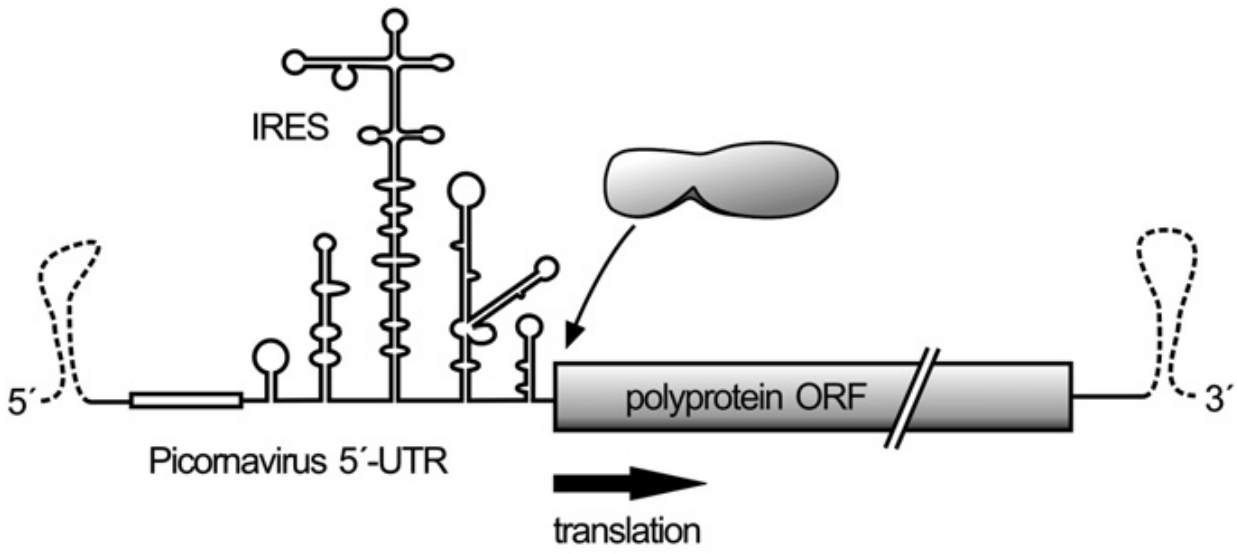


Figure 2. Picornavirus IRES elements comprise of RNA structures that direct a ribosome to an internal start site on the viral RNA, regardless of upstream sequences.

Niepmann et al 2009

1.3 40S Ribosomal Subunit

Ribosomes are large ribonucleoprotein complexes that translate mRNA into proteins in all living organisms (27). Eukaryotes have 80S ribosomes, each possesses a small (40S) and large (60S) subunit. 40S ribosomal subunit has an 18S RNA (1900 nucleotide) and around 33 proteins. Translation is the process of producing proteins that yield to assembly of small and large subunits to give 80S ribosomes. Translation initiation is typically composed of two stages: 48S initiation complex formation and the joining of 48 complexes with 60S subunits (28). Generally, 48S complexes form by a 'scanning' mechanism. In this mechanism 43S preinitiation complex which has 40S ribosomal subunit, the ternary complex (elF2-GTP-Met-tRNA^{Met}), elF3, elF1 and elF1A, binds to the 5' cap structure of mRNA and scans the 5' untranslated region to find the initiation codon. 48S complex is formed after the initiation codon recognition. When 48S complex is formed, the displacement of elFs is promoted and 60S subunits attaches to the 40S subunit.

The two subunits, 40S and 60S, carry out different roles in protein synthesis (29). 40S ribosomal subunit mediates the correct interactions between codons in the mRNA and the anticodons of the tRNAs. Therefore, they translate to ascertain the order of the amino acids in the protein being synthesized (30). 40S subunit possesses three binding sites for tRNA. The A site places the aminoacryl-tRNAs which are about to be combined into the growing polypeptide chain. In addition, the P site binds the peptidyl-tRNA and the E site positions deacylated tRNAs before they separate from the ribosome (30).

Previously, It has been shown that 40S ribosomal subunit is responsible for the attachment of the 43S complex to the IRES of HCV (Hepatitis C virus) and CSFV (Classical swine fewer virus) by itself (31). It is also indicated that the initiation factors elF4A, elF4B and elF4F are not required for the processes of ribosomal binding to the IRES of HCV and CSFV, placing of the ribosome at the initiation codon and the assembly of 40S and 60S ribosomal subunits to form 80S (31). In another study (32), it was demonstrated that 9nt segment of the mRNA of the homeodomain protein Gtx is 100% complementary to the 18S rRNA and this 9nt segment can function independently as IRES. In an earlier study, Mauro et al. (33) showed that this 9nt sequence could bind to 40S ribosomal subunit by crosslinking to its complement within the 18SrRNA. Furthermore, since some research revealed that many mRNAs have sequences which are complementary to 18S rRNA and it was proposed that the rRNA-like segments of mRNA might bind ribosomal proteins (34), some artificial complementarities were introduced to the central domain of plant 18S ribosomal RNA. It was observed that these artificial inserts can significantly increase the translation of a reporter mRNA both *in vitro* and *in vivo*. In addition, the translation-enhancing ability of the insert which is complementary to 18S rRNA is directly related to its ability to bind 40S ribosomal subunit without the help of initiation factors (35). Karpova et al. focused on the p40, the largest protein of 40S ribosome in their study (36). They revealed that p40 has an important role in protein synthesis since it is connected with ribosomes in growing cells and when the translation level decreases it separated from ribosomes. The first step in translation initiation on the HCV RNA is the formation of the binary complex of 40S subunits with the IRES and then this step is followed by the

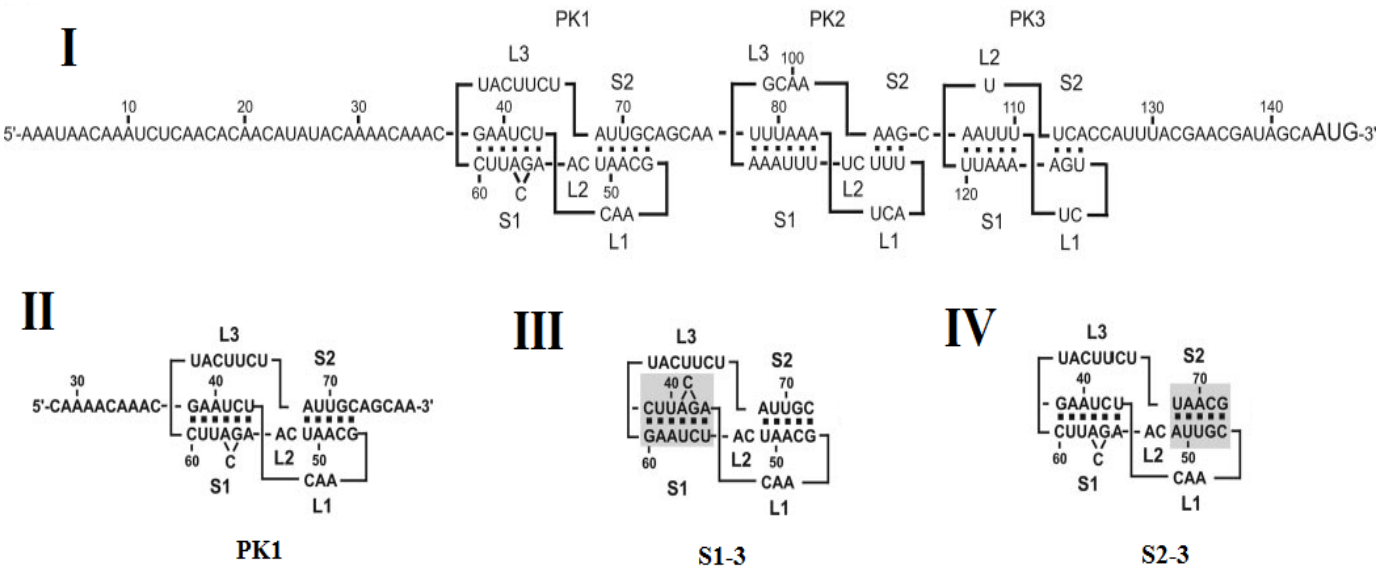
binding of elF3 and the ternary complex (elF2.Met-tRNA^{Met}.GTP). Their data indicate that p40 is entirely engaged in the binary complex formation. Moreover, translation of HCV is almost completely restrained, when the function of p40 was inhibited because of the blocked formation of the binary complex rather than by inhibition of elF3 binding to the 40S subunit.

1.4 Tobacco Etch Virus (TEV), PK1, S1-3 and S2-3

Tobacco etch virus (TEV) is a potyvirus which belongs to the picornavirus supergroup of positive-strand RNA viruses. The genomic RNA of TEV is a polyadenylated mRNA which is normally uncapped but comprises an IRES in the 5' leader sequence. The 143-nt TEV 5' leader is sufficient to complete cap-independent translation (37, 38). The TEV element is significant for its small size which represents one of the most compact viral elements promoting cap-independent translation, since IRES elements from picornaviral RNAs are normally big and highly structured (1). The 5' leader folds into a complex structure possessing two domains each of which has RNA pseudoknots. It was shown that mutations in the 5' proximal pseudoknot, PK1 that carries a nucleotide sequence complementary to a highly conserved region of 18 S RNA, reduced translation. PK1 (49bp) is an H-type pseudoknot with two stems, S1 (6 bp) and S2 (5 bp) which are joined by 3 loops L1 (3nt), L2 (2nt), and L3 (7nt) (Figure 1). Swapping S1 stem in PK1 such that the 5'-side of S1 became the 3'-side and the 3'-side became the 5'-side, so called S1-3, changed the original sequence of S1, while it preserved its potential for base pairing. S1-3 mutation decreased cap-independent translation to 7.4% of the wild-type TEV leader. Reversing S2 stem of PK1, S2-3 mutant, decreased cap-independent translation to 34% of the wild-type TEV leader, while it kept its potential for base pairing (1).

It has been shown that the TEV 5' leader promotes expression when a poly(A) tail was present to a greater extent than without the poly(A) tail (37). These data reveal that TEV 5' leader is functionally analogous to a cap, since it interacts with the poly(A)

tail to stimulate translation similar to the synergy previously described for a cap and a poly(A) tail. In addition, earlier quantitative binding studies have demonstrated that eIF4G binds the TEV leader and the PK1 RNA directly with higher affinity than eIFiso4G (39).



Gallie et al 2005

Figure 3. The predicted structure of TEV 5'-leader. **I**, the entire TEV 5'-leader (nt 1-143); **II**, the pseudoknot PK1 (bases: 28-77) of TEV 5'-leader; **III**, Mutation of Stem 1of PK1; **IV**, Mutation of Stem 2 of PK1

1.5 elF4F

Initiation of eukaryotic protein synthesis requires assembly of several factors. Eukaryotic translation initiation factor elF4F, a protein complex, facilitates recruitment of the ribosome to mRNA (12). elF4F is comprised of two proteins. elF4E, the small subunit of elF4F (25kD), is responsible for cap binding and a key protein involved in the regulation of protein synthesis (40, 41). The large subunit of elF4F is called elF4G, 171kD. elF4G interacts with some parts of the translational machinery including elF4A, elF4E, elF3, and poly(A) binding protein (PABP). As a result, it is considered as a scaffolding adapter molecule (40). Plants have a second form of elF4F, called elF(iso)4F, that functions correspondingly with elF4F. However, it consists of two different subunits than elF4F, elF(iso)4E (approximately 24kD) and elF(iso)4G (approximately 86kD) (42).

The two plant isoforms, elF4F and elF(iso)4F perform similar functions . They both promote translation initiation, stimulate ATP-dependent RNA helicase activity and operate as RNA-dependent ATPases (43-46). Lately, functional distinctions between these two isoforms have begun to be clarified. It was demonstrated that elF4F facilitated translation of an mRNA with a structured 5'-leader better than did elF(iso)4F (47). In addition, in vitro translation studies of tobacco etch virus (TEV) showed that in cap-independent translation, elF4G was used preferentially instead of elF(iso)4G (48). Furthermore, a quantitative binding study shows that elF4G binds the TEV leader and the PK1 containing region with higher affinity that the elF(iso)4G does (39). These studies suggest that TEV leader can distinguish between elF4G isoforms and preferentially recruit one isoform over the other. Previously, It has been indicated

that PABP and elF4B have a large effect on binding affinity of elF4F and elF(iso)4F to cap structure. In another study (49), the effect of PABP on the binding of elF4F and elF(iso)4F to IRES was investigated. Therefore, it was found that PABP has small effect on binding affinity between elF4F and elF(iso)4F and IRES.

1.6 elF4A

elF4A is a single polypeptide chain about 45kD which belongs to the family of "DEAD box" proteins (50). In this family, the proteins share seven conserved sequence motifs with approximately the same spacing between motifs (51). It was shown that the proteins that belong to DEAD box family have the ability to bind both ATP and nucleic acid (52). Most of the proteins in this group, referred to as helicases, possess RNA-dependent ATPase activity and ATP-dependent duplex RNA unwinding activity.

In most eukaryotic organisms, two different elF4A genes are observed, elF4A1 and elF4A2 (40). These two proteins show 90-95% sequence identity. When they were studied, it was found that they have the same biological activity in *in vitro* assays (40, 53, 54). Another isoform of elF4A, so called elF4A3, seems not to have a role in protein synthesis (55). elF4A3 shows 65% similarity to the other isoforms and has some of the elF4A activities, for example RNA-dependent ATP hydrolysis, ATP-dependent RNA duplex unwinding (55). Nevertheless, it repressed translation when added to reticulocyte lysates. Since the ratio of elF4A3 to elF4A1 is about 1:10 in the organism, it doesn't have much negative effect on the translation.

The most apparent function of the helicase activity of elF4A is to unwind regions of secondary and tertiary structure in the 5' end of the mRNA to facilitate 40S ribosome binding. However, there are other possibilities for its function. When the mRNA leaves the nucleus, it is covered with proteins and nucleic acids. Therefore, it is possible that the helicase activity of elF4A could be utilized to discharge the protein from the mRNA as it does to remove the RNA secondary structure. Previously, the same function of another helicase protein, NPH-II that belongs to the DEAD box family was shown (56). Another usage of the helicase activity function of elF4A is that the ATP-dependent movement of 40S ribosome from the 5'-cap to the 3' end of the mRNA to scan for the AUG start codon (40). elF4A is the only protein to bind and hydrolyze ATP in the mammalian translation system. Hence, it can be thought that the helicase activity as a motor of the elF4A-ribosome complex and the mRNA is moved along the complex as ATP is hydrolyzed. Another requirement for the helicase activity of elF4A is for the association of other initiation factors with either mRNA or the ribosome. This function could be used for both the initial binding of the proteins to the mRNA and the binding of the initiation factors-mRNA complex to the 40S ribosome (57). ATP hydrolysis by elF4A also functions to help the dissociation of factors from either the mRNA or 40S ribosome. One example is that elF4F is released from mRNA upon ATP hydrolysis (58, 59). Hydrolysis of ATP causes a conformational change in the elF4E, the small subunit of elF4F, making it release from the cap structure. This action permits the recycling of elF4F. Previous studies showed that elF4F molecules which are missing elF4A do not undergo a recycling process (59).

1.7 elF4B

The eukaryotic translation initiation factor elF4B is a dimeric protein with a size of 59kD. Essentially, it does not show any independent catalytic activity but stimulates the RNA-dependent ATP hydrolysis activity and ATP-dependent RNA helicase activity of elF4A and elF4F during translation initiation (60). In addition, elF4B has a function to arrange the assembly of the translational machinery through its interaction with elF4G, elF4A, elF3, PABP (poly(A)-binding protein) and RNA (61). elF4B is one of the least conserved initiation factors, even though its function is conserved between animals, plants and yeast.

In plants and animals PABP interacts with elF4B (62-64). The binding affinity of PABP for poly (A) RNA is enhanced by the interaction of elF4G elF4B with PABP (65). Furthermore, addition of elF4B to either elFiso4F or the elFiso4F-PABP complex has an effect to decrease the activation energy of binding of each to the 5'-cap structure in plants (12, 66). elF4B and PABP provide a lower energy barrier that may involve a conformational change which results in binding of elF4F to the 5' cap. Therefore, the stable recruitment of elF4F to an mRNA is promoted by elF4B and PABP together by increasing its binding to, and decreasing its dissociation from, the 5' cap. It is assumed that the interaction between PABP, elF4G and elF4B escorts the termini of an mRNA in close physical proximity to confirm the integrity of an mRNA as a prerequisite for recruitment of the 40S subunit (67, 68).

It is proposed that elF4A functions to unwind the secondary structure in the 5'-untranslated region of mRNA to facilitate binding of the 40S ribosomal subunit

to mRNA and allow for scanning of the 40S ribosome to the initiator AUG codon. Alone, elF4A showed little RNA helicase activity (69). elF4B is necessary to stimulate the ATPase and RNA helicase activity of elF4A and enhances ATP affinity of elF4A and the processivity of its helicase (70, 71). Briefly, elF4B operates to stabilize elF4F binding to the 5' cap, PABP binding to the poly(A) tail, mRNA binding to ribosomes, and stimulates elF4A helicase activity showing that even it doesn't have any helicase activity by itself, it carries out several functions through interactions with other factors and RNA to promote translation initiation.

1.8 Helicase Activity

In eukaryotic protein biosynthesis, binding of the 40S ribosomal subunit to mRNA is the rate- limiting step and a potential target for regulation (72-74). 5' cap and 3' poly (A) tail are found in most eukaryotic mRNAs. Initiation of eukaryotic protein synthesis requires at least three eukaryotic initiation factors, elF4A, elF4B, elF4F and hydrolysis of ATP to correctly bind and position mRNA on the 40S ribosome (50, 70, 75). Two models were suggested for the binding of mRNA to 40S ribosomal subunit (76). In the first model, the 5'-cap structure, m^7GpppN, is recognized by elF4F and then in combination with elF4A, elF4B and hydrolysis of ATP the unwinding of secondary structure in the 5'-untranslated region occurs. This unwinding process produces a single-strand RNA which facilitates the binding site for 40S ribosomal subunit. In the second model, first elF4E binds to the 5'-cap structure and

then joins with elF4G that is already bound to the 40S ribosomal subunit. In both models, after binding to the 5'-cap the initiation factors elF4F, elF4A and elF4B are required to unwind the secondary structure before reaching the initiation codon AUG.

Eukaryotic initiation factor 4A (elF4A), a DEAD box RNA helicase, in combination with elF4B operates to unwind secondary structure in the 5'-untranslated region of mRNA (77). It is indicated that elF4A is capable to function alone as an RNA helicase and there is a quantitative relationship between the initial rate of unwinding and the stability of duplex (71). Furthermore, the helicase activity of elF4A was studied in relation to substrate specificity. It was shown that the degree of unwinding of short RNA duplexes, around 10-15 base pairs, by elF4A relies only on the stability of the duplex rather that its length (60). In addition, elF4A, elF4F and elF4B have also been involved in cap-independent (IRES mediated) translation initiation (3).

1.9 Fluorescence Anisotropy

Fluorescence anisotropy measurements are dependent on the analysis of the rotational motions of species. It can be considered a competition between the molecular motion and the lifetime of fluorophores in solution.

If an immobilized fluorophore is excited by polarized light, its emitted light is also polarized. This polarized emission gradually returns to unpolarized fluorescence by several events including rotational diffusion of the fluorophore. The depolarization of the emitted light by rotational diffusion shows the median angular displacement of the fluorophore between the times of excitation to the time of emission. Binding of a second molecule, a conformational change in the fluorophore, and change of solvent viscosity are among the factors that affect the rate of rotational diffusion by the fluorophore. Therefore, the change in its rotational diffusion can be easily studied by monitoring the change of emitted polarized light from a fluorophore.

Anisotropy is directly related to the polarized light and is defined as a difference between intensities of vertically and horizontally polarized emissions divided by total fluorescence intensity. This technique is very valuable in binding studies, protein ligand interactions, monitoring conformational changes, determination of fluorophore lifetime, as well as offering insight into changes in molecular sizes of polymers and other molecules, and other properties inherent to biological systems (78-81).

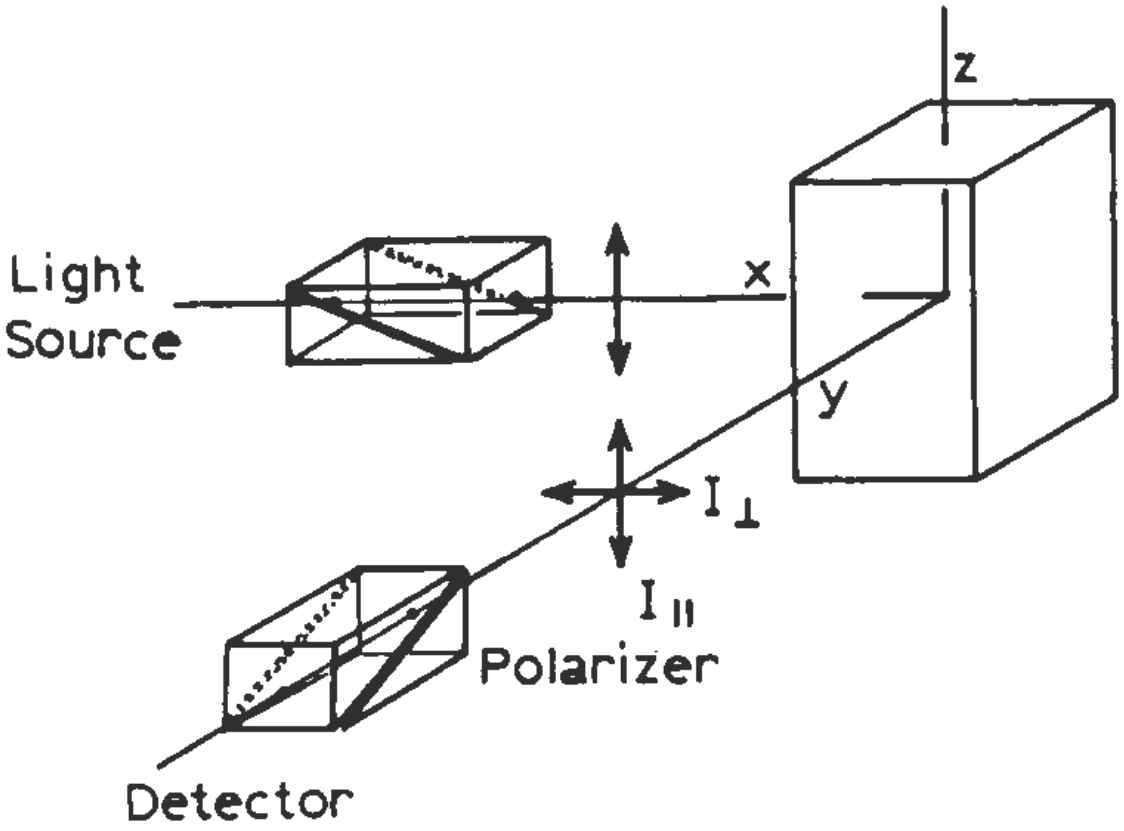


Figure 4. Fluorescence Anisotropy

2.0 Materials and Methods

2.1 Purification of eIF4F from Wheat Germ

Wheat germ eIF4F was isolated from the 0–40% ammonium sulfate fraction of the 120 mM KCl post-ribosomal supernatant as described previously (82). Equal amount of wheat germ and alumina were ground, suspended in buffer E and centrifuged. The supernatant was applied to a Sephadex G-25 column (Sigma). The active fractions were pooled and ultracentrifuged. The supernatant was brought to 40% ammonium sulfate saturation. The 0-40% ammonium sulfate fraction was applied to a 200 ml DEAE-cellulose column equilibrated in buffer B-40 (20 mM HEPES-KOH, pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% Glycerol, 40 mM KCl). The column was washed with buffer B-40 until the optical density returned to the baseline and developed with 40 to 150 mM linear KCl gradient in buffer B containing 20 mM HEPES/KOH, pH 7.6, 0.1 mM EDTA, 1 mM DTT, and 5% glycerol. The column was washed with buffer B-150 and then developed with a second 150 to 300 mM linear KCl gradient in buffer B. Fractions were collected in sterilized falcon tubes. The aliquots containing eIF4F fractions were pooled and dialyzed with buffer B-40. After dialyzed with buffer B-40 elF4F is applied to a 5 ml HiTrap SP column equilibrated in buffer B-40. The elF4F loaded column was washed with buffer B-40 and then developed with a 40 to 400 mM linear KCl gradient in buffer B. eIF4F elutes in 250 mM KCl and fractions of 1 ml were collected. The active fractions confirmed by binding assays were pooled and dialyzed in buffer B-40 overnight. After dialyzing, the samples were loaded to 2 ml m^7GTP-

sepharose column equilibrated in buffer B-40. The column was washed with buffer B-40, followed by buffer B-40 containing 0.1 mM GTP. eIF4F was retained on the column and eluted with B-40 containing 100 mM GTP. A centricon-10 microconcentrator (Amicon Corp.) was used to concentrate the active elF4F fractions. The concentration was confirmed by a Bradford assay using bovine serum albumin (BSA) as standard. 7.5% SDS-polyacrylamide gel electrophoresis was used to verify purity of the proteins.

2.2 Expression and Purification of eIF4B

eIF4B was expressed in *Escherichia coli* containing the constructed pET3d vector in BL21 (DE3) pLysS as described previously (4). The cells were cultured in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. When the optical density at 600 nm reached 0.5, expression was induced for 5 h with 0.1g/L isopropyl-1-thio-β-Dgalactopyranoside. After the induction, the cells were harvested by centrifugation at 5000 rpm for 5 min. Subsequent steps were performed at 4 °C. E. coli cells were disrupted by sonicator. The distrupted cells were suspended in buffer B-600 (20 mM Hepes/KOH (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 10% glycerol, and 600 mM KCl) containing 0.5 ml of aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 100 μg/ml soybean trypsin inhibitor.The disrupted cells were centrifuged at 45 000 rpm for 2h and supernatant was dialyzed against buffer B-50. After the dialysis, the protein was applied to a 5 mL HiTrap SP column. The column was washed with buffer B-50 (20 mM HEPES-KOH, pH 7.6, 0.1 mM EDTA, 1 mM

DTT, 10% Glycerol, 50 mM KCl) until the optical density returned to the baseline and developed with a 50 to 400 mM linear KCl gradient in buffer B (total volume of 100 mL). The protein elutes between 200 and 300 mM KCl fractions. The purity of the eIF4B was confirmed by 10% SDS-PAGE with Coomassie Brilliant Blue staining. The concentration was confirmed by a Bradford assay using bovine serum albumin (BSA) as standard.

2.3 Expression and Purification of elF4A

elF4A was expressed in *Escherichia coli* containing the constructed pET23d vector in BL21(DE3) pLyS as described elsewhere (83). A His-Bind kit from Novagen was used to purify eIF4A by the following manufacturers procedure. The cells were cultured in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. When the optical density at 600 nm reached 0.5, expression was induced for 4 h with 0.1g/L isopropyl-1-thio-β-Dgalactopyranoside. *E. coli* cells were disrupted by sonication. The disrupted cells were suspended in binding buffer (5.0 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9) containing 0.5 ml of aprotinin, 1.0 mM phenylmethylsulfonyl fluoride, and 100 μg/ml soybean trypsin inhibitor and centrifuged at 45,000 rpm for 2 h. The supernatant was loaded to a 10ml His-Band Nickel column. The column was washed with washing buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9) until the optical density returned to baseline. After washing, eIF4A was eluted by eluting buffer (100 mM imidazole, 0.5 M NaCl, 20 mM TriszHCl, pH 7.9). The aliquots of active elF4A were pooled and dialyzed against

buffer B-50. The protein was concentrated by using Centricon-10 microconcentrator (Amicon Corp.) The purity of protein was determined by 10% SDS polyacrylamide gel.

2.4 Purification of 40S ribosome from Wheat Germ

40S ribosomal subunit was isolated from wheat germ as described elsewhere (84). Briefly, 120g of wheat germ (Bob's Red Mill, Natural Foods, Inc.) was ground with 120g of alumina in a cold mortar. The powdered wheat germ was mixed thoroughly with 200ml of extraction buffer (20mM HEPES-KOH, pH7.6, 1mM $Mg(OAc)_2$, 2mM $CaCl_2$, and 6mM β-mercaptoethanol) containing 120mM KCl, transferred to centrifuge tubes and centrifuged for 20 min at 15,000g. The supernatant was applied to a Sephadex G-25 column equilibrated in Buffer A (20mM HEPES-KOH, pH7.6, 5mM $Mg(OAc)_2$, 6mM β-mercaptoethanol) and 10% glycerol) containing 120 mM KCl. The column was developed with the same buffer. The active fractions with greater than 90 units/ml at absorption 260nm were centrifuged for 3 h at 170,000g. After the centrifugation, the ribosomes were suspended in buffer A containing 120mM KCl and allowed to sit in high salt for 30min an ice bath and centrifuged for 5h at 150,000g. Ribosomes obtained from the 0.12 M KCl preparation of wheat germ were diluted to 35ml with room temperature buffer I (50mM Tris-HCl, pH 7.7, 2mM dithiothreitol, 0.1mM EDTA, 0.6M KCl, 3mM $MgCl_2$, and 5% sucrose by weight). The diluted ribosomes were incubated at 30^0C for 5 min. and were separated by centrifugation through a linear 10-30% sucrose gradient in buffer I for 4 h

at 150,000g. After centrifugation, 5 ml fractions were collected, and the fractions for 40S ribosome were pooled. The pooled fractions were dialyzed against 1L of buffer II (50mM Tris-HCl, pH 7.7, 1mM dithiothreitol, 0.1mM EDTA, 0.6M KCl, 5mM $MgCl_2$, 50mM KCl and 10% glycerol) and 40S ribosomal subunits were collected by centrifugation for 12h at 113,000g. The 40S ribosomal subunits were resuspended in buffer A containing 50mM KCl and divided into 0.05ml aliquots.

2.5 In Vitro PK1, S1-3, S2-3 mRNA Synthesis and Labeling

The pT7-luciferase (*luc*) reporter construct, in which the firefly *luc*-coding region is under the control of the T7 promoter in a pBluescript-derived vector, described elsewhere (1). The PK1-containing region (nt 28–77) and the mutants S1–3 and S2-3 were introduced into the HindIII and SalI sites within the polylinker and the *luc* reporter was introduced into the SalI and BamHI sites. Qiagen HiSpeed™Plasmid Purification kit was used to isolate DNA. DNA was further linearized with SalI, at a site immediately upstream of the *luc* open reading frame. The linearized DNA was treated with Proteinase K (100 µg/ml) and 0.5% SDS in 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$ for 30 min at 37 °C. After Proteinase K treatment, DNA was purified by extraction with TE saturated phenol:chloroform:isoamyl alcohol (25:24:1) at pH 8.0 followed by ethanol precipitation. 1% agarose gel was used to determine the purity of DNA. *In vitro* transcription of PK1, S1-3 and S2–3 was performed using Promega RiboMAX™ Large Scale RNA Production System T7. The concentration of RNA was determined by measuring the optical density at 260 nm using the absorbance at 260 nm

of 40 µg/ml RNA as 1. The purity of synthesized RNA was verified by measuring the absorbance ratio at 260/280nm. This absorbance ratio varied from 1.8 to 2.1 in different RNA preparations. After the purification, PK1, S1-3, S2-3 mRNAs are labeled using 3' EndTag™ DNA Labeling System from Vector Laboratories. Labeling was achieved in two steps. A thiol functional group was introduced to the 3' end of the mRNA followed by coupling of thiol-reactive fluorescein label to the 3' end of the mRNA.

2.6 Fluorescence Anisotropy Measurements

Fluorescence anisotropy measurements were performed using a Horiba Jobin Yvon FluoroLog®-3 equipped with excitation and emission polarizers. An L-format detection configuration was used to carry out the anisotropy experiments. The 40S ribosome–RNA interactions were studied by direct fluorescence anisotropy titration using a 10- mm path-length quartz cuvette. The 50 nM 5' fluorescein labeled PK1 RNA (^{FI}PK1), S1-3 RNA (^{FI}S1-3) and S2-3 RNA (^{FI}S2-3) were incubated with varying concentrations of 40S, 40S-eIF4F, 40S-eIF4B.elF4A.ATP, 40S-eIF4B.elF4A, and 40S-ATP complex in titration buffer. All samples were incubated at least 10 min before data were collected. The sample temperature was maintained at 25 °C for all experiments.

Interaction of 40S ribosome with ^{FI}PK1, ^{FI}S1-3 and ^{FI}S2-3 was determined by the enhancement in anisotropy of fluorescein labeled RNAs emission. The anisotropy of each sample was measured by excitation with vertically polarized light at 490 nm and the emission was measured at 519 nm in the horizontal and vertical directions. .

The excitation and emission slits were 3. The anisotropy data was fitted to equation 1 to determine the dissociation equilibrium constant (12).

$$r_{obs} = r_{min} + \{(r_{max} - r_{min})/(2 \times [^{FI}PK1])\}\{b - (b^2 - 4\ [^{FI}PK1]\ [40S])\text{^}\ 0.5\} \quad \text{(Eq 1)}$$

where, $b = K_d + [^{FI}PK1] + [40S]$, r_{obs} is the observed anisotropy for any point in the titration curve, r_{min} is the minimum observed anisotropy in the absence of 40S ribosome, r_{max} is the maximum anisotropy at saturation, $[^{FI}PK1]$ and [40S] are the PK1 RNA and ribosome concentrations. K_d is the equilibrium dissociation constant. For all equilibrium measurements, three independent titration experiments were performed, and the average value was reported. Nonlinear least squares fitting of the titration data were carried out using KaleidaGraph software (version 2.1.3; Abelbeck Software).

2.7 In Vitro Translation Assay:

Wheat germ extract was isolated as described previously (85). Translation was determined in luciferase assay buffer (25mM Tricine, pH 8.0, 5nM $MgCl_2$, 0.1mM EDTA supplemented with 33mM dithiothreitol, 0.25mM coenzyme A, and 0.5mM ATP) for luciferase activity. Different concentrations of TEV_{PK1}-luc-A_{50} were translated in a 100µl reaction mixture containing wheat germ extract, 25 units of RNase inhibitor, and a 5 µM complete amino acid mixture (Promega). Brome mosaic virus

RNA was used as a control for its luciferase activity. The reaction mixtures were incubated for 2 h at 22^{o}C , and light emission was measured after the addition of 0.5 mM luciferin using a Monolight 2010 Luminometer.

3.0 Results

3.1 40S Ribosome Binding to Fluorescein Labeled PK1 RNA

In their translation study, Zeenko and Gallie (1) showed that the PK1 within the TEV leader was required for cap-independent translation. PK1 (49bp) is an H-type pseudoknot with two stems, S1 (6 bp) and S2 (5 bp) which are joined by 3 loops L1 (3nt), L2 (2nt), and L3 (7nt). The two stems are separated by the L2 loop. PK1 is flanked by a 5'-proximal 37-nt sequence possessing little secondary structure. To correlate the requirement for 40S ribosome binding to PK1 in cap-independent translation, we investigated binding efficiency of 40S ribosomal subunit of wheat germ with 50nM PK1 RNA. Before starting the binding studies, a luciferase activity assay was performed in order to check the ribosome activity. Different concentrations of RNA were used to see a distinction in their light emmision. It can be seen in Fig.5 that with increasing concentration of RNA the relative light unit increases proposing that the 40S ribosome isolated from wheat germ is active. Fig. 6 shows the fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled PK1 RNA. The dissociation constant (K_d) for 40S-PK1 binding was 67 ± 6.4 nM, showing that there is a strong binding between 40S subunit of wheat germ ribosome and wild type PK1 RNA.

It has been demonstrated that mutation of S1 stem within PK1, so called S1-3, decreased cap-independent translation to 7.4% of the wild-type TEV leader, while it preserved its potential for base pairing. On the other hand, mutation of the S2 stem (S2-3) reduced cap-independent translation to 34% of the wild-type TEV leader. We studied the binding of 40S ribosomal subunit to S1-3 and S2-3 RNA, in order to determine whether the mutations in S1 and S2 stems of PK1 which essentially reduce cap-independent translation also reduce 40S ribosome binding. The binding of 40S ribosome to S2-3 mutant was reduced around 2 fold with the K_d of 138 ± 5 nM (Fig.7), when compared with binding to PK1 RNA. Furthermore, Fig.8 indicates that the dissociation constant, K_d, was found to be 489 ± 3nM for 40S-S1-3 titration which shows relatively weaker binding. The comparison graph shows the binding differences of PK1, S2-3, and S1-3 to 40S ribosomal subunit. (Fig. 9).

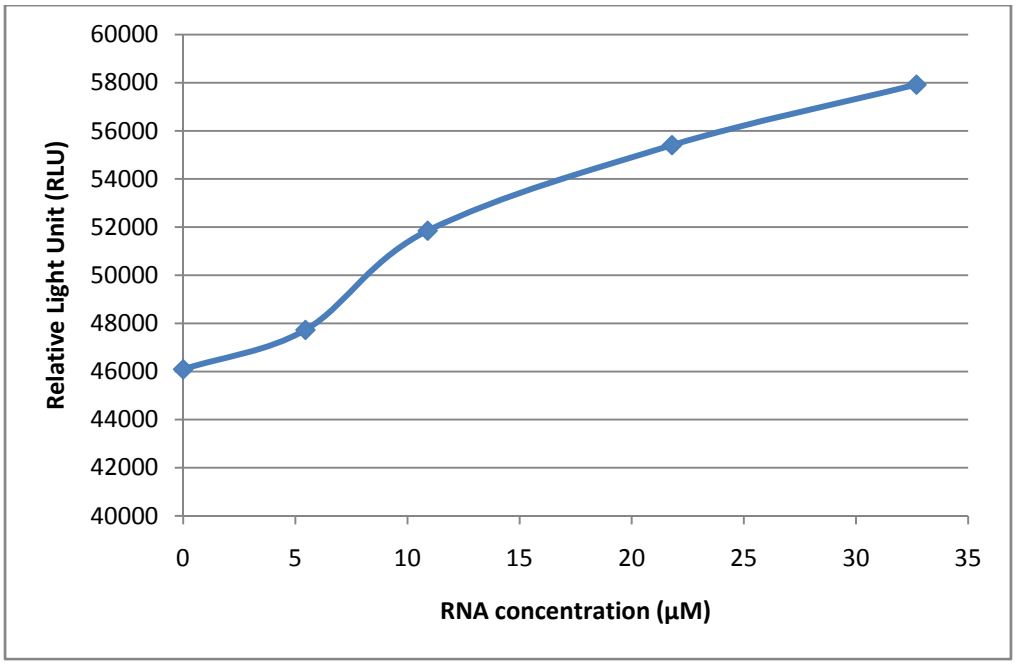


Figure 5. Luciferase Activity Assay

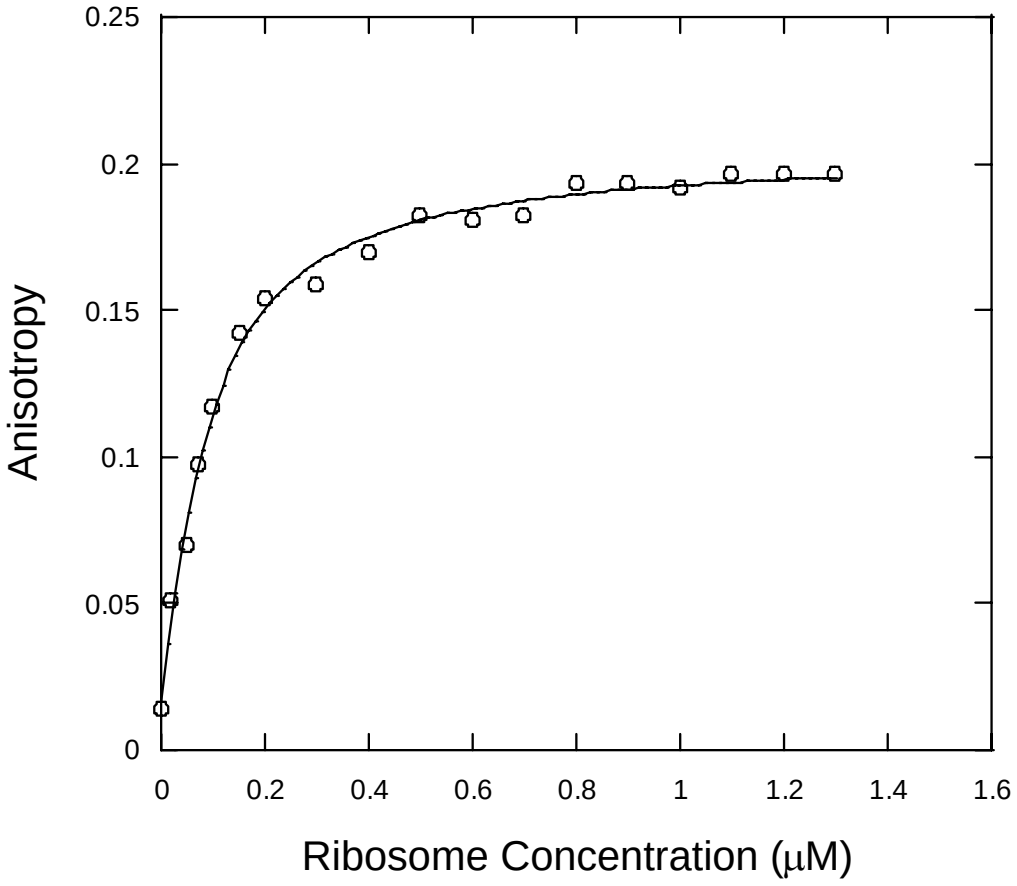


Figure 6. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled PK1 RNA. The anisotropy value of 40S ribosome is shown. The fluorescein labeled PK1 RNA concentration was 50 nM in titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

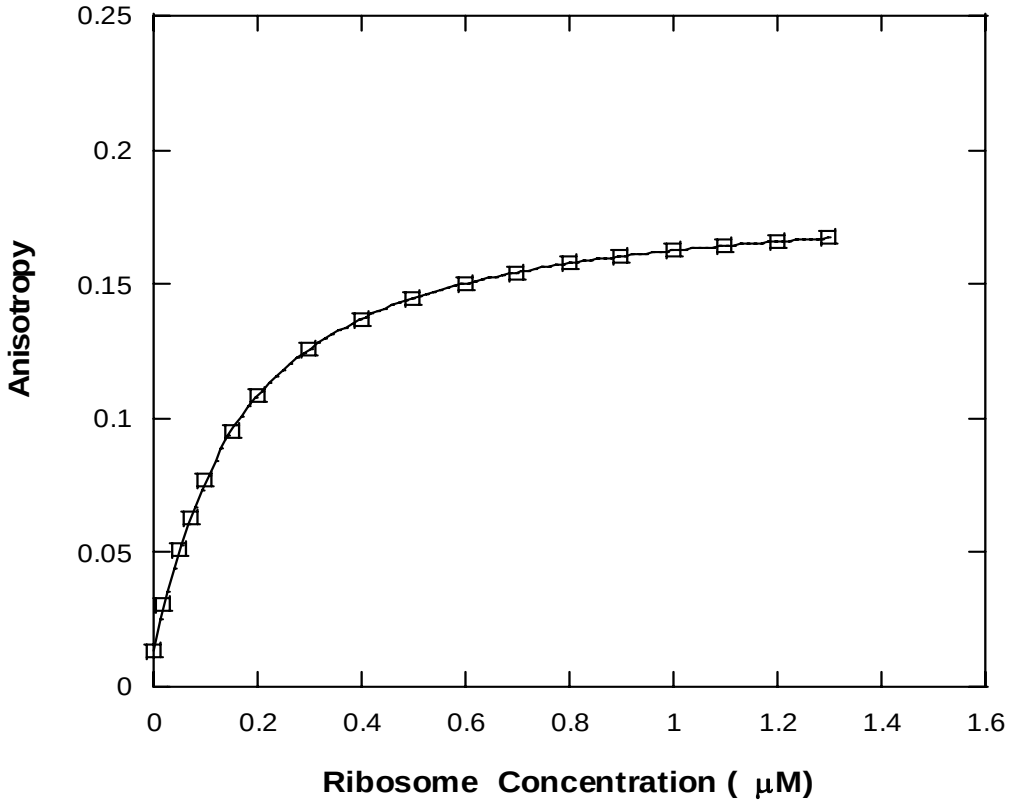


Figure 7. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S2-3 RNA. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S2-3 RNA concentration was 50 nM in Titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

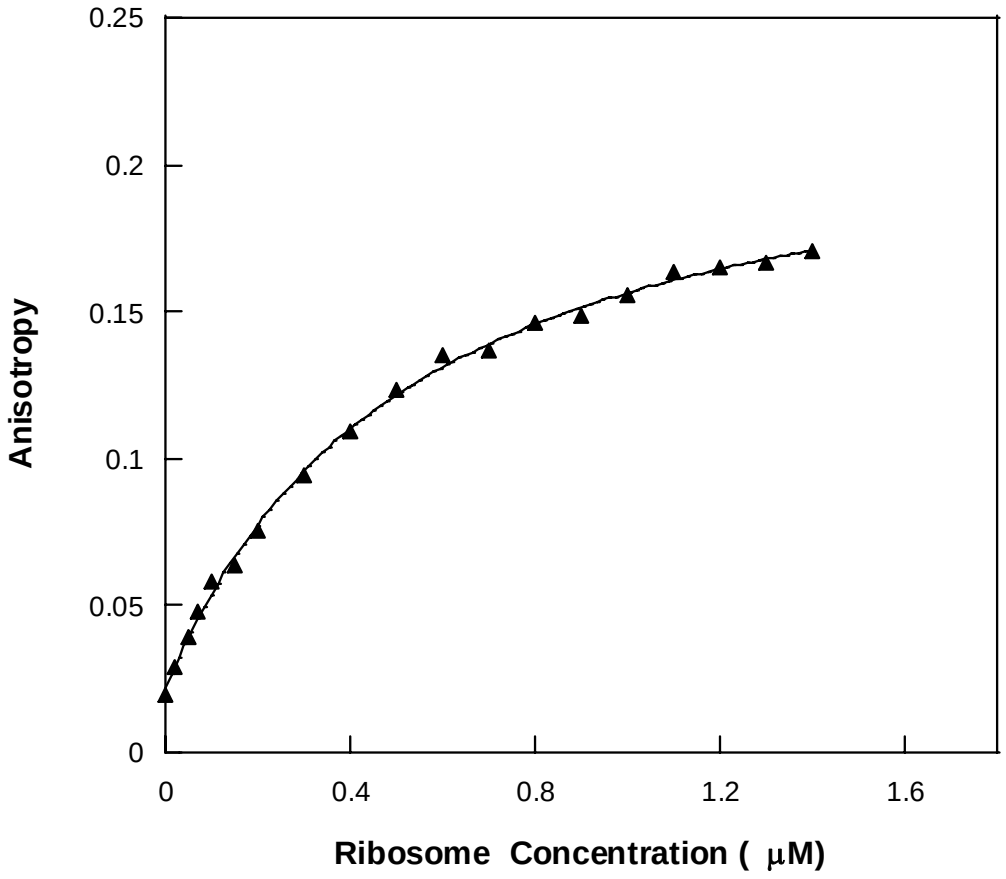


Figure 8. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S1-3 RNA. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S1-3 RNA concentration was 50 nM in titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

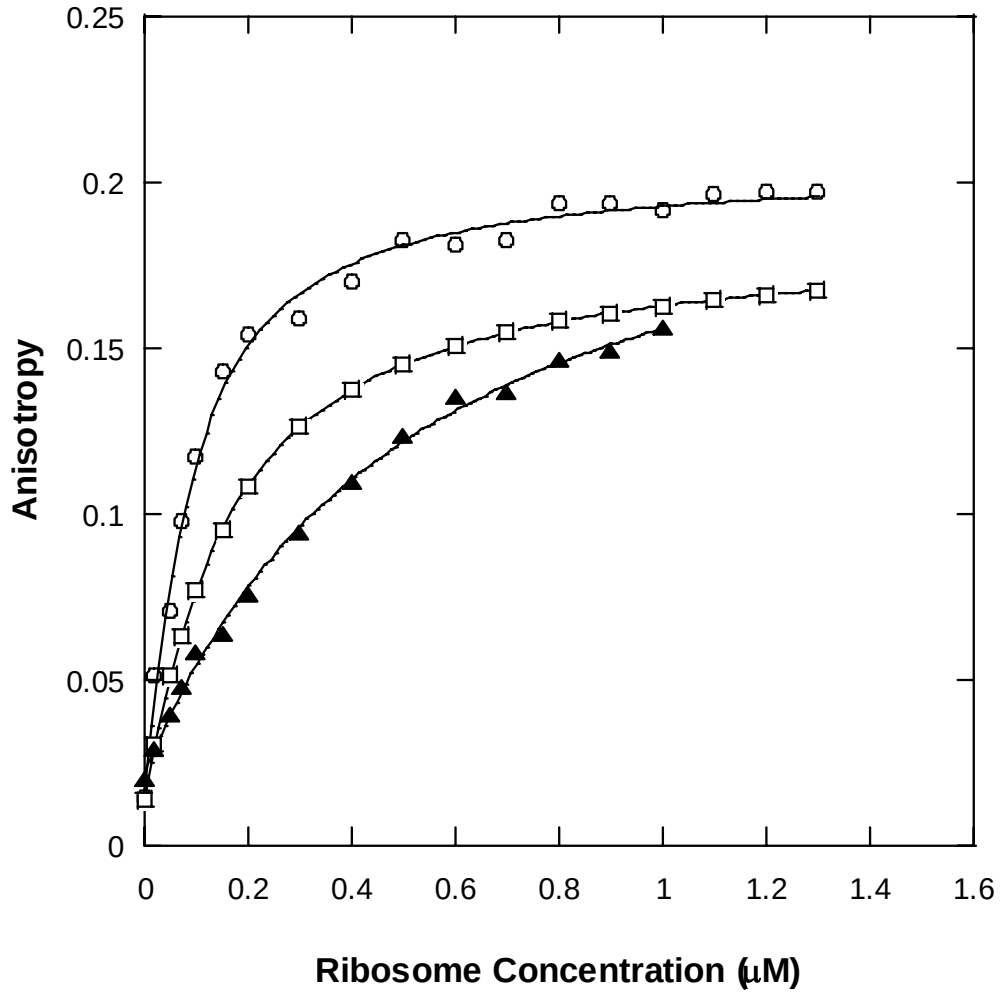


Figure 9. Fluorescence anisotropy measurements for the binding of 50 nM ^{FI}PK1 RNA, 50 nM ^{FI}S2-3 RNA and 50 nM ^{FI}S1-3 RNA with 40S ribosome. The anisotropy values of PK1 (—O—), S2-3 (—□—) and S1-3 (—▲—) are shown.

3.2 Effect of elF4F on Binding of 40S Ribosome to PK1 RNA

The wheat germ initiation factor elF4F is comprised of two initiation factors elF4E, the cap binding protein, and elF4G, the adapter protein that recruits other initiation factors engaged in stimulating 40S ribosome binding to mRNA. elF4F is a stable complex in plants and accordingly is isolated as a distinct complex when purified from wheat germ. The concentration of elF4F has an important role in both cap-dependent and cap-independent translation. In order to determine the effect of elF4F on the binding of 40S ribosome to PK1 RNA, the fluorescence anisotropy measurements for binding of fluorescein labeled PK1 RNA.elF4F complex with 40S ribosome were performed. 50 nM PK1 RNA was mixed with 1500 nM elF4F to make the complex then titrated with 40S ribosome at 25^{o}C. Fig.10 indicates that the translation initiation factor elF4F enhances the binding affinity of 40S ribosome to PK1 RNA 2.6 fold with Kd of 26± 2.3 nM. The effect of elF4F on the binding of 40S ribosome to S1-3 and S2-3 was also studied. Fig.11 shows a fluorescence anisotropy plot for the effect of elF4F on the interaction of 40S ribosomal subunit and S2-3 RNA. The binding affinity of 40S ribosome to S2-3 RNA is increased by 1.7 fold in the presence of elF4F (K_d= 83 ± 3.7 nM). The similar experiment was performed to examine the elF4F effect on the binding of 40S ribosome to S1-3 RNA. K_d for 40S ribosome binding to S1-3 RNA. elF4F complex was found to be 463 ± 29 nM (Fig.12). These results demonstrate that while the initiation factor elF4F enhances the binding affinity of 40S ribosome to PK1 RNA and S2-3 RNA, it does not show a significant effect on the interaction of 40S ribosome

with S1-3 RNA. Fig.13 shows the effect of elF4F on the binding affinity of PK1, S2-3, and S1-3 for 40S ribosome in one graph.

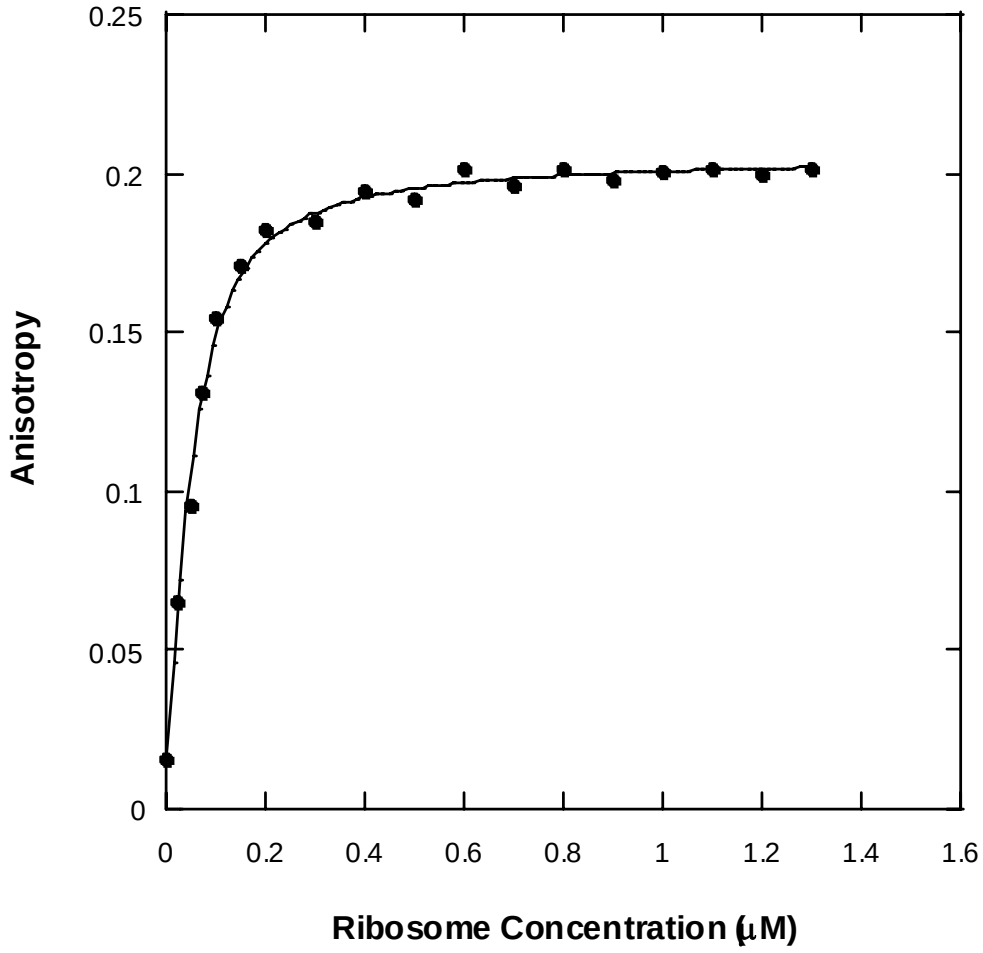


Figure 10. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled PK1.elF4F. The anisotropy value of 40S ribosome is shown. The fluorescein labeled PK1 RNA concentration was 50 nM and elF4F concentration was 1500 nM in titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

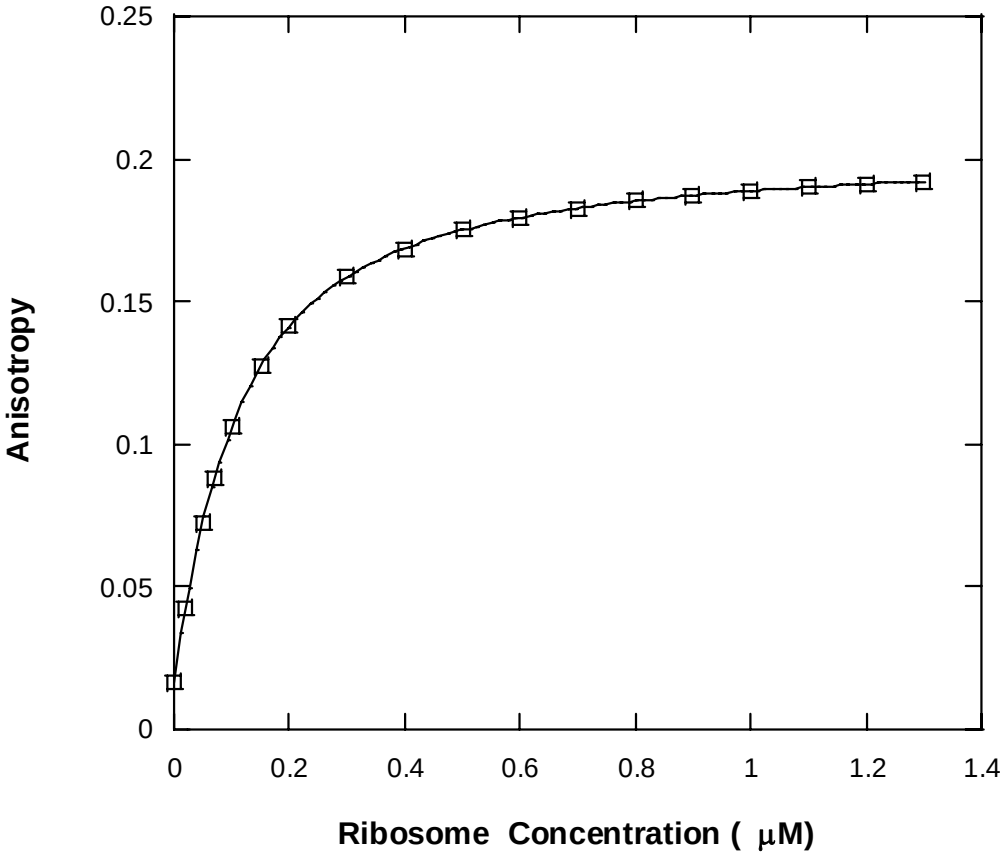


Figure 11. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S2-3 and elF4F. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S2-3 RNA concentration was 50 nM and elF4F concentration was 1500 nM in titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

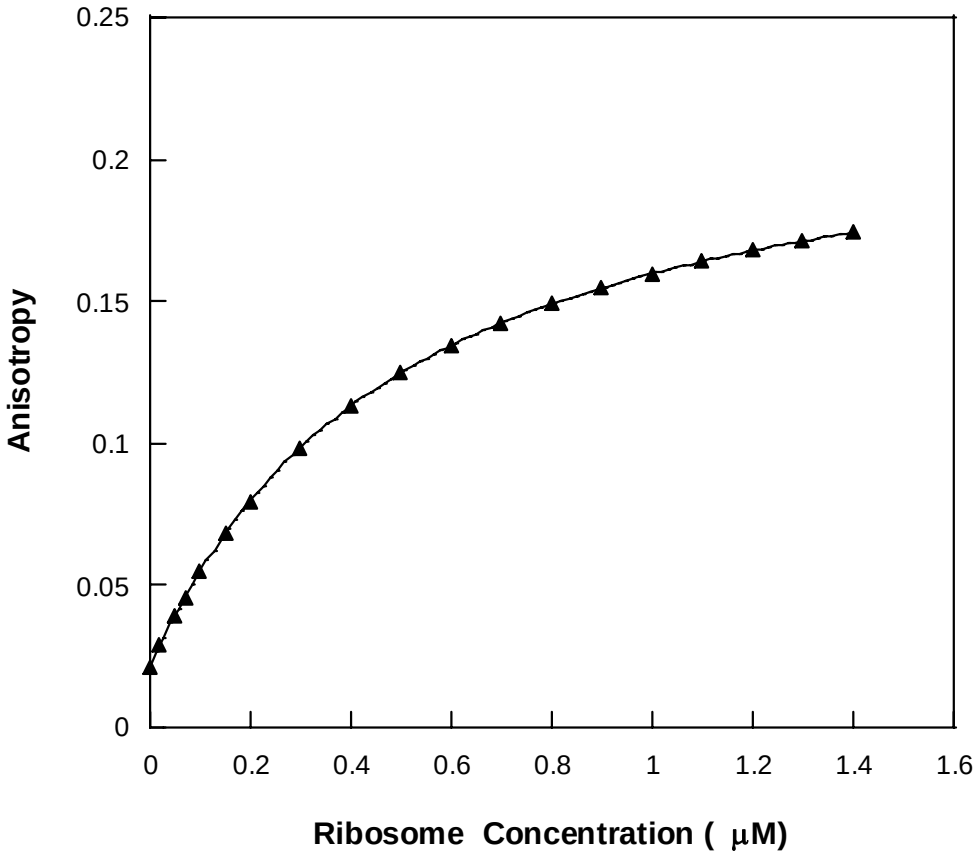


Figure 12. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S1-3 and elF4F complex. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S1-3 RNA concentration was 50nM and elF4F concentration was 4000 nM in titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

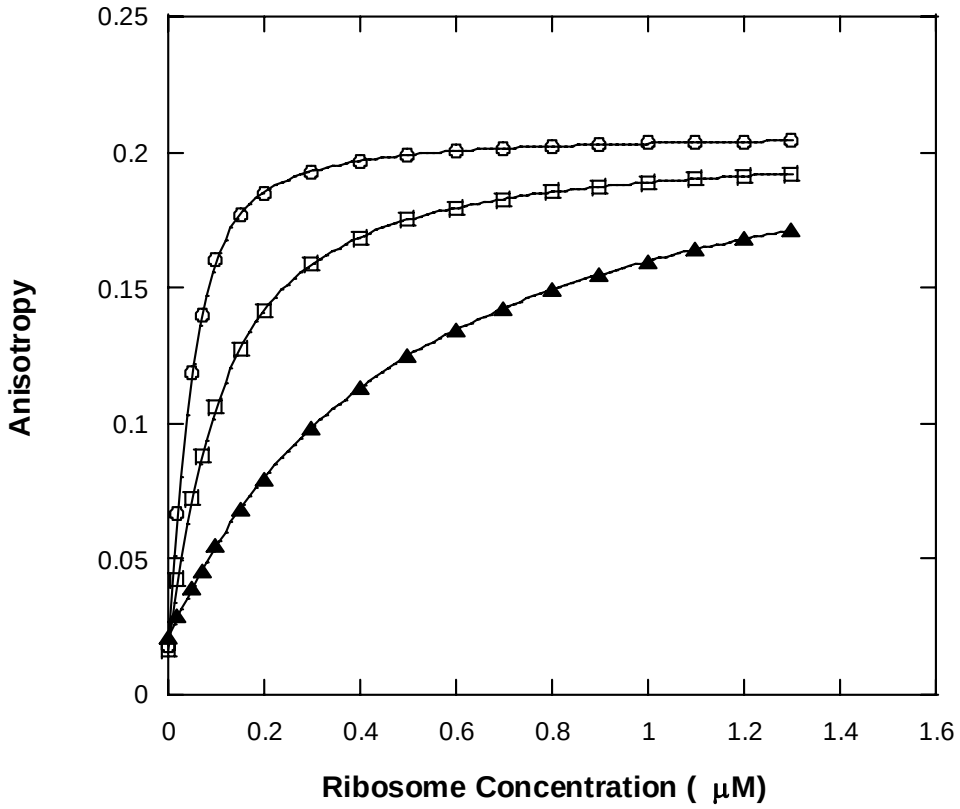


Figure 13. . Fluorescence anisotropy measurements for the binding of 40S ribosome with ^{FI}PK1.elF4F, ^{FI}S2-3.elF4F and ^{FI}S1-3.elF4F complexes. The anisotropy values of PK1 (—O—), S2-3 (—□—) and S1-3 (—▲—) are shown.

3.3 Effect of Helicase Activity on Binding of 40S Ribosome to PK1 RNA

In eukaryotic protein biosynthesis, binding of the 40S ribosomal subunit to mRNA is a critical and rate limiting step (83). Initiation of eukaryotic protein synthesis has need for several factors to correctly bind and position mRNA on the 40S ribosome (50). elF4A, elF4B, elF4F and hydrolysis of ATP are required in cap-dependent mRNA binding to the small ribosomal subunit (40S). elF4A is the prototype DEAD box RNA helicase possessing ATP binding and helicase motifs (51). elF4B is an RNA-binding protein that enhances the helicase activity of elF4A and elF4F (76, 77). Before the interaction of 40S ribosome and mRNA, elF4A, elF4B, and elF4F with the hydrolysis of ATP function to unwind 5'-proximal secondary structure in mRNA to facilitate ribosome binding. In order to understand whether helicase activity plays a role in the binding of the 40S small ribosomal subunit to mRNA in cap-independent translation, we studied the binding of 40S ribosome to unwound PK1 RNA. 50nM of fluorescein labeled PK1 RNA was mixed with elF4A, elF4B and ATP and incubated at 37^{o}C to allow the helicase reaction to complete. Following the helicase reaction, unwound PK1 RNA was titrated with 40S ribosome. Fig.14 shows a fluorescence anisotropy plot for the effect of helicase activity on the binding of 40S ribosome to PK1 RNA. It is observed that helicase activity of PK1 increased the binding affinity around 4 fold giving a K_d of 17 nM. In the same conditions, while the binding affinity of 40S ribosome with unwound S2-3 RNA was enhanced about 2 fold (Fig.15) with the K_d of 71 $\pm$ 4.8 nM, K_d was found to be 443 $\pm$ 30 nM for the binding between 40S ribosomal

subunit and unwound S1-3 RNA (Fig.16) indicating no significant change in the binding affinity. Fig 17 indicates the helicase activity effect on the binding of PK1, S2-3, and S1-3 to 40S ribosome in one graph.

After determining the effect of helicase activity on ribosome binding, it has been studied to verify whether the enhancement in the binding affinity is because of the helicase activity effect or the addition of the initiation factors are causing an increase in the binding affinity. Control experiments were performed. Fluorescence anisotropy measurements for binding of 40S ribosome to RNA in the presence of only ATP, only elF4A and elF4B and binding 40S ribosome to heat-melted RNA were performed (Table 1). 50 nM fluorescein labeled PK1 was mixed with elF4A and elF4B and incubated in the same conditions that the helicase reaction was performed before it was titrated with 40S ribosome. Fluorescence anisotropy data reveals that the addition of elF4A and elF4B does not have a significant effect on the binding affinity of 40S ribosome to PK1 RNA (Fig. 18). A similar control experiment was performed, in order to check if the ATP affects the binding affinity. Therefore, 50 nM of labeled PK1 RNA was mixed with ATP in the same conditions and titrated with 40S ribosome. Fig. 19 shows that ATP by itself doesn't have an effect on the 40S ribosome-PK1 RNA binding (K_d= 69 ± 3.4 nM). A positive control experiment was performed to verify that unwinding the secondary structure in mRNA increases the 40S ribosome ribosome binding to PK1 RNA. Fluorescein labeled RNA was heated at 90^0C to unwind the secondary structure and immediately placed on ice to prevent it from refolding. Following the unwinding process by heat, unwound RNA was titrated with 40S

ribosome. K_d was found to be 20 ± 1.8 nM indicating that unwinding by heat or helicase reaction has similar effects on the binding affinity (Fig.20). The initiation factor elF4F effect on binding of 40S ribosomal subunit to unwound PK1 RNA was also tested. There was not a significant change in the binding affinity as seen in Fig.21.

In addition, the same control experiments were also performed for S2-3 and S1-3 mutant RNAs (Table 2, Table 3). While the presence of only ATP or only elF4A and elF4B do not affect the binding of 40S ribosome to S2-3 RNA (Fig. 22, Fig. 23), the usage of unwound S2-3RNA by heat increased the binding affinity as well as the unwound S2-3 RNA by helicase reaction (Fig.24). Furthermore, Fig. 25 shows that the addition of initiation factor elF4F doesn't influence the binding of 40S ribosome to unwound S2-3 RNA. As seen in the Fig. 26-28, none of the control experiments had an effect on the binding affinity of 40S ribosomal subunit to S1-3 RNA.

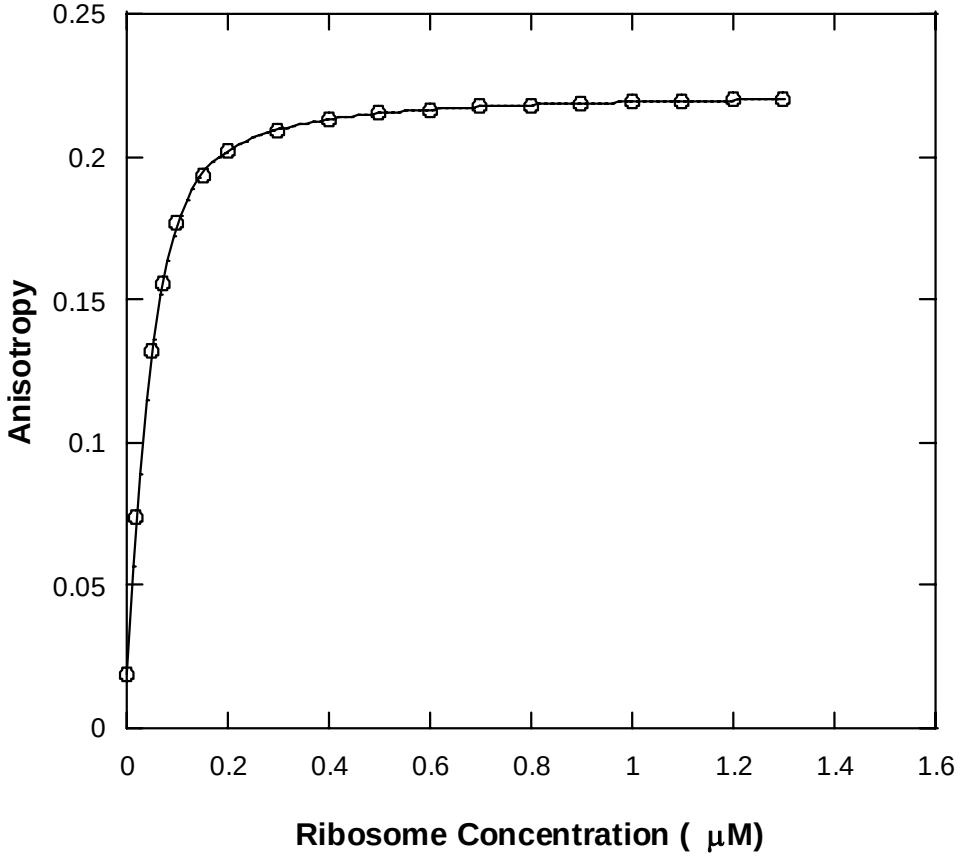


Figure 14. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled PK1 RNA in helicase reaction mixture with elF4A, elF4B and ATP. The anisotropy value of 40S ribosome is shown. The fluorescein labeled PK1 RNA concentration was 50 nM at 25 ^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

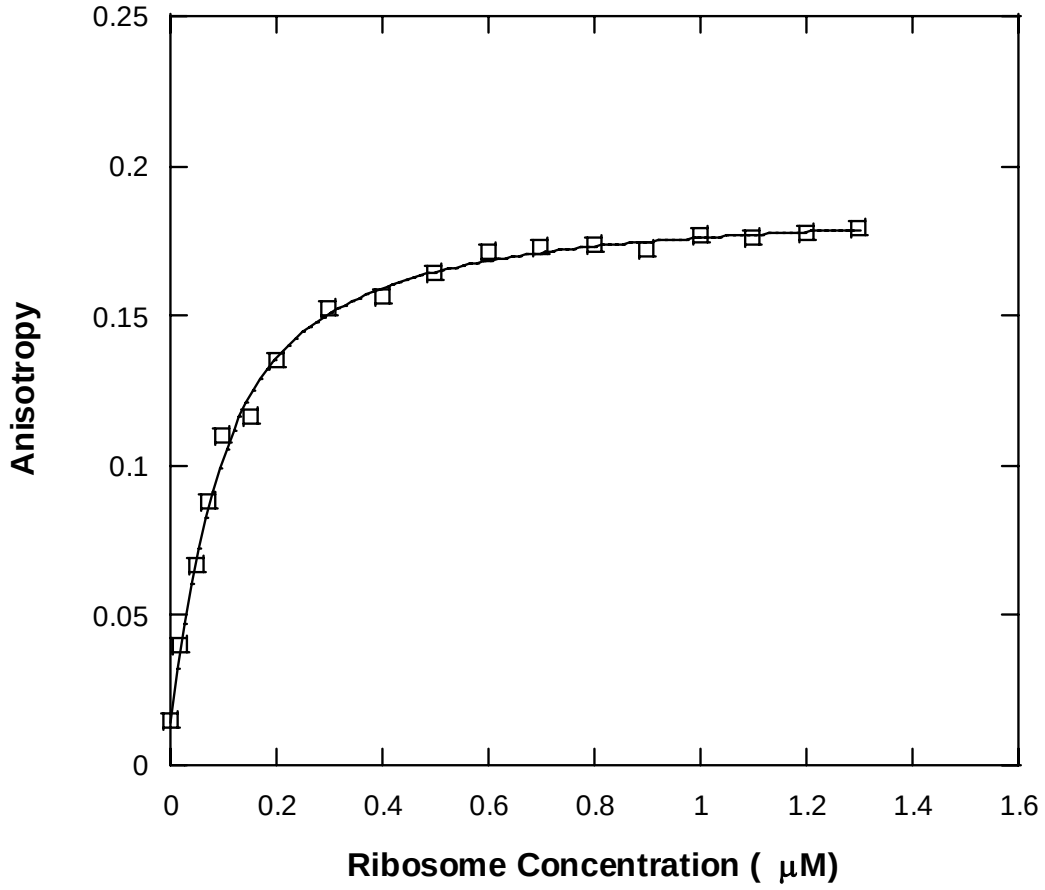


Figure 15. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S2-3 RNA in helicase reaction mixture with elF4A, elF4B and ATP. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S2-3 RNA concentration was 50 nM at 25 ^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

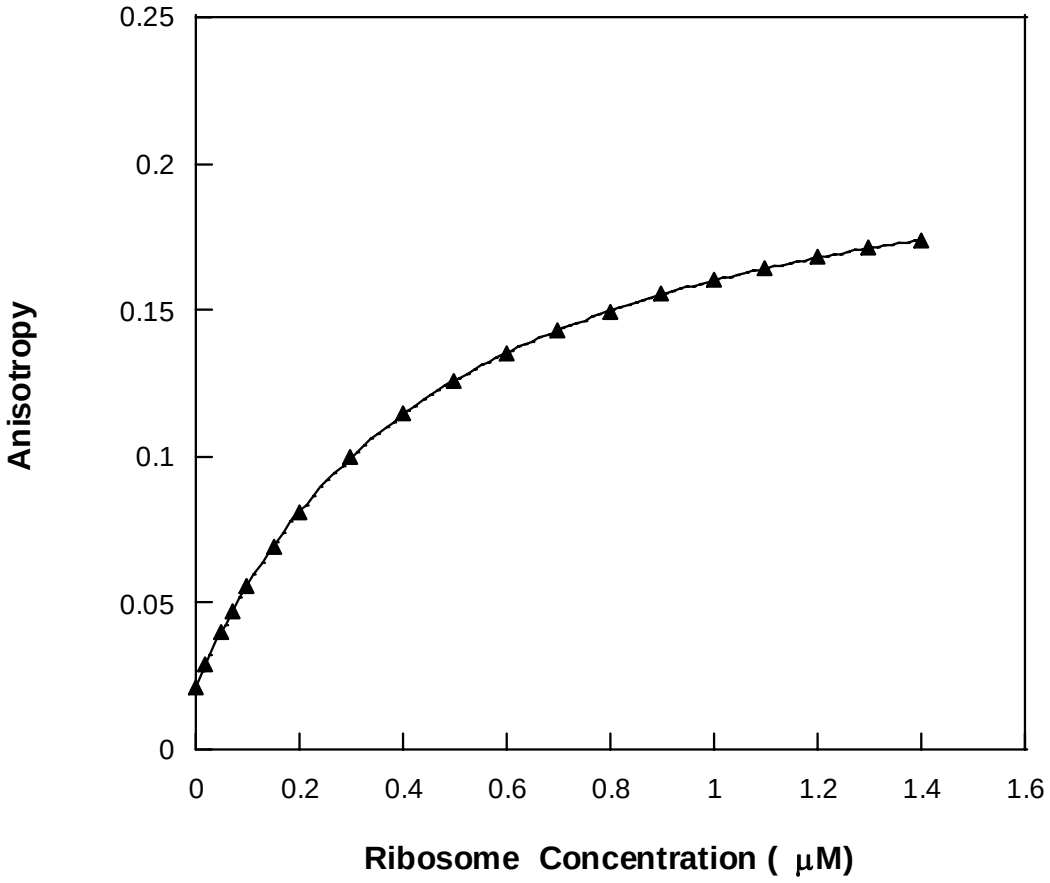


Figure 16. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S2-3 RNA in helicase reaction mixture with elF4A, elF4B and ATP. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S2-3 RNA concentration was 50 nM at 25 ^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

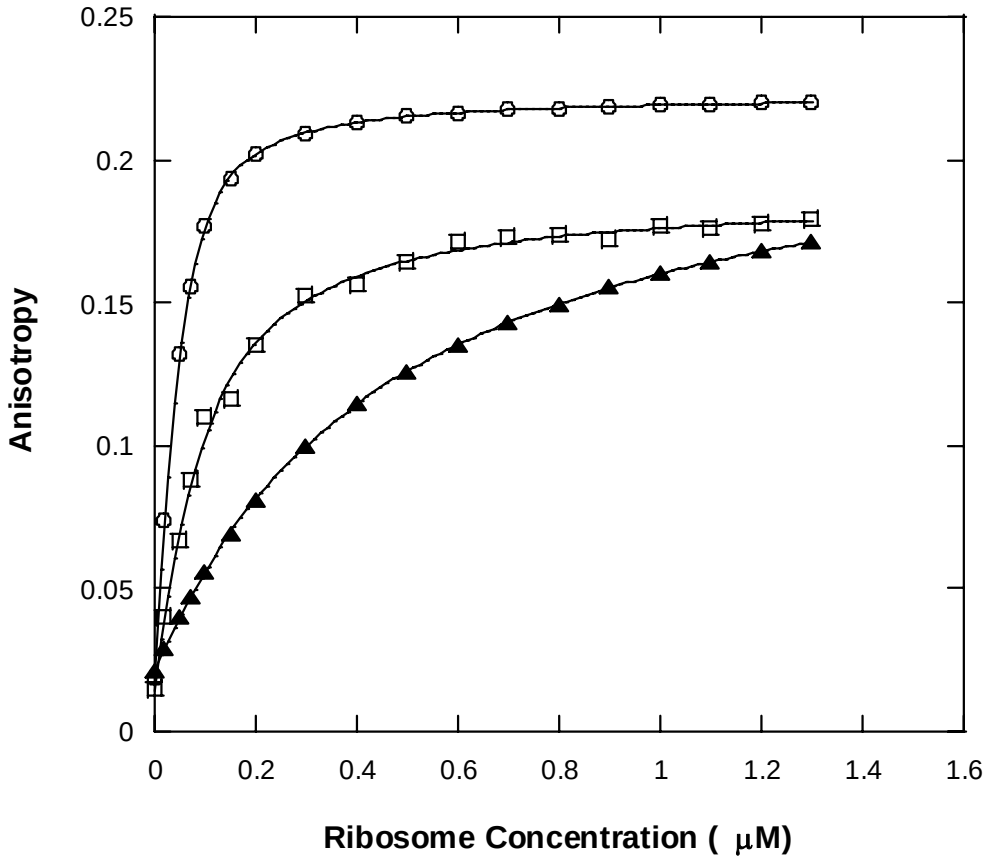


Figure 17. Fluorescence anisotropy measurements for the binding of 40S ribosome with ^{FI}PK1 RNA, ^{FI}S2-3 RNA and ^{FI}S1-3 RNA in helicase reaction mixture with elF4A, elF4B and ATP. The anisotropy values of PK1 (—O—), S2-3 (—□—) and S1-3 (—▲—) are shown. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

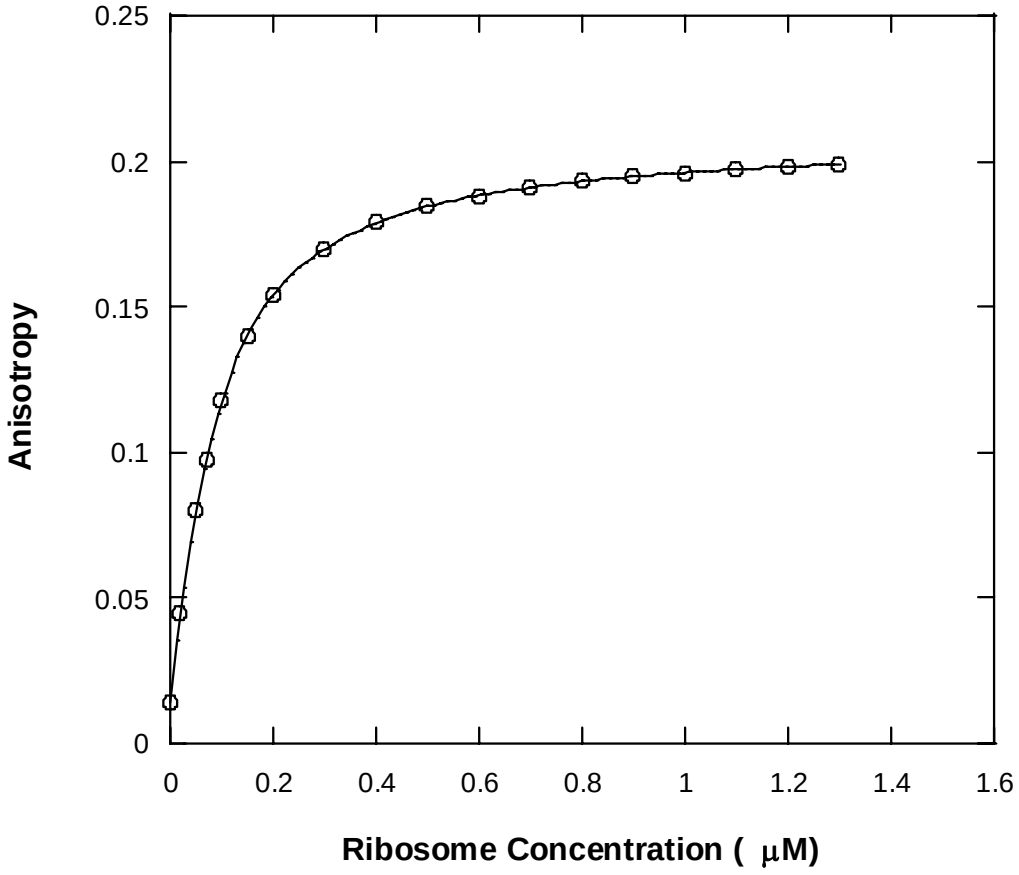


Figure 18. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled PK1 RNA in helicase reaction mixture with only elF4A, elF4B. The anisotropy value of 40S ribosome is shown. The fluorescein labeled PK1 RNA concentration was 50 nM at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

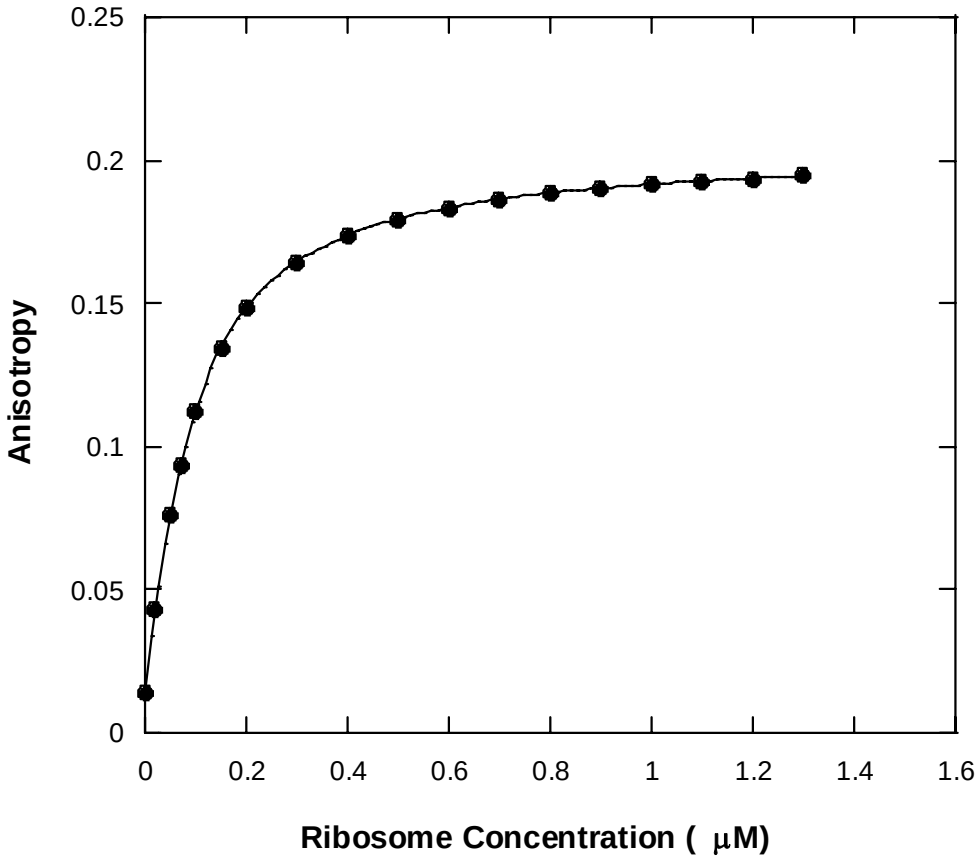


Figure 19. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled PK1 RNA in helicase reaction mixture with only ATP. The anisotropy value of 40S ribosome is shown. The fluorescein labeled PK1 RNA concentration was 50 nM at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

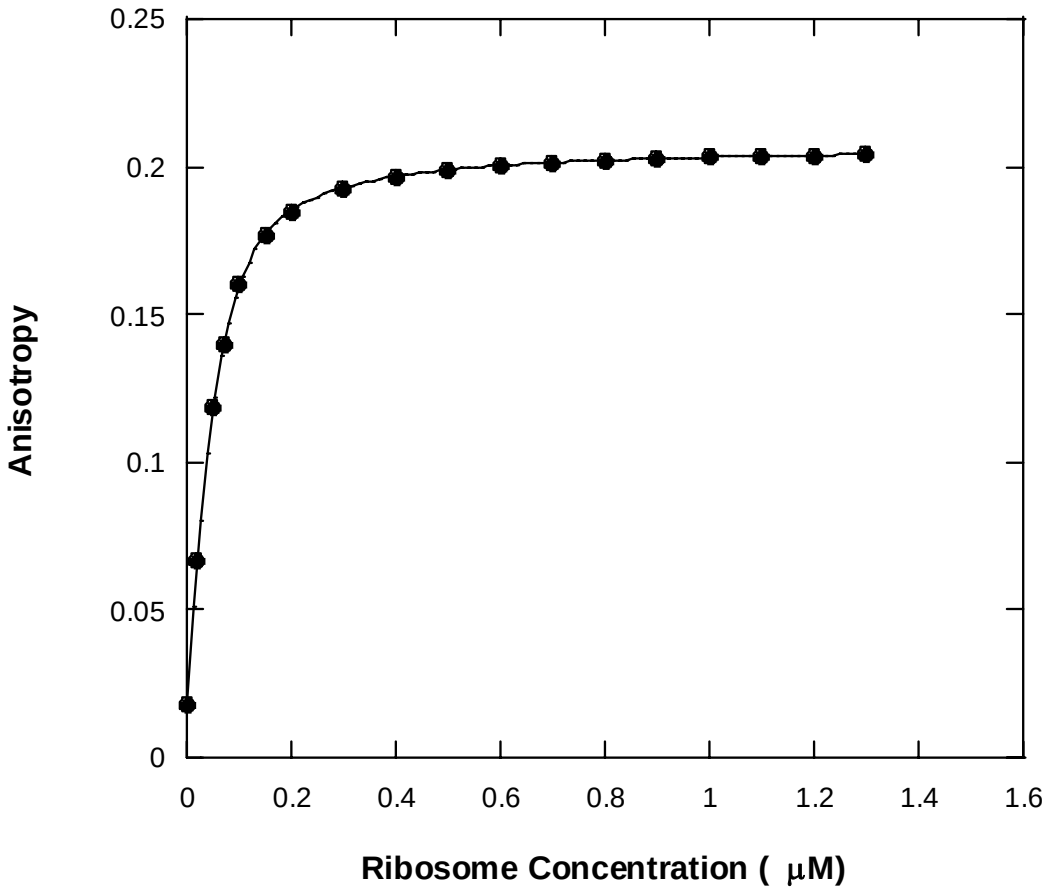


Figure 20. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled heat-melted PK1 RNA in titration buffer at 25°C. The anisotropy value of 40S ribosome is shown. The fluorescein labeled PK1 RNA concentration was 50 nM at 25°C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

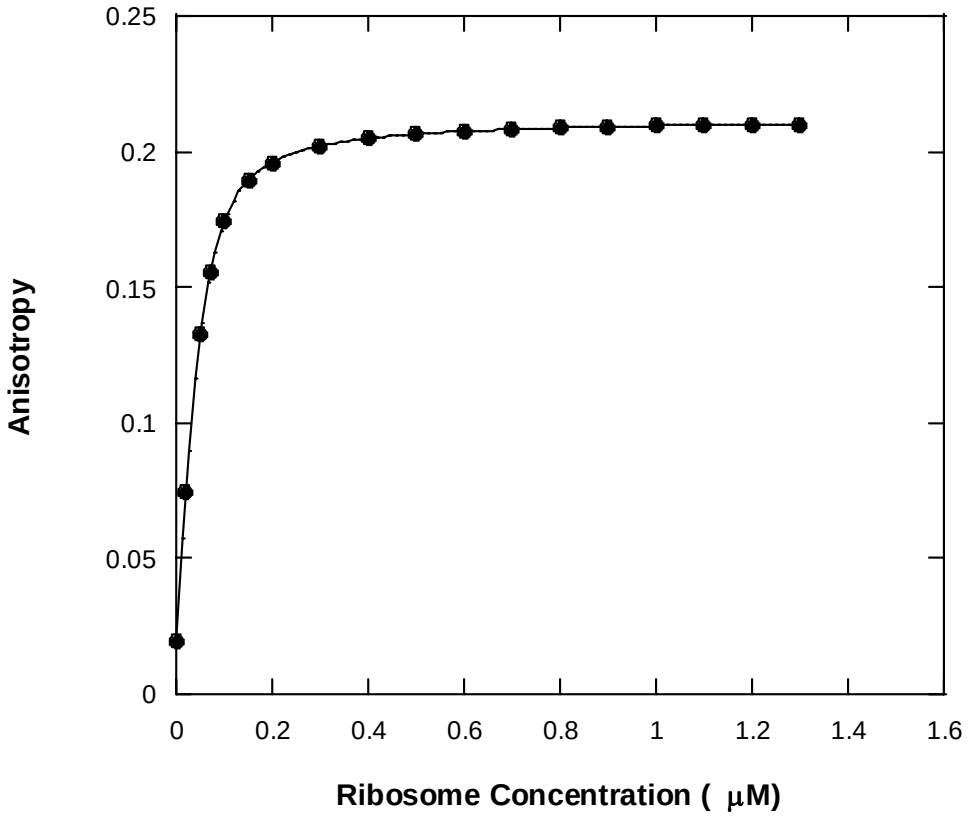


Figure 21. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled heat-melted PK1 RNA and elF4F complex. The anisotropy value of 40S ribosome is shown. The fluorescein labeled PK1 RNA concentration was 50 nM and elF4F concentration was 1500 nM in titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

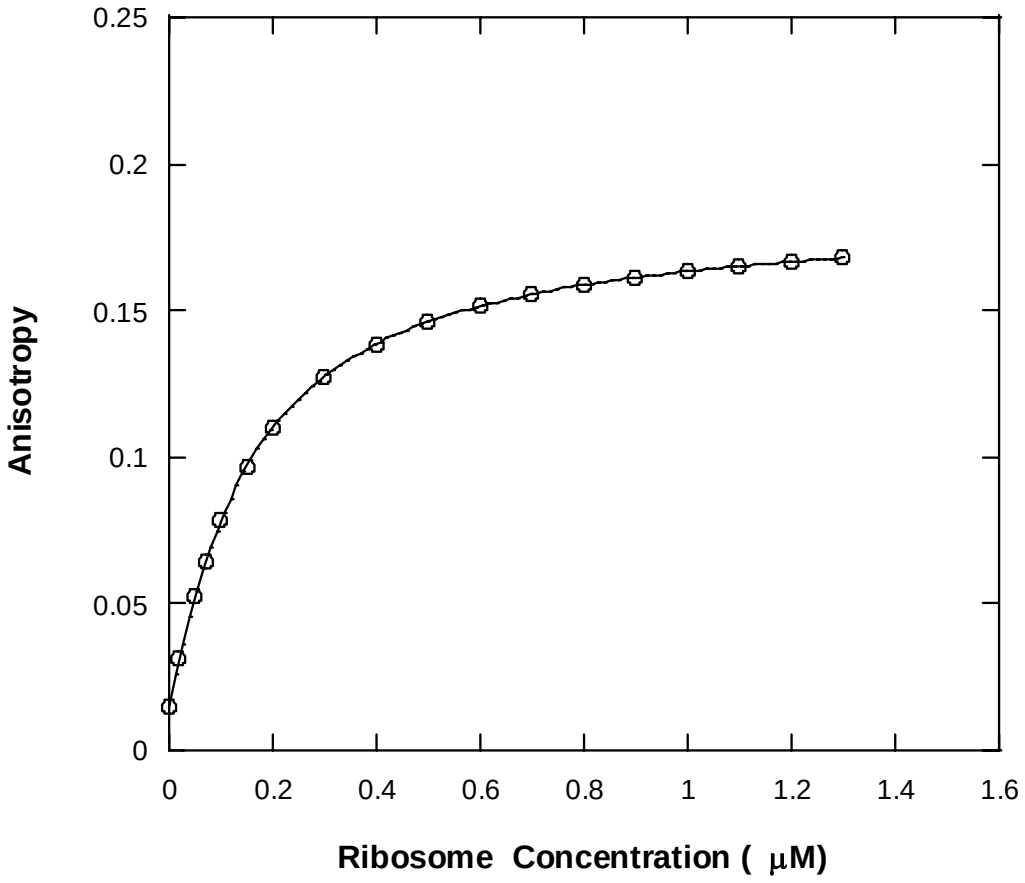


Figure 22. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S2-3 RNA in helicase reaction mixture with only elF4A, elF4B. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S2-3 RNA concentration was 50 nM at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

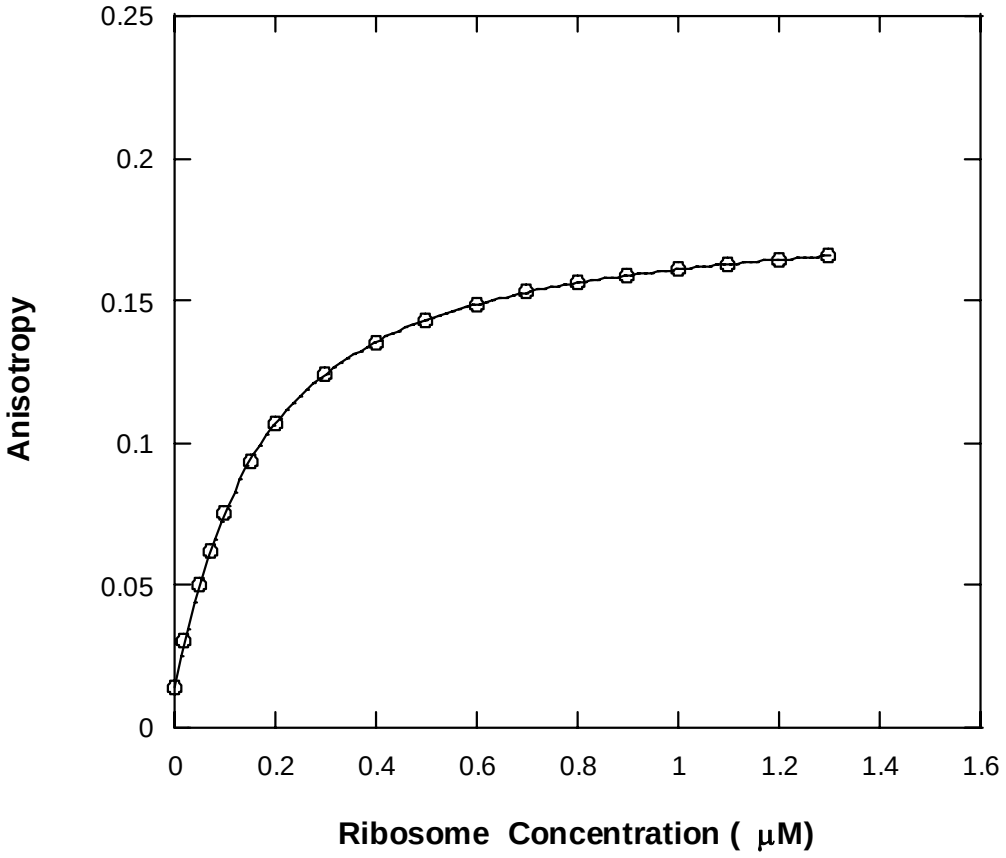


Figure 23. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S2-3 RNA in helicase reaction mixture with only ATP. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S2-3 RNA concentration was 50 nM at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

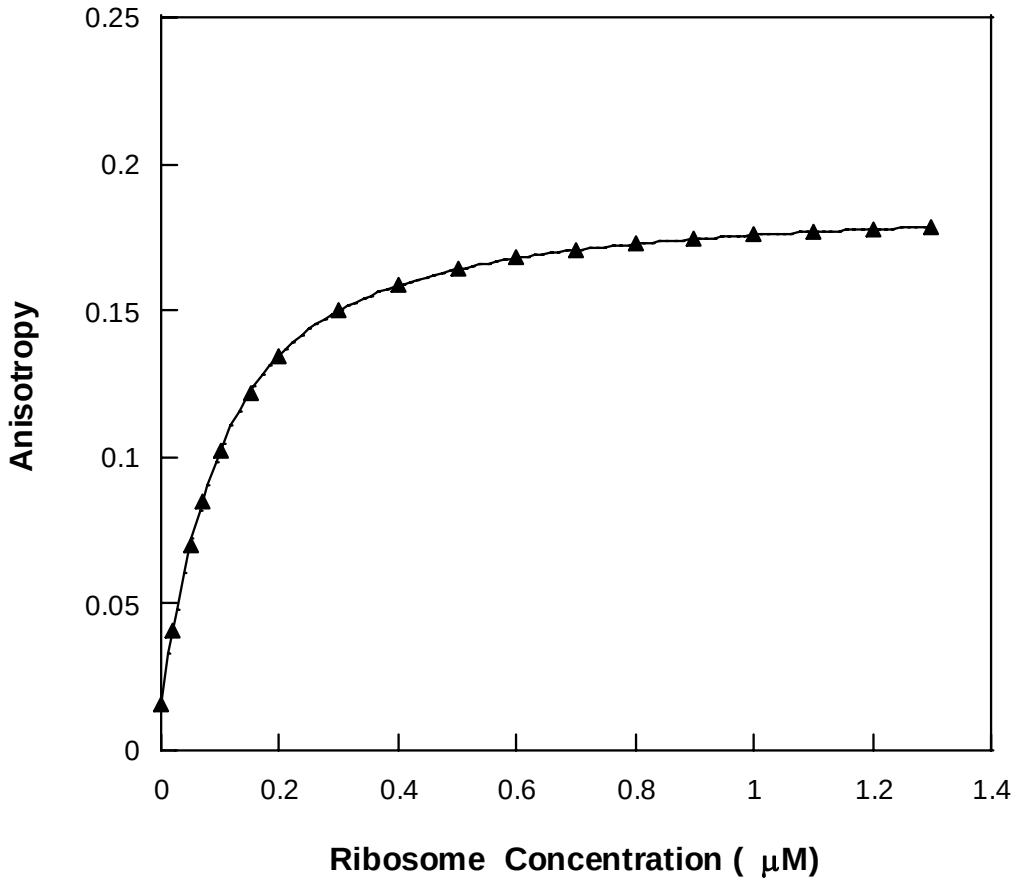


Figure 24. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled heat-melted S2-3 RNA. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S2-3 RNA concentration was 50 nM in titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

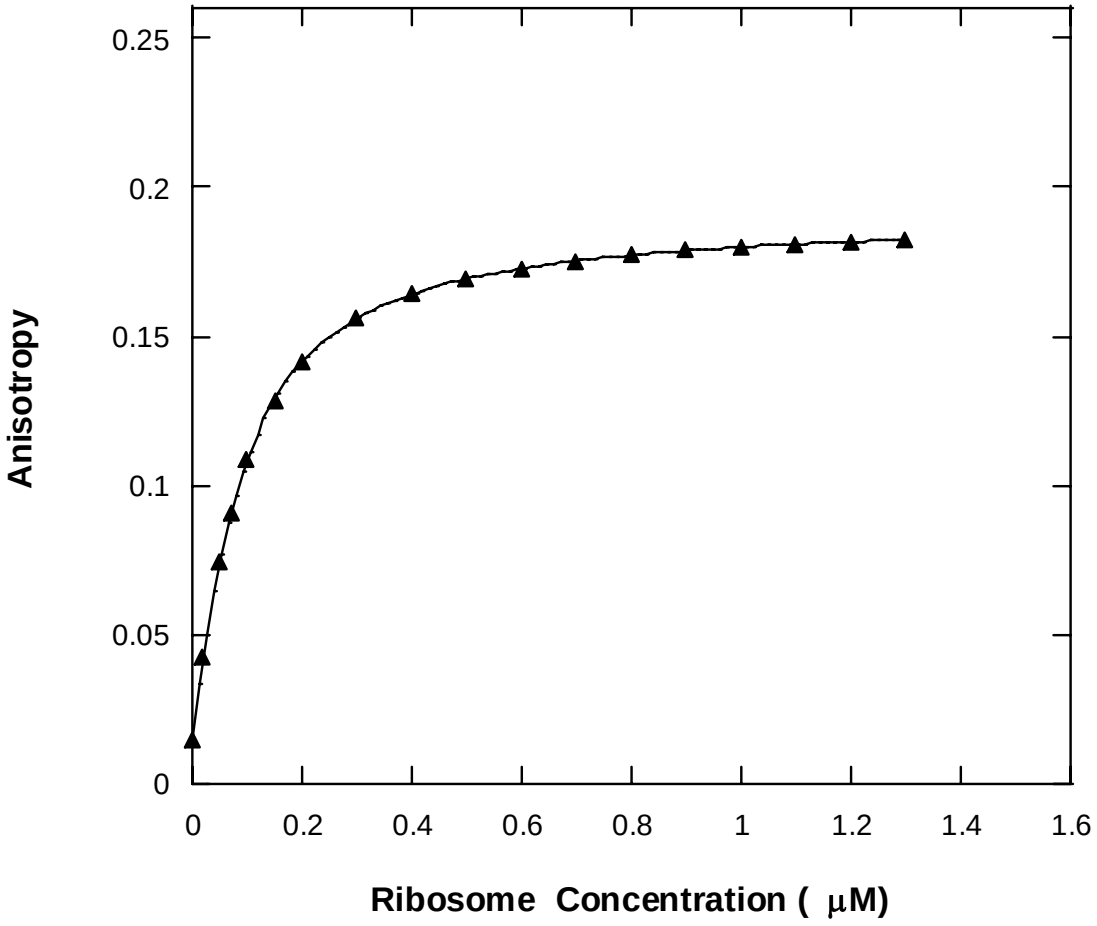


Figure 25. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled heat-melted S2-3 RNA and elF4F complex. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S2-3 RNA concentration was 50 nM and elF4F concentration was 1500 nM in titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

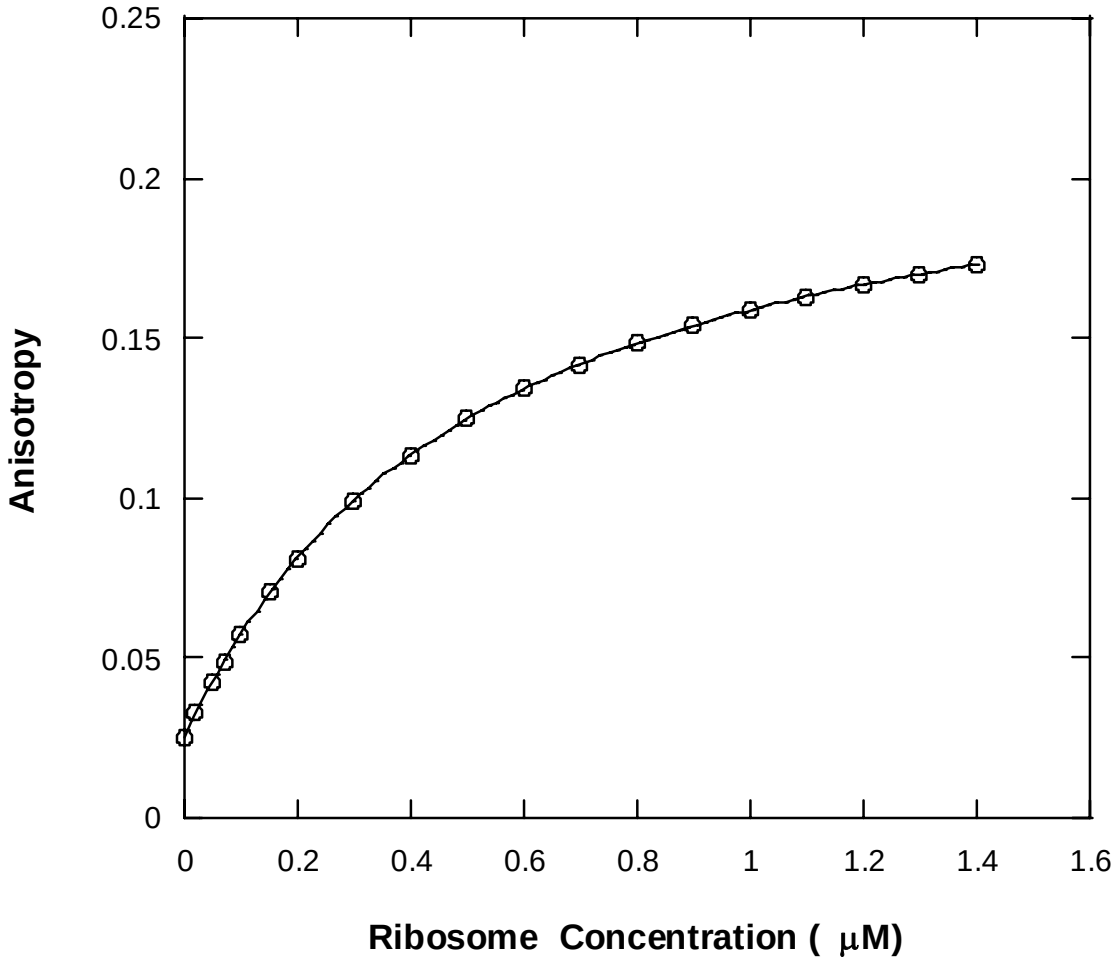


Figure 26. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S1-3 RNA in helicase reaction mixture with only elF4A, elF4B. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S1-3 RNA concentration was 50 nM at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

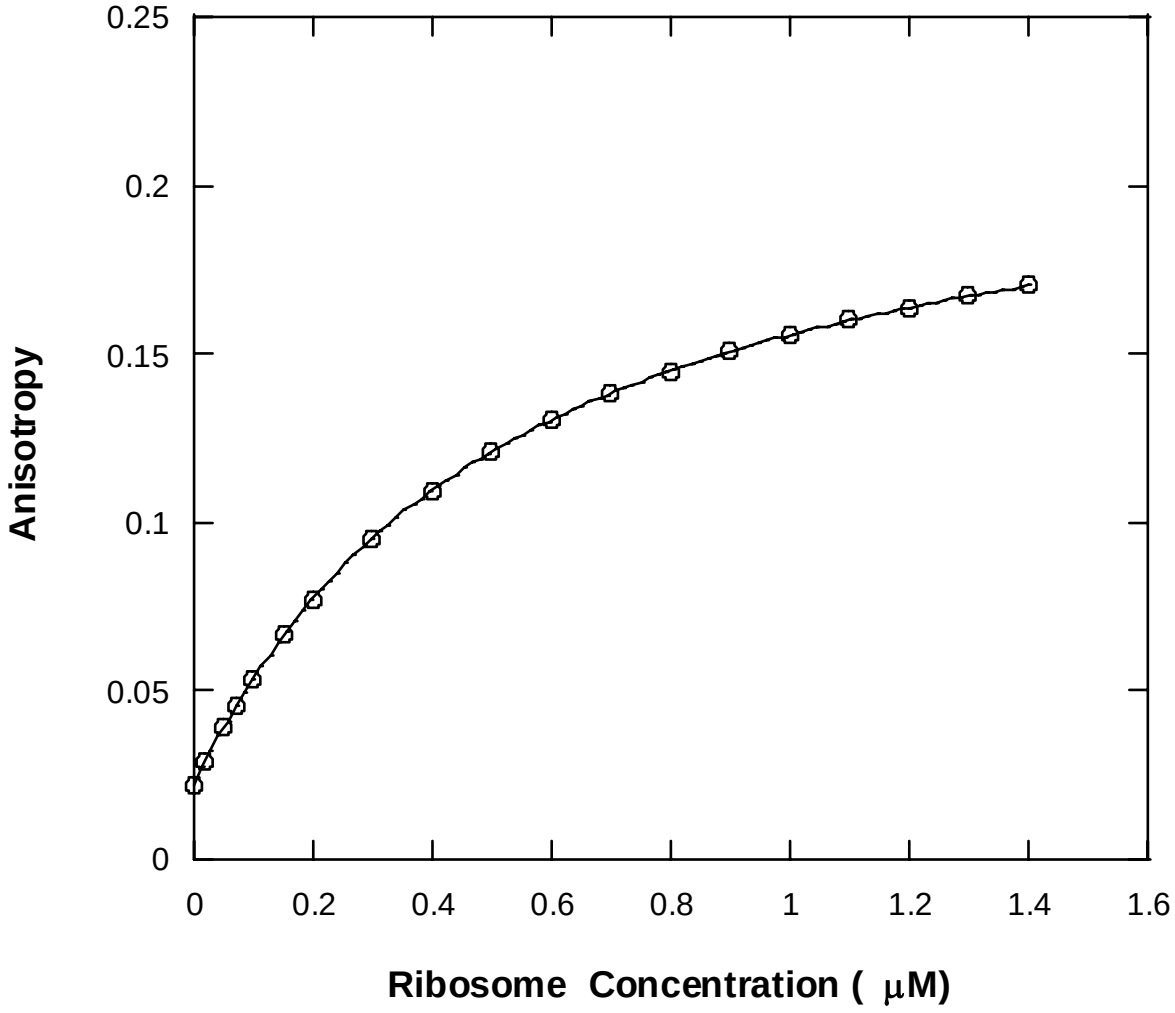


Figure 27. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled S1-3 RNA in helicase reaction mixture with only ATP. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S1-3 RNA concentration was 50 nM at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

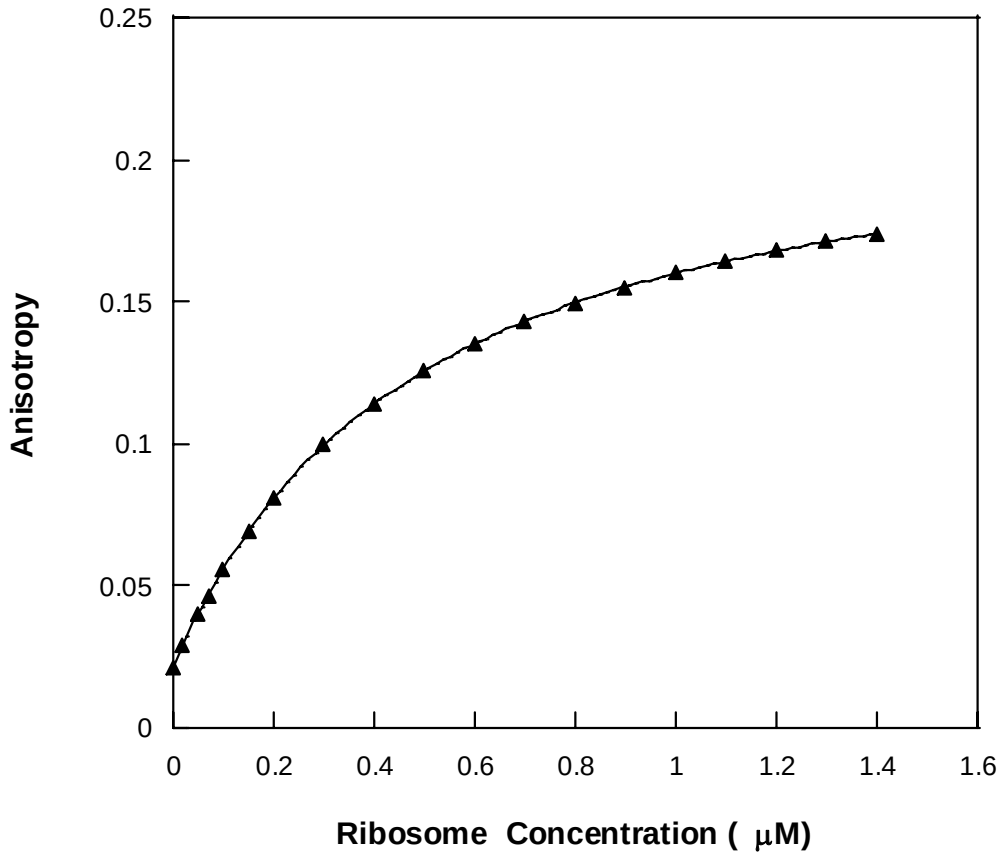


Figure 28. Fluorescence anisotropy measurements for the binding of 40S ribosome with fluorescein labeled heat-melted S1-3 RNA. The anisotropy value of 40S ribosome is shown. The fluorescein labeled S1-3 RNA concentration was 50 nM in titration buffer at 25^{o}C. The excitation and emission wavelengths were 490 nm and 519 nm, respectively.

Table 1. Dissociation Constant (K_d) for the interaction of 40S Ribosome with PK1 RNA, elF4F and helicase effect on the binding affinity and control experiments at 25 °C.

Complex	K_d (nM)
40S-PK1	67 ± 6.4
40S-PK1.4F	26± 2.3
40S-PK1 Helicase Activity	17 ± 1.6
40S-PK1 with ATP (control)	69 ± 3.4
40S-PK1.4A4B (control)	64 ± 3.1
40S-PK1 heat/melt (control)	20 ± 1.8
4F effect on heat/melt	14± 1.5

Table 2. Dissociation Constant, (K_d) for the interaction of 40S Ribosome with S2-3 RNA, elF4F and helicase effect on the binding affinity and control experiments at 25 °C.

Complex	Kd (nM)
40S-S23	138 ± 5
40S-S23.4F	83 ± 3.7
40S-S23 Helicase Activity	71 ± 4.8
40S-S23 with ATP (control)	142 ± 4.8
40S-S23.4A4B (control)	133 ± 4.5
40S-S23 heat/melt (control)	74 ± 5
4F effect on heat/melt	65± 4

Table 3. Dissociation Constant (K_d) for the interaction of 40S Ribosome with S2-3 RNA, elF4F and helicase effect on the binding affinity and control experiments at 25°C.

Complex	Kd (nM)
40S-S13	489 ± 31
40S-S13.4F	463 ± 29
40S-S13 Helicase Activity	443 ± 30
40S-S13 with ATP (control)	492 ± 28
40S-S13.4A4B (control)	476 ± 34
40S-S13 heat/melt (control)	447 ± 32

4.0 Discussion

Translation initiation starts subsequent to the assembly of an initiation complex of which the initiation codon of an mRNA and the anticodon of initiator tRNA are base-paired in the ribosomal "P" site. The mechanisms of initiation complex formation in prokaryotes and eukaryotes have similarities and significant differences. It is commonly accepted that translation initiation starts with separated ribosomal subunits.

In prokaryotes, the 30S ribosome, the small ribosomal subunit, binds mRNA and initiator tRNA in random order to form a complex. Following that step, conformational rearrangement which promotes codon–anticodon base-pairing at the P site and then joining of the large subunit, 50S, happens (86). Ribosome binding is an outcome of interactions between the 30S subunit and various recognition elements in the mRNA, such as the Shine-Dalgarno sequence (87). It does not depend on or get controlled by initiation factors. Since ribosome-binding sites can occur at any position within an mRNA, many prokaryotic mRNAs are polycistronic.

On the other hand, the 40S ribosome, the small ribosomal subunit, in eukaryotes needs a number of eukaryotic initiation factors, first to bind initiator tRNA (as a ternary complex with intiation factor eIF2 and GTP) to constitute a 43S complex and, subsequently, to bind mRNA to form a 48S complex (88). The most common mechanism for recruitment of an mRNA is mediated by a 5' cap structure (m^7GpppN, where N represents any nucleotide). This 5' cap structure functions as a molecular tag that marks the recruitment position of the 40S ribosome. This recruitment is mediated

by eIF4F (89). Because of the fact that eIF4E, the small subunit of eIF4F, specifically recognizes the 5' cap of mRNA, it is required for the start of cap-dependent translation initiation.

More than 20 years ago picornaviral mRNAs lacking a 5’ cap structure were found to be translated efficiently using highly structured internal ribosome entry site (IRES) elements (13, 14). IRESs are delineated by their ability to promote translation in the absence of 5’ cap, the binding site for elF4E. Studies showed that other RNA and DNA viruses contain IRES elements that initiate internal translation. Moreover, some cellular mRNAs have been found to possess IRES elements (18). mRNAs encoding translation initiation factors, transcription factors, oncogenes, growth factors, homeotic genes, survival proteins, and proteins involved in cell cycle progression and stress response are among the cellular mRNAs possessing an IRES element in mammalian systems (18). It is important to understand IRES regulated translation initiation in more detail.

Studies with Encephalomyocarditis virus (EMCV), which is ~450 nucleotides long, highly structured, and lies immediately upstream of the initiation codon, showed that translation initiation is ATP-dependent and uses the same set of canonical initiation factors as cap-mediated initiation (2,90). However, since it does not have a cap structure, there is no interaction between eIF4E and an m^7G cap. Instead, binding of the 43S complex to the EMCV IRES requires a peculiar cap-independent interaction between the eIF4G subunit of eIF4F and the IRES.

Hepatitis C virus (HCV) and pestiviruses such as classical swine fever virus (CSFV) and bovine viral diarrhea virus are another examples for IRES regulated

translation initiation. However, a number of properties differentiate HCV-like IRESs from EMCV-like IRESs and there were striking similarities between the mechanism of initiation on these viral IRESs and initiation on prokaryotic mRNAs. For example, they are ~100 nucleotides shorter than EMCV-like IRESs and include up to 30 nucleotides of coding sequence as well as functionally critical structures, such as a pseudoknot upstream of the initiation codon that are not found in the EMCV IRES. Studies (91-95) surprisingly showed that the mechanism of initiation regulated by HCV and CSFV IRESs diverged significantly from both cap- and EMCV IRES-regulated initiation. Interactions between 40S ribosome and HCV and CSFV IRESs revealed assembly of stable binary complexes. Initiation factors eIF4A, eIF4B, and eIF4F were not required for 40S binding to these mRNAs. 48S complexes formed in the absence of eIF4A, eIF4B, and eIF4F were competent to form 80S complexes active in methionylpuromycin synthesis. Moreover, translation of HCV and CSFV mRNAs differed from cap- and EMCV IRES-dependent mRNAs in that it required only Met–$tRNA_i^{Met}$, eIF2, and GTP to form 48S complexes.

While ribosome binding to eukaryotic mRNAs requires the participation of eukaryotic initiation factors eIF-4A, eIF-4B, and eIF-4F and the hydrolysis of ATP , similar to HCV, our results suggest that the 40S ribosomal subunit, the largest components of the 43S particle, binds directly to the PK1 RNA with dissociation constant (Kd) of 67 nM. To explore the specificity of the IRES RNA•40S interaction, we measured the affinity of the 40S subunit for TEV IRES RNA mutants, S2-3 RNA and to S1-3 RNA that have been previously characterized in terms of in vitro

translation initiation activity. S2-3 RNA shows about 30 % translation activity while S1-3 RNA shows about 7 % translation activity. Direct binding measurements of both wild type and mutant TEV IRES RNAs suggest that recruitment of the 43S particle to the TEV message is driven through the high affinity TEV RNA•40S interaction , involving a novel strategy of ribosome recruitment distinct from eukaryotic cap-dependent mechanisms.

Effect of the plant protein synthesis initiation factor eIF4F on binding interactions of 40S Ribosome with RNAs was also studied. The fluorescence anisotropy data showed that eIF4F increases 40S Ribosome binding affinity to PK1 RNA approximately 2.6 fold. This also suggests that the IRES RNA may act as a structural scaffold in which specifically placed recognition sites recruit the translational machinery.

eIF4A, a member of the DEA(D/H)-box RNA helicase family, unwinds secondary structures in the the 5` untranslated (5` UTR) region thereby facilitating scanning of the 40S subunit for the initiation codon. eIF4A itself does not have a strong ATPase activity and needs to be stimulated by eIF4G and eIF4B (51). The studies from our lab showed that eIF4B increases the ATP binding affinity of eIF4A and the ATP-dependent helicase activity (76, 83). To obtain direct evidence for initiation factor mediated RNA unwinding in cap independent initiation, we determined RNA helicase activity of 40S ribosome with TEV RNAs. The fluorescence anisotropy data revealed that in the presence of eIF4A, eIF4B, and hydrolysis of ATP, binding affinity of 40S ribosome to PK1 RNA increases 4 fold with K_d of 17 nM.

It has previously been shown (35) that 10 nucleotide sequence inserts that were complementary to plant 18S ribosomal showed efficient binding affinity to 40S ribosomal subunits and translated three times more efficiently than the same RNA without the inserts. It was observed that the insert that showed the highest ribosome binding and the greatest enhancement of translation was complementary to regions 1115-1124 of the plant ribosomal RNA. A sequence complementary to 18S nt 1123-1113 is included in L3 of TEV PK1 RNA (nt 61-67). Besides, if stem S1 is unwound additional three base pairs in the 5' side of L3 can base pair with 18S RNA. Similarly, if S2 is unwound, three additional base pairs could take place. These additional interactions may give an explanation for the high affinity of wt PK1 compared to the two mutants, S1-3 and S2-3. Further, this also a potential explanation for the increased affinity for the unwound RNA, either heat denatured or through helicase reaction. The S1-3 mutant loses three potential pase pairs on the 5' side of L3, which greatly reduces the ribosome binding affinity (and translation). The S2-3 mutant, however retains the 5' potential base pairs, but lacks 3' base pairing, implying that the 5' sequence is more important for ribosome binding. However, increasing the size of the L3 loop to extend complementarities with 18S RNA does not increase translation (1) and actually reduces the translational efficiency suggesting PK1 has the optimal base pairing.

Previous work had shown that the TEV 5' leader particularly required elF4G to promote cap-independent translation and that elF4A, elF4B and PABP further increased translation and binding. Here, we demonstrate that 40S ribosomal subunits are capable of high affinity binding in the absence of elFs, but that elF4F specifically increases binding for PK1 and S2-3 RNA, but not S1-3 RNA. These results suggest that

elF4F may interact with the upstream side of L3 or that it may have a part in stabilizing some needed tertiary structure of the RNA. elF4A and elF4B do not directly enhance ribosome binding, but when ATP is added to promote helicase activity or the RNA is thermally unwound, binding is enhanced.

The S1-3 mutant, although it has significant ribosome binding is translated very poorly. This mutant also has reduced elF4F binding. These results suggest that while complementarities of the IRES with 18S RNA are a novel recruitment strategy for 40S ribosomal subunits, other elements of the IRES are also crucial. The extents to which other factors and overall RNA structure influence ribosome binding present a testable model for future studies.

Because of the fact that viral protein synthesis is simpler than host cell protein synthesis, the results and information provided by this research has potential use to develop systems to produce desired proteins that are nutritionally beneficial and have other economic uses. In addition, it has potential to highlight the variety of strategies developed by viruses to perplex host protein synthesis so as to prefer synthesis of viral proteins. These evasions of host protein synthesis are now driving a number of fascinating studies demonstrating that translation involves complex but balanced interplay between the host cell translation machinery, the viral mRNA, and the viral proteins resulting from expression of the viral genome. Future studies will undoubtedly shed light on other new avenues in this fascinating and complex aspect of cell development.

APPENDIX

1.1 Cell media

1L Luria-Bertani (LB) media

Yeast Extract	**5g**
Peptone	**10g**
NaCl	**10g**
ddH_2O	**1000ml**

1.2 Buffers for Electrophoresis

Agarose Gel for DNA

50xTAE buffer pH 8.0

Tris Base	**242g**
EDTA	**18.6g**
Glacial Acetic Acid	**57.1g**
ddH_2O	**to 1000ml**

6x DNA sample loading buffer

Bromophenol Blue	**0.5ml**
1.0% Glycerol	**4.0ml**
50x TAE buffer	**0.2ml**
ddH_2O	**to 10 ml**

Agarose Gel for RNA

50xTAE buffer pH 8.0

5x MOPS buffer pH.8.0

0.2M MOPS	**10.5 g**
50 mM Sodium Acetate	**4.1 ml**
5 mM EDTA	**5 ml**
DEPC water	**to 250 ml**

RNA loading buffer

0.4% bromophenol blue	**0.02g**
1mg/ml ethidium bromide	**0.5ml**
1 mM EDTA	**0.02ml**
50% Glycerol	**2.5 ml**
DEPC water	**to 5 ml**

RNA sample buffer

deionized formamide	**10ml**
37% formaldehyde	**3.5ml**
5X MOPS buffer	**2ml**

1.3 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving Gel

Reagent	**Volume to make 5 ml**	**Volume to make 10ml**
30% Acrylamide stock*	2.0ml	4.0ml
ddH_2O	1.5ml	3.2ml
1M tris-HCl pH8.8	1.3ml	2.5ml
10%SDS	0.1ml	0.2ml
Ammonium Persulphate	0.1ml	0.1ml
10%TEMED (added last)	1.5µl	1.5µl

Stacking Gel

Reagent	**Volume to make 5ml**	**Volume to make 10 ml**
30% Acrylamide stock*	0.83ml	1.66ml
ddH_2O	2.72ml	5.54ml
0.5M Tris-HCl pH8.8	1.25ml	2.5ml
10%SDS	0.1ml	0.2ml
Ammonium Persulphate	0.1ml	0.1ml
10%TEMED (added last)	5µl	5µl

10x Tank buffer pH 8.8

SDS	**10g**
Glycine	**144.40g**
ddH_2O	**to 1000ml**

6x Protein Sample Loading Buffer

4x Stacking Gel buffer	**1.56ml**
SDS	**1.0g**
Glycerol	**5.0ml**
Bromphenol blue	**0.5ml**
1.0% β-mercaptoethanol	**2.5ml**
ddH_2O	**to 10 ml**

1.4 Determining the protein concentration by Bradford Method

Volume of the BSA	Volume of Diluent	Final BSA Concentration
300µl of (stock)	0µl	2,000µg/ml
375µl of (stock)	125µl	1,500µg/ml (A)
325µl of (stock)	325µl	1,000µg/ml (B)
175µl of (A)	175µl	750µg/ml (C)
325µl of (B)	325µl	500µg/ml (D)
325µl of (D)	325µl	250µg/ml (E)
325µl of (E)	325µl	125µg/ml (F)
100µl of (F)	400µl	25µg/ml (G)

1.5 Isolation of 40S ribosome from wheat germ

- Grind 120 g of wheat germ with 120 g of powdered alumina in a cold mortar.
- Mix the ground wheat germ with 300 ml of extraction buffer (20 mM HEPES-KOH, pH 7.6, containing 100 mM KCl, 1 mM $(MgOAc)_2$, 2 mM $CaCl_2$, and 5 mM 2-mercaptoethanol) and centrifuge for 10 min at 15,000g.
- Apply the supernatant (about 150 ml containing 450-550 A_{260} units/ml) to a 1.2-liter Sephadex G-25 column equilibrated in Buffer A (20 mM HEPES-KOH, pH 7.6, 5 mM $Mg(OAc)_2$, 6 mM 2-mercaptoethanol, and 10% glycerol) containing 120 mM KC1 and develop the column with the same buffer.
- Pool fractions with greater than 90 A_{260} units/ml and centrifuge for 20 min at 25,000g.
- Centrifuge the supernatant from this centrifugation (about 200-250 ml containing approximately 150 A_{260} units/ml) for 3 h at 170,000g.
- Suspend the ribosomes in Buffer A containing 120 mM KCl at a concentration of about 45 mg/ml (500 A_{260} units/ml).
- Dilute the ribosomes (45 mg/ml) with an equal volume of Buffer A containing 1.2 M KCl to give a final KCl concentration of about 0.6 M.
- Allow the ribosomes to sit in high salt for 30 min in an ice bath and then centrifuge for 5 h at 150,000g.
- Suspend the ribosomes in Buffer A containing 50 mM KCl and store in small aliquots at a concentration of 45 mg/ml at -70^{o}C.
- Dilute 3.5 ml ribosomes (containing 45 mg/ml) with 35 ml of room temperature buffer I (50 mM Tris-HCl, pH 7.7, 2 mM dithiothreitol, 0.1 mM EDTA, 0.6 M KCl, 3 mM $MgCl_2$, and 5% sucrose by weight) and incubate at 30°C for 5 min.

- Separate the ribosomal subunits by centrifugation through a linear l0-30% sucrose gradient buffer I for 4 h at 150,000g at 10°C.
- Collect 15ml fractions and pool those fractions with the highest absorbance at 260 nm in the 40S and 60 S regions of the gradient.
- Dialyze the pooled fractions for 4 h against 1 liter of buffer II containing 50 mM Tris-HCl, pH 7.7,5 mM MCl_2,, 50 mM KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol
- Collect the 40S ribosomal subunit by centrifugation for about 12 h at 113,000g.
- Suspend the 40S ribosomal subunit at a concentration of approximately 80 A_{260} units/ml in Buffer A containing 50 mM KCl, divide into 0.05-ml aliquots, fast froze in dry ice-acetone, and store at -70°C.

Buffer E (Extraction Buffer) pH 7.6

20 mM HEPES-KOH	**1.19 g**
1 mM $Mg(OAc)_2$	**0.0536 g**
2 mM $CaCl_2$	**0.0735 g**
6 mM DTT	**0.15 ml**
120 mM KCl	**2.237 g**
ddH_2O	**to 250 ml**

Buffer A pH 7.6

20 mM HEPES-KOH	**4.76 g**
5 mM $Mg(OAc)_2$	**1.07 g**
10% Glycerol	**100 ml**
6 mM DTT	**0.6 ml**
120 mM KCl	**8.95 g**
ddH_2O	**to 1000 ml**

Buffer I pH 7.7

50 mM Tris-HCl	**3 g**
0.1 mM EDTA	**0.2 ml**
3 mM $MgCl_2$	**0.3 g**
2 mM DTT	**0.2 ml**
0.6 M KCl	**22.37 g**
5% Sucrose	**25 g**
ddH_2O	**to 500 ml**

Buffer II pH 7.7

50 mM Tris-HCl	**6 g**
0.1 mM EDTA	**0.4 ml**
5 mM $MgCl_2$	**1 g**
1 mM DTT	**1 ml**
50 mM KCl	**3.73 g**
10% Glycerol	**100 ml**
ddH_2O	**to 1000 ml**

1.6 Buffers for wheat germ isolated elF4F

Buffer E (Extraction Buffer) pH 7.6

Buffer A pH 7.6

Buffer B pH 7.6

20 mM HEPES-KOH	**2.4 g**
0.1 mM EDTA	**0.4 ml**
1 mM DTT	**1 ml**
40 mM KCl	**3 g**
10% Glycerol	**100 ml**
ddH_2O	**to 1000 ml**

1.7 Luciferase Activity Assay Buffer

25 mM Tricine pH. 8	**1 ml**
0.1 mM EDTA	**0.008 ml**
5 mM $MgCl_2$	**0.2 ml**
1 mM coenzyme A	**5 ml**
10 mM ATP	**5 ml**
ddH_2O	**to 20 ml**

1.8 Helicase Activity Buffer

20 mM Tris-HCl pH 7.5	**2 ml**
1 mM MgOAc	**0.022 g**
2 mM DTT	**0.1 ml**
70 mM KCl	**0.52 g**
2u/µl RNAse inhibitor RNAsin	**4 ml**
ddH_2O	**to 100 ml**

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