CHAPTER III

INTERFERON-γ (IFN-γ) mRNA TRANSLATION IN WHEAT GERM LYSAE AND eIF2 BINDING IN VITRO
Ben Asouli et al (2002) from Dr. Kaempfer's laboratory in Hebrew University, Jerusalem, Israel have shown recently that wild type full length IFN-γ mRNA form (1264nt) activates PKR efficiently and facilitates eIF2α phosphorylation as compared to the truncated forms (469 and 203 nt) of IFN-γ mRNA in BHK-21 cells and in reticulocyte lysates. Further, they observed that the 5' terminal 203nt IFN-γ mRNA fragment is sufficient to activate PKR at least as efficiently as dsRNA. The activation of PKR is achieved by a pseudoknot present in the 5' terminal region of IFN-γ mRNA. Mutations that impair the pseudoknot stability reduce the ability to activate PKR and strongly increase translation efficiency of IFN-γ mRNA. Apparently, this is the first example of an mRNA that limits its own translation by activating PKR.

This laboratory is collaborating with Prof. Kaempfer's laboratory on certain aspects of eIF2 phosphorylation and I had a chance to work in his laboratory during August, 2000 for a month. I carried out some preliminary experiments during this period to determine the translational ability of wt and mutant forms of IFN-γ mRNAs in wheat germ lysate, their ability to activate wheat germ lysate PKR like activity (if any) and interaction with wheat germ eIF2 in vitro (by gel shift analysis).

*Translation of wt and mutant forms of IFN-γ mRNA in wheat germ lysates:* Wt IFN-γ mRNA and its mutants a4, d1, d2, d3 and d4 that are created by various substitutions or deletions in the 5’ UTR as described by Ben Asouli et al (2002) (the sequence information of these mRNAs is shown in Fig 28, Panel B) were translated in wheat germ lysates using labeled methionine. The labeled protein products were analyzed by autoradiography (Fig. 28, Panel A). Translation of wt IFN-γ mRNA is found to be relatively poor compared to the mutant forms of IFN-γ mRNA (compare lane 3 vs. 4-8). A control reaction containing luciferase mRNA translation is shown in lane 2. In the absence of added mRNA, no translation is observed in wheat germ lysates (lane 1). These results are essentially comparable to what has been observed by Kaempfer's group in cell-free translational systems derived from reticulocyte lysates.
Fig. 28. Translation of IFN-γ mRNAs in wheat germ lysate.

Panel A: Standard lysate protein synthesis assays (25 µl) were carried out in wheat germ lysate in the presence of IFN-γ mRNAs, wild type (wt) and mutants (a4, d1, d2, d3 and d4) (15 µg/ml) corresponding to the lanes 3 – 8 respectively using [35S] Methionine, at 25°C for 20 min. The reaction mixtures were terminated with the addition of SDS sample buffer and the proteins were separated by 10% SDS-PAGE as described in ‘Materials and Methods’. The figure is an autoradiogram.

Lanes: Mr, molecular weight marker; 1, WGL; 2, WGL + luciferase mRNA; 3, WGL + wt IFN-γ mRNA; 4, WGL + a4 IFN-γ mRNA; 5, WGL + d1 IFN-γ mRNA; 6, WGL + d2 IFN-γ mRNA; 7, WGL + d3 IFN-γ mRNA; 8, WGL + d4 IFN-γ mRNA.

Panel B: Sequence information of wt IFN-γ mRNA on its 5' UTR, a4 mutant and the deletions made in d1, d2, d3 and d4 mutants (from Dr. Kaempfer’s laboratory, Israel).
Wheat germ eIF2 phosphorylation in translating lysates: Since the reduced translation of IFN-γ mRNA is correlated to increased PKR activation and eIF2α phosphorylation in reticulocyte lysates (Ben Aouli et al, 2002), we have also tested the ability of these RNAs to activate wheat germ lysate PKR (if any). The activation of PKR was monitored by eIF2α phosphorylation. However, addition of these RNAs (wt or mutants) has not increased eIF2α phosphorylation in translating wheat germ lysates (data not shown). This may be because the wheat germ lysates may not have a potent PKR-like kinase. This is consistent with many of our earlier observations wherein we failed to detect any stimulation in wheat germ lysate PKR activity or eIF2α phosphorylation in the presence of double stranded RNA. Hence we think that the requirement of PKR inactivation for augmented translation of the mutant forms of IFN-γ mRNAs is not obligatory in wheat germ system.

eIF2-mRNA binding studies: Because eIF2 from yeast system has been shown to promote AUG initiation codon recognition (Yoon and Donahue, 1992; Pestova et al, 1998) and affinity of an mRNA for eIF2 is found correlating with its ability to compete in translation in reticulocyte lysates (Rosen et al, 1982; Ben-Asouli et al, 2000), we have also evaluated the ability of IFN-γ mRNA binding to purified wheat germ eIF2 in vitro. In these experiments, 5'-terminal 203 nt IFN-γ mRNA fragment (203 nt RNA) (which has been shown to act as efficiently as dsRNA to stimulate PKR by Ben-Asouli et al, 2002) was used. The RNA transcript was labeled using [α-32P] UTP during its transcription. Uniformly labeled 203 nt mRNA was incubated with increasing amounts of purified wheat germ eIF2 and the complexes were separated on 4% polyacrylamide gel and analyzed by autoradiography (Fig. 29). The migration of 203 nt mRNA is retarded with increasing concentrations of wheat germ eIF2. This finding suggests that wheat germ eIF2 forms a complex with IFN-γ mRNA. After establishing this point, further studies are carried out to identify the affinity between wheat germ eIF2 and different forms of IFN-γ mRNA. In these assays, the complex formation between labeled 203 nt IFN-γ mRNA and wheat germ eIF2 was studied in the absence and presence of unlabelled wt and mutants (d1-d4 and a4) of IFN-γ mRNAs. The replacement of labeled mRNA by unlabeled competitor mRNA suggests that the competitor mRNA has a higher affinity for eIF2. Our observations (Fig. 30)
Fig. 29. Binding of wheat germ eIF2 (WG eIF2) to mRNA (human IFN-γ mRNA 5’ – terminal 203 nt)

Uniformly [\textsuperscript{32}P] labeled human IFN-γ mRNA 5’ – terminal 203 nt transcript (0.08 pmol, 1.25 x 105 cpm/pmol) was incubated with out eIF2 (lane 1) or with wheat germ eIF2 (50, 100, 200, 400, 700 and 1000 ng corresponding to lanes 2 to 7 respectively). The reaction mixture was subjected to electrophoresis on a native gel to separate free human IFN-γ mRNA 5’ – terminal 203 nt from labeled RNA bound to wheat germ eIF2. The figure is an autoradiogram.
Fig. 30. Electrophoretic mobility shift assay using labeled human IFN-γ mRNA 5'-terminal 203 nt and wheat germ eIF2 in the presence and absence of unlabeled wild type and mutants of IFN-γ mRNAs.

Uniformly $^{32}$P labeled human IFN-γ mRNA 5'-terminal 203 nt transcript (0.08 pmol, 1.25 x 105 cpm/pmol) was incubated with out eIF2 or with 0.3 pico moles of wheat germ eIF2 in the absence or presence of unlabeled wild type (wt) and mutants (a4, d1, d2, d3, and d4 of IFN-γ mRNAs (0.005, 0.01, 0.02 and 0.05 pmol) (comp. RNA). The autoradiogram shows free and bound RNA. Two controls were used. One control was carried out in the presence of BSA (served as a protein control) and the other one is carried out in the absence of the competitor RNA. The samples were subjected to electrophoresis on a native gel to separate free RNA from bound RNAs. The figure is an autoradiogram.
indicate that the labeled complex formation is reduced efficiently in the presence of low concentrations of (0.01-0.05 p mol) of wt, d3 and a4 IFN-γ mRNAs. However, higher concentration of d1, d2 and d4 mRNAs are required to replace the labeled 203nt mRNA. These results suggest that the deletion mutants (d1, d2 and d4) have reduced affinity for eIF2 as compared to wt and the importance of the deleted regions in eIF2 binding.

These results on the binding between wheat germ eIF2 and different IFN-γ mRNA forms are essentially similar, except for d3 mutant mRNA, to what has been observed by Kaempfer’s group with reticulocyte eIF2. This suggests that plant and mammalian eIF2 discriminate different mRNA species essentially in the same manner.

These preliminary data support the idea that plant eIF2 behaves like mammalian eIF2 in its binding to mRNA but the physiological significance of this eIF2-mRNA interaction on the activation of PKR-like kinase or translational ability of mRNAs in plant systems has yet to be understood.