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AN INVESTIGATION ON THE EFFECTS OF INFLUENZA VIRUS INFECTION AS IT PERTAINS TO THE INITIATION OF TRANSLATION

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AN INVESTIGATION ON THE EFFECTS OF INFLUENZA VIRUS INFECTION AS IT PERTAINS TO THE INITIATION OF TRANSATION

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Morgan Hager McCoy

Lexington, Kentucky

Director: Dr. Thomas M. Chambers, Professor of Veterinary Science

Lexington, Kentucky

2004

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ABSTRACT OF DISSERTATION

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Like the majority of host cell mRNAs, the mRNAs of influenza virus are capped and polyadenylated. The NS1 protein of influenza has been implicated as a translational activator for both influenza and reporter gene mRNAs. Data is presented showing that influenza A virus infection resulted in an increased ratio of cap-dependent to cap-independent translation. This ratio increase was largely due to an increase in cap-dependent translation. These experiments employed a bicistronic reporter construct measuring cap-dependent and cap-independent translation in a single sample. Expression of NS1 alone resulted in a small, but reproducible increase in the ratio of cap-dependent to cap-independent translation. Additionally, with use of an NS1 deleted mutant influenza A virus (delNS1) it is shown that infection without NS1 expression produced less of a translation ratio increase compared to wild-type virus infection. Furthermore, expression of NS1 rescued a more wild-type ratio increase in delNS1 infected Vero cells. These results implicate NS1 as playing a role in increasing the ratio of cap-dependent to cap-independent translation in influenza A virus infected cells. Additionally, eIF4E-binding protein-1 (4E-BP1), a member of the protein
family that inhibits cap-dependent translation through their inhibition of the cap-binding protein, eukaryotic initiation factor 4E (eIF4E), is shown to be inactivated throughout the majority of the influenza A virus infection process.

KEYWORDS: Influenza virus, Cap-dependent translation, Cap-independent translation, NS1, IRES.

Morgan Hager McCoy

May 20, 2004
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Chapter 1: Introduction

Influenza A virus summary

Influenza virus is consistently one of the most devastating communicable diseases plaguing the worldwide human population. Each year in the United States over 100,000 people are hospitalized for influenza infection. Additionally, on average 30,000 people die each year in the U.S. resulting from complications of influenza infection (Simonsen et al., 2000; Thompson et al., 2003). Pandemics of influenza periodically sweep the world, infecting anywhere from 20%-40% of the total human population (Cox & Subbarao, 2000). These pandemics are theorized to occur due to the emergence of influenza virus strains with novel Hemagglutinin (HA) proteins for which people have no antibodies and are immunologically naïve (Kilbourne, 1977). The most notable pandemics known to have occurred are those of 1890, 1918, 1957 and 1968 (Reid & Taubenberger, 2003). Of these, the 1918 “Spanish Flu” has gained the most notoriety as a result of its unusually high mortality rate and young target population. The flu of 1918 was typical in that most of the case morbidity was specific to those under the age of 15 (Jordan, 1927). However, highly unusual was the high mortality rate of those under the age of 65 (Linder, F. E. and Grove, R. D., 43). Of the influenza-associated deaths of the 1918 virus, generally due to development and complications of pneumonia, 99% were people under the age of 65. Even more surprising was that people between the ages of 20 and 40 years were those most affected (Simonsen et al., 1998). Previous pandemics have generally occurred in 10-20 year intervals, however it has now been 36 years since the last such pandemic. Thus, it is theorized we are currently due for another such large-scale outbreak. Recent
predictions as to the severity of the next pandemic are dire. It is estimated that in the U.S. alone there will be up to 207,000 deaths, 734,000 hospitalizations, 42 million outpatient visits and 47 million additional illnesses. With this in mind and the estimated economic impact of 70-170 billion dollars, excluding disruptions to commerce and society, influenza virus poses a major threat to the world community as a whole (Meltzer et al., 1999).

Influenza A virus is one of five members of the Orthomyxoviridae family of viruses (Lamb & Krug, 1996). The other members of this virus family are influenza B virus, influenza C virus, thogotovirus and isavirus. All viruses in this family are characterized as having a segmented, single stranded, negative sense RNA genome. There are three known types of influenza virus: A, B and C. Under electron microscopy (EM) influenza C is distinguishable from its two cousins owing to its hexagonal glycoprotein arrangement. However, types A and B share such similar morphology they are indistinguishable under EM (Flewett & Apostolov, 1967; Herrler et al., 1981; Waterson et al., 1963). Influenza A and B viruses also exhibit very pleomorphic morphology when isolated from humans or animals and passaged only once in culture. When these viruses are regularly passaged in culture they take on a much more regular shape and size (Horne et al., 1960; Hoyle et al., 1961). Viruses of all three influenza types consist of a segmented negative sense RNA genome lined with nucleoprotein (NP) and encapsidated within a shell of matrix protein (M1). The capsids are enveloped by a host cell membrane derived lipid layer. The genomes of both types A and B consist of 8 total genomic RNA segments where as the genome of type C consists of only 7, due to its dual-function hemagglutinin-esterase-fusion (HEF)
glycoprotein. Thus, unlike influenza A and B viruses, which have two surface glycoproteins, HA and neuraminidase (NA), protruding from their envelopes, influenza C virus has only one surface glycoprotein which alone appears to perform functions similar to both the HA and NA (Herrler et al., 1988; Herrler & Klenk, 1991; Herrler et al., 1988; Herrler et al., 1981; Herrler et al., 1987; Herrler et al., 1985a; Herrler et al., 1985b; Rogers et al., 1986; Vlasak et al., 1987).

Of these three influenza types, A and B are the most common causes of outbreaks, with type A causing the majority of the infections (Simonsen, 1999). It is thought that due to its much larger host range including humans, swine, equids and a variety of water fowl, influenza A virus has a greater propensity for causing devastating outbreaks in the human population. Influenza B virus is a major cause of respiratory illness in humans. However, it appears to almost exclusively infect humans, although seals are a newly discovered host (Baine et al., 1980; Osterhaus et al., 2000). This dramatically reduces influenza B virus’ potential for genetic diversity through a process of genetic exchange with viruses of differing host species. Influenza A virus differs in that it is presumed to be largely an avian virus, which replicates asymptptomatically in the intestines of aquatic birds. Distinguishing the subtypes of influenza A virus are the two major antigenically significant proteins, HA and NA, of which there are currently 15 and 9 subtypes classified respectively (Webster et al., 1992; Webster et al., 1978). Of these subtypes, only a few (H1, H2, H3, H5, N1, N2) are known to infect humans. However, due to the susceptibility of pigs to some subtypes of both avian and human influenza A viruses, it is theorized that pigs are an intermediary for genetic reassortment between avian and human viruses in dually infected animals. This idea was first conjured in
1985 as a result of experiments analyzing the impact of viral NP protein on host specificity of ts mutant influenza A virus (Scholtissek et al., 1985). These suspicions were confirmed in 1992 when a phylogenetic study was performed comparing influenza A virus genomes isolated from avians, humans, and swine in Italy (Castrucci et al., 1993). The theory is that in a dually infected cell the two virus genomes may reassort with each other to produce novel combinations of gene segments, essentially generating a hybrid virus. Pigs are susceptible to both human and avian influenza viruses because of the terminal sugar linkages on the glycoprotein receptors of their tracheal epithelium. In humans, sialic acids of these receptors tend to be linked to a galactose by an $\alpha 2,6$ configuration, whereas in avians, the terminal sialic acid linkages are $\alpha 2,3$. Since pig sialic acids are found linked in both configurations, influenza viruses of both avian and human origin are capable of binding (Ito et al., 1998). Such virus promiscuity greatly enhances influenza A virus' power for genetic diversity and therefore survival.

Until 1997 it was thought humans could not be directly infected with an avian virus without going through a reassortment event in an intermediary host. However, during the 1997 Hong Kong outbreak 18 people were infected, of whom 6 died, with a completely avian influenza A virus (H5N1) (Lin et al., 2000). This incident proved that humans are not only at risk from viruses reassorted through intermediary hosts, but also from viruses through direct transmission from an avian reservoir. The Hong Kong virus of 1997 was particularly virulent because of its resistance to interferons alpha and gamma (IFN-\(\alpha\) and IFN-\(\gamma\)) and tumor necrosis factor alpha (TNF-\(\alpha\)), cytokines usually effective against influenza A virus replication. This resistance was conferred by the
H5N1 virus’ non-structural (NS1) protein, one of influenza A virus’ 10 proteins (Seo et al., 2002). This was discovered in an experiment where replacement of the H5N1 NS1 gene with that of a cytokine susceptible virus conferred a loss of resistance.

The influenza A virus genome consists of 8 segments which code for 10 proteins, all of which play essential roles during virus infection of normal hosts. Of the influenza proteins, 9 are present in the structure of the mature virion and 1, NS1, is present only in the infected host cell. Of the proteins present in the virion, the HA and NA are the two antigenic surface proteins against which the host mounts its primary antibody response (Laver & Valentine, 1969). The HA exists as a homotrimer, which during an infection binds to sialic acid glycoprotein receptors on the surface of the host cell and also mediates the virus/endocytic vesicle membrane fusion (White et al., 1981; Wiley & Skehel, 1977; Wiley et al., 1977; Wiley et al., 1981). The NA cleaves terminal sialic acid and terminal D-galactose or D-galactosamine structures and appears to be important in preventing the virus from self-aggregating, as well as from re-adsorption to the producer host cell (Gottschalk, 1957). These two surface proteins are anchored to a lipid bilayer derived from the cell membrane during egression from the host cell. Within this lipid layer also exists an ion channel protein formed by tetramers of the M2 protein, which is key in uncoating the genome during virus entry (Holsinger & Lamb, 1991; Sugrue & Hay, 1991). Beneath the lipid layer is a sheet of M1, which surrounds the viral ribonucleoprotein (vRNP) genome (Gregoriades, 1973; Lamb et al., 1985). The vRNPs are characterized as single genomic segments wound with a protective coat of the NP protein and complexed with the three polymerase proteins: PB1, PB2 and PA (Inglis et al., 1976; Lamb & Choppin, 1976). The nuclear export protein (NEP), formerly NS2,
was first thought to be a nonstructural protein present only in the infected host cell, however it is now thought that NEP is present in the virion and plays a role in the nuclear to cytoplasmic export of vRNPs during virion assembly (O’Neill et al., 1998; Richardson & Akkina, 1991; Yasuda et al., 1993). The 10 total proteins coded for by influenza A virus are transcribed from 8 full length mRNA transcripts and 2 alternatively spliced transcripts, which code for NEP and M2 (Inglis & Brown, 1981; Lamb et al., 1980). As will be described in more detail later, during an infection the nonstructural protein, NS1, plays a crucial role in shutting down the nuclear to cytoplasmic export of cellular mRNAs, as well as inhibiting the activation of the antiviral interferon cascade.

Once the virion has bound to a sialic acid glycoprotein receptor through HA interaction, the virus is endocytosed into the host cell. At this point the virus is enclosed and anchored to the interior membrane of the endosome. In the late endosome the pH decreases, resulting in a conformational change of the HA which leads to fusion of the virion and endosomal membranes which releases the viral genome in the form of vRNPs. The low pH of the endosome is crucial for activating the M2 ion channel, which then allows for the flow of protons from the endosome into the virion. It is thought this flow of protons is essential for disrupting the viral capsid and releasing the genomic segments into the cytoplasm. Treatment with amantadine, an M2 channel inhibitor, leads to the failed dissociation of M1 from the vRNPs (Martin & Helenius, 1991).

These vRNPs are then directed to the nucleus where the negative sense RNA is transcribed into mRNA as well as full length, positive sense template for genome synthesis. Even though influenza virus particles incorporate their own RNA-dependent RNA polymerase complexes (PB1, PB2 and PA), it was observed that inhibition of the
cellular RNA polymerase II protein inhibited viral replication (Lamb & Choppin, 1977; Mahy et al., 1972; Spooner & Barry, 1977). These observations are explained by the current model for influenza A virus transcription, in which capped cellular mRNA fragments are used as primers for viral mRNA synthesis (Bouloy et al., 1979; Bouloy et al., 1978). Inhibition of RNA polymerase II reduces the supply of capped mRNAs necessary for influenza A virus transcription and therefore, replication. The complex of PB1, PB2, PA, attached to vRNA and NP binds cellular mRNA via PB2’s interaction with the 5’ cap structure (Blaas et al., 1982; Braam et al., 1983; Ulmanen et al., 1981). This interaction appears to be independent of any base pairing between the cellular mRNA and the vRNA (Krug, 1981; Krug et al., 1980), which would explain the ability of the polymerase proteins to utilize the heterogeneous assortment of cellular mRNAs as primers. According to UV-cross-linking experiments, the PB1 protein is found as the leading protein of the polymerase complex and is likely responsible for nucleotide addition onto the growing viral mRNA (Braam et al., 1983). Additionally, PB1 is also thought to be responsible for the cap-dependent cleavage of the cellular mRNA primer, approximately 10-12 nucleotides down-stream of the cap structure, from the remaining cellular pre-mRNA (Li et al., 2001a). The role of the PA protein is not as well defined as the roles of PB1 and PB2. However, it has been suggested that the PA protein is essential in the transcription of complementary and genomic vRNA (Honda et al., 2002; Nakagawa et al., 1996).

Following transcription of mRNA and the synthesis of viral proteins, a switch from mRNA transcription to genomic replication occurs in influenza A virus infected cells (Barrett et al., 1979; Hay et al., 1982). It is likely that this RNA transcription switch is
regulated by viral protein synthesis, as the existence of free NP proteins is necessary for the transcription of complementary RNA (cRNA) as well as the genomic vRNA (Beaton & Krug, 1986; Shapiro & Krug, 1988). Although the mechanism for this requirement is not fully understood, it is presumed that the transcription process of cRNA and vRNA is dependent on their association with NP. Taken together, it has been postulated that there exist two separate polymerase complexes in the infected cell: one free of PA, which would be used for mRNA synthesis, and one complexed with PA for cRNA/vRNA synthesis. However, this theory has yet to be adequately studied and the true arrangement of the polymerase complex in vivo is not precisely understood. It should be noted the simplest complex needed for replication of a pseudo-genomic reporter construct, which contains influenza 5' and 3' terminal sequences is PB1, PB2, PA and NP (Huang et al., 1990).

**Cap-dependent translation initiation**

The initiation of translation has proven to be a highly regulated process in protein synthesis and its inhibition could be a point from which viral infections can be quashed (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., 2000). To summarize, during the initiation step the mRNA is loaded onto the 40S subunit of the ribosomal machinery, which then scans down the mRNA in a 5’ to 3’ direction until it locates the initiating AUG start codon (summarized in Fig. 1). The large ribosomal subunit is then recruited to the complex and elongation of the peptide continues. In eukaryotic systems the mRNA can be loaded onto the small ribosomal subunit through a cap-dependent mechanism or a cap-independent mechanism. In cap-dependent initiation the mRNA is loaded via the methyl-7-GTP cap structure located at the 5’ end of the mRNA.
Cap-dependent initiation begins with the formation of the ternary complex: 
methionyl-tRNA\textsubscript{i} (Met-tRNA\textsubscript{i}), eIF2 and GTP. Once the ternary complex has been 
formed it can then bind to the 40S ribosomal subunit. The resulting complex is often 
called the preinitiation complex or the 43S complex. It is known that preinitiation 
complex formation may occur in the absence of mRNA binding \{#1240\}. The methyl-7-
GTP cap structure of the mRNA is then bound to a member of the translation initiation 
complex termed eukaryotic initiation factor 4F (eIF4F). eIF4F is composed of three 
protein factors: eIF4E, the cap binding protein; eIF4G, a scaffolding protein; and eIF4A, 
a helicase.
Figure 1. Summary of cap-dependent initiation of translation. A. Dissociation of ribosome. B. Formation of ternary complex and 43S pre-initiation complex. C. mRNA binding to pre-initiation complex. D. Binding of large ribosomal subunit.
Each of the three components of the eIF4F complex has a specific function as it pertains to the initiation of translation. The cap binding protein, eIF4E, specifically binds to the 5’ cap of the mRNA and links it to the initiation complex (Sonenberg et al., 1978). There have been numerous forms of eIF4E found in various species, including two in humans (Rom et al., 1998). However, the purpose of these various forms is unknown.

Although eIF4E alone is capable of binding capped mRNA, when complexed with eIF4G, eIF4E is able to bind capped mRNA with 10-fold greater affinity (Haghighat & Sonenberg, 1997). The physical binding between eIF4E and the 5’ cap is done through intercalation of the m7G ring into a stacked set of two highly conserved tryptophan residues (Marcotrigiano et al., 1997; Matsuo et al., 1997). eIF4E is phosphorylated at serine 209, using mammalian eIF4E amino acid numbering, by the kinases Mnk1 and Mnk2 (Knauf et al., 2001; Waskiewicz et al., 1997). How this phosphorylation affects translation has yet to be definitively explained; although, frequently increased phosphorylation at this site coincides with higher levels of translation (Scheper & Proud, 2002). eIF4E phosphorylation has been linked to the down-regulation of cap-dependent translation, and furthermore, expression of a non-phosphorylatable mutant eIF4E partially reverses this effect (Knauf et al., 2001). However, another group has recently presented contradictory data, that dephosphorylation of eIF4E results in an increase in cap-independent translation (Dyer et al., 2003). eIF4E has been shown to be dephosphorylated during influenza infection (Feigenblum & Schneider, 1993). However, it is unclear how this dephosphorylation affects virus replication because of the uncertainty in how phosphorylation regulates eIF4E function. As stated previously, although high phosphorylation of eIF4E has often been found during times of high
translation, a cause and effect relationship has been difficult to establish. *Saccharomyces cerevisiae* lacking functioning endogenous eIF4E can be rescued for growth as efficiently by mutant, non-phosphorylatable eIF4E as the wild-type protein (McKendrick et al., 2001). Interestingly, a mutant strain of *Drosophila melanogaster* expressing only non-phosphorylatable eIF4E has been found viable. The only major phenotypic difference found between these mutant flies and their wild-type counterparts is a smaller body size. Surprisingly, the mutant flies maintain a wild-type cell count as well as body proportion when compared to wild-type flies. It appears their smaller body size is simply due to smaller individual cell size (Lachance et al., 2002).

In addition to phosphorylation, eIF4E is regulated through interaction with the eIF4E-binding proteins (4E-BP). When eIF4E is bound to 4E-BP it is sequestered from the initiation complex and therefore cannot play its part in initiating cap-dependent translation (Haghighat et al., 1995). The 4E-BPs are regulated through a phosphorylation hierarchy, whereby hypophosphorylation results in eIF4E binding and hyperphosphorylation results in eIF4E release (Fadden et al., 1997; Gingras et al., 1999; Lin et al., 1994; Pause et al., 1994). This is to say that when 4E-BP is highly phosphorylated cap-dependent translation is maintained at high levels, and conversely, when 4E-BP is under-phosphorylated cap-dependent translation is decreased.

The 4E-BPs are a group of proteins made up of three members, termed 4E-BP1, 2 and 3, which all share greater than 55% amino acid homology with one another (Pause et al., 1994; Poulin et al., 1998). Interestingly, the three members of the 4E-BPs are expressed at different levels depending on the cell type (Hu et al., 1994; Tsukiyama-Kohara et al., 1996). This hints at the possible evolution of different isoforms for
different tissues and/or functions. As stated above, the 4E-BPs bind to eIF4E and prevent it from associating with the initiation complex, specifically to eIF4G. This association with eIF4E is conferred by a conserved binding sequence motif “YXXXLΦ” in which X is any amino acid and Φ is an aliphatic amino acid, usually L but sometimes M or F (Altmann et al., 1997; Mader et al., 1995). 4E-BP1 is regulated through a complex hierarchy of phosphorylation events at six known sites and this regulation is as yet not fully understood. There are two phosphorylation sites, Thr-37 and Thr–46, which are located on the amino-terminus of the protein and are generally found phosphorylated during times of serum starvation in cell culture. Upon addition of serum, phosphorylation of these sites is only moderately increased (Gingras et al., 1999). This is in contrast to Ser-65 and Thr-70, which are located at the carboxy-terminus of the protein and are found dephosphorylated or weakly phosphorylated during serum starvation in cell culture. However, upon addition of serum, phosphorylation at these two sites is greatly increased (Gingras et al., 1999; Gingras et al., 1999). The current model is that the two “priming” sites, Thr-37 and Thr-46, are phosphorylated by the kinase FRAP/mTOR, a rapamycin sensitive kinase (Burnett et al., 1998; Gingras et al., 1999). Ser-65 and Thr-70 are phosphorylated by a kinase, or set of kinases, activated by the Akt/PKB kinases (Gingras et al., 1998; Kohn et al., 1998). The Akt/PKB kinases are activated via a PI3 kinase pathway and whether Akt/PKB activation affects FRAP/mTOR activation is not yet fully defined (Nave et al., 1999; Sekulic et al., 2000; West et al., 2002). Two other phosphorylation sites, Ser-83 and Ser 112, are not well understood and their phosphorylation may be cell line specific (Fadden et al., 1997; Fadden et al., 1998; Gingras et al., 1999; Gingras et al., 1998; Gingras et al., 1999;
Heesom et al., 1998). 4E-BP2 and 3 both maintain all the possible phosphorylation sites as 4E-BP1 except Ser112. However, not all of these remaining sites on 4E-BP2 and 3 are found phosphorylated in vivo. It is unclear how these phosphorylation patterns differ functionally from one another and how these differences may affect their function in vivo.

The scaffolding protein, eIF4G, of which there are two forms, eIF4GI and eIF4GII, seems to play the role of an adapter molecule for the eIF4F complex (Gradi et al., 1998; Yan et al., 1992). The two forms of eIF4G share 46% identity and function as homologs (Gradi et al., 1998). Through its multiple binding capabilities, eIF4G physically bridges the small ribosomal subunit to eIF4E via its interaction with another initiation factor, eIF3. The amino terminal portion (amino acids 1-634) of eIF4GI is responsible for binding eIF4E as well as poly(A)-binding protein (PABP) and is required for cap-dependent translation (Imataka et al., 1998; Lamphear et al., 1995; Mader et al., 1995). During picornavirus infections the amino terminus of eIF4GI is cleaved rendering it unable to bind to eIF4E (Lamphear et al., 1993). This cleavage, although abolishing cap-dependent translation, does not affect the translation of viral mRNAs, which are translated in a cap-independent manner through IRES initiation. The middle portion of eIF4G, residues 635-1039, contains binding sites specific to eIF4A, eIF3 (Imataka & Sonenberg, 1997) and RNA (Pestova et al., 1996). The carboxy-terminus has binding sites for a second eIF4A (Imataka & Sonenberg, 1997) as well as the kinases Mnk1 and Mnk2 (Pyronnet et al., 1999), both of which have been implicated in eIF4E phosphorylation (Knauf et al., 2001; Waskiewicz et al., 1997). eIF4G is also a phosphoprotein, however, the pathways involved in its phosphorylation and the
functional effects imposed on eIF4G are poorly understood (Bu et al., 1993; Donaldson et al., 1991; Feigenblum & Schneider, 1993; Morley & Pain, 1995a; Morley & Pain, 1995b; Morley & Traugh, 1990; Morley & Traugh, 1993; Tuazon et al., 1989). Interestingly, a physical interaction has been found between eIF4G and the influenza A virus NS1 protein (Aragon et al., 2000). How this association might affect virus replication is, however, not understood. A third member of the eIF4G family, p97, also known as NAT1 or DAP-5, is a protein homologous only to the carboxy-terminal two-thirds portion of eIF4GI and II (Imataka et al., 1997; Levy-Strumpf et al., 1997; Yamanaka et al., 1997). p97 appears to maintain all the eIF4G functions to which it is homologous, in that it can bind eIF4A, eIF3 and Mnk1. Additionally, p97 inhibits cap-dependent translation, presumably through its interaction with eIF4A and eIF3 to form inactive initiation complexes (Imataka et al., 1997).

The ATP dependent helicase, eIF4A, is important in unwinding the secondary structure of the mRNA’s 5’ untranslated region (UTR) (Lawson et al., 1988). mRNA containing a highly structured 5’ UTR, i.e. with multiple intra-chain base pairings to form stem-loop secondary structures, would prove difficult for ribosomal scanning and the need for eIF4A activity becomes apparent. Hypothetically, eIF4A activity becomes a potential player in the translation regulation of highly structured mRNAs; whereby higher eIF4A activity confers higher protein expression from those mRNAs. The opposite would also be true in that by decreasing eIF4A activity a decrease in protein expression from highly structured mRNAs would be observed. Two initiation factors, eIF4B and eIF4H, have been found to increase the ability of eIF4A to unwind the secondary structure of mRNA (Jaramillo et al., 1991; Richter-Cook et al., 1998; Rozen et al., 1990).
eIF4A is not processive, this is to say that eIF4A is capable of unwinding only 3-5 base pairs at a time without the assistance of eIF4B or eIF4H (Lorsch & Herschlag, 1998a; Lorsch & Herschlag, 1998b; Rogers et al., 1999). It is thought that through their interaction with RNA, eIF4B and eIF4H are able to prevent the reassociation of unwound RNA and therefore to promote the unwinding action of eIF4A.

**Cap-independent translation initiation**

In contrast to cap-dependent initiation, cap-independent initiation does not require the cap binding protein, eIF4E. Therefore, regulation inhibiting the activity eIF4E does not affect the initiation of cap-independent messages. Cap-independent messages are recruited to the initiation complex through sequence elements in the 5' UTR of the mRNA that are termed internal ribosome entry sites (IRES) (Belsham & Jackson, 2000). IRES, initially discovered in picornaviruses, are characterized as RNA sequences with a predicted high level of secondary structure (Pelletier & Sonenberg, 1988). It appears that through widely diverse and ill understood binding mechanisms, this secondary structure is essential for the mRNA’s interaction with the ribosome. Therefore, IRES obviate the role of eIF4E and the 5’ cap in translation initiation. Such is observed in many picornaviruses when early in infection eIF4G is cleaved, leaving eIF4E without its adapter to the ribosome (Etchison et al., 1982; Lee et al., 1985). Interestingly, most of the picornaviruses need the remaining initiation factors other than eIF4E for translation initiation (Imataka & Sonenberg, 1997; Lamphear et al., 1995; Pestova et al., 1996; Pestova et al., 1996). IRES are very diverse in their sequence, structure and effector protein binding and therefore, it should be understood that IRES characterization is still in its early stages. In recent years many eukaryotic mRNAs have
been identified, which are thought to contain IRES elements within their sequences. However, the existence of eukaryotic translation initiation through IRES has been questioned (Kozak, 2001). In her commentary, Kozak cites deficiencies in much of the current work claiming identification of eukaryotic IRES. In her opinion she describes poorly designed controls as well as faulty interpretations. Dr. Kozak describes the possibility of cryptic promoters and alternatively spliced mRNAs rather than true IRES to account for many investigators’ observations. However, in defense of their work many in the field of IRES translation submitted a response to Dr. Kozak’s criticisms (Schneider et al., 2001). The number of reports supporting IRES translation in eukaryotic cells far out number those questioning it. It is presumed that IRES-dependent mRNAs code for proteins important during periods where cap-dependent translation is shut down. Possible eukaryotic mRNAs translated in a cap-independent manner include, but are not limited to, mRNAs coding for proteins affecting apoptosis, translation, growth stimulation and RNA processing. In an analysis of 7000 cellular mRNAs, 200 were found associated with polysomes under conditions of total or near total eIF4G cleavage (Johannes et al., 1999). A web-based database for suspected IRES, both viral and cellular, is now available at http://ifr31w3.toulouse.inserm.fr/IRESdatabase/.

Presumably, stressed cells undergo the process of apoptosis in an attempt to minimize the damage done on neighboring tissues. Studies on cap-independent translation have begun to assess its importance in the cellular response to various stresses leading to apoptosis. It has been shown that many viral infections, including that of influenza A virus, result in apoptosis (Lin et al., 2002; Takizawa et al., 1993).
One of the hallmarks of apoptosis is the cleavage of eIF4G by activated caspase-3 (Marissen & Lloyd, 1998). This cleavage is reminiscent of the cleavage resulting from a picornavirus infection, although their cleavage profiles are different. Considering that cleavage of eIF4G favors cap-independent over cap-dependent translation in picornavirus infections (Imataka & Sonenberg, 1997; Lamphear et al., 1995), it is possible that caspase-3 cleavage of eIF4G during apoptosis also enhances cap-independent translation. Reports of cap-independent translation in apoptotic cells are conflicting. Interestingly, in one report, it appears that opposing factors involved in pro- and anti-apoptotic stimulation can both be translated in a cap-independent manner. In these studies reporter genes were put under the translational control of the IRES of different apoptotic factors. A reporter bearing the XIAP IRES is actively translated during conditions of transient cellular stress, such as serum starvation or low doses of γ-irradiation (Holcik et al., 1999; Holcik et al., 2000). Similarly, under another mild stress, anoxia, reporters bearing IRES from two pro-apoptotic factors, Apaf-1 and DAP5, can also be translated, though to a much lower magnitude than the XIAP IRES (Nevins et al., 2003). When treated with etoposide, a much stronger stress, translation of the XIAP IRES is relatively low compared to the greater amount of translation driven by the Apaf-1 and DAP5 IRES (Nevins et al., 2003). Results such as these suggest an intricate system of regulation within the realm of cap-independent translation so as to balance the cell's interests for survival or death. More specifically, during times of stress, when cap-dependent translation is inhibited, cap-independent translation directs the expression of whichever factors are the most appropriate for the given situation. Under light and likely transient periods of stress, it would benefit the cell to forgo
apoptosis if recovery is likely, and for this to occur, regulation favoring the IRES driven translation of anti-apoptotic factors would be necessary. However, if the stress were too taxing for the cell to overcome, such as etoposide treatment, IRES driven translation favoring pro-apoptotic factors would be appropriate. Unfortunately, and likely due to its intricate and precise regulation, the data supporting such an idea are confusing as not all reports agree on the finer details involved in cap-independent translation during apoptosis. For example, Henis-Korenblit and colleagues observed an enhancement in the translation driven by the XIAP IRES due to over expression of a particular eIF4G family protein cleavage fragment, DAP5/p86 (Henis-Korenblit et al., 2002). Yet in one of the previously mentioned studies, Nevins and colleagues found no such increase (Nevins et al., 2003). However, the fundamental principle of cap-independent translation being involved in the regulation of apoptotic factors during cellular stress lends credibility to the investigation of cellular cap-independent regulation in other circumstances, such as in the case of virus infection.

In addition to cell stresses, the position of cells in the cell cycle has been found to affect the level of cap-independent translation. Ornithine decarboxylase protein and the 58 kDa PITSLRE protein kinase have been reported to be preferentially translated by their IRES elements in the G2/M phase (Cornelis et al., 2000; Pyronnet et al., 2000). Additionally, a viral IRES, from hepatitis C virus (HCV), has been reported to be most active during the G2/M phase in HuH7 cells (Honda et al., 2000). Yet another report finds the HCV and the encephalomyocarditis virus (EMCV) IRES are the least active during the G2/M phase in HEK 293 cells (Venkatesan et al., 2003). Authors attribute their differing results to the fact two different cell lines were used in these studies.
These conflicting results support the idea that regulation of IRES driven translation is very complicated and is tightly controlled. Such results also suggest a cell line/tissue type-specificity relating to control of IRES driven translation. These specificities may possibly be attributed to the differences found in the levels of canonical and noncanonical translation factors found in individual cell lines.

The poliovirus IRES is one of the best studied IRES to date. Poliovirus is a member of the picornaviridae family of viruses, which consists of 6 virus genera: Enterovirus, Rhinovirus, Aphthovirus, Cardiovirus, Hepatovirus and Parechovirus. Poliovirus, a member of the Enterovirus genera, like all picornaviridae is a non-enveloped positive sense RNA virus. Due to the positive sense nature of the viral genome, the entire life cycle of the virus occurs within the cytoplasm of the host cell. This was demonstrated by infection of enucleated cells (Follett et al., 1975). Since then, much has been learned about the regulation and structure of the poliovirus, and other, IRES. As was the case in cell cycle and cellular stress signaling, cell type also appears to play a role in specific IRES translational activity. For example, a mutant virus consisting of the capsid proteins of a poliovirus strain and the IRES sequence of a rhinovirus loses replication efficiency in neuronal cells compared to the parental poliovirus (Gromeier et al., 1996; Gromeier et al., 1999). However, both the parental poliovirus strain and the mutant will replicate efficiently in HeLa cells. Such results implicate a tissue tropism that is dependent specifically on the IRES encoded in the genome. Again, as may be the case for the HCV and EMCV, since HeLa and neuronal cells may express different levels of proteins involved in translational regulation, tissue
tropism may be due to the availability of the appropriate cellular factors specifically needed for translation from a particular IRES.

The poliovirus IRES (Fig. 2) is approximately 450 nucleotides in length and although it was once thought the extreme 5’ end of the genome was only used to regulate the RNA genomic replication, more recent evidence suggests that translation efficiency can also be reduced following mutation of a stem-loop structure at position 21, well before the proposed IRES sequence (Gamarnik & Andino, 1998; Simoes & Sarnow, 1991). At the 3’ end of the IRES is a sequence of approximately 25 nucleotides common to all picornaviruses, which begins with a tract of approximately 10 pyrimidines and ends with a G-poor sequence. The first AUG 3’ of this 25 nucleotide sequence is considered the putative ribosomal entry site. However in the case of poliovirus this first AUG 3’ to the IRES sequence, at position 586, does not appear to be used at all (Ohlmann & Jackson, 1999; Pestova et al., 1994). Another AUG codon at position 743, approximately 160 nucleotides down-stream appears to be the site of the majority of all translation initiation (Dorner et al., 1982). Some have postulated that the first AUG following the IRES may be a site of ribosome entry from which scanning would then commence until a favorable AUG could be located (Belsham, 1992; Sangar et al., 1987). However, the evidence for such a hypothesis is unclear because mutation of the first AUG in the foot and mouth disease virus (FMDV), an Aphthovirus, IRES results in only a slight decrease in the virus’ infectivity. Conversely, mutation of the second, more efficient translation initiation site results in lethality (Cao et al., 1995). Whether the ribosome may be able to enter the RNA at a non-AUG codon remains to be studied. If so, a scanning hypothesis model could explain why mutation of the first
AUG results in only a partial decrease in virus replication and mutation of the true translation start codon is deleterious. More confusing are the results found in similar mutational experiments using both type I and II poliovirus (PV-1, PV-2) IRES. The first AUG 3’ of the PV-1 IRES, position 586, is required as mutation of it results in revertant mutations introducing AUG codons near the original AUG at nucleotide 586 (Pilipenko et al., 1992). However, mutation of the equivalent AUG in PV-2 IRES results in only a slight decrease in plaque size phenotype in tissue culture and a slight reduction in IRES driven translation (Meerovitch et al., 1991; Nicholson et al., 1991; Pelletier et al., 1988). Additionally, in support of ribosomal scanning 3’ of the IRES, insertion of hairpin structures between the two AUG codons results in reduced translation efficiency of FMDV and PV-1 IRES (Belsham, 1992; Hellen et al., 1994; Lopez de Quinto & Martinez-Salas, 1999; Pelletier & Sonenberg, 1988). However, it was noted the degree of inhibition was less than expected with addition of such structures in a ribosomal scanning model. Therefore, how the ribosome is recruited to the start codon is still largely unknown.
Figure 2. Diagram of the poliovirus IRES. (Belsham, G.J. & Jackson, R.J. (2000). Translation initiation on picornavirus RNA. In Translational Control of Gene Expression, pp. 869-900. Edited by Sonenberg, Hershey, and Mathews. Cold Spring Harbor, NY). Figure used with permission of publisher.
Additional cellular proteins have been found to enhance the translation driven by some IRES sequences. The La protein was found to enhance the translation efficiency of the PV-1 IRES in rabbit reticulocyte lysates (Meerovitch et al., 1993). However, the concentration of La necessary for observing such increases is very high and its physiological relevance for the PV-1 IRES has been challenged (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., 2000). During poliovirus infection La is redistributed from the nucleus to the cytoplasm of infected cells, implying a role in poliovirus IRES translation (Meerovitch et al., 1993). The polypyrimidine tract-binding protein (PTB), a protein involved in RNA splicing, is another protein found to enhance poliovirus IRES driven translation in rabbit reticulocyte lysates. However, this stimulation is low in comparison to the PTB stimulation of the rhinovirus IRES translation (Hunt et al., 1999; Hunt & Jackson, 1999). A cytoplasmic RNA binding protein termed poly(rC) binding protein-2 (PCBP-2) has also been found to increase the efficiency of translation from the poliovirus IRES. However, unlike the induction of La and PTB, PCBP-2 appears to be a much better inducer of poliovirus IRES driven translation. HeLa cell lysates depleted of PCBP-2 lose their capacity for poliovirus IRES translation. Furthermore, addition of recombinant PCBP-2 to the depleted HeLa cell extracts restores poliovirus IRES translation. Interestingly, depletion of PCBP-2 from HeLa cell extracts had no effect on EMCV or rhinovirus IRES driven translation (Blyn et al., 1997; Walter et al., 1999). Taken together these results illustrate the great diversity in the mechanisms supporting cap-independent translation and a great deal of investigation is needed for a more complete understanding of the system.
Regulation of host cell translation during influenza A virus infection

The host cell has developed an assortment of defense mechanisms, which are aimed at combating infectious agents. One mechanism in particular, which viral infections are known to induce is an interferon (IFN) response. IFN can be separated into two distinct categories: type I (α, β) and type II (γ). Type II IFN is commonly secreted by Th-1 lymphocytes and NK immune cells. However, type I IFN are secreted by a wide variety of cell types, including epithelial cells infected by influenza virus (Barber, 2001; Basler & Garcia-Sastre, 2002). In a broad sense, both types of IFN work to induce an antiviral state in activated cells. The most widely understood mode of type I IFN activation during a viral infection is mediated by the production of double stranded RNA. Double stranded RNA (dsRNA) is often an intermediate present in virus infected cells and can be produced during viral genome transcription from an RNA template for single stranded RNA viruses or from a double stranded RNA genome, as is the case for reovirus. dsRNA induces the activation of many transcription factors known as IFN regulatory factors (IRFs). IRFs are responsible for inducing the transcription of a variety of genes containing IFN stimulated response elements (ISRE). Such genes include PKR, 2’,5’-Oligoadenylate Synthetases/RNAse L and the Mx genes and are important in bringing the cell into an antiviral state as well as alerting neighboring cells to do the same. Additionally, dsRNA activation also leads to the production of more IFN, leading to a positive feedback loop through autocrine stimulation (Basler & Garcia-Sastre, 2002). As may be expected, many viruses, including influenza A virus, have developed strategies for impeding such antiviral counterattacks, which will be discussed later.
Early during influenza infection, the host cell’s protein synthesis machinery is assimilated and forced to selectively translate influenza mRNAs over their cellular counterparts, and host encoded protein synthesis is effectively shut down. To date there has been no identification of a single factor working to cause the host protein synthesis shut-down. However, the NS1 protein has some functions that are pertinent to translational control during an infection. NS1 enters the nucleus and shuts down the polyadenylation and nuclear to cytoplasmic export of cellular mRNAs (Chen et al., 1999; Li et al., 2001b). Subverting the host cell’s mRNA processing machinery is crucial for the virus, because the initial interferon-\(\alpha/\beta\)-independent IRF-3 and IRF-7 response activates the transcription of antiviral pre-mRNAs coding for numerous effector molecules (Kim et al., 2002; Navarro et al., 1998; Preston et al., 2001; Wathelet et al., 1998; Weaver et al., 1998; Yoneyama et al., 1998), which would bring the cell into an antiviral state. By the NS1 induced blockage of their processing, these antiviral pre-mRNAs are never expressed. NS1 performs this block by binding to and inhibiting the activities of both cleavage and polyadenylation specificity factor (CPSF) and poly(A)-binding protein II (PABII); two host factors important in the 3’ processing of cellular pre-mRNA (Chen & Krug, 2000; Chen et al., 1999; Li et al., 2001b; Nemeroff et al., 1998). NS1 binds to both of these cellular proteins via its carboxy-terminal region, or what is more commonly called the effector domain (illustrated in Fig. 3). The amino acid regions of NS1 binding these cellular proteins are located in close proximity: CPSF binds to amino acids centered around 186 and PABII binds to the region between amino acids 223-237 (Fig. 3) (Li et al., 2001b). Normally, during the processing of host pre-mRNA into mature exportable message, CPSF binds to a specific signal sequence,
AAUAAA, which is located upstream of the cleavage site in pre-mRNAs, and cleaves the mRNA at that site making it ready for synthesis of a poly(A) tail by poly(A) polymerase (PAP) (Colgan & Manley, 1997; Wahle & Kuhn, 1997). Once the pre-mRNA has been cleaved at the 3’ end, PAP synthesizes the poly(A) tail through the aid of PABII. By inhibiting CPSF, NS1 is able to abolish the addition of a poly(A) tail, a process necessary for nuclear to cytoplasmic export. In addition to inhibition of CPSF, NS1 also inhibits the processive elongation of the poly(A) tail by inhibiting the interaction between PAP and PABII. Thus, NS1 inhibits polyadenylation, and nuclear export of host mRNAs, through two different mechanisms.

**NS1 Protein**

![Figure 3. A schematic drawing of the influenza A virus NS1 protein](image)

Expression of NS1 has also been shown to selectively increase the translation of mRNAs bearing an influenza UTR as well as some co-transfected reporter genes devoid of such sequences (Aragon et al., 2000; de la Luna et al., 1995; Enami et al., 1994; Enami & Enami, 2000; Salvatore et al., 2002). By themselves, these actions of NS1 do not account for the observed rapid reduction in host encoded protein synthesis.
Furthermore, a mutant influenza virus devoid of NS1 expression is still able to induce a host protein synthesis shut-down (Salvatore et al., 2002).

Recently a cellular protein factor has been identified which also increases the translation of influenza mRNAs: guanidine-rich sequence factor 1 (GRSF-1). GRSF-1 is a member of the RNP superfamily of RNA binding proteins. These proteins are characterized as containing one or more RNA recognition motifs (RRM), of which GRSF-1 possesses three. The RRM is a conserved motif of approximately 80 amino acids with two highly conserved regions. This superfamily of proteins has been associated with a variety of processes, such as RNA processing, trafficking and translation (Bandziulis et al., 1989; Kenan et al., 1991; Kim & Baker, 1993). As the name implies, GRSF-1 increases the translation efficiency of mRNAs containing a particular guanine rich sequence. This sequence, AGGGU, is found in the 5’ UTR of influenza, which is conserved almost intact in all 8 gene segments, and is thought to be at least partly responsible for selective viral mRNA translation (Kash et al., 2002; Park et al., 1999a). In addition to the principle AGGGU sequence, there are some deviations (Table 1), which may still confer increased translation efficiencies (Kash et al., 2002). The mechanism by which GRSF-1 works to increase translation efficiency is unclear and although an importance has been established during a viral infection, the precise cellular purpose of this protein is currently unknown.
In addition to mRNA processing, protein kinase R (PKR) activation is inhibited during influenza A virus infection. Upon activation by dsRNA, PKR phosphorylates eukaryotic initiation factor 2α (eIF2α) thereby shutting down protein synthesis by interfering with the initiation of translation (Kaufman, 2000). Briefly, following an initiation event GTP is exchanged for GDP on eIF2 by a guanine nucleotide exchange factor, eIF2B, in order to recycle the initiation factor for subsequent translation initiation. However, when eIF2α is phosphorylated by PKR the eIF2/GDP/eIF2B complex is stabilized in such a way that no GTP exchange is performed and in essence eIF2α is inactivated. Although NS1 is capable of binding dsRNA (Lu et al., 1995), it remains unclear how this binding affects the NS1 induced inhibition of PKR activation. Infection with ts influenza viruses coding for defective NS1 or an influenza virus completely

<table>
<thead>
<tr>
<th>Segment</th>
<th>Sequence</th>
<th>GRSF-1 binding</th>
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<tbody>
<tr>
<td>NP</td>
<td>GGAGCAAAAGCAGGGUAGAUAAUAACUCAUCACUGAGUGACAUCAAAAUCAUG</td>
<td>+++</td>
</tr>
<tr>
<td>NP-A</td>
<td>GG--------------GUAGAUAAUCACUCAGUGAGCAUCAAAUAUCAUG</td>
<td>-</td>
</tr>
<tr>
<td>NP-B</td>
<td>GGAGCAAAACCAGG-----------------------------UCACUGAGACUCAAAUAUCAUG</td>
<td>+</td>
</tr>
<tr>
<td>NP-C</td>
<td>GGAGCAAAAGCAGGUAGUAUAAU---------------------GUGACAUCAAAUAUCAUG</td>
<td>+++</td>
</tr>
<tr>
<td>NP-14</td>
<td>GGAGCAAAACGAGGU------------------------------------------</td>
<td>++</td>
</tr>
<tr>
<td>NP-12</td>
<td>GGAGCAAAAGCAGG----------------------------------------</td>
<td>-</td>
</tr>
<tr>
<td>NPΔ3</td>
<td>GG-------------AGCAGGGUAGUAAU------------------GUGACAUCAAA----</td>
<td>+++</td>
</tr>
<tr>
<td>NPΔ6</td>
<td>GG--------------AGCAGGGUAGUAAU----------------GUGACAUCAAA----</td>
<td>++</td>
</tr>
<tr>
<td>NPΔ9</td>
<td>GG--------------AGGUGUAUAAU----------------GUGACAUCAAA----</td>
<td>+</td>
</tr>
<tr>
<td>ns1</td>
<td>GGAGCAAAAGCAGGGUGACAAAGCAUAAUG-----------------------</td>
<td>+</td>
</tr>
<tr>
<td>u14c</td>
<td>GGAGCAAAAGCAGGGCGACAAAGCAUAAUG-----------------------</td>
<td>+++</td>
</tr>
<tr>
<td>g13/u14c</td>
<td>GGAGCAAAAGCAGGGCGACAAAGCAUAAUG-----------------------</td>
<td>-</td>
</tr>
<tr>
<td>m1</td>
<td>GGAGCGAAAGCAGGUAGUAAUGUAAAGAUG------------------------</td>
<td>++</td>
</tr>
<tr>
<td>na</td>
<td>GGAGCGAAAGCAGGGGUUAUAA-----------------------------</td>
<td>+</td>
</tr>
<tr>
<td>PB1</td>
<td>GGAGCGAAAGCAGGGCGACAAAGCAUAAUG-----------------------</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1. Sequences analyzed for GRSF-1 binding. Listed are RNA sequences evaluated for their ability to bind GRSF-1 in vitro in gel-shift assays. (+) and (-) indicate a relative comparison for GRSF-1 binding. The shaded portion is the proposed GRSF-1 binding sequence. (Data are from Kash et al. 2002)
without NS1 protein are unable to inhibit the activation of PKR by dsRNA (Bergmann et al., 2000; Hatada et al., 1999). Further supporting a link between RNA binding ability of NS1 and repression of PKR activation, the ts mutant viruses mentioned above also exhibited a reduced ability for binding RNA (Hatada et al., 1999). A peptide fragment consisting of the first 73 amino acids of NS1 is capable of binding to dsRNA in vitro. Additionally, as is the case for the full-length protein, the 73 amino acid fragment is able to dimerize in vitro (Qian et al., 1995). This dimerization is likely necessary for the protein’s dsRNA binding capabilities since mutagenesis abolishing dimerization also leads to a loss in dsRNA binding ability (Wang et al., 1999). The three dimensional structure obtained from X-ray crystallography and nuclear magnetic resonance (NMR) solution reveal that each 73 amino acid fragment of the dimer is made up of 3 alpha helices (Chien et al., 1997). Studies have indicated that within each fragment only two amino acids, Arg-38 and Lys-41, are necessary for dsRNA binding. In these experiments, it was shown that replacement of Arg-38 with Lys did not affect the fragment’s ability to bind dsRNA. However, when Arg-38 was replaced with Ala, dsRNA binding was inhibited and therefore a positively charged amino acid at position 38 is likely necessary for nucleic acid binding (Wang et al., 1999). Further supporting that the 1-73 amino acid fragment retains all the RNA binding-associated properties of the full-length protein, pre-incubation of the fragment with dsRNA prior to incubation in reticulocyte extracts abolishes the ability of the dsRNA to inhibit translation (Qian et al., 1995). Based on the understood action of dsRNA and the counteraction of NS1 it is presumed this block in translation inhibition is due to the lack of dsRNA-mediated PKR activation in the reticulocyte extracts.
Interestingly, NS1 and the 1-73 amino acid fragment appear to not only bind dsRNA, but also bind poly(A) RNA as well as a stem-bulge in U6 small nuclear RNA (snRNA) (Qian et al., 1995). It is presumed NS1 requires highly structured RNA targets and for this reason, it is capable of binding the previously mentioned RNA molecules. However, since the secondary structures produced by these RNAs are difficult to predict with certainty a particular RNA binding structure has not been established for NS1. It should be noted that NS1 binding does appear to require RNA/RNA duplexes (Chien et al., 2004). This was established with experiments using DNA/DNA and DNA/RNA duplexes in gel-shift assays using the 1-73 amino acid fragment as a binding substrate. In these experiments only dsRNA was capable of binding to the fragment.

It is presumed that NS1 inhibits the PKR activation activity of dsRNA by sequestering it from the PKR enzyme. However, NS1 has also been found to physically bind to PKR. In these experiments NS1 was found to co-precipitate with PKR in influenza infected Hela cell lysates. The interaction was confirmed in a yeast two-hybrid system (Tan & Katze, 1998). Similar to the dsRNA binding domain, the PKR binding domain was mapped to the amino terminus of NS1. Additionally, a mutant NS1 protein incapable of inhibiting the activation of PKR was also unable to bind to the kinase. However, another group has presented data of their own yeast two-hybrid experiments and found no indication of NS1 binding to PKR (Falcon et al., 1999). Therefore, the physical interaction of PKR remains controversial and so would any implication of its effect on PKR activation.

In addition to the actions of NS1, a cellular PKR inhibitor, P58 inhibitor of protein kinase (P58\textsuperscript{IPK}), has been shown to be recruited during influenza A virus infections to
assist in the inactivation of PKR (Lee et al., 1990; Lee et al., 1992). P58\textsuperscript{IPK} is present in cells as an inactive protein complex which likely consists of both heat shock protein 40 (hsp40) (Melville et al., 1997; Melville et al., 1999) and P52\textsuperscript{IPK} (Gale et al., 1998). During influenza A virus infection P58\textsuperscript{IPK} is activated through its dissociation with hsp40 (Melville et al., 1999). Although its anti-P58\textsuperscript{IPK} activity has been established, how P52\textsuperscript{IPK} may be affected during influenza A virus infection has yet to be determined. Other than physical contact, the precise mechanism of PKR inhibition imposed by P58\textsuperscript{IPK} is not clear at this time. It does appear to involve the ATPase activity of heat shock protein 70 (Hsp70), a protein factor associated with P58\textsuperscript{IPK} (Melville et al., 1999). However, due to the fact hsp70 is a chaperone protein, it has been speculated that P58\textsuperscript{IPK} may somehow act as a co-chaperone in activating an hsp70 mediated re-folding of the PKR protein, rendering it non-functional. A non-functional PKR leads to a reduction in phosphorylation of eIF2\textalpha, which in turn leads to an increase in protein synthesis.

Research objectives

Therefore, as there is an apparent role for cap-independent translation in eukaryotic cells two research objectives were identified: 1. How do levels of cap-dependent translation compare to cap-independent translation in the host cell during influenza A virus infection? 2. How are 4E-BP1 and eIF4E, key factors in translation initiation, affected during influenza A virus infection?

To study the first objective, a dual luciferase assay was used, which measured the levels of expression of two individual reporter genes in a given sample (summarized in Fig. 4). In Promega's Dual-Luciferase Assay Reporter System the firefly (Photinus
pyralis) and renilla (Renilla reniformis) luciferases are measured in a single lysate by two consecutive chemical reactions. In order to maintain equal levels of the two reporter gene transcripts, a bicistronic reporter construct was employed, which transcribes a single mRNA coding for both the renilla and firefly luciferases. The translation of the renilla luciferase (Rluc) cistron was initiated by the 5’ cap structure. The translation of the firefly luciferase (Fluc) was initiated by the inter-cistronic poliovirus IRES. Therefore, by expressing two different luciferases, a comparison of the relationship between cap-dependent and cap-independent translation in cells under a particular treatment was made. Additionally, since the two messages are on a single mRNA, thereby automatically eliminating any variance in the respective levels of the two mRNA transcripts, direct measurements of cap-dependent and cap-independent translation in a single lysate were taken.

As stated previously, the dual luciferase assay uses two consecutive reactions for the measurement of the two luciferases in a given sample. The firefly and Renilla luciferases come from distinct evolutionary origins and because of this have distinct chemical requirements. Specifically, in Promega’s dual luciferase assay the Fluc uses a Beetle Luciferin substrate whereas the Rluc uses a coelenterazine substrate. Additionally, neither Rluc nor Fluc require any post-translational modification for activity; therefore, both luciferases are competent for a luciferase reaction directly following translation (de Wet et al., 1985; Wood et al., 1984). This eliminates the possibility of additional post-translation regulation affecting the activity of either of the enzymes, which lends to a more clearly defined measurement. The Fluc activity is first measured following addition of the sample lysate to the reaction mixture. A second reagent is then
added to the mixture, which quenches the first reaction and initiates the second reaction so the Rluc activity can be measured. The effect of influenza A virus infection on 4E-BP1 and eIF4E phosphorylation was measured using phosphorylation-specific antibodies. By comparing western blots using either the phosphorylation-specific or total protein antibodies, an evaluation of the phosphorylation state of the proteins was assessed in influenza A virus infected cells.
Summary of Dual Luciferase Protocol

Step 1
Transfect pcDNA3-rLuc-pIRES-fLuc

Step 2
Infect 8 hours post transfection

Step 3
Lyse cells

Step 4
Measure luciferase activities and calculate Rluc/Fluc ratio from a single lysate

Figure 4. A summary of the dual luciferase assay protocol
Chapter 2: Results

Infection post transfection of the bicistronic reporter construct was determined to be optimal at 8 hours post transfection

CHO cells were transfected with the bicistronic luciferase reporter plasmid and at various times post infection cells were harvested beginning at 1 hour post transfection and taken every hour until 11 hours post transfection. An additional time point at 24 hours post transfection was harvested as a positive control. As figure 5 illustrates very little luciferase activity was detectable until 6 or seven hours post transfection. At 8 hours post transfection both luciferase activities were detectable. However, when compared to the levels observed at 24 hours post transfection these activities were relatively insignificant. Therefore, in order to minimize the amount of luciferase present in the system prior to infection, 8 hours post transfection was determined to be the optimal time from which to begin the virus infections.
Figure 5. Luciferase expression post transfection of pcDNA3-rLuc-polIRES-fLuc and Untransfected Control (C) CHO Cells.

CHO cells were transfected with pcDNA3-rLuc-polIRES-fLuc or pcDNA3.1+ as a negative control. Cells were harvested at the indicated time post-transfection and analyzed for luciferase activities.
Transfection of CHO cells using TransFast transfection reagent did not inhibit infection

To test whether transfection of CHO cells would inhibit virus infection, CHO cells were either transfected in 24 well plates on glass cover slips with the control vector plasmid, pcDNA3.1+, or left untreated. Following an 8 hour transfection period the cells were infected with WSN (MOI=10). At 6 HPI the cells were fixed in 100% methanol at –20° C for 15 minutes and stained with a FITC conjugated antibody directed against influenza A virus NP protein. These cells were then visualized with confocal-microscopy (Fig. 6). No difference in the ratio of positive to negative cells in the transfected and non-transfected CHO cells was observed. Therefore, it was concluded that transfection did not affect the infection efficiency of CHO cells.

Figure 6. Effect of transfection on infection efficiency of CHO cells. CHO cells were either transfected with pcDNA3.1+ or left untreated. At eight hours post transfection both sets of cells were inoculated with WSN (MOI=10). At six hours post infection (HPI) the cells were fixed and stained with a FITC-conjugated antibody directed against the influenza NP protein. The upper left quadrant of each image is the FITC image only, the upper right quadrant is the negative evans blue stain and the lower right quadrant is the overlay of the two images. The antibody was tested for specificity against influenza NP protein on mock infected cells, which exhibited no reaction with the anti-NP antibody.
Influenza A/WSN/33 and influenza A/Memphis/88 infection resulted in an increased cap-dependent to cap-independent translation ratio

CHO cells were transfected with the bicistronic luciferase reporter plasmid at 8 hours prior to infection with influenza A/WSN/33 (H1N1) virus. Following infection with either WSN (MOI=10) or a mock inoculum, lysates were harvested at times 4, 6, 8, 10, 12, 14 and 16 hours post infection (HPI). These samples were analyzed using a dual luciferase reporter assay and the ratio of renilla luciferase (cap-dependent) activity to firefly luciferase (cap-independent) activity, i.e. Rluc/Fluc, was plotted against time (Fig.7a). By 16 HPI the ratio of cap-dependent to cap-independent translation in WSN infected cells had increased to greater than 6 times that of mock infected cells (P<0.0001). Another influenza A virus, influenza A/Memphis/88, was tested and at 16 HPI the ratio of Rluc/Fluc was 3.8 times that of mock infected cells (data not shown).

An increase in both luciferase activities was observed in WSN infected CHO cells early in infection, and as illustrated in figure 7b, was particularly dramatic with respect to cap-dependent translation. By 6 HPI, cap-dependent and cap-independent translation had respectively increased by factors of 3.8 and 1.6 times that of mock-infected cells (P<0.0001 for both). In mock infected cells a steady increase over time of cap-independent translation was observed. Conversely, in WSN infected cells following the initial increase, cap-independent translation underwent a steady decrease over time.

The fall off of luciferase activities at times ≥ 12 HPI is attributed to gradual loss of the monolayer due to cytopathic effect of the virus. To test whether the observed increase in the absolute values of the luciferase activities was due to an increase in translation or transcription, semi-quantitative RT-PCR was performed on CHO cells first transfected
with the bicistronic reporter plasmid and then infected with either WSN (MOI=10) or a mock inoculum. Since the firefly and renilla luciferases are encoded on a single mRNA, semi-quantitative RT-PCR analysis of only one of the two luciferases was necessary. As illustrated in figure 8a, mock-infected cells contained a greater amount of firefly luciferase message compared to WSN infected cells. β-actin was analyzed in these samples as a control and as figure 8b illustrates the mock infected cells exhibited higher levels of β-actin message compared to WSN infected cells. The observed lower levels of both β-actin and firefly luciferase messages in the virus infected cells can be attributed to the shut down of host cell mRNA transcription and possible mRNA degradation due to virus infection. This experiment was repeated and similar results were observed. Therefore, the observed increase in translation was not due to an increase in reporter gene transcription.
Figure 7. Cap-dependent to cap-independent translation ratio in WSN infected CHO cells. CHO cells were transfected with the pcDNA3-rLuc-polIRES-fLuc reporter plasmid and subsequently infected 8 hours later with either influenza A/WSN/33 (MOI=10) (♦) or a mock inoculum (●). Lysates of these cells were harvested at various times post infection and analyzed for luciferase activities as described in the methods section. (a) Mean ratios of renilla to firefly luciferase (Rluc/Fluc), i.e. cap-dependent to cap-independent translation. (b) Means of the individual luciferase activities. Standard deviations are indicated with error bars. This experiment was repeated and the results were similar.
Figure 8. Semi-quantification of reporter mRNA in WSN infected CHO cells. For relative quantification of reporter mRNA, CHO cells were transfected with pcDNA3-rLuc-pollRES-fLuc and infected as previously stated. At four hours post infection total RNA was isolated and semi-quantitative RT-PCR analysis was performed using firefly luciferase (a) and β-actin specific primers (b). In figure 8b overexposure of the gel was necessary to visualize the β-actin bands, shown beneath the original image. The cDNA dilution used for PCR is indicated. This experiment was repeated and the results were similar.
**WSN infection resulted in a shut down of host cell protein synthesis**

Since WSN infection of CHO cells resulted in an increase in the translation of the reporter genes, an $^{35}$S-pulse labeling experiment was performed on CHO cells that were either mock-infected or infected with WSN (MOI=10). Before harvesting at the respective time points (0, 2, 4 and 6 HPI), the CHO cells were starved for methionine (M) and cysteine (C) in M and C free media for 15 minutes. Following starvation these cells were treated with 100µCi of $^{35}$S-methionine/cysteine and labeled for 30 minutes. Following the labeling period the cells were harvested in SDS-PAGE loading buffer. Once all the time points had been collected, the samples were subjected to SDS-PAGE. The gel was dried onto filter paper and exposed to X-ray film overnight. As figure 9 illustrates, a dramatic reduction in cellular protein synthesis was observed in WSN infected CHO cells compared to their respective mock controls.
Figure 9. Shut-down of host protein synthesis in WSN infected CHO cells. CHO cells were infected with either WSN (MOI=10) or a mock inoculum and $^{35}$S pulse labeled prior to harvesting at the indicated time post infection. The lysates were then subjected to SDS-PAGE and autoradiography.
Expression of the Memphis/88 NS1 resulted in an increased ratio of cap-dependent to cap-independent translation

Since influenza NS1 has been implicated as an activator of translation (Salvatore et al., 2002), it was tested whether CHO cells transfected with both the bicistronic reporter plasmid and an NS1 expression plasmid would exhibit an increase in the cap-dependent to cap-independent translation ratio when compared to control vector transfected cells. As shown in figure 10a, the Rluc/Fluc ratio increased 1.3 times in cells transfected with the NS1 expression plasmid when compared to the control plasmid (P<0.0001). As illustrated in figure 10b, an increase in both renilla and firefly luciferase activities, 3.3 and 2.6 times respectively, was observed in NS1 transfected cells compared to controls (P=0.001 and 0.0019 respectively). Thus, similarly to WSN infection, a greater increase in cap-dependent translation was observed compared to the cap-independent form. The increase in reporter activity following transfection of the NS1 expression plasmid was consistent with other researchers’ results. Salvatore et al. have determined NS1 to be a translational activator based on reporter gene activities in NS1 expressing cells (Salvatore et al., 2002). The observations of the present study suggest the translation activation induced by NS1 is predominantly cap-dependent. To test whether the increase in the absolute values of the luciferase activities was due to an increase in translation rather than transcription, semi-quantitative RT-PCR was performed on CHO cells transfected with both the bicistronic reporter plasmid as well as with either the NS1 expression plasmid or a control vector. As illustrated in figure 11a, CHO cells expressing NS1 exhibited lower expression of firefly luciferase message compared to vector transfected cells. β-actin was also analyzed in these samples as a
control (Fig. 11b). The lower level of firefly luciferase message can be attributed to the NS1 imposed inhibition of post-transcriptional processing of cellular pre-mRNAs (Krug et al., 2003). This experiment was repeated and similar results were observed. Therefore, the observed increase in translation was not due to an increase in reporter gene transcription.
Figure 10. Cap-dependent to cap-independent translation ratio in NS1 expressing CHO cells. CHO cells were transfected with the pcDNA3-rLuc-pollRES-fLuc reporter plasmid together with an NS1 expression plasmid or a control vector plasmid. At twenty four hours post transfection the cells were lysed and analyzed for luciferase activities as described in the methods section. (a) Mean ratios of renilla to firefly luciferase (Rluc/Fluc), i.e. cap-dependent to cap-independent translation. (b) Means of the individual luciferase activities. Standard deviations are indicated with error bars. This experiment was repeated and the results were similar.
Figure 11. Semi-quantification of reporter mRNA in NS1 expressing CHO cells. For relative quantification of reporter mRNA, CHO cells were transfected with pcDNA3-rLuc-poliRES-fLuc and either an NS1 expression plasmid or a control vector plasmid. At twenty four hours post transfection total RNA was isolated and semi-quantitative RT-PCR analysis was performed using firefly luciferase (a) and β-actin specific primers (b). The cDNA dilution used for PCR is indicated. This experiment was repeated and the results were similar.
Infection with influenza A virus devoid of NS1 expression (delNS1) induced less of an increase in the ratio of cap-dependent to cap-independent translation compared to a wild-type virus

Based on the results from the previous NS1 expression experiment, it was hypothesized that an influenza virus devoid of NS1 expression would not induce an increase in the ratio of cap-dependent to cap-independent translation. An NS1 deleted mutant virus, delNS1, developed by Garcia-Sastre and co-workers was used to test this hypothesis. This group has established that an NS1 deleted influenza virus can replicate efficiently in type I interferon deficient systems such as Vero cells (Diaz et al., 1988; Garcia-Sastre et al., 1998). Therefore, Vero cells were first transfected with the bicistronic reporter plasmid and subsequently infected 8 hours later with delNS1 influenza virus (MOI=10). As shown in figure 12a, delNS1 infection of Vero cells produced less of an increase in the cap-dependent to cap-independent translation ratio compared to WSN infection. Although the delNS1 virus induced a statistically significant ratio increase in Vero cells (P<0.0001), it was only ~70% that of the WSN induction (Fig. 12a). The ratio increase was due to a relative decrease in cap-independent translation (P=0.0157). Additionally, the observed increase in luciferase activity following WSN infection of CHO cells was not observed following WSN infection of Vero cells (Fig 12b).
Figure 12. Cap-dependent to cap-independent translation ratio in delNS1 infected Vero cells. Vero cells were transfected with the pcDNA3-rLuc-polIRES-fLuc reporter plasmid and subsequently infected 8 hours later with either delNS1 influenza virus (MOI=10) (■) or a mock inoculum (○). Also, as controls, pcDNA3-rLuc-polIRES-fLuc transfected Vero cells were infected with either influenza A/WSN/33 (MOI=10) (▲) or a mock inoculum (△) and lysed 16 hours post infection. Lysates of these cells were analyzed for luciferase activities as described in the methods section. (a) Mean ratios of renilla to firefly luciferase (Rluc/Fluc), i.e. cap-dependent to cap-independent translation. (b) Means of the individual luciferase activities in the WSN infected Vero cells. Standard deviations are indicated with error bars. This experiment was repeated and the results were similar.
Expression of NS1 coupled with infection of delNS1 resulted in a wild-type increase in the ratio of cap-dependent to cap-independent translation

To confirm that NS1 was necessary to produce a more wild-type increase in the cap-dependent to cap-independent translation ratio, Vero cells were transfected with the bicistronic reporter plasmid as well as with either the NS1 expression plasmid or an empty control vector plasmid. These cells were then infected with delNS1 (MOI=10) or a mock inoculum, lysed at 16 HPI and analyzed for luciferase activities. DelNS1 infection coupled with NS1 expression rescued a more wild-type cap-dependent to cap-independent translation ratio increase when compared to the vector transfected/mock infected control cells (P<0.0001) (Fig. 13a). A slight ratio increase was again observed in cells expressing NS1 and in cells infected with delNS1 (P=0.0039 and 0.0055 respectively). As illustrated in figure 13b, an increase in both the renilla and firefly luciferase activities, by factors of 2 and 1.3 respectively, was observed in NS1 transfected/mock infected cells compared to controls (P=0.0001 and 0.0157 respectively). Additionally, expression of NS1 increased cap-dependent translation in Vero cells infected with delNS1 when compared to the vector transfected/delNS1 infected cells (P=0.0002). The more wild-type increase in the translation ratio rescued by expression of NS1 in Vero cells infected with delNS1 was specifically due to an increase in cap-dependent translation.
Figure 13. Rescue of a more wild-type translation ratio in delNS1 infected Vero cells by coupled NS1 expression. Vero cells were transfected with the pcDNA3-rLuc-polIRES-fLuc reporter plasmid as well as either an NS1 expression plasmid or a control vector plasmid. Three wells of each group were infected 8 hours later with delNS1 influenza virus (MOI=10) and 3 with a mock inoculum. Lysates of these cells were harvested at 16 hours post infection and analyzed for luciferase activities as described in the methods section. (a) Mean ratios of renilla to firefly luciferase (Rluc/Fluc), i.e. cap-dependent to cap-independent translation. (b) Means of the individual luciferase activities. Standard deviations are indicated with error bars. This experiment was repeated and the results were similar.
Addition of interferon-α did not increase reporter translation in NS1 expressing Vero cells

Since Vero cells are deficient in their expression of type I IFN, it was hypothesized this could be the reason Vero cells do not exhibit the dramatic increase in luciferase activity upon influenza A virus infection or transfection of an NS1 expression plasmid. Vero cells were transfected with both the bicistronic reporter plasmid and the NS1 expression plasmid. Cells were then either treated with INF-α or left untreated. As figure 14 illustrates, cells treated with INF-α exhibited less reporter gene activity compared to the untreated controls. Therefore, INF-α treatment of Vero cells did not rescue a more wild-type, NS1-induced increase in reporter gene translation compared to controls.

Figure 14. Reporter activity in interferon-alpha treated Vero cells expressing NS1. Vero cells were transfected with the pcDNA3-rLuc-polIRES-fluc reporter plasmid as well as the NS1 expression plasmid. Half of these cells were treated with IFN-α and half without treatment as indicated. At twenty four hours post transfection the cells were harvested and analyzed for luciferase activities.
Influenza infection resulted in reduced phosphorylation of 4E-BP1 in late infection in CHO cells

Since influenza A virus infected CHO cells exhibited high levels of translation, it was hypothesized that 4E-BP1 is deactivated during infection in order to enhance translation. Therefore, CHO cells were infected with WSN (MOI=10) or a mock inoculum and later harvested at 4, 8, 12 and 16 HPI in SDS-PAGE loading buffer. These lysates were then subjected to SDS-PAGE, transferred and western blotted individually with antibodies against 4E-BP1 phosphorylated at either S-65 or T-70. Following image analysis, these blots were washed and incubated with an antibody directed against total 4E-BP1 (Fig. 15). Once the blots were reanalyzed a comparison between phosphorylated and non-phosphorylated 4E-BP1 was performed. In infected cells, 4E-BP1 remained phosphorylated at both S-65 and T-70 at levels equivalent to mock infected cells until the later times post infection. At 16 HPI phosphorylation at S-65 had been reduced on the order of 30% compared to mock infected cells. However, no significant difference was noted with respect to T-70. Additionally, at 16 HPI faster migrating species, indicating dephosphorylation of 4E-BP1, were observed.
**Figure 15. 4E-BP1 phosphorylation in WSN infected CHO cells.** CHO cells were infected with WSN (MOI=10) or a mock inoculum. Cells were lysed at 4, 8, 12 and 16 HPI and subjected to SDS-PAGE, transferred and western blotted against (a) Phospho-S-65 4E-BP1 or (b) Phospho-T-70 4E-BP1. Both blots were then re-probed with an antibody against total 4E-BP1. A similar experiment, harvesting cells at 8 and 16 HPI was repeated 2 additional times and the results were similar.
Influenza infection did not affect the phosphorylation state of 4E-BP1 in MDCK cells

Since CHO cells were not permissive for influenza A virus infection in our system it was questioned whether, like CHO cells, MDCK cells would also exhibit high levels of 4E-BP1 phosphorylation. Therefore, MDCK cells were infected with WSN (MOI=10) and later harvested at 4, 8 HPI in SDS-PAGE loading buffer. These lysates were then subjected to SDS-PAGE in duplicate wells, transferred and western blotted individually with antibodies against 4E-BP1 either phosphorylated at S-65 or T-70. Following X-ray exposure and development, these blots were washed and incubated with an antibody directed against total 4E-BP1 (Fig. 16). Once the blots were reanalyzed a comparison between phosphorylated and non-phosphorylated 4E-BP1 was performed. 4E-BP1 remained phosphorylated at both S-65 and T-70 at levels equivalent to mock infected MDCK cells.
Figure 16. **4E-BP1 phosphorylation in WSN infected MDCK cells.** MDCK cells were infected with WSN (MOI=10) or a mock inoculum. Cells were lysed at 4 and 8 HPI and subjected to SDS-PAGE, transferred and western blotted against (a) Phospho-S-65 4E-BP1 or (b) Phospho-T-70 4E-BP1. Both blots were then re-probed with an antibody against total 4E-BP1. This experiment was repeated and the results were similar.
Chapter 3: Discussion

WSN infection of CHO cells resulted in an increase in the ratio of cap-dependent to cap-independent translation compared to mock infected cells (Fig. 7a). In order to understand why this ratio changed, the absolute luciferase values, in relative light units (RLU), at each of the time points must be examined (Fig. 7b). Infection with influenza virus initiated a dramatic increase in both of the reporter genes. Initially it was postulated this increase could be the result of transcriptional activation due to interaction of the virus with the CMV promoter on the reporter plasmid. However, according to the semi-quantitative RT-PCR results, the increase in luciferase activity was not due to transcriptional activation, but rather to an increase in translation of the available mRNA (Fig. 8). This is contrary to the well documented influenza induced shut-down of host encoded protein synthesis, which this study confirmed (Fig. 9). Therefore, the observed increase in luciferase translation in CHO cells was not likely due to a block or defect in the influenza induced protein synthesis shut-down mechanism. It was concluded the bicistronic reporter transcript exhibits increased translation efficiency during influenza infection. In an effort to explain these results the 5' UTR coded for by the bicistronic reporter construct was sequenced for the possible identification GRSF-1 binding sites.

A recently discovered cellular protein, GRSF-1, has been implicated in the selective translation of influenza mRNAs (Kash et al., 2002; Park et al., 1999b). The 5' UTR of influenza mRNAs contain a conserved sequence, which is recognized by GRSF-1. The conserved sequence, AGGGU, as well as some variations noted in table 1 have been found to bind GRSF-1 \textit{in vitro} in gel shift assays and it is proposed this
binding somehow increases the translation efficiency of mRNAs (Park et al., 1999b). In their work on GRSF-1, Park and colleagues have demonstrated that removal of GRSF-1 in HeLa cell extracts through immunodepletion reduces the translation of an mRNA containing the 5’ conserved sequence of the influenza A virus NP protein mRNA. Furthermore, by reintroducing the GRSF-1, they are able to rescue wild-type translation levels. Although sequencing the pcDNA3-rLuc-polIRES-fLuc UTR did not reveal one of the published GRSF-1 binding sites, a similar sequence was identified. The 5’ UTR coded for by pcDNA3-rLuc-polIRES-fLuc contains an AGGGA, which could account for the observed increase in translation of the luciferase reporters during influenza A virus infection of CHO cells. To assess the ability of this sequence in increasing translation efficiency, deletion and substitution mutant clones of pcDNA3-rLuc-polIRES-fLuc would need to be generated. The resulting plasmids would then be tested in a side-by-side comparison with the parental plasmid for their translation efficiency during influenza A virus infection.

Interestingly, CHO cells transfected with the NS1 expression plasmid together with the bicistronic reporter plasmid also exhibited an increase in the translation of the luciferase genes (Fig. 10b). Additionally, as in the influenza infected CHO cells, the observed translation increase in NS1 expressing cells favored cap-dependent translation over cap-independent translation (Fig. 10a). The increase in the translation of the luciferase reporters observed in the NS1 transfected cells implies NS1 plays a role in the previous observations of a virus induced translation increase. This translational increase was again confirmed with semi-quantitative RT-PCR (Fig. 11). In comparison, however, the amount of β-actin appeared not to have been as severely
reduced in the NS1 transfected cells compared to those levels observed in the influenza
A virus infected cells. These findings are attributed to the transfection efficiency of the
system. Since an MOI of 10 was used in the infection experiments, it is safe to
presume, which the fluorescence staining experiments later confirmed, that nearly
100% of the cells were infected with influenza A virus. Therefore, since infection with
influenza A virus shuts down transcription and nuclear to cytoplasmic export of cellular
mRNAs, a severe reduction in β-actin mRNA levels was observed. However, in the
NS1 expression experiment, transfection of the plasmid was not as efficient as the
infection, approximately 10%. If only 10% of a population of cells is expressing NS1, it
would be consistent that an analysis of the β-actin mRNA levels in a population would
not detect a drastic decrease in β-actin mRNA.

Interestingly, Vero cells infected with influenza A virus did not exhibit an increase
in the absolute values of the luciferase reporters as was the case in the CHO cells (Fig.
12b, 7b). However, an increase was observe in the absolute values of the luciferase
reporters following transfection of the NS1 expression plasmid in Vero cells (Fig. 13b).
It is unclear why such a discrepancy between these two cell lines would be observed.
However, given their different sources and composition, different translation regulatory
properties may be evident. Vero cells do not express type I IFN due to a deletion of
these genes from the genome. Although it seems contrary given type I IFN’s known
inhibition on translation, it was hypothesized lack of type I IFN could be responsible for
both an absence in increased levels of reporter gene translation during infection and a
lesser increase during NS1 expression. To test this, IFN-α was added to the cell culture
media following transfection of the NS1 expression plasmid, which led to a decrease in
reporter translation compared to controls (Fig. 14). Therefore, it was concluded the lack of an increase in reporter translation due to influenza A virus in Vero cells was not due to their inability to induce an IFN-α response. Nevertheless, influenza A virus infection of Vero cells did result in an increase in the ratio of cap-dependent to cap-independent translation compared to mock infected cells (Fig. 12a).

Although upon initial examination, it appeared that CHO and Vero cells behaved differently during influenza infection, a closer analysis revealed the translation ratio increases observed in both cell lines followed similar patterns. CHO cells infected with influenza A virus initially exhibited an increase in both the cap-dependent and cap-independent reporter. However, following this initial increase a small, but steady decrease over time in the cap-independent reporter, Fluc, was observed. This is in contrast to the cap-dependent reporter, Rluc, which continued to increase until a steady state level was reached (Fig. 7b). Cap-independent translation was diminished when compared to cap-dependent translation, which was increased to a peak level and then maintained. Upon examination of Vero cells infected with influenza A virus a similar trend is visible. Although no increase in reporter activity was observed in infected Vero cells, the activity of the cap-independent reporter decreased, whereas the activity of the cap-dependent reporter remained as high as the mock infected cells (Fig. 12b). Since cytopathic effect (CPE) resulting from virus infection was not controlled for, it is possible the lack of increased luciferase activity in Vero cells was due to virus infection-induced loss of the monolayer. Nevertheless, compared to mock controls it was specifically the inhibition of cap-independent translation that was fully responsible for the observed increase in the ratio of cap-dependent to cap-independent translation during influenza A
virus infection of Vero cells. Since an increase in cap-dependent translation was also observed, the negative regulation of cap-independent translation is only partly responsible for the increase in the ratio of cap-dependent to cap-independent translation in influenza A virus infected CHO cells.

Since there is an apparent role for NS1 in increasing the ratio of cap-dependent to cap-independent translation during influenza A virus infection, it was hypothesized that infection with delNS1, a virus devoid of NS1 expression, would not result in an increase in the ratio of cap-dependent to cap-independent translation. However, delNS1 infection of Vero cells did result in an increase in the translation ratio, yet this ratio increase was less than that observed in WSN infected Vero cells (Fig. 12a). Therefore, although NS1 appears not to be solely responsible for the increase in the ratio of cap-dependent to cap-independent translation in influenza A virus infected cells, it may be necessary for a wild-type increase. To confirm this, a rescue experiment was designed where NS1 expression was coupled with delNS1 infection of Vero cells. If NS1 expression is required for a wild-type increase in the ratio of cap-dependent to cap-independent translation in influenza A virus infected cells, coupling NS1 expression with delNS1 virus infection should result in a more wild-type translational response. This proved to be the case as NS1 expression coupled with delNS1 virus infection resulted in a more wild-type translation ratio increase (Fig. 13a). Consistent with the previous results in NS1 expressing cells, the rescued translation ratio increase was specifically due to an increase in cap-dependent translation (Fig. 13b). These data further solidify that NS1 expression plays a crucial role in the influenza A virus induced increase in the ratio of cap-dependent to cap-independent translation.
Although it is possible the relatively small translation ratio increase observe in NS1 expressing cells was due to low transfection efficiency, there is an alternative explanation, which may also explain why infection with delNS1 virus coupled with NS1 expression resulted in a more wild-type translation ratio. The NS1 protein is found in the nucleus and cytoplasm of infected cells (Greenspan et al., 1988). NS1 nuclear localization is presumably due to two separate nuclear localization signals (NLS), which aid in the translocation of NS1 from the cytoplasm to the nucleus during infection (Greenspan et al., 1988). NLS usually consist of short stretches of positively charged amino acids. In contrast, nuclear export signals (NES) often consist of short leucine-rich sequences. NS1 contains one such NES in the first 13 amino acids of the amino terminal portion of the effector domain (amino acids 134-147) (Greenspan et al., 1988). Since influenza A virus NS1 is found in both the cytoplasm and nucleus of infected cells, there must be a regulation strategy in place for trafficking NS1 in-and-out of the nucleus. Li and colleagues show that expression of an NS1 fragment containing one of the NLS (amino acids 1-134) results in total nuclear localization. Conversely, expression of an NS1 fragment containing one of the NLS and the NES (amino acids 1-147) results in complete cytoplasmic localization. However, the adjacent 14 amino acids (amino acids 148-161) appear to serve as an inhibitor for the NES as an NS1 fragment consisting of amino acids 1-161 is localized in both the nucleus and the cytoplasm of transfected cells. Addition of the second NLS leads to the complete nuclear retention of the protein fragment, which is also the case for the full-length protein (Li et al., 1998). Therefore, the 14 amino acid sequence (amino acids 148-161) of influenza A virus NS1 may serve as an inhibitor for cytoplasmic localization. The authors hypothesize that since
influenza infection results in NS1 cytoplasmic and nuclear localization, there is a viral protein(s) that is responsible for “unmasking” the NES from the 14 amino acid inhibitor (Li et al., 1998). However, as yet no influenza A virus protein has been implicated in performing this unmasking. A similar scenario has been described in adenovirus infected cells. The NES of the adenovirus E4 34-kDa protein is active only when the protein is bound to the E1B 55-kDa viral protein (Dobbelstein et al., 1997). Regulation of NS1 localization may explain the discrepancy in the cap-dependent to cap-independent translation ratio between cells expressing NS1 and cells both expressing NS1 and infected with delNS1 influenza virus. Although not tested, cells expressing NS1 alone may have the majority of NS1 localized in the nucleus, which could diminish any effect NS1 may have on translation. However, infection of delNS1 influenza coupled with NS1 expression may provide the necessary protein(s) for unmasking the NES of NS1, which could result in more cytoplasmic NS1 localization and therefore a greater effect on the ratio of cap-dependent to cap-independent translation. The localization of NS1 in NS1 expressing/delNS1 virus infected cells has not been established and therefore it is currently not known whether the localization of NS1 may contribute to the rescue of a wild-type translation ratio in these cells compared to cells expressing NS1 alone.

The rescue experiment in Vero cells illustrates the two components of the influenza A virus infection-induced increase in the ratio of cap-dependent to cap-independent translation. It is possible for the translation ratio increase to be due to an increase in cap-dependent translation. On the other hand, it is also possible for the translation ratio increase to be due to a decrease in cap-independent translation. For
the case of influenza A virus infection, it appears both components are needed for a wild-type increase in the ratio of cap-dependent to cap-independent translation. In the WSN infected Vero cells, there was no change in cap-dependent translation compared to mock infected cells. What was responsible for the ratio increase during WSN infection of Vero cells was the inhibition of cap-independent translation (Fig. 12b). Conversely, delNS1 infection of Vero cells resulted in a decrease in both cap-independent and cap-dependent translation compared to mock infected cells (Fig. 13b). Therefore, because delNS1 was unable to maintain a cap-dependent translation level equal to that of the mock infection, it induced less of a ratio increase. However, coupling NS1 expression with delNS1 infection resulted in an increase in cap-dependent translation and therefore a more wild-type increase in the translation ratio (Fig. 13b). Since delNS1 influenza virus is capable of reducing cap-independent translation there must be a viral protein(s) other than NS1, which is capable of inhibiting cap-independent translation. A possible mechanism for this will be discussed later.

CHO cells were chosen for their propensity for transfection and their ability to be infected by influenza A virus. Before beginning the infection/transfection experiments the optimal time for virus infection post transfection of the bicistronic reporter plasmid was determined. Due to the half lives of Fluc and Rluc, 3 and 5 hours respectively (Bronstein et al., 1994; Thompson et al., 1991), this was necessary to minimize the level of luciferase translated pre-infection. In a time course experiment where cells were harvested at various times post transfection, It was determined that 8 hours post transfection was optimal since, although both luciferase activities were measurable, they were relatively insignificant when compared to the activities measured at 24 hours
post transfection. Also, in the Promega Dual Luciferase Reporter Assay System Fluc produces approximately 53% more light than Rluc. Therefore, although the light output of Fluc and Rluc are not molar equivalents, the light output does represent the trend of cap-dependent and cap-independent translation in the system used. Since the 53% discrepancy was not adjusted for in these analyses, the amount of Fluc, i.e. cap-independent translation, has been overestimated. Furthermore, due to this overestimation, calculations of the ratio of cap-dependent to cap-independent translation have been conservative.

Although influenza A virus can infect CHO cells, a block in virus growth in CHO cells was observed in this study. This was discovered when attempting to grow a stock of WSN virus in CHO cells. Following infection, no hemagglutination (HA) titer at 1, 2 or 3 days post infection was observed. It is unclear why these results were observed especially in light of the observed production of viral proteins in the $^{35}$S pulse labeling experiment (Fig. 9). Additionally, there has been a previous report of WSN virus growth in CHO cells (Chelbi-Alix et al., 1998). Nevertheless, since a normal shut-down of host encoded protein synthesis was observed in CHO cells upon infection with virus grown in MDCK cells, it was concluded CHO cells would be an adequate model for studying influenza A virus translational control (Fig. 9). There is a range of possibilities for explaining the lack of virus growth in CHO cells. Perhaps the particular WSN strain used in these experiments is simply not well adapted for growth in CHO cells and although amplification is not possible, overwhelming the cells with a high MOI resulted in infection.
Some have suggested that transfecting cells with plasmid DNA results in a reduction in the efficiency of infection. However, the data presented here do not support this suspicion as is evident in the comparison of fluorescently stained transfected and non-transfected, influenza A virus infected CHO cells (Fig. 6). However, that is not to say that in all situations transfection has no effect on virus infection. A precise mechanism for reduced infection efficiency due to transfection would be difficult to analyze since a wide variety of transfection reagents and techniques are used throughout the scientific community. However, for the case of lipid transfection reagents, such as the TransFast Reagent (Promega) used in this work, it has been suggested the interferon system may be activated. If an interferon response is elicited prior to infection, it is plausible that cells would have ample time to activate the production of anti-viral molecules and in general, bringing the cell into an anti-viral state. Of the anti-viral proteins induced from a type I IFN response, there are three most highly understood: PKR, 2’,5’-Oligoadenylate Synthetase (OAS)/RNAse L and the Mx proteins (Basler & Garcia-Sastre, 2002). Although a basal level of PKR is expressed in cells for the purpose of acute activation from dsRNA, PKR gene expression is increased upon activation of type I IFN (Nakaya et al., 2001). An increase in PKR expression in conjunction with the appropriate activating substrate, including but possibly not limited to dsRNA, could lead to the phosphorylation of eIF2α and therefore an inhibition of translation. OAS/RNAse L leads to a degradation of cellular as well as viral mRNA (Justesen et al., 2000). Upon activation by type I IFN, OAS converts ATP into 2’,5’-oligoadenylates which in turn activate the degradation activity of RNAse L (Nakaya et al., 2001). Finally, induction of the Mx proteins has been shown to interfere
with viral RNA synthesis and vRNP transport (Krug et al., 1985; Weber et al., 2000). Taken together, lipid transfection reagent-dependent induction of any of the mentioned type I IFN effector molecules could result in the inhibition of viral infection.

According to experiments using the phosphorylation-specific antibodies, 4E-BP1 phosphorylation remained high during influenza A virus infection (Fig. 15 and 16). A reduction in the phosphorylation of 4E-BP1 was not observed until late in infection of CHO cells (Fig. 15). Additionally, it appeared that 4E-BP1 was highly phosphorylated in MDCK cells during influenza A virus infection (Fig. 16). Bolstering the phosphorylation-specific antibody data was the observation of faster migrating species of 4E-BP1 present in the influenza A virus infected CHO cells late in infection. Faster migration on SDS-PAGE of 4E-BP1 is indicative of a reduced phosphorylation state of the protein. Finally, in agreement with the theory as to the phosphorylation hierarchy of 4E-BP1, the band directly below the slowest (most highly phosphorylated) band is only visible when using the phospho-T-70 antibody and not the phospho-S-65 antibody. This would indicate that S-65 is either the last site phosphorylated or the first to be dephosphorylated.

Since many stresses, including viral infection, have been found to result in a dephosphorylation of the 4E-BPs (Gingras et al., 1996; Svitkin et al., 1998), observations of high phosphorylation levels are somewhat surprising. However, since influenza A virus appears to be adept at combating the cellular defenses, regulation favoring 4E-BP1 deactivation is not unreasonable. Highly phosphorylated 4E-BP1 would be beneficial for influenza virus replication since influenza A virus mRNAs are translated in a cap-dependent manner. Additionally, observations of a highly
phosphorylated 4E-BP1 are consistent with the high cap-dependent translation found 8-10 HPI in CHO cells. 4E-BP1 dephosphorylation has been associated with apoptosis and since influenza A virus induces apoptosis in cells (Horton et al., 2002; Lin et al., 2002; Takizawa et al., 1993; Tee & Proud, 2000), this is likely the reason why 4E-BP1 is dephosphorylated later in infection. Additionally, it remains to be investigated whether influenza virus infection is involved in up-regulating the phosphorylation of 4E-BP1. Since virus infection can be considered a cellular stress, it is possible the host cell’s defense system would lead to the dephosphorylation of 4E-BP1 in an attempt to shut down translation, much like it leads to the phosphorylation of eIF2α. In fact there is a report, which hints at the possibility of PKR activation leading to the dephosphorylation of 4E-BP1 (Jeffrey et al., 2002). PKR regulation leading to dephosphorylation of 4E-BP1 would be logical since both 4E-BP1 dephosphorylation and eIF2α phosphorylation have similar inhibitory effects on translation. As stated previously, eIF2α is phosphorylated by PKR upon activation by double stranded RNA. The effects, if any, dsRNA has on 4E-BP1 phosphorylation has not been determined. However, if dsRNA did lead to a dephosphorylation of 4E-BP1 through a PKR-dependent pathway, this would likely be inhibited by NS1 through the NS1-sequestering of dsRNA. Alternatively, the induction of the PKR inhibitor, P58IPK, would also likely inhibit any PKR induced dephosphorylation of 4E-BP1.

It has been shown that eIF4E is dephosphorylated in influenza infected cells (Feigenblum & Schneider, 1993). Moreover, eIF4E phosphorylation has been implicated in regulating the ratio of cap-dependent to cap-independent translation, albeit with conflicting results. eIF4E phosphorylation by over-expressed MNK1 has been
linked to the down-regulation of cap-dependent translation, and furthermore, expression of a non-phosphorylatable mutant eIF4E results in “partial reversal of this effect,” i.e. with non-phosphorylatable eIF4E, MNK1 activity has little effect on cap-dependent translation (Knauf et al., 2001). However, another group has recently presented data to the contrary, that dephosphorylation of eIF4E results in an increase in cap-independent translation (Dyer et al., 2003). eIF4E phosphorylation was a logical characteristic to analyze during influenza A virus infection. Unfortunately, detection of any consistent level of eIF4E phosphorylation in either mock or influenza infected CHO cells was not possible under serum free conditions. The validity of the phosphorylation-specific eIF4E antibody was assessed by treating CHO cells with 20% FBS for 2 hours prior to harvesting. There are two possibilities which would account for these negative results: 1) eIF4E is not phosphorylated in this system due to the use of a serum free cell culture medium throughout the experiments. 2) The level of eIF4E phosphorylation is simply below levels detectable with the phosphorylation-specific antibody. In either case, these results are inadequate to define how eIF4E phosphorylation is affected in influenza A virus infected CHO cells. Likewise, what effect this phosphorylation has on the ratio of cap-dependent to cap-independent translation during influenza A virus infection remains unknown.

Influenza A virus infection results in the preferential translation of influenza viral mRNAs, which are very similar in structure to the host cell mRNAs. Even though influenza A viral mRNAs are capped at their 5’ end and polyadenylated at their 3’ end, much like cellular mRNAs, there is a dramatic reduction in the amount of cellular protein synthesis compared to the high translation efficiency of viral mRNAs. Since influenza A
virus mRNAs require the same machinery that cellular capped mRNAs require, the method for selectively translating viral messages is not an obvious one. Poliovirus infection results in the cleavage of eIF4G (Etchison et al., 1982; Lee et al., 1985), a key protein in cap-dependent translation. Since poliovirus mRNA is translated cap-independently via an IRES, regulation shutting down cap-dependent translation makes good sense. Since influenza A virus requires cap-dependent translation for its own life cycle a different strategy must be employed for translating influenza proteins first and foremost over those of the host cell.

Due to its imposed inhibition of host cell pre-mRNA processing (Chen & Krug, 2000; Chen et al., 1999; Li et al., 2001b; Nemeroff et al., 1998), the influenza A virus NS1 protein was thought to be responsible for the dramatic shut down in host cell translation. However, this theory is likely not true for a variety of reasons. In addition to inhibiting cellular pre-mRNA processing, NS1 has also been shown to increase the translation efficiencies of influenza mRNAs (Aragon et al., 2000; de la Luna et al., 1995; Enami et al., 1994; Enami & Enami, 2000). Additionally, this report as well as others, have observed increased translation of reporter genes due to NS1 expression (Salvatore et al., 2002). Although a mechanism has not been proposed for these observations, it is possible that NS1 up-regulates the activity of a host cell translational enhancer. GRSF-1 would serve as a possible candidate since the sequence coding for the UTR of the bicistronic reporter used in these experiments contains a sequence similar to those published as the putative GRSF-1 binding sequence (Table 1) (Kash et al., 2002). If the UTR of the reporter used in these experiments does contain a binding site, this would indicate an association between GRSF-1 activity and NS1 expression.
Although NS1 may localize to the nucleus of the host cell, it is not likely that NS1 acts as a transcription factor for increasing expression of GRSF-1 mRNA. Even if GRSF-1 mRNA expression was up-regulated, the NS1 inhibition of cellular pre-mRNA processing would keep those mRNAs from maturing. Therefore, any effect NS1 may have on GRSF-1 would likely involve an increase in the activity of the available GRSF-1. NS1 might accomplish this through physical interaction with GRSF-1 or through interaction with GRSF-1 regulators. Since NS1 has been found physically associated with several cellular proteins including both nuclear (CPSF and PABII) and cytoplasmic (eIF4G and PABI) proteins (Aragon et al., 2000; Burgui et al., 2003; Chen & Krug, 2000; Chen et al., 1999; Li et al., 2001b; Nemeroff et al., 1998), such interaction is plausible. An NS1 induced activation of GRSF-1 would explain the observed increase in reporter gene translation in NS1 expressing cells. It would also imply a possible mechanism for the NS1 increase in translation of influenza mRNAs, which contain GRSF-1 binding sites (Kash et al., 2002).

Another model proposed to explain the influenza A viral shut-down of host protein synthesis involves the inhibitory effect of NS1 on cellular pre-mRNA processing (Chen & Krug, 2000; Chen et al., 1999; Li et al., 2001b; Nemeroff et al., 1998). Although one would expect to observe a decrease in protein production from a reduction in mRNA expression, this theory does not take into account the fully processed and mature cellular mRNAs in the cytoplasm of the cell upon infection. These mRNAs are still competent for translation and since cellular mRNA degradation does not occur until later in infection (Zurcher et al., 2000), after the observed shut-down of host encoded protein synthesis, inhibition of pre-mRNA processing is not likely
responsible. Additionally, since delNS1 influenza virus, which does not express NS1, can induce a shut-down in host protein synthesis (Salvatore et al., 2002), neither an NS1 activation of GRSF-1 nor its inhibition of pre-mRNA processing can account for the protein synthesis shut-down. Therefore, there is another factor, or set of factors, induced by influenza A virus infection, which shuts down host protein synthesis.

The requirement for cap-dependent translation in influenza A virus infected cells is self evident due to the cap-dependent translation of influenza mRNAs. During virus infections cells often attempt to shut-down cap-dependent translation, for which PKR is largely responsible. However, since the actions of NS1 and the recruitment of P58IPK inhibit PKR activation in influenza A virus infected cells this shut-down is thwarted. There have been previous observations of eIF2α dependent increases in IRES driven translation. In two of these studies, cells were stressed with either genotoxins or amino acid starvation (Fernandez et al., 2002; Subkhankulova et al., 2001). In these studies the activities of reporters translated via the c-myc, and cat-1 IRES, respectively, were analyzed. In the remaining study, cell differentiation leading to eIF2α phosphorylation was found to cause an increase in translation driven via the vascular endothelial growth factor (VEGF), c-myc and EMCV IRES (Gerlitz et al., 2002). Although there has been an observation of eIF2α phosphorylation during poliovirus infection, a direct comparison of the effects of phosphorylated and nonphosphorylated eIF2α on the efficiency of poliovirus IRES translation has not been conducted (O'Neill & Racaniello, 1989). The authors suggest that in addition to eIF4G cleavage, eIF2α phosphorylation may aid in shutting down host cell cap-dependent translation (O'Neill & Racaniello, 1989). It is therefore possible that the poliovirus IRES can continue to initiate translation even
under conditions of high eIF2α phosphorylation. However, if this theory is taken further to hypothesize that eIF2α phosphorylation enhances poliovirus IRES driven translation; a reasonable mechanism for explaining the observation of decreased cap-independent translation during influenza virus infection may be proposed. During influenza A virus infection PKR is inactivated, inhibiting the phosphorylation of eIF2α (Katze et al., 1988). Therefore, the lack of eIF2α phosphorylation may explain the observed reduction in cap-independent translation compared to mock infected cells. To test this theory, one might express a constitutively active PKR in influenza A virus infected cells. If the lack of eIF2α phosphorylation is responsible for the reduced cap-independent translation in infected cells, the resulting hyperphosphorylation of eIF2α should increase cap-independent translation. Increased phosphorylation of eIF2α is not consistent with increased levels of any translation using the traditional eIF2-GTP-met-tRNA as the vehicle for introducing the initiating amino acid for protein synthesis. Therefore, a nontraditional use of eIF2 or a novel protein factor would likely be necessary for initiating protein synthesis from IRES sequences during stress or cell differentiation as described above.

As a whole, these data provide evidence that influenza A virus infection not only inhibits the translation of host cell mRNA, but may also enhance the translation of particular mRNA subsets. These mRNA subsets may include those containing GRSF-1 binding sites in the 5’ UTR. However, the involvement of other translational up-regulators cannot be ruled out. Although the observations of high translation levels during infection and NS1 expression may be anomalous due to the UTR of the reporter transcript, they are consistent with previous reports. There is a possible translation-enhancing sequence in the UTR of pcDNA3-rLuc-polIRES-fluc transcript that may bind
GRSF-1 or another related protein. Being a host cell protein, GRSF-1 likely provides enhanced translation of host cell mRNAs as well. Identifying these mRNAs would be interesting in establishing a function for GRSF-1. Additionally, expression of the mRNA subset for which translation is enhanced during influenza A virus infection apparently does not inhibit virus replication. If such were the case then viral evolution would have likely selected for a different translation strategy. Therefore, it would be interesting to identify how the proteins originating from this subset of mRNA affect influenza A virus infection, if at all.

These data also indicate that in comparison to cap-dependent translation, cap-independent translation is partially inhibited during influenza A virus infection. Since it has been established that cap-independent translation is variably affected by cellular stresses, this indicates a possible role for cap-independent translation in combating influenza A virus infection. Perhaps there are mRNAs translated cap-independently coding for anti-influenza genes and somehow the translation of these mRNAs is meant to be up regulated during infection.
Chapter 4: Materials and Methods

Cells and viruses

All cell culture media contained antibiotics throughout all procedures (Cambrex Corporation). Chinese Hamster Ovary (CHO) cells were grown and maintained in HyQ-CCM5 serum free media (HyClone Laboratories, Inc.). Vero cells were grown and maintained in VP-SFM serum free media (Invitrogen Corporation). Madin Darby Canine Kidney (MDCK) cells were grown and maintained in EMEM with 10% FBS (HyClone Laboratories, Inc.). All counting of cells was performed on a hemicytometer. Influenza A/Memphis/88 (H3N2) was a kind gift from Robert Webster (St. Jude Children’s Research Hospital, Memphis, TN). delNS1 influenza was a kind gift from Adolfo Garcia-Sastre (Mount Sinai School of Medicine, NY, NY). Influenza A/WSN/33 (H1N1) and influenza A/Memphis/88 viruses were grown and titrated by TCID$_{50}$ assay (see description below) in MDCK cells in OptiMEM (Invitrogen Corporation) plus TPCK-treated trypsin (1µg/ml) (Sigma-Aldrich, Inc.). delNS1 influenza virus was grown and titrated by TCID$_{50}$ assay in Vero cells in VP-SFM plus TPCK-treated trypsin (1µg/mL).

Virus stock growth

For influenzas A/WSN/33 and A/Memphis/88 MDCK cells were grown to near confluency in 75cm$^2$ flasks in their normal growth medium. On the day of infection a 1:1000 dilution of the respective stock virus was made in OptiMEM. The cell monolayer was washed 2 times in PBS and incubated in a 500µL volume of the virus dilution. After a 1 hour adsorption period at 37°C, the monolayer was washed 1 time with PBS and 12 mL of OptiMEM plus TPCK-treated trypsin (1µg/mL) was added to the flask. The cells were then incubated at 37°C for approximately 2 days before the supernatant was
collected and clarified by centrifugation. The virus containing supernatant was then aliquoted and stored at –70°C. For delNS1 influenza virus Vero cells were grown in VP-SFM to near confluency in a 75cm² flask. On the day of infection a 1:1000 dilution of the stock virus was made in VP-SFM. The cell monolayer was washed 2 times in PBS and incubated in a 500µL volume of the virus dilution. After a 1 hour adsorption period at 37°C the monolayer was washed 1 time in PBS and 12 mL of VP-SFM plus TPCK-treated trypsin (1µg/mL) was added to the flask. The cells were then incubated at 37°C for approximately 2 days before the supernatant was collected and clarified by centrifugation. The virus containing supernatant was then aliquoted and stored at –70°C.

**Hemagglutination (HA) titering**

For each sample to be titered, 2 fold serial dilutions of the sample were made in a 50µl volume of PBS. 50µl of undiluted PBS was used as a negative control. 50µl of .5% processed chicken red blood cells (see recipes) was mixed with each of the dilutions and incubated at room temperature for 30 minutes. The plates were then examined for hemagglutination. Negatives were scored as having a red button of chicken red blood cells that ran when the plate was tilted. Positives were scored as having no button or a button, which did not run when the plate was tilted.

**TCID₅₀ assay of virus titer**

MDCK and Vero cells were grown to confluency in a 25cm² flask with growth media. The day prior to setting up the TCID₅₀ these cells were trypsinized and resuspended in 10 mL of growth media and plated on a 96 well tissue culture plate at 100µL/well. On the day of titration the virus to be titrated was diluted in 10 fold serial
dilutions in OptiMEM when titrating in MDCK and in VP-SFM when titrating in Vero cells. The 96 well plate of cells was washed 2 times in PBS and 8 replicate wells representing the dilutions spanning 10^{-1}-10^{11} were inoculated using a 50 µL inoculum volume. In addition 8 wells were mock infected with OptiMEM. Following a 1 hour adsorption incubation at 37° the inoculum was removed from the wells followed by washing with 100µL of PBS/well. OptiMEM or VP-SFM plus TPCK-treated trypsin (1µg/mL) was added at a volume of 100µL/well. The plate was allowed to incubate for 3 days at 37°C before staining. Briefly, the supernatant was removed from the plate and the formalin/crystal violet stain was added and incubated for 10-15 minutes at room temperature. The stain was removed and the plate was washed in gently running water. Once the plate had dried a TCID_{50} titer was calculated using the Reedy/Munch calculation method.

**Plasmids**

The bicistronic reporter plasmid, pcDNA3-rLuc-polIRES-fLuc, was a kind gift from Nahum Sonenberg (McGill University, Montreal, Quebec, Canada). The NS1 expression plasmid, pcDNA3.1+NS1, was made by cutting the NS1 open reading frame derived from influenza A/Memphis/88 out of a pATX vector (obtained from Robert Webster, St. Jude Children's Research Hospital, Memphis, TN) and cloning it into the pcDNA3.1+ vector (Invitrogen Corporation). For expression of only NS1, the resulting cloned plasmid was then mutagenized using the Quik Change Site-Directed Mutagenesis method (Stratagene) in two locations thereby inserting two stop codons near the 5' end of the NS2 transcript, which are silent in the NS1 transcript. The forward primer used was as follows:
5'CCAGGACATACTAT\underline{AGAGGATGT}\underline{AAAAATGCAATTGGGGTCC}3'. The reverse complement to this was used as the reverse primer (Integrated DNA Technologies, Inc.). The nucleotides in underlined bold represent the two sites mutated to adenosines. These two mutations inserted two stop codons, which correspond to the 14th and 17th amino acid residues of NS2. The pcDNA3.1+NS1 sequence was confirmed by sequencing on an ABI PRISM automated sequencer (Applied Biosystems Corporation). All plasmid preparations were done using the Plasmid Midi Kit purification system (Qiagen, Inc.). DNA concentrations were determined according to absorbance readings at 260 nanometers on a spectrophotometer (Milton Roy Company). NS1 expression was confirmed by immunocytochemistry with an anti-NS1 antibody (obtained from Adolfo Garcia-Sastre, Mount Sinai School of Medicine, NY, NY). The pcDNA3 and pcDNA3.1+ expression vectors utilize a cytomegalovirus derived promoter.

**DNA sequencing**

Polymerase chain reaction (PCR) of the desired segment was performed according to the table of primers below with the indicated annealing temperatures using PWO polymerase according to the manufacturer's specifications (Roche). 25µl of the PCR reaction was run on a 1% agarose gel and the band of interest was cut out and purified using the Qiaquick gel extraction kit according to the manufacturer’s specifications (Qiagen). The resulting gel extraction was then split into two sequencing reactions, one for each primer used in PCR, using BigDye (version I) according to the manufacturer's specifications (ABI). The reaction was ethanol precipitated by adding 1/10 the volume of 3M sodium acetate and 2 times the volume of 100% ethanol. The precipitate was spun down in a microcentrifuge at maximum speed for 30 minutes. The pellet was
washed with 75% ethanol and spun in a microcentrifuge at maximum speed for 10 minutes a total of two times. The pellet was dried under a vacuum and resuspended in 20µl of TSR buffer. The sample was then heated to 95°C for 5 minutes followed by a quick cooling on ice for 5 minutes and loaded on an ABI Prism 310 sequencer according to the manufacturer.

**Dual Luciferase Assays**

*CHO infection with influenza A/WSN/33 and influenza A/Memphis/88*

CHO cells were plated at a concentration of 1X10^5 cells/well on a 24 well plate the day prior to transfection. pcDNA3-rLuc-pollRES-fLuc (0.75µg) was transfected per well at 1:1 ratio with TransFast Transfection Reagent (Promega Corporation) according to manufacturer's specifications. Following an 8 hour incubation at 37°C, the monolayers were washed 2X with PBS and inoculated with either a mock inoculum or with approximately 10 TCID_{50} units per cell of influenza A/WSN/33 virus. After 1 hour of adsorption at 37°C, the virus was removed and the monolayers were washed 1X with PBS and incubated in fresh medium at 37°C. Except for the time 4 HPI (hours post infection) where mock and experimental wells were harvested in duplicate, at all other time points (6, 8, 10, 12, 14 and 16 HPI) mock and experimental wells were harvested in duplicate and quadruplicate respectively by lysis in 100µl of passive lysis buffer and frozen at -20°C. These lysates were analyzed by using the Dual-Luciferase Reporter Assay System (Promega Corporation) on a TD 20/20 luminometer (Turner Designs, Inc.) according to each manufacturer's specifications.

*Vero cell infection with delNS1 influenza virus*
Vero cells were plated at a concentration of $1 \times 10^5$ cells/well on 24 well plates 2 days prior to transfection. Three $\mu$g of pcDNA3-rLuc-polIRES-fLuc was transfected per well at 1:1 ratio with TransFast Transfection Reagent. More DNA was used in Vero cells due to less transfection efficiency observed compared to CHO cell transfection. Following an 8 hour incubation at 37°C, the monolayers were washed with PBS and inoculated with either a mock inoculum or with approximately 10 TCID$_{50}$ units per cell of delNS1 influenza virus. After a 1 hour adsorption at 37°C, the virus was removed, the monolayers were washed 1X with PBS and incubated at 37°C in fresh medium. For each time point 4, 8, 12, 13, 14 and 16 HPI, mock and experimental wells were harvested in duplicate and triplicate respectively and analyzed by using the dual luciferase assay as previously described.

**NS1 transfection experiment**

CHO cells were plated at $1 \times 10^5$ cells/well on a 24 well plate the day prior to transfection. pcDNA3-rLuc-polIRES-fLuc (0.75$\mu$g) was transfected at 1:1 ratio with TransFast Transfection Reagent into each of six wells. Additionally, 3 of these wells were transfected with 1$\mu$g each of pcDNA3.1+ control vector and 3 with 1$\mu$g each of pcDNA3.1+NS1 expression plasmid. At 24 hours post transfection, the cells were harvested and analyzed by using the dual luciferase assay as previously described.

**NS1 rescue experiment**

Vero cells were plated at a concentration of $2 \times 10^5$ cells/well the day before transfection. Twelve wells received 3$\mu$g each of pcDNA3-rLuc-polIRES-fLuc, 6 of which received 4$\mu$g each of pcDNA3.1+NS1 whereas the other 6 received 4$\mu$g each of pcDNA3.1+. Eight hours later, three wells of each set were either mock infected or
delNS1 influenza virus infected (MOI=10) as stated previously. At 16 HPI the cells were harvested and analyzed by using the dual luciferase assay as previously described.

*Vero infection with influenza A/WSN/33*

Vero cells were plated at a concentration of $2 \times 10^5$ cells/well the day before transfection. Six wells were transfected with 3µg each of pcDNA3-rLuc-polIRES-fLuc and, after an 8 hour incubation at 37°C, 3 wells were infected with influenza A/WSN/33 (MOI=10) and 3 wells were mock infected as previously described. Cells were harvested at 16 HPI and analyzed by using the dual luciferase assay as previously described.

*Semi-quantitative RT-PCR*

*CHO infection with influenza A/WSN/33*

CHO cells were plated, transfected with pcDNA3-rLuc-PolIRES-fLuc and infected with influenza A/WSN/33 as described for the dual luciferase assays. At 4 HPI cells were harvested and total RNA was extracted using the Rneasy Mini Kit according to the manufacturers specifications (Qiagen). To ensure no DNA contamination occurred an on-column DNA digestion was performed using the RNase-Free DNase Set (Qiagen). An equal amount of total RNA (0.8-1 µg) from mock infected and WSN infected CHO cells was reverse transcribed into cDNA using the GeneAmp RNA PCR kit with an oligo-d(T)$_{16}$ primer (Applied Biosystems). Prior to the addition of reverse transcriptase, the samples were heated to 75°C for 3 minutes then cooled on ice for 5 minutes. The samples were then reverse transcribed in a one step cycle: 42°C for 45 minutes, 99°C for 5 minutes, and 5°C for 5 minutes. The resulting cDNAs were serially diluted 2 fold and 2µl of each dilution from both mock and WSN infected cells was PCR amplified with
the Expand High Fidelity PCR System (Roche, Inc.) in duplicate reactions using either a firefly luciferase or a β-actin specific primer set according to the manufacturer’s specifications:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ - 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluc forward</td>
<td>AAGGCCCGGCCATTCTATC</td>
</tr>
<tr>
<td>Fluc reverse</td>
<td>ATAATCATAGGACCTCTCACACACAGTTTCG</td>
</tr>
<tr>
<td>β-actin forward</td>
<td>GGGTCAGAAGGATTCTATG</td>
</tr>
<tr>
<td>β-actin reverse</td>
<td>GGTCTCAAACATGATCTGGG</td>
</tr>
</tbody>
</table>

An equal volume of the PCR product for each dilution was loaded and run on a 1% agarose gel containing ethidium bromide. The gel was then visualized using a FluorChem 8800 Imaging System (Alpha Innotech Corp.).

**NS1 transfected CHO cells**

CHO cells were transfected with pcDNA3-rLuc-PolIRES-fLuc and either pcDNA3.1+NS1 or pcDNA3.1+ as described in the dual luciferase assay. Cells were harvested at 24 hours post transfection and the RNA was analyzed as described for the WSN infected CHO cells.

**eIF4E phosphorylation studies and antibodies**

CHO cells were plated at 4X10⁵ cells per well in 6 well plates 1 prior to infection. On the day of infection, the monolayers were washed 2X with PBS and inoculated with either WSN (MOI=10) or a mock inoculum. Following a 1 hour adsorption incubation at 37°C, the monolayers were washed 1X with PBS and replaced with fresh media. At 6 and 8 HPI the monolayers were washed 1X with PBS and lysed in 100μl of SDS-PAGE loading buffer (Bio-Rad Laboratories, Inc.). Twenty μL of each lysate were run on a
12% SDS-PAGE gel (Invitrogen Corporation) and transferred for 1 hour at a constant voltage of 100 to a PVDF membrane in a Tris-Glycine transfer buffer (see recipes) (Bio-Rad Laboratories, Inc.). The resulting membrane was western blotted against phospho-eIF4E according to the manufacturers specifications (Cell Signaling Technologies). Briefly, the membrane was blocked in blocking buffer (see recipes) for 1 hour at room temperature. The membrane was then rinsed briefly in TBS/T (see recipes) and then incubated with the phospho-specific eIF4E antibody at a 1:1000 dilution in primary dilution buffer (see recipes) overnight at 4°C under gentle rocking. The membrane was then washed 3X5 minutes in TBS/T and incubated in the secondary HRP conjugated antibody at a dilution of 1:10,000 in blocking buffer for 1 hour at room temperature under gentle rocking. The membrane was then washed 5-7X5 minutes in TBS/T. The membrane was then incubated for 5 minutes in SuperSignal (Pierce) and drained. Following this the membrane was visualized on an imager (AlphaInnotech). After imaging, the membrane was washed 3X5 minutes and then incubated with an antibody against the total eIF4E at a dilution of 1:1000 in primary dilution buffer. The membrane was washed as before and incubated with the secondary HRP conjugated antibody as previously indicated. The membrane was revisualized to detect the amount of total eIF4E. To confirm the validity of the phospho-specific antibody CHO cells were plated as described previously and stimulated with 20% FBS prior to analysis.

**4E-BP1 phosphorylation studies and antibodies**

CHO cells were plated at 4X10^5 cells/well in 6 well plates the day prior to infection. On the day of infection, the monolayers were washed 2X with PBS and inoculated with either WSN (MOI=10) or a mock inoculum. Following a 1 hour
adsorption incubation at 37°C, the monolayers were washed 1X with PBS and replaced with fresh media. At times 4, 8, 12 and 16 HPI the monolayers were washed 1X with PBS and lysed in 100µl of SDS-PAGE loading buffer. Twenty µL of each lysate were run on a 12% SDS-PAGE gel and transferred to a PVDF membrane according to the manufacturer’s specifications (Bio-Rad Laboratories, Inc.). The resulting membrane was then western blotted against with phospho-specific antibodies directed against either the S-65 or T-70 phosphorylation sites (Cell Signaling Technologies) as indicated earlier. Following image analysis, these blots were reprobed against the total 4E-BP1 (Cell Signaling Technologies) in a manner similar to that indicated previously.

For MDCK cell 4E-BP1 phosphorylation studies, cells were plated at 2x10⁵ cells/well the day before infection. The cells were then infected with WSN (MOI=10) or a mock inoculum as stated previously. Samples were lysed as stated previously at 4 and 8 HPI and analyzed for phosphorylation of 4E-BP1 as before. However, in this case rather than computer imaging, X-ray films were exposed and developed on a developer (Kodak).

Effect of transfection on CHO infection efficiency

The day before transfection, CHO cells were plated at 1x10⁵ cells/well on glass cover slips in 24 well plates. The cells were either transfected as before with pcDNA3.1+ control vector + transfection reagent or left untreated as a control. Eight hours post transfection, the cells were washed 1X with PBS and infected with approximately 10 TCID₅₀ units per cell of WSN as stated previously. Nine HPI the cells were fixed in 100% methanol at –20°C for 15 minutes. The cover slips were removed from the plates, glued to glass slides and stored at –20°C. For staining the slides were
incubated in a FITC-conjugated anti-influenza A virus NP antibody (Virostat) at a dilution of 1:5 in PBS and incubated in a moist chamber at 37°C for 1 hour. The slides were then washed 1 X 5 minutes in PBS. The slides were then washed 1 X 5 minutes in PBS + Evans blue followed by an additional 5 minute wash in fresh PBS. Cover slips were mounted onto the slides using mounting media (Invitrogen Corporation) and they were then stored at –20°C until viewed on a confocal microscope (Leica).

Statistics

T-tests, ANOVA, two-way ANOVA, and regression analysis and end point significance were performed using SAS software (SAS Institute, Inc.).

Recipes

Phosphate buffered saline (PBS) (10L)

NaCl................................. 80g
KCl................................. 2g
KH₂PO₄............................. 1.2g
Na₂HPO₄......................... 9.1g
MiliQ water................. to 10L

Physiological saline

NaCl................................. 8.5g
MiliQ water................. 1L

Packed chicken red blood cells (CRBC)

1. Collect chicken blood in a heparinized tube
2. Spin at 500 X G for 5 minutes at 4°C
3. Remove the supernatant and resuspend in 10mL physiological saline

4. Repeat steps 2 and 3

5. Remove supernatant and resuspend in 10mL of PBS

6. Spin at 500 X G for 20 minutes at 4°C

7. Remove supernatant and store at 4°C for up to 1 week

0.5% *Chicken red blood cells (CRBC)*

- PBS (cold)........................ 50mL
- Packed CRBC.................. 0.3mL

Store at 4°C for 1 week

**SDS-PAGE Running buffer (10X)**

- Tris base .................... 30g
- Glycine .................... 144g
- SDS ......................... 10g
- MiliQ water ................. to 1L

Dilute 1:10 in miliQ water before use

**Transfer buffer (10X)**

- Tris base .................... 30g
- Glycine .................... 144g
- MiliQ water ................. to 1L

**Transfer buffer (working solution)**

- Transfer buffer (10X) ...... 100mL
- Methanol ................. 200mL
MiliQ water.......................... 700mL

Tris buffered saline TBS (10X)

Tris base........................... 24.2g
NaCl................................. 80g
MiliQ water......................... to 1L
(Adjust pH to 7.6 with HCL)

TBS + tween-20 (TBS/T) (1L)

TBS (10X)............................ 100mL
MiliQ water.......................... 900mL
Tween-20............................ 1mL (equals 0.1%)

Blocking buffer

TBS (10X)............................ 15mL
MiliQ water.......................... 135mL
Nonfat dry milk..................... 7.5g
Tween-20............................ 0.15mL (equals 0.1%)

Primary antibody dilution buffer

TBS (10X)............................ 2mL
MiliQ water.......................... 18mL
Bovine serum albumin (BSA)...... 1g
Tween-20............................ 0.02mL (equals 0.1%)

Evans blue stock solution (2%)

2g Evans blue in 100mL MiliQ water

PBS + Evans blue
PBS................................. 300mL

Evans blue stock solution  0.3mL
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