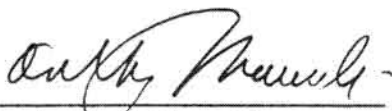


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Title of Thesis: The Viral and Eukaryotic Distribution of the Internal Ribosome
Entry Site (IRES) and its Potential as an Anti-viral Translation
Target

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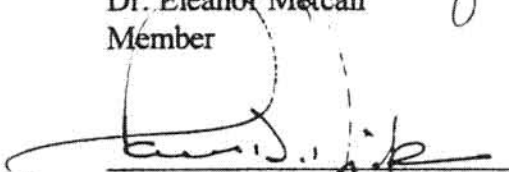
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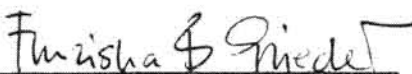
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A handwritten signature in black ink, appearing to read "D.T. Goshua". The signature is fluid and cursive, with the first name "D.T." being more distinct than the last name "Goshua".

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Abstract

Picornaviruses and flaviviruses are responsible for an enormous variety of medically important human and animal diseases which include the common cold (rhinoviruses), poliomyelitis (poliovirus), and hepatitis C. The majority of mRNAs initiate translation by the binding of ribosomes to the 5' cap structure, followed by ribosomal scanning of the mRNA until the appropriate AUG start codon is encountered. In contrast, translation of the mRNA of naturally uncapped picornaviruses and some flaviviruses, notably hepatitis C and pestivirus, is mediated by a mechanism involving internal initiation of translation. The sequence responsible for cap-independent, internal ribosome binding is within the 5' untranslated region (UTR) of the mRNA and forms a complex secondary structure, termed the internal ribosome entry site (IRES). This mechanism of internal initiation, once thought to be unique to viruses, is utilized by several eukaryotic mRNAs such as the ones encoding heavy chain immunoglobulin binding protein, BiP, *Drosophila Antp* protein, c-myc, and eIF4G. The serendipitous discovery of an IRES inhibitory RNA (I-RNA) in *Saccharomyces cerevisiae* has laid a solid foundation for investigating anti-IRES agents as anti-viral agents.

The Viral and Eukaryotic Distribution of the Internal Ribosome Entry Site (IRES) and its
Potential as an Anti-viral Translation Target

by
Alicia Tamara Crowder

Thesis submitted to the Faculty of the Department of Microbiology and Immunology
Graduate Program of the Uniformed Services University of the
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Dedications

To my son, James.

To Dr. Neil Grunberg, whose mentoring and caring has enabled me to build on my strengths and recognize my weaknesses. Thank you for giving me a compass.

To Alison Dr. O'Brien, whose guidance and support made this Master's thesis possible. Thank you for the opportunity to rotate in your laboratory. From you I have learned to be a better student, scientist and mother. May God bless you.

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List of Abbreviations

AAV	adeno-associated vector
<i>Antp</i>	Antennapedia gene of <i>Drosophila</i>
BiP	Heavy chain immunoglobulin binding protein
BVDV	bovine viral diarrhea virus
CAT	chloramphenicol acetyl transferase
c-myc	human proto-oncogene
CSFV	classical swine fever virus
CVB1	coxsackievirus B1
eIF	eukaryotic initiation factor
ER	endoplasmic reticulum
HCV	hepatitis C virus
HChV	hog cholera virus
HRV-14	human rhinovirus-14
HSP	heat shock protein
IRES	internal ribosome entry sequence
I-RNA	inhibitory-RNA
LUC	luciferase
PSF	protein splicing factor
PTB	polypyrimidine tract binding protein
PV	poliovirus
RRL	rabbit reticulocyte lysate
RSW	ribosomal salt wash
SAH	S-adenosylhomocysteine
TK	thymidine kinase
UFR	unusual folding region
UTR	untranslated region
VEGF	vascular endothelial growth factor

INTRODUCTION AND OVERVIEW

Selective Therapeutic Agents

The ideal therapeutic agent for an infectious disease targets a process critical to the pathogen's life cycle without adversely affecting the host. In the case of extracellular bacterial pathogens, this differentiation between pathogen and host is achieved by targeting the dissimilar biochemical requirements of the pathogen. Examples of such targets include inhibitors of bacterial cell wall synthesis. Nearly all bacteria possess a peptidoglycan layer consisting of β -1,4-linked repeating disaccharide units of N-acetyl-D-glucosamine (GlcNAc) and N-acetyl-D-muramic acid (MurNAc) (Anderson *et al.*, 1966). The antibiotics cycloserine and penicillin block peptidoglycan synthesis (Strominger *et al.*, 1967). Alternatively, the distinction between host and pathogen may exist as differences in analogous targets. An example of this type of selectivity is antibiotics that affect bacterial protein synthesis. Though both prokaryotes and eukaryotes utilize translation as the means for converting genetic information from mRNA into proteins, their translation machineries have differences which allow for specific targeting of the pathogen's system without affecting the host system. The eukaryotic ribosome, for example, is comprised of a 40S and 60S subunit, whereas the prokaryotic ribosome is comprised of a 30S and 50S subunit (Kozak, 1983). Streptomycin and related aminoglycosides target proteins specific to the 30S subunit causing misreading and errant protein synthesis (Garvin, *et al.*, 1974).

Bacteria and fungi are cells, possessing DNA, RNA, and their own cellular machinery for producing the macromolecules essential for life. Different targets and/or different affinities to targets that exist in the pathogen, but do not exist in the host achieve the selective action of therapeutic agents.

In contrast, viruses are not cells. They possess no cellular machinery, only DNA or RNA, and the genomic material may be either single or double stranded. Viruses have two distinct phases in their life cycle: an inert, extracellular, transmissible stage; and an intracellular, reproductive phase. Once inside a cell, the virus subverts the host's metabolic pathways and machinery to produce new virions. Because viral pathogens exist within the host cell and utilize host elements to complete their life cycle, developing selective antiviral agents has proven to be a difficult task. The purpose of this paper is to discuss the current state of knowledge of cap-independent translation, how to further elucidate the mechanism(s) of internal initiation, and the possibility of utilizing that information for targeting cap-independent translation for therapeutic intervention in human and animal viral diseases.

The Viral Replication Cycle

To cause infection, viruses must bind to and enter cells. Viral attachment proteins on the viral surface bind receptors (typically glycoprotein or glycolipid) on the plasma membrane of the host cell, resulting in attachment. The virus then either enters the cell via endocytosis or fuses with the plasma membrane (Knipe, 1994). Following penetration, capsid proteins are removed and the viral genome begins its replication process. Due to

their heterologous genomic nature, different families of viruses have adapted diverse mechanisms of transcription and translation (White and Fenner, 1994).

The proteins of viral pathogens are synthesized by the host cell's translation machinery. Most cytocidal viruses code for proteins that shut down host protein synthesis, subverting the cellular machinery for its own use. The shut down may be rapid or gradual, but either way provides a selective advantage by allowing viral message to bind ribosomes and initiate translation (White and Fenner, 1994). Conventional, cap-dependent viral translation proceeds as the 5' capped terminus of the viral mRNA binds to the 40S eukaryotic ribosomal subunit, which then moves down the mRNA scanning for the AUG initiator codon. The 40S subunit stops at the initiation codon, the 60S subunit binds with its associated initiation factors, and the viral mRNA is translated into protein (Knipe, 1994).

Role of Cap in Eukaryotic and Prokaryotic Translation

Cellular protein synthesis is a highly conserved process. In nearly all organisms, the “cellular components” and reactions of translation: ribosomes, aminoacyl-transfer RNA (tRNA) binding, peptide bond formation, and ribosome translocation are similar (Kozak, 1983). The process of translation has a beginning, middle, and an end that are termed initiation, elongation and termination, respectively. The primary distinction between prokaryotic and eukaryotic translation is the manner in which ribosomes interact with the mRNA and the selection of the AUG start codon. In prokaryotes, ribosomes engage the mRNA through the Shine-Delgarno site, a conserved region of six nucleotides found in the mRNA transcript, 4-7 nucleotides upstream of the start codon (McCarthy and Gualerzi, 1990). This sequence pairs with the 16S RNA in the small ribosomal subunit, correctly

aligning the ribosome with the correct AUG initiating codon (McCarthy and Gualerzi, 1990). Translation initiation of the majority of eukaryotic mRNAs also requires that the AUG initiator codon be correctly identified (Kozak, 1983). Initiation begins with binding of the 40S ribosomal subunit to a region of the mRNA known as the 5' cap. The cap structure defines the translational start site, and thus increases the efficiency of translation. It consists of a 7-methylguanosine residue joined to the 5' nucleoside of the mRNA via a 5'-5' triphosphate bridge. The initiation of translation of eukaryotic mRNA also requires nearly a dozen protein factors termed eukaryotic initiation factors (eIFs) (Kozak, 1983). The process of ribosome binding to mRNA is dependent upon eIF-4A, eIF-4B, eIF-4F and ATP hydrolysis. Upon binding to the mRNA, the 40S ribosomal subunits, with associated initiation factors, migrate down to the first (5' proximal) AUG initiator codon where a 60S subunit joins the 40S subunit and the first peptide bond is formed (Kozak, 1983; Harford, 1995). The initial "scanning" is a complex process determined by the 5'- and 3'-noncoding regions and the 3'-terminus poly (A) tail. Initiation factor eIF-4F consists of three polypeptides, p220 (eIF4G), p50 (eIF4A), and p24 (eIF4E) (Merrick, 1992). It is currently believed that eIF-4A, eIF-4B, and eIF-4E unwind the RNA secondary structure within the 5' UTR of the mRNA, allowing the 40S subunit to "scan" for the initiator codon (Harford, 1995). In general, the initiator codon of eukaryotes is defined as the AUG codon closest to the 5' terminus of the mRNA. This scanning model, developed by Marilyn Kozak, fits in with the monocistronic nature of most eukaryotic mRNAs (Kozak, 1983).

Until 1988 it was assumed that eukaryotic ribosomes could access mRNA initiation sites only via the cap-dependent scanning mechanism. The cap-independent translation mechanism of poliovirus did not fit the scanning model. In the conventional scanning model

of translation, a downstream cistron within a bicistronic mRNA would be inefficiently translated because few, if any, of the ribosomes would be able to continue scanning through the intercistronic spacer to the second, downstream cistron. Theoretically, under this circumstance, only the first, upstream cistron would be efficiently translated. In 1988, Pelletier and Sonenberg, utilizing a bicistronic mRNA construct, elegantly demonstrated that an internal sequence in the 5' untranslated region (UTR) of poliovirus RNA was responsible for cap-independent translation of the virus (Pelletier and Sonenberg, 1988).

Viruses Break the Rules: Cap Independent Translation

It had been assumed that eukaryotic ribosomes could only access mRNA initiation sites by first interacting with the 5' capped end of the mRNA, and then scanning for the appropriate initiation codon in a 5' – 3' direction. However, recent studies have shown that in virus-infected cells, different rules apply. Hewlett *et al.*, (1976) showed that in the Picornavirus family of viruses which includes polioviruses, RNA that is naturally uncapped at its 5' end is present. Recently it has been shown that eIF4G, a cap-binding protein complex necessary for cap-dependent translation, is cleaved by viral protease 2A, effectively shutting off cap-dependent translation in poliovirus-infected cells, though still allowing for poliovirus translation (Sonenberg, 1990). These results showed that poliovirus clearly does not translate via the conventional, cap-dependent mechanism; however, the precise mechanism remains perplexing. To better understand the mechanism of translation initiation by poliovirus mRNA, Pelletier and colleagues (1988) analyzed the effects of deletions within the 5' untranslated region (UTR) the efficiency of translation. In their study, Pelletier *et al.*, (1988) mapped a major domain within the poliovirus 5' UTR that appeared to be responsible

for translation. This domain became the focus of further investigations into the precise mechanism of the poliovirus translation process.

The scanning model proposed by Kozak (1983) was sufficient to explain the mechanism of translation of most mRNAs having caps and 5' UTRs in which upstream AUGs were rare. Therefore, the precise mechanism of translation of some viruses, poliovirus in particular, remained an enigma. Lacking a cap and possessing an extremely long 5' UTR with many upstream AUGs, it was hard to fit the scanning model of translation to poliovirus translation. In the last 10 years, researchers have accumulated evidence for the existence of an internal ribosome entry sequence (IRES) that enables translation via cap-independent ribosomal binding (Sonenberg, 1990; Oh and Sarnow, 1993). IRES-mediated initiation utilizes cellular translation machinery to assemble internal to the 5' end of the mRNA, near the appropriate AUG codon for the viral protein. The IRES can be thought of as the viral equivalent to the Shine-Delgarno sequence, which provides for a ribosome binding site near the appropriate AUG in prokaryotes (Harford, 1995). Picorna- and other viruses selectively inhibit only host protein synthesis by encoding a protease that cleaves eukaryotic initiation factor-4 γ (eIF-4 γ or eIF4G), a critical component of the cap binding initiation factor eIF-4F (Wyckoff, 1992). Cap-dependent translation is inhibited as a result of the picornavirus protease, but the IRES allows viral protein translation to proceed via cap-independent initiation. Inhibition of host translation, while proceeding with its own, provides the virus with a clear advantage during infection. The distinctive features of viral protein synthesis, particularly the IRES, in virally infected cells provide a potential target for therapeutic intervention (Harford, 1995).

HOST DEFENSE: Interferon

The subversive nature of viral replication poses an unusual problem for the host's defensive mechanisms. There exists, in effect, a translational war between virus and host. The cell has devised anti-viral defenses and the virus attempts to counter those measures. One way in which the host counters viral infection is through interferon synthesis. Interferons are regulatory glycoproteins secreted by virally infected cells that serve to protect uninfected cells against infection with the same or an unrelated virus. The synthesis of interferon is induced by dsRNA, which is produced by both DNA and RNA viruses at some point during infection (Peska *et al.*, 1987). There are 20 human interferons divided into three chemically distinct groups: interferon α , interferon β and interferon γ . The synthesis of interferons α and β is induced, among other stimuli, by virus infection (especially RNA viruses) and takes place in almost any cell. However, interferon γ is made only by T lymphocytes and NK cells following antigen-specific stimulation; increasing the efficiency with which target cells present viral peptide (Pestka *et al.*, 1987). Interferons are used clinically to treat certain viral diseases such as hepatitis A (Chang *et al.*, 1993).

The mechanisms by which interferons prevent viral proliferation are largely unknown. One mechanism involves inhibiting protein synthesis in the infected cells in two ways: (1) the production of a protein kinase, p68; and (2) the production of a catalytic enzyme, 2,5-A synthetase. The activities of both p68 and 2,5-A synthetase require dsRNA and inhibit protein synthesis by inducing the inhibition of ribosomal initiation and degradation of mRNA, respectively.

Protein kinase p68

The P1/eIF-2 α kinase, also termed p68, is produced constitutively at low levels in uninfected cells; however, its synthesis is upregulated by the presence of interferons (α , β , or γ). The role of p68 is to prohibit protein synthesis during viral infection (Jacobs and Langland, 1996). dsRNA, produced by the virus-infected cells, binds p68 causing it to autophosphorylate its own P1 subunit. Once phosphorylated, the p68 enzyme becomes activated and phosphorylates the α subunit of eukaryotic initiation factor eIF-2, inactivating it. Since intact eIF-2 is required for initiation of protein synthesis, interferon-induced p68 will inhibit synthesis of all proteins, viral and cellular (Jacobs and Langland, 1996).

Many viruses have developed countermeasures for circumventing the protein kinase, or p68, defense. Adenoviruses encode low molecular weight RNAs that bind p68, preventing binding of dsRNA and resulting activation. Poliovirus infection degrades p68, allowing the cellular translational machinery to resume function, however cap-dependent initiation has been inhibited by viral protease 2A, so the machinery replicates the poliovirus proteins (Jacobs and Langland, 1996).

2-5A Synthetase

Another interferon-induced enzyme, 2-5A synthetase, catalyzes the synthesis of short-lived oligonucleotides known as (2'-5')pppA(pA)_n, or 2-5A from ATP (Imai and Torrence, 1984). 2-5A then activates RNaseL, a cellular endonuclease that degrades mRNA, inhibiting host cell protein synthesis and inducing apoptosis. This 2-5A/RNaseL system, an antiviral mechanism, is particularly effective against picornaviruses (Chebath, *et al.*, 1987).

To counter the effects of interferon-induced enzymes, viruses have acquired genes that enable them to block specific interferon pathways. Hepatitis C, vaccinia, and Moloney murine sarcoma viruses are all associated with an inhibition in interferon system activation of 2-5A synthetase (Podevin *et al.*, 1997; Rivas *et al.*, 1998; Birnbaum *et al.*, 1993).

VIRAL COUNTERACTION - THE INTERNAL RIBOSOME ENTRY SITE

Overview of Picornavirus and the IRES

Members of the Picornaviridae family include polioviruses, coxsackieviruses, echovirus, enteroviruses, rhinoviruses, and hepatitis A virus (Fig. 1, adapted from Palmenberg, 1989; Jackson *et al.*, 1990). Though consisting of many distinct species, the Picornaviridae share many common features: the virion is a naked icosahedron with a positive, single-stranded RNA. The viral genome is approximately 7.4 kb long with a poly(A) tail at the 3' end (Ambros *et al.*, 1978; Rivera *et al.*, 1988). Since it has plus-stranded RNA, the primary sequence serves as mRNA to direct synthesis of virally encoded proteins. The RNA codes for a single polyprotein that is cleaved into three precursor polyproteins (P1, P2, and P3). Processing of these precursor proteins by viral proteases 2A, 3C and 3CD yields mature structural and non-structural proteins. Capsid proteins, VP1, VP2, VP3, VP4, are derived from P1. Nonstructural proteins, essential for replication, are derived from P2 and P3 (Krausslich and Wimmer, 1988). The mRNA of picornaviruses has an unusually long 5'-untranslated region (UTR) that, in contrast to nearly all mRNAs, is not

Human rhinovirus (HRV)

Enteroviruses

- Poliovirus (PV)
- Coxsackie A virus (CAV)
- Coxsackie B virus (CBV)
- Bovine enterovirus (BEV)

Aphthoviruses

- Foot and mouth disease virus (FMDV)

Cardioviruses

- Mengovirus
- Encephalomyocarditis virus (EMCV)
- Theiler's murine encephalomyelitis virus (TMEV)

Hepatitis A virus (HAV)

Fig. 1. Classification of the animal picornaviruses adapted from Jackson *et al.*, 1988. The principal genera are shown in large type. With the exception of mengovirus, there are several different strains of each species - human rhinovirus has over 100 different strains.

capped with a 7-methylguanosine cap (m^7GpppX). It is covalently linked to VPg, a virally encoded oligo-peptide (Krausslich and Wimmer, 1988; Jackson *et al.*, 1990).

In addition to degrading p68, some picornaviruses, including poliovirus and foot-and-mouth-disease virus, have evolved a unique mechanism for shutting down cellular mRNA. They do not need a cap on their 5' end to translate their mRNA and so have developed a means for inactivating the eukaryotic eIF-4F cap-binding mechanism via protease 2A (poliovirus) and Lb protease (foot and mouth disease) (Roberts, 1998).

Poliovirus - The First Evidence of IRES

The scanning model does not satisfactorily explain picornavirus translation due to several of the unique characteristics shared by the mRNA of the Picornaviridae. Specifically, these include: (i) the long 5' untranslated region spanning nucleotides (nt) 650-1,300, (ii) multiple AUGs upstream of the translation initiation site, and (iii) the absence of a 5' cap structure (Pelletier and Sonenberg, 1988). The exact mechanism of translation remained a mystery until 1988 when Pelletier and Sonenberg successfully demonstrated that poliovirus mRNA initiated translation via a cap-independent binding of host ribosomes to an internal sequence within the 5' noncoding region of the poliovirus genome. The first indication that the poliovirus 5'-untranslated region (UTR) was necessary and sufficient for translation initiation came from the observation that 5' UTR sequences were able to confer cap-independent translation to a heterologous gene (Trono *et al.*, 1988; Pelletier *et al.*, 1988). Using deletion mutagenesis, Pelletier and Sonenberg (1988) showed that an internal sequence of the 5' region (nucleotides 320 to 631) of poliovirus type 2 was sufficient for cap-independent translation in poliovirus-infected HeLa cell extracts. Cellular translation of

capped mRNAs is normally inactivated in poliovirus-infected cells because of cleavage of the cap-binding protein by viral protease 2A (Pelletier *et al.*, 1988).

Since demonstrating that the 5' UTR of poliovirus was adequate for cap-independent translation did not rule out the possibility that ribosomes bound the 5' end and then migrated toward some other, unidentified mediator of translation, Pelletier and his colleagues devised an elegant construct of a "bicistronic" mRNA. This construct would become the benchmark test for identification of IRES sequences. The general model consists of an upstream open reading frame encoding protein X, an intercistronic spacer containing the putative IRES, and a downstream open reading frame encoding protein Y. The presence of the intercistronic IRES allows for internal initiation of translation of the second cistron in spite of translation of the first, or in the absence of translation of the first (Fig 2, adapted from Belsham *et al.*, 1995).

The bicistronic constructs of Pelletier and Sonenberg contained the thymidine kinase (TK) gene of herpes simplex virus-1 as the first cistron and the bacterial chloramphenicol acetyl transferase (CAT) gene as the downstream, second cistron (Pelletier and Sonenberg, 1988). To determine whether internal initiation could occur *in vivo*, TK/CAT and TK/P2/CAT were subcloned into the expression vector pSV2 and those products then transfected into mock or polio-infected COS-1 cells. TK and CAT protein levels were determined by pulse-labeling with [³⁵S] methionine followed by immunoprecipitation analysis. In a parallel experiment, COS-1 cells without the bicistronic plasmid were infected with poliovirus, labeled with [³⁵S] methionine four hours post-infection, and subjected to immunoprecipitation analysis to show that neither TK or CAT were present in control (without bicistronic plasmid), infected cells (Pelletier and Sonenberg, 1988).

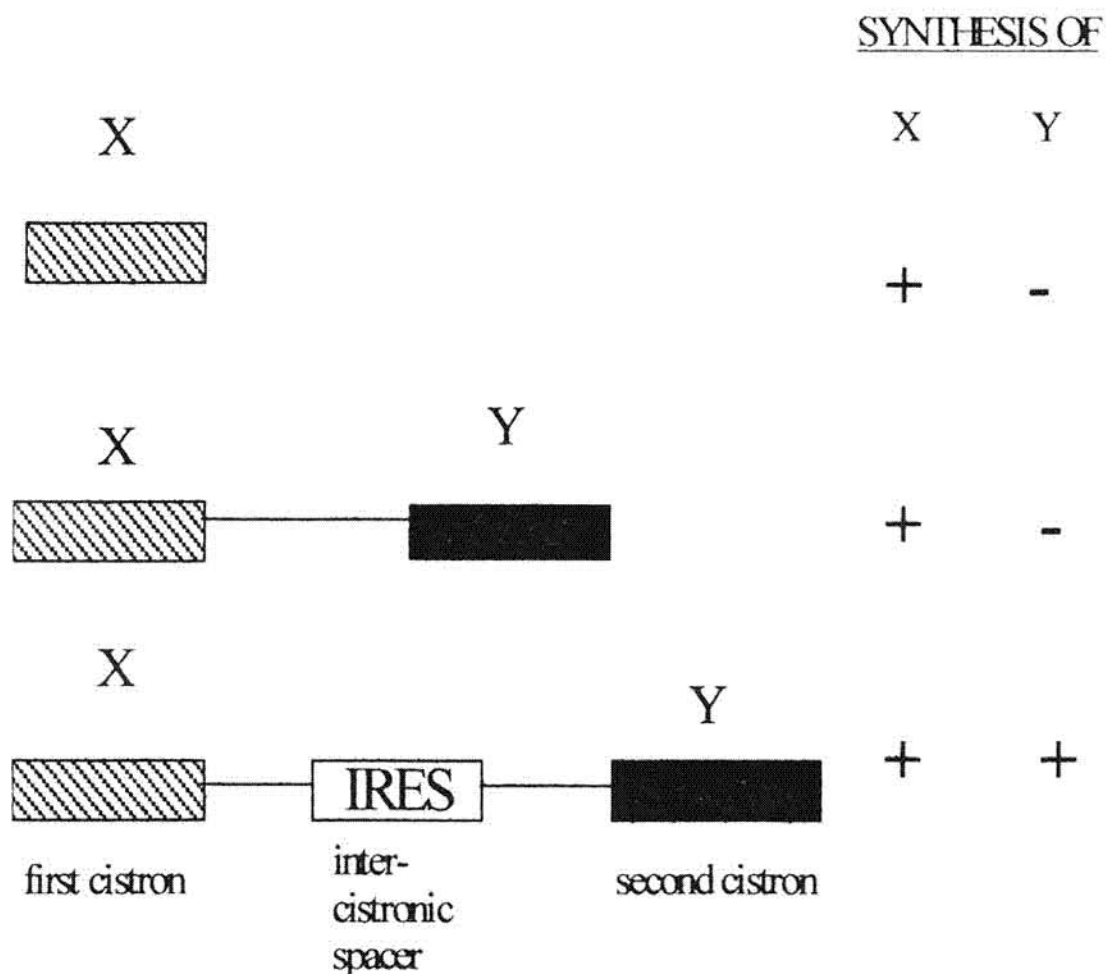


Fig. 2. Schematic of bicistronic construct adapted from Belsham, 1996. Plasmids are constructed which express the mRNA of X downstream of an SP& or T7 promoter alone or fused to the mRNA of Y. in both cases only X is translated via the scanning model. The downstream AUG codon of Y is not read. When an IRES is inserted as an intercistronic spacer, internal initiation takes place, allowing for the translation of Y.

This design allowed them to determine whether the internal sequence of the 5' UTR of poliovirus mRNA could itself direct internal initiation. Pelletier's group theorized that if the 5' UTR mediated internal initiation, abolishing initiation at the first cistron should not affect translation of the second cistron. The methodology showed that under conditions that did not allow for cap-dependent translation (poliovirus-infection), synthesis of the protein from the second cistron was not inhibited, whereas cap-dependent synthesis from the first cistron was completely inhibited (Pelletier and Sonenberg, 1988).

Since their *in vivo* experiments demonstrated the existence of internal initiation in poliovirus mRNA, Pelletier and Sonenberg attempted to demonstrate the results *in vitro*. If internal initiation were successfully reproduced in an *in vitro* system, it would justify the use of translation extracts for the identification of trans-acting factors involved in internal initiation of translation. Further, *in vitro* studies would lead to the elucidation of the molecular mechanism of the process of internal initiation. Translation studies were performed in a reticulocyte lysate using mRNAs synthesized *in vitro* in the SP6 transcription system (Pelletier and Sonenberg, 1988). Work by Shih, et al. (1978) had shown that translation of poliovirus mRNA was inefficient in the reticulocyte system, producing aberrant proteins. Later work by Meervitch, et al. (1993), suggested that the cellular factor(s) absent in reticulocyte lysates, but present in HeLa cell extracts were responsible for the mis-translation. Others had previously defined a sequence within the 5' UTR, at nucleotides 70-381, responsible for the inhibition of translation in the reticulate lysate system (Pelletier and Sonenberg, 1985). When the 5' UTR containing the inhibitory sequence was placed next to the CAT coding sequence, translation of CAT was reduced ~200-fold. Translation of TK mRNA gave a 46K protein corresponding to HSV-TK and

translation of TK/CAT gave the same pattern of proteins, indicating that the CAT was not translated in this construct. In contrast, when TK/P2/CAT was translated, CAT was synthesized. Though it must be noted that, in these studies, CAT rather than poliovirus translation was stimulated, the *in vitro* results clearly paralleled the *in vivo* experiments supporting the existence of an internal initiation site.

These data provided the first direct evidence that the 5' UTR of poliovirus could mediate internal ribosome binding and that the internal entry of ribosomes required an element located within the 5' UTR of PV RNA. This sequence element responsible for internal initiation was termed the ribosome landing pad (RLP) or, more commonly, the internal ribosome entry site (IRES) (Pelletier and Sonenberg, 1988).

To further support their finding that translation initiation of the TK and CAT cistrons occurred via independent mechanisms, Pelletier and Sonenberg subjected the engineered COS-1 cells to hypertonic conditions (Pelletier and Sonenberg, 1988). Translation of most cellular mRNAs is reduced under hypertonic conditions (Carrasco and Smith, 1975). Because poliovirus translation had been shown to be more resistant to hypertonic inhibition (Etchison *et al.*, 1982), Pelletier and Sonenberg reasoned that translation of a cistron preceded by the poliovirus 5' UTR would be increased relative to a cistron which was preceded by SV40. The bicistronic construct consisted of an upstream, TK gene, preceded by SV40 and a downstream, CAT gene, preceded by the 5' UTR of poliovirus. Under hypertonic conditions (excess~190-mM NaCl), translation of TK was inhibited 50-fold whereas CAT translation was slightly stimulated under the same conditions. Together, these results support a model of independent, internal access for ribosomes translating the two cistrons.

Mapping the IRES

To map the region responsible for internal initiation, a series of deletion mutants within the 5' UTR was constructed (Fig. 3, adapted from Pelletier and Sonenberg, 1988). The deletion mRNAs were translated in poliovirus-infected HeLa cells, which do not allow cap-dependent mRNA translations. As expected, there was no expression from the upstream TK cistron in any of the constructs; however CAT expression was observed in the TK/P2CAT construct, but not the bicistronic TK/CAT construct. Translation of the mRNAs of TK/P2CAT deletion constructs TK(Δ 5'-465)CAT and TK(Δ 3'-461)CAT was completely abolished which indicated that this region of the 5' UTR (nucleotides 140-630) contains the internal ribosome binding site (Fig 3). To further support the theory that the ribosomes were binding first to an internal site within the 5' UTR and then translocated to the initiator AUG, three *Xba*I linkers (5'CTCTAGAG3') were introduced position 70 or position 631 of the poliovirus 5' UTR (Fig. 3). The linkers have the potential to form a hairpin structure that interfered with the ability of the ribosome to bind, and would thus inhibit translation. Insertion of the linker derivatives into the 5' proximal region (position 70) had no effect on the translation of CAT; however, insertion at the 3' distal region (position 631) strongly inhibited P2CAT translation (Pelletier and Sonenberg, 1988). A second site mutation was not responsible for the inhibition, because removal of the *Xba*I linkers in the TK/P2CAT (X-70) construct restored translation to control levels (P2CAT mRNA without linkers). Taken together, these results illustrate that poliovirus translation is

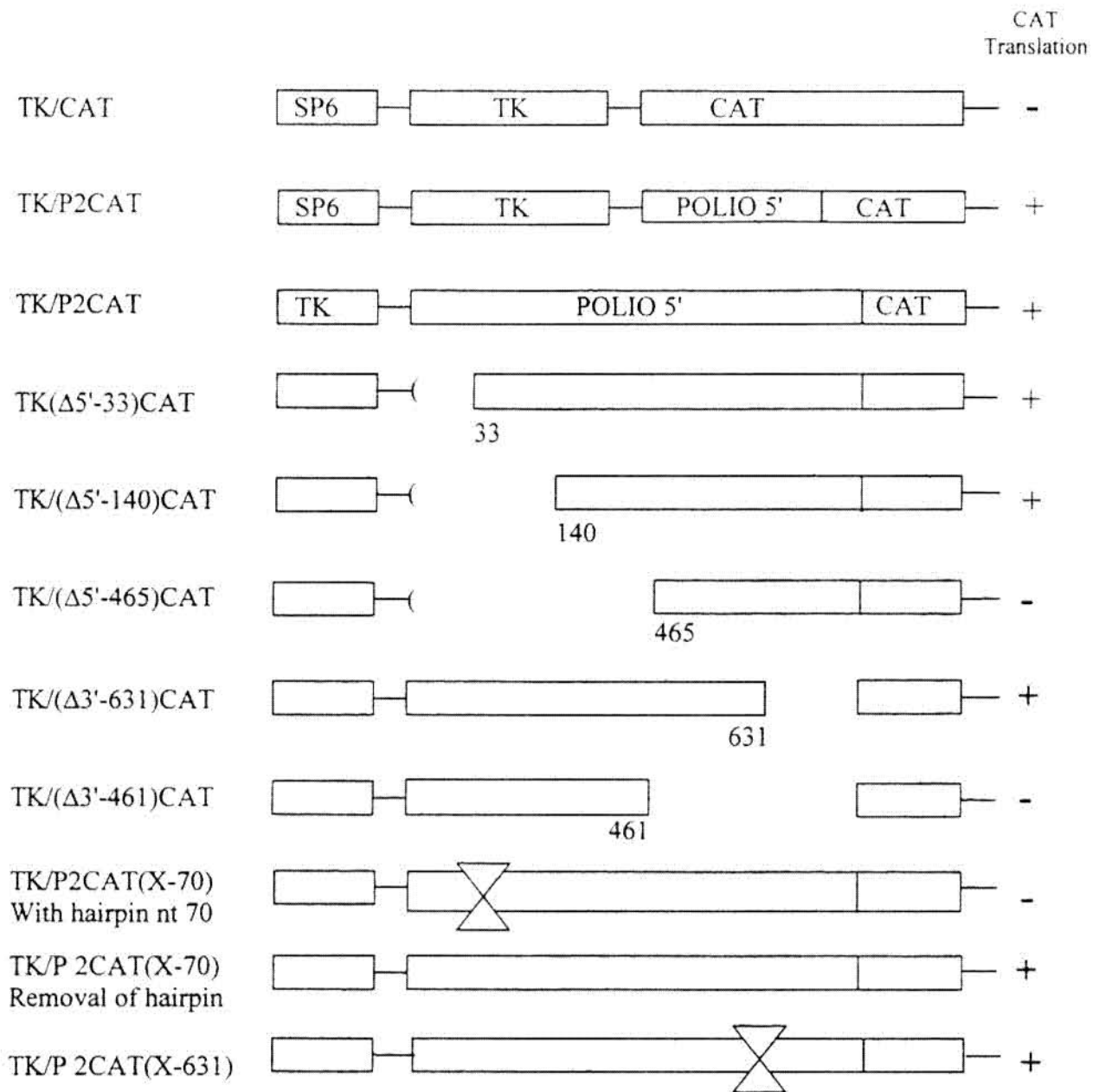


Fig. 3. Schematic representation of poliovirus 5' UTR deletion mutants used to map the region responsible for internal initiation (adapted from Pelletier, *et al.*, 1988). The results of CAT translation are shown in the column to the right. Hairpin insertions are represented with the hourglass figure.

initiated when ribosomes bind to the poliovirus 5' UTR upstream of position 631, but downstream of position 70, and then translocate to the AUG initiator at position 745 (Pelletier and Sonenberg, 1988).

This classical work was the first to present evidence that eukaryotic ribosomes could bind internally to the 5' UTR of poliovirus RNA. The internal initiation explains the ability of poliovirus to translate in spite of its peculiarities including the inactivation of the cap binding protein complex (eIF4F) and the extremely long 5' UTR containing several AUG codons. Pelletier and Sonenberg developed a model for cap-independent, internal binding of ribosomes to mRNA. According to this model, internal initiation does not require eIF4F, but does require that eIF4A and eIF4B, possibly in conjunction with an unknown factor, bind to the internal sequence of the mRNA to initiate translation.

Encephalomyocarditis virus IRES

At the same time Pelletier and Sonenberg were unraveling the mechanism of internal initiation in poliovirus RNA, Jang and colleagues had discovered an IRES in encephalomyocarditis virus (EMCV) RNA (Jang *et al.* 1988). All picornavirus RNAs are uncapped messengers and have very long, open reading frames (ORFs) beginning far from the 5' terminus of the nucleotide (N) sequence, at N743 in poliovirus type I RNA and N834 in EMCV RNA. According to the scanning model of translation, some of these upstream short ORFs should be translated and the downstream translation sites severely impeded by the upstream translation, especially if the reading frames overlap (Kozak, 1983). In a bicistronic mRNA with two non-overlapping reading frames (A and B), translation usually results in a greater yield of the first (A) than the second (B) reading frame (Kozak, 1983).

The initiation sites for translation of all picornavirus polyproteins are preceded by extremely long 5' UTRs (Jang *et al.*, 1988). In addition, EMCV and foot and mouth disease viruses (FMDV) contain a stretch of poly (C), of unknown function, located within the 5' UTRs. To determine whether the 5' UTR of EMCV contained an IRES, Jang and colleagues (1988) constructed a plasmid that would transcribe as a bicistronic mRNA. The first cistron contained the full 5' UTR of poliovirus upstream of the *sea* indicator gene, which encodes an oncogene product; and the second cistron contained a segment of the EMCV 5' UTR (nt 260-833) upstream of the entire coding sequence of poliovirus protease 2A. In rabbit reticulocyte lysates, translation of the second cistron was independent of the first, evidence for the existence of an IRES in the 5' UTR of EMCV (Jang *et al.*, 1988).

Jang and colleagues believed that the ribosome was either re-initiating translation of the second cistron without dissociating from mRNA or that it was re-entering by binding to an internal site on the second cistron. To determine the precise mechanism, the *sea* gene was placed under the translational control of a poliovirus 5' UTR. This poliovirus/*sea* construct was placed upstream of a second cistron consisting of the 5' UTR of EMCV (a putative IRES containing region) and polio protease 2A. The results showed that both the *sea* and protease 2A genes were translated efficiently (Jang *et al.*, 1988). In contrast, a bicistron that contained a deletion of nucleotides 260-563 in the EMCV 5' UTR failed to translate the 2A gene (Fig. 4. Jang *et al.*, 1988). These results supported the theory that the 5' UTR of EMCV includes an IRES site and that 40S ribosomal subunit was re-entering the translational machinery by binding to that internal site on the mRNA.

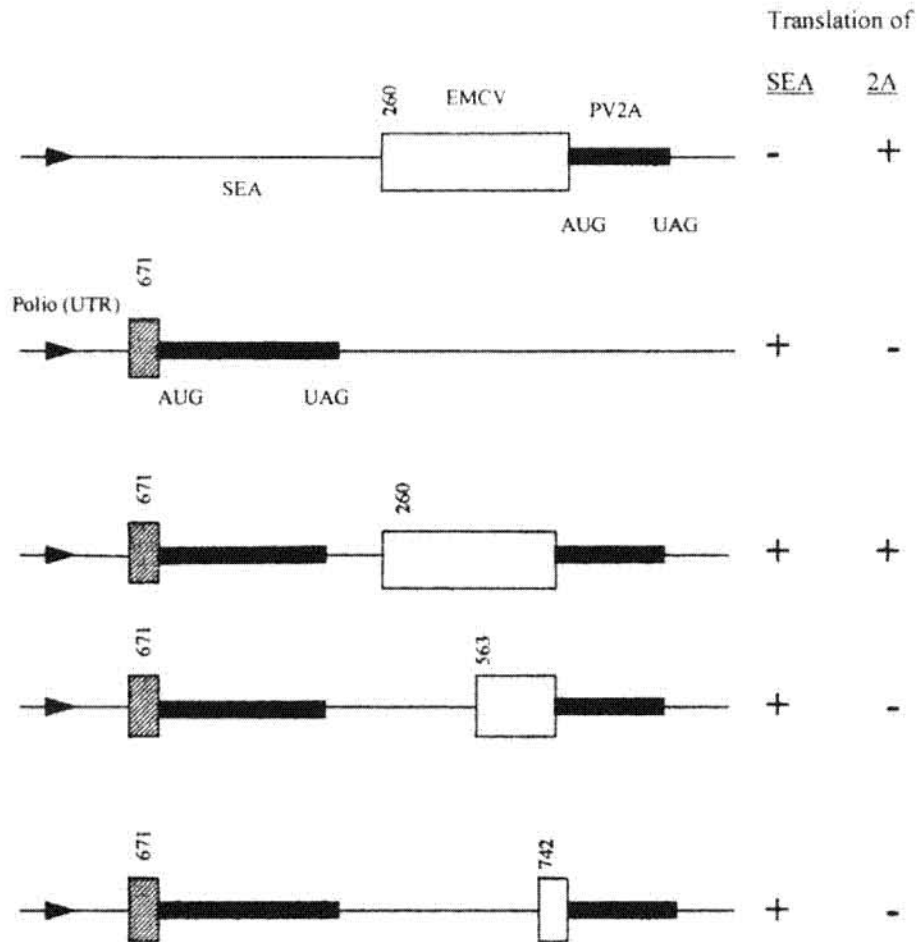


Fig. 4. Effect of intact and truncated EMCV 5' UTR on second gene translation, adapted from Jang *et al.*, 1988. The first gene *sea* is under the control of the truncated 5' UTR of poliovirus. The second gene, poliovirus 2A is under the control of the entire, or truncated, 5' UTR of EMCV. Translation results are in columns to right of constructs.

To insure that synthesis of 2A was not due to initiation on monocistronic mRNA degradation fragments, Jang demonstrated that the yield of 2A was as high as the monocistronic mRNA derivative tested (Jang *et al.*, 1988). Further, when deletions were made in the EMCV segment, the synthesis of 2A from the dicistronic mRNA required the entire segment, though synthesis from the monocistronic segment did not (Jang *et al.*, 1988). By using the poliovirus protease 2A, this set of experiments provided support for the ability of a picornavirus initiate internal translation as well as the ability of a picornavirus mRNA to be translated via this mechanism. These results clearly confirmed the presence of an internal initiation site within the 5' UTR of EMCV.

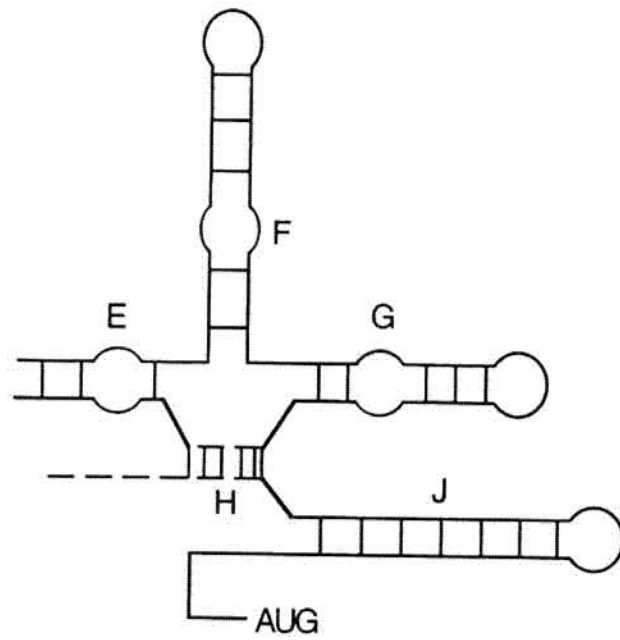
Characterization of Picornavirus IRESs

In classic work from two independent laboratories (Pelletier and Sonenberg, 1988; Jang *et al.*, 1988), the presence of an internal ribosome entry site allowing for cap-independent translation of mRNA within the 5' UTR of poliovirus and encephalomyocarditis virus was demonstrated. Further, these researchers establish the operational criteria for internal initiation, the bicistronic RNA assay. Poliovirus and EMCV belong to two distinct sub-groups of picornavirus. In later work, Brown and colleagues further characterized the IRES in EMCV by *in vitro* translation of mRNAs generated by *in vitro* transcription of EMCV cDNAs that had serial deletions from either the 5' or 3' end of the 5'UTR (Brown *et al.*, 1992). From this it was determined that efficient translation required regions downstream of nt 403 and upstream of nt 811 (Brown *et al.*, 1992). Four primary pieces of data provided evidence that the secondary structure, a stem-loop configuration, was essential for IRES function. First, the addition of short oligodeoxynucleotides to the

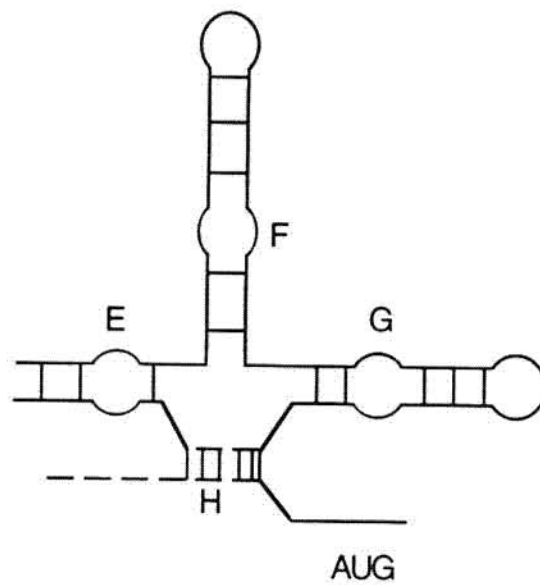
nucleotide segments 309-338 and 420-449 had no or little effect on translational efficiency of monocistronic mRNAs in rabbit reticulocyte lysate (Shih *et al.*, 1978). Second, insertion of linkers, or deletion of nucleotides of various sizes into sites within the 5' UTR had widely different effects on translational efficiency. A linker of 125 nt at nucleotide 490 did not affect translation, while deletion of three nucleotides at nt 560 completely abolished translation (Witherell *et al.*, 1995). Third, mutations within the secondary structure loops of the IRES drastically impaired translation, while substitutions that restored the secondary structure, restored the internal initiation capacity of the ribosome (Jang and Wimmer, 1990; Duke *et al.*, 1992). Finally, deletion analysis and nucleotide substitution demonstrated that the number of nucleotides in the region upstream of the initiating AUG were more important than the actual sequence (Jang and Wimmer, 1990).

Based on RNA sequence and proposed secondary structure differences, two IRES types were proposed within the family Picornaviridae (Schmid and Wimmer, 1994). Recently, three groups of IRES elements were suggested by Le and Maizel, (1998). Type 1 IRES elements are found in poliovirus, coxsackievirus, echovirus, and human rhinovirus. Type 2 IRES elements are found in EMCV, TMEV, and FMDV. Type 3 IRES elements are found in hepatitis A virus. There is very little sequence homology between these three classes of IRES; however all classes share of stem-loops E-H (Fig. 5, adapted from Le and Maizel, 1998).

Though they differ widely in sequence homology and biological activity, picornavirus IRESs have one common sequence motif. This sequence motif is the only known conservation between the two groups and has the designation Yn-Xm-AUG where Yn represents a pyrimidine rich region: Xm represents any of the nucleotides, but generally

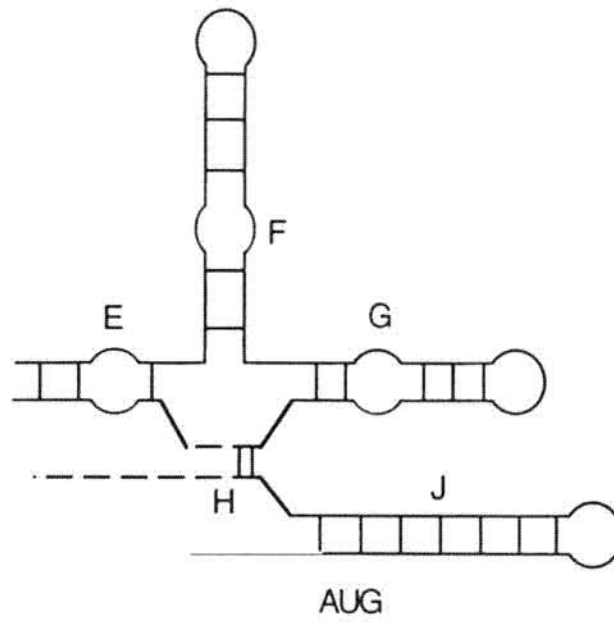


Type 1 IRES



Type 2 IRES

Fig. 5. Adapted from Le and Maizel, 1998. Schematic diagram of common structural core in the three types of the picornavirus IRES structures. The pseudoknot and domains E -H are the common structural elements that are conserved in all picornavirus elements.



Type 3 IRES

Fig. 5. Adapted from Le and Maizel, 1998. Schematic diagram of common structural core in the three types of the picornavirus IRES structures. The pseudoknot and domains E -H are the common structural elements that are conserved in all picornavirus elements.

numbers approximately 20 (Brown *et al.*, 1992). In all picornavirus IRESs, the ribosome entry site is an AUG codon located 20-25 nucleotides downstream of the polypyrimidine tract. The Xm tract may contain any sequence, but is always G-poor and most likely serves as an unstructured spacer. Insertions or deletions in this spacer region nearly always result in extremely reduced translational efficiency, yet translation is readily restored by nucleotide substitution (Pilipenko *et al.*, 1989). The two IRES groups proposed by Schmid and Wimmer (1994) had different requirements to function most efficiently *in vitro*. In rabbit reticulocyte lysate, type I IRES-dependent initiation required supplemental extracts from HeLa or Krebs II ascite cells; type 2 IRES- dependent initiation proceeded quite efficiently in RRL alone (Pelletier, 1989). This difference may have been due to polypyrimidine tract-binding protein (PTB) dependence discussed later in the paper. UV cross-linking revealed several RNA proteins that bound to IRES elements. Taken together, this information confirmed that cellular factors were required for efficient internal initiation.

OTHER PICORNAVIRUS IRESs

Coxsackievirus B3

Coxsackievirus B3 (CVB3) is a member of the *Enterovirus* genus of the *Picornaviridae* family, that causes myocarditis in humans and animals (Abelmann, 1973). Like other picornaviruses, the 5' UTR of CVB3 RNA contains the characteristics associated with an IRES site: it is extremely long (741 nucleotides) and forms a highly ordered secondary structure (Le and Maizel, 1997). To test for the presence of an IRES, synthetic

mono- and bicistronic RNAs were constructed by cloning a 2.08 kb cDNA fragment containing the CVB3 5' UTR and a truncated viral P1 polyprotein into pSPT18 (Yang *et al.*, 1997). The monocistronic plasmid was designated pSPT18(P1). The P1 polyprotein was under the control of the CVB3 5' UTR. The bicistronic plasmid contained the CAT gene and P1 cloned into pSPT18 and was designated pSPT18(CAT + P1). A second bicistronic plasmid pSPT18(TAC + P1) was constructed with the CAT in the reverse orientation (TAC) to destroy the open reading frame of the CAT gene and to block 5' end of the downstream P1 cistron to prevent ribosomal scanning. Translation products were analyzed and evaluated by SDS-PAGE. As in the other picornaviruses, when the complete 5' UTR was present, the CVB3 P1 polyprotein was translated equally as well as the monocistronic transcripts. As expected, there was no translation of the bicistronic plasmid with the reversed CAT. The downstream cistron, P1, was unaffected by the CAT gene, which indicated that translation occurred by an internal ribosome binding event.

To determine the location of the IRES within the 5' UTR of CVB3, mono- and bicistronic RNA transcripts were made from deletion mutant plasmids. The P1 translation products were then immunoprecipitated, and it was determined that deletions between nucleotides 530-630 abolished P1 translation >92%. Interestingly, abolishing nucleotides 1-63 also abolished P1 translation by as much as 90%. These two regions have the lowest level of secondary structure within the 5' UTR; and deletions between the two regions resulted in only slightly reduced translational efficiency (Wang, 1997).

At this point the data did not answer the question of whether the IRES structure was a discontinuous or continuous sequence. If discontinuous, the two regions (nt 1-63 and 529-630) were probably associated as a cluster within the tertiary structure of the RNA. If

the IRES sequence was continuous, nucleotides 529-630 were probably the core region of the IRES and nucleotides 1-63 the binding site for translation initiation factors (Yang *et al.*, 1997). Yang and colleagues contend that the segment from 530-630 is the actual core region and that the segment from 1-63 is the binding site for initiation factors. This view is strongly supported by the following points. First the space required for ribosomal docking is small. Based upon data that the bases inside the RNA helix are 0.25 nm apart, the binding site should be approximately 120 nt long. The 529-630 nt sequence plus flanking regions (total 196 nt) is of a suitable length for ribosome binding. Second, a polypyrimidine-rich tract UUCAUUUU lies between nucleotides 562-571 and there is a conserved AUG 19 nt downstream (Yang *et al.*, 1997). The presence of a polypyrimidine-AUG motif is conserved in the IRESs of picornaviruses. Finally, though the poliovirus IRES is reported to lie between nt 134-585, many internal regions have been deleted without affecting translation (Yang *et al.*, 1997). These data suggest that the 5' end of the UTR may not be the core region of the IRES, but rather a site for initiation factors to bind. The slightly enhanced translation product obtained by deletion of nucleotides 250-529 may be explained by an alteration in the secondary structure that enhances ribosomal and initiation factor binding (Yang *et al.*, 1997).

One problem that had not yet been addressed in other studies was whether or not the flanking regions of the IRES are also important for ribosome binding. Since the deletions were obtained using convenient restriction sites, it was possible that the secondary structure was being altered in such a way as to mis-lead researchers. Seven antisense oligonucleotides complementary to sequences within the CVB3 IRES were tested for their anti-translational ability. The translation products were analyzed by SDS-PAGE and quantitated with laser

densitometry. Both the SDS-PAGE and laser densitometry results suggest that the core of the IRES is the sequence element between nucleotides 529-630 and that the flanking regions facilitate ribosome orientation, binding, and thus, initiation of CVB3 translation (Yang *et al.*, 1997).

Using the GCG program, a secondary structural model of the CVB1 5' UTR was predicted (Yang *et al.*, 1997). This model has 11 stem-loops labeled A-K. Though the first 5 stem-loops, A-E resemble the folding pattern of poliovirus, the remaining loops differ widely (Yang *et al.*, 1997). Despite these differences between the IRES of poliovirus and CBV3, the successful translation of the P1 cistron in the bicistronic construct and results of the 5' UTR deletion mutagenesis studies strongly support for the presence of an IRES within the 5' UTR of CVB3.

Foot and Mouth Disease Virus IRES

Foot and Mouth Disease Virus (FMDV) is an aphthovirus in the picornavirus family. At 1,300 nt, the 5' UTR is the longest of the picornavirus family. It is unique in that polyprotein synthesis initiates at two different sites, AUG 11 and AUG 12, producing two gene products, proteases L and L' (Strebel and Beck, 1986).

Since FMDV belongs to the picornavirus family, it seemed likely that the virus also contained an IRES within its 5' UTR. Localization of the site was first derived from sequence homology to other picornaviruses (Pilipenko *et al.*, 1989). The first functional analysis of the internal ribosome entry site provided data that allowed the site to be characterized. Due to the extreme length of the 5' UTR, deletion mutagenesis would have been an unwieldy means of determining the critical regions of the IRES. To identify the

regions of the 5' UTR essential to translational efficiency, the entire 5' UTR region was mutagenized by random insertion of adapter fragments into DNase I-cleaved plasmid DNA (Kuhn *et al.*, 1990). Plasmid DNA was linearized, fused to synthetic *EcoRI* adaptors and recircularized, resulting in a 32-base pair fragment insertion. The position of the inserted *EcoRI* restriction site within the 5' UTR was then analyzed by sequence analysis. The truncated 5' UTRs were fused with the open reading frame of the *neo* gene of transposon Tn5. Using the *neo* gene as an indicator of translational activity, rather than the viral gene products, gave a more accurate quantitative analysis since viral genes are rapidly degraded by the autoproteolytic products of the viral genome. All mutants were sequenced and appropriate clones chosen for transcription and translation in rabbit reticulocyte lysates. As expected, some mutations had little or no effect on the translation of *neo*, while others reduced or completely abolished expression. To determine the effect the mutations would have *in vivo*, and to identify the contribution of different parts of the 5' UTR to translational efficiency, FMDV insertion and deletion mutants were ligated into a plasmid, transferred from the plasmid into an SV40 shuttle vector and then expressed in BHK-21 cells. The efficiency of *neo* translation was quantitated by *in situ* phosphorylation of kanamycin. Based upon the data from the insertion and deletion mutagenesis both *in vitro* and *in vivo*, it appeared that the regions involved in the internal binding of ribosomes were 450 nucleotides upstream of the AUG 11 start site (Kuhn *et al.*, 1990). Mutagenesis between nucleotides 0-360 and 660-750 appeared to have no deleterious effect; however mutagenesis between nucleotides 360-660 reduced translational efficiency >90% (Kuhn *et al.*, 1990). The data suggest that the region of the FMDV 5' UTR required for internal initiation is divided into

two functional domains between positions 360-660 and 750-805 (805= site of AUG 12). A proposed secondary structure was drawn that contained 5 hairpin stem-loops. Mutations in the middle of the IRES, corresponding to hairpin 3 (nt 360-660) were the most deleterious. Mutations within hairpin 4 (nt 750-805) reduced translational efficiency approximately 50%, suggesting that this region is less important (Kuhn *et al.*, 1990).

Rhinovirus IRES

According to the National Center for Health Statistics (NCHS) individuals in the United States acquire 1 billion colds a year. The NCHS reports that rhinoviruses cause between 30-35% of adult colds; and, in 1995, 22 million days lost from work and 7.9 million doctor visits were claimed to be due to the common cold (Benson and Marano, 1995). Therapeutics directed against rhinovirus are of particular interest due to the enormous economic impact of the common cold.

The first investigation of internal initiation of rhinovirus translation resulted in mapping the 3' boundary of the human rhinovirus-2 (HRV-2) 5' UTR (Borman and Jackson, 1992). First, truncations were made from the 3' end of the HRV-2 5' UTR, and placed upstream of a temperature-sensitive reporter gene, derived from influenza virus NS cDNA. *In vitro* transcripts were translated in rabbit reticulocyte lysate (RRL), with or without the addition of HeLa cell extracts. Analysis of translational efficiency led to the conclusion that the 3' ribosome entry site (the most 5'-proximal point where the ribosome can successfully bind for internal initiation) of the human rhinovirus 2 (HRV-2) IRES is between nt 554-568

(Borman and Jackson, 1992). Though this study stimulated translation of a reporter gene rather than a rhinovirus mRNA, the results strongly suggest the presence of an internal initiation site within the 5' UTR of HRV-2.

Later, Rojas-Eisenring (1995) and colleagues used mobility shift and UV cross-linking assays to identify the specific sequences in human rhinovirus-14 (HRV-14) that bound cellular factors. The IRES element of HRV-2 stretches from nt 10-568, and interacts with the cellular proteins p57 (PTB) and p97 (Borman and Jackson, 1992). To determine the boundaries of the IRES, four RNA probes were constructed with various 3' ends, each containing a portion of the linear, unstructured region between the last two, 3' stem-loop structures (Fig. 6). Probe A contained nucleotides 538-598, probe B contained nucleotides 538-591, probe C contained nucleotides 538-583, and probe D, the nucleotides spanning 538-575. The ability of the probes to form complexes with cell proteins was evaluated by mobility shift assays. Probes A and B exhibited the most specific and stable binding, suggesting that the sequences between nt 538 and 591 were the minimum required for RNA-protein binding.

To characterize the proteins that bound the rhinovirus 5' UTR, probes A, B, C, and, D were incubated with HeLa cells and ribosomal salt wash (RSW), and then UV cross-linked. All four probes cross-linked to at least 6 major proteins. However, the crosslinked proteins with probes A and B (*i.e.*, p57, p52, p97, and p68) were noticeably absent when probed with C and D. Based on the work done by Borman *et al.* (1992), it was suspected that the RNA sequence from nt 538-591, a region that is just upstream of the stem-loop

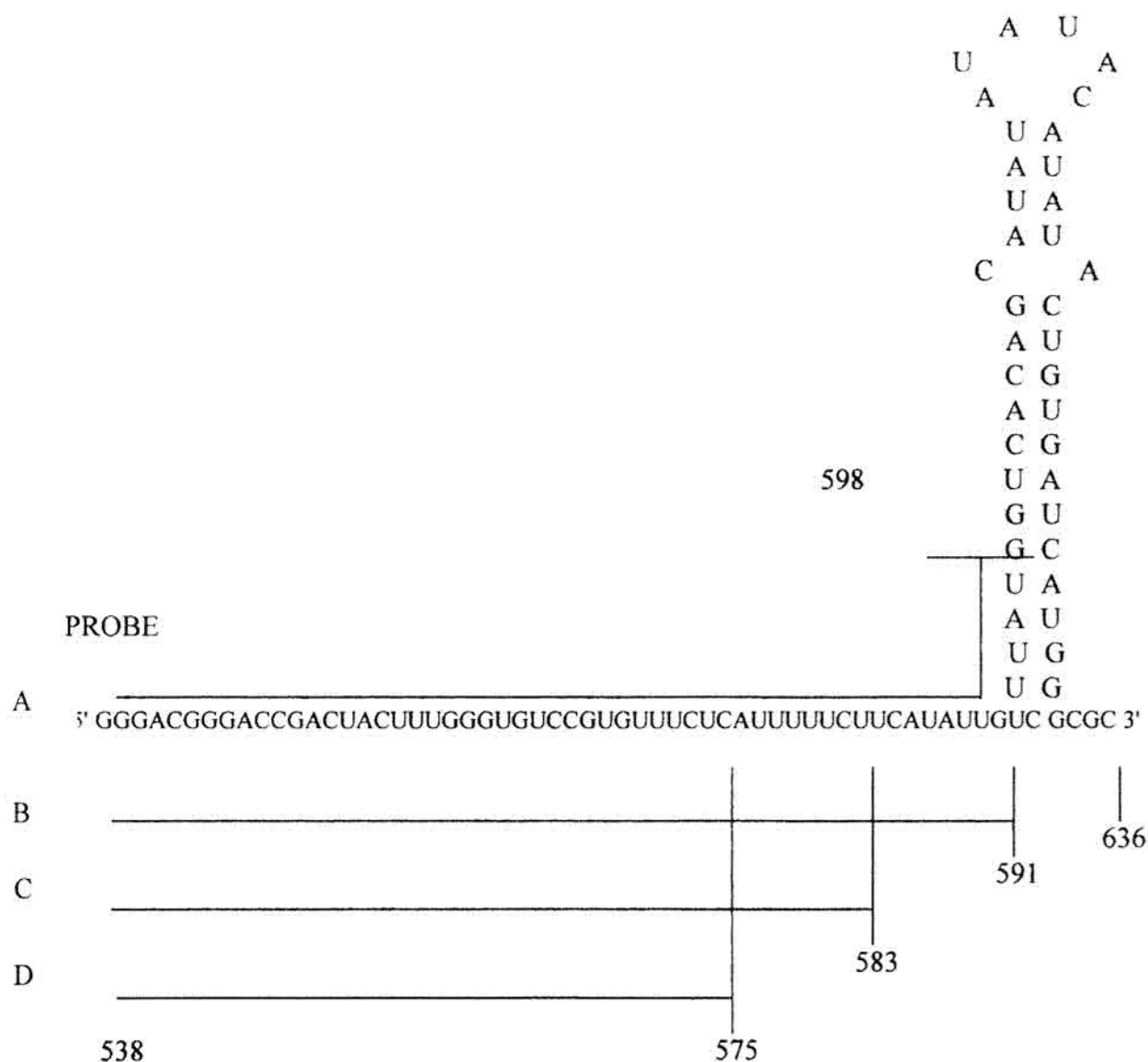


Fig. 6. Probes A, B, C, and D in the rhinovirus 5' UTR adapted from Rojas-Eisenring, *et al.*, 1995. The HRV-2 5' UTR sequence from nucleotides 536-636 is shown and corresponds to the 3' end of the UTR.

structure containing the initiating AUG codon, was the site of internal ribosome binding. To determine the identity of p57 and p52, on Western blots were performed on proteins cross-linked to probes A, B, C, and D with anti-La (α -p52) and anti-PTB (α -p57) antibodies. These results showed that proteins pPTB (p57), La (p52), p97, and p68 bind to the IRES element in HRV-14 as in other picornaviruses, and that nt 538-591 near the 3' end of the UTR are critical for cap-independent ribosomal binding in HRV-14 (Rojas-Eisenring *et al.*, 1995).

FLAVIVIRIDAE

Hepatitis C and Pestivirus Overview

The family Flaviridae contains approximately 70 members, including hepatitis C virus (HCV) and the pestiviruses, which include bovine viral diarrhea (BVDV), hog cholera virus (HChV), and classical swine fever virus (CSFV). Flaviviruses contain positive sense, single-stranded RNA genomes that have long 5' UTRs. HCV is the etiologic agent of blood-borne non-A, non-B hepatitis, and is the predominate infectious agent associated with post-transfusion hepatitis (Wang, 1993). In addition, clinical studies have shown a strong correlation between HCV infection and hepatic cancer (Saito *et al.*, 1990). Pestiviruses are the causative agents of several economically important animal diseases (Moenning, 1990).

Like the picornaviruses, the 5' UTRs of HCV and the pestiviruses contain extremely long 5' UTRs (HCV: 332 to 341 nucleotides, depending upon subtype; BVDV: 385 nucleotides; CSFV: 373 nucleotides) (Saito *et al.*, 1990; Rijnbrand *et al.*, 1997).

Additionally, these regions contain multiple AUG codons upstream of the true initiation site. These characteristics suggested that HCV and the pestiviruses might contain IRES elements.

Hepatitis C Virus (HCV) and Pestivirus IRESs

Tsukiyama-Kohara and colleagues first described the presence of an IRES in HCV (Tsukiyama-Kohara *et al.*, 1992). Structural similarities of the 5' UTR of HCV RNA to picornavirus RNAs suggested the presence of an IRES in HCV. To study the mechanism of translation, Tsukiyama-Kohara's group transcribed HCV RNA from cDNA. Since Pelletier (1988) showed that picornavirus translation was unaffected by the introduction of a cap structure to the 5' UTR, the mRNA activities of HCV RNAs with methylated caps and unmethylated caps were compared in the absence or presence of S-adenosylhomo-cysteine (SAH). SAH prevents methylation of GpppG-ended RNA, resulting in abrogation of cap-dependent translation. The mRNA of capped, methylated HCV RNA was similar to capped, unmethylated HCV RNA in an intact 5' HCV UTR, suggesting that HCV had a means of translating in a cap-independent manner (Tsukiyama-Kohara *et al.*, 1992). Evaluation of translation products derived from a plasmid construct, which lacked nucleotides 1-267 of the HCV 5' UTR, demonstrated that capped, methylated RNA was much more efficient than capped, unmethylated RNA. This result suggested that a segment within the 5' UTR, upstream of nt 270, has the ability to initiate protein synthesis independently of cap functions (Tsukiyama-Kohara *et al.*, 1992). The determinations that HCV RNA with an unmethylated, inactive cap can translate as well as with a methylated, active cap and that deletion of segments within the 5' UTR inhibited translation further supported the presence of an IRES within the 5' UTR of HCV mRNA.

To confirm that the 5' UTR contained an IRES, a capped, methylated bicistronic mRNA consisting of CAT mRNA and HCV mRNA, as the first and second cistrons, respectively, was synthesized (Tsukiyama-Kohara *et al.*, 1992). The translation experiments were performed in rabbit reticulocyte lysate (RRL) and coxsackievirus B1 (CVB1) infected HeLa cells. A member of the family Picornaviridae, coxsackievirus B1 has been shown to suppress cap-dependent translation in HeLa cells. If an IRES existed within the 5' UTR of HCV, there would be no translation of the first cistron (CAT), but the second cistron (HCV) would be translated in the CVB1-infected cells. In RRL, both the CAT and HCV mRNA were translated with equal efficiency, but in the CVB1-infected HeLa cells, only the HCV RNA was translated. These results confirmed both the existence of an IRES of the 5'UTR of HCV and the ability of HCV mRNA to be translated via internal initiation of translation (Tsukiyama-Kohara *et al.*, 1992).

Wang *et al.*, (1993) also utilized the bicistronic expression strategy of Pelletier *et al.*, (1988) to further support the conclusion of a functional IRES in HCV. In this work, the HCV 5' UTR was inserted between two reporter genes, CAT and luciferase (LUC). The bicistronic constructs were transfected into rabbit reticulocyte lysates and the translation products from the full length HCV 5' UTR, T7DC1-341, and three truncated constructs: T7DC323-29, T7DC29-323, T7DC29-332, were analyzed. As expected, the CAT translation product (the upstream cistron) was translated with equal efficiency from the full length and truncated constructs. Expression of LUC, the downstream cistron, was inhibited by the truncated UTR constructs. Both the anti-sense (T7DC323-29) and truncation of nine nucleotides at the 3' end of the UTR (T7DC29-323) significantly reduced translation of the LUC gene (Wang *et al.*, 1993). Hep2G cells, a cultured hepatoma cell line, were

transfected with the bicistronic constructs. Again, LUC activity was dramatically inhibited in T7DC323-29 and T7DC29-332. Together, the works of Tsukiyama-Kohara *et al.*, (1992) and Wang *et al.*, (1993), suggested that the region of the 5' UTR of HCV between nucleotides 29 and 332 is essential in directing translation via an internal ribosome entry site.

Once the role of the HCV 5' UTR had been established in internal initiation of translation, Wang and colleagues (1993) used a larger series of deletion constructs than Tsukiyama-Kohara's group to define the minimal region that was responsible for the activity of the IRES. The constructs included deletions from the 5' and 3' end within the 5' UTR of HCV, cloned in front of the LUC gene. The monocistronic RNAs were translated in an RRL system. The analysis of LUC activity indicated that the first 75 nucleotides on the 5' end of the UTR were critical for translation via internal initiation, perhaps by the contribution to secondary structure (Wang *et al.*, 1993).

Sequence analysis of the 5' UTR of the hepatitis C genome showed that the region consists of 330-341 nucleotides that are highly conserved. The HCV IRES has a Yn-Xm-AUG motif similar to picornaviruses. The helical structure associated with the pyrimidine-rich tract (Yn) is more important than the primary sequence, as shown by nucleotide substitutions that maintained the integrity of the secondary structure remained functional. Conversely, deletions or substitutions that disrupted the secondary structure did not maintain the ability to initiate internal translation (Wang, 1993).

Brown and colleagues (1992) unraveled the secondary structure of the 5' UTRs of HCV and pestivirus mRNAs using the FOLD computer program. Though the sequences of HCV and the pestiviruses are widely divergent, there is a highly conserved pestiviral structural domain within the HCV structure (Brown *et al.*, 1992). The conserved stem loop

structure is located between bases 125-323 of the HCV 5' UTR, the region shown by Tsukiyama-Kohara *et al.*, (1992) to be essential for ribosome entry in bicistronic constructs. This conserved secondary structural element found within the 5' UTR of HCV and the pestiviruses BVDV and HChV differs very much from the IRES of HAV, suggesting that they may have evolved independently of each other (Brown *et al.*, 1992). Interestingly, though they probably evolved differently, the 5' UTRs of HCV and HAV do share two features not found in pestiviruses: short, single-stranded, pyrimidine rich regions upstream of the ribosome "landing pad" and short, conserved regions that are complementary to 18S ribosomal RNA within the pyrimidine rich regions (Brown *et al.*, 1992; Le *et al.*, 1995). Further investigations, similar to this one, are needed to develop a model of the evolution of internal ribosomal entry sequences. One way to analyze the evolution of the IRES is to utilize the "split decomposition" technique of Dopazo *et al.* (1993). This method uses a clustering technique to illustrate different evolutionary dynamics (Dopazo *et al.*, 1993).

SPECIFIC INTERACTIONS OF TRANS-ACTING CELLULAR FACTORS WITH THE IRES

In addition to cis-acting elements, successful internal ribosome entry requires cellular, trans-acting factors. Dorner and colleagues (1984) determined that accurate translation of poliovirus mRNA in cell-free systems, such as rabbit reticulocyte lysates, require the addition of HeLa cell proteins. Translation products of poliovirus RNA from rabbit reticulocyte lysates (RRL) were less efficiently translated than those produced *in vivo*

or in poliovirus-infected HeLa cell extracts. This result suggested that cellular factors, perhaps not present in RRLs, were involved in the ability of ribosomes to initiate accurate, efficient, internal translation (Pelletier and Sonenberg, 1988).

P52/La

Two types of experiments, the mobility shift assay and poliovirus translation in RRL, have been used to investigate the trans-acting factors necessary for IRES translation. A mobility-shift assay permitted the characterization of a specific RNA-protein complex that forms between HeLa cell extracts and nucleotides 559-624 of the stem-loop structure of type 2 poliovirus (Meerovitch *et al.*, 1989). UV cross-linking assays identified the protein as a 52 kD protein (p52) (Meerovitch *et al.*, 1989). The p52 protein is abundant in HeLa cell extracts, but not in rabbit reticulocyte lysates (Meerovitch *et al.*, 1989). Purification and peptide microsequencing of p52 revealed that this protein is identical to the human La auto antigen (Meerovitch *et al.*, 1993). The human La auto antigen causes an auto-immune response in patients with lupus erythematosus (Tan, 1996). The human La autoantigen also binds to the 3' end of nearly all polymerase III transcripts and has been reported to stimulate translation by RNA polymerase III *in vitro* (Gottlieb and Steitz, 1989).

To examine the role of La in poliovirus infection, poliovirus translation accuracy was assessed in reticulocyte lysates, with and without recombinant La (Meerovitch *et al.*, 1993). The patterns of polypeptides synthesized were evaluated at 1 and 3 hours. Though normal poliovirus proteins were synthesized, at three hours, there were also several aberrant proteins, most notably, P2. The addition of La restored translational accuracy to, and increased the yield of the processed poliovirus proteins, three- to four-fold. The rescue

effect of La was similar to the effect seen when lysates were incubated with ribosomal salt wash and p52.

In addition, a bicistronic mRNA encoding both CAT and LUC, separated by the PV 5' UTR, was transcribed and translated in rabbit reticulocyte lysates alone, or with recombinant La. The addition of purified La protein enhanced LUC translation about five-fold. HeLa cell extracts immunodepleted with La antibodies failed to promote translation; and exogenous addition of La failed to restore translational efficacy, perhaps due to some other, uncharacterized cellular factor (Meerovitch *et al.*, 1993). Together, these data suggest that p52/La has the ability to enhance translation and suppress aberrant translation in poliovirus. La protein had no effect on EMCV translation, which indicated that its action is specific for poliovirus and that some other factor(s) must interact with the IRES elements of other picornaviruses (Svitkin *et al.*, 1994).

p57/PTB

A 57 kDa protein, termed polypyrimidine tract binding protein (PTB), was initially identified by UV cross-linking to the polypyrimidine tract in pre-mRNAs (Garcia-Blanco, *et al.*, 1989). UV cross-linking studies identified a 57 kD cellular protein that interacts with the IRES factors of encephalomyocarditis virus, foot and mouth disease, rhino-, polio-, and hepatitis A- viruses (Jang and Wimmer, 1990; Luz and Beck, 1991; Pestova, 1991; Chang *et al.*, 1993). The EMCV IRES contains four p57/PTB binding sites that correspond to two p57/PTB binding sites on the 5' UTR of FMDV (Chang *et al.*, 1993; Luz and Beck, 1991; Kaminiski and Jackson, 1998). Jang and Wimmer (1990) demonstrated, also via UV crosslinking, that the interaction of p57/PTB with the EMCV H domain binding site was

abrogated by deletion or disruption of the structure by nucleotide substitution in the 5' half of a stem-loop region termed the H domain. Further studies demonstrated that there is no apparent sequence conservation between RNA probes that are bound by p57/PTB. Disruption of the secondary structure of IRES regions bound by PTB destroyed IRES translation. Restoration of the structure restored the function of the domain, consistent with the finding that PTB recognition is dependent upon RNA structure (Garcia-Blanco *et al.*, 1989; Patton *et al.*, 1991). It is now apparent that PTB binding cannot be predicted based upon the presence of pyrimidine content (Patton *et al.*, 1991).

To characterize the binding of cellular proteins with the IRES of hepatitis A virus, Chang *et al.* (1993) used a UV cross-linking/label transfer assay. A 57 kD protein found in HeLa cell protein extracts reacted with anti-polypyrimidine tract-binding (PTB) protein in immunoblots, providing evidence that internal translation of HAV requires the cellular PTB protein (Chang *et al.*, 1993).

Hellen and colleagues (1993) demonstrated that p57 is identical to a polypyrimidine tract binding-protein (PTB), a predominately nuclear protein. As PTB interacts with a 100 kDa splicing factor (PSF) and other pre-mRNAs, one of its roles is probably to determine splice-site selection. Though depletion of PTB abrogated cap-independent translation, attempts to restore translational efficiency via addition of recombinant PTB were unsuccessful, somewhat clouding the conclusion that PTB was required for EMCV translation (Hellen *et al.*, 1993). Later, Hellen and colleagues (1994) demonstrated that depletion of PTB from HeLa cell lysates inhibited poliovirus and EMCV mRNA, but had no effect on the translation of beta-globin mRNA (cap-dependent translation). This finding

appeared to support the necessity of p57/PTB in internal initiation of poliovirus and EMCV replication.

To define the interaction of trans-acting proteins that might function as translation initiation factors in the IRES, Chang and colleagues analyzed the binding of ribosome-associated proteins from different cell types to the 5' UTR of HAV. Their results showed that, depending upon the cell type, four major proteins (p30, p 39, p57, and p110) bind the HAV 5' UTR. The observation that HAV grows better in some cells than others, may be a reflection of the expression of different 5' UTR binding proteins within different cell types.

EUKARYOTIC PROTEINS CONTAINING IRESs

As discussed in the Introduction, initiation of protein synthesis in eukaryotes involves the scanning and sequential binding of the 40S ribosomal subunit, along with translational initiation factors, and 60S ribosomal subunits to an mRNA, leading to the assembly of the 80S initiation complex at the appropriate AUG codon (Kozak, 1983). Several eukaryotic mRNAs including BiP, *c-myc*, eIF4G, and VEGF have been identified that can initiate cap-independent translation via IRES sequence elements (Yang *et al.*, 1997).

Human immunoglobulin heavy chain binding protein (BiP) IRES

The immunoglobulin heavy-chain binding protein (BiP) is a 78 kD protein found in the lumen of the endoplasmic reticulum (ER) (Macejak and Sarnow, 1990). BiP is abundant in the ER of most eukaryotic cells; however its transcription is strongly upregulated in

response to stress such as glucose starvation. The translation of BiP was known to be cap-dependent is also regulated at the post-transcriptional level.

Translation of the mRNA encoding the immunoglobulin heavy-chain binding protein (BiP) is enhanced in poliovirus-infected cells (Macejak and Sarnow, 1990). This observation made BiP a likely candidate for internal initiation of translation. To test whether or not the mRNA of BiP was translated by internal ribosome binding, Macejak and Sarnow constructed a plasmid to express bicistronic hybrid RNAs. Plasmid vectors, with either the promoter for T7 RNA polymerase or simian virus 40 (SV40), contained two open reading frames encoding CAT and LUC placed immediately downstream of the promoters. Three vectors were constructed containing different intercistronic spacer (ICS) sequences. The negative control contained a sequence from *Drosophila* (ED) as the intercistronic spacer. The positive control contained the 5' non-coding region of poliovirus (known to have an IRES) as the intercistronic spacer. To test for the presence of an IRES within BiP, the intercistronic spacer consisted of 220 nucleotides from the 5' UTR of BiP. Both cistrons were translated efficiently in CAT/BiP/LUC, but only the CAT cistron was translated in CAT/ED/LUC, suggesting that the 5' UTR of BiP mRNA contains an IRES sequence and can be translated via a cap-independent mechanism (Macejak and Sarnow, 1990). The presence of a hairpin structure in the 5' end inhibited translation of CAT, but not of LUC thus further supporting the existence of a cellular IRES (Macejak and Sarnow, 1990).

Expression of various bicistronic mRNAs, which contained various portions of the 5' UTR of BiP, revealed that the BiP IRES is located between nt 129-220 at the 3' end of the UTR. Le and Maizel identified a 92 nt unusual folding region (UFR) from nt 129-220, close to the initiator AUG in the BiP mRNA (Le and Maizel, 1997). Detailed analysis revealed

that the UFR could be folded into a Y-type stem-loop with an additional stem-loop in the 3'-end. The Y-type structure is conserved among many other BiP mRNAs, including those found in *Trypanosoma brucei*, *Giardia lamblia*, and the *Saccharomyces cerevisiae* karogamy gene (KAR2, a homolog of human BiP). The original work of Macejak and Sarnow (1990) was the first to demonstrate the presence of an IRES in eukaryotic mRNA, and provided the first evidence that translation by internal ribosome binding is not unique to picornavirus.

Yang and colleagues located the specific site on the 5' UTR responsible for ribosome binding by constructing bicistronic plasmids containing different regions of the BiP 5' UTR within the intercistronic spacer (Yang *et al.*, 1997). Their data indicated that the sequences that mediate internal initiation are located between nucleotides 120-220. In picornaviruses, internal initiation appears to require approximately 450 nucleotides of the 5' UTR and the UTR contains many nonfunctional AUG codons. The 5' UTR of BiP does not match this profile. It is only 220 nucleotides long, contains no AUG codons, and is the shortest known sequence involved in internal ribosome binding. A comparison of the sequences of the 5' UTRs between picornavirus and BiP did not reveal any similarity, suggesting that the secondary structure or the IRES determines function (Yang *et al.*, 1997).

Antennapedia Gene of Drosophila

The Antennapedia (*Antp*) gene of *Drosophila* is a homeotic gene with two promoters, P1 and P2. The resulting mRNAs contain 1512 nt (P1) and 1727 nt (P2) 5' UTRs. Transcription of the gene produces two transcripts composed of exons A, B, D, and E or exons C, D, and E from promoters P1 and P2, respectively. Exons A, B, and C contain

multiple AUG codons. If the scanning mechanism were used for initiation of Antp mRNA, the ribosomal complex would bind at the 5' end of the mRNA and scan 1730 nt, through 15 AUG codons, to initiate protein synthesis at the 16th AUG codon (Oh and Sarnow, 1993). To test whether or not ribosomes could use internal initiation sequences to initiate translation within the Antp gene, Oh and Sarnow (1993) utilized the bicistronic method described by Pelletier and Sonenberg (1988). They constructed plasmid vectors producing bicistronic transcripts of CAT, an intercistronic spacer (ICS) containing portions of the Antp 5' UTR, and LUC. When transfected into cultured SL2 cells, the entire 5' UTR of Antp P2 (exons C, D, and E) mediated translation most efficiently, but exon D alone was sufficient to mediate translation of LUC (Oh and Sarnow, 1993). The sequence of the noncoding exon D is present in transcripts initiated at both the P1 and P2 promoters. Although the *Antp* gene contains an exceptionally long 5' UTR (>1500 nt), the first 55 nt of exon D are required for Antp IRES function. In addition, this 55 nt sequence is highly conserved in three species of *Drosophila* even though the flanking regions are widely divergent (Oh, *et al.*, 1992; Oh and Sarnow, 1993). The common ancestor of these three species is believed to have existed 60 million years ago. The conservation of this 55 nt region of exon D suggests that the sequence may serve an important function in regulating transcription or translation (Hooper *et al.*, 1992).

c-myc

The human proto-oncogene, *c-myc*, has a fundamental role in various cellular events, including proliferation, differentiation and apoptosis (Nanbru *et al.*, 1997). *c-myc* contains two initiation codons CUG and AUG that encode two proteins, the 64 kDa c-Myc-1 and 67

kDa c-Myc2, respectively (Nanbru *et al.*, 1997). Transcription may be from one of four promoters (P0, P1, P2, and P3) yielding four different RNA 5' UTR sequences. Normally, most of the transcripts start at the P1 and P2 promoter. In a diseased state such as Burkitt's lymphoma, which has a c-myc chromosomal translocation, the P1 promoter is preferentially used and P0 mRNA accounts for approximately 10% of the total c-myc RNA (Eick *et al.*, 1990).

To evaluate the translational regulation of c-Myc expression by the P0, P1, and P2 promoters, the three leader sequences (1173, 524, and 363 nt upstream from the CUG initiator codon respectively) were fused to the CAT coding sequences: pSCT-MyCAT-P0, pSCT-MyCAT-P1, and pSCT-MyCAT-P2. In addition, two deletions were created in the 5' UTR of pSCT-MyCAT-P2, resulting in reduced leaders of 94 and 14 nt. These constructs were designated MyCAT- Δ 2 and MyCAT- Δ 1, respectively. Translation analysis clearly showed that cap-independent expression of CUG and AUG-initiated Myc-CAT proteins is conferred by the 5' UTR of P0, P1, and P2. Construction of bicistronic Myc-CAT DNAs and subsequent expression in COS-7 cells showed that an IRES is present in both the P0 and P2 5' leaders of c-myc mRNA. Further, the lack of translation in the deletion constructs demonstrated that the IRES structure is located between nucleotides 363 and 64 upstream from the CUG initiating codon (Nanbru *et al.*, 1997).

This study demonstrates the presence of an IRES in the 5' UTR of c-myc mRNA. It is, to date, the only proto-oncogene with the ability to initiate translation in a cap-independent manner. As in the case of human fibroblast growth factor, FGF-2, internal initiation of *c-myc* appears to be initiated in response to stress such as the cancer-prone disorder, Bloom's syndrome (Vagner *et al.*, 1995; West *et al.*, 1995). Nanbru and

colleagues (1997) postulate that *c-myc* is activated by an IRES-dependent translation pathway that permits a rapid response to stress stimuli such as heat shock and oxidative stress.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a growth factor that also exercises cap-independent initiation of translation under stressful conditions. VEGF acts primarily to promote compensatory angiogenesis under periods of oxygen shortage (Plate and Warnke, 1997). The option of internal initiation would provide VEGF with an advantage when eukaryotic initiation factor 4G (eIF4G) becomes rate limiting, as under hypoxia.

Using the standard bicistronic reporter gene constructs, Stein and colleagues (1998) determined that the 1,014 bp 5' UTR of VEGF contains a viable IRES sequence element (Stein *et al.*, 1998). The IRES element was not characterized with deletion mutants, but sequence analysis shows no homology to known cellular or viral IRES sequences. Further studies should include deletion analysis to determine the minimum region of the 5' UTR responsible for internal initiation; as well as predict secondary, stem-loop structure.

eIF4G mRNA

Cap-dependent translation initiation requires eIF4G initiation factors. eIF4G forms a heterotrimeric complex (termed eIF4F) comprised of a cap-binding protein (eIF4E), an RNA helicase (eIF4A) and eIF4B. This complex catalyzes the recognition of the mRNA cap, unwinding of the mRNA secondary structure, and binding of the mRNA to the 43S pre-initiation complex (Gan *et al.*, 1998). In mammalian cells infected by entero- or

rhinoviruses, viral proteases cleave eIF4G, effectively shutting down cap-dependent initiation, while allowing the cap-independent translation of viral proteins.

The 5' UTR of eIF4G resembles picornavirus IRES sequences in that it is extremely long and contains four upstream open reading frames. Using the 5' UTR upstream of a LUC reporter gene, and inserting the UTR between two cistrons of a bicistronic mRNA, Gan and Rhodes (1996) demonstrated the presence of an IRES in the 5' UTR of eIF4G. Recently, the IRES of eIF4G was further characterized. Deletion constructs from both the 5' and 3' ends of the UTR indicate that nucleotides 161-357 (196 nts) have IRES activity equal to the complete 5' UTR. Mutational analysis indicates that the AUG at position 369 is the initiation codon (Gan *et al.*, 1998).

Many of the aspects of the mechanism of internal initiation are unknown. Only recently has the specific interaction(s) of cellular factors with IRES elements been explored. One of the first studies of the influence of cellular proteins on internal initiation involved the 5' UTR of eIF4G (Gan and Rhodes, 1996). The change in translational specificity, from cap-dependent to cap-independent, mediated by viral proteolysis of eIF4G, led to the theory that the purpose of proteolytic eIF4G cleavage was not merely to terminate cap-dependent translation, but that, when cleaved, the product also served to direct internal initiation (Gan *et al.*, 1998). When cleaved, the N-terminal portion of eIF4G (the region bound to the cap-binding protein, eIF4E) is released. The C-terminal portion is bound to the remainder of the initiation complex, and though unable to initiate cap-dependent translation, does participate in, and enhance, internal initiation of picornavirus mRNA (Gan and Rhodes, 1996).

Human eIF4G mRNA contains four open reading frames, all upstream of the 4188-nt open reading frame that encodes the protein (Yan *et al.*, 1992). To test the manner of

translation, Gan and colleagues placed the first 357 nt of eIF4G mRNA upstream of LUC, driven by the SV40 promoter. The control vector was then transfected into a leukemia cell line and the expression of LUC quantified. LUC translation was enhanced 7.3-fold in the construct containing the 356 nt fragment of eIF4G (Gan and Rhodes, 1996). Results of a ribonuclease protection assay of luciferase mRNA showed that mRNA levels were similar in the control vector and eIF4G 5' UTR-containing vector, and not proportional to luciferase expression. This finding indicated that the 5' UTR acts at the level of translation, rather than transcription. To determine whether this observation of increased translation was unique to the leukemia cell line, three other cells lines were tested in the same manner. In all cases, the presence of the eIF4G 5' UTR increased levels of LUC translation (Gan and Rhodes, 1996). Taken together, these results strongly suggested the presence of an IRES within the 5' UTR of eIF4G.

To determine the mechanism of initiation codon selection for the eIF4G IRES, Gan and colleagues (1998) constructed three plasmids, each containing LUC reporter, the 5' UTR of eIF4G and an out of frame AUG at nt 298, 319, or 340. Luciferase expression was unaffected by the out-of-frame AUG at nt 298. In contrast, the out-of-frame AUGs introduced at nucleotides 319 and 340 significantly reduced LUC activity. LUC was not effectively translated from the out-of-frame start codons; however, the presence of some product prompted the authors to suggest that downstream AUGs may permit translation from alternative initiation sites. Because initiation codons can be recognized by 40S ribosomal subunits, regardless of whether they are located at nt 319, 340, or 369, it appears that eIF4G IRES-mediated translation involves ribosomal scanning. More recently, eIF4G has been implicated in directly recognizing and binding the IRES, and enabling eIF4A to

enter the mRNA-43 S ribosomal preinitiation complex (Pestova *et al.*, 1996). In an initiation reaction reconstituted *in vitro* from purified translation components, IRES-mediated initiation was dependent upon eIF4A (helicase) and the central third of eIF4G. In this study, UV cross-linking demonstrated that the central third of eIF4G bound strongly and specifically to the IRES of encephalomyocarditis virus with the same specificity as eIF4F (Pestova *et al.*, 1996). Later work by the same group used chemical and enzymatic probing to map the specific eIF4G binding site to the EMCV IRES, and reported that the target site for eIF4G binding is an oligo (A) loop, or bulge, at the junction of three helices (Kolupaeva *et al.*, 1998).

The preceding works strongly support the existence of an IRES in eIF4G mRNA. The presence of multiple upstream open reading frames would be expected to reduce mRNA translation, but instead stimulate translation. The increase in translation of the downstream LUC gene in the bicistronic construct is hard to explain without involving internal initiation. It is important to note, however, that these experiments involved stimulation of a reporter gene, not eIF4G itself.

The role of viral proteins on internal initiation has not been clearly elucidated. When eIF4G is cleaved by Lb protease, produced by foot and mouth disease virus, translation of uncapped transcripts is enhanced (Ohlmann *et al.* 1997). The 2A proteases of rhinovirus and enterovirus also stimulate translation of cap-independent RNAs (Borman *et al.*, 1995). Translational stimulation is clearly dependent upon enzymatic activity, as shown in experiments using proteolytically inactive mutants, or using wild-type proteinases in the presence of proteinase inhibitors (Ziegler *et al.*, 1995). Moreover, Ohlmann and colleagues

demonstrated that, for uncapped eIF4G mRNA, translation stimulation could be correlated with the presence of cleaved eIF4G (Ohlmann *et al.*, 1995).

The advantage conferred by the internal mechanism of initiation for eIF4G mRNA may be that it is merely a normal homeostatic mechanism that serves to maintain intracellular levels of eIF4G. If eIF4G levels decreased, as in proteolytic degradation, the ability of the cell to carry out cap-dependent translation would also decrease. Without the ability to translate via internal initiation, eIF4G would eventually be completely degraded and the cell would die. The ability of eIF4G to initiate its own translation independent of the traditional cap-dependent mechanism enables the cell to restore translational function following infection and clearly provides a survival advantage.

I-RNA

To date, serendipity has provided us with the only known inhibitor of viral IRES. This first discovery of an inhibitor of internal initiation of translation occurred as a result of trying to express an infectious poliovirus cDNA clone in *Saccharomyces cerevisiae*, hoping that poliovirus expressed proteins in yeast would shut-off the yeast cell transcription and translation. Instead, when the full-length, infectious clone of poliovirus cDNA under the control of the GAL 10 promoter was introduced into yeast, no viral protein synthesis was evident although intact viral transcripts were present (Coward and Dasgupta, 1992). Using poliovirus 5' UTR-CAT constructs both *in vivo* and *in vitro*, they showed that the yeast inhibitor required viral 5' UTR sequences to repress viral protein synthesis. This effect was demonstrated *in vitro* with cell free translation assays using yeast extracts and is apparently

due to a *trans*-acting factor since addition of yeast cell lysates to the HeLa cell translation reactions prevented translation of the viral RNA (Coward and Dasgupta, 1992).

Though the inhibitor also prevented the translation of other picornaviruses, including rhino-, hepatitis A, and Theiler's murine encephalomyelitis viruses, it was unable to prevent the translation of EMCV in HeLa cell lysates. A PV-EMCV chimera readily replicated in the hepatoma cell line expressing I-RNA. Additionally, the overall translation in the hepatoma cells was unaffected by the expression of I-RNA as determined by [³⁵S]methionine (Coward and Dasgupta, 1992). Based on the evidence, it seems that the cell system contains a functional PV receptor and that the inhibition seen in the cells transfected with the PV-HCV chimera was not due to an I-RNA-mediated disruption in general translational machinery, or in the PV receptor function. A member of the picornavirus family, EMCV RNA is translated by both HeLa cell lysates and rabbit reticulocyte lysates, in contrast to poliovirus RNA which is translated only by HeLa cell lysates. Together, the difference in internal initiation of translation requirements and the contrasting activity of the inhibitor on poliovirus and EMCV suggests that the inhibitor is interfering with a *trans*-acting factor specific for poliovirus RNA translation.

Characterization of the inhibitor revealed that it was heat stable, escapes phenol extraction, is resistant to proteinase K and DNase I treatments, and is sensitive to RNase A digestion, leading to the conclusion that the inhibitor is an RNA rather than a protein (Das *et al.*, 1994). Sequencing and purification revealed an RNA that is approximately 110 nt long, and is neither antisense to PV RNA nor has any sequence homology to poliovirus mRNA. Although 110 nt long, only the first 60 nucleotides were able to be sequenced with any certainty (Das *et al.*, 1994). A synthetic clone was prepared which produced RNA that

blocked poliovirus IRES-mediated translation, but not cap-dependent translation. Deletion analysis of poliovirus 5' UTR showed that this purified yeast inhibitor RNA (I-RNA) requires IRES sequences to effectively inhibit internal translation. A bicistronic construct, which contains poliovirus IRES between two cistrons, showed that I-RNA inhibited translation of the second cistron, but not the first. To further support the role of I-RNA as an inhibitor of poliovirus translation, HeLa cells were co-transfected with viral RNA and I-RNA. Translation of poliovirus RNA was almost completely blocked. This inhibition was reversed with the addition of an equimolar amount of an anti-sense I-RNA during co-transfection. Further, capping RNA prior to translation prevented I-RNA-mediated inhibition of translation (Das *et al.*, 1994).

UV cross-linking experiments revealed that the I-RNA interacts with a number of polypeptides known to be involved in internal initiation of translation, most notably a 52 kDa protein (the human La autoantigen) and a 57 kDa protein (the polypyrimidine tract binding protein) (Gottlieb and Steitz, 1989; Das *et al.*, 1994). Competition studies showed that the yeast I-RNA binds a 52 kDa protein similar to that bound by nucleotides 559-624 in the poliovirus 5' UTR. In a recent work, Das *et al.* reported that the addition of exogenous purified La protein reversed the I-RNA-mediated inhibition of poliovirus 5' UTR-dependent translation (Das *et al.*, 1996). However, the significance of p52/La and p57/PTB in IRES-mediated translation has not been clearly established. Yoo and Wolin (1994) reported a yeast homolog to human La autoantigen in *Saccharomyces cerevisiae*. Because the La protein is involved in transcription termination (Gottlieb and Steitz, 1989), it is possible that I-RNA may regulate transcription of pol III genes. It would be interesting to study the

interaction of yeast La protein with I-RNA. Such a study may help to determine the function of I-RNA in *Saccharomyces cerevisiae*.

Using nested deletions, Das *et al.* (1996) identified a region of I-RNA 16 nt long that inhibited PV IRES-mediated translation. This deletion construct, I-3 RNA, demonstrated that the minimum region required for inhibition is located between nt 30 and 45 (Fig. 7, from Das *et al.*, 1996). Since I-RNA shares no apparent homology with poliovirus RNA, and an antisense RNA with an exact complementary sequence to I-RNA binds p52 with equal affinity as the sense, it seems likely that the secondary structure of the I-RNA is responsible for the inhibitory activity (Das *et al.*, 1994).

Though there is very little sequence similarity between HCV and PV IRES elements, because the two bind similar polypeptides, Das and colleagues theorized that I-RNA might also inhibit HCV IRES-mediated translation (Das *et al.*, 1998). Using transfected hepatoma cells, and a hepatoma cell line that expressed I-RNA constitutively, they were able to determine the effect of I-RNA on HCV IRES-mediated translation during virus infection. Because no tissue culture system is available to study HCV infection, they used a PV-HCV chimera. The PV-HCV chimera consisted of the 5' cloverleaf structure of PV, followed by HCV IRES (nt 9 to 332), 123 amino acids of HCV core protein, followed by the entire PV open reading frame including the 3' UTR and poly (A) tail. Translation of the chimera is mediated by the HCV IRES. Hepatoma cells that expressed I-RNA constitutively were resistant to the chimera and *in vitro* translation of a bicistronic mRNA (CAT and LUC) flanked by HCV IRES sequence also showed specific inhibition. ³²P labeled I-RNA and HCV 5'UTR RNA UV cross-linked to cellular polypeptides revealed binding of p57/PTB, p57/La, and other polypeptides to I-RNA. Though these proteins are believed to be

					Inhibition of Translation	
I-RNA	1			60	(100)	
I-1 RNA	1	15		60	(65)	
	(	)				
I-2 RNA	1	16	30	60	(55)	
	(	)				
I-3 RNA	1		31	45	60	(0)
	(		)			
I-4 RNA	1			46	60	(6)
	(			)		
I-5 RNA	1	20		60	(52)	
	(	)				
I-6 RNA	1	25		60	(83)	
	(	)				
I-7 RNA	1	25		51	60	(93)
	(	)		(	)	
I-8 RNA	1	26		60	(0)	
	(	)				
I-9 RNA	1	29		46	60	(82)
	(	)		(	)	

Fig. 7. Adapted from Das et al., 1996. Schematic of nested deletions of I-RNA. Percentage translation inhibition is shown in column at right.

required for IRES-mediated translation, attempts to deplete a HeLa cell extract by passing over an I-RNA affinity column failed (Das *et al.*, 1998). To determine the precise role of cellular proteins in IRES-mediated translation, future studies should deplete a HeLa cell extract via an I-RNA affinity column and attempt to restore translation by addition of purified proteins.

The interaction of cellular proteins with the IRES of foot-and-mouth-disease virus has been studied using UV cross-linking (Meyer, *et al.*, 1995). This study indicated the importance of an 80 kDa protein, eIF-4B, in binding to the IRES structure. Attempts to separate ribosome-associated from non-ribosome-associated protein fractions led to a loss of cross-linking activity. The authors speculate that the loss of activity suggests that additional protein factors contribute to the interaction of eIF4B with the IRES of FMDV. It is possible that I-RNA interferes with the binding of cellular factors to initiation factors, inhibiting IRES-mediated translation. Additional work will most certainly include determining how the yeast La, eIF4B, and other cellular proteins interact with I-RNA as well as determining the three dimensional structure of I-RNA.

DISCUSSION

Internal ribosome binding requires the presence of *cis*-acting elements found in the 5' UTR of the mRNA, termed internal ribosome entry sites (IRES), and *trans*-acting, cell type-specific factor(s) such as p52/La and p57/PTB. The RNA-protein interaction involves the specific recognition of IRES sequences and the structural elements of the IRES within the 5' UTR by the cellular factors. Though first discovered in picornaviruses, this phenomenon is of general importance because it is not restricted to the picornaviruses. IRESs are found in

other viruses such as hepatitis C virus (HCV) and the pestiviruses, as well as some eukaryotic mRNAs such as those encoding the human immunoglobulin heavy chain binding protein (BiP), the Antennapedia gene of *Drosophila*, the proto-oncogene, *c-myc*, vascular endothelial growth factor (VEGF), and the eukaryotic initiation factor eIF4G.

Even though there are similarities between the viral and cellular IRESs, they operate in fundamentally different ways. Cellular IRES elements, such as BiP and eIF4G, contain a cap and poly (A) tail and are also capable of conventional, cap-dependent translation. More importantly, insertions of cellular IRESs do not stimulate translation of all uncapped mRNA in poliovirus-infected cells (Harford, 1995). Interestingly, eIF4G is the one factor common to both viral and cellular IRES processes. While it is clear that cleavage of eIF4G by the 2A and Lb viral proteases is a principal feature of host-cell shut off, it is unclear who benefits from the internal initiation mechanism of eIF4G. On one hand, the continued synthesis of eIF4G (via IRES) during infection may provide the host with the capacity to restore eIF4G levels and re-establish cap-dependent initiation, which could be an advantage to the host in terms of survival. Alternatively, one could argue that cleavage of eIF4G, through the action of viral proteases, provides more cleavage products (cleaved eIF4G) to increase cap-independently initiated viral translation. It is possible that the required concentration of eIF4G is lower for cap-independent than cap-dependent initiation. If so, the components of the translational machinery would then be more available for cap-independent translation, increasing eIF4G and thus completing an autocrine loop.

Heat shock is a physiological condition that can mediate the switch from cap-dependent to cap-independent initiation and allows for a more controlled study than possible with virus-infected cells (Lamphear and Panniers, 1990). During heat shock, as denatured

proteins accumulate, a protein kinase is released which acts to shut down cap-dependent protein synthesis, resulting in the rapid decrease in the synthesis of non-heat shock proteins (non-HSPs), but stable or increased synthesis of HSPs (Panniers, 1994). Cleavage products of eIF4G, eIF4A, and eIF4E can be readily separated by m7 GTP-Sepharose column chromatography (Lamphear *et al.*, 1995). To further elucidate the internal translation mechanism of eIF4G, future work should consider trying to rescue IRES-mediated initiation in heat shocked cells via the addition of proteolytic cleavage products of eIF4G, both with and without the addition of other initiation factors. eIF4G is the cellular translation factor critical to both viral and eukaryotic internal initiation of translation, and eIF4G itself has the capacity to translate via this mechanism. Unraveling the relationship between eIF4G and the IRES structure could reveal many underlying mechanisms of internal initiation of translation.

When compared with viral IRESs, little is known about cellular IRES elements. Le and Maizel (1997) suggest that the function of viral and cellular IRES elements is correlated with conserved, higher-order RNA structures. Because IRES-mediated internal initiation of translation is not unique to viruses, further characterization of the differences in the molecular mechanisms of viral and cellular internal initiation processes is important in combating the IRES containing viruses without risking damage to eukaryotic systems which utilize the mechanism of internal initiation.

The extensive stem-loop structure of IRES elements probably provide binding sites for cellular proteins which play a role as *trans*-acting factors during cap-independent translation. A comparison of the viral and cellular IRES structures indicates that they share folding shape, stem stacking, and sequence location at the 5' UTR. Cellular IRES stems A-D

correspond to stems E, F, G, and I/J of viral IRES. The "stem stacking" between stems B and C in the cellular IRES corresponds to stacking of viral stems F and G. Because the compact, tertiary structure of the IRES is formed by stem stacking, this element may prove to be one the most important properties of cellular and viral IRES elements (Le and Maizel, 1997)

The primary difference between the viral and cellular structural elements is the presence of an RNA "pseudo-knot" in viral IRES elements. Highly conserved and necessary for HCV IRES function, the pseudo-knot, which is composed of stems E and H, is found in all viral IRES elements (Wang, *et al.*, 1995). If the pseudo-knot proves to be required for the function of all viral IRESs, then this finding suggests a major difference between viral and cellular IRES-dependent internal initiation (Wang, *et al.*, 1995; Le and Maizel, 1998). Taking into account the complex interactions between ribosome and mRNA during translation, it is hard to imagine that the mechanism of translation via internal initiation differs completely from the cap-dependent initiation mechanism. It is highly probable that many of the cellular factors involved in cap-dependent translation initiation also play a role in cap-independent translation initiation.

While some studies had shown EMCV IRES-mediated translation to be PTB-independent, others had shown it to be PTB-dependent. Recently, the discrepancy in results regarding the necessity of p57/PTB in the IRES functioning of EMCV has been cleared up. Kaminski and Jackson (1998) report that the PTB requirement for internal initiation of translation in EMCV RNA is conditional, rather than absolute, upon a particular stem-loop structure in the IRES. Based on sequence and secondary structure published by Pilipinko *et al.* (1989) and Duke *et al.* (1992), the wild-type EMCV IRES contains an A-rich "bulge" in

the Y stem-loop structure. This bulge had the sequence UAAAAAA, which corresponds to the sequence published by Duke, et al. (1992). The EMCV constructs used in the experiments of Hellen et al. (1993), Jang and Wimmer (1990), Kaminiski (1990), as well as Coward and Dasgupta (1992) were oligo (A) loop expanded: UAAAAAAA. Kaminiski and Jackson (1998) speculate that the additional A residue was acquired either as an artifact of cloning or by replicative stuttering during cDNA propagation in *Escherichia coli*.

Using an RNA affinity column to specifically deplete rabbit reticulocyte lysate of p57/PTB, Kaminiski and Jackson (1998) examined whether the size of the A-rich bulge in the EMCV IRES influenced the PTB dependence of IRES-promoted translation. All constructs that had an IRES with a 7A bulge had PTB dependence. This was in contrast to the wild-type EMCV, containing a 6A bulge, which was completely PTB independent (Kaminiski and Jackson, 1998). Wild-type TMEV possesses "intermediate" PTB dependence. In an attempt to increase PTB dependence, the same strategy was used to expand the equivalent bulge in the TMEV IRES. The IRES-mediated translation of the TMEV mutants showed strongly increased PTB-dependence. This work appears to have resolved the controversy of the role of PTB in internal initiation of translation in EMCV.

These results suggest that the size of the A-rich bulge can determine the stability of folding, and thus, whether an IRES will require PTB for efficient internal initiation of translation. UV-crosslinking studies showed that the size of the bulge has no effect on PTB binding to the high-affinity site (Kaminiski and Jackson, 1998). PTB binds as effectively to IRESs that do not require PTB as it binds to those with complete dependence on PTB. The bulge is far downstream of the high-affinity PTB binding site in both the primary sequence and the secondary structure. Kaminiski and Jackson (1998) propose that the enlargement of

the A-rich bulge leads to a distortion of the spatial relationship between the contiguous stem-loops, diminishing IRES activity. According to this hypothesis, binding of PTB to its high-affinity site helps to refold the IRES to its correct, 3-dimensional structure, enabling translation to proceed. A PTB-independent IRES is one that does not require PTB binding to adopt the correct 3-dimensional folding structure, whereas a PTB-dependent IRES requires PTB binding to correct any mis-folding of the IRES (Kaminiski and Jackson, 1998). It should be noted that PTB interacts not only with its high-affinity site, but also with other sites on the EMCV IRES, two of which are very close to the A-rich bulge (Kolupaeva, *et al.*, 1996). If Kaminiski and Jackson are correct, mutation of the high-affinity PTB-binding site (but not the other sites) should inhibit IRES-mediated translation of a PTB-dependent IRES.

The identification of the oligo (A) bulge as the specific structural element within the IRES of EMCV responsible for eIF4G binding, is consistent with the general model for the interaction of RNA binding domains with RNA. Commonly, an RNA loop binds to the surface of a beta-sheet as an open structure; and the sequence of the loop interacts extensively with bound RNA, which remains exposed on the beta sheet RNA-binding surface where it is accessible for interaction with other RNA-binding proteins (Oubridge, *et al.*, 1994). Previous work demonstrated that eIF4G directly recognizes the EMCV IRES and that the interaction was important in recruiting the IRES to ribosomes (Pestova, *et al.*, 1996). A similar structural element comprising an oligo (A) loop occurs at the identical position (the Y-loop) in the IRESs of many other picornaviruses, including all cardio-, aphtho-, echo-, equine rhino-, and hepatitis A viruses (Pilipenko, *et al.*, 1989; Jackson and Kaminiski, 1995; Brown, *et al.*, 1991).

Though the phenomenon of internal initiation is relatively new, it provides an attractive target for therapeutic agents against picorna-, flavi-, and pestiviruses. Since Paterson *et al.* (1977) first used single-stranded DNA to inhibit translation of complementary mRNA in a cell free system, focus has been placed on the use of antisense oligonucleotides as antiviral agents (Cohen, 1991; James and Gibson, 1998; Gewirtz *et al.*, 1998). Strategies for inhibiting translation primarily target mRNA. "Antisense" therapy strategies rely on the reverse complementary base pairs between the agent and mRNA, and may be DNA oligonucleotides, RNA, RNA decoys or ribozymes. Hybridization between the target and agent creates a block that prevents the ribosomal complex from reading the message, arresting translation. Ribozymes are catalytic RNA molecules that recognize and cleave specific sequences, virtually ensuring destruction of the mRNA target. Following cleavage of the target mRNA, ribozymes are released, free to hybridize with another RNA (Castanotto *et al.* 1994). The site-specific cleaving moieties of ribozymes can be incorporated into single-stranded RNA molecules containing 5' and 3' ends designed to hybridize with the flanking sequence of a potential cleavage site within the target mRNA (Gewirtz *et al.*, 1998). Because of their ability to interact directly with, and destroy mRNA, ribozymes are an attractive candidate for antisense therapy of IRES-containing viruses. Currently, the I-RNA discovered by Coward and Dasgupta (1992) provides the best insight into development of IRES-translation targeted therapeutics. Though there is no significant sequence homology between I-RNA and poliovirus mRNA, an antisense RNA with the exact complementary sequence of I-RNA bound p52 as efficiently as the I-RNA (Das, *et al.*, 1994). Though the sequence of I-RNA shares an 89.5% homology with a region of the Japanese encephalitis virus genome, and 70-80% homology with regions of herpes-simplex,

Sindbis, Epstein-Barr, influenza, and dengue viruses, it is most likely the secondary structure of the I-RNA that is responsible for the inhibition of internal initiation (Das, *et al.*, 1992). The secondary structures of the IRES elements of HCV and PV are very similar. Das and colleagues have recently found a stem-loop structure within the I-RNA that is similar to portions of the HCV and PV IRES, though the sequences are quite different (Das *et al.*, 1998).

I-RNA readily binds many cellular factors, notably p52/La and p57/PTB. It is the stabilizing relationship between p57/PTB, the IRES structure and p52/La upon the eIF4G pre-initiation complex that may play the main role in the effectiveness of I-RNA in inhibition of internal initiation of translation. This inhibitory RNA is effective in inhibiting internal initiation of translation in rhino-, hepatitis A, and Theiler's murine encephalomyelitis viruses, all viruses that are intermediately to highly dependent upon PTB for stable IRES folding (Coward and Dasgupta, 1992; Kaminiski and Jackson, 1998). However, the internal initiation of wild-type EMCV, a PTB-independent virus, was unaffected by I-RNA. Work by del Angel *et al.* (1989) showed the binding of cellular protein complexes to regions of the IRES. Both complexes contained eIF2A, suggesting that cellular proteins are necessary to place the translation machinery on the IRES. It seems reasonable that I-RNA is acting to prevent binding of the translational machinery to the IRES. Evidence from phylogenetic analysis suggests that the critical sequence elements required for proper IRES functioning are actually short motifs throughout the primary sequence and unpaired regions of the secondary structure (Kaminiski and Jackson, 1998). It is possible that any mutation, even the addition of a nucleotide, could distort the three-dimensional space such that IRES activity would be compromised or abrogated. If this is correct, then the PTB binding could

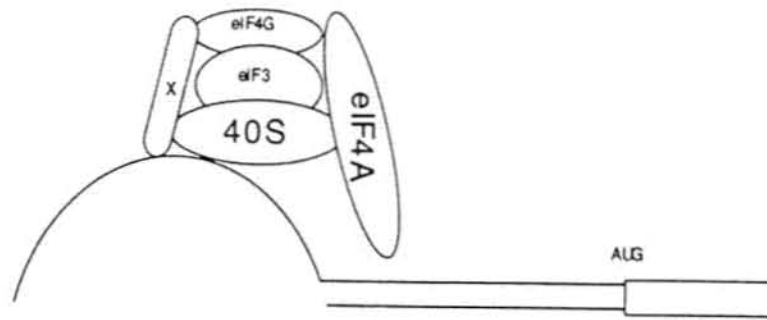
function to re-fold the IRES in the correct three-dimensional structure. One way to test this is to repeat the I-RNA inhibition experiment (Coward and Dasgupta, 1992) with an EMCV strain with an expanded oligo (A) bulge (PTB-dependent). If translation of a PTB-dependent IRES is affected by the presence of I-RNA, the I-RNA should now inhibit the IRES functioning.

Though it has been shown that I-RNA binds cellular factors directly, that does not exclude I-RNA binding to the IRES (Das *et al.*, 1996). Kaminiski and Jackson (1998) found that PTB binding at the high-affinity site was unaffected in EMCV, regardless of the presence of an additional adenosine nucleotide. Perhaps stable, secondary structure is dependent upon PTB binding at one or both of the other PTB-binding sites located on the bulge. UV crosslinking of I-RNA to EMCV mRNA could reveal whether or not the inhibitory action of I-RNA is due to binding directly to the stem-loop RNA equally well in PTB-dependent and PTB-independent IRESs.

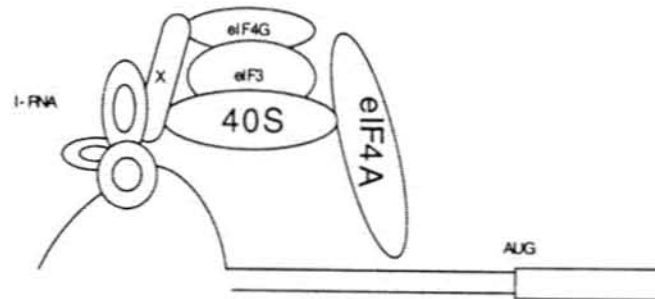
To synthesize proteins, mRNAs and ribosomes must be presented to each other in translation initiation. Protein-mRNA interactions precede the joining of the small ribosomal subunit (40S) with the mRNA. eIF4G, the translation factor responsible for bringing the IRES mRNA to the 40S ribosomal subunit, binds at the oligo (A) bulge (Pestova, *et al.*, 1996; Kolupaeva, *et al.*, 1998). The central third of eIF4G, containing the ribosomal binding domain, binds eIF3, a multi-meric complex that is bound to the 40S ribosomal subunit (Lamphear, *et al.*, 1995). Since capping RNA prior to translation prevented the I-RNA-mediated inhibition of translation (Das *et al.*, 1994), it is unlikely that I-RNA is directly interacting with eIF4G (which is necessary for both cap-dependent and cap-independent translation), but rather a cellular factor that assists eIF4G binding to the IRES, or the IRES.

It is possible that the disruption of secondary structure (or lack of stabilization in the absence of PTB, or presence of I-RNA) creates conditions that either prohibit eIF4G binding, or disrupt the structure such that eIF4G cannot correctly place the mRNA on the 40S ribosomal subunit (Fig. 8).

We can not discount the presence of other cellular factors that bind I-RNA such as p52/La, but the significance of this interaction remains to be shown. The La protein (p52) does stimulate poliovirus RNA translation, presumably by binding to stem-loop G within the 5' UTR (Svitkin, *et al.*, 1994). Additionally, deletion of the COOH half of La results in the loss of ability to stimulate translation (Svitkin, *et al.*, 1994). Possibly the N-terminal half of La binds the IRES, whereas the C-terminal half may be required for a protein-protein interaction. UV cross-linking studies reveal that p52/La is the major polypeptide competed for by I-RNA (Das, *et al.*, 1998). In general, cellular factors bind directly to RNAs either as a result of their recognition of specific sequences, secondary, or tertiary structure (Haller and Semler, 1995). The role of p52/La may be to "fine tune" the IRES-mediated translation by forming a bridge between eIF4G and the IRES and "guide" the eIF4G to the appropriate start codon. Though not absolutely critical for cap-independent translation, p52 may act as an additional stabilization factor during binding of the translational machinery to PTB-dependent IRES structures. Given that Meervitch and colleagues (1993) demonstrated that some successful protein translation proceeds in the absence of La, the IRES elements of entero- and cardioviruses bind the cellular protein p52, and the addition of p52 has been demonstrated to rescue aberrant protein translation in poliovirus, this hypothesis appears reasonable.



Successful binding of eIF4G complex to pseudoknot.



Translation cannot proceed in the presence of I-RNA.

Fig. 8. Possible interaction of eIF4G and I-RNA with conserved bulge in viral IRES. The upper figure diagrams interaction of the eIF4G-40S ribosomal subunit complex in the absence of I-RNA. Since eIF4G has been shown to contact the bulge directly, and p52/La has been shown to enhance translation efficiency, "X" may be the p52 protein. The presence of p52 may stabilize binding to the IRES. The lower figure diagrams the possible disruption of bulge secondary structure, and inhibition of "X". Presumably, I-RNA inhibits the binding of the 40S subunit to the IRES.

One approach to probe the interaction of I-RNA with the mRNA of IRESs is to make an I-RNA “knockout” in yeast cells. Exogenous DNA, when introduced into *Saccharomyces cerevisiae* cells readily integrates into chromosomal DNA by homologous recombination. A cloned gene can be manipulated *in vitro* so that the integration results in the replacement of the wild-type with a non-functional copy of the gene. URA could be used as a selectable marker for successful integration of the non-functional gene. This marker would be introduced into the I-RNA gene. It is unknown whether the I-RNA gene is essential for yeast cell growth. To avoid potential lethality of an I-RNA yeast cell knockout, the integration of the mutant I-RNA gene should be performed in diploid cells to produce strains that are heterozygous for the gene disruption. Meiosis of the heterozygous diploids will result in four haploid spores, two of which will carry the mutant allele and selectable marker and two of which will carry the wild-type allele. Assuming all four spores survive and yeast cell growth is not dramatically altered by the I-RNA gene knockout, IRES-containing virus cDNA could then be expressed in the knockout cells. This would be of benefit to study HCV, for which there is no adequate cell system available.

Following successful construction of knockout yeast, future studies could utilize I-RNA deletion mutant transfected cell lines by infecting with poliovirus, rhinovirus, or HCV and determining the effectiveness of various ribozyme constructs corresponding to the secondary structure of I-RNA. It is beyond the scope of this work to review in detail ribozyme selection and construction. Briefly, the synthetic mRNA target and ribozyme is synthesized by *in vitro* transcription from synthetic oligonucleotides and then reacted together to confirm that the ribozyme was synthesized correctly, and will hybridize to and

cleave the mRNA sequence. When the cleavage activity of the ribozyme against the target has been confirmed, the ribozyme is cloned into a virus vector such as a recombinant adeno-associated vector. Advantages to recombinant adeno-associated vectors (AAV) include stable transmission to daughter cells, absence of viral gene expression that might cause an undesirable immune response, and better stability allowing AAV vectors to be stored and handled like traditional pharmaceutical products (Welch *et al.*, 1998). Cells can be transduced with the AAV vector and challenged with IRES-containing viruses. The successful ribozyme construct will cleave the appropriate region of the target, abolishing viral translation. It is reasonable to expect that a single ribozyme construct will effectively inhibit internal initiation of translation in several of the IRES-containing viruses. The reader is referred to excellent reviews on ribozyme construction (Gewirtz *et al.*, 1998; James and Gibson, 1998; Gibson and Shillitoe, 1998).

Obstacles to successful ribozyme therapeutics include susceptibility to ribonucleases, weighing the optimization of the association between the ribozyme flanking sequences and the target mRNA, and balancing the rapid cleavage product dissociation with mismatched base-pairing. The susceptibility to ribonucleases can be overcome by modifying the nucleotides within the different regions or constructing a DNA/RNA chimera to increase serum half-life. The longer the flanking sequences surrounding the catalytic core, the more specific the ribozyme will be; however longer sequences are less likely to dissociate from the target. Keeping the flanking sequence to about 13 nt ensures rapid turnover following cleavage, but not mis-matched base pairing (Scanlon *et al.*, 1995; James and Gibson, 1998).

One of the most important questions raised by the discovery of I-RNA is what function does I-RNA have in the yeast? Possibly, yeast cells contain genes that initiate

translation internally, and the inhibitor RNA is used to regulate expression of these genes - post-transcriptionally. Evaluation of translational product production during a competition assay between I-RNA and phosphorylated eIF4F (activated) as well as de-phosphorylated (inactive) eIF4F, and another competing I-RNA with full-length and cleaved eIF4G could help to determine the action of the inhibitor on eukaryotic translation.

It seems incredible that two distinctly different mechanisms of translation could have evolved both in viral and eukaryotic systems. Experimental data suggests that the two systems are not entirely distinct and utilize the same cellular translational elements. Fortunately, the presence of a “pseudoknot” in viral IRES elements, that is absent in eukaryotic IRES elements, provides a target for translation-targeted therapy. The interaction of an inhibitory RNA, produced in yeast, with the pseudoknot structure of the viral IRES provides a model for ribozyme construction, specific to viruses utilizing the internal initiation of translation mechanism. Further unraveling the common features between the viral and eukaryotic IRES elements should allow us to develop better screening methods for the development of translation-targeted therapy. The discovery of a eukaryotic inhibitor of the picornaviral internal initiation mechanism is the first step towards the development of a unified model of cap-independent initiation.

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