THE ROLE OF VPg IN TRANSLATION OF CALICIVIRUS RNA

by

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APPROVAL

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ABSTRACT

Molecular mechanisms of *Norovirus* replication remain for the most part undefined, primarily due to the lack of cell culture and small animal model systems. However, sequence comparisons and studies using cultivable caliciviruses have lead to the description of many features of the viral genome. Genomes are positive sense RNA, where the genome itself serves as mRNA for the production of viral protein. Additionally, viral RNA is covalently attached at the 5' end to the viral protein VPg. VPg is required for infectivity of the RNA by transfection, and removal of VPg by proteinase K treatment reduces the ability of the RNA to be translated in vitro. Because of these data, and because viral RNA is presumably not translated by an IRES mechanism, it has been suggested that VPg plays a role in translation of viral RNA. Studies described herein were initiated to investigate the potential role for Norwalk virus (NV) VPg in this process. It was found that NV VPg binds translation initiation factor 3 (eIF3) directly and in cell lysates, and is present in complexes with other eIFs including the cap-binding protein eIF4E, the large scaffolding protein eIF4G, the S6 ribosomal protein, and eIF2α, a component of the ternary complex. VPg also inhibits translation of reporter RNAs in vitro, suggesting that the interactions observed between VPg and eIFs are relevant to translation. Regions of VPg responsible for interactions with eIFs were mapped, and it was found that interaction between VPg and the 40S ribosome is most likely that which is responsible for translation inhibition of the reporter RNAs. VPg directly binds 40S ribosomal subunits by sucrose density gradient centrifugation, and this interaction is likely mediated by the central domain of VPg, similar to binding properties observed for the universally conserved factor eIF1A. Finally, a recently discovered, cultivable murine norovirus – 1 (MNV-1) was used to ask if interactions between VPg and eIFs occur in infected cells. It was found that VPg of MNV – 1 coprecipitates with eIF4GI, the d subunit of eIF3, and eIF4E from infected cells, and that this VPg has similar binding properties as the NV VPg. Together the data support the hypothesis that VPg plays a role in translation of viral RNA during infection, and suggets a third mechanism of ribosome recruitment dependent upon protein-protein interactions between VPg and eIFs. These studies also highlight the possibility of using MNV – 1 as a molecular model for the study of human norovirus infection.

EXPRESSION OF CALICIVIRUS GENES

Caliciviruses

Calicivirus Classification

The family *Caliciviridae* is composed of four genera (Figure 1.1), including *Lagovirus*, *Vesivirus*, *Norovirus*, and *Sapovirus* (230). Classification is based on sequence comparisons between individual genomes. All calicivirus genomes consist of 7 – 8 kilobases of single stranded (ss) RNA of positive polarity (17, 151, 182, 105). Noroviruses are further divided into genogroups based on analysis of the capsid and RNA dependent RNA polymerase regions of the genome (113). Caliciviruses belonging to the *Norovirus* and *Sapovirus* genera predominantly infect humans, while lagoviruses and vesiviruses infect animals. Bovine caliciviruses and the recently discovered Murine Norovirus – 1 (MNV-1) are most similar to the human noroviruses, but enough sequence diversity exists to warrant their placement in separate genogroups (204, 253).

Disease pathology associated with individual viruses differs. Sapoviruses and most noroviruses cause acute gastroenteritis in humans. The *Vesivirus* feline calicivirus (FCV) causes respiratory disease, as well as vesiculation/ulceration of the epithelium of the oral cavity, in cats (264). Vesicular exanthema of swine virus (VESV) and San Miguel sea lion virus (SMSV) cause fever and oral and extremity vesicle formation in swine and sea lions, respectively. VESV and SMSV are also associated with pre-term abortions (254). The *Lagovirus* rabbit hemorrhagic disease virus (RHDV) is the cause of a highly contagious disease in rabbits that results in liver necrosis, hemorrhages, and high

mortality (73). Clearly disease related to caliciviruses is diverse, and classification of viruses based on symptoms alone would not be an accurate indication of common molecular features as comparisons based on sequence provide.

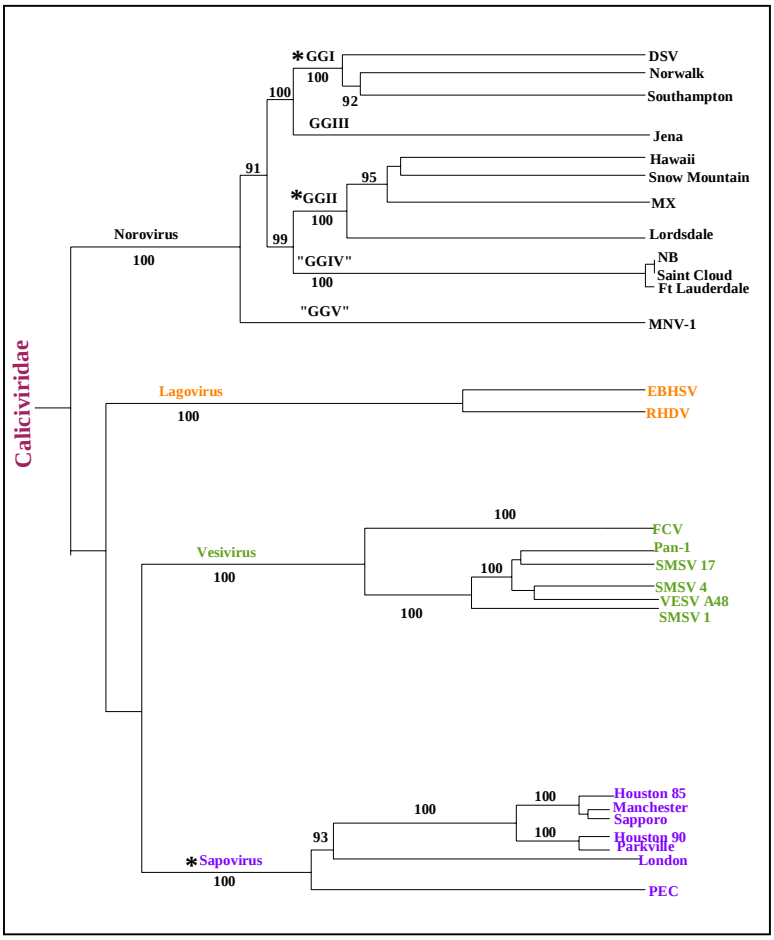

Figure 1.1: Phylogenetic classification of the family *Caliciviridae*. Asterisks indicate those viruses that infect humans. Diversity between genera was based on comparison of capsid gene sequences. Adapted from (112).

Norovirus Disease and Pathology

Norwalk virus (NV) belongs to the *Norovirus* genus and was originally described in 1972 from an outbreak at a school in Norwalk, Ohio (111). Identification was based on visualization of a 27 nm viral particle by immune electron microscopy of stool filtrate obtained from an individual affected by the outbreak. The incubation period is typically 24 to 48 hours (76) and clinical illness includes diarrhea, vomiting, low-grade fever, and nausea (43). Infection is self-limiting and symptoms last between 12 and 60 hours (76).

Pathology associated with Norwalk virus infection has been described to a certain extent since its initial identification. No gastric lesions are found in infected individuals (286), but pathology of the small intestine, including lesions of mucosal inflammation, mucosal villous shortening, crypt hypertrophy, absorptive cell damage, increased epithelial cell mitosis (286), blunting of villi, shortening of microvilli, dilation of the ER, and a decrease in brush border enzymatic activities are prominent (2). Symptoms are eliminated upon clearing of the infection (2). Lesions of the small intestine are characteristic of viral gastroenteritis in general and not one particular virus, because Hawaii virus (HV) causes the same pathology as NV (248).

Information about the immunity associated with *Norovirus* disease was acquired by the discovery that noroviruses can be transmitted easily by oral administration of stool filtrates from infected volunteers (43). Two forms of immunity, either of short or long duration, can be associated with *Norovirus* infection, but factors other than serum antibody must be important for immunity because not all volunteers make antibody in response to infection (207) and immunity to *Norovirus* does not appear to be present in

the general population (43). Though pre-existing antibody to NV does not seem to be associated with protective immunity in the community as a whole, antibody levels can become associated with protection after repetitive exposure to virus (110). However, immunity to one virus most likely does not confer immunity to another, at least across genogroups, in response to *Norovirus* infection. Cross-challenge studies demonstrate that infection with NV or HV fails to confer protection from subsequent disease caused by the other (295). Only one study has looked at the specific type of response mounted upon *Norovirus* infection, and it found that during Snow Mountain Virus (SMV) infection of human volunteers, a predominantly CD4+ cell-mediated Th1-type response was observed (167).

Information about immune response associated with noroviruses also comes from studies using virus-like particles (VLPs). VLPs are recombinant viral capsids identical to authentic virus particles that lack genetic material. They are produced from the expression of the structural proteins in insect cells using the baculovirus expression system (106). The capsid protein self-assembles into VLPs upon expression (106). They are morphologically and immunologically indistinguishable from native virus found in human feces. NV VLPs are immunogenic in mice when administered orally without adjuvant, where serum IgG and mucosal IgA are produced (261). When administered to human volunteers, all individuals develop significant rises in IgA anti-VLP antibody-secreting cells, and 90% experience rises in serum anti-VLP IgG (265), further suggesting the potential for the use of VLPs as a means to study immunity to *Norovirus* infection, or to use as potential vaccines themselves.

Epidemiology

Before the advent of cloning and RT-PCR in the early 1990's, illness would be attributed to virus only if a bacterium or parasite was not found in samples from infected individuals (76). Today, noroviruses are recognized as the etiologic agent in 93% of outbreaks of nonbacterial gastroenteritis (52), and are responsible for 23,000,000 infections, 50,000 hospitalizations, and 300 deaths each year in the US (185). Due to increased surveillance, the proportion of *Norovirus*-confirmed outbreaks increased from 1% in 1991 to 12% in 2000 (285). Likewise, *Norovirus* is the single most common cause of gastroenteritis in people of all ages and is as common as rotavirus in patients who consult their doctor concerning their symptoms (118). Genogroup II strains are responsible for more outbreaks than those of genogroup I (52).

Noroviruses are transmitted by several routes including person-to-person contact, exposure to airborne droplets of vomitus (181), and from consumption of contaminated food or water (52,53). Greater than 56% of food borne *Norovirus* outbreaks from 1991-2000 were associated with eating salads, sandwiches, and fresh produce, suggesting that contamination of food that requires no further heating prior to consumption is a source of infection (285). Oysters have been well-documented in outbreaks (156,53), but have not been frequently associated with disease in the United States in the last 10 years (285). Problems with *Norovirus* infection frequently occur in communal settings such as cruise ships, day care centers, nursing homes, and military settings, where people are eating and drinking material of a common source, as well as residing in close quarters (76).

Norovirus infection is considered an emerging infectious disease. For example, rates of bacterial food borne illness are declining but *Norovirus*-related infections are not, mostly because they are resistant to chlorination and freezing, they can persist easily in the environment, and require a very low inoculum to infect. Modern lifestyles make us more vulnerable, as communal settings such as nursing homes, and the consumption of prepared or processed foods, are more common. Additionally, people today travel more than ever before, making person-to-person spread more likely (284).

Norovirus Replication and Genome Expression

Knowledge of the basic steps of *Norovirus* replication including attachment, entry, gene expression, and maturation have been hampered by the lack of a cell culture system. Much information regarding processing and maturation of viral proteins has come from in vitro proteolytic studies. Many functional analogies have been made with viruses of the cultivable *Vesivirus* genus, and those of the *Picornaviridae* family. Information regarding cellular attachment was derived from binding studies using recombinant VLPs.

Norovirus Attachment to Cells

Norovirus attachment to cells is mediated by interaction with specific carbohydrate ligands found on mucosal surfaces of the gut, on red blood cells, and in bodily fluids such as saliva. These ligands are formed from the conversion of H type precursors through the activity of particular enzymes into A, B, H, and Lewis (Le) antigens (161). A diagram of these enzymatic reactions is found in Figure 1.2. Type 1 precursor (type 1 is shown, but 5 precursor types exist) is converted to Le_a by the FUT3 gene product, sialyl-Le_a by the

ST3GalIII and FUT3 gene products, or to H type 1 antigen by the FUT2 and FUT1 gene products. FUT2 is expressed in secretory tissues, like epithelium of lung and intestine, such that H type 1 antigen can be synthesized from the type 1 precursor and expressed on these surfaces. FUT1 is expressed in erythroid cells, such that H type 1 antigen is produced on red blood cells and therefore can be found circulating in the blood stream. Individuals with at least one functional FUT2 gene are called secretors (80% of European and white American population) (161), because not only are H type antigens found on gut and lung epithelium as a result of conversion from precursor through the activity of FUT2, but also in secretions such as saliva, tears, sweat, and semen (38). Individuals with two nonfunctional copies of FUT2 are considered nonsecretors, and H type 1 antigens are found only on red blood cells through the action of FUT1 (161). Individuals lacking FUT1 activity are rare and are said to be of the Bombay phenotype. These individuals do not express human blood group antigens on their red blood cells, but can potentially express these antigens in secretions through the activity of FUT2 (161).

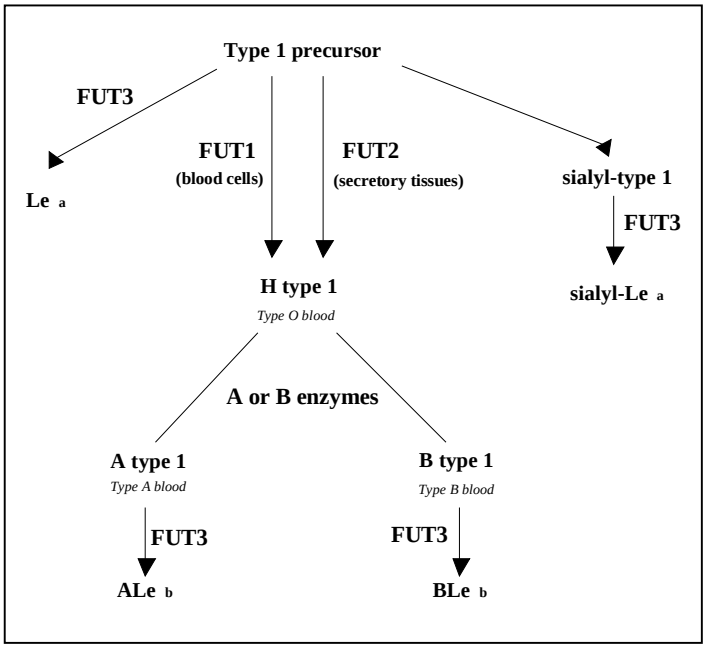

Figure 1.2: Flow chart of conversion of Type 1 precursor to A, B, H, and Lewis human blood group antigens by FUT and A/B gene families.

Once H type 1 is made, FUT3 gene product converts it to Lewis b (Le_b) antigen (not shown), or A and/or B enzymes either in secretory tissues or exclusively on red blood cells add N-acetylgalactosamine or galactose in α-1,3 linkage, respectively, to convert it to ALe_b or BLe_b antigens. The presence of A and B enzymes to produce A and B antigens depends on the genetics of the individual, and is what confers blood type. Obviously individuals expressing the A enzyme have type A blood, individuals expressing the B enzyme have type B blood, and individuals expressing both enzymes have type AB blood. Individuals lacking both enzymes cannot modify H type 1, and are therefore of blood type O. It is these antigens present on gut epithelium and in saliva that make individuals susceptible to *Norovirus* infection.

A significant breakthrough in the understanding of *Norovirus* infection of humans came with the discovery that NV VLPs bind surface epithelial cells of the gastroduodenal junction as well as to saliva of secretor individuals through H type 1 and/or H type 3 carbohydrate moieties (180). Since this discovery much attention has been paid to the details of interactions between caliciviruses, especially those of the *Norovirus* genus, and human blood group antigens. Using mostly recombinant VLPs, it has been shown that NV binds H type 1, H type 3, and Le_b antigens present in human saliva (81). Antisera from pre-infected humans or inoculated mice effectively block VLP binding to these antigens, suggesting that a mechanism for antibody-mediated neutralization is possible (81). Individuals of type O blood are more susceptible to NV infection because they lack enzymes to convert H type 1/3 to anything else (81, 97), and people of type B blood are resistant to NV infection (on the rare occasion they do become infected, they are asymptomatic) (97). Expression of the FUT2 susceptibility allele is fully penetrant against NV infection because nonsecretors do not develop an infection after challenge, regardless of dose (167). In contrast, infection with SMV (a genogroup II *Norovirus*) is not dependent upon blood group secretor status, because all volunteers in a recent study of every blood type, secretor phenotype and Lewis type became infected (166). In all, six distinct binding patterns have been described, and a description of interactions between *Noroviruses* and different human blood group antigens, such as A, B, H, or Lewis antigens, has been synthesized (95).

Genome Organization

Genome organization of caliciviruses differs between genera. Caliciviruses belonging to the *Vesivirus* and *Norovirus* genera encode three open reading frames, with nonstructural proteins encoded by open reading frame (ORF) 1, and the structural proteins VP1 and VP2 encoded by ORF 2 and ORF 3, respectively. The *Sapovirus* and *Lagovirus* genera encode two open reading frames, where ORF 1 encodes the nonstructural proteins and VP1, and ORF 2 encodes VP2 (37)(Figure 1.3).

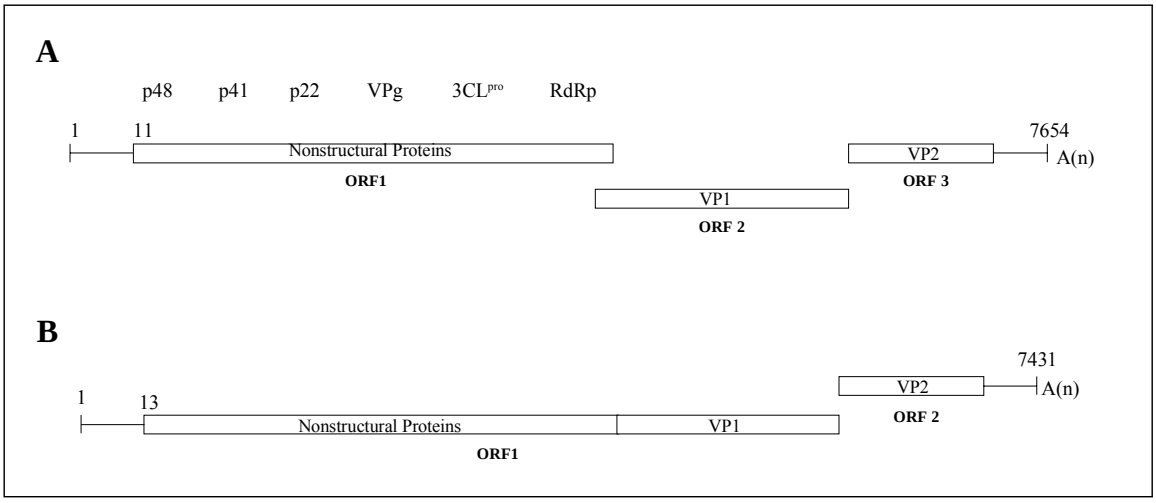

Figure 1.3: Genome organization of the *Caliciviridae*. A: Genome organization of the *Noroviruses* and *Vesiviruses*. Location of nonstructural proteins of the Norwalk virus strain are shown. B: Genome organization of the *Sapoviruses* and *Lagoviruses*. The Manchester strain of the *Sapovirus* genus is shown.

NV is the prototype strain of the *Noroviruses*, and its genome is composed of 7.7 kilobases of single stranded, positive sense RNA. ORF1 encodes the 6 nonstructural proteins. The nonstructural proteins are translated as one polyprotein (Figure 1.3) that is cotranslationally cleaved by the viral protease to form mature, active viral proteins with

functions in genome replication and assembly. Cleavage maps of the nonstructural proteins in ORF1 have been generated for many caliciviruses (14, 77, 189, 255, 256, 272). VP1 and VP2 are expressed from a subgenomic RNA that is coterminal with the 3' end of the genomic RNA (107). ORFs 1 and 3 are in the same reading frame. ORF1 extends into ORF2, and ORF3 starts within the ORF2 coding sequence (37). The genomic and subgenomic RNAs have a poly (A) tail of 110 nucleotides (107). The genome organization of caliciviruses makes it distinct from other positive sense ssRNA viruses, such as poliovirus, because the nonstructural proteins are encoded within the 5' region of the genome, followed by the structural proteins (37).

Many caliciviral genomes have been sequenced (28, 74, 78, 105, 107, 151, 169, 187, 204, 223, 238). Apart from obvious sequence similarities, structural features of the viral RNA are common. All genomes are polyadenylated at the 3' end. Likewise, the 5' untranslated region (UTR) of the genomic and subgenomic RNAs of all caliciviruses is very short compared to those of typical eukaryotic messages. The first AUG in the preferred Kozak context (see "Cellular Mechanisms of Translational Control") (130) for genomic and subgenomic RNA of NV, FCV, and RHDV is at nucleotide 11, 20, and 10, respectively (28, 151, 189). The 5'UTR of caliciviruses has not been shown to contain an internal ribosome entry sequence (IRES), and because the N terminal protein of ORF 1 is produced from this AUG close to the 5' end (37, 168, 291), and not from AUGs further downstream, it is unlikely one will be found (78). The 5'UTR between genomic and subgenomic RNAs is nearly identical (Figure 1.4)(78), suggesting a relevant function of this structure, possibly in translation or replication.

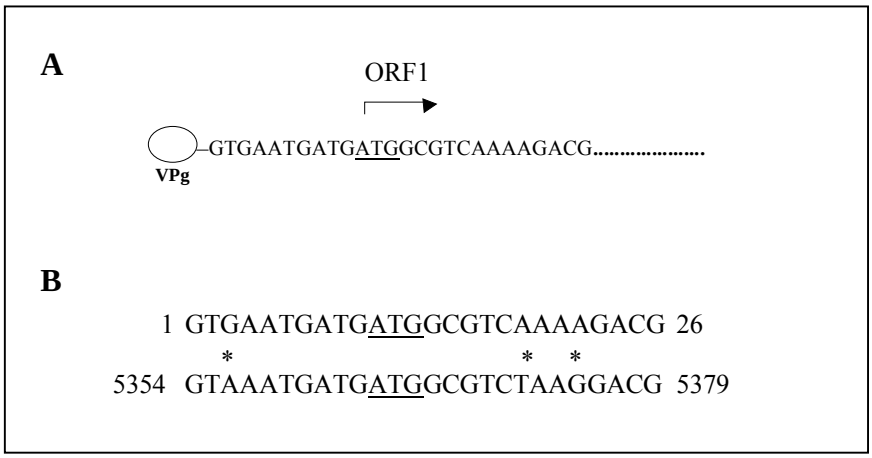

Figure 1.4: The 5' UTR of NV is 11 nucleotides and is 88% identical between genomic and subgenomic RNAs. A: The AUG initiator codon is at nucleotide 11 for NV. Of the three tandem AUGs in this region, the third is in appropriate Kozak context. B: The 5' UTR of the genomic and subgenomic RNAs are nearly identical in NV, and similar formats exist for other caliciviruses. Asterisks indicate nucleotide substitutions.

Norovirus Structural Proteins

Opening reading frames 2 and 3 encode the structural proteins of *Noroviruses*. ORF 2 encodes the major capsid protein VP1, and ORF3 encodes a minor structural protein VP2. The structural proteins are produced from direct translation of a subgenomic RNA that is 3' coterminal with the genomic RNA (107).

The structure of the NV capsid has been solved by x-ray crystallography to 3.4 angstrom resolution. Crystals of NV VLPs were grown produced from the expression of ORFs 2 and 3 using the baculovirus system in insect cells (226). VLPs are stabilized if ORF2 and ORF3 are expressed together in the baculovirus clone, as VP2 increases the stability of VP1 and protects it from disassembly and protease degradation (16). The capsid is composed of 90 dimers of 180 copies of VP1 that self-assemble upon expression. The capsid is of T=3 icosahedral symmetry and is composed of two

domains, S and P, that are connected by a flexible hinge. The S domain is made up of the amino terminal 225 residues and is involved in the formation of the icosahedral shell. The P domain is composed of residues 226 to 530 and forms the prominent protrusion emanating from the shell. The S domain is composed of a classical eight-stranded beta sandwich, like that of the plant tombus viruses (91,82). The P domain shares little resemblance with other viral capsids, and is therefore considered to be a modulator of strain diversity.

The P domain can be further divided into two subdomains. The P1 subdomain contains β strands and a well-defined α helix, though a specific function, apart from being responsible for dimer contacts and strain specificity along with the P2 subdomain, have not been defined (226). The P2 subdomain is the most variable and is located on the surface of the capsid. The fold of P2 is similar to the RNA binding domain 2 of the elongation factor-Tu (EF-Tu), a GTP binding protein involved in transporting aminoacyl tRNAs to the ribosome (242). By analogy it has been suggested that this domain could play a role in viral or cellular RNA translation or regulation of protein synthesis (226). In a patient chronically infected with a *Norovirus* for more than two years, RT-PCR of viral RNA from stool samples showed that most mutations occurred in the P2 subdomain of the capsid (199). Importantly, an intact P domain is necessary for receptor binding, further defining elements within the P domain as being essential for strain-specific binding (267).

Although common among plant viruses, virus capsids composed of a single structural protein are unusual for animal viruses, with the only known example of this phenomenon

being found in the *Nodaviridae* (94). The architecture of the NV capsid is similar to that of other animal caliciviruses including the primate calicivirus Pan-1 (228), RHDV (271), FCV, and SMSV (33, 227). They all consist of 90 dimers of capsid protein on a T = 3 icosahedral lattice, and for all, the P2 domain shows the most difference in size and shape (33).

A property of VLPs is that they assemble spontaneously in the absence of genomic RNA. Because there are no holes large enough in the capsid to engulf the RNA after assembly, it is unlikely that RNA is added after capsid formation. It is possible that genome packaging is concomitant with capsid assembly, and VP2, which is highly basic in nature and would likely bind negatively-charged RNA, may be involved in packaging. For FCV, there are an average of 1.38 copies of VP2 per particle (258).

NV VLPs undergo specific proteolytic cleavage with trypsin to generate a cleavage product of 32 kDa (native capsid protein is 58 kDa) (80). The cleavage product is identical in size to a peptide found in stool, suggesting it is biologically relevant. Only the soluble form, not that assembled into capsids, is susceptible to cleavage, suggesting that the cleavage product is not required for infectivity (80).

Creating monoclonal antibodies to VLPs and mapping the epitope they recognize is a prominent method to identify conserved and/or biologically relevant regions of the capsid between strains, as well as developing reagents for use in diagnostic assays (79). Monoclonal antibodies made to a genogroup I strain that cross-react with a genogroup II strain mapped to the C terminal P1 subdomain of the capsid protein (206). Likewise, a recent study has reported the generation of monoclonal antibodies that block binding of

NV VLPs to cells, and a putative epitope has been defined in the P2 subdomain of the capsid (170). However, some studies have noted inability to generate monoclonal antibodies capable of cross-reacting between the *Norovirus* and *Sapovirus* genera (114), indicating the potential for great diversity in relevant epitopes.

Norovirus Nonstructural Proteins

There are six nonstructural proteins encoded within ORF1 that are expressed as a single polyprotein of approximately 200 kDa. Nonstructural proteins are cotranslationally cleaved by the viral protease to form mature individual proteins. Nonstructural proteins for NV are encoded in the order NH_2-p48-p41-p22-VPg-Pro-RdRp-COOH. Designation of each component of the polyprotein is based on analogy and position in genome to the nonstructural proteins of the *Picornaviridae* (107). For example, the NV nonstructural protein found in the same position as in the poliovirus genome would receive the same designation as that of the poliovirus protein. Frequently proteins that occupy the same position in both virus families have sequence similarities and perform similar functions.

The N terminal protein of *Norovirus* genomes occupies a position equivalent to the 2AB region of picornavirus genomes. For the *Noroviruses* there is only one protein encoded in this region (p48 for NV) (14) but for the *Vesiviruses* and *Lagoviruses*, 2 proteins derive from this region (256, 291), and obvious sequence variation exists between genera (41, 223). However, the N terminus of NV p48 has distant sequence relatedness to the 2A protein of some picornaviruses, and two cellular proteins (Tig3 and H-rev107) implicated in cellular proliferation (96, 108). The C terminus of p48 has

similar function as the 2B protein of picornaviruses in that it associates with host cell membranes (55), and the FCV equivalent, p32, can be found associated with membranous replication complexes in infected cells (71). p48 of NV interacts with VAP-A, a SNARE-binding protein shown to play a role in regulated vesicle transport (50). Transient expression of p48 in cells disrupts cellular trafficking (50) and disorders the structure of the golgi apparatus (55).

p41 shares sequence motifs with the 2C protein of picornaviruses, as well as the superfamily 3 helicases. For picornaviruses, 2C has supposed helicase activity (63, 163, 263), and is thought to be involved in strand separation during replication (42, 269). p41 has nucleoside triphosphate (NTP) binding and hydrolysis activities, but to date helicase activity has not been detected (178, 220). The nucleotide binding motif GxxGxGKT/S is present in a relatively similar location in p41 of NV as in 2C (107).

p22 is a small protein whose function remains for the most part undescribed in *Norovirus* infection. It can be found in the 3A position of the genome by analogy to picornaviruses. During picornavirus infection 3A is found as a 3AB precursor, to presumably anchor VPg (3B) to membranes during replication (64). Mutation of 3A halts viral replication (64). p30, the FCV equivalent of p22, can be found as the precursor p30-VPg (like 3AB) in infected cells (256, 258) and p30-VPg and p30 alone can be found associated with membranous replication complexes isolated from FCV-infected cells (71).

VPg occupies a polyprotein position equivalent to the picornavirus 3B protein. VPg is covalently attached to the 5' nucleotide of genomic and subgenomic RNAs (24). VPg

of FCV can be found associated with replication complexes as described above, suggesting a role in replication (71), and a role for VPg in translation of viral RNA has been suggested (24, 46, 87). VPg will be discussed in greater detail in a subsequent section.

The protease of caliciviruses maps to a similar region of the picornavirus polyprotein as the 3C protease. The 3C-like protease ($3CL^{pro}$) of the *Caliciviridae* is the only protease responsible for maturation cleavages in the precursor polyprotein. The NV $3CL^{pro}$ resembles cellular chymotrypsin-like serine proteases (20), but the serine residue of the cellular enzyme has been replaced by a cysteine in the viral form (77); for NV the nucleophilic cysteine can be found at C^{1239} (77). The recognition sites of the protease on the polyprotein differ between genera. Proteolytic processing maps generated for many of the caliciviruses define the residues found either N or C terminal to each scissile bond. The protease can be expressed in vitro as a GST fusion protein and is active (193). In FCV infection, the protease cleaves the capsid protein precursor as well as the polyprotein (255).

The RNA dependent RNA polymerase (RdRp) of caliciviruses maps to the approximate region of the picornavirus polyprotein as the 3D RdRp of picornaviruses. The 3D RdRp has regions of homology with all known DNA and RNA polymerases (245). The crystal structure of the NV RdRp has been solved (196). Its structure is similar to that of the RHDV RdRp, except that the C terminus lies directly in the active site cleft; for RHDV and poliovirus, and other caliciviruses based on structure predictions, the C terminus lies more towards the front of the active site cleft (196). For

NV the location of the C terminal segment interferes with binding of dsRNA in the active site, suggesting a possible regulatory role for this domain in the initiation of RNA synthesis. Several residues in the C terminus of both the RHDV and NV RdRps are disordered, and it has been proposed that these residues become ordered through interaction with other components of replication complexes, such as RNA or other nonstructural proteins (174). It is possible the active form of the RdRp during infection is the protease-polymerase precursor (14, 71), as the presence of the protease dramatically increases the activity of the polymerase (281). The RdRp can be expressed using the baculovirus system (59) and in bacteria as a GST fusion protein (276). Both forms are active, suggesting that eukaryotic post-translational modifications are not necessary for function of the bacterial product.

Viral Protein Genome-Linked (VPg)

VPg is a general term for a protein encoded within a viral genome attached through a covalent linkage to the 5' end of genomic and subgenomic RNA. VPg plays a diverse array of roles in synthesis of progeny virus, including roles in replication, protein synthesis, and potentially the packaging of RNA into viral capsids. VPg proteins are attached to the genomes of many plant and animal viruses as described below.

VPg of Plant Viruses. VPg is linked through a tyrosine residue by a phosphodiester linkage to the 5' nucleotide of the positive sense RNA genome of the *Luteoviridae*, *Comoviridae*, and *Potyviridae* plant virus families (244). These VPgs are typically larger than VPg of other virus families (approximately 22 - 24 kDa). The function of VPg is

diverse for plant viruses. It plays a role in long distance movement of virus within the plant (47, 246), as well as possible roles in replication of viral RNA (93, 231, 252). For many viruses, the presence of VPg on RNA is required for infectivity (25, 159, 195), and a role for VPg in translation of viral RNA has been suggested (159, 160, 292). Most studies of the VPg of plant viruses have been conducted using the *Potyvirus* system.

VPg may function in potyviral RNA replication. VPg binds the RdRp in vitro, though no functional details were investigated (93). It is uridylylated by viral RdRp to form the VPg-pU-pU protein primer required for synthesis of RNA minus strands (231). By analogy to the VPg-pU-pU primers of the *Picornaviridae* family, it is thought that the primer anneals to the poly(A) tail to initiate minus strand synthesis in the *Potyviridae* family, though this has not been demonstrated in infected cells.

It is possible VPg of potyviruses plays a role in translation initiation. VPg of turnip mosaic virus (TuMV) binds the cap binding protein isoform eIF(iso)4E in vitro (292), and the precursor VPg-Pro binds eIF(iso)4E and eIF4E in plants (160). The interaction between potyvirus VPg and eIF(iso)4E is inhibited in the presence of m^7GTP, but not GTP alone (159), defining the specificity of the interaction. Importantly, mutant plants that do not express eIF(iso)4E are resistant to TuMV infection (48, 158). A direct role for potyvirus VPg in translation initiation complex recruitment has not been demonstrated, and it is unclear if the function of this interaction is simply to sequester eIF(iso)4E from cellular messages, or to direct translation of viral RNA (292). For many potyviruses, sequences or structures in the 5' UTR enhance translation of reporter RNAs in vitro (12, 26, 162, 197, 198), suggesting the possibility that translation of viral RNA is

IRES driven. It remains unknown if VPg remains attached to viral RNA during translation (237).

The nepovirus tomato black ring virus (TBRV) is closely related to potyviruses in genomic structure and genome expression strategy, with two major differences. The genome of TBRV is bipartite, and its VPg is smaller (only 6 kDa) (115). TBRV RNA can be translated efficiently in vitro without VPg, and remains associated with ribosomes in its absence (115). Clearly the role of VPg in plant virus translation is multifaceted and awaits further study.

VPg of Animal Viruses. Picornaviruses, such as poliovirus, are covalently attached by a phosphodiester bridge to the 5' terminal uridylic acid of their positive sense genomic RNA to a VPg (290). Picornavirus VPg is approximately 2 kD in size, and is therefore smaller than VPg of other virus families. VPg is not required for infectivity of viral RNA, as its removal still permits a productive viral infection by transfection of viral RNA (200). Obviously, poliovirus genomic RNA does not possess an m^7G cap structure (89, 201). An enzyme in uninfected HeLa cells cleaves VPg from poliovirus RNA upon entry into the cell, implying poliovirus mRNA is translated in a form that lacks VPg (4, 154, 200). Though VPg does not have to be removed before translation can occur (67), it is not involved in translation of picornaviral RNA as binding of translation machinery is instead directed by an IRES located approximately 400 – 800 nucleotides internal to the 5' end.

Poliovirus VPg is involved in replication of viral RNA, as it serves as a protein primer to synthesize RNA minus strands. VPg is uridylylated by poliovirus $3D^{RdRp}$ to the

form VPg-pU-pU (209). This primer anneals to the poly(A) tail of plus strands and initiates the synthesis of minus strands, and is hypothesized to involve intracellular membranes as anchors. VPg of picornaviruses are not interchangeable, because a chimeric picornavirus with poliovirus genomic sequence and HRV16 VPg was not able to replicate in cells (34). Additionally, VPg may serve as an encapsidation signal for the packaging of viral RNA (225).

Viruses of the family *Caliciviridae* are covalently linked at the 5' end of genomic and subgenomic RNAs to VPg (24, 46, 86, 188, 247). A role for VPg in translation of calicivirus RNA has been suggested. VPg is required for infectivity of genomic RNA because removal by proteinase K treatment abrogates the infectivity of RNA by transfection (24, 46, 86). Mutation of VPg within the FCV genome also results in loss of infectivity (193). Likewise, the removal of VPg from genomic RNA results in a dramatic decrease in the ability of the RNA to be translated in vitro (86). It is possible VPg plays the role of a cap analogue, because capped synthetic transcripts of FCV RNA are capable of being translated in vitro in the absence of VPg (257). However, translation of VPg-linked FCV RNA is not inhibited in the presence of m^7G cap analogue, suggesting that the mechanism by which VPg is involved in translation is distinct from understood cap-dependent mechanisms (86). The fact that caliciviral genomes have such short 5'UTRs and that they presumably lack IRES structures supports the idea of VPg being important in translation of RNA.

Like picornaviruses, VPg of caliciviruses may play a role in replication. VPg of RHDV is uridylylated in the presence of RNA template (without the requirement for

$3D^{RdRp}$) (174) presumably to create the VPg-pU-pU protein primer necessary for the synthesis of minus strands on intracellular membranes (71). Intracellular membranes are disrupted during calicivirus infection (71, 293), though localization of VPg on these membranes has not been demonstrated. In all, the role of VPg in calicivirus infection is multifaceted, and the details of the potential roles discussed here remain to be further defined.

Translation Initiation in Eukaryotes

Cap-Dependent Translation Initiation

Cap-dependent translation initiation is based on recruitment of the ribosome through interactions with the m^7G cap structure at the 5' end of the mRNA. A consequence of the cap-dependent mechanism is that the ribosome enters the mRNA only through the capped 5' terminus.

Translation begins with the assembly of the ternary complex consisting of eIF2-GTP-Met-$tRNA_i^{Met}$ (Figure 1.5). The ability of the ternary complex to assemble into a functional unit is dependent upon the status of eIF2 (30). eIF2 is bound to a GDP molecule after one round of translation initiation. It is non-functional for subsequent rounds of initiation in this form and requires that GDP be exchanged for a GTP molecule by the guanine exchange factor eIF2B. The Met-$tRNA_i^{Met}$ molecule is chosen by eIF2 in the cytoplasm to form the competent ternary complex.

Formation of the 43S complex includes binding of the ternary complex to the eIF3/40S ribosomal subunit complex. 40S ribosomes are rarely found alone in the

cytoplasm, and are instead bound to eIF3, presumably to prevent premature association between 40S and 60S subunits (109). Transfer of ternary complex to 40S/eIF3 is mediated by eIF1A (32). The 43S complex is stabilized by eIF3, eIF1, and eIF1A (176, 280).

The cap binding protein eIF4E is the rate-limiting factor in initiation (90). eIF4E binds the m^7G cap at the 5' end of the mRNA (250) as part of the eIF4F complex consisting of the RNA helicase eIF4A (210) and the large scaffolding protein eIF4G. Methylation of the G residue of the cap is essential for this interaction (147). Interaction between eIF4G and eIF3 brings the 43S complex to the 5' end of the message to form the 48S complex. eIF4B is an accessory helicase to eIF4A but is not part of the eIF4F complex.

Through the hydrolysis of an ATP molecule catalyzed by eIF5 (15, 123), and the helicase activities of eIF4A and eIF4B (239, 240), the 48S complex traverses the length of the UTR until an AUG in appropriate Kozak context is encountered (see section on "Translation Control by Cellular Mechanisms"). This process is called scanning (119), and although it has never officially been demonstrated that the 40S ribosome that assembles at the cap is the same one that is found at the AUG, adequate data supports this idea. For example, substantial evidence exists demonstrating that ribosomes have an inherent property to slide along mRNA upon entry at the 5' end of the message (120, 123, 147). Furthermore, ribosomes cannot bind circular polymers of nucleotides (121). eIF1 is recruited to the 40S ribosome, possibly by interaction with eIF3 (56), and with eIF1A facilitates the scanning process (13) and AUG recognition (212, 215), as well as in the

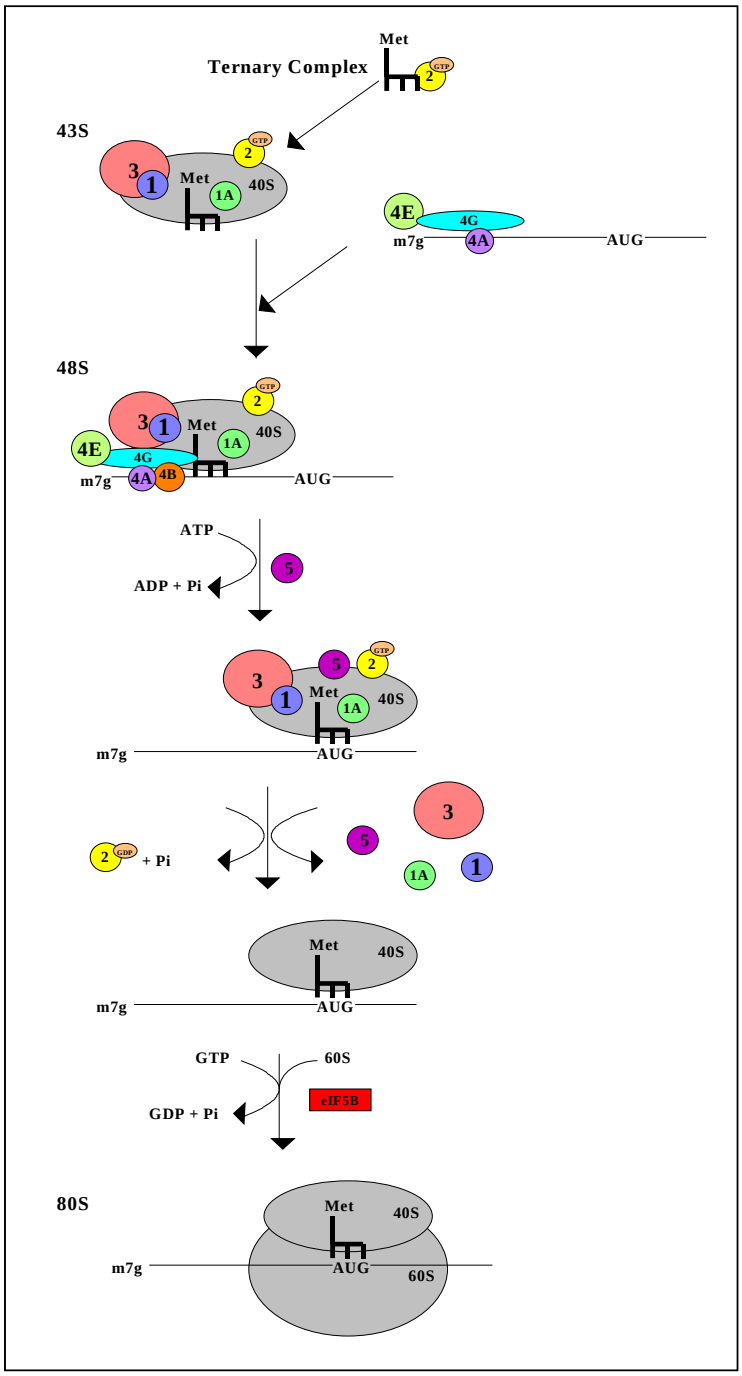

Figure 1.5: Cap-dependent translation initiation. eIFs and ribosomal subunits are represented by circles, not sized to scale. Mechanism details are outlined in the text. Adapted from (88).

joining of the 60S subunit to form the 80S complex. eIF1A enhances the ability of eIF1 to dissociate aberrantly assembled complexes on mRNA (212). Recent data suggests eIF1 and eIF1A bind the 40S ribosome independently of other eIFs, arguing against the idea that eIF1 is brought to the ribosome by interaction with eIF3 (172).

Once the AUG in an adequate context is recognized, base pairing between the AUG and the anticodon of the Met-tRNA$_i^{Met}$ molecule stalls the scanning complex. Through the hydrolysis of an eIF2-associated GTP molecule, eIF2 dissociates from the complex (275). Another molecule of GTP is hydrolyzed through the activity of eIF5B (153, 217), and the remaining eIFs release (179, 205, 275), leaving the 40S ribosome bound to the AUG. Interaction between eIF5B and eIF1A may promote the release of remaining initiation factors (205). The 60S ribosomal subunit then joins to form the functional 80S complex. It is the 80S complex that traverses the remainder of the message to catalyze the formation of peptide bonds between individual amino acids to form protein.

Cap-Independent Translation Initiation

Not all mRNAs are translated by the 5'end- or cap-dependent mechanism. Many viral RNAs (3, 103, 294), and even some cellular mRNAs (92, 173), are instead translated by an internal ribosome entry site, or IRES. An IRES is a segment of RNA internal to the 5' end of the mRNA that possesses extensive secondary structure and directs binding of the translation initiation machinery. Binding of the machinery is independent of the status of the 5' end. The specifics of IRES-dependent translation differ depending on the type of message being translated.

Picornavirus mRNA is translated by an IRES dependent mechanism (103). Encephalomyocarditis virus (EMCV) has been extensively studied in terms of its IRES element. The EMCV IRES is approximately 450 nucleotides in length (103). The IRES lies immediately upstream of the functional AUG codon, and therefore scanning is not required once the machinery is assembled (218). This IRES does not require the cap binding protein eIF4E; instead, the 43S complex is brought to the IRES directly by interaction between the central domain of eIF4G and the IRES (116, 117). The EMCV IRES requires eIF3, eIF4B, eIF4A, eIF2, and ATP to translate (36, 116, 214), and 40S subunits cannot contact the IRES in the absence of these factors (36). When eIF4G binds the IRES, it recruits eIF4A to cause a localized conformational change in the eIF4G binding region of the IRES (116) so that translation may progress.

The EMCV IRES is unique among picornaviruses in that its functional AUG is located immediately downstream of the IRES, where no scanning of the initiation complex is required. For coxsackie B3 virus, poliovirus, and hepatitis A virus, the AUG is located 112, 131, and 45 bases, respectively, downstream of the IRES (21, 22, 273, 297). For these IRES elements it is likely that either scanning or shunting guides the ribosome to the AUG after entry at the IRES (296). For the coxsackie B3 virus IRES, a region of the IRES base pairs with 18S rRNA of the 40S ribosome, reminiscent of the Shine-Delgarno interaction observed between prokaryotic mRNA and rRNA of the 30S ribosome. In vitro data show that after the 40S ribosome binds the IRES, the complex scans 180 nucleotides to the AUG (296). Similar mechanisms have yet to be determined for the other viruses.

The family *Flaviviridae* is composed of pestiviruses, flaviviruses, and hepaciviruses. Requirements for the translation of these mRNAs are distinct from those described for picornaviruses. The Hepatitis C virus (HCV) IRES is approximately 350 nucleotides in length and its IRES is more prokaryotic-like than others because it can bind the 40S ribosome in the absence of other eIFs (218). Cryo-electron microscopy studies of the HCV IRES - 40S ribosome complex show that IRES binding to the ribosome induces a pronounced conformational change in the 40S ribosome that closes the mRNA binding cleft. The IRES itself assumes a single conformation during this interaction (260). eIF3 binds stably to the IRES on its own, and together the 40S ribosome, eIF3, and eIF2 are sufficient to form the 48S complex. It is possible that the IRES itself functions in place of initiation factors that carry out similar activities during cap-dependent translation, because IRES RNA coordinates the interaction of eIF3 and eIF2 on the 40S ribosome that is required to position the Met-tRNA$_i^{Met}$ on the mRNA in the ribosomal P site (104). The 80S binding site comprises a series of discontinuous domains within the IRES (171).

Like HCV, the IRES of the flavivirus bovine viral diarrhea virus (BVDV) binds the 40S ribosome and eIF3 directly, and initiation does not require eIF4B, eIF4A, or eIF4F. Like HCV, eIF2, 40S, and eIF3 are sufficient for formation of the first peptide bond (213).

Porcine techovirus is a picornavirus, but its IRES element has more in common with that of HCV than other members of the *Picornaviridae*. It lacks a polypyrimidine tract 5' to the AUG, and its activity is not modified by eIF4G cleavage (see "Viral Mechanisms

of Translational Control"). It requires eIF2 and Met-tRNA$_i^{Met}$ to form the 48S complex, but directly binds 40S ribosomes and eIF3 (222).

The *Dicistroviridae* family, including the cricket paralysis virus (CrPV) and rhopalosiphum padi virus (RhPV), has recently been described. Both genomes are composed of 2 ORFs separated by an intergenic region (IGR) IRES. The CrPV IGR IRES assembles 80S ribosomes without Met-tRNA$_i^{Met}$, eIF2, or GTP hydrolysis, which is completely distinct from any other known mechanism (202). The IRES occupies the P site of the ribosome, directs the assembly of 80S complexes, sets the reading frame, and mimics Met-tRNA$_i^{Met}$ and peptidyl tRNA to support elongation and termination (101). In this way translation begins at the A site with a GCU codon, distinct from accepted mechanisms of ribosome assembly (102, 202).

The RhPV genome is also naturally bicistronic. The RhPV IGR IRES requires eIF2, eIF3, and eIF1, and is stimulated by eIF1A, eIF4F, and eIF4A; eIF4B is not needed in this system (268). eIF4G and eIF4A are not completely required, because their omission from reconstituted translation systems does not completely abrogate assembly of the 48S complex on the AUG. The fact that factors important for ribosome scanning, such as eIF1 and eIF1A, are required for translation by this IRES suggests that it is distinct from those currently described, and may have features in common with the scanning mechanism. Deletions made within the IRES demonstrate that no specific eIF binding sites exist, and that only deletion of an unstructured poly(U) region has any negative effect on IRES function (268).

Other cellular factors that bind the 5' terminal regions of viral ssRNA to direct translation are well-documented. Lupus antigen La is a human antigen that is recognized by antibodies from patients with autoimmune disorders (190) and was originally described because of its ability to bind the 3' poly(U) region of RNA polymerase III transcripts (261). Despite the fact that La is usually found in the nucleus, it binds the 5' UTR of HCV (235), coxsackie B3 (3) and poliovirus (190) RNA. In addition La stimulates IRES-mediated translation of HCV, coxsackie B3, and poliovirus in the cytoplasm (3, 235). Poly-r(C) binding protein (PCBP-2) is a primarily cytoplasmic RNA binding protein that binds poly-r(C) elements in RNA, and its activity is regulated by its phosphorylation status (155). PCBP-2 binds elements in the 5' UTR of picornavirus genomes (19, 61, 251, 279) but is only required for the translation of entero- and rhino-virus genera, and not for cardio- and aphthoviruses (279). Another protein that binds the 5' UTR of viral RNA is polypyrimidine tract binding protein (PTB). PTB is a pre-mRNA splicing factor (208) that has been demonstrated to interact with the genome and enhance translation of picornaviruses (5, 60, 84) and HCV (99). A commonality between these proteins is that their usual cellular function is altered in the presence of virus to enhance the translation of the viral genome. The mechanisms by which these factors influence translation have yet to be determined.

In summary, factor requirements for translation mediated by IRES elements differ between virus types, yet a common thread remains. The translation initiation complex machinery assembles internal to and is independent of the 5' end.

Selected eIFs

eIF3

eIF3 is the largest of the initiation factors, being 700 kDa in size and composed of 12 individual subunits. It was originally purified from rabbit reticulocyte lysates (15). Most mammalian eIFs can substitute in yeast except eIF3, which lacks subunits y and z, suggesting an important role for all twelve subunits in higher eukaryotes (11). eIF3 was originally identified as the factor that binds 40S ribosomes and keeps them from prematurely associating with 60S ribosomes (109). Most of the twelve subunits have been cloned and sequenced, and specific functions have been attributed to many (7, 8, 18, 58, 109, 184, 210). A polyclonal antibody to this complex has been produced, enabling the identification and assignment of individual subunits (192). The major role of eIF3 in the translation of capped messages is stabilizing the ternary complex before binding to the mRNA, and positioning the 40S ribosome at the 5' end of the mRNA through its interactions with the large scaffolding protein eIF4G. eIF3 is indispensable for the translation of specific viral RNAs translated by IRES elements.

eIF4G

eIF4G is a 200 kDa scaffolding protein that functions to unite the 40S ribosome with the 5' end of the mRNA. eIF4G is a component of the eIF4F complex, along with the helicase eIF4A and the cap binding protein eIF4E. eIF4G possesses binding sites for many factors. It binds the poly (A) binding protein (PABP) at its N terminus and this interaction circularizes the mRNA to promote recycling of ribosomes on the mRNA without the need for their disassociation and rebinding (98). eIF4G possesses two

binding sites for eIF4A, one binding site for eIF4E (175), and one binding site for eIF3. It also binds Mnk1, a kinase that phosphorylates eIF4E to its active form (232). An eIF4G isoform, eIF4GII, possesses 46% identity at the amino acid level, and has similar binding sites for eIF4E, eIF4A, and eIF3. Likewise, it can be found as a part of eIF4F. eIF4GII can be cleaved like eIF4GI by viral proteases into separate domains important for cap-independent initiation (see "Viral Mechanisms of Translational Control") (69). The region of eIF4GI that binds RNA and is critical for ribosome scanning has been defined (229).

eIF1A

eIF1A is one of two universally conserved translation initiation factors, with homologs found in eukaryotes, prokaryotes, and archea (150). Conservation between the prokaryotic and eukaryotic forms is approximately 21% (150), whereas archael and eukaryotic forms share as much as 38% identity (124). eIF1A is essential for the transfer of the ternary complex eIF2 – GTP – Met-$tRNA_i^{Met}$ to the 40S ribosomal subunit (32), and with eIF3 is required to form the stable 43S complex (15, 31, 54). eIF1A and its bacterial homolog IF1 interact with the second universally conserved eIF, eIF5B, and its bacterial homolog IF2, respectively (179, 205). eIF5B catalyzes the hydrolysis of a GTP molecule and joins the 60S ribosome with the 40S ribosome to assemble the 80S complex (153, 217). This interaction is suggested to be required to stabilize the interaction of the Met-$tRNA_i^{Met}$ to the P site of the ribosome by occupying the A site itself (35, 194). The solution structure of eIF1A has been solved (13). Like its homolog IF1, the central domain is composed of an oligonucleotide binding domain (13), and it is

this region that is important for binding to the 40S subunit (54). The amino acids flanking the immediate N and C termini of the oligonucleotide binding domain are not found in IF1, suggesting a role for these domains that is specific to eukaryotes (13). eIF1A acts synergistically with eIF1 to facilitate the scanning of the 43S complex along the length of the 5' UTR to locate the authentic initiator AUG (212) and in the absence eIF1A, the 43S complex stalls at the 5' end of the mRNA (212). In addition to interactions with eIF5B and the ternary complex, eIF1A also possesses binding sites for eIF3, eIF2, RNA, and the 40S ribosome (54, 205).

eIF4E

eIF4E is 25 kDa and binds specifically to the 5' m^7G cap on eukaryotic mRNA. The structure of eIF4E bound to m^7G has been solved by X-ray crystallography and NMR (183, 177). eIF4E is one of the three components of the eIF4F complex, which also includes the large scaffolding protein eIF4G and the RNA helicase eIF4A. Interaction between eIF4E and the m^7G cap, and between eIF4G and eIF3/40S brings the 40S ribosomal subunit to the 5' end of the mRNA. eIF4E is a rate-limiting factor in initiation (90), and a portion of it can be found in the nucleus (157) to possibly facilitate the transfer of capped mRNA to the cytoplasm for translation (44). The activity of eIF4E is regulated by its kinase Mnk1, which has a binding site on eIF4G. The phosphorylated form of eIF4E is involved in more cap complexes than the unphosphorylated form (233). At least three cellular peptides called the eIF4E binding proteins (4E-BP) regulate the function of eIF4E. eIF4E bound to a 4E-BP is incapable of associating with eIF4G and cap-dependent translation is inhibited. Hypophosphorylation of 4E-BPs renders them

capable of sequestering eIF4E, where their hyperphosphorylated forms are not (165). Importantly, eIF4E is only required for cap-dependent translation, and its availability and/or modifications have no effect on cap-independent translation (75).

Cellular Mechanisms of Translational Control

Translation is regulated at all stages, including initiation, elongation, and termination, but tight regulation at the initiation step is the most studied (142). Control at the level of initiation is influenced by many features of the mRNA. Most eukaryotic mRNA are capped and polyadenylated at the 3' end (243). The 5' UTR of most eukaryotic messages is approximately 90 nucleotides in length (134), and the 3' UTR are typically much longer (88). These common features play roles in control of the translation of the mRNA in which they are found.

Translational control of mRNA is influenced by the characteristics of the 5' UTR (or leader). The length of this sequence plays a pivotal role. Extending the leader enhances translation of the mRNA (135) because if it is too short (ie the AUG is too close to the cap) the amount of protein produced from initiation at this AUG is dramatically reduced (139). For example, a leader of 12 nucleotides is too short for initiation at the AUG found at nucleotide 13 (139). Long leader sequences may accumulate extra 40S subunits, and a moderately structured leader, irrespective of its sequence, is necessary and sufficient for efficient translation (140).

Secondary structure within the leader influences translation efficiency. A hairpin placed 14 nucleotides downstream of the authentic AUG facilitates recognition of an AUG in suboptimal context, presumably to prohibit the ribosome from scanning too fast,

and thereby facilitating its ability to locate the authentic AUG (138). Fourteen nucleotides is the optimal length because it is the approximate distance between the leading edge of the ribosome and its AUG recognition center (138). Relatively weak hairpins closer to the cap are more detrimental than those positioned further downstream (136) likely because the cap is no longer accessible to the ribosome. Strong hairpins can impede translation altogether when placed at the 5' end of the mRNA (131). Obviously, as the stability of the introduced secondary structure increases, the efficiency of ribosome binding decreases (122).

Context and placement of the AUG also influence translation efficiency. A purine (usually A) in position –3 (the A of the AUG is position +1), and a G in position +4 are optimal for recognition by the ribosome (144, 125, 127, 126, 128, 132, 130, 129). The purine in position –3 is the most conserved position in all eukaryotic mRNA (137). Initiator AUG codons that are not within this context can be overlooked by the passing ribosome, or used at an extremely inefficient level. If the authentic AUG is modified such that its context is no longer optimal, ribosomes will have a propensity to stop at a different AUG, likely one that is further downstream in the 5' UTR (124).

Placement of the AUG within the leader is also important. Most ribosomes initiate at the first AUG encountered. However, structures called upstream ORFs can regulate the influence of the position of the AUG. An upstream ORF is described as when the first AUG is followed shortly by a stop codon (1, 133, 248, 282), and another AUG for a longer cistron is found close to the stop codon for the upstream ORF. The scanning ribosome stops at the first AUG and initiates translation there. As it encounters the stop

codon for that cistron, the closely positioned AUG located right after the stop codon ensnares the scanning ribosome and uses it to translate the second cistron. This phenomenon is called reinitiation (146) and controls how much protein from the second cistron is produced.

Leaky scanning is another mechanism of translation regulation driven by placement of the AUG. In this case a scanning ribosome overlooks the first AUG in the sequence because it is not in optimal Kozak context and continues traversing until an AUG in appropriate Kozak context is encountered (125, 145, 139). Translation ensues from the second AUG instead. Occasionally, the ribosome stops at the first AUG despite its suboptimal context, and synthesizes protein encoded by that sequence. This mechanism of regulation is relevant if protein encoded by the first cistron is needed in lower quantities than that encoded by the second. Leakiness is suppressed when a modest amount of secondary structure is introduced downstream of the first AUG (141).

The 3'UTR and poly(A) tail of eukaryotic mRNA play roles in translational regulation as well. Synergism between the cap and poly(A) tail exists, and is a result of circularization of the mRNA (98, 191). Interactions between PABP bound to the poly(A) tail and eIF4E on the cap are mediated by the binding of PABP by eIF4G. Terminating ribosomes encounter the poly(A) tail, and because of their being in the vicinity of the cap, recycle on the same mRNA. This mechanism presumably maintains large polysomes (88). It has been suggested that interaction between a translation release factor eRF3 and PABP might bring the terminating ribosome close enough to the poly(A) tail to take advantage of interactions with the cap (274).

Viral Mechanisms of Translation Control

Viruses do not encode translation machinery and rely on the host cell for such factors (211). To facilitate the translation of their own genomes and down-regulate the translation of mRNAs associated with the host, viruses often interact with or modify components of the translation machinery. For example, the 2A protease of poliovirus, coxsackievirus, and rhinovirus, and the L protease of foot and mouth disease virus (FMDV), cleave the eIF4GI subunit of the eIF4F complex (152, 203, 278). The central fragment of eIF4GI that is generated from this cleavage event supports IRES mediated translation of the viral genome, while decreasing cap dependent translation of cellular mRNA (49, 219). The 3C and 2A proteases of poliovirus also cleave the poly(A) binding protein (PABP) and inhibit host cell translation (149). The 3C protease of FMDV cleaves the helicase subunit eIF4A of the eIF4F complex, but it is not known if this cleavage renders eIF4A inactive (164). The NSP3 protein of rotavirus is not proteolytically active but rather binds the 3' end of viral RNA. NSP3 interacts with eIF4GI and the circular conformation of the viral RNA generated as a result of the NSP3-eIF4GI interaction promotes the efficient translation of the viral RNA while inhibiting cellular protein synthesis (221, 277). The inhibition of protein synthesis is thought to be a result of competition between NSP3 and PABP for a similar binding site on eIF4GI (221). Likewise, the nonstructural protein NS1 of influenza virus interacts with eIF4GI and sequesters it for translation of viral RNA (6).

Investigation into the potential inhibition of cellular protein synthesis during calicivirus infection has recently begun. The FCV protease cleaves PABP in vitro and in

vivo (148). During FCV infection, cleavage of eIF4GI and eIF4GII is also observed (287), as is a global downregulation of host protein synthesis (148). The protease of the norovirus MD145 cleaves PABP in vitro, and inhibits translation of both endogenous and exogenous mRNA in HeLa cell extracts (148). In contrast to FCV, this protease does not cleave eIF4GI (148).

Murine Norovirus – 1 (MNV-1)

The human noroviruses do not grow in cell culture and a small animal model for their study does not exist (45), making investigation into the details of their molecular biology difficult. Recently, the murine norovirus – 1 (MNV-1) was discovered in the brains of infected mice (112). Infection is not lethal to wild-type mice, and viral RNA is cleared by three days. In Rag deficient mice, infection is persistent but animals do not die from infection, suggesting that an adaptive T and B cell response is not required for viral clearance. However, all mice lacking STAT-1, a signaling component of the innate immune system, succumb to lethal infection, suggesting that an intact innate immune system is necessary for viral clearance. Symptoms associated with lethal infection include encephalitis, vasculitis of the cerebral vessels, meningitis, hepatitis, and pneumonia. Arguably, the use of this system as a model for human disease is not warranted, as disease associated with human norovirus infection is acute gastroenteritis, and even though viral RNA can be detected in the gut of all infected mice, disease only occurs in immune deficient mice. However, using this system to study the molecular biology of viral replication is likely acceptable due to the conserved sequence identity

between MNV – 1 and the human noroviruses. MNV - 1 grows well in murine macrophages and dendritic cells in culture (293), and therefore investigation into the molecular biology of noroviruses can be explored using these systems.

THE GENOME-LINKED PROTEIN VPg OF THE NORWALK VIRUS BINDS EIF3, SUGGESTING ITS ROLE IN TRANSLATION INITIATION COMPLEX RECRUITMENT

Introduction

Cap-dependent translation initiates through a complex set of protein – protein and RNA – protein interactions that begin with binding of initiation factor eIF4F to the 5' terminal m^7G cap structure on the mRNA. eIF4F is composed of subunits eIF4E, eIF4GI, and eIF4A. Recruitment of the 43S preinitiation complex, consisting of the Met-$tRNA_i^{Met}$ – eIF2 – GTP ternary complex and eIF3 bound to the 40S ribosomal subunit, to capped mRNA is mediated primarily through interactions between eIF4GI and eIF3 (65, 83). This 48S complex scans to the first AUG in an appropriate context to initiate protein synthesis. Translation initiation can also be cap- and end-independent. This mechanism is exemplified by internal ribosomal entry sites (IRES) of picornavirus RNA genomes (216), although internal initiation on some cellular mRNAs also occurs (29, 85). IRES-mediated assembly of initiation complexes occurs through RNA – protein interactions, and protein synthesis begins at initiation codons downstream of the IRES. Thus, ribosome recruitment and translation initiation on IRES-containing mRNAs are independent of the 5' terminus and an m^7G cap.

Positive-strand RNA viruses in the families *Picornaviridae*, *Potyviridae*, *Luteoviridae*, *Comoviridae* and *Caliciviridae* lack m^7G cap structures. Instead, their RNAs are covalently linked at the 5' end to a small protein called VPg (viral protein genome-linked). VPg of the picornaviruses does not function in initiation of translation

on the viral RNA, as initiation is IRES driven (103). The potyviral VPg is multifunctional, and binds to eIF(iso)4E (292), but the consequences of this interaction with respect to translation initiation are not clear, as elements in the 5' UTR of the potyviral RNA direct cap-independent translation (26). In contrast to these examples, there is significant suggestive evidence of a role for VPg in initiation of protein synthesis on calicivirus RNA. Removal of VPg from calicivirus RNA results in loss of infectivity (24), and dramatically reduces translation of feline calicivirus (FCV) RNA in vitro (87). These observations suggested VPg was important in initiation of protein synthesis, perhaps functioning as a cap analogue, as proposed by Herbert and co-workers (87). However, a putative VPg-directed mechanism must deviate from that displayed by m^7G cap-dependent initiation, because addition of m^7G cap analogue to translation reactions of VPg-linked FCV RNA had no effect on protein synthesis.

The *Caliciviridae* include the prototype human calicivirus Norwalk (NV), FCV, rabbit hemorrhagic disease virus (RHDV), vesicular exanthema of swine virus (VESV), and others (37). Calicivirus RNA genomes are 7 – 8 kb in length, positive sense, polyadenylated and covalently linked to a 12 – 15 kDa VPg at the 5' ends of both genomic and subgenomic RNAs (37). The genomes of NV, FCV, and RHDV have been completely sequenced (28, 76, 107, 188). The first strong context initiation codon in each of the viral genomic RNAs is near the 5' terminus, at nucleotide 11, 20 and 10, respectively. The N-terminal protein encoded in the first open reading frame is expressed in vitro and in infected cells (37, 168, 291), suggesting the absence of an IRES that would initiate translation at downstream AUGs. Such features of calicivirus genomes raise

interesting questions regarding how translation on calicivirus RNA is initiated in the absence of a cap or an IRES.

NV and other human caliciviruses cause epidemic outbreaks of acute gastroenteritis (53, 76). These viruses do not grow in cell culture and thus the molecular mechanisms by which the proteins encoded in their genomes are expressed are not well understood. We formulated the hypothesis that initiation of protein synthesis on NV RNA proceeds by a unique mechanism that is dependent on interactions between VPg and the cellular translation machinery. In this study, we show that VPg directly binds to initiation factor eIF3. We also show that VPg inhibits cap-dependent and IRES-driven translation of reporter mRNAs in a dose-dependent manner. Based on data described herein, we propose VPg may function to recruit translation initiation complexes to calicivirus mRNA through direct protein – protein interactions with eIF3, and potentially other eIFs as well.

Materials and Methods

Yeast Plasmids and Transformations

Construction of Yeast Plasmids and Transformations. Matchmaker yeast two-hybrid vectors were purchased from Clontech. The activation domain vector pGADT7 carries the LEU2 nutritional marker and the DNA-binding domain vector pGBKT7 carries the TRP1 nutritional marker for selection in yeast. The oligo(dT)-primed MA104 cell cDNA library was cloned into pGADT7 (70). The sequence encoding VPg was amplified from a full-length NV cDNA clone (77) by PCR with two primers, VPg-EcoRI(+) 5'-

ccggaattcggaaagaacaaaggcaagacc-3' and VPg-BamHI(-) 5'-cgcggatccttcaaaattgatcttttcattataat-3'. Restriction enzyme sites are underlined. Amplification conditions consisted of 30 cycles of 94°C for 1 minute, 50°C for 30 seconds, and 72°C for 30 seconds. The resulting 400 bp fragment was cloned into pGBKT7 to generate pGBK-VPg. Binding domain plasmids were transformed into AH109 yeast cells by the lithium acetate/PEG method as described by Gietz and Woods (62). Two-hybrid interactions were scored by the ability of yeast to grow on SC-L-W medium and to activate reporter genes HIS3, ADE2, and MEL1. Activation of reporter gene expression was indicated by growth in the absence of histidine (H) and adenine (A), and by the ability to metabolize the chromogenic substrate X-α-gal (ICN). Approximately 6 X 10^9 pGBK-VPg yeast cells were transformed with 120 μg of the MA104 cDNA library by the lithium acetate/PEG procedure. Transformations were plated on SC-L-W-H-A medium and cultured for 2 – 4 days at 30°C. Colonies were re-streaked on the same selective medium with the addition of 400 μg X-α-gal.

Isolation of Plasmid DNA from Yeast and Identification of cDNA. Plasmid DNA was isolated from yeast as described previously (62). The activation domain plasmids with cDNAs encoding potential interactors were recovered by electroporation into DH10B cells and culture on LB agar containing 50 μg/mL ampicillin. Small-scale plasmid purifications were performed with the Eppendorf Perfect Prep kit. DNA was sequenced on an ABI 310 Genetic Analyzer with BigDye Terminator® chemistry.

Expression and Purification of GST-VPg
and GST-VPg Deletion Mutants

To construct vectors expressing GST fusion proteins, sequences encoding NV VPg were amplified by PCR from the full-length NV cDNA clone with two primers, VPg-EcoRI(+) and VPg-XhoI(-) 5'-ccgctcgagttcaaaattgatcttttcattataat-3' under the same conditions as described above. Sequences encoding VPg of SMV were amplified by PCR from a SMV cDNA clone (169) with two primers SMV-VPg (+) 5'-ccggaattcagtgacatcacgcctgaaggc-3' and SMV-VPg (-) 5'acgcgtcgacctcaaaactgagtttctcatt-3'. Amplification conditions were 25 cycles of 94°C for 15 seconds, 55°C for 15 seconds, and 72°C for 1 minute. The N terminus of NV VPg (amino acids 1 – 69) was amplified with VPg-EcoRI(+) and VPg-N term 5'ccgctcgagaccatcaccacctgcctgtacctc-3' to generate GST-VPg $_{1\text{-}69}$. The C terminus of NV VPg (amino acids 70 – 138) was amplified with VPg-EcoRI(+)C term 5'-ccggaattcggcataggagaaactgaaatgg-3' and VPg-XhoI(-) to generate GST-VPg $_{70\text{-}138}$. Amplification conditions were 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes, followed by a 5 minute extension at 72°C. Each PCR fragment was cloned into pGEX-4T-1 (Amersham) and expressed as a GST fusion protein in BL21(DE3) cells. Bacteria were cultured to an OD_{600} of 0.6 and recombinant protein expression was induced with 1 mM IPTG for 2 hours. Bacteria were collected by centrifugation and suspended in buffer containing 50 mM Tris-HCl pH 8, 2 mM EDTA, 1% Triton X-100 and 100 μg/mL lysozyme. The suspension was incubated for 15 minutes at 30°C and then sonicated three times on ice for 10 second pulses. Soluble and insoluble proteins were separated by centrifugation for 10 minutes at 12,000xg. The supernatant containing soluble protein was retained for the purification of

GST-VPg. GST was expressed as a control and purified under the same conditions described below. A 50% slurry of glutathione-Sepharose 4B beads (Amersham) was prepared following instructions provided by the manufacturer. One hundred microliters of prepared beads were mixed with 3 mL soluble bacterial cell lysates from either 50, 15, or 3 mL GST-VPg-induced culture (volume dependent on the experiment), and rocked for 10 minutes at room temperature. Beads were washed four times with cold phosphate buffered saline (PBS), and collected by centrifugation for 5 minutes at 500xg. GST-VPg was eluted from the beads by three 10 minute room temperature incubations in elution buffer containing 10 mM reduced glutathione and 50 mM Tris-HCl pH8. Beads were removed by centrifugation for 5 minutes at 500xg. The eluates were combined, and eluted proteins were evaluated for purity by SDS-PAGE. GST-VPg and GST were quantified with the Bio-Rad protein assay reagent with bovine serum albumin as standard.

GST Pull-Down Assay

GST-VPg pull-down assays employed purified eIF3, in vitro translated eIF3d, and native eIF3 in mammalian cell (CaCo-2) lysates. Purified eIF3 was obtained from HeLa cells by previously described methods (51, 186). ^{35}S-labeled eIF3d was translated in vitro as described below for luciferase RNA. Translation reactions were treated with 0.4 µg/µl RNAse A for 30 minutes at 37°C before use in the pull-down assay. CaCo-2 colon adenocarcinoma cells were grown to confluence in 60 mm dishes in MEM (Invitrogen) containing 15% fetal bovine serum (Atlanta Biologicals). The cells were washed twice with PBS, scraped from the plate and collected in 100 µl lysis buffer containing 50 mM

Tris-HCl pH 8, 15 mM NaCl, 140 mM KCl, 2% NP-40, and 6 μg/mL each of aprotinin, leupeptin, and pepstatin A. The lysate was brought to a final volume of 1 mL with wash buffer (20 mM Tris-HCl pH 7.5, 15 mM NaCl, 140 mM KCl, and 0.1% NP-40), mixed with the prepared glutathione sepharose 4B beads bound to GST-VPg or GST alone, or the indicated mutant, and rotated end-over-end for 4 hours at 4°C. In some experiments, the lysates were pretreated with 75 U/mL of S7 micrococcal nuclease (Roche) in the presence of 1 mM $CaCl_2$ for 15 minutes at 20°C. Nuclease digestions were terminated by addition of EGTA to a final concentration of 2 mM. The beads were collected by centrifugation for 5 minutes at 500xg at 4°C and washed three times with wash buffer. GST-VPg and interactors were eluted from the beads by three 10 minute incubations in elution buffer as before. Pull-down eluates were electrophoresed on SDS-10% or 8% polyacrylamide gels, and proteins were transferred to nitrocellulose for Western immunoblotting. The membranes were blocked in 10% Blotto (10% nonfat dry milk in PBS), and then incubated overnight at room temperature with goat anti-rabbit eIF3 (186) diluted 1:2000 in 0.5% Blotto. The reactivity of the anti-eIF3 antibody has been described previously (23, 186). This antiserum recognizes all of the subunits of eIF3, in addition to eIF4GI (49). The titer of antibody to the eIF3d subunit is low. Additional antibodies employed in Western blots include rabbit anti-eIF4GI (phospho) diluted 1:1000, rabbit anti-eIF4B diluted 1:1000, rabbit anti-eIF2α diluted 1:1000 and anti-S6 diluted 1:1000 (all from Cell Signaling Technologies). The membranes were washed three times in 0.5% Blotto, then incubated for 2 hours at room temperature with horseradish peroxidase-conjugated rabbit anti-goat, or goat anti-rabbit IgG diluted 1:3000

(Jackson ImmunoResearch Laboratories) in 0.5% Blotto. Proteins that bounds antibodies were detected by ECL (Amersham).

In Vitro Translation in RRLs

The Luciferase T7 DNA (Promega) served as template for synthesis of capped mRNA (m^7G-Luc). The luciferase gene was cloned into the pCITE4a+ vector (Novagen) downstream of the EMCV IRES to generate a template for synthesis of IRES-containing mRNA (IRES-Luc). The plasmid pEJ4 that contains the IGR-IRES of CrPV upstream of the firefly luciferase reporter was kindly provided by Dr. P. Sarnow (Stanford University Medical School). Templates were linearized with XmnI (T7 DNA), PvuII (pCITE-Luc) or BamHI (pEJ4) for 2 hours at 37°C, phenol-chloroform extracted and precipitated with 450 mM NH_4OAc and ethanol. M^7G-Luc mRNA was transcribed with the Ambion mMessage mMachine system, and IRES-Luc mRNA was transcribed with the Ambion T7 Megascript system following the provided protocols. Plasmid pETp66N was the template for synthesis of eIF3d RNA (8). Linearized DNA was transcribed using the Ambion T7 Megascript system following the provided protocols. RNA was heated to 65°C for 3 minutes and quenched on ice before use. Translation reactions were performed in Flexi-RRL (Promega) as recommended by the manufacturer. Two hundred and fifty nanograms of reporter RNA was translated in 25 µl reactions containing 16.5 µl of lysates, 4 µCi [^{35}S] methionine (1000 Ci/mmol; Amersham), 20 µM amino acid solution minus methionine, 70 mM KCl, and GST or GST-VPg as inhibitor. Translation reactions were incubated for 1 hour at 30°C. Translation products were resolved by SDS-PAGE and visualized by autoradiography. Luciferase bands were quantified by

densitometric analysis on a Bio-Rad Molecular Imager FX. Translation inhibition experiments were performed a minimum of three times.

Results

NV VPg Binds Translation Initiation Factor eIF3

Removal of VPg from calicivirus RNA results in loss of infectivity of transfected RNA (24). However, capped synthetic RNA transcribed from a full-length cDNA clone of FCV is infectious (257), indicating that m^7G can substitute for VPg. These observations suggest that VPg may function in initiation of translation on viral RNA. To test this hypothesis, we employed NV VPg as bait in a yeast two-hybrid screen of an epithelial cell cDNA library to identify binding partners of VPg that may suggest a role for this protein in translation of viral RNA. Approximately 1.3×10^6 yeast transformants were screened for cDNA clones encoding proteins that interacted with VPg. Eighty colonies were isolated, and the cDNA inserts of activation domain plasmids extracted from 22 colonies were sequenced. BLASTx searches with these cDNA sequences revealed similarities with thymosin β-10, RanBPM, and predominately hypothetical proteins predicted from sequencing projects. One cDNA encoded the eIF3d subunit (p66) of translation initiation factor eIF3. The impetus to perform the two-hybrid screen with VPg was to identify candidate binding partners that may point to a role for VPg in translation initiation. Therefore, although eIF3d was recovered only once in our screen, we chose to pursue further characterization of the potential interaction between VPg and eIF3.

eIF3 is a 700,000 Da complex formed by twelve non-identical subunits (88). Among other functions, eIF3 stabilizes binding of the Met-tRNA$_i^{Met}$ – eIF2 – GTP ternary complex to the 40S ribosomal subunit to form 43S preinitiation complexes (88). The eIF3d subunit is a 66,000 Da protein that is the major RNA binding subunit of eIF3 (8). The 1.6 kb cDNA encoding eIF3d isolated in the yeast two-hybrid screen lacked only the N-terminal 10 amino acids when compared with the published sequence (8). eIF3d in the activation domain plasmid was unable to activate reporter gene expression to allow growth on nutrient-deficient medium in the absence of VPg (data not shown). Likewise, VPg did not activate reporter gene expression in the absence of eIF3d, or in the presence of an irrelevant protein in the activation domain plasmid.

VPg Binds Purified Mammalian eIF3

A glutathione S-transferase (GST) pull-down assay utilizing a GST - VPg fusion protein and eIF3 purified from HeLa cells was performed to test whether VPg interacted with mammalian eIF3. GST – VPg, but not GST, bound to purified eIF3 (Figure 2.1A). The subunits pulled down in the eIF3 complex were defined by a banding pattern which is similar to that described by characterization of the eIF3 antibody (123). eIF3 subunits eIF3a, eIF3b, and eIF3c were clearly identified, as compared with input purified eIF3. Detectable levels of eIF3f and eIF3j also were apparent on longer exposures. Interestingly, the eIF3j subunit displayed strongly in the input eIF3 was absent in the eluates of GST – VPg. Asano and co-workers have reported that eIF3j is loosely associated with the eIF3 complex (107), yet whether VPg specifically displaces eIF3j, or any of the other subunits, from the holocomplex currently is not known. The interaction

of VPg and eIF3d in yeast suggested that the eIF3 complex might be bound to VPg through interaction with this subunit. The eIF3d antibody titer in the eIF3 polyclonal antibody is too low to allow detection of eIF3d in the pull-down experiments with cell lysates or with purified eIF3. Therefore, to test whether eIF3 bound to VPg through interactions with eIF3d, radiolabeled eIF3d translated in vitro was used in the pull-down assays. Figure 2.1B shows that eIF3d bound to GST – VPg, but not to GST, suggesting the eIF3 holocomplex may be bound at least partly through interactions between VPg and eIF3d.

The ability of VPg to interact with eIF3 in its native form in mammalian cell lysates was examined by pull-down assay with extracts prepared from CaCo-2 cells. GST – VPg, but not GST, bound eIF3 from CaCo-2 cell lysates (Figure 2.2A), similar to results obtained in the assay using purified eIF3. As before, subunits eIF3a, eIF3b, and eIF3c were clearly detected. Pre-treating the cell lysates with S7 nuclease prior to the pull-down assay did not disrupt the interaction, indicating that binding of VPg to eIF3 was not mediated by RNA (Figure 2.2B). eIF3 also was not detected by pull-down with GST fused to an irrelevant protein (data not shown). We cloned and expressed VPg of the Snow Mountain strain of human calicivirus (SMV) to test whether the interaction with eIF3 was unique to VPg of the NV strain. VPg of these two calicivirus strains share 68% amino acid identity (122). SMV VPg also bound eIF3 in a GST pull-down assay performed with CaCo-2 cell lysates (Figure 2.2C).

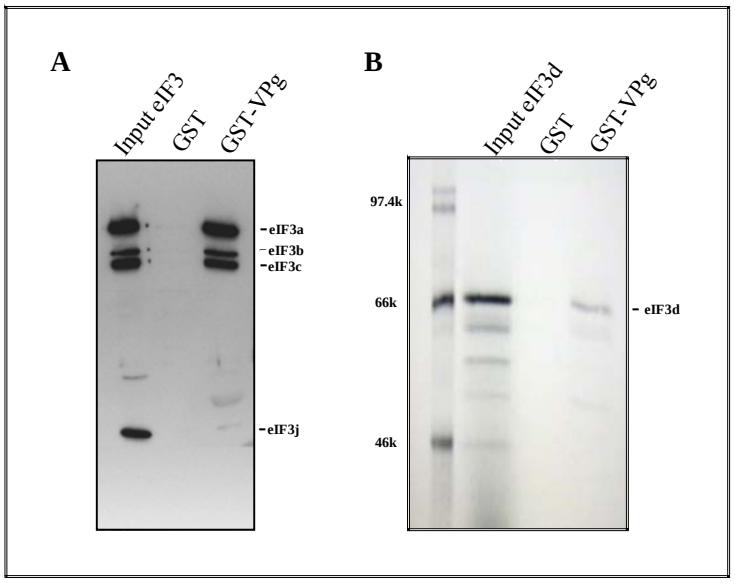

Figure 2.1: VPg binds initiation factor eIF3. Pull-down assays were conducted with GST or GST-VPg immobilized on glutathione-Sepharose 4B beads. A: Pull-down with purified eIF3. The input eIF3 lane represents 10% of the amount of protein used in the pull-down reaction, and the western blot was probed with anti-eIF3 polyclonal antibody. B: Pull-down with ^{35}S-labeled in vitro translated subunit eIF3d. The input eIF3d lane represents 25% of the amount used in the GST and GST-VPg pull-downs. Pull-down eluates were separated by 10% SDS-PAGE and exposed to film for autoradiography.

We constructed two GST fusion proteins that contained either the N-terminal domain (amino acids 1-69, GST-VPg$_{1\text{-}69}$) or the C-terminal domain (amino acids 70-138, GST-VPg$_{70\text{-}138}$) of VPg, in order to identify regions that may be important in mediating the interaction with eIF3. Figure 2.2D shows that GST-VPg$_{70\text{-}138}$ strongly bound eIF3 in the pull-down assay. The amount of eIF3 recovered in the pull-down eluate was similar to that observed with the full-length VPg. In contrast, GST-VPg$_{1\text{-}69}$ showed only slight reactivity with eIF3. These data suggest the eIF3 binding domain of VPg resides primarily in the C-terminal half of the protein, although some extension of the binding domain towards the N-terminus is possible. Taken together, the data presented above

show that VPg binds specifically to eIF3, binding is mediated primarily by the C-terminal half of the protein and the interaction is not dependent on RNA or other protein-protein interactions.

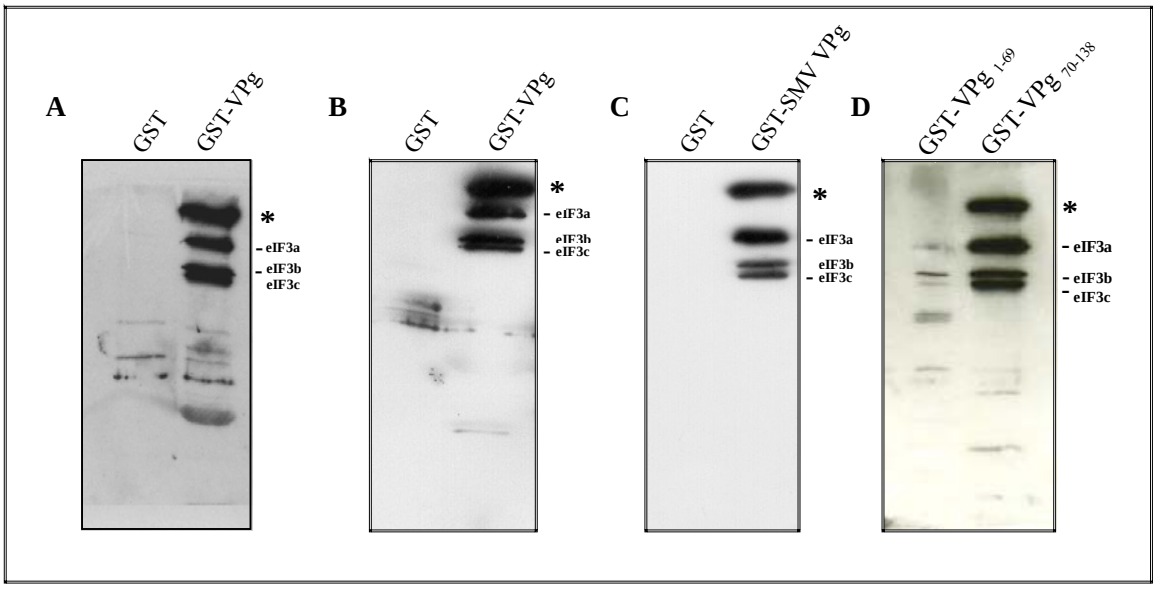

Figure 2.2: VPg binds eIF3 present in cell lysates. Pull-down assays were conducted with GST, GST-VPg, or GST-VPg mutant constructs immobilized on glutathione-Sepharose 4B beads and incubated with CaCo-2 cell extracts. A: CaCo-2 cell lysates. B: S7-treated CaCo-2 lysates. C: GST-SMV-VPg. D: VPg deletion mutants. Western blots were probed with anti-eIF3 polyclonal antibody. Asterisks indicate a protein likely to be eIF4GI.

Translation Initiation Factors Present in VPg Pull-Down Eluates

In cap-dependent initiation, the 43S complex is recruited to mRNA through interactions between eIF3 and the eIF4GI component of the cap-binding complex eIF4F (65, 83). A high molecular weight protein was detected in all of the GST – VPg pull-down assays in which CaCo-2 cell lysates were used (Figure 2.2, asterisks). We could not relate this protein to the standard eIF3 binding pattern, and it was never detected in

pull-down assays that employed purified eIF3. Previous characterization of the anti-eIF3 serum demonstrated reactivity to eIF4G (p220) (111). Based on the estimated molecular weight of the protein, characteristics of the eIF3 antibody and known eIF3 binding proteins, we tested whether this unknown protein might be eIF4GI. GST – VPg pull-down eluates were probed in a western immunoblot with an anti-eIF4GI antibody. The data shown in Figure 2.3A indicate that eIF4GI was present in eluates of GST – VPg, but not in eluates of GST, suggesting that the unknown band seen in the blots with the anti-eIF3 antiserum may be eIF4GI. Similar analyses of eluates from GST – VPg deletion constructs showed that eIF4GI was strongly present in eluates from GST – VPg_{70-138}, and significantly less so in the eluates of the N-terminal VPg domain GST – VPg_{1-69}. The excess of eIF4GI detected in eluates from GST – VPg_{70-138} may reflect the stronger interaction of this construct with eIF3. It should be noted that the purified eIF3 preparation also was probed for the presence of contaminating eIF4GI, and the result was negative. We currently do not know whether there is a direct interaction between VPg and eIF4GI, or whether eIF4GI was pulled down through its association with eIF3 in cell lysates, as yeast two-hybrid assays to address this question yielded inconclusive results (data not shown).

Since eIF4GI was strongly present in the pull-down eluates of full-length VPg and its C-terminal domain, we probed for additional components of translation initiation complexes including eIF2α, eIF4B, eIF4E, and the S6 protein of the 40S subunit (Figure 2.3B). Small amounts of eIF2α were detected in eluates from full-length VPg and GST – VPg_{70-138}. Similar results were observed for eIF4E. The S6 protein was detected in

eluates from all three constructs, consistent with an affinity of VPg for the 40S subunit (unpublished data). eIF4B was not detected in any of the eluates, with the exception of slight reactivity with GST alone. Taken together, the binding data suggest that several translation initiation factors are present in VPg-interacting complexes. The extent to which these interactions are direct with VPg, or mediated through eIF3, is not yet known.

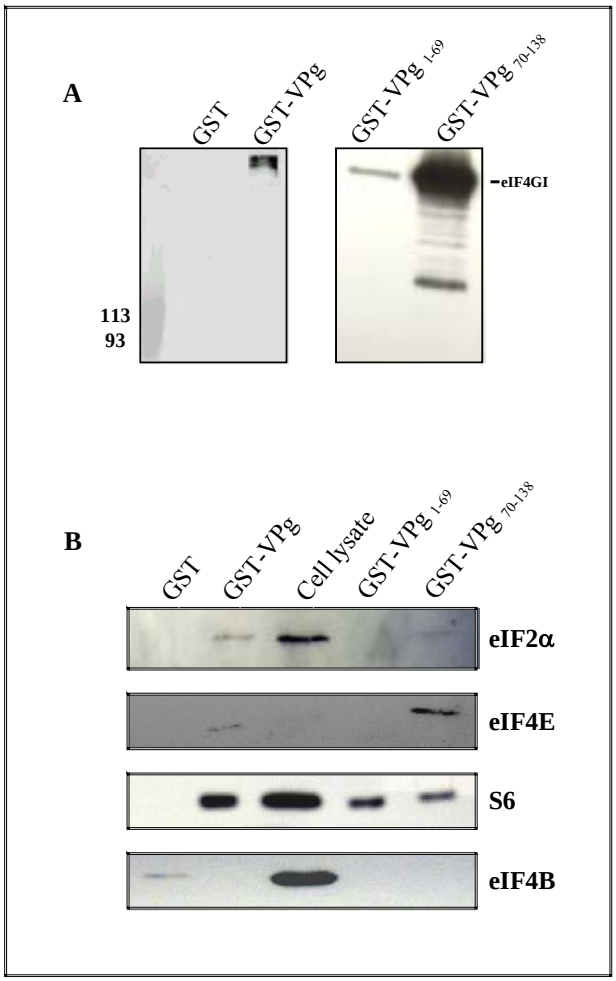

Figure 2.3: VPg interactions with eIF4GI and other initiation factors. Pull-down assays were conducted with GST, GST-VPg, or GST-VPg mutant constructs immobilized on glutathione-Sepharose 4B beads and incubated with CaCo-2 cell extracts. Western blots were probed with A: anti-eIF4GI, or B: anti-eIF2α, anti-eIF4E, anti-S6 or anti-eIF4B.

VPg Inhibits Translation of Reporter RNAs in Rabbit Reticulocyte Lysates

NV and other human caliciviruses do not grow in cell culture, making functional studies difficult. Therefore, to begin to understand the functional consequences of the interaction between VPg and eIF3, we investigated the effects of the presence of VPg on cap-dependent translation in vitro. A capped reporter RNA (m^7G-Luc) was translated in rabbit reticulocyte lysates (RRL) in the presence of increasing concentrations of GST – VPg or GST. Translation on m^7G-Luc RNA was inhibited in the presence of GST – VPg, but not GST, in a dose-dependent manner (Figure 2.4A).

Pestova and co-workers reported that canonical initiation factors were utilized in both cap-dependent initiation and translation initiation mediated by an IRES (126). The role of the eIF4F complex in translation of an EMCV IRES-containing mRNA was different from its role in m^7G-dependent translation, as assembly of functional initiation complexes did not require eIF4E binding to a 5' terminal cap. We reasoned that elucidating potential mechanisms of translation inhibition would aid in understanding the function of VPg, and thus asked whether VPg could inhibit translation of an mRNA under the control of an EMCV IRES (IRES-Luc). Increasing concentrations of GST – VPg or GST were added to IRES-Luc mRNA translation reactions. GST – VPg eliminated translation of the IRES-Luc RNA, as it did for the m^7G-Luc RNA (Figure 2.4B), also in a dose-dependent manner.

The RNA genome of cricket paralysis virus (CrPV) is a functionally dicistronic mRNA (133). Translation of the virus structural proteins encoded in the second open reading frame is mediated by an intergenic region (IGR) that functions as an IRES. The

IGR-IRES of CrPV is unique in that 80S ribosomes can assemble in the absence of the canonical eIFs (133). We tested whether VPg could inhibit translation of the CrPV IGR-IRES that does not require eIF3. As in the previous experiments, GST – VPg inhibited translation from this RNA in a dose-dependent manner, whereas GST alone had no effect (Figure 2.4C). Complete inhibition required approximately twice as much VPg as did the EMCV IRES, and although reproducible, the physiological relevance of this difference currently is not known. One interpretation of these data is that the interaction between VPg and eIF3 does not play a role in inhibiting translation. An extension of this prediction is that VPg inhibits translation of all three RNAs by the same mechanism, and the least common denominator for which we have shown an interaction with VPg is the 40S subunit. Although canonical eIFs are not required for the assembly of 80S ribosomes on the CrPV IGR-IRES, these factors are still present in the lysates used to translate the RNA, and the effects of these endogenous eIFs on translation from the IGR-IRES are not known. An alternative explanation is that the interactions between VPg and eIF3, and VPg and 40S, are not mutually exclusive, and that binding of VPg to either eIF3 or 40S subunits (or both) could inhibit translation of any of the reporter RNAs analyzed here. The mechanism of inhibition then would be dependent on the specific factor requirements of each RNA. The ability of VPg to inhibit translation of capped mRNA and mRNAs that contain IRES sequences with different eIF requirements suggest that VPg may be inhibiting translation through protein interactions common to cap-dependent and internal initiation.

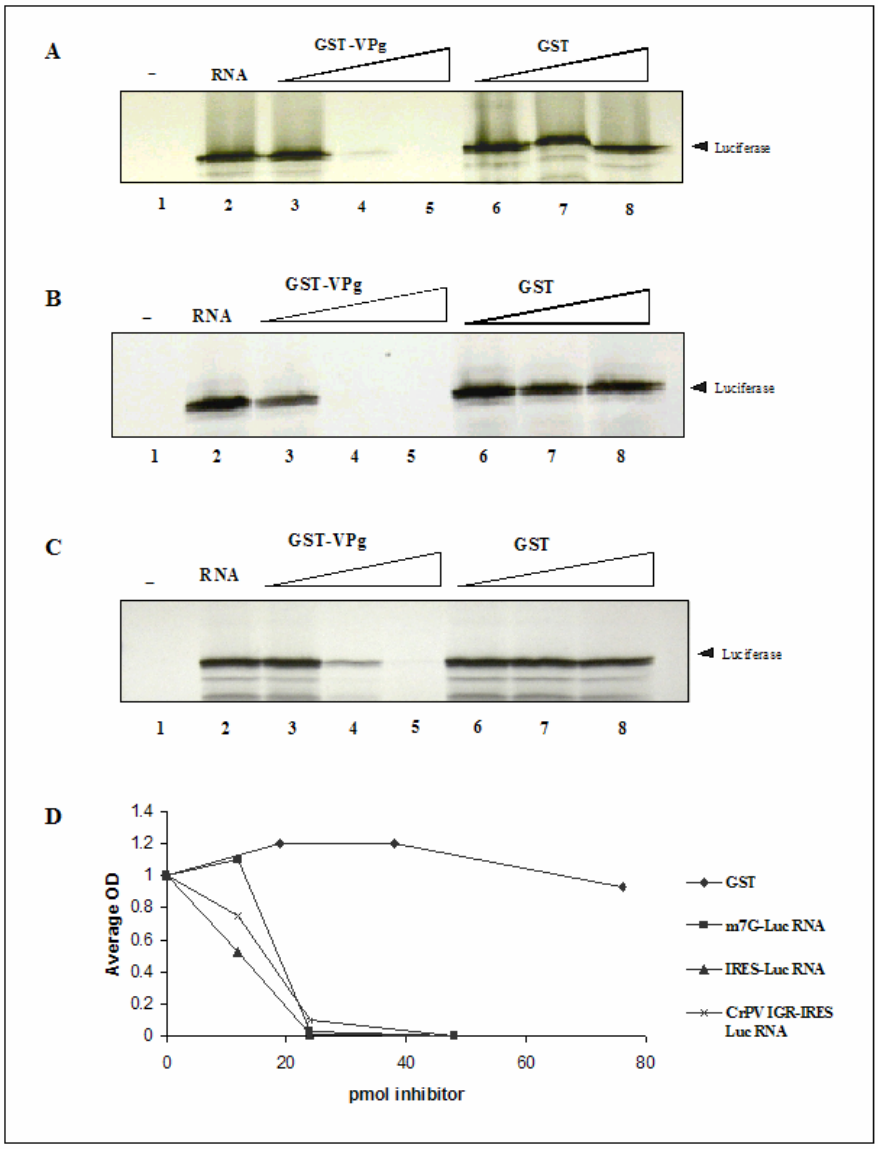

Figure 2.4: VPg inhibits translation of reporter RNAs. A: m^7G-Luc RNA, B: IRES-Luc RNA, and C: CrPV IGR-IRES RNA were translated in Flexi RRL without or with RNA (lanes 1 and 2) and with increasing concentrations of GST-VPg (lanes 3, 4, 5 = 12, 24, and 48 pmol, respectively), or increasing concentrations of GST (lanes 6, 7, and 8, = 19, 38, and 76 pmol, respectively). ^{35}S-labeled proteins were separated by SDS-10% PAGE and visualized by autoradiography. D: Average OD of luciferase synthesized in the presence of GST-VPg in the translation reactions shown in (A-C). m^7G-Luc (squares), IRES-Luc (triangles) and CrPV IGR-IRES (crosses). A control reaction where GST was used as the inhibitor in the IRES-Luc translation reactions is shown as a representative (diamonds). Data are normalized to 1.

Discussion

Mechanisms of eukaryotic ribosome recruitment to mRNA are distinguishable by their requirements for initiation factors and 5' end dependence (116, 126). The mechanism utilized by the majority of cellular and viral mRNAs is 5' end and m^7G cap dependent, and the 43S preinitiation complex is recruited to the mRNA through its interactions with the eIF4GI component of the cap-binding complex eIF4F. 5' terminus-independent internal initiation driven by an IRES, typified by those present in some positive strand RNA virus genomes, has different initiation factor requirements depending on the IRES analyzed (116, 126). We put forth the hypothesis that translation initiation on calicivirus RNA may proceed by a unique mechanism that involves protein-protein interactions between VPg and the cellular translation machinery. In this study, we have shown that recombinant VPg binds to purified eIF3, and to native eIF3 in mammalian cell lysates; the C-terminal half of VPg is the primary mediator of the interaction; and eIF4GI and other translation initiation factors are present in VPg-interacting complexes. These data, interpreted in the context of those previously reported for translation of the FCV genomic RNA, suggest that VPg may function as a cap analogue through protein-protein interactions distinct from those thus far described as important in m^7G cap-dependent or IRES-mediated translation initiation. Herbert and co-workers have postulated a role for VPg in direct ribosome recruitment (115), and our data so far support this model. Future studies will address the current hypothesis that the interactions described herein function as an end-dependent mechanism of ribosome

recruitment to the calicivirus RNA, mediated by interactions between a 5' terminus-linked viral protein VPg and host cell translation initiation complexes.

The organization of calicivirus genomic and subgenomic RNA, specifically the 5'-genome-linked protein and short 5' UTRs, led to the hypothesis that VPg may function in translation initiation. Generally, short 5' UTRs confer an inefficiency of translation on the mRNAs in which they are present (134, 139, 140). The 5' UTRs of all the calicivirus genomes sequenced to date are less than 20 nucleotides long. In addition, an equally short 5' UTR is present on the subgenomic RNA encoding the calicivirus capsid protein (37). It is possible then that a direct interaction between VPg and eIF3 bound to 40S subunits positions the ribosome precisely at the initiating AUG, and thus these viral RNAs would not be subject to the diminutive effects of a short 5' leader sequence on translation efficiency. Such a direct recruitment has been proposed for the HCV IRES, where 40S subunits and eIF3 directly bind the IRES in the absence of other initiation factors (218). In this study, VPg bound directly to purified eIF3. Furthermore, the ribosomal subunit protein S6 was detected in pull-down eluates of full-length VPg, and both the C- and N-terminal VPg domains. It may be that binding of 40S subunits is mediated through the N-terminal domain of VPg. It is tempting to draw a parallel with the HCV IRES with respect to these interactions functioning to position the ribosome at or near the initiator codon. Still, the mechanisms and initiation factor requirements must be different between the two systems, as the HCV IRES – eIF3 – 40S interaction is RNA-protein, whereas the VPg interactions are protein-protein.

The presence of additional components of translation initiation complexes, such as eIF4GI, eIF2α and eIF4E, when binding assays were performed with cell extracts instead of purified eIF3 suggests one alternative mechanism that involves VPg binding to multiple initiation factors (including 40S subunits) to assemble an initiation complex that more closely resembles that recruited by an m^7G cap. Asano and co-workers reported the presence of a multifactor intermediate complex composed of eIF3, eIF1, eIF5, and eIF2 – GST – Met-$tRNA_i^{Met}$ in cell extracts that exist free of 40S subunits (9). It is conceivable that the presence of one or more of the additional eIFs detected by immunoblot in the pull-down assays reflects their associations with eIF3 instead of direct interactions with VPg. Still, detection of components of eIF4F, including eIF4GI and eIF4E, point to the possibility of a complex set of interactions that finally would result in assembly of a functional initiation complex on VPg-linked RNA.

The effects of VPg on translation of reporter RNA in vitro were tested to investigate the functional consequences of the eIF3 interactions. VPg inhibited translation of a capped reporter RNA and a reporter RNA containing an EMCV IRES. We considered the possibility that the mechanism of inhibition of translation of these two RNAs may involve disruption of the interaction between eIF4GI and eIF3. eIF4GI was detected in GST – VPg pull-down eluates, but we were unable to show, or exclude, a direct interaction through a two-hybrid analysis of eIF4GI and VPg. These two proteins may bind the same sites on eIF3, leading to an inhibition of translation in the presence of VPg. The binding site(s) of eIF4GI on eIF3 is not known, but if the mechanism of translation inhibition of VPg includes preventing the eIF4GI – eIF3 interaction, the eIF3d subunit of

eIF3 may play a role. VPg may sequester eIF3, and potentially other initiation factors bound to eIF3, making them unavailable to form functional initiation complexes. Obvious candidates for inhibition targets include those required for cap-dependent translation, such as components of eIF4F. An alternative and equally plausible hypothesis is provided by the ability of VPg to inhibit translation mediated by the CrPV IGR-IRES, which can assemble 80S ribosomes from purified 40S and 60S subunits in the absence of any other eIF (289). These data suggest that inhibition of translation from this mRNA (and perhaps the others) by VPg is mediated through the 40S subunit. However, the presence of endogenous eIFs in the reticulocyte lysates used for these experiments complicates this straightforward interpretation. Thus the mechanism by which VPg is able to inhibit translation of reporter RNAs that have different factor requirements remains unclear at this point. Studies that add purified initiation factors to VPg-inhibited translation reactions to rescue protein synthesis are ongoing in order to define the inhibitory mechanism reported here.

VPg of positive-strand RNA viruses in families other than the *Caliciviridae* has been ascribed a number of functions (244). The picornavirus VPg is comparatively small (2 – 4 kDa), functions in protein-primed RNA synthesis (209) and is not necessary for infectivity of transfected viral RNA. The function of VPg linked to potyvirus genomes appears more complex. Potyviral VPg is larger than the calicivirus VPg (24 – 26 kDa). In addition to its role in RNA synthesis (93), VPg has been found in the nucleus (236), functions in long-distance cell-cell movement (246) and binds eIF(iso)4E (159, 292). The role of potyvirus VPg binding to eIF(iso)4E with respect to protein synthesis is not

known, yet a recent study has confirmed the importance of this interaction by showing that mutant *Arabidopsis thaliana* resistant to potyviral infection do not express eIF(iso)4E (158). Whether potyviral VPg functions in translation initiation remains to be established, as it is clear that sequences in the 5' UTR of potyviral RNA direct cap-independent translation enhancement (26). Thus far, there is no evidence that NV RNA contains an IRES to direct cap- or 5' terminus-independent translation. However, a direct interaction between FCV VPg and eIF4E has recently been described, and further supports the idea of VPg as a cap analogue (68). Assays to address directly if and how the NV VPg may function in ribosome recruitment are challenging. This virus does not grow in cell culture and, consequently, there is no source of NV VPg-linked RNA to perform recruitment studies. Efforts to establish a system with a cultivatable calicivirus from which native VPg-linked RNA can be obtained are in progress. Such a system will allow us to address direct translation initiation complex recruitment through functional assays.

DOMAINS OF VPg RESPONSIBLE FOR INTERACTIONS WITH eIFS

Introduction

Norwalk virus (NV) is the prototype *Norovirus* within the *Caliciviridae* family. Many details about translation of its viral RNA are insufficient due to the lack of cell culture or small animal model systems. However, investigations into these mechanisms using other viruses within the calicivirus family have begun to shed light on requirements for this process.

Calicivirus genomic and subgenomic RNA are covalently linked at the 5' end to a small viral protein VPg (24, 46, 87, 188, 247). VPg is thought to be important for viral protein synthesis because its removal from viral RNA by proteinase K treatment dramatically reduces the production of viral protein in RRL (87). Likewise, removal of VPg from purified genomic RNA of FCV renders it uninfectious by transfection (24). By analogy to its position on the RNA with the m^7G cap structure typical of eukaryotic mRNAs, it is proposed VPg may function as a cap analogue. Support for this idea comes from recent data demonstrating the VPg of FCV binds the cap binding protein eIF4E directly, and that this interaction is critical for translation in vitro (68). Additionally, m^7G cap can substitute for VPg on synthetic FCV transcripts (257). However, the mechanisms of translation mediated by these two structures are obviously distinct, as inclusion of the cap analogue m^7G in translation reactions of VPg-linked RNA in vitro does not negatively affect the production of viral protein (68, 87).

VPg of the *Caliciviridae* family is not the only VPg implicated in translation of viral RNA. VPg of potyviruses binds eIF(iso)4E in vitro and in vivo (160, 292). Mutant plants that lack expression of eIF(iso)4E are not susceptible to viral infection (48, 158), suggesting a biologically relevant role for this interaction. It remains unclear if the interaction between VPg and eIF(iso)4E directs translation of the viral RNA, or if it simply sequesters the eIF from cellular messages, as some potyvirus RNAs contain 5' UTRs that direct translation similar to IRES-dependent mechanisms (12, 26, 162, 197, 198, 292).

Important to the understanding of calicivirus replication came with the finding that VPg of viruses within the *Norovirus* genus interacts with components of the translation initiation apparatus. VPg of NV and SMV binds eIF3 in cell lysates, and NV VPg binds eIF3 directly (39). NV VPg is present in complexes with other eIFs, such as eIF2α, eIF4GI, eIF4E, and the S6 40S ribosomal protein (39), all suggestive of an important role for VPg in the translation process.

As described, translation initiation factors that bind the VPg from many virus types are coming to light. However, regions of VPg that are responsible for these interactions are only beginning to be described (39). In the studies presented here, we pursue the identification of domains of NV VPg that are important for interaction with individual eIFs, and extend interpretation of the data to propose factors important for translation of VPg-linked RNA. Domains of NV VPg responsible for interaction with eIF3, eIF4GI, and the S6 protein of the 40S ribosomal subunit were identified by pull-down assay. The data demonstrate that VPg can bind eIF4GI independently of eIF3, and we implicate

interaction between VPg and the S6 ribosomal protein as being important in the observed inhibition of translation of a capped reporter mRNA in vitro. Together the data suggest complex interactions between VPg and eIFs, including the 40S ribosome, are likely important for the translation of viral RNA.

Materials and Methods

Construction of VPg Mutant Clones

cDNA of all mutated VPg constructs were amplified from a full-length NV genomic clone, pSPNVF (77). DNA was amplified using the KOD enzyme (Novagen) and reactions were assembled following instructions provided by the manufacturer. Amplification conditions consisted of 25 cycles of 15 seconds at 98°C, 3 seconds at 56°C, and 5 seconds at 68°C. Amplification of all of the following products used VPg/XhoI(-) as the reverse primer (39). The forward primer for GST-$VPg_{87\text{-}138}$ was C term 87/EcoRI (+) 5'-ccggaattcaagagtaagaaacaccaacaag-3', for GST-$VPg_{104\text{-}138}$ was C term 104/EcoRI(+) 5'-ccggaattcggatcagacatcagaaaacgt-3', and for GST-$VPg_{121\text{-}138}$ was C term 121/EcoRI(+) 5'-ccggaattcgaatgggcagatgatgacagag-3'. To amplify GST-$VPg_{34\text{-}104}$, primers VPg 34-104/EcoRI(+) 5'-ccggaattcaaaaagatcagagaagaaaag-3' and VPg 34-104/XhoI(-) 5'-ccgctcgagtccagtcactagaccaagtgg-3' were used. Forward primers contain a restriction enzyme site for EcoRI, and reverse primers contain a restriction enzyme site for XhoI. Restriction sites are underlined. Amplification products were cloned into the pGEX-4-T1 vector (Amersham) with these enzymes. Constructs were transformed into

BL21-DE3 cells by heat shock. Recombinant proteins were expressed and purified as described (39).

GST Pull-Down Assays

Pull-down assays using GST – VPg mutants and CaCo-2 cellular extracts, and Western immunoblots were conducted exactly as described (39).

In Vitro Translation in RRLs

Translation reactions were performed using capped reporter mRNA (m^7G-Luc) with the addition of recombinant VPg protein as described (39).

Results

Domains of NV VPg Responsible for Interactions with eIF3, eIF4GI, and S6 Ribosomal Protein by Pull-Down Assay

To identify domains of VPg responsible for interactions with eIFs, we performed GST pull-down assays using GST - VPg deletion mutants and CaCo-2 cellular extracts as described (39). Figure 3.1 shows the regions of VPg that were cloned into the pGEX-4T-1 plasmid and expressed in *E. coli*. Deletions were made starting from the C terminus of the protein, as that is the region shown to potentially mediate interactions with eIF3 and eIF4GI (39). Recombinant proteins were expressed in the soluble fraction and were easily purified from other prokaryotic contaminants (data not shown). The GST – VPg fusion proteins were bound to glutathione Sepharose-4B and incubated with CaCo-2 intestinal cell extracts as described (39). Eluates were subjected to SDS-PAGE and

Western immunoblot with the indicated antibodies. Figure 3.2 shows the results of pull-down assays with these mutant constructs.

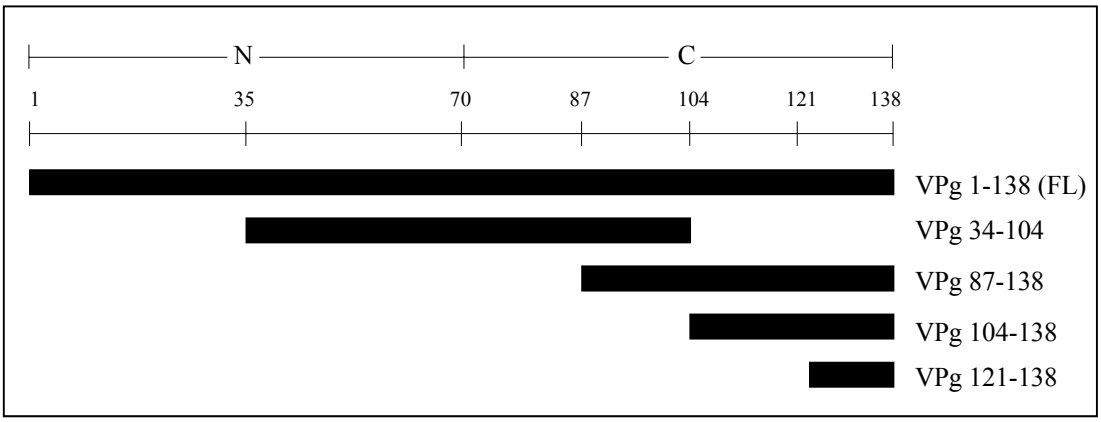

Figure 3.1: Domains of VPg cloned into the pGEX-4T-1 plasmid for expression in *E. coli* as GST fusion proteins.

Western immunoblot with anti-eIF3 serum showed the full-length VPg fusion protein (GST – $VPg_{1\text{-}138}$) brought down eIF3 as before (39). The VPg mutant encompassing amino acids 87 – 138 also brought down eIF3 by this assay. Mutants that eliminated amino acids from the N terminal region of the C terminal half of VPg abrogated binding, suggesting that interaction between VPg and eIF3 requires amino acids located near the central domain of VPg. However, further deletion of the C terminal end of VPg will have to be made before the minimal set of amino acids required for binding can be defined.

Western immunoblot of pull-down eluates with anti-eIF4GI showed that the full-length VPg fusion protein (GST – $VPg_{1\text{-}138}$) brought down eIF4GI as before (39). All mutants of VPg brought down eIF4GI except GST – $VPg_{34 - 104}$. The smallest domain of VPg that reacted with eIF4GI contained amino acids 121 – 138. Importantly, this domain

of VPg did not bind eIF3 in this assay, suggesting it is possible VPg binds eIF4GI directly, and that eIF4GI is not being detected as a result of interaction with eIF3.

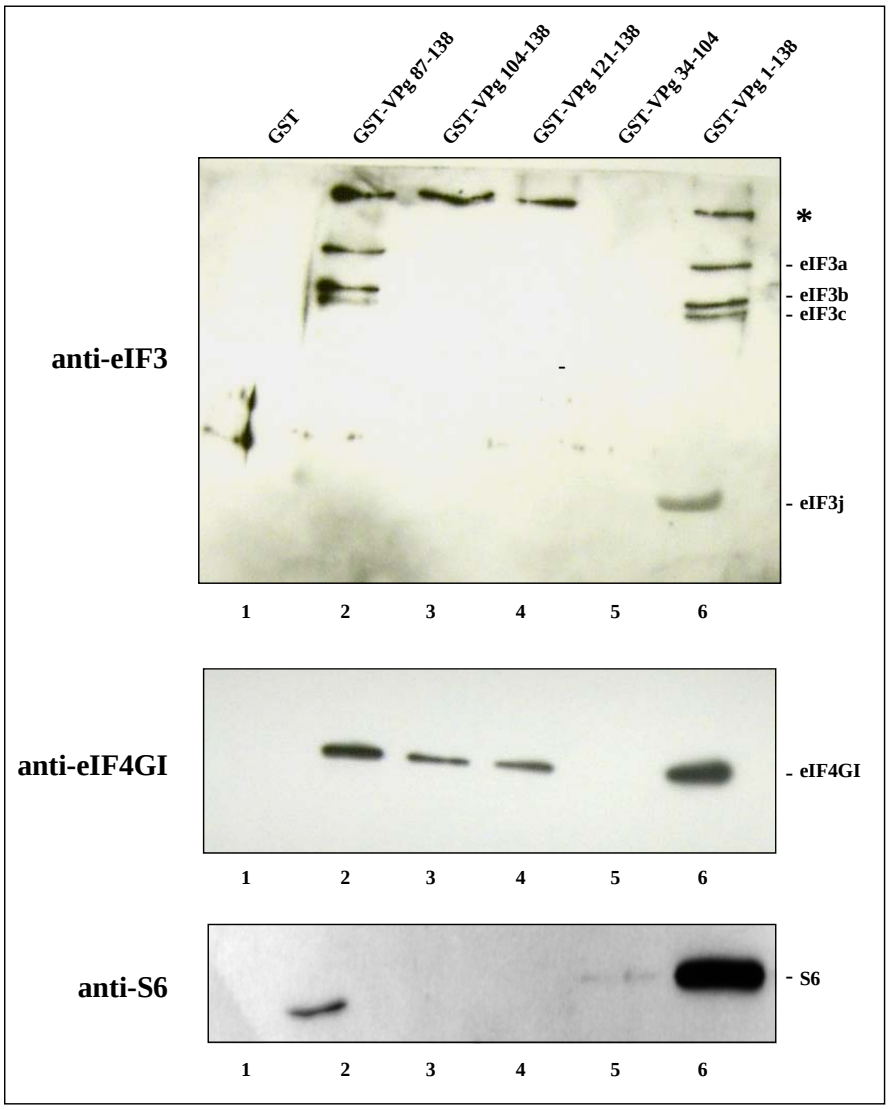

Figure 3.2: Western blots of GST – VPg mutant pull-down eluates with anti-eIF3, anti-eIF4GI, and anti-S6. Domains of VPg were expressed as GST fusion proteins as indicated in Figure 3.1. Recombinant protein was bound to glutathione Sepharose 4B and subjected to pull-down assay using CaCo-2 cellular extracts as described in Materials and Methods. The asterisk in the anti-eIF3 blot is eIF4GI.

Western immunoblot of pull-down eluates with anti-S6 showed that, as before, the full-length VPg fusion protein (GST – VPg_{1-138}) brought down this protein. The mutant GST – VPg_{87-138} also showed reactivity with S6. Slight reactivity with S6 was detected in eluates using the mutant encompassing amino acids 34 – 104. The interaction between this mutant and S6 may be sufficiently weak that detection by pull-down assay is not optimal. However, these binding data suggest that amino acids in the central region of VPg mediate interaction with S6, and by extension, the 40S ribosome. Data from Chapter 2 show that both the N and C terminal domains of VPg bind S6 equally well, supporting the idea that the binding site includes amino acids from both termini.

Domains of VPg Differentially Inhibit Translation of Capped Reporter mRNA

Our recent study demonstrated that GST – VPg inhibited translation of reporter RNAs with different eIF requirements in rabbit reticulocyte lysates (RRL) (39). The common denominator for all of the reporter mRNAs used in the study is the requirement for the 40S ribosome. To test which interactions between VPg and eIFs may be important for the observed translation inhibition, we employed the panel of GST – VPg deletion mutants in the translation inhibition assay. Figure 3.3 shows that the full-length VPg protein, GST – VPg_{1-138}, inhibited translation to completion, consistent with previous data (39). Mutants encompassing amino acids 104-138 and 121-138 did not inhibit translation to significant levels, or at least to levels that would suggest that their interactions with eIF4GI are important for the observed inhibition. The VPg mutant encompassing amino acids 87-138 inhibited translation to approximately 15% of the uninhibited control,

suggesting that interactions between this mutant and the eIFs it binds, such as eIF3, eIF4GI, and S6, are important for the translation of this RNA. However, the mutant encompassing amino acids 34-104 inhibited translation to levels similar to the full-length VPg, suggesting that the observed interactions with eIFs mediated by this mutant are sufficient to inhibit translation of the reporter mRNA. Interestingly, the only factor brought down by this mutant was S6. A summary of eIFs bound by the mutants, and the extent to which the mutant inhibited translation is shown in Figure 3.4. Taken together, the data indicate that interaction with S6, and by extension the 40S ribosome, is most likely the interaction relevant for impeding translation of the capped reporter mRNA.

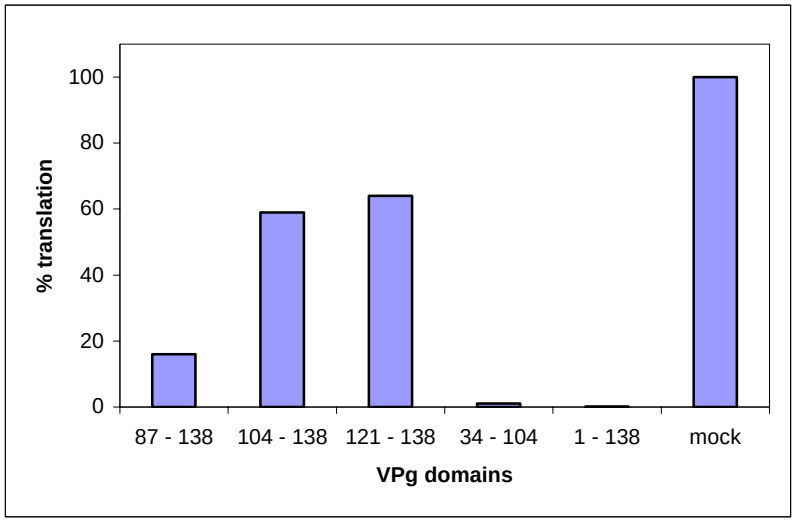

Figure 3.3: Inhibition of cap-dependent translation by GST – VPg deletion mutants. VPg deletion mutants were expressed as GST fusion proteins and included in translation reactions of capped reporter mRNA as described (39). Data for each deletion mutant are reported as percent translation as compared to the uninhibited control (mock).

Domain	eIF3	eIF4GI	S6	Strength of Inhibition
1 – 138	+++	+++	+++	+++
87 – 138	+++	+++	++	++
104 – 138	-	++	-	+
121 – 138	-	++	-	+
34 – 104	-	-	+	+++

Figure 3.4: Summary of binding of GST – VPg deletion mutants to eIFs and the relative extent to which it inhibits translation of capped reporter mRNA in RRL.

Discussion

Most eukaryotic mRNAs are capped, and translation is mediated by interaction between the cap and the cap binding protein eIF4E. eIF4E is a member of the eIF4F complex, which also contains eIF4GI and eIF4A. eIF4GI interacts with eIF3 bound to the 40S ribosome, and in this manner the ribosome and the Met-$tRNA_i^{Met}$ are brought to the 5' end of the message and translation can commence. Some cellular and viral mRNAs are not translated by the cap-dependent mechanism, and instead their translation is mediated by an IRES element found internal to the 5' end of the mRNA. Factor requirements for the translation of unique IRES elements differ, but a common feature between them is that they do not require the cap-binding protein eIF4E to initiate. Some IRES elements, such as that found in the intergenic region of the Cricket paralysis virus (CrPV) genome, do not require any eIFs or the initiator tRNA to initiate, and instead only require the 40S ribosome. Data presented in Chapter 2 demonstrate that GST – VPg inhibits translation of mRNA whose translation is mediated by all three mechanisms, including an mRNA containing the CrPV IRES element. This study was initiated to

define the regions of VPg that bind eIFs by pull-down assay, and ascertain which of these interactions is important for the observed translation inhibition.

The smallest domain of VPg capable of reacting with eIF3 by pull-down assay is the mutant encompassing amino acids 87-138. Data from Chapter 2 show that the C terminus of VPg most likely mediates this interaction, and the binding data with this mutant confirm this idea. However, deletions made from the C terminus will have to be tested in order to determine the minimum binding site for eIF3.

GST – $VPg_{121-138}$ included the minimal domain of VPg capable of reacting with eIF4GI by pull-down assay. Interestingly, this region of VPg binds eIF4GI independent of eIF3, suggesting that the observed interaction with eIF4GI may be direct, or at least not mediated by eIF3. Recent data with FCV shows that its VPg binds eIF4E, and that this interaction is necessary for translation in vitro (68). It is possible that eIF4GI comes down with VPg as a result of interaction with eIF4E, since it too is part of the eIF4F complex, and was also found in pull-down eluates with GST – VPg_{1-138} (39). However, because the identity between NV VPg and that of FCV is only 17%, and because GST – FCV VPg does not bind eIF3, eIF4GI, or S6 by pull-down assay in our hands (data not shown), it is possible that the mechanism of ribosome recruitment, and thus interactions with eIFs, differs between these two viruses. Another possibility, which is not mutually exclusive from the first, is that interaction between VPg and eIF4GI, with or without eIF4E, circularizes the viral RNA, as has been described for other viral and cellular mRNAs, to promote efficient translation. Pull-down assays with GST – VPg and

individual purified eIFs will need to be conducted before clear interpretations can be made.

The domain of VPg capable of inhibiting translation of a capped reporter mRNA in RRL to similar levels as full-length VPg was that including amino acids 34-104. The only protein detected in our pull-down assay that showed slight reactivity with this mutant was S6, a component of the 40S ribosome. The VPg mutant including amino acids 87-138, inhibited translation to approximately 15% of the uninhibited control and also brought down S6. These data suggest that interaction between VPg and the 40S ribosome is a mechanism by which translation of the capped reporter mRNA is inhibited. This idea is consistent with previous translation inhibition data for which the 40S ribosome is the common requirement for the translation of all reporter mRNAs tested. It is possible the reactivity of GST-VPg$_{87\text{-}138}$ with S6 is stronger than that for GST-VPg$_{34\text{-}104}$ because of association of 40S with eIF3 and eIF4GI, which were also found in eluates using this mutant.

The data presented here describe the domains of VPg that mediate interactions with eIFs. The data suggest that interaction between VPg and S6 is important for inhibition of translation of reporter RNAs. By extension, interaction between VPg and the 40S ribosome may be important for translation of VPg-linked viral RNA. It is possible that VPg binds the 40S ribosome directly, as GST-VPg$_{34\text{-}104}$ only reacted with S6. Studies to identify the binding site of VPg on the eIFs themselves are required to further define the mechanism of inhibition as well as the mechanism by which VPg-linked RNA is translated.

DOMAINS OF VPg RESPONSIBLE FOR INTERACTIONS WITH 40S RIBOSOMAL SUBUNITS

Introduction

Important to the appreciation of the initial steps of the protein synthesis pathway is the realization that two eIFs, eIF1A and eIF5B, are conserved within all domains of life. eIF1A is required for scanning and for transfer of the ternary complex to the 40S ribosome (13, 32), and eIF5B catalyzes the hydrolysis of a GTP molecule to join the 60S ribosome with the 40S ribosome to create the complete 80S complex (217). These events in the translation initiation process are pivotal to the pathway, and the presence of homologs to mediate these processes in eukaryotes, prokaryotes, and archea is obviously warranted.

The bacterial homolog of eIF5B is IF2. They share greater than 27% sequence identity and possess the same three-dimensional structure (241). The GTP-bound form of IF2/eIF5B promotes ribosomal subunit joining (217), and the current hypothesis is that GTP-induced changes enable IF2/eIF5B to bind both ribosomal subunits concomitantly to facilitate their unification. It has been suggested that IF2/eIF5B binds the small ribosomal subunit at a site where, along with eIF1A, it can direct positioning of the initiator tRNA to the correct location on the ribosome (241).

eIF1A shares 21% sequence identity with the bacterial initiation factor IF1 (150). These proteins are members of the S1 family of oligonucleotide binding fold proteins. IF1 increases the rate of ribosomal subunit association, and binds the A, or aminoacyl, site of the small ribosomal subunit to direct the initiator tRNA to occupy the P, or

peptidyl, site (27). Importantly, IF1 protects some of the same nucleotides as the A site aminoacyl-tRNA, confirming this idea (194). By analogy to IF1, eIF1A presumably occupies the A site of the 40S ribosomal subunit, and in so doing guides the Met-$tRNA_i^{Met}$ molecule to the P site to eventually base-pair with the AUG initiator codon. eIF5B is important in this reaction because it binds eIF1A (35, 205), and it is thought that this interaction stabilizes the binding of the initiator tRNA to the A site (35). Additionally, fragments of eIF5B that do not bind eIF1A are nonfunctional in vivo and in vitro, suggesting this is a biologically important interaction (35). eIF5B and eIF1A contact each other in two regions, one of which includes regions of both that are not found in their bacterial homologs, suggesting a eukaryotic-specific interaction (179).

Sequence conservation between NV VPg and eIF1A has been noted (255). Given that eIF1A is a universally conserved eIF, the discovery of a viral homolog would not be unexpected. Since eIF1A binds the 40S ribosome (54, 270), presumably through the A site to transfer the initiator tRNA directly to the P site, then by analogy a similar role for VPg could be predicted.

Previous data suggests that interaction between NV VPg and S6, and by extension the 40S ribosome, is important for the inhibition of translation of a capped reporter mRNA in vitro. In addition to shared sequence identity between VPg and eIF1A, a role for VPg in 40S subunit binding would confirm another similarity between these two proteins. This study was initiated to further dissect the potential interaction between NV VPg and 40S subunits, and to test if similar sites on VPg and eIF1A are responsible for this interaction.

Materials and Methods

Cloning of GST – VPg_{32-94}

Nucleotide sequence encoding amino acids 32 – 94 of NV VPg were amplified from a full-length NV genomic clone, pSPNVF (77) using the primers VPg 32-94/EcoRI(+) 5'-ccggaattcgagtacaaaaagatcagagaag-3' and VPg 32-94/XhoI(-) 5'-ccgctcgagttgttggtgtttcttactcttgg-3' (restriction sites are underlined). Reactions were assembled using the KOD enzyme (Novagen) according to instructions provided by the manufacturer. Amplification conditions consisted of 25 cycles of 94°C for 1 minute, 56°C for 15 seconds, and 68°C for 15 seconds. Amplification products were cloned into pGEX-4T-1 (Amersham) using EcoRI and XhoI. The construct was transformed into BL21-DE3 cells, and recombinant protein was expressed and purified as described (39). Cloning of GST – VPg_{Nterm} and GST – VPg_{Cterm} was performed as described in Chapter 3.

Purification of 40S Ribosomal Subunits

Ribosomal subunits were purified from one liter of HeLa cell suspension culture (National Cell Culture Center). The cell pellet was thawed on ice for two hours, then suspended in 5 mL lysis buffer containing 0.3 M NaCl, 15 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1% Triton X-100, 1 mg/mL heparin, and 1 mM DTT. The suspension was lysed for 30 minutes on ice. Lysates were clarified by centrifugation for 30 minutes at 14,000 rpm in a Sorvall SS34 rotor at 4°C. The supernatant was decanted into a thick-walled polycarbonate tube with cap. A 30% wt/wt sucrose cushion prepared in buffer containing 0.5 M KCl, 2 mM DTT, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, and 6 mM $Mg(OAc)_2$

was layered under the sample. Ribosomes were pelleted by centrifugation for 22 hours at 100,000xg (36,000 rpm) using a Ti70 rotor at 4°C. The pellet was suspended on ice in 300 µl resuspension buffer containing 6.8% sucrose (wt/wt), 20 mM Tris-HCl 7.5, 6 mM $Mg(OAc)_2$, 150 mM KCl, and 2 mM DTT. Absorbance of 1 µl of sample mixed with 99 µl water was determined at 260 nm. The sample was adjusted to 150 A_{260} units/mL by addition of the appropriate volume of resuspension buffer. Puromycin was added to 2 mM final concentration, and the sample was incubated for 10 minutes at 4°C, for ten minutes at 37°C, then chilled on ice. Sample was transferred to a 10 mL flask with stir bar, KCl was added to 0.5 M, and was stirred vigorously for 30 minutes at 4°C. The sample was divided into two equal parts, and each part was gently layered onto a 10 – 30% wt/wt sucrose gradient prepared in buffer containing 20 mM Tris-HCl pH 7.5, 6 mM $Mg(OAc)_2$, 0.5 M KCl, and 2 mM DTT. Gradients were prepared in polyallomer SW41 tubes. Samples were centrifuged for 18 hours at 64,000xg (23,000 rpm) using an SW41 rotor at 4°C. One mL fractions were collected from each gradient with an ISCO density gradient fractionator and absorbance was monitored at 254 nm. Fractions containing 40S ribosomal subunits as defined by the absorbance profile were pooled, adjusted to 8 mM $MgCl_2$, and precipitated with 0.7 volumes of ice-cold 100% ethanol. Ribosomes were centrifuged for 20 minutes at 14,000 rpm in a microcentrifuge at 4°C. Ethanol was removed and pellets were suspended in buffer containing 20 mM Tris-HCl pH 7.5, 70 mM KCl, 2 mM $Mg(OAc)_2$, and 1 mM DTT. Absorbance of 1 µl of sample mixed with 99 µl water was determined at 260 nm. The quantity of 40S ribosomal subunits was

calculated using the constant 50 pmol of subunits per 1 A_{260} unit. Subunits were devoid of eIF3 and eIF2α as evaluated by Western immunoblot with appropriate antibodies.

Sucrose Density Gradient Centrifugation

Seventeen pmol of purified 40S ribosomal subunits were incubated with 17 pmol recombinant GST fusion protein for 5 minutes at 37°C, then chilled on ice. Buffer containing 20 mM Tris-HCl pH 7.5, 70 mM KCl, 2 mM $Mg(OAc)_2$, and 1 mM DTT was added to 500 µl final volume. Each sample was gently layered onto a 10 – 30 % sucrose gradient (wt/vol) prepared in the same buffer as above. Samples were centrifuged for 3.5 hours at 38,000 rpm using an SW41 rotor at 4°C. One mL fractions were collected from each gradient and absorbance at 254 nm was monitored to locate 40S subunits within the gradient. Protein in each fraction was precipitated with 0.7 volumes of ice-cold 100% ethanol, centrifuged for 20 minutes at 14,000 rpm in a microcentrifuge at 4°C, and pellets were suspended in 1X sample buffer containing 50 mM Tris-HCl pH 8, 2% SDS, 0.l% bromophenol blue, 10% glycerol, and 100 mM β-mercaptoethanol. Proteins were separated by SDS-PAGE and transferred to nitrocellulose. Blots were blocked for 30 minutes in 10% Blotto, and incubated overnight at room temperature with anti-GST antibody (Amersham) at a dilution of 1:2000. Blots were washed in 0.5% Blotto and incubated with HRP-conjugated rabbit anti-goat antibody at a dilution of 1:3000 for two hours at room temperature. Blots were developed with ECL (Amersham).

Results

NV VPg Binds Purified 40S Subunits

Previous data suggest VPg binds the 40S ribosomal subunit (Chapter 3). To test if this interaction is direct, the ability of VPg to cosediment with 40S subunits was tested by sucrose gradient centrifugation. GST or GST – VPg were mixed with equimolar amounts of purified 40S subunits, then centrifuged through 10 – 30% sucrose gradients. Fractions were precipitated with ethanol, and subjected to SDS-PAGE and Western immunoblot with anti-GST antibody. Figure 4.1 shows the result of this experiment. 40S subunits were found in fractions 4 and 5 as defined by absorbance at 254 nm. GST – VPg, but not GST alone, sedimented in the same fractions with 40S subunits, suggesting a direction interaction between these two components.

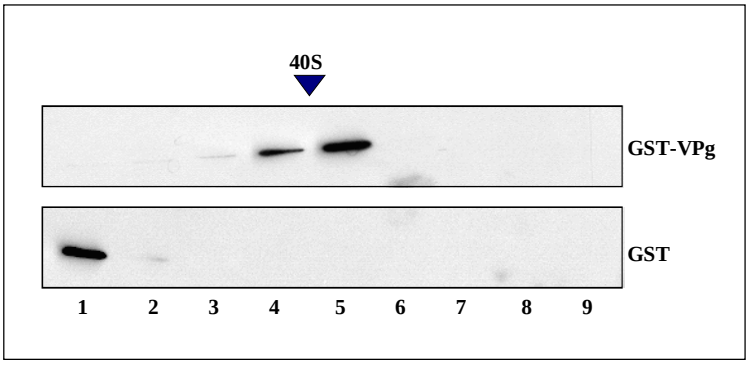

Figure 4.1: GST – VPg, but not GST alone, cosediments with purified 40S ribosomal subunits by sucrose density gradient centrifugation. GST – VPg and GST were mixed with equimolar amounts of purified 40S subunits, incubated for 5 minutes at 37°C, then gently loaded onto 10 – 30% sucrose gradients. Gradients were centrifuged for 3.5 hours at 38,000 rpm using an SW41 rotor at 4°C, and fractionated into 1 mL fractions with an absorbance monitor at 254 nm to localize 40S subunits. Fractions were precipitated with ethanol and subjected to Western immunoblot with anti-GST antibody. The arrow indicates the location of 40S subunits within the gradient. Sedimentation is from left to right.

GST – VPg Binds 40S Ribosomal Subunits
at 2 mM, but not 5 mM $MgCl_2$

To characterize the strength of the interaction between NV VPg and 40S ribosomal subunits, the ability of GST – VPg to cosediment with 40S subunits was tested at 2 mM and 5 mM $MgCl_2$. The physiological concentration of magnesium is 2 mM, and biologically relevant interactions are predicted to occur at this concentration. However, sucrose density gradient centrifugation can disrupt authentic interactions due to the nature of the assay, and increasing magnesium concentration to 5 mM can sometimes alleviate this effect by strengthening the observed interaction (31). GST – VPg was mixed with equimolar amounts of 40S subunits as before, and centrifuged through 10 – 30% sucrose gradients containing the indicated amount of $MgCl_2$. Figure 4.2 shows that GST – VPg cosedimented with 40S subunits at 2 mM, but not 5 mM $MgCl_2$, suggesting a physiological relevance to this interaction.

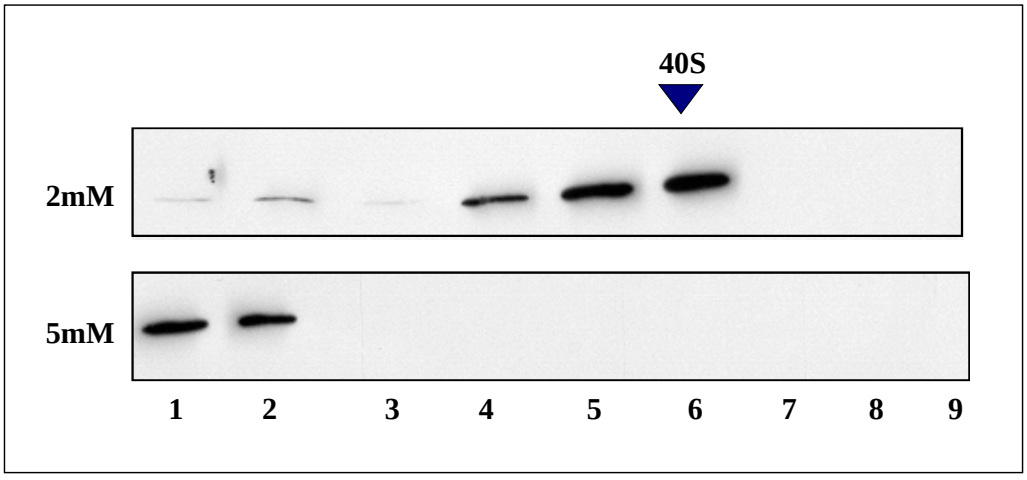

Figure 4.2: GST – VPg cosediments with purified 40S ribosomal subunits by sucrose density gradient centrifugation at 2 mM but not 5 mM $MgCl_2$. Experimental parameters are the same as those described in Figure 4.1. Sedimentation is from left to right.

Neither the N nor C Terminus of VPg is Sufficient
to Mediate Interactions with 40S Subunits

Different domains of VPg mediate differential interactions with eIFs (Chapter 3). We next extended those observations by asking what domains of VPg mediate the observed interaction with 40S subunits. GST – VPg_{Nterm} or GST – VPg_{Cterm} was mixed with equimolar amounts of purified 40S subunits and subjected to sucrose density gradient centrifugation as before. Fractions were probed by Western immunoblot with anti-GST antibody. The results in Figure 4.3 show that neither the N nor C terminal halves of VPg were sufficient for cosedimentation with 40S subunits.

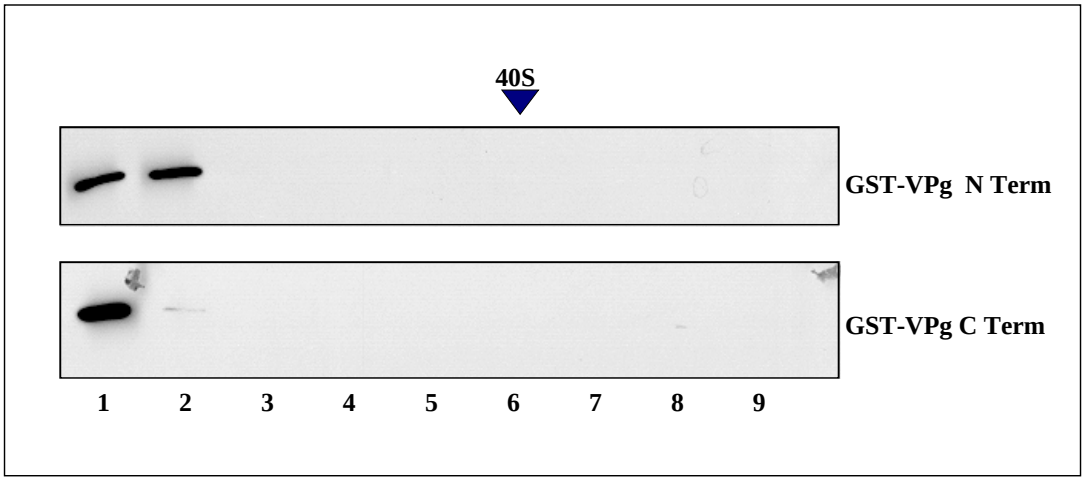

Figure 4.3: Neither GST – VPg_{Nterm} nor GST – VPg_{Cterm} cosediments with 40S ribosomal subunits by sucrose density gradient centrifugation at 2 mM $MgCl_2$. Experimental parameters are the same as those described in Figure 4.1. Sedimentation is from left to right.

GST – $VPg_{34\text{-}104}$ Weakly Cosediments with
40S Subunits at 5 mM $MgCl_2$

We next asked if the deletion mutant GST – $VPg_{34\text{-}104}$ mediated interaction with 40S subunits, as is suggested by data from Chapter 3. GST – $VPg_{34\text{-}104}$ was mixed with

equimolar amounts of 40S ribosomal subunits and subjected to sucrose density gradient centrifugation as before. Concentrations of 2 mM and 5 mM $MgCl_2$ were tested in this assay to assess if changes in salt concentration were necessary to stabilize a possible weak interaction. As shown in Figure 4.4, GST – VPg_{34-104} did not cosediment with 40S subunits at 2 mM $MgCl_2$, but a weak interaction was observed at 5 mM $MgCl_2$. These data suggest that although weak, it is possible that interaction between VPg and 40S ribosomal subunits is mediated by the central domain of VPg.

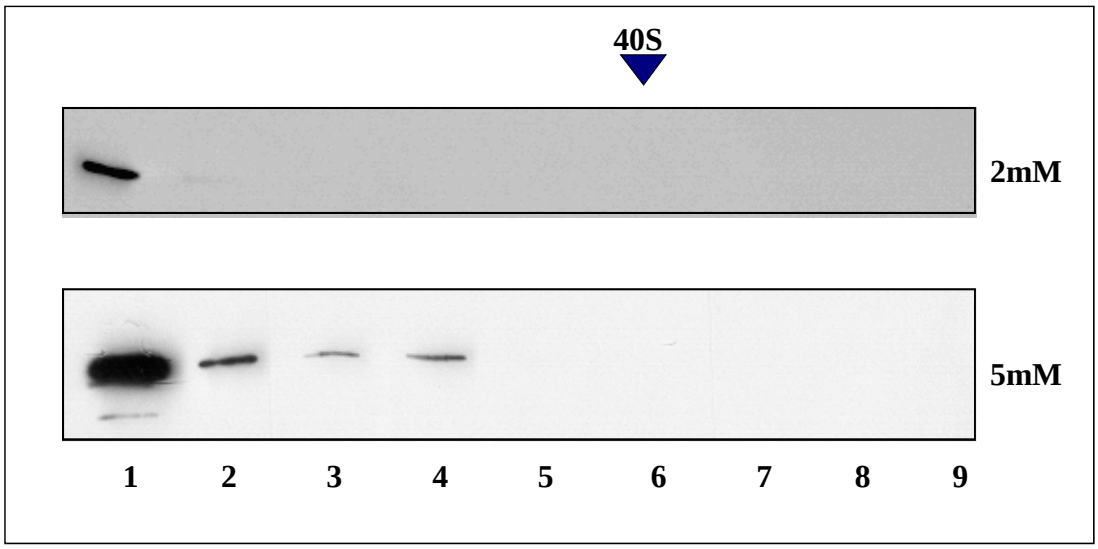

Figure 4.4: GST – VPg_{34-104} cosediments weakly with 40S ribosomal subunits by sucrose density gradient centrifugation at 5 mM $MgCl_2$. Experimental parameters are the same as those described in Figure 4.1. Sedimentation is from left to right.

GST – VPg_{32-94} Cosediments with 40S Subunits

The observation that VPg and eIF1A share sequence identity was first made by Sosnovtseva et al (Figure 4.5, 255). Additionally, VPg and eIF1A have similar charge distribution, where acidic amino acids compose the N termini, and basic amino acids compose the C termini. They are the same molecular weight, and they both bind RNA,

eIF3, and important to this study, the 40S ribosome. To extend the observation that VPg and eIF1A share many physical characteristics, we next asked if the 40S binding site on the proteins is mediated by the same amino acid region. The 40S binding site on eIF1A has been predicted based on analogy to IF1, and has recently been demonstrated to be located within the central domain of the protein (13, 54, 205).

```
VPg      -GKNKGKTKKGRGRKNNYNAFSRRGLS-DEEYEEYKKIREEKNGNYSIQEYLEDRQRYEE   58
eIF1A    MPKNKGKGGKNRRRGKNENESEKRELVFKEDGQEYAQVIKMLGNGRLEAMCFDGVKRLCH   60
           *****  *.* * :* *  .:* *  .*: :** :: :  ...      ::. :*  .

VPg      ELAEVQAGGDGGIGETEMEIRHRVFYKSKSK----KHQQEQRRQLGLVTGS--DIRKRKP   112
eIF1A    IRGKLRK--KVWINTSDIILVGLRDYQDNKADVILKYNADEARSLKAYGELPEHAKINET   118
           .:::   .  *. ::: :     *:.:.     *:: :: *.*        . : .:.

VPg      IDWTPPKNEWADDDREVDYNEKINFE   138
eIF1A    DTFGPGDDDEIQFDDIGDDDEDIDDI   144
           : * .::  : *   * :*.*:
```

Figure 4.5: NV VPg shares 18% sequence identity with eIF1A. Sequences were aligned with ClustalW (v1.82). Asterisks indicate identical residues and colons indicate conservative substitutions.

A GST – VPg deletion mutant was made that corresponds to the 40S binding site on eIF1A (Figure 4.6), and is similar to the GST – $VPg_{34\text{-}104}$ mutant except two conserved amino acids were added to the N terminus of the VPg sequence and the C terminus was shortened by 13 amino acids.

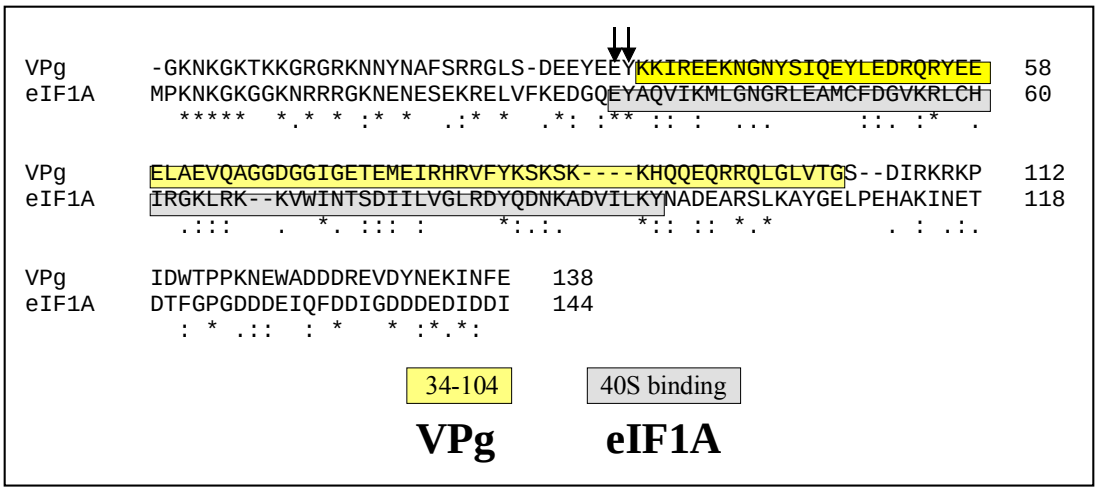

Figure 4.6: GST – VPg_{32-94} corresponds to the 40S binding site on eIF1A. VPg and eIF1A were aligned with ClustalW (v1.82). Highlighted in yellow are the amino acids of VPg that correspond to the GST – VPg_{34-104} mutant. Highlighted in gray are the amino acids of eIF1A that comprise the 40S ribosome binding site. Arrows indicate two conserved amino acids between the two proteins that are not found in GST – VPg_{34-104}. GST – VPg_{32-94} was made to include the amino acids in VPg that correspond to those in gray for eIF1A.

GST – VPg_{32-94} was subjected to the sucrose density gradient centrifugation assay as before, and fractions were probed with anti-GST antibody. Results in Figure 4.7 show that, at both 2 mM and 5 mM $MgCl_2$, GST – VPg_{32-94} cosedimented with 40S subunits, similar to what has been proposed for eIF1A (13, 54, 205). Importantly, cosedimentation was observed at physiological 2 mM $MgCl_2$, and not only at 5 mM $MgCl_2$, as was observed using GST – VPg_{34-104}. The presence of VPg in fractions 1 – 5, in addition to fraction 6, is possibly due to overloading of the gradient with VPg in excess of 40S subunits. Alternatively, the interaction between this mutant and 40S subunits is somewhat unstable. These data suggest that the central domain of VPg mediates

interactions with the 40S ribosome, and strengthen the idea that VPg and eIF1A are physically and functionally similar.

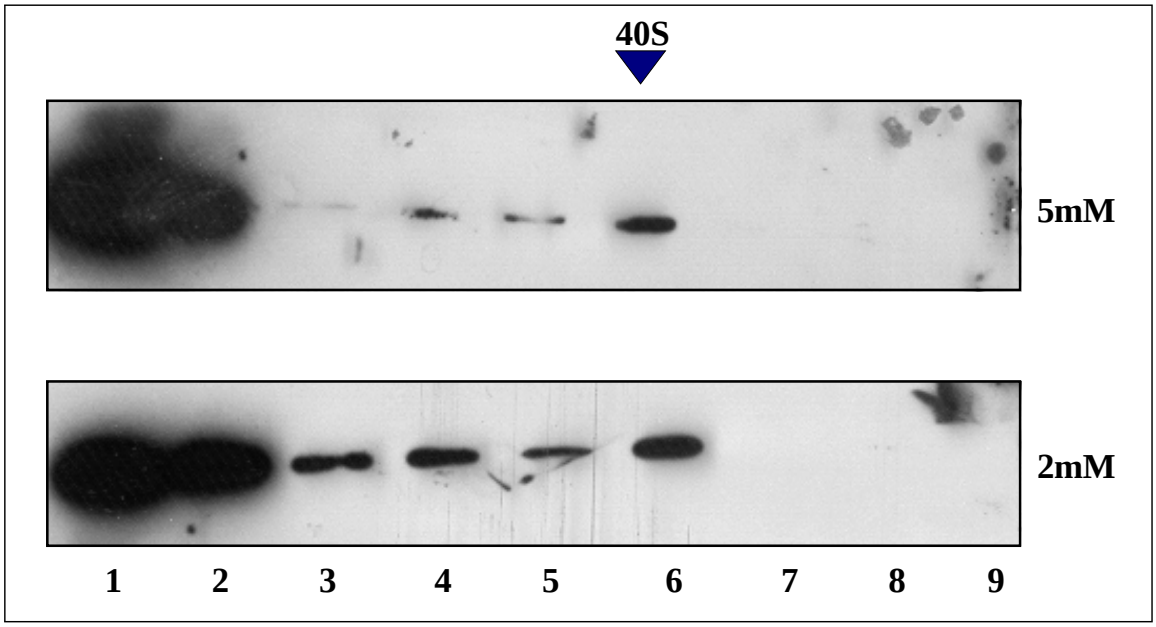

Figure 4.7: GST – VPg_{32-94} cosediments with purified 40S ribosomal subunits by sucrose density gradient centrifugation at 2 mM and 5 mM $MgCl_2$. Experimental parameters are the same as those described in Figure 4.1. Sedimentation is from left to right.

Discussion

This study was undertaken to test whether VPg binds 40S subunits in the absence of other eIFs. Here we show that GST – VPg cosediments with purified 40S ribosomal subunits at 2 mM $MgCl_2$ by sucrose density gradient centrifugation. Neither the N nor C termini of VPg are sufficient for this interaction, but the central domain of VPg, including amino acids that correspond to the 40S binding site on eIF1A, cosedimented with purified 40S subunits. These data suggest it is possible VPg shares common functional properties with eIF1A, in addition to sequence identity and other shared physical characteristics.

There are several common features between VPg and eIF1A. For instance, both factors bind eIF3 directly (39, 205). For eIF1A this interaction likely stabilizes the interaction of eIF3 with the ternary complex, as both components are required to generate stable 43S complexes (176). VPg may be involved in a similar interaction with eIF3 to stabilize complexes that assemble at the 5' end of the viral RNA. VPg may bind eIF3 as a means to recruit eIF4G for the circularization and enhanced translational efficiency of viral RNA, but data indicate VPg can bind eIF4GI independently of eIF3 (Chapter 3), suggesting a more complicated eIF recruitment pattern. It is possible that interaction between VPg and eIF3, and the interaction with 40S ribosomal subunits observed here, is a means to recruit the ribosome to viral RNA, and association of eIF3 is due to the fact that eIF3 and 40S subunits rarely exist independently of each other in cells (109).

Both eIF1A and VPg have polar termini. Their N termini are composed of acidic amino acids, and their C termini are composed of basic amino acids. It has been proposed that polarity in small proteins generates two distinct binding surfaces to permit interaction with at least two distinct types of molecules (40). eIF1A binds eIF2 through its basic N terminus, and its acidic C terminus binds eIF5B and the ternary complex (205). If these properties are similar for VPg, it is possible that interaction between VPg and eIF2 is important for ternary complex transfer to the 40S ribosome, and interaction with eIF5B is necessary for 60S subunit joining and eIF release. Further investigation into the binding properties of VPg will need to be explored before a complete comparison of VPg and eIF1A can be made.

Both eIF1A and VPg bind directly to 40S ribosomal subunits (270). Understanding the mechanism of interaction between these proteins with 40S ribosomes comes from an analysis of the two important domains of the 40S ribosomal subunit in protein synthesis. The peptidyl, or P site, is that which is occupied by the initiator tRNA molecule that will base pair with the AUG initiator codon. Figuratively speaking, the P site is the 5' site of the ribosome with respect to the mRNA. The aminoacyl, or A site, will be sterically positioned over the second codon of the cistron, and is therefore the 3' site of the ribosome with respect to the mRNA. The second appropriate aminoacyl-tRNA is base paired with this codon. After release of initiation factors, including eIF1A from the A site, and the joining of the 60S ribosome to form the catalytic 80S complex, the methionine residue on the tRNA in the P site is then transferred to the amino acid attached to the tRNA in the A site, catalyzed by the ribosome itself (262). The ribosome then moves one codon length of the mRNA, such that the tRNA attached to the growing peptide is now found in the P site, and the A site is available for binding by the third aminoacyl-tRNA. As described, the transfer of the initiator tRNA to the P site of the 40S ribosomal subunit is mediated by eIF1A (32). By analogy to IF1, eIF1A occupies the A site of the ribosome, and through interaction with IF2/eIF5B, the A site is sterically blocked from binding by the initiator tRNA, forcing it to bind to the P site instead. In this manner aberrantly assembled tRNA-40S ribosome complexes are avoided.

If VPg is a viral form of eIF1A, functions of eIF1A in ternary complex transfer could be extended to VPg. As described, eIF1A presumably binds the ribosomal A site and directs the positioning of the initiator tRNA into the P site of the ribosome (Figure 4.8A).

If VPg performs a similar role, it would be expected that interaction of VPg with the A site would similarly position the initiator tRNA into the P site of the ribosome (Figure 4.8B). It is possible the complex scans to reach the AUG found at nucleotide eleven, although it is known that scanning a 5'UTR of this length is inefficient when RNA is capped (139). Alternatively, a conformational change in the complex assembled on VPg would position the AUG at nucleotide eleven in the P site, so that no scanning would be required.

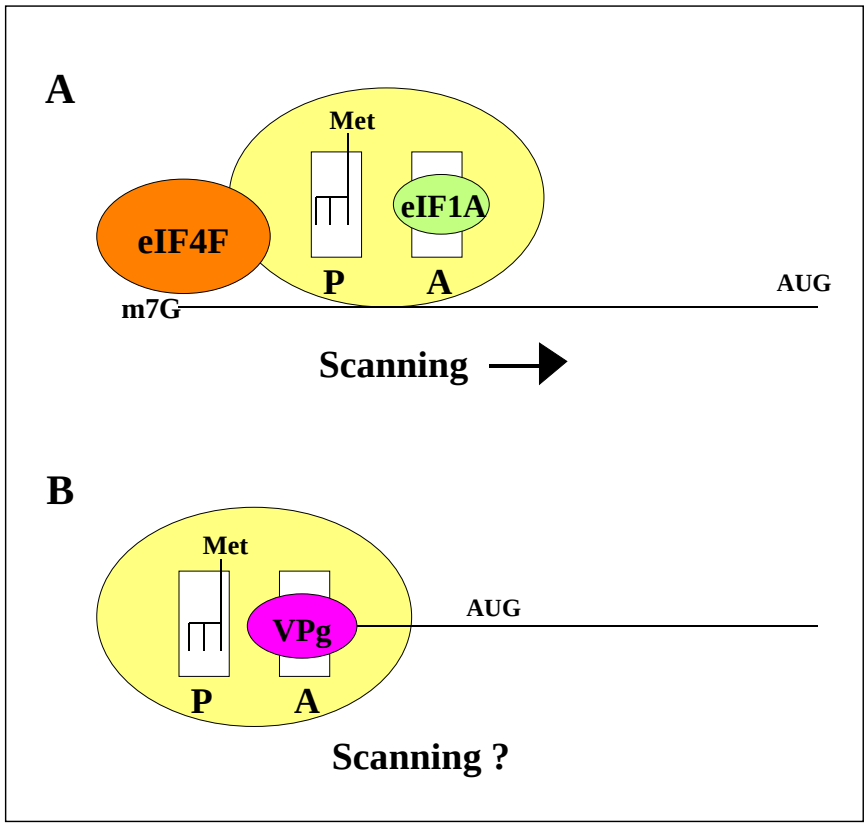

Figure 4.8: Schematic of transfer of initiator tRNA to the P site of the 40S ribosome by eIF1A (A) and a proposed mechanism for VPg (B). Drawing not to scale.

In summary, the data presented in this chapter demonstrate GST – VPg binds 40S ribosomal subunits directly, and that the central domain of VPg, including those amino acids that correspond to the 40S ribosome binding site on eIF1A, are potentially important for this interaction. Further characterization of the function of VPg with respect to eIF1A includes determining if VPg binds the A site of the ribosome, and ascertaining if VPg performs similar functions as eIF1A, including the transfer of the ternary complex to the 40S ribosome, scanning, and AUG codon recognition.

MURINE NOROVIRUS VPg BINDS TRANSLATION INITIATION FACTORS IN INFECTED CELLS

Introduction

Noroviruses constitute a genus in the family *Caliciviridae* and include viruses that are the most frequent cause of food borne viral gastroenteritis epidemics (72). Norovirus genomes are 7.7 kb positive-sense single-stranded RNA and are polyadenylated at the 3' end. The genome codes for three open reading frames. ORF1 encodes the nonstructural proteins synthesized as a polyprotein precursor. ORF2 and ORF3 encode the capsid protein VP1 and minor structural protein VP2, respectively, and both VP1 and VP2 are synthesized from a subgenomic RNA that is 3' co-terminal with the genomic RNA. Instead of a 7-methylguanosine (m^7G) cap at the 5' end, genomic and subgenomic RNAs are covalently linked to a viral encoded protein VPg. Data from animal caliciviruses indicate VPg is necessary for infectivity of native viral RNA (24), but an m^7G cap can confer infectivity on in vitro generated feline calicivirus (FCV) genomic RNA transcripts (257). These and other data (87) taken together suggested VPg functions in translation of viral RNA.

Most cellular mRNAs are translated by a cap-dependent mechanism driven by protein-protein and protein-RNA interactions between translation initiation factors (eIFs) and mRNA (88). Cap-dependent translation initiates with binding of eIF4F that consists of eIF4E, eIF4GI and eIF4A, to the m^7G cap structure. Recruitment of the 43S pre-initiation complex composed of eIF3, 40S ribosomal subunits, and initiator tRNA to mRNA occurs through interactions primarily between eIF4G and eIF3 (65, 83). This 48S

complex scans the mRNA to initiate translation at the first strong context AUG. Cap-independent translation is mediated by extensively structured regions of RNA called internal ribosome entry sites (IRES). These elements are found in picornavirus and other viral genomes and some cellular mRNAs, where initiation complexes assemble internal to the 5' end of the molecule and translation initiates downstream of the IRES (85). The fact that norovirus RNAs lack a m^7G cap, are 5' nucleotide-protein linked, and have 5' untranslated regions (UTR) of only 10 nucleotides suggest translation initiation on VPg-linked RNA proceeds in a manner distinct from cap-dependent and IRES-dependent mechanisms.

VPg of human norovirus binds directly to eIF3, is present in complexes with other eIFs, and inhibits translation of mRNAs that have different eIF requirements for functional initiation complex assembly in cell-free assays (39). Human noroviruses do not grow in cell culture and therefore, connecting in vitro eIF binding data with events that occur during native norovirus infections is difficult. A significant advance in the field was the discovery of a murine norovirus (MNV-1) that replicates in cultured macrophages and dendritic cells (112, 293). MNV-1 is currently the only norovirus that is known to grow in culture and it is closely related genetically to the human norovirus strains. Therefore, we used MNV-1 to address questions about the functional significance of interactions between VPg and eIFs in infected cells, and the role of VPg in recruiting translation machinery to norovirus RNA. The data show for the first time that VPg binds eIFs in the context of a norovirus infected cell, and that MNV-1 VPg has similar eIF binding properties as NV VPg.

Materials and Methods

Maintenance of RAW 264.7 Cells

RAW 264.7 cells (ATCC) were maintained in DMEM containing 10% FBS (Atlanta Biologicals), 10 mM Hepes, 4 mM L-glutamine, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate.

Construction of GST-MNV VPg and Pull-Down Assays

MNV-1 VPg was amplified from pGBKT7-MNV-VPg using the primers MNV-VPg/BamHI(+) 5'-cgcggatccggaaagaagggcaagaac-3' and MNV-VPg/XhoI(-) 5'-ccgctcgagctcaaagttgatcttctcg-3'. Reactions were assembled using the KOD enzyme (Novagen) according to instructions provided by the manufacturer. Amplification conditions consisted of 25 cycles of 98°C for 15 seconds, 58°C for 3 seconds, and 72°C for 5 seconds. Amplification product was cloned into pGEX-4-T1 (Amersham) using BamHI and XhoI restriction enzymes. Restriction sites are underlined. pGBKT7-MNV-VPg was created by amplification from pSPORT-T7-MNV1 (gift from H. Virgin, Washington University School of Medicine) by amplification using primers MNV-VPg (F-NdeI) 5'-caccatatgggaaagaagggcaagaacaag-3' and MNV-VPg (R-BamHI) 5'-cacggatcctcaaagttgatcttctcg-3' (restriction sites are underlined), and was cloned into pGBKT7 (Clontech) using NdeI and BamHI. Expression and purification of GST-MNV-VPg, as well as pull-down assays using RAW 264.7 cell lysates, were as described (39).

Immunoprecipitations

RAW 264.7 cells were grown to 90% confluence in 10 cm dishes, and infected with MNV-1 for 17 hours at a multiplicity of infection of 1.5 pfu/cell. Cells were harvested in 1 mL cold medium and pelleted for 5 minutes at 3000xg at 4°C. Pellets were washed twice in cold PBS-cmf, and lysed on ice for 30 minutes in 500 µL IP buffer containing 20 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1% NP-40, 10 mM β-glycerophosphate, 5 mM NaF, 2.5% glycerol, and one mini protease inhibitor cocktail tablet (Roche). Lysates were cleared by centrifugation for 5 minutes at 10,000xg at 4°C. Ten µl (2%) of each sample was retained as controls not subjected to immunoprecipitation. The remaining lysate was incubated with indicated antibody and rotated gently for two hours at 4°C. Anti-phospho-eIF4GI (Cell Signaling) was used at a dilution of 1:100, anti-eIF3d (Santa Cruz Biotechnology) was used at a dilution of 1:100, and anti-MNV-1 VPg (Kim Green, NIAID) was used at a dilution of 1:1000. Thirty µl Gamma Bind Plus Sepharose beads (Amersham) prepared in IP buffer were added, and complexes were rotated for one hour more at 4°C. Beads were collected for 2 minutes at 500xg at 4°C and washed four times with wash buffer containing 20 mM Tris-HCl pH 7.5, 50 mM NaCl, and 0.1% NP-40. Complexes were eluted from the beads by incubation on ice for 10 minutes with 20 µl elution buffer (0.75% wt/vol glycine in water, pH 2.2). The beads were collected for 2 minutes at 500xg at 4°C. Supernatants were transferred to fresh tubes containing 0.9 µl neutralization buffer (2M Tris base).

Western Immunoblot Analysis

Immunoprecipitation and pull-down samples were subjected to SDS-PAGE and transferred to nitrocellulose, blocked in 10% Blotto, and incubated with antibody overnight at room temperature. Antibodies used were anti-phospho-eIF4GI at 1:1000, anti-eIF3d and 1:1000, anti-S6 and anti-eIF4E (both from Cell Signaling) at 1:1000, anti-eIF3 (John Hershey, UC Davis) at 1:2000, and anti-MNV-1 VPg at 1:2000. Appropriate horseradish peroxidase-conjugated secondary antibodies were used at a dilution of 1:3000, and were incubated with blots for 45 minutes at room temperature. Blots were developed with ECL (Amersham).

Results

Recombinant MNV-1 VPg Binds eIF3, eIF4GI and S6 Ribosomal Protein in Vitro

VPg of genogroup I NV and genogroup II Snow Mountain virus (SMV) share 68% amino acid sequence identity and both bind to eIF3 in cell extracts (39). VPg sequences of MNV-1 and NV were aligned with ClustalW and found to share 54% identity (Figure 5.1). The N- and C-terminal domains showed the highest level of conservation, and flank a central variable region that includes a nine amino acid insertion in NV VPg. An overall 54% amino acid sequence identity and conservation in both the N- and C-terminal domains suggested MNV-1 could have similar eIF binding properties as NV VPg.

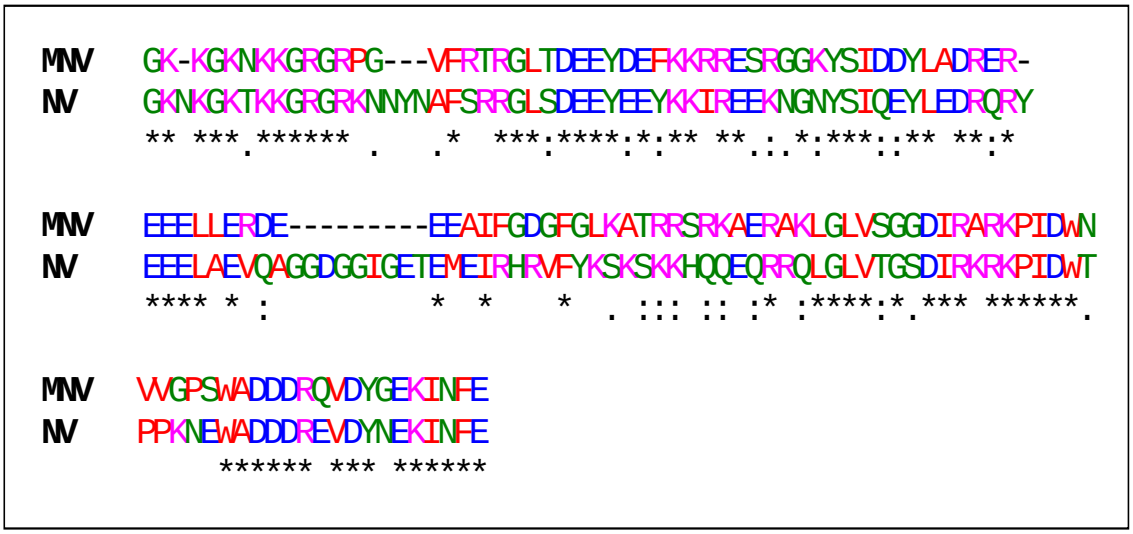

Figure 5.1: MNV-1 VPg and NV VPg share significant amino acid sequence identity. Sequences encoding MNV-1 VPg and NV VPg were aligned with ClustalW. Asterisks indicate identical residues.

MNV-1 VPg was expressed as a GST fusion protein in bacteria and used in pull-down assays with extracts prepared from RAW 264.7 macrophages as previously described (39). Proteins in pull-down eluates were analyzed by immunoblots probed with antibodies to eIF3, phospho-eIF4GI and S6 ribosomal protein. All three of these factors were detected in GST-VPg eluates (Figure 5.2). As reported before, eIF4GI was present in blots probed with anti-eIF3 because antibodies to eIF4GI are present in this serum (49). The lower molecular weight products that reacted with the eIF4GI antibody are degradation products of the full-length protein. These data show MNV-1 VPg and NV VPg have the same in vitro eIF binding properties.

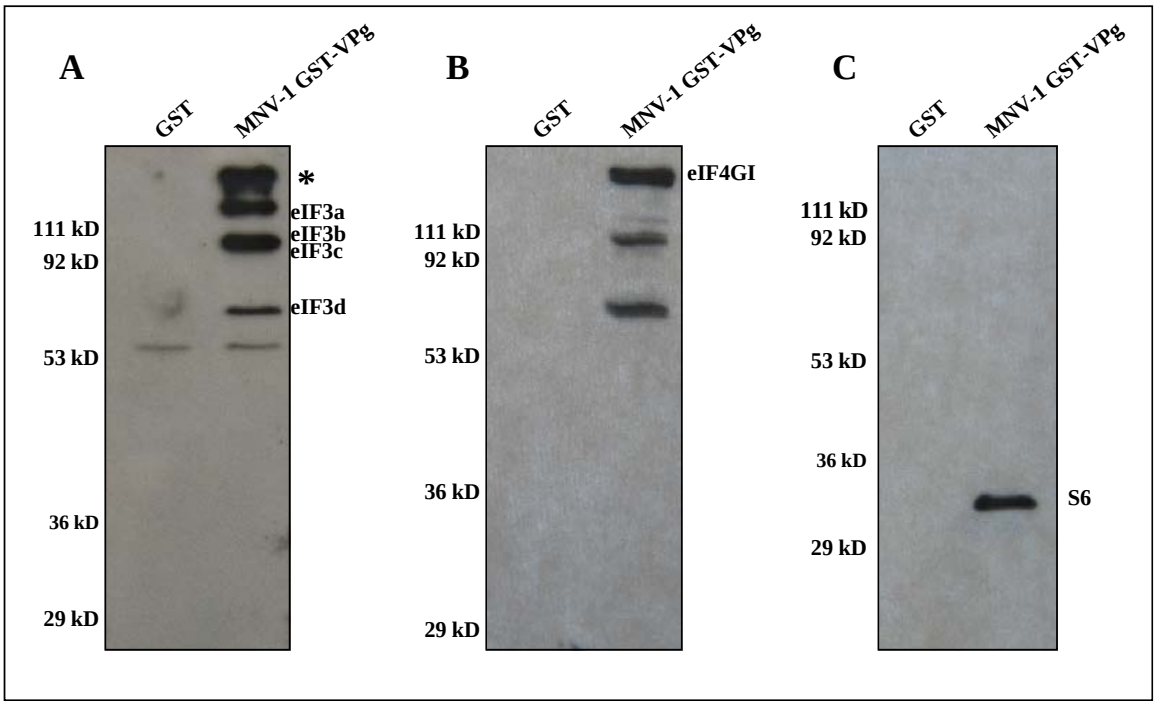

Figure 5.2: MNV-1 GST – VPg binds eIF3, eIF4GI, and S6 by pull-down assay. GST and MNV-1 GST – VPg were expressed in bacteria, purified, and bound to glutathione sepharose 4B beads and used in pull-down assays as described in the text. Samples were subjected to SDS-PAGE and immunoblot and probed with A: anti-eIF3, B: anti-phospho-eIF4GI, and C: anti-S6. Blots were developed with ECL. The asterisk in A indicates a protein identified as eIF4GI.

VPg and eIFs Co-immunoprecipitate from
MNV-1 Infected RAW 264.7 Cells

To test whether the interactions between VPg and eIFs observed by pull-down assay reflected interactions that occur in infected cells, RAW 264.7 cells were infected with MNV-1 and immunoprecipitations (IP) were performed following established protocols (57, 293).

Anti-VPg antibody precipitated VPg (Figure 5.3A, lane 4, middle panel) and co-precipitated eIF4GI (Figure 5.3A, lane 4, top panel). In the reciprocal experiment, anti-

eIF4GI antibody bound eIF4GI (Figure 5.3A, lanes 5 and 6, top panel) and co-precipitated MNV-1 VPg (Figure 5.3A, lane 6, middle panel). These data demonstrate interactions between VPg and eIF4GI occur in infected cells. In addition, three observations about eIF4GI can be made from these experiments. First, the anti-eIF4GI antibody recognizes only eIF4GI phosphorylated on serine 1108. There was a reproducible increase in the amount of phospho-eIF4GI in infected versus mock lysates (Figure 5.3A lanes 1,2, 5 and 6, top panels). The amount of eIF4GI phosphorylated at serines 1108, 1148 and 1192 increases in response to serum stimulation, and phosphorylation at these sites is modulated by the N terminal third of the protein (234). Potential mechanisms, including de-repression of eIF4GI by direct phosphorylation of the N terminus or alternatively, interactions with an eIF4GI binding partner to expose the C terminal domain to kinase activity were proposed. It is conceivable that MNV-1 infection could result in activation of kinases or pathways that specifically modulate the activity of eIF4GI in response to virus infection. An alternative and not mutually exclusive possibility is that direct interactions between eIF4GI and VPg alter the conformation of eIF4GI to a form where the C terminal serine residues are phosphorylated.

A second observation is that there were several eIF4GI degradation products in lanes of cell lysate (Figure 5.3A, lanes 1 and 2, top panel), or lanes where protein was immunoprecipitated with anti-eIF4GI antibody (Figure 5.3A, lanes 5 and 6, top panel). In contrast, anti-VPg antibody exclusively precipitated full-length eIF4GI (Figure 5.3A, lane 4 top panel). Assuming a direct interaction, this suggests a binding site for VPg could reside in the N-terminal two-thirds of eIF4GI, since the eIF4GI antibody recognizes

phospho-serine 1108 in the C terminal third of the protein. Finally, the presence of identical degradation products in mock versus infected lysates suggests that in contrast to FCV infection (287), eIF4GI is not detectably cleaved during MNV-1 infection. A similar protein degradation pattern was observed in immunoblots with antibody to total eIF4GI (data not shown). These data are consistent with reports showing recombinant norovirus 3C protease (MD-145 strain) does not cleave eIF4GI in vitro (148).

Since NV VPg binds the eIF3d subunit of eIF3 (39), we tested the ability of MNV-1 VPg to interact with eIF3d and by extension, the eIF3 holocomplex, in infected cells. Anti-eIF3d antibody bound eIF3d (Figure 5.3B, lanes 3 and 4, top panel) and co-precipitated MNV-1 VPg (Figure 5.3B, lane 4, bottom panel), suggesting the VPg-eIF3 interaction occurs not only in GST pull-down assays, but also in native norovirus infected cells.

Goodfellow and co-workers have recently shown that FCV VPg co-precipitates eIF4E in infected cells, and that this interaction is necessary for translation in vitro (68). To test if this interaction also occurs between MNV-1 VPg and eIF4E in infected cells, we immunoprecipitated VPg from infected cell lysates and probed samples by Western immunoblot for eIF4E. eIF4E was found in similar quantities in mock and infected cell lysates that did not receive antibody (Figure 5.3A, lanes 1 and 2, bottom panel) but only immunoprecipitated with VPg from infected cells (Figure 5.3A, lanes 3 and 4, bottom panel). These data suggest that interaction between MNV-1 VPg and eIF4E occurs in infected cells, similar to FCV infection. However, it is not known if this interaction is

direct, or if eIF4E is being detected as a result of interaction with other members of eIF4F.

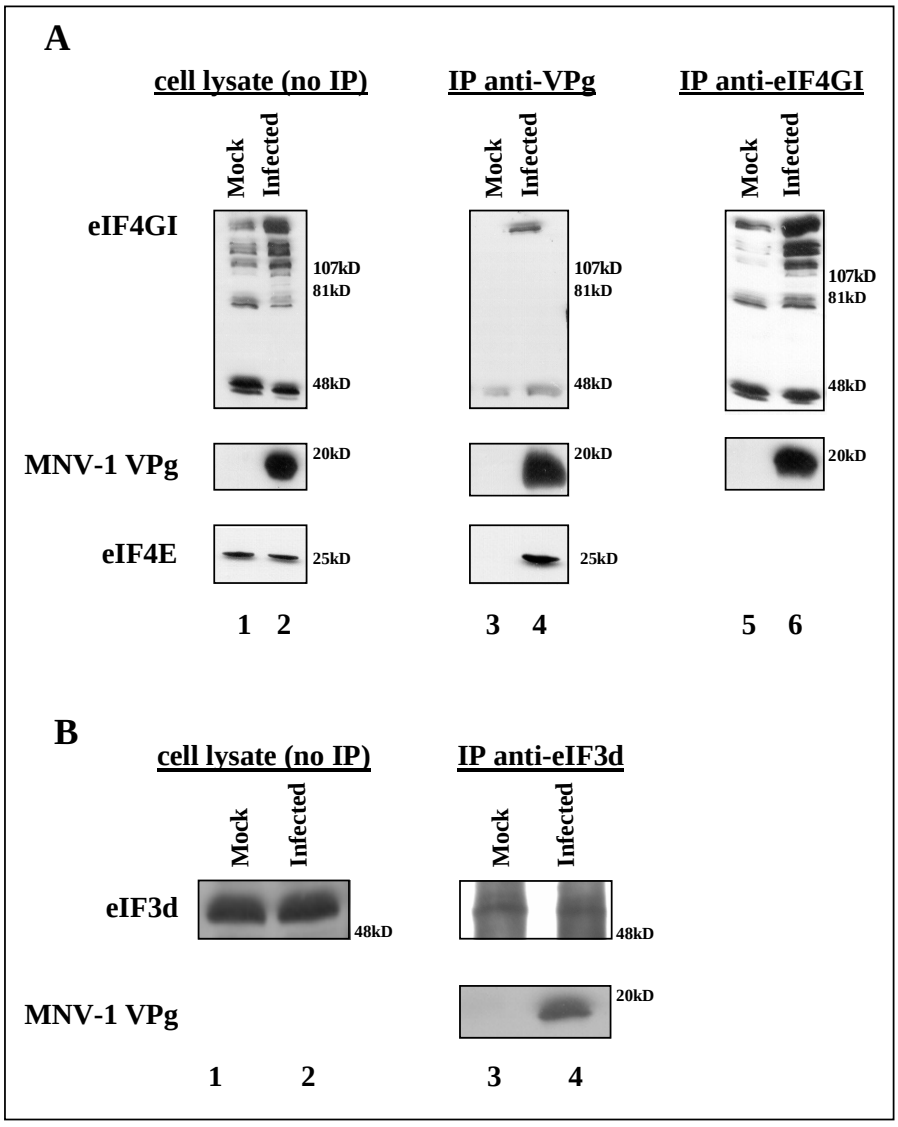

Figure 5.3: eIF4GI, eIF4E, and eIF3 co-immunoprecipitate with VPg from MNV-1 infected RAW 264.7 cells. Mock infected or infected cell lysates were subjected to immunoprecipitation with anti-MNV-1 VPg (Panel A, lanes 3 and 4), anti-phospho-eIF4GI (Panel A, lanes 5 and 6), or anti-eIF3d (Panel B, lanes 3 and 4). Immunoblots were probed with the relevant antibody indicated to the left of the panels. Lanes 1 and 2 in each panel are control lysates that did not receive antibody. Blots were developed with ECL.

Discussion

We and others have put forth the idea that VPg functions in translation initiation on VPg-linked RNA through interactions with eIFs. The data presented here provide the first experimental evidence of interactions between VPg and eIFs in norovirus infected cells and bolster the assertion that the interactions previously defined for NV VPg are relevant and functional.

eIF4GI was found in GST pull-down eluates of both NV VPg and MNV-1 VPg and in co-immunoprecipitates with VPg from MNV-1 infected cells. These assays do not prove a direct interaction between VPg and eIF4GI, but strongly suggest VPg interacts with one or more components of the eIF4F complex. We suggest in this regard, that VPg is a protein cap analogue with respect to binding interactions with eIF4F. However, we further suggest that the role of VPg in ribosome recruitment is more complex than providing a scaffold for eIF4F binding. VPg is a 15 kD protein covalently linked to the 5' end of an RNA with a 10 nucleotide 5' UTR. If VPg functions simply as a protein cap, then by analogy to capped mRNA, translation initiation at nucleotide 11 would be inefficient. Moreover, direct interactions between VPg and eIF3 point to VPg's potential involvement in 43S pre-initiation complex recruitment. Sequence conservation between VPg and initiation factor eIF1A, important in AUG codon recognition and ternary complex recruitment (215), has been noted for NV VPg (255), and a similar degree of conservation exists between MNV-1 VPg and eIF1A (data not shown). We have suggested a model of translation initiation on NV VPg-linked RNA that we now extend to MNV-1, whereby a direct interaction between VPg and eIF3 positions the ribosome at

the initiator AUG, possibly without ribosome scanning. This model implies numerous concerted interactions between norovirus VPg and eIFs additional to the interactions reported so far.

Studies with animal calicivirus strains in the *Vesivirus* genus of the family, particularly the feline calicivirus (FCV), have contributed a large body of data on mechanisms of calicivirus replication (71, 148, 256, 259). These data provide a good foundation on which to build models of norovirus replication by analogy. However, these analogies have limitations largely due to the genetic divergence between animal caliciviruses and noroviruses. For example, FCV VPg and NV VPg share only 17% amino acid identity, and thus far we have been unable to detect interactions between FCV VPg and eIF3 or eIF4GI in GST pull-down assays (unpublished data). We have not performed extensive binding studies with FCV VPg to determine what interactions may occur that are different from those observed with the norovirus VPg, but a recent study has shown that FCV VPg directly binds eIF4E (68). It is possible that specific interactions and mechanistic details differ between the animal caliciviruses and noroviruses. These observations exemplify the importance of MNV-1 as a model for human noroviruses, as interactions between MNV-1 VPg and eIFs precisely mimic those reported for NV VPg. Experiments to dissect the functional consequences of the in vivo interactions between MNV-1 VPg and eIFs are in progress.

SUMMARY AND CONCLUSIONS

Norovirus infection is recognized as the etiological agent in 93% of outbreaks of nonbacterial gastroenteritis, and is responsible for 23,000,000 infections, 50,000 hospitalizations, and 300 deaths each year in the US. Study into the replication cycle of these important viruses has traditionally been hampered by the lack of cell culture or small animal model systems. Information about basic molecular biology was attained by using in vitro systems, and many examples of expression and proteolytic processing of viral proteins are now understood. Functional analogies have been made with viruses of the cultivable *Vesivirus* genus, and these relationships have provided some insight into the mechanism of *Norovirus* replication. Importantly, previous studies of the genome-linked protein VPg of vesiviruses have suggested it plays a role in translation initiation on viral RNA. This information has led us to form the hypothesis that VPg of NV, and by extension VPg of other noroviruses, is important in this pathway as well. Its mechanism in translation initiation may be distinct from those currently described, as VPg is a protein and accepted mechanisms define nucleotides or modified nucleotides as functioning in ribosome recruitment. Studies described in this thesis were undertaken to investigate this idea.

In Chapter Two, basic questions were asked to address the potential role of NV VPg in translation initiation. Data from these studies demonstrate that NV VPg binds eIF3 directly, and is present in complexes with other eIFs, such as eIF2α, eIF4G, eIF4E, and S6, by pull-down assay. Additionally, VPg inhibits translation of reporter mRNAs with

different eIF requirements in vitro, suggesting that the interactions between VPg and eIFs play a functionally significant role with respect to translation.

In Chapter Three, studies were undertaken to identify the domains of VPg that are important in mediating the described interactions with eIFs. The data suggest that regions of the C terminal domain of VPg contact eIF4G independently of eIF3, and that the central domain of VPg potentially mediates interactions with S6, and by extension, the 40S ribosome. The panel of VPg mutants was tested in the translation inhibition assay using a capped reporter mRNA, and the data show that interaction between VPg and the 40S ribosome is likely a mechanism by which translation of capped reporter mRNA, as well as those mRNAs described and tested in Chapter Two, are inhibited. Further studies will need to be pursued to test if interaction between VPg and the 40S ribosome is important for translation of viral RNA.

VPg shares sequence identity and physical properties with eIF1A. In Chapter Four, studies were undertaken to further define common binding properties between these proteins. It was found that VPg binds 40S ribosomal subunits independent of other eIFs, and that this interaction is possibly mediated by the central domain of VPg, including the amino acids on VPg that correspond to the 40S binding site on eIF1A. Studies into the potentially common eIF binding properties shared by VPg and eIF1A may help define the ribosome recruitment mechanism used by VPg.

Finally, MNV-1 was used as a molecular model for NV to investigate if interactions between VPg and eIFs occur in infected cells. Data from this chapter demonstrate that VPg of MNV-1 co-immunoprecipitates with eIF4G and eIF3d in *Norovirus*-infected

cells, important for the confirmation of data presented in previous chapters. Importantly, it was shown that in vitro binding data between NV VPg and MNV VPg are similar, in that both proteins bring down eIF3, eIF4G, and S6 by pull-down assay, supporting the use of MNV-1 as a molecular model for human *Norovirus* study.

Together, the data presented here support the hypothesis that VPg is important for translation of viral RNA. These are the first studies to demonstrate that a VPg from the *Caliciviridae* family binds eIFs, and that direct contacts with the 40S ribosome are made. Importantly, these studies demonstrate that these interactions occur in infected cells. These data suggest a unique mechanism of translation initiation distinct from those currently described, where ribosome recruitment is mediated by a protein instead of a nucleotide structure. Further studies will need to be undertaken to understand the role of these factors in the translation of viral RNA.

FUTURE STUDIES

Data presented in previous chapters show that VPg of noroviruses associates with eIFs in vitro and in virus-infected cells. Though these studies have begun to characterize the function of VPg in the translation initiation process, further experiments are necessary to define if and how VPg mediates translation of viral RNA.

A more detailed description of VPg binding to eIFs is warranted. Data presented here begin to shed light on characterizing these interactions. However, it would be beneficial to understand what other eIFs bind VPg, either directly or indirectly. GST pull-down assays could be conducted as before, except individual purified eIFs, alone or in groups, could be tested for interaction with VPg. For example, a direct interaction between VPg and eIF2 would further support the hypothesis that VPg is a viral form of eIF1A and is involved in ternary complex transfer to 40S ribosome subunits. Additionally, it is possible that interaction between VPg and an eIF is stabilized in the presence of another, and pull-down assays using different combinations of eIFs would address this possibility. More extensive deletion mutants of VPg will be instrumental in creating an interaction map of VPg with eIFs, and will provide suggestive evidence of a ribosome recruitment mechanism.

Further investigation into the potential similarities between VPg and eIF1A would help define the role of VPg in translation of viral RNA. The 40S binding site on eIF1A has recently been described, and such information about VPg would be helpful in defining its role. The use of gel filtration instead of sucrose density gradient centrifugation may provide an alternative means to investigate these interactions, as the

conditions of this assay exert less force on protein complexes, and this method could be used to ask if VPg occupies the A site on 40S subunits as eIF1A does. Likewise, it would be necessary to investigate the possibility that VPg transfers the ternary complex to the 40S ribosome, or if it plays a role in scanning or AUG codon recognition. Data from such experiments would support or refute the hypothesis that VPg is a viral form of eIF1A.

Investigation into the mechanism of the VPg – 40S ribosome interaction is also warranted. To understand this mechanism, it would be valuable to know if scanning is required after complex assembly to position the AUG initiator codon in the P site, or if a conformational change of the assembled complex properly positions the AUG so that scanning is not required.

Finally, a study of the eIF requirements for translation of viral RNA is obvious. Data presented in this thesis suggest a role for VPg in translation initiation. However, demonstration that VPg is required for 48S complex assembly is necessary to confirm this idea. A source of norovirus VPg-linked RNA now exists, and methods for the purification of eIFs from mammalian cells have been extensively described. Mixtures of VPg-linked RNA with combinations of purified eIFs would be subjected to toe-print assay to ascertain requirements for proper 48S complex assembly. Incremental omission of individual eIFs, and the removal of VPg from the RNA, would provide information as to their importance in this process. Similarly, individual eIFs could be depleted from RRL or HeLa cell lysates, and the ability of VPg-linked RNA to translate in these lysates could be assessed to define minimum requirements for translation.

Together, experiments undertaken to examine the ideas presented here should provide a description of how the 40S ribosome is recruited to VPg-linked mRNA, and which interactions between eIFs and VPg are required for this process.

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