4. DISCUSSION.

Hepatitis E virus is the major cause of enterically transmitted non-A, non-B hepatitis in many developing countries and accounts for a substantial proportion of acute, epidemic and sporadic hepatitis in both children and adults (1, 5, 6). During HEV epidemics, unusually high rates of fulminant hepatitis and subsequent mortality have been observed among pregnant women (46-48). The extreme severity of hepatitis E in the pregnant patient results in high maternal and fetal mortality in fulminating acute hepatitis. On the other hand, several attempts to reproduce the fulminant hepatitis in experimental animal models has failed. Due to lack of a reliable culture system (32) and the limitations of animal models (40), HEV biology and pathogenesis are poorly understood. The replication and propagation of HEV in cell culture has been limited to a few laboratories and not easily available (43). Nevertheless, studies towards understanding the basic questions in HEV such as the virus biology and pathogenesis have been mainly addressed using subgenomic expression strategies.

The HEV is a simple virus with three open reading frames-ORF1 encoding the proposed nonstructural polyprotein containing enzymatic activities such as methyltransferase, protease, helicase and replicase, ORF2 encoding the viral capsid protein, and ORF3 encoding a small protein of undefined function. We reasoned that the ORF3 protein may play a role in modulating the host response to viral infection. A couple of years ago, our laboratory had shown that the ORF3 protein (pORF3) is phosphorylated at a single serine residue and is associated with the cytoskeletal and membrane fractions of the cell through an N-terminal hydrophobic domain. Further characterization of the pORF3-phosphorylating cellular kinases showed that pORF3 can
be phosphorylated by the extracellularly regulated kinases (ERK1/2) as well as by the stress-activated protein kinases or the N-terminal Jun kinases (SAPKs/JNKs). These earlier findings suggested that pORF3 might be involved in the modulation of cellular signaling pathways (28). To further understand and explore the properties and functions of pORF3 towards understanding host-virus interactions, we have studied the ability of pORF3 to interact with cellular signaling proteins and the consequent effects on modulation of any cell signaling pathway(s).

4.1. The ORF3 protein binds SH3 domain-containing proteins: The ORF3 protein has 123 amino acids and carries two PxxP motifs, called as P-1 and P-2 (Fig. 5) in its primary sequence. Such motifs are known to bind SH3 domains from different signaling proteins, mainly non-receptor protein tyrosine kinases, adapter proteins and cytoskeletal proteins (125). The P-2 motif is completely conserved among all HEV isolates. Moreover, it is homologous to that of SH3 domain-binding ligands which appears to be seven-residue peptides containing the consensus X-P-p-X-P, where X tends to be an aliphatic residue and the two conserved prolines are crucial for high affinity binding. The intervening scaffolding residue also tends to be a proline. In the SH3 binding-peptide, R-A-L-P-P-L-P-R-Y, the first arginine forms a salt bridge with an aspartate in the SH3 domain (108, 125, 226).

A number of protein-interaction modules have been identified, including Src homology 2 (SH2) and SH3, and PTB, WW, and PDZ domains (227). These domains share some common characteristics, such as being found in a wide variety of proteins, being able to fold independently, being involved in cellular signaling and conferring binding specificity. Among these, SH3 domains bind to proline-rich sites that adopt the
left-handed polyproline-II helix conformation. There is also specificity among SH3 domains for different binding sites, but the determinants of specificity are not fully characterized, as in the case of SH2 domains. There is often an overlap in the spectrum of sites that bind different SH3 domains. In addition, many proteins contain multiple SH3 domains while others target proteins containing multiple binding sites, resulting in increased affinity due to multidentate interactions. The SH3 domains also have specific preferences for arginine and leucine residues, as revealed by the use of combinatorial peptide libraries with the Src and phosphatidylinositol 3-kinase SH3 domains. These studies identified two classes of ligands for both SH3 domains: class I, with the consensus sequence RXLPPZP (Z= L for Src SH3; Z= R for PI 3-K SH3; X= no clear consensus), and class II, with the sequence XPPLPX. A class I or class II ligand is defined primarily by the N-terminal or C-terminal position, respectively, of the Arg residue with respect to the PPLP, hydrophobic core.

We tested the in vitro binding ability of pORF3 to a number of SH3 domains from the following molecules: Src family of non-receptor tyrosine kinases (Src, Hck, Fyn, Csk and Abl), phosphatidylinositol 3-kinase (p85α subunit), phospholipase Cγ (PLCγ), adapter proteins (Grb2, Crk, and p130Cas) and a cytoskeletal protein (Spectrin). In vitro GST pull-down and filter binding assays confirmed that E. coli expressed and purified-ORF3 protein binds to SH3 domains of Src, Hck, Fyn, Grb2, PLCγ and P85α. The binding to SH3 domain from Abl could not be reproduced with either the filter binding assay or with the ORF3 protein produced in insect cells.

In vivo expression-based assays for protein-protein interactions such as yeast two-hybrid system are powerful methods for studying the physical association of two proteins
of biological interest, or for characterizing the degree and nature of interactions between sets of proteins. While protein-protein interaction studies are useful for describing protein networks, the main goal of most interaction studies today is to learn about individual proteins-their potential partners, functions and interaction complexes.

To study the in vivo interaction between pORF3 and SH3 domains, we employed the yeast two-hybrid system to assay for the interaction between pORF3 and the Grb2-SH3 domain. Using all proper positive and negative controls, we were able to show in vivo interaction between pORF3 and Grb2-SH3 which was dependent upon the C-terminal proline-rich regions in pORF3. This confirmed the in vitro result.

The SH3 binding domain in pORF3 has been mapped by in vitro as well as in vivo assays to the P-2 motif located in the C-terminal part of the protein, between residues 104 to 113. In the GST pull-down assay, the pORF3Δ92-123 mutant showed no binding to any SH3 domain tested, while wild type pORF3 and the pORF3 from Mexican isolate bound well to SH3 domains from Grb2 and Src. Similarly, pORF3(1-80) mutant did not show any interaction with Grb2 in the yeast two-hybrid assay. Both assays confirmed that the P-2 region is the PxxP motif which binds to SH3 domains. This is not surprising because as mentioned earlier, this motif is completely conserved among all HEV isolates. Further it is homologous to that of SH3 domain-binding peptide ligands and forms a type II polyproline helix with three residues per turn. Structural and mutational studies have shown that each X-P pair fits into a hydrophobic pocket formed by aromatic amino acids within the SH3 domain, providing the principal binding energy. This structure is further stabilized by a salt bridge between a terminal arginine in the ligand and a conserved acidic residue in the SH3 domain. The SH3 ligands can bind
either as class I or class II peptides, depending upon whether the arginine residue is N-terminal or C-terminal to the proline-rich core (109, 162, 228). These key structural elements were found in P-2 and provided a structural basis for its interaction with various SH3 domains. This was further confirmed by molecular modeling of the complex of c-Src or the Grb2 SH3 domain with the P-2 peptide. Based on ligand preferences of many SH3 domains and the modeled structures, P-2 is likely to bind the SH3 domains as a class I peptide. Direct structural studies will address this issue in future.

In addition, most SH3-PxxP interactions involve ionic interactions between a basic residue located before or after the PxxP motif and a highly conserved acidic residue in the SH3 domain (D99 in Src) (129, 136). Therefore, some degree of specificity in peptide recognition has been described. For instance, Abl SH3 domain does not bind well to ligands of the Src family SH3 domains and vice versa (122). This appears to be due to the lack of an acidic residue in the binding pocket of the Abl SH3 domain. Similarly, pORF3 does not bind or binds weakly to some of the SH3 domains. We propose that selectivity in pORF3 binding to some but not all may be due to the P-2 sequence, RPSAPPLP which is also slightly different from the high affinity SH3 binding sequence RXLPPXP (where X is any amino acid). An extra amino acid, RPSA instead of RXL, N-terminal to the PPXP core might result in loss of binding to some, but not other SH3 domains. However, sequences similar to P-2 have been identified from proteins that bind either the Src or Grb2 SH3 domains (124, 125).

All possible assay systems have been used to demonstrate the interactions between pORF3 and cellular SH3 domains. Thus we conclude that pORF3 interacts with a number of SH3 domains from a variety of cell signaling proteins. In GST pull-down
and yeast two-hybrid assays, using pORF3 deletion mutants, the P-2 motif was found to be necessary for these interactions.

The combinatorial library approach and some mutational evidence suggested that in SH3 binding ligands, the PxxP motif is necessary for specificity but flanking residues are also important for high affinity interaction (119). Lee et al reported that full length HIV-1 Nef binds to Hck SH3 with the highest affinity ($K_D$ 250 nM) when compared with their natural ligands (122). In contrast to Hck, affinity of the highly homologous Fyn SH3 for Nef was too weak ($K_D$ 20 µM). It was shown that the specificity lies in a variable loop, positioned close to conserved SH3 residues. Although Nef contains a PxxP motif which is necessary for the interaction with Hck SH3, high affinity binding was only observed for the intact Nef protein. The binding of a peptide containing the Nef PxxP motif showed >300-fold weaker affinity for Hck SH3 than full-length Nef (122).

Similarly we have used a 30 mer peptide which contained the P-2 proline region of pORF3, and found that only about 1000 fold excess of this peptide was able to compete with wild type pORF3 when the peptide was preincubated with Hck SH3 domain. We propose that although the PxxP motif is critical for binding, there are other factors in the native protein which could be contributing in this interaction. HIV-1 Nef was used to evaluate the affinity of pORF3 towards Hck SH3 domain in the absence of direct affinity measurements between pORF3 and SH3 domains. Competition between pORF3 and Nef showed similar affinities of the two protein for the Hck SH3 domain, indicating that pORF3 is also likely to bind SH3 domains with an affinity in the low micromolar range.
4.2. pORF3 shows cytoplasmic distribution and colocalizes with Src and Grb2: The green fluorescent protein (GFP) from Aequorea victoria is a versatile reporter protein for monitoring gene expression and protein localization in a variety of cells and organisms. Despite many early successes using this reporter, wild type GFP was suboptimal for most applications due to low fluorescence intensity when excited by blue light (488 nm). Recent studies on GFP mutations improved the fluorescence intensity and higher expression levels yielding enhanced green fluorescent protein (EGFP). The latter provides greater sensitivity in most systems (239).

To monitor the ORF3 gene expression and protein localization in mammalian cells, we constructed a eukaryotic expression vector expressing EGFP-ORF3 fusion protein under the immediate early promoter of human cytomegalovirus (CMV). Huh7 cells were transiently transfected with either EGFP alone or EGFP-ORF3 vectors. Live Huh7 cells were monitored for EGFP-ORF3 as well as control EGFP expression and subcellular localization from early to late transfection. In the early phase of transfection, there was no difference between EGFP-ORF3 and EGFP, however, in the later phase of transfection, EGFP-ORF3 showed very distinct cytoplasmic localization while EGFP alone diffused all across the cell, including the nucleus (Fig. 17C). In earlier subcellular fractionation studies (28), it has been shown that pORF3 is cytoplasmic and not nuclear in its localization. It has further been shown that the protein fractionates significantly into the cytoskeletal and cytosolic fractions and to some extent in the membrane fractions as well. The imaging results presented here are consistent with earlier subcellular fractionation studies. Further localization studies using various standard marker proteins
for subcellular compartments are needed to fine map the distribution of pORF3 in an expressing cell.

After confirming cytoplasmic localization of the ORF3 protein in live cells, we have studied its colocalization with Src and Grb2. The interaction between pORF3 and SH3 domains from Src and Grb2 has been further supported by dual immunoflourescent staining of COS-1 cells transfected with pMT-ORF3. The cells were fixed and stained for transiently expressed pORF3 and endogenous Src or Grb2 with anti-pORF3 antibodies and anti-Src or anti-Grb2 antibodies respectively. In dual staining experiments, pORF3 showed significant cytoplasmic colocalization with Src and Grb2 (Fig. 18).

4.3. pORF3 binds pp60Src in a multimeric complex but does not activate the Src kinase in vitro: Most of the Src family tyrosine kinases are relatively restricted in their expression pattern, being found predominantly in haematopoietic cells (228). However, Src and Fyn are more ubiquitously expressed. Regulation of these kinases is very complex and number of studies has shown that Src is negatively regulated by phosphorylation of tyrosine 527 in its carboxy-terminal tail (130). A protein kinase, Csk, that phosphorylates tyrosine 527 was later identified (140). Further, mutational analysis showed that SH2 and SH3 domains also contribute to the regulation of Src kinase activity (128). In the cellular context, protein-protein interactions involving SH3 and SH2 domains occur in transient associations between proteins of signaling pathways resulting in the formation of multiprotein complexes. Formation of these complexes leads to enzymatic modifications and in turn amplification of intracellular signals.

Binding of ligands, including viral proteins, to either of the Src homology domains lead to the enzymatic activation of kinases (98, 108, 122, 174, 228). Among the viral proteins, Nef was shown to interact with number of SH3 domains from various cellular proteins. Moreover, addition of Nef to the downregulated Hck caused a large
increase in the catalytic activity of Hck. It was proposed that Nef-HckSH3 complex destabilizes and activates the Hck kinase by binding to and displacing the SH3 domain (122).

We also investigated the activation of Src in pORF3 expressing cells. Immunoprecipitation with anti-Src and anti-phosphotyrosine antibodies showed an increase in the hyperphosphorylated form of Src in pORF3 cells but not in control cells. In addition, in pORF3 expressing cells but not in the control cells, a second cellular protein (p80) was immunoprecipitated with both anti-Src as well as anti-phosphotyrosine antibodies. A cellular protein, called p81, was found in a complex with the transforming polyoma virus middle-T antigen and Src indicating increased activation of c-Src (145). Later studies showed that all mutant forms of middle-T antigen capable of transformation form complexes with c-Src and p81 (147). Using in vitro kinase assays, p81 was found to be the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-K). It turned out to be an essential component in middle-T antigen transformation of cells (148). Fukui et al. concluded from their findings that PI 3-kinase activity is not intrinsic to Src but is a property of another molecule complexed with Src. All transforming variants of Src were associated with PI 3-kinase activity, whereas this enzyme activity was hardly detectable in immunoprecipitates from cells infected with nontransforming c-Src (138-140). We have observed a similar protein (p80) in the immunoprecipitates from pORF3 expressing cells but not from the control cells.

Using an immunocomplex kinase assay, we further characterized the Src kinase activity, but found no increase in pORF3 cells. A number of controls included in this assay indicated that pORF3 indeed does not cause direct activation of Src. However, PI 3-kinase itself is involved in the cellular proliferation pathway and has been shown to be essential for the regulation of apoptosis versus cell survival pathways. It remains to be seen whether p80 immunoprecipitated from pORF3 cells is active and if it can provide a
growth advantage to these cells. Thus, PI 3-kinase activation and its role in the pORF3-mediated signaling pathways need to be studied to elucidate our current understanding of this viral protein.

4.4. pORF3 expression results in MAP kinase activation: pORF3 interacts with a number of SH3 domains other than Src. One of these is Grb2, an adapter protein which is an early messenger of many signaling pathways. It associates with tyrosine-phosphorylated protein kinase receptors via its SH2 domain and through its SH3 domain recruits Sos to the cell membrane, where it activates the Ras-GTP complex (154, 155). This activation in turn triggers the Ras-Raf-MAPK kinase pathway.

A number of signaling pathways have been identified so far and among these, the MAP kinase pathway has received particular attention because many of these enzymes translocate to the nucleus to activate transcription factors, in turn regulating cellular proliferation and differentiation (186, 201). The MAP kinase pathway is a major signaling cascade by which cells transduce extracellular signals into intracellular responses. The mammalian MAPK subtypes, by contrast, can be activated simultaneously via distinct parallel cascades in response to the same stimuli suggesting cross-talk between the signaling pathways (190, 192).

We have studied the ability of pORF3 to activate MAP kinase in the presence or absence of serum factors by three independent assay systems. These included in vitro immunocomplex MBP assay, p44/42 MAP kinase assays and an In Vivo MAP Kinase Assay. The immunocomplex assay measures ERK activity by phosphorylating MBP as a substrate while the p44/42 MAP kinase assay system estimates phosphorylation of the downstream effector, Elk-1. Both assays showed an increase in MAP kinase activation in pORF3 cells. The HEV ORF3 protein showed ERK activation in the absence or presence of serum when it was measured directly using MBP as a substrate. However, Elk-1 phosphorylation was found to be higher in pORF3 expressing cells compared to control
cells when the cells were starved of serum; in the presence of serum, no difference was observed. Using *In Vivo* MAPK assay system as well, we observed a 3-5 fold induction in reporter gene expression by MAP kinase phosphorylation in 293 as well as HepG2 cells. In addition, we have also studied the effect of pORF3 on MAPK localization. In pORF3 cells but not control cells, MAP kinase showed cytoplasm to nucleus translocation. These results taken together suggest that in the MAPK cascade, while the effects of pORF3 are largely at or upstream of ERK, a component of the serum response might work downstream as well.

Persistent hepatitis C virus (HCV) infection is associated with hepatocellular carcinoma (HCC). Although the mechanism of HCC is not clear, recent studies showed a close relationship between the development of HCC and the MAP kinase cascades (214-216). HCV core protein significantly activated MAPK/ERK cascade suggesting a critical role in HCV oncogenesis. In an independent investigation, HCV core was shown to activate ERK and Elk-1. Elk-1 activation was further enhanced by the tumor promoter, 12-O-tetradecanoyl phorbol 13-acetate (TPA), but not hepatocyte mitogens such as epidermal growth factor (EGF) and transforming growth factor α (TGF-α) in cells expressing HCV core protein (224). This might contribute to the development of the HCV-related liver disease through the activation of the MAPK/ERK cascade, including Elk-1, to induce the gene transcription required for cell-cycle progression, in addition to nonspecific hepatitis-induced cell proliferation (217, 226).

Another hepatitis virus, the hepatitis B virus (HBV), encodes a small protein, called HBx whose transcriptional transactivation effects have been studied and are well established (205). The mechanism by which HBx induces transcriptional transactivation is not well understood. Recent findings showed the ability of HBx to influence cell signaling cascades (206). It has been shown that HBx induces a high level of Ras activity by stimulating Ras-GTP complex, which transduces its signal downstream, activating Raf
and MAPK pathway. This might provide a pathway for increased survival of HBV-infected cells, leading to hepatocellular carcinoma.

The interaction of pORF3 has been demonstrated, in vitro, with SH3 domains of proteins that are upstream modulators of three important mitogenic signaling pathway. These include PI3-K/Akt pathway, the PLCγ/PKC pathway and Ras/Raf/MEK pathway. All these pathways lead to cell proliferation and differentiation.

PLCγ is involved in protein kinase C (PKC) activation and subcellular localization to the cytoskeleton. Activation of PKC in a number of cells has been shown to feed into MAPK activation depending upon the type of signal received at the cell surface (160, 161). Although we have shown in vitro interaction between pORF3 and PLCγ we have not assayed the PLCγ activation by any means. However, we have used Calphostin C, a PKC pathway inhibitor, to measure its effect on MAPK activation in pORF3-expressing cells. MAPK activation was reduced about 70% when pORF3 cells were treated with Calphostin C suggesting that the PKC pathway may contribute to MAPK activation in pORF3 expressing cells.

p85α is a subunit of PI 3-kinase and it interacts with pORF3 in vitro. PI 3-kinase is a heterodimeric protein composed of a noncatalytic p85 subunit, and catalytic p110 subunit. Signaling by a variety of receptors and non-receptor tyrosine kinases is mainly mediated by Ras, a membrane-associated GTPase (189). Expression of v-Src results in Ras activation and leads in turn to transformation of NIH 3T3 cells (216). Further, expression of dominant-negative mutants of Ras completely inhibited the transforming ability of v-Src and also inhibited the activation of MAP kinase ERK2 which is downstream of Ras. In Rat-2 fibroblasts and chicken embryo fibroblast cells, however, expression of dominant-negative Ras completely inhibited ERK2 activation but did not affect the transforming ability of v-Src, indicating the presence of other pathways (150).
Further studies showed that PI 3-kinase is activated independent of Ras and this led to the transformation of Rat-2 and embryonic chicken fibroblasts (154).

In preliminary experiments, we have used inhibitors of different signaling pathways, U0126 (MEK inhibitor), LY294002 (PI 3K/Akt inhibitor), Calphostin C (PKC inhibitor), TMB-8 (Ca++ mobilization inhibitor) and Genistein (Src protein tyrosine kinase inhibitor). After treating cells with these inhibitors, we measured MAP kinase activity in pORF3 cells by MBP assay. PI 3-K/Akt inhibitor, LY294002, and PKC inhibitor, Calphostin C reduced the pORF3-induced MAP kinase activity by about 60 to 70%, while the MEK inhibitor, U0126, had no effect. We also studied the GTP bound form of Ras, and observed no increase in Ras-GTP in pORF3 expressing cells. Both these results suggest that increased MAP kinase activity in pORF3 cells is not through Ras-Raf-MEK pathway. However, pORF3 might activate MAP kinase targeting two other upstream pathways, PKC and PI 3-K. These pathways need to be further elucidated.

4.5. A proposal: pORF3 increases cell survival. While there are many reports which demonstrate MAPK activation following viral infection, few have established and determined its significance for viral replication. The MAPK/ERK cascade responds to diverse extracellular stimuli and is activated immediately. In hepatocytes, the MAPK/ERK pathway responds to EGF and TGF-α with distinct kinetics. This signal activates various nuclear transcriptional factors (Elk-1, c-Myc, c-Jun) downstream of MAPK/ERK cascade to induce cell-cycle progression in hepatocytes (226). Hepatocyte growth factor (HGF) is a cytokine that acts as a mitogen in various target cells. In hepatocytes, HGF has been shown to promote cell survival through the PI 3-Kinase/Akt cascade which protect cells from apoptosis (240).

Although we have provided the first evidence of an activation of a particular intracellular signaling cascade by HEV ORF3 protein, the mechanism(s) by which it activates the MAPK is not yet established. Preliminary inhibitor experiments indicated
that pORF3 activates the MAPK cascade through either or both of the PKC and PI 3-K pathways, but not the Ras/Raf/ERK pathway. It is very likely that pORF3, through activated MAPK, might promote cell survival in virus-infected hepatocytes to delay apoptosis by CTLs.

It is not a coincidence that all the hepatitis viruses, HBV, HCV and HEV induce MAP kinase pathway. HCV and HBV cause persistent infection and the activation of MAPK/ERK cascade may be one mechanism linked to their persistence in hepatocytes. Nevertheless, in the case of virus-infected cells, induction of early cell death would severely limit virus replication, and many viruses have evolved strategies to avoid or delay apoptosis. By activating MAPK, the HEV ORF3 protein may delay cell death to facilitate the viral replication and propagation. In same viral infections, it has been shown that MAPK activation plays an important role in pathogenesis. For example, virus production and ribonucleoprotein (RNP) export from the nucleus during the life cycle of influenza A virus is been impaired following inhibition of the Raf/MEK/ERK signaling cascade (219). Borna disease virus is another example whereby the MEK-specific inhibitor UO126 efficiently blocks infection of cells with progeny virus without being toxic for the host cell (220). It is not known whether activation of MAPK cascade and the cell survival has any effect on HEV replication and propagation. These questions remain to be investigated.