CHAPTER 3

RESULTS
3. RESULTS.

3.1. pORF3 binds SH3 domains in vitro: Hepatitis E virus ORF3 protein has two conserved PxxP motifs, P1 (aa 75-86) and P2 (aa 104-113) in its primary sequence from all isolates including swine HEV. The Mexican isolate pORF3, however, does not contain the P1 motif. Similar motifs are found in other cellular and viral proteins and have been shown to bind SH3 domains of signaling proteins that play essential roles in cellular proliferation and differentiation. We tested the ability of pORF3 to interact with SH3 domains from a number of such signaling proteins. In a GST pull-down assay (see Fig. 11 upper panel for schematic illustration of GST pull-down assay), pORF3 expressed in *E. coli* (panel A) or insect cells (panel B), bound to SH3 domains from src-family tyrosine kinases Src, Hck and Fyn, and the p85α regulatory subunit of phosphoinositol-3-kinase (PI3-K), phospholipase Cγ (PLC-γ) and Grb2 (Fig. 11 A; lanes 1-3, 6-8). pORF3 did not show any binding to GST alone or SH3 domains from Crk, Csk, Spectrin and p130Cas (Fig. 11A; lanes 4, 5, 10, 11) while weak binding was observed with the SH3 domain of Crk (Fig. 11A; lanes 4) even though equivalent amounts of GST-SH3 fusion proteins were used to trap pORF3 on beads (Fig. 11C). The interactions were also evaluated independently by a filter-binding assay (see Fig. 12 upper panel for schematic representation of filter binding assay), the results being consistent with those from the GST pull-down assay (Fig. 12). The only exception was the SH3 domain of Abl which bound to *E. coli* expressed, but not to insect cell expressed pORF3 in the GST pull-down assay (Fig. 11; panels A, B; lane 12); no binding was observed in the filter-binding assay.
Figure 11: *In vitro* binding of pORF3 to SH3 domains. (A) *E. Coli* expressed and purified pORF3 was used for *in vitro* GST pull-down assay and probed with anti-pORF3 antibodies. (B) GST pull-down assay using pORF3 expressed in insect cell lines, was used as in panel A. (C) GST-SH3 domains, used in the pull-down assays, were resolved in SDS-PAGE and stained with Coomassie Blue.
GST pull-down assays

Western blot analysis of bound pORF3

A

B

C

WB: anti-ORF3

CB stain
**Figure 12**: Filter binding assay. pORF3 was biotinylated as illustrated above. GST-SH3 domains were separated on SDS-12%PAGE (bottom panel). Separated GST-SH3 domains were transferred to a nitrocellulose membrane and probed with biotinylated pORF3 (upper panel). Src, Hck, Fyn, p85α, PLC γ, Grb2, and Grb2 N’terminal show binding to pORF3 (lanes 2, 3, 4, 7, 8, 9, 10).
Filter Binding Assay

Biotinylation of pORF3

Separate all SH3 domains on SDS-PAGE

Probe with B pORF3

Filter Binding

CB stain
as well (Fig. 12 lane 13). The binding of pORF3 to other SH3 domains was both specific and reproducible between the two assays.

To test which of the two PxxP regions in pORF3 bound SH3 domains, we carried out GST pull-down assays using mutant ORF3 proteins (Fig. 13) and fusion proteins containing the Grb2 or Src SH3 domains. While the wild type pORF3 bound Grb2 and Src SH3 domains (Fig. 13, top and middle panel, lane 3), the Δ(92-123) or the Δ(78-123) mutants did not (Fig. 13, top and middle panel, lanes 6,7). A mutant of pORF3 in which serine-80 (in the P-1 region) was changed to alanine, as well as pORF3 from the Mexican isolate of HEV, containing a conserved P-2 region but a variant P-1 region, also bound the GST-SH3(Grb2) and GST-SH3(Src) fusion proteins (Fig. 13, top and middle panel, lanes 4 and 5, respectively). The Δ(92-123) protein did not bind to any of the GST-SH3 fusion proteins in our panel, in either the GST pull-down assay or the filter binding assay. These results showed that the P-2 region of pORF3 is necessary for its binding to SH3 domains. Purified wild type pORF3 and its mutants used in the binding assay are shown by western blotting with anti-hexahistidine tag antibodies (Fig. 13, bottom panel, 3-7). The GST fusion protein for the Grb2 and Src SH3 domains are also shown by Coomassie blue staining of SDS-polyacrylamide gels (Fig. 13, lanes 1 and 2).

3.2. Competition analysis of pORF3 binding to SH3 domains: The binding of pORF3 to SH3 domains was further characterized by competition with a 30-mer synthetic peptide. Though the peptide included the entire P-2 region and its flanking sequences, it competed very poorly with the full-length ORF3 protein for SH3 domain binding (Fig. 14 A). Only at a 1000-fold molar excess of the peptide there was upto 90% reduction in pORF3 binding, when the peptide was pre-incubated with the GST-
**Figure 13**: pORF3 binds to SH3 domains through its P-2 region. GST-Src and GST-Grb2 SH3 domains were tested for binding to wild type pORF3 as well as its mutants. Left panel shows Coomassie Blue stained GST-Src and GST-Grb2 SH3 domain fusion proteins. Upper and middle panel show pORF3 binding to GST-Grb2 and GST-Src SH3 domain, respectively. Wild type pORF3, pORF3(Mexican strain) and pORF3 S80A bind to both Grb2 and Src SH3 domains (top and middle panel lanes 3-5, respectively) while pORF3Δ92 and Δ78 mutants do not show any binding to these GST-SH3 fusion proteins (lanes 6,7).
SH3(Hck) beads prior to pORF3 binding. However, when pORF3 and the peptide were co-incubated with GST-SH3(Hck) beads, protein binding was reduced only by about 50% at a 1000-fold molar excess of the peptide. Thus compared to pORF3, a peptide encompassing the P-2 region bound SH3 domains with reduced affinity.

The interaction of HIV-1 Nef with SH3 domain-containing cellular proteins has been characterized in detail and the binding affinities have been measured. Since Nef binds the Hck SH3 domain with high affinity, we carried out a competition analysis for binding of pORF3 and Nef to the GST-SH3(Hck) target. The Nef protein was expressed in *E. coli* and purified as described in materials and methods. The results showed that pORF3 and Nef were equally effective in competing with each other for binding to the Hck SH3 domain (Fig. 14 B).

3.3. Yeast two-hybrid analysis: To test if the interaction of pORF3 with SH3 domains took place *in vivo* as well, we used the yeast two-hybrid system (a schematic representation of the yeast two-hybrid assay is shown in Fig. 15A) and chose Grb2 as an example of the interacting partner. The AD-Grb2 and BD-ORF3 constructs (Fig. 15B) were expressed singly or together in Y190 *Saccharomyces cerevisiae* cells carrying the *HIS3* and β-galactosidase (*lacZ*) reporter genes. An interaction between hybrid proteins was scored by the capacity of yeast cells to grow on medium lacking histidine (His⁺). The results are presented in Fig. 16A-G. All cells grew on YPD medium showing that the transformed yeast cells were viable and the expressed fusion proteins did not inhibit growth (Fig. 16B). Cells transformed with single vectors, either carrying the GAL4 DNA binding domain (with *trpJ* auxotrophic marker) or the GAL4 activation domain (with *leu3* auxotrophic marker) were able to grow on synthetic medium lacking tryptophan
**Figure 14**: Competition analysis of pORF3 binding to GST-Hck SH3 domain. (A) A peptide encompassing P-2 motif in pORF3 sequence, was used to compete with full length protein. The peptide was either pre-incubated with GST-Hck SH3 domain prior to addition of pORF3 (a; lanes 1-4), or the peptide and pORF3 were co-incubated with GST-Hck SH3 domain (b; lanes 6-9). The pORF3 retained on the beads was estimated by SDS-PAGE and Coomassie Blue staining. (B) Competition of pORF3 with Nef. Beads carrying GST-Hck SH3 domain were incubated with either 3 μg of Nef and increasing amounts of pORF3 (a; lanes 1-4), or with 3 μg of pORF3 and increasing amount of Nef (b; lanes 6-9). The proteins retained on beads were western blotted with anti-hexahistidine tag antibodies. In both competition assays, the bands were quantitated by densitometry using the Kodak image analyser and the resulting graphs are shown on the right.
A. Fold molar excess of peptide

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| GST-Hck | 10 | 100| 1000| MW
| pORF3   | 10 | 100| 1000| MW

B. Fold molar excess of pORF3

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| HIV-Nef | 10 | 100| 1000| MW
| pORF3   | 10 | 100| 1000| MW

% of control

Fold excess of peptide

Fold excess of competitor
Figure 15: A. Schematic illustration of the yeast two-hybrid system. His3 and LacZ genes are integrated in yeast strain Y190 and driven by GAL4 promoter which is activated only when DNA binding and activation domains of GAL4 come together and in turn drive the expression of HIS3 and LacZ genes. B. Plasmid constructs used in this study: (1) pGAD424, GAL4 activation domain; (2) pGBT9, GAL4 DNA-binding domain; (3, 4) pGBT-SNF1 and pGAD-SNF4, positive control plasmids; (5) pGAD-Grb2, containing Grb2 fused to the GAL4 activation domain; (6) pGBT-ORF3, containing ORF3 fused to the GAL4 DNA-binding domain; (7) pGBT-ORF3(1-80), containing ORF3 mutant encompassing amino acids 1 through 80.
A.

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B.

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1. PADH1 | GAL4-AD | MCS | TADH1 | pGAD424
2. PADH1 | GAL4-BD | MCS | TADH1 | pGBT9
3. PADH1 | GAL4-BD | SNF1 | TADH1 | pGBT9-SNF1
4. PADH1 | GAL4-AD | SNF4 | TADH1 | pGAD424-SNF4
5. PADH1 | GAL4-AD | Grb2 | TADH1 | pGAD424-Grb2
6. PADH1 | GAL4-BD | ORF3(1-123) | TADH1 | pGBT9-ORF3
7. PADH1 | GAL4-BD | ORF3(1-80) | TADH1 | pGBT9-ORF3(1-80)
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**Figure 16**: Yeast two-hybrid analysis of pORF3-Grb2 interaction. Panels show the plating pattern (A), growth on YPD (B), or on synthetic medium lacking tryptophan (C), leucine (D), or leucine, tryptophane and histidine (E), β-galactosidase expression in cells grown on YPD shown on a filter (F) or in a liquid β-galactosidase assay (G).
YPD
Trp −
Leu −
LTH −
Filter β-gal

Liquid β-gal assay
(SDTrp⁻) or leucine (SDLeu⁻), respectively (Fig. 16C,D). Only cells co-transformed with AD-Grb2/BD-ORF3 vectors or the positive control vectors (SNF1/SNF4) grew on synthetic medium lacking leucine, tryptophan and histidine (SDLeu⁻Trp⁻His⁻) (Fig. 16E). Over 100 His⁺ colonies were obtained on co-transformation with AD-Grb2 and BD-ORF3 vectors. Most of these cells also showed solid and liquid β-galactosidase activity. The results of only two representative colonies are shown (Fig. 16F,G). The binding of BD-ORF3 and AD-Grb2 fusion proteins was specific since (a) the former did not bind the GAL4 AD alone and the latter did not bind the GAL4 BD alone (b) the GAL4 activation and binding domains by themselves do not interact with each other, and (c) the BD-ORF3 fusion proteins expressed from two independent clones of plasmid pGBT9-ORF3 bound to the AD-Grb2 fusion protein. A truncated pORF3, lacking amino acid residues 80 to 123 and both the P-1 and P-2 regions, also did not show any interaction with full-length Grb2. These results confirmed that in vivo also, pORF3 bound a SH3 domain-containing protein, Grb2, and that this binding was dependent upon proline-rich regions in the C-terminal part of pORF3.

3.4. Subcellular distribution of pORF3 and its colocalization with SH3 domain-containing proteins, Src and Grb2 : pEGFP-ORF3 construct has been generated to study the subcellular distribution of pORF3 in live cells (Fig. 17A). Expression of EGFP-ORF3 was confirmed by immunoprecipitation using anti-pORF3 antibodies (Fig. 17B). In a transiently-transfected Huh7 cells, EGFP-ORF3 expression but not EGFP alone showed distinct cytoplasmic distribution (Fig17C). Following transfection, live Huh7 cells were monitored by confocal microscopy. Early in transfection there were no significant differences in the distribution of EGFP and EGFP-
Figure 17: Subcellular distribution of pORF3. A. pEGFP and pEGFP-ORF3 expression vector constructs. B. Expression and immunoprecipitation of EGFP and EGFP-ORF3 in COS-1 cells. C. Upper panel shows time point expression and localization of EGFP-ORF3 in live Huh7 cells, bottom panel shows EGFP alone. pORF3 showed a distinct cytoplasmic distribution at later phase of transfection while EGFP alone showed very homogenous distribution across the cell.
A. pEGFP-N3

[ M CS EGFP ]

SV40 poly A

pEGFP-ORF3

[ ORF3 EGFP ]

SV40 poly A

B. MW

Control

EGFP-ORF3

EGFP-ORF3

Live Huh7 cells

Live Huh7 cells
ORF3. However, at later time points while EGFP-ORF3 fusion protein showed distinct cytoplasmic localization (Fig. 17C upper panel), the control EGFP showed homogenous distribution across the cell, including nucleus (Fig. 17C bottom panel).

In dual staining experiments, we studied the colocalization of pORF3 and either Src or Grb2 in COS-1 cells transiently transfected with expression vector pMT-ORF3. The distribution of pORF3 in these cells was cytoplasmic and displayed punctate staining (Fig. 18D panels a, d). The Src protein was found all over the cell in the cytoplasmic as well as the nuclear compartments (Fig. 18D panel b), while the Grb2 protein primarily localized to the cytoplasmic regions (Fig. 18D panel e). Distinct orange or yellow staining regions were observed in the merged images indicating the colocalization of pORF3 with either Src or Grb2 (Fig. 18D panels c, f).

3.5. Molecular modeling of pORF3 binding to SH3 domains: The P-2 peptide was modeled on the three-dimensional structures of the c-Src and Grb2 SH3 domains (74, 75). The energy minimized solvated structures are shown in Fig. 19. For comparison, the binding surfaces of P-2 with Src and Grb-2 SH3 domains are shown along with those for the Src SH3-RLP2 (1RLQ) and Grb2 SH3-mSOS (1SEM) structures. The interaction energies for Src SH3 domain binding to P-2 and the RLP2 ligands were calculated to be -76.5 kcal/M and -70.3 kcal/M, respectively; the Grb2 SH3 domain bound the P-2 and mSOS ligands with interaction energies of -73.8 kcal/M and -85.8 kcal/M, respectively. The P-2 peptide binds to the hydrophobic recognition platform in the SH3 domain with the same conformation in both Src and Grb2. In both models, residues 2 to 8 of P-2 form a polyproline II (PPII) helix, with three residues per turn. Analogous to other PPII helices, the P-2 peptide shows a (i, i+3) arrangement of
Figure 18: Co-immunofluorescence staining of pORF3 with either Src or Grb2. After 48 hrs of post-transfection, the cells were fixed, permeabilized and stained with primary anti-pORF3 monoclonal antibodies (a, d), anti-Src (b) or anti-Grb2 (e) polyclonal antibodies. pORF3 was stained with Alexa 488 (green), Grb2 and Src proteins were stained with Alexa 594 (red). Images were merged to observe the co-localization in Adobe Photoshop (c, f).
prolines, with the flanking prolines in the PPLP sequence on the same face of the helix. Pro2, Pro5 and Pro8 in P-2 form one face of the helix that interacts with the SH3 domain. Ala4 and Leu7 are on the other face, while Ser3, Pro6 and His9 project away from the helix (Fig. 19, top). The ligand binding site on the c-Src SH3 domain has been shown to contain three binding pockets. The first one, formed by Tyr14 and Tyr60 interacts with Leu7 and Pro8 of the P-2 peptide. Residues Tyr16, Trp42, Pro57 and Tyr60 form the second pocket that makes hydrophobic interactions with Ala4 and Pro5 of P-2. The third pocket binds the Arg1 residue in a salt bridge with a conserved aspartate. While in the 1RLQ structure this interaction is with Asp23, the P-2 peptide interacts with the conserved Asp41 in the c-Src SH3 domain. Residues C-terminal to the proline-rich core. His9 and Val10 in P-2 or Arg8 and Tyr9 in the RLP2 ligand, do not bind the c-Src SH3 domain. Similar sets of contacts were found in the model for P-2 binding to the Grb2 SH3 domain. In the 1SEM structure Arg1 of the peptide interacts with Glu172, while the Arg1 of peptide P-2 interacts with the conserved Asp188 of the Grb2 SH3 domain.

Modeling of the P-2 peptide with c-Src and Grb2 SH3 domains and a comparison of these models with the 1RLQ and 1SEM structures showed that the P-2 region bound similarly in the two structures (Fig. 19). The proline-rich core of P-2 was buried in each of the SH3 domains and this binding caused minimal structural deformity of the SH3 domains. The arginine (Arg1) present at the N-terminus of the PXXP motif formed salt bridges in both structures, though with conserved residues other than those found in the PDB structures.

3.6. pORF3 binds but does not activate the Src kinase: The binding of proteins containing a PXXP motif to the SH3 domain of Src family protein tyrosine
Figure 19: Computer modeling of c-Src SH3 domain binding to the ORF3 P-2 peptide. The energy-minimized solvated models are shown for the Src SH3 with P-2 (1) or RLP2 (2), and for the Grb2 SH3 with P-2 (3) or mSOS (4). The SH3 domains are shown in grey. In the peptide ligand, the hydrophobic core is orange, the salt-bridging Arg is blue and the non-interacting residues are green. The upper panel shows side chains in P-2 interacting with Src SH3 binding pockets.
kinases can activate these kinases (109). We tested if pORF3 had a similar effect. Control cells or cells stably expressing pORF3 were labeled with $[^{32}\text{P}]$-orthophosphate and the lysates immunoprecipitated with anti-pORF3, anti-vSrc or anti-phosphotyrosine (pY) antibodies. Two differentially phosphorylated forms of pp60$^{\text{src}}$ (S1 and S2) were seen. While S2 was predominant in control cells (Fig. 20A, lane 4), the slower migrating S1 was predominant in pORF3-expressing cells (Fig. 20A, lanes 5, 6, 8, 9). Since S2 was missing from control (Fig. 20A, lane 7), but not from pORF3-expressing cells (Fig. 20A, lanes 8, 9) when immunoprecipitated with anti-pY, we propose that this may be pp60$^{\text{src}}$ phosphorylated at Tyr$^{527}$. In this inactive form of pp60$^{\text{src}}$, Tyr$^{527}$ is buried in the Src SH2 domain and would therefore be inaccessible to anti-pY antibodies (Fig. 20A lanes 7-9). The S1 band would then represent pp60$^{\text{src}}$ that is dually phosphorylated at Tyr$^{527}$ and Tyr$^{416}$.

Another phosphoprotein (p80) with an apparent mobility of 75-80 kDa was observed in pORF3 expressing cells, but not in control cells (Fig. 20A, upper panel, lanes 5, 6, 8, 9). Since p80 was immunoprecipitated with anti-Src or anti-pY but not with anti-pORF3, it appeared to be associated with pp60$^{\text{src}}$. Anti-Src or anti-pY antibodies were also able to immunoprecipitate pORF3, though with poor efficiency, from ORF3-1 and ORF3-4 cells, but not from control cells (Fig. 20A, lanes 5-6, 8-9 bottom panel). This apparent inefficiency of cross-immunoprecipitation might result from only a transient intracellular association between pORF3 and pp60$^{\text{src}}$. Thus, there appears to be a transient trimeric complex of pp60$^{\text{src}}$ with p80 and pORF3.
Figure 20: c-Src phosphorylation status in pORF3 expressing cells. A. Immunoprecipitation of [32P]-orthophosphate labeled cells with anti-pORF3 (lanes 1-3), anti-Src (lanes 4-6) and anti-phosphotyrosine (lanes 7-9) antibodies. Two differentially phosphorylated forms of pp60src were seen (S1 and S2). p80 indicates a coprecipitating cellular protein. B. c-Src in vitro kinase assay. Control and pORF3 expressing cells either grown in the presence of 10%FBS (lanes 1, 2), serum starved (lanes 3, 4), induced with 1 μM insulin for 1 h. (lanes 5, 6), or treated with 300 μM Genistein in the presence of insulin (lanes 7, 8). Upper panel shows the Src kinase assay using acid-denatured enolase, bottom panel shows the western blotting of c-Src for each reaction.
The activity of Src kinase was directly assayed by means of an immunoprecipitation kinase assay using acid-denatured Enolase as a substrate. No difference was observed between control and pORF3 expressing stable cell lines (Fig. 20B). Control and ORF3 cells were grown in the presence of 5% FBS (Fig. 20B, lanes 1, 2) or serum starved for 6 hrs (Fig. 20B, lanes 3, 4), followed by induction with 1 μM insulin (Fig. 20B, lanes 5, 6). As a control, some serum-starved and insulin-induced cells were treated with 300 μM Genistein (Fig. 20B, lanes 7, 8). The cells were lysed and in vitro immunoprecipitation kinase assay was performed for Src activity as described in Materials and Methods.

3.7. pORF3 expression results in MAP kinase activation and nuclear translocation: The control and ORF3 stable cell lines were assayed for Erk activity by means of two independent assays. In a commercially available assay, using the transcription factor Elk-1 as a substrate, lysates from two independent ORF3 cell lines showed higher activity than control cells (Fig. 21 top, lanes 5-7). However, no significant difference in the Erk activity was observed if the cells were not starved for serum prior to lysate preparation (Fig. 21 top, lanes 1-3). In an immunocomplex kinase assay as well, using myelin basic protein as a substrate, increased Erk activity was observed in the ORF3 cell lines compared to the control cells (Fig. 21, bottom panel). In this assay, however, no effect of serum starvation was observed upon Erk activity.

The localization of MAP kinase isoforms in ORF3 cells was evaluated by indirect immunofluorescent labeling and confocal microscopy. Cells were stained with anti-p44/42 for total ERK isoforms, or with anti-phospho p44/42 for the dually phosphorylated and activated form of ERK. Cells were stained either directly after
Figure 21: Effect of pORF3 on ERK activity. Control cells (lanes 1 and 5) and pORF3 expressing cells (lanes 2, 3, 4, 6, and 7) were grown in the presence of serum (lanes 1-3) or were serum-starved for 6 hours (lanes 5-7). The lysates were then assayed for ERK activity by means of either the p44/42 MAPK Assay System (Cell Signaling Technology) using Elk-1 as a substrate (upper panel), or by an immunocomplex kinase assay using myelin basic protein as a substrate (lower panel). The molecular size markers shown are 30 and 46 kDa (upper panel) and 21.5 kDa (lower panel).
growth in serum, or were first starved in serum-free medium to reduce the background of activated ERK. When stained with anti-p44/42, the control cells showed only diffuse, non-specific staining (Fig. 22A, E). With anti-phospho p44/42 and when grown in serum, the control cells showed largely perinuclear staining (Fig. 22C); when starved for serum, these cells showed only low levels of diffuse and non-specific staining (Fig. 22G). The cells expressing pORF3, on the other hand, showed cytoplasmic and largely perinuclear staining with anti-p44/42 antibodies, whether grown with serum or starved of it (Fig. 22B, F). When the pORF3-expressing cells were stained with anti-phospho p44/42, the signal was mainly nuclear (Fig. 22D, H) whether the cells were grown in serum or starved of it. These results suggest that pORF3 expression promoted translocation of the ERK subfamily of enzymes to the nucleus. Since these enzymes undergo a cytoplasm-to-nucleus translocation on activation by dual phosphorylation, which is the form of the enzyme detected with anti-phospho p44/42 antibodies, these results further confirmed the increased activity of ERK in pORF3-expressing cells, compared to control cells.

3.8. pORF3 increases MAP kinase activity *in vivo* : It has been previously shown (192) that MAP kinase activation leads to phosphorylation of transcription factors of the Elk family and to an increase in their transcriptional activity. Figure 22 summarizes the *in vivo* MAPK assay and its components. The first step is to cotransfect three vectors into mammalian cell line: a plasmid containing your gene of interest, the appropriate transactivator vector (TA) such as pTet-ELK, -ATF, -JUN, or -CREB for the signaling pathway of interest, and the reporter vector, pTRE-Luc. Transcription of reporter gene depends on the phosphorylation of TetR-TA fusion protein by the gene of interest. This effect could be either direct or indirect (upstream in the signaling pathway)
Figure 22: Effect of pORF3 on the localization of ERK. Control cells (A,C,E,G) and pORF3-expressing cells (B,D,F,H) were grown in the presence of serum (A-D) or were serum-starved for 6 hours (E-H). The cells were then fixed, stained with either anti-p44/42 (A,B,E,F) or anti-phospho p44/42 (C,D,G,H) antibodies, and viewed by confocal microscopy, as described in Material and Methods.
Control ORF3 Control ORF3

+ serum

- serum

anti-p44/42 anti-phospho p44/42
depending upon the kinase functionality of a gene. As shown in Fig. 23 transactivator plasmid expresses a fusion of the TetR-TA, the pTRE-Luc reporter construct contains the tetO sequence, upstream of the luciferase reporter. When the transactivator expression vector and pTRE-Luc are cotransfected into mammalian cells, the TetR portion of the TetR-TA fusion protein binds to TRE in the absence of Tetracycline or Doxycycline (Tc, Dox), bringing the transactivator in close proximity to the minimal promoter. Once the TA is phosphorylated, the promoter is activated and luciferase is expressed. In contrast, the presence of Tc or Dox alters the DNA binding ability of TetR so it can not bind TRE (Fig. 23). This provides a negative control to determine the specific activity of a gene of interest on a signaling pathway.

Therefore, we utilized this system to study the pORF3-inducible MAP kinase activity in vivo as well (MAPK In Vivo Assay Kit; Clontech, USA.). HEK293 and HepG2 cells were transiently-transfected with pMT-ORF3 or the pMT3 control vector together with pTRE-Luc and pTet-Elk1. pORF3 expression (Fig. 24 lane 8) but not the control (Fig. 24 lane 6) increased the MAP kinase activity and Elk1 phosphorylation as determined by an approximately four-fold increase in luciferase activity in HEK293 cells. Moreover, treatment of ORF3-transfected cells with 2 μg/ml Doxycycline for about 24 hrs dramatically reduced down the luciferase activity (Fig. 24 lane 9), whereas there was no significant effect on the activity in control cells (Fig. 24 lane 7). This showed that the effect of pORF3 on MAP kinase activation is specific. Of the controls, cells alone showed no luciferase activity, whereas pTRE-Luc either alone or when cotransfected with pTetElk1 showed basal level luciferase activity (Fig. 24 lanes 2, 3 respectively). For negative control, co-transfection of cells with pTRE-Luc and pTet-Neg resulted in
Figure 23: Schematic representation of the in vivo MAPK assay. This is a tet-off system, based on co-transfection of cells with three vectors including gene of interest, transactivator and the reporter vectors. While there is no transcription of reporter gene in the presence of Doxycycline/Tetracycline, phosphorylation of the transactivator-TetR fusion either directly or indirectly by the gene of interest activates the transcription of reporter gene in the absence of Doxycycline/Tetracycline.
Transactivator Vector

Vector expressing your target gene

Reporter Vector

Cotransfect mammalian cells

+ Dox

- Dox

**Negative control:**
- TetR cannot bind to TRE
- No luciferase expression

**pTet-Transactivator**

**pTarget**

**pTRE-Luc**

**TetR** binds to TRE

No phosphorylation

- No luciferase expression

Kinase phosphorylates activation domain of transactivator

Transcription

- Phosphorylated transactivator induces luciferase expression
Figure 24: In vivo MAP kinase assay. 293 or HepG2 cells were either tested for luciferase activity without any transfection (lane 1) or after 48 hrs. post-transfection with pTRE-Luc alone (lane 2), pTRE-Luc and pTet-Elk1 (lane 3), pTRE-Luc and pTet-Neg (lane 4), pTRE-Luc and pTet-Off (lane 5), pTRE-Luc, pTet-Elk1 and pMT3 (lane 6), pTRE-Luc, pMT-ORF3 and pTet-Elk1 (lane 8). Control pMT3 vector and pMT-ORF3 transfected cells were treated with 2 μg/ml Doxycycline for 24 hrs. (lanes 7 and 9 respectively).
minimal luciferase activity as well (Fig. 24 lane 4). pTet-Off contains TetR, which is fused to the negatively charged C-terminal activation domain of VP16 protein of the herpes simplex virus. Unlike the in vivo kinase transactivators, VP16 is a strong transactivator, which does not depend on kinase activity. Therefore, in the absence of tetracycline or doxycycline, the TetR and VP16 fusion protein binds to the TRE and activates transcription of the luciferase gene. Thus, cells co-transfected with pTRE-Luc and pTet-Off showed a five-fold increase in luciferase activity (Fig. 24 lane 5).