RESEARCH REPORT
SWINE HEALTH

Title: Investigating the role of PB1-F2 in the pathogenicity of circulating strains of SIV – **NPB #10-161**

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INDUSTRY SUMMARY

The objectives of this study were to initiate an investigation into the impact of a newly identified influenza virus protein, PB1-F2, in swine lineage influenza virus (SIV) pathogenicity. PB1-F2 was first identified in human and avian lineage influenza virus strains, and was shown to play a crucial role in the pathogenicity of these virus strains by initiating the induction of a host cell pathway, termed apoptosis, that results in cell death via localization to cellular mitochondria. While most swine lineage influenza viruses encode the capacity to express PB1-F2, the proteins are substantially different at the primary amino-acid sequence level from PB1-F2 proteins that have been studied. In this work, we utilized an SIV strain isolated from clinically ill pigs and humans at a 2007 Ohio state fair that is representative of a group of currently circulating strains to investigate the role of swine lineage PB1-F2 in induction of cell death of swine and human cells. An antibody specific for swine lineage PB1-F2 was created and utilized to examine PB1-F2 in SIV infected cells. Multiple untagged and tagged plasmid clones were additionally created to further investigate the PB1-F2 protein from SIV. Additionally, clones were created in reverse genetics plasmids in which the PB1-F2 ORF was mutated such that recombinant viruses can be created that do not express PB1-F2 to identify the function of PB1-F2 in SIV replication and pathogenicity. Unlike the previously described mitochondrial localization of human lineage influenza PB1-F2, swine lineage PB1-F2 was found not to substantially localize to this cellular organelle, suggesting it may play an alternative role in SIV infection. SIV PB1-F2 was instead found to localize to the nucleus of infected cells. Localization of PB1-F2 to the nucleus likely impacts its function. PB1-F2 expression in infected cells was also differentially regulated in a strain specific manner at the level of protein synthesis. This regulation involves two sequence-dependent elements located in the PB1-F2 open reading frame (ORF) and downstream of the PB1-F2 ORF in the PB1 gene. Understanding the mechanisms involved in PB1-F2 sub-cellular localization and translational regulation will give insight into predicting PB1-F2 expression and virulence potential in swine influenza viruses.

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KEYWORDS

Swine influenza virus, PB1-F2 protein, sub-cellular localization, apoptosis, viral pathogenesis, viral replication, translational regulation

SCIENTRIFIC ABSTRACT

The influenza A PB1-F2 protein is translated through a second open reading frame (ORF) in the PB1 gene segment and has been implicated in regulation of polymerase activity, immunopathology, susceptibility to secondary bacterial infection, and induction of apoptosis. The majority of work examining PB1-F2 function in infection has been performed with human and avian isolates. As the recent H1N1 2009 pandemic highlighted, swine origin influenza viruses have the capacity to infect human hosts on a large scale. Thus, it is necessary to have a proper understanding of the potential risk that pathogenicity factors from swine isolates may have on both human and swine health. Studies examining PB1-F2 from swine isolates have been focused primarily on pH1N1 viruses, which does not naturally express PB1-F2, that were engineered to encode a PB1-F2 ORF. These recombinant viruses were then studied to determine the impact of swine origin PB1-F2 on replication and pathogenicity. However, to our knowledge, experimental evidence of pH1N1 PB1-F2 protein expression from recombinant viruses has not been demonstrated. In this study we have found that PB1-F2 proteins from swine and human isolate influenza viruses have substantial differences in their cellular localization patterns and expression levels during viral infection. We provide compelling evidence that PB1-F2 protein expression is regulated at the translational level with swine isolate PB1-F2 expressed at very low levels relative to human isolate PB1-F2. Translational regulation of PB1-F2 expression was mapped to two independent regions within the PB1 mRNA, with one located within the PB1-F2 ORF and the other located downstream of the PB1-F2 ORF. Our data suggest that the presence of an intact PB1-F2 protein coding sequence alone is not predictive of PB1-F2 expression in infected cells and instead, PB1-F2 expression is differentially regulated at the translational level in an isolate-specific manner dependent on RNA sequences present in the PB1 gene.

INTRODUCTION

Influenza A viruses (IAV) are important human, animal, and bird pathogens of the *Orthomyxoviridae* family. These enveloped viruses contain a negative-sense, segmented RNA genome consisting of 8 RNA segments encoding 10 or 11 proteins. The segmented nature of IAV allows for viral gene reassortment leading to the continual production of novel viruses. In the case of SIV, following a stable period from 1930 to 1997, in which one major strain (classical H1N1) was the predominant virus isolated from swine herds, several severe outbreaks of influenza occurred throughout the U.S. in which multiple new virus strains were identified to be the causative agents. These strains included triple reassortant viruses with gene segments from classical swine, human, and avian origin (Vincent, Ma et al. 2008). Viruses from the triple reassortant lineage have now become endemic in the swine population. As a result of the acquisition of the avian and human genes, circulating SIV strains appear to be able to more easily reassort the surface HA and NA genes that are the predominant antigens during an immune response, making it difficult for swine herds to develop immunity against all circulating strains of SIV.

In the spring of 2009, a previously unidentified strain of influenza A virus was identified as the etiologic agent behind outbreaks of flu-like illness in humans in North America. This strain was sequenced and found to contain genes from four different sources, including North American swine influenza, North American avian influenza, European swine influenza, and Asian swine influenza (Garten, Davis et al. 2009). This particular combination of gene segments had not previously been detected in swine or any other species. Within a very short time, the virus spread throughout the world, resulting in laboratory confirmed cases in over 200 countries, over 15,000 confirmed deaths, and leading the World Health Organization to declare the virus as a pandemic strain . This virus, H1N1 (2009) has now been the focus of intense investigation in animal models, and has been found to cause symptoms similar to circulating strains of SIV in swine (Itoh, Shinya et al. 2009; Lange,

Kalthoff et al. 2009; Vincent, Lager et al. 2009). Interestingly, a recent study suggests that H1N1 (2009) is not very pathogenic in a mouse model compared to other triple reassortant circulating strains of SIV, although the viruses share some similarity at the genome segment level (Belser, Wadford et al.). The differences in pathogenicity between these viruses have not yet been mapped to specific gene segments.

PB1-F2 is a newly identified influenza virus protein (Chen, Calvo et al. 2001). Many influenza viruses express PB1-F2 from an alternative open reading frame of the PB1 encoding gene segment 2 (Chen, Calvo et al. 2001),(Zell, Krumbholz et al. 2007). The PB1-F2 C-terminus contains an alpha-helical mitochondrial targeting signal (MTS) that has been implicated in its localization to the inner and outer leaflets of the mitochondrial membrane (Chen, Calvo et al. 2001), (Yamada, Chounan et al. 2004). This localization results in qualitative changes in mitochondrial morphology and loss of mitochondrial membrane potential resulting in induction of apoptosis and cell death (Yamada, Chounan et al. 2004), (Chen, Calvo et al. 2001), (Zamarin, Ortigoza et al. 2006). PB1-F2 was recently shown to interact with ANT3 and VDAC1, two proteins that are components of the permeability transition pore complex of mitochondria, suggesting that PB1-F2 may directly permeabilize mitochondria (Zamarin, Garcia-Sastre et al. 2005). The apoptotic effects of PB1-F2 have been reported to occur more readily in monocytes than epithelial cells (Zamarin, Garcia-Sastre et al. 2005), (Coleman 2007). This may suggest that PB1-F2 targets cells of the immune system to prevent viral clearance leading to increased pathogenicity. Alternatively, as secondary infections by viral and bacterial pathogens play a crucial role in the mortality of IAV infection, this may be an important factor in the increased susceptibility of IAV infected hosts to secondary infection. In support of this hypothesis, it was recently shown that expression of the pandemic 1918 IAV PB1-F2 protein enhances the pathogenicity of viral and secondary bacterial infections in mice (Zamarin, Garcia-Sastre et al. 2005).

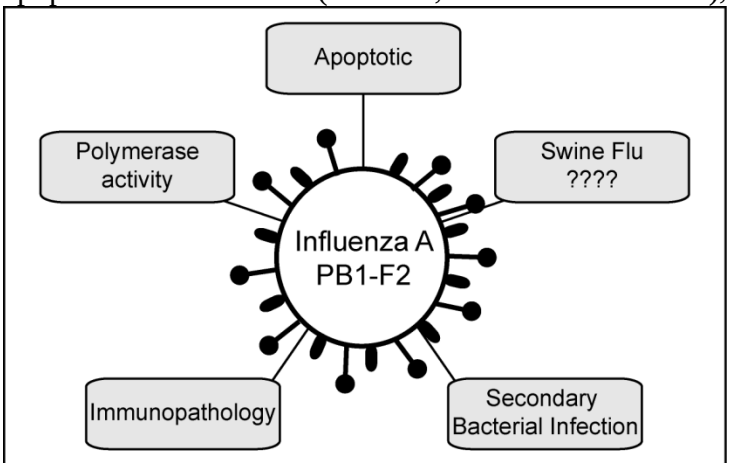

Fig.1. PB1-F2 plays significant roles in influenza pathogenicity. PB1-F2 has been shown to increase pathogenicity through the mechanisms listed above, which are associated with a more severe disease.

Very little is known about the role of PB1-F2 in SIV infection. Classical SIV strains harbor stop codons in multiple places in the PB1-F2 open reading frame, and therefore either do not express the protein, or express a truncated version of the protein that does not contain the C-terminal MTS. However, many currently circulating strains of SIV are predicted to express PB1-F2. A recent study examined the PB1-F2 protein from two European SIV isolates, and found that they were expressed during in vitro infection of MDCK cells, and that the PB1-F2 protein localized to the mitochondria of transfected HeLa cells (Zell, Krumbholz et al. 2007). Similar to classical SIV strains, the recently emergent pandemic H1N1 (2009) PB1-F2 open reading frame harbors stop codons, and does not express PB1-F2 (REF). A recent study showed that mutation of the stop codons in H1N1 (2009) so that it expresses functional PB1-F2 results in altered expression of pro-inflammatory cytokines and modulation of the immune response, but does not significantly alter virus virulence in mouse or ferret models (Hai, Schmolke et al.). Importantly, the PB1-F2 protein from H1N1 (2009) differs at the amino-acid level when compared with the PB1-F2 of other circulating SIV strains and that of the 1918 PB1-F2, suggesting there may be differences in the virulence potential of PB1-F2 depending on its primary sequence. Additionally, the experiments performed in this single study did not examine the virulence of H1N1 (2009) PB1-F2 in swine cells. There are clearly gaps in our knowledge with regards to the importance of PB1-F2 in SIV immune evasion and virulence in swine cells and in pigs that are important to address.

The influenza A virus**,** A/SW/OH/511445/2007 H1N1 (OH07) was isolated from swine during an outbreak of respiratory illness in pigs and people. Viruses isolated from affected people were identical to the swine isolates. OH07 was evaluated in a pig pathogenesis and transmission study and shown to induce severe clinical disease, severe lung pathology, and was shed at high titers from the nose. Transmission was highly

efficient from infected pigs to contact pigs. OH07-like H1 SIV is a predominant type of virus currently circulating in the U.S. swine population (Vincent A. L. 2009). The factors leading to its increased virulence in pigs or its propensity to infect humans are unknown.

OBJECTIVES

The objectives of this proposal were to define the molecular properties of mitochondrial targeting and apoptosis-induction of the PB1-F2 protein from SIV, and elucidate the function of these properties in cells and animals.

Objective 1. Elucidate the molecular properties of OH07 PB1-F2 protein *in vitro*.
Objective 2. Understand the translational regulation of influenza PB1-F2
Objective 3. Examine the role of PB1-F2 in viral replication and cell death in swine cells.
Objective 4. Determine the role of PB1-F2 on replication and pathogenicity of OH07 virus in an *in vivo* model.

Our long term goals are (i) to delineate the mechanism behind translational regulation and subcellular localization of PB1-F2 (ii) to elucidate the function of the PB1-F2 protein and importance of subcellular localization during SIV infection **in swine** to expand our understanding of SIV infection, and (iii) to utilize the information gained in this proposal to examine the effect of decreased expression and altered subcellular localization of PB1-F2 in attenuating a cross-protective modified-live SIV vaccine developed at NADC.

MODIFICATIONS OF PROJECT FROM ORIGINAL PROPOSAL

Due to the low levels of PB1-F2 expression and change in subcellular localization relative to human origin PB1-F2 proteins in swine influenza virus discovered during the course of this project, it was modified to include an objective and goal towards understanding the mechanism in which PB1-F2 expression is controlled. This modification is necessary as it is impossible to determine the function of PB1-F2 if expression levels are to low to have biological relevance. Once we have defined how PB1-F2 expression is regulated we will address our original goal of understanding the function of PB1-F2 in SIV infection and virulence.

MATERIALS AND METHODS

Cells and viruses: Madin-Darby Canine Kidney (MDCK) and Porcine Kidney 15 (PK-15) cells were maintained using eagles modified essential media while human alveolar basal epithelial cells (A549) were maintained in F-12K media both containing 10% FBS and penicillin-streptomycin incubated at 37 °C. The viruses used in this study are A/OH/511445/2007 (OH07) and a recombinant A/PR/8/34 (PR8). All infections were carried out using Eagles modified essential media with 2% bovine serum albumin fraction V (BSA), penicillin-streptomycin, and 1µg/ml of TPCK treated trypsin (infection media) and were incubated at 37 °C.

Infections and Transfections. Cells were plated and incubated overnight then infected for 12h with either OH07 or PR8 influenza viruses at MOI=2. Transfections were performed using Lipofectamine 2000 per the manufacturer's instructions.

Immunofluorescence Microscopy. Samples were washed three times with phosphate buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 10 min. The cells were washed again with PBS and then either permeabilized with 0.2% Triton X-100 in PBS, or were directly stained with primary antibodies in 2% BSA in PBS for 30 min. Following primary antibody incubation the cells were washed again and stained with Alexa Flour conjugated secondary antibodies (Invitrogen) for 30 min in 2% BSA in PBS. The cells were then washed and mounted onto slides using Prolong Antifade reagent with or without DAPI (4',6-diamidino-2-phenylindole dihydrocloride) (Invitrogen) and visualized using a Zeiss Axiovert 200 inverted microscope equipped with fluorescence optics. Images were then prepared using Photoshop and Illustrator (Adobe). The primary

antibodies used in this study are: human α-Mitochondria (Immunovision), mouse α-NP, mouse α-FLAG (Sigma), rabbit α-PB1-F2 OH07 (GenScript), rabbit α-PB1-F2 PR8 (Dr. Peter Palese, Mount Sinai School of Medicine New York, NY), and goat α-PB1 (SantaCruz Biotech).

Immunoblotting. Cells were seeded on 35-mm (2.5×10^5) or 60-mm (5.0×10^5) dishes the day before infection and incubated overnight at 37°C. Cells were harvested at indicated times and lysed with 100 □L 2X SDS protein loading buffer (125 mM Tris-HCl [pH 6.8], 200 mM DTT, 4% SDS, 0.2% Bromophenol blue, 20% Glycerol). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose by electroblotting in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol [pH 8.3]). Nitrocellulose containing transferred proteins was blocked for 15 min with 5% nonfat skim milk or 5% bovine serum albumin (BSA) as per antibody specification in Tris-buffered saline (20 mM Tris, 137 mM NaCl [pH 7.6]) containing 0.1% Tween-20 (TBS-T) and then incubated overnight with primary antibodies in TBS-T containing 1% milk or 5% BSA. Blots were washed three times for 15 min each with TBS-T, followed by 4 h incubation with AP-conjugated secondary antibodies in TBS-T containing 1% milk. Blots were washed three times and exposed to Western Lightning Plus enhanced chemiluminescence substrate (Perkin Elmer) or Lumi-Phos™ WB Chemiluminescent Substrate (Thermo Scientific). Images were collected using a ChemiDoc XRS camera (Bio-Rad), and protein bands were quantified using Quantity-One software (Bio-Rad). All experiments were independently performed at least 3 times and representative results are shown.

Plasmids. The full set of PR8 and MX/09 reverse genetics plasmids were obtained from Dr. Richard Webby and Dr. Amy Vincent, respectively. PR8, OH07, and MX/09 PB1 chimeric plasmids were created as described in results.

RESULTS

Objective 1. Elucidate the molecular properties of OH07 PB1-F2 protein *in vitro*.

SIV PB1-F2 protein is substantially divergent from previously studied PB1-F2 proteins. At the genesis of this project, the pandemic swine lineage influenza virus of 2009 was emerging, and it was determined that this virus was less pathogenic than previous

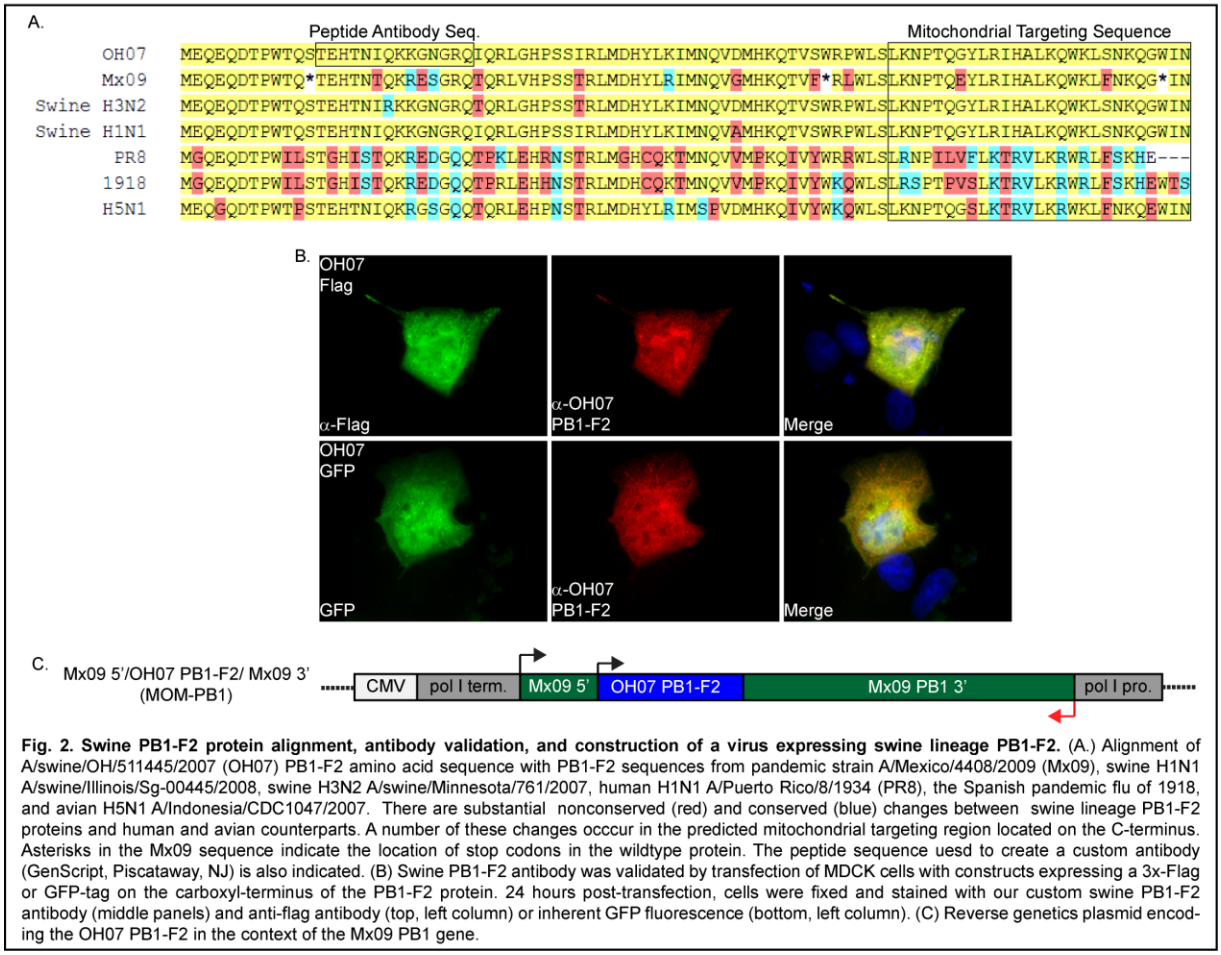

Fig. 2. Swine PB1-F2 protein alignment, antibody validation, and construction of a virus expressing swine lineage PB1-F2. (A.) Alignment of A/swine/OH/511445/2007 (OH07) PB1-F2 amino acid sequence with PB1-F2 sequences from pandemic strain A/Mexico/4408/2009 (Mx09), swine H1N1 A/swine/Illinois/Sg-00445/2008, swine H3N2 A/swine/Minnesota/761/2007, human H1N1 A/Puerto Rico/8/1934 (PR8), the Spanish pandemic flu of 1918, and avian H5N1 A/Indonesia/CDC1047/2007. There are substantial nonconserved (red) and conserved (blue) changes between swine lineage PB1-F2 proteins and human and avian counterparts. A number of these changes occcur in the predicted mitochondrial targeting region located on the C-terminus. Asterisks in the Mx09 sequence indicate the location of stop codons in the wildtype protein. The peptide sequence uesd to create a custom antibody (GenScript, Piscataway, NJ) is also indicated. (B) Swine PB1-F2 antibody was validated by transfection of MDCK cells with constructs expressing a 3x-Flag or GFP-tag on the carboxyl-terminus of the PB1-F2 protein. 24 hours post-transfection, cells were fixed and stained with our custom swine PB1-F2 antibody (middle panels) and anti-flag antibody (top, left column) or inherent GFP fluorescence (bottom, left column). (C) Reverse genetics plasmid encoding the OH07 PB1-F2 in the context of the Mx09 PB1 gene.

pandemic strains and also expressed a truncated (11 amino acids) PB1-F2 protein. The lack of PB1-F2 expression was postulated to contribute to lower pathogenicity of this virus. We compared protein sequences of swine lineage PB1-F2 with that of the predicted full-length pandemic PB1-F2 protein (Mx09), as well as previously studied human and avian lineage PB1-F2 proteins using L-align (EMBnet) software. We found that while the PB1-F2 protein from the strain utilized in our studies (OH07) is similar to other swine influenza isolates endemic to the United States including the predicted sequence of full-length pandemic PB1-F2 protein, there are a large number of sequence differences between OH07 PB1-F2 and other strains that have been previously studied, including PR8, the 1918 pandemic strain, and an avian H5N1 virus (Figure 2A).

To allow analysis of OH07 PB1-F2, an antibody was designed against a peptide sequence within the protein (GenScript, indicated in Figure 2A). This antibody was tested by immunofluorescence on MDCK cells transfected with constructs we created that contained either a FLAG or GFP tag fused in frame with the OH07 PB1-F2 carboxyl terminus (Figure 2B). In these experiments, FLAG and GFP expression and PB1-F2 staining using this antibody were found to overlap (Figure 2B). This data suggests that the antibody specifically recognizes OH07 PB1-F2.

OH07 PB1-F2 localization and expression levels in infected cells. Because previous data indicated that human and avian lineage PB1-F2 was a mitochondrial targeting protein, we examined the localization of swine PB1-F2 in infected cells. MDCK, PK-15, and A549 cells were infected with OH07 or PR8 viruses and stained for NP, PB1-F2, or mitochondria, with respective antibodies. We found that swine-derived PB1-F2 localizes primarily in the nucleus of infected cells. When comparing the localization of OH07 PB1-F2 with mitochondria staining, it is clear that PB1-F2 does not colocalize to areas in which mitochondrial staining is observed (Figure 3, middle rows). However, as previously reported, the PR8 PB1-F2 protein colocalizes with mitochondria in cells infected with the human lineage PR8 influenza isolate (Figure 3, top rows). These data indicate that unlike the previously

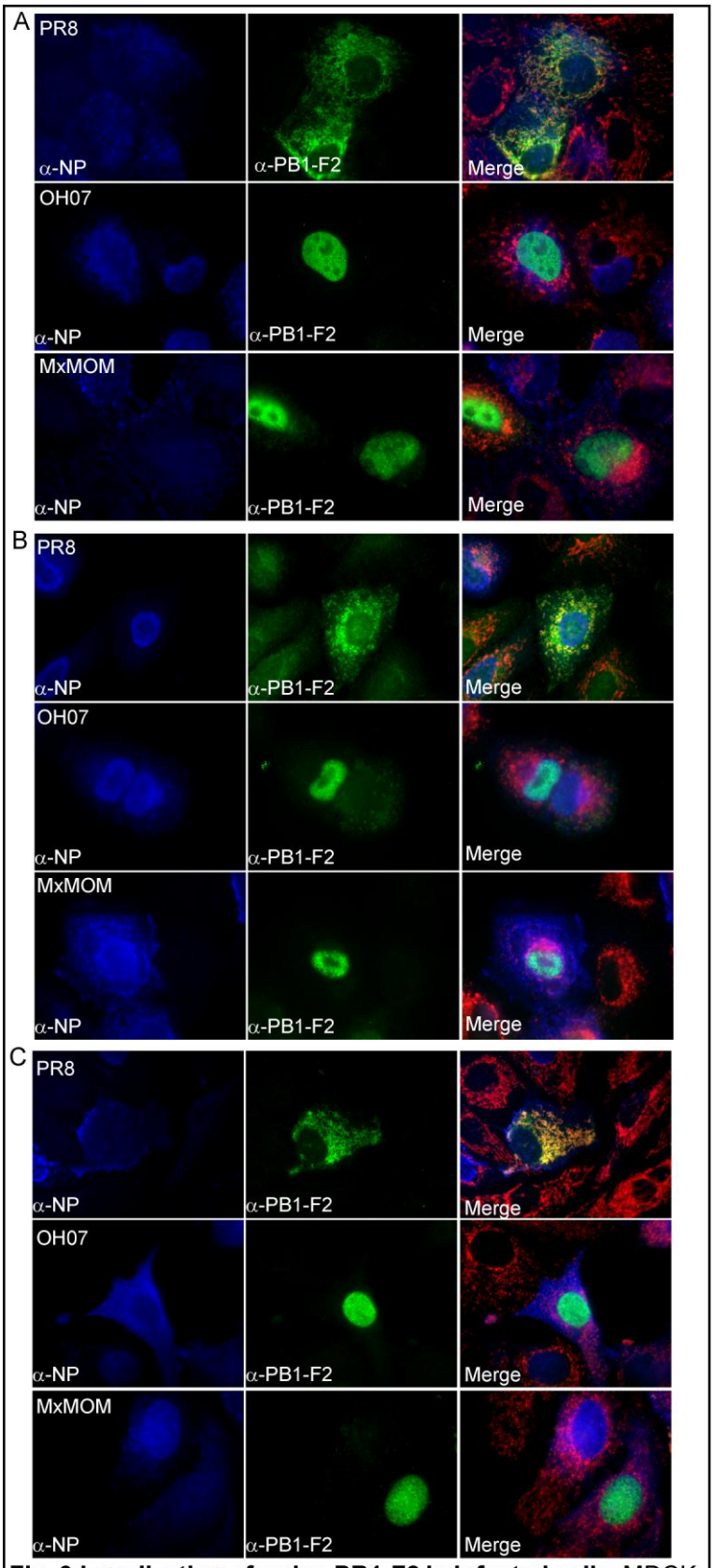

Fig. 3.Localization of swine PB1-F2 in infected cells. MDCK (A), A549 (B), and PK-15 (C) cells were infected with PR8 (top rows), OH07 (middle rows), and MxMOM (bottom rows). At 12 hours post-infection, cells were immunostained with NP (blue), PR8 or OH07 PB1-F2 (green), and mitochondrial (red) antibodies followed by Alexa fluor-conjugated secondary antibodies.

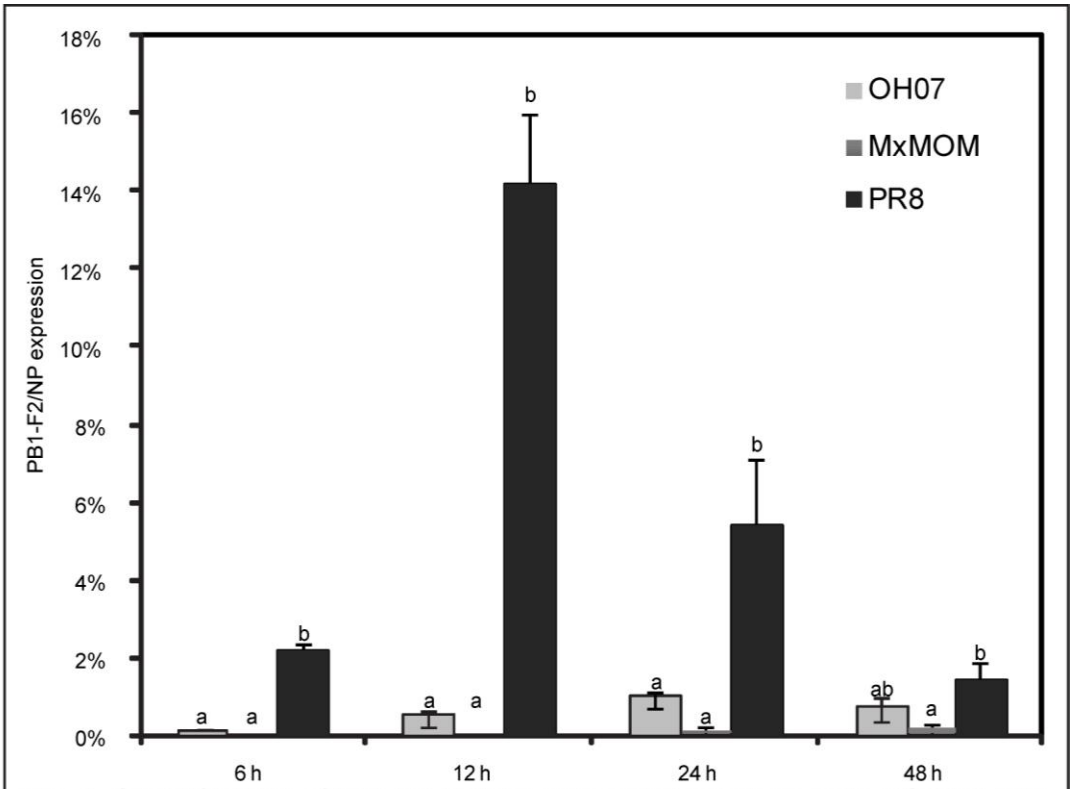

Fig. 4. Quantification of PB1-F2 protein expression levels in cells infected with human and swine lineage viruses. MDCK cells were infected with OH07, MxMOM, or PR8 viruses at a MOI of 2. At 6, 12, 24, and 48 h p.i. cells were immunostained with NP and PB1-F2 antibodies followed by Alexa fluor-conjugated secondary antibodies. NP and PB1-F2 expressing cells were counted and the percentage of PB1-F2 expressing infected cells was determined and plotted on a graph. Experiments were repeated three times and standard deviation and statistical significance were determined. Statistically significant differences are denoted by different letters.

studied human and avian lineage PB1-F2 proteins, swine PB1-F2 does not substantially localize to cellular mitochondria. Because PB1-F2 apoptosis likely depends on mitochondrial localization, this data suggests that swine lineage PB1-F2 may not possess the apoptosis capability reported for other PB1-F2 proteins.

In the process of examining the localization of swine lineage OH07 PB1-F2, we found that very few infected cells expressed the PB1-F2 protein relative to the previously studied human lineage PR8 PB1-F2 (Figure 4). This decrease in expression was found throughout virus infection and was cell-type independent. This decrease in PB1-F2 expression may occur via changes in mRNA expression, altered regulation of PB1-F2 translation, or increased PB1-F2 degradation. Because PB1-F2 is encoded from a second ORF in the same mRNA that codes for the polymerase protein PB1, it is highly unlikely that there are substantial differences in the transcription of this RNA during infection. Moreover, addition of proteasome inhibitors during infection was not able to rescue OH07 PB1-F2 expression (data not shown). This suggests that there are differences in the regulation of translation between some swine lineage PB1-F2 proteins and the human and avian lineage strains that have been studied to date.

PB1-F2 expression from an OH07/Mx09 chimeric virus. In addition to studying the PB1-F2 protein from the swine lineage OH07 virus, we wanted to determine if decreased translational regulation may also impact expression of the swine PB1-F2 protein from the 2009 pandemic virus. Although the pandemic virus encodes a truncated PB1-F2, rapid genetic change such that stop codons mutate to code for amino-acids may create a strain with the predicted capacity to express full-length PB1-F2. Our observations that OH07 does not express substantial PB1-F2 even though it codes for a full-length protein raise the possibility that the pandemic strain may also not express PB1-F2 even if mutated to do so. In fact, although several studies have been performed in which the pandemic virus was mutated such that it should express full-length PB1-F2, protein expression was not demonstrated (Hai, Schmolke et al. ; Ozawa, Basnet et al. ; Pena, Vincent et al.). OH07 PB1-F2 is highly homologous to the predicted expressed pandemic PB1-F2 protein, sharing 86% homology at the amino-acid level. This suggests it is a good surrogate for examining regulation of 2009 pandemic PB1-F2 expression. Unfortunately, we were unable to rescue OH07 virus using our recombinant plasmid system. We also could not examine the expression of PB1-F2 by creating a mutant pandemic virus that expressed the full-length PB1-F2

protein because the peptide used to produce our swine PB1-F2 antibody covers a rare region of poor homology between OH07 and the 2009 pandemic strains and therefore, the antibody is not predicted to recognize the pandemic PB1-F2 protein (Figure 2A). Therefore, in order to examine the expression of PB1-F2 in the background of the pandemic virus, we created a reverse genetics plasmid in which the OH07 PB1-F2 ORF was inserted into a pandemic strain A/Mexico/4408/2009 (Mx09) PB1 gene such that all of the sequences 5' and 3' of the PB1-F2 ORF were derived from the Mx09 strain while the PB1-F2 ORF sequence was derived from OH07 (see Figure 2C.). Recombinant viruses were rescued containing this 5' Mx09-OH07/PB1-F2-3' Mx09 (MOM) PB1 gene segment in which all other segments were derived from Mx09. Examination of PB1-F2 expression in cells infected with this virus revealed that similar to what we had seen with OH07, PB1-F2 was only minimally expressed and was primarily nuclear localized in the pandemic virus genetic background (Figure 3 and 4). This further suggests that some swine lineage PB1-F2 proteins are not produced at substantial levels, even though they appear genetically capable of being expressed.

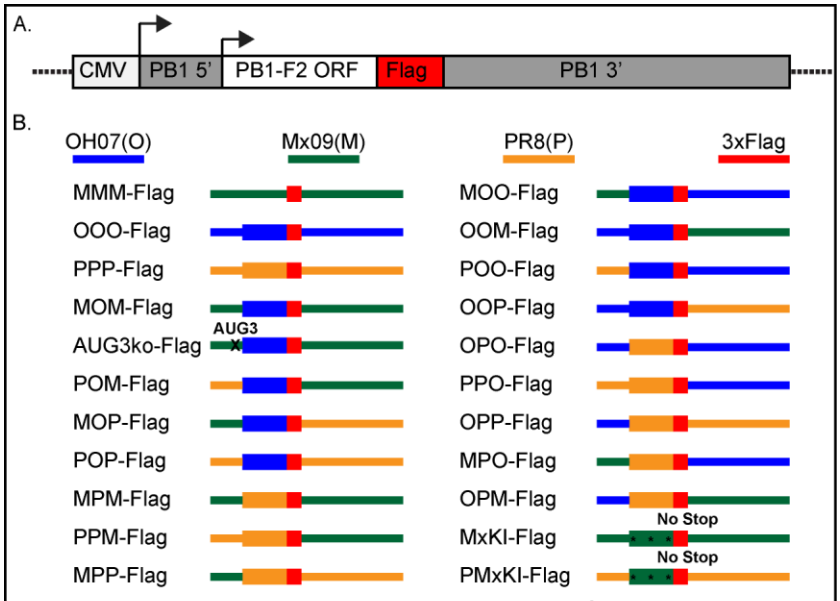

Fig. 5. Mapping PB1-F2 translational regulation using 3xflag-tagged PB1 chimeric genes. (A) Illustration of the strategy used to create chimeric PB1 genes expressing a 3xFlag PB1-F2 protein. Constructs divide the PB1 mRNA into the 5' RNA region prior to PB1-F2 ORF, the PB1-F2 ORF, and the 3' RNA region after the PB1-F2 ORF and will examine all possible combinations of the PB1 RNA from OH07, Mx09, and PR8 to identify sequences or structures influencing PB1-F2 translation. Each construct will express a full-length PB1-F2 protein tagged with flag. The flag-tag is inserted such that it does not interrupt expression of full-length PB1. (B). Constructs created or under construction using the strategy in A. Orange segments represent sequences from PR8, green segments represent sequences from Mx09, blue segments represent sequences from OH07, and red segments represent the 3X Flag tag.

Objective 2. Understanding the translational regulation of influenza PB1-F2

Regulation of swine lineage PB1-F2 protein translation. There are two primary methods of translational regulation, that of trans regulation by other proteins which might inhibit or activate translation via interaction with viral RNA, and that of cis regulation in which the viral RNA contains regulatory sequences that effect translation efficiency. Therefore, it was possible that PB1-F2 expression was regulated by another viral protein, and that this regulation was strain-specific. To examine this possibility, we created two additional recombinant viruses. One of these viruses was a monoreassortant which contains the PR8 PB1 (and PB1-F2 encoding) gene with all other genes from the Mx09 strain (PR8PB1/Mx). The other virus contains the MOM PB1 (and PB1-F2 encoding) gene combined with 7 PR8 genes (MOMPB1/PR8). Experiments were carried out in which cells were infected with PR8, PR8PB1/Mx, MOMPB1/Mx, or MOMPB1/PR8. At 12 h post-infection, cells were fixed and immunostained with antibodies against PB1-F2 and virus protein NP. The number of infected cells expressing PB1-F2 in each case was determined relative to total infected cells. In these experiments, the expression of PB1-F2 in MOMPB1/PR8 infected cells did not substantially differ from that expressed in MOMPB1/Mx infected cells (data not shown). Likewise, the expression of PB1-F2 in PR8PB1/Mx was similar to that in PR8 infected cells (data not shown). These data suggest that the regulation of PB1-F2 expression at the translational level is contained within the PB1 gene, and is not dependent on other viral proteins.

To examine cis regulation of PB1-F2, we needed to first design an experimental strategy that would accomplish two goals. First, because the PB1 gene encodes for a component of the influenza viral polymerase, there are potential differences in rates of infection and protein expression as a result of changes introduced to examine PB1-F2 expression into the PB1 gene. Therefore, we needed to prevent viral polymerase regulation of

PB1-F2 expression by removing our experiments out of the context of viral infection. Second, because of differences in amino-acid sequence within the high and low expressing PB1-F2 proteins we were studying it was necessary in our infection experiments to use different antibodies to PB1-F2 proteins. Because antibodies can bind proteins with different affinities under different preparation conditions, we needed to create a strategy in which the same antibody could be utilized to detect PB1-F2 expression levels from all influenza virus strains. To achieve both of these goals, we created plasmids that contained the full PB1 gene driven by the cytomegalovirus promoter (Figure 5A). Within the PB1-F2 ORF of this plasmid, we inserted a 3X Flag tag, such that any PB1-F2 protein that was expressed could be detected with the same FLAG-specific antibodies (Figure 5A). The FLAG tag was inserted such that no stop codons were introduced into the PB1 protein. In this way, we could use PB1 expression as an internal control for PB1-F2 expression. As a proof of concept for this strategy, we first created three plasmids that contained the PB1 gene from PR8 (PPP-Flag), OH07 (OOO-Flag), and the Mx09/OH07/Mx09 chimera (MOM-Flag) (Figure 5B). Each of these constructs was transfected into cells, and at 18 h post-transfection cells were counted based on PB1 and PB1-F2 Flag expression. The percent of PB1 expressing cells that also expressed PB1-F2 Flag was determined and plotted on a graph (Figure 6A). Similar to what we determined in infected cells, PPP-Flag expressed very high levels of PB1-F2, whereas OOO-Flag and MOM-Flag expressed only low levels of PB1-F2. These data provide support that our strategy for determining cis regulation of PB1-F2 translation mirrors what is seen in infected cells, further suggest that PB1-F2 translation is regulated in cis, and importantly, confirm our findings that suggest some swine lineage influenza viruses, likely including the recent 2009 pandemic strain, express only minimal levels of PB1-F2 even when they are mutated to contain the full-length PB1-F2 ORF.

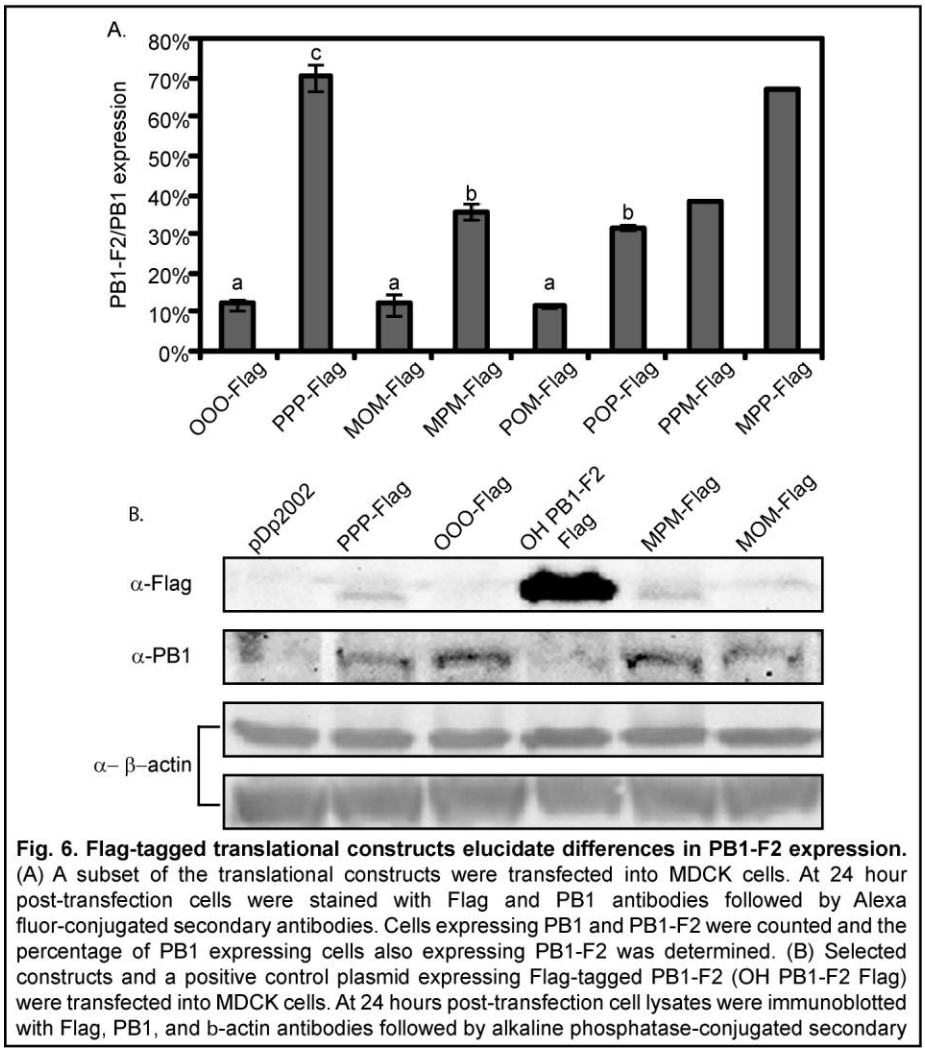

Fig. 6. Flag-tagged translational constructs elucidate differences in PB1-F2 expression. (A) A subset of the translational constructs were transfected into MDCK cells. At 24 hour post-transfection cells were stained with Flag and PB1 antibodies followed by Alexa fluor-conjugated secondary antibodies. Cells expressing PB1 and PB1-F2 were counted and the percentage of PB1 expressing cells also expressing PB1-F2 was determined. (B) Selected constructs and a positive control plasmid expressing Flag-tagged PB1-F2 (OH PB1-F2 Flag) were transfected into MDCK cells. At 24 hours post-transfection cell lysates were immunoblotted with Flag, PB1, and b-actin antibodies followed by alkaline phosphatase-conjugated secondary

The PB1-F2 ORF and 3'UTR sequences contribute to decreased protein translation. Translational regulation in cis can occur by a number of mechanisms. Because PB1-F2 translation initiation is thought to occur as a result of ribosomal leaky scanning (Chen, Calvo et al. 2001), it is possible that differences within the predicted Kozak sequence may impact the levels of PB1-F2 expression between virus strains. Alternatively, there are five AUG codons within the 5' end of the PB1 gene, and the presence of these codons has been shown to impact the levels of PB1-F2 expression in the PR8 PB1 gene (Wise, Foeglein et al. 2009). It is therefore possible that differences in leaky scanning among these AUG codons between viral strains may impact PB1-F2 expression. Moreover, many genes, particularly those of virus origin, have also been shown to contain sequences or structures that regulate translation of proteins from internal ORFs (Moss, Priore et al.), and it is

possible that this may occur within the PB1 gene, impacting PB1-F2 translation. To begin to identify the mechanism of cis regulation of PB1-F2 translation, we have created a number of additional chimeric constructs using our PB1/Flag gene approach. Each construct is designed such that it contains a 5' RNA portion prior to the PB1-F2 ORF, the PB1-F2 ORF, and the 3' RNA portion after the PB1-F2 ORF from either PR8, OH07, or Mx09. We have created constructs in which the sequences from these three strains were interchanged to make all possible combinations of chimeric PB1 genes (Figure 5B). The constructs are named based on the virus (PR8=P, Mx09=M, and OH07=O) from which each gene segment originated (eg. PPP is a construct in which the 5' RNA is derived from PR8, the PB1-F2 ORF is derived from PR8, and the 3' RNA is derived from PR8). Additionally, a construct expressing a mutated Mx09 mRNA that express the full length PB1-F2 is currently under construction (Figure 5B). We have completed a number of experiments in which selected constructs were transfected into MDCK cells to determine the impact of these three PB1 gene regions on PB1-F2 expression. These constructs include the parental plasmids already mentioned as well as MPM-Flag, POM-Flag, POP-Flag, PPM-Flag, and MPP-Flag. Transfected cells were immunostained for PB1 and PB1-F2 Flag expression and counted to determine the percentage of PB1 expressing cells that were also expressing PB1-F2-Flag. In these experiments, the POM-Flag construct expressed PB1-F2-Flag at levels similar to OOO-Flag and MOM-Flag (Figure 6A). This suggests that the 5' RNA portion of PR8 mRNA is not able to rescue expression of an otherwise swine lineage PB1-F2. This is an important finding because it largely rules out differences in the efficiency of leaky scanning with regard to initiation at the PB1-F2 AUG, as well as ruling out major impacts of three of the other AUGs located in the PB1 5' RNA region. This finding also suggests that an RNA sequence or structure located downstream of the PB1-F2 AUG is regulating PB1-F2 protein expression. In support of this, the MPM-Flag, POP-Flag, and PPM-Flag expressed significantly higher levels of PB1-F2 Flag expression then OOO-Flag and MOM-Flag. However, these levels were still substantially lower than what was seen with PPP-Flag. These data suggest that RNA from both the PR8 PB1-F2 ORF and the 3' UTR region contribute to upregulation of swine lineage PB1-F2 translation, but neither is sufficient to increase expression to full PPP-Flag levels. Interestingly, of clones tested so far, only the MPP-Flag clone expressed levels of swine lineage sequence containing PB1-F2 Flag protein that were comparable to PPP-Flag. This further suggests that there are sequences or a structure within the PB1-F2 ORF and 3' RNA that regulate expression of PB1-F2 translation.

Objective 3. Examine the role of PB1-F2 in viral replication and cell death in swine cells.
Objective 4. Determine the role of PB1-F2 on replication and pathogenicity of OH07 virus in an *in vivo* mouse model.

Once we fully understand the mechanism that controls the expression and subcellular localization of PB1-F2, we will create viruses in which localization and expression levels of PB1-F2 are modulated. These viruses will then be used in assays examining the effect PB1-F2 expression and localization has on replication and pathogenicity in cellular and animal models, allowing further definition of its role in the influenza virus lifecycle.

DISCUSSION

Since the discovery of influenza A virus's 11th protein, PB1-F2, very little experimental work has been done to understand the molecular properties of this protein isolated from natural reservoir species, particularly swine. Most published experimental work has focused on two lab-adapted strains, PR8 and WSN34. This focus may have inadvertently created a bias in our understanding of the phenotype of the protein that may not be indicative of wild-type isolates. In an effort to fill this gap we have examined a swine influenza isolate representative of those seen in nature. This isolate shares little homology in the PB1-F2 coding sequence to the influenza strains that have been previously studied, but is highly homologous to the PB1-F2 coding sequences of currently circulating SIV strains.

When swine, human, or dog cells were infected with OH07, we anticipated that the localization of the swine PB1-F2 protein would be similar to those described in avian and human influenza viruses. To our surprise the PB1-F2 protein in swine influenza infected cells appears to be localized, not to the mitochondria like has

previously been reported for PR8 and WSN34, but instead to the nucleus of infected cells. This differed from transfected cells, in which the protein was dispersed throughout the cell. We hypothesize this difference is likely the result of the presence of viral proteins sequestering PB1-F2 in the nucleus in infected cells. The difference in localization would suggest that there are potential differences in PB1-F2 function, in which the swine PB1-F2 protein may play a larger role in viral replication then it does in viral pathogenesis. The mechanism and consequence of mitochondrial versus nuclear PB1-F2 localization in influenza infection will be investigated in future studies.

An equally striking difference between previously studied PB1-F2 proteins and swine origin PB1-F2 proteins investigated in our studies was drastic differences in PB1-F2 protein expression levels. The human PB1-F2 expressing PR8 virus was found to express several fold higher levels of PB1-F2 protein when compared to the swine PB1-F2 expressing viruses. Such a difference in expression levels in wildtype viruses, to our knowledge, has not been previously described. These findings strongly suggest that PB1-F2 expression in influenza viral infection is likely strain-specific and that simply possessing the PB1-F2 ORF is not the sole determinant of PB1-F2 expression. Our findings additionally suggest that differences in PB1-F2 expression occur primarily at the translational level, with little to no regulation occurring through the proteasome degradation pathway or transcriptional regulation.

Utilizing translational clones to examine what dictates PB1-F2 expression, we have shown that the 5′ region prior to the PB1-F2 ORF appears to have little effect on the translation regulation of the PB1-F2 protein. This result is somewhat surprising because the PB1-F2 protein is thought to be expressed through ribosomal scanning of the PB1 mRNA (Wise, Foeglein et al. 2009), which would make the upstream sequence a prime target for translational regulation. However, sequence in the PB1-F2 ORF and 3′ region after the PB1-F2 ORF appear to play a significant role in regulating PB1-F2 expression and independently resulted in a two- to three-fold difference in PB1-F2 expression and up to a six-fold difference when combined, with higher levels being associated with human influenza PB1 sequence and lower levels being associated with swine PB1 sequence in these regions. It is unclear at this time if these two elements work to regulate PB1-F2 expression independently or together, although this will be the focus of future study. Moreover, whether these elements work at the initiation or elongation step of translation will also be determined in future work.

With this data we plan to further map the identified regions to a minimal sequence that is both necessary and sufficient for PB1-F2 translational regulation. Once identified, this sequence will be incorporated into both the PR8 and Mx09 reverse genetic systems to modulate the expression of the PB1-F2 protein and examine the impact of PB1-F2 in swine influenza viruses. We will additionally determine the molecular mechanism used to regulate PB1-F2 by investigating potential interaction of the RNA elements with each other and cellular and viral regulatory proteins. With this data we will be able to more accurately assess the potential risk that PB1-F2 poses for both human and swine population as well as better understand and predict the translation of PB1-F2 in influenza A viruses.

REFERENCES

"CDC 2009, posting date. 2009 H1N1 flu: International Situation Update."

Belser, J. A., D. A. Wadford, et al. "Pathogenesis of Pandemic Influenza A (H1N1) and Triple-Reassortant Swine Influenza A (H1) Viruses in Mice." J Virol.

Chen, W., P. A. Calvo, et al. (2001). "A novel influenza A virus mitochondrial protein that induces cell death." Nat Med **7**(12): 1306-1312.

Chen, W., P. A. Calvo, et al. (2001). "A novel influenza A virus mitochondrial protein that induces cell death." Nat Med **7**(12): 1306-1312.

Coleman, J. R. (2007). "The PB1-F2 protein of Influenza A virus: increasing pathogenicity by disrupting alveolar macrophages." Virol J **4**: 9.

Garten, R. J., C. T. Davis, et al. (2009). "Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans." Science **325**(5937): 197-201.

Hai, R., M. Schmolke, et al. "PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models." J Virol **84**(9): 4442-4450.
Hai, R., M. Schmolke, et al. "PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models." J Virol.
Itoh, Y., K. Shinya, et al. (2009). "In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses." Nature **460**(7258): 1021-1025.
Lange, E., D. Kalthoff, et al. (2009). "Pathogenesis and transmission of the novel swine-origin influenza virus A/H1N1 after experimental infection of pigs." J Gen Virol **90**(Pt 9): 2119-2123.
Moss, W. N., S. F. Priore, et al. "Identification of potential conserved RNA secondary structure throughout influenza A coding regions." RNA **17**(6): 991-1011.
Ozawa, M., S. Basnet, et al. "Impact of amino acid mutations in PB2, PB1-F2, and NS1 on the replication and pathogenicity of pandemic (H1N1) 2009 influenza viruses." J Virol **85**(9): 4596-4601.
Pena, L., A. L. Vincent, et al. "Restoring PB1-F2 into the 2009 Pandemic H1N1 Influenza Virus has minimal effects in swine." J Virol.
Vincent A. L., S., S. L., Lager, K. M., Gauger, P.C., Loiacono, C., and Y. Zhang (2009). "Characterization of an influenza A virus isolated from pigs during an outbreak of respiratory disease in swine and people during a county fair in the United States." Veterinary Microbiology **Available online 6 Jan. 2009**.
Vincent, A. L., K. M. Lager, et al. (2009). "Absence of 2009 pandemic H1N1 influenza A virus in fresh pork." PLoS One **4**(12): e8367.
Vincent, A. L., W. Ma, et al. (2008). "Swine influenza viruses a North American perspective." Adv Virus Res **72**: 127-154.
Wise, H. M., A. Foeglein, et al. (2009). "A complicated message: identification of a novel PB1-related protein translated from influenza A segment 2 mRNA." J. Virol.: JVI.00826-00809.
Yamada, H., R. Chounan, et al. (2004). "Mitochondrial targeting sequence of the influenza A virus PB1-F2 protein and its function in mitochondria." FEBS Lett **578**(3): 331-336.
Zamarin, D., A. Garcia-Sastre, et al. (2005). "Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1." PLoS Pathog **1**(1): e4.
Zamarin, D., M. B. Ortigoza, et al. (2006). "Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice." J Virol **80**(16): 7976-7983.
Zell, R., A. Krumbholz, et al. (2007). "Prevalence of PB1-F2 of influenza A viruses." J Gen Virol **88**(Pt 2): 536-546.