The Use of Reverse Genetics to Clone and Rescue Infectious, Recombinant Human Parainfluenza Type 3 Viruses

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THE USE OF REVERSE GENETICS TO CLONE AND RESCUE INFECTIOUS,  
RECOMBINANT HUMAN PARAINFLUENZA TYPE 3 VIRUSES  

by 

Jason P. Roth 

A dissertation submitted in partial fulfillment  
of the requirements for the degree  
of  
DOCTOR OF PHILOSOPHY  
in  
Bioveterinary Science  

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2009
ABSTRACT

The Use of Reverse Genetics to Clone and Rescue Infectious, Recombinant Human Parainfluenza Type 3 Viruses

by

Jason P. Roth, Doctor of Philosophy
Utah State University, 2009

Major Professor: Dr. Dale L. Barnard
Department: Animal, Dairy, and Veterinary Sciences

Reverse genetics is a discipline that involves the use of genetic manipulation and modification to study an organism’s altered phenotype. In this study, infectious recombinant viruses were rescued from altered cDNA clones encoding the antigenome of human parainfluenza virus type 3 and the resulting phenotypes were examined. In one clone, the gene for the enhanced green fluorescent protein was inserted into the virus antigenome to be expressed during viral replication, resulting in infected cells emitting green fluorescence. Viral titers, mRNA replication, and genomic replication for the virus expressing the enhanced green fluorescent protein were reduced when compared to the human parainfluenza virus type 3 wild-type strain. In addition, the sensitivity of the virus expressing the enhanced green fluorescent protein to antiviral compounds is increased when compared to the wild-type strain, which may lead to the identification of false positive antiviral compounds. An assay that measures the enhanced green fluorescent protein as a direct indicator of virus replication can be shortened to 3 days in duration and
is a more robust assay compared to assays that measure cellular viability. In other clones, mutations were introduced into the phosphoprotein gene to eliminate the expression of the D domain of the PD protein in order to understand its function. The titers of two recombinant knockout viruses that are deficient in the expression of the D domain are reduced when compared to the wild-type strain in both MA-104 and A549 cells. In MA-104 cells, viral mRNA transcription and genomic replication of the two knockout viruses are reduced when compared to the wild-type strain. In A549 cells, cellular expression and secretion of antiviral cytokines infected with the two knockout viruses are either reduced or remain unchanged when compared to the wild-type strain. These results suggest that the D domain may play a role in viral RNA synthesis and not in counteracting the host cell’s antiviral response. The results of these studies shed light on the influence an additional gene has on viral replication and possible functions of the D domain.

(166 pages)
To Malinda, Cy, and Dawson who supported me through this endeavor blindly.
ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Dale Barnard, who gave me the idea in the beginning and many more ideas along the way. Thank you for your expertise and sound advice with my difficult questions. Most importantly, thank you for all the help with my editorial deficiencies. Also, thank you for the extra time and patience when I needed them the most.

I would also like to thank my committee members, Dr. John Morrey, Dr. Joseph Li., Dr. Steven Aust, and Dr. Thomas Bunch, for all of their helpful insights and critical feedback on tough issues. Also, thank you for pushing me to my limits. Thanks to the ADVS department, faculty, and staff for their administrative help and patience. I would also like to thank Dr. Smee, Dr. Morrey, and Dr. Sidwell for the financial support through contract N01 AI-30048 from the Virology Branch, NIAID, NIH.

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Last, but not least, thank you to my family who has endured this difficult road with me and has not looked back. Special thanks to my wife; without you none of this would have happened. Thank you for your patience and understanding along the way.
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Jason P. Roth
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<td>HPIV-3</td>
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<td>NP</td>
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<td></td>
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<tr>
<td>P</td>
<td>phosphoprotein</td>
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</tr>
<tr>
<td>M</td>
<td>matrix protein</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>fusion protein</td>
<td></td>
</tr>
<tr>
<td>HN</td>
<td>hemagglutinin-neuraminidase protein</td>
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</tr>
<tr>
<td>L</td>
<td>large protein (polymerase)</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>guanosine</td>
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<tr>
<td>rHPIV3</td>
<td>recombinant HPIV-3</td>
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<td>WT</td>
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<td>EGFP</td>
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<td>QRT-PCR</td>
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<td>CPE</td>
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<td>--------------</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>SV40</td>
<td>simian virus 40</td>
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<td>SARS</td>
<td>severe acute respiratory syndrome</td>
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<td>UTR</td>
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<td>CAT</td>
<td>chloramphenicol acetyl transferase</td>
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<tr>
<td>ES</td>
<td>editing site</td>
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<td>VSV</td>
<td>vesicular stomatitis virus</td>
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<td>MeV</td>
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<td>SeV</td>
<td>Sendai virus</td>
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<td>RSV</td>
<td>respiratory syncytial virus</td>
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<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>H</td>
<td>hemagglutinin</td>
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<tr>
<td>A</td>
<td>adenosine</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<td>lactate dehydrogenase</td>
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<td>2-p-(iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>human parainfluenza virus type 1</td>
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<td>CARD</td>
<td>caspase recruitment domain</td>
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<tr>
<td>MAVS</td>
<td>mitochondrial antiviral signaling protein</td>
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<td>IPS-1</td>
<td>interferon-beta promoter stimulator 1</td>
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<td>TANK</td>
<td>TRAF family member-associated NT-κB activator</td>
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<td>TANK binding kinase 1</td>
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<td>IKKe</td>
<td>IkappaB kinase</td>
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<td>regulated on activation normal T cell expressed and secreted</td>
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<td>IFNAR</td>
<td>IFN receptors</td>
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<td>JAK-STAT</td>
<td>Janus kinase-signal transducers and activators of transcription</td>
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<td>ISGF-3</td>
<td>IFN-stimulated gene factor 3</td>
<td></td>
</tr>
<tr>
<td>ISRE</td>
<td>IFN-stimulated response elements</td>
<td></td>
</tr>
<tr>
<td>ISG</td>
<td>IFN-stimulated genes</td>
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</tr>
<tr>
<td>SI</td>
<td>selective index</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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</tr>
<tr>
<td>HeLa</td>
<td>Human cervical carcinoma cells</td>
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</tr>
<tr>
<td>MA-104</td>
<td>Embryonic African green monkey kidney cells</td>
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</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
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</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>2-thio-6-azauridine</td>
<td>2-[(2R,3R,4S,5R)-3,4-Dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-3-sulfanylidene-1,2,4-triazin-5-one</td>
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<td>ribavirin</td>
<td>1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2,4-triazole-3-carboxamide</td>
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ribamidine 1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2,4-triazole-3-carboximidamide

selenazofurin 2-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,3-selenazole-4-carboxamide

EICAR 1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-5-ethynylimidazole-4-carboxamide

3-deazaguanosine 6-amino-1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-5H-imidazo[4,5-c]pyridin-4-one

NEAA non-essential amino acids

RT-PCR reverse transcriptase-polymerase chain reaction

PCR polymerase chain reaction

PFU plaque forming units

MOI multiplicity of infection

Ara-C cytosine β-D-arabinofuranoside

ANOVA analysis of variance

RT reverse transcriptase

FAM 6-carboxy-fluorescein

EC₅₀ 50% effective concentration

DMSO dimethyl sulfoxide

IC₅₀ 50% inhibitory concentration

RACE rapid amplification of cDNA ends

S.D. standard deviation
<table>
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<th>Full Name</th>
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<tr>
<td>BPIV-3</td>
<td>bovine parainfluenza virus type 3</td>
</tr>
<tr>
<td>A549</td>
<td>Human lung epithelial carcinoma cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered phosphate</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inhibitory protein</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>EO</td>
<td>eotaxin</td>
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CHAPTER 1
INTRODUCTION

Reverse genetics is a discipline that involves the use of genetic manipulation and modification to study an organism’s altered phenotype. Reverse genetics has been particularly useful when studying viruses because of the ease of manipulation, due to their relatively small genomes, and their short and quick replication rates leading to rapid phenotypic expression. To study RNA viruses by reverse genetics, their complete genomes must first be reverse transcribed into a cDNA clone, which can then be manipulated in many ways. To rescue or create an infectious, recombinant RNA virus, the cDNA clone must be converted back into RNA. Negative-sense RNA viruses are problematic because their viral RNA lacks the signals recognized by eukaryotic host cells for transcription and translation. Therefore, no viral positive or negative-sense genomic RNA strands, viral mRNA strands, or viral proteins are produced from transfected naked negative-sense viral genomic RNA alone to induce an infection. To circumvent these problems, viral proteins necessary for viral mRNA transcription and genomic replication, which are supplied by the virus during normal replication, need to be present during the artificial rescue of an RNA virus from a cDNA clone. Once an infectious, negative-sense RNA virus has been rescued, the phenotype of the recombinant virus can be studied.

Human parainfluenza virus type 3 (HPIV-3), a non-segmented negative-sense RNA virus, was chosen for this project. HPIV-3 has a genome length of 15,462 nt and encodes eight known proteins, nucleocapsid protein (NP), phosphoprotein (P), C protein, PD protein, matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein
(HN), and large protein (L). In the middle of the P gene, an editing site exists where the viral polymerase, L, stops and stutters, adding non-templated guanosine (G) residues. This leads to the expression of three fusion proteins with similar N-terminal ends and differing C-terminal ends; P, PD, the putative W protein, and perhaps a theoretical V protein. The NP, P and L proteins, which are necessary for all aspects of viral RNA synthesis, were present for the successful rescue of the recombinant HPIV-3 cDNA clones used in the present study.

In the current study, reverse genetics was used to construct an infectious, recombinant (r)HPIV3 clone, which contained three genetic markers to distinguish the recombinant viruses from the wild-type (WT) virus and served as the backbone for all other recombinant viruses. In the first phase of this study, the gene for the enhanced green fluorescent protein (EGFP) was inserted into the rHPIV3 antigenome (rHPIV3-EGFP) and used as a marker for active viral replication. The hypothesis for this phase of the research was that the expression of the additional gene from the viral genome would not attenuate the rHPIV3-EGFP virus; the replication rates and antiviral sensitivity profiles would be similar to the rHPIV3 and HPIV-3 WT viruses. The following aims were used to test the hypothesis:

1. To detect any differences in the replication rates of the three viruses, HPIV-3 WT, rHPIV-3, and rHPIV3-EGFP, viral titers and replication curves are obtained for each virus.

2. To detect any differences in viral mRNA transcription and viral genomic replication rates between HPIV-3 WT and rHPIV3-EGFP, each process was measured by quantitative reverse transcription polymerase chain reaction (QRT-PCR).
3. To detect any differences in antiviral sensitivity and cytopathic effect (CPE) between HPIV-3 WT and rHPIV3-EGFP, the sensitivity of each virus to antiviral compounds and CPE was measured by neutral red (NR) absorption.

4. To investigate the possibility of substituting the HPIV-3 WT virus with rHPIV3-EGFP virus in antiviral screening, viral expressed EGFP was measured and evaluated against three cellular viability detection assays. In addition, a panel of antiviral compounds, known to inhibit HPIV-3, was used to inhibit the rHPIV3-EGFP virus and viral replication was assayed both by EGFP fluorescence and NR dye uptake assays.

In the second phase of this study, an rHPIV3 virus (rHPIV3-NT) was constructed to express a hexahistidine tag on the C-terminal end of the P protein to trace all proteins expressed from the P gene start codon. In addition, two other rHPIV3 viruses were constructed using the rHPIV3-NT cDNA backbone; one that eliminated the editing site in the P gene (rHPIV3-ΔES) and a second that was deficient in the expression of the D domain by a nonsense mutation (rHPIV3-ΔD). The hypothesis for this second study was that the V protein is expressed from the P gene and that the D domain counteracts the host cell’s antiviral response and inhibits the expression of interferon (IFN)-β. The following aims were used to test this hypothesis:

1. To detect proteins expressed from the P gene start codon, the hexahistidine tagged proteins in rHPIV3-NT virus lysates were detected with an anti-hexahistidine antibody conjugated to horseradish peroxidase (HRP).
2. To detect any differences in the replication rates of the three viruses, rHPIV3-NT, rHPIV3-ΔES, and rHPIV3-ΔD, viral titers and replication curves are obtained for each virus replicated in both MA-104 and A549 cells.

3. To detect any differences in viral mRNA transcription and viral genomic replication rates between rHPIV3-NT, rHPIV3-ΔES, and rHPIV3-ΔD in MA-104 cells, each process was measured by QRT-PCR.

4. To detect any differences in cytokine expression from A549 cells infected with rHPIV3-NT, rHPIV3-ΔES, or rHPIV3-ΔD, the extracellular levels of cytokines secreted from infected A549 cells were measured by enzyme-linked immunosorbent assay (ELISA).
1. **Forward vs. reverse genetics**

Forward genetics, or classical genetics, is a discipline that involves the study of an organism’s phenotype by naturally manipulating the organism’s genotype. Gregor Mendel, “The Father of Genetics,” was the first scientist to recognize that phenotypic traits of pea plants followed Laws of Inheritance, which are passed on through genes (Mendel, 1866). Even though these laws do not govern the world of viruses, the principles he laid out can be applied to viruses. Generally, each virus has a specific niche where it grows most efficiently, but when the virus is transferred to a hostile environment a small population of viruses may be able to replicate and survive in the new environment. For example, drug-resistant mutant viruses arise when the WT virus is replicated in cell culture in the presence of antiviral compounds. After several passages of increasing drug concentration, the new drug-resistant mutant virus may replicate normally in the presence of the antiviral compound at high concentrations; whereas the WT virus can not. Another example is when a WT virus is replicated through several passages in cell culture at a lower temperature than normal. In this case, the new cold-adapted, temperature-sensitive mutant virus may not replicate at the original or normal temperature, whereas the WT virus can replicate at both temperatures. In both cases, the new phenotype of the mutant viruses can be traced back to natural mutations located in the viral genome.
On the other hand, the discipline of reverse genetics involves the deliberate manipulation of an organism’s genome to study its phenotype. The “Central Dogma” of gene expression starts with an organism’s genetic material, which is transcribed into mRNA and then translated into protein. The multitudes of proteins expressed by an organism are what ultimately interact with each other in complex mechanisms and lead to the organism’s phenotype. The use of reverse genetics to study viral phenotypes can be particularly useful because of the ease of manipulation, due to their relatively small genomes, and their short and quick replication rates leading to rapid phenotypic expression. The largest obstacle to overcome when using reverse genetics to study viruses is the successful rescue of an infectious, recombinant virus.

2. **Rescuing infectious, recombinant viruses**

2.1. **DNA viruses**

Viruses containing DNA genomes were the first to be genetically altered using full-length DNA clones and homologous recombination technologies. The first virus, whose manipulated DNA genome produced infectious, recombinant progeny, was simian virus 40 (SV40) (Goff and Berg, 1976). SV40 was an excellent model to start the recombinant virus revolution because of its small genome size of 5243 nt, therefore it was relatively easy to sequence and manipulate. Also, the DNA genome of SV40 is infectious alone because it encodes eukaryotic promoters and is transcribed and replicated in the nucleus using the host cell’s replication and transcription machinery (Barbanti-Brodano et al., 1970; Buchman et al., 1981). The genome of SV40 resembles
eukaryotic DNA so much that it has served as an excellent model to study and understand eukaryotic gene regulation, mRNA transcription, and DNA replication.

In contrast, the genome sizes of most DNA viruses are too large to clone and manipulate directly, so the use of homologous recombination has been used to manipulate larger DNA viruses. To genetically modify the genomes of herpes, pox, and adenoviruses, intact viral DNA is co-transfected with recombinant DNA, which is surrounded by viral sequences (Jones and Shenk, 1978; Mackett et al., 1982; Post and Roizman, 1981). The result is homologous recombination between the two DNA strands and recovery of a mixture of wild-type and recombinant viruses, which can be separated using screening methods.

2.2. RNA Viruses

2.2.1 Positive-sense single-stranded RNA viruses

Positive-sense single-stranded (ss)RNA viruses have the distinct advantage of being in the same sense as mRNA, positive-sense by definition, therefore the viral genomes are recognized by the host cell’s transcriptional machinery, which is used for virus replication during a natural infection. The genomic RNA of positive-sense viruses with small-to-medium sized genomes is infectious alone and results in the formation of infectious virus progeny. To genetically manipulate these viruses, the RNA genome must be converted into a cDNA clone first, which encodes the viral genome, and then it can be modified using molecular biology techniques. Recombinant viral genomic RNA can then be copied from the modified cDNA clone and transfected into cells where infectious,
recombinant virus progeny are recovered. For example, the bacteriophage Qβ was the first ssRNA virus to be rescued from a recombinant source, followed by Sindbis and Semliki forest viruses (Liljestrom et al., 1991; Rice et al., 1987; Taniguchi et al., 1978). Notably, poliovirus can be rescued from transfected cDNA clones alone, without the need to transcribe and transfect the viral RNA genomic intermediate (Racaniello and Baltimore, 1981).

Positive-sense, ssRNA viruses with larger genomes will encapsidate their RNA genomes with viral protein for protection. For example a recombinant severe acute respiratory syndrome (SARS) virus can only be successfully rescued in the presence of the viral nucleocapsid protein (Yount et al., 2003). In addition, positive-sense, single-stranded retroviruses are unique in that their virus genome is replicated through a double-stranded DNA intermediate and integrates into the host cell’s genome. Therefore, transfection of the full-length viral cDNA genome alone leads to the rescue of infectious, recombinant virus progeny (Wei et al., 1981). Taking advantage of the fact that retroviral genomes integrate in the host cell’s genome, recombinant retroviruses have been widely used for eukaryotic gene expression and gene therapy.

2.2.2  Non-segmented, negative-sense ssRNA viruses

Compared to positive-sense ssRNA viruses, the ability to rescue infectious, recombinant, non-segmented, negative-sense ssRNA viruses are more complex. Not only do these viruses replicate in the cytoplasm of the infected cells, but the negative-sense RNA genome is not recognized by the host cell’s transcriptional machinery because of its negative polarity (Friedman et al., 1981). Therefore, the viral proteins necessary for viral
RNA synthesis are packaged into the virion in active transcriptase-replicase complexes for immediate replication upon infection (Storey et al., 1984). This means that during the rescue of a recombinant negative-sense virus all necessary components for viral replication need to be present, which include the viral RNA genome, NP proteins, P proteins, and L proteins. The NP proteins encapsidate the single-stranded viral RNA genome, not only for protection but they also interact with themselves to facilitate assembly of the helical nucleocapsids (Buchholz et al., 1993). In addition, the NP protein interacts with the P protein during all aspects of viral RNA synthesis and the M protein during virion packaging (Coronel et al., 2001; Curran et al., 1993). The P protein acts as a cofactor that facilitates the interaction of the viral L protein with the encapsidated viral RNA during viral RNA synthesis (Horikami et al., 1992). While the C-terminal end of the P protein is necessary for viral RNA synthesis, a domain on the N-terminal end of the P protein chaperones newly synthesized NP proteins to encapsidate the naked viral genomic RNA (Fig. 2-1) (Curran et al., 1995). The L protein is the last and largest viral protein expressed from the genome and is solely responsible for RNA polymerase catalytic activity (Poch et al., 1990).

During the infancy of rescuing negative-sense ssRNA viruses, a basic replication system involving minigenomes was set up to optimize the conditions of rescuing infectious virus from cDNA. Minigenomes contain the viral 3’ and 5’ untranslated regions (UTRs) of a particular virus flanking a single reporter gene, such as chloramphenicol acetyl transferase (CAT) or luciferase, to measure viral RNA synthesis. Initial experiments used this system to confirm that the presence of the NP, P, and L proteins are required for CAT or luciferase expression and activity, although each study
reported using differing ratios of the three NP, P, and L support plasmids (Durbin et al., 1997b; Kato et al., 1996). Minigenomes also aided in the application of the “Rule of Six” in the recovery of infectious, recombinant ssRNA viruses. The “Rule of Six” suggests that viral replication is most efficient when the length of the viral genomes is a factor of six ribonucleotides, most likely due to a single nucleocapsid protein binding to six genomic ribonucleotides (Calain and Roux, 1993). Minigenomes that were a factor of six ribonucleotides exhibited greater CAT activity compared to minigenomes in any of the other five possibilities (Durbin et al., 1997b).

Furthermore, the transfection of the viral genome in cDNA form will not induce the rescue of a virus. The cDNA clone needs to be transcribed into viral genomic RNA so viral NP, P, L proteins can interact with it and induce the rescue of the recombinant virus. Therefore, the eukaryotic cells that will host the rescue operation are infected with a vaccinia virus that has been engineered to express a T7 RNA polymerase, vTF7-3 (Fig. 2-1. Schematic of the HPIV-3 phosphoprotein. Three domains in the phosphoprotein (P) include the interaction with unincorporated nucleocapsid (NP*) for viral genomic RNA encapsidation and interaction with the large protein (L) and encapsidated viral RNA genome (NP:RNA) for viral RNA synthesis. An editing site (ES) exists in the middle of the P gene where the viral polymerase adds additional non-templated G residues. This leads to the expression of three fusion proteins, P, PD, W, and perhaps the hypothetical V*. The C protein is translated by ribosomal choice.)
To control and inhibit the replication of vTF7-3, which may interfere with the rescue of the RNA virus, and select for the recombinant RNA virus, the cytosine β-D-arabinofuranoside (Ara-C) and/or rifampicin antiviral compounds are added to the infected cells (Kato et al., 1996). In addition, the host cells are co-transfected with the viral genomic cDNA plasmid and support plasmids that contain the genes for the viral NP, P, and L proteins, whose transcription is driven by a T7 promoter. The mRNA transcribed from the three support plasmids is further translated into the NP, P, and L proteins. On the other hand, the T7 RNA polymerase will transcribe the viral genomic cDNA plasmid into an RNA strand that is longer than the actual viral RNA genome should be. To transcribe the viral RNA genome to the proper length and a factor of six ribonucleotides, the T7 promoter is strategically placed immediately upstream of the first nucleotide of the viral genome, separated by two G nucleotides (Durbin et al., 1997a). On the other end of the genome, an antigenomic hepatitis delta virus ribozyme is positioned immediately following the last nucleotide of the viral genome (Perrotta and Been, 1991). The ribozyme will self-cleave itself from the viral RNA genome leaving the full-length virus RNA genome intact. Another important aspect of the viral RNA genome during the rescue of a negative-sense RNA virus is the polarity of the viral cDNA clone. Infectious, recombinant ssRNA viruses that were rescued from positive-sense, antigenomic cDNA clones resulted in higher titers than ssRNA viruses rescued from negative-sense, genomic cDNA clones, even though the viral RNA genome was itself negative-sense (Durbin et al., 1997a; Kato et al., 1996). With all of the factors in place, which includes the expression of the viral NP, P, and L proteins and the transcription of the positive-sense viral RNA antigenome that has been excised to the
Fig. 2-2. Methodology for rescuing a non-segmented, negative-sense RNA virus.
1. Infection with the vaccinia virus vTF7-3, which expresses a T7 RNA polymerase.
2. Transfection and transcription of four plasmids encoding an rHPIV3 cDNA clone, nucleocapsid protein (NP), large protein (L), and phosphoprotein (P) driven by a T7 promoter. NP, L, and P subsequently translated into proteins.
3. Encapsidation of the genomic RNA by NP.
4. Assembly of an active transcriptase-replicase complex through the interaction of the L, P, and encapsidated RNA.
5. Natural infection induced and replication of the virus takes place.
6. Infectious virions bud out of the plasma membrane of a eukaryotic cell.
proper length, virus replication should be induced and infectious, recombinant virus
progeny should be released from the infected cells (Fig. 2-2).

Several infectious recombinant viruses have been successfully rescued from
cDNA clones, starting with the first negative-sense ssRNA virus, rabies, followed by
vesicular stomatitis virus (VSV), measles virus (MeV), Sendai virus (SeV), and HPIV-3
(Durbin et al., 1997a; Garcin et al., 1995; Lawson et al., 1995; Radecke et al., 1995;
Schnell et al., 1994). In addition, the human respiratory syncytial virus (RSV) expresses
the M2 protein that is also necessary for viral RNA synthesis and is needed for the
successful rescue of an infectious, recombinant RSV virus (Collins et al., 1995).

2.2.3 Segmented, negative-sense ssRNA
viruses

The techniques used to successfully rescue segmented, negative-sense ssRNA
viruses are even more complex because of the multiple genomic segments. The first
successful rescue of a recombinant segmented virus used minigenomes to manipulate
individual segments and rescued recombinant viruses with only one altered segment,
leaving the other segments in natural form. First, the cDNA clone for one segment was
cloned, manipulated, and transcribed into RNA in vitro. Next, the RNA segment was
reconstituted with nucleoprotein and polymerase protein to form a ribonucleoprotein in
vitro. Finally, the ribonucleoprotein was transfected into cells infected with a helper
virus that provided all other proteins and segments necessary for virus replication to
rescue infectious virions (Enami et al., 1990). A drawback to this technique is the
presence of two viruses, the helper virus and the recombinant virus, but with creative
engineering the recombinant virus can be selected for (Enami and Palese, 1991). In
addition, the first segmented virus to be completely rescued from cDNA clones was the Bunyamwera virus that contains three genomic segments (Bridgen and Elliott, 1996). This virus was rescued in a similar way to non-segmented ssRNA viruses, with viral genomic RNA and viral replication proteins transcribed by T7 RNA polymerase and self-cleaved to the proper length by the hepatitis delta ribozyme.

On the other hand, rescuing an 8-segment influenza virus proved to be difficult using these techniques. But, recombinant influenza viruses were rescued from cDNA clones encoding all viral genomic segments using the host cell’s RNA transcription system, since influenza viruses replicate in the nucleus of infected cells (Spooner and Barry, 1977). The eukaryotic RNA Pol I promoter was used to drive the transcription and replication of the cDNA clones encoding the viral genomic segments and terminated by either eukaryotic termination sequences or hepatitis delta virus ribozyme cleavage (Fodor et al., 1999; Neumann et al., 1999). In addition, the eukaryotic RNA Pol II promoter was used to drive the transcription and expression of the viral proteins necessary for viral replication from support plasmids. In this system, eight plasmids encoding the eight genomic segments were co-transfected with four plasmids encoding the viral proteins necessary for viral replication and recombinant influenza viruses were successfully rescued. Not only did this system eliminate the need for a helper virus for successful rescue and subsequent selection, but it also eliminated the need for the cytotoxic infection of the recombinant vaccinia virus to supply the T7 RNA polymerase. This system was further optimized by cloning the RNA Pol I driven genomic segments between RNA Pol II promoter and polyadenylation sequences, therefore viral segmented genomic RNA and viral mRNA transcription occurred simultaneously from the same
plasmid, similar to a natural infection (Hoffmann et al., 2000). This approach reduced the number of transfected plasmids from twelve to eight and still resulted in successful rescue. Even with all the technological advancements in reverse genetics, only three segmented, negative-sense ssRNA viruses have been rescued, the two mentioned above and the 6-segment Thogoto virus (Wagner et al., 2001).

2.2.4 Segmented, double-stranded RNA viruses

The genomes of double-stranded (ds)RNA viruses are segmented, similar to influenza viruses, but dsRNA viruses replicate in the cytoplasm, which further increases the complexity of successful rescue from cDNA clones. Initial studies to rescue infectious, recombinant dsRNA viruses resembled the early system to rescue segmented negative-sense RNA viruses where individual recombinant viral RNA genomic segments were transcribed in vitro. Recombinant viruses were rescued with the transfection of the individual RNA segment and the aid of a helper virus, which was separated from the recombinant virus using screening techniques (Roner et al., 1997). Recently, a recombinant Bluetongue dsRNA virus has been rescued by co-transfecting all viral RNA segments, which were transcribed in vitro (Boyce et al., 2008). In addition, the T7 promoter/hepatitis delta ribozyme system has been used to successfully rescue a segmented dsRNA reovirus completely from cDNA, without the need to co-transfect support plasmids encoding the viral proteins necessary for virus replication (Kobayashi et al., 2007).
3. Reverse genetics applications

3.1. Viral induced expression of a foreign gene

One application using reverse genetics is to express a protein, which is foreign to the viral host, from a distinct gene on the virus genome. For the foreign gene to be recognized and expressed from the viral genome it must mimic a viral gene by encoding the necessary viral mRNA regulation sequences. The genomes of the *Paramyxovirinae* subfamily, which are non-segmented, negative-sense, ssRNA viruses, contain a series of 6–7 distinct gene units, which may contain one or more open reading frames (ORF). The main proteins expressed from the distinct gene units are NP, P, M, F, HN or hemagglutin (H), and L; also SH for rubulaviruses (Fig. 2-3A). The series of gene units are flanked by 3’ leader and 5’ trailer UTRs that are essential for viral transcription and replication regulation (Calain and Roux, 1995). Each gene unit is separated by semi-conserved intergenic regions that consist of gene end, intercistronic, and gene start sequences (Fig. 2-3B). Therefore, when inserting a foreign gene as a distinct gene unit, the new gene unit must contain the gene end, intercistronic, and gene start sequences to be effectively expressed through viral mRNA transcription.

During viral mRNA synthesis, the viral RNA polymerase recognizes the gene end sequence and stutters, adding non-templated adenosine (A) residues to create a poly-A tail. The viral RNA polymerase then reengages viral mRNA transcription at the gene start sequence for the next gene unit. The viral RNA polymerase will sometimes fail to reengage mRNA transcription for the downstream gene, which results in fewer mRNA transcripts for downstream genes compared to upstream genes transcribed from the same
template. This phenomenon is called transcriptional polarity and ultimately leads to less protein expressed from the downstream gene units in a gradient fashion (Wertz et al., 1998). This phenomenon can be used advantageously for regulation purposes by cloning the new foreign gene unit into various locations on the viral genome. For example, if the expression of the foreign gene is to be upregulated then the gene unit could be cloned towards the 3’ end of the viral genome, but if the foreign gene is to be downregulated then the gene unit could be cloned towards the 5’ end of the viral genome. Furthermore, if the foreign gene unit is to be cloned as the first gene on the viral genome then a second viral regulatory sequence needs to be taken into consideration. Past the 3’ leader UTR are three G ribonucleotides equally separated by five ribonucleotides, which correspond to one complete turn of the 3-dimensional helical encapsidated RNA genome. This

Fig. 2-3. Organization of the major genes units encoded in the *Paramyxovirinae* genomes. (A) The viral genomes consists of the following major features in order: 3’ leader (le), nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) or hemagglutinin (H), large protein (L), and 5’ trailer (tr). (B) Semi-conserved gene end, intercistronic, and gene start sequences of HPIV-3 at the gene junctures NP-P (1), P-M (2), M-F (3), F-HN (4), and HN-L (5).
location is thought to co-regulate viral replication perhaps through the assembly and binding of the L-P complex with the encapsidated RNA genome (Tapparel et al., 1998).

This method has been used to effectively express a foreign gene from the genome of infectious, recombinant ssRNA viruses for many purposes. For example, a recombinant SeV, which is non-pathogenic to humans thus a prime candidate for vaccine development and gene transfer, has been used as a expression vector in gene transfer to deliver foreign eukaryotic genes to neurons and airway epithelium (Shirakura et al., 2003; Yonemitsu et al., 2000). In addition, several recombinant paramyxoviruses have been developed that express other viral surface proteins to be used as vaccines. The ebolavirus glycoprotein and the RSV fusion protein have been expressed from recombinant HPIV-3 and SeV viruses, respectively, and have resulted in protective immunity against ebolavirus and RSV, respectively (Bukreyev et al., 2006; Zhan et al., 2007). A third area where the insertion of a foreign gene into a recombinant virus has been used is for the expression of a reporter gene for the purpose of tracing the viral infection. A recombinant HPIV-3, strain JS, has been engineered to express the EGFP protein and was used to trace the infection of HPIV-3 exclusively to the apical surface of ciliated airway epithelium by attaching to α2-6-linked sialic acid receptors (Zhang et al., 2005).

### 3.1.1 Antiviral assays detecting virus expressed reporter genes

Another benefit of a recombinant virus that expresses a reporter gene is the ability to detect and measure virus replication in real-time, which is directly linked to the expression of the foreign reporter gene. This measurement of real-time replication has direct implications in antiviral compound screening in vitro. When using the traditional
NR dye uptake assay to measure antiviral compound effectiveness, complete cell
destruction is necessary to obtain effective and reliable assay results. A major drawback
to this type of assay for some viruses is the inability to produce detectable amounts of
CPE or long incubation times that are needed for some viruses to completely lyse the cell
monolayer. Viral incubation times may extend up to 14 days for some viruses and is
problematic because the cell control monolayer may not survive that long. Other viruses,
such as SARS and influenza, do not suffer from this problem because of their short
incubation periods and lytic nature of their replication cycle (Barnard et al., 2004; Smee
et al., 2001). On the other hand, HPIV-3 and RSV form syncytia during the onset of
infection and the syncytia lyse later (Lin et al., 1999). The early syncytia formation is
problematic because, even though viral CPE can be seen within days, syncytia cells
absorb similar amounts of NR compared to intact cells (Smee et al., 2002). Therefore, it
is necessary for the syncytia to completely lyse before the detection assay is conducted.

A detection system, such as a recombinant virus expressing a reporter gene that
can be measured in real-time may shorten the amount of time needed to complete the
antiviral assay and allow direct CPE measurement. This has been accomplished for
antiviral compound screening purposes by inserting the gene for the green fluorescent
protein into the genomes of recombinant ebolavirus and cytomegalovirus (Marschall et
al., 2000; Towner et al., 2005). In addition, the luciferase gene has been inserted into the
genome of a recombinant West Nile virus (Puig-Basagoiti et al., 2005). HPIV-3 exhibits
a 6–8 day incubation period and, therefore, is a candidate for such a purpose (Lin et al.,
1999). A recombinant HPIV-3 virus has been constructed that expresses a green
fluorescent protein and was used to screen an antiviral compound library (Mao et al.,
2008). But it was not reported to have been extensively studied and validated as a suitable replacement for the wild-type virus in cell-based antiviral assays.

3.1.2 Traditional antiviral detection assays

Traditionally, to measure the efficacy of antiviral compounds, several cell viability detection systems involving colorimetric, luminescence, or fluorescence have been used. The colorimetric gold standard uses neutral red, which is absorbed by lysosomes of intact cells and can be measured empirically by a spectrophotometer (Anderson and Orci, 1988; Finter, 1969; Lowik et al., 1993; McLaren et al., 1983; Paragas et al., 2004; Smee et al., 2002). One major problem with neutral red is the formation of thread-like crystals that form on the cell monolayer, most likely caused by poor solution preparation and may be accelerated by certain compounds. The neutral red crystals can not be eliminated with numerous rinses and are readily dissolved in the extraction process, thereby increasing the absorbance values and skewing the results. In addition, because of the colorimetric nature of the assay, certain test compounds may have absorption spectra similar to neutral red, which may also skew the results. Even though neutral red can be added directly to the test medium, it also requires lengthy absorption times, subsequent washes, and extraction with ethanol, making this method time consuming and labor intensive. The washes are also problematic because, depending on the cell type used in the assay, the cell monolayer could be washed away.

A second detection method uses the substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Pauwels et al., 1988; Watanabe et al., 1994). MTT is a yellow tetrazolium salt that is reduced by a mitochondrial reductase in cells to
form water-insoluble purple formazan crystals, which can be measured empirically (Mosmann, 1983). MTT is similar to neutral red in that it is absorbed by intact cells, but differs by being chemically modified once inside a cell. One problem with the MTT assay is that the presence of phenol red in the test medium can interfere with the spectrophotometer measuring the absorbance, and it is recommended that the cell monolayer be incubated in phenol-free medium or at least be replaced with phenol-free medium during absorption. One major disadvantage of the MTT assay is that the purple formazan crystals are formed through an enzymatic reaction inside the cell. Not only could the antiviral compound being tested inhibit virus replication, it may also inhibit purple crystal formation, thus skewing the absorbance values.

A third colorimetric assay measures the activity of lactate dehydrogenase (LDH) that has leaked out of dead or dying cells (Baba et al., 2005; Mori et al., 1995; Watanabe et al., 1995). A substrate, 2-p-(iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT), is reduced by LDH, in a NADH-coupled reaction, to form a water-soluble red formazan, which can be measured empirically (Hinman and Blass, 1981). A difference between the LDH assay and the two methods mentioned above is that the supernatant, which contains LDH that has leaked from dead or dying cells, is removed from the cell monolayer during the LDH assay. This assay increases the handling of the samples but the incubation times and solubility issues are reduced.

Another type of assay is the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI), which measures the adenosine triphosphate (ATP) generated by intact cells (Noah et al., 2007). When luciferase and luciferin are added to the test medium that contains ATP, light is emitted and the amount of light generated is
proportional to the health of the cell monolayer. The benefits to this assay are the direct addition of reagents to the test medium, short incubation, and high sensitivity.

Even though the luminescence assay solves the handling and time disadvantages of the colorimetric assays, a major issue arises that is present in the colorimetric assays as well. A major problem with most cell viability assays is that they are designed to detect any cell death and do not differentiate between drug toxicity and viral CPE. By conducting a parallel drug toxicity assay, the two factors can be mathematically separated, although it is assumed that the differences measured in the assay are due to drug toxicity and CPE independently. This assumption ignores the possibility of positive or negative compounding effects from potential interactions between the drug and virus. Therefore, a detection system that can directly measure a change in virus replication independently of drug toxicity would be preferable. Foreign reporter proteins that are expressed from the genomes of recombinant viruses and detected in real-time can measure virus replication directly regardless of drug toxicity and could be a possible solution to the problem.

3.2. Characterization of viral accessory proteins using recombinant knockout viruses

A second application using reverse genetics is to study the functions of viral genes through mutational analysis. The cDNA clone for a ssRNA virus can serve as backbone for genetic manipulation and infectious, recombinant viruses can be rescued from it, assuming the mutation is not deleterious to the virus. The recombinant parent and mutant viruses can then be measured using traditional and modern virological
techniques and compared to each other. If any differences in growth characteristics, for example, are seen between the two recombinant viruses, the change may be attributed to the mutations of the mutant virus. For the most part, this method has been used to study the accessory proteins expressed by ssRNA viruses because they are generally not essential for virus replication, even though they may play part in replication in some way. Most other genes in the viral genome are essential for viral replication and any deliberate knockouts or mutations may render the recombinant virus uninfectious. Generally, the accessory proteins are involved in counteracting the host cell’s antiviral response, discussed later.

The accessory proteins are expressed from mRNA transcribed from the P gene of most paramyxoviruses genomes. The P gene on the HPIV-3 genome contains additional ORFs that encode for the C, W, and PD proteins, in addition to the P protein (Fig. 2-2) (Galinski et al., 1986; Galinski et al., 1992). Other paramyxoviruses encode for more accessory proteins, such as, the V protein expressed by all paramyxoviruses except HPIV-3 and HPIV-1, two Y and a second C’ protein expressed by SeV and HPIV-1, and the I protein expressed by rubulaviruses (Giorgi et al., 1983; Matsuoka et al., 1991). The translation of the C and Y proteins in initiated by ribosomal choice or shunting and the V, W, I, PD proteins are transcribed by mRNA editing (Latorre et al., 1998).

3.2.1 C proteins

The C protein is about 170 amino acids long, overall basic in charge, and a small percentage of sequence homology is shared by all members of the Paramyxovirinae. All members of this subfamily express the C protein in some way. The individual C proteins
expressed by respiroviruses, morbilliviruses, and henipaviruses are translated by ribosomal choice (Bellini et al., 1985; Galinski et al., 1986; Giorgi et al., 1983). The start codon for the C protein is approximately 10 ribonucleotides downstream of the start codon for the P protein (Fig. 2-2). During the initiation of protein translation, the ribosome may read through the start codon for the P protein and initiate translation at the start codon for the C protein. Also, the SeV and HPIV-1 viruses encode for a second C’ protein whose start codon is upstream of the P protein start codon and is also translated by ribosomal choice (Giorgi et al., 1983). In addition, the SeV and HPIV-1 viruses encode for two Y proteins, also located on the 5’ end of the P gene mRNA, but translation is initiated by ribosomal shunting (Latorre et al., 1998). Ribosomal shunting occurs when the ribosome “jumps” a portion of the mRNA and initiates translation at a start codon further downstream; at least 80 ribonucleotides downstream of the P protein start codon for the SeV virus. For the members of the rubulaviruses and avulaviruses, ribosomal choice or shunting does not occur, but the P protein is translated as a fusion protein with the C-terminal cofactor end of the P protein fused to a C-like protein N-terminal end (McGinnes et al., 1988; Thomas et al., 1988).

The C proteins seem to be involved in some aspect of regulating viral RNA synthesis. The titers of recombinant HPIV-3 and SeV viruses, which are deficient in the expression of their C proteins, are restricted in cell culture (Durbin et al., 1999; Kurotani et al., 1998). However, the specific aspect of viral RNA synthesis is different for each virus. The SeV virus C protein regulates genomic replication, whereas the HPIV-3 virus C protein regulates viral mRNA transcription (Cadd et al., 1996; Malur et al., 2004).
3.2.2 Proteins expressed by mRNA editing

The P gene of all members in the Paramyxovirinae subfamily contains an mRNA editing site where the viral polymerase stops and stutters, adding non-templated G residues during viral mRNA transcription. This results in frame shifts in the open reading frame of the mRNA and leads to the translation of three fusion proteins with identical N-terminal ends and different C-terminal ends. For HPIV-3, the proteins expressed in this way are the P, W, and PD (Fig. 2-2) (Galinski et al., 1992). The V and I proteins are also expressed in this manner by other members of the Paramyxovirinae subfamily (Cattaneo et al., 1989; Thomas et al., 1988; Vidal et al., 1990). The stop codons for the W protein, expressed in respiroviruses, morbilliviruses, and henipaviruses, and the I protein, expressed in rubulaviruses and avulaviruses, appear shortly after the editing site and lead to the removal of the C-terminal cofactor end of the P protein. The main functions of the W and I proteins have been theorized to be that of the remaining N-terminal ends. In respiroviruses, morbilliviruses, and henipaviruses, the function of the W protein has been attributed to chaperoning unincorporated NP proteins to encapsidate naked genomic RNA. In rubulaviruses and avulaviruses, the function of the I protein has been attributed to the functions of the C protein.

Another fusion protein is the V protein, which is characterized by a highly conserved, C-terminal, cysteine-rich domain that binds two zinc atoms to form a zinc-finger structure (Paterson et al., 1995). It is expressed by most members of the Paramyxovirinae, except for HPIV-3 and HPIV-1 (Cattaneo et al., 1989; Thomas et al., 1988; Vidal et al., 1990). Even though the cysteine-rich domain is not encoded anywhere within the HPIV-1 genome, it is located in the HPIV-3 antigenome in the same reading
frame as the W protein downstream of 2 or 3 stop codons, depending upon the virus strain (Fig. 2-1 and 2-4) (Galinski et al., 1992; Matsuoka et al., 1991). Although the HPIV-3 antigenome may contain the ribonucleotide sequence that encodes the cysteine-rich domain characteristic of the V protein, it is still not known if it is expressed at all. In the only study reporting research pertaining to the HPIV-3 V protein, the D and V domains were silenced individually and simultaneously (Durbin et al., 1999). No attenuation in viral titers was seen when the individual or simultaneous knockout viruses were used in vitro or individually in vivo, but attenuation was seen when the cistrons were silenced simultaneously in vivo. These data suggest that the D and V domains were expressed and that together they played a role in the host cell’s antiviral response. The main function of the V protein expressed by other paramyxoviruses has been attributed to counteracting the antiviral response, discussed later. To date no virus-specific function of the V protein has been found.

More recently, the PD protein of HPIV-3, which is the fusion of the N-terminal end of the P protein and the C-terminal D domain, was detected in vitro. Three nuclear localization signals were identified within the PD protein that directed the intracellular

Fig. 2-4. Protein alignment of the respiroviruses V domain. The amino acid sequence of the V domain aligned from Sendai virus (SeV), Bovine and Human parainfluenza type 3 viruses (B or HPIV-3). The conserved amino acids shared by all members of the Paramyxoviridae family, except HPIV-3 and HPIV-1 (star).
localization of the PD protein to the nucleus, but its function remains a mystery (Wells and Malur, 2008).

3.2.3 Host cell’s antiviral response

When a ssRNA virus infects a cell, the virus produces intermediate structures in the host cell’s cytoplasm that are unique to the replicating virus and the host cell can recognize them as foreign. In response, protein receptors inside the infected host cell, which bind to the viral intermediate RNA structures, initiate the primary interferon pathway that ultimately expresses interferons, which are secreted from the infected cell. Two of the viral structures that the host cell can recognize as foreign are ds RNA and 5’-triphosphate or uncapped ssRNA. The cellular proteins that bind these structures through a RNA helicase domain are the melanoma differentiation associated gene (MDA)-5 and the retinoic acid inducible gene (RIG)-1, both of which initiate the primary interferon pathway. While MDA-5 binds viral dsRNA intermediates, RIG-1 binds the 5’-triphosphate ends of ssRNA, which are characteristic of viral transcribed mRNA and genomic RNA, and also short dsRNA intermediates, which are characteristic of viral RNA secondary structures (Fig. 2-5) (Hornung et al., 2006; Kang et al., 2002; Kato et al., 2008; Pichlmair et al., 2006). When these proteins bind viral RNA, their N-terminal caspase recruitment domain (CARD) interacts with mitochondrial antiviral signaling (MAVS) or IFN-β promoter stimulator (IPS)-1 proteins (Kawai et al., 2005; Seth et al., 2005). In turn, MAVS/IPS-1 activates TRAF family member-associated NF-κB activator (TANK) binding kinase (TBK)-1 and IkappaB kinase (IKK)ε, which in turn, leads to the homo- or hetero-dimerization of the interferon regulatory factors (IRF)-3 and -7 and their
translocation into the nucleus (Kumar et al., 2006; Sun et al., 2006). Once in the nucleus, IRF-3/7 dimers promote the expression of IFN-β and the regulated on activation normal T cell expressed and secreted (RANTES) proteins, both of which are secreted from the infected cell (Au et al., 1995; Lin et al., 2000).

Extracellular IFN-β then activates the secondary interferon pathway in an autocrine and paracrine fashion by binding to type 1 IFN receptors (IFNAR), which in turn activates the Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathway (Fig. 2-5) (Levy and Darnell, 2002). Ultimately, STAT-1 and -2 proteins are phosphorylated, dimerized, and translocated to the nucleus where they associate with IRF-9 to form IFN-stimulated gene factor (ISGF)-3 complexes. Finally, this complex binds to IFN-stimulated response elements (ISREs) to promote the expression of IFN-stimulated genes (ISGs). For example, the 2’-5’ oligoadenylate synthetase enzyme is an ISG that is expressed in response to viral invasion and activates RNaseL, which degrades all RNA, viral and cellular, inside the infected cell (Zhou et al., 1993).

3.2.4 Viral antiviral response antagonists

The paramyxovirus V and C proteins work in concert to inhibit both the primary and secondary IFN response. To initiate the primary IFN antiviral response, cells infected with paramyxoviruses primarily use the RIG-1 protein sensor (Kato et al., 2006). To counteract this response, the SeV virus C protein inhibits some aspect of the RIG-1 signaling pathway but not the MDA-5 signaling pathway (Fig. 2-5) (Strahle et al., 2007). As a second measure, the paramyxovirus V proteins also inhibit the antiviral response by
Fig. 2-5. Primary and secondary interferon responses. The primary interferon response is initiated by the presence of either uncapped RNA or dsRNA, which activates RIG-1 or MDA-5 sensors. These sensors then activate MAVS/IPS-1, which in turn activates the TBK-1/IKKe kinases. These kinases then phosphorylate IRF-3/7, which dimerizes and translocates to the nucleus. Once in the nucleus the IRF-3/7 transcription factors induce the expression of IFN-β, which is secreted out of the cell. Once outside the cell, the secondary interferon response is activated by IFN-β binding to the IFNAR receptor, which activates the JAK/STAT pathway. The Jak-1/TYK-2 kinase phosphorylates STAT-1/2, which dimerizes and translocates to the nucleus and interacts with IRF-9 to form the ISGF-3 transcription factor. This transcription factor then binds to ISRE sequences and induces the expression of ISGs. The paramyxovirus V proteins bind and inhibit MDA-5, thus preventing the primary IFN response. The Me V protein also inhibits IRF-7 to prevent IFN-β expression. The SeV C protein interferes with RIG-1 signaling, thus preventing the primary IFN response. The paramyxovirus C protein also prevents the secondary IFN response by binding and inhibiting STAT activation.
binding to MDA-5 and, thus, competing with and preventing dsRNA binding (Fig. 2-5) (Andrejeva et al., 2004; Childs et al., 2007; Childs et al., 2009; Komatsu et al., 2007). In addition, the MeV V protein competes with IRF-7 for binding to and phosphorylation by IKKα, therefore, preventing the primary IFN response that is initiated by both MDA-5 and RIG-1 (Pfaller and Conzelmann, 2008). On the other hand, the single-stranded genomic RNA for RSV interacts with the cellular La protein that prevents the binding of and detection by RIG-1, thus, inhibiting the primary IFN antiviral response (Bitko et al., 2008).

Furthermore, the paramyxovirus C proteins interfere with secondary IFN signaling of the infected host cell by binding and inducing ubiquitination of STAT1 and, thus, proteosome-mediated degradation of STAT1 (Fig. 2-5) (Didcock et al., 1999; Garcin et al., 2002; Palosaari et al., 2003). Also, the HPIV-3 virus C protein binds and prevents the phosphorylation of the STAT proteins, therefore, inactivating STAT and preventing the secondary IFN antiviral response (Malur et al., 2005). In conclusion, paramyxoviruses have developed multifaceted and complex mechanisms, involving viral and cellular proteins, in which to control the host cell’s antiviral response and propagate the virus progeny.

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CHAPTER 3

A RECOMBINANT, INFECTIOUS HUMAN PARAINFLUENZA VIRUS TYPE 3 EXPRESSING THE ENHANCED GREEN FLUORESCENT PROTEIN FOR USE IN HIGH-THROUGHPUT ANTIVIRAL ASSAYS¹

Abstract

The ability to rescue an infectious, recombinant, negative-stranded RNA virus from a cDNA clone has led to new opportunities for measuring viral replication from a viral expressed reporter gene. In this study, the EGFP gene was inserted into the HPIV-3 antigenome and a recombinant, infectious virus was rescued. Maximum EGFP expression levels, measured by fluorescence, were seen at day 3. Comparison of a 3-day, viral expressed EGFP fluorescence assay to a 7-day, neutral red assay, based on complete cell destruction in virus infected MA-104 cells, yielded Z´-factor values of 0.83 and 0.70, respectively. A 3-day, endpoint EGFP-based antiviral assay and a 7-day, endpoint neutral red based antiviral assay were run in parallel to establish antiviral sensitivity profiles of 23 compounds based on selective index values. Using a selective index threshold of 10, the EGFP-based antiviral assay had a sensitivity of 100% and a specificity of 54%. Thus, the use of an EGFP-based antiviral assay for testing potential antiviral compounds against HPIV-3 in a high-throughput format may be justified.

¹ Coauthored by Jason P. Roth, Joseph K.-K. Li, Donald F. Smee, John D. Morrey, and Dale L. Barnard, Antiviral Res. 82, 12-21.
1. Introduction

The advent of therapeutic antiviral compounds for human treatment begins with in vitro testing and the successful inhibition of a virus prior to further assessment. To measure the efficacy of these drugs, cell-based antiviral assays involving colorimetric and luminescence detection systems have been used. Colorimetric assays often measure absorption of NR or MTT, which are taken up by intact cell cells as indicators of cell health. Neutral red measures membrane integrity by being absorbed by lysosomes, while MTT, once inside the cell, is reduced from a yellow tetrazolium salt into water-insoluble purple formazan crystals by a mitochondrial reductase (Barnard et al., 2004; Finter, 1969; Watanabe et al., 1994). The time consuming and labor intensive nature of these methods have proven to be drawbacks (Smee et al., 2002). An alternative type of detection method is the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI), which measures the ATP generated by intact cells (Noah et al., 2007). It is claimed that this assay is more sensitive, less time consuming, and less labor intensive. Even though the luminescence assay may solve the disadvantages of the colorimetric assays, several issues arise that are also common to colorimetric assays as well.

A major problem with most cell viability assays is that they are designed to detect any cell death and do not differentiate between drug toxicity and viral CPE. By conducting a parallel drug toxicity assay, the two factors can be mathematically separated, although it is assumed that the differences measured in the assay are due to drug toxicity and CPE independently. This assumption ignores the possibility of positive or negative compounding effects from potential interactions between the drug and virus. A second problem is the inability for some viruses to produce detectable amounts of CPE
and/or the long incubation times needed to reach complete cell monolayer destruction, which is often required for accurate dye uptake assays. For the colorimetric and luminescent antiviral assay to be effective and reliable complete destruction of the virus infected cell controls is necessary to obtain significant signal-to-background ratios compared to uninfected cell controls. In addition, viral incubation times may extend up to 14 days for some viruses, which is problematic because the uninfected cell controls may not survive that long. A third problem is virus induced syncytia formation of the infected cells that do not completely lyse until the later stages of the virus infection. Early syncytia formation is problematic because they absorb similar amounts of neutral red or MTT compared to uninfected cells, even though viral CPE can be visually detected within a few days (Smee et al., 2002). Therefore, it is necessary to wait for complete destruction of the virus induced syncytia so dye uptake assays can differentiate between uninfected cells and cells impacted by syncytial formation. Therefore, a detection system that can directly measure changes in virus replication independently of drug toxicity in a short amount of time would be preferable.

One strategy for enhancing sensitivity and decreasing the incubation times of an assay would be to construct a recombinant virus that expresses a reporter gene. For example, a gene for a fluorescent protein could be cloned directly into a viral genome that would be expressed in proportion to virus replication and allow direct measurement of virus replication independently from drug toxicity. This has been accomplished for antiviral compound screening purposes by inserting the gene for the green fluorescent protein into the genomes of the ebolavirus and cytomegalovirus (Marschall et al., 2000; Towner et al., 2005). Thus, for syncytial forming viruses, such as HPIV-3, a similar
approach would be very useful in reducing the time needed to complete an antiviral assay.

Two rHPIV3 viruses have been constructed that express a green fluorescent protein, but they have not been extensively studied and validated for use in cell-based antiviral assays. One such virus, strain JS, was used to trace the infection of the recombinant HPIV-3 virus in lung epithelium (Zhang et al., 2005). The second virus, strain 47885, was used to screen a library of potential antiviral compounds, yet specific details on replication differences of the recombinant virus expressing the fluorescent protein, compared to the wild-type strain were not discussed (Mao et al., 2008). In this paper, an rHPIV3 expressing EGFP, rHPIV3-EGFP, was rescued and evaluated for its use in antiviral assays by comparing it side-by-side with both the HPIV-3 WT and the recombinant HPIV-3 strains. Slight differences in virulence between the rHPIV3-EGFP virus and the HPIV-3 WT virus in cell culture may validate the possibility of substituting the rHPIV3-EGFP for the HPIV-3 WT virus in primary, high-throughput antiviral assays.

2. Materials and methods

2.1. Cells and viruses

Human cervical carcinoma cells (HeLa) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained at 37°C and 5% CO2 in minimal essential medium (MEM, HyClone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories), 0.1 mM non-essential amino acids (NEAA, Invitrogen, Carlsbad, CA), and 1 mM sodium pyruvate (Invitrogen). Embryonic African green monkey kidney cells (MA-104) were obtained from the ATCC and
maintained at 37°C and 5% CO2 in MEM supplemented with 10% FBS. A recombinant
vaccinia virus that expresses the bacteriophage T7 RNA polymerase, vTF7-3, generously
provided by Dr. Bernard Moss, was propagated in HeLa cells (Fuerst et al., 1986).
HPIV-3 WT, isolate 14702, (J. Bouvin, Hosp. St. Justine, Montreal, Canada) was
propagated in MA-104 cells. During the antiviral assays, MA-104 cells were incubated
in MEM supplemented with 2% FBS and 50 μg/ml gentamicin (Sigma Chemical
Company, St. Louis, MO).

2.2. Antiviral compounds

2-[(2R,3R,4S,5R)-3,4-Dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-
3-sulfanylidene-1,2,4-triazin-5-one (2-thio-6-azauridine) was obtained from Sigma and
the remainder of the antiviral compounds, including 1-[(2R,3R,4S,5R)-3,4-dihydroxy-
5-(hydroxymethyl)oxolan-2-yl]-1,2,4-triazole-3-carboxamide (ribavirin),
1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2,4-triazole-
3-carboximidamide (ribamidine), 2-[(2R,3R,4S,5R)-3,4-dihydroxy-
5-(hydroxymethyl)oxolan-2-yl]-1,3-selenazole-4-carboxamide (selenazofurin),
1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-5-ethynylimidazole-
4-carboxamide (EICAR), 6-amino-1-[(2R,3R,4S,5R)-3,4-dihydroxy-
5-(hydroxymethyl)oxolan-2-yl]-5H-imidazo[4,5-c]pyridin-4-one (3-deazaguanosine), and
1-(4-methoxybenzyloxy) adenosine, were obtained from the repository of the NIAID
Antiviral Substances Program, NIH. Also obtained from the repository were CS-978,
CS-1164, CS-1196, CS-1227, PSI-0194, PSI-5067, PSI-5095, PSI-5098, PSI-5452,
PSI-5449, PSI-5741, PSI-5746, PSI-5747, PSI-5852, and PSI-5990 nucleoside analog
compounds that were submitted by and used with permission from Dr. Michael Otto of Pharmasset, Inc. The DAS181 fusion protein, also obtained from the repository, was submitted by and used with permission from Dr. Fang Fang of NexBio, Inc. (Malakhov et al., 2006).

2.3. **Plasmid construction**

Three overlapping cDNA strands, encompassing viral bases 1–5267, 5249–11366, and 11284–15453, were generated from RNA isolated from a HPIV-3 WT, strain 14702, infection. Forward and reverse primers were derived from the consensus sequence between three known HPIV-3 strains, 47885, JS, GPv (Galinski, 1991; Ohsawa et al., 1998; Stokes et al., 1992). To generate the 1–5267 cDNA segment the

5’-CCGACGTCTTATTAATTAAATACGACTCACTATAGGACCACAAACAAAGAGAGAAACCTT-3’ forward primer, which contains AatII and PacI restrictions sites (bolded) and a T7 promoter (underlined) and the 5’-GGTCACCACAAGAGTTAGA-3’ reverse primer were used. To generate the 5249–11366 cDNA segment the

5’-TCTAACTCTTGGTGACC-3’ forward primer, which contains a natural BstEII restriction site (bolded) and the 5’-ATTCACTCCCAAGGGCAATA-3’ reverse primer were used. To generate the 11284–15453 cDNA segment the

5’-AGAATGGTTATTCACCTGTC-3’ forward primer and the

5’-GAGAAGCACTCTGTGTGGTAT-3’ reverse primer, which contains a mutated DraIII restriction site (bolded) with the two mutations, A to C and T to G (underlined), were used. The cDNA segments were inserted into the SmaI site of pUC19 (New England Biolabs, NEB, Ipswich, MA). Clones were sequenced in both directions to
assure accuracy. An SphI site in the 5249–11366 cDNA segment was destroyed by mutating A to G, viral base position 8635, using the QuikChange® XL Site-Directed Mutagenesis (Stratagene, La Jolla, CA) kit and the forward primer, 5’-CTTAGGAGCAAAGCGTGCTCAGAAAATGGACACTG-3’, and reverse primer, 5’-CAGTGTCCATTCTGAGCAGCAGCTTGGCTCCTAAG-3’.

To confirm the sequence of the 3’ end of the HPIV-3 WT genome, a poly(A) tail was added to the 3’ end of the isolated HPIV-3 WT RNA using the Poly(A) Tailing Kit (Ambion, Austin, TX). The tailed RNA was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using a 60-nt poly(T) oligonucleotide, as the forward primer, and 5’-TCGTTTTAGATCCTTCTCAATCA-3’, as the reverse primer.

To sequence the 5’ end of the HPIV-3 WT genome, the SMART™ RACE cDNA Amplification Kit (Clontech) was used. An HPIV-3 WT specific primer, 5’-GGAAGGAGCCATCGGCAAATCAGAAG-3’, was used to prime cDNA synthesis and also used in the polymerase chain reaction (PCR) amplification step as the forward primer. The PCR products from both the 3’ and 5’ reactions were sequenced to complete the HPIV-3 WT 14702 genome (GenBank accession no. EU424062).

Two oligonucleotides were generated to contain a 14 base pair overlap between each other,

5’-TTTTTGTGCGCCCAATACGCAAACCGCCTCTCCCGGCCTTTGGCCGTTAAT TAAAGAGGGTGACCCCTGCACAGAGTGCC-3’ and

5’-TTTTGTAAAAAACCCCTCAAGACCCCTTTAGAGGCCAACAGGTTATGCG TAGTTAGGTCACCAGGGCCTGTGAGCAG-3’. The oligonucleotides were annealed together and extended using Sequenase™ Version 2.0 DNA polymerase (USB
Corporation, Cleveland, OH). The fragment was inserted into the SmaI site of pUC19 and named pUC19-A. The completed segment contained the PacI, BstEII, DraIII, SmaI, and KpnI sites (bolded), a T7 termination sequence (underlined), and two vaccinia virus termination sequences flanking each end (italicized). A second set of oligonucleotides were annealed, extended, and inserted into pUC19 in the same manner,

5’-ACCAACACAGAGTGCTTCTCTTTGGGTGGGTCCGCGATGGCATCTCCACCTCCCTCGGTGTCGACCT-3’ and
5’-GGCCGGTGACCTCCCTTAGCCATGCCGAGTGACGACGTCCCCTCTCTCTCGGATGCCCAGGTCGGACCAGCGA-3’.

The completed segment, pUC19-R, contained the KpnI and DraIII restriction sites (bolded), the antigenomic hepatitis delta virus ribozyme (underlined) (Perrotta and Been, 1991), and the viral bases 15435–15462 (italicized). pUC19-R, digested with DraIII and KpnI, resulted in a 108 nt segment that was inserted into the same sites of pUC19-A and was renamed pUC19-B. After destroying the native SphI site in pUC19-B and renamed pUC19-C, an adapter, using the oligonucleotides

5’-GTGACCGCGCATGCCACAGA-3’ and 5’-GTGGGTCGACGCGGCG-3’, was inserted into the DraIII and BstEII sites of pUC19-C to encode a SphI site (bolded) and renamed pUC19-D. Next, the 5249–11366 cDNA segment was digested with BstEII and SphI, inserted into the same sites of pUC19-D, and renamed pUC19-F. Then, the 1–5267 cDNA segment was digested with PacI and BstEII, inserted into the same sites of pUC19-F, and renamed pUC19-G. Finally, the 11284–15453 cDNA segment was digested with DraIII and SphI, inserted into the same sites of pUC19-G, and renamed pUC19-H.
The 1–5267 cDNA segment, digested with AatII and BstEII, was inserted into the same sites of pACYC177 (NEB) and named p177-1Gen. The open reading frame of EGFP was amplified by PCR using pEGFP (Clontech, Mountain View, CA) as the template and the forward, 

5’-TTGACTAGAAGGTCAAGAACCTGCAAGTCGACTCTAGAGGAT-3’, and reverse,

5’-TTGACCTTCTAGTCGAATGCTTTAATCCTAAGTTTTTCTTTATTTATTAACCG GCCTGCTAGTTGGAAT-3’, primers. Both primers contain a DrdI restriction site (bolded) on their 5’ ends. The reverse primer also includes the HPIV-3 WT gene end, intercistronic, and gene start signals (underlined). The 868 bp band was purified and inserted into the SmaI site of pUC19, which was renamed pUC19-EGFP. pUC19-EGFP, digested with DrdI, resulted in an 852 bp band that was inserted into the same site of p177-1Gen and named p177-1Gen-E. A 1–6119 antigenomic cDNA segment, containing EGFP, was digested out of p177-1Gen-E with PacI and BstEII, inserted into the same sites of pUC19-F, and named pUC19-I. Finally, the 11284–15453 cDNA segment was digested with DraIII and SphI, inserted into the same sites of pUC19-I, and renamed pUC19-J.

Three PCR products encompassing the NP, P, and L genes were inserted into the SmaI site of pUC19 and named pUC19-NP, pUC19-P, and pUC19-L. The following three sets of forward and reverse primers were used: NP,

5’-GAAGGTCAAGAAAAGGGAACTCT-3’ and

5’-TTGATTCGATTAGTTGCTTTCA-3’; P, 5’-TGATGGAAAGCGACGCTAAA-3’ and 5’-GGATCATTGGCAATTGTTGA-3’; L, 5’-GCGTGCAGAAAATGGACA-3’
and 5'-CCTTAGGCTTAAAGATAAAGGTTAGGA-3’. The start codon (bolded) for the HPIV-3 WT accessory C protein, located on the P forward primer, was mutated from T to C (underlined) to silence its expression. pUC19-NP, pUC19-P, and pUC19-L were digested with SalI and KpnI and the 1.5, 2, 7 kb bands, respectively, were inserted into the same sites of pTNT™ (Promega) and named pTNT-NP, pTNT-P, and pTNT-L.

2.4. Rescue of infectious virus from cDNA

HeLa cells, seeded in a 12-well plate, were infected with 5.4 x 10⁵ plaque forming units (PFU) of vTF7-3 at 1 multiplicity of infection (MOI) for 1 h. The virus and medium were removed and replaced with Opti-MEM® (Invitrogen), containing 0.1 mM NEAA. The three support plasmids, 0.8 μg of pTNT-NP, 1.6 μg of pTNT-P, and 0.04 μg of pTNT-L, were cotransfected along with 0.4 μg of either pUC19-H or pUC19-J, using Lipofectamine™ 2000 (Invitrogen) for 4-5 h at 37°C. MEM supplemented with 20% FBS, 0.1 mM NEAA, 1 mM sodium pyruvate, and 250 μg/mL of Ara-C (Sigma) was added to each transfection and incubated for 48 h at 37°C. The transfected cells were scraped from the tissue culture wells and the lysates were frozen at -80°C. The rescued rHPIV3, pUC19-H transfection, and rHPIV3-EGFP, pUC19-J transfection, viruses were amplified on MA-104 cells, supplemented with 250 μg/mL Ara-C, for 3–4 days at 37°C. The infected cells were scraped from the tissue culture wells and the lysates were frozen at -80°C. Each virus was purified by picking agarose plugs over isolated plaques on MA-104 cells in the absence of Ara-C. Each plug was placed in MEM and frozen at -80°C. The medium, containing the plug and isolated virus, was used to infect MA-104 cells to amplify the virus for 3–4 days at 37°C. The purification and amplification steps
were repeated two more times. The rHPIV3, rHPIV3-EGFP, and HPIV3 WT viruses were amplified on MA-104 cells for 3 days at 37°C. The infected cells were scraped from the tissue culture wells and the lysates were frozen at -80°C, which were used for further testing. Sequencing of the 5’ ends of the genomic RNA, isolated from rHPIV3 and rHPIV3-EGFP infections, was repeated using the SMART™ RACE cDNA Amplification Kit to confirm the DraIII genetic markers.

2.5. **Viral infectious assays**

2.5.1 **Plaque assay**

Duplicate dilutions of HPIV-3 WT, rHPIV3, and rHPIV3-EGFP, were used to infect MA-104 cells in quadruplicate. Each virus was absorbed to MA-104 cells for 2 h, after which, the virus was removed and replaced with an overlay of 1% SeaPlaque® low-melting agarose (ISC BioExpress®, Kaysville, UT) supplemented with MEM and 0.2% sodium bicarbonate and incubated for 2–3 days at 37°C. Cells were fixed with 3.6% formaldehyde for 2 h at room temperature, after which, the formaldehyde and agarose overlay was removed and 0.5% crystal violet was added for 5 min. After removal of the dye and one rinse with phosphate buffered saline (PBS), the stained plaques were counted. Viral titers were compared and statistically analyzed by unpaired, two-tailed Student's t-test using the Microsoft® Office Excel 2003 software (Redmond, WA). In addition, plaques produced by rHPIV3-EGFP were also photographed using an Eclipse TS100 microscope (Nikon, Melville, NY), CoolSNAP digital camera, and RS Image™ software, version 1.7.3, (both from Roper Scientific, Photometrics, Tucson, AZ). Fluorescent photographs were taken with the same equipment except under UV light and
the B-2A fluorescent filter combination was used, which incorporates excitation wavelengths between 450 and 490 nm and emission filter wavelengths greater than 515 nm.

2.5.2 One-step growth curve

Duplicate 12-well plates were seeded with MA-104 cells and infected with 1.4 x 10^6 PFU of HPIV-3 WT, rHPIV3, and rHPIV3-EGFP viruses, separately, at an MOI = 2. After the virus was absorbed to MA-104 cells for 2 h at 37°C, it was replaced with fresh MEM supplemented with 2% FBS, and the cells incubated at 37°C. Individual wells containing infected cells were scraped from the tissue culture wells and the lysates harvested every 6 h starting at the time of virus exposure and frozen at -80°C. At time 0, virus was added but then immediately removed and replaced with fresh medium. Each time point for each virus was plaque titered in quadruplicate following the same method as described above. Each growth curve was compared to the other two curves, individually, and statistically analyzed by analysis of variance (ANOVA) using the Microsoft® Office Excel 2003 software.

2.5.3 Cytopathic effect assay

Ninety-six-well plates were seeded with MA-104 cells and infected with 3.9 x 10^3 PFU of either the HPIV-3 WT or rHPIV3-EGFP virus in duplicate at an MOI = 0.1 in quadruplicate wells. The plates were incubated at 37°C and on each day, including the day of infection, the cells were stained with 0.034% neutral red for 2 h at 37°C, washed once with PBS, and the NR extracted with ethanol:Sörenson’s citrate buffer for 30 min while rocking at room temperature. Absorbances at 540 and 405 nm were read with an
Opsys MR™ spectrophotometer equipped with Revelation Quicklink software, version 4.24 (both from Dynex Technologies, Chantilly, VA). The results were compared and statistically analyzed by ANOVA.

2.5.4 QRT-PCR assay

Ninety-six-well plates were seeded with MA-104 cells and infected with $7.8 \times 10^4$ PFU of HPIV-3 WT and rHPIV3-EGFP viruses, separately, in duplicate at an MOI = 2. At specific time points; 0, 12, 24, and 36 h, uninfected and infected cells were harvested using CellsDirect Resuspension and Lysis Buffers (Invitrogen). Each lysate was used as the template for two different reverse transcriptase (RT) reactions. One reaction used a primer specific for the HPIV-3 genome, 5’-ATTATAAAAACCTTAGGAGTAAAG-3’, and the other reaction used an Oligo(dT)20 primer (Invitrogen). The primers used to PCR amplify the cDNA products from the RT reactions include 5’-CGTTATAGTGCTGACCACAAGAATAA[FAM]G-3’ and 5’-ATGGAAGACCAGACGTGCAC-3’, for genomic replication, and 5’-CGATTAAGGAAAGCGGATTAAGTAAT[FAM]G-3’ and 5’-GAGACACAAATTAGGCAGGAGAT-3’, for L gene transcription. Platinum® Quantitative PCR SuperMix-UDG (Invitrogen), 200 nM of the forward and reverse LUX™ primers (Invitorgen), and 1/10th of the RT reaction were mixed and added, in triplicate, to Hard-Shell 96-well skirted PCR plates (Bio-Rad Laboratories, Hercules, CA). The reaction was run on a DNA Engine Opticon 2 Real-Time PCR Detection System (MJ Research, Waltham, MA). The Opticon Monitor™ software, version 3.1.32 (Bio-Rad Laboratories), was used to calculate relative expression differences, Delta-CT,
at each time point for each virus, using the 0 h for each virus as the baseline calibrator (Pfaffl, 2001). For each assay, the two curves were compared and statistically analyzed by ANOVA.

2.6. Antiviral sensitivity assay

An antiviral CPE assay was used to evaluate the antiviral sensitivity profiles of the HPIV-3 WT and rHPIV3-EGFP viruses (Barnard et al., 2001; Cavanaugh et al., 1990). Briefly, three compounds: ribavirin (positive control), 2-thio-6-azauridine, and DAS181, were plated in four 10-fold dilutions in five replicates on 96-well plates seeded with MA-104 cells using starting concentrations of 1000, 100, and 1 μg/mL, respectively. Two of five replicates were toxicity controls with no virus added, while the other three replicates were infected with 3.9 x 10³ PFU of either the HPIV-3 WT or the rHPIV3-EGFP virus at an MOI = 0.1. The plates were incubated at 37°C for 7 days and, after which, the cells of each plate were stained with NR following the same method as described above. The assays were completed three times. Fifty percent effective concentrations (EC₅₀) were calculated by linear regression using percents of untreated, uninfected cell and untreated, infected virus controls. EC₅₀ values were compared and statistically analyzed by the unpaired, two-tailed Student's t-test.

2.7. EGFP expression assays

Ninety-six-well plates were seeded with MA-104 cells and infected with 3.9 x 10⁴ PFU of rHPIV3-EGFP in duplicate at an MOI = 1. On each plate, quadruplicate four 10-fold dilutions of virus were plated and the cultures incubated at 37°C. Each day for 8 days, including the day of infection, the medium was removed and the cells were washed
with PBS once and fresh PBS added. On the day of infection, the virus was added but then immediately removed and the cells washed with PBS. EGFP fluorescence was measured with the FMax® fluorometer, using the 485 nm excitation and 538 nm emission filters, and recorded with SOFTmax® PRO software, version 1.3.1, (both from Molecular Devices, Union City, CA).

Duplicate 96-well plates were seeded with MA-104 cells. On each plate, 16 wells were infected with $3.9 \times 10^3$ PFU of rHPIV3-EGFP at an MOI = 0.1, while 16 wells were left uninfected as a cell control and 16 wells were left unseeded as a no-cell background control. The plates were incubated for 3 days at 37°C. After incubation, the uninfected and infected cells and unseeded wells were washed with PBS, replaced with fresh PBS, and fluorescence was measured using the FMax® fluorometer. The traditional NR-based assay and Vybrant® MTT Cell Proliferation Assay (Invitrogen) were completed following the same 96-well plate format mentioned above, except that each assay was conducted after complete infected cell lysis on day 7. The cells for the NR assay were stained following the same procedure described earlier, while the manufacture’s quick protocol was followed for staining of the cells for the Vybrant® MTT assay. The absorbance values for cells treated with MTT were measured at 540 nm, Opsys MR™ spectrophotometer, and Revelation Quicklink software. The CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was also performed using the same 96-well format except that MA-104 cells were seeded on white, half area 96-well plates with a clear bottom. Therefore, plating volumes were reduced by 50% and the CellTiter-Glo® reagent was reduced by 75%, while otherwise following the manufacture’s protocol. Luminescence
was measured using the Centro LB 960 luminometer and recorded with MikroWin 2000 software, version 4.34 (both by Berthold Technologies, Oak Ridge, TN).

2.8. **EGFP-based antiviral assay**

Six 96-well plates were seeded with MA-104 cells to evaluate the NR and EGFP assays in parallel. A format was used allowing seven compounds to be tested per plate. Each compound was plated using four 10-fold dilutions in triplicate starting at 1000 μg/mL for ribavirin (positive control) and ribamidine; 100 μg/mL for 2-thio-6-azauridine, 3-deazaguanosine, 1-(4-methoxybenzyloxy) adenosine, selenazofurin, and EICAR; 100 μM for CS-978, CS-1164, CS-1196, CS-1227, PSI-0194, PSI-5067, PSI-5095, PSI-5098, PSI-5452, PSI-5449, PSI-5741, PSI-5746, PSI-5747, PSI-5852, and PSI-5990; and 1 μg/mL for DAS181. Compounds that were reconstituted in dimethyl sulfoxide (DMSO) were diluted down to working concentrations of 0.5% or less DMSO to eliminate cell toxicity due to the DMSO. Two of the replicates were infected with $3.9 \times 10^3$ PFU of rHPIV3-EGFP, at an MOI = 0.1, while the remaining replicate served as a toxicity control with no virus added. Three of the plates were incubated at 37°C for 3 days, after which, the toxicity and cell control cells were stained with NR following the same procedure described earlier. NR fluorescence was measured with the FMax® fluorometer, using the 544 nm excitation and 612 nm emission filters. The untreated virus control and treated, infected cells were washed with PBS, fresh PBS was added, and the fluorescence measured with the FMax® fluorometer, using the 485 nm excitation and 538 nm emission filters. The other three plates were assayed using the traditional colorimetric NR assay. After incubation for 7 days at 37°C, the cells were stained with
NR following the same procedure described earlier. For the NR assay, EC₅₀ and 50% cell inhibitory concentrations (IC₅₀) were calculated by linear regression from percents of untreated, uninfected cell and untreated, infected virus controls. For the EGFP assay, EC₅₀ values were calculated by linear regression using percents of untreated, infected virus controls and IC₅₀ values were also calculated by linear regression using percents of untreated, uninfected cell control. The EC₅₀ and IC₅₀ values for each compound for both assays were compared and statistically analyzed by unpaired, two-tailed Student's t-test. An SI was calculated for each compound for each assay using the formula: SI = Mean IC₅₀/Mean EC₅₀. Compounds were sorted into positive, SI ≥10, and negative, SI <10, categories for the combination of NR and EGFP assays. Using the NR assay as the gold standard, sensitivity, true positives/(true positives+false negatives), and specificity, true negatives/(true negatives+false positives), were calculated.

3. Results

3.1. The successful insertion of the EGFP gene into the HPIV-3 antigenome and rescue of an infectious, recombinant HPIV-3 expressing the fluorescent protein

A DrdI restriction site was found between the N gene’s start signal and start codon of the HPIV-3 WT antigenome that was used to facilitate the insertion of the EGFP gene. Therefore, both the forward and reverse primers that were used to amplify the EGFP open reading frame were designed to contain a DrdI restriction site on their 5’ ends. The HPIV-3 WT gene end, intercistronic, and gene start signals were also encoded onto the reverse primer so that the inserted EGFP gene would be recognized as an
addition HPIV-3 WT gene (Fig. 3-1A). The “Rule of Six” was followed to generate an 852 bp EGFP gene segment. The rule suggests that viral replication is most efficient when the viral genome length is a factor of six ribonucleotides, which is most likely due to a single nucleocapsid protein binding to six genomic ribonucleotides (Calain and Roux, 1993; Durbin et al., 1997). In constructing the recombinant HPIV-3 expressing EGFP, the presence of three G ribonucleotides, which are equally separated by five ribonucleotides starting 79 ribonucleotides from the 5’ end of the antigenome, had to be manipulated. This location represents one complete turn of the 3-dimensional helical structure of the nucleocapsid encased RNA genome and is thought to co-regulate viral replication perhaps through the assembly and binding of the viral polymerase-phosphoprotein complex with the nucleocapsids (Tapparel et al., 1998). The EGFP forward primer interrupted this natural pattern but the problem was solved by adding the three G residues in the forward primer at positions 11, 17, and 23. Before the addition of the EGFP gene segment into the antigenome, the 1–5267 cDNA segment was cloned into the pACYC177 plasmid to circumvent multiple DrdI restriction sites located in the pUC19 plasmid. The resulting 1–6119 cDNA segment, now encoding the gene for EGFP, was then cloned into the pUC19 plasmid, already containing the 5249–11366 cDNA segment. Finally, the addition of the 11284–15453 cDNA segment to the construct resulted in a complete, infectious, recombinant HPIV-3 virus, expressing the EGFP gene.

To demonstrate the successful rescue and isolation of two rHPIV3 strains, one with and one without the EGFP gene insertion, sequences surrounding three genetic markers were aligned and compared to the HPIV-3 WT virus, isolate 14702 (Fig. 3-1B).
RNA isolated from rHPIV3-EGFP, rHPIV3, and HPIV-3 WT infections was amplified by RT-PCR. The sequences generated from the 5’ rapid amplification of cDNA ends (RACE) RT-PCR, containing the DraIII restriction site, confirmed the A to C and T to G mutations. These two mutations created a unique DraIII restriction site present only in the recombinant viruses and allowed for the insertion of the final 11284–15453 cDNA segment and completion of the recombinant viruses. The third genetic marker was also confirmed by aligning sequences generated from the 5’ end of the L gene from all three viruses. The A to G mutation eliminated one of two natural SphI restriction sites located...
in L gene portion of the HPIV-3 WT virus. The second SphI restriction site was used to insert both the 5249–11366 and 11284–15453 cDNA segments. These PCR products were also digested with SphI and separated on an agarose gel to show that HPIV-3 WT was digested in the presence of SphI, while the two recombinant viruses were not (Fig. 3-1C).

Plaques formed by rHPIV3-EGFP were stained with crystal violet and analyzed by bright field microscopy. The viral induced syncytia absorbed more crystal violet compared to surrounding uninfected cells (Fig. 3-2A). The syncytia from the same plaque were visualized by fluorescent microscopy and had high concentrations of green fluorescence (Fig. 3-2B). On the other hand, plaques formed by HPIV-3 WT did not produce fluorescence, data not shown. This result demonstrates a direct correlation between viral growth, syncytia formation, and EGFP expression.

3.2. rHPIV3-EGFP replication is slightly attenuated due to the additional gene

The infectious virus present in the stocks of all three viruses was plaque titered and the means, ± standard deviation, of duplicate assays were found to be: 2.9 ± 0.41 x 10^7 PFU/mL for HPIV-3 WT, 2.8 ± 0.22 x 10^7 PFU/mL for rHPIV3, and 1.9 ± 0.49 x 10^7 PFU/mL for rHPIV3-EGFP. The infectious virus titer for rHPIV3 was not significantly different compared to HPIV-3 WT whereas, rHPIV3-EGFP was significantly lower compared to both HPIV-3 WT and rHPIV3 (p<0.01). The addition of the EGFP gene into the HPIV-3 antigenome appeared to attenuate rHPIV3-EGFP compared to either the WT or recombinant strains. However, the process of creating and rescuing the recombinant virus and/or the presence of the three genetic markers did not cause
Fig. 3-2. MA-104 cells showing an rHPIV3-EGFP induced plaque. (A) Plaque stained with crystal violet and photographed under bright field microscopy. (B) Same plaque photographed under fluorescent microscopy. Scale bar, 50 µm.
attenuation of rHPIV3 because no significant reduction in virus titer was seen. For all subsequent experiments the volume of virus inocula were adjusted so that equal PFUs were added. In addition, the replication kinetics of the three viruses, HPIV-3 WT, rHIPV3, and rHPIV-EGFP were measured to confirm the attenuation of rHPIV3-EGFP compared to the wild-type and recombinant viruses. The growth curves for rHPIV3 and HPIV-3 WT were very similar, with no significant differences (Fig. 3-3A). However, the growth curve for rHPIV3-EGFP was significantly delayed compared to the growth curves for both HPIV-3 WT and rHPIV3 (p<0.01). During the initial stages of infection, the attenuated growth of rHPIV3-EGFP compared to both the wild-type and recombinant viruses can be seen, yet it appears that the replication of the rHPIV3-EGFP virus may recover and amplify itself to similar levels compared to the other two viruses during the later stages of replication. This result confirmed that the addition of an additional gene into the HPIV-3 genome may be the cause of attenuation.

The CPE produced by rHPIV3-EGFP and HPIV-3 WT viruses in infected MA-104 cells was monitored for 7 days and measured by NR uptake until complete infected cell lysis occurred, verified by microscopic examination. Complete cell lysis induced by both viruses occurred at the same time on day 7 and no significant difference in either curve was detected (Fig. 3-3B). This result contradicted previous results showing attenuation in the replication of the rHPIV3-EGFP virus, but the result supports the idea that rHPIV3-EGFP is able to recover and replicate up to HPIV-3 WT standards.

To determine how the additional gene may have contributed to the attenuation seen during the onset of infection, a QRT-PCR assay was completed to measure genomic replication and L gene transcription. An HPIV-3 specific primer that annealed to the
Fig. 3-3. Infectious assays comparing the recombinant HPIV-3 virus, expressing EGFP, to the wild-type and recombinant viruses. (A) Single step growth curve. The growth curve for rHPIV3 (▲) was not significantly different compared to the growth curve for HPIV-3 WT (■), while the growth curve for rHPIV3-EGFP (●) was significantly different compared to the growth curves for HPIV-3 WT and rHPIV3 (p<0.01). (B) Time course of HPIV-3 WT (■) and rHPIV3-EGFP (●) induced CPE. No significant differences were detected. Relative expression differences of HPIV-3 WT (■) and rHPIV3-EGFP (●) for L gene transcription (C) and genomic replication (D) measured by QRT-PCR. Significant reductions were seen in rHPIV3-EGFP genomic replication and L gene transcription compared to HPIV-3 WT (p<0.01). All Y-axis values on all graphs represent the mean ± S.D. of duplicate assays.
intergenic sequence between the fusion and hemagglutinin-neuraminidase genes of the viral, negative-sense RNA, only allowing binding to viral, genomic RNA rather than viral mRNA or viral, positive-sense, antigenomic RNA, was used as a primer for the RT reaction. An Oligo(dT)20 primer was used to prime the RT reaction for the L gene transcription measurement, binding only viral mRNA and not viral genomic RNA. Relative expression differences were calculated and normalized, using the 0 h for each virus as the baseline calibrator, according to the calculations developed by Pfaffl (2001). A calibrator was used to normalize the amount of mRNA transcripts or genomic copies generated during the infections with the amount that was added at the time of infection for each virus. L gene transcription (Fig. 3-3C) and genomic replication (Fig. 3-3D) were significantly reduced in an rHPIV3-EGFP infection compared to the HPIV-3 WT infection (p<0.01). The additional gene present in the HPIV-3 genome caused a reduction in the amount of viral mRNA transcripts and genomic copies that are normally generated in a WT infection.

3.3. *rHPIV3-EGFP is slightly more sensitive to antiviral compounds*

To further study the consequences of the attenuation detected for rHPIV3-EGFP replication, three known antiviral compounds that inhibit HPIV-3 were tested. The three compounds include two nucleoside analogs, ribavirin and 2-thio-6-azauridine, and a recombinant fusion protein between a sialidase catalytic domain and cell surface-anchoring sequence, DAS181 (Malakhov et al., 2006). EC$_{50}$ values, which are the concentration of compounds that inhibit 50% of virus replication, were calculated for each compound for both HPIV-3 WT and rHPIV3-EGFP viruses. The mean, ± standard
deviation, of three replicates were found to be: 35 ± 2.5 μg/mL and 19 ± 4.9 μg/mL for ribavirin, respectively; 1100 ± 58 ng/mL and 630 ± 75 ng/mL for 2-thio-6-azauridine, respectively; and 53 ± 2.3 ng/mL and 13 ± 2.3 ng/mL for DAS181, respectively. The rHPIV3-EGFP virus was significantly more sensitive to inhibition by these compounds than was the wild-type virus (p<0.05). This supports the idea that the rHPIV3-EGFP virus was attenuated.

3.4. Using EGFP expression as a measure of viral infectivity leads to a faster and more robust assay

To determine the earliest possible day that a potential EGFP-based assay could be completed, EGFP expression by rHPIV3-EGFP was measured. The fluorescence emitted from the viral expressed EGFP was measured each day in rHPIV3-EGFP infected MA-104 cells at various MOIs of virus. EGFP expression rose in a dose-dependant manner beginning at day 1, peaked on day 3 regardless of MOI, and leveled off thereafter (Fig. 3-4). Even though, the infection at MOI = 1 resulted in the greatest fluorescence, a large amount of fluorescence was still detected for the other three MOIs as well. The infection at MOI = 0.1 was equivalent to the concentration of virus used in typical antiviral assays, so this concentration of virus was used in further testing.

To compare the 3-day, EGFP-based assay to the traditional NR-based assay, Vybrant® MTT Cell Proliferation, and CellTiter-Glo® Luminescent Cell Viability Assays, the Z’-factors, signal-to-background, and signal-to-noise ratios were calculated (Table 3-1). The Z’-factor is a statistical calculation that assesses the quality of a high-throughput screening assay and predicts the potential of the assay if the number of
samples were scaled up. \(Z\)'-factors were computed for each assay and compared using two different fitness tables (Kraybill, 2005; Zhang et al., 1999). Ultimately, a higher \(Z\)'-factor value means the assay is more robust when it is used in a high-throughput format. The 3-day, EGFP-based assay, 0.83, proved to be more robust than the other three 7-day assays: 0.70 for the NR-based assay, 0.73 for the CellTiter-Glo\textsuperscript{®} Luminescent Cell Viability Assay, and 0.50 for the Vybrant\textsuperscript{®} MTT Cell Proliferation assay. According to the fitness tables, the EGFP-based, NR-based, and CellTiter-Glo\textsuperscript{®} Luminescent Cell Viability Assays were all considered to be good to excellent assays. On the other hand, the Vybrant\textsuperscript{®} MTT Cell Proliferation assay was considered to be borderline excellent/marginal on one table and at the recommended minimum level for the second table. For the signal-to-background ratios, the EGFP assay, with a value of 241, again
Table 3-1
Evaluation of the viral expressed EGFP detection method compared to three types of viral CPE detection methods using the rHPIV3-EGFP virus.

<table>
<thead>
<tr>
<th></th>
<th>3-Day Assay</th>
<th>7-Day Assay</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Viral expressed EGFP fluorescence</td>
<td>Colorimetric neutral red uptake</td>
</tr>
<tr>
<td><strong>Z’-factor\textsuperscript{a}</strong></td>
<td>0.83</td>
<td>0.70</td>
</tr>
<tr>
<td><strong>Signal-to-background</strong></td>
<td>241</td>
<td>65</td>
</tr>
<tr>
<td><strong>Signal-to-noise</strong></td>
<td>4057</td>
<td>301</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The Z’-factor is a statistical calculation that assesses the quality of a high-throughput screening assay and predicts the potential of the assay if the number of samples were scaled up.

proved to be superior showing excellent signal to background signal separation. The NR assay, value of 65, resulted in good separation, whereas both the CellTiter-Glo\textsuperscript{®} Luminescent, value of 6, and Vybrant\textsuperscript{®} MTT assays, value of 7, resulted in poor separation of signal to background signal. In addition, the EGFP assay, with a signal-to-noise ratio of 4057, again proved to be superior to the other three assays, signal-to-noise ratios of: 301 for NR, 59 for CellTiter-Glo\textsuperscript{®}, and 60 for Vybrant\textsuperscript{®} MTT, by showing excellent signal to background variability separation.

3.5. *Comparison of a 7-day, NR-based antiviral assay and a 3-day, EGFP-based antiviral assay*

To investigate the practicality of using rHPIV3-EGFP in an antiviral screening assay, a panel of 23 antiviral compounds were selected that had shown antiviral activity against HPIV-3 in past screens (data not shown). A standard 7-day, NR-based assay and
a 3-day, EGFP-based assay, using the same virus stock, were compared in parallel using 23 compounds, which included 22 nucleoside analogs and the one fusion protein, DAS181 (Table 3-2). An SI value ≤3 was considered not active, SI values between 4 and 9 slightly active, between 10 and 49 moderately active, and ≥50 highly active. Compounds with SI values ≥10 dictated if an antiviral drug would be further evaluated in additional assays. Using the threshold SI value of 10, the 3-day, EGFP-based assay had a sensitivity of 100% and specificity of 54%, compared to the 7-day NR assay. Using the 7-day NR assay as the gold standard, six compounds were falsely identified as selective inhibitors of virus replication using the rHPIV3-EGFP virus in the antiviral assay, which led to the 54% specificity. These six compounds showed an increase in the SI value over the threshold of 10 in the EGFP assay but under the threshold in the NR assay. Of these six, PSI-5449 was not active in the NR assay but was moderately active in the EGFP assay. An additional four, ribamidine, selenazofurin, PSI-5852, and PSI-5095, were considered slightly active in the NR assay and moderately active in the EGFP assay. The remaining compound, CS-1196 was considered slightly active in the NR assay and highly active in the EGFP assay. A factor that contributed to the differences in selectivity detected in each assay was the lack of toxicity found in cells exposed to compound in the EGFP assay. The toxicity of a drug is determined by the concentration at which it is lethally toxic to 50% of the cells present in the assay, termed IC$_{50}$. No toxicity was observed for all six compounds falsely identified as selective inhibitors in the EGFP assay and the IC$_{50}$ values for four out of the six compounds were significantly decreased in the 7-day NR assay (p<0.05). The difference in toxicity was probably due to the accumulation of toxicity during the 7-day incubation period of the NR assay. On the
Table 3-2
Panel of antiviral compounds tested by the traditional 7-day, colorimetric, neutral red uptake assay and 3-day, EGFP fluorescence assay using the rHPIV3-EGFP virus. Continued on next page.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>7-Day colorimetric neutral red uptake assay</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>EC$_{50}^a$</td>
</tr>
<tr>
<td>EICAR$_c^c$</td>
<td>0.81 ± 0.061</td>
</tr>
<tr>
<td>DAS181$_c^c$</td>
<td>0.013 ± 0.0015</td>
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<tr>
<td>PSI-5067$_c^c$</td>
<td>0.76 ± 0.032</td>
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<tr>
<td>PSI-5452$_c^c$</td>
<td>0.84 ± 0.14</td>
</tr>
<tr>
<td>2-Thio-6-azauridine$_c^c$</td>
<td>0.6 ± 0.095</td>
</tr>
<tr>
<td>PSI-5746$_c^c$</td>
<td>6.4 ± 0.46</td>
</tr>
<tr>
<td>CS-1164$_c^c$</td>
<td>6.7 ± 0.21</td>
</tr>
<tr>
<td>Ribavirin$_c^c$</td>
<td>20 ± 3.6</td>
</tr>
<tr>
<td>PSI-5990$_c^c$</td>
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<tr>
<td>PSI-5747$_c^c$</td>
<td>8.3 ± 2.3</td>
</tr>
<tr>
<td>PSI-5852$_c^c$</td>
<td>9.9 ± 2.8</td>
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<tr>
<td>Selenazofurin$_c^c$</td>
<td>4.9 ± 1.4</td>
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<tr>
<td>Ribamidine$_c^c$</td>
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<tr>
<td>PSI-5095$_c^c$</td>
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<td>CS-1196$_c^c$</td>
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<td>PSI-5449$_c^c$</td>
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<td>PSI-5098$_c^c$</td>
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<td>CS-1227$_c^c$</td>
<td>38 ± 4.2</td>
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<td>3-Deazaguanosine$_c^c$</td>
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<tr>
<td>PSI-0194$_c^c$</td>
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<tr>
<td>CS-978$_c^c$</td>
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<tr>
<td>PSI-5741$_c^c$</td>
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<tr>
<td>1-(4-methoxybenzoyloxy)</td>
<td>&gt;31</td>
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<tr>
<td>adenosine$_c^c$</td>
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</table>

$^a$ Mean of three independent assays ± S.D.

$^b$ SI = Mean IC$_{50}$/Mean EC$_{50}$.

$^c$ µg/mL.

$^d$ Significant difference compared to the EC$_{50}$ or IC$_{50}$ of the 7-day NR uptake assay (p<0.05).

$^e$ µM.
Table 3-2 Continued

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<tr>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>SI</th>
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<tr>
<td>0.35 ± 0.025&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&gt;100</td>
<td>&gt;280</td>
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<tr>
<td>0.011 ± 0.0024</td>
<td>&gt;1</td>
<td>&gt;89</td>
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<tr>
<td>0.86 ± 0.059</td>
<td>&gt;100&lt;sup&gt;d&lt;/sup&gt;</td>
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</tr>
<tr>
<td>0.76 ± 0.085</td>
<td>&gt;100&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&gt;130</td>
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<tr>
<td>0.89 ± 0.13&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>5.1 ± 0.68</td>
<td>&gt;100</td>
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<tr>
<td>10 ± 3.3</td>
<td>&gt;100</td>
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<tr>
<td>31 ± 2.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&gt;1000&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>9.8 ± 1.9</td>
<td>&gt;100</td>
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<td>6.8 ± 0.46</td>
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<td>2.9 ± 0.97&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>3.5 ± 0.1</td>
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<td>68 ± 2.6</td>
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<td>43 ± 2.5</td>
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other hand, when the rHPIV3-EGFP virus was measured by the EGFP fluorescent assay the resulting EC\textsubscript{50} values were significantly lower (p<0.05) for three out of the six drugs compared to the NR assay. This result will also contribute to the higher selectivity detected for these compounds in the EGFP assay compared to the selectivity of these compounds evaluated in the NR assay. The combination of an increased IC\textsubscript{50} and a decreased EC\textsubscript{50} undoubtedly increased the SI for these six compounds and falsely suggested further evaluation, explaining the low specificity of the EGFP assay. Overall, for most other compounds a trend is seen when an increase in the SI value was detected in the EGFP fluorescent assay compared to the NR assay. In most cases, the increased SI can be contributed to either a significant decrease in the EC\textsubscript{50}, significant increase in the IC\textsubscript{50}, or a combination of both scenarios.

4. Discussion

The purpose of this study was to investigate the possibility of substituting rHPIV3-EGFP for HPIV-3 WT in the initial screening of potential antiviral compounds. First, attenuation of either the rHPIV3 or rHPIV3-EGFP, compared to HPIV-3 WT, was studied to determine if loss of virulence had occurred due to either the assembly or rescue of a recombinant clone or the addition of the EGFP gene. The rescued rHPIV3 virus has three genetic mutations that made it distinguishable from HPIV-3 WT. Although these three genetic markers were detected in the rescued virus, they did not attenuate the recombinant virus. Two of these markers reside in the 3’ untranslated region of the HPIV-3 antigenome and could have disrupted regulatory promoters to attenuated rHPIV3; but they did not. On the other hand, the addition of the EGFP gene into the
rHPIV3 virus, which increased the length of the viral genome by only 5% and added a seventh, distinctive gene unit, did significantly attenuate the rescued rHPIV3-EGFP virus. Even though the attenuation was statistically significant, only a 1.5-fold reduction in rHPIV3-EGFP titers was seen. The attenuation may be of no significance because a wild-type virus grown in varying cell culture conditions: the age of the cells, confluency of the cell monolayer, variation in the media formulations, or incubation in other varying conditions, may inhibit or accelerate virus replication. A 10-fold, or greater, reduction in virus titer would indicate severe attenuation and would suggest that the new virus was indeed biologically different than the wild-type strain. In support of this point, the CPE produced by rHPIV3-EGFP was not significantly different compared to the CPE produced by HPIV-3 WT throughout the duration of a viral infection. Although, decreased viral titers and slower viral replication was detected for the rHPIV3-EGFP virus, CPE produced by each virus remained the same.

The attenuation of rHPIV3-EGFP can be attributed to the combination of the small increase in genome length and the addition of a foreign gene, which contributed to the significantly reduced viral genomic replication and mRNA transcription. The viral polymerase terminates and reinitiates transcription at each gene junction inconsistently, resulting in a reduction of downstream gene transcription and expression in a gradient fashion (Cattaneo et al., 1987; Homann et al., 1990). Thus, the first of six viral genes, NP, should be expressed at significantly higher levels compared to the last gene, L. Therefore, the insertion of the EGFP gene into the first viral gene position should result in a reduction in mRNA transcription in all downstream viral genes due to the inconsistency of the viral polymerase to reinitiate viral mRNA transcription. This
phenomenon was confirmed when transcription of the L gene was reduced in the
rHPIV3-EGFP virus infection compared to the HPIV-3 WT virus infection. In addition,
genomic replication was also reduced, perhaps due to the overall decline in the
expression of necessary viral replication proteins. A significant reduction in viral
transcription could also lead to a reduction in translation of the viral transcripts. Overall,
less viral proteins would be available for replication purposes resulting in less efficient
viral replication. Thus, less virions would be assembled because of the reduction in viral
proteins and genomic RNA strands resulting in an attenuated virus. Furthermore,
ribavirin and 2-thio-6-azauridine inhibit inosine monophosphate dehydrogenase and
orotidine monophosphate decarboxylase, respectively, and can be classified as nucleoside
analogs, which may be incorporated into the viral RNA strands and interfere with further
protein translation and genome replication (De Clercq, 1993; Sidwell, 1996). The
reduced EC$_{50}$ values are probably related to the reduction in mRNA transcription and
genomic replication; therefore, less compound is needed to incorporate into the RNA
strands and inhibit viral expression. On the other hand, DAS181 eliminates the host cell
receptor needed for viral entry and inhibits virion binding and fusion (Malakhov et al.,
2006). The reduced EC$_{50}$ values are most likely due to the reduction in viable virions
produced by the attenuated virus; therefore, less compound is required to prevent virion
attachment. The consequence of a slightly attenuated virus is the possibility of a reduced
EC$_{50}$ value, which may increase the SI value above the threshold of 10 and result in a
false positive, meaning that the same compound may not inhibit the wild-type virus as
much. This consequence is acceptable in the initial screening of a high-throughput assay
because the false positive compounds would be retested in the presence of the wild-type
virus and if they were true false positives they would be eliminated in the second round of screening.

The potential of using a recombinant virus that expresses a reporter gene to measure viral replication leads to the possibility of detecting the virus earlier in the assay. Assays that use dyes or enzymes, like NR, Vybrant® MTT, and CellTiter-Glo®, are only measuring the health of a cell and for these assays to be most accurate the maximum difference between signal and background needs to be achieved. This occurs for these types of assays when the virus has completely lysed the infected cell monolayer or when cell membranes have become non-functional. The length of time needed to reach this point depends upon the virus. The virus used in this study, HPIV-3, requires 7 days to achieve complete cell destruction at the MOI used. However, using an assay that measures a viral expressed reporter gene, the incubation time is only limited to when the reporter gene reaches acceptable or maximal signal-to-background ratio levels. For rHPIV3-EGFP, EGFP expression is detectable 24 h post-infection and reaches its peak at 3 days in a dose responsive manner. The characteristic syncytia formation of the HPIV-3 virus might be the reason that abundant EGFP expression levels are achieved and remain for 5 days after the maximum expression levels are reached. Even the lowest dose of virus shown could potentially be used in the antiviral assay, but for the purpose of this study we chose to use a concentration of virus that was equivalent to the concentration of virus used in typical antiviral assays. The broad range of EGFP detection possibilities, in both the length of time and concentration of virus, could potentially be used in experiments that need more defined parameters.
The 3-day, EGFP-based assay was evaluated for use in high-throughput assays using Z’-factor analysis and other parameters. It had a good to excellent Z’-factor value in addition to very high signal-to-background and signal-to-noise ratios. The Z’-factor takes into account the variability of both the signal and background and the difference between the signal and background. Thus, the virus-expressed EGFP gene is very suitable for this type of assay because only the infected cells will fluoresce and any background measurement seen is due to autofluorescence of the plate, medium, or cells, which can be subtracted from the signal. On the other hand the NR, Vybrant® MTT, and CellTiter-Glo® luminescent assays all measure any intact cells infected or not infected with virus. This can be problematic if the virus does not lyse the cell monolayer completely because the background measurements are raised, reducing the sensitivity and validity of the assay. For example, the CellTiter-Glo® luminescent assay was very susceptible to this phenomenon because even when the cells of the virus controls were completely lysed, as determined visually, significant luminescence was measured when compared to the no-cell control (data not shown), thus the poor signal-to-background and signal-to-noise ratios for the luminescent assay.

When the 3-day, EGFP assay was evaluated with a panel of known inhibitors of HPIV-3 WT replication, the assay resulted in excellent sensitivity and marginal specificity. When the rHPIV3-EGFP virus was used in an antiviral assay and fluorescence was measured, approximately an equal number of false positives and true positives would have passed the initial round of antiviral drug screening. However, this is of no concern because in the second round of antiviral drug testing, HPIV-3 WT virus would be used with results being measured by the NR uptake assay. In addition, active
compounds could also be further evaluated and validated by virus yield reduction assay or modified plaque reduction assay (Barnard et al., 2004; Matrosovich et al., 2006). Thus, the true, false positives would be detected and eliminated from the drug screening process. It would be more of a concern if the sensitivity were marginal because that would mean that false negative compounds would be eliminated from further testing.

However, some of the differences seen between the SI values determined from the EGFP-based and NR-based assays were due to differences in drug toxicity measured at 3 and 7 days, respectively. The compounds in question seem to be less toxic on day 3 than on day 7, which implies that the toxicity effects accumulate over time. In addition, cell growth may be slowed leading to apparent cell growth inhibition due to depletion of nutrients and acid build up in the medium after 7 days of incubation. These two phenomena probably contributed to the apparent increase in toxicity as measured by the IC50 values in the 7-day, NR assay. Furthermore, because of the additional factors contributing to cell toxicity a more accurate assay for detecting cell toxicity is conducted in follow-up studies for active compounds using rapidly-dividing MA-104 cells, which are incubated for 3 days in the absence of virus and measured by NR uptake. In essence, the 3-day, EGFP assay may be more accurate compared to the 7-day, NR assay for measuring cell toxicity.

The development of the EGFP assay has increased the sensitivity and quality of the antiviral assay, while shortening the duration and significantly decreasing, although not completely eliminating, the time consuming and labor intensive nature of the NR dye uptake assay. Overall, the use of the rHPIV3-EGFP virus in initial antiviral drug testing reduces the amount of time needed to obtain results and may be beneficial when testing
numerous compounds in a high-throughput format. These conclusions warrant the replacement of the HPIV-3 WT virus with the rHPIV3-EGFP virus in initial antiviral testing. Finally, additional research to improve the 3-day, EGFP assay might include scaling-up to a 384-well plate format and the development of a non-green fluorescent dye to replace NR in cell toxicity measurements.

References


(H1N1 and H3N2) virus activities and toxicities of compounds. J. Virol. Methods 106, 71-79.


CHAPTER 4
RECOMBINANT, INFECTIOUS HUMAN PARAINFLUENZA TYPE 3 VIRUSES DEFICIENT IN EXPRESSION OF THE D DOMAIN OF THE PD PROTEIN SHOW A REDUCTION IN VIRAL REPLICATION

Abstract

The HPIV-3 P gene contains an editing site that leads to the expression of the viral proteins, P, PD, the putative W, and theoretical V proteins. In this study, a rHPIV3 cDNA clone was engineered to express a hexahistidine tag on all proteins expressed from the P gene start codon through mRNA editing. The P, PD, and W proteins were identified but the V protein was not detected. Also in this study, the involvement of the D domain in viral replication and/or the host cell’s antiviral response was investigated by rescuing two knockout rHPIV-3 viruses, rHPIV3-ΔES and rHPIV3-ΔD, which are deficient in the expression of the D domain of the PD protein by separate mechanisms. The growth kinetics of the two knockout viruses were attenuated, compared to the parent virus, when each virus was replicated in both MA-104 cells and A549 cells. Also, a significant reduction in viral mRNA transcription and genomic replication, suggesting attenuation, was seen in both knockout viruses. In addition, cytokine expression from A549 cells infected with either knockout virus was unchanged or reduced compared to cells infected with the parent virus. These data suggest that the D domain may be involved in some aspect of viral replication and likely not involved in the host cell’s antiviral response.
1. Introduction

All members of the *Paramyxovirinae* subfamily express additional accessory proteins from the P gene by mRNA editing and/or ribosomal choice, in addition to the P protein. The C proteins, expressed by the respiroviruses, are transcribed by ribosomal choice, where the ribosome scans the mRNA bypassing the first start codon and initiates translation at a second start codon further downstream. For HPIV-3, the start codon for the C protein is approximately ten ribonucleotides downstream from the start codon of the P protein (Galinski et al., 1986). On the other hand, viral fusion proteins are expressed by mRNA editing, which occurs when non-templated G residues are added by the viral polymerase at an editing site located in the middle of the P gene during viral mRNA transcription. The result of mRNA editing is a pool of P gene mRNA with differing G insertions and leads to the translation of fusion proteins with identical N-terminal ends and different C-terminal ends. For HPIV-3, the proteins expressed by mRNA editing are the P, PD, putative W, and hypothetical V proteins (Galinski et al., 1992; Vidal et al., 1990).

The functions of the accessory proteins have been attributed to regulation of viral RNA synthesis and/or inhibition of the host cell’s antiviral response. For example, the C proteins are involved in both regulating viral RNA synthesis and counteracting the antiviral response of the infected host cell. The titers of recombinant HPIV-3 and SeV viruses, which are deficient in the expression of their C proteins, are restricted in cell culture (Durbin et al., 1999; Kurotani et al., 1998). However, the specific aspect of viral RNA synthesis is different for each virus. The SeV virus C protein regulates genomic replication, whereas the HPIV-3 virus C protein regulates viral mRNA transcription.
(Cadd et al., 1996; Malur et al., 2004). In addition, the SeV C protein inhibits some aspect of the RIG-1 signaling pathway, but not the MDA-5 signaling pathway, thus preventing the primary antiviral response and IFN-β expression from the infected host cell (Strahle et al., 2007). Also, the SeV C protein interferes with the secondary antiviral response by binding and inducing ubiquitination of STAT1; leading to proteosome-mediated degradation of STAT1 and inhibition of IFN signaling (Garcin et al., 2002). The HPIV-3 C protein also interferes with the secondary antiviral response by binding and preventing the phosphorylation and activation of STAT1 and, thus, inhibiting IFN signaling (Malur et al., 2005).

Furthermore, the V protein works in concert with the C protein to inhibit the primary antiviral response and IFN-β expression. The V protein is characterized by a highly conserved C-terminal cysteine-rich domain that binds two zinc atoms to form a zinc-finger structure (Paterson et al., 1995). It is expressed by most members of the Paramyxovirinae, except for HPIV-3 and HPIV-1 (Paterson et al., 1995; Vidal et al., 1990). Even though the cysteine-rich domain is not encoded anywhere within the HPIV-1 genome, it is located in the HPIV-3 antigenome in the same reading frame as the W protein downstream of 2 or 3 stop codons, depending upon the virus strain (Chapter 3; Galinski et al., 1992; Matsuoka et al., 1991). The V proteins for most paramyxoviruses inhibit the primary antiviral response by binding MDA-5 and, thereby, preventing dsRNA binding and further signaling of the antiviral response and inhibiting IFN-β expression (Andrejeva et al., 2004; Childs et al., 2007). Also, the MeV V protein interferes with IFN-β expression by competing with IRF-7 for binding to and
phosphorylation by IKKα, which is common to both RIG-1 and MDA-5 signaling pathways (Pfaller and Conzelmann, 2008).

Although the HPIV-3 antigenome may contain the nucleotide sequence that encodes for the cysteine-rich domain characteristic of the V protein, it is still unknown if it is expressed. In the only study reporting research pertaining to the HPIV-3 V protein, the D and V domains were silenced individually and simultaneously (Durbin et al., 1999). No attenuation in viral titers was seen when the individual or simultaneous knockout viruses were used in vitro or individually in vivo, but attenuation was seen when the domains were silenced simultaneously in vivo. These data suggest that the D and V domains were expressed as proteins and that they played a role in the host cell’s antiviral response. More recently, the PD protein of HPIV-3, which is the fusion of the N-terminal end of the P protein and the C-terminal D domain, was detected in vitro. Three nuclear localization signals were identified within the PD protein that directed the intracellular location of the PD protein to the nucleus (Wells and Malur, 2008). Alternatively, if HPIV-3 does not express the V protein, then perhaps the PD protein, which is only expressed by HPIV-3 and bovine (B)PIV-3, is expressed to replace the V protein during the viral infection and interferes with the primary antiviral response to reduce IFN-β expression. In this study, we investigated if the V protein is expressed from the start codon of the P gene through the editing site. We also identified possible roles of the D domain in the viral replication cycle by generating knockout viruses deficient in the expression of the D domain of the PD protein.
2. Materials and methods

2.1. Cells and viruses

MA-104 were obtained from the ATCC (Manassas, VA) and maintained at 37°C and 5% CO₂ in MEM (Hyclone Laboratories, Logan, UT) supplemented with 10% FBS (Hyclone Laboratories). Human lung epithelial carcinoma cells (A549) were obtained from the ATCC and maintained at 37°C and 5% CO₂ in Dulbecco's modified eagle's medium (DMEM, Hyclone Laboratories) supplemented with 10% FBS. A recombinant vaccinia virus that expresses the bacteriophage T7 RNA polymerase, vTF7-3 was generously provided by Dr. Bernard Moss (Fuerst et al., 1986). During all assays, MA-104 and A549 cells were incubated in MEM supplemented with 2% FBS and 50 µg/ml gentamicin (Sigma Chemical Company, St. Louis, MO).

2.2. Plasmid Construction

The nucleotide sequence that encodes six histidine amino acids was inserted into the rHPIV3 antigenome immediately following the P gene start codon using the QuikChange® XL Site-Directed Mutagenesis (Stratagene, La Jolla, CA) kit and the previously reported 1–5267 cDNA segment (Chapter 3). The forward, 5’-CTCAATCAATAGAGAGTTGATGATCACCATCACCATCACGAAAGCGATGCTAAAAACTATC-3’, and reverse, 5’-GATAGTTTTTAGCATCGCTTTCGTGATGGTGATGGTGATGCATCAACTCTCTATTGATTGAG-3’, primers were also used in the reaction to insert the nucleotide sequence for the hexahistidine tag, underlined, at position 1787. The resulting clone was sequenced for accuracy and renamed pUC19-NT. Two additional clones were also
engineered to eliminate the P gene editing site and silence the D domain of the PD protein using pUC19-NT as the template and QuikChange® XL Site-Directed Mutagenesis kit. The forward, 5’-CTTCAACACATCAAGAAGATGACAAAAAGAGGGAAAGACTGGTTT-3’, and reverse, 5’-AAACCAGTCTTTCCCTTTTGTATCTTTCTTGATGTGTGAAG-3’, primers were used to delete 18 nucleotides at positions 2495–2512 from the antigenome. The clone was sequenced for accuracy and renamed pUC19-DS. Also, the forward, 5’-CAAAAGAATTTAAAAAGGGGGAAGGTGAAAGACTGGTTTAAGAAATCA AAAG-3’, and reverse, 5’-CTTTTGATTTCTTAAACCAGTCTTTCACTTTTCCCCCTTTTTTATTCTTTTG-3’, primers were used to mutate a single nucleotide from G to T, underlined, at position 2514. The resulting clone was sequenced for accuracy and renamed pUC19-SD. The assembly of the complete antigenomes for each mutant clone was followed as previously described and they were renamed pUC19-L, which included a hexahistidine tag, pUC19-V, which destroyed the editing site, and pUC19-X, which silenced the D domain of PD protein (Chapter 3).

2.3. Rescue of infectious virus from cDNA

MA-104 cells, seeded in a 12-well plate, were exposed to vTF7-3 for 1 h at 1 x 10^5 PFU, which equals to a MOI of 1. The virus and medium were removed and replaced with Opti-MEM® (Invitrogen, Carlsbad, CA). Three support plasmids, 1 μg of pTNT-NP, 2 μg of pTNT-P, and 0.05 μg of pTNT-L were cotransfected along with 0.5 μg of either pUC19-L, pUC19-V, or pUC19-X using Lipofectamine™ 2000 (Invitrogen)
for 6 h at 37°C (Chapter 3). MEM supplemented with 2% FBS and 250 μg/mL of Ara-C (Sigma, St. Louis, MO) was added to each transfection and incubated for 3 days at 37°C. The transfected cells were scraped from the tissue culture wells and the virus lysates frozen at -80°C. The rescued viruses, rHPIV3-NT by pUC19-L transfection, rHPIV3-ΔES by pUC19-V transfection, and rHPIV3-ΔD by pUC19-X transfection, were amplified in MA-104 cells supplemented with 250 μg/mL Ara-C for 3 days at 37°C. The infected cells were scraped from the tissue culture wells and the virus lysates frozen at -80°C. Each virus was purified by removing agarose plugs containing virus plaques in MA-104 cells in the absence of Ara-C. Each plug was placed in MEM and frozen at -80°C. The medium, containing the plug and isolated virus, was used to infect MA-104 cells to amplify the virus for 3 days at 37°C. The purification and amplification steps were repeated one more time. The rHPIV3-NT, rHPIV3-ΔES, and rHPIV3-ΔD viruses were amplified a final time in MA-104 cells for 3 days at 37°C. The infected cells were scraped from the tissue culture wells and the virus lysates were frozen at -80°C until used in each experiment. Viral genomic RNA was purified and sequenced to identify mutations contained in the P gene using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen) and the 5’-GCAACACCAGATGATGAAGAAGA-3’ forward primer for cDNA synthesis. The cDNA products were amplified using AccuPrime™ Pfx SuperMix (Invitrogen), the forward primer, mentioned above, and the 5’-GGATTACACCAAGATTCTGCAATAG-3’ reverse primer, and the amplification products were then sequenced.
2.4. Western blot analysis

Twelve-well plates were seeded with MA-104 cells and infected separately with one of the following viruses: rHPIV3-NT, rHPIV3-ΔES, or rHPIV3-ΔD, at an MOI = 0.1 (3 x 10^4 PFU). Each virus was absorbed to the cells for 2 h at 37°C; after which the virus was removed and replaced with fresh MEM supplemented with 2% FBS. The plates were incubated at 37°C for 2 days. Protein from each infection was extracted using M-PER® Mammalian Protein Extraction Reagent and concentration was measured using the colorimetric BCA Protein Assay Kit (both from Pierce, Thermo Fisher Scientific, Rockford, IL). The assay was measured with a SpectraMax® Plus Spectrophotometer (wavelength 562 nm) and the data analyzed with SOFTmax® PRO software, version 4.3 (both from Molecular Devices, Sunnyvale, CA). A mixture of 20 μg of each protein sample, NuPAGE® LDS Sample Buffer and Sample Reducing Agent was heated at 70°C for 10 min (Invitrogen). The resulting mixtures were then loaded on a NuPAGE® Novex® 4-12% Bis-Tris Gel (Invitrogen) and electrophoresed at 200V for approximately 1 h in NuPAGE® MOPS SDS Running Buffer and Antioxidant (Invitrogen). The proteins in the gel were transferred to nitrocellulose using the iBlot™ Dry Blotting System and Gel Transfer Stacks (Invitrogen). The unbound protein binding sites on the nitrocellulose membrane were bound with SuperBlock® T20 (TBS) Blocking Buffer (Pierce) for 1 h with rocking at room temperature. Hexahistidine tagged proteins were detected by the following chemiluminescent procedure. The Monoclonal Anti-polyHistidine Peroxidase (Sigma) antibody (4 μg/mL) was incubated with the nitrocellulose membrane for 1 h with rocking at room temperature. The membrane was washed three times with Tris buffered saline (TBS) containing 0.05% Tween-20 (Pierce)
for 5 min with rocking at room temperature. The antibody bound proteins were then incubated with the SuperSignal® West Pico Chemiluminescent Substrate (Pierce) to detect any chemiluminescence. The membrane was then photographed with the Image Station 2000R camera using a 1 min exposure and the image was analyzed with the 1D Image Analysis software (both from Eastman Kodak Company, New Haven, CT). The 1D Image Analysis software was also used to estimate the molecular weight of each band in each lane by comparison to the BenchMark™ His-tagged Protein Standards (Invitrogen).

2.5.  **MA-104 replication plaque assay**

Two 48-well plates were seeded with MA-104 cells and infected separately with 10-fold dilutions of rHPIV3-NT, rHPIV3-\(\Delta ES\), and rHPIV3-\(\Delta D\) viruses and plated in triplicate. Each virus was absorbed for 2 h at 37°C; after which the virus inocula were removed and replaced with an overlay of 1% SeaPlaque® low-melting agarose (ISC BioExpress®, Kaysville, UT) supplemented with 1X MEM (Invitrogen). The plates were incubated for 2 days at 37°C to allow plaque formation. Cells were then fixed with 3.6% formaldehyde for 2 h at room temperature. The formaldehyde and agarose overlays were then removed and 0.5% crystal violet was added to each well and incubated for 5 mins at room temperature. After removal of the dye and rinsing one time with PBS, the stained plaques were counted. Viral titers were compared and statistically analyzed using unpaired two-tailed Student's t-tests. The resulting titers of each virus were used to calculate and adjust the volumes of virus inocula to equalize the PFU of each virus used in all subsequent experiments.
2.6. A549 replication plaque assay

Two 12-well plates were seeded with A549 cells and the cells were infected separately with one of the following viruses: rHPIV3-NT, rHPIV3-ΔES, and rHPIV3-ΔD, at an MOI = 0.1 (4.8 x 10^4 PFU). Each virus was absorbed to the cells for 2 h at 37°C. The virus was then removed, replaced with fresh MEM supplemented with 2% FBS, and incubated at 37°C for 3 days. The infected A549 cells were scraped from the tissue culture wells and the virus lysates frozen at -80°C. Each virus was plaque titered in triplicate, following the method described above, and comparisons were completed using unpaired, two-tailed Student's t-tests. To verify if any reversions had occurred in the genomic sequences of either knockout virus the viral genomic RNA was amplified and sequenced following the methods described above and compared to the parent virus.

2.7. MA-104 and A549 one-step replication curves

Two 12-well plates were seeded with MA-104 and A549 cells separately and infected with either rHPIV3-NT, rHPIV3-ΔES, and rHPIV3-ΔD viruses separately at an MOI = 3 (9.1 x 10^5 PFU for MA-104 cells and 1.4 x 10^6 PFU for A549 cells). Each virus was absorbed to the cells for 2 h at 37°C. The virus inocula were then removed, replaced with fresh MEM supplemented with 2% FBS, and incubated at 37°C. The infected cells were scraped from individual tissue culture wells at 2, 12, 24, and 48 h post virus exposure and frozen at -80°C. Each time point for each virus was quantified by plaque assay in triplicate as described earlier. Statistical comparisons of the MA-104 and A549
growth curves were compared by two-factor ANOVA and pairwise multiple comparisons were accomplished by Tukey's honestly significant difference tests.

2.8. **MA-104 viral QRT-PCR assay**

Three 96-well plates were seeded with MA-104 cells and infected separately one of the following viruses: rHPIV3-NT, rHPIV3-ΔES, and rHPIV3-ΔD at an MOI = 3 (5.7 x 10^3 PFU). At 0, 12, 24, and 48 h post virus exposure, RNA from uninfected and infected cells was harvested using CellsDirect™ Resuspension and Lysis Buffers kit (Invitrogen). RNA from each lysate was used as a template for two different RT reactions using the SuperScript™ III CellsDirect™ cDNA Synthesis System kit (Invitrogen). A primer specific for the HPIV-3 genome, 5’-AATTATAAAAAACTTAGGAGTAAAG-3’, was used in one reaction and an Oligo(dT)20 primer (Invitrogen) was used in a second reaction. LUX™ primers (Invitrogen) were used to PCR amplify the cDNA products from the RT reactions and included 5’-CGTTATAGTGCTGCCACAAAGAATAA[FAM]G-3’ and 5’-ATGGAAGACCAGACGTGCATC-3’ primers for genomic replication, and 5’-CGATTAAGGAAAGCGACCTGTAAGTAAT[FAM]G-3’ and 5’-GAGACACAAATTAGGCGGAGAT-3’ primers for L gene transcription. The contents of the Platinum® Quantitative PCR SuperMix-UDG kit (Invitrogen), 200 nM of the forward and reverse primers, and one-third of the RT reaction, described above, were mixed. The reaction mixtures were then added to duplicate wells of Hard-Shell 96-well skirted PCR plates (Bio-Rad Laboratories, Hercules, CA). The reaction mixtures were incubated in a DNA Engine Opticon 2 Real-Time PCR Detection System (MJ Research,
Waltham, MA). The Opticon Monitor™ software, version 3.1.32 (Bio-Rad Laboratories) was used to calculate the relative expression differences, Delta-CT, at each time point for each virus using time 0 for each virus as the baseline calibrator (Pfaffl, 2001). Data points outside the median ± three times the interquartile range were eliminated from further analysis. Statistical comparisons of the viral mRNA transcription and genomic replication curves were compared by two-factor ANOVA and pairwise multiple comparisons were accomplished by Tukey's honestly significant difference tests.

2.9. A549 cytokine ELISA

Three 24-well plates were seeded with A549 cells and infected separately with the following viruses: rHPIV3-NT, rHPIV3-ΔES, and rHPIV3-ΔD at an MOI = 0.1 (3.5 x 10^4 PFU). Each virus was absorbed for 2 h at 37°C. The virus inocula were removed, replaced with fresh MEM supplemented with 2% FBS, and incubated at 37°C for 24 h. The tissue culture medium for the infected and uninfected wells was removed and stored at -80°C. Cellular RNA was isolated from the monolayer from the same tissue culture wells and used for the QRT-PCR as discussed and shown below. Cytokine levels present in the tissue culture media were measured by enzyme-linked immunosorbent assay (ELISA) using the VeriKine™ Human Interferon-Beta ELISA Kit (PBL InterferonSource, Piscataway, NJ) to measure IFN-β levels secreted by A549 cells. The assay was measured on a SpectraMax® Plus384 spectrophotometer (wavelength 450 nm) and the data analyzed with SOFTmax® PRO software. IFN-β expressed from MA-104 cells infected with rHPIV3-NT at an MOI = 0.1 (2.5 x 10^4 PFU) was also assayed for using the same procedure and ELISA kit. A human multiplex cytokine array was used to measure the
expression levels of interleukin (IL)-1α, IL-6, IL-8, IL-10, IL-12p70, monocyte chemotactic protein (MCP)-1, macrophage inhibitory protein (MIP)-1α, MIP-1β, RANTES, IFN-γ, tumor necrosis factor (TNF)-α, and eotaxin (EO) cytokines also present in the same supernatants following the manufacturer’s protocol (Cytokine Research Institute, Logan, UT). Chemiluminescence was measured with a Quansys imager and the data analyzed with Q-view™ software, version 2.0 (both from Quansys Biosciences, Logan, UT). Concentrations of the cytokines present in the tissue culture media were determined either by linear or exponential regression analysis. Data points outside the median ± three times the interquartile range for IFN-β and the median ± one and a half times the interquartile range for all other cytokines were eliminated from further analysis. The concentrations of each cytokine expressed from A549 infected and uninfected cells were compared for significance using unpaired, two-tailed Student's t-tests.

2.10. A549 interferon-β QRT-PCR assay

The CellsDirect™ One-Step qRT-PCR kit (Invitrogen) was used to amplify mRNA encoding the IFN-β and β-actin genes from total RNA isolated from infected and uninfected A549 cells 24 h after virus exposure. FAM labeled forward primer (200 nM) and unlabeled reverse primer (250 nM) for the IFN-β D-LUX™ Select Gene Expression Assay (Invitrogen) along with JOE labeled forward primer (100 nM) and unlabeled reverse primer (125 nM) for the β-actin Certified LUX™ Primer Set (Invitrogen) were added to the QRT-PCR mixture. Also, an additional 1 unit of Platinum® Taq DNA Polymerase (Invitrogen) and 3 mM of magnesium sulfate (MgSO₄) was also added to the QRT-PCR mixture. Following Dnase I treatment of the total RNA samples, one-quarter
of each sample was added to duplicate wells of Hard-Shell 96-well skirted PCR plates. The reaction mixtures were incubated in a DNA Engine Opticon 2 Real-Time PCR Detection System. Opticon Monitor™ software was used to calculate the relative expression of Delta-Delta-C_{T} of IFN-β during each infection using β-actin as an internal control and the IFN-β expressed in uninfected cells as the baseline calibrator (Pfaffl, 2001). Data points outside the median ± three times the interquartile range were eliminated from further analysis. Relative expression levels for IFN-β for each virus infection were compared and statistically analyzed using unpaired, two-tailed Student's t-tests.

3. Results

3.1. The successful rescue of infectious, recombinant HPIV-3 viruses expressing a hexahistidine tag without the D domain of the PD protein

The rHPIV3-NT parent clone was constructed to contain six codons, each of which encoded a histidine amino acid, CAU or CAC, immediately downstream of the P gene start codon. This addition created a hexahistidine tag on all proteins expressed from the P gene start codon, which included the known P and PD proteins and an unidentified, putative W protein. The nucleic acid sequence that encoded the hexahistidine tag was added to the complete viral antigenome to contain a total of 18 nucleotides to conform to the “Rule of Six.” The rule suggests that viral replication is most efficient when the viral genome length is a factor of six ribonucleotides, which is most likely due to a single nucleocapsid protein binding to six genomic ribonucleotides (Calain and Roux, 1993;
Durbin et al., 1997). Two other knockout clones, also containing the hexahistidine tag, were made to silence the expression of the D domain of the PD protein using two different approaches. In the first approach, 18 nucleotides, following the “Rule of Six,” were eliminated from the P gene of the rHPIV3-NT antigenome that surrounded the editing site, which formed the rHPIV3-ΔES clone. In the second approach, a single nucleotide was substituted to create a stop codon and silenced only the D domain of the PD protein, which formed the rHPIV3-ΔD clone. Sequence analysis of genomic RNA isolated from separate infections of the three rHPIV3 clones and converted to DNA showed an intact editing site in the rHPIV3-NT clone, the elimination of the editing site in the rHPIV3-ΔES clone, and a single substitution mutation in the rHPIV3-ΔD clone (Fig. 4-1A).

In addition, the proteins expressed from these clones could be detected with an anti-HIS antibody. The rHPIV3-NT clone, with no modification to the editing site, should have expressed the two predicted proteins, P and PD, and any other possible proteins that are expressed from the P gene start codon, for example the putative W protein. Western blot analysis of the rHPIV3-NT infected cell lysates, probed with an anti-HIS antibody, showed the presence of a P protein at 78 kilodaltons (kDa), the PD protein at 50 kDa, and the W protein at approximately 36 kDa (Fig. 4-1B). Other possible proteins expressed by rHPIV3-NT through the P gene start codon, namely the V protein, were not detected by western blot. For infected cell lysates exposed to the rHPIV3-ΔES clone, only a single band at 77 kDa representing the P protein with six amino acids removed was detected; notably absent were the W and PD protein bands. For rHPIV3-ΔD infected cell lysates two bands were detected; one at 78 kDa,
representing the P protein, and a second band at 36 kDa, representing the W protein. No PD protein band was detected in the rHPIV3-ΔD infected cell lysates. The relative expression levels of the individual proteins produced by rHPIV3-NT could not be compared to each other nor estimated because only cell lysates were analyzed. The P protein is a structural protein and is packaged with virions that are released from the infected cell into the cell culture medium. However, relative expression levels between all three recombinant viruses were estimated and used as a guide to judge the effectiveness of the mutations. The rHPIV3-ΔES clone expressed only the P protein resulting in a dramatic increase in the intensity of the P protein band, 78 kDa (Fig. 4-1B).
During replication of the rHPIV3-ΔD clone, the PD protein was truncated to a size equal to that of the putative W protein. The intensity of the W protein band, derived from the rHPIV3-ΔD infected cell lysates, increased slightly although there was no change in the intensity of the P protein band.

3.2. Attenuation of the knockout clones
rHPIV3-ΔES and rHPIV3-ΔD in MA-104 and A549 cells

To compare the replication kinetics of the two knockout clones, each virus was amplified in MA-104 cells and A549 cells. In MA-104 cells, peak titers for all three recombinant viruses were measured by plaque assay. The titers of the infectious rHPIV3-ΔES and rHPIV3-ΔD viruses were significantly attenuated when compared to the parent strain rHPIV3-NT (p<0.001) (Table 4-1); virus titers were reduced by over 50%. On the other hand, the peak titers of rHPIV3-ΔES and rHPIV3-ΔD were not significantly different when compared to each other. MA-104 cells have been shown to be completely susceptible to an HPIV-3 infection resulting in 100% CPE, which is most likely due to the lack of IFN-β expression (Chapter 3 and Appendix A). Thus, HPIV-3 replication in MA-104 cells progresses unchallenged and any differences seen in the replication kinetics of the knockout viruses compared to the parent virus could be attributed to the kinetics of virus replication. Therefore, the similar lower viral titers of the two recombinant viruses not expressing the D domain of the PD protein, suggests that the D domain may facilitate virus replication. Even though it would seem that the D domain might not be necessary for virus replication, it may be needed for optimal and efficient virus replication. In support of this, a similar trend in lower viral titers was seen
for each virus when infecting A549 cells. The titers for rHPIV3-ΔES and rHPIV3-ΔD were significantly attenuated compared to the titer for rHPIV3-NT (p<0.001) (Table 4-1). The titers of the two knockout viruses were also significantly different from each other, with the rHPIV3-ΔES virus being the most attenuated (p<0.001). A549 cells appear to be a more restrictive cell line for supporting virus replication based on the significant difference in the viral titers of rHPIV3-NT grown in A549 and MA-104 cells (p<0.001) (Table 4-1), probably due to the fact that A549 cells express IFN-β (Fig. 4-4A and D). Knockout virus titers were attenuated compared to the titer of the parent virus in A549 cells, suggesting that the D domain could facilitate viral replication and/or interfering with the host cell’s antiviral response.

One-step viral replication curves were completed to further understand the attenuation of infectious knockout virus production in both MA-104 and A549 cells. The results resembled the titer results. The viral replication curves generated in MA-104 cells demonstrate an attenuation of rHPIV3-ΔES and rHPIV3-ΔD in virus production when compared to the replication curve for the parent virus rHPIV3-NT (p<0.001) (Fig. 4-2A).

Table 4-1
3-day titers of the rHPIV3-NT parent type and rHPIV3-ΔES and rHPIV3-ΔD knockout viruses in MA-104 and A549 cells.

<table>
<thead>
<tr>
<th>Virus</th>
<th>MA-104 Cells</th>
<th>A549 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Titera</td>
<td>p-value vs. NT</td>
</tr>
<tr>
<td>rHPIV3-NT</td>
<td>7.5 ± 0.5 X 10^7</td>
<td>p &gt; 0.05</td>
</tr>
<tr>
<td>rHPIV3-ΔES</td>
<td>3.6 ± 1.4 X 10^7</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>rHPIV3-ΔD</td>
<td>3.7 ± 0.6 X 10^7</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

a Mean of duplicate independent assays ± S.D.
But no significant differences were observed in the viral production of the two knockout viruses, rHPIV3-ΔES and rHPIV3-ΔD. In addition, the viral replication in A549 cells was also significantly reduced for the two knockout viruses, rHPIV3-ΔES and rHPIV3-ΔD, when compared to virus production of the parent virus rHPIV3-NT (p<0.001) (Fig. 4-2B). In contrast to the finding for infected MA-104 cells, virus replication of the two knockout viruses, rHPIV3-ΔES and rHPIV3-ΔD, was significantly different in A549 cells (p<0.001). These results support the notion that the knockout viruses are attenuated and suggest that the D domain facilitates the virus replication process in either cell type or may interfere with the antiviral response produced by A549 cells.

In both the peak titer assay and one-step replication curve in A549 cells, the titer of the rHPIV3-ΔES virus was significantly reduced compared to the titer of the rHPIV3-ΔD virus (Table 4-1 and Fig. 4-2B). One possible explanation for this could be that the rHPIV3-ΔD knockout virus, which has a single point mutation to silence the expression of the D domain, is reverting itself back into an rHPIV3-NT-like virus and expressing the D domain. This would lead to an increase in viral titers of rHPIV3-ΔD, which occurs when it is compared to the titer of the rHPIV3-ΔES virus. However, when the genomic RNA for the rHPIV3-ΔD virus, along with the other two viruses, was isolated and sequenced, no changes in the original mutations were seen (data not shown). In addition, when rHPIV3-ΔD was replicated in both MA-104 and A549 cells and rHPIV3-ΔES was replicated in MA-104 cells, a consistent 50% reduction in viral titers was detected (Table 4-1). In contrast, a full-log reduction in the viral titer of rHPIV3-ΔES was observed in A549 cells. This suggests that the replication of
Fig. 4-2. Single-step replication curves of the two knockout viruses compared to the parent virus. Replication of the parent virus, rHPIV3-NT (♦), and knockout viruses, rHPIV3-ΔES (■) and rHPIV3-ΔD (▲), in MA-104 cells (A) and A549 cells (B). All Y-axis values on all graphs represent the mean ± S.D. of duplicate assays.
rHPIV3-ΔES was even more restricted in A549 cells and confirms that no back mutations occurred in the rHPIV3-ΔD virus. This result might be explained by the absence of six amino acids from the middle of the P protein in the rHPIV3-ΔES virus, which may be necessary for a luxury function of the P protein. This phenomenon only occurred in the restrictive A549 cells infected with the rHPIV3-ΔES virus and not in MA-104 cells. Hence, the six amino acid deletion may have interfered with the host cell’s antiviral response.

3.3. Viruses deficient in the expression of the D domain show a reduction in genomic replication and viral gene transcription

To further investigate if the knockout viruses deficient in the expression of the D domain of the PD protein are attenuated viruses, viral genomic replication and gene transcription were measured by QRT-PCR at various time points during viral replication in MA-104 cells. During the RT reaction, different primers were used to measure viral gene transcription and genomic replication independently of each other. In both cases, viral gene transcription and genomic replication were significantly reduced in both knockout viruses rHPIV3-ΔES and rHPIV3-ΔD when compared to the viral RNA synthesis of the parent virus rHPIV3-NT (p<0.001) (Fig. 4-3). On the other hand, no significant differences were seen in viral gene transcription and genomic replication of the two knockout viruses, rHPIV3-ΔES and rHPIV3-ΔD. These results not only support the notion that the two knockout viruses are attenuated, but also that the D domain may facilitate all aspects of viral RNA synthesis.
Fig. 4-3. Viral RNA synthesis of the two knockout viruses compared to the parent virus measured by QRT-PCR. Viral mRNA transcription (A) and genomic replication (B) from the parent virus, rHPIV3-NT (♦), and knockout viruses, rHPIV3-ΔES (■) and rHPIV3-ΔD (▲), in MA-104 cells. All Y-axis values on all graphs represent the mean ± S.D. of triplicate assays.
3.4. *A549 cells infected with viruses deficient in the expression of the D domain have decreased expression of inflammatory cytokines*

To investigate the other putative functions of the D domain, cytokine expression in A549 cells infected with each knockout virus, rHPIV3-ΔES and rHPIV3-ΔD, was measured and compared to cytokine expression of A549 cells infected with the rHPIV3-NT parent virus. Time course evaluation of IFN-β expressed from A549 cells infected with rHPIV-NT showed peak expression at 24 h, therefore cytokine expression studies were measured at this time point (see Appendix A). Of the cytokines measured by ELISA, only five cytokines, IFN-β, IL-6, IL-8, RANTES, and MCP-1, were significantly increased from infected cells when compared to uninfected cell controls (p<0.01) (Fig. 4-4A, B, and C). The expression levels of the cytokines IFN-β, IL-6 and RANTES were significantly decreased in the cells infected with both knockout viruses when compared to cells infected with the parent strain (p<0.01) (Fig. 4-4A and B). On the other hand, the expression levels of the IL-8 and MCP-1 cytokines of A549 cells infected with all three viruses were similar (Fig. 4-4B and C). To confirm the reduction in IFN-β expression levels in cells infected with the knockout viruses, IFN-β mRNA levels were measured by multiplex QRT-PCR. IFN-β mRNA levels were significantly reduced in A549 cells infected with either knockout viruses when compared to the levels of IFN-β transcribed in cells infected with the parent virus (p<0.05) (Fig. 4-4D). These data confirm that the knockout viruses show attenuation and suggest that the D domain may not be involved in the A549 cell’s antiviral response because of a reduction or no change in cytokine expression.
Fig. 4-4. Cytokine profile expression in infected A549 cells measured by ELISA and QRT-PCR. Only the IFN-β (A), IL-6 (A), RANTES (B), IL-8 (B), and MCP-1 (C) cytokines expressed from infected A549 cells resulted in significant increases compared to uninfected cell controls (white bars, 4) (p<0.01). Significant decreases were seen in the levels of cytokines expressed from A549 cells infected with either rHPIV3-ΔES (grey bars, 2) or rHPIV3-ΔD (diagonal bars, 3) compared to cells infected with rHPIV3-NT (black bars, 1) (*** and *) (p<0.01). A significant increase was seen in the levels of the IFN-β (A) and RANTES (B) cytokines expressed from A549 cells infected with rHPIV3-ΔES compared to cells infected with rHPIV3-ΔD (*** (p<0.01). (D) IFN-β mRNA transcription, measured by QRT-PCR, in cells infected with either rHPIV3-ΔES or rHPIV3-ΔD were significantly reduced compared to cells infected with rHPIV-NT (*) (p<0.05). All Y-axis values on all graphs represent the mean ± S.D. of triplicate assays.
As noted above, rHPIV3-ΔES virus titers in A549 cells were dramatically reduced compared to not only the parent rHPIV3-NT virus titers, but more importantly the rHPIV3-ΔD knockout virus. Levels of IFN-β and RANTES expression from A549 cells infected with the rHPIV3-ΔES virus were significantly increased compared to the cytokines expressed from A549 cells infected with rHPIV3-ΔD (p<0.01) (Fig. 4-4A and B). However, IFN-β mRNA levels in cells infected with either knockout viruse were transcribed at similar levels (Fig. 4-4D). These results may suggest that the six amino acids deleted from the P protein of the rHPIV3-ΔES knockout virus may influence the host cell’s antiviral response.

4. Discussion

One of the objectives of this study was to investigate the possibility of the expression of additional proteins containing the N-terminal end of the P protein of HPIV-3. The P gene contains an editing site where the viral RNA-dependent RNA-polymerase stops and stutters adding non-templated G nucleotide residues (Appendix B) (Galinski et al., 1992). These additions lead to a pool of P gene mRNA with differing G insertions and three different C-terminal ends fused to an identical N-terminal end. Adding the nucleotide sequence that encodes for a hexahistidine tag immediately downstream of the start codon of the P gene would label the proteins expressed from the P gene that share the N-terminal end. This would allow the identification of the P and PD proteins and might identify the putative W protein and/or the theoretical V protein (Galinski et al., 1986; Wells and Malur, 2008). The V protein is expressed by all other members of the Paramyxoviridae family and is characterized by a
conserved cysteine-rich domain that forms a zinc finger (Paterson et al., 1995). In HPIV-3, this same domain, although semi-conserved, is also encoded for within the P gene in the same reading frame as the W protein downstream of 2–3 stop codons, depending upon the virus strain, thus allowing for the potential expression of the V protein (Galinski et al., 1992). In this study using hexahistidine tags, the proteins found to be expressed from the P gene start codon during an rHPIV3-NT infection were the P, PD, and W proteins; no V protein was detected by western blot. According to the nucleotide sequence, the calculated molecular weight of the P, PD, and W protein should be 68, 43, and 28 kDa, respectively. However, the observed molecular weights in our study appeared to be 78, 50, and 36 kDa, respectively. This discrepancy might be attributed to high levels of phosphorylation and other post-translational modifications. Although no band, representing the V protein, was detected, it could still exist at extremely low expression levels that are beyond levels of detection. Low levels of expression could be possible due to ribosomal frameshifts and/or readthrough mechanisms as seen in retrovirus systems (Leger et al., 2007). In addition, the V protein could also be expressed from a different initiation codon either by internal ribosome binding sites, which are present in picornaviruses and hepatitis C viruses, or by ribosomal shunting exhibited by adenoviruses and paramyxoviruses (Boehringer et al., 2005; Junemann et al., 2007; Latorre et al., 1998; Xi et al., 2005).

To understand the fitness of a virus relative to various mutants of the parent virus, measuring the titer of each virus could offer clues on the possible differences in the replication cycles of the viruses or possible consequences of mutations. In this study, the results from both the virus titration and viral replication curve assays suggest that the lack
of expression of the D domain from the PD protein results in an attenuated virus. When the expression of the D domain was silenced in two different ways, the resulting infectious viral titers of the two knockout viruses, rHPIV3-ΔES and rHPIV3-ΔD, were significantly reduced compared to the parent virus rHPIV3-NT when grown in two different cell lines, MA-104 and A549 cells (p<0.001). Since attenuation was observed in both cell lines, A549 cells that express IFN-β and MA-104 cells that do not, the results of this study suggest that the D domain is involved in some aspect of viral replication and not necessarily involved in countering the host cell’s antiviral response. On the other hand, if attenuation had been seen in A549 cells and not in MA-104 cells, that observation would have suggested that the D domain of the PD protein played a role in the countering the host’s cell antiviral response. The involvement of the D domain in some aspect of viral replication is supported by the results that both viral genomic replication and viral mRNA transcription from both knockout viruses were significantly reduced when compared to the parent strain (p<0.001). By measuring both major viral RNA synthesis processes, viral mRNA transcription and genomic replication, any differences between the knockout viruses and the parent virus in either process would suggest involvement of the D domain in that particular process. But, since a reduction was detected in both processes, the function of the D domain may influence and/or play a general role in viral RNA synthesis.

Durbin et al. (1999) published a study in which they also knocked out the D and V domains encoded for in the P gene. In their study, they reported no attenuation in the titers of the knockout viruses, deficient in the expression of the D and V domains individually, in vitro or in vivo. But when the D and V domains were silenced
simultaneously, they detected attenuation in the viral titers only in vivo. In contrast, attenuation in viral titers was observed for both knockout viruses deficient in the expression of the D domain in this study. The major difference between the two viruses used in each study was the position of the stop codon in relation to the remaining sense codons of the D domain. Durbin et al. (1999) introduced three stop codons into the nucleotide sequence that encodes the PD protein at amino acid positions 304, 305, and 306. In the current study, only one stop codon was introduced into the nucleotide sequence that encodes the PD protein at amino acid position 245, which immediately follows the editing site located within the P gene. With the stop codon located immediately following the editing site, it is ensured that the complete D domain is silenced and that any differences seen, compared to the parent virus, can be attributed to the D domain. In contrast, Durbin et al. (1999) introduced the stop codons in the middle of the D domain. Therefore attenuation can only be attributed to the C-terminal end of the D domain and not to the complete D domain. In addition, Wells and Malur (2008) identified three total nuclear localization signals encoded in the PD protein, located at amino acid positions 225–241, 266–272, and 340–346. From the results of these three studies, it can be concluded that the deletion of both nuclear localization signals located in the D domain of the PD protein results in an attenuated virus, but the deletion of the nuclear localization signal at amino acid position 340–346 alone does not attenuate the virus.

Although the attenuation of the knockout viruses deficient in the expression of the D domain in A549 cells can be explained by a reduction in viral RNA synthesis it could have also been attributed to an increase or decrease in the host cell’s antiviral response.
An increase in cytokine expression levels from A549 cells infected by the knockout viruses would have suggested that the D domain interfered with the host cell’s antiviral response. Theoretically, cytokine expression levels would be reduced during a normal infection when the viral D domain is expressed if the D domain interfered with the host cell’s antiviral response. On the other hand, the absence of the D domain should have allowed the host cell’s antiviral response to operate freely and an increase of cytokines expressed from infected A549 cells would have been seen. Therefore, more cytokines present would have induced greater control over virus replication by the infected cells manifested by a reduction in viral titers. But in the current study, cytokine expression levels of the knockout viruses were either significantly reduced or unchanged (p<0.01), suggesting that the D domain does not perturb the host cell’s antiviral response, but instead leads to an attenuated virus via viral RNA synthesis regulation. An attenuated virus should induce a weaker antiviral response from infected cells resulting in reduced cytokine expression because the host cells would need a less aggressive immune response to control a weaker infection.

The host cell’s antiviral response can be initiated by intracellular uncapped mRNA and/or dsRNA, which is detected by RIG-1 and MDA-5 (Hornung et al., 2006; Kang et al., 2002; Kato et al., 2008; Pichlmair et al., 2006). RIG-1 and MDA-5, bound to dsRNA, induce a cascade that leads to the dimerization and translocation of the transcription factor IRF-3 into the nucleus, which initiates the expression of IFN-β and RANTES (Au et al., 1995; Lin et al., 2000). Then it is no surprise that in this study, the IFN-β and RANTES expression levels for all three RNA viruses were identical. Whereas, an IRF-3 binding site located in the promoter region of MCP-1 and IL-6 has not
been found, the IL-8 promoter does contain an IRF-3 binding site (Samuel et al., 2008; Sutcliffe et al., 2009; Wagoner et al., 2007). Therefore, IL-8 should have also been expressed from A549 cells infected with the three rHPIV3 viruses in a similar pattern compared to IFN-β and RANTES in this study. Although, IL-8 is expressed as a result of the presence of dsRNA and the IFN primary response, IFN-β induction of the Jak/STAT signaling pathway leads to inhibition of IL-8 expression, among a multitude of other pathways (Laver et al., 2008). This might explain why no significant difference in IL-8 expression was detected in A549 cells infected with any of the three viruses because IL-8 cytokine levels were being controlled by IFN-β expression.

Six amino acids, IKKGGK at position 238–243 of the P protein, were deleted from the P protein in the rHPIV3-ΔES virus, which encoded a portion of a bipartite nuclear localization signal, amino acid position 225–241 (Wells and Malur, 2008). The replication kinetics of this knockout virus in A549 cells was further attenuated when compared to the replication of the same virus in MA-104 cells. On the other hand, the degree of reduction in viral titers of the rHPIV-ΔD knockout virus in both MA-104 and A549 cells were similar. In contrast, an increase in the expression of the IFN-β and RANTES cytokines induced from A549 cells infected with the rHPIV3-ΔES knockout virus was detected when compared to the levels of RANTES and IFN-β detected in cells infected with the rHPIV-ΔD knockout virus. Using the same logic mentioned previously, these results might suggest that the nuclear localization signal, located on the N-terminal side of the editing site in the P gene for the P, PD, and W proteins, is involved in counteracting the host’s cell antiviral response in a new, novel way. The host cell’s primary IFN-β response is initiated by dsRNA in the cytoplasm and finishes with the
translocation of cellular transcription factors to the nucleus and IFN-β expression. Perhaps the nuclear localization signal present on the viral P, PD, and W proteins allows the translocation of these viral proteins to the nucleus where their N-terminal ends interact with host cell’s transcription factors and directly interfere with IFN-β expression.

However, the rHPIV3-ΔES virus is also deficient in the expression of the W protein and that could also explain the further attenuation and increased induction of cytokine expression in A549 cells infected with the rHPIV3-ΔES virus. The contradicting results of different IFN-β protein expression levels and similar IFN-β mRNA transcription levels of the two knockout viruses in A549 cells, might suggest that the W protein may influence cellular protein synthesis. Both cellular and viral protein synthesis occur in the cytoplasm and the translational machinery is used concurrently by both entities. Thus, it would not be out of the realm of possibility that HPIV-3 might encode a viral protein that “hijacks” a component of the host cell’s protein synthesis machinery to preferentially promote viral protein synthesis. In the absence of the W protein, an increase in cellular protein expression would be seen. In addition, cellular and viral mRNA transcription are separate processes that occur in different compartments of the cell. Therefore, cellular mRNA transcription may not be directly influenced by the viral replication machinery and no change in cellular mRNA transcription would be seen in the absence of the W protein. Even though, this theory is supported by the significant difference in IFN-β protein expression (p<0.01) and no difference in mRNA transcription between the knockout viruses, a majority of the amino acid sequence representing the W protein is also present in the P protein. Therefore, the W protein’s function could be present and active, even though no 36 kDa W protein is directly expressed by the
rHPIV3-ΔES virus. In addition, if the W protein did influence cellular protein expression a more dramatic difference in viral titers may have been detected in the replication of rHPIV3-ΔES in MA-104 cells, similar to the full-log reduction in viral titers detected in A549 cells. Overall, a combination of the two processes, direct interference with IFN-β expression or the viral W protein “hijacking” the host cell’s translational machinery, might explain the differences in cytokine expression and viral titers in A549 cells observed between the two knockout viruses in the current study.

In summary, three proteins, P, PD, and W, were found to be expressed from the P gene start codon within the HPIV-3 genome, but expression of the V protein from the same start codon was not detected. Also, the lack of expression of the D domain from two knockout viruses resulted in a reduction in viral genomic replication and viral mRNA transcription. In addition, the unchanged or reduced extracellular levels of cytokines expressed from A549 cells infected with each knockout viruses confirms the notion that the D domain may be involved in viral RNA synthesis rather than negatively perturbing the host cell’s antiviral response. The PD protein contains three nuclear localization signals and has been localized to the cellular nucleus (Wells and Malur, 2008). The main functions occurring in a cell’s nucleus are cellular mRNA transcription and cellular genomic DNA replication with an abundance of protein-nucleic acid complexes and co-factors involved with each process. Therefore, the PD protein may enter the nucleus to recruit necessary cellular proteins and/or co-factors to aid in viral RNA synthesis occurring in the cytoplasm. For example, the cellular La protein, which is responsible for cellular RNA protection and mainly found in the nucleus, has been found to bind the single-stranded genomic RNA of RSV (Bitko et al., 2008; Rinke and Steitz, 1982). The
HPIV-3 PD protein may recruit the cellular La protein to bind to the single-stranded genomic RNA of HPIV-3 for protection and increase the efficiency viral RNA synthesis.

Further research is needed to investigate why the PD protein needs to translocate to the nucleus and to identify what cellular and/or viral proteins the PD protein interacts with to promote viral RNA synthesis. In addition, the bipartite nuclear localization signal, which is present on the N-terminal end of the P, PD, and W proteins, needs to be further studied to determine what role it plays in the viral replication cycle by counteracting the host cell’s antiviral response.

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CHAPTER 5
SUMMARY

The purposes of this study were to develop a high-throughput antiviral assay and investigate the function of the D domain for HPIV-3 using reverse genetics. The two main focuses of the current study were the insertion of the foreign EGFP gene into the viral antigenome and silencing the expression of the D domain. Reverse genetics techniques were used to successfully construct an rHPIV3 cDNA clone that was amplified directly from viral genomic RNA isolated from an HPIV-3 WT infection. The rHPIV3 cDNA clone was then used as a backbone for genetic manipulation during each phase of the current study. In the first phase, the gene encoding for EGFP was inserted into the rHPIV3 antigenome, rHPIV3-EGFP, upstream of the NP gene’s start codon; therefore EGFP was expressed as the first gene. In the second phase, two rHPIV3 clones were engineered to be deficient in the expression of the D domain, in unique mechanisms. In one clone, 18 nucleotides surrounding the editing site in the P gene were removed, rHPIV3-ΔES, and in the second clone, a single ribonucleotide was changed to create a nonsense mutation that silenced the expression of the D domain, rHPIV3-ΔD.

In both phases of the current study, the replication of the altered rHPIV3 viruses were significantly attenuated in the permissive MA-104 cell line compared to their respective parent viruses, HPIV-3 WT from which rHPIV3-EGFP was derived and rHPIV3-NT from which rHPIV3-ΔES and rHPIV3-ΔD were derived. In addition, both viral genomic replication and viral mRNA transcription were also significantly reduced in MA-104 cells infected with the rHPIV3-EGFP, rHPIV3-ΔES and rHPIV3-ΔD viruses.
compared to their respective parent viruses. These results suggest that the presence of the additional EGFP gene in rHPIV3-EGFP and the silenced expression of the D domain in both rHPIV3-ΔES and rHPIV3-ΔD may have negatively influenced all aspects of viral RNA synthesis, which led to the attenuation.

Furthermore, the rHPIV3-EGFP virus was evaluated for use in antiviral assays as a substitute for HPIV-3 WT. The time frame to complete an antiviral assay for HPIV-3 WT in MA-104 cells is approximately seven days and both viruses exhibited similar amounts of CPE, measured by NR dye uptake, when compared to each other over that same 7-day time frame. In addition, the expression of EGFP in MA-104 cells infected with rHPIV3-EGFP peaked at three days, which would allow for a more efficient and quicker assay if the rHPIV3-EGFP virus is to be used in an antiviral assay. To further test the fitness of the EGFP fluorescent assay the Z’-factor, signal-to-background, and signal-to-noise ratios were measured and compared to three other 7-day cell fitness assays, NR dye uptake, CellTiter-Glo® Luminescent Cell Viability Assay, and Vybrant® MTT Cell Proliferation assay. When the scores for Z’-factor, signal-to-background, and signal-to-noise were compared between all four detection assays, the 3-day viral expressed EGFP fluorescent assay was more robust and superior to the other three 7-day indirect CPE detection methods.

When the HPIV-3 WT and rHPIV3-EGFP viruses were replicated in MA-104 cells and exposed to known antiviral inhibitors of HPIV-3, the rHPIV3-EGFP virus was more susceptible to inhibition than HPIV-3 WT, which led to reductions in EC50 values. This may be due to the additional resources and time necessary to express the foreign EGFP gene from the viral genome, which slows viral RNA synthesis and attenuating the
virus. Ultimately, if the rHPIV3-EGFP virus were to be used in antiviral assays, lower EC₅₀ values might be obtained that would raise SI values and could increase the number of false-positive antiviral compounds passing the initial round of screening. In the realm of high-throughput antiviral screening though, this consequence is acceptable because during the second round of antiviral compound screening the HPIV-3 WT virus would be used and false-positive compounds would be eliminated from further examination. In fact, when the rHPIV3-EGFP virus was tested against a panel of known HPIV-3 inhibitors in MA-104 cells a decrease in EC₅₀ values was generally detected. The decrease in EC₅₀ values ultimately increased the SI values for identical antiviral compounds when measuring viral replication by EGFP fluorescence compared to the traditional NR dye uptake assay.

In addition, the replication of the rHPIV3-ΔES and rHPIV3-ΔD knockout viruses in the more restrictive, IFN-β expressing A549 cell line were also attenuated, which may suggest that the D domain may perturb the host cell’s antiviral response, in addition to influencing viral RNA synthesis. But, taking into account that both rHPIV3-ΔES and rHPIV3-ΔD knockout viruses were attenuated in both the non-IFN-β expressing MA-104 and IFN-β expressing A549 cells, heavily favors the theory that the D domain plays a role in viral RNA synthesis and not in disrupting the host cell’s antiviral response. Also, a reduction or no change in cytokine expression levels was detected in A549 cells infected with either knockout virus compared to the rHPIV3-NT parent virus. This result further supports the idea that the D domain may be involved in viral RNA synthesis rather than disrupting the host cell’s antiviral response.
On the other hand, when the rHPIV3-ΔES knockout virus was used to infect A549 cells, a full-log reduction in viral titers was detected compared to a 50% viral titer reduction of the same virus in MA-104 cells. Also, the expression of the IFN-β and RANTES cytokines from A549 cells infected with the rHPIV3-ΔES knockout virus were increased compared to the identical cytokines expressed from A549 cells infected with rHPIV3-ΔD. These data suggests that the six amino acids, which make up a portion of a nuclear localization signal, removed from the rHPIV3-ΔES virus may play an additional luxury role in disrupting the A549 cell’s antiviral response.

In conclusion, the similar methods used in reverse genetics have allowed the investigation of two different hypotheses involving the HPIV-3 virus. The first hypothesis was found to be incorrect in that the insertion of a foreign gene into the viral genome to be expressed as an additional gene did attenuate the virus. But along with further evaluation, it was concluded that the attenuation of the rHPIV3-EGFP virus was acceptable and the rHPIV3-EGFP virus could be used as a suitable replacement for the HPIV-3 WT virus in antiviral assays. To improve the assay, further research is needed to find a non-green fluorescent dye to measure cell toxicity induced by the antiviral compounds. If one were found, compound cellular toxicity and effectiveness against the HPIV-3 virus could be measured in the same cell culture well and perhaps scaled up to 384-well plates for high-throughput screening.

The second hypothesis was also found to be false in that the D domain expressed from the P gene of HPIV-3 may be involved with viral RNA synthesis rather than disrupting the host cell’s antiviral response. Knowing that the PD protein, whose C-terminal end is the D domain, translocates to the cellular nucleus and that cellular
mRNA transcription and cellular genomic replication occur there, the PD protein might recruit cellular nuclear proteins or cofactors that assist in viral RNA synthesis. Further research is needed to determine exactly what cellular and/or viral proteins the PD protein interacts with and what the purpose of the PD protein in the cellular nucleus is. Also, additional research is needed to further investigate the exact function of the nuclear localization signal present on the N-terminal end of the HPIV-3 P, PD, and W proteins. It seems that the signal may be involved in some aspect that disrupts the host cell’s antiviral response rather than influencing viral RNA synthesis. Perhaps once inside the nucleus, the N-terminal end of the P, PD, and W proteins directly interferes with IFN-β expression.
APPENDICES
APPENDIX A

TIME COURSE OF IFN-β EXPRESSED FROM A549 AND MA-104
CELLS INFECTED WITH RHPIV3-NT

Introduction

Gao et al. (1999) determined that an HPIV-3 infection of A549 cells induced IFN-β, although peak expression of IFN-β during an infection was not shown in that study. In addition, no research could be found that reported the expression of IFN-β from MA-104 cells infected with any virus. The current study was undertaken to determine when the expression of IFN-β from A549 cells, infected with the rHPIV3-NT parent virus, was maximal during the course of the infection. In addition, the expression levels of IFN-β from MA-104 cells infected with rHPIV3-NT was also determined.

Materials and Methods

Two 24-well plates were seeded with A549 and MA-104 cells and infected with the rHPIV3-NT viruses at an MOI = 0.1 (3.7 x 10^4 PFU for A549 cells and 2.5 x 10^4 PFU for MA-104 cells). The virus was absorbed for 2 h at 37°C. The virus inocula were removed, replaced with fresh MEM supplemented with 2% FBS, and incubated at 37°C for 16-32 h. Every 4 h, starting at 16 h and ending at 32 h post virus infection, the tissue culture medium for the infected and uninfected wells was removed and stored at -80°C. Fresh MEM was added to the infected and uninfected tissue culture wells and incubated for another 4 h. Cytokine levels present in the tissue culture media were measured by ELISA using the VeriKine™ Human Interferon-Beta ELISA Kit (PBL InterferonSource) to measure IFN-β levels secreted by A549 and MA-104 cells. The level of detection of
IFN-β for the kit is 25-2000 pg/mL. The assay was measured on a SpectraMax® Plus spectrophotometer (wavelength 450 nm) and the data was analyzed with SOFTmax® PRO software.

**Results**

As expected, expression of IFN-β from A549 cells infected with rHPIV3-NT increased dramatically over time compared to mock infected A549 cell controls. Peak expression of IFN-β occurred at 24 h post infection (Fig. A-1). In contrast, infected MA-104 cells expressed levels of IFN-β below detectable limits of the assay during the rHPIV3-NT infection similar to mock infected MA-104 cell controls.

![Fig. A-1](image-url). Time course of IFN-β expression from A549 and MA-104 cells infected with rHPIV3-NT. IFN-β expression from infected A549 cells (♦), mock infected A549 cells (■), infected MA-104 cells (▲), and mock infected MA-104 cells (●) measured by ELISA. Y-axis values for infected cells represent the mean ± S.D. of duplicate assays.
Conclusions

Since peak expression of IFN-β occurred at 24 h post infection, IFN-β and other cytokine expression levels were measured at this time point (Fig. 4-4). The fact that MA-104 cells do not express IFN-β might explain why MA-104 cells are more susceptible to HPIV-3 infections, which result in 100% CPE.

Reference

APPENDIX B
SCREENING OF A CDNA LIBRARY AMPLIFIED FROM THE P GENE IN AN HPIV-3 WT INFECTION

Introduction

Galinski et al. (1992) identified an RNA editing site in the P gene of HPIV-3 at nucleotide position 791–805. They reported that the viral polymerase stops and stutters at this particular location adding 1–12 G residues indiscriminately when they screened a pool of approximately 50 clones. They also reported no insertion of additional G residues at a second possible editing site, nucleotide position 1121–1136. The current study was undertaken to confirm the insertion of non-templated G residues at nucleotide position 791–805 and to identify any other possible editing sites downstream of the known editing site to nucleotide position 1519 in the P gene using modern techniques.

Materials and Methods

MA-104 cells were seeded in 12-well plates and infected with HPIV-3 WT at an MOI = 0.1 (3 \times 10^4 PFU). The virus was absorbed for 2 h at 37°C. The virus inoculum was removed, replaced with fresh MEM supplemented with 2% FBS, and incubated at 37°C for 2 days. Total RNA, cellular and viral, was isolated from the infected MA-104 cells using the RNeasy® Plus Mini Kit (Qiagen). A cDNA library was constructed by converting all mRNA into cDNA using the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen) and the Oligo(dT)20 primer supplied in the kit. HPIV-3 P gene mRNA was amplified by PCR using the AccuPrime™ Pfx SuperMix (Invitrogen), the forward primer, 5’-TCTTCAACACATCAAGAAGATGACA-3’, and the
5’-GTGCCTCCATAAGTGGGTCAAAC-3’ reverse primer. The PCR products were
inserted into the SmaI site of pUC19 and transformed into electrocompetent cells.
Plasmid DNA was isolated from total of 105 clones and sequenced.

**Results**

The unedited P gene (+0G) was the most prevalent mRNA species detected in the
P gene cDNA library, which represented 60% of the total clones screened (Table A-1).
The other mRNA species found in the cDNA library had between 1 and 5 non-templated
G residues added with rates ranging from 4.8% to 13.3% of the total clones screened.
Accounting for the fact that 3 nt equals 1 amino acid, the mRNA species can be
combined into groups that express the same protein. The mRNA species with +0 and +3
G insertions account for 67.6% of the mRNA transcripts and express the P protein (Table
A-1). The mRNA species with +1 and +4 G insertions account for 18.1% of the mRNA

<table>
<thead>
<tr>
<th>Number of Non-templated G Residues Added</th>
<th>P protein (+0 and +3 G)</th>
<th>W protein (+1 and +4 G)</th>
<th>PD protein (+2 and +5 G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Clones</td>
<td>71</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>Percentage of Clones</td>
<td>67.6%</td>
<td>18.1%</td>
<td>14.3%</td>
</tr>
</tbody>
</table>

Table A-1
Amplitude of non-templated G residue insertion during RNA editing in the P gene of the
HPIV-3 WT virus.
transcripts and express the W protein. The mRNA species with +2 and +5 G insertions account for 14.3% of the mRNA transcripts and express the PD protein. In addition, the alignments of all 105 cDNA clones did not exhibit any other editing sites between nucleotides 699–1519, which would have been seen by the insertion of additional non-templated nucleotides (Fig. A-2).

Conclusions

Although, Galinski et al. (1992) reported the insertion of 1–12 non-templated G residues that the HPIV-3 viral polymerase adds at the editing site of the P gene, the current study found the insertion of only 1–5 non-templated G residues in the editing site. However, this study confirmed that the amplitude of insertions does occur indiscriminately, even though +1 G was slightly favored. In addition, no other nucleotide insertions or editing sites in the P gene within nucleotide position 699–1519 were found.

Reference

Fig. A-2. Alignment of selected cDNA library clones showing unedited (+0G) and non-templated G insertions (+1G–+5G). The alignment does not indicate any other nucleotide insertions or editing sites downstream of the main editing site to nucleotide position 1519.
Fig. A-2. Continued
Fig. A-2. Continued
APPENDIX C

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