Investigating the mechanisms of influenza polymerase host adaptation

Division of Investigative Sciences
Faculty of Medicine

Submitted for the degree of
Doctor of Philosophy

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Supervised by Professor Wendy Barclay

June 2014
Declaration of Originality

I confirm that all work presented here is my own and that the use of all material from other sources has been properly and fully acknowledged.

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Abstract

An avian virus can become adapted to humans by mutating or recombining with currently circulating human viruses. These viruses have the potential to cause pandemics in an immunologically naïve human population. Host restriction involves multiple determinants, however, influenza polymerase is considered to play an important role. The heterotrimeric polymerase complex (PA, PB1 and PB2) associates with viral RNA and nucleoprotein (NP) to form a ribonucleoprotein (RNP) complex responsible for viral replication and transcription. Host specific genetic signatures have been identified on all of the polymerase subunits and on NP, but the PB2 protein arguably carries the dominant determinants of host range.

Avian-origin influenza polymerase activity can be dramatically increased in human cells with the PB2 E627K substitution. This has been suggested to stabilise the interaction between the NP and PB2 components of the vRNP complex in the nuclei of infected cells. However, we demonstrate that a variety of adaptive PB2 substitutions including E627K did not enhance the stability of the vRNP in human cells, but rather increased the amount of replicated RNA, and that resulted in more PB2-NP co-precipitation.

The introduction of many adaptive PB2 mutations enhances avian influenza polymerase activity in an in vitro reconstituted polymerase assay. However, only some of these mutations have been detected in viruses that are found circulating in nature. We explored whether the polymerase assay truly predicts viral growth and investigated viral selection pressures that might favour some adaptive mutations over others. We used reverse genetics to create a series of viral variants carrying mutations in the PB2 gene and carried
out virological assays and also analysed the effects of the mutations in vivo. Some mutations that increased in vitro polymerase activity led to attenuated virus replication and resulted in an increase in interferon activation. These data increase our understanding of the host range barrier and why certain adaptive mutations may or may not have emerged.
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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>50-92</td>
<td>A/Turkey/England/50-92/91</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BVDV</td>
<td>Bovine viral diarrhea virus</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
</tr>
<tr>
<td>DI RNA</td>
<td>Defective interfering RNA</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide triphosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>Eng195</td>
<td>A/England/195/09</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HPAI</td>
<td>Highly pathogenic avian influenza</td>
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<tr>
<td>HPI</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>ISA</td>
<td>Infectious salmon anaemia virus</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LPAI</td>
<td>Low pathogenic avian influenza</td>
</tr>
<tr>
<td>M1</td>
<td>Matrix protein 1</td>
</tr>
<tr>
<td>M2</td>
<td>Matrix protein 2</td>
</tr>
<tr>
<td>MA</td>
<td>Mouse adapted</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-derby canine kidney</td>
</tr>
<tr>
<td>Mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MTS</td>
<td>Mitochondrial targeting signal</td>
</tr>
<tr>
<td>Mx</td>
<td>Myxovirus-resistance protein</td>
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<tr>
<td>N/A</td>
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<tr>
<td>NA</td>
<td>Neuraminidase</td>
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<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
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<td>NEP</td>
<td>Nuclear export protein</td>
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<td>NES</td>
<td>Nuclear export signal</td>
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<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS1</td>
<td>Non-structural protein 1</td>
</tr>
<tr>
<td>NS2</td>
<td>Non-structural protein 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PA</td>
<td>Polymerase acidic protein</td>
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<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
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<td>PB1</td>
<td>Polymerase basic protein 1</td>
</tr>
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<td>PB1-F2</td>
<td>Polymerase basic protein 1-frame 2</td>
</tr>
<tr>
<td>PB2</td>
<td>Polymerase basic protein 2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PFU</td>
<td>Plaque forming units</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcriptase PCR</td>
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<tr>
<td>RLU</td>
<td>Relative light units</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>SAPO</td>
<td>Specified animal pathogens order</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>svRNA</td>
<td>small viral RNA</td>
</tr>
<tr>
<td>TAP</td>
<td>tandem-affinity purification</td>
</tr>
<tr>
<td>Vic</td>
<td>A/Victoria/3/75</td>
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<tr>
<td>vRNA</td>
<td>Viral RNA</td>
</tr>
<tr>
<td>vRNP</td>
<td>Viral ribonucleoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
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X-gal  5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
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1. Introduction

1.1 Origins of the influenza virus

Historical literature suggests an influenza-like illness has caused epidemics throughout human history. There is speculation that influenza was responsible for the outbreak which devastated Athens in 430 BC, the sweating sickness that debilitated the French and English armies during the Hundred Years war and the outbreak at the court of Mary Tudor in 1562 (Potter, 2002).

However, historical data on influenza are difficult to interpret, not least because the symptoms are similar to those of other respiratory diseases. The first convincing record of an influenza pandemic, agreed by all authors, was an outbreak in 1580. The pandemic began in Russia and spread to Europe via Africa. In Rome, over 8,000 people were killed, and several Spanish cities were almost wiped out. In the ensuing centuries several influenza pandemics, identified by clinical symptoms, are reported to have occurred. However, it was not until 1932, when the influenza virus was first isolated in the laboratory, that the history of this infection could be recorded and confirmed by laboratory diagnosis (Potter, 2002).

1.2 Host reservoirs

In addition to humans, influenza A viruses have been isolated from a variety of hosts, including pigs, horses, birds, whales, seals, minks, cattle, and even cats, tigers and dogs.
The relationship between influenza A viral subtypes and the hosts they can infect is shown in Figure 1.

Figure 1. Host range of influenza A virus. Wild aquatic birds are the natural host reservoir of influenza A virus. The viral subtypes which infect other species originate from this reservoir. The subtypes that infect cats, humans, horses, migratory birds, mink, poultry, seals, swine, and whales are shown. Solid lines represent confirmed transmission pathways; dotted lines show suspected transmission events (Wahlgren, 2011).
Influenza A viruses are classified according to the subtypes of the haemagglutinin (HA) and neuraminidase (NA) surface proteins. The HA and NA genes are particularly variable in sequence and at least 16 HA and 9 NA subtypes are perpetuated in the wild waterfowl natural reservoir, as well as an as yet undetermined number in bats. (Fouchier et al., 2005; Tong et al., 2012; Tong et al., 2013).

All the influenza subtypes circulating in the human population originate from the natural host reservoir of aquatic birds (Scholtissek, 1994). However, only a very limited number of influenza A subtypes have actually become established in the human population; these are the H1N1, H1N2, H2N2 and H3N2 subtypes (Taubenberger and Morens, 2010). Successful inter-species transmission is a relatively infrequent event as an emerging influenza virus requires adaptation before sustained transmission within the human population can be attained. A degree of adaptation may first be achieved in intermediate hosts such as poultry or pigs (Russell and Webster, 2005).

The effects of influenza A virus infection are host dependent. Wild birds typically exhibit no symptoms when infected with influenza; this is indicative of the optimal level of adaptation achieved by the virus in this host. The virus primarily infects the intestinal tract at a temperature close to 41°C. The intestinal replication causes the virus to be shed at high concentrations in faeces. Consequently, water courses and lakes become contaminated with infectious virus particles which facilitates both intra- and inter-species transmission of the virus (Webster et al., 1978).
The influenza virus also primarily infects the intestinal tract in poultry. However, the clinical outcome of infection in poultry can be very different to the outcome in wild birds. Poultry infected with influenza can exhibit low or highly pathogenic forms of disease. The low pathogenic form causes mild symptoms such as ruffled feathers and a drop in egg production, indeed, the infection may not even be detected. In contrast, the highly pathogenic form causes severe symptoms and typically affects multiple internal organs. The mortality rate can reach 90-100 %, often within 48 hours and the virus spreads rapidly through flocks particularly under intensive farming conditions (Spackman, 2008).

The severity of disease caused by influenza in humans can vary considerably. The most common symptoms are chills, fever, sore throat, muscle pains, headache, coughing and weakness/fatigue, but disease may be life threatening for individuals with underlying health conditions (http://www.cdc.gov/flu/keyfacts.htm). The virus establishes an upper respiratory tract infection and virus particles are transmitted through the air by coughs or sneezes. In clinical studies, particles laden with virus have been detected in exhaled breaths of patients and in air samples from healthcare settings at the peak of seasonal influenza. In addition the virus can be transmitted by direct contact or through contact with contaminated surfaces (Nicas et al., 2005; Tellier, 2009).

1.3 Influenza Pandemics

New influenza viruses are constantly evolving. Two separate mechanisms are responsible for this antigenic change: antigenic shift and antigenic drift.
Antigenic drift occurs because mutations are introduced during viral replication. The viral RNA-dependent RNA polymerase is error prone since it doesn't possess proof reading ability. Mutations that are detrimental to the virus are not selected; however, mutations that give the virus an advantage over its parental strain are positively selected for. The sequential introduction of mutations over time can alter the replication, virulence and transmissibility of the virus. Epidemics occur yearly in the human population because this is sufficient time for the virus to genetically drift from the previously circulating virus so individuals are no longer protected against re-infection.

In contrast, antigenic shift is the result of whole genome segments being replaced by recombination between two or more viruses. In order for this to be achieved a single host cell must be infected with more than one virus at the same time. The influenza virus possesses a segmented genome and thus segments from different viruses can be packaged together into new virus particles. This leads to the generation of a virus with a novel composition of gene segments from more than one viral strain.

Influenza pandemics are infrequent but reoccurring events. They occur when a virus with at least partially novel genomic makeup crosses the species barrier or recombines to become infectious in the human population. The emergence of pandemic influenza strains, since the beginning of the twentieth century, is shown in Figure 2.
Figure 2. Timeline of human influenza pandemics. In the 20\textsuperscript{th} century there were four influenza pandemics, in 1918, 1957, 1968 and 1977. The most recent pandemic was in 2009 (Watanabe et al., 2012).

The most severe pandemic in recent history was the 1918 H1N1 'Spanish flu'. It is estimated that this pandemic caused 40-50 million deaths globally and that between 500 million and 1 billion people (30-50 \% of the world's population) were infected (Niall et al., 2002). In addition, unlike seasonal influenza outbreaks, which disproportionately kill the very young and the elderly, the 1918 virus predominately killed young healthy adults. Experiments undertaken in cynomolgus macaques show the reconstructed 1918 virus causes a fatal illness induced by an aberrant innate immune response with dysregulation of the antiviral response which has been termed a 'cytokine storm' (Kobasa et al., 2007). However, the majority of deaths during the 1918 influenza pandemic may not have been caused by the influenza virus acting alone. Many victims succumbed to secondary bacterial pneumonia caused by common upper respiratory-tract bacteria (Morens et al., 2008).
Sequencing data suggests the 1918 virus was entirely novel to humans and that it originated from an avian influenza strain that had infected and adapted to replicate in humans (Taubenberger, 2006). However, intriguingly, the source of the virus remains unknown. None of the 1918 viral gene segments are similar to contemporary avian influenza genes and yet the viral gene sequences isolated from wild birds collected circa 1918 are very similar to those from contemporary avian viruses (Fanning et al., 2002; Reid et al., 2003).

In 1957 the H2N2 'Asian' pandemic emerged and caused two million deaths and considerable morbidity globally (Cox and Subbarao, 2000; Scholtissek et al., 1978b). The virus responsible was a reassortant formed from human and avian viruses. The HA, NA and PB1 genes were of avian origin (Kawaoka et al., 1989; Scholtissek et al., 1978b), whereas the remaining five genes were from the human H1N1 strain circulating at the time (Kawaoka et al., 1989). This virus displaced the H1N1 viruses that had been circulating since 1918.

In 1968 a novel H3N2 virus completely replaced the H2N2 viruses that had been circulating. The 'Hong Kong' pandemic killed approximately one million people worldwide and infected an estimated 500,000 Hong Kong residents. This pandemic was also caused by a reassortant virus. The virus was composed of avian-origin HA and PB1 genes, together with six genes from the human H2N2 strain that had been circulating previously (Scholtissek et al., 1978b).

The 1977–1978 'Russian' influenza pandemic infected mostly children and young adults. Serological and genetic analysis revealed the virus was identical to the H1N1 virus that had been present in the human population in the early 1950s and most adults exhibited considerable immunity to the virus (Nakajima et al., 1978). Thus, it is considered likely that
the virus was accidently released from a laboratory in Russia or Northern China, although this has never been proven. Unlike the 1957 and 1968 pandemics, the H1N1 strain responsible for the 1977 pandemic did not replace the circulating viruses. Thus, since 1977 both H1N1 and H3N2 viruses have circulated in the human population (Taubenberger and Morens, 2009).

The most recent influenza pandemic was in 2009. The virus responsible for crossing the species barrier into humans had previously been circulating in swine and hence the virus was termed 'swine flu'. The H1N1 virus is a complex reassortant virus with genes from avian and several swine viruses (Figure 3). The virus is the result of recombination between a triple reassortant North American swine virus with a Eurasian avian like swine virus. The triple reassortant North American swine virus has genes of avian, swine and human origin. Thus, the resulting pandemic H1N1 virus had PB2 and PA genes of North American avian virus origin, a PB1 gene from the human H3N2 virus, HA, NP and NS genes originating from a classical swine virus and NA and M genes of Eurasian avian-like swine virus origin (Dawood et al., 2009; Schnitzler and Schnitzler, 2009; Smith et al., 2009).

The virus was excellent at transmitting within the human population. In addition, as the virus harboured surface proteins that were similar to those of the 1918 virus but distinct from those of recent circulating seasonal H1N1 viruses, it was antigenically novel to most of the human population and there was hardly any preexisting immunity to the strain. Approximately 300,000 people were killed by the disease with a disproportionate number of children and young adults (Dawood et al., 2012), however the virus mostly caused a mild,
self-limiting upper respiratory tract illness and as such was less virulent than initially feared (Girard et al., 2010; Schnitzler and Schnitzler, 2009).

Figure 3. Origin of the 2009 H1N1 pandemic influenza virus. The novel H1N1 virus arose when a triple reassortant virus, containing gene segments from avian, human and swine viruses, recombined with a Eurasian swine virus (Garten et al., 2009).

The fear that an influenza pandemic will originate directly from an avian influenza virus was heightened in 1997 when it was first reported that humans could be infected with the highly pathogenic H5N1 avian influenza strain. Since this time, the avian H5N1 virus has frequently infected humans and caused severe disease. There have been 648 confirmed cases of human infection and 384 cases were fatal, as of December 2013 (http://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). The mortality rate is extremely high for influenza and a pandemic originating from this virus could have devastating consequences. This viral subtype has been detected in
avian species globally (Feare, 2010) and it has been isolated from humans in many countries including Indonesia, Egypt and Vietnam. Although, currently, human to human transmission of this highly pathogenic avian influenza virus is infrequent, there is concern the introduction of further mammalian host adaptive mutations could generate a highly pathogenic H5N1 strain capable of transmitting efficiently between humans (Russell et al., 2012).

Two landmark studies in 2012 further fuelled concerns of a H5N1 influenza pandemic. Both studies reported aerosol transmission of highly pathogenic H5 viruses in ferrets which are the best small animal model for influenza research. Mammalian adaptive mutations which enabled the transmission events were selected upon serial passage of genetically engineered H5 viruses in ferrets (Herfst et al., 2012; Imai et al., 2012).

In March 2013 the first reported cases of human infection with H7N9 influenza virus in Eastern China were announced, raising concern that this viral subtype could cause a pandemic (Gao et al., 2013). As of November 2013 there have been 134 confirmed cases of human infection, most infected individuals developed a severe respiratory illness and 43 cases were fatal (http://www.who.int/csr/don/2013_11_06/en/index.html).

Sequence analysis revealed the virus originated from a recombination event between H7 and N9 viruses, possibly from aquatic birds, and enzootic H9N2 viruses from chickens (Gao et al., 2013). It is now known that many infected individuals had a history of contact with, or exposure to poultry before disease onset (Li et al., 2013), suggesting a zoonotic origin of infection. However, there is concern that this virus has the ability to be transmitted
between humans as some family clusters were identified (Li et al., 2013). Furthermore, it has been shown that the virus has the capability to transmit by aerosol in ferrets (Zhu et al., 2013).

1.4 Classification

Influenza A viruses belong to the Orthomyxoviridae family, which is comprised of enveloped viruses with negative-sense and segmented single-stranded RNA genomes. By convention, mRNA is considered positive-sense, and as such the viral RNA packaged into the virions of the Orthomyxoviridae family is complementary to mRNA. Thus, before protein synthesis can start, the viruses must undergo transcription to generate mRNA. In contrast to most RNA viruses which replicate in the cytoplasm, viruses in the Orthomyxoviridae family replicate in the nucleus.

There are five genera in the Orthomyxoviridae family: the influenza viruses A, B and C, Thogotovirus and Infectious Salmon Anaemia virus (ISA) (Pringle, 1996). Influenza viruses are divided into the classes A, B and C based on the serological reactivity of their NP (nucleoprotein) and M (matrix) proteins. The structural features and genome organization of influenza A, B and C viruses suggest they share a common ancestor. Influenza A viruses infect a broad range of bird and mammalian species, whereas influenza B and C viruses are found mainly in humans, although there is increasing evidence that influenza B and C viruses can also infect other species such as seals for influenza B and pigs or dogs for influenza C (Bodewes et al., 2013; Osterhaus et al., 2000; Webster et al., 1992).
Influenza A viruses are named according to several factors in the following order: genus, species from which the virus was isolated, location of isolation, isolate number, year of isolation and the hemagglutin and neuramindase subtype. For example, a H5N1 subtype virus isolated from a turkey in England in 1991 is named: influenza A/turkey/England/50-92/91 (H5N1). For human isolates, the species the virus was isolated from is normally omitted.

1.5 Virion structure and genomic organization

Influenza virions are approximately 80-120 nm in diameter and can be spherical or filamentous, depending on strain and culture conditions. A filamentous morphology is typical of clinical isolates, whereas a spherical morphology is more common in laboratory-passaged influenza viruses (Ada et al., 1957; Bourmakina and Garcia-Sastre, 2003; Elleman and Barclay, 2004). The basic structure of a spherical influenza virion is shown in Figure 4. The lipid envelope of the virion is derived from the plasma membrane of the infected host cell (Kates et al., 1962). Embedded in the membrane are three types of viral glycoproteins: the rod-shaped HA protein, the mushroom-shaped NA protein and small amounts of the ion channel protein M2 (Zebedee and Lamb, 1988). The HA forms trimers of identical subunits and the NA form tetramers of identical subunits. The matrix protein M1 is found lining the underneath of the lipid bilayer (Nermut 1972, Schulze 1972). Small amounts of nuclear export protein (NEP), also known as non-structural protein 2 (NS2), are found associated with M1 inside the virion (Richardson and Akkina, 1991; Yasuda et al., 1993).
Figure 4. 3D representation of an influenza virion. The viral glycoproteins include the rod-shaped HA protein (blue) and the mushroom-shaped NA protein (pink). The ion channel protein M2 is shown in purple. The lipid envelope is shown in brown, the underlying matrix M1 protein in pink and the viral ribonucleoprotein complexes are shown in green. Each complex consists of a viral genome segment associated with a viral polymerase complex and multiple copies of the viral nucleoprotein (http://www.cdc.gov/flu/images.htm).

A study by Calder et al. used electron cryotomography to investigate the morphology of frozen-hydrated influenza virions. This approach showed the matrix layer is formed by an ordered helix of the M1 protein and that it is this layer which is responsible for determining the morphology of the virion. The matrix layer changed from its helical, membrane
associated form to a multilayered coil inside the virion when the virus was incubated at a low pH. Viral ribonucleoprotein complexes were seen assembled at one end of the virion and NA and HA typically formed clusters at the opposite end (Figure 5) (Calder et al., 2010).

Recently, three-dimensional electron microscopy has been used to study the structure of influenza virus filaments 'budding' from cells. Intriguingly, this approach identified a variety of filamentous particles, most of which did not contain ordered ribonucleoprotein complexes at their leading ends. Thus, filaments which appear to contain no genetic material may play a unique role in viral infection (Vijayakrishnan et al., 2013).
Figure 5. Tomogram sections of frozen-hydrated influenza virions. A has been colored to identify ribonucleoprotein complexes (red), multilayered-coil (blue), NA (purple), and HA (green) (Calder et al., 2010).

The influenza genome is organised into 8 RNA segments and encodes at least 14 proteins (Table 1). Recently, it has been reported that segment 3, which was previously thought only to encode PA, also encodes a second protein which has been termed PA-X. Segment 3 contains a second open reading frame which is accessed by ribosomal frameshifting. The frameshift product, PA-X, is composed of the endonuclease domain of the viral PA protein and the C-terminal domain encoded by the X-ORF. PA-X appears to play a role in virulence. Intriguingly, if deleted, the pathogenicity of the influenza virus is increased in mice. PA-X is thought to be implicated in changing the kinetics and magnitude of host gene expression and resulting immune responses (Jagger et al., 2012).
Table 1. Proteins encoded by the influenza genome.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Protein</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PB2</td>
<td><strong>Polymerase basic protein 2</strong> - Cap-binding, part of polymerase complex</td>
</tr>
<tr>
<td>2</td>
<td>PB1</td>
<td><strong>Polymerase basic protein 1</strong> - RNA-dependent RNA polymerase, part of polymerase complex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Virulence factor, pro-apoptotic protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Interacts with polymerase complex, role unclear</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>Role unclear</td>
</tr>
<tr>
<td></td>
<td>PA</td>
<td>Role unclear</td>
</tr>
<tr>
<td>3</td>
<td>PA</td>
<td><strong>Polymerase acidic protein</strong> - Endonuclease, part of polymerase complex</td>
</tr>
<tr>
<td></td>
<td>PA-X</td>
<td>Role unclear</td>
</tr>
<tr>
<td>4</td>
<td>HA</td>
<td><strong>Haemagglutinin</strong> - Major surface glycoprotein involved in receptor binding and membrane fusion</td>
</tr>
<tr>
<td>5</td>
<td>NP</td>
<td><strong>Nucleoprotein</strong> - Binds viral RNA, required for transcription and replication</td>
</tr>
<tr>
<td>6</td>
<td>NA</td>
<td><strong>Neuraminidase</strong> - Surface glycoprotein, cleaves sialic acid allowing release of virions from cells</td>
</tr>
<tr>
<td>7</td>
<td>M1</td>
<td><strong>Matrix protein 1</strong> - Structural component of virion, viral budding</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td><strong>Matrix protein 2</strong> - Ion channel, viral budding</td>
</tr>
<tr>
<td></td>
<td>M42</td>
<td><strong>Matrix protein 42</strong> - Can compensate for loss of M2 expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mRNA 3 is a further spliced transcript, to date it has not been shown to express a protein</td>
</tr>
<tr>
<td>8</td>
<td>NS1</td>
<td><strong>Non-structural protein 1</strong> - IFN antagonist, inhibits cellular mRNA export, increases viral mRNA translation</td>
</tr>
<tr>
<td></td>
<td>NS2/NEP</td>
<td><strong>Non-structural-protein 2</strong> - Nuclear vRNP export, role in replication</td>
</tr>
</tbody>
</table>
1.6 Structure of influenza viral ribonucleoprotein complex

Each viral genome segment is associated with one heterotrimeric viral RNA polymerase composed of the viral proteins PA, PB1 and PB2, as well as multiple copies of nucleoprotein (NP), forming a viral ribonucleoprotein (v)RNP complex that serves as the template for both transcription and replication (Figure 6A). NP binds strongly to RNA in a sequence-independent manner. It is estimated that one NP molecule binds to every 24 nucleotides (Ortega et al., 2000).

Isolated vRNPs are very flexible and unfortunately in negative stain electron microscopy it is difficult to distinguish between the polymerase proteins and NP (Klumpp et al., 1997). However, in 2001 electron microscopy was used to analyse a minimal vRNA of 248 nucleotides associated with 9 copies of NP and the trimeric polymerase (Martin-Benito et al., 2001); the polymerase proteins were identified with specific antibodies (Area et al., 2004). This model was advanced in 2009 when a high-resolution cryo-electron microscopy reconstruction of this minimal vRNP was obtained with a resolution of 12 Å for NP and 18 Å for the polymerase proteins (Coloma et al., 2009; Ruigrok et al., 2010) (Figure 6B).
Figure 6. Structural models of influenza RNPs (A) Schematic of influenza RNP. The viral RNA (shown in black) is looped into a hairpin like structure which associates with many NP molecules and a single heterotrimeric viral polymerase composed of PB1, PB2 and PA (Compans et al., 1972; Portela and Digard, 2002). (B) Cryo-electron microscopy reconstruction of a mini-RNP with 9 NP molecules and the 3 polymerase proteins. The known atomic structure of NP was docked into the EM structure (Coloma et al., 2009; Ruigrok et al., 2010).

In 2012, cryo-EM reconstructions of authentic vRNPs were published by two research groups. At a 20 Å resolution, the final models confirm that each RNA segment is folded into a double-helical hairpin structure leading to and away from the polymerase that is located
at one end of the RNP. The rest of the RNA is tightly wrapped around NP (Arranz et al., 2012; Moeller et al., 2012) (Figures 7A & B).

Figure 7. Structural organization of influenza RNPs. (A) Cryo-EM reconstruction of a RNP (Arranz et al., 2012). The viral polymerase complex is located at the bottom of the RNP and is shown in green and orange. (B) Cryo-EM reconstruction of a RNP (Moeller et al., 2012). The viral polymerase is highlighted in red and orange.

All eight vRNA segments have conserved sequences of 12 and 13 nucleotides at their 3' and 5' ends, respectively. These sequences are partially complementary and thus the two extremities of each RNA segment form a partial duplex. This region constitutes the promoter region of each segment and it is here that the polymerase binds.

Initially it was observed by electron microscopy that both ends of viral RNA were involved in polymerase binding and the structure of the vRNA promoter was considered to be a 'panhandle', formed by the two RNA termini (Hsu et al., 1987). However, further studies
suggested this model was an oversimplification of the promoter structure. A study by Fodor et al in 1995 proposed the promoter forms a 'fork' structure in which there is double-stranded region but both termini are in a single-stranded conformation (Fodor et al., 1995). More recently, site-directed mutagenesis has been undertaken to thoroughly analyse the promoter structure and it has been proposed that the promoter is folded into a more complicated 'corkscrew' conformation (Figure 8) (Flick and Hobom, 1999; Flick et al., 1996).

![Figure 8](image_url) 'Corkscrew model' for vRNA promoter. A partial duplex is formed between the nucleotides at the 3' and 5' ends of the viral RNA and this constitutes the polymerase binding site (Flick and Hobom, 1999).

### 1.7 Influenza lifecycle

The influenza virus undergoes a complex replication cycle which is illustrated in Figure 9. The virus has developed numerous strategies to avoid the host immune response as well as
to use the host cell machinery for its own purpose. It can take as little as 6 hours for one round of viral replication (Kamps, 2006).

**Figure 9. Schematic of the influenza life cycle.** The virus initially enters the cell via receptor-mediated endocytosis. A series of processes are then triggered by the virus to release the vRNP complexes into the cytoplasm. The vRNPs are subsequently transported to the nucleus, the site of viral transcription and replication. Viral mRNA is exported into the cytoplasm for translation. Proteins made early in the viral lifecycle are transported back into the nucleus to ensure the viral genome is transcribed and replicated at a high rate. Late in the infection cycle the newly synthesised vRNP complexes are transported out of the nucleus and the viral proteins made late in the infection cycle (M1 and NS2/NEP) aid in this process. The assembly and budding of progeny virions occurs at the plasma membrane (Neumann et al., 2009).
1.7.1 Viral entry

The major glycoprotein on the surface of the virion, HA, attaches to sialic acid-containing glycoprotein and glycolipid receptors on the cell surface (Gottschalk, 1959). Following attachment the virus enters the cell via receptor-mediated endocytosis. Clathrin-dependent as well as clathrin-independent pathways have both been described for influenza (Matlin et al., 1981; Sieczkarski and Whittaker, 2002). NA has been shown to be involved in efficient viral uptake (Matrosovich et al., 2004), though its precise role is not known, it could be important for helping the virus access the apical surface of target cells that are otherwise occluded by mucus.

The low pH of endosomes triggers a series of events which promote the uncoating and entry of the virus RNP into the cytoplasm. It induces a conformational change in the HA protein (Skehel and Wiley, 2000) which mediates fusion of the viral membrane and the endosome membrane (Stegmann et al., 1990). The M2 protein acts as an ion channel in the viral membrane and the acidic environment of the endosome opens up the M2 ion channel. H+ ions flow into the virion and cause an acid-induced dissociation of the M1 protein from the RNPs. These processes culminate in the release of free RNPs into the cytoplasm of the infected cell (Whittaker et al., 1996).

1.7.2 Nuclear import

One of the characteristics of the influenza virus life cycle which is unusual for an RNA virus is its dependence on nuclear functions. The incoming virus must deliver its genome and
necessary viral proteins to the nucleus which is the site of replication for influenza. The vRNP complexes are too large to enter the nucleus by passive diffusion. Instead nuclear entry is facilitated by the importin-α/β heterodimer. Importin-α binds the NLS within NP, while importin-β mediates translocation of the vRNP through the nuclear pore complex (Cros et al., 2005; O’Neill et al., 1995; O’Neill and Palese, 1995). Monomeric PB2 and NP synthesised within the host cell are also imported by this classical pathway through direct interaction with importin-α (Gabriel et al., 2008; O’Neill and Palese, 1995; Resa-Infante et al., 2008). In contrast, PB1 and PA are trafficked via the non-classical import pathway by direct binding to the importin-β homologue RanBP5 (Deng et al., 2006a; Fodor and Smith, 2004).

1.7.3 Transcription and replication

The influenza polymerase and NP are the minimal viral components required for transcription and replication of the viral genome (Huang et al., 1990). Using very short RNA templates of 74 nucleotides or less, NP was found to be dispensable, and its role is now thought to be in elongation of the natural viral RNAs (Honda et al., 1998; Lee et al., 2002; Turrell et al., 2013). Viral RNA is wrapped around a scaffold of oligomerised NP (Honda et al., 1988; Huang et al., 1990).

The influenza polymerase first transcribes viral RNA into mRNA which is 5’ capped and 3’ polyadenylated. mRNA is then exported into the cytoplasm where it is translated into viral protein. For replication, a full-length copy of the vRNA, termed complementary RNA (cRNA),
is made as a replicative intermediate. This cRNA molecule is then copied back into full-length vRNA (Figure 10).

**Figure 10. Transcription and replication of influenza genomic RNA.** The 12 conserved nucleotides at the 3’ end of the vRNA and 13 at the 5’ end are shown. The cap stolen from host cell pre-mRNAs is shown at the 5’ end of the mRNA and the poly-A tail is at the 3’ end (Mikulasova et al., 2000).

Transcription of the influenza genome is thought to occur in a cis-acting manner, the polymerase attached to the viral RNA segment upon nuclear entry is responsible for transcribing it (Jorba et al., 2009; Scholtissek and Rott, 1970). Viral mRNAs mimic cellular mRNA and must have the appropriate 5’ and 3’ modifications, namely a 5’ methylated cap and a poly-A tail. These modifications regulate nuclear export, prevent degradation by exonucleases, promote translation into protein and facilitate interactions with cellular factors (Fodor, 2013).

Initiation of influenza transcription is primer-dependent. However, the virus does not generate its own primers to start transcription; instead it uses cellular RNAs as primers for initiating transcription. The virus obtains the 5’ primer through a process termed 'cap-
snatching’. Host pre-mRNAs are bound by the cap-binding domain located in the PB2 subunit (Blaas et al., 1982; Ulmanen et al., 1981) and cleaved after 10-15 nucleotides by the endonuclease located in the PA subunit (Dias et al., 2009; Yuan et al., 2009). The cap-binding and endonuclease activities of the polymerase proteins are only activated if viral genomic RNA is bound (Hagen et al., 1994). These short capped oligomers are then used as primers for the transcription of viral mRNAs.

The influenza protein PB1 is responsible for RNA chain elongation. PB1 contains an SDD (Serine, Aspartic acid, Aspartic acid) motif common to all RNA-dependent RNA polymerases which is likely to form the active site (Muller 1994). Mutations can be introduced into this motif which prevent the transcriptional and replicative activities of the polymerase in a cell-based activity assay (Biswa and Nayak, 1994). Chain elongation proceeds until a sequence of 5 to 7 uridine residues located near the 5' end of the vRNA. This poly-U stretch is the signal for polyadenylation (Luo et al., 1991). The polymerase stops at this region and is unable to move further, presumably due to a steric hindrance as PB1 remains bound to the 5' end of the vRNA (Fodor et al., 1994; Tiley et al., 1994). The polymerase 'stutters' and repeatedly copies the sequence forming a poly(A) tail (Hay et al., 1977; Poon et al., 1998; Robertson et al., 1981). The resulting viral mRNAs are thus not complete copies of the influenza vRNA.

The two smallest genomic segments, segment 7 (M) and 8 (NS), can be spliced. Unspliced segment 7 encodes the M1 matrix protein; however 3 alternatively spliced versions of segment 7 have also been identified. These include the transmembrane ion channel M2, a transcript giving rise to a small peptide M3 (Shih et al., 1995) and a further variant form M42
which has been identified recently but is not expressed by all strain of the virus (Wise et al., 2012). Unspliced segment 8 encodes NS1, the main viral antagonist of the innate immune response. A spliced version of segment 8 encodes NEP which has been implicated in nuclear export of RNPs as well as the regulation viral transcription and replication (Paterson and Fodor, 2012).

The virus relies on cellular machinery for the process of splicing. Indeed, it has been shown that influenza mRNA can be spliced without the presence of other viral proteins (Lamb and Lai, 1982, 1984). However, there is some evidence to indicate viral proteins, especially NS1, can be involved in regulating splicing (Garaigorta and Ortin, 2007; Robb et al., 2010; Smith and Inglis, 1985). Timing of splicing is affected by accumulation of viral proteins. For example, on segment 7 M3 is the favoured spliced mRNA until accumulated polymerase binding to the RNA termini masks the M2 donor site and in conjunction with the host cell splicing factor ASF2 favours M2 production later in the replication cycle (Shih and Krug, 1996). Control of splicing of segment 8 by use of a suboptimal splice acceptor site is important for optimal virus replication so that NEP does not accumulate too early (Chua et al., 2013).

Virus RNA replication occurs in two steps. First full length copies of the viral gene segment, termed complementary RNA (cRNA), are synthesized. These cRNAs are then used to generate large amounts of new viral gene segments. In contrast to viral transcription, the products of viral replication are not modified at the 5' and 3' ends; they are neither capped nor polyadenylated and initiation of both vRNA and cRNA synthesis occurs in a primer-independent manner (Hay et al., 1982; Young and Content, 1971).
cRNA synthesis is initiated at the 3’ end of the template vRNA and elongation of the product produces a full length cRNA, with the polymerase reading through the poly-U stretch accurately (Figure 11B). In contrast, vRNA synthesis is initiated three nucleotides downstream of the 3’ end. After synthesizing the first two nucleotides into a pppApG primer this primer re-aligns to the 3’ end of the template and elongation proceeds from there (Figure 11A) (Deng et al., 2006b).
Figure 11: Initiation strategies used by influenza polymerase for cRNA and vRNA synthesis. The vRNA and cRNA promoters are illustrated in the corkscrew conformation (Neumann and Hobom, 1995). (A) vRNA synthesis is not initiated exactly at the 3' end of the cRNA promoter. Instead, initiation occurs 3 nucleotides downstream. The pppApG dinucleotide synthesized is then repositioned to the 3' end for elongation. (B) In contrast cRNA synthesis is initiated at the terminal end of vRNA (Deng et al., 2006b).
Influenza transcription and replication are known to be tightly controlled processes; however, as yet there is limited understanding of the control mechanisms. Initially upon infection the virus acts as a transcriptase and maximal rates of mRNA synthesis occur relatively early. Later in infection the polymerase is mainly responsible for replicating the viral genome and large amounts of vRNA are made (Naffakh et al., 2008).

There was initially thought to be a 'switch' which controlled the transition from transcription to replication. When cells were treated with cycloheximide, a drug which prevents protein synthesis, influenza replication, but not transcription was inhibited. These results suggest that replication requires de novo protein synthesis (Barrett et al., 1979; Hay et al., 1977; Taylor et al., 1977).

The influenza nucleoprotein (NP) has been put forward as the 'switch' in a number of studies. NP associates with viral RNA and it has been proposed that NP promotes replication by changing the panhandle structure of the viral promoter (Beaton and Krug, 1986; Fodor et al., 1994; Klumpp et al., 1997). Furthermore, biochemical studies have suggested that NP can interact with the polymerase and potentially prevent the polymerase from stealing host cell pre-mRNA caps (Biswas et al., 1998; Mena et al., 1999; Newcomb et al., 2009).

However, more recently it has been shown that NP is not required for replication and transcription of short viral RNA-like templates. This implies that NP is not involved in the switch between transcription and replication, instead, NP is thought to act as an elongation factor for the polymerase (Turrell et al., 2013). Furthermore, the very concept of a switch
In vitro studies have demonstrated that vRNPs synthesize both cRNA and mRNA without any additional viral or cellular proteins. cRNA is not initially detected in infected cells, however, it has been suggested that cRNA synthesised in cells is degraded by host nucleases until enough influenza polymerase and NP has been synthesised to stabilise it. Indeed, with pre-expression of the influenza polymerase proteins and NP, cRNA can be detected from the start of infection. (Vreede and Brownlee, 2007; Vreede et al., 2004).

A trans-model has been proposed for the production of vRNA from the cRNA template based on a series of complementation experiments. In this model the cRNA is protected from degradation by a resident polymerase complex which binds to the promoter. However, it is a non-resident polymerase complex which accesses the 3' cRNA promoter and functions as the replicative enzyme (Jorba et al., 2009).

Recently, the influenza protein NEP has been implicated in the control of viral transcription and replication. NEP can affect synthesis of mRNA, cRNA and vRNA, although as yet there is no consensus for the mechanism by which NEP does this or the true role during replication. Expression of NEP in a cell-based polymerase activity assay has been shown to have varying effects on the synthesis of the 3 main RNA species (Bullido et al., 2001; Manz et al., 2012; Robb et al., 2009).

A further study has suggested that NEP could regulate viral RNA synthesis by promoting synthesis of small viral RNAs (svRNA). svRNAs map to each of the 5' ends of the vRNA segments and are 22-27 nucleotides in length. They require NEP in addition to the 3
polymerases and NP for their production. They have been implicated in enhancing vRNA synthesis. The depletion of svRNA has minimal impact on mRNA and cRNA levels but leads to a dramatic reduction in vRNA synthesis in a segment-specific manner. svRNAs could promote the replication step cRNA→vRNA by associating with trans-acting RNA polymerase (Perez et al., 2010; Umbach et al., 2010). Mechanistically, svRNAs have been suggested to interact with a novel RNA binding channel in the influenza polymerase protein PA (Perez et al., 2012).

1.7.4 Viral assembly and budding

The viral RNA formed during the replication cycle remains encapsidated with NP and bound by the three polymerase proteins in a vRNP complex. This entire complex is thought to be transported out of the nucleus via the CRM1 dependent pathway through nuclear pores. The current model of vRNP export proposes the formation of a 'daisy-chain' complex. M1 binds to the negative-sense vRNPs and NEP and forms a bridge between them. In turn, NEP binds to CRM1 in a RanGTP dependent manner, possibly through its leucine-rich nuclear export signal and through this 'daisy-chain' complex, the vRNPs are transported into the cytoplasm (Akarsu et al., 2003; Baudin et al., 2001; Boulo et al., 2007; O'Neill et al., 1998). Live cell imaging has been used to observe the movement of vRNPs during the viral life cycle. Intriguingly, NP preferentially localizes to the apical side of infected nuclei suggesting that vRNPs exit the nucleus in a polarized manner (Elton et al., 2005; Loucaides et al., 2009).

Influenza is an enveloped virus and it forms viral particles from the host cell's plasma membrane. The viral proteins found within the envelope (HA, NA and M2) mature through
the ER and trans-Golgi network before locating to the plasma membrane where they associate with lipid rafts. The M1-vRNP complexes localise to these lipid raft domains and M1 links the envelope proteins and vRNPs (Nayak et al., 2004). Virus particles can be produced that contain no or a few vRNPs, but virus particles cannot form without the envelope proteins (Nayak et al., 2009).

The manner in which viral genomic segments are packaged into virions has been described in two models. The first predicts that viral genomic segments are packaged into virions randomly and the second predicts they are specifically packaged into virions and the order they are packaged in is dictated by packaging signals. Since packaging signals have been found in the 5' and 3' non-coding and coding regions of some of the viral segments, the specific packaging model is currently favoured (Fujii et al., 2005; Fujii et al., 2003; Liang et al., 2005; Muramoto et al., 2006; Watanabe et al., 2003). However staining for expression of viral proteins does suggest some fluidity in the process since it is clear that not every cell infected expresses a full complement of viral genes (Brooke et al., 2013).

It is currently not well understood how influenza A viruses achieve membrane scission. M1 forms virus-like particles when overexpressed in mammalian cells (Gomez-Puertas et al., 2000) and so it was initially thought to be the driving force for viral budding. However, additional studies have suggested that HA, NA and M2 are also required for viral budding (Chen et al., 2007a; Gomez-Puertas et al., 2000; Rossman et al., 2010). The contrasting results may be due to the different strains of virus used.
In the study by Rossman et al, M2 was found to play a crucial role in viral budding. A highly conserved amphipathic helix was identified within the cytoplasmic tail of the protein which was capable of mediating a cholesterol-dependent alteration in membrane curvature. Furthermore, M2 was observed accumulating at the neck of budding virions and the authors found mutating the amphipathic helix prevented membrane scission and virus release (Rossman et al., 2010).

After the virions have budded from the cell, they must be actively released from the plasma membrane. Newly formed virions are still able to interact with the host cell as HA on the surface of the virion interacts with sialic acid. The viral protein NA is required to cleave these sialic acids from glycoproteins and glycolipids (Palese et al., 1974).

1.8 Influenza polymerase and NP

Our mechanistic understanding of the influenza polymerase has been limited by an absence of complete detailed structural information. The structure of the influenza polymerase has been remarkably difficult to determine. Three-dimensional images of the polymerase complex associated with vRNA and NP have been derived from tomography of images obtained at high resolution electron microscopy or cryo-electron microscopy. These indicate the three polymerase subunits are tightly associated and form a compact structure (Area et al., 2004; Arranz et al., 2012; Coloma et al., 2009; Martin-Benito et al., 2001; Moeller et al., 2012) (Figure 12).
Figure 12. 3D model of influenza virus polymerase. (A) Polymerase complex bound with a short vRNA segment and NP molecules, obtained by 3D reconstruction from electron micrograph images. Red = N-terminal region of PB2, blue = C-terminal region of PA, green = C-terminal region of PB1. (B) Polymerase complex within a vRNP obtained by 3D reconstruction from cryo-electron micrograph images. The C terminus of PB2 was identified by Nanogold labeling (Au). NP is shown in green.

The main reason for the absence of detailed structural information on the polymerase is an overwhelming difficulty in producing purified polymerase proteins in sufficient quantities. However, since 2007, because of heightened efforts and advances in expression and crystallization technologies, a series of X-ray and NMR structures of domains within PA, PB1, and PB2 have been determined. The structurally defined regions are shown in Figure 13.
Figure 13. Structurally defined regions of influenza polymerase proteins. Regions for which structure has been obtained at high resolution are boxed (Boivin et al., 2010).

The structure of the PA C-terminal domain bound to a short PB1 N-terminal peptide has been solved. The PB1 fragment is grasped tightly in a highly conserved cleft (He et al., 2008; Obayashi et al., 2008). The structure the N-terminal domain of PA has also been solved. This revealed that the endonuclease activity of the polymerase, required for stealing host cell pre-mRNAs, is located in this protein (Dias et al., 2009; Yuan et al., 2009).

The crystal structures which have been obtained for PB2 were achieved using a technique called ESPRIT. In this high-throughput approach random truncations of the PB2 gene were screened to identify fragments which could be solubly expressed by E.coli. The C-terminal
domain of PB2 (residues 678-759) was solved and was found to contain a bi-partite nuclear localisation sequence (Tarendeau et al., 2008). In addition, the central domain of PB2 (residues 320-483) has also been crystallized (Guilligay et al., 2008). This novel folded structure contains the binding site for capped cellular pre-mRNAs. The third PB2 domain which has been determined spans residues 538–678 and it is also contains of a novel folded structure (Tarendeau et al., 2008). This domain incorporates residue 627 which has been so widely cited for its role in host adaptation. The residue lies at the centre of solvent exposed region (Figure 14). A glutamic acid (E) is present in this position in almost all avian isolates; however, replacing this residue with a lysine (K) can dramatically overcome the block to replication in mammalian cells (Hatta et al., 2001; Shapira et al., 2009b). The glutamic acid forms a region of negative charge which the introduction of a lysine disrupts.

Figure 14. The nature of the amino acid at position 627 affects the electrostatic potential at the surface of PB2. Crystal structure of PB2 harbouring (A) a glutamic acid (B) a lysine at position 627 (Tarendeau et al., 2008). Positive and negative electrostatic potentials are shown in blue and red, respectively (Ye et al., 2006).
It has proved especially difficult to determine the structure of PB1 and it remains largely uncharacterized. Only the N- and C-terminal fragments of PB1 are known at an atomic resolution and these have been determined in complex with other polymerase proteins (He et al., 2008; Obayashi et al., 2008; Sugiyama et al., 2009). In the central region of PB1 are conserved motifs which are typical of RNA-dependent polymerases suggesting it has a classic polymerase fold (Muller et al., 1994; Poch et al., 1989).

Unlike the polymerase proteins, the near complete structure of NP has been solved and to a resolution of 3.2 Å (Ye et al., 2006). The crystal structure reveals a head domain, a body domain and a tail loop (Figure 15). This tail loop can be inserted into a neighboring molecule to facilitate oligomerisation. NP oligomerisation is independent of RNA binding (Elton et al., 1999; Turrell et al., 2013). Single amino acid mutations in the tail loop can completely abolish nucleoprotein oligomerisation. The tail loop is flexible which allows NP to form different structures. An RNA-binding groove has been identified between the head and body domains; it is lined with basic residues that are highly conserved.
Figure 15. Crystal structure of NP. Basic residues that are likely to be involved in RNA binding are indicated. Positive and negative electrostatic potentials are shown in blue and red, respectively (Ye et al., 2006).

Considerable work has been undertaken to map the interactions between the different polymerase proteins and also between polymerase proteins and NP and a range of different approaches have been used to achieve this. The N-terminal domain of PB1 has been shown to bind PA. This was determined by functional studies and characterized crystallographically (He et al., 2008; Obayashi et al., 2008) The C-terminal domain of PB1 has been shown to interact with both the C-terminal and the N-terminal domains of PB2. (Biswas and Nayak, 1996; Gonzalez et al., 1996; Ohtsu et al., 2002; Poole et al., 2004; Sugiyama et al., 2009; Toyoda et al., 1996).

Despite several attempts, largely involving biochemical approaches no direct interaction had been demonstrated between PA and PB2 (Digard et al., 1989; Ohtsu et al., 2002; St Angelo
et al., 1987). However, recently bimolecular fluorescence complementation was used to detect an interaction between the N-terminus of PA and PB2. This approach is able detect weak or transient interactions in vivo (Hemerka et al., 2009).

A direct interaction between NP and the polymerase has been confirmed using biochemical approaches (Biswas et al., 1998; Labadie et al., 2007; Mehle and Doudna, 2008; Newcomb et al., 2009; Poole et al., 2004; Rameix-Welti et al., 2009) and a high cryo-resolution electron microscopy reconstruction has enabled this interaction to be visualised (Figure 7) (Moeller et al., 2012). In addition, it has been shown that three amino acids (R204, W207, and R208) found at the top of the head domain of NP are necessary to enable a functional interaction with the viral polymerase (Marklund et al., 2012).

All the polymerase subunits have been implicated in binding to model vRNA and cRNA promoters using cross-linking studies (Deng et al., 2005; Fodor et al., 1994; Fodor et al., 1993; Hara et al., 2006; Jung and Brownlee, 2006; Pritlove et al., 1995) and attempts have been made to define the interacting domains.

Regions within the C-terminus of PB1 required for viral RNA promoter binding have been identified (Jung and Brownlee, 2006; Kerry et al., 2008; Li et al., 1998); however, other studies have implicated the N-terminus of PB1 in vRNA promoter binding (Gonzalez and Ortin, 1999a). The regions of PB1 responsible for binding to the cRNA promoter are the core of the protein containing the enzymatic active site as well as the N-terminus (Gonzalez and Ortin, 1999b). The use of surface plasmon resonance confirmed an interaction between the crystalised C-terminal domain of PB2 and the 5' vRNA promoter (Kuzuhara et al., 2009).
Furthermore, the N-terminal region of PA has been shown to be involved in viral RNA promoter binding (Hara et al., 2006).

The observation that all three polymerase proteins interact with the promoter suggests there is a considerable degree of cooperativity between the proteins to enable promoter binding and recognition.

1.9 Influenza host range

To become established in other hosts, avian influenza viruses must overcome species barriers and adapt to interact successfully with host-specific factors to achieve efficient viral replication. The enormous capability for genetic variation by the influenza virus occasionally enables this transformation.

1.9.1 The role of haemagglutinin in influenza host adaptation

The influenza glycoprotein haemagglutinin (HA), plays an important role in determining the host range of influenza viruses. Although influenza HA proteins recognize oligosaccharides containing terminal sialic acid (SA) on the surface of epithelial cells, they have different receptor specificities. Human viruses preferentially recognise SA linked to galactose by α2,6 linkages, whereas avian viruses preferentially recognise SA linked to galactose via α2,3 linkages (Figure 16) (Matrosovich et al., 1997; Rogers and Paulson, 1983; Suzuki, 1994).
Figure 16. Characteristics of avian influenza HA and NA which restrict human transmission. (A) Avian influenza virus HA proteins (green) recognise SA linked to galactose by α2,3 linkages, whereas human influenza viruses have HAs that are adapted to recognise α2,6 linkages (blue). Mutations at the receptor binding site of HA adapt the human virus for increased binding to α2,6 linked sialic acid receptors. (B) Some poultry adapted avian influenza virus HAs undergo fusion at a higher pH than human transmissible virus HAs. This renders them susceptible to premature inactivation in the environment and can decrease the likelihood of transmission. Mutations that stabilize HA overcome this and inevitably lead to fusion in the late endosome rather than early endosomes. (C) Amino acid deletions within the stalk domain (pink short stalk NA) are often identified following the transmission of influenza from wild birds into chickens. However, deleting residues in the stalk domain of NA compromises respiratory droplet transmission in ferrets, possibly by reducing the
ability of the virus to penetrate the mucus barrier or by decreasing virion release from human cells. Human transmissible viruses have NA with full length stalk (purple long stalk NA).

In humans, $\alpha_{2,6}$ receptors are mainly expressed on cells of the ciliated epithelium along the upper regions of the respiratory tract (Baum and Paulson, 1990; Thompson et al., 2006; van Riel et al., 2010), whereas $\alpha_{2,3}$ receptors are found in the lower region of the respiratory tract (Shinya et al., 2006). Avian influenza viruses replicate more efficiently deep in the lungs where the $\alpha_{2,3}$ receptors are more prevalent (van Riel et al., 2006). The paucity of $\alpha_{2,3}$ receptors in the upper respiratory tract is thought to restrict human to human transmission of avian influenza viruses. To achieve sustained human transmission, avian viruses must adapt to gain $\alpha_{2,6}$ binding in humans. It has also been proposed that mucus in the human airway is rich in soluble $\alpha_{2,3}$ receptors and acts as a soluble receptor decoy to inhibit replication and spread of avian influenza viruses. However the evidence for an excess of $\alpha_{2,3}$ over $\alpha_{2,6}$ SA in human mucus is poor, and the samples analysed have been often obtained from patients with conditions that might affect mucus constituents such as cystic fibrosis. We and others found both $\alpha_{2,3}$ and $\alpha_{2,6}$ SA in secretions obtained from human airway cells (Kesimer et al., 2009; Roberts et al., 2011). Thus, although an attractive hypothesis, a role for sialic acids in mucus in driving human adaptation of influenza virus HA is unproven.

Specific amino acid substitutions within the receptor binding site of HA can alter the receptor preference from $\alpha_{2,3}$ SA linkages to $\alpha_{2,6}$ SA linkages. Replacing glutamine with leucine at position 226 and/or replacing glycine with serine at position 228 can change the preference from $\alpha_{2,3}$ to $\alpha_{2,6}$ receptors for H2 and H3 serotypes (Rogers and Paulson, 1983;
For the 1918 and 2009 pandemic H1N1 (pH1N1) viruses replacing glutamic acid with aspartic acid at position 190 (H3 numbering) is crucial in altering receptor preference and an aspartic acid instead of glycine at residue 225 also affects receptor specificity (Gamblin et al., 2004; Glaser et al., 2005; Soundararajan et al., 2009).

Several studies have attempted to identify mutations that alter the receptor preference of H5 HA. One approach has been to analyse the glycan receptor binding of H5 HAs with natural variations in the receptor-binding site (Yamada et al., 2006). In another approach the classic H1, H2 and H3 HA humanising mutations were introduced into H5 viruses, however these mutations alone are insufficient to improve virus transmissibility (Maines et al., 2011).

In 2012 two studies reported aerosol transmission of highly pathogenic H5 viruses in ferrets, which express a similar distribution of SA as humans. In both studies, mutations predicted to switch preference of HA from α2,3 to α2,6 SA were engineered into the viruses. As seen before (Maines et al., 2011) these were insufficient to facilitate transmission between ferrets. However further adaptive mutations mapping to HA which enabled the transmission events were selected upon serial passage of the viruses in ferrets (Herfst et al., 2012; Imai et al., 2012). These included changes to a glycosylation site near the receptor binding region that likely affect the affinity of HA for the two different types of SA, and also changes in the stalk region of HA that were proven to (Imai et al., 2012) or likely to (Herfst et al., 2012) affect the pH at which HA underwent fusion. Biophysical measurements indicate that the altered H5 HA exhibits a small increase in its affinity for human receptor and a considerable reduction in affinity for avian receptor (Xiong et al., 2013). However, the quantitative switch
to α2,6 binding was not as significant as in the strains which have previously caused pandemics within the human population (Tharakaraman et al., 2013). Indeed, although aerosol transmission was achieved, the H5N1 infected sentinel animals shed virus later after exposure than for similar 2009 pH1N1 influenza transmission experiments.

In similar studies with H9N2 influenza virus, mutations in HA that were required to support ferret transmission included both mutations that affected SA binding specificity as well as a mutation the stem region of the HA protein that affected pH stability (Sorrell et al., 2009).

From these studies it is now apparent that reassortment in which a novel HA gene is introduced into a human adapted genetic backbone is not sufficient on its own to generate a pandemic influenza virus. Rather the reassorted virus would also need to undergo mutation of the HA gene to adapt it for human receptor binding and increase its stability in the transmission environment.

1.9.2 The role of neuraminidase in influenza host adaptation

The viral glycoprotein HA binds to sialic acid-containing receptors on target cells, whereas, the viral glycoprotein neuraminidase (NA) removes sialic acid residues from cellular receptors and extracellular inhibitors, facilitating the mobility of virions and their release from cells. An optimal balance between the activities of HA and NA is required for efficient viral replication and transmission (Baum and Paulson, 1991; Gimsa et al., 1996; Lakdawala et al., 2011; Yen et al., 2011). This balance can be disrupted by reassortment or transmission to
a new host that bears a different set of receptors. To restore the functional balance, compensatory mutations may need to be selected.

Xu et al. compared the HA and NA activities of 2009 pH1N1 human viruses and swine progenitor viruses. A consistent functional HA and NA balance was observed in the human viruses but not the swine progenitor viruses (Xu et al., 2012; Yen et al., 2011).

Neuraminidase enzyme activity resides in the head domain of the mushroom shaped protein which extends from the virion or infected cell surface on a stalk. Amino acid deletions within the stalk domain have been identified following the transmission of influenza from wild birds into chickens (Banks et al., 2001; Hossain et al., 2008; Sorrell and Perez, 2007; Steensels et al., 2007). For example the H5N1 influenza viruses mostly have 19 amino acids deleted from NA (Munier et al., 2010). The longest deletion reported is 35 amino acids in a H7N7 virus (Li et al., 2011).

Deleting residues in the stalk domain of NA reduces its ability to release virions from the surface of cells (Castrucci and Kawaoka, 1993; Matrosovich et al., 1999). It is not clear why this is selected for in poultry but the reduction in receptor destroying activity could counterbalance the reduced binding of the HA to sialic acids expressed in these hosts (Baigent and McCauley, 2001; Mitnaul et al., 2000; Wagner et al., 2002). Indeed a deletion in the stalk domain of NA enhanced viral replication and pathogenicity in chickens (Hoffmann et al., 2012). We recently showed that an otherwise mammalian adapted virus with the NA gene from a poultry adapted HPAI H5N1 virus harbouring a stalk deletion was compromised in respiratory droplet transmission between ferrets (Blumenkrantz et al., 2012).
This deficit was overcome by lengthening the stalk region of NA to that seen in typical human transmissible strains.

Further evidence of NA host adaptation was observed during and after the 1957 H2N2 pandemic when the emerging virus containing an N2 NA from an avian virus was introduced into humans. The ability of the new NA to cleave α2,6 SA was enhanced compared with avian N2 NA, demonstrating adaptation to the α2,6 SA receptor specificity of humans (Baum and Paulson, 1991; Kobasa et al., 1999).

To conclude, in addition to reassortment that introduces novel antigens to which the human population lack immunity, a prerequisite for a pandemic virus is for adaptive changes in both HA and NA that facilitate efficient interaction with host sialic acids, and affect protein stability (Figure 16).

1.9.3 Influenza polymerase and host range

It is well documented that polymerases from avian strains of influenza do not function well in the mammalian host and that mutations which increase polymerase activity are a prerequisite for host adaptation (Beare and Webster, 1991; Naffakh et al., 2008). Host specific genetic signatures have been identified on all of the polymerase subunits and on NP (Figure 17). Some of these adaptive mutations have arisen during natural evolution, some have been identified in bioinformatic studies comparing genetic sequences of avian and mammalian strains and others have been identified through serially passaging avian viruses.
in mice. However, our molecular understanding of why avian polymerases are restricted in human cells and how these adaptive mutations overcome this restriction remains limited.

There is evidence to suggest that PB2, PA and NP proteins often co-evolve within strains, most likely as a result of the important physical and functional interactions the proteins have with each other (Naffakh et al., 2008; Obenauer et al., 2006). The incompatibility of reassortant vRNPs may further limit interspecies transmission (Long et al., 2013). Activity of polymerase constellations from different viruses is often studied \textit{in vitro} in a reconstituted polymerase assay in which a minigenome is amplified and expressed by cloned polymerase proteins (Pleschka et al., 1996).
Figure 17. Linear representation of polymerase proteins and NP showing functionally characterised domains and residues implicated in host adaptation. Many different mammalian host range determinants have been localised to the polymerase proteins and NP, some of these have arisen naturally, some have been identified in bioinformatic studies and other have appeared through the serial passage of avian viruses in mice. Green = interaction with viral proteins, red = involved in nuclear localization, purple = involved in nuclear export, orange =interaction with cellular proteins, yellow = MxA resistance, blue = RNA binding. (Manz et al., 2013b).
PB2

PB2 is the polymerase protein which arguably carries the dominant determinants of host range. In 1977 Almond first identified the PB2 segment as being responsible for the restriction of an avian fowl plague virus in mammalian cells (Almond, 1977). In 1993 Subbarao showed that the restriction of a reassortant virus containing the PB2 gene from an avian influenza strain could be overcome through serial passage of the virus in mammalian cells which selected for a single mutation, PB2 E627K (Subbarao et al., 1993).

Position 627 in PB2 is a particularly remarkable host-associated genetic signature. A glutamic acid (E) is present in this position in almost all avian isolates; however, replacing this residue with a lysine (K) can dramatically overcome the block to replication in mammalian cells, increase pathogenicity of avian virus in mice and facilitate transmission between guinea pigs or ferrets (Chen et al., 2007b; Gao et al., 2013; Hatta et al., 2001; Shinya et al., 2004; Steel et al., 2009).

The 627K signature is present in the 1918 pandemic virus and it is likely that this mutation arose early in the emergence of the pandemic as the PB2 gene is avian-like apart from at this residue (Taubenberger et al., 2005). This mutation was also present in two subsequent influenza pandemic viruses which emerged in 1957 and 1968 because these reassortant viruses retained the PB2 gene from the 1918 pandemic virus (Guan et al., 2010). A lysine at position 627 in PB2 has been identified in several other avian strains of influenza that have infected humans. 32 % of H5N1 strains isolated from humans since 1997 have the 627K
adaptation (Long et al., 2013), as do the majority of recent H7N9 human isolates from China in 2013 (Liu et al., 2013).

Currently the mechanism for the observed enhancement in polymerase activity is unclear. The 627 E→K switch has been suggested to stabilise the interaction between the NP and PB2 components of the vRNP complex in the nuclei of infected cells (Labadie et al., 2007; Mehle and Doudna, 2008; Ng et al., 2012; Rameix-Welti et al., 2009). Moreover, it has been suggested that the effect of the E627K mutation is dependent on the origin of the NP protein. The selection of the 627K mutation during passage in mammalian MDCK cells of a reassortant virus with the majority of segments from a 1997 H5N1 virus only occurred when the genome constellation contained the NP of a more recent H5N1 virus but not with the 1997 NP (Bogs et al., 2011). However, it cannot be excluded that certain virus constellations do not accommodate the nucleotide changes required for the E627K switch. Indeed, work recently published from our group indicated the RNA sequence in the 627K region may play an important cis acting role that depends on the sequence of other RNA segments in the virus. This role might be for example in vRNA packaging. We showed that a modern H5N1 virus that had naturally selected the 627K mutation was able to tolerate either the Lys or Glu codon switch in birds, whereas a historic H5N1 virus from 1991 did not tolerate the Lys codon, unless reassorted with gene segments from either the modern H5N1 virus or a lab adapted H1N1 strain (Long et al., 2013). These findings suggest polymerase activity in avian cells is independent of residue 627, yet alternative selection pressures of RNA sequence may determine the evolution of amino acids in this region.
The crystal structure of the 627 containing domain of PB2 (residues 535-684) contains a unique structure composed of an alpha helix encircled by a loop which is located next to a highly basic groove (Figure 18). Position 627 is located within the loop structure (Kuzuhara et al., 2009). The helical bundles in this domain of PB2 share structural similarity with activator 1, a subunit of the DNA replication clamp loader and itself an RNA-binding protein. Interestingly, the nature of the amino acid at position 627 in PB2 has been implicated in affecting the way in which the polymerase interacts with the viral RNA promoter. The RNA binding activity of the PB2 domain containing amino acid 627 was determined by surface plasmon resonance and the presence of a lysine shown to enhance RNA binding ability in comparison to glutamic acid (Kuzuhara et al., 2009). It was also suggested in this study that the 627 domain has a preferentially higher affinity for the vRNA promoter in comparison with capped and non-capped RNA oligomers. However, the promoter binding activity of the polymerase has also been studied in vitro using a UV-cross-linking approach. Here, the presence of a lysine was shown to significantly reduce vRNA and cRNA promoter binding, corresponding with a reduced ability to initiate replication in vitro (Nakazono et al., 2012). Despite these conflicting results in vitro, where host factors are lacking, the concept that promoter binding in the cell nucleus is affected by the PB2 E627K mutation is supported by Paterson et al. (2013) as described below.
Figure 18. Structure of PB2 627 domain. Position 627 is located within a loop encircling an alpha helix. This unique structure is positioned alongside a highly basic groove (Kuzuhara et al., 2009).

Temperature has also been implicated in the selection of PB2 627K. Human influenza A viruses replicate in the upper respiratory tract which is approximately 33°C, whereas avian influenza viruses are adapted to replicate in the intestines of birds at a temperature of approximately 41°C. Indeed avian influenza virus replication in human airway epithelial cells is compromised at lower temperatures and this is at least partly accounted for by PB2 627E (Hatta et al., 2007; Scull et al., 2009). In a mammalian cell-based polymerase assay, a polymerase containing PB2 627K was shown to be more active at 33°C than a polymerase with PB2 627E. This difference in polymerase activity was not as apparent at 37°C. Thus, the cold sensitivity of an avian influenza polymerase could be controlled at least in part by the residue at position 627 in PB2 (Massin et al., 2001).
In a further study, influenza polymerases containing either the human or avian signature at position 627 were generated using a baculovirus expression system and their in vitro activities compared at different temperatures. At high temperatures the glutamic acid containing polymerase remained active, whereas the introduction of a lysine considerably reduced activity (Aggarwal et al., 2011). Thus, the nature of the amino acid at position 627 may alter the temperature-dependent enzymatic activity of the influenza polymerase. However, temperature cannot be the sole factor in determining host range restriction, as influenza polymerases containing 627E are less active in human cells than polymerases containing 627K even at high temperatures (Bussey et al., 2010; Massin et al., 2001).

Indeed, it is unlikely that polymerase host adaptation is achieved solely through altering interactions between viral proteins or RNAs; interactions with host-specific factors must play an important role. Once the virus has been transmitted to a new species, it is confronted with changes in the cellular microenvironment that might influence every step of the replicative cycle. Two studies using polymerase activity assays in heterokaryons of human and avian cells suggest an important involvement for cellular factors. The authors of the first study, Mehle and Doudna, concluded that mammalian cells express a dominant inhibitory factor which restricts the function of a polymerase harbouring a glutamic acid at position 627 of PB2 (Mehle and Doudna, 2008). However, a later study from our laboratory came to a different conclusion using a similar experimental strategy, and showed that the activity of a typical avian polymerase in avian cells was not inhibited upon fusion with human cells (Moncorgé et al., 2010). Rather, Moncorgé et al. observed that the activity of an avian polymerase in human cells was enabled upon fusion with avian cells, but not
human cells. This suggested that factors within an avian cell, which are not present in human cells, can facilitate the activity of an avian polymerase. It is possible that both interpretations of these experiments are correct and that virus polymerase activity is affected by both positive and negative factors that are present in different cell types at different concentrations. Until the identity of all such factors is resolved, further interpretation is difficult.

There are already a number of cellular factors whose interactions with the influenza polymerase have been implicated in host range. A study by Bortz et al. used a functional genomics approach to describe a network of human proteins that modulate influenza polymerase activity (Bortz et al., 2011). Cellular factors were identified that differentially regulate the activity of a polymerase in human cells depending on the amino acid at position 627 in PB2, including the DEAD box RNA helicase 17 (DDX17). Knockdown of DDX17 in human cells inhibited a human-adapted (627K) polymerase but intriguingly increased the activity of a PB2 627E containing polymerase. It was also shown that the chicken DDX17 homologue was required for efficient avian (627E) and human (627K) virus infection in chicken cells, but further work is required to fully understand the role played by DDX17 in influenza polymerase host range.

The restriction of an avian influenza polymerase in a mammalian cell-based polymerase assay can be overcome if artificial mutations (in particular G3A, U5C and C8U) are introduced into the 3’ viral promoter (Crescenzo-Chaigne et al., 2002; Neumann and Hobom, 1995) (Figure 19). These mutations result in perfect base pairing between the terminal nucleotides of the 3’ and 5’ ends of vRNA, which is likely to alter the structure of
both vRNA and cRNA promoters and affect the manner and affinity with which the polymerase binds to the vRNA and/or cRNA promoters (Tiley et al., 1994). The effects of these mutations are dependent on the residue at position 627 in PB2. The dramatic enhancement in polymerase activity in mammalian cells when position 627 is glutamic acid, is not observed when a lysine is introduced (Crescenzo-Chaigne et al., 2002). Paterson et al. have recently shown that enhancement of activity for a polymerase with 627E replicating a mutated promoter was not due to increased panhandle complementarity but was specific for the 3’ promoter sequence (Paterson et al., 2013).

![Nucleotide sequence and predicted 'corkscrew' conformation of (A) WT and (B) mutated 3-5-8 vRNA and cRNA viral promoters.](image)

(Red: mutated residues and complementary mutations)
Despite considerable focus on PB2 E627K, this mutation is not essential for mammalian adaptation since mutations at several other residues within PB2 can overcome the host range block. The 2009 pH1N1 virus contained the typical avian virus motif of glutamic acid at position 627 and substituting this for a lysine did not enhance viral replication or pathogenicity in mice (Herfst et al., 2010; Jagger et al., 2010; Zhu et al., 2010). Instead, the pH1N1 PB2 protein harbours the mutations G590S and Q591R, located on the same surface of the crystallized PB2 fragment as 627. It has been proposed that these mutations mimic the effects of a lysine at position 627, masking the negative charge of the glutamic acid to re-establish a positively charged patch on the surface of the domain (Mehle and Doudna, 2009; Tarendeau et al., 2008; Yamada et al., 2010). However, it should be noted that the work of Foeglein et al. implies that charge is not the only determinant of function. No obligatory correlation was observed between the electrostatic potential of the domain and transcriptional activity in mammalian cells (Foeglein et al., 2011).

Interestingly, replication of avian influenza virus in ostrich and emu, members of the ratitae genus, also selected for mutation of PB2 at residue 591 (Yamada et al., 2010). This suggests that this genus of birds which are quite phylogenetically distinct from anseriformes (ducks) and galliformes (chickens), may express similar relevant host factors as mammalian species that exert restriction on unadapted avian virus at the level of the polymerase.

A further marker of host range identified in the pH1N1 virus is residue 271 in PB2. Most avian influenza viruses have a threonine at this position, whereas an alanine is present in the pandemic strain and in previous human influenza virus PB2 genes. This mutation has been shown to enhance polymerase activity in human cells (Bussey et al., 2010; Foeglein et
al., 2011) and enhance viral replication in mice (Bussey et al., 2010). In addition, back mutation to threonine abolished guinea pig transmissibility of recombinant virus, and, in conjunction with an 'avianizing' mutation at the receptor binding site of HA, abrogated transmission between ferrets (Zhang et al., 2012). The mechanism for increased polymerase activity bestowed by mutation at residue 271 is completely unknown.

The PB2 mutation E158G was identified by passage of a pH1N1 virus in mice. Although sequence database searches only identified a single human pH1N1 virus with this mutation (A/Auckland/1/2009), its introduction considerably increased pH1N1 polymerase activity, viral growth in a mouse cell line and morbidity and mortality in the mouse model. In addition, this mutation increased the morbidity and mortality of two H5 viruses in mice (Ilyushina et al., 2010; Zhou et al., 2011; Zhou et al., 2013). The mechanism by which this mutation confers increased polymerase activity is also unknown.

PB2 D701N is an additional mutation which has been strongly implicated in mammalian host adaptation. This mutation was identified when an avian H7 virus was serially passaged in mice. The mouse adapted virus replicated much more efficiently than the parental strain in mammalian cells (Gabriel et al., 2005b; Gabriel et al., 2008). In addition, PB2 701N has been shown capable of expanding the host range of avian H5N1 to mice and humans (de Jong et al., 2006; Li et al., 2005b), as well as to compensate for the absence of 627K and restore transmissibility between guinea-pigs (Gao et al., 2009; Steel et al., 2009). Most recently, the introduction of PB2 D701N into the pH1N1 virus enhanced viral replication, pathogenicity in mice and led to more efficient transmission in ferrets (Zhou et al., 2013). The fact that 701N did this but 627K did not suggests that 701N acts by a different mechanism than the 627 and
590 mutations. Although the PB2 701N residue is not present in human circulating influenza strains, it is present in some human H5N1 isolates and in the dominant Eurasian swine H1N1 lineage. This may explain how the avian virus that crossed from ducks into swine in the late 1970s in Europe has adapted to replicate in pigs (Qi et al., 2012).

Amino acid 701 resides in a domain which has been co-crystallized with human importin-α5 and is implicated in the nuclear localization of the PB2 polymerase subunit (Tarendeau et al., 2007). There is increasing evidence that components of the nuclear import machinery play an important role in influenza virus host adaptation and that avian influenza polymerases must adapt to interact with human importin-α isoforms to enable efficient viral replication in human cells (Gabriel et al., 2005b; Gabriel et al., 2008; Gabriel et al., 2011; Hudjetz and Gabriel, 2012; Resa-Infante et al., 2008). It has been suggested that this could be achieved by two distinct mechanisms; to increase nuclear entry of vRNP components, and/or to facilitate the use of an importin-α as a cofactor to enhance vRNP activity. Since most of these observations have been based on experiments whereby importin-α isoforms have been silenced, another interpretation of the data is that yet another cofactor whose nuclear transport is controlled by importin-α is required for the polymerase to function optimally in mammalian cells.

It has been shown that avian influenza viruses undergo a switch in importin-α dependency upon avian–mammalian adaptation. For virus replication in a mammalian cell, avian viruses particularly depend on the expression of importin-α3, whereas mammalian viruses depend on importin-α7. This switch to importin-α7 dependency enhances efficient viral replication and is mediated by the PB2 D701N mutation. It has been suggested that PB2 627K also
facilitates an optimal interaction with particular importin-α isoforms within the vRNP complex in mammalian cells (Hudjetz and Gabriel, 2012). Silencing importin-α1 and -α7 reduced the activity of polymerases with PB2 627K but not 627E. Moreover, mice lacking importin-α7 were less susceptible to human-like (627K) but not avian-like (627E) influenza virus infection (Hudjetz and Gabriel, 2012).

A novel study found that PB2 mutations can affect the dynamics of the viral polymerase in human cells. A correlation between low polymerase activity and slow diffusional mobility of the polymerase was observed. A PB2 627E containing polymerase exhibited significantly slower diffusion in human but not avian nuclei compared to a PB2 627K containing polymerase. In addition, host-range mutations at positions 271, 588, 636 and 701 were also shown to enhance both polymerase activity and mobility in human cells. This correlation is further support for the proposition that interactions of the viral polymerase with cellular factors influence its activity (Foeglein et al., 2011).

In conclusion, several different amino acids in PB2 confer increased polymerase activity and are able to overcome host restriction. However, it is not clear what finally determines which ones are selected for in nature in different virus genetic backgrounds, and in different ecological niches during zoonosis and host range adaptation.

PA

The PA protein encoded by RNA segment 3 consists of an N terminal domain that contains endonuclease activity essential for cap snatching, and a C terminal domain that interacts
with PB1. The role of PA in host adaptation is less well characterised than PB2, however, it is increasingly considered to exert an important influence on host range. One recent study found the restriction of an avian influenza polymerase in human cells could be overcome if the avian PA subunit was replaced with human-origin PA subunits. Interestingly, reassortants with the pH1N1 PA proteins were the most active (Mehle et al., 2012). Like the PB2 segment, the PA segment of pH1N1 2009 virus was recently derived from an avian viral source (Garten et al., 2009). It might be assumed therefore that it has evolved to accommodate host adapting mutations for replication in pigs and humans and indeed several residues in pH1N1 PA have been shown to be involved in host adaptation (Figure 17). The PA mutations T85I, G186S and L336M in particular are thought to affect host range (Bussey et al., 2011).

There has been considerable effort to identify markers of host range in PA. A number of groups have serially passaged the pH1N1 virus in mice and identified mutations in PA which increase polymerase activity and pathogenicity (Ilyushina et al., 2010; Sakabe et al., 2011; Zhu et al., 2012). In a similar approach, an isoleucine at the PA residue 97 was selected following the serial passage of a low-pathogenic avian H5N2 virus in mice (Song et al., 2009). This mutation increased polymerase activity and replication in mice but not chickens.

Recently a second viral protein generated from mRNA of segment 3 was discovered, termed PA-X (Jagger et al., 2012). This protein comprises of the first 191 amino acids containing the endonuclease domain of PA, but then through a frameshift gains a novel C terminus of 61 amino acids whose sequence is unique from that of PA. The function of the protein appears to be in controlling host gene expression since it destroys host mRNAs through the
endonuclease activity that it retains, and this can have profound effects on pathogenicity. The PA-X sequence is quite variable between different virus strains, and is often truncated in viruses from swine or dogs (Shi et al., 2012). However there is no experimental evidence as yet that this newly discovered influenza A virus proteins plays a role in determining host range, although it is tantalizing that the endonuclease activity is more potent in PA-X genes from avian than human influenza virus strains (Desmet et al., 2013).

**PB1**

In PB1, amino acid 375 is a host range signature amino acid. Most avian strains have an asparagine at this position, whereas most human influenza strains have a serine (Taubenberger et al., 2005). The 1918, 1957 and 1968 human pandemics were caused by viruses harbouring PB1 gene segments from different origins and strikingly in all these gene segments the avian signature asparagine was substituted for a serine at position 375 (Naffakh et al., 2008). However, although this residue appears to play an important role in host range, it does not strictly map as a host range determinant. A serine is found in several avian viruses at this position and some human H3N2 viruses contain an asparagine at position 375 (Taubenberger et al., 2005). It is likely that compensatory mutations can be introduced in PB1 or a different viral protein. The mechanism by which mutations at PB1 residue 375 might affect polymerase activity is unknown.

**NP**
The interaction between NP and the host cell protein importin-α was one the first to be described in the literature (O’Neill et al., 1995). It is now evident that NP makes many interactions with the host cell (Mayer et al., 2007) and undoubtedly affects host range. Although introducing an avian NP gene into a human strain of influenza did not attenuate viral growth in experimentally infected human volunteers, the growth of this virus was hindered in the respiratory tracts of squirrel monkeys (Clements et al., 1992; Tian et al., 1985). Furthermore, Scholtissek showed that although most temperature sensitive (ts) highly pathogenic influenza virus mutants could be rescued by human H3N2 viruses in chicken cells, those with a ts defect in the NP gene could not. Reassortant viruses containing the NP gene of a human strain could only be rescued in a mammalian cell line (Scholtissek et al., 1978a).

Host specific signatures have been localised to the NP gene in bioinformatic studies (Chen et al., 2006; Finkelstein et al., 2007) and some have been implicated in facilitating evasion from interferon stimulated MxA restriction (see below) (Manz et al., 2013a). In addition, the NP N319K mutation has been shown to enhance avian viral replication in mammalian cells by altering the manner in which the protein interacts with importin-α isoforms (Gabriel et al., 2007; Gabriel et al., 2005b; Gabriel et al., 2008; Gabriel et al., 2011).

1.9.4 The role of NEP in influenza host adaptation

Segment 8 encodes NS1, the main viral antagonist of the innate immune response. In addition a spliced version of this segment encodes nuclear export protein (NEP/NS2) which has recently been implicated in the mammalian adaptation of some avian influenza viruses,
especially those without PB2 E627K. A series of adaptive mutations within NEP, including M16I, enhanced the ability of a highly pathogenic H5N1 avian virus to replicate in mammalian cells by overcoming the block to vRNA amplification (Manz et al., 2012). Moreover, Manz identified NEP mutations capable of enhancing avian influenza polymerase activity in several circulating strains of influenza including the pH1N1 virus. NEP was shown to interact with the polymerase proteins PB1 and PB2 (Manz et al., 2012) and has previously been documented to affect synthesis of mRNA, cRNA and vRNA (Bullido et al., 2001; Manz et al., 2012; Robb et al., 2009), which has led to speculation that NEP acts as a regulator that controls viral replication. Indeed, disruption of splicing control in segment 8 that results in increased NEP production was highly deleterious for virus fitness in vitro, suggesting that premature stimulation of vRNA replication is normally precluded by the poor splice consensus in this mRNA (Chua et al., 2013).

1.9.5 The role of antagonizing or evading host defenses in host adaptation

The innate immune response is critical in the early stages of a novel viral infection. The expression of type I or type III interferon (IFN) up-regulates several hundred host cell genes many of which are capable of suppressing viral replication and spread. IFN expression is a potent early response against influenza infection and so the virus has co-evolved gene products to minimise IFN expression and the signalling pathways it induces (Figure. 20).
Figure 20. Influenza proteins counteract IFN expression and the signalling pathways it induces.

Influenza virus encodes several viral proteins that act as antagonists to counter the induction of transcription from the interferon gene promoter. These include NS1, PB2 and PB1-F2. The major interferon antagonist is NS1. NS1 from both avian and human influenza viruses is capable of binding to RIG-I/TRIM25/viral RNA complex and preventing RIG-I activation. NS1 also acts in the nucleus to counter the processing of recently induced host mRNAs including interferons and interferon stimulated genes (ISGs). NS1 from some avian and some human influenza viruses lack this activity but there is not good evidence at present that this determines host range. PB2 from avian influenza viruses do not localize to mitochondria due to sequence variation at the N terminus. This may compromise their ability to counter MAVS. A major anti-influenza virus ISG is MxA. MxA targets the vRNPs and inhibits its nuclear import. Avian virus vRNPs are more efficiently targeted than those of human adapted viruses due to mutations in NP protein.

The main viral antagonist of the innate immune response is the non-structural protein NS1. This protein possesses an array of antagonistic capabilities, interacting with a plethora of host factors to block IFN induction by preventing the activation of RIG-I, or inhibiting IKKβ as
well as inhibiting the expression of cellular genes by interfering with transcription, stability, processing or export of mRNA from the nucleus (Hale et al., 2008; Marazzi et al., 2012). Thus it seems likely that NS1 could play a role in determining host range that depends on its efficiency at controlling the induced interferon response. Indeed, phylogenetic studies support a role for the variation in the sequence of NS1 according to the host origin of the virus and recently, a sophisticated sequence feature analysis supported the notion that NS1 is a host range determinant (Noronha et al., 2012). Moreover, the NS1 gene appears to be particularly flexible, showing large natural variation and also the capacity to accept sequence changes in a way that makes it particularly adept to accommodate adaptive mutations such as might be required to pass from one host species to another (Heaton et al., 2013). Nonetheless direct evidence for a deficiency in the ability of avian influenza NS1 to control interferon in human cells is lacking.

IFN-β expression induced by influenza virus in human cells is largely dependent on activation of RIG-I, whose optimal downstream signalling relies on its being ubiquitinylated by TRIM25 (Gack et al., 2007). In human cells, NS1 controls RIG-I activation by binding to TRIM25 (Gack et al., 2009), and avian virus NS1 proteins appear to also be able to do this (Rajsbaum et al., 2012). This latter finding was surprising bearing in mind that chicken cells appear to lack RIG-I (Barber et al., 2010) and instead rely on Melanoma Differentiation-Associated protein 5 (MDA5) to sense influenza infection and mount an IFN response (Karpala et al., 2011; Liniger et al., 2012). However, ducks which are the reservoir host for influenza, express RIG-I and so presumably the avian virus NS1 proteins control IFN induction in ducks by interacting with a duck TRIM25 homologue, although this is not yet shown. In addition some but not all human influenza viruses control the expression of
induced genes in infected cells, including IFN-β, by binding to the host cell factor CPSF30 and inhibiting the processing and nuclear export of host mRNAs (Nemeroff et al., 1998). This property appears to be present in some but not all avian influenza viruses, and can be acquired by NS1 mutations during natural evolution (Twu et al., 2007).

Taking together the findings that most avian virus NS1 protein can bind human TRIM25 and CPSF30, it was not entirely surprising to find that a panel of avian NS1 proteins controlled IFN-β expression comparably to the NS1 proteins of human strains both when expressed exogenously and in the context of an infectious virus (Hayman et al., 2007). However further investigations have revealed variation in the efficiency with which different avian NS1 proteins inhibited IFN-β production in human cells that may support the hypothesis that a lack of interferon control contributes to host range restriction (Hayman et al., 2007; Mukherjee et al., 2012; Munir et al., 2011). Knepper et al. recently showed that the NS1 protein from the newly emerged H7N9 influenza virus associated with more than 200 human infections in China in 2013 had an increased capacity for interferon antagonism in human cells compared to a typical chicken H7 virus (Knepper et al., 2013). On the other hand, the virus that crossed from pigs to humans in 2009 and sparked the H1N1 pandemic was partially deficient in the ability to control the induced host response because its NS1 protein lacked CPSF30 binding (Hale et al., 2010a; Shelton et al., 2013).

Human cells compromised in their ability to respond to interferon become permissive for plaque formation by avian viruses (Hayman et al., 2007). This could suggest that the host-range barrier imposed upon avian influenza viruses infecting human cells is due to an inability to outpace the antiviral immune response rather than an inability to combat the
response. An alternative explanation is that a negative factor(s) that targets the avian polymerase is induced by interferon. It would be interesting to visualise the balance between polymerase activity and the innate immune response in a temporal manner.

Other viral gene products in addition to NS1 have also been implicated in controlling the innate immune response. In addition to its role as part of the heterotrimeric polymerase complex, PB2 has been shown to localise to the mitochondria and to interact with the mitochondrial antiviral signaling protein (MAVS) to inhibit interferon induction (Graef et al., 2010; Iwai et al., 2010). The ability to localise to the mitochondria is determined by the amino acid at position 9 in PB2; this residue is within a predicted mitochondrial targeting signal (Carr et al., 2006). Typically seasonal human influenza viruses have an asparagine at residue 9 and localise to the mitochondria, whereas avian influenza viruses have an aspartic acid and do not. A human influenza virus mutated to prevent mitochondrial accumulation induced higher levels of interferon and was attenuated in mice (Graef et al., 2010). This suggests that the inability of an avian virus PB2 to regulate the innate immune response may contribute to the restriction of avian polymerase activity in mammalian cells.

PB1-F2, a small protein encoded by the +1 alternate open reading frame of the PB1 gene, can also influence the innate immune response and viral pathogenicity. This protein also localises to the mitochondria and interacts with MAVS to inhibit interferon induction. It has been demonstrated that the single amino acid substitution N66S in PB1-F2 enhances the virulence of an H5N1 virus as well as the 1918 pandemic virus in mice (Conenello et al., 2011; Varga et al., 2011). PB1-F2 containing 66S binds to MAVS more efficiently than PB1-F2 containing 66N and this enhances the inhibition of interferon expression (Varga et al., 2012).
It appears PB1-F2 is required for prolonged shedding of virus in ducks but hardly affects virulence in avian species (Schmolke et al., 2011). In a significant proportion of human and swine influenza viruses PB1-F2 becomes truncated over time suggesting a detrimental function in these hosts and that PB1-F2 is a relic of the avian origin of these viruses (Zell et al., 2007).

In human cells expression of interferon induces hundreds of genes including MxA, a dynamin-like large GTPase. Human MxA protein is a major restriction factor for influenza but can also block replication of many RNA viruses, e.g. orthomyxoviruses (Thogoto Virus), paramyxovirus (Measles virus), bunyaviruses (LaCrosse virus) or rhabdoviruses (Vesicular Stomatitis virus) as well as DNA hepadnaviruses (Hepatitis B virus) (Haller and Kochs, 2011). MxA binds to various viral nucleocapsid proteins and is thought to prevent replication by oligomerizing and sequestering these proteins. How human MxA blocks influenza replication is not fully understood but recent work suggests it retains incoming viral genomes in the cytoplasm (Xiao et al., 2013). How MxA can target so many different viruses remains a mystery.

The sensitivity of influenza virus to MxA is determined by the NP protein and avian strains of influenza are typically more sensitive to MxA than human strains (Dittmann et al., 2008; Zimmermann et al., 2011). This might be explained if avian Mx homologues were not active against influenza, and acquisition of resistance to human MxA by amino acid substitutions in NP was selected when the virus crossed into humans. The role of chicken Mx in restricting influenza replication has been controversial, but there is a lack of convincing evidence to
support Mx as an important restriction factor in birds (Benfield et al., 2008; Ko et al., 2002; Sironi et al., 2008).

A series of adaptive mutations in the 1918 pandemic and 2009 pH1N1 NP genes have been identified which provide some level of escape from human MxA. When the adaptive mutations found within the 1918 pandemic NP gene were introduced into an MxA-sensitive H5N1 NP they rendered it resistant to inhibition in the reconstituted minigenome polymerase assay. However in the context of infectious recombinant virus, these mutations hindered viral growth in mammalian cells deficient for Mx and avian cells (Manz et al., 2013a). Thus, it is likely that evading MxA comes at a fitness cost to replication and that compensatory mutations allow human adapted viruses to evade MxA restriction whilst retaining viral fitness in the new host.

1.10 Influenza vaccines and anti-virals

The most effective method of combating influenza infection currently available is vaccination and vaccines are available which provide protection against the viral strains in circulation. However, because the virus is subject to considerable antigenic drift, the proteins present on the surface of the virion are highly variable and change yearly (Hilleman, 2002). Due to these frequent changes, new influenza vaccines must be developed each year. Currently vaccination is advised for the elderly, and individuals with certain pre-existing health problems that predispose them to severe outcomes.
Vaccines which protect against currently circulating strains of influenza will not provide protection against novel pandemic strains and unfortunately vaccine production is a time consuming process. During a pandemic, as occurred in 2009, it can be months before a vaccine is produced and the necessary safety trials are performed (Girard et al., 2010). A delay of several months from when a novel pandemic strain emerges until the new vaccine is distributed could have devastating consequences. Vaccine strains are currently amplified in embryonated eggs, alternative approaches using tissue culture are being developed in an attempt to speed up production (Barrett et al., 2011).

Research is also continually ongoing to develop a universal influenza vaccine. Studies are being undertaken in an attempt to develop vaccines with more broadly immunogenic, cross reactive immune responses post-vaccination. A universal vaccine would reduce the need for seasonal influenza vaccines. This would be beneficial as the uptake rate for the seasonal vaccine remains low; many people are reluctant to receive vaccinations every year (Chiu et al., 2013; Gilbert, 2012; Kaur et al., 2011).

Due to the challenges faced with vaccine development there is considerable demand for compounds to be produced which are effective in treating influenza. However, there are at present only two classes of antivirals licensed for the treatment of influenza infection in the West. The first class contains the M2 channel blockers, the adamantanes, and the second class contains the neuraminidase inhibitors (Boltz et al., 2010). The adamantane derivatives amantadine and its analog rimantadine are inhibitors of the M2 ion channel and block acidification of the virion after endosomal internalisation. This prevents the vRNPs from being released into the cytoplasm (Pinto and Lamb, 1995). Unfortunately, there is evidence
the drug can occasionally cause severe side effects such as neurotoxicity (De Clercq, 2006) and resistance to adamantanes can be acquired by circulating influenza viruses through a single amino acid change (Belshe et al., 1988; Bright et al., 2005; Bright et al., 2006; Rahman et al., 2008). Importantly the acquisition of resistance is not accompanied by a fitness cost so that resistant strains are readily propagated through the community.

The second class of licensed antivirals, the neuraminidase inhibitors, includes zanamivir (trade name Relenza) and oseltamivir (trade name Tamiflu). These drugs inhibit the enzymatic activity of NA by binding within its active site. Inhibiting neuraminidase prevents virions from being released from the cell surface. However, resistance to oseltamivir has been observed to develop quickly in human clinical isolates, in addition to H5N1 strains isolated from patients (Kiso et al., 2004). Zanamivir resistance has also been observed, although less frequently in treated patients. Even without the issue of drug resistance, the beneficial effects of NA inhibitors on disease progression are only observed if the drug is taken within 48 hours of disease onset and even then, they only shorten the course of the disease for on average one day (Burch et al., 2009).

Despite the obvious drawbacks associated with these antivirals, many countries have stockpiled these drugs for the interim period in a pandemic before a vaccine becomes available. This demonstrates the urgent need to develop novel approaches to treating influenza from which the virus is less likely to escape.

The viral RNA polymerase represents a promising drug target, its activities are distinct from those of the host cell, it plays an essential role in the lifecycle of the virus, it harbours
multiple enzymatic activities and its genes are highly conserved. Although the lack of structural information has restricted the development of polymerase inhibitors, an increasing number are being designed.

Ghanem et al, have demonstrated that the interaction between PB1 and PA can be disrupted via a 25 amino acid peptide that corresponds to the PA-binding domain of PB1 (Ghanem et al., 2007) and recently, small molecule inhibitors of influenza A and B viruses that act by disrupting subunit interactions of the viral polymerase have been developed (Muratore et al., 2012). Furthermore, the influenza NP protein has also been identified as a druggable target. Nucleozin, a small-molecule inhibitor has been shown to prevent nuclear accumulation of NP by triggering its aggregation (Kao et al., 2010).

By far the most promising polymerase inhibitor is Favipiravir. It is a pyrazine derivative which has considerable in vitro and in vivo anti-influenza activity (Furuta et al., 2002; Takahashi et al., 2003). It is effective against numerous influenza strains, including H1N1, H2N2, H3N2, a mouse adapted H5N1 (Sidwell et al., 2007) and a highly pathogenic H5N1 virus in a mouse model (Kiso et al., 2010). This anti-viral agent has the advantages of oral administration and it has been reported to be on the brink of phase III clinical trials (Furuta et al., 2013). However, its mechanism of action is not fully understood (Baranovich et al., 2013) and the best results in mice came from a very high dose (300 mg/kg) (Kiso et al., 2010).
In an alternative approach, as the influenza virus life cycle is dependent on its host cell, cellular functions that are required for viral replication are being considered as potential targets for antiviral therapy. In recent years there has been considerable effort to identify host cell factors involved in the influenza replication cycle.

Originally techniques to identify interactions between viral proteins and cellular binding partners were employed. The yeast-two-hybrid assay revealed that NP interacts with importin-α isoforms (O'Neill and Palese, 1995) and that NS1 interacts with CPSF30 (Nemeroff et al., 1998). Shapira used this approach to identify 87 cellular proteins which interact with the A/PR/8/34 (H1N1) (PR8) virus and 66 cellular proteins which interact with the A/Udorn/72(H3N2) virus (Shapira et al., 2009a). Encouragingly 56 of the interactions identified were shared by the two viruses. A further approach to identify interactions between viral proteins and cellular binding partners has been to isolate protein complexes bound to viral proteins and to determine their identity by mass spectrometry. Mayer et al. found 41 human proteins which interact with the vRNP complex using this method (Mayer et al., 2007).

Since the advent of RNA interference (RNAi) technology, five different genome wide RNAi screens have been undertaken to identify host factors required for influenza replication. The first study was carried out in Drosophila cells. Hao et al. created a virus in which the influenza HA gene was replaced with the vesicular stomatitis virus glycoprotein so that the virus could infect Drosophila cells (Hao et al., 2008). 104 genes involved in viral replication were identified.
More recently, three RNAi screens undertaken in human cells have been published. Karlas et al. transfected human A549 cells with a genome wide-library of siRNAs and then infected these cells with influenza virus (Karlas et al., 2010). They stained for the viral protein NP and quantified expression using microscopy. Additionally, they added supernatant onto reporter cells containing a viral-like luciferase reporter to measure the amount of virus released. 168 hits were identified by at least two siRNAs per gene.

In a further RNAi screen in human cells, Brass et al., identified 129 required cellular factors and four restriction factors (Brass et al., 2009). The restriction factor IFITM3 was investigated in more detail together with its paralogs IFITM1 and IFITM2 and the IFITM proteins have now been characterised as a group of influenza virus restriction factors. Additionally in a further screen, Konig et al. identified 295 factors that were required for efficient influenza virus replication.

Shapira et al., undertook an integrative genomics approach (Shapira et al., 2009a). Initially a yeast-two-hybrid screen was undertaken to identify factors which interact with the viral proteins. Next, transcriptional responses were analysed in human cells infected with influenza virus. These data sets were integrated and the hits further tested for their involvement in viral replication.

There is very little overlap in the factors identified in the various screens. Indeed, only a single gene (COPA) was identified in the four screens carried out in human cells. Although this is a perhaps little disappointing, it should not have been unexpected. A similar lack of overlapping genes has been seen for RNAi screens undertaken for HIV (Brass et al., 2008;
Bushman et al., 2009; Konig et al., 2008; Zhou et al., 2008) and different cell lines, siRNA libraries, viruses and readouts were used in the siRNA screens.

These screens are able to provide us with crucial information on the pathways and cellular functions required by influenza viruses. For example, very recently, a human genome-wide RNAi screen has identified an essential role for inositol pyrophosphates in the Type-I interferon response (Pulloor et al., 2014). However, much more work is required to characterise the interactions between the virus and the host. A more thorough understanding of this interplay is critical so that we can identify the parts of the replication cycle that the virus might have difficulty escaping from. Hopefully, this information will facilitate the development of more effective anti-viral agents.

1.11 Aims

Typical avian influenza A viruses do not replicate efficiently in mammals. Many adaptive mutations have been mapped to the polymerase complex (PB1, PB2 and PA) as well as the nucleoprotein (NP) which are required for transcription and replication of influenza viral RNA, however the mechanism/s of host adaptation are unknown. Further understanding of the molecular mechanisms of host adaptation is of paramount importance and could have a significant impact on anti-influenza drug design.

Polymerase activity is often measured using a cell-based assay in which the active enzyme is reconstituted from its constituent parts expressed from plasmids. Using this approach we
wish to investigate if different adaptive PB2 mutations enhance polymerase activity by a universal mechanism.

The PB2 mutation E627K dramatically increases avian-origin influenza polymerase activity in human cells. Previously others have proposed that an increased biochemical stability of the PB2-NP complex explains the enhanced polymerase activity observed with PB2 E627K in human cells. An aim of this study is to determine if other host-adaptive mutations affect the stability of the polymerase-NP interaction.

Further aims are to address why certain mutations, which enhance activity in the reconstituted polymerase assay, are not selected for by the virus in nature and to explore whether the polymerase assay truly reflects viral fitness. These data will help risk assess the likelihood of different avian influenza viruses crossing the host range barrier and increase our understanding of the relative balance between virus replication and the host innate response.

1.12 Selected methods for the study of influenza

1.12.1 Reverse genetics

Originally recombinant influenza viruses were generated using helper viruses. However, as it was necessary to subsequently remove these helper viruses with a strong selection it was difficult to generate viruses with growth deficiencies (Enami et al., 1990; Neumann et al., 1994). The advent of reverse genetics has greatly aided influenza research by enabling
influenza A viruses to be artificially generated from cloned cDNAs (Fodor et al., 1999; Neumann et al., 1999).

In this study we used the twelve-plasmid 'rescue system' which was originally described by Neumann et al. (Figure 2). In this approach, plasmids encoding each of the viral gene segments flanked by the human RNA polymerase I promoter and the mouse RNA polymerase I terminator are transfected into 293T cells. The method exploits the RNA polymerase I which transcribes uncapped, non-polyadenylated ribosomal RNA. Hence, the resulting viral RNAs are unmodified exact copies of the viral genome. The 293T cells are also transfected with RNA polymerase II expression plasmids encoding the three polymerase proteins and NP. The resulting proteins are able to transcribe and replicate the RNA polymerase I vRNA transcripts. Thus, the influenza proteins and vRNA can be produced in sufficient amounts to enable viral assembly and production. 293T cells can be transfected easily but they don't readily support viral growth. Thus, 293T cells are typically co-cultured with Madin-Darby canine kidney epithelial (MDCK) cells which allow for rapid viral production.
Figure 21. Influenza twelve-plasmid 'rescue system'. Plasmids encoding each of the viral gene segments flanked by the human RNA polymerase I promoter and the mouse RNA polymerase I terminator are transfected into 293T cells and the resulting viral RNAs are unmodified exact copies of the viral genome. The 293T cells are also transfected with Pol II expression plasmids encoding the three polymerase proteins and NP. These proteins are able to transcribe and replicate the RNA polymerase I vRNA transcripts. Thus, influenza proteins and vRNA are produced in sufficient amounts to enable viral assembly and production. Typically 293T cells are co-cultured with MDCK cells which allow for rapid viral production (Neumann and Kawaoka, 2001).

1.12.2 Minireplicon assay

The minireplicon assay is a cell-based influenza polymerase activity assay. It is widely used to compare the activities of polymerases from different strains of influenza in different cell types. In this approach, cells are transfected with plasmids that use species-specific
polymerase I promoters to direct synthesis of minigenomes containing a reporter gene flanked by viral noncoding sequences. The cells are also transfected with expression plasmids encoding influenza NP, PA, PB1, and PB2 proteins. The vRNA-like minigenome is encapsidated by NP, and recognized, transcribed, and replicated by the polymerase proteins. The level of reporter gene expression is a measure of polymerase activity, the more active the polymerase, the greater the expression of the reporter gene (Figure 22).

Figure 22. Schematic diagram of a minireplicon assay. Cells are transfected with expression plasmids encoding PB1, PB2, PA and NP as well as a species-specific viral-like reporter vector expressing negative-sense RNA flanked by viral conserved non-coding sequences that are the minimal viral promoters required for transcription and replication of viral RNA. The vRNA reporter is replicated and transcribed only in the presence of a functional influenza polymerase and in this example the level of luciferase expression is a measure of polymerase activity.
2. Investigating effects of adaptive PB2 mutations on avian polymerase activity *in vitro*

2.1 Introduction

The influenza polymerase is considered to play an important role in determining host range. Host specific genetic signatures have been identified on all of the polymerase subunits and on NP, but the PB2 protein arguably carries the dominant determinants of host range (Almond, 1977; Chen et al., 2006; Finkelstein et al., 2007; Subbarao et al., 1993). The introduction of mammalian host adaptive PB2 mutations (e.g., E627K, D701N and Q591K) into avian viruses has been shown to support enhanced viral replication and pathogenicity in mammalian model systems (Gabriel et al., 2007; Gabriel et al., 2005a; Hatta et al., 2001; Mehle and Doudna, 2009; Yamada et al., 2010).

The molecular basis for polymerase host range adaptation is not fully elucidated and it is possible that adaptation by different genetic mutations is achieved by distinct mechanisms. The key host-range mutations which will be investigated are shown in Figure 23. Some of these mutations have arisen naturally; some have been identified in bioinformatic studies and others have appeared through the serial passage of avian viruses in mice. The different mutations have been investigated in different assays and to different extents. The manner in which some of the mutations affect polymerase activity has been studied in both human and avian cells, whereas for others, their effect on polymerase activity has only been assessed in human cells. Furthermore, the effect some of these mutations have on polymerase activity is yet to be determined.
Although many of these mutations have been shown to enhance avian virus polymerase activity in human cells, the mechanism(s) by which this achieved has not been determined and is it not clear how universal it is. The mutations are scattered throughout the primary sequence of PB2, but as the complete structure of PB2 has not been determined at an atomic resolution it is not possible to establish where these mutations are located. In order to investigate whether all the mutations enhance polymerase activity via a universal mechanism we investigated whether the mutations enhance polymerase activity in a host specific manner.

An idea popular in the literature is that the enhanced polymerase activity observed with PB2 E627K is due to an increased biochemical stability of the vRNP in human cells (Labadie et al., 2007; Mehle and Doudna, 2008; Ng et al., 2012; Rameix-Welti et al., 2009). We wished to investigate if this mechanism also explains how other naturally or experimentally occurring PB2 mutations adapt avian virus polymerase for mammalian replication.

2.2 Results

2.2.1 Adaptive PB2 mutations do not enhance polymerase activity via a universal mechanism

In order to investigate whether adaptive PB2 mutations (Figure 23) influence polymerase activity in a host-dependent manner it was necessary to introduce these mutations individually into an avian PB2. The avian polymerase used was derived from A/Turkey/England/50-92/91 (50-92). This is a highly pathogenic H5N1 virus that was isolated
during an outbreak in domestic birds in Norfolk, England, in 1991 (Wood et al., 1994). It is a
typical avian influenza strain, and unlike modern genotype Z-like H5N1 viruses, there is no
evidence that humans or other mammals were infected by the 50-92 virus or its derivatives
during the outbreak. The adaptive mutations were introduced into the gene by site directed
mutagenesis on the pCAGGS PB2 expression plasmid. This was achieved using an
overlapping PCR approach using pCAGGS internal primers and a pair of overlapping primers
containing the desired mutation.

The mammalian host specific PB2 signatures introduced were A44S, E158G, A199S, T271A,
throughout the PB2 protein (Bussey et al., 2010; Chen, 2006; Ilyushina et al., 2010; Mehle
and Doudna, 2009; Naffakh et al., 2000; Subbarao et al., 1993; Zhou et al., 2011).
We first investigated how this panel of mutations influence polymerase activity in human, swine and avian cells. Although influenza virus polymerase activity has been studied in human and avian cells for many years by use of a minigenome assay (described in chapter 1), similar investigations in pig cells had not been reported until recently. However, in 2012 Moncorgé et al. cloned the swine RNA polymerase I promoter and developed the first minigenome assay for pig cells (Moncorge et al., 2012).

Minireplicon assays were undertaken in human (293T), swine (NTr) and avian (DF-1) cells with species-specific firefly luciferase reporters to investigate how the adaptive PB2 mutations affect polymerase activity. After measuring the kinetics of polymerase activity (data not shown), it was determined that Firefly luciferase activities should be obtained 12
hours after transfection to allow sufficient time for protein expression and vRNP formation without the possibility of polymerase activity plateauing (Figure 24).

Almost all of the introduced PB2 mutations significantly enhanced 50-92 polymerase activity in human and swine cells, whereas only a subset of the PB2 mutations significantly enhanced polymerase activity in avian cells. For example, introducing E158G dramatically enhanced polymerase activity in human, swine and avian cells, whereas the introduction of E627K only resulted in a significant enhancement in activity in human and swine cells. The observation that some PB2 mutations enhance polymerase activity in a mammalian specific manner whereas others affect activity in human, swine and avian cells suggests there are different mechanisms of enhancing activity.
Figure 24. Polymerase activity supported by PB2 mutants. 293T (A), NPTr (B) and DF-1 (C) cells were transfected with pCAGGS 50-92 PB1, PA, NP and WT or mutated PB2 as well as pCAGGS Renilla and a viral-like firefly luciferase minigenome expressing plasmid. Luciferase production was measured 12 hours post transfection. Values were normalised to Renilla expression and to the
activity of the WT polymerase. Results are expressed as the mean ± standard deviation of triplicate independent samples and ordered based on polymerase activity in 293Ts. The statistical significance of differences in polymerase activity compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P <0.05; **P <0.01; ***P <0.001).

2.2.2 Unstable polymerase-NP interaction is not responsible for avian influenza polymerase restriction in human cells

Previously, co-immunoprecipitation experiments demonstrated that the interaction between PB2 627E and NP either when expressed alone or in the context of a reconstituted RNP complex in human cells was weak. The introduction of a lysine at position 627 in PB2 was shown to strengthen this interaction and thus presented as a mechanism to facilitate mammalian host-range adaptation (Labadie et al., 2007). In addition Ng et al. recently reported differences in the interactions between the C terminus of PB2 with either 627 E or K and NP using purified components and biophysical techniques (Ng et al., 2012). In contrast, Mehle and Doudna showed that a PB2 harbouring the avian signature glutamic acid at position 627 bound NP as well as PB2 627K when just these two proteins were expressed but the interaction between PB2 627E and NP in the context of a reconstituted RNP complex was diminished in human cells (Mehle and Doudna, 2008). These latter findings suggested that the species-restricted activity of PB2 627E was the result of a defect(s) in vRNP assembly or conformation that precludes binding to NP within the RNP complex.
We aimed to investigate if other PB2 host range determinants stabilised the PB2-NP interaction in human cells in the manner reported for E627K. The PB2 mutations which enhanced polymerase activity most dramatically in human cells (E158G, T271A, M535T, G590S Q591R, E627K and D701N) were introduced into a TAP-tagged PB2 in the pCAGGS expression vector. The C-terminal TAP tag ensured all PB2 mutants were readily and equally detected regardless of the introduction of host range mutations and the presence of the tag reduced polymerase activity only 2-fold (Figure 25).

Figure 25. Activity of 50-92 with untagged or TAP-tagged PB2. 293T cells were transfected with pCAGGS 50-92 NP, PB1, PA and WT or E627K PB2 (untagged or TAP-tagged), as well as a viral-like firefly luciferase reporter plasmid and pCAGGS Renilla. Firefly luciferase production was measured 12 hours post transfection. Values were normalised to Renilla expression and to the activity of the untagged polymerase. Results shown are the mean ± standard deviations of triplicate samples.

Initially, it was investigated whether the introduced PB2 host range determinants affected the amount of PB2 precipitated by NP when these two proteins were expressed alone in 293T cells. 293T cells were transfected with pCAGGS 50-92 NP and mutated or WT TAP-
tagged PB2. At 12 hours post transfection cells were resuspended in cell lysis buffer. Immunoprecipitations were performed with protein A-agarose and an antibody specific for NP. Immunoprecipitated proteins were washed, dissolved in SDS sample buffer and analysed by Western blotting using an antibody directed against NP and an anti-Flag antibody to detect PB2 (Figure 26).

We demonstrated that the introduced PB2 host range determinants did not affect the amount of PB2 precipitated by NP when these two proteins were expressed alone in 293T cells; this is in accordance with Mehle and Doudna (Mehle and Doudna, 2008).

**Figure 26. Effect of PB2 mutations on PB2–NP interaction in 293T cells.** 293T cells were transfected with pCAGGS 50-92 NP and WT or mutated PB2-TAP. 12 hours post transfection cell lysates were prepared and subjected to NP immunoprecipitation prior to Western blot analysis. The levels of co-immunoprecipitated PB2 were normalised to precipitated NP using ImageJ.

We then investigated whether the introduced PB2 host range determinants affected the amount of PB2 precipitated by NP in the context of the entire vRNP complex. 293T cells were transfected as in the minireplicon assay to reconstitute active vRNPs and co-
immunoprecipitation and western blotting was undertaken as described above (Figure 27).

In the reconstituted RNP complex, those PB2 mutants that resulted in increased polymerase activity (Figure 27C) also increased the amount of PB2 precipitated by NP (Figure 27A 27B). The two results were proportionally correlated for all the introduced PB2 mutations. Moreover, a proportionate increase in precipitated PB1 and PA was also observed. It was confirmed by qRT-PCR that the precipitated RNP complex also contained a proportionate increase in negative sense viral-like RNA (Figure 27D). Thus it appeared that, as reported by others, mutations in PB2 that adapted avian polymerase for increased activity in mammalian cells did so by enhancing the interaction between NP and polymerase in the vRNP complex.
Figure 27. Effect of PB2 mutations on polymerase–NP interaction in 293T cells. (A) 293T cells were transfected with pCAGGS 50-92 NP, PB1, PA and WT or mutated PB2-FLAG and a viral-like firefly luciferase reporter plasmid. 12 hours post transfection cell lysates were prepared and subjected to NP immunoprecipitation prior to Western blot analysis. (B) The levels of co-immunoprecipitated PB2 in (A) were normalised to precipitated NP using ImageJ. (C) Polymerase activity supported by mutated PB2-FLAG compared to WT PB2-FLAG. Values were normalised to Renilla expression and to the activity of the WT polymerase. Results shown are the mean ± standard deviations from triplicate samples. The statistical significance of differences in polymerase activity compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P < 0.05; **P < 0.01; ***P < 0.001). (D) Level of
vRNA isolated from cell immunoprecipitates analysed in (A) for PB2 E158G-FLAG and PB2 E627K-FLAG normalised to PB2 WT.

However it occurred to us that the increased signal of precipitated polymerase proteins might also reflect an increased amount of vRNP produced by more active polymerases as more vRNA molecules are made. To test this, we engineered a mutation into the conserved SDD motif of the catalytic site of the 50-92 polymerase subunit PB1 (D446Y) (Biswas and Nayak, 1994) that would prevent transcription and replication of the template viral-like RNAs. In this case vRNP complexes formed in transfected cells could only be formed from input RNA and expressed proteins rather than from amplified RNA products. Initially it was confirmed that the activity of a 50-92 polymerase harbouring PB1 D446Y was abolished in a minireplicon assay in 293T cells (Figure 28).

![Figure 28. PB1 mutation D446Y inhibits 50-92 polymerase activity.](image)

293T cells were transfected with pCAGGS 50-92 NP, FLAG-tagged PB2 WT or 627K, PA and PB1 WT or PB1-D446Y as well as a viral-like firefly luciferase reporter plasmid and pCAGGS Renilla. Firefly luciferase production was measured 12 hours post transfection. Values were normalised to Renilla expression. Results shown are the mean ± standard deviations from triplicate samples.
The co-immunoprecipitation assay was repeated with PB1 D446Y (Figure 29). In this instance the PB2 host range mutations did not affect the amount of PB2 precipitated by NP compared with WT avian PB2. This demonstrated that the PB2 host range mutations did not affect the stability of the PB2-NP interaction. Without amplification of viral RNA there was no increase in RNP complex formation and precipitation of the heterotrimeric polymerase complex by NP was not enhanced over that seen with vRNP complexes formed with wild type avian PB2.

Figure 29. In the absence of transcription/replication PB2 mutations do not affect amount of PB2 precipitated by NP. 293T cells were transfected with pCAGGS 50-92 NP, PB1 D446Y, PA and WT or mutated PB2-FLAG and a viral-like firefly luciferase reporter plasmid. 12 hours post transfection cell lysates were prepared and subjected to NP immunoprecipitation prior to Western blot analysis. The levels of co-immunoprecipitated PB2 in were normalised to precipitated NP using ImageJ.

The conclusion that this co-immunoprecipitation assay is simply a measure of the number of vRNP complexes formed was reiterated using a different approach. The G3A, U5C and C8U
mutations (3-5-8 mutations) were introduced into the 3’ arm of the viral promoter of the luciferase reporter (Figure 30). The 3-5-8 mutations result in perfect base pairing between the terminal nucleotides of the 3’ and 5’ ends of vRNA which is likely to alter the structure of both vRNA and cRNA promoters by either stabilising the panhandle conformation and/or melting stem loop structures in the corkscrew conformation (Neumann and Hobom, 1995).

![Figure 30](image)

Figure 30. Nucleotide sequence and predicted 'corkscrew' conformation of (A) WT and (B) mutated 3-5-8 vRNA and cRNA viral promoters. (Red: mutated vRNA residues and complementary mutations in cRNA)

These mutations have been shown to enhance the amplification and expression of the minigenome driven by an avian polymerase in human cells (Crescenzo-Chaigne et al., 2002; Neumann and Hobom, 1995). This was confirmed in a minireplicon assay with the 50-92 polymerase in 293T cells, a 43-fold enhancement in luciferase reported signal was observed with the 3-5-8 reporter compared with the WT reporter (Figure 31).
Figure 31. Mutated 3-5-8 viral-like firefly luciferase reporter enhances luciferase activity. 293T cells were transfected with pCAGGS 50-92 NP, PB1, PA and PB2 (WT) as well as a WT or mutated 3-5-8 firefly luciferase reporter plasmid. Firefly luciferase production was measured 12 hours post transfection. Values were normalised to Renilla expression. Results shown are the mean ± standard deviations from triplicate samples. The statistical significance of differences in polymerase activity compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P < 0.05; **P < 0.01; ***P < 0.001).

The co-immunoprecipitation assay was undertaken in the presence of the WT and mutated 3-5-8 luciferase reporters. The amount of PB2, PA and PB1 precipitated by NP was much greater when vRNP complexes were formed with the 3-5-8 promoter mutant viral-like RNA compared with the WT minigenome (Figure 32).
Figure 32. Mutated 3-5-8 viral-like firefly luciferase reporter enhances luciferase activity and the amount of PB2 precipitated by NP. 293T cells were transfected with pCAGGS 50-92 NP, PB1, PA and PB2 (WT) as well as a WT or mutated 3-5-8 firefly luciferase reporter plasmid. 12 hours post transfection cell lysates were prepared and subjected to NP immunoprecipitation prior to Western blot analysis. The levels of co-immunoprecipitated PB2 were normalised to precipitated NP using ImageJ.

Taken together the results can be explained by the idea that in human cells where avian virus polymerase is only poorly active, few vRNA molecules are produced and few nascent vRNP complexes form, therefore only a small amount of the heterotrimeric polymerase is precipitated by NP. However, by introducing mutations into the PB2 protein which increase polymerase activity or by mutating the viral promoter sequence, more RNP complexes are assembled around the RNA promoter and more PB2, PA and PB1 will be precipitated with NP. Our findings suggest that previous interpretations of this assay by other groups working in this field were preliminary because they did not take into account the possibility of an
increase in replication resulting in an increase in numbers of vRNP complexes (Labadie et al., 2007; Mehle and Doudna, 2008; Ng et al., 2012; Rameix-Welti et al., 2009). We suggest the restricted activity of an avian polymerase in human cells is not explained by the instability of the PB2-NP complex.

2.2.3 Adaptive PB2 mutations proportionally enhance synthesis of mRNA, cRNA and vRNA

If the restricted activity of an avian polymerase in human cells is not explained by the instability of the PB2-NP complex, then why are avian influenza polymerases restricted in mammalian cells and how do host adaptive mutations help to overcome this restriction? The influenza polymerase is responsible for both transcription and replication and replication of viral RNA involves the production of a positive-sense cRNA intermediate. To assess the effects of the PB2 host range mutations on viral genome replication and transcription, the levels of viral mRNA, cRNA and vRNA were separately measured by reverse transcription and real-time quantitative PCR (Figure 33).
Figure 33. The level of viral mRNA, cRNA, and vRNA synthesised by different RNA polymerase PB2 mutants. 293T (A), NPtr (B) and DF-1 (C) cells were transfected with pCAGGS 50-92 PB1, PA, NP and WT or mutated PB2 as well as pCAGGS Renilla and a viral-like firefly luciferase minigenome expressing plasmid. The level of viral mRNA, cRNA, and vRNA synthesised by different RNA polymerase PB2 mutants was normalised to that of the WT polymerase. Expression of 50-92 NP mRNA was used as an internal control. Results shown are the mean ± standard deviations from triplicate samples. The statistical significance of differences in polymerase activity or RNA synthesis compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P <0.05; **P <0.01; ***P <0.001).
Regardless of whether the mutation was host specific or not, an increase in polymerase activity correlated with proportionally enhanced synthesis of all 3 viral RNA species in human, avian and swine cells. This confirms that the mutations are working by at least increasing the level of replication, rather than just by increasing transcription of mRNA from a constant amount of vRNA template.

2.2.4 Investigating how adaptive PB2 mutations enhance viral replication

In the minireplicon assays undertaken so far in this chapter, a minigenome reporter which expresses negative-sense firefly luciferase RNA has been used. In this approach the ability of the polymerase to transcribe and replicate a vRNA template is assessed. In an attempt to determine whether the adaptive PB2 mutations enhance a particular step in the replication process, a minigenome reporter which expresses positive-sense firefly luciferase RNA was constructed. With this construct the ability of a polymerase to transcribe and replicate a cRNA template can be assessed. (Figure 34).
Figure 34. The balance between viral transcription and replication. (A) With a negative sense viral-like reporter the ability of a polymerase to transcribe and replicate a vRNA template is assessed. (B) With a positive sense viral-like reporter the ability of a polymerase to transcribe and replicate a cRNA template is assessed.

A minireplicon assay was undertaken in 293T cells with the positive sense firefly luciferase reporter and the panel of mutant PB2 constructs (Figure 35).
Figure 35. Polymerase activity supported by PB2 mutants with a positive sense viral-like reporter.

293T cells were transfected with pCAGGS 50-92 PB1, PA, NP and WT or mutated PB2 as well as pCAGGS Renilla and a positive sense firefly luciferase viral-like reporter plasmid. Luciferase production was measured 12 hours post transfection. Values were normalised to Renilla expression and to the activity of the WT polymerase. Results are expressed as the mean ± standard deviation of triplicate samples. The statistical significance of differences in polymerase activity compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P < 0.05; **P < 0.01; ***P < 0.001).

As viral replication feeds back on itself it is difficult to distinguish between the two steps of viral replication. However, the level of enhancement observed with the PB2 adaptive mutations in the presence of the positive sense viral-like reporter was much less than with the negative sense reporter (Figure 24 & Figure 35). The E627K mutation enhanced polymerase activity 5 fold with the positive sense reporter which can be contrasted with the 176 fold enhancement observed with the negative sense reporter. These results appear to
suggest that the WT avian polymerase is restricted at generating a functional cRNP and that the adaptive PB2 mutations support this particular step of replication.

2.3 Discussion

Almost all of the introduced PB2 mutations significantly enhanced 50-92 polymerase activity in human and swine cells, whereas only a subset of the PB2 mutations significantly enhanced polymerase activity in avian cells. The observation that some PB2 mutations enhance polymerase activity in a mammalian specific manner whereas others affect activity in human, swine and avian cells suggests there are different mechanisms of enhancing activity. This concept is supported by studies in which certain cumulative PB2 mutations enhance polymerase activity in an additive manner (Bussey et al., 2010; Foeglein et al., 2011; Li et al., 2009).

Furthermore, the work of Bussey et al. suggests that host adaptive mutations in PA can facilitate different mechanisms of enhancing activity. They showed that although singly introducing humanising mutations into positions 85, 186 and 336 of an avian PA significantly increased avian polymerase activity in human cells, singly introducing avian signature amino acids into pH1N1 PA at these positions only slightly decreased the activity of a human polymerase in human cells (Bussey et al., 2011). Indeed a proteomic analysis of host proteins interacting with polymerase subunits identified more than 300 human proteins that bound to PA alone (Bradel-Tretheway et al., 2011) suggesting that this viral protein intimately associates with the host cell.
Only Mehle et al. have investigated the effects of PA mutations on polymerase activity in both human and avian cells. Mehle identified several PA mutations which significantly enhanced polymerase activity in human cells (P400L, M423I, V476A, T552S, V630E), whereas in avian cells the activity of all the mutants was similar to WT (Mehle et al., 2012).

When avian viruses infect mammals certain PB2 mutations, such as 627K, are commonly selected for, whereas other PB2 mutations are identified much less frequently. For instance E158G, which was selected upon the serial passage of an avian virus in mice, only appears in a single human isolate (A/Auckland/1/2009) in a sequence database search (Zhou et al., 2011). In addition PB2 535T mutation is not seen in viral strains circulating in nature.

This absence of PB2 E158G and M535T substitutions in natural isolates is intriguing; especially as in a cell-based assay these mutations enhance polymerase activity to a greater extent than mutations such as T271A and D701N which have frequently been observed in viral isolates (Bussey et al., 2010; Chen, 2006; Finkelstein et al., 2007; Li et al., 2005a; Miotto et al., 2008; Qi et al., 2012). This leads us to question why these mutations are not selected for more frequently in nature. It will be necessary to address whether the polymerase assay truly reflects viral replication and to consider what selective pressures drive host adaptation. There may be a cost associated with these mutations in the context of whole virus.

An idea popular in the literature is that the enhanced polymerase activity observed with PB2 E627K is due to an increased biochemical stability of the vRNP in human cells (Labadie et al., 2007; Mehle and Doudna, 2008; Ng et al., 2012; Rameix-Welti et al., 2009). We wished to
investigate if this mechanism also explains how other naturally or experimentally occurring PB2 mutations adapt an avian virus polymerase for mammalian replication.

However, our findings suggest that previous interpretations of this assay by other groups working in this field were preliminary because they did not take into account the possibility of an increase in replication resulting in an increase in numbers of vRNP complexes (Labadie et al., 2007; Mehle and Doudna, 2008; Ng et al., 2012; Rameix-Welti et al., 2009). Unlike Labadie, we did not find a primary defect in the interactions between PB2 with 627E and NP when expressed alone, out of context of the rest of the vRNP components. Similarly Mehle and Doudna (Mehle and Doudna, 2008) also did not find a difference in the direct PB2-NP interaction with PB2 627 E or K. Different strains of virus were used as source of polymerase in these three studies, as well as different protein tags and different orders and conditions for precipitation and detection of interacting partners, and these variables may explain the discrepant results. Although Ng et al. (2012) like and co-workers Labadie, found a difference in PB2-NP interaction dependent of residue 627, their work utilized fragments of PB2 and was not performed in the context of a host cell so may not be directly comparable with these other studies. Thus, we suggest the restricted activity of an avian polymerase in human cells is not explained by the instability of the PB2-NP complex.

In support, Fodor and co-workers recently showed restriction of polymerase bearing PB2 627E in human cells occurred independently of NP. A significant difference in the activities of polymerases bearing PB2 627E or 627K was observed in the presence of a short template which can be copied in the absence of NP (Paterson et al., 2013).
It was shown by qRT-PCR that regardless of whether the mutation was host specific or not, an increase in polymerase activity correlated with proportionally enhanced synthesis of all 3 viral RNA species in human, avian and swine cells. This confirms that the mutations are working by at least increasing the level of replication, rather than just by increasing transcription of mRNA from a constant amount of vRNA template. Furthermore, the results with the positive sense viral-like reporter, appear to suggest that the WT avian polymerase is restricted at generating a functional cRNP and that the adaptive PB2 mutations support this particular step of replication.

This is supported by the 2012 publication from Manz et al. They showed that avian influenza polymerases, lacking the human signature PB2-E627K, were incapable of generating usable cRNP complexes in human cells. Both avian and human-like polymerases synthesized cRNA to equivalent extents, but the cRNP complexes produced by the avian influenza polymerase were unable to support vRNA synthesis.

Although influenza virus polymerase activity has been studied in human and avian cells for many years by use of a minigenome assay, this approach was not possible in pig cells until 2012 when Moncorgé et al. cloned the swine RNA polymerase I promoter and developed the first minigenome assay for pig cells (Moncorge et al., 2012). Swine influenza lineages often originate from avian or human influenza viruses (reviewed in (Brown, 2008; Van Reeth, 2007)), implying that pigs are susceptible to infection with both types of influenza viruses. As a consequence, pigs have been 'accused' of acting as intermediate hosts for the mammalian adaptation of avian influenza viruses or the generation of new reassortants between avian and human influenza strains that can cause pandemics (Brown, 2000;
Castrucci et al., 1993; Ito et al., 1998; Kida et al., 1994; Kida et al., 1988; Ma et al., 2008; Scholtissek et al., 1985; Webster et al., 1971; Yasuda et al., 1991). Thus, it is important to investigate the consequences of mammalian host range determinants in pig cells.

In this study it was observed that PB2 mutations which enhance avian-origin influenza polymerase function in human cells also increase activity in pig cells. PB2 mutations selected in nature when avian-origin viruses have crossed into swine, such as D701N, G590S & Q591R, and T271A (Liu et al., 2012; Mehle and Doudna, 2009; Moncorge et al., 2012), increased influenza polymerase function in pig cells. The G590S & Q591R and T271A mutations naturally appeared in swine in the generation of the TRIG cassette and the enhancement of avian polymerase activity in pig cells is in line with their role in replication and virulence of influenza viruses in mice (Bussey et al., 2010; Liu et al., 2012). Indeed, the results of the polymerase assays suggest that from a polymerase point of view, pigs do not appear to be a more appropriate host for avian-origin influenza viruses than humans.

Intriguingly, PB2 E627K which enhances avian influenza polymerase activity in pig cells is rarely detected in porcine influenza viruses derived directly from an avian source (Moncorge et al., 2012). The presence of other adaptive mutations may determine whether certain host adaptive mutations are selected for.
3. Investigating effects of adaptive PB2 mutations on avian virus growth

3.1 Introduction

The introduction of many adaptive PB2 mutations enhanced avian influenza polymerase activity in a cell based assay in Chapter 2. However, only some of these mutations have been detected in viruses that are found in nature. In this chapter we wished to explore whether the polymerase assay truly predicts viral growth and to investigate viral selection pressures that might favour some adaptive mutations over others. In order to achieve this we began to consider viral factors that can affect influenza virus host range that were not present in the in situ polymerase reconstitution assays employed in Chapter 2.

NEP can regulate the synthesis of vRNA, cRNA and mRNA and such regulation is independent of its nuclear export function (Bullido et al., 2001; Gorai et al., 2012; Manz et al., 2012; Robb et al., 2009). Speculation that NEP acts as co-factor to regulate viral replication has been heightened since the work of Manz showed that adaptive mutations in NEP can overcome avian polymerase restriction in human cells and that NEP interacts with the polymerase proteins PB1 and PB2 (Manz et al., 2012)

Many functions have been ascribed to the primary protein encoded by influenza segment 8, NS1. Crucially, it plays an important role in the suppression of host innate immune responses (Hale et al., 2008). However, it has also been implicated in the regulation of viral RNA synthesis (Falcon et al., 2004; Wang et al., 2010; Wolstenholme et al., 1980). Indeed, NS1 has been shown to co-immunoprecipitate with vRNP complexes from virus infected
cells, as well as to interact specifically with the NP protein in vRNP complexes (Marion et al., 1997; Robb et al., 2011). We wished to explore whether the addition of NEP and NS1 alters the activities of the PB2 mutant polymerases.

A further aim of this chapter was to explore the effects of the PB2 mutations in the context of the whole virus. To achieve this we used reverse genetics to create a series of viral variants carrying mutations in the PB2 gene. We carried out virological assays as well as measures of transcription and replication in the context of replicating virus in order to further characterise these mutations.

3.2 Results

3.2.1 NEP and NS1 affect activity of 50-92 PB2 mutants differently

Recently the influenza proteins NEP and NS1 have been implicated in playing a role in viral transcription and replication. Therefore we decided to introduce expression plasmids encoding these proteins into the minireplicon assay system to determine how they affect polymerase activities of PB2 mutants.

Initially it was necessary to introduce the 50-92 NEP gene into a pCAGGS expression plasmid. The avian 50-92 NEP gene was amplified from the Pol I plasmid containing A/Turkey/England/50-92/91 segment 8 cDNA with primers that introduced a 5' NotI site and a 3' MluI site. The amplified sequence was ligated into pCAGGS 50-92 PB2 which had been previously digested with NotI and MluI to remove the 50-92 PB2 coding sequence.
Next an increasing concentration of NEP, NS1 or both plasmids were titrated into human 293T cells co-transfected with a viral-like firefly reporter plasmid and the plasmids required to reconstitute the 50-92 polymerase. The addition of NEP considerably enhanced the activity of the 50-92 polymerase (Figure 36A). In contrast, the introduction of NS1 slightly decreased polymerase activity (Figure 36B). The addition of both NEP and NS1 significantly increased polymerase activity, presumably due to the enhancing properties of NEP (Figure 36C).
Figure 36. NEP and NS1 modulate 50-92 polymerase activity in human cells. 293T cells were transfected with pCAGGS 50-92 PB1, PA, PB2 and NP as well as a negative sense firefly luciferase viral-like reporter plasmid. In addition, cells were transfected with an increasing concentration of pCAGGS NEP (A), NS1 (B) NEP and NS1 (C) or an empty pCAGGS control. Luciferase production was measured 12 hours post transfection. Results are expressed as the mean ± standard deviation of triplicate samples. The statistical significance of differences in polymerase activity compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P <0.05; **P <0.01; ***P <0.001). The amount of NEP titrated in ranged from 10 ng to 200ng and the amount of NS1 titrated in ranged from 10 ng to 100 ng.
To investigate the effects of NEP and NS1 on the activity of 50-92 polymerases containing adaptive PB2 mutations, minireplicon assays were undertaken in the presence of these viral proteins. The amount of NEP which enhanced 50-92 polymerase activity most significantly was used (20 ng) and for consistency this amount of NS1 was also used.

Addition of NEP enhanced the activity of certain PB2 mutants, whereas it reduced the activity of others (Figure 37A). The presence of NEP increased minireplicon expressed of polymerases containing the adaptive mutations T271A, D701N, G590S Q591R and M535T. However, NEP decreased transcription/replication of polymerases containing E158G and E627K (Figure 37A). This suggests that there is a balance between the initial level of polymerase activity and the regulation of NEP.

Addition of NS1 reduced the activity of all PB2 mutants, except the polymerase containing E627K for which addition NS1 unexpectedly enhanced activity (Figure 37B). This enhancement was observed with a range of NS1 concentrations (data not shown). Addition of both NEP and NS1 enhanced the activity of all polymerases containing host-adaptive PB2 mutations except those containing M535T and E158G (Figure 37C). Intriguingly, these are the two PB2 mutations that are typically not observed in natural isolates.

Overall, these results underscore the complex nature of influenza polymerase host adaptation and highlight the important role viral proteins not typically included in minireplicon assays can have on polymerase activity.
Figure 37. NEP and NS1 affect activity of 50-92 PB2 mutants differently in human cells. 293T cells were transfected with pCAGGS 50-92 PB1, PA, PB2 (WT or mutated) and NP as well as a negative sense firefly luciferase viral-like reporter plasmid. In addition cells were transfected with 20 ng pCAGGS NEP (A), NS1 (B) NEP and NS1 (C) or an empty pCAGGS control. Luciferase production was measured 12 hours post transfection. Results are expressed as the mean ± standard deviation of triplicate samples. The statistical significance of differences in polymerase activity compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P <0.05; **P <0.01; ***P <0.001).
3.2.2 Rescuing recombinant 50-92 viruses containing adaptive PB2 mutations

In order to take into account further factors which could affect host range such as host genes and host innate immune responses, a panel of adaptive PB2 mutations were introduced into the 50-92 virus using reverse genetics. Initially site directed mutageneisis was performed on the 50-92 PB2 Pol I rescue plasmid to singly introduce the mutations E158G, T271A, G590S & Q591R, E627K and D701N. Viruses were then generated using the 12 plasmid rescue system. Viruses contained the NP, PB1, PB2, PA and NS of A/Turkey/England/50-92/91 (50-92) and the HA, NA and M of A/PR/8/34 (PR8). PR8 is a well characterised vaccine strain and generating recombinant viruses with this genetic constellation rather than as whole HPAI viruses allows experimentation in containment level 3 rather than SAPO 4 laboratories. This is based on risk assessment that postulates that viruses with PR8 HA, NA and M are unlikely to infect humans since the wild type PR8 virus itself was non-virulent in human challenge studies. On the other hand, PR8 NA and HA and M facilitate efficient viral entry into cultured human cells such as the A549 lung cell line and also into mice. All viruses were rescued on the first attempt during co-culture of transfected 293T cells with MDCK cells, except the PB2 E158G containing virus which was rescued on the third attempt.

3.2.3 Investigating growth of recombinant 50-92 viruses with adaptive PB2 mutations

Initially the viruses were titrated on MDCKs in a standard agar plaque assay. Attenuated viruses often form small plaques. The results from analysing plaque size suggested that polymerase activity did not always correlate with viral growth. The E158G mutation
dramatically enhances polymerase activity in the minireplicon assay (Chapter 2 Figure 24). However, the virus containing this mutation formed smaller plaques than the WT virus (Figure 38). In contrast, an enlargement in plaque size was observed for all other PB2 mutant viruses compared to WT, although the size of the plaques formed by the 50-92 PB2 M535T virus were rather variable. The largest plaques were formed by the 50-92 PB2 D701N virus.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Average (mm)</th>
<th>Range (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50-92 WT</td>
<td>2.2</td>
<td>1.7 - 3.0</td>
</tr>
<tr>
<td>50-92 PB2 E158G</td>
<td>0.5</td>
<td>0.2 - 1.3</td>
</tr>
<tr>
<td>50-92 PB2 T271A</td>
<td>1.6</td>
<td>0.8 – 3</td>
</tr>
<tr>
<td>50-92 PB2 M535T</td>
<td>1.3</td>
<td>0.2 - 1.3</td>
</tr>
<tr>
<td>50-92 Q590S &amp; R591T</td>
<td>2.0</td>
<td>1.3 - 2.5</td>
</tr>
<tr>
<td>50-92 E627K</td>
<td>1.9</td>
<td>1.3 – 3.0</td>
</tr>
<tr>
<td>50-92 D701N</td>
<td>2.8</td>
<td>1.3 - 3.4</td>
</tr>
</tbody>
</table>

Figure 38. Plaque assay of recombinant 50-92 viruses with adaptive PB2 mutations. 20 plaques were measured per mutant virus.

To test the effects of the PB2 mutations on viral growth, a multi-cycle growth curve was performed on human A549 cells infected at an MOI of 0.01. The supernatant was titrated on MDCKs in a standard agar plaque assay to determine viral titres (Figure 39). These results
again suggested an enhancement in polymerase activity measured in a minireplicon assay does not always with viral growth. Introducing the M535T mutation into PB2 significantly enhanced avian polymerase activity (44 fold) in a minireplicon (Chapter 2 Figure 24). However, the growth rate of the 50-92 virus containing this mutation was slower than WT (**P <0.01 at 72 hours post infection).

Furthermore the mutations which enhanced polymerase activity most significantly in a minireplicon assay did not enhance viral growth most significantly. Introducing E158G into PB2 significantly enhanced avian polymerase activity (156 fold) in a minireplicon (Chapter 2 Figure 24). However, the virus containing this mutation only reached a titre significantly greater than WT at 24 hours post infection (Figure 39). This can be contrasted with the adaptive mutation D701N. Introducing D701N into PB2 enhanced avian polymerase activity only 32 fold in a minireplicon assay (Chapter 2 Figure 24); however the virus containing this mutation grew to the highest maximum viral titre (Figure 39). The maximal viral titre for 50-92 PB2 E158G was 4.3x10^5 PFU/mL, whereas for 50-92 PB2 D701N it was 2.0 x10^7 PFU/mL.
**PB2 mutation** | 24 HPI | 48 HPI | 72 HPI | Maximum viral titre PFU/ml |
--- | --- | --- | --- | --- |
WT |  |  |  | 2.17 $\times 10^5$ |
E158G | *** | ns | ns | 4.3 $\times 10^5$ |
T271A | ns | * | ** | 5.5 $\times 10^6$ |
M535T | ** | ns | ** | 5.8 $\times 10^4$ |
G590S Q591R | ** | ns | *** | 9.7 $\times 10^6$ |
E627K | *** | ** | * | 8.3 $\times 10^6$ |
D701N | ** | ns | ** | 2.0 $\times 10^7$ |

**Figure 39.** Multi-cycle growth of recombinant 50-92 viruses with adaptive PB2 mutations. A549 cells were infected with the mutant PB2 viruses at an MOI of 0.01. The viral titres released into supernatants at 24 hour time points were assessed by titration in MDCK cells by plaque assay. Results are expressed as the mean ± standard deviation of triplicate samples. The statistical significance of differences in viral titre compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*$P<0.05$; **$P<0.01$; ***$P<0.001$) HPI = hours post inoculation.
3.2.4 Investigating the role of interferon in controlling the growth of recombinant 50-92 viruses with adaptive PB2 mutations

The 50-92 PB2 E158G virus and to a degree the 50-92 PB2 M535T virus formed small ‘fuzzy’ plaques on MDCKs. Such morphology is characteristic of viruses that induce high levels of interferon (IFN) or are unable to control the IFN (Hayman et al., 2006; Mazur et al., 2008; Talon et al., 2000). There are three distinct classes of IFN, which include type I IFN (mainly α/β), type II IFN (γ) and type III IFN (λ). Type II IFN is involved in establishing the adaptive immune response. Type III IFN has been implicated in controlling influenza virus infection, however, the type I IFN response is the most important for restricting influenza virus replication (Garcia-Sastre et al., 1998a; Garcia-Sastre et al., 1998b; Szretter et al., 2009). The expression of type I IFN in host cells upregulates several hundred genes capable of efficiently suppressing viral replication and spread (Hale et al., 2010a).

Fodor et al. and Iwai et al. have suggested that the PB2 protein plays a role in direct control of interferon induction (Graef et al., 2010; Iwai et al., 2010). Fodor’s group have shown that some PB2 proteins can interact with MAVS, and this relies on sequences in the N terminus of the protein that contains a mitochondrial targeting signal (MTS). Carr et al. had previously shown that the 50-92 PB2 protein was not efficiently targeted to mitochondria of human cells in contrast to the PB2 proteins derived from human adapted strains with intact MTS (Carr et al., 2006). However it remained a possibility that mutations in other regions of the 50-92 PB2 protein affected mitochondrial location and impacted on the ability of PB2 to control interferon induction. We hypothesized that the ability of the 50-92 PB2 E158G and M535T proteins to control the IFN response might be compromised. The ability of
exogenously expressed PB2 proteins to block expression of luciferase driven from the IFNβ promoter induced by expression of MAVS in human 293T cells was compared. As a positive control the inhibition of the same stimulus by the 50-92 NS1 protein was monitored as was inhibition in the presence of a PB2 protein derived from a representative human H3N2 circulating strain isolated in Australia in 1975 (A/Victoria/3/75) (Victoria) (Figure 40). There was no specific reduction of PB2 E158G or M535T to inhibit Luciferase expression, but the general inhibition compared to NS1 was minimal.

![Luciferase graph]

**Figure 40. Investigating the ability of mutant 50-92 PB2 proteins to control the IFN response.** IFNβ induction by pCAGGS MAVS was measured in the presence of pCAGGS 50-92 PB2, pCAGGS Victoria
PB2 or pCAGGS 50-92 NS1 24 hours post transfection. pCAGGS Renilla was used as a transfection control. Results are expressed as the mean ± standard deviation of triplicate samples. The statistical significance of differences in luciferase expression in the presence of NS1 or PB2 compared to in the presence of empty pCAGGS were assessed by a two-tailed, unpaired Student’s t-test (*P <0.05; **P <0.01; ***P <0.001).

An alternative mechanism for the fuzzy plaque phenotype of the E158G mutant virus was that it drove a higher interferon induction that was not adequately controlled by its interferon antagonist viral proteins. We wished to compare levels of type I IFN induced by the panel of viruses. A549 cells were infected with the viruses at an MOI of 3. At 8 hours post infection the levels of IFN mRNA, as well as viral mRNA, cRNA and vRNA were measured by reverse transcription and real-time quantitative PCR. The 50-92 PB2 E158G and M535T viruses induced the most IFNβ mRNA expression (Figure 41A). Interestingly, these were the two viruses that reached lower maximal titres than all other mutant viruses in a multi-cycle growth curve (Figure 39).

Cytoplasmic viral RNA species that contain triphosphate groups at their 5’ ends have been identified as the major PAMP for influenza (Hale et al., 2008). However, these results suggest that the level of M gene vRNA synthesised by the PB2 E158G and M535T viruses does not explain the high levels of IFNβ observed. For instance, there was no significant difference between the vRNA synthesised by the PB2 E158G and E627K viruses, however the PB2 E158G induced significantly more IFNβ (P <0.001) (Figure 41). Furthermore, the PB2 E627K viruses synthesised significantly more vRNA than the PB2 M535T virus (P <0.001), however the M535T virus induced significantly more IFNβ (P <0.01). These results suggest
that the increase in IFN induction did not correlate with an enhancement in viral titre or in vRNA accumulation.

Figure 41. Synthesis of IFNβ mRNA induced by recombinant 50-92 viruses with adaptive PB2 mutations. (A) A549 cells were infected with recombinant 50-92 viruses with adaptive PB2 mutations at an MOI of 3. 8 hours post infection the level of IFN β mRNA synthesised in the presence of different PB2 mutant viruses was determined and normalised to that synthesised by the WT virus. (B) The levels of viral m gene mRNA, cRNA, and vRNA synthesised by different PB2 mutant viruses
were determined and normalised to that of the WT virus. Results shown are the mean ± standard deviations from triplicate samples. The statistical significance of differences in RNA synthesis compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

In order to investigate the level of IFN expression at the protein level, an A549 cell line containing a stably integrated firefly luciferase under the control of the IFNβ promoter (A549 IFN-Luc) (Hayman et al., 2006) was infected with the 50-92 viruses at an MOI of 3. Luciferase production was measured 24 hours post infection. The 50-92 PB2 E158G and M535T viruses induced a higher IFN signal than mock at 24 hours post infection.

![Figure 42. IFNβ expression induced by recombinant 50-92 viruses with adaptive PB2 mutations.](image)

Figure 42. IFNβ expression induced by recombinant 50-92 viruses with adaptive PB2 mutations. An A549 IFN-Luc reporter cell line was infected with the 50-92 viruses at an MOI of 3, or with serum-free DMEM for mock. Luciferase activity was measured 24 hours post infection. Values were normalised to mock. Results are expressed as the mean ± standard deviation of triplicate samples.
Next it was investigated whether the IFN induced by the 50-92 PB2 E158G and M535T viruses is sufficient to inhibit viral growth. Viral growth was compared in an A549-NPro cell line at an MOI of 0.01. This cell line constitutively expresses bovine viral diarrhea virus (BVDV) NPro which completely abolishes IFN-β induction through destabilising IRF-3 (Hilton et al., 2006).

In the absence of an anti-viral response; the 50-92 PB2 E158G and M535T viruses were able to replicate more efficiently and grew to higher maximal titres than in the A549 cells (Figures 43 and 39). However, the 50-92 WT and E627K viruses also grew to higher titres in A549-NPros (Figure 43) compared to in A549s (Figure 39). This can be explained by a study from Young et al. They observed that WT mumps viruses formed significantly larger plaques on IFN-nonresponsive cells; implying that even the WT virus cannot completely evade the IFN response (Young et al., 2009). Previously, it was shown that a range of viruses form bigger plaques and grow to higher titres in IFN-nonresponsive cells (Young et al., 2003). This supports the concept that viruses typically face a race between virus growth and the induction of an antiviral state. In addition it suggests that the IFN response acts as a constant selection pressure to ensure viruses are maximally fit in their replicative ability, whilst maintaining mechanisms to circumvent the IFN response. Overall, these observations suggest the degree and timing of viral IFN induction is of considerable importance in terms of pathogenesis and host range.

Thus, it is difficult to conclude whether the interferon induced by the viruses restricts their growth. However, as the 50-92 PB2 M535T virus grows better than the WT virus in A549-
NPors, but worse in A549s, this could suggest that the growth of this virus can be restricted by the IFN it induces.

<table>
<thead>
<tr>
<th>PB2 mutation</th>
<th>24 HPI</th>
<th>48 HPI</th>
<th>72 HPI</th>
<th>Maximum viral titre PFU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td>4.8 x10^6</td>
</tr>
<tr>
<td>E158G</td>
<td>Ns</td>
<td>*</td>
<td>ns</td>
<td>4.0 x10^6</td>
</tr>
<tr>
<td>M535T</td>
<td>**</td>
<td>*</td>
<td>ns</td>
<td>3.3 x10^6</td>
</tr>
<tr>
<td>E627K</td>
<td>***</td>
<td>**</td>
<td>ns</td>
<td>1.6 x10^7</td>
</tr>
</tbody>
</table>

**Figure 43. Growth of 50-92 PB2 E158G, M535T and E627K viruses in A549-NPors.** Cells were infected with the 50-92 viruses at an MOI of 0.01. The virus released into supernatant at 24 hour time points was assessed by titration in MDCK cells. Results are expressed as the mean ± standard deviation of triplicate samples.

**3.2.5 Detecting defective interfering (DI) RNAs**
The exact nature of the influenza viral PAMPs that induce expression of type I IFN is not completely clear. IFNβ expression in human cells is largely dependent on RIG-I and in a study by Rehwinkel et al. it was shown that all of the eight influenza genomic vRNA segments interact with RIG-I. However, it was also shown that the different influenza segments associate with RIG-I to different extents and it that short RNAs preferentially interact with RIG-I (Figure 44) (Rehwinkel et al., 2010). Defective interfering (DI) RNAs were found to specifically and preferentially associate with RIG-I in the context of an influenza infection. DI RNAs are generated through mistakes in virus replication; they have sequences of the parental vRNA deleted but retain the 5' and 3' end-specific sequences of the progenitor vRNA that contain the cis-acting signal directing them to be amplified and packaged into virions (Davis et al., 1980; Von Magnus, 1954).
Figure 44. Differential recognition of viral RNA by RIG-I. Viral RNAs produced during an influenza infection of infection are shown. These RNAs include vRNA, cRNA, mRNAs, defective interfering RNA (DI RNAs) and small viral RNAs (svRNA). The RNAs which interact with RIG-I during infection are shown in red, with a darker colour and thicker line representing a stronger association (Baum and Garcia-Sastre, 2011).

We compared the amount of DI RNAs produced by the different 50-92 viruses upon infection in A549s cells. 8 hours post infection RNA was extracted and PCR performed with primers specific to the 5' and 3' ends of the PB2 gene. A variety of short PCR products were produced by each of the 50-92 viruses (Figure 45). However, the 50-92 PB2 M535T virus
produced considerably more of these short products than any other virus. This virus grew to the lowest titre in A549s (Figure 4) and yet induced large amounts of IFN (Figure 41). These findings suggest that mutant viruses which induce high amounts of IFN do so partially due to the generation of aberrant PAMPs.

![Figure 45. Defective interfering RNAs produced by recombinant 50-92 viruses with adaptive PB2 mutations. A549 cells were infected with recombinant 50-92 viruses with adaptive PB2 mutations at an MOI of 3. 8 hours post infection the amount of DI RNAs made by the different viruses was compared using with primers specific to the 5' and 3' ends of the PB2 gene. The expected size of PB2 is 2.3 kb.]

3.3 Discussion

The presence of NEP increased the activity of polymerases containing the adaptive mutations T271A, D701N, G590S Q591R and M535T. However, NEP decreased the activity of
polymerases containing E158G and E627K (Figure 37A). This suggests that there is a balance between the initial level of polymerase activity and the regulation of NEP.

Addition of both NEP and NS1 enhanced the activity of all polymerases containing host-adaptive PB2 mutations except those containing M535T and E158G (Figure 37C). Intriguingly, these are the two PB2 mutations that are typically not observed in natural isolates. However, even in the presence of NEP and NS1 these mutant polymerases were still more active than the WT polymerase in human cells. This implies that minireplicon assay results are an oversimplification of influenza polymerase activity that there must be a further explanation for why these mutations are not observed in natural isolates.

In order to investigate this we singly introduced a panel of PB2 mutations into the 50-92 PB2 gene and generated recombinant viruses using a reverse genetics approach. We compared the growth rates of the mutant viruses in human A549 cells. Interestingly, we found that an enhancement in polymerase activity in a minireplicon assay does not always correlate with an enhancement in viral growth. For instance, introducing the M535T mutation into PB2 significantly enhanced avian polymerase activity in a minireplicon, however, the growth rate of the 50-92 virus containing this mutation was slower than WT. In addition, the PB2 E158G mutation dramatically enhanced avian polymerase activity in a minireplicon, however, the growth rate of the 50-92 virus containing this mutation was only slightly greater than WT.

Within the literature there is some support for the observation that an enhancement in polymerase activity measured in a minireplicon assay does not always correlate with an enhancement in viral growth. For instance, Bussey et al. identified a mutation in PA (L336M)

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which increased the activity of an avian polymerase in mammalian cells in a minireplicon assay but did not increase growth in A549s cells. However, many published studies are investigating mutations which widely circulate in nature and thus these adaptive mutations are most likely selected for because they do not have deleterious side effects.

Although the 50-92 PB2 E158G and M535T viruses reached lower maximal titres than all other mutant viruses in a multi-cycle growth curve, these viruses induced the most IFNβ expression. This IFN could have inhibited viral growth since replication was rescued in cells lacking an interferon response. Cytoplasmic viral RNA species that contain triphosphate groups at their 5′ ends have been identified as the major PAMP for influenza (Hale et al., 2008). Although the level of M gene vRNA synthesised by the 50-92 PB2 E158G and M535T viruses did not explain the enhancement in IFN expression, an increase in small RT-PCR products containing PB2 gene segments termini, presumed to be the DI RNAs, was observed for the 50-92 PB2 M535T virus. Although this mutation has the ability to confer a replicative advantage in a minireplicon assay, a consequence of this mutation is a disproportionate upregulation of IFN which could ultimately hinder viral growth.

In the minireplicon assay the luciferase gene replicated is approximately 1.5kb. However, the PB2 gene segment is much larger at 2.5 kb and many DI RNAs were produced by the PB2 M535T containing viral polymerase replicating this segment. Thus would be interesting to investigate the ability of the polymerases to replicate and transcribe a larger reporter gene. As yet the reason for the higher level of IFN induced by the 50-92 PB2 E158G containing mutation remains unclear since this virus did not appear to direct synthesis of PB2 derived
small RNAs. DI RNAs can also be derived from other gene segments and this could be probed using different set of PCR primers (Duhaut and McCauley, 1996).

The generation of DI RNAs by influenza viruses is suspected of competing with the replication of the wild-type virus (Compans et al., 1972; Honda et al., 1998; Lee et al., 2002). DI RNAs produced by influenza virus have been identified in patients by deep sequencing primary specimens collected from human nasopharyngeal specimens (Saira et al., 2013). Defective RNAs have also been observed in experimentally infected embryonated chicken eggs (Jennings et al., 1983). Tapia et al. infected mice with strains of influenza virus either lacking DI RNAs or with a high DI RNA content. Mice infected with the high DI RNA virus initially lost weight more rapidly than those infected the WT virus and this correlated with an increase in IFN β expression in the lungs but not an increase in viral titre. These mice then went on to recover weight more rapidly than those infected with the WT virus (Tapia et al., 2013).

Interestingly, a recent study investigated the dynamics of IFN production and response within foci of infection. Several RNA viruses including influenza A were investigated (Chen et al., 2010). Surprisingly, it was found that only a limited number of cells within infected foci were responsible for the production of IFN. Whereas, in contrast many cells, including those yet to be infected, upregulated IFN stimulated genes (ISGs). Thus although IFN is only secreted by a small number of cells, it is considered critical in upregulating an anti-response in the surrounding uninfected cells, thereby slowing the spread of the infection. Mechanistically, the secreted IFN binds to receptors on the surface of nearby cells, thereby initiating a signalling pathway culminating in the upregulation of many ISGs and the
expression of anti-viral effectors (Randall and Goodbourn, 2008). It would be interesting to visualise the anti-viral response of the recombinant 50-92 PB2 mutants by monitoring the upregulation of ISGs and to determine whether the viruses which induce more IFN do so by upregulation of ISGs in only a limited number of cells.
4. Role of PB2 residue 158 in host range and pathogenicity of 2009 H1N1 pandemic influenza virus

4.1 Introduction

Mice are often used as a mammalian model for studying influenza virus host range and pathogenicity even though they are not naturally infected with the virus. They can however succumb to infection when infected intranasally and exhibit weight loss, which is used as a measure of illness (Lu et al., 1999; Maines et al., 2009; Ward, 1997). Influenza viruses are often serially passaged in mice to investigate the molecular determinants of host range and pathogenicity in mammals. The genetic basis for increased virus growth and virulence can be assessed.

Ilyushina et al. reported the first mouse adaptation experiments on pandemic 2009 H1N1 isolates. They investigated the adaptation of two pandemic isolates, A/CA/04/09 and A/TN/1-560/09 and identified numerous mutations within HA and the polymerase proteins and NP which contributed to their adaptation and enhanced virulence in mice (Ilyushina et al., 2010).

In the study two mouse-adapted variants for both A/CA/04/09 and A/TN/1-560/09 were derived in parallel. The amino acid at PB2 residue 158 was substituted in one out of two of the viruses for both strains. In one mouse adapted A/CA/04/09 strain the E158G substitution had been selected and in A/TN/1-560/09 this position was mutated to 158A.
In a completely different study, serial passage generated a 2009 pH1N1 virus (A/New York/1682/2009) that was highly pathogenic for mice. In this approach PB2 E158G was identified for its ability to increase the morbidity and mortality of the pandemic strain in mice (Zhou et al., 2011).

In Chapter 3 it was observed that the PB2 E158G mutation significantly enhanced avian (50-92) polymerase activity compared to WT in a minireplicon assay in human and avian cells (***P <0.001). However, in Chapter 3 it was observed that the recombinant 50-92 virus containing this mutation induced high levels of IFN, and did not grow to high titres in interferon competent cells. This could explain why this mutation has only been identified in a single human influenza isolate and four avian influenza virus isolates in nature (Zhou et al., 2011).

We aimed to characterise the E158G mutation in the 2009 pH1N1 strain A/England/195/09 (E195) available in our laboratory. This virus was generated by reverse genetics from a representative pandemic influenza A H1N1 isolate. We wished to determine if this virus displayed similar characteristics to the 50-92 PB2 E158G virus and to further explore the role of this mutation in viral host range and pathogenicity
4.2 Results

4.2.1 PB2 E158G enhances E195 polymerase activity in a minireplicon assay

A/England/195/09 (E195) WT and E195 PB2 E158G viruses had been rescued previously in the laboratory by R. Elderfield. In addition pCAGGS expression plasmids encoding E195 NP, PB1, PB2 and PA had also been generated previously. However, to investigate the effect of PB2 E158G on polymerase activity in a minireplicon assay it was necessary to introduce PB2 E158G into a pCAGGS expression plasmid. The E195 PB2 coding sequence was amplified from the Pol I plasmid containing A/England/195/09 segment 1 cDNA with primers that introduced a 5' NotI site and a 3' MluI site. The amplified sequences were ligated into pCAGGS 50-92 PB2 which had been previously digested with NotI and MluI to remove the 50-92 PB2 coding sequence.

Minireplicon assays were undertaken in human (293T) and avian (DF-1) cells with species-specific firefly luciferase viral-like reporters (Figure 46). The PB2 E158G mutation significantly enhanced E195 polymerase activity in human and avian cells, further suggesting that this is not a mammalian specific adaptation. Previously, this mutation was seen to significantly enhance 50-92 polymerase activity in human, swine and avian cells (Section 2.2.1).
Figure 46. E195 polymerase activity is enhanced by PB2 E158G. 293T and DF-1 cells were transfected with pCAGGS E195 PB1, PA, NP and PB2 (WT or E158G) as well as pCAGGS Renilla and a viral-like firefly luciferase minigenome expressing plasmid. Luciferase production was measured 12 hours post transfection. Values were normalised to Renilla expression and to the activity of the WT polymerase. Results are expressed as the mean ± standard deviation of triplicate samples. The statistical significance of differences in polymerase activity compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P<0.05; **P<0.01; ***P<0.001).

4.2.2 Effect of NEP and NS1 on activity of E195 WT and PB2 E158G polymerases

Recently the influenza proteins NEP and NS1 have been implicated in playing a role in viral transcription and replication. Therefore we decided to introduce expression plasmids encoding these proteins into the minireplicon assay system to determine how they affect the activities of the E195 WT and PB2 E158G polymerases.
Addition of NEP, NS1 or both together did not statistically significantly affect the activities of either the WT or mutant polymerases (Figure 47). Thus these results do not explain why the PB2 E158G mutation has only emerged in a single natural isolate.

**Figure 47.** Effect of NEP and NS1 on E195 WT and PB2 E158G polymerases. 293T cells were transfected with pCAGGS E195 PB1, PA, PB2 (WT or mutated) and NP as well as a negative sense firefly luciferase viral-like reporter plasmid. In addition cells were transfected with 20 ng E195 pCAGGS NEP (A), NS1 (B) NEP and NS1 (C) or an empty pCAGGS control. Luciferase production was measured 12 hours post transfection. Results are expressed as the mean ± standard deviation of triplicate samples. The statistical significance of differences in polymerase activity compared to WT were assessed by a two-tailed, unpaired Student’s t-test.

**4.2.3 PB2 E158G restricts E195 virus growth**

The E195 WT and PB2 E158G recombinant viruses were initially titrated on MDCKs in a standard agar plaque assay. A reduction in plaque size was observed for the E195 PB2
E158G virus (Figure 48). Although the E158G mutation significantly enhanced polymerase activity in the minireplicon assay, these results suggest this mutation does not enhance viral growth. Furthermore, the E195 PB2 E158G virus formed 'fuzzy', indistinct plaques and such morphology is characteristic of viruses that induce high levels of IFN. The viruses were rescued from DNA a second time and the same plaque morphology was observed.

We then compared the multicycle growth of the E195 WT and E195 PB2 E158G viruses in human A549 cells. Cells were infected at an MOI of 0.01 and supernatant was sampled at 24 time points post infection (Figure 49). The WT virus displayed a growth rate considerably greater than the PB2 E158G virus, with a statistically significant enhancement in viral titre at all 3 time points tested.
Figure 49. Multi-cycle growth of E195 WT and PB2 E158G viruses in A549s. Cells were infected with the E195 viruses at an MOI of 0.01. The virus released into supernatant at 24 hour time points was assessed by titration in MDCK cells. Results are expressed as the mean ± standard deviation of triplicate samples. The statistical significance of differences in viral titre compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P <0.05; **P <0.01)

4.2.4 Investigating the role of IFN in controlling the growth of E195 PB2 E158G

We compared the ability of the E195 WT and PB2 E158G proteins to control the IFN response as this could explain the different growth rates of the two viruses. The ability of exogenously expressed PB2 proteins to block expression of luciferase driven from the IFNβ promoter induced by expression of MAVS in human 293T cells was compared. As a control the inhibition of the same stimulus by the E195 NS1 protein was monitored as was inhibition in the presence of a PB2 protein derived from a representative human H3N2 circulating
strain isolated in Australia in 1975 (A/Victoria/3/75) (Victoria) (Figure 50). The E195 PB2 WT and E158G proteins could not inhibit the level of luciferase expression. The E195 NS1 protein does not exhibit CPSF30 binding and so is unable to restrict host gene expression therefore this protein did not inhibit luciferase expression (Hale et al., 2010b).
Figure 50. Investigating the ability of E195 PB2 WT and E158G proteins to control the IFN response.

IFNβ induction by pCAGGS MAVS was measured in the presence of pCAGGS E195 PB2, pCAGGS Victoria PB2 or pCAGGS E195 NS1 24 hours post transfection. pCAGGS Renilla was used as a transfection control. Results are expressed as the mean ± standard deviation of triplicate samples. The statistical significance of differences in luciferase expression in the presence of NS1 or PB2
compared to in the presence of empty pCAGGS were assessed by a two-tailed, unpaired Student’s t-test (*P <0.05; **P <0.01; ***P <0.001).

We then compared the levels of type I IFN induced by the 2 viruses. A549 cells were infected with both viruses at an MOI of 3 PFU/cell. At 6 and 24 hours post infection the levels of IFNβ mRNA, as well as viral mRNA, cRNA and vRNA were measured by reverse transcription and real-time quantitative PCR (Figure 51).

The E195 PB2 E158G virus induced significantly more luciferase expression than WT at 24 hours post infection. Cytoplasmic viral RNA species that contain triphosphate groups at their 5’ ends have been identified as the major PAMP for influenza (Hale et al., 2008). However, the E195 PB2 E158G virus synthesised significantly less vRNA than WT at 24 hours post infection and yet it induced significantly more IFN β. Thus, the increase in IFN β did not correlate with an enhancement in viral titre or in vRNA accumulation.

Interestingly however, the stoichiometry of RNA species synthesised by the PB2 E158G mutation was altered. A significant increase in cRNA synthesis and decrease in mRNA and vRNA synthesis was observed compared to WT. This altered stoichiometry may explain why the growth of the E195 PB2 E158G virus is restricted and why this mutation has not emerged in viral strains circulating in humans.
**Figure 51. Synthesis of IFNβ mRNA induced by E195 WT and PB2 E158G viruses.** A549 cells were infected with E195 WT and PB2 E158G viruses at an MOI of 3, or with serum-free DMEM for mock. 6 and 24 hours post infection the level of viral m gene mRNA (A), cRNA (B) and vRNA (C) synthesised by the E195 PB2 E158G was determined and normalised to that of the WT virus. In addition the level of IFNβ mRNA synthesis induced by the 2 viruses was determined and normalised to that in mock infected cells at the same time (D). Results shown are the mean ± standard deviations from triplicate samples. The statistical significance of differences in RNA synthesis compared to WT were assessed by a two-tailed, unpaired Student’s t-test (*P < 0.05).

In order to confirm the E195 PB2 E158G virus infection resulted in more IFN protein being produced than for WT virus, an A549 cell line containing a stably integrated firefly luciferase under the control of the IFNβ promoter (A549 IFN-Luc) (Hayman et al., 2006) was infected
with both viruses at an MOI of 3. Luciferase production was measured 12 and 24 hours post infection.

The E195 PB2 E158G virus induced significantly more luciferase expression than the WT virus at 24 hours post infection indicating that IFNβ levels would be also increased (Figure 52).

![Figure 52. IFNβ expression induced by E195 WT and PB2 E158G viruses.](image)

Next it was investigated whether the IFN induced by the E195 PB2 E158G virus is sufficient to inhibit viral growth. Viral growth was compared in an A549-NPro cell line at an MOI of...
This cell line constitutively expresses bovine viral diarrhea virus (BVDV) NPro which completely abolishes IFN-β induction through destabilising IRF-3 (Hilton et al., 2006).

In the absence of an anti-viral response; the E195 WT and PB2 E158G viruses were able to replicate more efficiently and grew to higher maximal titres than in the A549 cells (Figures 53 and 49). This suggests that the IFN response acts as a constant selection pressure to ensure viruses are maximally fit in their replicative ability, whilst maintaining mechanisms to circumvent the IFN response.

Growth of the E195 PB2 E158G virus remains restricted in the A549-NPro cell line in comparison to the WT virus. This suggests that the IFN produced may be a side effect of an unfit virus rather than the explanation for why growth of the virus is hindered.

**Figure 53. Growth of E195 WT and PB2 E158G viruses in A549-NPros.** Cells were infected with the E195 viruses at an MOI of 0.01. The virus released into supernatant at 24 hour time points was assessed by titration in MDCK cells. Results are expressed as the mean ± standard deviation of triplicate samples.
4.2.5 Detecting Defective interfering (DI) RNAs

Defective interfering (DI) RNAs were found to specifically and preferentially associate with RIG-I in the context of an influenza infection. DI RNAs are generated through mistakes in virus replication. These are considered an important influenza viral PAMP that induces expression of type I IFN.

We compared the amount of DI RNAs produced by the E195 WT and mutant viruses upon infection in A549s cells. 8 hours post infection RNA was extracted and PCR performed with primers specific to the 5' and 3' ends of the PB2 gene. No short PCR products were produced by either of the E195 viruses (Figure 54). The process was repeated 24 hours post infection and PCR was performed with primers specific to the 5' and 3' ends of the PB2, PB1 and PA genes in an attempt to conclusively establish if short PCR products were produced by either of the viruses. Here, short PCR products, presumed to be DIs were detected (Figure 54). However, the E195 PB2 E158G virus did not generate more DIs than the WT virus which suggests this is not the explanation for the observed increase in IFN expressed.
Figure 54. Investigating if defective interfering RNAs are generated by E195 WT and PB2 E158G viruses. A549 cells were infected at an MOI of 3. 8 and 24 hours post infection the amount of DI RNAs made by the different viruses was compared using with primers specific to the 5' and 3' ends of the PB2, PA and PB1 genes.

4.2.6 Growth and pathogenicity of E195 WT and PB2 E158G viruses in mice

To test whether the phenotypes of the mutant viruses seen in vitro manifested in vivo, we evaluated the growth and pathogenicity of these viruses in a mouse model. Groups of 5, 6-8
week old BALB/c mice were infected intranasally with 2\times10^5\text{ PFU} and body weight was monitored daily for 10 days (Figure 55).

Mice infected with the E195 PB2 E158G virus initially lost weight more rapidly than those infected the WT virus (Figure 55A). This reduction in weight loss was statistically significant on days 2 and 3 post infection (**\textit{P}<0.01). However, mutant virus-infected mice reached their maximal weight loss (90.6%) on day 3 post infection and began to recover 4 days post infection, whereas WT virus-infected mice reached their maximal weight loss (90.1%) on day 7 post infection and began to recover 8 days post infection.

On days 2 and 6 post-infection one group of mice for each virus was sacrificed. The lungs were harvested and homogenized before titration to determine viral load by plaque assay or amount of IFN\alpha expression by ELISA.

The viral titre was higher at day 2 than day 6 for both WT and mutant virus-infected mice (Figure 55B). The viral titre was higher in the WT virus-infected mice (1.7 \times10^7\text{ P.F.U} \text{ lung}^{-1}) compared to the mutant virus-infected mice (4.4 \times10^6\text{ P.F.U} \text{ lung}^{-1}) at day 2 post infection, although this difference was not statistically significant. Again, at day 6 post infection, the viral titre was higher, but not statistically significant, in the WT virus -infected mice.

IFN\alpha expression was significantly higher than mock at day 2 post infection for both WT and mutant virus-infected mice (Figure 55C). IFN\alpha expression was significantly higher in the mutant virus-infected mice (298 pg lung g^{-1}) compared to the WT virus-infected mice (168
pg lung $g^{-1}$) at day 2 post infection. At day 6 post infection there was no significant difference in IFNα expression compared to mock for both viruses.

**Figure 55. Infection of mice with E195 WT and PB2 E158G viruses.** Groups of five mice were infected with $2 \times 10^5$ P.F.U of each virus or PBS (Mock). (A) Mice were monitored daily for weight loss. Mean weight loss is plotted with standard error shown by the error bars. On days 2 and 6 post-infection one group of mice for each virus was sacrificed. The lungs were harvested and homogenized before titration to determine viral load by plaque assay or amount of IFNα expression by ELISA. Each animal’s viral load per gram of lung tissue or pg of IFNα per gram of lung tissue is
plotted with mean indicated. The statistical significance of differences compared to WT were assessed by a two-tailed, unpaired Student's t-test (*P < 0.05; **P < 0.01).

4.3 Discussion

Initially the effect of PB2 E158G on E195 polymerase activity was assessed in a cell-based assay. As reported by Zhou et al. for a different strain of pH1N1 2009 virus the PB2 E158G mutation significantly enhanced E195 polymerase activity in human and avian cells. However, in our hands in contrast to the reports from Zhou et al., the E195 PB2 E158G virus was attenuated compared to the WT virus. The E195 PB2 E158G virus formed smaller and less distinct plaques on MDCKs and grew to lower titres in human A549 cells.

Furthermore, the E195 PB2 E158G virus induced significantly higher levels of IFNβ mRNA synthesis and IFNβ protein expression compared the E195 WT virus. However, the high level of IFN induced by the E195 PB2 E158G mutant does not appear to restrict viral growth in cell culture.

The stoichiometry of RNA species synthesised by viruses containing the PB2 E158G mutation was altered. A significant increase in cRNA synthesis and decrease in mRNA and vRNA synthesis was observed for the E195 PB2 E158G viruses compared to WT. This altered stoichiometry may explain why the growth of the E195 PB2 E158G virus is restricted and why this mutation has not emerged in viral strains circulating in humans. Influenza transcription and replication are known to be tightly controlled and regulated processes
(Elton et al., 1999; Fodor et al., 1994; Tiley et al., 1994) and so altering the balance between these processes is likely to be deleterious for the virus.

To test whether the phenotypes of the mutant viruses seen in vitro manifested in vivo, we evaluated the growth and pathogenicity of these viruses in a mouse model. Mice infected with the E195 PB2 E158G virus initially lost weight more rapidly than those infected the WT virus and IFNα expression was significantly higher in the mutant virus-infected compared to the WT virus-infected mice at day 2 post infection. However, there was no significant difference in viral titre between the 2 viruses at this time point. This suggests that much of the weight loss in the infected mice was driven by an immunopathologic response of high cytokines levels induced by this apparently ‘mouse adapted’ virus rather by direct damage from virus replication. The disparity between the polymerase assay results and the viral growth and in vivo data suggests caution must be taken when analysing minireplicon assay results as they may not truly reflect viral growth or whether a mutation is likely to be selected for in nature.

The PB2 E158G mutation was previously identified as a marker of pathogenicity for the 2009 pH1N1 virus in mice (Ilyushina et al., 2010; Zhou et al., 2011) and the in vivo data described in this chapter clearly supports this. However, the effect of the E158G mutation on viral replication is less certain.

In the study by Zhou et al, it was observed that the E158G mutation enhanced viral replication in vitro and in vivo. The introduction of this mutation enhanced viral polymerase activity in a minireplicon assay in human cells and the 2009 pH1N1 virus containing this
mutation grew to higher titres in mice. In the Ilyushina study 2 mouse-adapted variants of A/CA/04/09 (H1N1), A/CA/04/09-MA1 and A/CA/04/09-MA2 (MA for mouse adapted), were derived in 2 parallel lung-to-lung passage experiments. The MA1 and MA2 viruses differed by a single mutation (E158G) in PB2 and had 4 further amino acid changes in common. Ilyushina et al concluded that E158G provided a replicative advantage both in vitro and in vivo and indeed introducing E158G significantly enhanced polymerase activity in a minireplicon assay, however, the effect of this mutation on viral growth were less clear.

A significant difference in time to death of mice infected with MA1 (E158G) versus MA2 ($P < 0.05$) was observed. The day of death (mean ± SD) for MA1 was 7.4 ± 0.2 and for MA2 it was 8.4 ± 0.5. However, there was no significant different in viral titres for MA1 and MA2 in the lungs, brain, spleen and blood in groups in mice euthanized 3 days post infection. In an MDCK growth curve viral titres were measured at 6 12 hour intervals post infection. MA1, in which the E158G mutation was introduced, exhibited significantly greater growth than MA2 at only a single time point ($P < 0.05$).

In this chapter the E195 polymerase containing this mutation was more active in a cell-based activity assay. However, our in vivo data did not indicate that this mutation provided a replicative advantage and in human A549 cells the mutant virus was severely attenuated compared to WT virus. It may be that other compensatory mutations or sequence differences between the strains of viruses used in these studies explain the difference in outcome for the E158G mutation. On the other hand the difference may depend on the way experiments have been performed for example, the dose, volume and route of virus used for mouse inoculation and mouse strain.
Reside 158 is located in a structurally undefined and mainly uncharacterised region of PB2. However, this region was been implicated in NP-binding in studies using PB2 deletion mutants and the E158G residue is predicted to lie in an hydrophilic loop that is exposed on the surface of PB2 (Zhou et al., 2011). In addition, the surrounding residues are highly conserved (Zhou et al., 2011). E158G could facilitate an enhanced interaction with a viral or cellular factor, although this cellular factor is unlikely to be host specific because in chapter 2, the E158G mutation enhanced avian polymerase activity in human, swine and avian cells.
5. Discussion

5.1 Enhancing avian influenza polymerase activity in human cells

In humans, traditional avian influenza viruses don’t replicate efficiently and rarely cause disease following exposure. There are many host range restrictions but one of the most important is at the level of the polymerase. Avian influenza polymerases are hardly active in human cells but the molecular explanation for this is not known.

In order for an avian polymerase to overcome the host range barrier and become active in human cells adaptive mutations may be introduced into the polymerase proteins and especially PB2. In Chapter two it was shown that numerous mutations within PB2 enhance avian polymerase activity in human cells. It was shown that that not all these PB2 mutations enhance polymerase activity via a universal mechanism, however the mechanisms themselves remain elusive.

The influenza polymerase is responsible for both transcription and replication but it is not known how the polymerase becomes committed to either process. Replication of viral RNA involves the production of positive-strand cRNA intermediates. A common belief in the field is that the ‘switch’ from vRNA transcription to replication requires the accumulation of a viral product that could be NP, viral polymerase or NEP (Beaton and Krug, 1986; Fodor et al., 1994; Robb et al., 2009). Vreede et al. have proposed that replication of vRNA into cRNA may occur right from the beginning of infection, with polymerase acting in cis but that the cRNA only accumulates once it is protected from degradation by a polymerase complex which binds to its promoter (Vreede et al., 2004).
In order to investigate the effects the PB2 mutations have on transcription versus replication qRT-PCR was performed to measure synthesis of m, v and cRNA in a cell-based polymerase activity assay with a viral-like reporter (Chapter two). An increase in polymerase activity correlated with proportionally enhanced synthesis of all three RNA species. This suggests the mutations are working by at least increasing the level of replication, rather than just by increasing transcription of mRNA from a constant amount of vRNA template. A cell-based polymerase activity assay was undertaken where the ability of a polymerase to transcribe a cRNA template rather than a vRNA template was assessed. The avian polymerase was much more active in this system suggesting that the restriction lies at the provision of a bona fida cRNP.

For many years the point at which the avian polymerase was restricted in mammalian cells was not clear. However, a recent study by Manz et al. used a complementation approach to show that polymerases containing PB2 627E can undergo transcription but not replication in human cells. It was shown that these PB2 627E containing polymerases produce defective cRNPs that cannot be used to synthesise vRNA. In contrast, a polymerase containing a lysine at position 627 in PB2 was able to generate a cRNP that acted as a template for vRNA synthesis (Manz et al., 2012).

A trans-complementation model has been proposed for the production of vRNA from the cRNA template and it has gained considerable support from researchers in the field. In this model, as suggested by Vreede et al., the cRNA is protected from degradation by a resident polymerase complex which binds to the promoter. However, Jorba et al. then propose that it is a non-resident polymerase complex which accesses the 3' cRNA promoter and functions
as the replicative enzyme (Jorba et al., 2009). The model is supported by the work of Manz et al. 2012. In their study after viral infection in the presence of cycloheximide, vRNA was only observed with pre-expression of NP and catalytically active polymerase (Manz et al., 2012). Furthermore, in the study by Moeller et al, negative stain EM micrographs revealed RNP complexes with a branched arrangement in which a smaller nascent RNP appears to bud from a larger full-length RNP and a polymerase residing at the junction of the smaller RNA with the full-length RNP was observed (Moeller et al., 2012). Finally recent work from Fodor’s group using purified cRNPs showed a requirement for a trans acting polymerase before vRNA was synthesized (York et al., 2013).

The implications from these studies are that the 3’ end of the cRNA template needs to be released by the vRNP-associated polymerase to be copied by the trans-acting polymerase. Jorba et al. speculate that the soluble polymerase must interact with the resident polymerase bound to the 5’ cRNA in order to gain access to the 3’ promoter (Jorba et al., 2009). Interestingly, Manz et al. showed that the supply of a trans-acting PB2 627K containing polymerase could not replicate a cRNP produced a polymerase harbouring PB2 627E (Manz et al., 2012). Thus perhaps exposure of the 3’ promoter for the soluble polymerase is only facilitated when the resident polymerase binds in the correct orientation. Perhaps when the resident influenza polymerase is of avian origin it binds too strongly or in the incorrect orientation or is unable to interact with a cellular factor capable of separating the 5’ and 3’ cRNA. This would prevent vRNA being synthesised from the cRNA template (Figure 56). It would be interesting to investigate these protein-RNA and RNA-RNA interactions potentially by using FRET in vivo (Zhao and Rueda, 2009).
Figure 56. Avian influenza polymerases are restricted in synthesis of vRNA in human cells. Avian influenza polymerases undergo efficient transcription but not replication in human cells. Avian influenza polymerases with PB2 627E produce a cRNP that cannot be used as a template to further synthesise vRNA.
The recent study from Fodor’s group is not quite in line with the model proposed by Jorba. They also suggested that a non-resident polymerase complex is required for replication of cRNA because purified cRNPs were unable to give rise to vRNA unless a second polymerase complex purified from ether insect cells or mammalian cells was supplied. However, in the Fodor study the second exogenously supplied complex could activate replication even if it carried mutations in PB1 that rendered it enzymatically inactive (York et al., 2013). This suggests that the trans-activating polymerase could act cooperatively with the resident polymerase, which then carries out vRNA synthesis.

As yet there is no model for the production of cRNA from the vRNA template; it is not clear whether this step is carried out in cis or in trans. However, the in vitro work of Vreede demonstrated that vRNPs can synthesise cRNA without any additional viral or cellular proteins and that vRNPs introduced into the cell by infecting virions have the capability to produce both mRNA and cRNA early on during infection. This suggests vRNA is synthesised by the polymerase acting in cis (Vreede and Brownlee, 2007; Vreede et al., 2004). Furthermore, Manz et al. showed that pre-expressed, catalytically inactive polymerase allowed the accumulation of cRNA in cycloheximide-treated, virus-infected cells (Manz et al., 2012).

In Chapter two it was shown that the activity of a typical avian polymerase was artificially enhanced when a triple mutation was introduced into the 3’ viral promoter of the luciferase reporter. The 3-5-8 mutations result in perfect base pairing between the terminal nucleotides of the 3’ and 5’ ends of vRNA which is likely to alter the structure of both vRNA and cRNA and decrease the stability of the stem loop structures in the corkscrew
conformation (Figure 57). However, Paterson et al. have recently shown that enhancement of activity for a polymerase with 627E replicating a mutated promoter is not due to increased panhandle complementarity but is specific for the 3’ promoter sequence (2013). These mutations could correctly position the polymerase on the 3’ vRNA promoter or could alter how the resident polymerase binds to the cRNA template in the trans-complementation model of vRNA synthesis.

Figure 57. Nucleotide sequence and predicted ‘corkscrew’ conformation of (A) WT and (B) mutated 3-5-8 vRNA and cRNA viral promoters. (Red: complementary mutations).

The effects of this triple mutation are known to be dependent on the nature of the residue at position 627 in PB2 (Crescenzo-Chaigne et al., 2002). Indeed, we observed a dramatic
enhancement in polymerase activity when a glutamic acid is at position 627, but not when a lysine is introduced, in fact polymerase activity actually decreased in the presence of the 3-5-8 reporter (Figure 58). The activity of the PB2 E158G containing polymerase was also restricted with the 3-5-8 reporter. This suggests that certain adaptive mutations may correctly position the polymerase on the viral promoter.

Figure 58. Polymerase activity supported by PB2 WT and mutants with a WT or mutated 3-5-8 viral-like firefly luciferase reporter in human cells.

5.2 The role of NEP in viral replication

NEP can regulate the synthesis of influenza vRNA, cRNA and mRNA and such regulation is independent of its nuclear export function. Currently, the reported effects of NEP on polymerase activity are not consistent between different research groups. Initially NEP was shown to reduce the levels of minireplicon-derived vRNA, cRNA, and mRNA molecules (Bullido et al., 2001). However, in a further study co-expression of NEP resulted in a
decrease in mRNA and an increase in cRNA and vRNA accumulation (Robb et al., 2009). NEP has also been shown to enhance polymerase activity and levels of vRNA, mRNA and cRNA produced by an avian virus polymerase in human cells (Manz et al., 2012). Speculation that NEP acts as co-factor to regulate viral replication has been heightened since the work of Manz showed that adaptive mutations in NEP can overcome avian polymerase restriction in human cells and that NEP interacts with the polymerase proteins PB1 and PB2 (Manz et al., 2012).

The discrepancies regarding NEP in the literature may be explained by the results in Chapter three. It was observed that there is a balance between the initial level of polymerase activity and the regulation by NEP. The presence of NEP increased activity of avian virus polymerases containing the adaptive PB2 mutations T271A, D701N, G590S Q591R and M535T. However, NEP decreased activity of the most active polymerases, those containing E158G and E627K. Furthermore, we found that the effects of NEP were dose dependent and that a high concentration of NEP decreased the level of mRNA, vRNA and cRNA synthesised by all polymerases (data not shown), this may explain previous results (Bullido et al., 2001).

H. Zhou (Barclay lab) has been investigating the role of NEP on influenza polymerase activity. The effects of NEP on the activity of polymerases from human (pandemic H1N1 virus A/England/195/09, a seasonal H3N2 virus A/Victoria/3/75), swine (avian like H1N1 swine virus A/England/453/2006) and avian (H1N1 virus A/Duck/Bavaria/77) viruses were investigated in a minireplicon assay. The effects of NEP on the different v, c or m RNA species were also detected and compared by qRT-PCR.
The swine (453) and avian (Bav) polymerases were poorly active in human cells in a minireplicon assay, compared to the activities of the two human influenza polymerases (195 and Vic) (Figure 59A). Addition of the 453 or Bav NEP increased the levels of cRNA, vRNA and mRNA synthesized by the 453 polymerase (Figure 59B). However, in contrast, with human adapted polymerases (VIC & 195), NEP promoted the accumulation of cRNA but the decrease of mRNA (Figures 59C & D). This suggests that cRNA synthesis is regulated by NEP to maintain a suitable level of influenza virus replication in infected cells. The level and timing of NEP expression is likely to be critical in ensuring optimal levels of cRNA synthesis. Indeed, disruption of splicing control in segment 8 that results in increased NEP production was highly deleterious for virus fitness in vitro, suggesting that premature stimulation of vRNA replication is normally precluded by the poor splice consensus in this mRNA (Chua et al., 2013).

NEP could regulate viral RNA synthesis by promoting synthesis of small viral RNAs (svRNA). svRNAs map to each of the 5' ends of the vRNA segments and are 22 - 27 nucleotides in length. They require NEP in addition to the polymerase complex and NP for their production (Perez et al., 2010). They have been implicated in enhancing vRNA synthesis, in a segment-specific manner. svRNAs might promote the replication step cRNA→vRNA by associating with trans-acting RNA polymerase via their proposed interaction with a novel RNA binding channel in PA (Perez et al., 2010; Perez et al., 2012; Umbach et al., 2010).
Figure 59. NEP effects the stoichiometry of RNA species synthesised by influenza polymerase. (A) Minireplicon assay in human 293T cells with polymerases from a human pandemic H1N1 virus (A/England/195/09), a seasonal H3N2 virus (A/Victoria/3/75), a swine virus (A/England/453/2006) and an avian H1N1 virus (A/Duck/Bavaria/77). The level of viral mRNA, cRNA, and vRNA synthesised by (B) 453, (C) 195 and (D) Vic polymerases upon the addition of NEP normalised to that of the WT polymerase in 293T cells.

5.3 Does polymerase activity correlate with pathogenicity?

In Chapter four the E195 PB2 E158G virus induced weight loss in mice more rapidly than the WT virus; however, the mutant virus did not achieve a higher titre than the WT virus. Thus, the increase in pathogenicity observed did not correlate with an increase in viral replication. Rather it appeared that the mutant virus did not effectively control the innate immune response early in infection leading to higher cytokine levels that may have caused immunopathology and weight loss but eventually led to a more rapid virus clearance.
Several previous studies have concluded that an increase in polymerase activity correlates with an increase in virulence (Gabriel et al., 2005b; Grimm et al., 2007; Leung et al., 2010). Grimm et al. compared PR8 strains with high and low virulence and assessed polymerase activity, viral growth and pathogenicity (Grimm et al., 2007). In a minigenome assay, the activity of the polymerase from the highly virulent strain was 2-fold greater than the polymerase from the less virulent strain.

In a study by Gabriel et al. a range of mammalian adaptive PB2 mutations were introduced into an avian influenza strain and polymerase activity in a minireplicon assay was plotted against the median lethal dose (LD$_{50}$) of the mutant viruses in mice (Figure 60). It was concluded that there was a positive correlation between polymerase activity and pathogenicity, and as such, enhancing polymerase activity facilitates mammalian adaptation (Gabriel et al., 2005b).

![Figure 60. Correlation of polymerase activity measured in a minireplicon assay and median lethal dose (LD$_{50}$) in mice (Gabriel et al., 2005b).](image-url)
However it is important to note that Gabriel et al. found that the viruses whose polymerases were the most active were not actually the most virulent (Gabriel et al., 2005a). Intriguingly, this suggests that 'excessive' polymerase activity could be deleterious for the virus. Perhaps if the polymerase were too active it would trigger too great an immune response for the virus to control.

The work of Bussey has challenged the concept that host adaptive mutations always increase viral replication and therefore pathogenicity. Several PA mutations identified had a considerable effect on polymerase activity and host adaptation but no or only a minimal effect on viral pathogenicity (Bussey et al., 2011). Moreover, Bussey, identified a mutation in PA (L336M) which increased the activity of an avian polymerase in mammalian cells in a minireplicon assay, but did not increase viral growth in A549s cells and yet caused considerable enhancement in pathogenesis in the mouse model. It would be interesting to measure the level of IFN induced by this virus.

A study by Zeng et al. compared the numbers of host inflammatory genes induced by the 2009 pH1N1 virus (Mexico/4108) and the H5N1 virus (A/Vietnam/1203/04). A greater number of inflammatory genes were induced by the H5N1 virus despite both viruses displaying similar productive viral growth in the human epithelial cell line (Zeng et al., 2011). This suggests that the H5N1 strain is more pathogenic than the 2009 pH1N1 strain in a manner independent of polymerase activity.
It is necessary to have a delicate balance between influenza viral replication, protein expression, and assembly. Belicha-Villanueva et al. generated viruses with the G3A/C8U promoter mutations that have previously been demonstrated to increase polymerase activity in a minireplicon assay (Belicha-Villanueva et al., 2012; Neumann and Hobom, 1995). When the promoter mutations were introduced into an individual gene segment (either PB1 or PA), the polymerase preferentially replicated the mutated segments and as such, there was a considerable decrease in the level of vRNA detected for all the other segments. This disrupted the stoichiometry at the viral RNA and/or protein levels. An increase in immunostimulatory RNAs was observed. Correspondingly, there was an increase in type I IFN induction and an increase in the ratio of noninfectious versus infectious virus particles. These results illustrate how an uncoordinated upregulation of viral RNA replication can be deleterious to the virus.

Avian viruses have often been serially passaged in mice to identify viral markers of mammalian adaptation (Gabriel et al., 2005b; Gabriel et al., 2008; Zhou et al., 2011). Typically mutations that increase viral pathogenicty are further characterized. However, host adaptation and pathogenicity are distinct concepts and must be considered as such. In evolutionary terms, extreme pathogenicity can lead to unwanted side effects and be counterproductive for virus survival. Thus, mutations which increase polymerase activity without increasing pathogenicity would be the most beneficial to the virus.

Ebola is highly efficient at evading the immune system and exhibits a high rate of replication, however, such a high level of virulence limits the duration over which transmission is possible. Thus, the high mortality rate of ebola is actually an evolutionary
disadvantage (Casillas et al., 2003). If the pathogen kills the host too frequently or too quickly (as ebola does), then the parasite is not transmitted as successfully and cannot become as established.

The most successful pathogens strike a balance between replication and transmission. Seasonal human influenza viruses are highly adapted for human transmission and often leave individuals healthy enough to come into regular contact with other individuals. Similarly avian influenza viruses are endemic and largely asymptomatic in the wild bird reservoir. Successful pathogens that kill their host or result in high disease burden are transmitted via alternative mechanisms. For instance, malaria is transmitted by mosquitoes and *Bacillus anthracis* (anthrax) can form spores that survive time, heat and desiccation for decades while waiting for a new host.

### 5.4 Balance between polymerase accuracy and speed

In this study it was observed that several adaptive mutations which increase polymerase activity in a minireplicon assay are not selected for by the virus in nature. In addition, although it is likely that several of the adaptive mutations enhance polymerase activity via different mechanisms, many viruses identified in nature contain very few of the adaptive mutations we have investigated. Thus we wished to explore the balance between polymerase accuracy and speed. Can a polymerase ever replicate too quickly?

RNA viruses have an extremely high mutation rate. It has been estimated that there are between 0.4 and 1.1 nucleotide errors per genome in each replication cycle (Drake et al.,
1998). This is thought to be at least a hundred-fold higher than those predicted for DNA viruses (Drake, 1993; Drake et al., 1998). This difference is due to the inability of the polymerases in RNA viruses to undergo proofreading (Steinhauer et al., 1992). It has been suggested that the most likely explanation for the high mutation rates observed with RNA viruses is a trade-off with replication speed. Belshaw et al. hypothesise that viruses are able to replicate either quickly or accurately; but not both (Belshaw et al., 2008). The M535T PB2 mutant we studied in Chapter 3 might be an example whereby high replication rate measured in the polymerase assay came at the cost of deleterious mistakes that led to defective viruses, containing short RNAs that induced high levels of interferon. We measure inaccurate vRNA replication simply by assessing the length of RNA segments produced by RT-PCR on infected cell lysates. In future, deep sequencing might also be used to assess error rates in full length genomes. Zhang et al. recently described a novel method for this using measles viruses engineered to contain an additional open reading frame in GFP (Zhang et al., 2013).

The ability of RNA viruses to replicate rapidly and produce large numbers of mutations is a powerful advantage in overcoming host range. However, accumulating increasing numbers of mutations will ultimately lead to lethal mutagenesis. This is because many mutations do not support viral replication. There is a mathematical measurement which has been termed the 'error threshold', which is the threshold at which the genetic information that must be maintained (Figure 61) (Belshaw et al., 2008).
Figure 61. Model of error catastrophe. Most viruses in a normal population are viable. However, a small increase in the mutation rate is predicted to push the virus population into error catastrophe (the mutagenized population, Right). Here, the virus makes too many errors during replication and the virus population is not viable. (Crotty et al., 2001).

Studies strongly suggest that RNA viruses evolve near to the error threshold. The first evidence came from studying poliovirus and vesicular stomatitis virus. Cells infected with these viruses were treated with base analogs, such as 5-azacytidine, which act as mutagens and are introduced into the growing RNA chain. Upon the treatment of infected cells with these drugs the rate of mutagenesis increased only slightly, however, the production of new infectious virus particles was dramatically reduced. In contrast, when the experiment was repeated with a DNA virus, the rate of mutagenesis was increased to a much greater extent before a large number of infectious particles were produced (Crotty et al., 2000; Holland et al., 1990; Lee et al., 1997; Loeb et al., 1999).
Crotty et al. clearly showed how near poliovirus evolves to error catastrophe through treating infected cells with ribavirin, a mutagen. Infected-cells treated with a concentration of ribavirin that lead a 9.7-fold increase in the mutation rate caused a 99.3% loss in poliovirus infectivity (Figure 62). Infectivity is reduced to 70 % less than WT when only 2 mutations per genome are introduced and infectivity is almost eliminated when 7 mutations per genome are introduced (Crotty et al., 2001).

![Graph showing the relationship between mutation frequency and genomic RNA infectivity.](image)

**Figure 62. Relationship between mutation frequency and genomic RNA infectivity.** There is a dramatic reduction in RNA genome infectivity when the frequency of mutations introduced is only slightly greater than normal; this implies that poliovirus evolves near to the error threshold (Crotty et al., 2001).

An implication of the observation that RNA viruses evolve close to their error threshold could be that adaptive mutations which increase polymerase activity in a minireplicon assay may not be selected for by the virus, as this could push the polymerase past the error threshold. It is likely that many more mutations can be tolerated in the reporter gene used in the minireplicon assay than can be tolerated in an actual influenza virus genome.
segment. It would be interesting to investigate the rate at which mutations accumulate in a reporter gene replicated by different influenza polymerases. In addition, there may be factors other than speed which affect the rate at which mutations accumulate. In certain situations this could be very beneficial to virus and may explain why certain strains of influenza are able to overcome the host range barrier more readily than others.

5.5 Significance and Implications

There are at present only two classes of antivirals licensed for treatment of influenza infection. The first class contains the M2 channel blockers, the adamantanes and the second class contains the neuraminidase inhibitors (Boltz et al., 2010). Unfortunately, considerable resistance to both drug classes has been reported. Thus, there is urgent need for new drugs from which the virus is less likely to escape.

The influenza polymerase is one potential target for antiviral development. Indeed, it represents a promising drug target, its activities are distinct from those of the host cell, it plays an essential role in the lifecycle of the virus, it harbours multiple enzymatic activities and its genes are highly conserved.

By far the most promising polymerase inhibitor is Favipiravir. It is a pyrazine derivative which has considerable in vitro and in vivo anti-influenza activity (Furuta et al., 2002; Takahashi et al., 2003). It is effective against numerous influenza strains, including H1N1, H2N2, H3N2, a mouse adapted H5N1 (Sidwell et al., 2007) and a highly pathogenic H5N1 virus in a mouse model (Kiso et al., 2010).
As discussed previously the viral mutation rate is close to the error threshold, and therefore even a slight increase in the mutation rate can negatively affect viral fitness. Although Favipiravir’s mechanism of action is still not entirely certain, it is a nucleoside analog and its antiviral activity may be caused by its introduction into viral RNA increasing the mutation rate of the virus. In a study undertaken by Baranovich et al. cells infected with seasonal and 2009 pH1N1 influenza were treated with increasing concentrations of Favipiravir (Baranovich et al., 2013). Treatment resulted in an increasing loss of specific virus infectivity and an increase in the mutation frequency was observed. These findings suggest that lethal mutagenesis is an important mechanism by which Favipiravir inhibits influenza virus replication.

A drug which forces a virus past its error threshold would make an effective anti-viral. However, it is important to consider what the properties of a virus mutant resistant to a drug such as Favipiravir would be. Ribavirin is an example of a drug which pushes poliovirus beyond the error threshold by increasing the rate of mutagenesis. In a study by Pfeiffer et al. a poliovirus mutant resistant to ribavirin was selected by passing the virus in the presence of increasing concentrations of the drug (Pfeiffer and Kirkegaard, 2003). Resistance to this drug was achieved through a single amino acid substitution, G64S, in the polymerase. This mutation increased the fidelity of the virus and the polymerase made fewer errors in the absence of drug compared with the WT virus. Growth of this virus was tested in mice to investigate if increased fidelity continued to be advantageous in an environment of increased complexity. However, the mutant virus exhibited reduced growth compared to WT and also did not invade the motor neurons which is the mechanism by
which poliovirus leads to poliomyelitis. This attenuation was likely due to the high fidelity (Pfeiffer and Kirkegaard, 2005). This is further evidence that a high rate of mutation is advantageous to the virus in vivo.

In this study we gained further understanding of the molecular mechanisms of influenza polymerase host adaptation. This understanding is of paramount importance and could have a significant impact on anti-influenza drug design. It is likely that polymerase host-adaptive mutations are found in functionally important sites that could be potential targets for antiviral drug development. A cellular factor required for influenza virus replication could be efficiently targeted by a drug. In this study we suggest that there are different mechanisms of enhancing polymerase activity and this could have implications for anti-viral drug design. If a drug was designed to target a particular mechanism of host adaptation, this may limit the drug’s effectiveness against other strains of influenza.

In this study we determined that polymerase assay results do not necessarily always reflect viral fitness. Some mutations that increased in vitro polymerase activity led to attenuated virus replication that correlated with an increase in interferon activation. These data help risk assess the likelihood of different avian influenza viruses crossing the host range barrier and increase our understanding of the relative balance between virus replication and the host innate response.

There is considerable surveillance of avian and swine influenza viruses in order to monitor outbreaks which pose particular risks to human health. Infected animals can be quarantined or culled to minimise animal to human transmission. Thus, it is important to know as much
as possible in terms of which influenza strains have pandemic potential and are most likely to cross host range barriers.
6. Materials and Methods

6.1 Materials

6.1.1 Cell lines

Table 2: Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td><strong>293T</strong></td>
<td>Human kidney cells expressing large T-antigen of SV40</td>
<td>ATCC</td>
</tr>
<tr>
<td><strong>DF-1</strong></td>
<td>Chicken fibroblast cells</td>
<td>ATCC</td>
</tr>
<tr>
<td><strong>NPT</strong></td>
<td>Newborn pig trachea cells</td>
<td>I. Brown, AHVL A</td>
</tr>
<tr>
<td><strong>MDCK</strong></td>
<td>Madin darby canine kidney cells</td>
<td>ATCC</td>
</tr>
<tr>
<td><strong>A549</strong></td>
<td>Adenocarcinomic human alveolar basal epithelial cells</td>
<td>ATCC</td>
</tr>
<tr>
<td><strong>A549-NPro</strong></td>
<td>Adenocarcinomic human alveolar basal epithelial cells constitutively expressing bovine virus diarrhea NPro</td>
<td>R. Randall, St Andrews</td>
</tr>
</tbody>
</table>

6.1.2 Plasmid vectors

All plasmids used in this study contain genetic antibiotic resistance to Ampicillin.

Table 3: Plasmid vectors used in this study
<table>
<thead>
<tr>
<th>Plasmid</th>
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<th>Source</th>
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</thead>
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<tr>
<td><code>pCAGGS-50-92 NP</code></td>
<td>Express avian influenza 50-92 NP protein</td>
<td>Barclay Lab</td>
</tr>
<tr>
<td><code>pCAGGS-50-92 PB1</code></td>
<td>Express avian influenza 50-92 PB1 protein</td>
<td>Barclay Lab</td>
</tr>
<tr>
<td><code>pCAGGS-50-92 PB1</code></td>
<td>Express avian influenza 50-92 PB1 protein mutated</td>
<td>This study</td>
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<td><code>446Y</code></td>
<td></td>
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<td>Express avian influenza 50-92 PA protein</td>
<td>Barclay Lab</td>
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<tr>
<td><code>pCAGGS-50-92 NS1</code></td>
<td>Express avian influenza 50-92 NS1 protein</td>
<td>Barclay Lab</td>
</tr>
<tr>
<td><code>pCAGGS-50-92 NEP</code></td>
<td>Express avian influenza 50-92 NEP protein</td>
<td>This study</td>
</tr>
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<td><code>pCAGGS-50-92 PB2</code></td>
<td>Express avian influenza 50-92 PB2 protein</td>
<td>Barclay Lab</td>
</tr>
<tr>
<td><code>pCAGGS-50-92 PB2 44S</code></td>
<td>Express avian influenza 50-92 PB2 protein mutated</td>
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<tr>
<td><code>158G</code></td>
<td></td>
<td></td>
</tr>
<tr>
<td><code>pCAGGS-50-92 PB2</code></td>
<td>Express avian influenza 50-92 PB2 protein mutated</td>
<td>This study</td>
</tr>
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<td>Construct</td>
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<td>Origin</td>
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<tr>
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<tr>
<td>pCAGGS-50-92 PB2 702R</td>
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<td>pCAGGS-E195 NP</td>
<td>Express human influenza pH1N1 E195 NP protein</td>
<td>M. Mura (Barclay Lab)</td>
</tr>
<tr>
<td>Plasmid Name</td>
<td>Description</td>
<td>Author(s)</td>
</tr>
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<td>pCAGGS-E195 PB1</td>
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<td>M. Mura (Barclay Lab)</td>
</tr>
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<td>pCAGGS-E195 PB2</td>
<td>Express human influenza pH1N1 E195 PB2 protein</td>
<td>M. Mura (Barclay Lab)</td>
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<td>Express human influenza pH1N1 E195 PB2 protein mutated E158G</td>
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<td>M. Smith (Barclay Lab)</td>
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<td>pCAGGS-Victoria PB2</td>
<td>Express human influenza Victoria PB2 protein</td>
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<td>pHOM1-Firefly</td>
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<td><strong>IFN_Luc</strong></td>
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<td>Expression plasmid for human MAVS</td>
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<td><strong>Pol I-50-92 PB2</strong></td>
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<td><strong>Pol I-50-92 PB2 E158G</strong></td>
<td>Pol I vector containing A/Turkey/England/50-92/91 segment 1 cDNA mutated E158G</td>
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<td><strong>Pol I-50-92 PB2 Q590S G591R</strong></td>
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<td>This study</td>
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<td><strong>Pol I-50-92 PB1</strong></td>
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<td><strong>Pol I-PR8 HA</strong></td>
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<td>Pol I vector containing A/England/195/09 mutated</td>
<td>R. Elderfield</td>
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### 6.1.3 Antibodies

Table 4: Antibodies used in this study

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<thead>
<tr>
<th>Antibody</th>
<th>Origin</th>
<th>Dilution</th>
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<tr>
<td>anti-NP</td>
<td>Rabbit</td>
<td>1:2000 for western blot</td>
<td>Gift from Paul Digard</td>
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<tr>
<td>anti-PA</td>
<td>Mouse</td>
<td>1:400 for western blot</td>
<td>Gift from Juan Ortín</td>
</tr>
<tr>
<td>anti-PB1</td>
<td>Rabbit</td>
<td>1:400 for western blot</td>
<td>Gift from Juan Ortín</td>
</tr>
<tr>
<td>anti-Flag</td>
<td>Mouse</td>
<td>1:2000 for western blot</td>
<td>Sigma (F3165)</td>
</tr>
<tr>
<td>anti-vinculin</td>
<td>Goat</td>
<td>1:1000 for western blot</td>
<td>Santa Cruz Biotechnology (SC7649)</td>
</tr>
<tr>
<td>HRP conjugated anti-</td>
<td>Donkey</td>
<td>1:5000 for western blot</td>
<td>Santa Cruz Biotechnology (SC2020)</td>
</tr>
<tr>
<td>goat IgG</td>
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</tr>
<tr>
<td>HRP conjugated anti-</td>
<td>Mouse</td>
<td>1:5000 for western blot</td>
<td>Sigma (A2074)</td>
</tr>
<tr>
<td>rabbit IgG1 (heavy chain-specific)</td>
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<tr>
<td>HRP conjugated anti-mouse IgG</td>
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<td>1:10000 for western blot</td>
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### 6.1.4 Oligonucleotides

Table 5: Main oligonucleotides used in this study. Oligonucleotide primers were synthesised by MWG Eurofins and stock solutions of 100 pmol/μl were made using sterile water.

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<th>Sequence</th>
<th>Use (sequencing)</th>
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<tbody>
<tr>
<td>4715fw</td>
<td>CTGCTAACATGTTGATGCTCTC</td>
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<tr>
<td>85rev</td>
<td>GGTATTTGTGAGCCAGGGCATTG</td>
<td>Sequencing primer of pCAGGS (reverse)</td>
</tr>
<tr>
<td>M13fw</td>
<td>GTAAAACGACGGCCAGT</td>
<td>Sequencing primer of minigenome reporter plasmids (forward)</td>
</tr>
<tr>
<td>M13rev</td>
<td>GGAAACAGCTATGACCAGT</td>
<td>Sequencing primer of minigenome reporter plasmids (reverse)</td>
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<td>To sequence the E195 PB2 gene (forward)</td>
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<tr>
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<td>GATGGGACATTGGTCTTTGG</td>
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</tr>
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<td>To sequence the 50-92 PB2 gene (reverse)</td>
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<table>
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<td>50-92_NEP_MluI_rev</td>
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<td>Name of primer</td>
<td>Sequence</td>
<td>Use (mutagenesis)</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------------------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>pHOM1_Firefly_cR NA_rev1</td>
<td>CCTGTTTTCTACTCCCCCCCACTTT CGGAGGTC</td>
<td>To clone human Pol I minigenome reporter plasmid containing the positive-sense firefly coding sequence and a mouse Pol I terminator (reverse)</td>
</tr>
<tr>
<td>50-92_PB1_D446Y_fw</td>
<td>CCAATCTTCTGATTATTTCGCTCT CATAGTAATGCACCC</td>
<td>Mutagenic primer to introduce D446Y mutation into pCAGGS-50-92 PB1 (forward)</td>
</tr>
<tr>
<td>50-92_PB1_D446Y_rev</td>
<td>GAGAGGCAAAATAATCAGAAGATT GGAGTCCATCCC</td>
<td>Mutagenic primer to introduce D446Y mutation into pCAGGS-50-92 PB1 (reverse)</td>
</tr>
<tr>
<td>50-92_PB2_A44S_fw</td>
<td>GAGAAGAATCTTCACTTACATGAGATT GAAGTGGATGGGCC</td>
<td>Mutagenic primer to introduce A44S mutation into pCAGGS-50-92 PB2 (forward)</td>
</tr>
<tr>
<td>50-92_PB2_A44S_rev</td>
<td>CTTCATCCTAAGTGAAGGATTCTTTCATCCC CTCCGTCTTCTCG</td>
<td>Mutagenic primer to introduce A44S mutation into pCAGGS-50-92 PB2 (reverse)</td>
</tr>
<tr>
<td>50-92_PB2_E158G_fw</td>
<td>GTGCTAAAGGAGCACAAGATGTCTCATGGAGG</td>
<td>Mutagenic primer to introduce E158G mutation into pCAGGS-50-92 PB2 (forward)</td>
</tr>
<tr>
<td>50-92_PB2_E158G_rev</td>
<td>GCCATGCAGATCTTAGTGCTAAAGGAGCACAAGATGTCTCATGGAGG</td>
<td>Mutagenic primer to introduce E158G mutation into pCAGGS-50-92 PB2 (reverse)</td>
</tr>
<tr>
<td>50-92_PB2_A199S_fw</td>
<td>GACTGTAAGATTCTCCTTATAATG GTGGCATACATGTTGG</td>
<td>Mutagenic primer to introduce A199S mutation into pCAGGS-50-92 PB2</td>
</tr>
<tr>
<td>50-92_PB2_A199S_rev</td>
<td>GAGGAGCTTCAGGACTGTAAGAT TTCTCCTTTAATGGTG</td>
<td>Mutagenic primer to introduce A199S mutation into pCAGGS-50-92 PB2 (reverse)</td>
</tr>
<tr>
<td>50-92_PB2_T271A_fw</td>
<td>GTTAGGAGAGCAGCAGTATCAGC AGACCGTTGG</td>
<td>Mutagenic primer to introduce T271A mutation into pCAGGS-50-92 PB2 (forward)</td>
</tr>
<tr>
<td>50-92_PB2_T271A_rev</td>
<td>CTGATACCTGCTGCTCTCCTAACGA TATTCTAGCAG</td>
<td>Mutagenic primer to introduce T271A mutation into pCAGGS-50-92 PB2 (reverse)</td>
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<tr>
<td>50-92_PB2_L475M_fw</td>
<td>GAGATGTCAATGAGAGGAGTGA GAGTCAGCAAATG</td>
<td>Mutagenic primer to introduce L475M mutation into pCAGGS-50-92 PB2 (forward)</td>
</tr>
<tr>
<td>50-92_PB2_L475M_rev</td>
<td>CTCACTCCTCTCATTGAGCATCTCT GTGCTGGGGG</td>
<td>Mutagenic primer to introduce L475M mutation into pCAGGS-50-92 PB2 (reverse)</td>
</tr>
<tr>
<td>50-92_PB2_A588I-fw</td>
<td>CCTAAGGCTATTAGAGGCCAGTA TAGTGGATTTGTG</td>
<td>Mutagenic primer to introduce A588I mutation into pCAGGS-50-92 PB2 (forward)</td>
</tr>
<tr>
<td>50-92_PB2_A4558I_rev</td>
<td>CTGGCCTCTAATAGCCTTAGGAA CCAAGGATTGAAG</td>
<td>Mutagenic primer to introduce A588I mutation into pCAGGS-50-92 PB2 (reverse)</td>
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<tr>
<td>50-92_PB2_G590S&amp;Q591R_fw</td>
<td>CTGCCAAGAGCGGTATAGTGGA TTTGTAGGACGC</td>
<td>Mutagenic primer to introduce G590S &amp; Q591R mutations into pCAGGS-50-92 PB2 (forward)</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Sequence</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>50-92_PB2_G590S&amp;Q5_91R_rev</td>
<td>CACTATACGGCTTTCTGGCAGCC TTAGGAACCAAG</td>
<td>Mutagenic primer to introduce G590S &amp; Q591R mutations into pCAGGS-50-92 PB2 (reverse)</td>
</tr>
<tr>
<td>50-92_PB2_A684S-fw</td>
<td>GAGGGAACATCTGGAGTGGAAT CTGGGTATTG</td>
<td>Mutagenic primer to introduce A684S mutation into pCAGGS-50-92 PB2 (forward)</td>
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<tr>
<td>50-92_PB2_A684S_rev</td>
<td>GAAGACCCAGATGAGGGAACT CTGGAGTGGAATC</td>
<td>Mutagenic primer to introduce A684S mutation into pCAGGS-50-92 PB2 (reverse)</td>
</tr>
<tr>
<td>50-92_PB2_K702R-fw</td>
<td>GCAAAGAAGACAGAAGATATGG ACCAGCGACGCATC</td>
<td>Mutagenic primer to introduce K702R mutation into pCAGGS-50-92 PB2 (forward)</td>
</tr>
<tr>
<td>50-92_PB2_K702R_rev</td>
<td>GGTCCATATCTTCTGCTCTTTGG CCTGAATTAGAAATCC</td>
<td>Mutagenic primer to introduce K702R mutation into pCAGGS-50-92 PB2 (reverse)</td>
</tr>
<tr>
<td>pHOM1_358_Firefly-fw</td>
<td>AAATTTAAATTTGAATTCTATGAC TTTGTCACCCTG</td>
<td>Mutagenic primer to introduce G3A, U5C and C8U mutations in the 3' promoter sequence of pHOM1-Firefly (forward)</td>
</tr>
<tr>
<td>pHOM1_358_Firefly_rev</td>
<td>GGAAACAGCTATGACCATGATTA CGCC</td>
<td>Mutagenic primer to introduce G3A, U5C and C8U mutations in the 3' promoter sequence of pHOM1-Firefly (reverse)</td>
</tr>
<tr>
<td>PolI_50-92_PB2_E158G-fw</td>
<td>GCAGATCTTAGTGCTAAGGAGC ACAAGATGTTCATC</td>
<td>Site-directed mutagenesis primer to introduce E158G mutation into Pol I-50-</td>
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<tr>
<td></td>
<td>Forward Primer Sequence</td>
<td>Reverse Primer Sequence</td>
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<tr>
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<td>-------------------------</td>
<td>-------------------------</td>
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<tr>
<td>Poll_50-92_PB2_E158G_rev</td>
<td>GATGACATCTTGTCCTTATAGC</td>
<td>ACTAAGATCTGC</td>
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<td>Poll_50-92_PB2_T271A_fw</td>
<td>CGTGGGAGAGCAGCTGATCAG</td>
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<td>Poll_50-92_PB2_T271A_rev</td>
<td>GGGTCTGCTGATCTGCTGCTCT</td>
<td>CCTAACG</td>
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<tr>
<td>Poll_50-92_PB2_G590S_Q591R_fw</td>
<td>CTAAGGCTGCCAGAAGCCGGTAT</td>
<td>AGTCGATTTG</td>
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<tr>
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<td>CAAATCCACTATACCGCTTCTGG</td>
<td>CAGCCTTAG</td>
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<tr>
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<td>AGGATG</td>
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<td>Poll_50-92_PB2_E627K_rev</td>
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<td>CTGCTG</td>
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<td>Poll_50-92_PB2_D701N_fw</td>
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<td>AAACAAAAGATATGGACCAC</td>
</tr>
<tr>
<td>Name of primer</td>
<td>Sequence</td>
<td>Use (qRT-PCR)</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------------------------------------</td>
<td>--------------------------------------------</td>
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| Oligo(dT)20                    | TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TT
<table>
<thead>
<tr>
<th>PCR_fwd</th>
<th>PCR_rev</th>
<th>E195_M_qRT-PCR</th>
<th>E195_M_cRNA_RT</th>
<th>E195_M_vRNA_RT</th>
<th>HU_IFNβ_qRT-PCR_fwd</th>
<th>HU_IFNβ_qRT-PCR_rev</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CACTAATCAGGCATGAAAACAGA ATGG</td>
<td>AGTAGAAACAAGGTAGTTTT</td>
<td>CACTAATCAGGCATGAAAACAGA ATGG</td>
<td>AAACCTCATGAGCAGTCTGCA</td>
<td>AGGAGATCTTCAGTTTCGGAGG</td>
</tr>
</tbody>
</table>

Amplify E195 M coding region in qRT-PCR (forward)  
Reverse transcribe E195 segment 7 cRNA  
Reverse transcribe E195 segment 7 vRNA  
Amplify Human IFNβ coding region in qRT-PCR (forward)  
Amplify Human IFNβ coding region in qRT-PCR (reverse)

### 6.1.5 Viruses

Table 6: Viruses used in this study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Comments</th>
<th>Source</th>
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<tbody>
<tr>
<td>50-92 PB2</td>
<td>Recombinant virus segments 4 (HA), 6 (NA) and 7 (M) are from PR8 and segments 1 (PB2), 2 (PB1), 3 (PA), 5 (NP), and 8 (NS) are from A/turkey/England/50-92/1991(H5N1)</td>
<td>M. Mura (Barclay Lab)</td>
</tr>
<tr>
<td>50-92 PB2 158G</td>
<td>Recombinant virus segments 4 (HA), 6 (NA) and 7 (M) are from PR8 and segments 1 (PB2), 2 (PB1), 3 (PA), 5 (NP), and 8 (NS) are from A/turkey/England/50-92/1991(H5N1)</td>
<td>This study</td>
</tr>
<tr>
<td>Virus Code</td>
<td>Description</td>
<td>Notes</td>
</tr>
<tr>
<td>------------</td>
<td>-------------</td>
<td>-------</td>
</tr>
<tr>
<td>50-92 PB2 271A</td>
<td>Recombinant virus segments 4 (HA), 6 (NA) and 7 (M) are from PR8 and segments 1 (PB2), 2 (PB1), 3 (PA), 5 (NP), and 8 (NS) are from A/turkey/England/50-92/1991(H5N1)</td>
<td>This study</td>
</tr>
<tr>
<td>50-92 PB2 535T</td>
<td>Recombinant virus segments 4 (HA), 6 (NA) and 7 (M) are from PR8 and segments 1 (PB2), 2 (PB1), 3 (PA), 5 (NP), and 8 (NS) are from A/turkey/England/50-92/1991(H5N1)</td>
<td>M. Mura (Barclay Lab)</td>
</tr>
<tr>
<td>50-92 PB2 590S 591R</td>
<td>Recombinant virus segments 4 (HA), 6 (NA) and 7 (M) are from PR8 and segments 1 (PB2), 2 (PB1), 3 (PA), 5 (NP), and 8 (NS) are from A/turkey/England/50-92/1991(H5N1)</td>
<td>This study</td>
</tr>
<tr>
<td>50-92 PB2 627K</td>
<td>Recombinant virus Segments 4 (HA), 6 (NA) and 7 (M) are from PR8 and segments 1 (PB2), 2 (PB1), 3 (PA), 5 (NP), and 8 (NS) are from A/turkey/England/50-92/1991(H5N1)</td>
<td>This study</td>
</tr>
<tr>
<td>50-92 PB2 701N</td>
<td>Recombinant virus Segments 4 (HA), 6 (NA) and 7 (M) are from PR8 and segments 1 (PB2), 2 (PB1), 3 (PA), 5 (NP), and 8 (NS) are from A/turkey/England/50-92/1991(H5N1)</td>
<td>This study</td>
</tr>
<tr>
<td>E195</td>
<td>All gene segments from A/England/195/09</td>
<td>R. Elderfield (Barclay Lab)</td>
</tr>
<tr>
<td>E195 PB2 158G</td>
<td>All gene segments from A/England/195/09</td>
<td>R. Elderfield (Barclay Lab)</td>
</tr>
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</table>
6.1.6 Buffers and culture media

Table 7: Buffers and culture media used in this study

<table>
<thead>
<tr>
<th>Solution</th>
<th>Recipe</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein loading dye (x6)</td>
<td>375 mM Tris-HCl pH 6.8, 6 % SDS, 30 % glycerol, 9 % 2-Mercaptoethanol, 0.03 % bromophenol blue</td>
<td>Protein gel electrophoresis</td>
</tr>
<tr>
<td>Immunoprecipitation lysis buffer</td>
<td>50 mM potassium phosphate pH 8.0, 200 mM NaCl, 0.75 % IGEPAL, 1 mM DTT, 2 mM EDTA, 5 µl/ml RNAsine, 25 % glycerol, one Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche)/10 ml</td>
<td>Lysing cells for immunoprecipitations</td>
</tr>
<tr>
<td>Immunoprecipitation wash buffer</td>
<td>50 mM potassium phosphate pH 8.0, 200 mM NaCl, 0.1 % IGEPAL, 1 mM DTT, 2 mM EDTA,</td>
<td>Washing buffer for immunoprecipitations</td>
</tr>
<tr>
<td><strong>Crystal violet solution</strong></td>
<td>5 µl/ml RNAsine, 25 % glycerol one Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche)/10 ml</td>
<td>Crystal violet staining</td>
</tr>
<tr>
<td><strong>Crystal violet solution</strong></td>
<td>100 ml Crystal violet stock solution 300 ml ethanol 1.6 L water</td>
<td>Crystal violet staining</td>
</tr>
<tr>
<td><strong>Lysogeny Broth (LB)</strong></td>
<td>1 % Oxoid tryptone 0.5 % Oxoid yeast extract 0.5 % NaCl 0.1 % glucose</td>
<td>Culturing transformed bacterial cells</td>
</tr>
<tr>
<td><strong>Lysogeny Broth (LB)</strong></td>
<td>Lysogeny broth 1.5 % (w/v) Difco Agar 1 % Ampicillin</td>
<td>Culturing transformed bacterial cells</td>
</tr>
<tr>
<td><strong>LB agar</strong></td>
<td>100 ml 10 x Earle’s minimal essential medium (EMEM) 28 ml 7.5 % BSA 1 % glutamine (200 mM) 20 ml 7.5 % NaHCO₃ 10 ml 1M HEPES 5 ml 1 % DEAE Dextran (Sigma) 1 % penicillin-streptomycin (5000 IU/ml) 2 % Agarose (Oxoid)</td>
<td>Plaque assays</td>
</tr>
<tr>
<td>Buffer Name</td>
<td>Ingredients</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>SDS running buffer (x10)</td>
<td>30.25 g Tris-base</td>
<td>Buffer for gel electrophoresis in western blot (wet system)</td>
</tr>
<tr>
<td></td>
<td>144 g glycine</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 g SDS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ddH₂O to 1 litre</td>
<td></td>
</tr>
<tr>
<td>SOC medium</td>
<td>2 % Oxoid tryptone</td>
<td>Recovery medium used during transformation of <em>Escherichia coli</em> XL10-Gold cells</td>
</tr>
<tr>
<td></td>
<td>0.5 % Oxoid yeast extract</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mM NaCl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 mM KCl</td>
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</tr>
<tr>
<td></td>
<td>10 mM MgCl₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mM MgSO₄</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 mM glucose</td>
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<tr>
<td>TAE buffer</td>
<td>40 mM Tris-acetate pH 8</td>
<td>DNA gel electrophoresis</td>
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<tr>
<td></td>
<td>1 mM EDTA</td>
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<tr>
<td>Western blot wash buffer</td>
<td>PBS</td>
<td>Buffer for washing membrane during antibody staining of proteins in western blots analysis</td>
</tr>
<tr>
<td></td>
<td>0.1 % Tween 20</td>
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</tr>
<tr>
<td>Western blot transfer buffer</td>
<td>5.85 g Tris-base</td>
<td>Buffer for transfer of proteins to PVDF membrane in western blot (wet system)</td>
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<tr>
<td></td>
<td>29 g glycine</td>
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</tr>
<tr>
<td></td>
<td>1 g SDS</td>
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</tr>
<tr>
<td></td>
<td>250 ml ethanol</td>
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</tr>
<tr>
<td></td>
<td>dd H₂O to 1 litre</td>
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### 6.2 Molecular cloning techniques and nucleic acid preparation

### 6.2.1 Standard polymerase chain reaction conditions
PCR reactions were carried out in a total volume of 50 μl. Reaction mixtures contained 50 ng of plasmid DNA, 50 pmol of sense and anti-sense oligonucleotide primers, 5 μl of 10X Thermo Pol buffer (New England Biolabs), 1 μl of dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP), and 1 μl of Vent polymerase (New England Biolabs). Standard PCR conditions are presented in Table 7.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Process</th>
</tr>
</thead>
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<td>1</td>
<td>96 °C</td>
<td>3 min</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>2</td>
<td>96 °C</td>
<td>30 sec</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>55 °C</td>
<td>30 sec</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>72 °C</td>
<td>1 min/Kb</td>
<td>Elongation</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>Go to step 2, repeat 30 times</td>
</tr>
<tr>
<td>6</td>
<td>72 °C</td>
<td>4 min</td>
<td>Final extension</td>
</tr>
<tr>
<td>7</td>
<td>4 °C</td>
<td></td>
<td>Hold</td>
</tr>
</tbody>
</table>

6.2.2 Agarose gel electrophoresis

DNA samples were resuspended in loading buffer (New England Biolabs) and separated on 1 % agarose gels diluted with 0.5X TAE buffer and supplemented with 1 μg/ml Gel Red (Cambridge Bioscience). Gels were run in 0.5X TAE buffer. Samples were run alongside a 1 kb DNA size ladder (Invitrogen) at 100 - 150 V for a sufficient time to achieve clear separation of the bands. DNA was visualised using a UV transiluminator.
6.2.3 DNA product purification

The SV Gel and PCR Clean-Up system (Promega) was used to isolate PCR products and DNA excised from gels following manufacturer’s instructions.

6.2.4 DNA digestion by restriction endonucleases

36 μl PCR purified DNA (or 3 μg of purified plasmid DNA in 36 μl of water) was digested with 2 μl each restriction enzyme (NEB) and 4.4 μl of the appropriate 10X buffer (NEB) at 37 °C for 3 hours. For analytical digests, 1 μg of DNA was digested with 1 μl of each restriction enzyme and 2 μl of the appropriate 10X buffer at 37 °C for 3 hours.

6.2.5 DNA ligation

Typically, 6 μl of the digested insert and 1 μl of the digested vector were ligated with 1 μl T4 DNA ligase buffer and 1 μl T4 DNA ligase enzyme (NEB) in a total volume of 10 μl. The ligation reactions were incubated at room temperature for 1 hour or overnight.

6.2.6 Transformation of competent bacterial cells

XL10-Gold ultracompetent cells (Agilent Technologies) were gently thawed on ice and 45 μl aliquots were prepared. 5 μl of ligated DNA was added to an aliquot of the ultracompetent cells and the transformation reaction was incubated on ice for 30 minutes. The tubes were
heat-pulsed in a 42 °C water bath for 30 seconds prior to a 2 minute incubation on ice. 0.5 ml of room temperature SOC broth was added to each tube and the tubes were incubated with shaking at 37 °C for 1 hour. The transformation reactions were spread on LB agar plates containing 100 µg/ml ampicillin and incubated at 37 °C overnight.

6.2.7 Small scale plasmid purification

A single bacterial colony was picked from LB agar plates containing 100 µg/ml ampicillin and grown overnight in 5 ml LB containing the same antibiotic at 37 °C, with shaking. 300 µl of the bacteria suspension was mixed with 700 µl sterile 80 % glycerol and stored at -80 °C as a glycerol stock. The remaining bacterial cells were harvested by centrifugation at 3000 rpm for 10 minutes at 4 °C. The supernatant was discarded. Plasmid DNA was purified using the QIAprep spin miniprep kit (QIAGEN) following the manufacturers’ instructions. The membrane was washed and plasmid eluted with 36 µl of warm sterile water in a 1.5 ml Eppendorf tube. Plasmids were stored at -20 °C to prevent DNA degradation.

6.2.8 Large scale plasmid purification

250 ml LB medium containing 100 µg/ml ampicillin was inoculated with 100 µl from a glycerol stock and grown overnight with shaking at 37 °C. The bacterial cells were harvested by centrifugation at 3000 rpm for 30 minutes at 4 °C. Plasmids were recovered using the high-speed plasmid purification maxi kit (QIAGEN) according to the manufacturer’s instructions. The plasmids eluted were 500 µl of warm sterile water in a 1.5 ml Eppendorf tube. Plasmids were stored at -20 °C to prevent DNA degradation.
6.2.9 Measuring DNA and RNA concentration

DNA and RNA concentration was measured using the NanoDrop® ND-1000 (NanoDrop Technologies).

6.2.10 DNA sequencing

Sequencing of constructs was conducted by the MRC DNA Core Genomics Laboratory, Imperial College London, using the ABI3730xl instrument. 600 ng DNA was sequenced using 3.2 pmol primer in a total volume of 10 µl. Sequencing results were analysed using Geneious v5.1.

6.2.11 Plasmid construction

6.2.11.1 Site-directed mutagenesis

Site directed mutagenesis on the 50-92 rescue plasmids was performed using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). Site directed mutagenesis was achieved by overlap extension PCR for all other constructs. Two mutagenic primers, containing the mutation with complete complementary to each other were designed. Each primer was used in a separate reaction with an outer flanking primer designed to one end of the region of interest using the PCR protocol in Table 8. The 2 PCR products were purified and equal amounts were used as templates in the proceeding PCR
reaction. 30 PCR cycles as in Table 8 were performed before addition of the primers. This PCR protocol was then repeated to amplify the full length product.

**6.2.11.2 Positive-sense firefly minigenome reporter construction**

A positive-sense firefly minigenome reporter (pHPOM1-Firefly-cRNA) was constructed from the negative-sense firefly minigenome reporter by overlapping PCR. Initially the reporter gene was amplified using the primers pHOM1_Firefly_cRNA_fw and pHOM1_Firefly_cRNA_rev. The Pol I promoter was amplified using the Pol I plasmid internal primer MI3fw and pHOM1_Firefly_cRNA_rev1. The mouse terminator was amplified using the Pol I plasmid internal primer MI3rev and pHOM1_Firefly_cRNA-fw1. The amplified sequences were sequentially annealed and amplified. The final extended sequence was digested with Xhol and BamHI and ligated into pHOM1-Firefly which had previously been digested with Xhol and BamHI to remove the Pol I promoter, the negative-sense luciferase coding sequence and the mouse terminator.

**6.2.11.3 Cloning 50-92 PB2 into Strep-Flag tagged expression plasmid**

The 50-92 PB2 coding sequences containing host adaptive mutations were amplified from pCAGGS with the primers 50-92_PB2_NotI-fw and 50-92_PB2_XhoI_nostop_rev which introduced a 5’ NotI site and 3’ Xhol site and removed the stop codon. The amplified sequences were ligated into pCAGGS-50-92 PB2-Strep-Flag tag which had been previously digested with NotI and Xhol to remove the PB2 coding sequence.
6.2.11.4 50-92 NEP expression plasmid construction

The 5092 NEP coding sequence was amplified from the Pol I plasmid containing A/Turkey/England/50-92/91 segment 8 cDNA (Pol I-50-92 NS) with the primers 50-92 NEP_NotI_fw and 50-92_NEp_MluI_rev. The primers introduced a 5' NotI site and a 3' MluI site. The amplified sequence was ligated into pCAGGS 50-92 PB2 which had been previously digested with NotI and MluI to remove the 50-92 PB2 coding sequence.

6.2.11.5 E195 PB2 expression plasmid construction

The E195 PB2 coding sequence was amplified from the Pol I plasmid containing A/England/195/09 segment 1 cDNA (Pol I-E195 PB2) with the primers E195_PB2_NotI_fw and E195_PB2_MluI_rev. The primers introduced a 5' NotI site and a 3' MluI site. The amplified sequences were ligated into pCAGGS 50-92 PB2 which had been previously digested with NotI and MluI to remove the 50-92 PB2 coding sequence.

6.3 Cell lines and cell culture techniques

6.3.1 Cell lines, media and maintenance

Human embryonic kidney (293T), chicken fibroblast (DF-1), newborn pig trachea (NTr), adenocarcinomic human alveolar basal epithelial cells (A549) and Madin-Darby Canine Kidney Epithelial (MDCK) were passaged using standard tissue culture techniques. Cells were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) with pyruvate and L-Glutamine,
supplemented with 10 % heat inactivated Foetal Calf Serum (FCS) and 1 % penicillin and streptomycin and maintained at 37 °C, 5 % CO₂. Media for A549-luc cells was supplemented with 2 mg/ml Geneticin (G418). Media for A549-NPro cells was supplemented with 2 μg/ml Puromycin.

6.3.2 Transfections

Typically transfections were performed on 80 % confluent monolayers of cells plated 24 hours beforehand. 30 minutes prior to transfection the media on the cells was removed and replaced with DMEM supplemented with 2 % FBS. Cells were transfected with Lipofectamine 2000 (Invitrogen). For transfections in a 24 well cell culture plate, 2.5 μl of Lipofectamine 2000 transfection reagent was mixed with 97.5 μl of Optimem (Invitrogen). Separately, plasmid DNA was mixed with 50 μl of Optimem, before the two mixtures were combined and incubated at room temperature for 20 minutes. The transfection mixture was then added dropwise to cells and the cell culture media was replaced with DMEM containing 10 % FBS 3 hours post transfection. Quantities were increased or decreased as appropriate for larger or smaller scale transfections.

6.3.2 Minireplicon assay

Typically, cells were transfected in 24 well plates with plasmids encoding PB1, PB2, PA and NP proteins (NP: 160 ng, PB1 and PB2: 80 ng, PA: 20 ng) together with a reporter expressing plasmid (80 ng) and a Renilla expression plasmid. When DF-1 and NPTr cells were used, twice the normal amount of DNA was transfected. Cells were lysed with 200 μl of Passive
lysis buffer (Promega) and luciferase activity was measured using a FLUOstar Omega plate reader (BMG Labtech).

6.3.3 IFN assays

Typically, cells were transfected in 24 well plates with plasmids encoding PB2 or NS1 proteins (50 ng), together with a Luciferase reporter plasmid with an IFN-β promoter (25 ng), a plasmid expressing MAVS (25 ng) and a Renilla expression plasmid. Cells were incubated for 24 hours and lysed with 200 μl of Passive lysis buffer (Promega). Luciferase activity was measured using a FLUOstar Omega plate reader (BMG Labtech).

6.4 Protein manipulation

6.4.1 Immunoprecipitations

293T cells seeded in 6 well plates to reach 80 % confluency were transfected with plasmids encoding 50-92 PB1, PB2, PA and NP proteins (NP: 640 ng, PB1 and PB2: 320 ng, PA: 80 ng) together with 320 ng pHOM1-Firefly. 12 hours post transfection, cells were washed with PBS, detached and collected by centrifugation. Cells were lysed in 500 μl of cell lysis buffer (50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM DTT, 2 mM EDTA, 0.75 % IGEPAL, 25 % glycerol, one Complete Mini EDTA-free protease inhibitor cocktail tablet (Roche)/10 ml, 5 μl/mL RNasin (Promega)). Cells were incubated for 1 hour on ice prior to centrifugation at 16,000 xg for 30 minutes at 4 °C. Supernatants were collected and incubated with 3μl of a rabbit polyclonal antibody specific for NP for 30 minutes on ice followed by incubation with 40 μL protein-A agarose (Santa Cruz) at 4 °C for 2 hours on a rotating device. The agarose
was washed 4 times with wash buffer (50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM DTT, 2 mM EDTA, 0.1 % Igepal CA-630 (Sigma), 25 % glycerol, one Complete Mini EDTA-free protease inhibitor cocktail tablet/10 ml, 5 µl/mL RNasin), prior to dilution in 6X sample buffer (375 mM Tris-HCl pH 6.8, 6 % SDS, 30 % glycerol, 9 % 2-Mercaptoethanol, and 0.03 % bromophenol blue).

6.4.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples diluted in sample buffer were heated at 100 °C for 10 minutes. Protein samples were loaded onto Tris HCL 4 – 15 % linear gradient gels (Bio-Rad) alongside a Novex sharp pre-stained protein standard (Invitrogen) and run at 120 V in running buffer (27.6 mM Tris base, 0.2 M glycine, 0.1 % SDS, pH 8.8).

6.4.3 Western blotting

Proteins were transferred onto 0.45 μm nitrocellulose membrane (Anachem) for 1 hour at 100 V in chilled transfer buffer. Membranes were blocked in 5 % milk solution (milk powder, 0.1 % tween, dissolved in PBS) for 1 hour at room temperature prior to incubation with primary antibody in milk solution for 2 hours at room temperature. Membranes were then washed 3 times in wash buffer (0.1 % tween in PBS) prior to a 1 hour incubation at room temperature with a HRP-conjugated secondary antibody diluted in milk solution. Following 3 washes, the membranes were incubated with ECL Plus Western Blotting Detection System reagents (Amersham) and developed using a Fuji X-ray Film processor.
6.5 RNA manipulation

Whilst working with RNA, care was taken to minimise RNase contamination. RNaseZAP (Ambion) was routinely used to remove RNase from all surfaces and equipment.

6.5.1 RNA extraction

Total RNA from $1 \times 10^5$ cells was purified using an RNeasy mini kit (Qiagen), following manufacturer's instructions. RNA was subject to an on-the-column treatment with RNase-Free DNase (Qiagen), following manufacturer's instructions.

6.5.2 Reverse transcription

Purified total RNA was subject to first-strand cDNA synthesis with gene specific primers or oligo(dT)20 which reverse transcribes all mRNA. A reaction mixture consisting of 500 ng RNA, 1 µl 10 mM dNTP mix (Invitrogen) and 2 pmol of the gene specific primers or 500 ng of oligo(dT) and RNase-free water up to 13 µl was incubated at 65°C for 5 minutes. The reaction was then incubated at 4 °C for 1 minute prior to the addition of 4 µl 5X First-Strand Buffer, 1 µl 1 M DTT, 1 µl RNaseOUT and 1 µl SuperScript III (Invitrogen). Reactions were then incubated at 55 C for 60 minutes, followed by inactivation at 70 °C for 15 minutes. RNA complementary to the cDNA was removed by incubation with 2 units of RNase H (Invitrogen) at 37 °C for 20 minutes. The resulting cDNA was stored at -20 °C.

The pHPOM1-Firefly, pSPOM-Firefly and pCKPOM1-Firefly reporter plasmids contain the luciferase gene in the reverse orientation between the 5' and 3' terminal promoter
sequences of the influenza virus segment 8. The Luc_vRNA_RT primer, which is complementary to the luciferase coding region, was used to reverse transcribe luciferase vRNA. The Luc_cRNA_RT primer, which is complementary to the 3' portion of the segment 8 cRNA, was used to reverse transcribe luciferase cRNA.

To determine the accumulation of PR8 segment 7 cRNA and vRNA in infected cells specific primers were used. The PR8_M_vRNA_RT primer, which is complementary to the M gene coding region, was used to reverse transcribe segment 7 vRNA. The PR8_M_cRNA_RT primer, which is complementary to the 3' portion of the segment 7 cRNA, was used to reverse transcribe segment 7 cRNA.

To determine the accumulation of E195/50-92 segment 7 cRNA and vRNA in infected cells specific primers were used. The E195_M_vRNA_RT primer, which is complementary to the M gene coding region, was used to reverse transcribe segment 7 vRNA. The E195_M_cRNA_RT primer, which is complementary to the 3' portion of the segment 7 cRNA, was used to reverse transcribe segment 7 cRNA.

6.5.3 Real-time quantitative PCR analysis

The synthesized single stranded cDNAs were subject to real-time quantitative PCR analysis (Applied Biosystems® Viia™ 7 Real-Time PCR System) with two gene specific primers. The PCR reaction mix was prepared in a MicroAmp 96-well reaction plate (Applied Biosystems). Each reaction contained 10 µl Fast SYBR Green Master Mix (2X) (Applied Biosystems), 25 ng cDNA, 200 ng of each forward and reverse primer and RNase-free water to a total volume of
20 μl. The plate was centrifuged briefly prior to an initial denaturation at 95 °C for 20 seconds, before 40 cycles of 0.1 seconds at 95 °C, 20 seconds at 60 °C, followed by 15 seconds at 95 °C and 1 second at 60 °C.

6.5.4 Detecting DI RNAs

A549 cells, in triplicate, were infected with an MOI of 3. Following inoculation for an hour at 37 °C virus was removed and serum-free DMEM was added. At 8 h post-infection cells were lysed with RLT buffer (Qiagen). Total RNA was purified using an RNeasy mini kit (Qiagen). Reverse transcription was performed using a primer complementary to the 3' portion of segment 1 vRNA. The synthesized single stranded cDNAs were subject to PCR analysis with primers specific to the 3' and 5' portions of segment 1. Amplified PCR products were run on an agarose gel.

6.5.5 Viral RNA extraction

Viral RNA was extracted using the QIAamp viral RNA mini kit (QIAGEN) according to manufacturers' instructions. 140 μl of virus stock was lysed using the AVL buffer containing RNAsin. This was bound to a silica membrane, washed and eluted in 30 μl of AVE elution buffer. vRNA was stored at -80°C.

6.5.6 Sequencing viral RNA
Purified RNA was subject to first-strand cDNA synthesis with random hexamers. 11 µl RNA, 1µl 10mM dNTP mix (Invitrogen) and 1 µl random hexamers (Invitrogen) were incubated at 65 °C for 5 minutes, prior to a 1 minute incubation at 4 °C. 4 µl 5X First-Strand Buffer, 1 µl, 1M DTT, 1 µl RNaseOUT and 1 µl SuperScript III (Invitrogen) were then added. Reactions were incubated at 25 °C for 5 minutes, 50 °C for 60 minutes and 70 °C for 15 minutes. PCR reactions were undertaken with the primers E195_PB2_F and E195_PB2_R or 50-92_PB2_F and 50-92_PB2_R as appropriate. Products were purified using a PCR purification kit (Promega) and sent for sequencing at the MRC DNA Core Genomics Laboratory, Imperial College London.

6.6 Virus manipulation

6.6.1 Rescue of Influenza virus

Rescue of influenza virus was conducted at the appropriate Biological containment level, after risk assessment of the pathogen to be generated, set by the Advisory Committee on Dangerous Pathogens (ACDP). BCL 3 was used for all virus rescues in this study.

Co-culture virus rescue: A plasmid reverse genetics approach was used to rescue mutant viruses. 12 plasmids (8 Pol I plasmids and 4 protein expression plasmids) were transfected into 293T cells, using serum free DMEM in 6 well plates, with cells of 70 - 80 % confluency. 20 µl Fugene was added to 200 µl Optimem and incubated for 5 minutes at room temperature. The DNA mix (listed in Table 9) was then added and incubated with the Fugene mix for a further 20 minutes at room temperature, before being added, drop-wise,
to the cell monolayer. The transfections were incubated at 37 °C for 6 hours. The transfection mix was then replaced with serum free DMEM containing NEAA.

Table 9: Co-culture virus rescue plasmids

<table>
<thead>
<tr>
<th>Pol I plasmid</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pol I-50-92 PB1</td>
<td>1 µg</td>
</tr>
<tr>
<td>Pol I-50-92 PB2</td>
<td>1 µg</td>
</tr>
<tr>
<td>Pol I-50-92 PA</td>
<td>1 µg</td>
</tr>
<tr>
<td>Pol I-50-92 NS</td>
<td>1 µg</td>
</tr>
<tr>
<td>Pol I-50-92 NP</td>
<td>1 µg</td>
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<tr>
<td>Pol I-PR8 NA</td>
<td>1 µg</td>
</tr>
<tr>
<td>Pol I-PR8 HA</td>
<td>1 µg</td>
</tr>
<tr>
<td>Pol I-PR8 M</td>
<td>1 µg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Helper plasmid</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCI-Victoria PB1</td>
<td>1 µg</td>
</tr>
<tr>
<td>pCI-Victoria PB2</td>
<td>1 µg</td>
</tr>
<tr>
<td>pCI-Victoria PA</td>
<td>0.5 µg</td>
</tr>
<tr>
<td>pCI-Victoria NP</td>
<td>2 µg</td>
</tr>
</tbody>
</table>

MDCK co-culture: The next day transfected 293T cells were resuspended in 1mL DMEM with 2 % FBS. The resuspended 293T cells were transferred into a 25 cm² flask of 70 % confluent MDCKs in DMEM with 2 % FBS. These cells were allowed to adhere for 6 hours at 37 °C. Cells were then washed in serum-free DMEM and 5 ml serum-free DMEM with NEAA’s and 1
μg/ml trypsin was added. Cultures were incubated at 37 °C for 2-3 days until cytopathic effect was observed, or haemagglutination activity was observed by a Haemagglutination assay. Virus rescues were harvested, cell debris removed by centrifugation at 3000 rpm for 5 minutes, and resulting aliquots stored at -80 °C.

6.6.2 Plaque assay

12 well plates of confluent MDCKs were washed with serum free DMEM. Virus was diluted in a 10-fold serial dilution using serum free DMEM. 200ul of each viral sample was added to a well of MDCKs for 1 hour at 37 °C and 5 % CO₂. Medium was then removed and 2 % agarose overlay medium containing 1 μg/ml trypsin was added. Cells were incubated at 37 °C and 5 % CO₂ for 3 days and then visualised by crystal violet staining.

6.6.3 Haemagglutination assay

Virus was diluted in a two-fold serial dilution using PBS in 96 well V-bottomed plates. An equal volume of 0.5 % chicken blood cell suspension (diluted in PBS) was then added to each well. After at least 1 hour incubation on ice, the resulting HA titre was determined, as the well prior to the first well displaying a blood pellet.

6.6.4 Virus infection

Viruses were plaqued on MDCK cells in triplicate prior to use in growth curves.
**Multi-cycle growth curve:** Confluent A549 cells in 6 well plates were washed with PBS and infected with an MOI of 0.01 in 500 µl. The cells were incubated at 37 °C and 5 % CO₂ for 1 hour, the inoculum was removed, the cells were washed with PBS and serum free DMEM containing 1 µg/ml trypsin was added. The cells were then incubated 37 °C and 5 % CO₂. 24, 48 and 72 hours post infection a 200 µl aliquot was removed and stored at -80 °C. Cell culture supernatants were titrated on MDCKs by plaque assay.

**Single-cycle growth curve:** Confluent A549 cells in 6 well plates were washed with PBS and infected with an MOI of 3 in 500 µl. The cells were incubated at 37 °C and 5 % CO₂ for 1 hour, the inoculum was removed, the cells were washed with PBS with serum free DMEM containing 1 µg/ml trypsin was added. The cells were then incubated 37 °C and 5 % CO₂. 200µl cell culture supernatants were removed at various time points post infection and were titrated on MDCKs by plaque assay.

### 6.6.5 Growth of virus stocks

MDCK cells grown to 70 % confluency in 75 cm² flasks were infected with virus at an MOI of 0.001 in 2 ml serum free DMEM for one hour at 37 °C. On removal of the initial inoculum, 12 ml serum free DMEM containing 1 µg/ml trypsin was added. Cultures were incubated at 37 °C for 2 - 3 days until cytopathic effect was observed, or haemagglutination activity was observed by a Haemagglutination assay. Virus rescues were harvested, cell debris removed by centrifugation at 3000 rpm for 5 minutes, and resulting aliquots stored at -80 °C.
6.6.6 IFN-β luciferase assay

The A549 IFN-luc cell line was previously generated in the laboratory by L. Hartgroves. This cell line contains the human IFNβ luciferase reporter plasmid. This plasmid is maintained in the cell line by G418 selection.

Confluent A549-luc cells in 24 well plates were washed with PBS and infected with an MOI of 3 in 50 µl serum free DMEM. Cells were incubated with the inoculum at 37 °C and 5 % CO₂ for 1 hour before the cells were washed with PBS and serum free DMEM containing 1 µg/ml trypsin was added. Cells were then incubated 37 °C and 5 % CO₂. At 12 and 24 hours post-infection cells were lysed with 200 µL of Passive lysis buffer (Promega) and luciferase activity was measured using a FLUOstar Omega plate reader (BMG Labtech).

6.6.7 Mouse pathogenicity experiment

Female BALB/c mice, 6–8 weeks old, were housed in groups of five and inoculated with 2x10⁵ PFU of virus diluted in PBS or PBS alone (Mock) under isofluorane anaesthetic. Mice were weighed daily and would have been euthanized upon loss of more than 20 % of the start weight. On days 2 and 6 post-infection groups of five animals were sacrificed, the lungs individually harvested and frozen at -80 °C. Lungs were weighed and 500 ml PBS added prior to homogenization. Viral load was assessed by plaque assay of lung homogenates on MDCK cells. The amount of expressed IFNβ in the lung homogenates was assessed using the VeriKine Mouse Interferon Alpha ELISA kit (R&D).
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