HHS Public Access

Author manuscript

Curr Top Microbiol Immunol. Author manuscript; available in PMC 2015 October 24.

Published in final edited form as:

Curr Top Microbiol Immunol. 2014 ; 385: 243–274. doi:10.1007/82_2014_388.

Molecular Determinants of Influenza Virus Pathogenesis in Mice

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Abstract

Mice are widely used for studying influenza virus pathogenesis and immunology because of their low cost, the wide availability of mouse-specific reagents, and the large number of mouse strains available, including knockout and transgenic strains. However, mice do not fully recapitulate the signs of influenza infection of humans: transmission of influenza between mice is much less efficient than in humans, and influenza viruses often require adaptation before they are able to efficiently replicate in mice. In the process of mouse adaptation, influenza viruses acquire mutations that enhance their ability to attach to mouse cells, replicate within the cells, and suppress immunity, among other functions. Many such mouse-adaptive mutations have been identified, covering all 8 genomic segments of the virus. Identification and analysis of these mutations have provided insight into the molecular determinants of influenza virulence and pathogenesis, not only in mice but also in humans and other species. In particular, several mouse-adaptive mutations of avian influenza viruses have proved to be general mammalian-adaptive changes that are potential markers of pre-pandemic viruses. As well as evaluating influenza pathogenesis, mice have also been used as models for evaluation of novel vaccines and anti-viral therapies. Mice can be a useful animal model for studying influenza biology as long as differences between human and mice infections are taken into account.

1 Introduction

Waterfowl are the natural hosts for most influenza viruses, harboring 16 of the 18 known hemagglutinin (HA) and 9 of the 11 known neuraminidase (NA) subtypes of influenza A viruses. A limited number of influenza A virus subtypes have become established in humans and other mammalian hosts and have been responsible for tens of millions of human deaths following their emergence in humans, during the initial pandemic phase and during subsequent sustained seasonal transmission in the endemic phase. Besides influenza viruses that are endemic human pathogens, several avian- or swine-origin subtypes are capable of

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zoonotic infection, with the potential of causing another pandemic. Therefore, the mechanisms of influenza pathogenicity are therefore the subject of intense research, especially the molecular mechanisms that allow non-human influenza viruses to become adapted to efficient human infection and transmission. Mice are a popular animal model for influenza research. We focus this review on molecular determinants that confer increased pathogenicity to influenza viruses in mice.

As with any disease model, the mouse has both advantages and disadvantages for the study of influenza pathogenesis. The relatively low cost of mice, the ease of handling, the wide range of mouse-specific reagents, and the availability of many inbred, transgenic, and knockout mouse strains make them an attractive model. On the other hand, some aspects of influenza infection and disease differ between mice and humans, complicating the application of mouse-based research to human health.

Initial attempts to characterize influenza viruses from infected humans involved transmission experiments between humans and many different species, including mice; however, despite an early claim of mouse infection that was not reproduced (Gibson and Connor 1918), “all such attempts were entirely unsuccessful until the ferret was used” (Smith et al. 1933). However, shortly after transmission to ferrets was demonstrated, several groups demonstrated that outbred mice could be infected with both swine and human influenza viruses (Andrewes et al. 1934; Francis 1934; Shope 1935). These early investigators observed signs that included weight loss, lethargy, increased respiratory effort, and loss of appetite, as well as lung pathology with extensive consolidation and fluid accumulation. Significant mortality was seen in mice following infection with high doses of the viral filtrates. Notably, there was evidence of adaptation of some, but not all, influenza viruses, with some isolates showing a marked increase in mice virulence after repeated passages in mice (Andrewes et al. 1934; Francis 1934; Francis and Magill 1935; Shope 1935; Stuart-Harris 1939) while other isolates were virulent even without mouse adaptation (Shope 1935). All these findings still hold true today.

2 Influenza Infection of Mice

While some human seasonal influenza A and B viruses can replicate in the murine respiratory tract, mice are not a natural host for influenza viruses, and adaptation through repeated passage is usually required to increase the virulence of the human-origin viruses for this species (Andrewes et al. 1934; Beaudette et al. 1957; Francis 1934; Francis and Magill 1935; Hirst 1947; Shope 1935). Importantly, even non-mouse-adapted influenza viruses that cause minimal or no disease in mice are often capable of some replication in the respiratory tract (Hirst 1947), since otherwise mouse passage would not allow adaptation to occur. The adaptation process increases the ability to replicate and may also increase virulence, either directly as a result of increased replication or through other factors.

A number of mouse-adapted strains of virus are commonly used, with A/Puerto Rico/8/1934(H1N1) (PR8) (Francis 1934; Francis and Magill 1935) and A/WSN/1933(H1N1) (WSN) (Stuart-Harris 1939) being the most common in early studies. The WSN virus was adapted through repeated mouse brain passage, resulting in a strain that in mice is

neurotropic and highly virulent (Stuart-Harris 1939). PR8 is also widely used in reassortment as a recipient for HA and NA genes from other influenza viruses. A reassortant virus (X-31) containing HA and NA from an H3N2 pandemic virus, A/Aichi/2/1968(H3N2), and remaining gene segments from PR8 (Kilbourne et al. 1971) is often used as a prototype H3N2 virus in mouse studies.

The virulence of non-mouse-adapted strains of the influenza virus to mice varies widely (reviewed in Bouvier and Lowen 2010). Contemporary seasonal H3N2 and former (pre-2009 pandemic) seasonal H1N1 viruses generally replicate poorly in mice, even after extensive passage. However, pandemic H1N1 viruses including the 1918 H1N1 pandemic virus (Tumpey et al. 2005) and the 2009 A(H1N1)pdm09 pandemic strain (H1N1pdm09) (Belser et al. 2010; Maines et al. 2009) replicate efficiently in mouse respiratory tissues and cause disease in mice without prior adaptation, although some adaptation was required to derive mouse-lethal H1N1pdm09 viruses. Some swine viruses are also pathogenic to mice without adaptation (Belser et al. 2010), as are a number of avian influenza (AI) viruses (AIV). AI viruses are divided into “low-pathogenicity AIV (LPAIV)” and highly pathogenic AIV (HPAIV) based on their pathogenicity in chickens, and some members of both groups are able to infect and cause disease in mice without adaptation (Driskell et al. 2010). In general, LPAIV are less likely to be highly virulent in mice than are HPAIV; the latter, especially those of the H5N1 HPAI lineage, often cause very severe disease in mice (Gao et al. 1999; Gubareva et al. 1998; Lu et al. 1999). However, even some LPAI viruses, such as H7N9, can cause severe disease in mice, albeit at higher doses than required for many HPAI viruses (Xu et al. 2013).

Mice are typically inoculated with influenza virus by intranasal droplets; aerosol infection may also be used, and may be associated with increased morbidity and mortality (reviewed in Gustin et al. 2012). Depending on the virus, volume of inoculum, and level of sedation, after intranasal inoculation the virus initiates replication in the nasal turbinates and may then descend to the lower respiratory tract, which is the main site of replication. Unlike humans and ferrets, mice infected with influenza virus do not exhibit sneezing, coughing, or marked fever. When disease is present, the most common manifestations are weight loss, ruffled fur, shivering, hunched posture, hypothermia, and reduced activity. Weight loss, measured daily over the course of infection, is a readily quantifiable parameter used to evaluate morbidity. Pathologic and histopathologic evidence of lung edema, epithelial cell damage, and inflammation are often apparent. Viral lung titers are commonly used as an indicator of viral replication, especially when evaluating immunity or antiviral treatment. Depending on the challenge dose, mortality (including euthanasia based on humane endpoints) may be used as a measure of disease severity (Lu et al. 1999; Matsuoka et al. 2009a; Mount and Belz 2010; Narasaraju et al. 2009; O’Donnell and Subbarao 2011; Raut et al. 1975; Tripp and Tompkins 2009; van der Laan et al. 2008; Ward 1997; Xu et al. 2013). Mice have also been used to model the comparative ability of influenza viruses of different subtypes to use the eye as a portal of entry and initiation of infection (Belser et al. 2009).

Although most viruses infectious for mice replicate primarily in the respiratory tract, some viruses are capable of efficient extrapulmonary dissemination. One factor that permits systemic spread is the presence of a polybasic cleavage site in HA (discussed further below),

which allows viral replication outside the respiratory tract. H5N1 HPAI viruses, which usually possess this polybasic cleavage site, frequently show systemic spread after a few days of infection, including to the brain (Lu et al. 1999; Spesock et al. 2011). Similarly, the neurotropic H1N1 strain WSN is capable of spread outside the respiratory tract (Ward 1996). In these cases, neurological signs such as ataxia and hind-limb paralysis may be seen, and the disease is generally fatal.

Even in susceptible mice infected with mouse-adapted strains of influenza, the virus is rarely spontaneously transmitted between animals, even by direct contact (Lowen et al. 2006). In the occasional cases where influenza transmission between mice has been reported (Andrewes et al. 1934; Eaton 1940; Edenborough et al. 2012; Price et al. 2014; Schulman 1967, 1968; Schulman and Kilbourne 1962; 1963a, b), the transmission is much more efficient through direct contact than via aerosol spread (Price et al. 2014), and transmission only occurs among a minority of animals.

3 Host Genetics Influencing Susceptibility to Influenza

Mouse strains differ markedly in their resistance to influenza infection. Most laboratory mice are much more susceptible to influenza viral infection than wild mice (Haller 1981; Lindenmann 1964), since laboratory strains carry a large deletion in the myxovirus resistance 1 (MX1) gene (Staeheli et al. 1988), an interferon (IFN)-induced gene that confers protection against many viruses (Haller et al. 2007). However, even strains of mice that lack MX1 have very different susceptibilities, with A/J mice being more susceptible than the widely-used C57BL/6 and BALB/c strains (Srivastava et al. 2009) and DBA mice being still more susceptible (Alberts et al. 2010; Pica et al. 2011; Srivastava et al. 2009; Trammell et al. 2012).

Several recent reviews (Horby et al. 2012, 2013; Korth et al. 2013; Trammell and Toth 2008) have discussed genetic variations of mice associated with influenza susceptibility. With the exception of MX1 (Ferris et al. 2013), the function and relative importance of most of these genetic factors are not well understood, although some are potentially associated with inflammation, such as Tnfrsf21 (Boivin et al. 2012), IL16, and Nox4 (Ferris et al. 2013). Importantly, crosses between resistant and susceptible mice display a gradient of resistance, suggesting that resistance is polygenic rather than involving a limited number of genes (Boon et al. 2011; Bottomly et al. 2012; Ferris et al. 2013; Horby et al. 2013).

A major factor influencing the severity of influenza disease in mice is the inflammatory response induced by the virus, with increased inflammation being associated with increased disease severity (Askovich et al. 2013; Brandes et al. 2013; Cilloniz et al. 2010; Song et al. 2013a; Wyde and Cate 1978; Wyde et al. 1978). Many genes associated with inflammation are strongly upregulated following influenza infection of mice, especially following highly virulent viruses (Boon et al. 2011; Josset et al. 2012; Kash et al. 2006; Korth et al. 2013; Trammell and Toth 2008). Many of these genes are differentially regulated in different mouse strains (Alberts et al. 2010; Ding et al. 2008), with susceptible strains showing a more robust inflammatory response to influenza infection than do the more resistant strains (Alberts et al. 2010; Boon et al. 2011; Srivastava et al. 2009; Trammell et al. 2012),

suggesting that at least one reason for differential influenza susceptibility may be variations in immune responses. However, the genetic loci involved are as yet unclear, and the increased inflammation may be secondary to increased viral load, which may be controlled by other host factors (Boon et al. 2011; Ferris et al. 2013).

One advantage of the mouse as a model system is the relative ease of generating mutant mice with alterations in specific genes. Knockout and transgenic mice have demonstrated the importance of many genes in influenza pathogenesis. Examples include transgenic mice in which MX1 expression was restored, conferring greatly increased influenza resistance, and knockout mice lacking RIG-I (Kato et al. 2006) or the IFN-inducible gene IFITM3 (Everitt et al. 2012), which are highly susceptible to influenza infection. Conversely, mouse knockouts lacking IL-17RA, IL-15, CC-chemokine receptor 2 (CCR2), or a combination of tumour necrosis factor (TNF) and IL-1 receptors, were relatively resistant to mortality due to influenza virus infection (reviewed in Medina and Garcia-Sastre 2011), further suggesting that at least in some cases influenza mortality in mice may be due to an overactive inflammatory response. It is important to note that while complete knockout of a gene can demonstrate the role of that gene in the response to influenza, it does not tell us about differential responses associated with allelic variation.

4 Viral Determinants of Influenza Virulence in Mice

Many components of influenza viruses contribute to their ability to infect mice. Identification of the viral molecular determinants involved in mouse virulence can be approached in a number of ways. By analyzing strains of influenza with differential virulence in mice (e.g., viruses before and after mouse adaptation), genetic changes associated with mouse virulence can be identified. However, specific genetic changes in these viruses are not necessarily associated with mouse virulence; some changes may have been selected as a result of other laboratory properties, such as growth in eggs, or may simply represent genetic drift. For example, the PR8 lineage of the virus, which has been grown in the laboratory for many decades, has diverged into multiple strains with different genome sequences, all of which are still capable of causing disease in mice to varying extents (Blazejewska et al. 2011; Grimm et al. 2007; Liedmann et al. 2013).

Another approach to identifying mouse virulence-associated variants is to introduce specific mutations into viruses, and then test the resulting strains for their ability to infect mice. This reverse genetics approach may also be used to confirm the functional importance of the variants found in mouse-adapted viral strains. A disadvantage with this approach is that it becomes difficult to test multiple mutations that act together to alter virulence.

In spite of the technical difficulties involved, a number of mouse adaptation determinants have been identified, spanning all eight segments of the influenza genome (summarized in Table 1). Some of these variants may be specific to mice, while others represent adaptations to mammals versus birds, or even broader virulence determinants that are important for infecting cells in general. Many of the general mammalian determinants have been reviewed recently (Basler and Aguilar 2008; Pepin et al. 2010; Reperant et al. 2012).

4.1 Hemagglutinin

Influenza viruses enter cells after binding to cell-surface receptors consisting of sialic acid (SA) moieties. Sialic acid is a complex structure that can be attached to galactose in a number of different linkages (α2,3; α2,6; α2,8). Influenza viruses of different host origins preferentially bind to different SA configurations, with human viruses preferring SA with an α2,6 linkage to galactose while avian viruses preferentially bind SA with α2,3 linkages (Ge and Wang 2011; Imai and Kawaoka 2012; Shinya et al. 2006). The distribution of SA in the host is therefore an important factor determining influenza pathogenesis. In humans, α2,6-linked SA dominate in the upper airways (Shinya et al. 2006), and human influenza viruses mainly bind to α2,6-linked SA. In mice, α2,3-linked SA are found in both the upper and lower respiratory tract (Ibricevic et al. 2006), so that many human wild-type viruses are poorly or not infectious for mice.

As the protein required for receptor binding, HA is a critical component of influenza infectivity. Multiple studies have linked variants in the HA segment of the influenza genome with enhanced mouse infectivity and virulence (Brown 1990; Kobasa et al. 2004; Pappas et al. 2008; Rudneva et al. 1986; Uraki et al. 2013; Watanabe et al. 2013). In some cases, mouse adaptation of human influenza viruses has been associated with a shift in receptor preference from to α2,6- to α2,3-linked SA (Ilyushina et al. 2010; Keleta et al. 2008). However, several lines of evidence suggest that this is not the sole reason for mouse adaptation. As noted above, even viruses that are avirulent in mice may replicate in the lungs (Hirst 1947). Some viruses that preferentially bind α2,6-linked SA, such as the 1918 pandemic H1N1, replicate efficiently and cause significant disease in mice (Qi et al. 2009; Tumpey et al. 2005), while many LPAIV that preferentially bind α2,3-linked SA cause only minimal morbidity in this species (Driskell et al. 2010).

HPAIV generally have a polybasic cleavage site in their HA (Bosch et al. 1981; Webster and Rott 1987), while HA from mammalian viruses and LPAIV usually have only a single basic residue at their cleavage site (Garten and Klenk 1999; Klenk and Garten 1994). The presence of the polybasic residues allows the HA to be cleaved by enzymes that are expressed in a range of tissues, whereas the standard cleavage site can only be cleaved by enzymes in the respiratory tract. Since HA cleavage is required for viral infectivity, the polybasic cleavage site broadens the tissue range of the virus and is strongly associated with the systemic spread and increased infectivity and virulence in birds and mammals, including mice (Stienke-Grober et al. 1992; Suguitan et al. 2012; Tanaka et al. 2003; Zhang et al. 2012). In H7 viruses, a similar phenotype has arisen on several occasions through non-homologous recombination between HA and other viral or host genes (reviewed in Belser and Tumpey 2014). Similarly, sequence variation at the cleavage site of the WSN strain that confers a broader range of enzyme cleavage is associated with increased neurovirulence (Sun et al. 2010). However, not all HPAIV with a polybasic cleavage site are highly virulent in mice (Gao et al. 1999; Hiromoto et al. 2000; Hu et al. 2012; Katz et al. 2000; Lu et al. 1999).

Mutations in residues in three regions that surround the receptor-binding site (the 190-helix, the 220-loop, and the 130-loop; reviewed in Imai and Kawaoka 2012) have been associated with mouse adaptation (Govorkova et al. 2000; Hiromoto et al. 2000; Ilyushina et al. 2010;

Keleta et al. 2008; Koerner et al. 2012; Narasaraju et al. 2009; Ping et al. 2010, 2011; Smirnov et al. 2000; Song et al. 2013b). This is probably due to changes in receptor binding preference (Imai and Kawaoka 2012; Pekosz et al. 2009).

One mouse-adaptive change of particular interest is at position 222 (in H1 numbering; 225 in H3 numbering). A D222G mutation has repeatedly appeared during mouse adaptation of H1N1 viruses (Ilyushina et al. 2010; Seyer et al. 2012; Smee et al. 2007; Song et al. 2013b; Xu et al. 2011a) and, at least in some cases, increased mouse virulence (Abed et al. 2011; Zheng et al. 2010). This variant is associated with increased pathogenicity of H1N1pdm09 in humans (Baldanti et al. 2011; Kilander et al. 2010) and increases α2,3-linked SA binding of HA (Abed et al. 2011; Belser et al. 2011; Chutinimitkul et al. 2010).

HA is a glycoprotein, with multiple sites of carbohydrate attachment. Loss of glycosylation sites from HA is a common observation during mouse adaptation (Chen et al. 2007; Reading et al. 2007; Shilov and Sinitsyn 1994; Smee et al. 2007; Ward 1996, 1997). Studies with natural variants and reverse genetics viruses have shown that virulence in mice decreases as the number of glycans increases (Kaverin et al. 2002; Medina et al. 2013; Reading et al. 2009; Rudneva et al. 2005; Sun et al. 2013a; Vigerust et al. 2007; Yen et al. 2009). In one exception to the rule, adding glycosylation sites to the HA of H1N1pdm09 viruses increased viral virulence in mice, perhaps related to increased inflammation due to enhanced innate immune recognition (Zhang et al. 2013). Some of this effect may be due to changing receptor specificity (Das et al. 2011; Ohuchi et al. 1997; Sun et al. 2013a; Wang et al. 2009; Yen et al. 2009; Zhang et al. 2013) and thus may be specific to mouse infection. However, increasing glycosylation also renders influenza virus more susceptible to innate immune control (Kaverin et al. 2002; Reading et al. 2007, 2009; Shilov and Sinitsyn 1994; Tate et al. 2011; Vigerust et al. 2007), so that loss of glycosylation is probably an adaptive factor for mammalian infections in general. The effect of glycosylation is complicated by the ability of glycans to shield antigenic sites from antibody recognition, so that increasing glycosylation may confer some protection against population immunity while reducing viral infectivity (Das et al. 2011; Job et al. 2013; Medina et al. 2013; Rudneva et al. 2005); however, since mice infected with influenza are generally immunologically naïve to the virus, this is less likely to affect mouse adaptation.

Other changes in HA associated with mouse virulence, including 78, 162, 321, and 354 in HA1 (Keleta et al. 2008; Koerner et al. 2012; Ping et al. 2011; Seyer et al. 2012; Xu et al. 2011b) and 58, 154, and 156 in HA2 (Keleta et al. 2008; Krenn et al. 2011; Ping et al. 2010), may alter HA stability and fusion efficiency (Smeenk et al. 1996; Ward 1997; Zaraket et al. 2013), which in turn may alter cell tropism. Other changes in HA may be associated with altered inflammatory response to the virus (Brown and Bailly 1999; Watanabe et al. 2013; Xu et al. 2011a).

4.2 Neuraminidase

While HA binds to SA, NA cleaves SA from glycoprotein carbohydrate chains, allowing newly-formed virions to escape from the cell surface. NA activity must balance HA activity for optimal viral entry and exit, and it is therefore not surprising that the NA segment affects mouse virulence (Gen et al. 2013; Pappas et al. 2008; Tumpey et al. 2004; Zhang et al.

2011). In some cases, enzymatic activity of NA has been linked to mouse virulence. For example, NA contains a stalk region that varies in length between and within influenza subtypes and influences its enzymatic function (Castrucci and Kawaoka 1993; Matsuoka et al. 2009b; Zhou et al. 2009). NA segments from either H5N1 (Matsuoka et al. 2009b; Zhou et al. 2009) or H9N2 viruses (Sun et al. 2013b) containing a short stalk confer increased mouse pathogenicity to reassortant influenza viruses, compared to NA with full-length stalks. Loss of a glycosylation site near the catalytic site of NA was found in two mouse-adapted viruses (Brown and Bailly 1999; Li et al. 1993) and may alter the enzymatic activity of NA (Brown and Bailly 1999) or even change HA functionality (Goto and Kawaoka 1998).

NA mutations, such as H275Y (H274Y in N2 numbering), associated with resistance to the NA inhibitor antiviral drug oseltamivir, are occasionally observed in viruses isolated from humans, At least on the H1N1pdm09 background, the H275Y variant is associated with increased virulence and infectivity in mice even in the absence of antiviral treatment (Ferraris et al. 2012; Song et al. 2013b), especially when compensatory mutations (such as V234M and R222Q in NA (N2 numbering) and K153E in HA) are present (Bloom et al. 2010; Song et al. 2013b).

4.3 Ribonucleoprotein Complex

Within an influenza A virion, each genomic segment is associated with a set of proteins comprised of NP and the RNA-dependent RNA polymerase complex (PB2, PB1, and PA). This complex is collectively termed the ribonucleoprotein (RNP) complex (reviewed in Noda and Kawaoka 2010), and acts as a single unit throughout much of the viral life cycle. Variants in RNP components are frequently linked to mouse adaptation, and in several cases the same functional adaptation (such as improved nuclear import) may be achieved through mutations in any of several components of the RNP.

PB2 is a major virulence determinant of influenza viruses, and mouse-adapted viruses frequently contain mutations in this gene. One of the most common changes as avian influenza viruses are adapted to mouse infection is an E627K change in PB2 (de Jong et al. 2013; Kim et al. 2010; Ping et al. 2010; Song et al. 2011; Tian et al. 2012; Wang et al. 2012; Wu et al. 2009; Zhang et al. 2011), and this change is strongly linked to increased mouse virulence in H5N1 (Chen et al. 2007; Hatta et al. 2001; Li et al. 2009; Maines et al. 2005) and H7N7 (Munster et al. 2007) HPAI viruses, H7N9 LPAI viruses (Mok et al. 2014; Zhang et al. 2014), and the 1918 H1N1 (Qi et al. 2012). Many H5N1 HPAIV and H7N9 LPAIV isolated from zoonotic human infections possess this substitution, whereas viruses isolated from avian species generally do not (Fonville et al. 2013; Manz et al. 2013). The same effect was seen in an equine H7N7 virus as it became mouse adapted (Shinya et al. 2004, 2007). Less commonly found during mouse adaptation, but also strongly associated with increased mouse virulence, is a D701N variant in PB2 (Gabriel et al. 2005; Li et al. 2005; Ping et al. 2010, 2011; Zhou et al. 2013). Both of these changes (E627K and D701N) are considered to be general markers of mammalian, not just mouse, virulence (Gabriel et al. 2007, 2013; Steel et al. 2009; Subbarao et al. 1993). Polymerase complexes which possess the E627K mutation are more active in mammalian cells (Subbarao et al. 1993), at least in part due to

their interaction with importin-α isoforms (Gabriel et al. 2008, 2011; Hudjetz and Gabriel 2012). Importins are components of the nuclear import pathway (Whittaker et al. 2000) that are required for influenza virus nuclear entry and therefore replication (Hutchinson and Fodor 2012). Species-specific differences in importins affect influenza RNP nuclear import, and mutations in the RNP that enhance nuclear import in a new host appear to be strongly adaptive. As well as PB2, changes in NP (N319K) alter interactions with importins and therefore affect mouse adaptation and host range in general (Gabriel et al. 2005, 2008, 2011). These and other compensatory changes (e.g. PB2 Q591K/L) (Gabriel et al. 2013; Yamada et al. 2010) mean that the E627K and D701N variants are not absolutely required for mouse virulence (Li et al. 2009; Manz et al. 2012; Xu et al. 2012; Yamada et al. 2010).

As well as interactions with importins, adaptive changes in PB2, including E627K, may play other roles in polymerase functions, such as enhancing the ability of the polymerase to act at the lower temperatures associated with mammalian respiratory tracts vs. avian gastrointestinal tracts (reviewed in Manz et al. 2013).

Mouse-adaptive substitutions in NP seem to be rare, but include D34N and D480N of H3N2 (Ping et al. 2011). Mouse-adaptive changes in the PB1 and PA genes include several that compensate for the lack of E627K in an avian-origin RNP complex, such as L472V and L598P in PB1 (Xu et al. 2012). Multiple other substitutions in these genes that have been associated with changes in mouse virulence have been described (Hiromoto et al. 2000; Ilyushina et al. 2010; Liedmann et al. 2013; Manz et al. 2013; Ping et al. 2011; Song et al. 2013b; Zhang et al. 2011). The mechanisms by which these substitutions affect mouse virulence are not known, although many of the mutations are at sites involved in the protein-protein interactions involved in polymerase complex assembly (Manz et al. 2013; Ping et al. 2011). The PB1 gene also encodes a small protein known as PB1-F2, expressed from an alternate open reading frame. The significance of this protein in influenza virus mouse adaptation and virulence will be addressed below.

4.4 M1 and M2

M1 and M2 are produced by differential splicing from the M gene segment. M1 is an internal structural protein that helps mediate virion assembly, interacting with HA and NA as well as the RNP. M2 is an ion channel that is required for viral entry into cells and subsequent replication (reviewed in Rossman and Lamb 2011). Early investigations into mouse adaptive mutations identified changes in M1 (A41V and T139A) as important virulence determinants for the mouse-adapted WSN strain (Ward 1995, 1996, 1997). The T139A mutation has also been iden-tified in the mouse-adapted A/FM/1/47-MA(H1N1) strain (Brown and Bailly 1999; Smeenk and Brown 1994; Smeenk et al. 1996). Changes in M1 and in M2 have also been observed during mouse adaptation of H2 (Govorkova et al. 2000), H3N2 (Brown et al. 2001; Ping et al. 2010), and H9N2 (Zhang et al. 2011) viruses, although the functional importance of these changes is unclear. Using reverse genetics, N30D and T215A in the M1 protein were shown to increase the virulence of H5N1 virus in mice (Fan et al. 2009). A V15I/T substitution in M1 protein was a common amino acid substitution found in H5N1 viruses exhibiting high virulence in mice (Katz et al. 2000).

Functional domains of M1 and M2 are not well understood as yet, and the mechanism(s) by which these substitutions in M1 and M2 may alter mouse pathogenicity are not yet clear.

4.5 NS1 and NEP

NS1 is an important non-structural protein expressed from a transcript from the NS gene segment. Alternate splicing of this transcript produces a second protein, nuclear export protein (NEP; also known as NS2) that has several roles in viral replication (Paterson and Fodor 2012). While NS1 is non-essential for viral replication in cultured cells (Garcia-Sastre et al. 1998), it is critical for natural infection because of its ability to inhibit the host innate immune response (Donelan et al. 2003) (reviewed in Hale et al. 2008) through species-specific interactions with multiple host proteins (Rajsbaum et al. 2012). Therefore, it is not surprising that mutations in NS1 are frequently observed during mouse adaptation of both human (Brown et al. 2001; Forbes et al. 2012; Ping et al. 2011) and avian (Dankar et al. 2011; Zhang et al. 2011) influenza viruses. For example, adaptation of the human H3N2 virus to mice resulted in 12 different NS1 mutations, most of which enhanced the ability of the virus to block IFN induction in mice (Forbes et al. 2012). However, NS1 mutations can apparently alter mouse virulence without affecting the IFN response (Forbes et al. 2012; Steidle et al. 2010).

Reassortant and reverse genetics experiments have provided more detailed information on the amino acid residues in NS1 associated in mouse pathogenesis, identifying a number of functional motifs that interact with host proteins and affect NS1 activity. One such motif is the four-residue C-terminal PDZ domain ligand. This region interacts with multiple members of the PDZ domain family of proteins, which are often involved in localized intracellular signaling. The PDZ domain ligand sequence has different canonical sequences in human (typically RSKV) and avian (ESEV) NS1 proteins, and the avian sequence binds to more human PDZ domain proteins than does the human sequence, potentially disrupting multiple protein-protein interactions (Obenauer et al. 2006). In mice, the avian sequence confers increased virulence to low-virulence viruses (Fan et al. 2013; Jackson et al. 2008; Zielecki et al. 2010). However, the impact of this sequence is context-dependent (Hale et al. 2010; Zielecki et al. 2010), so that in some cases effects are only seen when certain other changes are present (Fan et al. 2013).

Another functional motif in NS1 includes residues F103 and M106, which are involved in binding to the cleavage and polyadenylation specificity factor (CPSF30), which is required for 3’ end processing of cellular pre-mRNAs and is involved in IFN induction following influenza virus infection. H5N1 HPAI viruses containing NS1 without this motif are significantly less virulent in mice, showing greatly reduced systemic spread (Dankar et al. 2011, 2013; Spesock et al. 2011). Similarly, an R184G mutation in NS1, which also affects CPSF30 binding, reduces the mouse virulence of PR8; however, this mutation alters mouse virulence without apparently altering the IFN response (Steidle et al. 2010).

Other NS1 variants that have been linked with increased mouse virulence include P42S, which increases the ability of H5N1 NS1 to block IFN responses in mice (Jiao et al. 2008), and a deletion of residues 80–84 combined with a D92E change in H5N1 NS1, which increased virulence by an unknown mechanism (Long et al. 2008).

NEP, which plays several roles in viral replication, may be an important factor in the adaptation of AIV to human cells (Manz et al. 2012; Paterson and Fodor 2012). NEP mutations have been observed during mouse adaption, but the functional importance of these changes is not known (Forbes et al. 2012; Manz et al. 2013).

4.6 PB1-F2

PB1-F2 is a small gene that is expressed from an alternative open reading frame of segment 2, which also encodes the PB1 protein. Although multiple functions have been proposed for PB1-F2, several groups have found that this gene can antagonize IFN expression (Conenello et al. 2011; Dudek et al. 2011; Le Goffic et al. 2010; Varga et al. 2011, 2012). PB1-F2 is non-essential for viral replication and is frequently truncated in influenza viruses of mammals, but is usually full length in avian influenza viruses, suggesting that it may play an important role in the viral pathogenesis in birds versus mammals (Pasricha et al. 2013; Zell et al. 2007). In mice, the expression of full-length PB1-F2 (compared to a truncated protein) increases the virulence of several influenza viruses, including H5N1 and reassortant viruses expressing PB1-F2 from 1918 H1N1 or H5N1 viruses in the context of PR8 and WSN (Belser et al. 2010; Dudek et al. 2011; Leymarie et al. 2013; McAuley et al. 2007, 2013; Zamarin et al. 2006) (reviewed in Basler and Aguilar 2008; Conenello and Palese 2007; Krumbholz et al. 2011). The presence of full-length PB1-F2 in the PR8 virus is associated with increased susceptibility to secondary bacterial pneumonia (Alymova et al. 2014; Huber 2012; Iverson et al. 2011; McAuley et al. 2007). For reasons that are as yet unclear, the influence of PB1-F2 on pathogenicity is virus-dependent (McAuley et al. 2010) (reviewed in Krumbholz et al. 2011). For example, restoring full-length PB1-F2 expression to H1N1pdm09 viruses (which contain a stop codon 11 aa after the protein start site) does not alter virulence in mice (Meunier and von Messling 2012; Ozawa et al. 2011; Wang et al. 2010).

Analysis of reassortant and reverse-genetic viruses has shown that 66S in PB1-F2 is associated with high mouse virulence in H5N1 HPAIV and in 1918 H1N1, while 66N is associated with low-virulence viruses (Conenello et al. 2007, 2011; Schmolke et al. 2011), probably by altering IFN responses (Conenello et al. 2011; Varga et al. 2011). Several other residues located between 62 and 82 of PB1-F2 (L62, I68, L69, V70, R75, R79, and L82) have also been linked to increased inflammation, secondary bacterial infection, and lung pathology in mice (Alymova et al. 2011, 2014).

5 Conclusions

Are mice a good model for human influenza infection and disease? First, it is important to note that mice are used for many aspects of the influenza virus research other than the pathogenesis studies we discuss here. For example, mice are widely used to evaluate antibody and cell-mediated immune responses to influenza and are often a first-line preclinical model used to evaluate novel vaccines (Bodewes et al. 2010; Tripp and Tompkins 2009; van der Laan et al. 2008), and antiviral therapies including therapeutic antibodies (Mancini et al. 2011), and antiviral drugs (Barnard 2009; Gubareva et al. 1998; Ison et al. 2006; Sidwell and Smee 2000). It is also worth noting that mouse experiments are

not performed in vacuum; potentially interesting host or viral genetic determinants can be further analyzed in other models, such as ferrets and non-human primates.

If mice are a useful model for human infection with influenza virus adaptation and pathogenesis, then the changes associated with mouse adaptation should also be adaptive for humans. In many cases, mouse adaptive changes seem to be similar to those selected as avian viruses adapt to human infection (Brown et al. 2001; Pepin et al. 2010; Reperant et al. 2012). In general, the changes associated with mouse virulence can be divided into three groups. First are changes in the way the virus interacts with its receptors, which includes many of the HA and NA adaptive changes. These are most likely to be mouse-specific, since SA distribution in mice is different from humans (Ibricevic et al. 2006; Shinya et al. 2006). Nevertheless, some of these changes are informative for human disease. For example, the D222G (D225G in H3 numbering) substitution in HA is a mutation associated with mouse adaptation (Ilyushina et al. 2010; Seyer et al. 2012; Smee et al. 2007; Song et al. 2013b; Xu et al. 2011a), and also has been associated with severe disease in humans (Baldanti et al. 2011; Kilander et al. 2010). Similarly, some mouse-adaptive changes in the HA from H3N2 viruses mirror the changes associated with human adaptation of avian H3N2 (Keleta et al. 2008).

A second group of molecular changes associated with mouse adaptation and virulence is of those found in viral proteins required for replication. Examples of such changes include PB2 E627K and D701N, and related compensatory mutations. These are often associated with mouse adaptation, and are also repeatedly seen in H5N1 and H7N9 viruses isolated from humans, while the same viral subtypes isolated from birds are less likely to have these mutations. These mutations therefore do seem to be broadly relevant to mammalian infection, including humans (Gabriel et al. 2007, 2013; Manz et al. 2013; Steel et al. 2009).

A third group includes changes in non-essential proteins such as PB1-F2 and NS1. Such mutations are commonly seen during mouse adaptation and have major effects on virulence in mice. These proteins tend to modulate the host response to infection. Again, in many cases, these interactions seem to be broadly similar for mammalian infection. However, some interactions seem to be different for mice and humans, since mouse adaptation of human viruses leads to multiple mutations in NS1 (Brown et al. 2001; Forbes et al. 2012; Ping et al. 2011). This is not surprising, since genes associated with immune function evolve more rapidly than those involved with basic cellular functions (Hughes et al. 2005), so that such targets are more likely to differ between different mammalian species. For these genes, the overall pattern of change may be significant while the precise mutations involved may not always extrapolate to virus adaptation to humans.

Thus, studies of influenza infection in mice can in many ways parallel or predict virus adaptation to and pathogenesis in humans (Pepin et al. 2010; Reperant et al. 2012). Overall, mice are a convenient and cost-effective animal model that, as long as observations are interpreted in the light of physiology and evolution, can enhance our understanding of influenza virus virulence determinants for mammals, including humans.

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Table 1

Summary of influenza virus variants and mutations that affect replication and virulence in mice

Protein	Virus	Mouse	Mutation	Effect	Reference(s)
HA	A/Vietnam/1203/2004(H5N1)	BALB/c	Deletion of polybasic cleavage site	Reduced respiratory and systemic replication. Reduced proinflammatory cytokines in lungs	Suguitan et al. (2012)
	A/Hong Kong/156/97(H5N1)	ddY	P211T	Decreased virulence	Hiromoto et al. (2000)
	Reassortant WSN-HA + A/Hong Kong/1/68(H1N1xH3N2)	BALB/c	T160N, P162S	Increased virulence	Keleta et al. (2008)
	A/Aichi/2/68(H3N2)	BALB/c	G218E	Increased virulence	Narasaraju et al. (2009)
	A/Mallard duck/Pennsylvania/10218/84(H5N2)		S203F, E273G, L320P	Adaptation and virulence	Smirnov et al. (2000)
	A/California/04/09(H1N1pdm09), A/Tennessee/1-560/09(H1N1pdm09)	BALB/c	D222G	Mouse adaptation and virulence	Ilyushina et al. (2010)
	A/Brisbane/59/2007(H1N1)	BALB/c	T89I, N125T, D221G	Mouse adaptation and virulence	Xu et al. (2011a)
	A/Hamburg/04/09(H1N1pdm09)	BALB/c	D222G K163E	Mouse adaptation and pathogenicity	Seyer et al. (2012)
	A/Hong Kong/1/68(H3N2)	CD-1	G218W	Mouse adaptation and virulence	Ping et al. (2010)
	A/Hong Kong/1/68(H3N2)	CD-1	T156N	Mouse adaptation and virulence	Ping et al. (2010)
	Reassortant WSN-HA + A/Hong Kong/1/68(H1N1xH3N2)	BALB/c	N154S	Mouse adaptation	Keleta et al. (2008)
	A/Vietnam/1203/2004(H5N1) NS1-deleted	Outbred	K58I	Increased immunogenicity	Krenn et al. (2011)
	A/black duck/New Jersey/1580/78(H2N3)		E216D, K307R, T318I	Mice adaptation and virulence	Govorkova et al. (2000)
	A/JapanxBellamy/57(H2N1)		K25T, S203F		
M1	A/black duck/New Jersey/1580/78(H2N3)		N30D, Q214H	Mice adaptation and virulence	Govorkova et al. (2000)
	A/JapanxBellamy/57(H2N1)		M179K		
	A/Wisconsin/33		A41V + T139A	Mice adaptation neurovirulence	Ward (1995)
	A/FM/1/47(H1N1)		T139A	Mice adaptation and virulence	Brown and Bailly (1999)
	A/duck/Guangxi/53/2002(H5N1)	BALB/c	N30D + T215A	Increased virulence	Fan et al. (2009)
M2	A/black duck/New Jersey/1580/78(H2N3), A/JapanxBellamy/57(H2N1)		N93S	Mice adaptation and virulence	Govorkova et al. (2000)

Protein	Virus	Mouse	Mutation	Effect	Reference(s)
NS1	Reassortant A/Hong Kong/1/68(H3N2) x PR8	BALB/c	F103L + M106I	Increased infectivity and virulence	Dankar et al. (2011)
	A/Puerto Rico/8/34(H1N1)	PKR KO, IFNAR/ IL28R KO	R184G	Increased replication in mice lungs	Steidle et al. (2010)
	A/Aichi/2/68(H3N2)	BALB/c	D125G	Increased virulence	Narasaraju et al. (2009)
	A/Hong Kong/156/97(H5N1)	ddY	D101N	Increased virulence	Hiromoto et al. (2000)
	A/Duck/Guangxi/12/03(H5N1)	BALB/c	P42S	Increased virulence	Jiao et al. (2008)
	A/Duck/Guangxi/27/03(H5N1)	BALB/c	S42P	Attenuation	
	A/Duck/Shandong/093/2004(H5N1)	BALB/c	E92D	Decreased virulence	Long et al. (2008)
PA	A/Hong Kong/156/97(H5N1)	ddY	G631S	Increased virulence	Hiromoto et al. (2000)
	A/Hamburg/04/09(H1N1pdm09)	BALB/c	F35L	Mouse adaptation and pathogenicity	Seyer et al. (2012)
PB1	A/Hong Kong/156/97(H5N1)	ddY	H456Y, S712P	Increased virulence	Hiromoto et al. (2000)
	A/Cambodia/P0322095/2005(H5N1)	BALB/c	473L + 598L	Decrease in mouse infectivity	Xu et al. (2012)
	A/Wisconsin/33(H1N1) PB2K627E	BALB/c	L473V + L598P	Restore mouse infection/replication	
	Reassortant A/California/07/2009(H1N1pdm09) + WSN	BALB/c	473L	Decrease in mouse infectivity	
PB1-F2	A/Viet Nam/1203/2004(H5N1)	C57BL/6/A	N66S	Increased virulence	Schmolke et al. (2011)
				No effect	
	Reassortant A/WSN/33(H1N1) + A/HK/156/97(H5N1) PB1, A/Brevig Mission/ 1918(H1N1)	C57BL/6	N66S	Increased virulence	Conenello et al. (2007)
	Reassortant A/Puerto Rico/8/34(H1N1) + A/Wuhan/359/95(H3N2) PB1	DBA/2	P62L + H75R + S82L	Increased virulence	Alymova et al. (2011)
	A/Puerto Rico/8/34 (H1N1)	BALB/c	I68T + L69Q + V70G	Reduced primary viral infectivity and secondary bacterial pneumonia	Alymova et al. (2014)
PB2	A/Hong Kong/1/68(H3N2)	CD-1	K482R, D701N, D740N	Increased mouse virulence	Ping et al. (2011)
	A/Hong Kong/1/68 (H3N2)	CD-1	D701N	Mouse adaptation and virulence	Ping et al. (2010)
	A/Hong Kong/156/97(H5N1)	ddY	N701D	Increased virulence	Hiromoto et al. (2000)
	A/Seal/Massachussetts/1/80(H7N7)	BALB/c	D701N	Increased virulence	Gabriel et al. (2005)

Protein	Virus	Mouse	Mutation	Effect	Reference(s)
	A/duck/Guangxi/22/2001(H5N1)	BALB/c	D701N	Adaptation and increased virulence	Li et al. (2005)
	A/New York/1682/2009(H1N1pdm09)	BALB/c	D701N	Increased virulence	Zhou et al. (2013)
	A/chicken/Netherlands/621557/03(H7N7)	BALB/c	E627K	Mouse adaptation and increased virulence	de Jong et al. (2013)
	A/chicken/Guangdong/Ts/2004(H9N2), A/chicken/Guangdong/V/2008(H9N2)	BALB/c	E627K	Reduced host antiviral response	Tian et al. (2012)
	A/chicken/Shandong/16/05(H9N2)	BALB/c	M147L + E627K	Increased virulence	Wang et al. (2012)
	A/Hong Kong/483/97(H5N1) A/Hong Kong/486/97(H5N1)	BALB/c	627K	Increased virulence	Chen et al. (2007), Hatta et al. (2001)
	A/Netherlands/33/03(H7N7) A/Netherlands/219/03(H7N7)	BALB/c	627K	Increased virulence	Munster et al. (2007)
	A/Shanghai/2/2013(H7N9)	BALB/c	E627K	Increased virulence	Mok et al. (2014)
	A/Anhui/1/2013(H7N9)	BALB/c	E627K	Increased replication and virulence	Zhang et al. (2014)
	Reassortant 1918 H1N1 x avian H1N1	BALB/c	E627K	Increased mouse virulence	Qi et al. (2012)
	A/equine/London/1416/73(H7N7)	BALB/c	E627K	Adaptation and increased neuronal invasion	Shinya et al. (2007)
NP	A/Hong Kong/1/68(H3N2)	CD-1	D34N, D290N, D480N	Increased mouse virulence Adaptation	Ping et al. (2010)
	A/Hong Kong/156/97(H5N1)	ddY	E127K	Increased virulence	Hiromoto et al. (2000)
	A/Seal/Massachussetts/1/80(H7N7)	BALB/c	N319K	Mouse adaptation	Gabriel et al. (2005, 2008)
NA	r A/Lyon/969/2009(H1N1pdm09) +/− NA from A/Lyon/1337/2007(H1N1)	Balb/cByJ	H275Y	Increased infectivity	Ferraris et al. (2012)
	A/California/04/09(H1N1pdm09)	Balb/c	H275Y	Increased virulence	Song et al. (2013b)