Viral determinants of influenza A virus host range

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Typical avian influenza A viruses are restricted from replicating efficiently and causing disease in humans. However, 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. It is critical that we understand the molecular basis of host-range restriction and how this can be overcome. Here, we review our current understanding of the mechanisms by which influenza viruses adapt to replicate efficiently in a new host. We predominantly focus on the influenza polymerase, which remains one of the least understood host-range barriers.

Introduction

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 nine 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, 2013). Viruses from this primordial reservoir can infect new species including humans, pigs, horses and sea mammals; however, this usually only results in sporadic cases of infection. It is very rare that zoonosis leads to a sustained epidemic with transmission within the human population.

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.

Role of HA in influenza host adaptation

The influenza glycoprotein HA plays an important role in determining the host range of influenza viruses. Although all influenza HA proteins recognize oligosaccharides containing terminal sialic acid (SA) on the surface of epithelial cells, they have different receptor specificities. Human viruses preferentially recognize SA linked to galactose by 2,6 linkages, whereas avian viruses preferentially recognize SA linked to galactose via 2,3 linkages (Matrosovich et al., 1997; Rogers & Paulson, 1983; Suzuki, 1994).

In humans, 2,6 receptors are expressed mainly on cells of the ciliated epithelium along the upper regions of the respiratory tract (Baum & Paulson, 1990; Thompson et al., 2006; van Riel et al., 2010), whereas 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 2,3 receptors are more prevalent (van Riel et al., 2006). The paucity of 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 2,6 binding in humans. It has also been proposed that mucus in the human airway is rich in soluble 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 2,3 over 2,6 SA in human mucus is poor, and the samples analysed have often been obtained from patients with conditions that might affect mucus constituents such as cystic fibrosis. We and others found both 2,3 and 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 SAs 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 2,3 SA linkages to 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 2,3 to 2,6 receptors for H2 and H3 serotypes (Rogers & Paulson, 1983; Viswanathan et al., 2010). 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 HAAs with natural variations in the receptor-binding site (Yamada et al., 2006).
et al., 2006). In another approach, the classic H1, H2 and H3 HA humanizing 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 that 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 probably 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 that in the strains that 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 those in 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 a mutation that affected SA binding specificity as well as a mutation in the stem region of the HA protein that affected 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.

Role of NA in influenza host adaptation
The viral glycoprotein HA binds to SA-containing receptors on target cells, whereas the viral glycoprotein NA removes SA 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 (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.

In a comparison of 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).

NA enzyme activity resides in the head domain of the mushroom-shaped protein that 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 & Perez, 2007; Steensels et al., 2007). For example, H5N1 influenza viruses mostly have 19 aa deleted from NA (Munier et al., 2010). The longest deletion reported is 35 aa in an 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 & 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 SAs expressed in these hosts (Baigent & McCauley, 2001; Mitnaut 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 highly pathogenic avian influenza H5N1 virus harbouring a stalk deletion was compromised in respiratory droplet transmission between ferrets (Blumenkrantz et al., 2013). 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 & Paulson, 1991; Kobasa et al., 1999).

To conclude, in addition to reassortment, which introduces novel antigens to which the human population lacks immunity, a prerequisite for a pandemic virus is for adaptive changes in both HA and NA that facilitate efficient interaction with host SAs and affect protein stability (Fig. 1).

Role of influenza polymerase and nucleoprotein (NP) in host adaptation
Each negative-sense viral genome segment is associated with one heterotrimeric viral RNA polymerase composed of the proteins PA, PB1 and PB2, as well as multiple copies of NP, forming a viral ribonucleoprotein (vRNP) complex. All eight viral RNA (vRNA) segments have conserved sequences of 12 and 13 nt 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, which is thought to fold into a ‘corkscrew’ conformation (Flick & Hobom, 1999; Flick et al., 1996). This region constitutes the viral promoter, and it is here that the polymerase binds. The influenza polymerase and NP are the minimal viral components required for transcription and replication of the viral genome (Huang et al., 1990). 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 (Fig. 2).

It is well documented that polymerases from avian strains of influenza do not function well in the mammalian host and that mutations that increase polymerase activity are a prerequisite for host adaptation (Naffakh et al., 2008). Host-specific genetic signatures have been identified on all of the polymerase subunits and on NP. Some of these adaptive mutations have arisen during natural evolution, often 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 in vitro in a reconstituted polymerase assay in which a minigenome is amplified and expressed by cloned polymerase proteins (Pleschka et al., 1996). Limitations of this assay are that it does not usually include expression of other viral proteins that might affect polymerase activity such as the non-structural protein NS1 or nuclear export protein (NEP), and also that the vRNP has to be assembled de novo inside the transfected cells rather than delivered ready-assembled as it would be from an incoming virion. Nonetheless, this convenient assay does stringently recapitulate the inability of avian virus polymerase to function optimally in a human cell. In our recent study, a series of mammalian-adaptive PB2 signatures were introduced singly into a typical avian PB2 gene and their effects on polymerase activity were compared in human, swine and avian cells. Certain PB2 mutations enhanced polymerase activity in all cell types, whereas others enhanced activity only in mammalian cells; this suggests that there are different pathways to enhancing polymerase activity (Cauldwell et al., 2013). However, only some of the adaptive mutations that enhanced activity in this in vitro system have been detected in viruses that are found in nature. The polymerase assay may not truly predict viral growth, and viral selection pressures that might favour some adaptive mutations over others should be investigated. Here we discuss the various adaptive strategies by which avian influenza polymerase restriction in human cells can be overcome and relate them to host-cell factors where possible (Fig. 2).

PB2
PB2 is the polymerase protein that 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). Later, Subbarao et al. (1993) showed that the restriction of a reasortant 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.

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., 2007; Gao et al., 2013; Hatta et al., 2001; Shinya et al., 2004; Steel et al., 2009).

There is very strong selection of PB2 627K in zoonotic infections. 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 that emerged in 1957 and 1968, because these reassortant viruses retained the PB2 gene from the 1918 pandemic virus (Scholtissek et al., 1978b). A lysine at position 627 in PB2 has been identified in several other avian strains of influenza that have infected humans. Thirty-two per cent 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).

The complete protein structure of PB2, or indeed any of the polymerase proteins, has not been determined. However, the crystal structure of an independently folded domain from residues 538–676 has been determined (Tarendreau et al., 2008). It exhibits a unique fold and residue 627 lies at the centre of a solvent exposed surface. The glutamic acid forms a region of negative charge which the introduction of a lysine disrupts.

Currently, the mechanism for the observed enhancement in polymerase activity is unclear. The 627 E→K switch was suggested to stabilize the interaction between the NP and PB2 components of the vRNP complex in the nuclei of infected cells (Labadie et al., 2007; Mehele & 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 an historic H5N1 virus from 1991 did not tolerate the Lys codon, unless reassorted with gene segments from either the modern H5N1 virus or a laboratory-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 limit the evolution of amino acids in this region.

It also seems unlikely that a poor interaction between PB2 and NP explains the host range defect for avian polymerases bearing PB2 627E. We recently showed that a variety of adaptive PB2 mutations including E627K did not enhance the stability of the vRNP in human cells but rather increased the amount of replicated RNA, indirectly resulting in more PB2–NP co-precipitation (Cauldwell et al., 2013). Furthermore, Paterson et al. (2013) recently showed that restriction of polymerase bearing PB2 627E in human cells occurred independently of NP.

The crystal structure of the position 627-containing domain of PB2 (residues 535–684) contains an α-helix encircled by a loop, which is located next to a highly basic groove. 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. 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 presence of lysine 627 enhanced PB2 binding to RNA measured by surface plasmon resonance (Kuzuhara et al., 2009). However, when the promoter binding activity of the polymerase was studied in vitro using a UV-cross-linking approach, the presence of a lysine was shown to significantly reduce vRNA and cRNA promoter binding, and reduce the 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.

Temperature has 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 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 enzymic activity of the influenza polymerase. However, temperature cannot be the sole
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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. In a new species, the virus 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 & Doumdna (2008), concluded that mammalian cells express a dominant inhibitory factor that restricts the function of a polymerase harbouring a glutamic acid at position 627 of PB2. 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, these authors 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 that 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. (2011) used a functional genomics approach to describe a network of human proteins that modulate influenza polymerase activity. Cellular factors were identified that differentially regulated the activity of 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 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 understand fully 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 & Hobom, 1995). 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. (2013) recently showed 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.

For many years, the point at which the avian polymerase was restricted in mammalian cells was not clear. However, a recent study by Mänz et al. (2012) 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 cannot produce a cRNP that can be used to synthesize vRNA; cRNPs formed but they were defective and could not be used (Fig. 3).

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 & Krug, 1986; Fodor et al., 1994; Robb et al., 2009). Vreebe et al. (2004) 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. However, for the next step of producing vRNA from the cRNA template, it has been suggested by at least two independent studies that a non-resident polymerase complex is required to transactivate the polymerase (see Fig. 3). In the most recent study from Fodor’s group, purified cRNPs were unable to give rise to vRNA unless a second polymerase complex purified from either insect cells or mammalian cells was supplied. The second exogenously supplied complex could activate replication even if it carried mutations in PB1 that rendered it enzymically inactive, but this is in contrast to work from Ortin’s group in which the trans-acting polymerase was proposed to access the 3′ cRNA promoter and function as the replicative enzyme (Jorba et al., 2009; Moeller et al., 2012; York et al., 2013).

Why this transactivation step should be deficient for an avian virus polymerase in human cells remains unknown, but it is tempting to speculate that a host-cell factor in human cells acts either to enhance this step for human-adapted viruses or to inhibit it for avian influenza strains.

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 contains 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 & 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 superorder, also selected for mutation of PB2 at residue 591 (Yamada et al., 2010). This suggests that this group of birds, which are quite phylogenetically distinct from Anseriformes (ducks) and Galliformes (chickens), may express similar relevant host factors to those of 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; Cauldwell et al., 2013; 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, eliminated transmission between ferrets (Zhang et al., 2012). The mechanism for increased polymerase activity bestowed by mutation at residue 271 is completely unknown,

**Fig. 3.** 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 synthesize vRNA.
but our observation that it only enhances avian polymerase activity in mammalian cells implies involvement of a host factor (Cauldwell et al., 2013).

The PB2 mutation E158G was identified by passage of the pH1N1 virus in mice. Although sequence database searches only identified a single human pH1N1 isolate 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, 2013), and it increased avian influenza polymerase activity in a cell-based polymerase assay in both human and avian cells (Cauldwell et al., 2013). The mechanism by which this mutation confers increased polymerase activity is also unknown.

PB2 D701N is an additional mutation that 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., 2005, 2008). In addition, PB2 701N has been shown to be capable of expanding the host range of avian H5N1 to mice and humans (de Jong et al., 2006; Li et al., 2005), as well as compensating for the absence of 627K and restoring 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 and 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 from 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).

Aa 701 resides in a domain that has been co-crystallized with human importin-α5 and is implicated in the nuclear localization of the PB2 polymerase subunit (Tarenadeau 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., 2005, 2008, 2011; Hudjetz & 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 & 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 & Gabriel, 2012).

A novel study found that PB2 mutations can affect the dynamics of the viral polymerase in human cells. A PB2 627E-containing polymerase exhibited significantly slower diffusion in human but not avian nuclei compared with a PB2 627K-containing polymerase. In addition, host-range mutations at positions 271, 588, 636 and 701 also enhanced 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).

Although influenza virus polymerase activity has been studied in human and avian cells for many years with a minigenome assay, this approach was not possible in pig cells until 2012 when we cloned the swine RNA polymerase I promoter and developed the first minigenome assay for pig cells (Moncorgé et al., 2013). Swine influenza lineages originate from avian or human influenza viruses (reviewed by Brown, 2008; Van Reeth, 2007), implying that pigs are susceptible to infection with both types of influenza viruses. As a consequence, pigs have been described 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, 2013). Thus, it is important to investigate the consequences of mammalian host-range determinants in pig cells.

Using the minigenome polymerase assay in pig cells, we found that two typical avian influenza polymerases were poorly active, but that the same PB2 mutations that enhanced avian influenza polymerase activity in human cells also increased activity in pig cells (Cauldwell et al., 2013; Moncorgé et al., 2013). These results suggest similar host range restrictions in pig and human cells and imply that the potential for avian influenza viruses to efficiently replicate and transmit in pigs is perhaps lower than had previously been assumed.

In conclusion, several different amino acids in PB2 confer increased polymerase activity and overcome host restriction. However, it is not clear what determines which adaptive mutations 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 cleaving 5’ methylated mRNA cap-containing oligonucleotides from host-cell pre-mRNAs for use as primers for viral transcription. This ‘cap snatching’ mechanism occurs in the nucleus and the 9–17 nt capped RNA fragments are used to ‘prime’ mRNA transcription using viral genomic RNAs as a template. The role of PA in host adaptation is less well characterized than that of PB2; however, it is increasingly considered to exert an important influence on host range. One recent study found that 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. 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. Several residues in pH1N1 PA have been shown to be involved in host adaptation including T85I, G186S and L336M (Bussey et al., 2011). A number of groups have serially passaged the pH1N1 virus in mice and identified further 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 selected following the serial passage of a low-pathogenic avian H5N2 virus in mice increased polymerase activity and replication in mice but not chickens (Song et al., 2009).

Only Mehle et al. (2012) have investigated the effects of PA mutations on polymerase activity in both human and avian cells. Several PA mutations that significantly enhanced polymerase activity in human cells (P400L, M423I, V476A, T552S and V630E) had no effect in avian cells. The enhancing activity conferred by PA derived from highly human-adapted seasonal influenza viruses mapped to residue 552; a position previously identified by bioinformatic analysis as a determinant of host range (Chen et al., 2006; Finkelstein et al., 2007; Tamuri et al., 2009; Taubenberger et al., 2005).

These various reports suggest that multiple residues in PA contribute to mammalian host adaptation. This concept is supported by the work of Bussey et al. (2011) who showed that although singly introducing humanizing 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. 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.

The work of Bussey et al. (2011) has challenged the concept that host-adaptive mutations that enhance viral replication automatically lead to increased pathogenicity. Several of the PA mutations studied had a considerable effect on polymerase activity but no or only a minimal effect on viral pathogenicity in mice (Bussey et al., 2011). Thus, pathogenicity and host adaptation are distinct concepts. Similarly, in studies mapping host-range-adapting mutations in several different polymerase genes of an H7 virus passaged in mice, Gabriel et al. (2005) found that several mutations that increased polymerase activity did not increase mouse virulence.

Recently, a second viral protein generated from mRNA of segment 3 was discovered, termed PA-X (Jagger et al., 2012). This protein comprises the first 191 aa containing the endonuclease domain of PA, but then through a frameshift gains a novel C terminus of 61 aa 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 protein plays a role in determining host range, although it is tantalizing that endonuclease activity is more potent in PA-X genes from avian than human influenza virus strains (Desmet et al., 2013).

PB1

In PB1, aa 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 cases 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 of the first to be described in the literature (O’Neill et al., 1995). It is now evident that NP has 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 et al. (1978a) 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.

Host-specific signatures have been localized to the NP gene in bioinformatic studies (Chen et al., 2006; Finkelstein et al., 2007; Tamuri et al., 2009) and some have been implicated in facilitating evasion from IFN-stimulated MxA restriction (see below) (Mänz et al., 2013). In addition, the NP N319K mutation has been shown to enhance avian viral replication in mammalian cells by enhancing the interaction with importin-α isoforms (Gabriel et al., 2005, 2007, 2008, 2011).

**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 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 (Mänz et al., 2012). Moreover, Mänz et al. (2012) 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, and has previously been documented to affect synthesis of mRNA, cRNA and vRNA (Bullido et al., 2001; Mänz 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).

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 nt 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, 2012; Umbach et al., 2010).

**Role of antagonizing or evading host defences 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 IFN upregulates 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 minimize IFN expression and the signalling pathways it induces (Fig. 4). 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 retinoic acid-inducible gene 1 (RIG-I), or inhibiting IkB kinase β 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 IFN 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 IFN 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 tripartite motif-containing protein 25 (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 also to 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 has not yet been 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 (cleavage and polyadenylation specific factor 4, 30 kDa subunit) 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 proteins 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 inhibit IFN-β production in human cells that may support the hypothesis that a lack of IFN control contributes to host-range restriction (Hayman et al., 2007; Mukherjee et al., 2012; Munir et al., 2011). Knepper et al. (2013) 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 IFN antagonism in human cells compared with a typical chicken H7 virus. In contrast, 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., 2010; Shelton et al., 2013).

Human cells compromised in their ability to respond to IFN 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 IFN. It would be interesting to visualize 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 localize to the mitochondria and to interact with the mitochondrial antiviral signalling protein (MAVS) to inhibit IFN induction (Graef et al., 2010; Iwai et al., 2010). The ability to localize 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 localize 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 IFN 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 alternative ORF of the PB1 gene, can also influence the innate immune response and viral pathogenicity. This protein also localizes to the mitochondria and interacts with MAVS to inhibit IFN 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 IFN 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 IFN 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) and rhabdoviruses (vesicular stomatitis virus), as well as DNA hepadnaviruses (hepatitis B virus) (Haller & 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, 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 for 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 a 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 (Mänz et al., 2013). 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.

Conclusions

Influenza viruses interact with host-cell factors at every stage of their replication cycle. Differences in these factors between species mean that the virus–host relationship is unbalanced when a zoonotic infection first occurs. This unbalance may be so severe that the virus cannot establish infection in the new host species, and this results in host-range restriction. The emergence of the novel H7N9 influenza A virus in Eastern China in March 2013 and the ensuing 251 human infections and 67 deaths confirmed by the World Health Organization as of 5 February 2014 (http://www.who.int/csr/don/2014_02_05bis/en/) highlight the permanent threat posed by influenza viruses. Further understanding of the molecular mechanisms of influenza host range is crucial if we are to determine which influenza strains have pandemic potential and are most likely to cross host-range barriers.

We hope that insight into the interplay between viral proteins and cellular factors will lead to new strategies for inhibiting virus multiplication. There is an urgent need for new drugs from which the virus is less likely to escape. The influenza polymerase is a promising target for antiviral development, as an inhibitor of the influenza polymerase would minimize the generation of viral escape mutants. In addition, drugs that target host factors essential for virus replication could also mitigate the emergence of drug resistance.

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Viral determinants of influenza A virus host range

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