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MINIREVIEW

Adaptation of Avian Influenza A Virus Polymerase in Mammals To Overcome the Host Species Barrier

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Avian influenza A viruses, such as the highly pathogenic avian H5N1 viruses, sporadically enter the human population but often do not transmit between individuals. In rare cases, however, they establish a new lineage in humans. In addition to well-characterized barriers to cell entry, one major hurdle which avian viruses must overcome is their poor polymerase activity in human cells. There is compelling evidence that these viruses overcome this obstacle by acquiring adaptive mutations in the polymerase subunits PB1, PB2, and PA and the nucleoprotein (NP) as well as in the novel polymerase cofactor nuclear export protein (NEP). Recent findings suggest that synthesis of the viral genome may represent the major defect of avian polymerases in human cells. While the precise mechanisms remain to be unveiled, it appears that a broad spectrum of polymerase adaptive mutations can act collectively to overcome this defect. Thus, identification and monitoring of emerging adaptive mutations that further increase polymerase activity in human cells are critical to estimate the pandemic potential of avian viruses.

Although the natural reservoirs of influenza A viruses lacking adaptive mutations that increase polymerase activity in mammalian cells (1–53) are wild birds (54), mammals are frequently infected with influenza viruses of avian origin. These zoonotic transmissions can cause severe disease in different mammals, including cats, dogs, horses, pigs, and humans (55–57), due to the lack of preexisting immunity in these species to the new influenza virus strain (Fig. 1). Fortunately, most of these infections are so-called dead-end infections and are not further transmitted within the new species due to several barriers. However, on rare occasions influenza A viruses can indeed break the species barrier and establish an entirely new virus lineage in a mammalian species, as exemplified by the human pandemic of 1918 (58), the Eurasian classical swine influenza virus lineage (59), H3N8 influenza viruses in horses (56), and possibly H17N10-like influenza viruses in bats (60). Although 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified in wild birds, human infection has been documented only for H1, H2, H3, H5, H7, and H9 (61–64) and only H1, H2, and H3 have been stably introduced into the human population (56, 65, 66). In the majority of cases, human pandemics were a result of genetic reassortment events whereby the circulating human virus strains acquired one or more gene segments from avian or swine sources (54). However, since humans in certain regions of the globe are constantly exposed to H5N1 influenza viruses, there exists a serious concern that this subtype might acquire mutations to stably cross the species barrier and start a new pandemic (67, 68).

REPLICATION OF INFLUENZA A VIRUSES

Influenza viruses belong to the family of *Orthomyxoviridae*, possessing a single-stranded negative-sense RNA genome that is comprised of eight segments (69). The ends of each genome segment are short complementary elements that form the viral promoter and are recognized by the viral RNA-dependent RNA polymerase, which is composed of the three subunits PB2, PB1, and PA (70–74). Together, the viral polymerase, the nucleoprotein NP, and the viral RNA genome form the ribonucleoprotein (RNP) complex, which is responsible for viral mRNA synthesis and genome replication. Upon infection, viral RNPs (vRNPs) are released into the cytoplasm (75) and transported into the nucleus, where the RNPs perform all of their enzymatic functions (76, 77). First, primary transcription is initiated and viral mRNA is synthesized from the viral genome (vRNA) (Fig. 2). vRNA also serves as a template for synthesis of a full-length copy (cRNA), which serves as a template for subsequent synthesis of new viral genomes (78). Viral mRNA synthesis is primed using a cellular capped pre-mRNA which is bound by the PB2 subunit (30, 79) and cleaved by the endonucleolytic domain of the polymerase subunit PA (39, 80). Synthesis of both c- and vRNA is apparently primer independent (81–83). Recent evidence suggests that mRNA is synthesized by viral polymerase complexes which are resident on vRNPs (in "*cis*"), whereas vRNA is synthesized from the cRNA template using soluble viral polymerase complexes (in "*trans*") (84). However, the mechanism of cRNA synthesis and the regulation of the switch between transcription and genome replication remain largely unknown (85). The viral nucleoprotein (NP) is one major candidate to regulate the switch between transcription and replication (86), presumably by direct interaction with the viral polymerase (87), although this role of NP has been questioned more recently (88).

Interestingly, in addition to the viral nucleoprotein, the nuclear export protein NEP (NS2) has been shown to be involved in the regulation of polymerase activity (11, 89, 90). In contrast to NP, it is not an essential component of the RNP complex, as it is not required for transcriptional activity in a polymerase reconstitution assay (Fig. 3). In this system, the effect of NEP on viral RNA synthesis was shown to be concentration dependent. While very high NEP concentrations completely abrogated polymerase activity (11), recent data demonstrate that small amounts of NEP stim-

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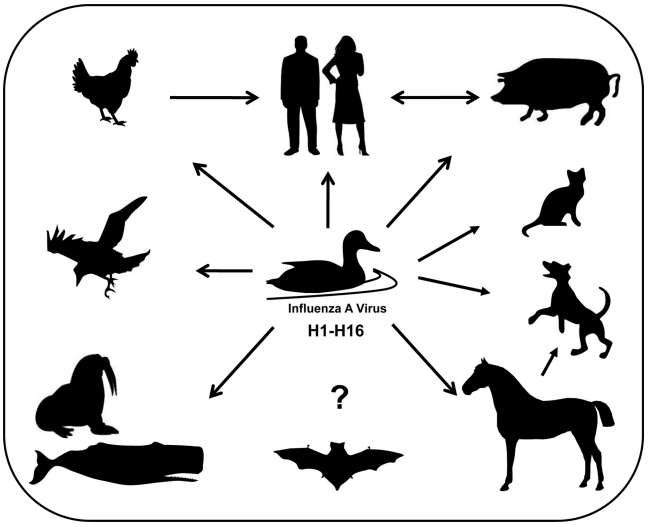

FIG 1 Host range of influenza A viruses. Wild water birds represent the natural reservoir of influenza A viruses, from which they can be transmitted to a wide variety of other hosts, including horses, cats, dogs, whales, seals, wild flying birds, chicken, pigs, and humans. Only recently, influenza A virus has also been detected in bats, although the origin is unclear.

ulate the synthesis of vRNA and cRNA and, depending on the experimental system, also mRNA (11, 89). The mechanism by which NEP enhances vRNA and/or cRNA synthesis and regulates viral polymerase activity remains unknown, but it appears to occur independently of its previously described function as a mediator of vRNP nuclear export (11, 89).

The discovery of small viral RNAs (svRNAs) generated during viral replication (91, 92) has furthermore paved the way for a new understanding of influenza virus polymerase regulation. svRNAs are 22 to 27 nucleotides in length, corresponding to the 5′ ends of the genomic viral RNA segments, and are synthesized from the 3′ end of the cRNA by the viral polymerase. Functional characterization indicates that svRNAs directly interact with the PA subunit and could provide a segment-specific guide for the viral polymerase to the cRNA templates, thereby promoting synthesis of new genomic vRNAs (93). This suggests that svRNAs are involved in the switch from mRNA transcription to genome replication (91) and that NEP mediates the generation of these svRNAs by stimulating cRNA synthesis (93).

During a replication cycle, at least 10 different viral proteins are expressed to establish efficient propagation of the virus in the infected host (40, 94–97). Besides the requirement of these proteins for essential functions during the viral life cycle, multiple interactions with host factors, such as DNA-dependent RNA polymerase II (Pol II), are required to snatch cap structures from host mRNAs (98) and to function as part of the cellular splicing machinery (99, 100) as well as to function as factors for nuclear trafficking (101). In the case of transmission of avian influenza viruses to mammalian hosts, introduction of adaptive mutations into viral proteins is essential to ensure optimal functionality and virus-host protein interactions in the cellular environment of the new host.

VIRAL FACTORS IMPORTANT IN HOST ADAPTATION

The hemagglutinin (HA) is one of the key factors which determines the host range of influenza A viruses, since it mediates at-

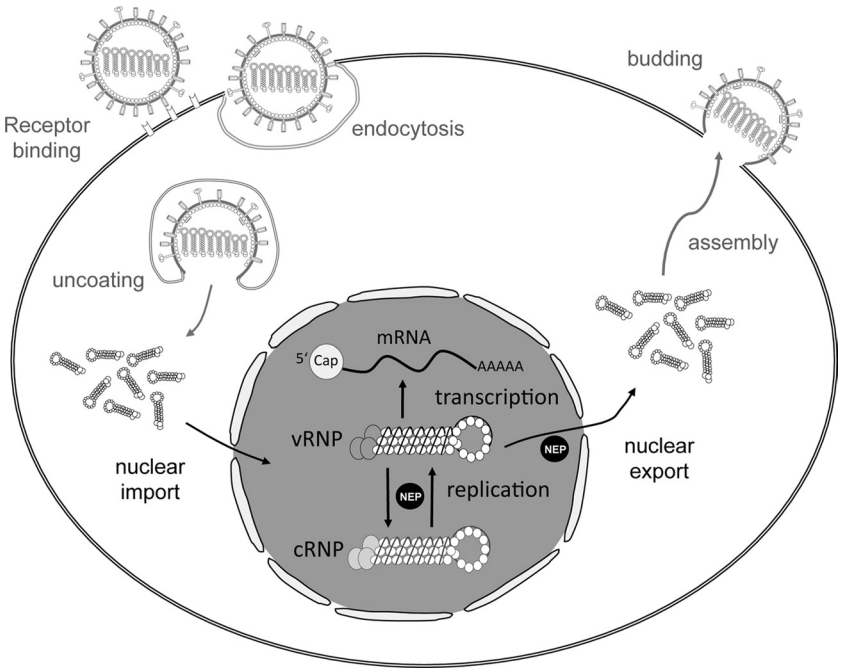

FIG 2 Illustration of the influenza A virus replication cycle. Influenza A virus particle binds to the cellular receptor and enters the cell by endocytosis. The viral ribonucleoproteins (vRNPs) are released into the cytoplasm upon acidification of the endosome. vRNPs are transported into the nucleus, where transcription and replication occur. Replication is supported by the viral nuclear export protein NEP. For genome replication, negative-sense viral RNA is transcribed into plus-sense cRNA that is complexed by the viral polymerase and NP to form the cRNP and serves as a template for vRNA synthesis. After export from the nucleus, vRNPs are assembled into new viral particles at the plasma membrane and released from the cell.

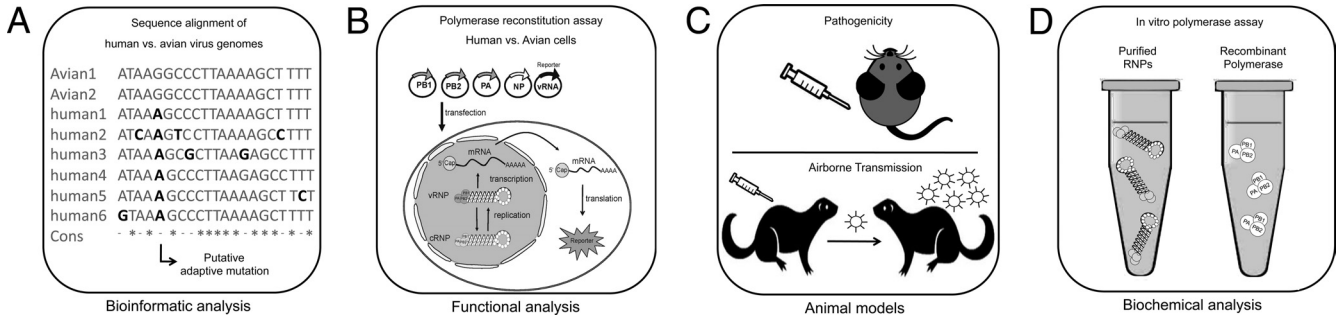

FIG 3 Experimental systems for identification of adaptive mutations of influenza A viruses. Several experimental systems are used to identify adaptive mutations that facilitate host adaptation. These include the following. (A) Bioinformatics analysis of bird- and human-derived influenza virus genome sequences. Such analyses are frequently used to identify unique nucleotide changes that occur only in humans but not in avian species that are selected in the specific host. (B) The polymerase reconstitution assay allows functional analysis of adaptive mutations in the polymerase subunits or NP by reverse genetics. Expression plasmids for the polymerase subunits PB2, PB1, and PA as well as NP and a viral genome analog, harboring a reporter gene instead of the viral protein, are transfected into cells, and reporter gene activity can be measured 24 h later. With this approach, the activities of polymerases from different influenza virus strains can be compared and analyzed in human and avian cells. (C) Different animal models, including mice and ferrets, are used to determine pathogenicity and airborne transmissibility of bird- and human-derived influenza A virus strains. (D) Biochemical analysis can be performed with virion-derived RNPs or recombinant proteins from different expression systems to study polymerase activity *in vitro*.

tachment and entry of the virus into target cells. Adaptation from avian to human hosts has been shown to target three main properties of HA. A switch in receptor specificity from avian (α-2,3-linked) to human (α-2,6-linked) sialic acids as well as stabilization of the stalk region to allow endosomal membrane fusion at the optimal pH is crucial for efficient transmission between mammals (67, 68) and host adaptation (102). Other important factors include appropriate HA glycosylation, the length of the stalk region in the neuraminidase (NA), and specific differences in codon usage (103–106).

As an antagonist of the alpha/beta interferon (IFN-α/β)-mediated host immune response, the NS1 protein plays a critical role during zoonotic transmission (107). The nonessential viral protein PB1-F2 was shown to be able to antagonize the interferon response (108). Additionally, the viral nucleoprotein NP mediates escape from restriction by the interferon-stimulated human MxA protein (50, 109, 110). Polymerase activity of various avian influenza A viruses was shown to be strongly impaired in mammalian cells, restricting replication of avian viruses in mammals (10, 111, 112). Low polymerase activity results not only in fewer copies of vRNA for packaging into new viral particles but also in reduced mRNA synthesis and expression of viral proteins. Perhaps most important for zoonotic viruses, low polymerase activity results in fewer opportunities for the virus to create new variant genomes containing potentially beneficial mutations. For this reason, mutations in the polymerase subunits that increase transcriptional activity are fundamental for avian influenza viruses to adapt to the human host.

THE DEFECT OF AVIAN H5N1 POLYMERASES REMAINS ELUSIVE

The molecular basis underlying the low polymerase activity of avian influenza A viruses in human cells remains a mystery. However, several mechanisms have been postulated to be responsible for this constraint. One popular hypothesis suggests that cellular factors are responsible for the inhibition: either the presence of a negative factor in mammalian cells which leads to inhibition of avian polymerases or the absence of a positive factor in mammalian cells—or the inability of avian viruses to bind such a positive factor—needed for high polymerase activity has been proposed to account for the restriction of avian polymerases in human cells (113, 114). Furthermore, it has long been unclear which transcriptional activity of avian polymerase—synthesis of viral mRNA, cRNA, or vRNA or of all three—is restricted in human cells. Recent work has provided evidence that vRNA synthesis, and thereby vRNP accumulation, is significantly reduced in avian influenza virus-infected cells, possibly due to the generation of defective complementary RNPs (cRNPs), which also results in reduced mRNA synthesis (11). These results suggest that only one enzymatic process may be constrained in human cells; however, further work is required to more precisely characterize this defect in avian polymerases.

ADAPTIVE MUTATIONS IN THE VIRAL POLYMERASE

The significance of enhanced polymerase activity during cross-species transmission of avian viruses to humans is highlighted by the occurrence of numerous adaptive mutations in the viral polymerase proteins during natural infections of mammals (including humans), as well as in experimental infection of animal models. Based on different experimental systems (Fig. 3), adaptive mutations conferring enhanced polymerase activity in mammals have been identified in all three subunits of the viral polymerase, NP, and NEP (Fig. 4) (115, 116). Interestingly, most mutations reside in the PB2 subunit, where they localize in two clusters: in the N and C termini of the protein.

The most often-observed and well-described mutation codes for a lysine instead of a glutamate at position 627 of the PB2 protein (PB2-E627K) and is solely sufficient for replication of several avian influenza viruses in mammals (3, 10, 12, 111). Amino acid 627 is located in the C-terminal region of the PB2 protein which includes the eponymous "627 domain" in addition to the importin alpha-binding domain (117). The change from glutamate to lysine at this position has been proposed to influence host adaptation on multiple levels. It has been shown to increase transmissibility of avian viruses between mammals (118), presumably due to increased polymerase activity at lower temperatures, as found in the human upper respiratory tract (33 to 35°C) compared to the avian intestinal tract (38 to 40°C) (12, 119). In addition, assembly

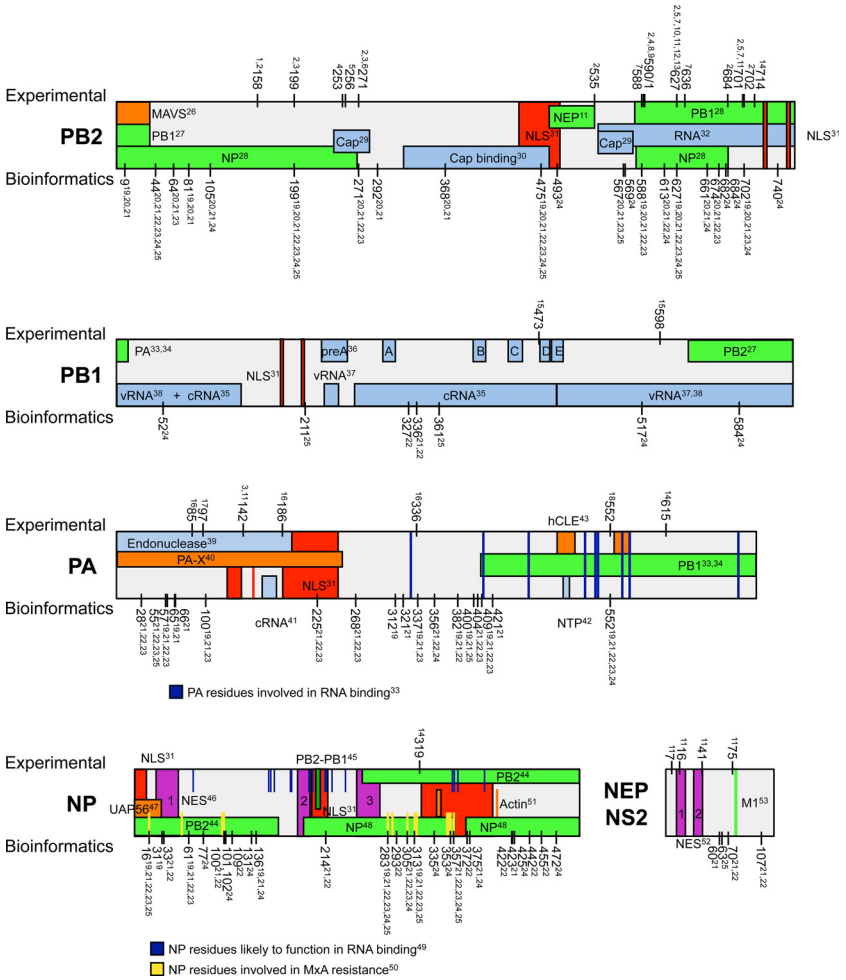

FIG 4 Described mutations increasing polymerase activity in mammalian cells. Published data were analyzed to screen for predicted host-adaptive amino acids (Bioinformatics) and mutations experimentally shown to increase activity of an avian influenza virus polymerase in the context of mammalian cells or to increase pathogenicity in a viral infection (Experimental) (115, 116). Functional domains are indicated in green (interaction with viral proteins), red (involved in nuclear localization), purple (involved in nuclear export), orange (interaction with cellular proteins), yellow (MxA resistance), and blue (RNA binding).

of new vRNPs was suggested to be impaired during replication of avian influenza virus in mammals due to unstable binding between NP and the PB2 subunit, a defect that was rescued by the mutation PB2-E627K (113, 120–122). Furthermore, the emergence of this adaptive mutation in mammalian cells and mice was shown to be dependent on the origin of the viral nucleoprotein (123). Whereas NP of avian origin provoked the appearance of E627K, NP of a human-derived H5N1 virus did not (123). These data indicate a strong correlation between NP and PB2 in host adaptation and the occurrence of adaptive mutations in either protein. However, there is also a deviating interpretation proposing that the reduced binding between PB2 and NP is an indirect observation and is a consequence of the restricted avian influenza virus RNA replication rather than of an alteration in NP-PB2 binding affinity (2).

The interaction of the viral polymerase with host cellular factors also seems to be affected by the species-specific amino acid at PB2 627. Structural investigations of the PB2 627 domain revealed that the change from glutamate to lysine alters the electrostatic surface potential of this domain. Interestingly, a change in shape and surface potential is also seen for the SR polymorphism at positions 590 and 591 in PB2, which is suggested to compensate for the lack of PB2-E627K in the 2009 pandemic H1N1 virus (4, 8, 9, 124). The change in surface potential is hypothesized to influence the interaction with cellular factors, such as α-importins (32, 125–127). Furthermore, in the case of the PB2-binding DEAD box RNA helicase DDX17/p72, the human form of the protein has been shown to stimulate mRNA and vRNA synthesis of human-adapted H5N1 polymerases (PB2-627K) but not their avian precursor, while chicken DDX17 supports growth of avian but not human-adapted H5N1 virus (128). However, it remains to be shown whether the species-specific activity of DDX17 is a determining factor driving human adaptation of avian H5N1 viruses.

Nonetheless, the precise function during species transmission of the PB2 C terminus, which harbors most of the identified adaptive mutations (Fig. 4), remains largely unknown. Strong homology to the cellular RNA-binding clamp-loader complex and increased RNA-binding activity of the recombinant PB2 C-terminal domain harboring the human 627K signature suggest a role in RNA binding (32). However, decreased binding to the viral promoter and a decrease in primed *in vitro* polymerase activity assays

was observed with full-length PB2 627K (129, 130), leaving the role of the 627 domain in RNA binding unresolved.

Several other important polymerase activity-enhancing mutations are found in the PB2 C-terminal domain (2, 7, 9, 14, 18, 118) (Fig. 4). The adaptive mutation PB2-701N is located in the well-defined importin alpha-binding domain and can partially compensate for the lack of PB2-627K in avian viruses (118). The mutation could be shown to influence the interaction with various importin alpha isoforms, which are responsible for the nuclear import of PB2 as well as of other viral proteins (125, 126). Mutation to PB2-701N causes avian viruses to switch dependency from importin alpha 3 to importin alpha 7, which increases viral replication and pathogenicity in mice (126, 131). On the molecular level, recent data suggest that PB2-701N, together with PB2-714R, increases the cap-binding efficiency of the PB2 subunit but decreases primed *in vitro* polymerase activity, an effect also observed with PB2-627K (132). Adaptive mutations in the N terminus of PB2 include exchanges at positions PB2-158G, -199S, -253N, -256G, and -271A (1–6). However, mechanistic insights into the effects of these mutations on polymerase activity and enhanced replication of avian influenza viruses in mammals are not yet available.

In the PA subunit, adaptive mutations seem to cluster in the endonuclease-containing N terminus (39) (Fig. 4). This region overlaps with the newly identified frameshift product PA-X that modulates host response to infection (40). However, it is not clear whether such N-terminal adaptive mutations impact PA-X function. Interestingly, the N terminus of PA is also associated with viral genome promoter binding (41, 133) and regulation of cRNA/vRNA synthesis (134), although it remains to be shown whether these mutations might affect the RNA-binding feature of PA. While additional mutations were identified at various positions in PA, including 336M, 552S, and 615N (3, 11, 14, 16–18), in PB1 only mutations at positions 473 and 598 were shown to increase polymerase activity of avian polymerases in mammalian cells (15).

Although the mutation PB2-E627K has been shown to efficiently adapt the polymerase of avian viruses to human cells, only 40% of H5N1 influenza viruses isolated to date from humans have acquired this mutation (13/01/14 NCBI database) (135), indicating that other mechanisms of adaptation to humans might have evolved. The lack of PB2-E627K can be partially compensated by mutations at other positions in the polymerase subunits, but none of these mutations has a comparable potency to increase polymerase activity (11, 13), and it is conceivable that mutations in other viral factors are required to overcome species barriers.

ADAPTIVE MUTATIONS IN THE VIRAL NUCLEOPROTEIN

To date, few adaptive mutations in NP have been identified as required for the efficient growth of avian influenza viruses in mammalian hosts (Fig. 4). These include N319K, which enhances viral replication in mammalian cells (14, 136) by affecting interaction of NP with host importin-a isoforms (126, 131). Although several other NP mutations were obtained by adaptation of human influenza A virus strains to mice or guinea pigs (137–140), it remains to be shown whether these mutations are also required for avian strains to efficiently replicate in mammalian cells. Multiple potentially adaptive mutations in NP were identified *in silico* by sequence comparisons of avian and human influenza A viruses (Fig. 4). Some of these adaptive mutations (G16D, L283P, F313Y, Q357K) are required to escape from restriction by the interferon-stimulated human MxA protein (50).

NEP: A NEW ADAPTIVE FACTOR

Recent work suggests that NEP is required for the adaptation of some avian H5N1 viruses, specifically those lacking the PB2 E627K mutation (11). Single mutations in NEP from human H5N1 isolates (Fig. 4) were found to be sufficient for NEP to stimulate viral RNA synthesis from avian polymerases in human cells to overcome polymerase restriction and, in conclusion, facilitate adaptation to the new host (11).

The adaptive mutations found in H5N1 human isolates are situated in both the N terminus (M16I and Y41C) and the C terminus (E75G) of NEP (Fig. 4). Interestingly, the C terminus of NEP alone is sufficient to regulate viral polymerase activity (89, 93). This suggests that the N terminus may act as a regulatory domain whose tertiary conformation relative to that of the C-terminal domain determines the protein's cofactor activity. Adaptive mutations may thus affect the interaction between the N-terminal and C-terminal domains, thereby increasing the polymerase activity-enhancing property of NEP through conformational changes. However, further investigations are required to substantiate this hypothesis.

MUTATIONS IN THE NONCODING REGIONS (NCRs)

Although naturally occurring adaptive mutations in the noncoding regions of the viral genomes are not known, artificial introduction of cRNA promoter-like bases into the 3′-end vRNA promoter, especially at positions 3 (G ★ A), 5 (U ★ C), and 8 (C ★ U), referred to as the up-promoter, is also able to rescue the restricted avian polymerase activity in the polymerase reconstitution assay in human cells (2, 83, 141–144).

As expected, during viral infection with recombinant viruses bearing the up-promoter mutations in either the PA- or PB1-encoding segment, segment-specific increases in cRNA and mRNA synthesis were observed (145). However, despite elevated PA or PB1 protein expression, these viruses were attenuated (145) and might explain why this strategy to increase polymerase activity is not observed in natural isolates.

It was shown that the up-promoter mutations lead to increased base pairing in the promoter (141). This might positively influence polymerase activity by changing the promoter structure. Alternatively, the nature of the nucleotides might be the important factor for increased RNA replication, as was suggested by an extensive promoter study (142).

Despite extensive studies on the promoter regions (first and last 12 to 13 nucleotides of the viral genome), very little is known about the impact of mutations in the remaining noncoding regions (NCRs) downstream of the polymerase-binding site (146). Besides their involvement in packaging (147, 148), few studies addressed the regulatory role of these NCRs in translation, mRNA transcription, and RNA replication. Intriguingly, these studies indicated a strong impact of the NCRs on genome replication (149–152), but further detailed analyses are needed to demonstrate a possible involvement of the NCRs in adaptation processes.

CONCLUDING REMARKS

A contribution of the polymerase complex, and particularly the PB2 subunit, in host adaptation to mammals has been known since the 1970s (153). Interestingly, a comparable pattern of adap-

tive mutations in PB2 was found not only in mammals but also in different flightless bird species (Ratitae), including ostrich, emu, and rhea. These include the mutations PB2-591K, -627K, and -701N (154, 155), suggesting related adaptive mechanisms occurring in these species after infection with avian influenza A viruses.

However, the number of adaptive mutations arising in the polymerase subunits also indicates a certain flexibility to overcome the restricted polymerase activity of avian viruses after species transmission and might explain the appearance of multiple compensatory mutations in influenza A virus strains, which have not adopted the human-like PB2-E627K mutation, to successfully replicate in human cells (11, 14, 18).

The ability of each of PB2-627K, NEP, and the artificial up-promoter to almost completely rescue the defect of an avian influenza virus polymerase in mammalian cells raises the hypothesis of a functional linkage of these three strategies, especially considering that adaptive mutations in NEP were observed only in human H5N1 isolates harboring PB2-627E (11). Interestingly, in all three cases, viral genome replication is boosted (2, 11, 89, 145), which corresponds to a potential defect in producing sufficient quantities of vRNA. To date, the exact defect of the avian polymerase in producing insufficient amounts of vRNA has not been known but could involve different steps in viral replication such as insufficient or inappropriate encapsidation of vRNA and/or cRNA, sequence errors introduced into noncoding regions during replication of vRNA, reduced promoter clearance by altered secondary structures, or localization of the RNP to an inappropriate cellular compartment. Furthermore, it remains to be shown whether host-specific factors contribute to this defect.

Increasing evidence indicates that NEP plays a highly versatile role during influenza virus infection, being involved in early and late phases of viral infection. It seems intriguing that a low-molecular-mass (14.8-kDa) protein such as NEP can harbor interaction sites for at least five different viral and cellular proteins (PB1, PB2, M1, CRM1, F1Fo-ATPase). These different interactions must be tightly regulated by as-yet-unknown mechanisms that could involve phosphorylation (156) and sumoylation (157) of NEP.

Clearly, further work is required to elucidate mechanistical insights into the adaptation of the avian influenza virus polymerase in mammals. The fact that the viral polymerase adapts in different bird species, but also that the polymerase PB2 and PA genes of the 2009 pandemic were of avian origin and partially adapted to humans in swine, highlights the necessity of investigating the role of different animal species in the adaptation process of avian influenza A virus to humans. Unraveling the precise function of NEP in viral replication and transcription processes might further help to elucidate the defect of the avian influenza virus polymerase in mammals, which would substantially increase our knowledge about influenza virus host adaptation.

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