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Differential Polymerase Activity in Avian and Mammalian Cells Determines Host Range of Influenza Virus▽

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As recently shown, mutations in the polymerase genes causing increased polymerase activity in mammalian cells are responsible for the adaptation of the highly pathogenic avian influenza virus SC35 (H7N7) to mice (G. Gabriel et al., Proc. Natl. Acad. Sci. USA 102:18590–18595, 2005). We have now compared mRNA, cRNA, and viral RNA levels of SC35 and its mouse-adapted variant SC35M in avian and mammalian cells. The increase in levels of transcription and replication of SC35M in mammalian cells was linked to a decrease in avian cells. Thus, the efficiency of the viral polymerase is a determinant of both host specificity and pathogenicity.

Influenza A viruses have wild aquatic birds as natural hosts, in which they occur with a large variety of different strains defined by 16 HA and 9 NA subtypes (15). Since the host barrier is not an insurmountable obstacle for these viruses, they can occasionally be transmitted from their natural reservoir to terrestrial birds and mammals, including humans. Most of these transmissions are transient. On rare occasions, the viruses adapt to the new species and give rise to a new lineage. Adaptation requires multiple mutations and may involve gene reassortment after coinfection with another virus. By these mechanisms, the human H1N1, H2N2, and H3N2 viruses, which caused the pandemics in 1918, 1957, and 1968, respectively, were generated (24, 26). More recently, avian influenza viruses of subtypes H5N1, H7N7, and H9N2 were transmitted from chickens directly to humans, posing a severe pandemic threat (2, 6, 16, 22). Species specificity has long been known to be a multifactorial trait depending on most viral genes and many host factors (9, 10, 18, 20). However, there is increasing evidence that some viral proteins are particularly important for host adaptation, among them the viral polymerase, which catalyzes both the transcription of viral genomic RNA (vRNA) to mRNA and the replication of vRNA with cRNA as an intermediate. The polymerase, a heterotrimeric complex consisting of subunits PB2, PB1, and PA (1), is active in the nucleus. Nuclear and cytoplasmic host proteins serve as cofactors of the polymerase (3, 4). Several important markers of host range and pathogenicity have been identified in the polymerase genes. Numerous studies have shown that adaptation of an avian virus to a mammalian host was linked to the PB2 mutation E627K (11, 14, 21). However, other mutations in the polymerase proteins or the associated NP protein may also be involved. Thus, when comparing the avian SC35 strain (H7N7) with its mouse-adapted variant SC35M, we previously identified six mutations in SC35M responsible for the increased virulence in mice (L13P and S678N in PB1, D701N and S714R in PB2, K615N in PA, and N319K in NP). By showing that the increase in virulence correlated with enhanced polymerase activity in mammalian cells, we also provided an explanation for the mechanism underlying the adaptation process (7). It was not clear, however, from this study whether the increased polymerase activity of SC35M was host dependent and whether replication or transcription, or both, were affected. Here, therefore, we have analyzed mRNA, cRNA, and vRNA synthesis in avian and mammalian cells. Furthermore, we have compared the pathogenicity of SC35 and SC35M in chicken embryos.

Transmission and replication of SC35 and SC35M in avian and mammalian cells. To compare the transcription and replication properties of SC35 and SC35M, we performed primer extension assays as described previously (5, 25). In primary chicken embryo fibroblasts, mRNA, cRNA, and vRNA levels of SC35M were only 40%, 30%, and 70% of SC35 levels, respectively (100%) (Fig. 1A). In quail fibrosarcoma (QT6) cells, SC35M activities were also reduced (Fig. 1B). In human embryo kidney (293T) cells, however, SC35M showed significantly higher transcription and replication activities than SC35 (Fig. 1C). Results obtained for monkey kidney (Vero) cells were similar to those for 293T cells (Fig. 1D). These data confirm our previous observations showing that SC35M has a replicative advantage over SC35 in mammalian cells (7). They also demonstrate that, vice versa, in avian cells SC35 replicates more efficiently than SC35M.

Effects of individual mutations on transcription and replication. To find out how individual mutations acquired during the adaptation process contributed to the functional differences in avian and mammalian cells, we studied a series of recombinant SC35 mutants comprising, on the one hand, single-gene reassortants (SGR viruses) of SC35 in which one complete polymerase gene was replaced with the corresponding SC35M gene and, on the other hand, SC35 recombinants with SC35M-specific single point mutations (SPM viruses) (7).

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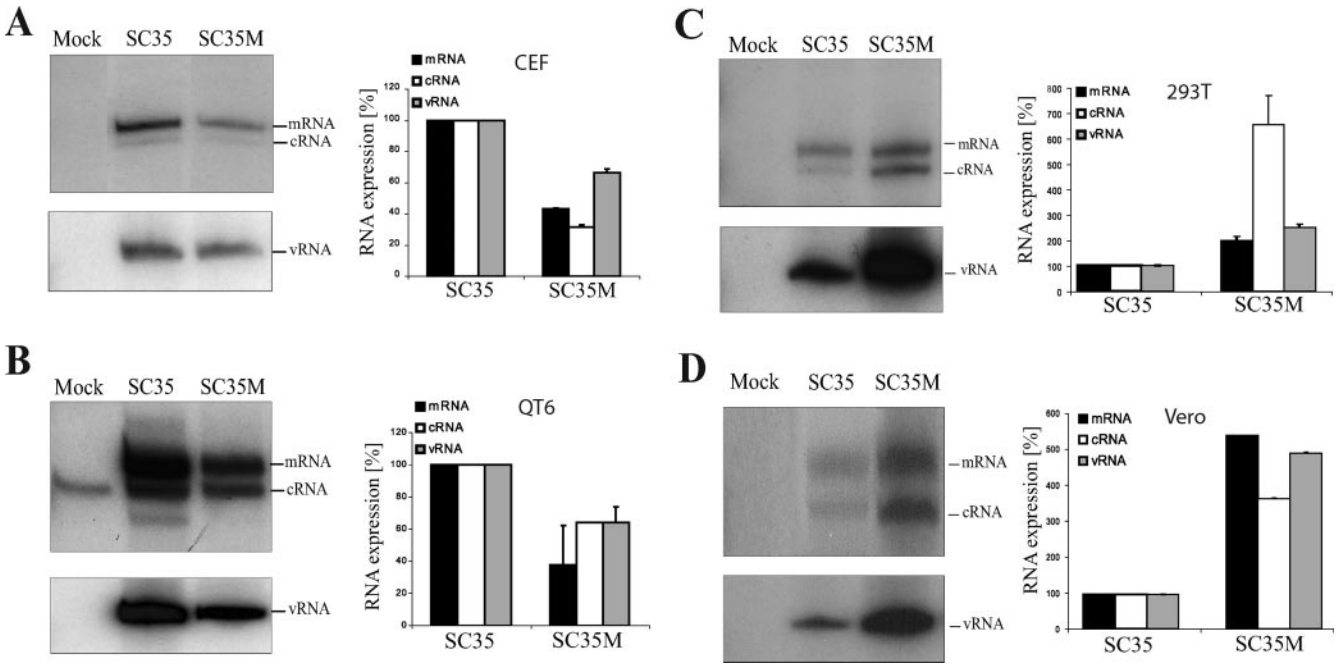

FIG. 1. Transcription and replication activities of SC35 and SC35M in avian and mammalian cells. mRNA, cRNA, and vRNA levels in SC35- and SC35M-infected chicken embryo fibroblasts (CEF) (A), quail fibrosarcoma (QT6) cells (B), human embryo kidney (293T) cells (C), and African green monkey kidney (Vero) cells (D) were determined by primer extension. Primer extension analysis was performed 14 h after inoculation at a multiplicity of infection of 0.1. Transcription products of three independent experiments were quantified using TINA 2.0 software. The results shown are derived from three independent experiments.

First, we examined the SGR viruses for transcription and replication properties in QT6 cells. SC35-PB2$_{SC35M}$, SC35-PA$_{SC35M}$, and SC35-NP$_{SC35M}$ showed mRNA, cRNA, and vRNA levels similar to those of SC35. However, SC35-PB1$_{SC35M}$ had lost the high transcription and replication properties of SC35 (Fig. 2A). The reductions in transcription and replication levels observed with SC35-PB1$_{SC35M}$ were similar to the reductions obtained with SC35M, indicating that PB1 of SC35 is a crucial factor for transcription and replication in avian cells. We then analyzed SPM viruses with PB1 point mutations in QT6 cells. SC35-PB1$_{13P}$ showed mRNA and vRNA levels similar to those of SC35, whereas cRNA levels were reduced. However, the mRNA and vRNA levels of SC35-PB1$_{678N}$ were higher than those of SC35, while cRNA levels were unchanged (Fig. 2A).

To determine which of the SC35M-specific mutations contribute to the increased transcription and replication levels in mammalian cells, we then analyzed RNA levels in Vero cells. With SC35-PB1$_{SC35M}$ and SC35-NP$_{SC35M}$, mRNA, cRNA and vRNA levels were increased, while SC35-PA$_{SC35M}$ showed only vRNA enhancement (Fig. 2B). Therefore, the PB1 or NP gene segment of SC35M, when introduced into the SC35 background, significantly enhanced both transcription and replication activities in mammalian cells. The PB2 and PA gene segments of SC35M enhanced replication slightly, whereas mRNA levels remained unchanged. Finally, to throw light on the effects of the individual mutations in PB1 and PB2, we analyzed the respective PB1 and PB2 SPM viruses. SC35-PB1$_{13P}$ had increased mRNA and cRNA levels, while all RNA species were strongly enhanced with SC35-PB1$_{678N}$. SC35-PB2$_{701N}$ and SC35-PB2$_{714R}$ also showed increased mRNA and cRNA levels, but there was no significant effect on vRNA (Fig. 2B).

Taken together, the data shown in Fig. 2A and B demonstrate that, in Vero cells, all mutations elevated transcription and replication efficiently; none of them affected transcription or replication exclusively. With some mutations, including the PB1 mutation S678N, the PB1 double mutation L13P S678N, and the PB2 double mutation D701N S714R, mRNA, cRNA, and vRNA levels were elevated to similar extents. With other mutants, enhancement was disproportionate. PA mutation K615N and NP mutation N319K stimulate vRNA synthesis more than mRNA and cRNA synthesis. The PB2 mutations D701N and S714R do not enhance vRNA levels as much as mRNA and cRNA levels. However, except for PB1 mutation L13P, all mutations caused similar mRNA and cRNA shifts, suggesting that mRNA and cRNA synthesis are closely linked. This observation is interesting because it is compatible with the proposal that mRNA and cRNA are synthesized simultaneously early in infection (25), but it does not rule out the classical model implicating a switch from early transcription to subsequent replication (8, 13, 19). PB2 mutations D701N and S714R and NP mutation N319K, which are convergent with H5N1 strains (7), also enhance the polymerase activity of reconstituted RNP complexes in mammalian cells when transferred into the PB2 and NP genes of an unrelated avian virus (Fig. 3). This result demonstrates that these mutations act in mammalian cells independently of the context of the virus genome. Whereas each individual mutation alters polymerase activities quite significantly in mammalian cells, the effects are

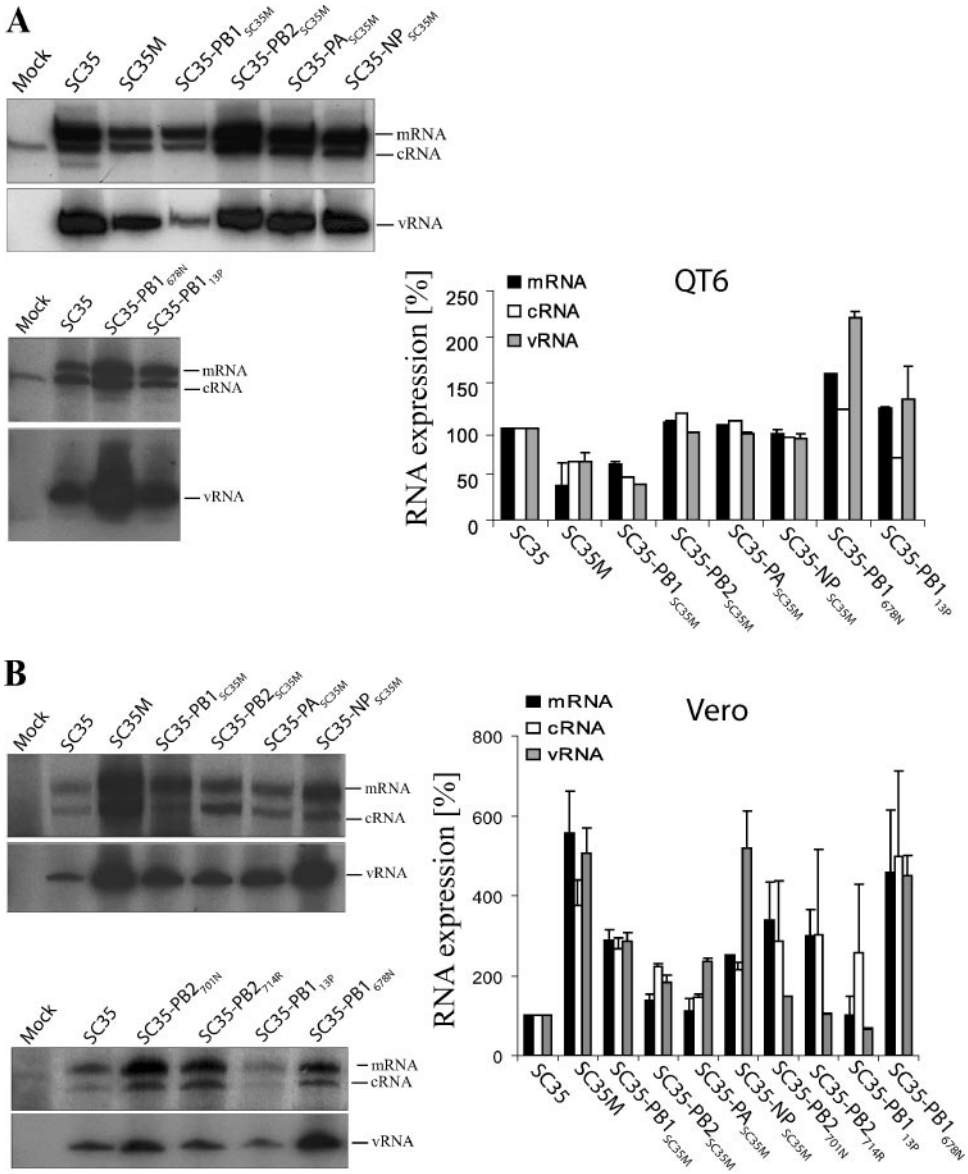

FIG. 2. Transcription and replication activities of SGR and SPM viruses in avian and mammalian cells. Quail fibrosarcoma (QT6) cells (A) and African green monkey kidney (Vero) cells (B) were infected with SC35, SGR viruses (SC35-PB1$_{SC35M}$, SC35-PB2$_{SC35M}$, SC35-PA$_{SC35M}$, and SC35-NP$_{SC35M}$), and SPM viruses (SC35-PB2$_{701N}$, SC35-PB2$_{714R}$, SC35-PB1$_{13P}$, and SC35-PB1$_{678N}$) at a multiplicity of infection of 0.1. At 14 h postinfection, mRNA, cRNA, and vRNA levels were determined by primer extension. The results shown are derived from three independent experiments.

less distinct in avian cells. Only the PB1 double mutation L13P S678N reduced transcription and replication activities to the levels observed with SC35M. Interestingly, the PB1 mutation S678N alone raised these activities in avian as well as mammalian cells, suggesting that the enhancing effect of this mutation is host independent. Most mutations, however, did not alter RNA synthesis in avian cells. These observations support the notion that the virus, when crossing the species barrier, goes through a phase that allows gradual acquisition of adaptive mutations without losing fitness for the old host. Such conditions should favor the development of the constellation of mutations necessary for optimal growth in mammalian cells.

Pathogenicity of SC35 and SC35M in chicken embryos. It has been reported previously that both SC35 and SC35M are pathogenic for chickens (12). However, that study was performed with adult chickens and at a high inoculation dose of 10^6 PFU that did not allow quantitative assessment of the virulence of these viruses. Therefore, we determined the 50% egg lethal dose (ELD_{50}) and the mean time to death (MDT) for SC35 and SC35M in 11-day-old embryonated chicken eggs as described previously (17, 24). Eggs were inoculated with 10-fold virus dilutions ranging from 10^6 to 10^{-2} PFU. SC35M showed lower virulence (ELD_{50}, 11.5 $\log_{10}$ PFU) and mortality (MDT, 96 h) in chicken embryos than SC35 (ELD_{50}, 0.2 $\log_{10}$

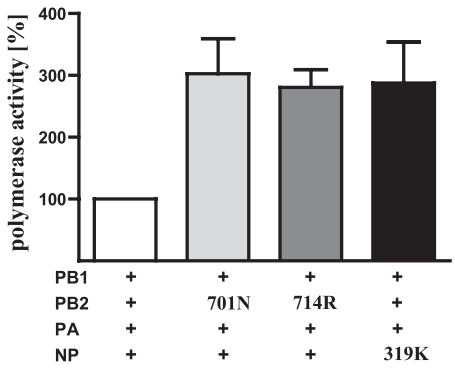

FIG. 3. Effects of PB2 mutations D701N and S714R and NP mutation N319K on RNP activities of A/Fowl plague virus (FPV)/Rostock/34 H7N1 in mammalian cells. 293T cells were transfected as indicated with plasmids containing FPV genes PB2, PB1, PA, and NP and FPV mutant genes PB2 D701N, PB2 S714R, and NP N319K. Polymerase activity was determined 24 h after transfection by measuring luciferase activity (7). The baseline value is the result of the cotransfection of plasmids pPol1-NP-Luc and pRSV-lacZ and was subtracted from each measurement. The results shown are derived from four independent experiments.

PFU; MDT, 24 h). These data demonstrate that the reduced transcription and replication activity of SC35M in avian cells corresponds to diminished virulence, while the high transcription and replication properties of SC35 in avian cells correspond to increased virulence in chicken embryos. Replication efficiency is therefore a determinant not only of host range but also of pathogenicity.

Our results suggest that the mutations observed with SC35M mediate the adaptation of the viral polymerase complex to host factors. This concept is supported by a recent study in which the structure of the C-terminal domain of PB2 was elucidated. It revealed that the mutation sites 701 and 714 are exposed at the surface of the molecule, where they interact with a bipartite nuclear localization signal that promotes transport into the nucleus by binding to importin α5 (23). Structural analysis of the NP protein has shown that the mutation site 319 is also located at the surface of the molecule, and it has been proposed that it may mediate binding to other subunits of the polymerase (27). Thus, it appears that the mutations responsible for adaptation to a new host alter interaction with host factors by affecting the contact site either directly, or indirectly by inducing structural changes in other subunits of the polymerase.

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