The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host

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Mammalian influenza viruses are descendants of avian strains that crossed the species barrier and underwent further adaptation. Since 1997 in southeast Asia, H5N1 highly pathogenic avian influenza viruses have been causing severe, even fatal disease in humans. Although no lineages of this subtype have been established until now, such repeated events may initiate a new pandemic. As a model of species transmission, we used the highly pathogenic avian influenza virus SC35 (H7N7), which is low-pathogenic for mice, and its lethal mouse-adapted descendant SC35M. Specific mutations in SC35M polymerase considerably increase its activity in mammalian cells, correlating with high virulence in mice. Some of these mutations are prevalent in chicken and mammalian isolates, especially in the highly pathogenic H5N1 viruses from southeast Asia. These activity-enhancing mutations of the viral polymerase complex demonstrate convergent evolution in nature and, therefore, may be a prerequisite for adaptation to a new host paving the way for new pandemic viruses.

Materials and Methods

Cells and Viruses. We grew 293T human embryonic kidney cells in DMEM supplemented with 10% FCS and Madin–Darby canine kidney (MDCK) cells in MEM containing 10% FCS. We produced chicken embryo fibroblasts by digesting pieces of 11-day-old chicken embryos in buffered saline containing 2.5% trypsin (GIBCO/BRL); the cells were then cultured in MEM with 10% FCS. We propagated influenza A viruses in 11-day-old embryonated chicken eggs. The growth curves were determined in three independent experiments by plaque titration (9). SC35, SC35M, and their mutants were handled under biosafety level 3 conditions.

Plasmids. To clone all eight genes of SC35 and SC35M, we amplified each segment by RT-PCR from isolated RNA (10) and ligated it into plasmid pHW2000 (11). For reporter assays, we used an influenza minigene containing luciferase as reporter protein (pPolI-NP-luc) and for standardization a β-gal-expressing plasmid (pRSV-β-gal). The plasmid pPolI-NP-Luc expresses a negative-sense RNA transcript carrying the complete ORF of the firefly luciferase gene (accession no. AF053462) flanked by the noncoding regions of the NP segment of influenza A/WS/33 virus (23 nt of the viral RNA 5′ end and 45 nt of the 3′ end; accession no. M30746).

Mutagenesis. We introduced mutations of SC35M into the cDNAs from SC35 or vice versa by site-directed mutagenesis with the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s protocol. We verified the presence of the introduced mutation and the absence of additional unwanted mutations by sequencing the whole cDNA.

Rescue of Recombinant Viruses. We transfected 293T cells with a mixture of eight plasmids encoding all eight influenza gene segments (1 µg each) and Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. We removed the medium 6 h later and replaced it with DMEM containing 0.2% BSA. After another 42 h, we plated the supernatant onto MDCK cells. We plaque-purified rescued viruses twice and inoculated them in 11-day-old hen eggs for propagation of stock virus. We ascertained the identity of propagated mutant viruses by sequencing amplicons of each viral gene segment by using RT-PCR.

Growth Curves. We inoculated chicken embryo fibroblasts, Vero cells, A549 (human lung epithelial cell line) and LA-4 (mouse adenoma cells), respectively, with virus at a multiplicity of infection of 10−4 and incubated them in the appropriate medium containing 0.2% BSA at 37°C. At time points 0, 8, 24, 48, 72, 96, and 120 h, we collected supernatants and determined the plaque titer on MDCK cells.

Luciferase Assay After Transfection. We transfected 293T cells with the pHW2000 plasmids constructs encoding the polymerase genes PB2, PB1, PA, and NP and the pPolI-NP-Luc and pRSV-β-gal plasmids (1 µg each) plus Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. After 6 h, we replaced the medium with DMEM (10% FCS). At 24 h after transfection, we:

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Abbreviations: HA, hemagglutinin; HPAIV, highly pathogenic avian influenza virus; MDCK, Madin–Darby canine kidney; NA, neuraminidase; NP, nucleoprotein; SGR, single-gene reassortant.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ266094–DQ266101).

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harnessed and lysed the cells. We then assayed luciferase activity by using the Luciferase assay system detection kit (Promega). This assay was standardized against the β-gal activity (resulting from co-transfection of the pRSV-β-gal plasmid), which was determined with a β-gal enzyme assay system (Promega). The baseline value is the result of the co-transfection of pPol1-NP-Luc plus pRSV-β-gal and was subtracted from each measurement. Each luciferase activity value is the average of four independent experiments.

Luciferase Assay with Recombinant Virus. We transfected 293T cells with 1μg of pPol1-NP-Luc plasmid and for standardization with 1μg of pRSV-β-gal plasmid plus Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. For infection, we washed the cells with PBS and inoculated them with influenza virus at a multiplicity of infection of 0.1. After 45 min, we washed the cells twice with PBS 10 h after transfection and incubated them with DMEM (0.2% BSA) for 14 h. We then harvested the cells 14 h after infection and assayed luciferase activity.

Animal Experiments. The animal experiments were performed according to the guidelines of the German Animal Protection law. All animal protocols were approved by the Regierungspräsidium Tübingen and the Regierungspräsidium Giessen. We anesthetized 4- to 6-week-old, female BALB/c mice (obtained from the animal breeding facility of the Friedrich-Löffler-Institut and bought from Charles River Laboratories) with 100 mg/kg ketamine and xylazine and inoculated them intranasally with 10 μl of infectious virus diluted in PBS. The BAS were determined by intranasal inoculation with a 10-fold dilution series of virus (10^6 plaque-forming units/ml) and calculated the LD50 as described in ref. 12.

Tree Calculation. We downloaded the nucleotide sequences from the Los Alamos Influenza Database (virus names and accession numbers in Table 3, which is published as supporting information on the PNAS web site) (13) and aligned them by using the PILEUP program of the WISCONSIN package, version 10.2 (Genetics Computer Group, Madison, WI). For tree calculation, we performed a neighbor-joining analysis following bootstrap calculation (1,000 samples) by using the programs SEQBOOT, DNADIST, NEIGHBOR, and CONSENSE from PHYLIP 3.6 (14). The regions of PB2, PA, and NP sequences used for calculation were nucleotides 1039–2268, 51–2169, and 46–1542, respectively.

Results

Generation of Recombinant SC35 and SC35M Viruses. Recombinant SC35 and SC35M viruses were generated by reverse genetics (11) after their genes were cloned into the plasmid pHW2000 (11). By using appropriate plasmid combinations, we then obtained single-gene reassortants (SGR) containing seven genes of SC35 plus one of SC35M and vice versa. According to the nomenclature we used, an SGR of SC35 with the NP gene of SC35M was designated SC35-NPSC35M. After directed mutagenesis, we also rescued recombinant viruses with point mutations. A virus containing the SC35 genes with the mutation L13P→I, observed in PB1 of SC35M, was named SC35-PB1L13P. Its reciprocal counterpart is the virus SC35M-PB1L13P. The low-pathogenic SC35 differs from the highly pathogenic SC35M by 9 amino acid exchanges. There are three amino acid exchanges in PB2, two in PB1, and one in NA.

Growth in Chicken and Mammalian Cells. We studied the growth of SC35 and SC35M in avian and mammalian cells. In chicken embryo fibroblasts, both viruses grow to high titers (>10^7 plaque-forming units/ml) without notable difference (Fig. 1A). This result corresponds to high pathogenicity of SC35 and SC35M in chickens (7, 8). In mammalian cell lines, like monkey kidney cells (Vero) (Fig. 1B), mouse lung adenoma cells (LA-4) (Fig. 1C), and human epithelial lung cells (A549) (Fig. 1D), the mouse-adapted SC35M grows to titers 2–4 logs higher than SC35. This difference demonstrates a considerable growth advantage of SC35M in mammalian cells.

Activity of Reconstituted Ribonucleoprotein Complexes. To investigate whether the SC35M polymerase mutations change enzymatic activity, we analyzed reconstituted ribonucleoprotein complexes in 293T cells by a luciferase minigene assay. SC35M has a polymerase
activity of 600% compared with SC35 (Fig. 2A, columns a and b). To assign this increase to single mutations, we investigated several combinations. The SC35M PB2 and NP increased polymerase activity to 300% and 220%, respectively, whereas PB1 and PA did not (Fig. 2A, columns c–f). PB2 and NP of SC35M together with PA and PB1 of SC35 have a similar polymerase activity of 600% as the authentic polymerase complex of SC35M (Fig. 2A, column g). By contrast, the reciprocal constellation of SC35 PB2 and NP with SC35M PB1 and PA showed the low polymerase activity of SC35 (Fig. 2A, column h), stressing the importance of the combination of PB2 and NP from SC35M for enhanced replication. Replacing one gene of the SC35M polymerase complex lowered its activity (Fig. 2A, column i–l). This finding implies that, in addition to PB2 and NP, the homologous combination of PB1 and PA of SC35M (Fig. 2A, column b) or SC35 (Fig. 2A, column g) is required. Thus, PB1 and PA have to match for the high polymerase activity of SC35M. We then introduced the three SC35M PB2 mutations into the SC35 PB2 plasmid. PB2 701N and 714R each increase activity up to 300% (Fig. 2B, columns d and e); stepwise combination of 701N and 714R in PB2 and 319K in NP leads to further rises (Fig. 2B, columns f, j, k, m, and n). Taken together, mutations in SC35M PB2 (701N and 714R) and NP (319K) considerably enhance polymerase activity in mammalian cells in an additive manner. In contrast, PB2 333I is a down-regulating mutation. Lys-627 in PB2 is a well known determinant of mammalian host range (15). The HPAIV SC35 has glutamic acid in this position like most avian isolates. The amino acid replacement 627K in the PB2 plasmid of SC35 raised the polymerase activity to 2,000% (Fig. 4, which is published as supporting information on the PNAS web site). Thus, this host range mutation, which is not observed in SC35M, also enhances polymerase activity.

**Polymerase Activity of Recombinant Virus.** In the luciferase-minigene assay performed with live virus, SC35M showed an increase in polymerase activity of 420% compared with SC35 (Fig. 2C; see also Fig. 5, columns a and b, which is published as supporting information on the PNAS web site). SGRs containing one polymerase gene segment of SC35M, SC35-PB1SC35M, SC35-PB2SC35M, SC35-PASc35M, and SC35-NPsc35M have increased polymerase activity ranging between 200% and almost 1,000% (Figs. 2C and 5, columns c, f, g, and h). The PB1 single-point mutants SC35-PB113P and SC35-PB1678N showed enhanced activities of 270% and 170%, respectively (Figs. 2C and 5, columns d and e). However, the reciprocal SC35M PB1 mutants, SC35M-PB113L and SC35M-
PB1_{701N} possess activities of even 1,000% and 1,400% (Figs. 2C and Fig. 5, columns o and p). The PB2 point mutants SC35-PB2_{701N} and SC35-PB2_{714R} have an increase in activity (180% and 170%), even higher in combination with SC35M NP: SC35-PB2_{701N}-NPSC35M and SC35-PB2_{714R}-NPSC35M (310% and 290%) (Figs. 2C and 5, columns i-l). Combination of the two PB2 mutations in SC35-PB2_{701N+714R} further enhances polymerase activity to 450% but not with SC35M NP as in SC35-PB2_{701N+714R}-NPSC35M (230%) (Figs. 2C and 5, columns m and n). Therefore, efficient polymerase activity is achieved by the combination of PB2 701N or PB2 714R with SC35M NP. Removal of PB2 701N in SC35M (SC35M-PB2_{701D}) did not change polymerase activity. However, replacement of 714R as in SC35M-PB2_{714S} lowered it (150%) (Figs. 2C and 5, columns r and s). Therefore, PB2 714R is essential for high polymerase efficiency. SC35M-PB2_{333T} without the suppressing mutation 333I has an LD_{50} of 1.4, which is even lower than that of SC35M (LD_{50} = 2.8). Therefore, both the polybasic cleavage site and the polymerase mutations contribute to high pathogenicity of SC35M.

**Correlation of Polymerase Activity with Pathogenicity.** Pathogenicity increases with rising polymerase activity (Fig. 2C). The most virulent viruses (SC35-PB2_{701N+714R}, SC35M-PB2_{701D}, SC35M, and SC35M-PB2_{333T}) have activities >4-fold higher than avirulent viruses: However, three viruses (SC35-PB1_{333T}, SC35M-PB1_{111D}, and SC35M-PB1_{678S}) with excessive activity (900%) are not of the highest virulence in mice. Thus, it appears that high pathogenicity requires a polymerase activity optimum.

**SC35M Polymerase Mutations in Mammalian Isolates.** To find out whether the amino acid exchanges observed in SC35M occur also in other strains, we looked at corresponding positions in published sequences (13). These positions are highly conserved and in most instances contain the same amino acid as SC35. However, some isolates have mutations at positions PB2 701 and 714, PA 615, or NP 319 as does SC35M, although the resulting amino acid exchanges were not always identical (Table 2). Interestingly, most of these strains are mammalian viruses that have been recently transmitted from birds and are still in adaptation to the new host. From their sequences and those of representative strains (16–18), phylogenetic trees were calculated to see whether the SC35M-like mutations are inherited in related lineages or whether they occur independently.

Among the 694 sequences analyzed, 664 contained aspartate as SC35 and 30 asparagine at position PB2 701 (Table 2). PB2 701N is present in 28 mammalian and two related avian isolates (Fig. 3A) and comprise several clades of H1N1, H1N2, and H3N2 swine isolates, H3N8 and H7N7 equine isolates, the mouse-adapted variant of pandemic A/HK/1/68 (H3N2), and the clade of H5N1 HPAIV from southeast Asia, including human isolates, like A/HK/97/98 and A/HK/488/97. Moreover, PB2 701N was also found in A/VietNam/3046/2004 (6) and A/Tiger/Suphan/Thail./T1-1/04 (19), which were isolated during outbreaks in Vietnam and Thailand. Remarkably, among the strains found is A/Swine/Germany/2/81, an avian-like swine virus (2). This isolate belongs to a lineage originating from an avian virus that was transmitted as a whole into swine (20). Thus, PB2 701N might support the adaptation to the mammalian host.

At position PB2 714, most viruses have the serine present in SC35, whereas amino acid exchanges are observed in four mammalian strains and 16 avian H5N1 isolates from 2001 (Table 2). The latter ones are highly pathogenic for chickens and guails, and they

### Table 2. Database search for SC35M mutations in natural strains

<table>
<thead>
<tr>
<th>Viral gene</th>
<th>Amino acid position</th>
<th>Total no. sequences</th>
<th>No. same as SC35</th>
<th>No. same as SC35M</th>
<th>No. different</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB2</td>
<td>701</td>
<td>694</td>
<td>664 (Asp)</td>
<td>30 (Asn)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>714</td>
<td>694</td>
<td>674 (Ser)</td>
<td>—</td>
<td>16 (Ile), 4 (Gly)</td>
</tr>
<tr>
<td>PA</td>
<td>615</td>
<td>650</td>
<td>600 (Lys)</td>
<td>1 (Asn)</td>
<td>49 (Arg)</td>
</tr>
<tr>
<td>NP</td>
<td>319</td>
<td>1,101</td>
<td>1,082 (Asn)</td>
<td>19 (Lys)</td>
<td>—</td>
</tr>
</tbody>
</table>

Comparison of amino acids in sequences of natural strains at positions corresponding to SC35M mutations. Shown are the number of all sequences in the database, the number of sequences with the same amino acid as SC35 and SC35M, and the number of sequences with a different amino acid at the same position (with the amino acids shown in parentheses). —, not applicable.
replicate well in mouse lung in contrast to their precursors, the Gs/Gd-like strains (21). One of these strains, A/Goose/Guangdong/1/96 (22), has serine at position PB2 714 as does SC35 (Fig. 3A). In contrast to the Gs/Gd-like viruses, the H5N1 isolates appear to have increased pathogenicity for mammals (21), supporting the view that PB2 714I is a host range marker.

PA 615R was found only in A/Duck/Shanghai/08/2001 (H5N1). Among 650 isolates, 600 isolates harbor lysine as SC35, 1 isolate harbors Asparagine, and 49 isolates harbor arginine (Table 2). PA 615R was found in several clusters of human H1N1, H5N1, and H9N2 isolates (Fig. 3B), including H5N1 HPAIV isolates of known high pathogenicity for mice and humans, like A/HK/483/97, A/HK/485/97, and A/HK/491/97 (23). The strains A/Teal/HK/W312/97 (H6N1) and A/Quail/HK/G1/97 (H9N2), proposed donors of internal genes of H5N1 viruses (24–26), also were found, emphasizing the relevance of PA 615R for host change.

NP 319K in SC35M, was observed in 19 of 1,101 sequences (Table 2 and Fig. 3C); the others contain asparagine. Fourteen isolates are human and equine strains. Five isolates are highly pathogenic for chickens and closely related to the highly pathogenic human H5N1 isolate A/HK/156/97 (27). Remarkably, this virus and other human H5N1 isolates (A/HK/482/97, A/HK/486/97, and A/HK/538/97) (23, 28) harboring NP 319K also carry PA 615R. Therefore, these changes in combination might be responsible for higher virulence in mammals. In general, the phylogenetic trees of PB2, PA, and NP demonstrate (Fig. 3) that the amino acids corresponding to SC35M exchanges are distributed over unrelated clusters. Therefore, these changes occurred independently. This finding demonstrates convergent evolution of the polymerase complex in different virus lineages during adaptation to the mammalian host.

**Discussion**

The molecular basis of adaptation of influenza A viruses to a new host species is poorly understood. To address this problem, we have analyzed the HPAIV SC35 and its mouse-adapted variant SC35M, which differ by seven amino acid exchanges in the polymerase, one amino acid exchange in HA, and one amino acid exchange in NA. Comparison of LD50 in mice revealed that all polymerase mutations contribute independently to virulence. Two SC35M mutations in PB2 (701N and 714R) and one in NP (319K) enhance polymerase activity. The amino acids found at positions PB2 701 and 714, NP 319, and PA 615 of SC35 are highly conserved and therefore present in most viruses analyzed. However, SC35M-like amino acid exchanges were found in some mammalian strains that have only recently been derived from avian viruses and in H5N1 HPAIV isolates that are also pathogenic for mammals. These observations support the concept that mutation of the polymerase complex is critical for adaptation to the new environment once the virus has been transmitted to a new host. The functional role of these mutations is not fully understood, but it is reasonable to assume that they are necessary for optimized interaction of the polymerase with the host leading to enhanced replication and transcription. Our data therefore underline that host factors are important for polymerase action. PB1 13P and 678N are localized within the PA-binding site (residues 1–25; α-domain) and the PB2-binding site (residues 600–757), respectively (29). Therefore, PB1 13P and 678N could improve the interplay of polymerase subunits and NP in a new host.
environment. SC35M PB2 333I is located within the cap-binding region (30) and might therefore affect viral transcription. The virus without this mutation, SC35M-PB2333IT, exceeds even the high virulence of SC35M.

HPAIV possesses polybasic cleavage sites in HA recognized by ubiquitous proteases of the subtilisin family (31–33). Such a cleavage site that is a marker for high pathogenicity is present in SC35 and SC35M. However, it is interesting that SC35M-HAmonobasic is more virulent for mice than SC35 but less virulent than SC35M. This finding highlights the importance of the polybasic cleavage site and the SC35M polymerase mutations in SC35M pathogenicity.

PB2 amino acid 627 is a known determinant of replication efficiency (34, 35) and host specificity (15). The substitution PB2 E627K was attributed to increased pathogenicity in avian H5N1 influenza viruses. Thus, there is evidence in several cases that PB2 627K is involved in adaptation to mammalian hosts. Introduction of 627K into the SC35 PB2 plasmid also raised the polymerase activity excessively (2,000%), although this mutation is not to be compatible with high virulence, indicating that an optimal polymerase activity is needed in the mammalian host. However, the luciferase activity as indirect marker corresponds to overall mRNA synthesis. Therefore, these mutations may lead to an imbalanced high transcription/replication ratio.

The finding of SC35M-like mutations in strains transmitted recently from birds and still undergoing adaptation points to convergent evolution (38) of the influenza polymerase complex in nature. Remarkably, the strains A/Teal/HK/W312/97 (H6N1) and A/QuakerH/G/1/97 (H9N2), proposed donors of internal genes of these H5N1 viruses (24–26), also harbor PA 615R. Human H5N1 isolates, which caused disease (23, 28), carry PA 615R and NP 319K. This combination might increase the virulence additionally. Experimental studies similar to the present work are needed to prove this concept.

Taken together, we demonstrated that the polymerase-enhancing mutations of SC35M contribute to increased virulence in mice and found convergent evolution of SC35M-like mutations in mammalian and H5N1 HPAIV strains. Therefore, the enhanced activity of viral polymerase enables HPAIV to adapt to a mammalian host. The viral polymerase may be the driving component of early evolution of influenza A viruses in a new host and pave the way for new pandemic viruses.