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Highly Pathogenic Avian Influenza Virus Subtype H5N1 Escaping Neutralization: More than HA Variation

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Influenza A viruses are one of the major threats in modern health care. Novel viruses arise due to antigenic drift and antigenic shift, leading to escape from the immune system and resulting in a serious problem for disease control. In order to investigate the escape process and to enable predictions of escape, we serially passaged influenza A H5N1 virus *in vitro* 100 times under immune pressure. The generated escape viruses were characterized phenotypically and in detail by full-genome deep sequencing. Mutations already found in natural isolates were detected, evidencing the *in vivo* relevance of the *in vitro*-induced amino acid substitutions. Additionally, several novel alterations were triggered. Altogether, the results imply that our *in vitro* system is suitable to study influenza A virus evolution and that it might even be possible to predict antigenic changes of influenza A viruses circulating in vaccinated populations.

Influenza A viruses are one of the major threats in modern health care. Besides seasonal epidemics caused by influenza viruses, pandemic viruses, like the 2009 H1N1 swine origin influenza A virus, significantly impact human and animal health. The segmented negative-strand RNA genome of influenza A virus comprises 8 segments ranging in size from approximately 850 nucleotides (nt) to 2,350 nt. Novel viruses arise due to antigenic drift and antigenic shift, the former caused by continuous mutation of the genomes as a result of the error rate of the viral RNA-dependent RNA polymerase and the latter the result of reassortments of genome segments during infection of a single host with different influenza A viruses (8).

The economic consequences, in addition to the zoonotic implications, of highly pathogenic avian influenza virus (HPAIV) H5N1 are still important. Endemic situations affecting Southeast Asia and Egypt are still an unsolved problem (9). Some countries tried to combat the animal disease by vaccination of poultry with inactivated vaccine preparations. For Egypt, vaccination of household/village poultry provided by the government was suspended in July 2009 because of limited impact on H5N1 HPAI incidence (9). Similar to vaccination in humans, in birds, a nonsterile immunity arises. As a consequence, antibodies and viruses coexist. In turn, so-called "escape mutants" resulting from antigenic drift of the viruses are selected (40). These escape mutants are less susceptible to vaccine-induced neutralizing antibodies. Related to vaccination programs and sometimes deficiencies in the programs, the occurrence of escape variants in poultry has been described for Central America, Indonesia, China, and Egypt (9, 11, 15, 19, 24, 31, 32).

In different studies, antigenic epitopes in the hemagglutinin (HA) protein were identified by sequencing and structural mapping after generating escape variants *in vitro* using monoclonal antibodies (21, 33, 37) or polyclonal (rabbit- or mouse-derived) antiserum (3, 23). However, polyclonal antisera from chickens were never used before to generate escape variants, although vaccine escape is a serious problem in influenza virus eradication programs for poultry, especially for HPAIV H5N1 (12).

The goal of the present study was to define hot spots in the viral genome, where mutations that enable immunoescape might preferentially or even mandatorily occur in a population with nonsterile immunity (e.g., in a vaccinated poultry flock). Moreover, we aimed to simulate and estimate the dynamics of immunoescape, i.e., to get a comprehensive view of adaptations with regard to the chronological succession of occurrence. To this end, we applied serial virus passaging under serum pressure, full-length virus genome deep sequencing using a Genome Sequencer FLX, and analysis of the viral diversity. Phenotypic *in vitro* and *in vivo* characterization confirmed escape and unveiled attenuation of the viruses. Sequencing revealed mutations already found in natural isolates, proving the *in vivo* relevance of the *in vitro*-induced amino acid substitutions. Most importantly, several novel amino acid alterations were identified.

MATERIALS AND METHODS

Ethics statement. This study was carried out in strict accordance with the recommendations and guidelines of the German Animal Welfare Act. The protocol and the animal trial were approved by the Committee on the Ethics of Animal Experiments of the Federal State of Mecklenburg-Western Pomerania, Germany (registration and approval number LALLF MV/TSD/7221.3-1.1-003/07).

Viruses. The ancestor virus for passaging was the 3rd passage of the reference strain A/cygnus cygnus/Germany/R65/2006 (H5N1) (45). All experiments using HPAI H5N1 virus were conducted in biosafety level 3+ containment facilities at the Friedrich-Loeffler-Institut (FLI), Greifswald-Insel Riems, Germany.

Cell culture. The principal steps for passaging virus under positive serum pressure were as follows. Virus and serum were preincubated at room temperature in 1 ml Dulbecco modified Eagle medium supplemented with 5% fetal calf serum for 1 h by gentle shaking. Madin-Darby canine kidney (MDCK) cells (Collection of Cell Lines in Veterinary Medicine, FLI, Insel Riems, Germany; RIE1061) were seeded in a 6-well plate

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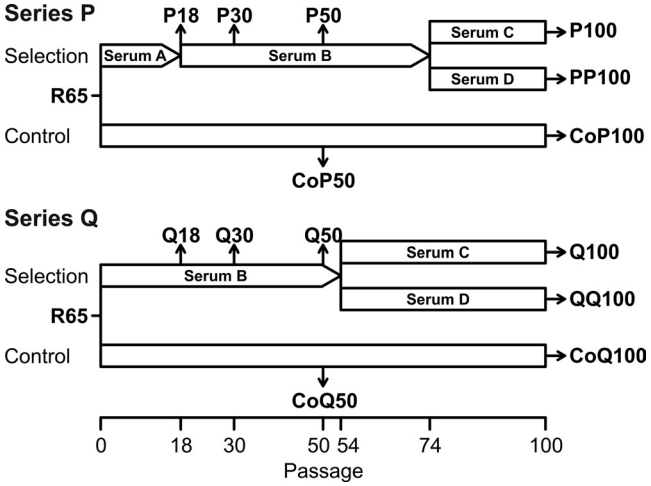

FIG 1 Schematic of virus passaging. The design of the passaging experiments is shown, accompanied by the designations of sera and virus isolates for series P and Q. The arrows labeled P*nn*/Q*nn* (where P is a series P virus isolate, Q is a series Q virus isolate, and *nn* is the number of passages before isolation) illustrate virus isolates that were characterized in detail. Note that beginning with introduction of sera C and D, two parallel passages were conducted, resulting in two isolates each of the P(P)100 and Q(Q)100 viruses.

(35-mm diameter) and incubated in a humidified 5% CO_2-air atmosphere at 37°C for at least 1 h. Then, the medium was replaced by the mixture of serum and virus and incubated at 37°C for an additional hour. By adding 1 ml cell culture medium, the final volume of 2 ml per well was achieved. Three different concentrations of serum were tested on one 6-well plate. After 48 to 72 h, the cell culture supernatant was harvested. The serum-virus mixture with the maximum serum concentration that still allowed virus replication, assessed by cytopathic effect, was used for continuation of passaging. Serum/virus ratios were continuously increased to expose minimum virus to maximum serum-induced pressure. Two independent groups of viruses that were serially passaged 100 times (series P and Q) were generated. While for escape group P three different sera were used successively, escape group Q was passaged in the presence of only two different sera consecutively (Fig. 1 shows the passaging scheme and virus nomenclature). When sera C and D were introduced (Fig. 1), the viruses were passaged in parallel twice, resulting in two isolates each for the viruses passaged 100 times. All sera were used as long as possible, i.e., until they were used up, in order to enable analysis of the long-term impact of the serum on the generation of escape variants. In parallel, to determine the pure passaging effect without additional impacts that might be exhibited by components contained in serum obtained from a naïve donor, control viruses were generated by passaging the ancestor virus 100 times without any serum addition. In Table S1 in the supplemental material, all isolates generated in this study are compiled, with their complete designations.

Sera. The sera used to implement neutralizing pressure originated from chickens vaccinated with a commercial inactivated vaccine of the H5N2 subtype (Nobilis Influenza H5N2; Intervet, Unterschleißheim, Germany) only (serum A) or vaccinated and afterwards challenged with A/cygnus cygnus/Germany/R65/2006 (H5N1) (sera B, C, D, and E).

Animal experiment. The appropriate test to assess the pathogenicity of a certain virus strain in avian species is the determination of the intravenous pathogenicity index (IVPI), according to the Office International des Epizooties (OIE) standard protocol (30). The IVPI represents the mean clinical score of 10 6-week-old chickens inoculated intravenously. Viruses are classified as HPAIV if the IVPI is greater than 1.2 after 10 days of evaluation (when birds are scored as 0 [healthy], 1 [sick], 2 [severely sick], or 3 [dead]). For this purpose, groups of 10 specific-pathogen-free chickens (Lohmann Tierzucht, Cuxhaven, Germany) were infected intravenously with the different escape and control viruses at 10^5 50% tissue culture infective doses ($TCID_{50}$) per animal.

***In vitro* neutralization assays.** The virus neutralization test was performed according to a previously described procedure (36) with a few modifications. In brief, serum samples were heat inactivated for 30 min at 56°C, and 3-fold serial dilutions were prepared in a 50-μl volume of cell culture medium in 96-well plates. The diluted serum samples were mixed with an equal volume of medium containing influenza virus or escape mutants at a concentration of 10^2 $TCID_{50}$/well. After 1 h of incubation at 37°C in a 5% CO_2 humidified atmosphere, 100 μl of MDCK cells at 1.5×10^5/ml was added to each well. The plates were incubated for 3 days at 37°C and 5% CO_2. Virus replication was assessed by visually scoring the cytopathic effect without staining. Each assay was validated by comparison with positive- and negative-control sera from chickens. In addition, infectivity was confirmed by titration of the virus dilutions used. Moreover, virus neutralization by serum C was measured using an avian cell line (QM9; Collection of Cell Lines in Veterinary Medicine, FLI Insel Riems, Germany; RIE0999). Here, neutralization of the ancestor virus, control viruses CoQ100/CoP100, and the escape variant viruses P100, PP100, Q100, and QQ100 was assessed.

Cell binding assay. The cell binding assay was performed as described previously (17) but using chicken instead of turkey erythrocytes. In brief, erythrocytes were pretreated with different concentrations (0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0 μg/ml) of receptor-destroying enzyme (RDE) (cholera filtrate; Sigma-Aldrich, Taufkirchen, Germany) for 60 min at 37°C. Pretreated erythrocytes (2% [vol/vol]) were incubated with 4 hemagglutinating units of the influenza A viruses to be tested, and subsequently, agglutination was measured.

Immunofluorescence assay of monoclonal HA antibody affinity. To test the binding affinities of different H5-specific monoclonal antibodies (provided by M. Dauber, Friedrich-Loeffler-Institute, Riems, Germany) (see Table S3 in the supplemental material), MDCK cells were infected with the ancestor virus; control viruses CoQ100/CoP100; escape variant viruses P100, PP100, Q100, and QQ100; or, to monitor nonspecific binding, influenza virus A/chicken/Brescia/1902 [H7N7]). After 24 h of incubation, the cells were fixed with a methanol-acetone mixture (1:1) for 30 min. The fixed cells were incubated with the monoclonal antibodies diluted 1:5 in phosphate-buffered saline (PBS) for 60 min at room temperature. Subsequently, the cells were incubated for 60 min at room temperature with Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Darmstadt, Germany) diluted 1:1,000 in PBS as a secondary antibody. To control the staining conditions, one well per virus was incubated with an anti-NP monoclonal antibody (ATCC HB-65; 1:20 dilution in PBS; 60 min; room temperature). The results of immunofluorescence analysis were scored from − (no specific staining) to ++ (strong staining of all infected cells).

Sequencing and sequence assembly. For preparation of DNA for sequencing the influenza A genome segments on the Genome Sequencer FLX instrument, the protocol of Höper and coworkers (18) was applied with some modifications. For reverse transcription of the RNA genome segments, a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) was used, and for amplification, iProof High-Fidelity Master Mix (Bio-Rad Laboratories GmbH, München, Germany) was used. The sequencing libraries were generated using the method of Wiley and colleagues (46), followed by binding the DNA to library capture beads and recovery of the single-stranded template DNA (sstDNA) library. For bead-bound clonal amplification of the DNA libraries, the libraries were subjected to duplicate emulsion PCRs (emPCRs) with the GS emPCR kit I (Roche), according to the manufacturer's instructions, with 2 copies per bead. After bead recovery and enrichment, the beads were sequenced using a GS LR70 sequencing kit (Roche) and the appropriate instrument run protocol. The resulting sequencing reads were sorted according to the genome segments to which they were related and were subsequently assembled into one contig (i.e., a set of overlapping sequencing reads) per segment using the GS FLX sequence assembly software

newbler (version 2.3; Roche). During the assembly, the primer sequences were trimmed off the raw data. To analyze the viral diversity within the isolates, we performed mapping of the raw sequencing reads along the appropriate reference sequences using the GS FLX reference mapper software (version 2.3; Roche).

In addition, for selected viruses, a portion of the HA was sequenced by Sanger sequencing. For reverse transcription (RT)-PCR amplification prior sequencing and for the sequencing reaction, the primers R65esc_HAclust_rev (5′-GAG TTT ATC GCC CCT ATT GG-3′) and R65esc_HAclust_for (5′-TCA ATC CAG CCA ATG ACC TC-3′) were used. The gel-purified PCR products were used as input for the sequencing reaction with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany). Subsequently, nucleotide sequences were determined with a 3130 Genetic Analyzer (Applied Biosystems).

Sequence analyses. Sequences were aligned using Multalin (4) (http://multalin.toulouse.inra.fr/multalin/multalin.html) with the symbol comparison table set to "identity." The alignments were put out in html format and further processed with R (35). Similarity searches were conducted using WU-BLAST at the European Bioinformatics Institute (EBI) (27) (http://www.ebi.ac.uk/Tools/) against the uniref100 database.

HA trimeric structure. The reference three-dimensional (3D) structure (accession no. 2FFP) was obtained from the NIAID Influenza Research Database (IRD) through the website at http://www.fludb.org (39). For visualization of the altered amino acids, Jmol (http://www.fludb.org) was used.

Clustering of mutations. Mutation clusters in the sequences were identified using R. To this end, the positions of the single base exchanges as identified in the alignments imported from Multalin were extracted. Subsequently, the total number of mutations per complete genome was summed up, and the theoretical number of mutations per nucleotide, assuming random distribution, was calculated. Clusters were identified with Pearson's chi-square test comparing the number of mutations actually located in a stretch of nucleotides with the theoretical number of mutations in a stretch of equal length. The window size for this test was calculated by determining the number of mutations per base that were induced in the control passages and subsequently dividing the reciprocal of this value by 5 for enhanced selectivity. The window was slid along the sequences by steps of 3 nucleotides, and the chi-square test was conducted for every position of the window. All consecutive windows for which the chi-square test was significant with an α value of $\leq 0.05/n$ (where n is the number of windows along a given genome segment) were defined as a mutation cluster. If two neighboring clusters overlapped each other, they were fused into a single cluster.

Nucleotide sequence accession numbers. The newly determined nucleotide sequences are available from the GISAID EpiFlu database (http://www.gisaid.org) under the accession numbers EPI287205 to EPI287348 and EPI309743 to EPI309750 (see Table S1 in the supplemental material).

RESULTS

We passaged HPAIV H5N1 strain A/cygnus cygnus/Germany/R65/2006 (45) in the presence of maximum concentrations of neutralizing polyclonal chicken sera. Starting with the same ancestor virus, 2 independent series (P and Q) of escape variants were generated. In parallel, control viruses, i.e., viruses passaged without immunogenic pressure, were isolated. The passaging scheme is summarized in Fig. 1.

Validation of escape. Prior to all further analyses, we tested whether the mutants successfully escaped neutralization by the antisera. To this end, virus neutralization assays were performed. The neutralizing activities of the sera used for passaging (sera A, B, C, and D), as well as one additional unrelated chicken serum (serum E), were measured. Figure 2 summarizes the results of the neutralization assays (for numerical values, see Table S2 in the supplemental material). The areas of the circles represent the neutralizing activities, i.e., the larger the circle, the stronger the neutralizing activity of the serum against the virus. As expected, neutralization of the escape mutants by the sera used for passaging was reduced compared to the activities of these sera against the ancestor virus (Fig. 2; see Table S2 in the supplemental material). The neutralizing activity of an unrelated chicken serum against the escape variants from both series was also reduced. On the other hand, all control viruses were neutralized to the same extent as the ancestor virus. Neutralization of the viruses in avian cells (QM9) was also determined but did not differ significantly from neutralization in MDCK cells (data not shown). Interestingly, none of the escape mutants became completely insensitive to neutralization by any of the sera (Fig. 2; see Table S2 in the supplemental material).

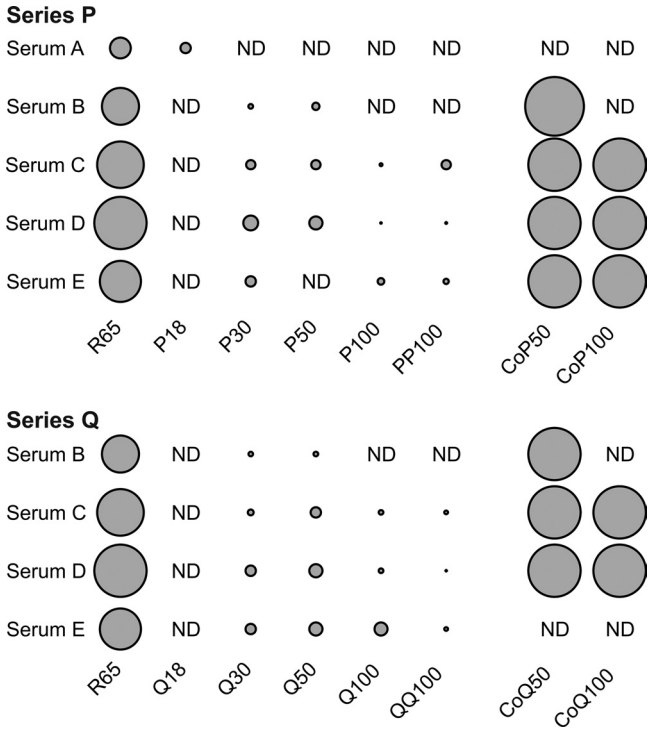

FIG 2 Neutralizing activities of different antisera against escape and control viruses, as determined by virus neutralization assay. Sera A, B, C, and D were used during passaging; serum E was not. The circled areas represent the neutralizing activities of the respective sera (rows) against the different virus isolates (columns). The larger the circle, the stronger the neutralizing activity is. ND, not determined.

In order to rule out the possibility that the escape was in fact due to altered receptor avidity, as recently reported by Hensley and coworkers (17), we performed cell binding assays with the ancestor virus R65, and both the control and escape variant viruses were isolated after 100 passages. Despite the different concentrations of RDE that were used during pretreatment of the erythrocytes, the hemagglutination exhibited by the selected viruses remained unaltered throughout (data not shown). Therefore, we concluded that the observed escape was not caused by changes in receptor avidity.

Additionally, to further characterize the escape variants in comparison to the ancestor and control viruses, we examined the recognition of the HA proteins by different H5-specific monoclonal antibodies. Antibody binding was visualized by immunofluorescence (for a summary of the results, see Table S3 in the supplemental material). The monoclonal influenza A virus NP-specific

antibody that was used as a control detected NP protein equally well regardless of the viral strain. On the other hand, all three anti-H5 antibodies recognized the ancestor virus R65 and the CoQ100 virus. The CoP100 virus was detected by only one out of the three H5-specific antibodies. Interestingly, that antibody failed to detect the escape variants from the Q series while still detecting the ancestor and both control viruses (CoP100 and CoQ100), as well as the escape variants of the P series (see Table S3 in the supplemental material). As an additional control, we included an influenza A virus of the subtype H7N7, which was readily detected by the NP antibody but not by the H5-specific antibodies. In conclusion, the observed mutations that were introduced during virus passaging led to reduced antibody affinity, i.e., they were the cause of the observed immune escape.

Sequence analyses. To get deeper insights, we sequenced the complete genomes of the escape variants and control viruses to a median depth of roughly 300 (the sequence depth is the number of times every nucleotide was sequenced). Sequence analyses were focused on the coding portion of the genome segments. With a single exception (P-series HA A502G, causing amino acid substitution K168E), none of the mutations found in the control viruses matched positions mutated in the escape variants, and *vice versa*. The K168E substitution was regarded as an adaptation to the cell line used for passaging. Therefore, it was not taken into account for further analyses. Besides the aforementioned A502G mutation, we did not detect any further hint at the genome sequence level that points to possible adaptations of the virus as a result of the switch to the mammalian cell line. All sequence deviations, both synonymous and nonsynonymous, are compiled in Table S4 in the supplemental material.

Sequence dynamics of escape. Sequence analyses revealed a progressive deviation of the escape mutants from the ancestor virus genome sequences. This was also found for the control viruses, albeit to a lesser extent and at different positions. All variations that were detected throughout the experiment are compiled in Table S4 in the supplemental material. Both in the escape and in the control viruses virtually no back mutations were observed. After 100 passages, 19 (7 silent) and 22 (6 silent) deviations were found distributed across the genome in the P- and Q-series control viruses, respectively. In the escape mutants, we detected 49 (16 silent) and 59 (23 silent) mutations in the P and Q series, respectively. Figure 3 summarizes all detected mutations from both the P and Q series schematically.

No more than 3 (2 in the HA and 1 in the M2 protein) of the 32 amino acid substitutions arose during use of serum A in the P-series escape mutants (Fig. 4). This serum was the only one derived from a chicken that was vaccinated twice with an inactivated H5N2 vaccine. All other sera were sampled from chickens that were first vaccinated with an inactivated H5N2 vaccine and subsequently challenged with infectious HPAIV H5N1.

The time required for manifestation of escape mutations after the introduction of a new serum proved to be short, in general. Nevertheless, additional mutations were induced during prolonged exposure of the virus to the serum (Fig. 4 and 5). In the P escape series HA gene, serum B induced 5 mutations, which were all manifested after passaging the virus 12 times (passages 19 to 30). No additional mutations were induced by the same serum from the 31st to the 50th passages. Likewise, in the Q escape series, where the same serum was used even longer (until passage 54), 6 of the 9 mutations induced by serum B were already detected after passage 18, another 2 occurred by passage 30, and 1 additional mutation arose by the 50th passage.

In an attempt to define more precisely the passage in which the base exchanges became manifest, we sequenced a part of the HA gene completely spanning one of the clusters with the most base exchanges (nucleotides 363 to 568) (see below). Therefore, we analyzed escape viruses isolated after 1 and 5 passages from both the P and Q series. However, this attempt revealed that by the 5th passage not a single position in the analyzed region had changed the consensus. Hence, we concluded that it would not easily be possible to track the changes more precisely.

Distribution of the mutations. Remarkably, neither in the control nor in the escape viruses were any mutations in the nuclear export protein (NEP) coding region detected. Beyond this, it was clearly visible that in the escape viruses the mutations were not evenly distributed across the genome but that there was a bias toward different segments and, within the segments, toward specific regions. We sought to identify regions where the density of amino acid substitutions was especially high in order to enable predictions of mutations that facilitate escape.

Using Pearson's chi-square test, we first compared the number of nonsynonymous mutations located in the respective gene with the theoretical number of mutations for that open reading frame, if the observed mutations were stochastically distributed across the genome segments. In the escape mutants, the nonsynonymous mutations primarily appeared in the HA genes ($\alpha = 0.027$ and $\alpha = 0.033$ for P and Q, respectively). On the other hand, in the control viruses, a genome segment in which mutations preferentially occurred could not be identified.

Second, mutational hot spots within the segments were identified, i.e., regions in which the number of mutations was significantly higher than expected for an even distribution across the genome. We were able to identify 15 regions where the mutation density was especially high. These mutational clusters were detected in the PB2-, PA-, HA-, M1-, and M2-encoding genes (Fig. 3). Interestingly, only one pair of clusters of the control and escape viruses in the HA genes overlap (Fig. 3). They are located in the control viruses from nt 558 to 649 (2 nt/2 amino acid substitutions; P series N198S; Q series R205K) and in the escape mutants from 570 to 685 (4 nt/4 amino acid substitutions; P series P210S, T211A, and G217E; Q series A201E).

In 4 of the identified cluster regions, a significant difference was found between the numbers of mutations in the escape and control viruses (Pearson's chi-square test). These cluster regions were located in the HA (nt 78 to 310, 6 sites, $\alpha = 0.014$; nt 363 to 568, 9 sites, $\alpha = 0.0024$; nt 726 to 985, 8 sites, $\alpha = 0.0044$) and the PA (nt 996 to 1099, 4 sites, $\alpha = 0.043$) genes. Strikingly, in the controls, no single mutation was detected in the regions of the HA gene that correspond to the 3 escape mutant clusters (Fig. 3). Moreover, due to the extraordinarily high proportion (82%) of nonsynonymous mutations in these clusters, there was a significant difference (Pearson's chi-square test) between the escape and control viruses even at the amino acid level (amino acids [aa] 26 to 104, 5 amino acid substitutions, $\alpha = 0.023$; aa 121 to 190, 9 amino acid substitutions, $\alpha = 0.0019$; aa 242 to 329, 5 amino acid substitutions, $\alpha = 0.023$).

We detected several amino acid substitutions in the so-called internal proteins (Fig. 3). Thirty-five (58.3%) of the overall 60 amino acid substitutions that were detected emerged in internal proteins.

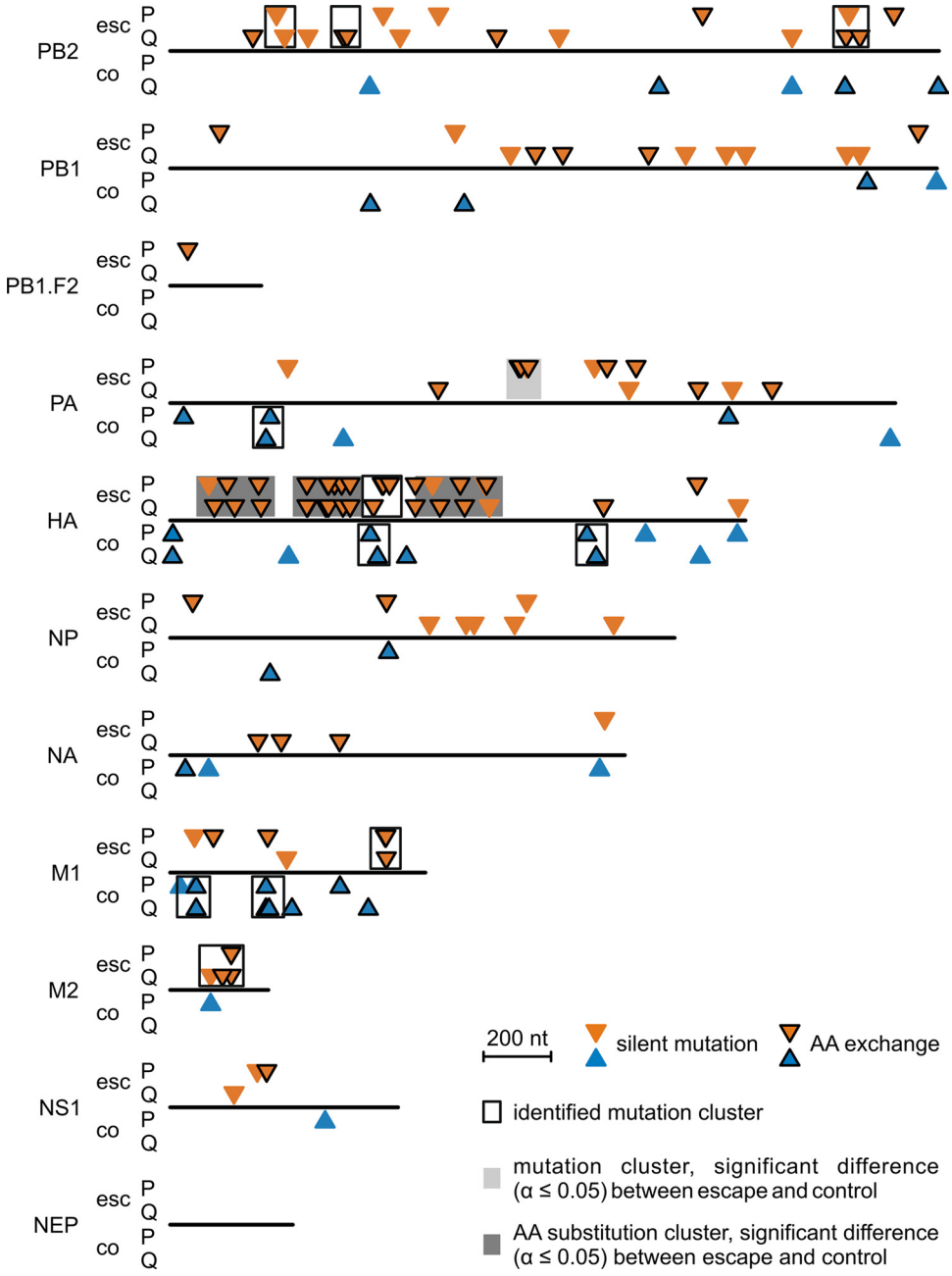

FIG 3 Overview of all mutations detected in the escape and control virus sequences and localization of the mutation clusters. The 11 genes of the influenza A virus are illustrated as horizontal lines; the detected mutations are depicted as triangles at the appropriate positions (orange, escape; blue, controls).

In-depth sequence analyses. In order to assess the *in vivo* relevance of the detected amino acid substitutions, we analyzed the peptide sequences of the amino acid substitution clusters in the HA by BLAST. These analyses revealed that a substantial portion of the *in vitro*-induced amino acid substitutions also occur in natural isolates, although not always in the combinations in which they originated in our experiment. Moreover, we analyzed the structural and functional roles of the substituted amino acid residues (Table 1; see Fig. S1 in the supplemental material). This analysis revealed that a substantial portion of the substituted amino acids are located in previously defined antigenic epitopes. Moreover, four of the substituted amino acids are part of the HA stalk that was also shown to be antigenically important (5). In addition, we found several substitutions of amino acids which until now have not been implicated in HA antigenicity. Interestingly, these residues seem to be predominantly substituted in later passages (Table 1 and Fig. 4 and 5).

Both escape series, but none of the controls, had the same R61G substitution in the M2 protein (Fig. 4 and 5). In order to assess the *in vivo* relevance of the R61G substitution, we also conducted BLAST analyses with the mutated M2 amino acid sequences. This analysis revealed that the R61G substitution also

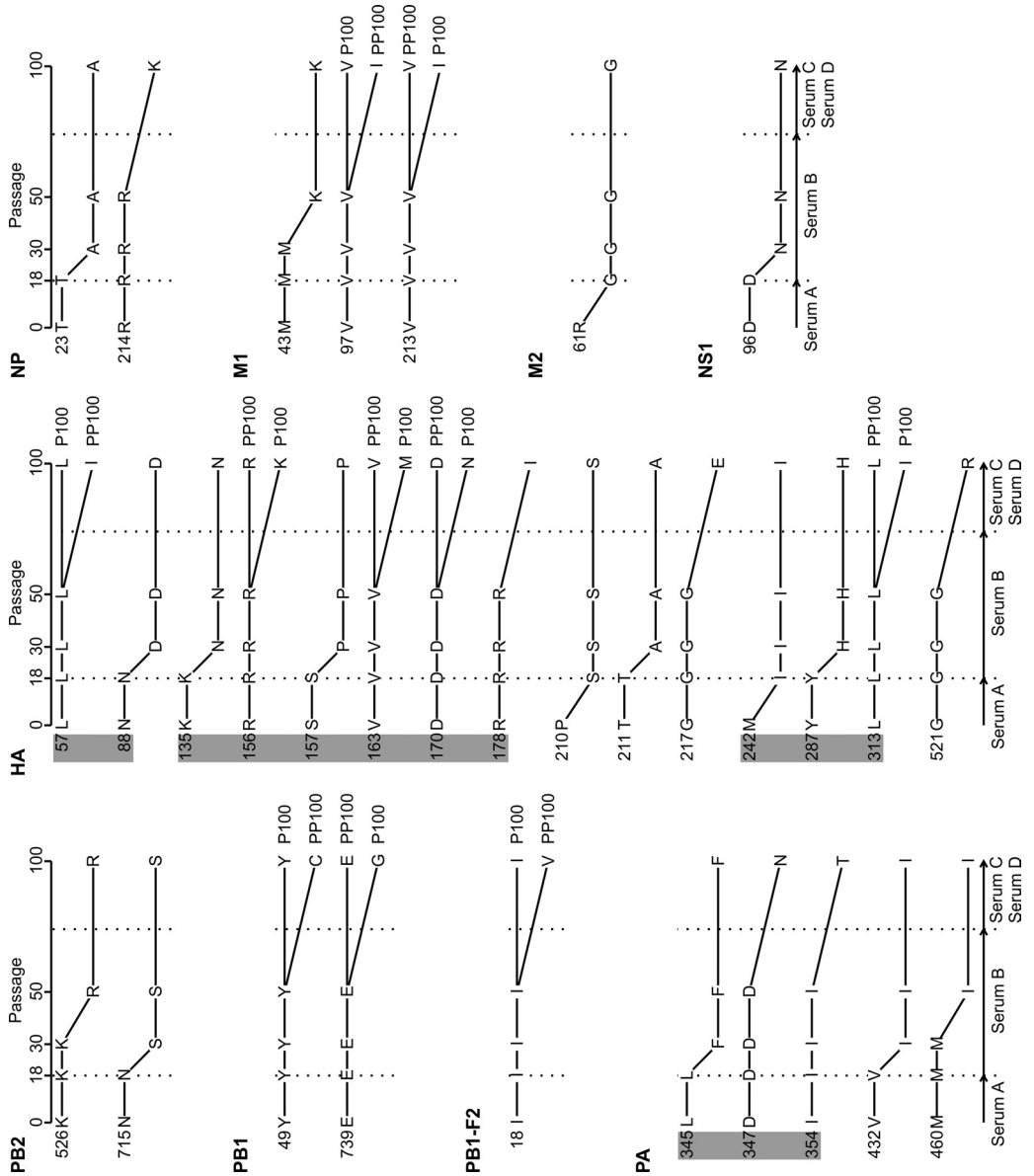

FIG 4 Exact localization and chronology of amino acid substitutions in the series P escape mutants. Shown are the positions at which amino acid substitutions were deduced. At passage 0, the ancestor amino acids are shown. At the following passages, the amino acids deduced from the sequences of the escape mutants are depicted. The gray rectangles link amino acid positions in the identified clusters (for details on the clusters, see the text).

occurs naturally. Amino acid R61 is involved in the formation of a highly conserved amphipathic helix adjacent to the transmembrane domain of M2 (41).

Several of the amino acid substitutions that were deduced for the internal proteins lie within peptides previously described as immunogenic (22). For instance, five of the total of eight amino acid substitutions that were detected in the PA protein are located within such immunogenic peptides (Fig. 6). Two of these peptides encompass the PA substitution cluster, and one additional peptide lies in close proximity to the cluster.

Analysis of viral diversity. The quasispecies theory states that a virus population is composed of a predominant master sequence and minor portions of mutant sequences (6). The sequence depth that was achieved enables detection of the different variants that constitute the virus population. In this study, variability under the consensus sequence was frequently detected in the sequences established for the control viruses and to a lesser extent in those of the escape viruses. Table S4 in the supplemental material summarizes all variants that could be detected accompanying the base exchanges that were found in the consensus sequences.

Analysis of the diversity of the escape mutant sequences revealed that at some positions the nascent consensus base existed in the genome mixture before the consensus transition. For instance, the P-series sequences contained 10% thymine (T) at PA position 1033 before the C (cytosine)→T changeover between passages 18 and 30 (see Table S4 in the supplemental material). Correspondingly, in some instances, the previous consensus base remained in the virus population after the change, as in Q-series HA at position

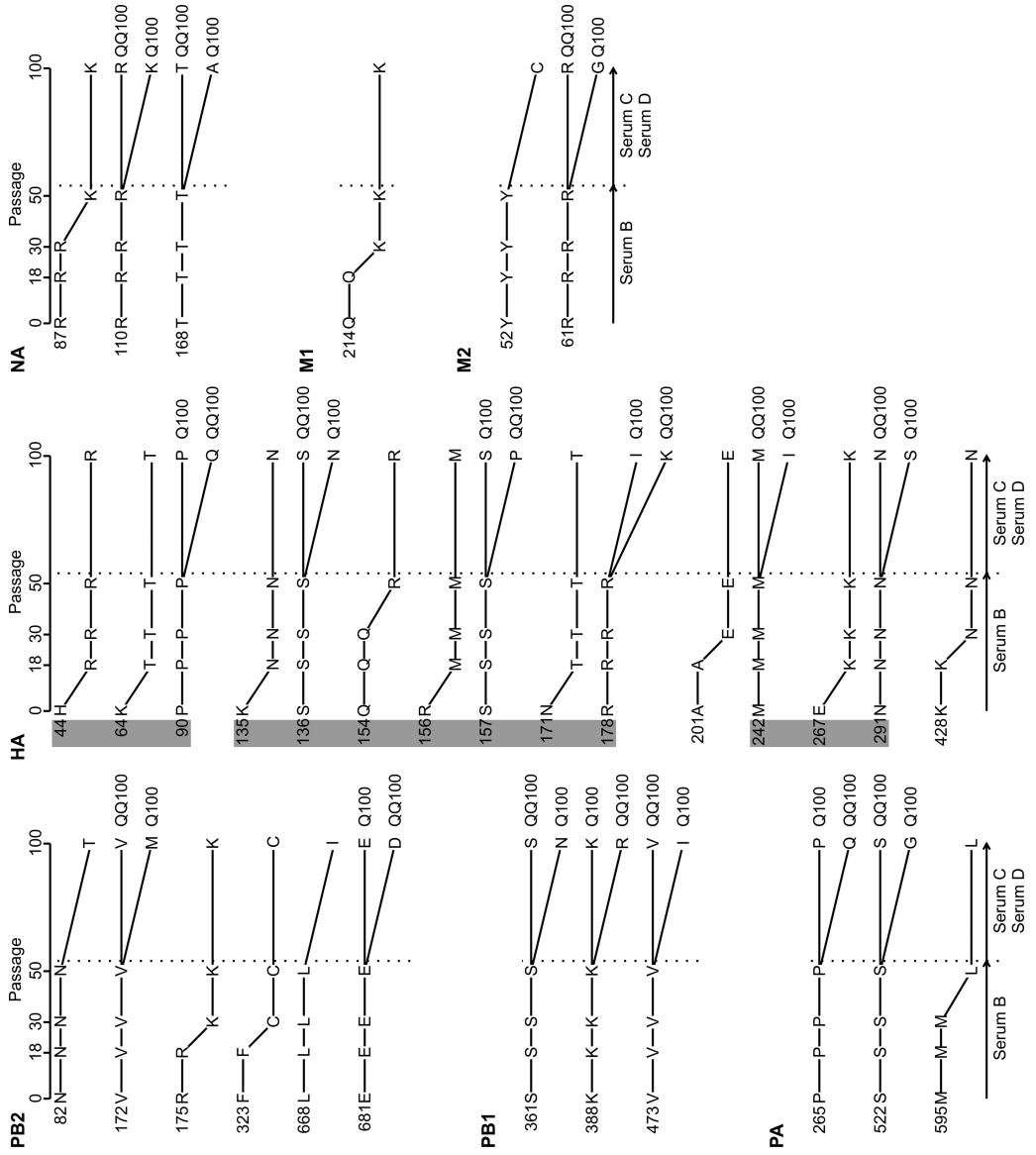

FIG 5 Exact localization and chronology of amino acid substitutions in the series Q escape mutants. Shown are the positions at which amino acid substitutions were deduced. At passage 0, the ancestor amino acids are shown. At the following passages, the amino acids deduced from sequences of the escape mutants are depicted. The gray rectangles link amino acid positions in the identified clusters (for details on the clusters, see the text).

602, where 22% of the C remained after the C→A (adenine) consensus transition after passage 30.

Two back mutations in the PA nucleotide sequences from Q-series controls (positions 258 and 343) were seen. The reference base guanine (G) at position 258 changed to A until passage 50, but still 38% of G were present (see Table S4 in the supplemental material). After 100 passages, the consensus was G again, with roughly 13% of A remaining in the mixture. The same was true for the aforementioned position 343, where the reference base was A, changing to G after 50 passages, with roughly 50% of A remaining at that position. After 100 passages, the consensus was A again, but 13% of all sequences still had a G at position 343. All the back mutations that were detected can be attributed to fluctuations in the genome segment mixture.

Phenotypic characterization of the viruses. In order to analyze the impacts of the mutations we found, the sampled virus isolates were phenotypically characterized. For *in vivo* characterization, the IVPI in chickens was determined. Compared with the IVPI of 2.97, classifying the ancestor virus as highly pathogenic (38), the generated isolates were attenuated (Fig. 7). The majority of the isolated mutant viruses were classified as low pathogenic. Only the P-series escape viruses isolated after passages 18 and 30 and the P-series control virus isolated after passage 50 were still highly pathogenic (IVPI ≥ 1.2).

DISCUSSION

In 1950, Archetti and Horsfall (1) reported on the emergence of antigenic variants of influenza A virus during serial passage *in ovo*. They induced the antigenic changes by passaging the viruses in the presence of immune sera against different but related strains. Sim-

TABLE 1 Summary of amino acid substitutions in the HA proteins and involvement of the respective amino acid residues in protein structures

Amino acid[a]	Amino acid residue		Detected after passage		Domain[b]	Subdomain[c]	Antigenic epitope[d]	Feature[e]
	R65	Escape	P	Q				
44	H	R		18	HA1	ST	C	
57	L	I	100		HA1	ST	E	
64	K	T		14	HA1	VE		AS
88	N	D	30		HA1	VE	E	
90	P	Q		100	HA1	VE		
135	K	N	30	18	HA1	RB	A	
136	S	N		100	HA1	RB		
154	Q	R		50	HA1	RB		AS
156	R	K	100	18	HA1	RB	B	AS
157	S	P	30	100	HA1	RB	B	AS
163	V	M	100		HA1	RB	B	
170	D	N	100		HA1	RB	D	GS
171	N	T		18	HA1	RB	D	GS
178	R	I	100	100	HA1	RB		
201	A	E		30	HA1	RB	D	AS
210	P	S	18		HA1	RB		
211	T	A	30		HA1	RB		
217	G	E	100		HA1	RB	D	
242	M	I	18	100	HA1	RB	D	
267	E	K		18	HA1	RB		
287	Y	H	30		HA1	VE		
291	N	S		100	HA1	ST		
313	L	I	100		HA1	ST		
428	K	N		30	HA2	ST		AS, AH
521	G	R	100		HA2	ST		

[a] Italics, boldface, and underlining mark the detected amino acid exchange clusters.
[b] Part of protein domain according to reference 13.
[c] Part of subdomain according to reference 13; RB, receptor binding site; VE, vestigial enzyme subdomain; ST, stalk subdomain.
[d] Involvement in formation of antigenic epitopes according to reference 7.
[e] Further detailed features of the amino acid; AS, antigenic site according to reference 7; GS, glycosylation site according to reference 7; AH, conserved α-helix in the stalk region according to reference 44.

ilarly, in the present study, escape mutants of HPAIV H5N1 were bred *in vitro* through 100 passages in the presence of selective antisera. In addition to the results of Archetti and Horsfall, which we confirmed, we were able to precisely determine the genome sequences of the resulting viruses, thereby adding significantly to our knowledge of immune escape.

While Cleveland and colleagues (3) argue that so many sequential passages are not natural conditions, we assume that, especially

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            231                                  *                                300
       R65  AYVDGFEPNG CIEGKLSQMS KEVNARIEPF LKTTPRPLRL PDGPPCSQRS KFLLMDALKL SIEDPSHEGE
H5-PA-1852       EPNG CIEGKLSQMS KEVNARIEPF LKTTPRPLRL PDGPPCSQRS KFLLMDALKL SIEDPSHEGE
H5-PA-1945
H5-PA-1904                                                              KL SIEDPSHEGE

            301                                        * *       *          370
       R65  GIPLYDAIKC MKTFFGWKEP NIVKPHEKGI NPNYLLTWKQ VLAELQDIEN EEKIPKTKNM KKTSQLRWAL
H5-PA-1852  GIPLYDAIKC MKTFFGWKEP NIVKPHEKGI NPNYLLAWKQ VLAELQDIEN E
H5-PA-1945                               I NPNYLLAWKQ VLAELQDIEN EEKIPKTK
H5-PA-1904  GIPLYDAIKC MK

            577                  *                                           645
       R65  WGME MRRCLLQSLQ QIESMIEAES SVKEKDMTKE FFENKSETWP IGESPKGVEE GSIGKVCRTL LAKSV
H5-PA-2202             QSLQ QIESMIEAES SVKEKDMTKE FFENKSETWP IGESPKGVEE GSIGKV
```

FIG 6 Alignment of the amino acid sequence deduced for the ancestor (R65) PA and the antigenic PA peptides identified previously (22). The amino acids in boldface constitute the amino acid substitution cluster (for details, see the text). Amino acid residues labeled with asterisks are replaced in the escape mutants in the course of passaging. R65, amino acid sequence for R65 PA; H5-PA-1852, H5-PA-1904, H5-PA-1945, and H5-PA-2202, amino acid sequences of antigenic PA peptides identified previously (22).

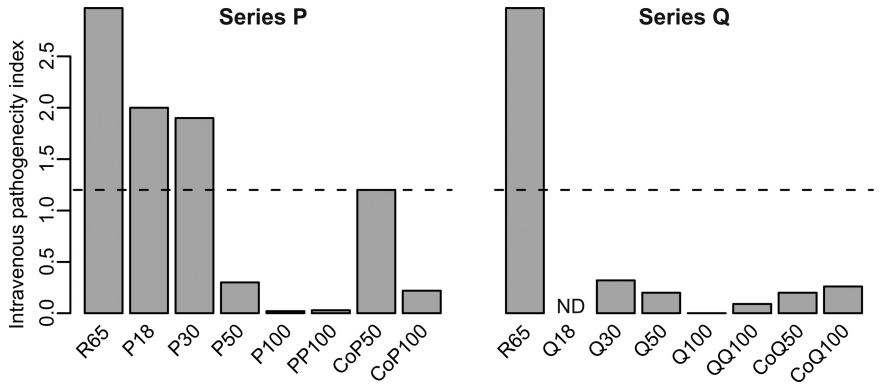

FIG 7 IVPI of the ancestor virus and isolates generated in this study. The dashed line indicates the threshold for the discrimination of high- and low-pathogenic viruses. ND, not determined.

in modern poultry farms (with 100,000 or even more individuals in direct contact), repeated passaging of virus in a flock with nonsterile immunity is possible. In addition, the authors (3) argue that polyclonal antiserum would not be able to select escape variants, because the presence of two or more antibodies against discrete epitopes cannot be compensated for by viral antigenic drift. However, Lambkin and colleagues (23) were able to select escape mutants with polyclonal sera from immunized mice. Here, we show that the same is possible using chicken sera for even 100 consecutive selective passages. The fact that no complete escape was achieved is consistent with the idea that antigenic drift arises through an epitope-biased antibody response (3). Furthermore, it was stated that the occurrence of substitutions that provide complete escape from the sum of all different antibodies in a polyclonal antiserum is unlikely (3). With our experimental setup, we were able to prove this for HPAIV H5N1 for the first time, even through 100 selective passages.

One drawback of our system, of course, is the attenuation of the isolates, as reflected in the IVPI. Possibly, this attenuation is caused by alterations in the viral polymerase proteins, as recently speculated (25). In addition, transmissibility as a property of the virus may be reduced without undermining the virus' success in infecting cells, because transmission is guaranteed by the passaging system. However, the attenuation of the isolates emphasizes that the polybasic cleavage site, which is considered characteristic of HPAIVs, is not the only determinant of pathogenicity. This was previously shown, not only *in vitro* (2), but also for natural isolates (reviewed in reference 26). Different studies provided hints of the impact of the so-called internal proteins on pathogenicity (16, 25). The data presented here additionally imply a possible role of the internal proteins in immunoescape.

The two key players that affect escape of the virus are mutations that randomly occur in the virus genome and the selection of mutants by the host immune system. The different passaging schemes used for series P and Q place emphasis on those regions of the genome that are of high importance for escape by randomizing the interplay of the aforementioned two factors that impact escape. For instance, we were able to define amino acid substitution clusters in the HA protein. These clusters conform to the well-defined regions involved in the formation of the immune epitopes A, B, C, D, and E (7). Within these clusters, 5 amino acid residues (aa 135, 156, 157, 178, and 242) may be addressed as important immunodominant sites because they are concurrently attacked by different sera. The importance of two of these residues is stressed by recent studies. Amino acid residues 157 and 178 were identified as relevant in the context of monoclonal immune characterization (37). More importantly, residue 157 was shown to be under positive selection in both avian and human hosts (7). Additional substitutions were found outside the main immune epitopes. This supports a very recent description of heterosubtype neutralizing antibodies that bound to epitopes in the HA stem region (5), implying that this region plays an important and so far underestimated role in humoral immunity and subsequent immunoescape.

Sequence analysis revealed that serum A, obtained from an animal that was only vaccinated but not challenged, through the first 18 passages induced only three amino acid substitutions. Two of these were detected in the HA protein and an additional one in the M2 protein. On the other hand, in series Q, serum B induced 6 amino acid substitutions in the HA within the first 18 passages. In addition, in both series P and Q, serum B induced diverse substitutions in PB2, PA, NA, NP, M1, and NS1. Hence, it seems that sera from animals challenged with infectious virus contained a broader spectrum of antibodies. Possibly, virus replication leads to induction of antibodies against multiple peptides, including those originating from internal proteins. The high incidence of amino acid substitutions in the hemagglutinin was not surprising, as it is the main immunogenic protein, and previous attempts to isolate escape mutants showed that HA is a main determinant of escape (15). While the role of HA in escape from host immunity was investigated in several studies, none of them addressed the impact of the host immune system on the so-called internal proteins and the role of these proteins in escape. Therefore, we focused on analysis of the changes in the internal proteins. Roughly 58% of all deduced amino acid substitutions were located in proteins other than HA. The amino acid substitutions in internal proteins, too, map within peptides that were shown to be antigenically active in humans by mapping immunogenic peptides using whole-genome-fragment phage display libraries and human patient sera (22). Thus, it seems very likely that the transformations we detected are not secondary adaptive mutations but in fact are consequences of antibody pressure. However, how this pressure may be transmitted in our *in vitro* system is so far unknown. One possibility is postentry neutralization of the virus caused by antibodies, as previously reported (34). The authors found that HA antibodies inhibit the viral ribonucleoprotein complex and thereby transcription and expression of viral genes. This is consis-

tent with the numerous substitutions in the amino acid sequences deduced for the viral polymerase proteins (Fig. 3). Another mechanism may be intracellular neutralization of virus by immunoglobulin A antibodies, as shown previously (28). However, we cannot rule out the possibility that the mutations in the internal proteins are compensatory modifications that reestablish necessary protein-protein interactions that were abolished by preceding mutations. In addition, these changes may simply be random substitutions that were manifested over the passages. This issue needs to be addressed in future studies.

Our finding that both escape series, but neither of the controls, ended up with the R61G substitution in the M2 protein implies that it might be an immunogenically important residue. This is emphasized by the fact that it was one out of the three substitutions induced by serum A. In addition, there are previous reports (47) about a monoclonal antibody directed against the M2 protein of human influenza A virus isolates. Moreover, the possibility that the R61G substitution in the cytoplasmic domain might have been induced by the immune pressure is reinforced by a recent study (22). The authors of the study used whole-genome-fragment phage display libraries and human patient sera to map immunogenic epitopes in all influenza A virus proteins. They detected antibodies not only directed against the ectodomain of the protein, but also directed against the cytoplasmic domain of the M2 protein. However, Nayak and colleagues (29) did not detect neutralizing activity in sera from chickens immunized with recombinant Newcastle disease virus expressing the HPAIV H5N1 M2 protein. The speculation about the immunogenicity of the cytoplasmic portion of the M2 protein is further fueled by the *in vivo* relevance of the R61G substitution proven by the BLAST results showing that this substitution also occurs naturally.

In both the control and escape isolates, sequence variation was detected under the consensus sequence. Especially in the control isolates, where broader variance was detected, the future consensus base could be found before, or the disappearing base remained in the mixture after, the manifestation in the consensus. This implies that the changes in the escape consensus sequences were also most likely initially included in the virus mixture and became manifest due to selection by antibody pressure. Hence, variants preexisting in the virus population seem to have been the basis for the quick escape of the virus set under immune pressure. This is in accordance with the theory of virus evolution that any of the viruses within real viral populations could compensate for perturbations in the environment (6). This immediate selection might in turn lead to diminished variability of the escape isolates, as detected in our sequence data. To our knowledge, this is the first time that it has been directly shown for an influenza virus.

While the list of perfect BLAST hits for the ancestor HA peptides corresponding to the escape cluster regions contained hundreds of entries from locations distributed all over Africa, Asia, and Europe, the best hits for these peptides from the escape variants always came from viruses isolated in Egypt or its neighboring countries and from China. In accordance with this, BLAST analysis of the M2 protein with the R61G substitution also identified a protein cluster containing solely sequences from China. Both in Egypt and in China, HPAIV H5N1 is endemic, and extensive vaccination of poultry is conducted (31, 32). These results support the theory that nonsterile immunity induced by vaccination furthers antigenic drift, as was previously shown, for instance, for foot and mouth disease virus (20).

The *in vitro* model presented reveals the basics of immunoescape of influenza A viruses by providing a look at the complete set of sites involved. Based on our data, prediction of immunoescape in vaccinated poultry is feasible, and further analysis tools can be tailored, as was recently published in a proof of concept (43).

Clearly, our model allows simulation of genetic drift leading to immunoescape as it occurs in avian populations. Also, in human health care, where boosting of host responses against more conserved instead of natural immunogenic determinants is propagated (10, 42), escape variants with significant impact emerge. This is reflected in the requirement for annual updates of influenza vaccine components to antigenically match the circulating strains. We assume that even prediction of possible changes in the antigenicity of influenza A viruses in the human population might be feasible with our model, using suitable sera (e.g., children's sera [14]).

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