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A Unique Multibasic Proteolytic Cleavage Site and Three Mutations in the HA2 Domain Confer High Virulence of H7N1 Avian Influenza Virus in Chickens

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ABSTRACT

In 1999, after circulation for a few months in poultry in Italy, low-pathogenic (LP) avian influenza (AI) H7N1 virus mutated into a highly pathogenic (HP) form by acquisition of a unique multibasic cleavage site (mCS), PEIPKGSRVRR*GLF (asterisk indicates the cleavage site), in the hemagglutinin (HA) and additional alterations with hitherto unknown biological function. To elucidate these virulence-determining alterations, recombinant H7N1 viruses carrying specific mutations in the HA of LPAI A/chicken/Italy/473/1999 virus (Lp) and HPAI A/chicken/Italy/445/1999 virus (Hp) were generated. Hp with a monobasic CS or carrying the HA of Lp induced only mild or no disease in chickens, thus resembling Lp. Conversely, Lp with the HA of Hp was as virulent and transmissible as Hp. While Lp with a multibasic cleavage site (Lp_CS445) was less virulent than Hp, full virulence was exhibited when HA2 was replaced by that of Hp. In HA2, three amino acid differences consistently detected between LP and HP H7N1 viruses were successively introduced into Lp_CS445. Q450L in the HA2 stem domain increased virulence and transmission but was detrimental to replication in cell culture, probably due to low-pH activation of HA. A436T and/or K536R restored viral replication *in vitro* and *in vivo*. Viruses possessing A436T and K536R were observed early in the HPAI outbreak but were later superseded by viruses carrying all three mutations. Together, besides the mCS, stepwise mutations in HA2 increased the fitness of the Italian H7N1 virus *in vivo*. The shift toward higher virulence in the field was most likely gradual with rapid optimization.

IMPORTANCE

In 1999, after 9 months of circulation of low-pathogenic (LP) avian influenza virus (AIV), a devastating highly pathogenic (HP) H7N1 AIV emerged in poultry, marking the largest epidemic of AIV reported in a Western country. The HPAIV possessed a unique multibasic cleavage site (mCS) complying with the minimum motif for HPAIV. The main finding in this report is the identification of three mutations in the HA2 domain that are required for replication and stability, as well as for virulence, transmission, and tropism of H7N1 in chickens. In addition to the mCS, Q450L was required for full virulence and transmissibility of the virus. Nonetheless, it was detrimental to virus replication and required A436T and/or K536R to restore replication, systemic spread, and stability. These results are important for better understanding of the evolution of highly pathogenic avian influenza viruses from low-pathogenic precursors.

Avian influenza viruses (AIV) belong to the genus *Influenza A virus* in the family *Orthomyxoviridae*. They contain a single-stranded RNA genome composed of eight gene segments encoding at least 10 viral proteins (1). The viral proteins can be allocated to three categories: surface proteins (hemagglutinin [HA], neuraminidase [NA], and matrix protein 2 [M2]), internal proteins (tripartite polymerase [PB2, PB1, and PA], nucleoprotein [NP], matrix protein 1 [M1], and nuclear export protein [NEP]), and nonstructural proteins (NS1 and sometimes PB1-F2) (1, 2). Currently, AIV are classified into 16 HA (H1 to H16) and 9 NA (N1 to N9) subtypes, which are known to infect birds, whereas influenza viruses of subtypes H17N10 and H18N11 have been identified in bats (3). While AIV of all subtypes induce subclinical or only mild disease in poultry, the virulence of H5 and H7 subtypes varies from asymptomatic to highly lethal infections (4). Major outbreaks of fowl plague are caused by highly pathogenic (HP) AIV subtypes that evolve from low-pathogenic (LP) ancestors after circulation in domesticated birds (5). Therefore, infections by either LP or HP H5 and H7 viruses are notifiable to the World Organization for Animal Health (formerly the Office International des Epizooties [OIE]) (6). Genetic changes that accompanied shifts of LP to HP AIV have been successfully studied using reverse genetics in several of these outbreaks (5). AIV pathogenicity for domestic poultry has been associated with changes in the proteolytic cleavage site (CS) of the HA protein from a monobasic to a multibasic motif (mCS). This alteration results in HA cleav-

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ability by ubiquitous host furin-like proteases, not only in the respiratory and digestive tracts, but throughout the body, inducing systemic infection. Besides the CS, amino acids in its vicinity, mostly in the HA1 domain, were also important in some H5 viruses (7, 8). Moreover, virulence markers that extend beyond the HA exist, mainly in the H5N1 subtype (9–11). Thus, the virulence of H5 AIV is determined multigenically, requiring an optimal gene constellation for full virulence or host adaptation (12). However, compared to H5 viruses, the virulence determinants of recent H7 viruses remain poorly understood.

In March 1999, LPAIV H7N1 resulted in 199 outbreaks in domestic poultry in Italy, marking the largest epidemic of LPAI reported in a Western country. After 9 months, an HPAIV emerged directly from the LP precursor in chickens and turkeys in an area with high poultry density (13). Over 413 outbreaks were reported, and 13 million birds were culled, which disrupted the poultry-marketing system for months before eradication was achieved (14). Within a short period, the virus had infected a wide range of hosts, including chickens, turkeys, guinea fowls, quails, pheasants, ostriches, ducks, geese, sparrows, doves, and Sakr falcons (14). Until now, little was known about the molecular basis for the high pathogenicity of the virus (13, 15, 16). The presumptive LPAIV precursor has a monobasic CS motif, PEIPKGR*GLF (asterisk indicates the cleavage site), while the HPAIV specifies a multibasic motif, PEIPKG**SRVR**R*GLF (multibasic amino acids in the cleavage site are shown in boldface) (13, 14). Although the amino acid composition of this mCS is peculiar to H7 viruses, it complies with the minimum RXK/RR*GLF motif recognized by furin-like proteases (17). The contributions of this motif and other virulence determinants of the Italian HPAIV H7N1 have not been fully identified and therefore were investigated using reverse genetics and *in vivo* experiments in chickens.

MATERIALS AND METHODS

Ethics statement. All challenge experiments were conducted in the biosafety level 3-plus (BSL3+) animal facilities of the Friedrich-Loeffler-Institut (FLI), adhering to the German Regulations for Animal Welfare after approval by the authorized ethics committee of the State Office of Agriculture, Food Safety, and Fishery in Mecklenburg-Western Pomerania (LALLF M-V) under registration number TSD/7221.3-1.1-018/07. Specific-pathogen-free (SPF) embryonated chicken eggs (ECE) were purchased from Lohmann Company (Cuxhaven, Germany) and handled following the guidelines of the OIE (6). All experiments were supervised and approved by the commissioner for animal welfare at the FLI, representing the Institutional Animal Care and Use Committee (IACUC).

Viruses and cells. The LP H7N1 A/chicken/Italy/473/1999 virus (here designated Lp), HP H7N1 A/chicken/Italy/445/1999 virus (here designated Hp), and HP A/chicken/Germany/R28/2003 virus (H7N7) were obtained from the repository of the FLI. Primary chicken embryo kidney (CEK) cells and a chicken fibroblast line (DF1) were used to establish replication kinetics. Madin-Darby canine kidney type II (MDCKII) cells were used for virus titration and propagation and, in combination with 293T cells, for rescue of viruses by reverse genetics. Quail muscle (QM9) cells (CCLV-RIE 999), a subline of QM7 (ATCC CRL-1062), were used for the fusion assay. All cell lines were provided by the Cell Culture Collection in Veterinary Medicine of the FLI.

Virus isolation, propagation, and characterization. All LPAI viruses with a monobasic CS were handled in biosafety level 2 (BSL2) facilities, while all viruses with an mCS were handled in BSL3+ containments at the FLI. All viruses were propagated in cell cultures and/or 9- to 11-day-old SPF ECE, as described previously (6). Full genome sequences of the Lp and Hp H7 viruses were established after plaque purification (18).

Generation of recombinant virus mutants. Viral RNAs of the Hp and Lp viruses were amplified and cloned into plasmid pHWSccdB (19) to obtain an 8-plasmid-based reverse genetics system for generation of recombinant viruses. Introduction of the selected mutations into the respective gene segments of Lp and Hp viruses was performed by site-directed mutagenesis (the oligonucleotides are available upon request) according to the QuikChange protocol (Invitrogen). All viruses and/or mutants were rescued (20) and propagated in SPF ECE. Confirmation of the introduced changes and absence of unwanted genetic alterations of the generated viruses were verified by sequencing of reverse transcription (RT)-PCR amplicons obtained from viral RNA, as described previously (18).

Plaque assay. MDCKII cells were incubated with 10-fold serial dilutions of viruses for 1 h at 37°C. The cells were washed twice with phosphate-buffered saline (PBS) and overlaid with 1.8% agar in Dulbecco's modified Eagle's medium (DMEM) with 4% bovine serum albumin (BSA). In the case of Lp viruses, 2 μg/ml of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma) was added. The plates were incubated at 37°C with 5% CO_2 for 3 days, fixed with 10% formaldehyde containing 0.1% crystal violet, and used for determination of viral titers, expressed as PFU per milliliter (8), or for measurement of plaque size by using Nikon NIS-Elements software. The mean plaque size for each virus was expressed as a percentage of the plaque size of the Lp in the presence of trypsin.

Replication kinetics. CEK and DF1 cells were inoculated at a multiplicity of infection (MOI) of 0.001 for 1 h, followed by treatment with citrate buffer, pH 3.0, for 2 min to inactivate extracellular virions. After two washing steps with PBS, minimal essential medium (MEM) containing 0.2% bovine serum albumin (Sigma) was added, and the infected cells were incubated for 1, 8, 24, 48, and 72 h at 37°C and 5% CO_2. At the indicated time points, the cells and supernatant were harvested and stored at −80°C. The titers of progeny viruses were determined by plaque assay.

Animal experiments. Four- to 8-week-old White Leghorn chickens were used. The birds were allocated to separate groups. Six birds per group were inoculated with $10^{4.5}$ PFU/bird of each virus via the oculonasal route. Furthermore, four sentinel birds were added 1 day postinoculation (dpi) to study the transmissibility of the viruses. The intravenous pathogenicity index (IVPI) was determined for selected viruses following the OIE manual (6). Clinical examination of all birds was done daily over a 10-day observation period. Clinical scoring was performed according to the standard protocols (6) as healthy (0), sick (1), severely sick (2), or dead (3). Moribund birds were euthanized and scored as dead on the next observation day. The arithmetic mean of clinical signs for all chickens was calculated each day by summing up the clinical scores of all animals and dividing by the number of inoculated chickens in each group, amounting to 6. Moreover, the pathogenicity index (PI) was calculated as the sum of the daily arithmetic mean values divided by 10 (the number of observation days). Thus, the PI for a given virus ranged from 0 (avirulent) to 3 (highly virulent). The extent of viral shedding was analyzed by titration of tracheal and cloacal swabs taken at 2, 4, 6, 8, and 10 dpi by plaque assay using MDCKII cells and expressed as PFU/ml. Serum samples collected from surviving chickens at the end of the experiments were tested using an enzyme-linked immunosorbent assay (ELISA) kit (ID Screen Influenza A Antibody Competition Multispecies; IDvet, Montpellier, France) for specific detection of influenza virus NP antibodies according to the manufacturer's manual.

Histopathology and immunohistochemistry. To study the tropism and pathological alterations after infection of birds by different recombinant viruses, samples from trachea, lungs, pancreas, liver, kidneys, spleen, heart, proventriculus, cecum, duodenum, bursa of Fabricius, thymus, and brain from two birds at 4 dpi were fixed in 10% neutral buffered formalin and then embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (HE) and screened for microscopic lesions. Another section from each organ was stained for immunohistochemical examination using primary rabbit anti-NP antibodies (1:750) and biotin-

ylated goat anti-rabbit IgG1 (Vector, Burlingame, CA, USA) as the secondary antibody (1:200), as previously described (21).

Heat stability. Aliquots of 1 ml (~10^6 PFU/ml) virus-containing allantoic fluids were incubated at 50°C for 0, 1, 2, 3, and 4 h. HA activity was analyzed by HA test against 1% chicken erythrocytes in duplicate, as previously described (6). The infectivity of viruses was assessed by plaque assay. The results are shown as the mean of values obtained in two independent trials.

pH stability. PBS with different pHs (4.0, 4.5, 5.0, 5.5, 6.0, 7.0, and 7.4) was mixed with ~10^5 PFU of selected viruses and used for incubation of CEK cells for 30 min at 37°C. The cells were washed twice with PBS before MEM with 0.2% BSA was added to each well for 8 h. Cells and supernatants were harvested, and the virus titers were determined using a plaque assay as described above.

Fusion assay. The effects of different mutations on the fusogenic activities of the recombinant viruses were studied in avian cells as previously described (22), with modifications. Briefly, QM9 cells in 24-well plates were transfected using Lipofectamine with 3 μg pHWS plasmid containing HA from Hp, HA of Lp with a cleavage site from Hp, or HA of Lp with a cleavage site from Hp with the T430, L450, or R536 single HA mutant. The cells were incubated for about 24 h. Thereafter, the medium was removed, and the cells were washed twice with PBS. PBS fusion buffer at pH 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, and 6.0 was added for 4 min. The cells were then washed with PBS, and MEM with 5% fetal calf serum (FCS) was added for 4 h at 37°C. The cells were fixed with methanol-acetone (1:1) for 15 min and stained with anti-H7 chicken antiserum and Alexa Fluor anti-chicken goat antibodies. The pH threshold was the highest pH value at which fusion was observed.

Western blotting. CEK cells were inoculated with selected viruses at an MOI of 1 and incubated for 6 h at 37°C. Lysates of about 10^6 cells were separated in a discontinuous sodium dodecyl sulfate-10% polyacrylamide gel and transferred to a nitrocellulose membrane using a TransBlot cell (Bio-Rad). The blots were incubated for 1 h with 5% low-fat milk diluted in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.25% Tween 20) and subsequently for an hour with chicken antiserum, which was obtained after challenge infection of vaccinated birds with HPAIV A/chicken/Italy/445/99 (H7N1) at a dilution of 1:1,000 and peroxidase-conjugated chicken IgY-specific goat IgG (Dianova, Hamburg, Germany) at a dilution of 1:20,000 in TBS-T. After repeated washings, antibody binding was detected by luminescence (Supersignal West Pico chemiluminescent substrate kit; Pierce, ThermoScientific, Rockford, IL, USA) in a Bio-Rad Versa Doc System with Quantity One software.

Sequence analysis and molecular modeling. The full genome sequence of Lp generated in this study was submitted to the Global Initiative on Sharing All Influenza Data (GISAID) under accession numbers EPI624433 to EPI624435 and EPI624437 to EPI624441. HA gene sequences of all Italian H7 influenza viruses were retrieved from GenBank and GISAID for comparison and calculation of the prevalences of mutations in LP and HP viruses. All the sequences were aligned with MAFFT (23) and BioEdit (24) and further edited manually. HA of isolate A/chicken/Italy/445/1999 H7N1 was used for tertiary-structure generation using Swiss Model (http://swissmodel.expasy.org/) and edited with RasTop version 2.7.1 (http://rasmol.org/). In this study, amino acid positions of HA proteins are according to the H7 numbering based on the mature protein after removal of the signal peptide. In the three-dimensional (3D) structure, the CS region is shown as ^{315}PEIPKGR*G^{322} for Lp, ^{315}PEIPKGSRVRR*G^{326} for Lp_CS445, and ^{315}PEIPKRRRR*G^{324} for Lp_CS28.

Statistics. Inter- and intragroup variations for the number of positive birds after examination of viral shedding in oral and cloacal swabs at 4 dpi were compared using pairwise exact Fisher tests with Bonferroni correction. An analysis of variance (ANOVA) with *post hoc* Tukey test was utilized to compare replication kinetics and heat stability. Statistical differences for other analyzed variables (e.g., the amount of virus excretion, plaque size, and pathogenicity index) between and within groups were evaluated using the Kruskal-Wallis test and Wilcoxon tests with Bonferroni correction, respectively. Significant differences in clinical scoring between groups were assessed by comparing the mean clinical scores per bird during a 10-day observation period. A P value of <0.05 was considered to be significant, and all analysis was done using R version 2.14.0 from the R Foundation for Statistical Computing, available at the R Project website (http://www.r-project.org).

RESULTS

Generation of recombinant viruses and mutants. All gene segments of Lp and Hp were cloned, and recombinant viruses were successfully rescued in 293T/MDCKII cell cultures and propagated for one passage in SPF ECE. A total of 16 recombinant viruses were generated, as illustrated in Fig. 1, with titers in the allantoic fluids of $10^{5.6}$ to $10^{7.9}$ PFU/ml (data not shown). Comparison of the full genome sequences of Lp and Hp revealed 34 nonsynonymous mutations in all coding regions, with the exception of the PB1-F2, M2, and NEP genes (data not shown).

Virulence determinants of H7N1 Hp virus are located within the HA protein in conjunction with a multibasic proteolytic cleavage site. To determine whether the virulence determinants of Italian H7N1 viruses are located in the HA protein, 7 recombinant chimeric viruses of the parental Lp and Hp viruses were generated (Fig. 1). In addition to the Lp and Hp recombinant viruses, two Lp viruses carrying the HA (Lp_HA445) or mCS (Lp_CS445) from Hp or the mCS from HPAIV A/chicken/Germany/R28/03 (H7N7) (Lp_CS28) and two Hp viruses carrying the HA from Lp (Hp_HA473) or with a monobasic CS (Hp_mono) were rescued (Fig. 1). The virulence was investigated by oculonasal inoculation of chickens with $10^{4.5}$ PFU.

All birds infected with Hp_mono or Hp_HA473 showed very mild disease, if any, and none of the birds died, resembling the low virulence of Lp (Fig. 2A to C).In contrast, Lp_HA445 killed all primarily infected and contact birds within 4 and 6 dpi, respectively, which was 2 days earlier than the parental Hp (Fig. 2H and I). Interestingly, Lp_CS445 was significantly less virulent than Hp, and only 3 out of 6 primarily infected birds and 1 out of 4 sentinel birds died (Fig. 2E). Nevertheless, the IVPI value of Lp was 0.0, whereas Lp_CS445, Lp_HA445, and Hp had IVPI values of 2.8, 3.0, and 3.0, respectively, reflecting high pathogenicity. To study whether the increase in the number of basic amino acids in the CS of Lp is sufficient for high virulence of Lp, Lp_CS28, possessing the CS motif PEIPK**RRR**R*GLF with up to 5 basic amino acids, as found in HPAI H7N7 viruses in the Netherlands, Germany, and Belgium (25), was generated. Surprisingly, none of the primarily infected chickens died but showed only transient mild to moderate clinical signs. However, two contact birds died at 8 dpi (Fig. 2D); direct sequencing of the HA from a virus in the brain of one of the two dead birds showed only one nonsynonymous mutation located in the signal peptide (data not shown). Successful infection of all surviving birds was verified by the development of anti-NP antibodies by the end of the experiment (data not shown). Thus, the determinants of virulence of the Italian HPAIV H7N1 are located within the HA gene segment. However, the unique mCS motif was not sufficient to exhibit full virulence and transmissibility of the LPAIV H7N1, and therefore, further mutations in the HA contribute. Interestingly, an increase in the number of basic amino acids in the CS of Lp, as is present in HPAIV H7N7, resulted in a decrease in virulence compared to Lp_CS445, suggesting that the latter cleavage site is very specific to the Italian viruses.

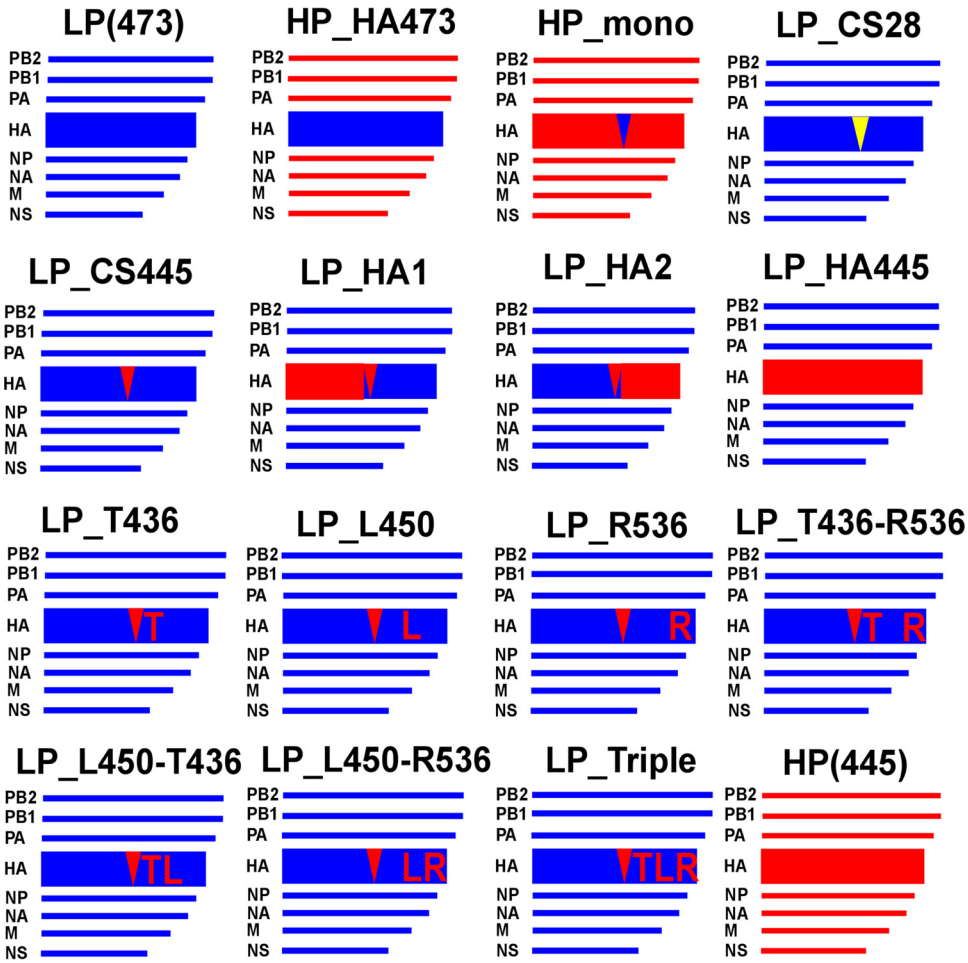

FIG 1 Diagrams of the recombinant viruses generated in this study. Gene segments or mutations of the LP H7N1 virus are illustrated in blue, whereas those from the HP H7N1 virus are in red. The triangles in the HA indicate the cleavage site motif: blue (mCS motif PEIPKGR*G from Lp), red (mCS PEIPKGSRVRR*G from Hp), or yellow (mCS PEIPKRRRR*G from HP H7N7).

Specific mutations in HA2 are required for full virulence and transmission of HPAIV H7N1 in chickens. Comparison of the HA protein sequences from Lp and Hp AIV revealed five mutations in the HA1 domain (N-2S in the signal peptide, T112A, A128T, A210E, and I249S), as well as five mutations in the HA2 domain (I398V, H423L, A436T, Q450L, and K536R). Two chimeric Lp_CS445 viruses carrying the HA1 (Lp_HA1) or HA2 (Lp_HA2) from Hp were generated (Fig. 1). Lp_HA2 killed all primarily infected birds within 5 dpi and all contact birds within 8 dpi, resembling Hp, while Lp_HA1 killed primarily infected chickens within 9 dpi and only two out of four contact chickens, presumably indicating decreased transmissibility of the chimeric virus (Fig. 2F and G). Nevertheless, the IVPI values of both chimeric viruses were at the maximum level of 2.9 for Lp_HA1 and 3.0 for Lp_HA2.

Q450L with or without the combination of A436T and/or K536R is required for exhibition of full virulence and transmission of Italian H7N1 in chickens. As Lp_HA2 showed virulence comparable to that of the parental Hp, we further explored the frequencies of the five mutations (I398V, H423L, A436T, Q450L, and K536R) in HA2 among the Italian H7N1 viruses collected during the early outbreak from 1999 to 2000 deposited in GenBank and GISAID. Of a total of 149 viruses, 66 LPAIV and 83 HPAIV sequences were available (Table 1). All the viruses except three LPAI viruses isolated from August to October 2000 (about 8 months after the emergence of the HPAI outbreak) specified V398, and all the viruses possessed L423 (except for Lp used in this study and another virus, which had H423 and F423, respectively), assuming that these 2 amino acids do not play a role in the virulence of the Italian strains. In contrast, all the LPAI viruses possessed alanine and lysine and all the HPAI viruses possessed threonine and arginine in positions 436 and 536, respectively, while 75 out of 83 HPAI viruses (~90%) possessed 450L, implying that those 3 amino acids have a pivotal role in virulence. For evaluation, we generated seven Lp_CS445 viruses carrying single, double, or triple amino acid alterations of T436, L450, and/or R536 (Fig. 1 and 3). Only the virus carrying L450 alone or in combination with T436 killed all primarily infected and contact birds (Fig. 3A, E, and G), indicating a central role of the amino acid in the virulence of the Italian viruses. Interestingly, sequence analysis indicated that 8 HPAI viruses specified Q450, resembling the LPAI viruses; they were isolated very early during the outbreak, from 12 October 1999, and circulated (in parallel with other L450-possessing viruses) for a limited time, to 2 July 2000. All virus sequences

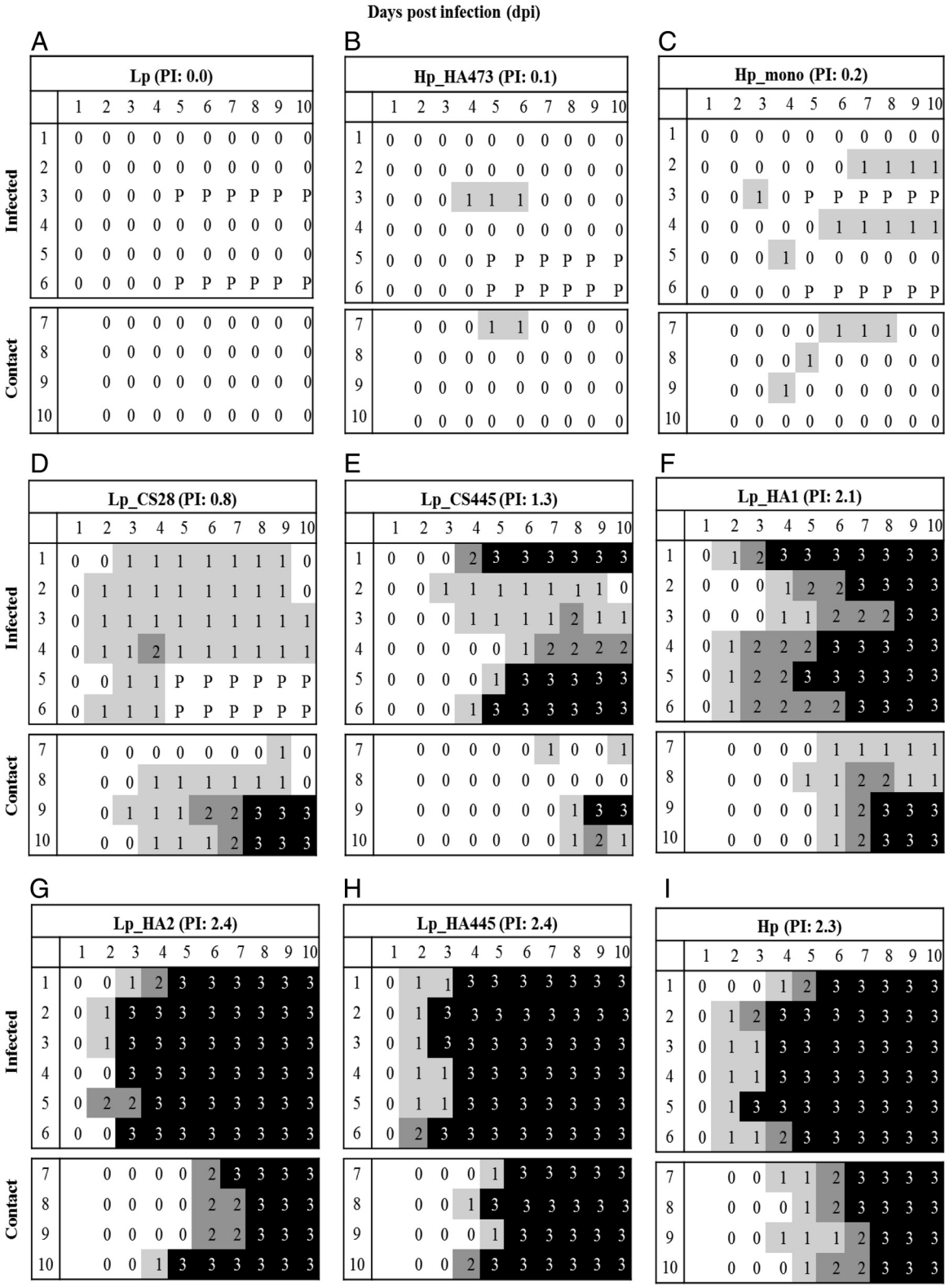

FIG 2 Clinical findings in chickens inoculated with H7N1 carrying different cleavage site motifs or HA proteins. Shown is clinical scoring after oculonasal inoculation of 4- to 6-week-old specific-pathogen-free White Leghorn chickens with $10^{4.5}$ PFU/ml of the indicated viruses. Chickens without clinical signs were scored 0 (white boxes). A score of 1 (light gray boxes) was applied to chickens with one of the following clinical signs: depression, ruffled feathers, diarrhea, sneezing, coughing, conjunctivitis, discharges, or cyanosis of the comb, wattle, or shanks. These chickens were categorized as ill. Severely ill chickens showed two or more clinical signs and were scored 2 (dark gray boxes), whereas dead chickens were scored 3 (black boxes). The PI was calculated as the mean sum of the daily arithmetic mean values divided by 10, the number of observation days. "P" stands for pathology; the birds were killed and taken for necropsy.

TABLE 1 Mutations in HA2 of the Italian LP and HP H7N1 viruses

Position	Amino acid	No. of sequences with mutation[a]		
		LP (n = 66)	HP (n = 83)	Total
398	I	3	0	3[b]
	V	63	83	146
423	H	0	0	0
	L	65	83	148[c]
436	A	66	0	66
	T	0	83	83
450	Q	66	8[d]	74
	L	0	75	75
536	K	66	0	66
	R	0	83	83

[a] The total was 149 sequences, excluding the Lp and Hp viruses used in this study.
[b] Three Lp viruses with I398 were isolated from August to October 2000, about 8 months after emergence of the HP.
[c] One low-pathogenic virus contained F423.
[d] Eight HPAI viruses carrying Q450 were isolated during the very early outbreak from 12 October 1999 and still circulated for a limited time, to 2 July 2000. Thereafter, all the viruses contained the three mutations.

deposited in the GISAID thereafter had the three mutations. Interestingly, sequence analysis of all H7 HA (n = 1,506) in GISAID indicated that these mutations were very specific to the Italian HPAI H7N1 viruses; while none of the viruses had L450, only 2 viruses (0.1%) had R536, and 85 viruses (5.6%) had T436 (Table 2). Moreover, among other influenza virus subtypes analyzed in this study (n = 46,709), L450 was surprisingly prevalent in all H11 to H16 viruses, and T436 is very common (≥99.4) in H3 and H14 viruses (Table 2), while no single influenza virus contains a combination of the three mutations, T436, L450, and R536.

Virus excretion increased after the introduction of the mCS and L450 when combined with T436 and R536. For detection of viral excretion from experimental birds, swabs were collected from all surviving birds and examined by plaque assay. Birds infected with Lp, Hp_HA473, or Hp_mono excreted no or only a very limited amount of viruses orally at 4 dpi, and only one swab from the contact birds in the Hp_mono-infected group was positive at 4 dpi (Fig. 4A). All the birds showed AIV NP-specific serum antibodies at the end of the experiment (data not shown). The number of shedders, as well as the amount of shed virus, was higher in Lp_CS445- and Lp_CS28-infected birds, with 5 out of 6 birds each. Also, all Lp_HA1- and Lp_HA2-infected birds excreted virus, as did the Lp_Triple-, Lp_HA445-, and Hp-infected

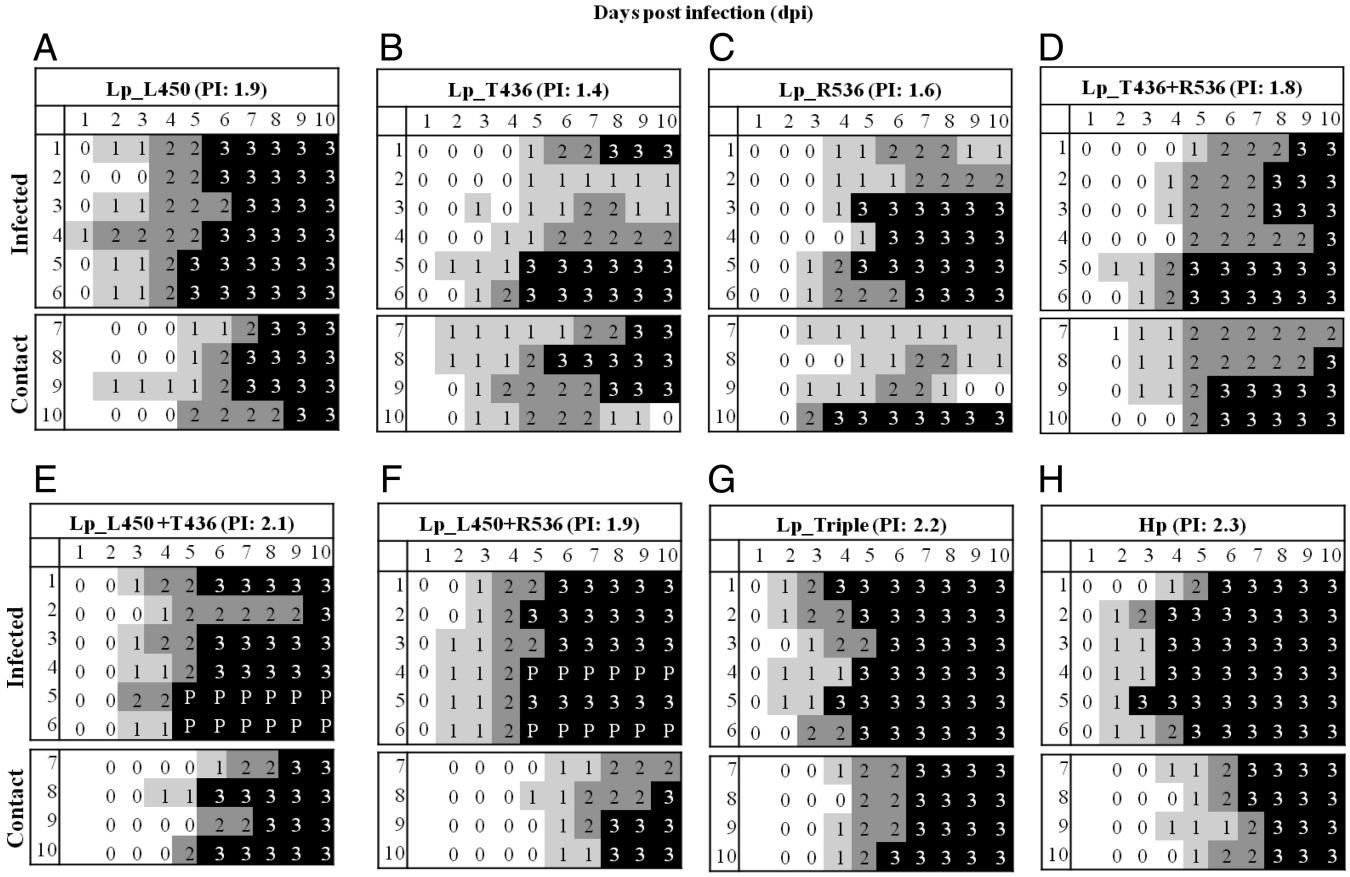

FIG 3 Clinical findings in chickens inoculated with H7N1 carrying multibasic cleavage sites and specific mutations in the HA2 domain. Shown is clinical scoring after oculonasal inoculation of 4- to 6-week-old specific-pathogen-free White Leghorn chickens with $10^{4.5}$ PFU/ml. Chickens without clinical illness were scored 0 (white boxes). A score of 1 (light gray boxes) was applied to chickens with one of the following clinical signs: depression, ruffled feathers, diarrhea, sneezing, coughing, conjunctivitis, discharges, or cyanosis of the comb, wattle, or shanks. These chickens were categorized as ill. Severely ill chickens showed two or more clinical signs and were scored 2 (dark gray boxes), whereas dead chickens were scored 3 (black boxes). The PI was calculated as the mean sum of the daily arithmetic mean values divided by 10, the number of observation days. "P" stands for pathology; the birds were killed and taken for necropsy.

TABLE 2 Prevalences of HA2 mutations among all avian influenza virus subtypes

Subtype	No. of sequences	HA2 mutation at position [no. (%) of sequences]: 436 A	436 T	436 Other (only H)	450 Q	450 L	450 Others	536 K	536 R	536 Others
H1 avian	450	0	1 (0.2)	449 (99.8)	450 (100)	0		0	0	S 449 (99.8)
H1 human	18,713	0	4 (0.02)	18,694 (99.9)	18,702 (99.9)	0		0	0	S 18,680 (99.8)
H2 avian	376	0	0	376 (100)	376 (100)	0		0	0	S 375 (99.7)
H2 human	93	0	0	93 (100)	93 (100)	0		0	0	S 91 (99.9)
H3 avian	1,186	7 (0.6)	1,179 (99.4)		1,184 (99.8)	0		1 (0.1)	0	Q 1,183 (99.9)
H3 human	15,216	3 (0.03)	15,207 (99.9)		15,211 (99.97)	4 (0.03)		0	0	Q 15,208 (99.9)
H4	1,069	0	0	1,064 (99.5)	0	0	A 1,069 (100)	1 (0.1)	0	Q 1,068 (99.9)
H5	4,244	0	0	4,242 (99.95)	4,238 (99.86)	2 (0.05)		0	0	S 4,228 (99.6)
H6	1,196	0	0	1,196 (100)	1,189 (99.4)	7 (0.6)		0	0	S 1,186 (99.2)
H7	1,506	1,421 (94.4)	85 (5.6)[a]		1,505 (99.9)	0		1,503 (99.9)	2 (0.1)	0
H8	91	0	0	91 (100)	1 (1.1)	0	R 90 (98.9)	0	0	Q 90 (98.9)
H9	1,428	0	0	1,426 (99.86)	0	0	A 1,401 (98.1)	0	0	S 1,426 (99.86)
H10	499	457 (91.6)	41 (8.2)[b]	0	499 (100)	0		491 (98.4)	8 (1.6)	
H11, H13, H16	493	0	1 (0.2)	492 (99.8)	0	493 (100)		0	0	S 459 (93.1)
H12	119	0	0	119 (100)	0	119 (100)		0	0	Q 119 (100)
H14	17	0	17 (100)	0	0	17 (100)		10 (58.8)	0	Q 7 (41.2)
H15	13	13 (100)	0	0	0	13 (100)		13 (100)	0	
Total	46,709	1,901 (4.1)	16,535 (35.4)	0	43,448 (93.0)	655 (1.4)		2,019 (4.3)	10 (0.01)	

[a] A total of 85 non-Italian H7N1 viruses possessed the virulence marker T436: 24 equine H7N7 viruses from 1956 to 1977 harboring anomalous CS as a result of recombination in the HA PENSTHKQLTHHMRKKR*GLF, PENSIHKQLTHHMRKKR*GLF, or PEAPAHKQLTHHMRKKR*GLF"; 15 strains from the United States in 2004 to 2010 (12 H7N2 and 1 H7N3), Singapore in 1994 (1 H7N1), and Korea in 2010 (1 H7N7) carrying monobasic CS; 42 LPAI H7N2 viruses from New York in 2004 to 2006 carrying PEKPKKRGLF; and only 4 viruses containing mCS (2 H7N7 [Germany, 1979, and Australia, 1985], 1 H7N2 [Tasmania/Australia, 2007], and 1 H7N6 [Australia, 2007]).
[b] H10 viruses of wild-bird origin from the United States in 2009.

animals (Fig. 4A). Interestingly, the number of shedders in the double-mutant-infected birds was lower than in the Lp_CS445-, Lp_CS28-, or single-mutant-infected chickens, especially in oral swabs. Birds infected by the single or double mutants excreted about 10 to 100 times less virus than chickens infected with the triple mutant. At 4 dpi, the majority of birds in all the groups excreted viruses through the cloaca, except for the Hp_HA473-infected (all the birds were negative) and Lp473-infected (only two birds were positive) groups. The amounts of cloacally excreted virus were comparable to oral excretion. The triple mutant was orally excreted in significantly larger amounts than the single or double mutant. Contact birds in groups inoculated with viruses possessing the mCS excreted the largest amount of viruses at 6 dpi, which decreased thereafter in the surviving chickens (data not shown). Together, the mCS and the triple mutations in HA2 increased virus excretion, indicating efficient replication of the virus in chickens.

Tissue tropism of the LPAI H7N1 virus was enhanced by the addition of mCS plus T436 or L450, but not R536. To investigate tissue tropism, internal organs of infected birds were collected 4 dpi and subjected to histopathological and immunohistochemical examination. No pathological changes and no influenza virus NP antigen were detected in organs from birds infected with Lp. Hp_HA473 was detected only in lungs and Hp_mono only in cecal tonsils and kidneys. Birds infected with Hp, Lp_HA445, Lp_HA1, or Lp_HA2 showed hemorrhages in the proventriculus, necrosis in the pancreas, airsacculitis, hemorrhages in the cecal tonsils, and/or multifocal necrosis and perivascular multifocal infiltration in the brain. Influenza virus NP was detected in all organs collected from birds infected with Hp or Lp_HA445, indicating the HA can modulate the tissue tropism of the HPAI H7N1 virus (data not shown). Likewise, the triple mutant carrying the three HA2 mutations showed systemic and extensive spread resembling that of Hp (Fig. 4B). On the other hand, the insertion of mCS (CS445 and CS28) in Lp enabled systemic spread of the virus to the heart, lungs, trachea, spleen, kidneys, thymus, pancreas, and brain, but to a lesser extent than the triple-mutant virus (data not shown). Viruses containing T436 or L450 were further detected in the bursa, cecal tonsils, gizzard, and proventriculus, while R536 was detected only in the bursa, kidney, and brain (Fig. 4B and data not shown). In conclusion, the insertion of the mCS conferred systemic spread on the LPAIV, and the extent was further increased by acquiring T436 or L450. On the other hand, NP antigen could be detected in only a small number of organs of R536-infected chickens, indicating a minimal role of the mutation alone in virus spread within the host.

The virulence-determining L450 mutation is detrimental to virus replication in avian cell culture, which is restored by T436 and R536. All the viruses replicated efficiently in CEK (Fig. 5A) and DF1 (data not shown) cells, reaching maximum virus titers at 24 hours postinfection (hpi), which decreased gradually thereafter. The Hp titer was significantly higher than the titers of other viruses, particularly at 24 hpi. Interestingly, the virus carrying L450 had a significantly decreased titer in cell culture, but its replication efficiency was restored by acquisition of T436 and R536 (Fig. 5A).

Incubation of the viruses at 50°C did not influence their HA activity, but infectivity of the virus in cell culture was significantly reduced in the absence of R536. The activity of the HA of AIV may be impaired at high temperature (e.g., in the environment or in feverish birds) (26). To investigate whether the mutations in HA2 might influence the heat stability of the viruses, selected viruses were incubated at 50°C for 0, 1, 2, 3, and 4 h. Reductions in the HA activity and infectivity were compared by HA test and plaque assay, respectively. Neither reduction in the HA activity nor correlation between the HA titer and plaque titers was observed. Incubation of Lp, Hp_HA473, Lp_T436, Lp_L450, Lp_R536, Lp_Triple, and Hp did not affect the HA activity in terms of the HA titer (data not shown). Conversely, all the viruses showed significantly reduced infectivity, as indicated by decreased

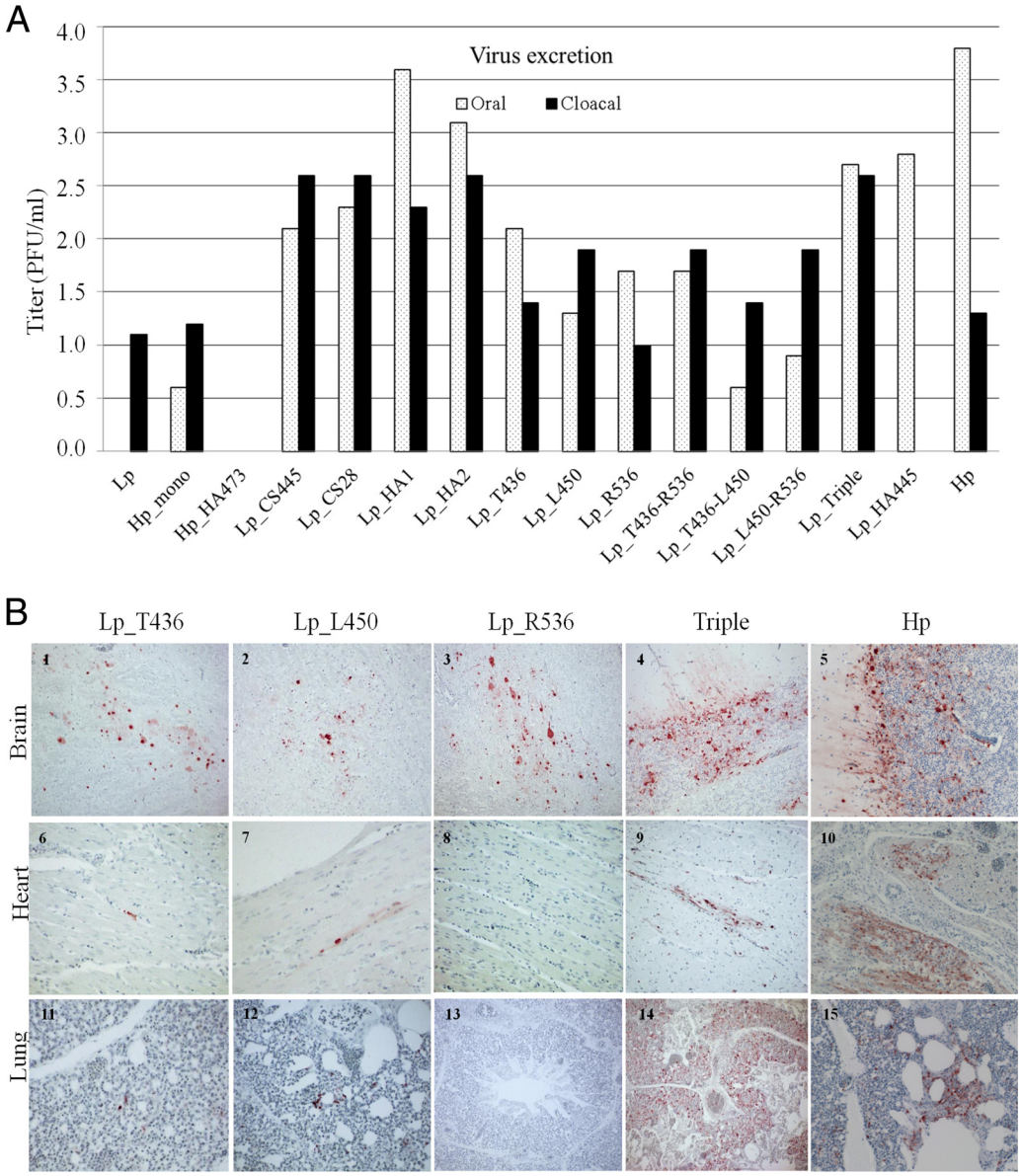

FIG 4 Virus shedding and tissue tropism 4 days postinoculation of SPF chickens. (A) Mean amounts of virus excreted at day 4 postinoculation from the oral and cloacal swabs as estimated by plaque assay on MDCK cells. (B) Detection of influenza virus NP by immunohistochemistry in lungs, heart, and brain (bright red staining); data on the full tropism of all viruses are available upon request.

PFU titers after 4 h postincubation. The Lp_Triple virus was as stable as the Lp_R536 and Hp viruses, while Lp and Lp_T436 lost infectivity significantly. Lp_L450 and Hp_HA473 had moderate reductions in infectious titers (Fig. 5B). These results may indicate that R536 is more important than the other mutations for heat stability of the virus.

Mutations in HA2 did not change the resistance of the Italian H7N1 viruses to different pHs. Resistance of influenza viruses to low pH is known to be crucial for persistence of the virus in the environment (26). Incubation of Lp, Lp_T436, Lp_L450, Lp_R536, Lp_Triple, and Hp viruses at pHs from 4 to 7.4 for 30 min did not change the infectivity of the viruses in cell culture. All viruses remained comparably active regardless of the pH value and grew at the same level after 8 h of incubation on CEK cells at 37°C (Fig. 5C).

In addition to HA1, mutations in HA2, and insertion of the mCS also significantly altered the plaque size of H7N1 viruses. All viruses with mCS replicated well without the addition of trypsin on MDCKII cells. Conversely, Lp virus required trypsin for induction of significantly bigger plaques than in the absence of trypsin (Fig. 5D). There was no statistical significance to the sizes of plaques of the Hp and Lp in the presence of trypsin ($P = 0.9964$). However, Lp_HA1, Lp_HA2, Lp_CS445, and single or triple HA2 mutants produced significantly bigger plaques than Lp.

L450 reduced the pH of membrane fusion in QM9 cells. To investigate the impact of HA2 mutations on the pH activation of H7N1 virus at pH 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, and 6.0, QM9 cells were transfected with plasmids containing HA from Hp or Lp HA with the mCS and the single mutation T430, L450, or R536 1 day

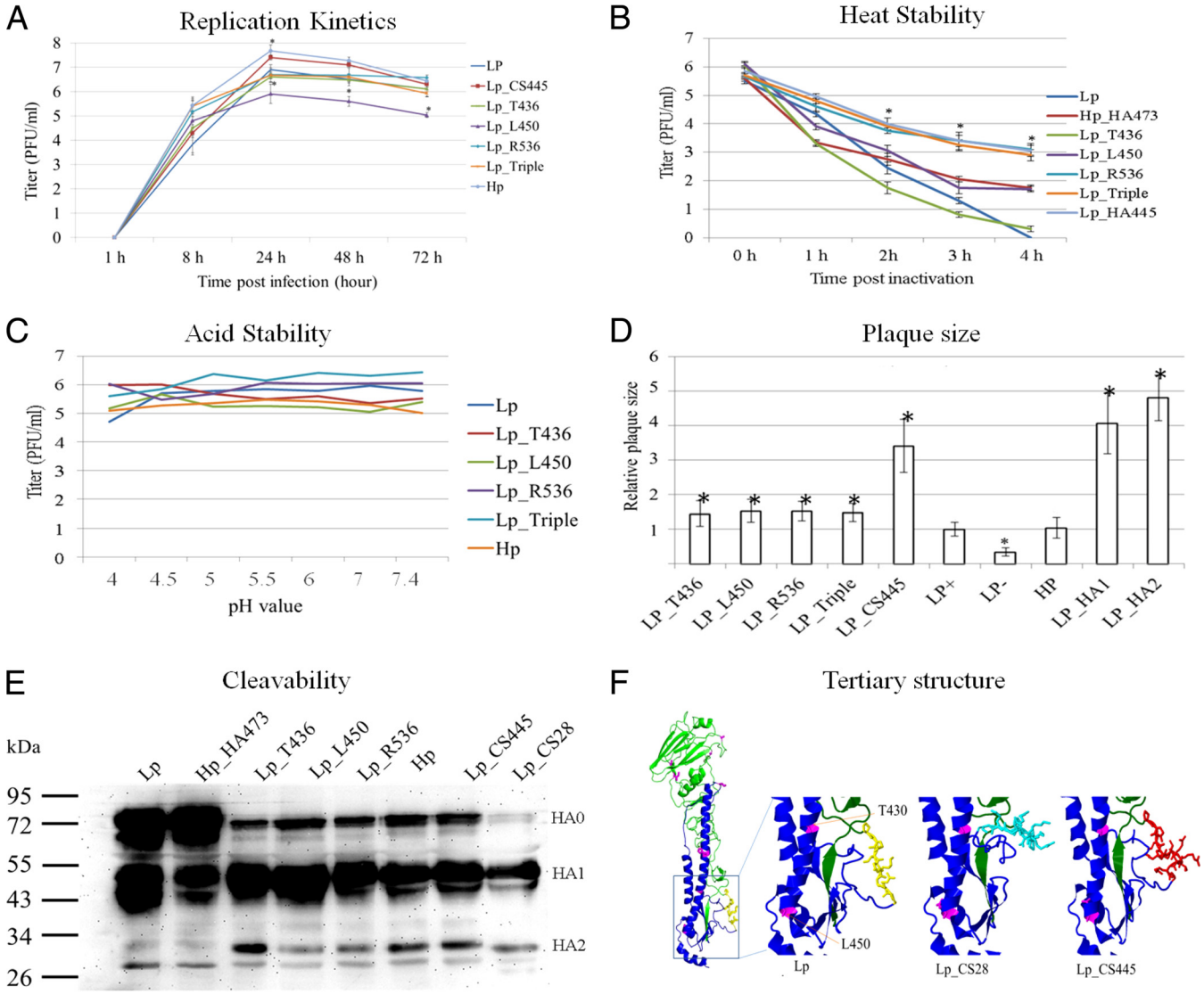

FIG 5 *In vitro* characterization of selected viruses reverse engineered in this study. (A) Replication kinetics as estimated by plaque test at 1, 8, 24, 48, and 72 h postinoculation of CEK cells at an MOI of 0.001 PFU/cell. (B) Heat stability at 50°C for 1, 2, 3, and 4 h. (C) Stability after incubation of viruses at pH 4, 4.5, 5, 5.5, 6, 7, or 7.4. (D) Sizes of plaques at MDCKII cells. (E) Cleavability of the HA of recombinant viruses 6 h after inoculation of CEK cells at an MOI of 1 in the absence of trypsin. (F) Predicted tertiary structure of the HA protein showing mutations in the HA of Lp versus Hp (pink) and variable cleavage site motifs (yellow in Lp, cyan in Lp_CS28, and red in Hp). The error bars represent the standard deviations of different replicates. The asterisks indicate *P* values of < 0.05.

before activation and fixed 4 h postactivation. The highest pH at which cell-cell membrane fusion was induced in cells transfected with HA from Hp or Lp_CS445 with T436, L450, or R536 was 5, 4.8, 4.6, or 4.8, respectively.

Mutations in HA2 did not enhance the cleavability of HA. The cleavabilities of the HA proteins were compared using Western blot analyses of CEK cells inoculated at an MOI of 1 for 6 h. The Lp and Hp_HA473 were not efficiently cleaved in the absence of trypsin, whereas viruses possessing mCS445 were cleaved into HA1 and HA2 without appreciable differences between them. Lp_CS28 was cleaved most efficiently (Fig. 5E). Therefore, the mutations in HA2 had no obvious influence on the cleavability of the HA protein.

Molecular modeling showed that T436 and L450 reside in the long HA2 coiled-coil helix. Mutations in HA1 (except for one mutation in the signal peptide) are in the head domain (Fig. 5F), whereas mutations in HA2 are located in the stem domain or in the C-terminal region (K536R). T436 is sterically closer to the cleavage site than L450, which resides in the distal tip of the long coiled-coil helix (Fig. 5F). R536 is located immediately after the membrane anchor region, the first amino acid of the cytoplasmic tail (27). Insertion of CS445 probably increased the exposed surface of the CS of Lp473 more than insertion of CS28 with five basic amino acids (Fig. 5F).

DISCUSSION

In this report, we determined the virulence markers of HPAIV H7N1 in chickens, which emerged in Italy in 1999 after extensive circulation of the LPAIV precursor for about 9 months. The HPAIV differs from the LPAIV ancestor by a unique multibasic

cleavage site and 34 mutations in the whole genome, including 10 nonsynonymous mutations in the HA protein. Our findings indicated that the HA protein is the virulence determinant of the virus in chickens, where the mCS alone was not sufficient to express high virulence after oculonasal inoculation. Three mutations in the HA2 domain act synergistically to maintain high replication efficiency, full virulence, effective transmission, and systemic spread in chickens.

In this study, LPAIV carrying the complete HA of HPAIV H7N1 killed all infected chickens, whereas chickens infected with HPAIV carrying HA from LPAIV H7N1 survived, indicating that HA is the prime determinant of virulence, which is in accordance with the central dogma for pathogenicity of AIV (28). Another study (as well as our unpublished data) showed that the NS1 gene segment of Italian HPAIV did not increase the virulence of LPAIV H7N1 in chickens (29). Altering the mCS of HPAIV into a monobasic motif resulted in low virulence, while introduction of the authentic mCS into LPAIV increased virulence, enhanced systemic spread, and increased viral excretion in chickens, but to levels lower than those observed with authentic HPAIV. This indicates that the mCS is required for virulence but is not sufficient for exhibition of full virulence. It has been reported that few H5N1/H5N2 viruses (30), as well as an American H7N2 virus (31) with mCS, exhibited only mild clinical signs, if any, in chickens. Increasing the number of basic amino acids from 3 or 4 in the parent viruses to 5 or 6 changed some H5/H7 viruses into HP phenotypes in chickens, as estimated by the IVPI (31–33). Interestingly, the LPAIV H7N1 in this study, harboring the mCS motif PEIPKR RRR*G from HPAIV H7N7 with 5 basic amino acids, was even less virulent than the mCS PEIPKGSVRR*G-containing virus. This probably indicates that the motif is either incompatible or suboptimal for the exhibition of full virulence of Lp and an additional number of basic amino acids or further mutations elsewhere (e.g., in HA2) are required. It is worth pointing out that the IVPI of viruses containing the authentic mCS in this study did not correlate with the pathogenicity index after inoculation via the oculonasal, natural route. Hence, all viruses with the authentic mCS induced an IVPI that complies with the OIE definition for HPAIV (IVPI $>$ 1.2) but showed remarkable differences in virulence and transmissibility after oculonasal infection from viruses containing the HA from HPAIV. Such a discrepancy has previously been observed in viruses of H4/H10 subtypes or anomalous H7N3 viruses (6). Moreover, formation of a loop structure bulging out from the HA may make the CS more accessible to the furin active site (34). The tertiary-structure modeling predicted that Lp_CS28 has a distorted loop in the cleavage site region, whereas Lp_CS445 seems to be protruded from the HA stem region slightly more than CS28, although it was cleaved less efficiently than CS28 *in vitro*. Nevertheless, the two mCS motifs provided similar tropism and replication efficiencies. Thus, the difference in virulence could not be explained by amino acid sequences in the CS alone but was attributed to ancillary mutations in the HA, raising the question of whether the mCS is a "true" virulence marker or just a facilitator for viral spread in multiple organs.

In addition to the authentic mCS, mutations in HA2 were required for the exhibition of full virulence in chickens. T436 and L450 are located in the stalk domain, not directly in the vicinity of the CS, whereas R536 resides in the cytoplasmic tail. The Q450L substitution proved to influence virulence and transmission. A possible explanation is that the replacement of glutamine (polar hydrophilic) in the Lp virus by leucine (nonpolar hydrophobic) in the Hp virus may affect the folding of the HA and/or interaction with other residues (e.g., in the HA1 stalk region or trimers). Since the HA2 stem region is highly conserved among influenza viruses and contains antigenic regions (35), subsequent conformational changes may lead to masking/altering of antigenic sites or immunogenic epitopes. The effects of the immune response on virus replication and virulence were not investigated in this study. A mutation in the stalk region (among other mutations in the internal gene segments) was also obtained after serial passages of a similar Italian HPAIV H7N1 in ferrets, which rendered the virus transmissible by air (36). Mutations in the stem region, very close to T436, destabilized the structure of HA2 (37), suggesting that stabilization probably requires R536 and L450.

It is known that the fusogenic activity of influenza virus is triggered by the acidic pH in the endosomes, which results in irreversible conformational changes that facilitate fusion of the viral envelope with the endosomal membrane. The threshold of pH activation of influenza viruses differ but mostly range from 4.6 to 6.0 (26). Impaired Lp_L450 virus replication in cell culture may be due to the reduced pH of activation compared to Hp. In H5N1 viruses, reduced pH of activation in cell culture by 0.3 to 0.4 unit correlated with high virulence in poultry and altered virus replication *in vitro* (38, 39). Moreover, in line with the environmental stability of the chimeric viruses at different pHs in this study, the Italian Lp and Hp H7N1 field viruses were stable at pH 5 and 7 for several hours (40).

According to the available gene sequences, viruses isolated during the early HPAI H7N1 outbreaks already contained the mCS, T436, and R536 and a few weeks later acquired the L450 mutation. Therefore, we hypothesize that the emergence of the early HPAIV was a gradual but very fast process. First, the virus acquired (i) the mCS, which was important to facilitate systemic spread and increase replication, cleavability, and excretion; (ii) T436, enhancing tissue tropism further; and (iii) R536, conferring HA stability. Thereafter, the virus acquired L450, resulting in maximized virulence and bird-to-bird transmission. Taken together, these three unique HA2 mutations synergistically increase the fitness of the H7N1 1999 Italian HPAIV *in vivo* and *in vitro*.

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