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Generation of Live Attenuated Novel Influenza Virus A/California/7/09 (H1N1) Vaccines with High Yield in Embryonated Chicken Eggs[▽]

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Several live attenuated influenza virus A/California/7/09 (H1N1) (CA09) candidate vaccine variants that possess the hemagglutinin (HA) and neuraminidase (NA) gene segments from the CA09 virus and six internal protein gene segments from the cold-adapted influenza virus A/Ann Arbor/6/60 (H2N2) virus were generated by reverse genetics. The reassortant viruses replicated relatively poorly in embryonated chicken eggs. To improve virus growth in eggs, reassortants expressing the HA and NA of CA09 were passaged in MDCK cells and variants exhibiting large-plaque morphology were isolated. These variants replicated at levels approximately 10-fold higher than the rate of replication of the parental strains in embryonated chicken eggs. Sequence analysis indicated that single amino acid changes at positions 119, 153, 154, and 186 were responsible for the improved growth properties in MDCK cells and eggs. In addition, the introduction of a mutation at residue 155 that was previously shown to enhance the replication of a 1976 swine influenza virus also significantly improved the replication of the CA09 virus in eggs. Each variant was further evaluated for receptor binding preference, antigenicity, attenuation phenotype, and immunogenicity. Mutations at residues 153, 154, and 155 drastically reduced viral antigenicity, which made these mutants unsuitable as vaccine candidates. However, changes at residues 119 and 186 did not affect virus antigenicity or immunogenicity, justifying their inclusion in live attenuated vaccine candidates to protect against the currently circulating 2009 swine origin H1N1 viruses.

Human infections with the swine origin influenza virus A (H1N1) were first detected in April 2009 and spread across the globe, resulting in WHO declaring a pandemic on 12 June 2009 for the first time in the past 41 years. More than 296,471 people have had confirmed infections with this novel H1N1 virus, and there have been at least 3,486 deaths as of September 18, 2009. In the last century, an influenza H1N1 virus caused the devastating 1918–1919 pandemic; this pandemic was characterized by a mild outbreak in the spring of 1918, followed by a lethal wave globally in the fall of that year which killed as many as 50 million people worldwide (20, 29). The 2009 H1N1 viruses circulating globally since April 2009 have not caused a significant rise in mortality related to influenza. Nucleotide sequence analysis suggested that E627 in PB2, a deletion of the PDZ ligand domain in NS1, and the lack of the PB1-F2 open reading frame in the 2009 H1N1 viruses may contribute to the relatively mild virulence (20, 26, 27). Recent animal studies have shown that the 2009 H1N1 influenza viruses did not replicate in tissues beyond the respiratory tract and did not cause significant mortality in the ferret model; however, the 2009 H1N1 viruses are capable of infecting deep in the lung tissues and caused more significant lesions in the lung tissues of animals, including nonhuman primates, than typical seasonal strains (13, 17, 19). Children and young adults are particularly susceptible to the 2009 H1N1 virus infection because they have no or low immunity to the novel 2009 H1N1 strains (11, 13). The widespread and rapid distribution of the 2009 H1N1 viruses in humans raises a concern about the evolution of more virulent strains during passage in the population. One fear is that mutant forms of the 2009 H1N1 viruses may exhibit significantly increased virulence (2, 19). Therefore, there is an urgent need to develop an effective vaccine to control the influenza pandemic caused by the swine origin H1N1 viruses.

Live attenuated influenza vaccine (LAIV) has been licensed in the United States annually since 2003. The seasonal vaccine protects against influenza illness and elicits both systemic and mucosal immune responses, including serum hemagglutination inhibition (HAI) antibodies that react to antigenically drifted strains (3, 4). A critical attribute of an effective pandemic vaccine is its capability to elicit an immune response in immunonaive individuals; LAIV has been shown to offer protection following a single dose in young children. However, two doses of vaccines are recommended for children younger than 9 years of age who have never been immunized with influenza vaccines. In order to produce LAIV to protect against the newly emerged swine origin H1N1 influenza virus, we have produced several 6:2 reassortant candidate vaccine strains that express the hemagglutinin (HA) and neuraminidase (NA) gene segments from influenza virus A/California/4/09 (A/CA/4/09) (H1N1) or A/CA/7/09 (H1N1), as well as the six internal protein gene segments (PB1, PB2, PA, NP, M, and NS) from cold-adapted A/Ann Arbor/6/60 (H2N2) (AA60) virus, which is the master donor virus for all influenza virus A strains in trivalent seasonal LAIV. Initial evaluation of these candidate vaccine strains indicated that they did not replicate as effi-

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TABLE 1. HA sequences and reassortant virus titers of novel H1N1 variants

WT virus (passage history)	6:2 Reassortant variant	Amino acid at indicated position[a] (H3 position)				% of clones with change(s)[c]	Titer[b] of reassortant in eggs (log_{10} FFU/ml ± SE)
		191 (194)	197 (200)	222 (225)	223 (226)		
A/CA/4/09 (MDCK, twice)	WT	L	T	D	Q	NA	NA[c]
	V1					100	Not rescued
A/CA/4/09 (egg, twice)	V2	I				42	7.4 ± 0.09
	V3				R	50	7.8 ± 0.15
	V7	I			R	8	7.8 ± 0.40
A/CA/7/09 (egg, twice)	WT	L	A	D/G	Q/R	NA	NA
	V4			D	Q	45	7.8 ± 0.27
	V5			G	Q	34	7.4 ± 0.14
	V6			D	R	21	7.7 ± 0.14

[a] Amino acid changes from the wild type A/CA/4/09 or A/CA/7/09 are shown.
[b] Virus titers are mean titers from at least three virus stocks.
[c] NA, not applicable.

ciently as seasonal H1N1 influenza vaccine strains in embryonated chicken eggs. In this report, we describe directed modifications of the HA gene segment that improved vaccine yields in eggs, resulting in a number of vaccine candidates that are available for human use.

MATERIALS AND METHODS

Generation of recombinant viruses. Wild-type (WT) influenza A (H1N1) viruses, A/CA/4/09 isolated from MDCK cells and A/CA/7/09 that was amplified in eggs, were received from the Centers for Disease Control and Prevention (CDC). Influenza virus A/CA/4/09 viral RNA isolated from egg-adapted virus was provided by Zhiping Ye of the U.S. Food and Drug Administration (FDA). The HA and NA gene segments of A/CA/4/09 and A/CA/7/09 were amplified by reverse transcription-PCR (RT-PCR) using primers that are universal to the HA and NA gene end sequences and cloned into pAD3000 vector (12). Site-directed mutagenesis to introduce specific changes into the HA gene was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the HA sequence was confirmed by sequencing analyses. The 6:2 reassortant candidate vaccine strains were generated by cotransfecting eight cDNA plasmids encoding the HA and NA of the H1N1 virus and the six internal gene segments of cold-adapted AA60 into cocultured 293T and MDCK cells. The reassortants used for vaccine manufacture were produced in serum-free Vero/chicken embryo kidney (CEK) cells by electroporation. Viruses were propagated in the allantoic cavities of 10- to 11-day-old embryonated chicken eggs (Charles River SPAFAS, Franklin, CT). The HA and NA sequences of the rescued 6:2 reassortant viruses were verified by sequencing RT-PCR cDNAs amplified from viral RNA.

Virus titration. Virus titers were measured by the fluorescence focus assay and expressed as log_{10} FFU (fluorescent focus units)/ml (8). Virus plaque morphology was examined by plaque assay as previously described (14).

Receptor binding assay. Chicken red blood cells (cRBCs) (HEMA Resource and Supply, Inc.) were desialylated and resialylated as previously described (21). cRBCs (100 μl at 10% cRBCs) were incubated with 50 mU *Vibrio cholerae* (Sigma, St. Louis, MO) at 37°C for 1 h to remove sialic acid (SA). After three washes with 1 ml phosphate-buffered saline (PBS), cells were resuspended in 1 ml PBS containing 1% bovine serum albumin and incubated with 2.5 mU of α2,3-N-sialyltransferase (Calbiochem, La Jolla, CA) or 2 mU of α2,6-N-sialyltransferase (Calbiochem, La Jolla, CA) plus 1.5 mM CMP-SA (cytidine-5′-monophospho-*N*-acetylneuramine acid; Sigma, St. Louis, MO) for 1.5 h at 37°C. The resialylated cRBCs were resuspended to 0.5% (vol/vol) in PBS after washing three times with PBS. Hemagglutination assays were carried out in V-bottomed 96-well microtiter plates for the binding activity. Fifty microliters of serially twofold-diluted viruses were incubated with 50 μl 0.5% cRBCs or α2,3-SA- or α2,6-SA-resialylated cRBCs at room temperature for 60 min. The hemagglutination titer was defined as the reciprocal of the highest dilution that agglutinated the RBCs.

Ferret studies. Eight- to 10-week-old male and female ferrets (n = 3/group) from Simonsen Laboratories (Gilroy, CA) were used to assess virus replication in the respiratory tract and to evaluate virus immunogenicity. Ferrets were housed individually and inoculated intranasally with 7.0 log_{10} FFU of virus per 0.2-ml dose. Three days after infection, ferrets were euthanized, and the lungs and nasal turbinates (NT) were harvested. Virus titers in the lungs and NT were determined by the 50% egg infectious dose (EID_{50}) assay and expressed as EID_{50} per gram of tissue (log_{10} EID_{50}/g). Ferrets that were assigned to immunogenicity studies were bled on days 14, 21, and 28 postinfection, and sera were assayed for antibody titers by HAI. Infection of ferrets with WT A/CA/7/09 was performed in the biosafety level 3 facility at the NIAID, NIH.

Serum antibody detection by HAI assay. The HAI assay was used to determine the levels of H1N1-specific antibody against homologous and heterologous viruses in ferret sera postinfection. Prior to serological analysis, ferret sera were treated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) that was reconstituted in 10 ml of 0.9% NaCl per vial. Serum (0.1 ml) was mixed with 0.15 ml receptor-destroying enzyme, incubated at 37°C for 18 h, and adjusted to a final 1:4 dilution by adding 0.15 ml of 0.9% sodium citrate, followed by incubation at 56°C for 45 min. Strain-specific serum HAI antibody titers were determined using 0.5% turkey RBCs (tRBCs), and the HAI titers are presented as the reciprocal value of the highest serum dilution that inhibited hemagglutination.

RESULTS

Generation of reassortant candidate vaccine strains. The HA and NA gene segments from WT A/CA/4/09 (MDCK isolate) were cloned from viral RNA following RT-PCR amplification. A total of 12 cDNA clones from each HA or NA segment were found to be identical by nucleotide sequence analysis. Plasmids representing HA and NA of WT A/CA/4/09 were transfected into cultured cells together with plasmids representing the six internal protein gene segments of cold-adapted AA60; however, viable reassortant progeny were not recovered on either MDCK cells or eggs inoculated with the transfected culture supernatants. The HA cDNAs cloned from WT A/CA/4/09 egg isolate were heterogeneous at amino acid residues 191 and 223 (Table 1). The corresponding H3 number based on the H3 HA 3D structure is also provided in the table. From 12 clones analyzed, three different variants were identified and named variant 2 (V2), V3, and V7. Forty-two percent of clones had the L191I change (V2), 50% had the Q223R change (V3), and one clone (8%) had both the L191I and Q223R change (V7). When plasmids representing these alternative HA sequences were combined with the NA of A/CA/4/09 and transfected into 293T/MDCK cells with the plasmids encoding the six internal protein gene segments of cold-adapted AA60, reassortant viruses were readily rescued.

The HA plasmid cloned from A/CA/7/09 that did not have amino acid changes at residue 222 or 223 could be rescued to

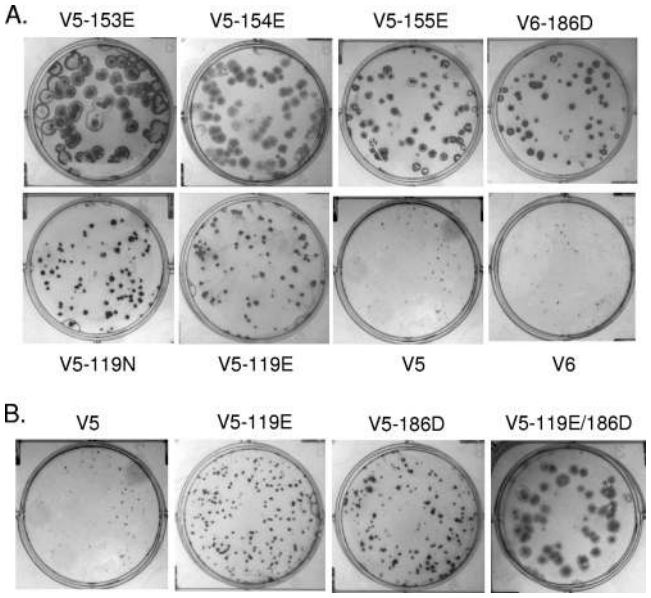

FIG. 1. (A) Plaque morphology of V5, V6, and MDCK-passaged variants containing introduced amino acid changes. Plaque assay was performed in MDCK cells incubated at 33°C for 4 days and immunostained with polyclonal antiserum against influenza A virus. (B) Plaque morphology of double mutant V5-119E/186D compared with that of single 119E and 186D mutants.

produce progeny virus (V4 in Table 1). This indicated that the T197A change in A/CA/7/09 was responsible for the efficient rescue of the 6:2 reassortant. Other A/CA/7/09 HA clones had an additional change of either D222G or Q223R, and viruses containing D222G (V5) or Q223R (V6) in HA were also rescued. Due to the high degree of similarity between the HAs of A/CA/4/09 and A/CA/7/09 and because the NA sequences were identical between these two strains, the variants derived from A/CA/7/09 were developed further.

Selection of candidate vaccine strains with more efficient growth in embryonated chicken eggs. As shown in Table 1, the rescued A/CA/4/09 and A/CA/7/09 reassortant viruses replicated in embryonated chicken eggs at titers of 7.4 to 7.8 $\log_{10}$ FFU/ml, at least 10-fold lower than typical seasonal H1N1 vaccine strains. Furthermore, the recovered A/CA/7/09 reassortants formed very small plaques (<1 mm) in MDCK cells. To improve virus growth in MDCK cells and eggs, A/CA/7/09 reassortants (V5 and V6) were passaged twice in MDCK cells at a multiplicity of infection of 4.0, 0.4, or 0.04, and then the viral supernatant was examined by plaque assay. All the MDCK-passaged viruses contained plaques that were much larger (2 to 4 mm) than those of the parental viruses (Fig. 1). The HA gene segments of 12 plaques from MDCK-passaged V5 and V6 reassortants were sequenced. Individual HA segments from isolates with large-plaque morphology exhibited single amino acid substitutions at one of the following positions: K119N or K119E (K119N/E), K153E, and K154E derived from V5 and A186D derived from V6.

The HA sequence of A/CA/7/09 was also compared with those of two earlier H1N1 viruses that originated from swine, A/swine/Iowa/1/1976 and A/swine/Iowa/1931; two recent human H1N1 viruses, A/South Dakota/6/07 (A/SD/6/07) and A/Brisbane/59/07; and the 1918 H1N1 A/South Carolina/1/18 virus. As shown in Fig. 2, K119, K153, and K154 are highly conserved among the swine H1N1 viruses and the 1918 H1N1 virus. A/SD/6/07 and A/Brisbane/59/07 also contain K119 and K154 residues. The amino acids at position 186 are more diverse among the six viruses shown in Fig. 2. A previous study (5) demonstrated that the G155E change was responsible for high rates of growth in eggs. The isolation of mutants with the K153E and K154E mutations indicates that a negatively charged E residue in the 153-to-155 region is preferred for replication of A/CA/7/09 virus in MDCK cells, as well as in eggs. To determine whether the G155E change previously reported for A/New Jersey/76 virus (5) also improved replication of A/CA/7/09 reassortant virus in eggs, G155E was introduced into V5 and V6 (V5- and V6-155E). The viruses containing mutations at positions 153, 154, and 155 indeed grew much better than V5 or V6 in eggs (Table 2). To confirm that the amino acids identified in the large-plaque isolates conferred a growth advantage in eggs, each of the identified mutations was introduced into the HA plasmid of A/CA/7/09 and reassortant viruses were rescued. Moreover, the A186D change identified in V6 was also introduced into V5 and V5-119E. Similarly, the 119N mutation found in V5 was introduced into V6 and V6-186D to evaluate the influence of single and double amino acid changes on virus replication in eggs. HA sequence analysis also revealed that A/CA/09 strains contained a unique glycosylation site at amino acid 278 (Fig. 2). To determine whether this additional glycosylation site altered virus growth, a T278K change was introduced into V5 and V6.

The rescued viruses were examined for their growth in eggs. As shown in Table 2, most of the variants containing the introduced HA mutations grew significantly better than V5 and V6. V5-278K and V6-278K had titers of 7.6 and 7.7 $\log_{10}$ FFU/ml, similar to the titers of V5 and V6. Thus, removal of the position 278 glycosylation site had minimal impact on virus replication levels in eggs. The changes at the position 119, 186, 153, 154, 155, and 186 sites increased virus titers by 0.2 to 1.2 $\log_{10}$ FFU/ml. The combined 119E and 186D change in V5 resulted in a slightly higher titer than either individual (119E or 186D) mutation.

Evaluation of reassortant variants for their antigenicity. To determine whether any of the amino acid changes introduced into the H1 HA affected virus antigenicity, A/CA/7/09 variants were assayed by HAI using ferret sera collected at various times postinoculation (Table 3). The cold-adapted viruses, A/CA/4/07 with the 223R residue (V3), A/CA/7/09 with the 222G residue (V5), and A/CA/7/09 with the 223R residue (V6), reacted similarly to the four ferret reference sera from animals immunized with WT A/CA/4/09 and cold-adapted A/CA/4/09 (V3), A/CA/7/09 (V5), and A/CA/07/09 (V6). The variants with amino acid changes introduced into V5 at positions 119 and 186 exhibited levels of antigenicity similar to that of V5; however, viruses with the differences at positions 153, 154, and 155 exhibited much lower reactivity with postinfection ferret serum, and a reduction in titer of more than fourfold was detected. Thus, the mutations at residues 153 to 154 should be excluded from the vaccine strain and the changes at positions 119 and 186 can be introduced into the vaccine strains without affecting virus antigenicity.

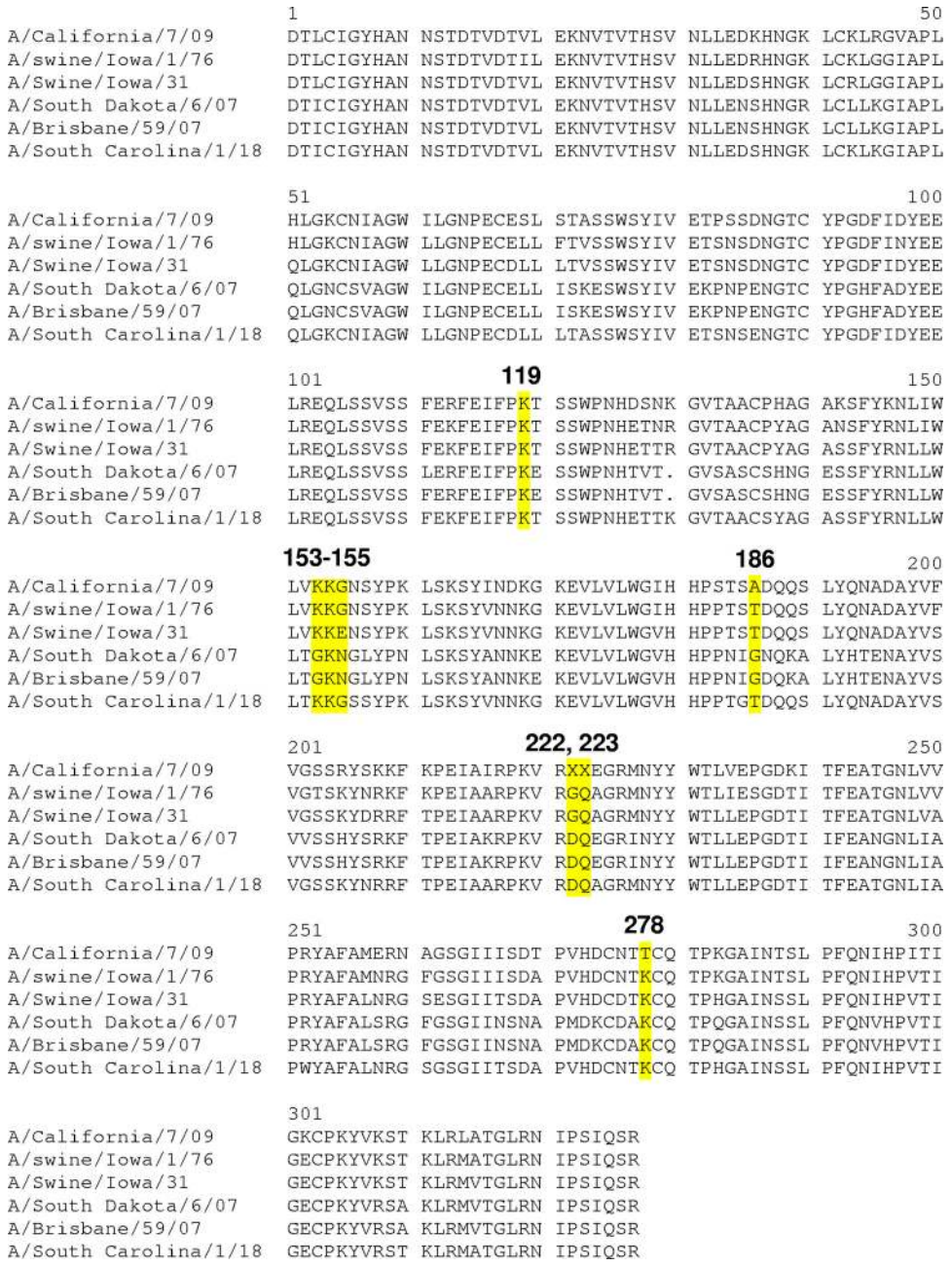

FIG. 2. HA1 protein sequence comparison of A/CA/7/09, A/swine/Iowa/1/76, A/swine/Iowa/31, A/SD/6/07, A/Brisbane/59/07, and A/South Carolina/1/18 (GenBank nucleotide sequence accession numbers FJ969540, CY022069, CY009628, EU516090, CY030230, and AF117241, respectively). The amino acids at positions 119, 153, 154, 155, 186, 222, 223, and 278 are highlighted in yellow.

A/CA/7/09 candidate vaccines are attenuated but immunogenic in ferrets. To evaluate A/CA/7/09 candidate vaccine variants for their attenuation phenotype and their ability to induce antibody responses, ferrets were inoculated intranasally with 7.0 $\log_{10}$ FFU of virus in a 0.2-ml dose, and titers of virus replication in the upper and lower respiratory tracts of ferrets on day 3 were determined by EID_{50} assay. As shown in Table 4, all A/CA/7/09 variants replicated efficiently in the NT tissues, but virus was not detected in the lungs. In contrast, WT virus replicated at high titers in both NT and lungs of ferrets. These data confirmed that these vaccine candidates are attenuated in ferrets, a phenotype that is conferred by the six internal protein gene segments of AA60.

Ferret serum was collected on day 14 after intranasal inoculation, and antibody titers were evaluated by HAI assay (Table 4). All V5 variants were very immunogenic and induced H1N1-specific antibody responses with HAI titers ranging from 256 to 1,024. Several V6 variants were also evaluated for their immunogenicity and were found to be less immunogenic than V5. Variants with the mutations at positions 119 and 186 maintained levels of antigenicity similar to that of the WT virus. Consistent with the antigenicity data presented in Table

TABLE 2. A/CA/7/09 HA variants identified from MDCK cells and introduced by reverse genetics

Virus	Amino acid at indicated position[a] (H3 position)								Virus titer[b] in eggs (log_{10} FFU/ml ± SE)
	119 (122)	153 (156)	154 (157)	155 (158)	186 (189)	222 (225)	223 (226)	278 (280)	
V5	K	K	K	G	A	G	Q	T	7.4 ± 0.14
V6						D	R		7.7 ± 0.14
V5-119N	N								8.3 ± 0.06
V5-119E	E								8.3 ± 0.07
V5-153E		E							8.3 ± 0.05
V5-154E			E						8.6 ± 0.05
V6-186D					D	D	R		8.4 ± 0.06
V5-186D					D				8.3 ± 0.15
V5-119E/186D	E				D				8.5 ± 0.06
V6-119N	N					D	R		7.9 ± 0.20
V6-119N/186D	N				D	D	R		8.1 ± 0.09
V5-155E				E					8.4 ± 0.11
V5-278K								K	7.6 ± 0.19
V6-155E				E		D	R		8.4 ± 0.19
V6-278K						D	R	K	7.7 ± 0.27

[a] Amino acid changes from V5 or V6 are shown.
[b] Virus titers are mean titers from at least three virus stocks.

3, sera from ferrets infected with the 154E and 155E variants did not react well with WT virus, V5, and V5 variants with the 119E and 186D changes. Interestingly, the 186D change resulted in a greater reduction in reactivity with 154E and 155E variants. These data demonstrate that the K119E and A186D substitution mutations in the HA of A/CA/07/09 (H1N1) conferred high rates of growth in eggs without altering virus antigenicity or immunogenicity, making them attractive as potential vaccine candidates for the swine origin 2009 H1N1 virus.

Receptor binding specificity of A/CA/7/09 variants. Growth of WT A/CA/4/09 and A/CA/7/09 viruses in eggs resulted in amino acid changes D222G and Q223R in the HA receptor binding site. These positions have been previously identified in other H1N1 strains following egg passage and are responsible for HA receptor binding specificity. To determine whether the variants have different receptor binding specificities, the V4, V5, V6, V5-119, and V5-186 variants were evaluated for their receptor binding specificities by the RBC binding assay (Table 5). V4 (without the residue 222 and 223 changes), representing the WT HA sequence, bound preferentially to α2,6-SA over α2,3-SA. V5 (D222G) bound to α2,3-SA- and α2,6-SA-resialylated RBCs equally well. However, V6 (Q223R) only bound α2,3-SA-resialylated cRBCs and lost its ability to bind to α2,6-SA, confirming that these two residues affect virus receptor binding specificity. The V5-119E/N and V5-186D viruses exhibited binding specificities similar to that of V5. The double HA mutant V5-119E/186D bound to α2,6-SA better than to α2,3-SA. The 119E and 186D residues introduced into V6 did not restore binding to α2,6-SA. Thus, the alterations at positions 119 and 186 do not significantly modify virus receptor binding specificity.

TABLE 3. Antigenicities of A/CA/7/09 HA variants

Infecting virus	Antibody titer[a] in serum of ferrets immunized with:			
	A/CA/4/09 WT	A/CA/4/09 ca 223R (V3)	A/CA/7/09 ca 222G (V5)	A/CA/7/09 ca 223R (V6)
CA04 V3	8,192	**1,024**	2,048	1,024
CA07 V5	8,192	1,024	**1,024**	1,024
CA07 V6	4,096	1,024	1,024	**512**
V5-119N	8,192	512	2,048	1,024
V5-119E	4,096	512	1,024	512
V5-153E	256	<32	128	64
V5-154E	512	<32	128	128
V5-155E	256	<32	128	128
V5-186D	8,192	1,024	2,048	1,024
V5-278K	4,096	512	1,024	512
V5-119E/186D	8,192	1,024	2,048	1,024

[a] HAI assay was performed with tRBCs. Each value is the reciprocal of the highest serum dilution that inhibited hemagglutination. Bold font indicates homologous. ca, cold adapted.

DISCUSSION

One of the most challenging tasks in producing a large quantity of swine origin 2009 H1N1 influenza vaccine is to produce a 6:2 reassortant vaccine seed capable of efficient replication in eggs, the substrate for current influenza vaccine manufacture, while retaining the appropriate antigenicity and immunogenicity. Using classical virology and reverse genetics technology, we were able to rapidly generate H1N1 LAIV vaccine candidates and improve their growth in eggs by approximately 10-fold. We identified two novel sites in the HA molecule that improved vaccine production in eggs without affecting vaccine antigenicity and immunogenicity. Such an approach should be applicable for future influenza vaccine development as well.

The receptor binding specificity of the HA and the activity of the NA are major determinants of the host range of influenza. Human and avian influenza viruses differ in receptor binding specificity. The HA of avian viruses preferentially binds to α2,3-linked sialyl-galactosyl moieties on the cell surface, whereas the HA of human viruses preferentially binds to α2,6-linked moieties. Moreover, the specific shape of the glycan can also influence the binding specificity (6). Receptor binding properties are also associated with viral transmission from animals to humans (1, 28). Egg adaptation of human influenza viruses increases their affinity for α2,3-SA (avian-like receptor) and concomitantly impairs their ability to bind to α2,6-SA

TABLE 4. Replication of A/CA/7/09 HA variants in the respiratory tract of ferrets and their immunogenicity[a]

Virus	Virus titer (log_{10} EID_{50}/g ± SE) in:		Geometric mean titer of HAI antibody to the indicated antigen						
	NT	Lung	V5	V5-119E	V5-119N	V5-186D	V5-119E/186D	V5-154E	V5-155E
V5	4.0 ± 0.23	<1.5	**512**	406	406	323	323	64	256
V5-119E	4.2 ± 0.33	<1.5	813	**645**	645	323	406	64	256
V5-119N	4.5 ± 0.33	<1.5	256	203	**256**	256	323	64	161
V5-186D	4.9 ± 0.20	<1.5	813	645	645	**645**	645	128	256
V5-119E/186D	4.7 ± 0.29	<1.5	512	406	512	512	**645**	64	256
V5-154E	5.7 ± 0.29	<1.5	161	323	323	64	81	**512**	1024
V5-155E	5.0 ± 0.20	<1.5	128	203	161	51	64	203	**1024**
A/CA/7/09 WT	7.8 ± 0.40	5.9 ± 0.30	512	512	645	645	406	102	323

[a] Ferret serum was collected on day 14 postinfection, and HAI assay was performed with tRBCs. Each value for the HAI assay is the reciprocal of the highest serum dilution that inhibited hemagglutination. Bold font indicates homologous.

(human-like receptor) (18). Single amino acid changes at positions 138, 163, 187, 189, 190, 194, 225, and 226 (H3 numbering) in the HA receptor binding region have previously been identified as being responsible for receptor binding preference to either human-like or avian-like receptor (18, 22–25). Egg adaptation of WT A/CA/4/09 and A/CA/7/09 selected virus variants with amino acid changes predominantly at position 222 (H3 position 225) or 223 (H3 position 226). The D225G change also resulted in virus binding to the α2,3-SA as found in the 1918 H1N1 A/New York/1/18 virus (10). The receptor binding assay employed here confirmed that the HA with the D222G change reduced the binding preference to α2,6-SA because of the loss of the critical contact of D222 with the base region of α2,6-SA, as predicted by the HA structure (28). The Q223R change resulted in complete loss of α2,6-SA binding ability. In addition, L191I (H3 position 194) in the HA of A/CA/4/09 and T197A (H3 position 200) in the HA of A/CA/7/09 also facilitated viral rescue and amplification in eggs. Residue L191 is located in the glycan-binding region and is conserved in the 2009 H1N1 and other human H1N1 influenza strains (27). Except for A/CA/4/09 that has T197, other swine, avian, and human virus H1 HAs all possess A197. Taking this feature into account, the failure to rescue A/CA/4/09 with T197 and the successful rescue of A/CA/7/09 with A197 without an additional change at residue 222 (H3 position 225) or 223 (H3 position 226) are both understandable.

Despite the presence of the egg adaptation residues (222G or 223R in the HA) that enabled the virus to be rescued and amplified in eggs, none of the 6:2 reassortant vaccine strains (V2 to V7) replicated to titers as high as observed for previously generated seasonal H1N1 human influenza vaccine strains, which can normally reach titers of 8.5 to 9.0 log_{10} FFU/ml in eggs. We confirmed that A/CA/7/09 H1 HA is indeed human adapted and binds with high affinity to α2,6-SA, even though it retains affinity to α2,3-SA better than other human H1N1 HAs (7, 27). The ability to bind to α2,3-SA may explain why the swine origin H1N1 viruses are capable of replicating in the intestine and can cause gastrointestinal symptoms. Interestingly, the 2009 H1N1 vaccine strains also replicate poorly in MDCK cells, which have both α2,6-SA and α2,3-SA. Very limited rounds of replication in MDCK cells selected viral variants that had single amino acid changes in the HA; all the variants showed much improved replication in both MDCK cells and eggs. Amino acid substitutions at HA residue 153, 154, or 155 involved a positively charged lysine residue that changed to a negatively charged glutamate residue. The negatively charged amino acid may have allowed the virus to contact the glycan better, as it is known that the K153 (H3 position 156) residue is positioned to contact the glycan (27). However, the changes at these three residues located at or near the antigenic and receptor binding sites significantly affected viral antigenicity, as previously shown for the H1N1 1976 swine virus (5, 16) and H3 viruses (15, 30). Therefore, the variants with mutations at positions 153, 154, and 155 could not be considered as vaccine candidates. However, we found that K119E/N combined with the A186D change had no impact on virus antigenicity. S183 (H3 position 186), T184 (H3 position 187), and A186 (H3 position 189) preceding the 190 helix are unique to the 2009 H1N1 strains; these residues are positioned to interact with D187 (H3 position 190) (9, 27). Although residue 119 is adjacent to the antigen binding region, the K119E/N change does not affect viral antigenicity. Therefore, the viruses with the 119E/N and/or 186D change in the HA were suitable as candidate vaccine strains. It is not known how the amino acid changes at residues 119 and 186 improve the growth of 2009 H1N1 virus. We speculate that these mutations may improve HA membrane association and facilitate HA incorporation into virions, in addition to some alterations in receptor binding properties of the HA. Further experimental data are needed to prove this speculation.

Oligosaccharide attachment provides a mechanism for evading immune responses by covering antibody binding regions. Comparison of 2009 H1N1 viruses with seasonal human H1N1

TABLE 5. Receptor binding specificities of A/CA/7/09 vaccine variants[a]

Virus	Hemagglutination titer in RBCs with indicated treatment			
	Untreated	α2,3-SA resialylated	α2,6-SA resialylated	Desialylated
V4	128	16	128	<2
V5	512	64	64	<2
V6	512	512	<2	<2
V5-119N	512	64	64	<2
V5-119E	512	128	128	<2
V5-186D	512	32	64	<2
V5-119E/186D	512	16	64	<2

[a] Each value is the reciprocal of the highest serum dilution that inhibited hemagglutination.

influenza viruses showed that the A/CA/09 viruses lost glycosylation sites at residues 127 and 160 but gained a glycosylation site at residue 278. Both position 127 and 160 are near the receptor binding and antibody binding regions, and the A/swine/Iowa/31 and A/swine/Iowa/76 (H1N1) strains also lack these two glycosylation sites. The loss of these two glycosylation sites might have contributed to the antigenic differences between swine and human influenza viruses. Our data indicate that acquisition of the position 278 glycosylation site in the HA of the A/CA/7/09 strain does not affect viral growth and antigenicity because this glycosylation site is not in the critical antibody and receptor binding site.

In summary, using classical virology techniques and reverse genetics, we were able to produce 2009 H1N1 influenza vaccine candidates that replicate efficiently in eggs to meet the vaccine demand. In addition, we have demonstrated that the candidate vaccines with amino acid changes at residue 119 and/or 186 maintained the cold-adapted, temperature-sensitive (data not shown), and attenuated phenotypes that are conferred by the six internal protein gene segments of cold-adapted AA60. These vaccine strains are antigenically matched to the WT H1N1 viruses and are very immunogenic in ferrets, making them appropriate vaccine strains aimed at protecting against the swine origin 2009 H1N1 influenza viruses. A/CA/7/09 V5-119E/186D has been used to manufacture the 2009 H1N1 LAIV for human use.

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REFERENCES

1. **Aytay, S., and I. T. Schulze.** 1991. Single amino acid substitutions in the hemagglutinin can alter the host range and receptor binding properties of H1 strains of influenza A virus. J. Virol. **65:**3022–3028.
2. **Belshe, R. B.** 2005. The origins of pandemic influenza—lessons from the 1918 virus. N. Engl. J. Med. **353:**2209–2211.
3. **Belshe, R. B., C. S. Ambrose, and T. Yi.** 2008. Safety and efficacy of live attenuated influenza vaccine in children 2–7 years of age. Vaccine **26**(Suppl. 4)**:**D10–D16.
4. **Belshe, R. B., K. M. Edwards, T. Vesikari, S. V. Black, R. E. Walker, M. Hultquist, G. Kemble, and E. M. Connor.** 2007. Live attenuated versus inactivated influenza vaccine in infants and young children. N. Engl. J. Med. **356:**685–696.
5. **Both, G. W., C. H. Shi, and E. D. Kilbourne.** 1983. Hemagglutinin of swine influenza virus: a single amino acid change pleiotropically affects viral antigenicity and replication. Proc. Natl. Acad. Sci. USA **80:**6996–7000.
6. **Chandrasekaran, A., A. Srinivasan, R. Raman, K. Viswanathan, S. Raguram, T. M. Tumpey, V. Sasisekharan, and R. Sasisekharan.** 2008. Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin. Nat. Biotechnol. **26:**107–113.
7. **Childs, R. A., A. S. Palma, S. Wharton, T. Matrosovich, Y. Liu, W. Chai, M. A. Campanero-Rhodes, Y. Zhang, M. Eickmann, M. Kiso, A. Hay, M. Matrosovich, and T. Feizi.** 2009. Receptor-binding specificity of pandemic influenza A (H1N1) 2009 virus determined by carbohydrate microarray. Nat. Biotechnol. **27:**797–799.
8. **Forrest, B. D., M. W. Pride, A. J. Dunning, M. R. Capeding, T. Chotpitayasunondh, J. S. Tam, R. Rappaport, J. H. Eldridge, and W. C. Gruber.** 2008. Correlation of cellular immune responses with protection against culture-confirmed influenza virus in young children. Clin. Vaccine Immunol. **15:** 1042–1053.
9. **Gambaryan, A. S., A. I. Karasin, A. B. Tuzikov, A. A. Chinarev, G. V. Pazynina, N. V. Bovin, M. N. Matrosovich, C. W. Olsen, and A. I. Klimov.** 2005. Receptor-binding properties of swine influenza viruses isolated and propagated in MDCK cells. Virus Res. **114:**15–22.
10. **Glaser, L., J. Stevens, D. Zamarin, I. A. Wilson, A. Garcia-Sastre, T. M. Tumpey, C. F. Basler, J. K. Taubenberger, and P. Palese.** 2005. A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J. Virol. **79:**11533–11536.
11. **Hancock, K., V. Veguilla, X. Lu, W. Zhong, E. N. Butler, H. Sun, F. Liu, L. Dong, J. R. Devos, P. M. Gargiullo, T. L. Brammer, N. J. Cox, T. M. Tumpey, and J. M. Katz.** 10 September 2009. Cross-reactive antibody responses to the 2009 pandemic H1N1 influenza virus. N. Engl. J. Med. [Epub ahead of print.] doi:10.1056/NEJMoa0906453.
12. **Hoffmann, E., G. Neumann, Y. Kawaoka, G. Hobom, and R. G. Webster.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. USA **97:**6108–6113.
13. **Itoh, Y., K. Shinya, M. Kiso, T. Watanabe, Y. Sakoda, M. Hatta, Y. Muramoto, D. Tamura, Y. Sakai-Tagawa, T. Noda, S. Sakabe, M. Imai, Y. Hatta, S. Watanabe, C. Li, S. Yamada, K. Fujii, S. Murakami, H. Imai, S. Kakugawa, M. Ito, R. Takano, K. Iwatsuki-Horimoto, M. Shimojima, T. Horimoto, H. Goto, K. Takahashi, A. Makino, H. Ishigaki, M. Nakayama, M. Okamatsu, D. Warshauer, P. A. Shult, R. Saito, H. Suzuki, Y. Furuta, M. Yamashita, K. Mitamura, K. Nakano, M. Nakamura, R. Brockman-Schneider, H. Mitamura, M. Yamazaki, N. Sugaya, M. Suresh, M. Ozawa, G. Neumann, J. Gern, H. Kida, K. Ogasawara, and Y. Kawaoka.** 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature **460:**1021–1025.
14. **Jin, H., B. Lu, H. Zhou, C. Ma, J. Zhao, C. F. Yang, G. Kemble, and H. Greenberg.** 2003. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. Virology **306:**18–24.
15. **Jin, H., H. Zhou, H. Liu, W. Chan, L. Adhikary, K. Mahmood, M. S. Lee, and G. Kemble.** 2005. Two residues in the hemagglutinin of A/Fujian/411/02-like influenza viruses are responsible for antigenic drift from A/Panama/2007/99. Virology **336:**113–119.
16. **Kilbourne, E. D., W. Gerhard, and C. W. Whitaker.** 1983. Monoclonal antibodies to the hemagglutinin Sa antigenic site of a/pr/8/34 influenza virus distinguish biologic mutants of swine influenza virus. Proc. Natl. Acad. Sci. USA **80:**6399–6402.
17. **Maines, T. R., A. Jayaraman, J. A. Belser, D. A. Wadford, C. Pappas, H. Zeng, K. M. Gustin, M. B. Pearce, K. Viswanathan, Z. H. Shriver, R. Raman, N. J. Cox, R. Sasisekharan, J. M. Katz, and T. M. Tumpey.** 2009. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science **325:**484–487.
18. **Matrosovich, M., A. Tuzikov, N. Bovin, A. Gambaryan, A. Klimov, M. R. Castrucci, I. Donatelli, and Y. Kawaoka.** 2000. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. J. Virol. **74:**8502–8512.
19. **Munster, V. J., E. de Wit, J. M. van den Brand, S. Herfst, E. J. Schrauwen, T. M. Bestebroer, D. van de Vijver, C. A. Boucher, M. Koopmans, G. F. Rimmelzwaan, T. Kuiken, A. D. Osterhaus, and R. A. Fouchier.** 2009. Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. Science **325:**481–483.
20. **Neumann, G., T. Noda, and Y. Kawaoka.** 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature **459:**931–939.
21. **Nobusawa, E., H. Ishihara, T. Morishita, K. Sato, and K. Nakajima.** 2000. Change in receptor-binding specificity of recent human influenza A viruses (H3N2): a single amino acid change in hemagglutinin altered its recognition of sialyloligosaccharides. Virology **278:**587–596.
22. **Robertson, J. S.** 1987. Sequence analysis of the haemagglutinin of A/Taiwan/1/86, a new variant of human influenza A(H1N1) virus. J. Gen. Virol. **68**(Pt. 4)**:**1205–1208.
23. **Robertson, J. S., J. S. Bootman, R. Newman, J. S. Oxford, R. S. Daniels, R. G. Webster, and G. C. Schild.** 1987. Structural changes in the haemagglutinin which accompany egg adaption of an influenza A (H1N1) virus. Virology **160:**31–37.
24. **Rogers, G. N., and J. C. Paulson.** 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology **127:**361–373.
25. **Rogers, G. N., J. C. Paulson, R. S. Daniels, J. J. Skehel, I. A. Wilson, and D. C. Wiley.** 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature **304:**76–78.
26. **Shinde, V., C. B. Bridges, T. M. Uyeki, B. Shu, A. Balish, X. Xu, S. Lindstrom, L. V. Gubareva, V. Deyde, R. J. Garten, M. Harris, S. Gerber, S. Vagasky, F. Smith, N. Pascoe, K. Martin, D. Dufficy, K. Ritger, C. Conover, P. Quinlisk, A. Klimov, J. S. Bresee, and L. Finelli.** 2009. Triple-reassortant swine influenza A (H1) in humans in the United States, 2005–2009. N. Engl. J. Med. **360:**2616–2625.
27. **Soundararajan, V., K. Tharakaraman, R. Raman, S. Raguram, Z. Shriver,**

V. Sasisekharan, and R. Sasisekharan. 2009. Extrapolating from sequence—the 2009 H1N1 "swine" influenza virus. Nat. Biotechnol. **27:**510–513.

28. **Srinivasan, A., K. Viswanathan, R. Raman, A. Chandrasekaran, S. Raguram, T. M. Tumpey, V. Sasisekharan, and R. Sasisekharan.** 2008. Quantitative biochemical rationale for differences in transmissibility of 1918 pandemic influenza A viruses. Proc. Natl. Acad. Sci. USA **105:**2800–2805.
29. **Tumpey, T. M., A. Garcia-Sastre, J. K. Taubenberger, P. Palese, D. E. Swayne, M. J. Pantin-Jackwood, S. Schultz-Cherry, A. Solorzano, N. Van Rooijen, J. M. Katz, and C. F. Basler.** 2005. Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J. Virol. **79:**14933–14944.
30. **Wiley, D. C., and J. J. Skehel.** 1987. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. **56:**365–394.