

## Rescue of an Influenza A Virus Wild-Type PB2 Gene and a Mutant Derivative Bearing a Site-Specific Temperature-Sensitive and Attenuating Mutation

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Live attenuated influenza A virus vaccines are currently produced by the transfer of attenuating genes from a donor virus to new epidemic variants of influenza A virus, with the selection of reassortant viruses that possess the protective antigens (i.e., the two surface glycoproteins) of the epidemic virus and the attenuating genes from the donor virus. The previously studied attenuated donor viruses were produced by conventional methods such as passage of virus at low temperature or chemical mutagenesis. The present paper describes a new strategy for the generation of a donor virus bearing an attenuating, non-surface-glycoprotein gene. This strategy involves the introduction of attenuating mutations into the cDNA copy of the PB2 polymerase gene by site-directed mutagenesis, transfection of *in vitro* RNA transcripts of PB2 cDNA, and recovery of the transfected PB2 gene into an infectious virus. An avian-human influenza A virus PB2 single-gene reassortant virus (with an avian influenza A virus PB2 gene) that replicates efficiently in avian tissue but poorly in mammalian cells was used as a helper virus to rescue a transfected synthetic RNA derived from a human influenza A virus PB2 gene. The desired human influenza A virus mutant PB2 transfectant was favored in this situation because the avian influenza A virus PB2 gene restricts viral replication in mammalian cells in culture, the system used for rescue, thereby providing strong selection for the virus bearing the human influenza A virus PB2 gene. We validated the feasibility of this approach by rescuing the PB2 gene of the wild-type influenza A/Ann Arbor/6/60 virus and a mutant derivative that had a single amino acid substitution introduced at position 265 by site-directed mutagenesis. Previously, this amino acid substitution had been shown to specify both a temperature-sensitive (*ts*) and an attenuation (*att*) phenotype. The rescued mutant 265 PB2 transfectant virus exhibited the *ts* and *att* phenotypes, which confirms that these phenotypes were specified by this single amino acid substitution. The transfectant virus was immunogenic and protected hamsters from subsequent challenge with wild-type virus. The cDNA copy of this influenza A/Ann Arbor/6/60 virus mutant 265 PB2 gene will be used as a substrate for the introduction of additional attenuating mutations by site-directed mutagenesis.

Protective immunity to influenza A virus in humans is directed primarily against the surface glycoproteins (the hemagglutinin [HA] and neuraminidase [NA] proteins) of the virus. Live attenuated influenza A vaccine viruses must be antigenically similar to newly emerged epidemic wild-type (wt) viruses with respect to these two proteins in order to be effective. A critical breakthrough in influenza A virus genetics was achieved with the recent demonstration that a synthetic HA or NA RNA transcribed from a cDNA copy of the gene can be rescued by a helper virus following transfection into infected cells in culture (10, 11). This important advance has made it possible to introduce site-specific mutations into a cDNA copy of an influenza A virus gene (5) and to study the effect of these mutations on virus virulence in animals (2). Mutations separately introduced into the regulatory or coding regions of the influenza A virus NA or HA gene have been shown to significantly attenuate the resulting mutant viruses for mice (14). However, since the HA and NA genes of a live, attenuated influenza A vaccine virus must be derived from the new epidemic or pandemic virus, the optimal sites for introducing attenuating mutations would appear to be non-surface-glycoprotein (or internal protein) genes that can subsequently be transferred by reassortment during mixed infection from

the vaccine donor virus to each new epidemic virus as it appears in nature. This process of reassortment is rapid and predictable and is currently being employed to generate experimental live, attenuated cold-adapted (ca) reassortant influenza A virus vaccines by transfer of attenuating genes from an attenuated ca donor virus (12). Therefore, in order to employ transfection-rescue technology to engineer attenuated influenza A vaccine viruses, it is necessary to develop a method for rescue of a nonglycoprotein (internal protein) gene bearing attenuating site-specific mutations. An influenza A virus wt nonstructural gene had been rescued previously by transfectant technology (6), but the rescue of an attenuated derivative of the nonstructural gene has not been described. We describe the development of a technique to rescue a wt as well as a mutant attenuating PB2 gene of influenza A virus.

We have previously described the isolation and phenotypic characteristics of an avian-human influenza A single-gene reassortant (SGR) virus that derives its PB2 gene from the avian influenza A/Mallard/New York/6750/78 virus and its remaining genes from the human influenza A/Los Angeles/2/87 (A/LA/2/87) (H3N2) virus (3). This PB2 SGR virus replicates efficiently in avian cells, but its growth in mammalian cells is highly restricted by the avian influenza A virus PB2 gene. We reasoned that this fortuitous host-range restriction could be exploited for use in a general method wherein the avian PB2 gene would be efficiently replaced by a human

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influenza PB2 gene synthesized *in vitro* from cDNA into which one or more attenuating mutations had been introduced by site-directed mutagenesis. This would make it possible to introduce, at will, a series of previously identified attenuating mutations into the PB2 gene and thereby bring genetic engineering to the construction of attenuated vaccine viruses. Progeny viruses selected in this manner are referred to as transfectant viruses and are similar to SGR viruses in that the PB2 gene is derived from cDNA and the remaining genes come from a wt virus such as the influenza A/LA/2/87 virus. We validated the feasibility of our PB2 transfection-rescue approach by using the PB2 gene of the influenza A/Ann Arbor/6/60 (A/AA/6/60) wt virus and its mutant equivalent present in the influenza A/AA/6/60 ca vaccine donor virus.

The influenza A/AA/6/60 ca virus, which is a promising candidate live attenuated influenza A donor virus, has three phenotypes, namely, temperature sensitivity (*ts*), cold adaptation (*ca*), and attenuation (*att*) for the respiratory tracts of ferrets or other experimental animals (12, 16). The PB1 and PB2 genes are independently responsible for the *ts* phenotype of the influenza A/AA/6/60 ca virus. The *ca* phenotype is specified by the PA gene while the *att* phenotype is specified independently by the PB1, PB2, and PA genes (16). The PB2 protein of the influenza A/AA/6/60 ca virus differs from the wt protein in only 1 amino acid, which is serine in place of asparagine at position 265 (4). This mutation is presumed to be responsible for the *ts* and *att* phenotypes specified by the A/AA/6/60 ca PB2 gene. By rescuing transfectant viruses bearing a synthetic equivalent of the influenza A/AA wt or a ca mutant PB2 gene, we were able to show that the single amino acid substitution at amino acid 265 indeed specified the *ts* and *att* phenotypes.

## MATERIALS AND METHODS

**Viruses.** The isolation and phenotypic characterization of the avian-human influenza A/LA/2/87 PB2 SGR virus has been described previously (3). The A/AA/6/60 wt virus (passage level 1PI) from which the cDNA was derived was kindly provided by H. F. Maassab, University of Michigan (8). The purified ribonucleoprotein (RNP) preparations were derived from the avian influenza A/Duck/Oklahoma/4/77 (H1N4) virus which was amplified in the allantoic cavities of 11-day-old embryonated eggs. The human influenza A wt A/LA/2/87 (H3N2) and A/Korea/82 (H3N2) viruses were described previously, as were three reassortant viruses, A/Korea/82 PB2 SGR and two 6-2 ca reassortant viruses which derived the HA and NA genes from the A/Korea/82 or A/LA/2/87 virus and the remaining six internal genes from the A/AA/6/60 ca virus (3, 16). The influenza B/AA/1/86 wt virus was also grown in the allantoic cavity of 11-day-old embryonated eggs.

**Cells.** Transfections were carried out in primary chick kidney cell monolayers prepared from kidneys of 3- to 5-day-old specific-pathogen-free chicks as described previously (6). Transfection harvests were plaqued onto Madin-Darby canine kidney (MDCK) cell monolayers (17). Virus titrations were carried out in MDCK cell cultures as previously described (13).

**Plasmids.** The PB2 gene of the A/AA/6/60 wt virus was amplified from virion RNA in two segments (nucleotides 1 to 1248 and 1219 to 2341) by reverse transcription with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) followed by the polymerase chain reaction (PCR). The nucleotide sequence of the insert was compared with the published sequence of the A/AA/6/60 wt gene and had no coding changes (4). Plasmid DNA was digested overnight

with *Hga*I and filled in with Klenow fragment before use as a template in transcription reactions.

**Site-directed mutagenesis.** The procedure outlined for the Muta-gene phagemid *in vitro* mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.) was followed. The insert from the plasmid pT3AAwtPB2 was cloned into pTZ18U and was used to transform DH5 $\alpha$  F' cells. The oligonucleotide designed for mutagenesis was as follows: nucleotide (nt) 812 5' CAGC CAGGAGCATAGTGA 3'. The nucleotide sequence of the recovered mutagenized A/AA/6/60 PB2 cDNA was confirmed to differ from the wt PB2 plasmid only at nt 821, where a G replaced an A to predict an amino acid substitution of serine for asparagine.

**RNP preparation.** Ribonucleoprotein (RNP) was prepared from virus purified from allantoic fluid harvested from 750 embryonated eggs inoculated with the avian influenza A/Duck/Oklahoma/4/77 virus as described previously (15).

**Transfection system.** Transfections were done with DEAE-dextran in a modification of the technique described previously by Luytjes et al. (11). Briefly, primary chick kidney cell monolayers in 35-mm Primaria (Becton Dickinson Co., Lincoln Park, N.J.) dishes were infected with the helper virus (PB2 SGR) at a multiplicity of infection of 30 to 100 for 1 h for the wt PB2 gene at 37°C or for 3 h at 32°C for the mutant PB2 gene. The transcription reaction mixture (50  $\mu$ l) contained 2.5  $\mu$ g of linearized and Klenow fragment-filled plasmid, 1 mM (each) deoxynucleoside triphosphates (Pharmacia Inc., Piscataway, N.J.), 0.1 M dithiothreitol, 150 U of T3 polymerase (Stratagene, La Jolla, Calif.), and 25 to 35  $\mu$ l of RNP and was incubated at 37°C for 55 min. Following the addition of DNase (Promega Corp., Madison, Wis.) and 100  $\mu$ l of phosphate-buffered saline with 1 mg of gelatin per ml to the transcription mix, the 150- $\mu$ l volume was transferred to the preinfected, DEAE-dextran-treated primary chick kidney cells and incubated for 1 h before being refed with 1 ml of minimal essential medium-4% bovine serum albumin (Quality Biological Inc., Gaithersburg, Md.). The cultures were maintained at 37°C for the wt gene and 32°C for the mutant gene and were harvested 22 h later. The cells were pelleted, and supernatant was plaqued onto MDCK monolayers. Plaque progeny underwent one additional plaque passage on MDCK cells and were then amplified in the allantoic cavities of embryonated eggs.

**Identification of the PB2 gene present in the transfection harvests.** Following proteinase K (Boehringer Mannheim, Indianapolis, Ind.) digestion, RNA was extracted from the allantoic fluid cultures of each plaque progeny by phenol-chloroform extraction. A 1-kb segment of the PB2 gene was amplified by PCR with a set of oligonucleotide primers designed for sequences conserved among the PB2 genes of A/AA/6/60, A/Duck/Oklahoma/4/77, and A/Mallard/New York/6750/78 (nt 1110 [positive sense] 5' GAGTTCACAAT GGTG 3' and nt 2000 [negative sense] 5' TTGTTGTAGT TGAATA 3'). Amplification of the gene segment from the A/Duck/Oklahoma/4/77 virus was inefficient. The PCR-amplified DNA was divided into four aliquots, and three of the aliquots were digested for 1 h at 37°C with *Bst*XI, *Ksp*632I, or *Hha*I. The uncut DNA and the digested samples were electrophoresed in a 1% agarose gel, and the patterns of migration were compared with the patterns of control PB2 DNA derived from the helper virus, the virus used to generate the RNP, and the plasmid. The pattern of digestion with the three enzymes was different for the three possible parental genes and allowed easy identification of the source of the PB2 gene in the putative transfectant viruses.

**Efficiency of plaque formation.** The efficiency of plaque formation was determined at 32, 38, 39, or 40°C in three

separate experiments with MDCK cells as described previously (19). Virus titers were expressed as mean log<sub>10</sub> PFU per milliliter for three experiments.

**Genotype analysis.** The parental origin of each gene segment in the transfectant viruses was determined by electrophoresis of purified virion RNA as described in the legend to Fig. 1A.

**Sequence analysis.** The PB2 gene was amplified in two separate reactions as overlapping segments from virion RNA by PCR (nt 1 to 1240 and 1141 to 2341) with avian myeloblastosis virus reverse transcriptase to synthesize the first strand and conventional methods of PCR amplification. The nucleotide sequences of the PCR products were determined with the CircumVent thermal cycler dideoxy DNA sequencing kit (New England Biolabs, Beverly, Mass.) with <sup>35</sup>S-dATP (Amersham Corp., Arlington Heights, Ill.). The entire length of the gene was sequenced with 13 oligonucleotide primers that were synthesized in an Applied Biosystems model 380A DNA synthesizer.

**Levels of replication and protective efficacy of viruses in hamsters.** The level of virus replication in the respiratory tract of hamsters and the abilities of the A/LA/2/87 AA wt and mutant (mt) 265 PB2 transfectant viruses to induce an antibody response and protect animals from challenge were compared as described in the footnotes to Tables 2 and 3. Titers of hemagglutination-inhibiting antibodies in sera were measured against the A/LA/2/87 wt virus as previously described (3).

**RESULTS**

**Rescue of the AA wt and mutant PB2 genes.** We first sought to rescue the A/AA/6/60 wt PB2 gene into the helper virus by transfecting a synthetic wt PB2 virion RNA into primary chick kidney cells infected with the helper virus and characterizing progeny viruses that produced plaques in MDCK cell monolayer cultures. A virus present in the transfection harvest that produces plaques on MDCK cells could have received its PB2 gene from the PB2 SGR helper virus (i.e., a host-range mutant PB2 gene), from residual PB2 gene RNA present in the RNP preparation, or from RNA transcribed from the PB2 cDNA (the desired gene). Of 23 plaques from the AA wt PB2 transfection, 2 (9%) derived their PB2 gene from the pT3AAwtPB2 plasmid, 11 (48%) derived it from the helper virus, and 10 (43%) derived it from the virus from which the RNP was purified. The two plaques, designated A/LA/2/87 AA wt PB2 clones 12A3 and 19A1, that represented AA wt PB2 transfectant viruses, were further plaque purified and genotyped (Fig. 1A), and their nucleotide sequence was determined and compared with that of the plasmid. Analysis of the

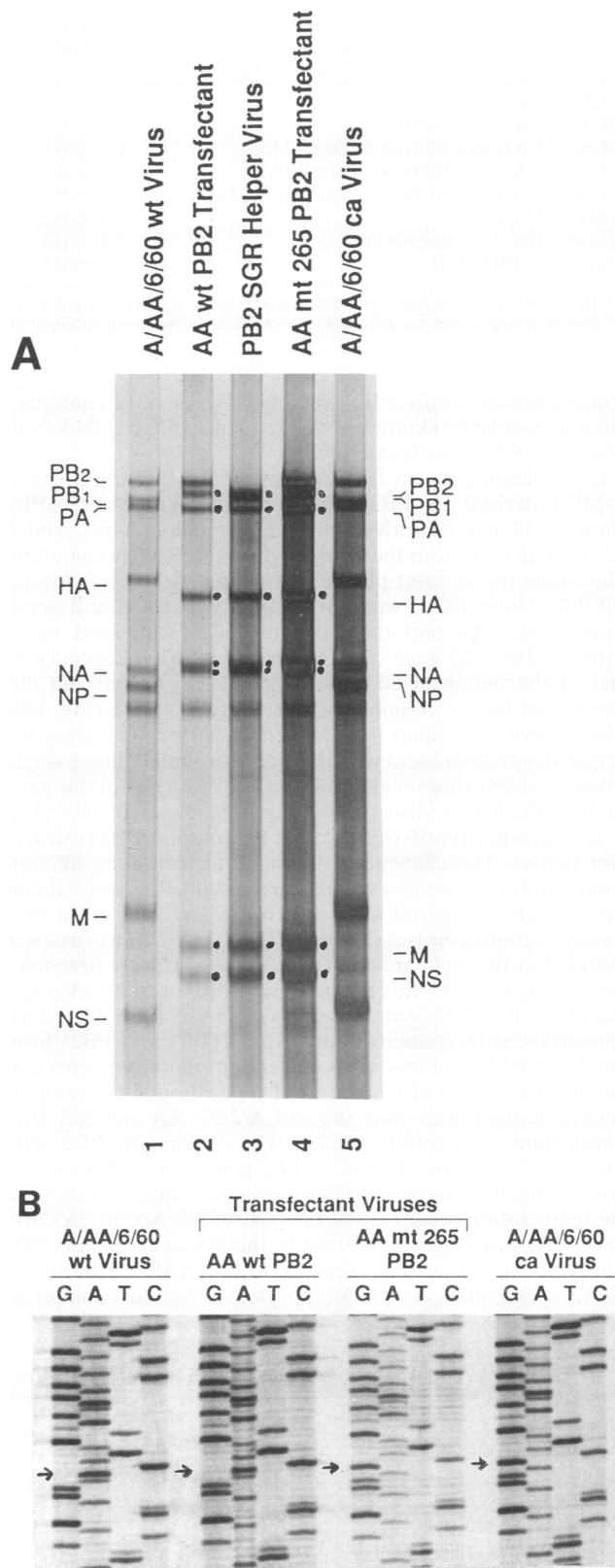


FIG. 1. (A) Genotyping gel showing the origin of the RNA gene segments of the A/LA/2/87 AA wt PB2 (lane 2) and A/LA/2/87 AA mt 265 PB2 (lane 4) transfectant viruses flanked by the virion RNAs of the A/AA/6/60 wt (lane 1) and A/AA/6/60 ca (lane 5) viruses and the helper virus (lane 3). The 16-cm gels contained 2.6% acrylamide and 6.0 M urea. Electrophoresis was carried out for 16 h at 0°C and 100 V constant voltage. Viral gene segments were visualized after ammoniacal silver staining (1, 6). The gene assignments on the left indicate positions of the A/AA/6/60 virus genes, and those on the right indicate positions of the A/Mallard/New York/6750/78 × A/LA/2/87 PB2 SGR helper virus genes. Dots indicate the A/LA/2/87 genes present in the helper and transfectant viruses. (B) Nucleotide sequence of the AA PB2 gene across nt 805 to 849. Arrows indicate nt 821, which is an A in the A/AA/6/60 wt virus and a G in the A/AA/6/60 ca virus. The transfectant viruses show identical sequences as the respective parent viruses.

TABLE 1. Comparison of the *ts* phenotypes of parent, reassortant, and transfectant viruses

Virus tested	Virus titer at 32°C <sup>a</sup>	Log <sub>10</sub> reduction in PFU/ml at indicated temp compared with permissive temp (32°C) <sup>a</sup>			Shutoff temp (°C) <sup>b</sup>
		38°C	39°C	40°C	
A/LA/2/87 wt	7.4 ± 0.1	0 ± 0.1	0.0 ± 0.1	0 ± 0.1	≥41
A/LA 2/87 6-2 ca reassortant	7.2 ± 0.3	0.7 ± 0.7	5.1 ± 1.7	6.5 ± 0.3	39
A/LA/2/87 AA wt PB2 transfectant 12A3	6.2 ± 0.1	0.0 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	≥41
A/LA/2/87 AA wt PB2 transfectant 19A1	5.9 ± 0.2	0.2 ± 0.1	0.5 ± 0.1	1.2 ± 0.5	≥41
A/LA/2/87 AA mt 265 PB2 transfectant 10A1	5.9 ± 0.2	0.4 ± 0.1	1.3 ± 0.2	4.9 ± 0.6	40
A/Korea/82 wt	6.8 ± 0.2	0.0 ± 0.2	0.2 ± 0.2	0.5 ± 0.3	≥41
A/Korea/82 6-2 ca reassortant	6.4 ± 0.2	1.7 ± 0.6	4.8 ± 1.1	5.7 ± 0.2	39
A/Korea/82 PB2 SGR	6.6 ± 0.3	1.0 ± 0.6	5.2 ± 0.7	5.9 ± 0.3	39

<sup>a</sup> Titers and titer reductions are expressed as mean log<sub>10</sub> PFU per milliliter ± standard error from three experiments.

<sup>b</sup> Shutoff temperatures are defined as the lowest restrictive temperature at which there is a ≥2-log<sub>10</sub> reduction in titer from the titer at the permissive temperature.

sequence of the entire PB2 gene of the virus 12A3 confirmed the sequence to be identical to the sequence of the cDNA from which the RNA was transcribed.

Of 24 plaque progeny from the AA mt 265 PB2 transfection, 1 (4%) derived its PB2 gene from the pT3AAmt265PB2 plasmid, 17 (71%) derived it from the helper virus, and 6 (25%) derived it from the virus used for the RNP preparation. The single transfectant plaque, designated A/LA/2/87 AA mt 265 PB2 clone 10A1, was further plaque purified and genotyped (Fig. 1A), and the PB2 gene was sequenced in its entirety. The PB2 gene of 10A1 was identical in sequence to that of the mutagenized pT3AAmt265PB2 plasmid and differed from the wt plasmid sequence only at nt 821 (Fig. 1B). The number of plaques that derived their PB2 gene from the helper virus is consistent with our previous finding that a single amino acid substitution at amino acid position 627 in this gene enabled the helper virus to replicate in mammalian cells (18).

**Temperature sensitivity of AA wt and mutant PB2 transfectant viruses.** The efficiencies of plaque formation at different temperatures of the two wt and one mutant PB2 transfectant viruses were compared with each other and with those of a variety of control viruses (Table 1). The A/LA/2/87 wt virus was included in the comparison because it most closely resembles the genotypes of the three transfectant viruses, being identical except in the PB2 gene (Fig. 1A). The A/LA/2/87 6-2 ca reassortant virus contains both *ts* genes (PB1 and PB2) from the A/AA/6/60 ca donor virus and should therefore represent the maximum level of restriction of replication at the nonpermissive temperature that the A/LA/2/87 AA mt 265 PB2 transfectant virus could manifest. The A/Korea/82 PB2 SGR virus, which contains the AA ca PB2 gene in a background of genes from an influenza A H3N2 virus, was included because the temperature sensitivity of the A/LA/2/87 AA mt 265 PB2 transfectant virus, also conferred by the AA ca-equivalent PB2 gene (identical in coding sequence to the ca PB2 gene) in an H3N2 background, would be expected to be most similar to

that of the A/Korea/82 PB2 SGR virus. As described previously (12), the A/LA/2/87 and A/Korea/82 wt viruses replicated to similar titers at permissive and restrictive temperatures (Table 1). The 6-2 gene ca reassortant viruses that derive their HA and NA genes from the wt viruses A/LA/2/87 or A/Korea/82 and six internal genes from the A/AA/6/60 ca donor virus showed a marked restriction in replication at 39°C. The A/LA/2/87 AA wt PB2 transfectant viruses behaved like the wt viruses, i.e., with no difference in level of replication at 40°C, whereas the A/LA/2/87 AA mt 265 PB2 transfectant virus was markedly restricted at 40 but not at 39°C. The A/LA/2/87 and A/Korea/82 ca reassortant viruses each derive two genes from the A/AA/6/60 ca virus that independently confer the *ts* phenotype (namely, the PB1 and PB2 genes) while the A/LA/2/87 AA mt 265 PB2 transfectant virus has only the mutant ca-equivalent PB2 gene, and this could explain the observation that the shutoff temperature for the A/LA/2/87 AA mt 265 PB2 transfectant virus was 1°C higher than that of the 6-2 ca reassortant viruses. The A/LA/2/87 AA mt 265 PB2 transfectant virus was also less restricted than the A/Korea/82 PB2 SGR at 39°C for reasons that are not understood.

**Level of replication in the respiratory tracts of hamsters.** The levels of replication of one of the A/LA/2/87 AA wt PB2 transfectant viruses and the A/LA/2/87 AA mt 265 PB2 transfectant virus in the upper and lower respiratory tracts of hamsters were compared (Table 2). These two viruses differed in sequence by only one amino acid, and thus the effect of the single mutation on replication *in vivo* could be directly assessed. Because the A/LA/2/87 AA mt 265 PB2 transfectant virus had a shutoff temperature of 40°C, and the body temperature of hamsters is 38.7 ± 0.3°C (7), we predicted, at best, a mild to moderate degree of attenuation of this virus for hamsters. As expected, the A/LA/2/87 AA mt 265 PB2 transfectant virus showed a modest restriction of replication compared with the A/LA/2/87 AA wt PB2 transfectant virus (Table 2), and both transfectant viruses were less restricted in repli-

TABLE 2. The A/LA/2/87 AA mt 265 PB2 transfectant virus is attenuated for the upper and lower respiratory tracts of hamsters<sup>a</sup>

A/LA/2/87 virus administered	No. of animals tested/day	Mean peak titer (log <sub>10</sub> TCID <sub>50</sub> /g of virus present in indicated tissue)					
		Nasal turbinates			Lungs		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
AA wt PB2 transfectant	6	4.8 ± 0.2	5.9 ± 0.1	5.4 ± 0.3	3.6 ± 0.7	3.6 ± 0.7	4.4 ± 0.6
AA mt 265 PB2 transfectant 12A3	6	4.2 ± 0.3	5.1 ± 0.2	4.9 ± 0.1	2.3 ± 0.4	2.7 ± 0.5	3.1 ± 0.6

<sup>a</sup> Anesthetized 5-week-old female Golden Syrian hamsters received 10<sup>5.0</sup> 50% tissue culture infective doses (TCID<sub>50</sub>) of virus in 0.1 ml intranasally. A 5% (wt/vol) suspension of nasal turbinates and a 10% (wt/vol) suspension of lung tissue were made, and the virus titer in each was determined in MDCK tissue culture. The titers are reported as mean log<sub>10</sub> TCID<sub>50</sub> per gram ± standard error.

TABLE 3. The A/LA/2/87 AA mt 265 PB2 transfectant virus is immunogenic and protects hamsters from wt virus challenge<sup>a</sup>

Immunizing virus	No. of animals tested	Serum HAI antibody titer at day 28 <sup>b</sup>	Mean titer (log <sub>10</sub> TCID <sub>50</sub> /g) of virus present in indicated tissue <sup>c</sup>	
			Nasal turbinates	Lungs
B/AA/1/86	7	≤3.0 ± 0.2	6.0 ± 0.1	3.7 ± 0.7
A/LA/2/87 AA wt PB2 transfectant 12A3	6	7.6 ± 0.2	2.6 ± 0.6	≤1.5 ± 0
A/LA/2/87 AA mt 265 PB2 transfectant	6	6.8 ± 0.2	2.7 ± 0.5	1.6 ± 0.2

<sup>a</sup> Anesthetized 5-week-old female Golden Syrian hamsters were immunized with 10<sup>5.0</sup> TCID<sub>50</sub> of virus in 0.1 ml intranasally and were challenged 31 days later with 10<sup>5.0</sup> TCID<sub>50</sub> of A/LA/2/87 wt virus in 0.1 ml intranasally. One day later, at the expected peak of replication of the A/LA/2/87 wt virus, the hamsters were sacrificed, a 5% (wt/vol) suspension of nasal turbinates and a 10% (wt/vol) suspension of lung tissue were made, and the virus titer in each was determined in MDCK tissue culture.

<sup>b</sup> Serum hemagglutination-inhibiting (HAI) antibody titer measured against A/LA/2/87 wt virus and expressed as mean log<sub>2</sub> titer ± standard error.

<sup>c</sup> Titers are expressed as mean log<sub>10</sub> TCID<sub>50</sub> per gram ± standard error.

cation than the 6-2 gene ca reassortant viruses evaluated previously (12). The differences in virus titers achieved over the 3 days postinoculation in the lungs ( $P = 0.02$ ) and in the nasal turbinates ( $P = 0.002$ ) of the 18 hamsters that received the A/LA/2/87 AA mt 265 PB2 and A/LA/2/87 AA wt PB2 transfectant viruses were highly significant (Student's  $t$  test). This indicated that the PB2 gene bearing a single mutation at amino acid position 265 specified the attenuation phenotype in hamsters. Despite its restricted replication in the lungs of hamsters, the A/LA/2/87 AA mt 265 PB2 transfectant virus was immunogenic and induced significant resistance to subsequent challenge with the A/LA/2/87 wt virus (Table 3).

## DISCUSSION

The strategy that we describe for the rescue of transfectant influenza A viruses with a synthetic PB2 gene has two important implications. First, we have demonstrated that the avian influenza A PB2 SGR virus (with all other genes derived from a human influenza A virus) can be used as a helper virus to rescue a synthetic human influenza A virus PB2 gene bearing a site-specific attenuating mutation. Thus, it should be possible to generate an engineered mutant PB2 gene containing additional attenuating mutations that specify the desired balance between attenuation and immunogenicity and the desired genetic stability. Mutations in addition to the one identical to that present in the AA ca PB2 gene will be required to generate a genetically stable vaccine virus because  $ts$  mutants, even those with two  $ts$  mutations, have been shown to undergo genetic alteration following replication in vivo, resulting in the emergence of a  $ts^+$  virulent virus (19). We have recently identified 10 separate amino acid substitutions that independently specify the  $ts$  phenotype for the PB2 gene of influenza A viruses (9). The introduction of one or more of these mutations into the AA mt 265 PB2 gene described here should yield a gene that specifies a greater degree of attenuation and a greater stability of the  $ts$  phenotype following virus replication in a fully susceptible host. Alternatively, or additionally, mutations known to be associated with attenuation in vivo could be introduced into the 3' and 5' noncoding regions of the PB2 gene (14). It should also be possible to introduce attenuating mutations into internal genes other than the PB2 gene with a similar transfection-rescue-selection technology. For example, a  $ts$  mutant virus can be used as a helper virus to rescue a wt gene, as has been reported with the nonstructural gene of an influenza A virus (6). The limitation of using  $ts$  helper viruses is that the rescue of attenuating genes will be restricted to mutant genes that have the  $ts^+$  phenotype, such as genes that bear non- $ts$  attenuating mutations or mutations in the regulatory sequences. These genes could then be used in conjunction

with an attenuating PB2 gene in an attenuated donor virus. Both attenuating genes would be transferred by reassortment from such a donor virus to newly emerged antigenic variants of influenza A virus as they appear in nature. It is important to emphasize that the transfection-rescue system is currently inefficient, with only a minority of progeny bearing the synthetic gene. It is possible that as more mutations are inserted into the PB2 gene, it may become more defective and the rescue system could become even more inefficient or incapable of rescuing such a defective gene. Studies are currently under way to increase the genetic stability of the helper virus so that it is less likely to lose its host-range restriction phenotype and thereby increase the efficiency of the transfection-rescue system.

The second important finding reported in this paper is the confirmation that the single amino acid difference between the A/AA/6/60 wt and ca PB2 genes indeed confers the  $ts$  and  $att$  phenotypes. Previous studies using an A/Korea/82 AA ca PB2 SGR virus had demonstrated that the PB2 gene segment of the A/AA/6/60 ca virus conferred  $ts$  and  $att$  phenotypes independently of the other genes (16), but the mutation(s) responsible for these phenotypes was not known. Sequence analysis revealed that the A/AA/6/60 ca PB2 gene differs from the wt gene at one nucleotide that results in a single amino acid substitution at position 265 (4). In addition, four other nucleotide differences that did not result in an amino acid substitution were present in the coding region. Our study clearly demonstrated that the single amino acid substitution at residue 265 conferred the  $att$  and  $ts$  phenotypes.

The two AA wt PB2 transfectant viruses were  $ts^+$  viruses similar to the A/LA/2/87 and A/Korea/82 wt viruses. In contrast, the A/LA/2/87 AA mt 265 PB2 transfectant virus exhibited an approximately 100,000-fold reduction in plaque titer at 40°C. The shutoff temperature of the A/LA/2/87 6-2 gene ca reassortant virus was lower than that of the A/LA/2/87 AA mt 265 PB2 transfectant virus, and this suggests that the A/AA/6/60 ca PB1 gene contributes significantly to the overall temperature sensitivity of the A/LA/2/87 6-2 gene ca reassortant virus. The observation that the A/Korea/82 ca PB2 SGR virus was more temperature sensitive than the A/LA/2/87 AA mt 265 PB2 transfectant virus was unexpected. There are three possible explanations for this finding. First, the gene constellation in which the A/LA/2/87 AA ca PB2 gene is present could influence the level of temperature sensitivity of the virus. Second, the four noncoding changes (from the A/AA wt to A/AA ca PB2 sequence) that were present in the PB2 gene of the A/Korea/82 PB2 SGR virus, but were not present in the plasmid from which the PB2 gene of the A/LA/2/87 AA mt 265 PB2 transfectant virus was derived, could contribute to the level of temperature sensitivity of the virus. The third possibility is that the PB2 gene (or other gene segments) of the

A/Korea/82 PB2 SGR virus sustained additional mutations in the process of generating the SGR virus and these new mutations contributed to the increased temperature sensitivity of the A/Korea/82 PB2 SGR virus. We cannot yet distinguish among these three possibilities but consider the first possibility to be the most likely to account for the different levels of temperature sensitivity of the A/Korea/82 PB2 SGR and A/LA/2/87 AA mt 265 PB2 transfectant viruses.

The presence of the single amino acid substitution in the PB2 gene of the A/LA/2/87 AA mt 265 PB2 transfectant virus also resulted in attenuation of the virus for the upper and lower respiratory tracts of hamsters, as evidenced by the reduction in the amount of virus produced over the 3 days tested, compared with that of the A/LA/2/87 AA wt PB2 transfectant virus. The mutation conferred only about a 10-fold reduction in replication in the lower respiratory tract, and thus it is clear that the single amino acid substitution present in the A/LA/2/87 AA mt 265 PB2 transfectant virus does not sufficiently attenuate the virus for this site. The AA mutant ca-equivalent PB2 gene can now serve as a substrate for the introduction of additional mutations to produce an attenuated influenza A donor virus that can confer, by gene reassortment, the desired balance between attenuation and immunogenicity, and genetic stability upon new variants of influenza A viruses as they appear in nature.

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