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Sequential Addition of Temperature-Sensitive Missense Mutations into the PB2 Gene of Influenza A Transfectant Viruses Can Effect an Increase in Temperature Sensitivity and Attenuation and Permits the Rational Design of a Genetically Engineered Live Influenza A Virus Vaccine

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We have previously described a strategy for the recovery of a synthetic influenza A virus wild-type (*wt*) PB2 gene (derived from influenza A/Ann Arbor/6/60 [AA] virus) into an infectious virus. It was possible to introduce an attenuating temperature-sensitive (*ts*) mutation at amino acid residue 265 of the AA *wt* PB2 gene and to rescue this mutant gene into infectious virus. Application of this new technology to influenza A virus vaccine development requires that multiple attenuating mutations be introduced to achieve a satisfactorily attenuated virus that retains the attenuation (*att*) phenotype following replication in vivo. In this report, we demonstrate that putative *ts* mutations at amino acids 112, 556, and 658 each indeed specify the *ts* and *att* phenotypes. Each of these mutations was introduced into a cDNA copy of the AA mutant mt265 PB2 gene to produce three double-mutant PB2 genes, each of which was rescued into an infectious virus. In general, the double-mutant PB2 transfectant viruses were more *ts* and attenuated in the lower respiratory tracts of hamsters than the single-mutant transfectant viruses, and the *ts* phenotype of two of three double-mutant PB2 transfectant viruses was stable even after prolonged replication in the upper respiratory tracts of immunocompromised mice. Two triple-mutant PB2 transfectant viruses with three predicted amino acid substitutions resulting from five nucleotide substitutions in the cDNA were then generated. The triple-mutant PB2 transfectant viruses were more *ts* and more attenuated than the double-mutant PB2 transfectant viruses. These results indicate that sequential introduction of additional *ts* mutations into the PB2 gene can yield mutants that exhibit a stepwise increase in temperature sensitivity and attenuation compared with the preceding mutant(s) in the series. Furthermore, the level of temperature sensitivity of the transfectant viruses correlated significantly with the level of attenuation of these viruses in hamsters. Although the triple-mutant PB2 transfectant viruses were attenuated in hamsters, intranasal administration of these viruses elicited a vigorous serum hemagglutination-inhibiting antibody response, and this was associated with resistance of the lower respiratory tract to subsequent *wt* virus challenge. These observations suggest the feasibility of using PB2 reverse genetics to generate a live influenza A virus vaccine donor strain that contains three attenuating mutations in one gene. It is predicted that reassortant viruses derived from such a donor virus would have the properties of attenuation, genetic stability, immunogenicity, and protective efficacy against challenge with *wt* virus.

A wide variety of conventional approaches have been used in attempts to develop a candidate live attenuated influenza A virus vaccine (14). Each of these approaches involves the selection or production of attenuating mutations in one or more genes of an influenza A virus that do not encode the two protective antigens of the virus (the hemagglutinin [HA] and neuraminidase [NA] glycoproteins). These attenuating genes are then transferred from the attenuated donor virus, by genetic reassortment, to new epidemic or pandemic influenza A virus variants that appear in nature. Reassortant viruses that bear the HA and NA genes of the wild-type (*wt*) virus and the attenuating gene(s) of the donor virus are then selected as the new candidate vaccine virus. Although many approaches for the production or identification of attenuating genes have been investigated, most have been unsuccessful because of incomplete attenuation of candidate vaccine viruses bearing these genes or instability of the attenuation (*att*) phenotype of satisfactorily attenuated strains following replication in vivo (14). Only one candidate vaccine developed by using these conventional techniques, the cold-adapted influenza A vaccine, still shows promise and is in an advanced stage of development as a vaccine for use in humans (11). The success of this vaccine likely stems from the presence of three attenuating gene segments in the donor strain (22). In the context of the many unsuccessful attempts at vaccine development, there is a need for a more rational approach to vaccine design that involves the sequential introduction of specific, defined attenuating mutations into a virus to produce a donor strain that is satisfactorily attenuated and that exhibits a stable *att* phenotype during replication in vivo. The present study describes one such approach.

By using the techniques of reverse genetics, a process by

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which a synthetic influenza A virus RNA gene that is transcribed in vitro from a cDNA copy of the gene is rescued into an infectious virus, it is possible to introduce site-specific mutations into selected gene segments (3, 4). Several studies have demonstrated that attenuating mutations can be introduced into the noncoding or coding regions of the HA and NA genes of influenza A viruses (1, 16), but such attenuating genes cannot be used to confer attenuation on new epidemic or pandemic viruses, since vaccine viruses must contain the HA and NA genes of the newly emerged antigenic variant to be useful in immunization.

We have previously reported the development of a successful strategy for the rescue of a synthetic influenza A virus *wt* gene that encodes an internal viral polymerase protein, PB2. This strategy was also applied successfully to the rescue of a mutant PB2 gene bearing a single site-specific mutation (at amino acid [aa] 265) that conferred the temperature-sensitive (*ts*) and *att* phenotypes upon the transfectant virus (25). We have been exploring the possibility of sequentially introducing additional mutations into the same gene segment in order to generate a single attenuating influenza A virus PB2 gene segment that might be used alone in a vaccine donor strain or in combination with another attenuating gene segment. The PB2 gene, and its associated *att* phenotype, could then be readily transferred from the attenuated donor virus to new antigenic variants of influenza A virus as they emerge (9, 15). The major assumption inherent in this approach was that the PB2 transfection rescue system would operate efficiently for selection of PB2 gene segments containing more than one *ts* mutation. A second assumption was that putative *ts* mutations identified by genetic and sequence analysis in a series of influenza A *ts* viruses (7, 10, 31) would confer the *ts* phenotype when introduced into the closely related but nonhomologous influenza A/Ann Arbor/6/60 (AA) virus PB2 gene. It was also unclear whether two or more mutations that independently conferred the *ts* phenotype on transfectant viruses would be additive and produce a virus that was more attenuated than viruses bearing only a single mutation. This study establishes the feasibility of the PB2 transfection rescue system for the rescue of double- and triple-mutant *ts* transfectant viruses and demonstrates that the sequential introduction of mutations into the PB2 gene can lead to a progressive increase in temperature sensitivity and attenuation.

MATERIALS AND METHODS

Viruses. The helper virus used in transfection experiments, as described previously, was a PB2 single-gene reassortant virus that derived its PB2 gene segment from the avian influenza A/Mallard/NY/78 virus and the remaining seven gene segments from the human influenza A/Los Angeles/2/87 (H3N2) (A/LA/2/87) virus. This single-gene reassortant virus is useful as a helper virus because of its host range restriction phenotype; i.e., the helper virus replicates efficiently in avian cell culture but replicates very poorly in mammalian cell culture (2, 25). Thus, the recovery of transfectant viruses bearing the PB2 gene of a human influenza A virus is favored in mammalian cell culture. The generation of the A/LA/2/87 AA *wt* PB2 transfectant virus and the A/LA/2/87 AA mutant mt265 PB2 transfectant virus by transfection rescue techniques has been described previously (25). The isolation and passage histories of the A/LA/2/87 *wt* virus which was used as a challenge virus and the influenza B/Ann Arbor/1/86 (B/AA/1/86) virus which was used as an immunologically irrelevant control virus for immunogenicity and challenge experiments have been described previously (2, 25).

Cells. Transfections were carried out in primary chicken kidney cell monolayers prepared from kidneys of 3- to 5-day-old specific-pathogen-free chicks as indicated previously (25). Transfection harvests were subsequently plaqued on Madin-Darby canine kidney (MDCK) cell monolayers. Virus titration for quantitation of virus (50% tissue culture infective dose [$TCID_{50}$]) produced in vitro or in vivo and the plaque assay for determining the effect of temperature on plaque formation and for cloning transfectant virus progeny were performed in MDCK cell monolayers as previously described (12).

Plasmids. pTZ18U plasmid DNA with the full-length PB2 gene from the AA virus (pT3AA*wt*PB2) generated as described previously (25) was used as the template for site-directed mutagenesis to generate the mt112, mt556, and mt658 PB2 plasmids, which each contained a single amino acid substitution at the indicated position of PB2. pTZ18U plasmid DNA containing the full-length PB2 gene from the AA virus bearing a single nucleotide substitution at position 821 (amino acid change at position 265) (pT3AAmt265) was generated as described previously (25) and was used as the template for site-directed mutagenesis to generate the double-mutant mt265+112, mt265+556, and mt265+658 PB2 plasmids. The mt265+556 and mt265+658 PB2 plasmids were then used as templates for further site-directed mutagenesis to generate the triple mutants mt265+112+556 and 265+112+658, respectively. Mutated PB2 plasmid DNA was digested overnight with *Hga*I (New England Biolabs, Beverly, Mass.) and filled in with Klenow fragment (New England Biolabs) before use as a template in transcription reactions as previously described (25).

Site-directed mutagenesis. The procedure outlined for the Muta-gene phagemid in vitro mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif.) was followed, using oligonucleotides designed to achieve the substitutions listed in Table 1.

Ribonucleoprotein preparation. Ribonucleoprotein was prepared from virus purified from 500 embryonated eggs inoculated with the avian influenza A/Duck/Oklahoma/4/77 virus as described previously (18, 25).

Transfection system. T3 polymerase ribonucleoprotein transcription reaction mixtures were incubated at 37°C for 55 min, treated with DNase for 5 min, and then transfected into DEAE-dextran-treated primary chicken kidney cells infected with the PB2 single-gene reassortant helper virus by a modification (25) of the technique described by Luytjes et al. (8). Transfection mixtures were incubated at 32°C overnight and were harvested 22 to 24 h later.

Generation and nucleotide sequence analysis of PB2 transfectant viruses. By using a method described previously, PB2 transfectant viruses that derived their PB2 gene from the plasmid were selected in MDCK cells, which are restrictive for replication of the PB2 single-gene reassortant helper virus, at 32°C (25). Plaque progeny, which underwent a total of three plaque-to-plaque passages on MDCK cell monolayers, were amplified first in MDCK cells and next in the allantoic cavity of embryonated eggs. The nucleotide sequence of the coding region of the PB2 gene of each transfectant virus was determined as previously described (25).

Efficiency of plaque formation. The efficiency of plaque formation was determined at 32, 36, 37, 38, 39, and 40°C in two to four separate experiments using MDCK cell monolayers as described previously (27). Virus titers were expressed as mean $\log_{10}$ PFU per milliliter.

Level of replication, immunogenicity, and protective efficacy of transfectant viruses in hamsters. The level of virus replication and the immunogenicity of the *wt* and mutant PB2 transfectant viruses were determined in female golden Syrian hamsters as indicated in the footnotes to Tables 3 and 4, respectively. Titers of hemagglutination-inhibiting antibodies in sera were measured against the A/LA/2/87 *wt* virus as described previously (17).

Level of genetic stability of double-mutant transfectant viruses after prolonged replication in nude mice. The A/LA/2/87 AA *wt* virus or a double-mutant PB2 transfectant virus derived from it was inoculated intranasally into 4- to 6-week-old female nude (*nu*/*nu*) BALB/c or nude (*nu*/*nu*) NIH Swiss mice ($10^{5.0}$ or $10^{6.0}$ $TCID_{50}$ in a 0.05- or 0.1-ml inoculum, respectively), and virus was allowed to replicate in these mice for 14 days. The AA mt265+112 PB2 transfectant virus was evaluated in 15 nude BALB/c and 100 nude NIH Swiss mice, while the other two double-mutant transfectant viruses were each evaluated in 120 nude NIH Swiss mice. Four additional nude BALB/c mice were infected with the A/LA/2/87 AA *wt* PB2 transfectant virus. The animals were sacrificed after 14 days, and lungs and nasal turbinates were removed and homogenized. Each specimen of lung and nasal turbinate suspension was cultured for virus, and the resulting isolates were then tested for their efficiencies of plaque formation at 32 and 39°C (for mt265+556 and mt265+658) or 39.5°C (for mt265+112) to determine the *ts* phenotype of virus isolated after prolonged replication in vivo. The choice of the nonpermissive temperature was based on the temperature at which the replication of the mutant PB2 transfectant virus was completely restricted. The *ts* phenotype of isolates that produced plaques at the restrictive temperature was evaluated further by characterization of plaque progeny (amplified at the permissive temperature) for efficiency of plaque formation at 32 or 39.5°C. Those isolates that produced plaques efficiently at restrictive temperature were considered to contain *ts*+ revertant virus.

RESULTS

Generation of single-, double-, and triple-mutant PB2 transfectant viruses. We previously described the rescue of the AA *wt* PB2 gene into infectious virus designated A/LA/2/87 AA *wt* PB2 transfectant virus. Similarly, a mutant derivative of the gene with a substitution mutation at aa 265 was rescued into infectious virus that was designated A/LA/2/87 AA mt265 PB2 transfectant virus (25). The mutation at aa 265 conferred the *ts* and *att* phenotypes on the transfectant virus, indicating that

TABLE 1. Nucleotide and predicted amino acid substitutions inserted by site-directed mutagenesis[a] into the AA PB2 gene rescued into transfectant viruses

A/LA/2/87 AA PB2 transfectant virus	Nucleotides and predicted amino acid change at indicated residue[b]								No. of plaques tested	No. of plaques with AA PB2 gene
	112		265		556		658			
	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid		
wt	CCA	Pro	AAC	Asn	AAC	Asn	TAC	Tyr	24	2
Single mutants										
mt112	TCA	Ser	—[c]	—	—	—	—	—	19	3
mt265	—	—	AGC	Ser	—	—	—	—	23	1
mt556	—	—	—	—	GAC	Asp	—	—	15	3
mt658	—	—	—	—	—	—	CAC	His	16	11
Double mutants										
mt265+112	TCA	Ser	AGC	Ser	—	—	—	—	8	8
mt265+556	—	—	AGC	Ser	GAC	Asp	—	—	31	2
mt265+658	—	—	AGC	Ser	—	—	CAC	His	7	7
Triple mutants[d]										
mt265+112+556	AGC	Ser	AGC	Ser	GAC	Asp	—	—	19	1
mt265+112+658	AGC	Ser	AGC	Ser	—	—	CAC	His	22	7

[a] Mutagenic oligonucleotide primers were designed to achieve the changes listed.
[b] The coding region of the PB2 gene in each selected and cloned transfectant virus was confirmed to differ from that of the *wt* AA PB2 gene only at the indicated sites (underlined).
[c] —, codon identical to that of the *wt* virus.
[d] Triple-mutant PB2 genes had five nucleotide substitutions encoding three *ts* mutations.

this mutation originally identified in the PB2 gene of the AA cold-adapted donor virus specified these phenotypes (25). We next selected three additional separate putative *ts* mutations for introduction into the AA *wt* PB2 gene (Table 1): (i) at aa 112, the site of a mutation that was present in three independently derived influenza A/Udorn/72 *ts* mutants; (ii) at aa 556, the site of a mutation that was present in another influenza A/Udorn/72 *ts* mutant in a region with homology to the cap-binding motifs of human and yeast proteins; and (iii) at aa 658 (in an influenza A/Great Lakes/65 virus PB2 gene), the site of a mutation that was present in the *ts*-1E mutant virus, which was previously evaluated as a vaccine candidate (7, 23). In this manner, it was possible to determine if *ts* mutations identified in a different influenza A virus (A/Udorn/72 or A/Great Lakes/65) could specify the *ts* phenotype when introduced by reverse genetics into a related but nonhomologous AA *wt* PB2 gene. Each of the mutant PB2 genes was successfully rescued into infectious virus; in each instance, viral progeny of several plaques were shown to possess a PB2 gene with the predicted amino acid mutation (Table 1). Progeny of a single positive plaque were selected from each group, and these clones were designated A/LA/2/87 AA mt112 clone 12D1, mt556 clone A, and mt658 clone 8B1, respectively.

Following demonstration that a mutation at aa 112, 556, or 658 specified the *ts* phenotype (Table 2), we sought to combine two mutations in the AA PB2 gene to determine whether the transfection rescue system would operate efficiently enough to rescue presumably more defective double-mutant viruses. Three different double-mutant PB2 transfectant viruses were successfully rescued, and in each instance, a single representative clone (designated A/LA/2/87 AA mt265+112 clone 6A1, mt265+556 clone 30A1, and mt265+658 clone 4A1) was selected for further study, plaque purified, and confirmed by nucleotide sequencing to differ in its sequence from the A/LA/2/87 AA *wt* PB2 gene only at the predicted amino acid residues 265 and 112, 556, or 658. In addition, we recovered two triple-mutant viruses that contained three amino acid substitutions, namely, aa 265, 112, and 556 and aa 265, 112, and 658, as a result of five nucleotide substitutions (Table 1). The observation that the PB2 reverse genetics system worked as efficiently for rescue of an AA double- or triple-mutant PB2 gene as for an AA single-mutant PB2 gene (Table 1) was encouraging because it clearly established the technical feasibility of inserting multiple *ts* mutations into the PB2 gene.

Sequential introduction of two or three *ts* mutations into the PB2 gene of transfectant viruses results in a progressive increase in temperature sensitivity. The efficiencies of plaque formation of the PB2 transfectant viruses with one, two, or three predicted amino acid substitutions in the PB2 gene were compared at different temperatures with that of the A/LA/2/87 AA *wt* PB2 transfectant virus (Table 2). The A/LA/2/87 AA mutant PB2 transfectant viruses bearing a single mutation showed a restriction of replication at 39 and 40°C that was greater than that of the AA *wt* PB2 transfectant virus. The finding that each of the three putative *ts* mutations (aa 112, 556, and 658) specified the *ts* phenotype confirmed that these amino acid substitutions were indeed *ts* mutations as had been shown previously for the aa 265 mutation. The substitution at aa 556 effected the greatest suppression of replication at an elevated temperature. The addition of a second mutation (at aa 112, 556, or 658) to the AA mt265 PB2 transfectant virus led to a restriction of replication at an elevated temperature greater than that conferred by a single *ts* mutation in one of the three instances, i.e., mt265+112. However, an increase in temperature sensitivity was clearly seen for the triple-mutant PB2 transfectant viruses, which displayed a shutoff temperature that was 1 degree lower than that of its parental double-mutant PB2 transfectant virus. Thus, the addition of two predicted amino acid substitutions to a PB2 gene bearing a single amino acid substitution led to further restriction of replication in vitro at an elevated temperature over the level of restriction imposed by one or two of these mutations. Reduction in the level of replication of mutant transfectant viruses at 37 and 38°C correlated significantly with the number of *ts* mutations (Fig.

TABLE 2. Addition of sequential mutations[a] into the PB2 gene of influenza A virus yields transfectant viruses that are progressively more *ts*

A/LA/2/87 AA PB2 transfectant virus	Titer at 32°C[b]	Mean $\log_{10}$ reduction in titer (PFU/ml) at indicated temp compared with titer at permissive temp (32°C)					Shutoff temp (°C)[c]
		36°C	37°C	38°C	39°C	40°C	
wt	6.7 ± 0.1	−0.1	0.1	0.6	1.6	**4.2**	40
Single mutants							
mt112	6.5 ± 0.1	0.2	0.5	1.0	**2.7**	5.3	39
mt265	6.3 ± 0.1	0.1	0.5	0.9	**2.9**	5.2	39
mt658	6.2 ± 0.1	0.2	0.4	1.0	**4.3**	≥5.5[d]	39
mt556	6.1 ± 0.1	0.4	0.9	**2.0**	5.0	≥5.4[d]	38
Double mutants							
mt265+658	6.1 ± 0.0	0.4	1.0	1.5	**≥5.4**[d]	≥5.4[d]	39
mt265+112	6.3 ± 0.1	0.4	1.0	**2.7**	5.5	≥5.6[d]	38
mt265+556	6.1 ± 0.0	0.6	1.1	**2.1**	≥5.4[d]	≥5.4[d]	38
Triple mutants							
mt265+112+658	5.5 ± 0.2	1.3	**2.8**	≥4.8[d]	≥4.8[d]	≥4.8[d]	37
mt265+112+556	5.9 ± 0.1	1.4	**≥5.2**[d]	≥5.2[d]	≥5.2[d]	≥5.2[d]	37

[a] Indicated by amino acid position in the PB2 gene.
[b] Mean of two to eight experiments ± standard error.
[c] The shutoff temperature, indicated by boldface numbers, is defined as the lowest restrictive temperature at which there is a ≥2 $\log_{10}$ reduction in titer from the titer at the permissive temperature of 32°C.
[d] No plaques were observed at the indicated temperature in any of the experiments.

1). The single- and triple-mutant transfectant viruses bearing the aa 556 mutation were more *ts* at 37 and 38°C than their counterparts (Fig. 1), indicating that the specific mutation itself is also an important determinant of the level of temperature sensitivity exhibited by the transfectant virus.

Introduction of two or three additional *ts* mutations into the PB2 gene of transfectant viruses results in greater degrees of restriction of replication in the upper and lower respiratory tracts of hamsters. The replication of the *wt* and mutant PB2 transfectant viruses in hamsters was next studied (Table 3). The A/LA/2/87 AA single-mutant PB2 transfectant viruses replicated in the upper respiratory tract (nasal turbinates) to a level similar to that of the A/LA/2/87 AA *wt* PB2 transfectant virus; the level of replication of these mutants in the warmer lower respiratory tract (lungs) also did not appear to be diminished significantly. The AA double-mutant PB2 transfectant viruses, on the other hand, replicated to lower titer in the nasal turbinates and were not detectable in the lungs. Similarly, the triple-mutant PB2 transfectant viruses were not detectable in the lungs and exhibited a greater degree of attenuation in the upper respiratory tract than the double-mutant transfectant viruses. It should be noted that the AA *wt* PB2 transfectant

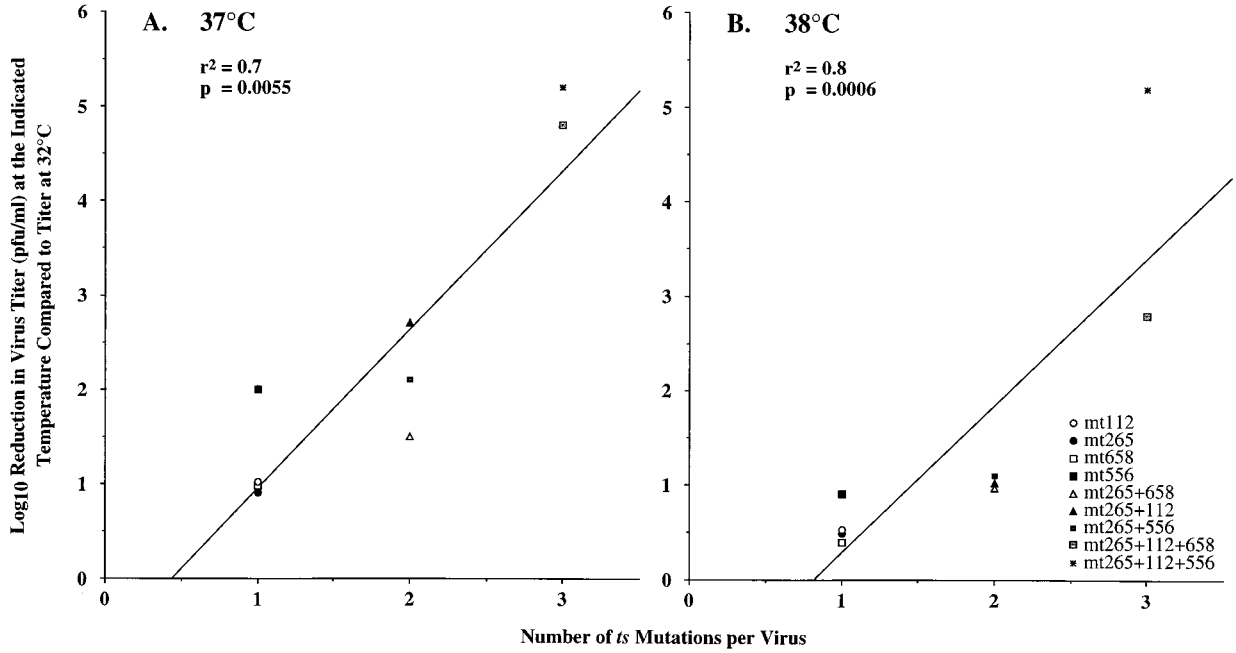

FIG. 1. Correlation between the number of *ts* mutations introduced into the PB2 gene and restriction of plaque formation at 37 and 38°C in MDCK cell culture.

TABLE 3. Relationship of the number of *ts* mutations present in the PB2 genes of transfectant viruses and the restriction of replication in the respiratory tracts of hamsters

A/LA/2/87 AA PB2 transfectant virus administered[a]	Expt[b]	Shutoff temp (°C)[c]	Virus in indicated tissue			
			Nasal turbinates		Lungs	
			Peak titer[d]	% of animals in which virus was detected[e]	Peak titer	% of animals in which virus was detected
wt	1–4	40	5.5	98	2.9	49
Single mutants						
mt112	1–3	39	5.3	100	2.3	26
mt265	1–3	39	5.2	100	2.1	35
mt658	1, 3	39	5.6	100	2.5	42
mt556	1, 3	38	5.1	97	1.6	6
Double mutants						
mt265+658	3	39	4.9	100	≤1.5	0
mt265+112	2, 3	38	3.9	93	≤1.5	0
mt265+556	3	38	4.3	100	≤1.5	0
Triple mutants						
mt265+112+658	4	37	3.5	83	≤1.5	0
mt265+112+556	4	37	2.9	75	≤1.5	0

[a] On day 0, $10^{5.0}$ $TCID_{50}$ of the indicated virus was administered intranasally in a 0.1-ml inoculum to anesthetized 5-week-old golden Syrian hamsters. On days 2 through 5, nasal turbinates and lungs were harvested and homogenized, and mean virus titers in the tissues were determined.

[b] Data from four experiments are summarized.

[c] Defined as the lowest restrictive temperature at which there is a ≥2 $\log_{10}$ reduction in titer from the titer at the permissive temperature of 32°C.

[d] Expressed as mean $\log_{10}$ $TCID_{50}$ per gram. The lowest detectable titers in nasal turbinates and lungs were 1.2 and 1.5 $\log_{10}$ $TCID_{50}$/g, respectively.

[e] Calculated as number of animals with virus detected in the indicated tissue/total number of animals tested. The range of the number of animals tested in each group was 18 to 84.

virus appears partially attenuated for the lungs of hamsters for reasons that remain undefined. Possibly, this reflects a mixed polymerase gene constellation effect observed previously for influenza A virus reassortants that derived one of their polymerase protein genes from one parent and the other two polymerase protein genes from the other parent (5). Therefore, the effects of the introduced *ts* mutations on replication in the lower respiratory tracts of hamsters must be interpreted in this context. The level of temperature sensitivity of the transfectant viruses correlated significantly with the peak level of virus replication in the upper and lower respiratory tracts of hamsters (Fig. 2), indicating that the level of temperature sensitivity achieved by site-directed mutagenesis was the predominant determinant of attenuation. The incremental nature of the restriction of replication conferred by the addition of second and third *ts* mutations is presented in Fig. 3. It is apparent that the addition of second- and third-site mutations to mt265 led to progressive increases in the level of attenuation for the upper respiratory tracts of hamsters and that the splays in restriction of replication manifested by the double- and triple-mutant viruses suggest that specific mutations can make different contributions to the level of attenuation manifested by the transfectant virus.

PB2 transfectant viruses with two or three *ts* mutations in the PB2 gene are immunogenic and protect the lower respiratory tracts of hamsters from *wt* virus challenge. We next evaluated whether selected AA double- and triple-mutant PB2 transfectant viruses were immunogenic and exhibited protective efficacy in vivo. Since a single-mutant PB2 transfectant virus (AA mt265) was previously shown to be immunogenic and protective in hamsters (25), we focused our evaluation on one of the AA double-mutant PB2 transfectant viruses (AA mt265+112) and the two triple-mutant PB2 transfectant viruses (Table 4). The influenza A/LA/2/87 *wt* virus was chosen as the virus to be used for challenge since it bears HA and NA genes that are homologous with those of the transfectant viruses and replicates to considerably higher titer in hamster lungs than the A/LA/2/87 AA *wt* PB2 transfectant virus, which possesses an AA PB2 gene in an A/LA/2/87 virus background (Tables 3 and 4). This mixture of polymerase protein genes from two different viruses might result in partial attenuation of the AA *wt* PB2 transfectant virus for hamsters as described above. The challenge virus was administered 30 days after immunization with the A/LA/2/87 AA *wt*, mt265+112, mt265+112+556, or mt265+112+658 PB2 transfectant virus. The immunologically irrelevant influenza B/AA/1/86 *wt* virus was also used to immunize mice which served as negative controls in the evaluation of protective efficacy. Lungs and nasal turbinates were harvested on the day following challenge because preliminary experiments indicated that peak titers following infection with the A/LA/2/87 *wt* challenge virus were achieved at this time. Despite the attenuation of the double- and triple-mutant PB2 transfectant viruses in hamsters (Table 3), the viruses, except for the triple mt265+112+556, were highly immunogenic, and each of these viruses induced resistance of the lower respiratory tract to *wt* virus challenge (Table 4).

PB2 transfectant viruses bearing two mutations in the PB2 gene display stability of the *ts* phenotype after prolonged replication in the respiratory tracts of mice. The level of stability of the *ts* phenotype of the double-mutant PB2 transfectant viruses was evaluated by determining the *ts* phenotype of viruses isolated after prolonged replication (14 days) in vivo in an immunocompromised host (i.e., T-cell-deficient nude [*nu/nu*] mice). Day 14 was selected as the time for harvesting lungs and nasal turbinates because data from the evaluation of live influenza A virus vaccines in humans suggested that vaccine viruses can replicate for up to 11 days in fully susceptible seronegative humans (30), a considerably longer time than the 6 to 8 days of virus replication observed in immunocompetent rodents. The A/LA/2/87 AA *wt* and mutant PB2 transfectant

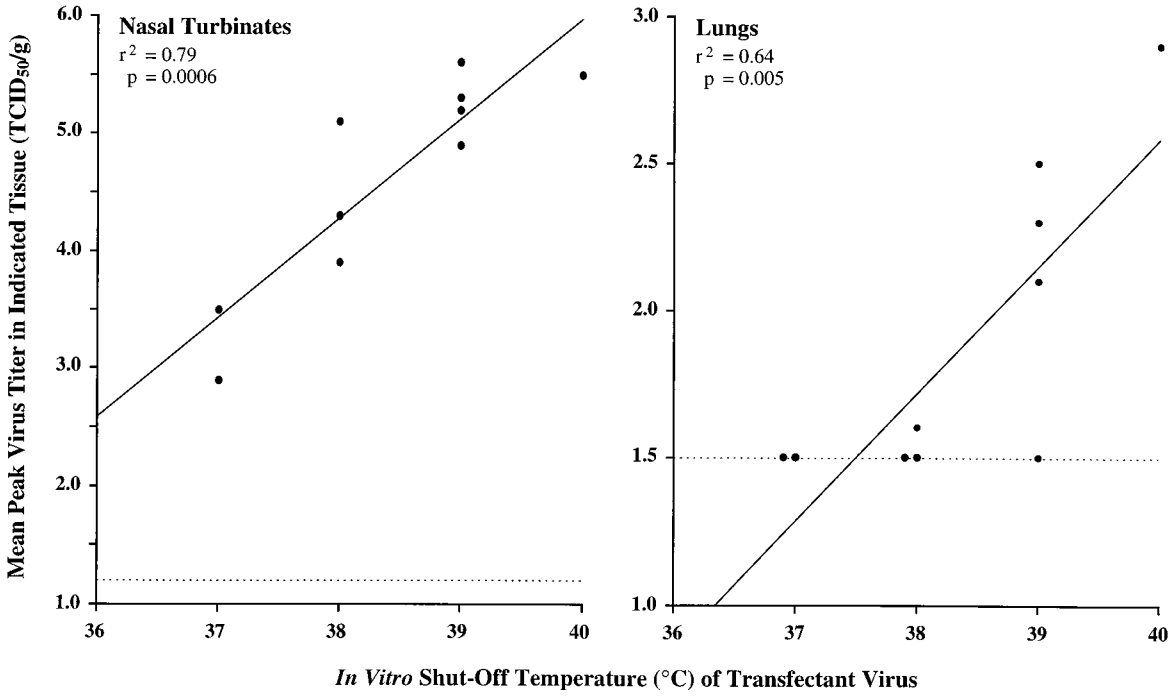

FIG. 2. Correlation between the level of temperature sensitivity of the AA PB2 transfectant viruses and level of replication in the respiratory tracts of hamsters. Dotted lines indicate the lower limit of detection.

viruses could not be recovered from the lungs of nude mice after 14 days; however, isolates were recovered at that time from the nasal turbinates. The single-mutant PB2 transfectant virus isolates were not studied systematically in mice because two of the four mutants and the AA *wt* PB2 transfectant virus as well produced some plaques at 40°C (Table 2). We therefore concentrated our efforts on characterization of the double-mutant PB2 transfectant viruses, two of which showed complete restriction of replication at 39°C, whereas replication of the remaining mutant, mt265+112, was completely shut off at 39.5°C (data not shown) (Table 2). Isolates obtained from each of four animals infected with the A/LA/2/87 AA *wt* PB2 transfectant virus retained the *ts* phenotype as expected (data not shown). The mt265+112 virus was administered to 115 nude mice, but only 10 isolates were recovered; all were *ts*. The mt265+556 virus was administered to 120 mice, 69 isolates were obtained, and all were *ts*. Similarly, the mt265+658 virus was administered to 120 mice, 89 isolates were obtained, and 7 produced plaques at 39°C. The original nasal turbinate tissue homogenates of the seven mice that yielded isolates with *ts*+ virus were next tested, and five of the seven specimens failed to produce plaques at 39°C (all were positive at 32°C). In addition, plaque progeny of these five isolates picked at 39°C and amplified at 32°C were all *ts*. These five isolates and their corresponding original nasal turbinate homogenates were therefore considered free of *ts*+ virus. The remaining two animals had *ts*+ viruses in the original nasal turbinate tissue homogenates, at a titer that was 5- to 20-fold lower at 39°C than the titer at 32°C. Plaque progeny picked from the isolates at 39°C were *ts*+ after amplification at 32°C. Thus, only 2 of 89 (2%) of isolates from animals infected with mt265+658 contained *ts*+ virus. The triple-mutant transfectant viruses were not studied because the frequency of isolating *ts*+ viruses from the double-mutant viruses was so low.

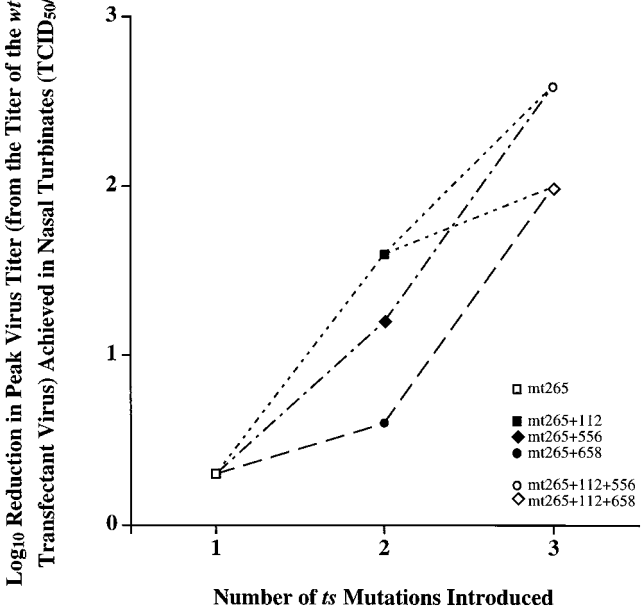

FIG. 3. Progressive increase in attenuation for the upper respiratory tracts of hamsters with sequential addition of *ts* mutations to the mt265 transfectant virus.

DISCUSSION

It has traditionally been difficult to satisfactorily attenuate influenza A viruses by conventional techniques. The major impediment to the generation of a satisfactorily attenuated donor virus has been our inability to add or subtract attenuat-

TABLE 4. Infection with PB2 transfectant viruses with one to three *ts* mutations induces antibodies and resistance to virus replication in the upper or lower respiratory tracts of hamsters during subsequent challenge with *wt* virus[a]

Immunizing virus	Expt[b]	No. of animals	Serum hemagglutination-inhibiting antibody titer (reciprocal of mean $\log_2$ ± SE on day 28)	Response to A/LA/2/87 *wt* virus[c] challenge (virus titer [mean $\log_{10}$ $TCID_{50}$/g ± SE] in indicated tissue[d])	
				Nasal turbinates	Lungs
B/AA/1/86	2, 3	12	<2.0 ± 0.0	6.2 ± 0.1	5.2 ± 0.7
A/LA/2/87 AA PB2 transfectant viruses					
wt	2	6	6.7 ± 0.6	2.7 ± 0.5	2.2 ± 0.7
Single mutant mt265[e]	1	6	6.8 ± 0.2	2.7 ± 0.5	1.6 ± 0.2
Double mutant mt265+112	2	6	5.5 ± 0.2	2.8 ± 0.6	2.1 ± 0.6
Triple mutants					
mt265+112+658	3	6	5.3 ± 0.3	5.6 ± 0.2	≤1.5 ± 0.0
mt265+112+556	3	6	3.8 ± 0.6	5.7 ± 0.5	2.4 ± 0.8

[a] On day 0, $10^{5.0}$ $TCID_{50}$ of the indicated virus was administered intranasally in a 0.1-ml inoculum to anesthetized 5-week-old golden Syrian hamsters. On day 28, the animals were bled, and on day 30, they were similarly challenged with $10^{5.0}$ $TCID_{50}$ of *wt* A/LA/2/87 virus.

[b] Data from three experiments are summarized.

[c] The A/LA/2/87 *wt* challenge virus is not a transfectant virus and therefore replicates to a higher titer than the A/LA/2/87 *wt* AA PB2 transfectant virus bearing an AA PB2 gene in a background of gene segments derived from A/LA/2/87 virus (compare Tables 3 and 4).

[d] Animals were sacrificed 1 day after challenge, and lung and nasal turbinate homogenates were quantitated for virus titer. The lowest detectable titers in nasal turbinates and lungs were 1.2 and 1.5 $\log_{10}$ $TCID_{50}$/g, respectively.

[e] Results previously reported (25) are included for comparison.

ing mutations to incompletely attenuated or overattenuated vaccine viruses in a controlled fashion. In some instances in which a satisfactory level of attenuation was achieved by combining two genes with attenuating mutations in one donor virus, the attenuation phenotype of the virus was not sufficiently stable following replication in fully susceptible seronegative individuals (6, 13, 27). The findings from the present study suggest that it may be possible to overcome these difficulties.

The use of PB2 reverse genetics for influenza A virus vaccine development now allows us to optimize attenuation by stepwise addition of attenuating mutations to one gene segment until a satisfactory level of attenuation has been reached. A systematic design and assessment of the effect of each serially added mutation is possible with this technique because each new mutant gene differs from its predecessor by one defined mutation, and the biological properties of transfectant viruses bearing these mutant genes can be evaluated directly in vivo at each stage of development. In the present study, we observed that although specific single mutations resulted in only a very modest increase in temperature sensitivity and attenuation, an incremental increase in the level of temperature sensitivity and attenuation could be achieved by the addition of a second and a third *ts* mutation to a PB2 gene segment that already had a single *ts* mutation. In this way, it was possible to produce two triple mutants with a 37°C shutoff temperature of replication in vitro. It is important to note that a previously evaluated influenza A virus with a 37°C shutoff temperature appeared satisfactorily attenuated in humans, but it was not stable with respect to its *ts* and *att* phenotypes after replication in seronegative humans (19, 27). From the results of extensive evaluation of *ts* viruses in humans, it is reasonable to suggest that the triple-mutant viruses with a 37°C shutoff temperature will likely be satisfactorily attenuated even in seronegative infants and children (19). Although it is possible to combine attenuating genes into one virus by the process of genetic reassortment, there are practical limits to the number of viral genes that can be transferred efficiently in a timely manner from a donor virus to a new antigenic variant of influenza A virus. The advantage of adding mutations to the same gene segment rather than distributing mutations among several gene segments is that the transfer of a single attenuating gene segment (with accompanying identifying markers such as temperature sensitivity) to a *wt* virus, by reassortment, will be easier than transfer of several gene segments that specify optimal attenuation only when present as a constellation. This technical advantage should permit the rapid generation of new reassortant vaccines in a timely manner to meet production deadlines for vaccines to be used against a predicted influenza virus epidemic or pandemic. While we anticipate that reassortant viruses bearing the triple-mutant AA PB2 gene in different *wt* virus backgrounds will be reproducibly attenuated, this can be ascertained only after several such reassortants are generated and studied systematically.

Immunization of hamsters with virus containing one, two, or three *ts* mutations in the PB2 gene induced hemagglutination-inhibiting antibodies and protected hamsters against *wt* virus challenge. The lower respiratory tract was protected even by immunization with the highly defective triple-mutant viruses. Although the triple-mutant PB2 transfectant viruses did not protect the upper respiratory tract from a locally administered large inoculum of challenge virus, this is not likely to pose a problem for use of the triple-mutant transfectant viruses in humans for two reasons. First, attenuated influenza viruses replicate and are shed from the human respiratory tract for 7 to 11 days following vaccine administration (30), while in the hamster model, these viruses are cleared rapidly, i.e., by days 5 to 6. The longer duration of influenza virus replication in humans should induce a greater mucosal antibody response. Second, influenza virus vaccines are likely to be used in a two-dose format to immunize young seronegative infants and children (11). The longer duration of virus replication in susceptible humans and the use of a two-dose schedule should result in higher levels of antibody than was observed in hamsters. Hence, we anticipate that vaccine viruses containing a triple-mutant PB2 gene will be sufficiently immunogenic in humans, but this remains to be demonstrated.

Another obstacle to influenza A virus vaccine development is instability of the desired *att* phenotype following prolonged replication in a susceptible host (26, 27). In the present study,

we evaluated the stability of the *ts* phenotype rather than the *att* phenotype itself because the cumulative experience with the transfectant viruses indicated that the *ts* mutations themselves specified attenuation and therefore are an appropriate surrogate marker for the *att* phenotype. The *ts* or *att* phenotype of influenza A viruses can be modified by reversion of mutant sequences as well as by the development of intragenic or extragenic suppressor mutations (21, 26, 27, 29). Since diverse genetic mechanisms can act to alter the *ts* and *att* phenotypes, it is not surprising to observe that viruses with single *ts* mutations, or even those with two *ts* mutations on different genes, readily undergo genetic modification resulting in the loss of the *ts* or *att* phenotype following replication in vivo (13, 24, 26, 27). In the present study, we evaluated the double-mutant PB2 transfectant viruses for stability of the *ts* phenotype, and two of the three double-mutant PB2 viruses exhibited complete stability of this phenotype even after prolonged replication in an immunocompromised host. The third transfectant exhibited a high but not complete stability. Previous findings with *ts* mutant viruses that had *ts* lesions on two different gene segments demonstrated that they, like single-lesion mutants, also lose their *ts* phenotype following replication in vivo, with >50% of isolates exhibiting a loss of the *ts* phenotype after 12 to 15 days of replication in immunocompromised animals (13). The present findings suggest that the *ts* phenotype of a single gene with two *ts* mutations is more stable genetically, with 2% or fewer isolates manifesting a loss of the *ts* phenotype, than a virus with a *ts* mutation on two separate gene segments. These observations have several implications for the direction of influenza A virus vaccine development. First, since *ts* mutants with two *ts* mutations can lose their *ts* and *att* phenotypes in vivo, it seems reasonable to conclude that more than two *ts* mutations will be needed to provide a satisfactory level of genetic stability of the *ts* phenotype. Fortunately, the present findings indicate that the PB2-specific host range selection system is efficient enough to isolate PB2 transfectant viruses containing three *ts* mutations, and it is reasonable to suggest that the triple-mutant transfectant viruses that have a 37°C shutoff temperature and that possess five mutations contributing to the *ts* and *att* phenotype will be even more stable than the double-mutant transfectant viruses. The triple-mutant transfectant virus that is of most interest as a vaccine donor virus is mt265+112+556 because both of the double-mutant viruses with a pair of these mutations were completely stable in nude mice. Second, since *ts*+ revertants of two different *ts* PB2 mutants developed suppressor mutations on the PA gene (27, 28), it seems reasonable to explore the possibility of combining, by reassortment, a triple-mutant PB2 gene with a PA gene that itself bears an attenuating mutation. It is anticipated that the presence of a mutant PA gene should decrease the likelihood of the development of PA mutations that suppress the *ts* phenotype specified by the mutant PB2 gene. It is interesting to speculate that the stability of the *ts* phenotype specified by *ts* PB2 and PB1 genes of the cold-adapted influenza A reassortant viruses in vivo is a partial consequence of the presence of an attenuating non-*ts* mutation on the PA gene (28).

In this study, we observed that the PB2 reverse genetics technique can be used to introduce additional attenuating mutations into an already mutant PB2 gene and that this resulted in a parallel increase in temperature sensitivity and level of attenuation. Clearly, the PB2 reverse genetics system is a powerful tool for the construction of live attenuated influenza A virus vaccines for use in humans. Also, since infectious virus can be rescued from RNA transcribed from the full-length genome of a nonsegmented negative-strand virus (20), it is reasonable to suggest that the principles established in this study could be applied to vaccine development for such viruses. For example, when rescue techniques are established for nonsegmented negative-strand RNA viruses that cause severe disease in the respiratory tract, e.g., respiratory syncytial virus and parainfluenza virus type 3, it should be possible to attenuate these viruses by the introduction of *ts* mutations by site-directed mutagenesis in their polymerase protein genes.

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