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NOTES

Imparting Temperature Sensitivity and Attenuation in Ferrets to A/Puerto Rico/8/34 Influenza Virus by Transferring the Genetic Signature for Temperature Sensitivity from Cold-Adapted A/Ann Arbor/6/60

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The four temperature-sensitive (*ts*) loci identified in the PB1 and PB2 gene segments of cold-adapted A/Ann Arbor/6/60 influenza virus, the master donor virus for influenza A virus (MDV-A) FluMist vaccines, were introduced into a divergent A/Puerto Rico/8/34 influenza virus strain. Recombinant A/Puerto Rico/8/34 virus with these four introduced *ts* loci exhibited both *ts* and *att* phenotypes similar to those of MDV-A, which could be used as a donor virus for manufacturing large quantities of inactivated influenza virus vaccine against potential pandemic strains.

Influenza virus causes significant morbidity and mortality in humans and domestic animals; annual epidemics of influenza are responsible for over 40,000 deaths every year in the United States alone (16, 18). Infrequent pandemics caused by the H1N1, H2N2, and H3N2 subtypes of influenza A virus during the 20th century resulted in significantly higher rates of influenza-related morbidity and mortality. The virulent nature of potential pandemic strains such as these requires the virus to be handled under conditions involving higher levels of biosafety containment and requires significant changes for the safe production of vaccines against the pandemic strains.

The present licensed inactivated influenza virus vaccines in the United States are produced by growing virus with appropriate antigenic characteristics in embryonated chicken eggs, with subsequent chemical inactivation and formulation of the main antigenic components. However, not all of the strains are suitable for vaccine production; some are limited by their relatively low yields in embryonated chicken eggs. To overcome this obstacle, high-growth reassortants can be prepared by reassorting the relevant hemagglutinin (HA) and neuraminidase (NA) gene segments with the high-yield A/Puerto Rico/8/34 (PR8; H1N1) strain (10). Producing a high-growth reassortant virus containing the six internal gene segments of PR8 and the HA and NA gene segments from the currently circulating wild-type (wt) strain following coinfection can be cumbersome and time-consuming (19).

The recovery of infectious influenza virus from plasmids (5, 7, 12, 14) should make the preparation of reassortant viruses for vaccine production more efficient. In addition, plasmid rescue, combined with routine molecular biology techniques, can be used to genetically modify the vaccine strains to impart additional safety properties to vaccine production and use (6). We have recently described the genetic signature for the temperature-sensitive (*ts*) phenotype of cold-adapted A/Ann Arbor/6/60 (A/AA/6/60) (9), the master donor virus used to produce FluMist influenza type A virus vaccines (MDV-A). The *ts* phenotype maps to five amino acids encoded on three different gene segments, segments for PB1 (K391E, E581G, A661T), PB2 (N265S), and NP (D34G) (9), and has been shown to be genetically stable following passage in humans and immunocompromised animals (13). These amino acids in combination enable efficient virus replication at 33°C but effectively shut off replication at 39°C.

Sequence alignments of the PB1, PB2, and NP genes of cold-adapted A/AA/6/60 (MDV-A) and PR8 (6) revealed that of the five loci responsible for the *ts* phenotype of MDV-A, only the NP (D34G) locus was identical between these two strains. To determine whether transferring the genetic signature for the MDV-A *ts* phenotype to a divergent strain would impart similar biological properties, the other four *ts* loci were introduced into the PR8 PB1 (pHW191-PB2) and PB2 (pHW192-PB1) expression plasmids (6) by site-directed mutagenesis with the use of the *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.).

To examine whether the MDV-A *ts* mutations introduced into the PB1 and PB2 genes of PR8 conferred the *ts* phenotype to the modified PR8 viral polymerase proteins in vitro, a minigenome assay was performed at permissive (33°C) and nonpermissive (39°C) temperatures. A minigenome (Flu-CAT) containing chloramphenicol acetyltransferase (CAT) CAT open reading frame flanked by the 5′ and 3′ ends of the influenza virus promoter sequences was constructed. Transcription of the CAT gene by the Pol I polymerase generated negative-strand CAT RNA, and the production of CAT protein was dependent on the expression of the influenza virus polymerase proteins PB1, PB2, PA, and NP (15). HEp-2 cells

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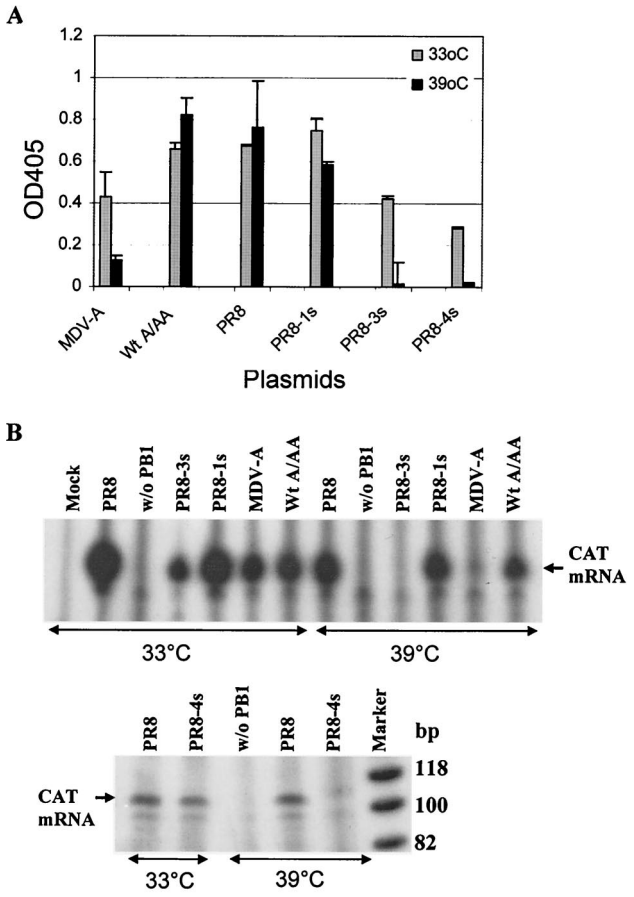

FIG. 1. Analysis of the effects of *ts* mutations on polymerase protein function in a minigenome assay. (A) HEp-2 cells were transfected with PB1, PB2, PA, NP, and a Flu-CAT minigenome and incubated at 33 or 39°C for 18 h, and cell extracts were analyzed for CAT reporter gene expression. OD405, optical density at 405 nm. (B) CAT mRNAs synthesized in transfected cells were examined by a primer extension assay. CAT mRNAs expressed in PR8-1s- and PR8-3s-transfected cells are shown in the upper panel, and those expressed in PR8-4s-transfected cells are shown in the lower panel. Wt A/AA, wt A/AA/6/60; w/o, without.

in six-well plates were transfected with 1 μg of each plasmid carrying the genes encoding PB1, PB2, PA, or NP together with 1 μg of the FLU-CAT minigenome by use of Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.). After overnight incubation at 33 or 39°C, the transfected cells were lysed, and the expression of the CAT protein was detected with the CAT enzyme-linked immunosorbent assay kit (Roche Biochemicals, Indianapolis, Ind.). As shown in Fig. 1A, the polymerase proteins from MDV-A produced slightly lower levels of CAT at 33°C than did those from wt A/AA/6/60 and PR8 but greatly reduced levels of CAT expression at 39°C compared to those at 33°C. The proteins from both wt A/AA/6/60 and PR8 expressed high levels of CAT at both 33 and 39°C, results consistent with the non-*ts* phenotype of these viruses. The introduction of the MDV-A PB2 mutation (N265S) into PR8 PB2 (PR8-1s) alone had very little effect on CAT expression at either the permissive (33°C) or the restrictive (39°C) temperature. The introduction of the three MDV-A *ts* loci (K391E, E581G, and A661T) into PR8 PB1 (PR8-3s) resulted in slightly reduced activity at 33°C compared to that of the parental PR8; however, there was a 98% reduction in CAT protein expression at 39°C compared to that at 33°C. A combination of both the PR8 PB1 and the PR8 PB2 mutant plasmid DNAs (PR8-4s) resulted in a reduction in polymerase activity at 33°C compared to that of PR8-3s; however, the levels of CAT expression at 39°C for PR8-4s and PR8-3s were too low to evaluate whether the combination had additional thermal instability. The level of CAT mRNA expressed in the transfected cells was examined by the primer extension method as described by Fodor et al. (4) and was found to correlate with CAT protein activity (Fig. 1B). These data demonstrate that the introduction of the four MDV-A *ts* loci into the PR8 PB1 and PB2 gene segments produced a viral polymerase whose mRNA synthesis was impaired in a temperature-dependent manner. The alterations in the PB1 gene segment had a greater impact on temperature sensitivity than those in the PB2 gene segment.

To evaluate the effects of the mutations on the replication of the virus, recombinant parental PR8 and modified PR8 viruses were generated by plasmid rescue. The cocultured COS7 and MDCK cells were transfected with eight plasmids carrying the PB2 or PB2-1s, PB1 or PB1-3s, PA, HA, NP, NA, M, and NS genes derived from PR8 by the eight-plasmid rescue technique described previously (6, 7). A total of four recombinant viruses were obtained (Table 1). Recombinant virus PR8-1s contains one *ts* substitution mutation in PB2, N265S. PR8-3s carries three *ts* mutations in the PB1 gene, K391E, E581G, and A661T. PR8-4s is a combination of PR8-1s and PR8-3s and contains the four mutations present in both PB1 and PB2. The three modified PR8 mutants grew to titers of ≥9.2 $\log_{10}$ PFU/ml in embryonated eggs at 33°C (Table 1). The *ts* phe-

TABLE 1. Amino acid residues of PR8 and PR8 mutants at the five MDVA *ts* loci and their *ts* phenotypes[a]

Virus	Amino acid residue at MDV-A *ts* locus[a]:					Virus titer ($\log_{10}$ PFU/ml) at indicated temp (°C)[b]			
	PB1			PB2 (265)	NP (34)				
	391[c]	581	661			33	37	38	39
PR8	K	E	A	N	G	9.6	9.5	9.5	9.0
PR8-1s	K	E	A	**S**	G	9.4	8.9	7.7	**7.4**
PR8-3s	**E**	**G**	**T**	N	G	9.2	8.8	7.8	**5.2**
PR8-4s	**E**	**G**	**T**	**S**	G	9.5	7.8	**7.1**	**4.4**
MDV-A	E	G	T	S	G	8.6	7.0	**6.4**	**<4.0**
wt A/AA/6/60	K	E	A	N	D	8.7	8.7	8.9	8.3

[a] Amino acid changes are shown in boldface.
[b] A titer of <4.0 $\log_{10}$ PFU/ml was assigned when no virus was detected at $1:10^4$ dilutions. Titers that define shutoff temperatures are shown in boldface.
[c] Numbers indicate positions of amino acids.

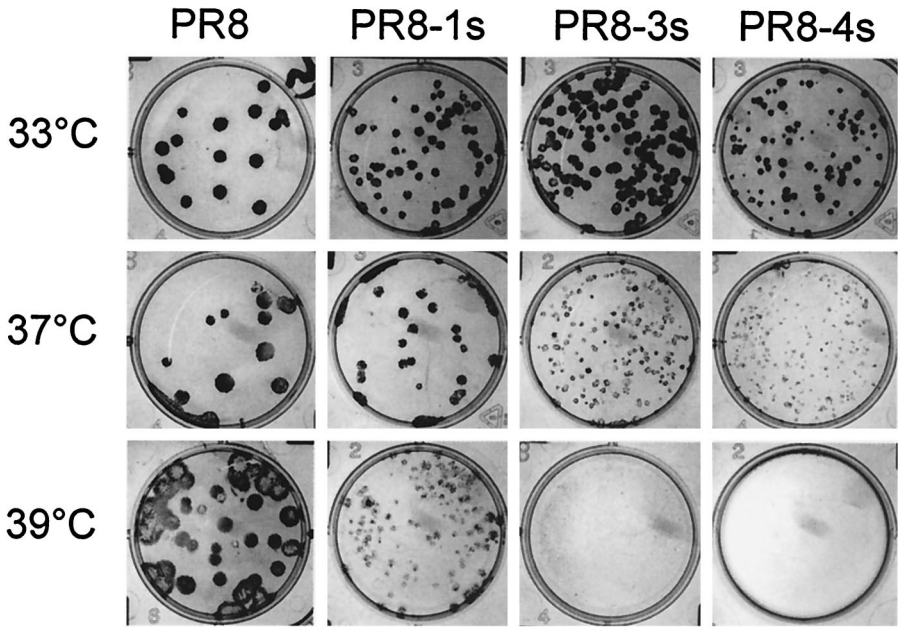

FIG. 2. Plaque morphology of PR8 mutants at various temperatures. MDCK cells were infected with the viruses indicated at the top of the figure and incubated at 33, 37, and 39°C for 3 days. Virus plaques were visualized by immunostaining and photographed.

notypes of these modified PR8 viruses were examined by plaque assays of MDCK cells at various temperatures (33, 37, 38, and 39°C), and the plaques were immunostained with anti-influenza A virus polyclonal antibodies (9). PR8 did not exhibit the *ts* phenotype; the difference in titers at 33 and 39°C was only 0.6 $\log_{10}$ PFU/ml. In contrast, PR8-1s and PR8-3s exhibited the *ts* phenotype at 39°C, with their titers at this temperature being reduced by 2.0 and 4.0 $\log_{10}$ PFU/ml, respectively, compared to those at 33°C. Additionally, PR8-4s, containing all four *ts* loci in the PB1 and PB2 gene segments, exhibited the *ts* phenotype at 38 and 39°C, with reductions of 2.4 and 5.1 $\log_{10}$ PFU/ml, respectively. In comparison, the MDV-A *ts* phenotype was similar to the PR8-4s *ts* phenotype. MDV-A titers were reduced by 2.2 and >4.4 $\log_{10}$ PFU/ml at 38 and 39°C, respectively.

Although virus titer reductions were not greater than 2.0 $\log_{10}$PFU/ml at 37°C for any of the three modified PR8 strains, all of these strains formed smaller plaques (Fig. 2). A significant reduction in plaque size at 37°C was observed for PR8-3s, and a much greater size reduction was observed for PR8-4s. PR8-1s exhibited a slight reduction in plaque size at 37°C and a greater reduction at 39°C. At 39°C, a few pinpoint-sized plaques were observed for both PR8-3s and PR8-4s. These three modified PR8 strains also exhibited reduced protein synthesis at 39°C, as determined by metabolic labeling of the infected MDCK cells (data not shown). Therefore, consistent with our previous results (9), the *ts* loci in both the PB1 and the PB2 genes contributed to the observed MDV-A *ts* phenotype, and PB1 had a greater impact than PB2.

Ferrets, an animal model used to evaluate the pathogenicity of human influenza virus strains, were used to evaluate whether the PR8 *ts* mutants were attenuated in the respiratory tract. Animals were inoculated with 8.5 $\log_{10}$ PFU of virus in a volume of 1.0 ml, and the nasal turbinate and lung tissues were harvested 3 days later. No influenza-like symptoms were observed in any of the infected animals. The lung tissue homogenates were serially diluted and titrated in 10-day-old embryonated chicken eggs at 33°C. The titers of virus recovered from the lung tissues were expressed as 50% egg infectious dose per gram of tissue ($\log_{10}$ EID_{50}/g). Virus replication in nasal turbinates was determined by a plaque assay of MDCK cells at 33°C and expressed as $\log_{10}$ PFU per gram of tissue. PR8 replicated to a titer of 6.6 $\log_{10}$ PFU/g in the nasal turbinates, and PR8-1s and PR8-3s titers were only slightly lower (5.9 and 5.8 $\log_{10}$ PFU/g, respectively). The replication of PR8-4s in the nasal turbinates was reduced by approximately 2.0 $\log_{10}$ PFU/g compared to that of PR8; however, the quantity of virus recovered from the nasal turbinates of PR8-4s-infected animals was similar to that recovered from the nasal turbinates of animals inoculated with MDV-A.

PR8 was not attenuated in the lower respiratory tracts of ferrets; an average of 4.9 $\log_{10}$ EID_{50} of virus per g of lung

TABLE 2. Replication of PR8 mutants in ferrets[a]

Group	Virus	Virus titer in:	
		Lungs ($\log_{10}EID_{50}$/g)[b]	NT ($\log_{10}$ PFU/g)[c]
1	PR8	4.9 ± 0.3	6.6 ± 0.1
2	PR8-1s	3.8 ± 0.4	5.9 ± 0.2
3	PR8-3s	1.7 ± 0.1	5.8 ± 0.3
4	PR8-4s	NVD	4.6 ± 0.2
5	MDV-A	NVD	4.6 ± 0.1
6	wt A/AA/6/60	4.4 ± 0.1	5.4 ± 0.1

[a] Values are means ± standard errors for four ferrets given doses of 8.5 $\log_{10}$ PFU.
[b] NVD, no virus detected. The detection limit was 1.5 $\log_{10}$ EID_{50}/g.
[c] NT, nasal turbinates. The titers for groups 4 and 5 were statistically different from that for group 1.

tissue was recovered from these animals (Table 2). However, PR8-1s was less virulent than the parental strain and replicated to only 3.8 $\log_{10}$ EID_{50}/g of ferret lung tissue, a 2.0-$\log_{10}$ reduction. Very limited replication in ferret lungs was detected for PR8-3s, and no virus was detected in the lung tissues of animals inoculated with PR8-4s. With regard to the controls, MDV-A did not replicate in ferret lungs and wt A/AA/6/60 replicated to a titer of 4.4 $\log_{10}$ EID_{50}/g. The data obtained in this ferret study indicate that the replication of PR8 carrying the *ts* loci derived from MDV-A was attenuated in the lower respiratory tracts of ferrets. PR8-4s induced a high level of antibody in ferrets and provided protection against challenge virus infection (data not shown).

In this study, we demonstrated that transferring the amino acid genetic signature of the MDV-A *ts* phenotype into the divergent PR8 strain imparted the biological properties of temperature sensitivity in vitro and attenuation in ferrets. Recombinant PR8 carrying these *ts* loci grew well in embryonated chicken eggs, but its replication was restricted at temperatures higher than 37°C in cell culture, and it was not recovered from the lungs of infected ferrets. The type of recombinant virus that produces high yields in eggs but has several properties of an attenuated virus could be used as a reassortant donor for the HA and NA genes of potential highly pathogenic pandemic strains. These attenuation properties in conjunction with genetic modification of the HA gene, such as the deletion of the multiple basic HA1-HA2 cleavage site (11), could produce a vaccine seed with enhanced safety properties compared to those of the vaccines that are presently available.

Although PR8 and MDV-A were very divergent, the importation of the minimal number of *ts* loci resulted in a virus that exhibited the characteristic MDV-A *ts* phenotype; it is likely that these *ts* loci could also impart the *ts* phenotype and reduced virulence properties to influenza A virus strains other than PR8. This speculation will be tested after additional strains with the five introduced MDV-A *ts* loci are made. A previous report also demonstrates that the sequential introduction of the *ts* mutations identified in other influenza virus strains into the PB2 gene of the A/AA/6/60 reassortant results in increased *ts* and *att* phenotypes (17). Our results indicate that the five MDV-A *ts* loci distributed in the three PR8 segments indeed interact and impart the biological properties of temperature sensitivity and attenuation to this divergent strain. The mapping of the genetic basis of the *ts* phenotype of MDV-A and the results obtained for the modified PR8 *ts* mutants revealed that PB1 had a greater effect on *ts* than PB2 or NP did (17). The contribution of the NP gene segment to the *ts* phenotype of PR8 was not addressed, since the G34 residue is already present in the parental PR8 strain.

Various studies have indicated that there is no association between PR8 attenuation in animal models and that in humans (2). Although PR8 is highly pathogenic in mice, it does not infect humans (2). The use of PR8 as a master donor virus for live attenuated influenza virus vaccines was explored in the 1970s. However, the reassortants between PR8 and H3N2 strains had unpredictable degrees of virulence in mice and humans; some of these reassortants regained virulence in humans (2, 3, 8). In addition, H1N1/PR8 reassortant viruses used for the production of inactivated vaccines have escaped and produced lethal infections in camels (1). Therefore, it is apparent that the modified PR8 strain that expresses the *ts* phenotype and is nonvirulent in ferrets could provide a safety advantage over the original PR8 strain as a donor virus for the production of influenza virus vaccines.

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