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Production and Level of Genetic Stability of an Influenza A Virus Temperature-Sensitive Mutant Containing Two Genes with *ts* Mutations

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Temperature-sensitive (*ts*) reassortant vaccine strains derived from the A/Udorn/72 *ts*-1A2 donor virus were not sufficiently stable genetically in humans. We therefore sought to produce a new, more stable donor virus. We had previously identified a stable *ts* virus with a *ts* P3 gene and in the current study identified another relatively stable single-lesion *ts* virus with a *ts* mutation in the NP gene. A new *ts* reassortant virus was constructed by mating these two single mutants and by isolating three reassortant progeny, clones 20, 53, and 55, that contained both a *ts* P3 and a *ts* NP gene. These reassortant progeny possessed a 37 to 38°C shutoff temperature and were as restricted in their replication in hamster lungs as the A/Udorn/72 *ts*-1A2 virus. All isolates from the lungs and nasal turbinates of hamsters were temperature sensitive. An in vitro stress test was used to determine whether the new *ts* P3 *ts* NP reassortant virus would undergo loss of its *ts* phenotype after replication at semipermissive temperature. Clone 20 and 55 reassortants underwent progressive loss of their *ts* phenotype in vitro, although at a rate slightly less than that of the A/Udorn/72 *ts*-1A2 virus. The level of genetic stability after replication in vivo was assessed in cyclophosphamide-treated hamsters in which virus replication continued for up to 15 days. Again, both the A/Udorn/72 *ts*-1A2 and the new *ts* P3 *ts* NP reassortant clone 55 manifested a progressive loss of temperature sensitivity after 7 days of replication. Clone 55 virus lost temperature sensitivity significantly less rapidly than the A/Udorn/72 *ts*-1A2 virus. These results indicated that, although the new *ts* P3 *ts* NP reassortant virus was more stable than the A/Udorn/72 *ts*-1A2 virus, it nevertheless underwent progressive loss of temperature sensitivity after replication in vitro and in vivo. Therefore, it does not appear to be a satisfactory donor virus. This experience plus that gained earlier with other *ts* mutants of influenza A virus suggest that influenza A virus mutants that rely solely upon their *ts* phenotype for attenuation are unlikely to exhibit the phenotypic stability required of a vaccine virus. Other genetic techniques are needed to produce more stable influenza A virus strains.

Temperature-sensitive (*ts*) mutants of influenza A virus have been developed and evaluated for their level of attenuation and immunogenicity in humans (1). Genes that bear a *ts* mutation were transferred by genetic reassortment into new influenza A wild-type viruses as they emerged in nature (1). The goal of this approach to the production of a live influenza A virus vaccine was the identification of a set of *ts* genes that conferred a satisfactory level of attenuation on each new wild-type virus into which these genes were transferred. It was also important that such viral reassortants be stable genetically after replication in humans. It was possible to identify a set of genes that conferred a satisfactory level of attenuation on new influenza A viruses (5, 7, 8, 11). The *ts* polymerase 1 (P1) and *ts* polymerase 3 (P3) genes present in our most promising *ts* donor virus, A/Udorn/72 *ts*-1A2 (H3N2) virus, did not exhibit the desired genetic stability (22). Specifically, the A/Alaska/77 *ts*-1A2 (H3N2) reassortant lost its *ts* phenotype after replication in a seronegative child (22), and the ts^+ virus isolated from the child regained its virulence for adult volunteers (M. D. Tolpin et al., submitted for publication). This indicated the need for a more stable donor virus.

In this report we describe our attempt to produce such a donor virus. To accomplish this,

it was necessary to produce additional *ts* mutants, to evaluate the level of genetic stability of these new *ts* viruses in hamsters, to construct a new *ts* reassortant virus containing the two most stable *ts* genes, and to compare the level of genetic stability of the new double-lesion virus with the A/Udorn/72 *ts*-1A2 virus in vitro and in vivo. The in vivo study was performed in hamsters immunologically compromised by cyclophosphamide administration so that virus replicated for up to 15 days. In this manner it was possible to examine the tendency for a virus to lose its temperature sensitivity after replication for relatively extended periods of time in the hamster's respiratory tract. Although we were successful in producing a mutant more stable genetically than the A/Udorn/72 *ts*-1A2 virus, this new *ts* mutant still underwent a progressive loss in its level of temperature sensitivity after in vitro or in vivo replication.

MATERIALS AND METHODS

Viruses. The isolation and cloning of the A/Udorn/307/72 wild-type (H3N2) virus clone 3A1 were described previously (16, 17). Influenza A/Victoria/75 *ts*-1A2 reassortant clone 65 ($H3_{75}N2_{65}$ or $_{68}$) was produced by mating the A/Udorn/72 *ts*-1A2 reassortant with the A/Victoria/3/75 ($H3_{75}N2_{75}$) wild-type virus as previously described (12). The A/Victoria/75 *ts*-1A2 clone 65 reassortant contained the *ts* P3 gene from the A/Udorn/72 *ts*-1A2 parent (4). The A/Udorn/72 *ts*-1[E] reassortant clone 24 ($H3_{72}N2_{72}$) virus possessed a *ts* P3 gene and a *ts* NP gene and was produced and characterized as previously described (3, 17). The virus suspensions used were grown in the allantoic cavity of 10-day-old specific-pathogen-free embryonated hen's eggs (SPAFAS, Storrs, Conn.)

Mutagenesis and isolation of *ts* mutants. An allantoic fluid suspension of the A/Udorn/72 virus was inoculated into primary bovine kidney cell monolayers (Flow Laboratories, McLean, Va.) at a multiplicity of approximately 0.1. After adsorption, medium containing 5-fluorouracil (Sigma Chemical Co., St. Louis, Mo.) at a concentration of either 100 or 200 μg/ml was added, and the infected monolayers were incubated at 34°C for 48 h. At these concentrations of 5-fluorouracil, virus yield was reduced approximately 100-fold from control cultures. Harvested medium containing virus was plated on bovine kidney cell monolayers at 34°C and 1,170 plaques were picked. Each plaque was inoculated into 24-well Co-Star plates containing Madin-Darby canine kidney (MDCK) tissue and incubated at 34 or 39°C. Those plaques producing cytopathic effect or hemadsorption or both at 34°C, but not at 39°C, were further cloned in primary bovine kidney monolayer cultures by a previously described method of plaque purification (14) or by direct "plaque-to-plaque" passage of the initial plaque material in bovine kidney tissue.

EOP, genetic analysis of *ts* mutants, and infectivity titrations. The efficiency of plaque formation (EOP) of a virus at various temperatures was assayed by plaque titration on MDCK monolayers on 12-well Linbro plastic plates obtained from Flow Laboratories, using Liebowitz medium 15 (M. A. Bioproducts, Walkersville, Md.) with trypsin and 0.8% agarose as previously described (18). To assess whether two *ts* viruses shared a *ts* mutation on the same gene, the plate complementation assay, as previously described (20), was used with a panel of *ts* mutant viruses whose locus of the *ts* mutation was known (15, 19–21). Infectivity of viral inocula and homogenates of hamster's lungs or nasal turbinates was determined on MDCK monolayers on 24-well Co-Star plastic plates as previously described (22).

Genotyping of A/Udorn/72 *ts*-368 virus by PAGE. To ascertain the locus of its *ts* mutation, the A/Udorn/72 *ts*-368 mutant was mated with the A/WSN/33 wild type, and *ts* reassortant viruses bearing the WSN hemagglutinin were isolated in the presence of A/Udorn/72 antiserum as previously described (20, 21). The parental origin of the genes in the WSN *ts* reassortants was determined by polyacrylamide gel electrophoresis (PAGE) as previously described (20, 21).

The genotype of the A/Udorn/72 *ts*-368 X A/Victoria/75 *ts*-65 reassortant progeny was also analyzed by PAGE. A 20-cm gel containing 3% acrylamide and 6 M urea that was run at 29°C and 100 V for 19 h was able to show differences in the rate of migration of the viral RNA segments encoding for the three polymerase proteins, the hemagglutinin, and the neuraminidase of the parental viruses.

Hamster studies. Four- to 6-week-old normal female outbred Golden Syrian hamsters (Charles River, Lakeview, Newfield, N.J.) were anesthetized with pentobarbitol and inoculated intranasally with 0.1 ml of virus (approximately $10^{4.5}$ 50% tissue culture infective doses [$TCID_{50}$]). Groups of 6 to 10 hamsters were sacrificed daily for 4 days after virus administration, and homogenates of the individual lungs and nasal turbinates were prepared as previously described (Tolpin et al., submitted). The titer of virus in individual lung or nasal turbinate homogenates was determined daily for 4 days after viral inoculation. The mean $\log_{10}$ titer was expressed as $TCID_{50}$ per gram of tissue. Virus isolates obtained in MDCK cultures were characterized for the presence of ts^+ "revertant" virus by plaque titration at 34 and 39°C. Those isolates yielding virus that produced plaques at 39°C were scored as positive for ts^+ revertant virus.

Separate groups of hamsters were administered cyclophosphamide (Mead Johnson and Co., Evansville, Ind.) intraperitoneally according to the following schedule: experiment 1—100 mg/kg on day 2, 50 mg/kg on day 5, and 150 mg/kg on day 7; experiment 2—150 mg/kg on day 2, 100 mg/kg on day 5, 100 mg/kg on day 8, 100 mg/kg on day 11, and 100 mg/kg on day 14. Fifteen hamsters from each virus group were sacrificed on days 4, 7, and 9 in experiment 1 and on days 9, 12, and 15 in experiment 2. Isolates were obtained as described above, and the EOP of the virus present in the isolate was characterized at 34, 36, 37, 38, and 39°C on MDCK monolayer cultures.

In vitro stress test. T-25 Co-Star flasks containing MDCK cells were inoculated with virus at a multiplicity of 0.1. Separate flasks were incubated at 34 to 36.5°C for 2 to 3 days until almost complete cytopathic effect was present. The virus from the flask incubated at 36.5°C was harvested, diluted 1:10, and passaged to another flask which was incubated at 37°C. In this

TABLE 1. Comparison of growth and genetic stability of A/Udorn/72 *ts* mutants in the lungs of hamsters with that of the A/Udorn/72 *ts*-1[E] and wild-type viruses[a]

Influenza A/Udorn/72 virus	Virus replication		Genetic stability (no. of isolates ts^{+b}/no. tested)
	Maximum level of virus replication (mean $\log_{10}$ $TCID_{50}$/g)	Reduction of replication (wild type minus *ts*)	
Expt A			
ts-368	3.9 ± 0.5[c]	4.2	7/31
ts-651	6.4 ± 0.2	1.7	1/31
ts-1[E]	5.7 ± 0.2	2.4	5/27
Wild type	8.1 ± 0.1		
Expt B			
ts-50	4.7 ± 0.3	2.8	29/31
ts-623	4.7 ± 0.4	2.8	21/35
ts-559	5.1 ± 0.3	2.4	2/26
ts-783	5.4 ± 0.2	2.1	18/31
ts-588	5.6 ± 0.2	1.9	19/27
ts-568	5.8 ± 0.3	0.7	25/27
ts-230	6.7 ± 0.2	0.8	13/35
ts-1[E]	5.4 ± 0.2	2.1	5/26
Wild type	7.5 ± 0.2		

[a] None of the *ts* mutants tested produced plaques at 39°C.

[b] ts^+ is defined as a hamster isolate that produces plaques at restrictive temperature (39°C).

[c] Values represent the mean $\log_{10}$ titers ± standard error and are expressed as $TCID_{50}$ per gram of lung. Eight to 10 hamsters were sacrificed daily for 4 days after receiving $10^{4.5}$ $TCID_{50}$ of virus intranasally. The lowest level of virus detectable was $10^{2.0}$ $TCID_{50}$/g. For calculation of mean $\log_{10}$ titers, tissues with $10^{2.0}$ $TCID_{50}$/g were considered to contain $10^{1.5}$ $TCID_{50}$. Daily mean titers were calculated, and the maximum level achieved over the 4-day period is included.

manner serial passages were performed starting at 36.5°C and progressing to 37, 38, and 39°C. The harvest taken at each temperature was tested for its EOP at 36, 37, 38, 39, and 40°C.

RESULTS

Genetic stability of the A/Udorn/72 *ts* mutants in hamster lungs. The A/Udorn/72 wild-type virus was mutagenized with 5-fluorouracil, and nine *ts* mutants were isolated from 1,170 plaques. These nine *ts* mutants failed to produce plaques at 39°C in MDCK monolayer culture and were next evaluated for their level of replication and genetic stability in hamster lungs (Table 1). As a first step, the level of genetic stability of the *ts* mutants was compared with that of the A/Udorn/72 *ts*-1[E] virus that contains a *ts* P3 and a *ts* NP gene. The A/Udorn/72 *ts*-1[E] virus was chosen for such a comparison because this virus replicated in the hamster lungs but was unstable in that approximately 25% of lung isolates contained ts^+ virus (17). In volunteers, this reassortant exhibited definite, but not complete, attenuation (22). Therefore, we sought to identify *ts* mutants that were at least as stable as the *ts*-1[E] virus.

Three A/Udorn/72 *ts* mutants, *ts*-368, *ts*-651, and *ts*-559, were as stable as the *ts*-1[E] virus (Table 1). Of these three *ts* mutants, the A/Udorn/72 *ts*-368 mutant was chosen as one parent in the production of a new double-lesion *ts* parent for several reasons. First, *ts*-368 was the most restricted mutant in its replication in the lung (Table 1). Second, complementation analysis suggested that the *ts*-368 mutant contained only one *ts* gene, the NP gene. This was confirmed by segregational analysis in which the genotype of eight WSN × *ts*-368 *ts* reassortants was determined. The genotype of one WSN reassortant, *ts* 218, is presented in Fig. 1. It received seven WSN RNA segments, with the NP gene being derived from the *ts*-368 parent, and behaved in complementation analysis like its *ts* parent. This identifies the NP gene as the locus of the *ts*-368 mutation. Third, by complementation and segregational analysis (data not presented), *ts*-651 and *ts*-559 viruses were suspected of having a *ts* mutation on the gene that codes for hemagglutinin or neuraminidase surface glycoprotein. A *ts* mutation in either of these genes would not be an acceptable locus of a *ts* mutation in an influenza A *ts* donor virus, and further evaluation of these mutants was not pursued. In this manner, the single-lesion *ts*-368 NP mutant which was at least as restricted and as stable as the double-lesion *ts*-1[E] virus was selected as one parent in the production of a new double-lesion *ts* parent.

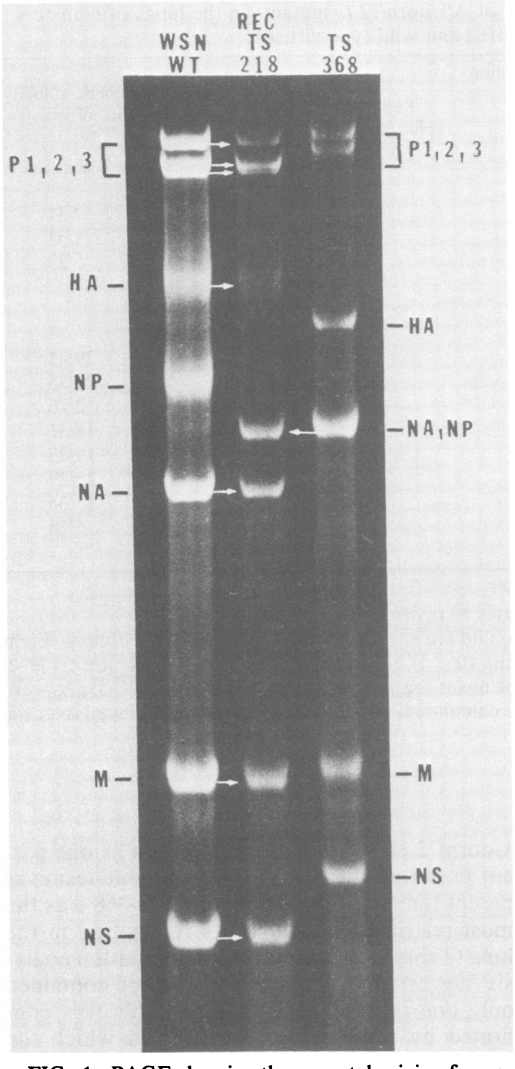

FIG. 1. PAGE showing the parental origin of genes in the WSN reassortant. The migration of viral RNA from parent viruses, WSN wild-type (left lane), and A/Udorn/72 *ts*-368 (right lane) was compared with that of the reassortant virus WSN *ts*-218 (middle lane) by electrophoresis at room temperature on a 2.6% polyacrylamide gel (11 cm) in the presence of 4.5 M urea for 8 h at 70 V. Arrows indicate the parental origin of genes in the reassortant.

Production and characterization of a new double-lesion *ts* mutant. The A/Victoria/75 *ts*-1A2 reassortant clone 65 has a *ts* P3 gene, a 38°C shutoff temperature, and a high level of genetic stability in that none of 44 hamster lung isolates were ts^+ (4, 12). This virus was mated with the newly produced A/Udorn/72 *ts*-368 mutant, and the *ts* reassortant progeny were characterized for the presence of the *ts* P3, the *ts* NP, or both *ts* genes as previously described (14). Eighty-seven plaque progeny were tested and 35 were *ts*. Twelve had the *ts* P3 gene, 20 had the *ts* NP gene, and 3 had both of the *ts* genes. The genetic characterization of cloned suspensions of each of the three double-lesion viruses and representative single-lesion viruses is presented in Table 2. Reassortant clones 55, 53, and 20 each failed to complement their parent viruses, which indicated that they contained both the *ts* NP gene (from the A/Udorn/72 *ts*-368 virus) and the *ts* P3 gene (from the A/Victoria/75 *ts*-1A2 clone 65 virus). It could not be ascertained by complementation or antigenic analysis (Table 3) whether clones 76 and 63 were indeed reassortant viruses. However, they represented parallel passaged single-lesion progeny viruses containing the *ts* P3 or *ts* NP gene, respectively, and it was thus useful to compare their EOP and other biological properties with those of the double-lesion reassortant viruses.

TABLE 2. Complementation-recombination between *ts* progeny viruses and the A/Victoria/75 *ts*-65 and A/Udorn/72 *ts*-368 parent viruses

Progeny clone	Ratio[a] of no. of plaques observed at 39°C to expected no. of cells dually infected with the indicated pairs of virus	
	A/Victoria/75 *ts*-65[b]	A/Udorn/72 *ts*-368[b]
55	0.0008	0.0006
53	0.002	0.0003
20	0.0003	0.0001
76	0.0003	25
63	178	0.0009

[a] The number of dually infected cells was estimated on the assumption that the distribution of the virus to the cell fits the Poisson distribution, using the formula $(1 - e^{-mA})(1 - e^{-mB})$ (number of cells), where mA and mB were the multiplicities of infection of the two infecting viruses determined at the permissive temperature of 34°C. A value of approximately 1 was arbitrarily taken to indicate that complementation-recombination had occurred, and values less than 0.1 indicated the absence of complementation-recombination.

[b] Clone 65 contains a single *ts* mutation on the P3 gene. Clone 368 contains a *ts* mutation on the NP gene. The ratio of the expected number of plaques to the observed number in matings of these two viruses was 21.

The antigenic characterization and the EOP of the A/Udorn/72 *ts*-368 × A/Victoria/75 *ts*-65 progeny viruses is presented in Table 3. The three double-lesion viruses were 10- to 700-fold more restricted in replication at 37°C than the parallel passaged progeny clones 76 (*ts* P3) and 63 (*ts* NP). This greater degree of temperature sensitivity was reflected in a greater restriction of replication in hamster pulmonary tissue (Table 4). Importantly, the three new double-lesion mutants exhibited the same level of replication

TABLE 3. EOP of *ts* progeny viruses and the A/Victoria/75 *ts*-65 and A/Udorn/72 *ts*-368 parent viruses

Influenza A virus	Clone designation	Putative locus of *ts* lesion	Surface antigen[a]		Log_{10} reduction[b] of virus titer (PFU/ml) at indicated restrictive temp (°C)				Shutoff temp (°C)[c]
			HA	NA	36	37	38	39	
A/Victoria/75 *ts*-65 × A/Udorn/72 *ts*-368 progeny	55	P3, NP	H3-72	N2-72	1.2	3.6	>5.2	>5.2	37
	53	P3, NP	H3-75	N2-72	0.8	2.3	>5.0	>5.0	37
	20	P3, NP	H3-72	N2-72	0.4	1.8	>5.6	>5.6	38
	76	P3	H3-75	N2-65 or 68	0.3	0.2	>5.1	>4.9	38
	63	NP	H3-72	N2-72	0.3	0.9	>4.7	>5.3	38
A/Victoria/75 *ts*-65 parent	65	P3	H3-75	N2-65 or 68	0.1	0.6	>4.6	>5.6	38
A/Udorn/72 *ts*-368 parent	368	NP	H3-72	N2-72	0.5	1.6	3.9	>6.2	38

[a] HA, Hemagglutinin; NA, neuraminidase.
[b] Average value of two to five tests.
[c] Shutoff temperature is defined as the lowest temperature at which a 100-fold or greater reduction of virus titer is observed.

TABLE 4. Comparison of growth and genetic stability of single- and double-lesion *ts* progeny viruses in the lungs and nasal turbinates of hamsters with growth and stability of the A/Udron/72 *ts*-1[E] and wild-type viruses

Influenza A virus	*ts* lesion in indicated gene	Shut-off temp (°C)	Virus replication [maximum level of virus titer (mean log_{10} $TCID_{50}$/g)][a]		Genetic stability	
			Lungs	Nasal turbinates	No. of isolates ts^+[b]/no. tested (lungs)	No. of isolates ts^+/no. tested (nasal turbinates)
Clone derived from mixed infection with *ts*-65 and *ts*-368 virus						
55	P3, NP	37	2.7 ± 0.7	5.1 ± 0.3	0/2	0/20
53	P3, NP	37	2.5 ± 0.6	5.6 ± 0.3	0/6	0/28
20	P3, NP	38	2.5 ± 0.5	5.1 ± 0.3	0/1	0/17
76	P3	38	3.5 ± 0.7	6.0 ± 0.5	0/24	0/20
63	NP	38	5.8 ± 0.4	4.9 ± 0.2	0/2	1/19
A/Udorn/72 *ts*-1[E]	P3, NP	38	4.2 ± 0.5	5.8 ± 0.4	8/25	0/32
A/Udorn/72 wild type		>40	8.1 ± 0.2	6.1 ± 0.6		

[a] Values represent mean log_{10} titers ± standard error and are expressed as $TCID_{50}$ per gram. Eight hamsters were sacrificed daily for 4 days after receiving $10^{4.5}$ $TCID_{50}$ of virus intranasally. The lowest level of virus detectable was $10^{2.0}$ $TCID_{50}$/g of lungs and $10^{2.3}$ $TCID_{50}$/g for nasal turbinates. For calculation of mean log_{10} titer, tissues with $<10^{2.0}$ $TCID_{50}$/g of lungs or $<10^{2.3}$ $TCID_{50}$/g of nasal turbinate were considered in the calculations to contain $10^{1.5}$ $TCID_{50}$ for lungs or $10^{1.8}$ $TCID_{50}$ for nasal turbinates. Daily mean titers were calculated including only animals from which virus was recovered, and the maximum level achieved over the 4-day period is included.
[b] ts^+ is defined as an isolate that produces plaques at restrictive temperature (39°C).

in lungs and turbinates and were approximately 80-fold more restricted in the lungs than the *ts*-1[E] virus. The level of restriction of replication of the three double-lesion reassortants in the lungs was comparable to that of the A/Udorn/72 *ts*-1A2 and its reassortants (9, 12–14). Of 12 isolates from the lungs of hamsters infected with one of the three double-lesion *ts* mutants, none was ts^+. In contrast, 8 of 25 isolates from the lungs of *ts*-1[E] virus-infected hamsters were ts^+, and this is significantly greater than that present in the isolates of the new double-lesion viruses ($P < 0.05$, Fisher exact test).

The parental origin of P1, P2, P3, HA, and NA genes in the A/Udorn/72 *ts* 368 × A/Victoria/75 *ts* 65 progeny was determined by PAGE. The origin of the gene coding for the M protein in both parental viruses was from the A/Udorn/72 virus (4), and thus the inheritance of this gene in the *ts* reassortants could not be assessed. De-

TABLE 5. Alteration of *ts* phenotype of *ts* viruses by growth at semipermissive temperatures

Virus	Virus grown at indicated temp (°C)	Log_{10} reduction of virus titer (PFU/ml) at indicated restrictive temp (°C) from titer at permissive temp (34°C)					Shutoff temp (°C)
		36	37	38	39	40	
A/Udorn/72 *ts*-1A2	34	1.1	>3.7	>5.1	>5.1	NT[a]	37
	36.5	0.9	>3.8	>3.8	>3.8	NT	37
	36.5, 37	0.3	0.7	1.4	>4.4	NT	39
	36.5, 37, 38	−0.2	0.2	0.5	2.1	3.9	39
	36.5, 37, 38, 39	0.0	−0.1	0.1	0.4	1.6	>40
A/Udorn/72 *ts*-20	34	0.4	>4.2	>4.2	>4.2	NT	37
	36.5	0.6	>4.4	>5.4	>5.4	NT	37
	36.5, 37	0.4	0.9	>5.1	>5.1	NT	38
	36.5, 37, 38	0.3	0.3	0.6	>4.4	NT	39
	36.5, 37, 38, 39	0.0	0.0	0.4	0.9	3.4	40
A/Udorn/72 *ts*-55	34	1.8	>4.9	>4.9	>5.9	NT	37
	36.5	0.1	3.6	>4.6	>4.6	NT	37
	36.5, 37	0.3	1.2	>6.3	>6.3	NT	38
	36.5, 37, 38[b]	0.0	0.1	1.0	>5.3	NT	39

[a] NT, Not tested.
[b] Virus from flask harvested at 38°C was amplified at 34°C before its EOP was determined.

spite many attempts with various sets of conditions, the parental origin of the NP and NS genes could not be determined by PAGE. However, this analysis did confirm the presence of the *ts* P3 gene in clones 55, 53, 20, and 76 (data not presented). Again, it is not possible to determine whether clones 63 and 76 were indeed reassortants because they had genotypes identical to their corresponding single-lesion parents at the five distinguishable loci. The three double-lesion reassortants had three distinct genotypes, indicating that they were three independently derived clones.

Comparison of the genetic stability of the new *ts* P3 *ts* NP double-lesion viruses with the A/Udorn/72 *ts*-1A2 viruses in vitro and in vivo. Since both the A/Udorn/72 *ts*-1A2 virus and the new *ts* P3 *ts* NP double-lesion reassortants did not lose their *ts* phenotype after replication in normal hamsters, additional tests were used to compare the relative genetic stability of these two defective viruses. An in vitro stress test was performed in which the *ts* virus was grown at semipermissive temperature, and the resulting virus yield was characterized by determining its EOP. The virus harvested was then serially passaged at higher temperatures, and the EOP of each harvest was determined (Table 5). The *ts*-1A2 virus manifested a loss of temperature sensitivity after replication at 37°C, and the virus present in the 37°C harvest fluid had a 39°C shutoff temperature. This indicated that a change in shutoff temperature of two degrees (i.e., from 37 to 39°C) occurred after replication at 37°C. This degree of change was also observed for the A/Alaska/77 *ts*-1A2 (H3N2) and A/Hong Kong/77 *ts*-1A2 (H1N1) reassortants that were similarly analyzed (data not presented). The *ts*-1A2 virus passaged at 39°C exhibited a shutoff temperature of greater than 40°C (Table 5). Clones 20 and 55 also underwent a progressive loss of temperature sensitivity after passage at semipermissive temperatures, but failed to manifest the 2-degree jump (37 to 39°C shutoff temperature) seen with the *ts*-1A2 reassortants after replication at 37°C. Clone 20 retained some temperature sensitivity even after passage at 39°C. Clone 55 appeared more defective than clone 20 in that amplification (at 34°C) of the virus grown at 38°C was required before sufficient virus was obtained to measure its EOP. In addition, virus harvested after passage at 39°C did not produce plaques at 34°C.

To assess the genetic stability of the double-lesion viruses in vivo, hamsters were administered an immunosuppressive drug (cyclophosphamide) to impair the immunologically mediated clearance of the virus from their respiratory tract. Isolates from the nasal turbinates were obtained and their EOP was determined (Table 6). Clone 55 was chosen for this study because it appeared to be slightly more defective than clone 20 in the in vitro stress test. Treatment of hamsters with cyclophosphamide prolonged virus shedding so that it was comparable to that seen in fully susceptible double-seronegative children who were administered vaccine virus (2, 23). Without the drug, hamsters normally cleared the virus by day 7 after infection. In this manner, the genetic stability of the two double-lesion viruses could be assessed in vivo. Only isolates from the nasal turbinates were charac-

TABLE 6. Replication of influenza A/Udorn/72 *ts*-1A2 and *ts*-55 viruses in cyclophosphamide-treated hamsters

Influenza virus administered	Day after virus administered	No. of isolates tested	No. of isolates with indicated shutoff temp (°C)[a]		
			37	38	39
ts-1A2	4	6	4	2	0
	7	7	7	0	0
	9	24	12	12	0
	12–15	7	0	2	5
ts-55	4	10	10	0[b]	0
	7	10	10	0	0
	9	22	18	4	0
	12–15	5	2	2	1

[a] *ts*-1A2 and *ts*-55 viruses administered to hamsters each had a 37°C shutoff temperature.
[b] Statistically significant difference ($P < 0.05$, Fisher exact test) between values at the ends of the brace.

terized because too few isolates were obtained from the lungs to permit a comparison of the two viruses. Progressive loss of temperature sensitivity of the two viruses was first detected on day 9 (Table 6). At this time, 50% of the *ts*-1A2 isolates had an altered EOP compared with 22% of the *ts*-55 isolates ($P < 0.05$, Fisher exact test). By days 12 to 15, all of the *ts*-1A2 isolates manifested a decreased level of temperature sensitivity, as did 60% of the clone 55 isolates.

DISCUSSION

The induction of *ts* mutation by chemical mutagenesis has been successfully used to attenuate respiratory viruses for humans (1). For influenza A virus, an acceptable balance between the attenuation of a *ts* virus and its immunogenicity has been achieved even for fully susceptible doubly seronegative individuals (1). For respiratory syncytial virus it has been possible to attenuate the virus for adults and for children, although an acceptable level of attenuation for fully susceptible individuals has not been achieved (1). The major problem with this approach to the production of attenuated viruses has been the loss of the temperature sensitivity of the virus after replication in fully susceptible individuals (22; Tolpin et al., submitted). The influenza A/Alaska/77 *ts*-1A2 reassortant virus was able to escape from its *ts* phenotype during replication in humans by developing mutations not only on the gene bearing the *ts* mutation but also on another gene that presumably suppressed the *ts* phenotype of the protein encoded by the *ts* mutant gene (22). These observations indicate that the *ts* phenotype of a virus can be corrected not only by true reversion but also by extragenic and probably intragenic suppressor mutation. This is a partial explanation for the ease with which the *ts* phenotype can be modified, or lost, even in a virus that contains two *ts* genes.

In the present study we examined the possibility of producing a virus more stable genetically than the A/Udorn/72 *ts*-1A2 donor virus. This was attempted in a manner analogous to the method in which the A/Udorn/72 *ts*-1A2 virus itself was produced, i.e., by constructing a double *ts* mutant by mating two stable single-lesion *ts* mutants. The most stable *ts* gene we have is the *ts* P3 gene derived from the A/Great Lakes/68 *ts*-1 (H2N2) virus. This gene was present in both A/Udorn/72 *ts*-1A2 donor virus and the new *ts* P3 *ts* NP reassortant virus produced in the present study (6, 14). A relatively stable virus bearing a *ts* NP gene was identified, and the *ts* NP gene was combined by genetic reassortment with the stable *ts* P3 gene. This new *ts* P3 *ts* NP potential donor virus proved to be as restricted in replication in the lungs of hamsters as the *ts*-1A2 virus. Next, tests were developed to assess the tendency of the *ts* P3 *ts* NP donor virus to lose its *ts* phenotype after replication in vitro and in vivo and to compare this with that of the *ts*-1A2 donor virus. The new *ts* P3 *ts* NP reassortant viruses were slightly more stable than the *ts*-1A2 virus in vitro and significantly more stable in vivo. However, the *ts* P3 *ts* NP reassortants still underwent progressive loss of the *ts* phenotype after replication in vitro at semipermissive temperatures and in vivo in immunosuppressed hamsters after 9 to 15 days of replication. This magnitude of loss of the *ts* phenotype was sufficient to preclude more extensive evaluation in humans of the *ts* P3 *ts* NP donor virus and reassortants derived from it. Ideally, one would like to identify a *ts* virus that would retain its phenotype under these stresses. Even by introducing two genes bearing our most stable *ts* mutations into a single virus, we have not been able to achieve a satisfactory level of

genetic stability. This conclusion has been reached after extensive efforts to generate a large number of *ts* mutations (6, 19). Considering the experience with the *ts*-1[E] and *ts*-1A2 viruses in humans and the *ts* P3 *ts* NP mutants evaluated in the present study, it seems reasonable to conclude that vaccine viruses that rely solely on their *ts* phenotype as their attenuating property will not be sufficiently stable (22, 23; Tolpin et al., submitted). It is important to emphasize that such considerations do not necessarily apply to other *ts* viruses such as the A/Ann Arbor/6/60 cold-adapted reassortants since evidence suggests that the *ts* phenotype of these viruses is not the sole factor contributing to the attenuation of the virus for humans (10).

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