JOURNAL OF VIROLOGY, Apr. 1997, p. 2772–2778 Vol. 71, No. 4
0022-538X/97/$04.00+0

Genetically Engineered Live Attenuated Influenza A Virus Vaccine Candidates

NEIL T. PARKIN,* PEGGY CHIU, AND KATHLEEN COELINGH
AVIRON, Mountain View, California 94043

Received 30 September 1996/Accepted 9 December 1996

We have generated new influenza A virus live attenuated vaccine candidates by site-directed mutagenesis and reverse genetics. By mutating specific amino acids in the PB2 polymerase subunit, two temperature-sensitive (*ts*) attenuated viruses were obtained. Both candidates have 38°C shutoff temperatures in MDCK cells, are attenuated in the respiratory tracts of mice and ferrets, and have very low reactogenicity in ferrets. Infection of mice or ferrets with either mutant conferred significant protection from challenge with the homologous wild-type virus. Three tests for genetic stability were used to assess the propensity for reversion to virulence: 14 days of replication in nude mice, growth at 37°C in tissue culture, and serial passage in ferrets. One candidate, which contains mutations intended to reduce the ability of PB2 to bind to cap structures, was stable in all three assays, whereas the second candidate, which contains mutations found only in other *ts* strains of influenza virus, lost its *ts* phenotype in the last two assays. This approach has therefore enabled the creation of live attenuated influenza A virus vaccine candidates suitable for human testing.

Influenza viruses are responsible for annual epidemics of respiratory disease associated with excess morbidity and mortality, particularly in the elderly and others with high-risk conditions (10, 12, 13). Many of the more serious effects of infection can be mitigated by prior vaccination against influenza with an inactivated, parenterally administered vaccine (22). However, the efficacy of the inactivated vaccine is suboptimal in children and in the elderly (11, 18, 35, 37). An alternative is vaccination with live attenuated viruses, which can be administered intranasally. Such live vaccines induce an immune response which more closely mimics that raised after natural infection and theoretically should be more protective and longer lasting than that induced by injection of the killed vaccine (14–17). Provided that the attenuating mutations lie in genes which encode proteins other than the hemagglutinin (HA) or neuraminidase (NA), the attenuated "donor" strain can be used on an annual basis to generate vaccines by reassortment with the HA and NA genes of currently circulating wild-type viruses.

A candidate live influenza virus vaccine, the cold-adapted variant of A/Ann Arbor/6/60, has been developed by Maassab et al. (26). This vaccine appears to be efficacious in children and young adults but may be too attenuated to stimulate an ideal immune response in individuals who have been exposed to many influenza virus infections during their lifetimes (39, 40). While the vaccine has phenotypes of cold adaptation, temperature sensitivity, and attenuation in ferrets and humans, the mechanism for these phenotypes is not well understood. The stability of the attenuation may be explained by evidence that there are mutations in as many as four genes (6, 19, 20, 43).

An established basis for attenuation of influenza viruses is temperature sensitivity (32). Such temperature-sensitive (*ts*) viruses are able to replicate only in the upper respiratory tract (URT), which is usually several degrees cooler than the lower respiratory tract (LRT). They are thus unable to cause LRT disease but are capable of stimulating a local and systemic immune response as a result of their replication in the URT. Previously described *ts* vaccine candidates have not been found to be genetically stable and have not been developed further (48, 51). All future candidates must therefore be highly resistant to reversion in order to be useful.

Our approach toward the goal of an improved live vaccine is to introduce discrete mutations into the genome of a wild-type influenza virus to attenuate its virulence. Previously, we (36) and others (45, 46) have described the generation by reverse genetics techniques of *ts* mutants of influenza A virus bearing mutations in the PB2 polymerase subunit. We expect that by combining several different types of mutations, each of which has a small effect on replication in the host, the level of attenuation can be fine-tuned appropriately. The greatest level of genetic stability will be achieved by combining a sufficient number of independently acting mutations which, because of the nature of the defect they impose on viral gene function(s), are unlikely to revert.

One potential way to generate a stable attenuated mutant would be to target, by site-directed mutagenesis of multiple nucleotides within multiple codons, essential functional sites within a viral protein which interacts directly with a host factor. Such mutations are less likely to be suppressible by a second mutation in a viral gene than are those which target residues that interact with another viral protein or RNA. Their effects should therefore be more stable. We have attempted to do this by targeting the cap-binding function of the viral polymerase subunit PB2. Binding to the cap 1 structure at the 5′ end of host cell mRNAs followed by endonucleolytic cleavage of the mRNA 10 to 14 nucleotides downstream is the mechanism by which influenza virus RNA polymerase generates primers for transcription of its mRNAs (4, 38, 50). Our results indicate that viruses containing these mutations are more stable than a comparably attenuated *ts* strain without mutations in the potential cap-binding domain, suggesting that this approach may be useful in generating a live attenuated influenza virus vaccine.

MATERIALS AND METHODS

Cells and viruses. Primary chicken kidney (PCK) cells were obtained from 3-day-old chicken kidneys as described previously (24). MDCK cells were ob-

* Corresponding author. Present address: Virologic, 270 E. Grand Ave., South San Francisco, CA 94080. Phone: (415) 638-1100, ext. 238.

tained from the American Type Culture Collection and maintained in Eagle minimal essential medium containing 10% fetal bovine serum and antibiotics.

Influenza virus A/Los Angeles/2/87 (H3N2) and the PB2 host range helper virus, a single-gene-reassortant (SGR) virus containing the PB2 gene from A/Mallard/New York/6750/78 (H2N2) and the remaining seven segments from A/LA/2/87 (lot E-287) described by Clements et al. (5), were obtained from L. Potash (DynCorp/PRI, Rockville, Md.). The ts1A2 vaccine candidate was obtained from Brian Murphy (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases), plaque purified once in MDCK cells, and grown in specific-pathogen-free eggs (SPAFAS) at 33°C.

DNA manipulations and site-directed mutagenesis. The PB2 gene of A/LA/2/87 was cloned and sequenced as described previously (36). The P112S mutant PB2 cDNA was generated by cassette mutagenesis with fragments amplified by PCR. A primer which contained the sequence of a nearby unique restriction site (*Tth*III1), as well as the sequence of the mutation, was used in conjunction with a primer of the opposite sense distal to a unique restriction site in the pUC19 vector (*Bam*HI). All other mutations were generated with the Chameleon mutagenesis kit (Stratagene, La Jolla, Calif.) and appropriately designed oligonucleotides, the sequences of which are available upon request. Combination mutations were constructed either by subcloning, when restriction sites allowed it, or by additional rounds of site-directed mutagenesis. The following mutants were assembled by these methods: E65G/P112S/N265S (3ts), E65G/P112S/N265S/N556D/Y658H (5ts), W552F/W557F/W564 (3WF), and E65G/P112S/N265S/W552F/W557F/W564F (3ts/3WF). The nucleotide sequences of all junctions of clones assembled by subcloning and the complete cDNA sequence of the 5ts and 3ts/3WF clones were determined. No changes apart from those introduced intentionally were found.

Reverse genetics. Transfection of PCK cells with altered PB2 genes, using the PB2 single-gene reassortant helper virus, were performed as described previously (36). Transfectant viruses were identified after selection in MDCK cells by reverse transcription-PCR and restriction enzyme digestion, plaque purified, and amplified in specific-pathogen-free eggs.

Phenotypic analysis. Temperature sensitivity was assayed by plaque assay in MDCK cells at various temperatures; stringent temperature control was achieved by incubating the six-well dishes in watertight containers submerged in waterbaths regulated with Lauda immersion circulators (36). Replication in mice and replication and reactogenicity in ferrets were carried out as described previously (36). Briefly, the animals were anesthetized and infected intranasally; 3 days after infection, nasal turbinates and lungs were removed, homogenized in a volume of medium to approximate a 10% (wt/vol) homogenate, and subjected to titer determination by infectivity assays in MDCK cells (mice) or eggs (ferrets).

HI assay. Serum samples taken from the tail veins of immunized mice were treated with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan) and heated to 56°C for 30 min before use. Hemagglutination inhibition (HI) was performed with 8 HA units of A/LA/2/87 and 0.5% chicken erythrocytes, as described previously (2).

Assays for genetic stability. (i) Nude mice. Studies with nude mice were performed at Sierra Biosource, Inc. (Gilroy, Calif.). Forty BALB/c *nu/nu* mice (3 to 4 weeks old) were anesthetized and infected intranasally with 10^5 PFU of ts1A2 virus (due to the low titer obtained from eggs, this is the maximum possible dose in 50 μl) or with 10^6 PFU of 5ts or 3ts/3WF. The mice were sacrificed 13 or 14 days later; in a previous study, replication was still detected at this time (46). Homogenates (approximately 10% wt/vol) prepared from nasal turbinates and lungs were subjected to titer determination by the 50% tissue culture infective dose ($TCID_{50}$) assay in MDCK cells at 34°C in 96-well plates. Once the cytopathic effect (CPE) was complete in the wells of the 96-well plate, the medium was harvested and pooled (when more than one well was positive). To determine if virus in this pool was still *ts*, it was subjected to titer determination by the $TCID_{50}$ assay at 34 and 37°C (ts1A2), or 34 and 39°C (5ts and 3ts/3WF). The choice of nonpermissive temperature was based on the shutoff temperature of the infecting virus. Viral pools which showed evidence of reversion were examined by plaque assay to confirm the phenotypic alterations.

(ii) Tissue culture stress test. MDCK cells were infected in 35-mm dishes at low multiplicity of infection (approximately 0.01) and incubated at temperatures ranging from 34 to 38°C (33, 49). Supernatants were harvested 2 to 3 days later from cultures incubated at the highest temperatures still showing CPE (36°C for ts1A2, 37°C for 5ts and 3ts/3WF). Viruses present in these supernatants were then tested for changes in the *ts* phenotype by plaque assay at various temperatures.

(iii) Serial passage in ferrets. All ferret studies were performed in the laboratory of H. F. Maassab, University of Michigan. Virus from the nasal turbinate homogenates of four ferrets infected with 3ts/3WF or 5ts was amplified in eggs, pooled, and used to infect two new ferrets each; after another amplification in eggs, this process was repeated. The ferrets were sacrificed 3 days after infection in each cycle. The body temperatures of the live, infected ferrets were recorded twice daily. The *ts* phenotype was assessed after each cycle of infections by performing plaque assays at 34°C and 38°C in MDCK cells with the egg fluid from the infectivity titer determinations of turbinate homogenates. After the last cycle, two plaques were picked from each of the two animals turbinate virus pools (grown at 34°C), amplified in eggs, and subjected to titer determination at 34, 38, and 39°C.

TABLE 1. Mutations introduced into A/LA/2/87 PB2

Mutation[a]	Wild-type codon	Mutant codon
E65G	GAA	GGC
P112S	CCA	AGC
N265S	AAC	TCG
I310T	ATA	ACG
N556D	AAT	GAC
Y658H	TAC	CAC
W552F	TGG	TTC
W557F	TGG	TTC
W564F	TGG	TTC

[a] Mutations are named by using the single-letter code for the wild-type amino acid, followed by its position and the mutant amino acid.

RESULTS

Rationale for selection of PB2 mutations. We chose the PB2 gene as a target for mutagenesis for several reasons: mutations in PB2 have been associated with *ts* and attenuated (*att*) phenotypes (29, 43, 52), several *ts* mutations in PB2 have been molecularly characterized (6, 20, 23), and a helper virus for rescue of PB2 into infectious influenza viruses has been described (45). We chose to generate the following mutations: (i) mutations associated with *ts* phenotypes, for which it was possible to introduce new codons that would require more than one nucleotide change to reencode the wild-type amino acid (E65G, P112S, N265S, or I310T); (i) mutations which are associated with marked attenuation in other *ts* viruses of particular interest (Y658H, found in the PB2 gene of the *ts* mutants ts1A2 and ts1E) (23); or (iii) mutations which have potential functional importance by virtue of their location within a region of partial sequence similarity with the cellular cap-binding protein, eIF-4E (7). Because biophysical studies (21) and mutational analysis (1) have implicated tryptophan residues in the binding of eIF-4E to the cap structure, the tryptophan residues at positions 552, 557, and 564 were mutated to phenylalanine, in each case by introducing two nucleotide changes per codon. The N556D and Y658H mutations could not be engineered such that more than one nucleotide change would be required for reversion to the wild-type amino acid. The precise nature of the nucleotide changes made at each codon is summarized in Table 1.

Initially, PB2 genes bearing each individual mutation were rescued with the single-gene reassortant helper virus and tested for temperature sensitivity in MDCK cells. Most of the single-mutant viruses, including the P112S, N265S, and Y658H mutants, were all only mildly affected. None, with the exception of I310T, had shutoff temperatures less than 40°C, although some (e.g. E65G, N556D, and W557F) showed a 10- to 20-fold reduction in plaque number at 40°C relative to the wild-type PB2 transfectant control (data not shown). The I310T transfectant virus had a shutoff temperature of 39°C. Analysis of the ability of these single-mutant viruses to replicate in the respiratory tracts of mice did not reveal evidence of non-*ts*, attenuating effects (data not shown). Since previous studies have strongly suggested that an acceptable level of attenuation is approached when the shutoff temperature is 38°C or lower (31), combination mutants were created to accentuate the *ts* phenotype.

Phenotypes of combination mutant transfectant viruses in vitro and in vivo. Transfectant viruses containing the following combinations of mutations in PB2 were generated: E65G/P112S/N265S (3ts), E65G/P112S/N265S/N556D/Y658H (5ts), W552F/W557F/W564 (3WF), and E65G/P112S/N265S/W552F/W557F/W564F (3ts/3WF). The two triple mutants (3ts and

TABLE 2. Temperature-dependent growth properties of PB2 combination mutants in MDCK cells

Virus	Titer at 34°C (log_{10} PFU/ml ± SEM)	Reduction in log_{10} PFU/ml (relative to that at 34°C) at[a]:				Shut-off temp (°C)[a]
		37°C	38°C	39°C	40°C	
LA wt	8.57 ± 0.09	−0.02	0.18	0.21	0.37	>40
3WF	8.45 ± 0.21	−0.05	0.02	1.08[b]	**4.62**[b]	40
3ts	8.30 ± 0.08	0.23	0.33	0.92[b]	**3.08**[b]	40
5ts	7.24 ± 0.06	1.06	**3.39**[b]	**4.39**[b]	**>5.54**	38
3ts/3WF	7.29 ± 0.24	0.35[b]	**2.25**[b]	**4.76**[b]	**>6.68**	38

[a] The shutoff temperature is defined as the lowest temperature at which a reduction in the EOP of 100-fold is observed (indicated by numbers in boldface type).
[b] Reduced plaque size.

3WF) were *ts* with a shutoff temperature of 40°C, and they formed small plaques at 39°C (Table 2). The 5ts and 3ts/3WF transfectants both had shutoff temperatures of 38°C, and their efficiency of plaque formation (EOP) and plaque size were slightly reduced at 37°C as well (Table 2).

We next examined the ability of the transfectant viruses containing multiple mutations in their PB2 genes to replicate in the respiratory tract of mice. Three-week-old BALB/c mice were infected intranasally with 10^5 PFU of each virus and analyzed for virus replication in lungs and nasal turbinates 3 days postinfection. The replication of the mutant viruses is summarized in Table 3. The two triple-mutant viruses, which both had a shutoff temperature of only 40°C, grew very poorly (3WF) or not at all (3ts) in mouse lungs. This suggests that the restriction of replication is due to factors other than simply thermosensitivity, since the core body temperature of a mouse is approximately 37°C. Neither 5ts nor 3ts/3WF viruses were detected in lungs, and 3ts/3WF also showed a significant reduction in nasal turbinate titer (300-fold compared to the wild-type).

Ferrets can be used as an animal model to assess the virulence and reactogenicity of influenza viruses (25, 42). Attenuation of replication in nasal turbinates and lungs can be quantitated as above, and body temperature can be monitored as a measure of reactogenicity. The average normal body temperature of ferrets is approximately 39°C (102.2°F), although there can be some variation in the upper and lower limits of the normal temperatures in individual ferrets (e.g., 101.0 to 103.0°F). We have used a body temperature of 39.9°C (103.8°F) or above as an operational definition for fever (25a). A virulent virus usually induces a fever on the first day after infection and is usually also able to replicate in the lungs.

The reduction in virus titers in lungs and nasal turbinates of ferrets infected with 5ts or 3ts/3WF was similar to that seen in mice, with 3ts/3WF replicating about 75-fold less well than the wild type in nasal turbinates (Table 3). Only one of eight ferrets infected with either virus became febrile, both with delayed onset (day 2 after infection). In contrast, all 12 ferrets infected with the wild-type transfectant control developed fevers on the first day after infection (Table 3).

Protection from homologous wild-type virus challenge. To determine whether immunization by 5ts or 3ts/3WF stimulates a protective immune response, an immunization-challenge study was performed in mice. Mice were infected on day 0, as described above, with 5ts, 3ts/3WF, or the parental wild-type virus, A/LA/2/87; on day 28, the mice (including an unimmunized, age-matched control group) were bled and then challenged with 10^5 PFU of A/LA/2/87. Viral titers in lungs and turbinates were measured 3 days later (Table 4).

The titer of serum antibodies which could inhibit hemagglutination of the challenge virus was below the level of detection in the HI assay (<1:40) for 5ts- or 3ts/3WF-infected mice. The HI titer in mice infected with A/LA/2/87, however, was approximately 1:80.

Mice infected with the wild-type virus A/LA/2/87, which replicates to high titers in the lungs and nasal turbinates, were effectively protected from infection following challenge with the same virus; replication was reduced by more than 10,000-fold in the lungs (only 1 of the 10 mice had detectable virus in the lung homogenate) and was undetectable in turbinates (Table 4). The experimental vaccines both evoked a similar protective immune response, as shown by the 1,000- to 2,000-fold reduction in titers achieved by the challenge virus in lungs or nasal turbinates (only 50 to 80% of the samples were positive, compared to 100% for the unimmunized controls). Thus, in spite of its lower replication in turbinates, the degree of protection conferred by 3ts/3WF is equal to that conferred by 5ts.

A similar immunogenicity and protection study was also performed in ferrets. Six animals per group were immunized with 10^7 egg infective doses (EID_{50}) of 5ts or 3ts/3WF. In this experiment, none of the infected animals developed a febrile response (compared to one of eight [Table 3]). Two of these ferrets were sacrificed after 3 days to measure the titers in

TABLE 3. Replication of PB2 combination mutant viruses in BALB/c mice and ferrets

Virus	Shut-off temp (°C)	Log_{10} $TCID_{50}$/g tissue[a] ± SEM in:		No. of ferrets with fever/no. infected	Log_{10} EID_{50}/g of tissue[b] ± SEM in:		No. of ferrets with positive lungs/no. infected
		Mouse NT[c]	Mouse lungs		Ferret NT	Ferret lungs	
LA wt	>40	5.42 ± 0.09	4.21 ± 0.26	12/12	7.64 ± 0.16	3.33 ± 0.31	3/12
3WF	40	5.62 ± 0.10	2.13 ± 0.10[d]	2/2	6.35 ± 1.35	≤2.75	0/2
3ts	40	4.93 ± 0.10[d]	≤1.95	ND[e]	ND	ND	ND
5ts	38	4.39 ± 0.08[d]	≤1.95	1/8	6.54 ± 0.16[d]	≤2.75	0/8
3ts/3WF	38	2.96 ± 0.13[d]	≤1.95	1/8	5.77 ± 0.23[d]	≤2.75	0/8

[a] There were 10 mice per group except for the wild type, for which there were five mice per group in each experiment; results shown are pooled from four experiments and thus represent data from 20 mice. The limit of detection of the $TCID_{50}$ assay is 2.2 log_{10} $TCID_{50}$ per g; negative samples were assigned a value of 1.95 and included in the calculation of mean titers.
[b] The limit of detection of the EID_{50} assay is 3.0 log_{10} EID_{50} per g; negative samples were assigned a value of 2.75 and included in the calculation of mean titers.
[c] NT, nasal turbinates.
[d] $P < 0.001$ with respect to the wild type (wt).
[e] ND, not done.

TABLE 4. Response of immunized mice or ferrets to challenge with A/LA/2/87

Immunizing virus	Log_{10} $TCID_{50}$/g of tissue[a] ± SEM		Response of ferrets to immunization			Response of ferrets to challenge	
	Mouse NT[b]	Mouse lungs	No. with fever/ total no.	Day 3 NT titer (log_{10} EID_{50}/g)	Day 21 HAI titer[e] (range)	No. with fever/ total no.	Day 3 NT titer (log_{10} EID_{50}/g)
None	6.10 ± 0.13	6.27 ± 0.36	0/4		<8	2/2	8.50
3ts/3WF	3.02 ± 0.41[c]	3.27 ± 0.41[c]	0/6	6.34	768 (512–1,024)	0/4	≤2.75
5ts	3.12 ± 0.34[c]	2.75 ± 0.33[c]	0/6	7.60	896 (512–1,024)	1/4	≤2.75
A/LA/2/87	≤1.95[c]	2.02 ± 0.08[c]	ND[d]	ND	ND	ND	ND

[a] Groups of 10 mice were used.
[b] NT, nasal turbinates.
[c] $P < 0.001$ with respect to titers from the same tissue of unimmunized animals.
[d] ND, not done.
[e] Reciprocal of the mean.

nasal turbinates and lungs. Both groups of immunized ferrets developed a good HAI response, measured 21 days after infection (range, 1:512 to 1:1,024 [Table 4]). The remaining four ferrets, as well as two previously uninfected controls, were then challenged on day 21 with 10^8 EID_{50} of A/LA/2/87 (wt). The unimmunized controls both developed fevers and had high virus titers in the nasal turbinates. Ferrets immunized with 3ts/3WF were completely protected from fever, and only one of four 5ts-immunized animals developed a fever, which was very mild (Table 4). No virus was detected in the nasal turbinates of the 5ts- or 3ts/3WF-infected ferrets. Thus, the immunized ferrets were protected from challenge, at least with respect to URT virus replication and febrile reactions. No virus was detected in the lungs of any of the ferrets after challenge, including the two unimmunized animals (data not shown); thus, conclusions about protection of the LRT cannot be drawn reliably from this experiment.

Genetic stability. The main advantage of the genetic engineering method of attenuating virulence over previously used empirical ones is the potential to be able to design a mutant virus with a high level of genetic stability. As a point of comparison in some of our stability tests, we have used the *ts* mutant, ts1A2, which was previously found to be genetically unstable in a seronegative young vaccinee (34, 47, 48). This virus has a shutoff temperature of 37°C and contains mutations in both the PB1 and PB2 genes (23, 27, 28).

We have used three assays for genetic stability: replication in immunodeficient mice, growth at elevated temperatures in cell culture, and multiple passage through ferrets. After each test, the *ts* phenotype was reevaluated; in the ferret passage test, the passaged viruses were also evaluated for the attenuation phenotype.

The first assay exploits the prolonged virus replication (at least 14 days) that occurs in nude mice (46), which approximates the duration of shedding in completely susceptible humans (up to 11 days) (51). Forty BALB/c *nu/nu* mice (3 to 4 weeks old) were anesthetized and infected intranasally with ts1A2, 5ts, or 3ts/3WF. The mice were sacrificed 13 to 14 days later. Homogenates prepared from nasal turbinates or lungs were used to infect MDCK cell cultures at 34°C. None of the lung homogenates contained detectable virus (≥2.2 log_{10} $TCID_{50}$/g). Virus was detected in 30 of 40 nasal turbinate samples from ts1A2-infected mice, 21 of 40 5ts-infected mice, and 23 of 36 3ts/3WF-infected mice (four mice died on days 3 to 4 due to unknown causes). To determine if the recovered viruses retained the *ts* phenotype, their infectivity in MDCK cells at permissive (34°C) and nonpermissive (37°C for ts1A2, 39°C for 5ts and 3ts/3WF) temperatures was compared.

Both 5ts and 3ts/3WF retained their *ts* phenotypes; the differences in the $TCID_{50}$ per milliliter between 34 and 39°C for the inoculum or for the pools of viruses recovered from the MDCK cell cultures infected with the turbinate homogenates were all greater than 1,000-fold (range, 1,000- to >100,000-fold).

In contrast, 10% (3 of 30) of the isolates from ts1A2-infected mice showed a reduction in titer at 37°C compared to 34°C of less than 1,000-fold (range, 20- to 200-fold); this reduction in titer, while indicating that the viruses are still *ts*, was significantly smaller than that observed for the inoculating virus or for the other isolates (>2,000-fold reduction). That this was indeed indicative of a profound alteration in the phenotype of the virus was later confirmed by plaque assays, where plaques formed by the revertant isolates were larger and more numerous at the nonpermissive temperature (37°C) than were those of the controls (data not shown).

While the differences in frequency of reversion between ts1A2 (3 of 30) and either recombinant virus (0 of 21 or 0 of 23) are not statistically significant, the data suggest that 5ts and 3ts/3WF are more genetically stable than ts1A2.

The second test for genetic stability involves growth in MDCK cells at temperatures which approach the shutoff temperature. This temperature "stress test" has been used previously to isolate ts^+ revertants of other *ts* vaccine candidates, such as ts1A2 (33), or of a *ts* virus containing the PB2 gene of the A/AA/6/60 *ca* strain (49). MDCK cells were infected at low multiplicity of infection and incubated at 34, 36, 37, or 38°C. Supernatants were harvested from cultures incubated at the highest temperatures and still showing CPE (36°C for ts1A2, 37°C for 5ts and 3ts/3WF). Virus present in these supernatants was then tested for changes in the *ts* phenotype by plaque assay at various temperatures. Since the probability of a specific genetic change occurring which causes a phenotypic change may vary and may be low enough that it would not be detected after only one round of replication at high temperature, the experiment was performed at least twice for each virus.

In three independent experiments, the phenotype of 3ts/3WF was unchanged after growth at 37°C (the reduction in the PFU per milliliter at 38 versus 34°C remained greater than 500-fold); however, 5ts showed loss of the *ts* phenotype every time the experiment was performed (the reduction in the PFU per milliliter at 38 versus 34°C was less than 10-fold). This result is striking in relation to the stability observed in nude mice and suggests that the stress test is a more rigorous test for genetic stability. The shutoff temperature of ts1A2 grown at 36°C increased in one of two experiments (the reduction in PFU per milliliter at 37 versus 34°C was 10-fold, compared to >10,000-fold for the original ts1A2 virus); in the second case, an additional round of replication at 37°C was required before reversion was observed.

The third genetic stability assay is multiple passage in ferrets. Virus present in the nasal turbinate homogenates of four of the ferrets infected with 3ts/3WF or 5ts (Table 3) was amplified in eggs, pooled, and used to infect two additional ferrets

TABLE 5. Genetic stability of 3ts/3WF and 5ts in ferrets

Infection	Animal no.	5ts			3ts/3WF		
		Peak ferret temp (°F)[a]	*ts* phenotype of recovered virus		Peak ferret temp (°F)[a]	*ts* phenotype of recovered virus	
			Log_{10} reduction 38 vs. 34°C	Log_{10} reduction 39 vs. 34°C		Log_{10} reduction 38 vs. 34°C	Log_{10} reduction 39 vs. 34°C
First infection	1	103.0	0.8	ND[b]	102.8	>3.9	ND
	2	102.8	0.7	ND	**104.2**	>3.8[c]	ND
	3	102.6	0.9	ND	103.2	2.3[d]	ND
	4	103.2	0.4	ND	103.0	3.8[d]	ND
Second infection (pool of animals 1–4)	5	**104.4**	0.5	ND	103.6	2.7[d]	ND
	6	**103.8**	0.4	ND	102.8	2.6[d]	ND
Third infection (pool of animals 5 and 6)	7	103.2	0.9	ND	102.8	1.4[d]	ND
	8	**103.8**	0.3	ND	103.6	2.3[d]	ND
Animal 7							
plaque A		ND	0.7[d]	1.7[d]	ND	>3.9	>4.9
plaque B		ND	0.7[d]	2.5[d]	ND	>3.8	>4.8
Animal 8							
plaque A		ND	0.4	0.7	ND	>3.5	>4.5
plaque B		ND	0.0[d]	0.9[d]	ND	>2.7	>3.7

[a] Temperatures above 103.6°F (bold type) are considered febrile; normal body temperatures ranged from 101.0 to 103.0°F.
[b] ND, not done.
[c] This isolate was plaque purified and reamplified before titer determination due to the low titer of the original egg stock.
[d] Small plaques.

each; this process was repeated once more, giving a total of three sequential passages in ferrets. The body temperatures of the infected ferrets was recorded for 3 days following each infection; the peak temperatures are indicated in Table 5.

The two ferrets infected with 5ts virus passaged once in ferret turbinates (animals 5 and 6 in Table 5) both showed a febrile response, as did one of the two ferrets (animals 7 and 8) in the next cycle. All three febrile responses were observed on the first day after infection. In contrast, none of the ferrets infected with turbinate-derived 3ts/3WF virus (one or two passages, animals 5 to 8) developed fevers (Table 5).

The *ts* phenotype of viruses in the egg fluids obtained after each cycle of infection was determined by plaque assays in MDCK cells at 34 and 38°C. Since this test was performed with the egg material obtained directly from the titer determination of the turbinate homogenates and was not biologically cloned, the data are reflective only of the pool of viruses present. For this reason, the EOP at 38°C was used as an indication of the presence of revertants in the population, and complete characterization of the shutoff temperatures was not performed. However, two plaques were picked from the 34°C plates infected with virus derived from the last cycle of ferret infections (animals 7 and 8) and analyzed at 38 and 39°C (Table 5).

Compared to the 1,000-fold reduction in EOP at 38 versus 34°C observed for the original 5ts virus (Table 2), isolates of 5ts recovered after one, two, or three passages in ferrets showed a reduction in the severity of the *ts* phenotype (<10-fold [Table 5]). Plaque-purified isolates showed similar phenotypes. Analysis of the PB2 gene from these clonal viruses by RT-PCR and restriction enzyme digestion indicated that all five of the restriction enzyme sites which were introduced along with the missense mutations were still present. Thus, the emergence of ts^+ viruses during the experiment was not due to contamination with wild-type A/LA/2/87 virus or other, unrelated ts^+ virus.

In contrast, viruses recovered from ferrets infected with 3ts/3WF retained the *ts* phenotype, with all but one pool of viruses showing a reduction in EOP at 38°C of at least 100-fold (Table 5). The virus pool recovered from ferret 7, while demonstrating a lower reduction (25-fold), still formed small plaques at high temperature. In addition, the two plaques picked from the 34°C plate were just as *ts* as the input virus (compare the reduction in EOP in Table 5 to those in Table 2).

DISCUSSION

The results presented in this report demonstrate that influenza A viruses generated by rational design have the potential to serve as live attenuated vaccines in humans. Two candidate vaccine strains, 5ts and 3ts/3WF, were found to be *ts* in MDCK cells, attenuated in the respiratory tracts of mice and ferrets, immunogenic in mice and ferrets, and almost completely nonreactogenic in ferrets. In addition, one of the two viruses, 3ts/3WF, was genetically stable in three different assays, in which other viruses (ts1A2 or 5ts) lost their temperature sensitivity.

The basis for the *ts* phenotype caused by the E65G, P112S, N265S, I310T, N556D, or Y658H mutation, originally identified in the PB2 genes of other *ts* strains, is unknown. When tested singly, none of these substitutions (except I310T) had a strong effect on the phenotype of the transfectant viruses: while the viruses formed small plaques at 39 or 40°C, they had shutoff temperatures over 40°C (our unpublished results). Thus, in spite of these mutations being the only ones found in the PB2 genes of the original *ts* strains, some of which had shutoff temperatures of 39°C or lower, the effect of most of these single-amino-acid substitutions in the context of the A/LA/2/87 background is subtle. This suggests either that the original *ts* viruses, which were generated by chemical mutagenesis, had mutations in other genes which also contributed to their phenotypes, or that the effect of these mutations is highly dependent on the genetic background of the virus. Nonetheless, the phenotype of each mutant was affected to some extent as evidenced by the reduction in plaque size and number at

39°C (E65G) or 40°C (P112S, N265S, N556D, and Y658H) relative to the wild-type control.

The *ts* phenotype of the single tryptophan-to-phenylalanine mutants (i.e. W552F, W557F, and W564F) was unanticipated. Mutations of this type have not been described in any *ts* strain of influenza virus, and analogous mutations in the yeast cap-binding protein, eIF-4E, were not reported to be *ts* (1). It is possible that the cap-binding activity of PB2 containing the 3WF mutations, or of other mutations in the same region, does indeed reduce the affinity of PB2 for the cap structure. This would be consistent with the prediction that a virus which contains mutations which reduce the affinity of a viral protein (PB2) for a host factor (the cap structure) should be more genetically stable than those which affect its interaction with other viral proteins. The temperature sensitivity of the viruses may or may not be directly related to any possible effects on cap binding; we are currently conducting experiments to address this issue.

The complete protection from challenge afforded by previous infection with the wild-type virus, compared to the lower level of protection observed with the attenuated viruses, may be related to a higher level of circulating antibody titers, which in turn may be a result of the ability of the virus to replicate in the lungs of the infected mice (9). A weak HAI response was detected in the sera of mice infected with the parental wild-type virus, but none was detected in mice infected with either recombinant (HAI titer, <1:40). Thus, the ability of the immunizing virus to replicate in turbinates and lungs confers greater protection than does replication in turbinates only. However, the results obtained in the protection study with ferrets were different; all the immunized ferrets were completely protected from replication in the nasal turbinates, and all developed a high titer of reactive antibodies detected in the HAI test. Thus, the two model systems differ in their response to the attenuated vaccine candidates; it is presently unclear which model is more predictive of the human situation.

It is clear from the results summarized in Table 5 that the 5ts transfectant virus is not genetically stable in ferrets. The fever profiles, combined with the clear change in *ts* phenotype after only one passage in ferrets, demonstrate that 5ts is able to rapidly revert to virulence. In a sense, this result is similar to that obtained in the MDCK stress test, since the temperature in the nasal turbinate of a ferret is estimated to be 37°C (2°C cooler than the core body temperature of 39°C). This genetic instability is of considerable interest. The rapidity with which revertants arose was surprising, since the *ts* phenotype is due to five separate mutations. However, it is not known how many independent functions are affected. Thus, reversion could theoretically occur as a result of fewer than five, and perhaps as few as one, second-site suppressor mutations. The mechanism of reversion is currently being addressed by sequence analysis and functional assays.

The propensity of the 5ts virus to lose its *ts* phenotype after growth at 37°C in vitro or in the nasal epithelium of ferrets would seem to exclude it as a vaccine candidate. However, it is unlikely that the virus would grow under similar conditions in humans, since the temperature in the upper airway is likely to be closer to 35°C and since lung replication is unlikely. In addition, since the ferrets were seronegative and thus more permissive for influenza virus replication than would be mildly seropositive humans, they may not accurately reflect the genetic stability of the virus in the probable target population, i.e., older adults and the elderly.

There are several advantages of a genetic engineering approach to the development of a live attenuated influenza virus vaccine. Given a sufficient number of mutations from which to choose, it is feasible to generate, by combination of the right kind and number of mutations, a panel of genetically stable mutants possessing different degrees of attenuation. This "fine-tuning" of virulence should enable the selection of a live vaccine strain optimally suited to any target population. The ability to target functional domains of viral proteins, especially those which interact with host as opposed to viral factors, and to introduce more than one nucleotide change per codon is particularly important in the design of genetic stability.

The level of attenuation of our most stable virus, 3ts/3WF, may not be optimal for vaccination of the elderly population, where the clinical need is currently the greatest. The level of replication observed in the nasal turbinates of seronegative naive mice and ferrets was reduced 75- to 300-fold compared to the wild-type control (Table 3); the amount of replication in the URT of a person who has some immunological memory as a result of previous exposures to related influenza viruses would be expected to be even lower. While preclinical models, again in naive animals, suggest that the virus can protect from homologous viral challenge, only clinical trials in adults or the elderly will answer the question whether the virus is appropriately attenuated and sufficiently immunogenic for use in seropositive individuals.

Another potential drawback of the PB2-based engineering approach is that only one gene contains attenuating mutations. The success of the cold-adapted vaccine in children and young adults is probably a result of specific sequences in at least four of the genes which encode internal proteins (6, 19, 20, 43). Mutations which lie in different genes are likely to affect different functions, reducing the likelihood of phenotypic reversion by a single suppression event. However, it is possible that multiple independently acting mutations in a single gene can provide the same level of genetic stability as single mutations distributed among multiple genes. The targeting of the cap-binding function of PB2, as shown here, or of other functions such as endonuclease activity (41), nuclear targeting (30), or interaction with PB1 and/or PA (3, 8, 44), should allow the engineering of such a virus.

ACKNOWLEDGMENTS

We thank David Stec and Ying Ge for preparation of the ribonucleoproteins from influenza virus A/PR/8/34; Jin Zhu and Wendy Davies-Rodrigues for technical assistance; Kim Mann and Lily Chung for providing cell culture support; and Tai-An Cha and the Core Sequencing Facility for DNA sequencing. In addition, we thank Richard Spaete and all members of the Respiratory Virus Research Group at Aviron for their comments and suggestions; H. F. Maassab, L. Herlocher, and Suzanne Powell (University of Michigan) for performing the ferret studies; and Chris Simonsen (Sierra Biosource Inc.) and Jim Russell for advice on the mouse studies.

REFERENCES

1. **Altmann, M., I. Edery, H. Trachsel, and N. Sonenberg.** 1988. Site-directed mutagenesis of the tryptophan residues in yeast eukaryotic initiation factor 4E. J. Biol. Chem. **263:**17229–17232.
2. **Barrett, T., and S. C. Inglis.** 1985. Growth, purification and titration of influenza viruses, p. 119–150. *In* B. W. J. Mahy (ed.), Virology: a practical approach. IRL Press, Oxford, England.
3. **Biswas, S. K., and D. P. Nayak.** 1996. Influenza virus polymerase basic protein 1 interacts with influenza virus polymerase basic protein 2 at multiple sites. J. Virol. **70:**6716–6722.
4. **Braam, J., I. Ulmanen, and R. M. Krug.** 1983. Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. Cell **34:**609–618.
5. **Clements, M. L., E. K. Subbarao, L. F. Fries, R. A. Karron, W. T. London, and B. R. Murphy.** 1992. Use of single-gene reassortant viruses to study the role of avian influenza A virus genes in attenuation of wild-type human influenza A virus for squirrel monkeys and adult human volunteers. J. Clin. Microbiol. **30:**655–662.
6. **Cox, N. J., F. Kitame, A. P. Kendal, H. F. Maassab, and C. Naeve.** 1988.

Identification of sequence changes in the cold-adapted, live attenuated influenza vaccine strain, A/Ann Arbor/6/60 (H2N2). Virology **167:**554–567.
7. **de la Luna, S., C. Martinez, and J. Ortin.** 1989. Molecular cloning and sequencing of influenza virus A/Victoria/3/75 polymerase genes: sequence evolution and prediction of possible functional domains. Virus Res. **13:**143–155.
8. **Detjen, B. M., C. St. Angelo, M. G. Katze, and R. M. Krug.** 1987. The three influenza virus polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. J. Virol. **61:**16–22.
9. **Fazekas, G., B. Rosenwirth, P. Dukor, J. Gergely, and E. Rajnavolgyi.** 1994. IgG isotype distribution of local and systemic immune responses induced by influenza virus infection. Eur. J. Immunol. **24:**3063–3067.
10. **Fedson, D. S.** 1994. Influenza and pneumococcal vaccination of the elderly: newer vaccines and prospects for clinical benefits at the margin. Prev. Med. **23:**751–755.
11. **Foster, D. A., A. Talsma, A. Furumoto-Dawson, S. E. Ohmit, J. R. Margulies, N. H. Arden, and A. S. Monto.** 1992. Influenza vaccine effectiveness in preventing hospitalization for pneumonia in the elderly. Am. J. Epidemiol. **136:**296–307.
12. **Glezen, W. P.** 1982. Serious morbidity and mortality associated with influenza epidemics. Epidemiol. Rev. **4:**25–44.
13. **Glezen, W. P., A. A. Payne, D. N. Snyder, and T. D. Downs.** 1982. Mortality and influenza. J. Infect. Dis. **146:**313–321.
14. **Gorse, G. J., and R. B. Belshe.** 1990. Enhancement of anti-influenza A virus cytotoxicity following influenza A virus vaccination in older, chronically ill adults. J. Clin. Microbiol. **28:**2539–2550.
15. **Gorse, G. J., R. B. Belshe, and N. J. Munn.** 1988. Local and systemic antibody responses in high-risk adults given live-attenuated and inactivated influenza A virus vaccine. J. Clin. Microbiol. **26:**911–918.
16. **Gorse, G. J., R. B. Belshe, and N. J. Munn.** 1991. Superiority of live attenuated compared with inactivated influenza A virus vaccines in older, chronically ill adults. Chest **100:**977–984.
17. **Gorse, G. J., E. E. Otto, D. C. Powers, G. W. Chambers, C. S. Eickhoff, and F. K. Newman.** 1996. Induction of mucosal antibodies by live attenuated and inactivated influenza virus vaccines in the chronically ill elderly. J. Infect. Dis. **173:**285–290.
18. **Govaert, T. M., C. T. Thijs, N. Masurel, M. J. Sprenger, G. J. Dinant, and J. A. Knottnerus.** 1994. The efficacy of influenza vaccination in elderly individuals. A randomized double-blind placebo-controlled trial. JAMA **272:** 1661–1665.
19. **Herlocher, M. L., D. C. DeBorde, M. W. Shaw, and H. F. Maassab.** 1989. Origin of *ts* phenotype expression of cold-adapted influenza virus A/AA/6/60, p. 387–401. *In* D. Kolakofsky and B. Mahy (ed.), Genetics and pathogenicity of negative strand viruses. Elsevier Biomedical Press, Amsterdam, The Netherlands.
20. **Herlocher, M. L., H. F. Maassab, and R. G. Webster.** 1993. Molecular and biological changes in the cold-adapted "master strain" A/AA/6/60 (H2N2) influenza virus. Proc. Natl. Acad. Sci. USA **90:**6032–6036.
21. **Ishida, T., M. Katsuta, M. Inoue, Y. Yamagata, and K.-I. Tomita.** 1983. The stacking interactions in 7-methylguanine-tryptophan systems, a model study for the interaction between the "cap" structure of mRNA and its binding protein. Biochem. Biophys. Res. Commun. **115:**849–854.
22. **Kilbourne, E. D.** 1993. Inactivated influenza vaccines, p. 565–581. *In* S. A. Plotkin and E. A. Mortimer (ed.), Vaccines. The W. B. Saunders Co., Philadelphia, Pa.
23. **Lawson, C. M., E. K. Subbarao, and B. R. Murphy.** 1992. Nucleotide sequence changes in the polymerase basic protein 2 gene of temperature-sensitive mutants of influenza A virus. Virology **191:**506–510. (Erratum, **145:**302, 1993.)
24. **Maassab, H. F.** 1959. The propagation of multiple viruses in chick kidney cultures. Proc. Natl. Acad. Sci. USA **45:**1035–1039.
25. **Maassab, H. F., A. P. Kendal, G. D. Abrams, and A. S. Monto.** 1982. Evaluation of a cold-recombinant influenza virus vaccine in ferrets. J. Infect. Dis. **146:**780–790.
25a. **Maassab, H. F.** Personal communication.
26. **Maassab, H. F., M. W. Shaw, and C. A. Heilman.** 1993. Live influenza virus vaccine, p. 781–801. *In* S. A. Plotkin and E. A. Mortimer (ed.), Vaccines. The W. B. Saunders Co., Philadelphia, Pa.
27. **Massicot, J. G., B. R. Murphy, F. Thierry, L. Markoff, K. Y. Huang, and R. M. Chanock.** 1980. Temperature-sensitive mutants of influenza virus. Identification of the loci of the two *ts* lesions in the Udorn-ts-1A2 donor virus and the correlation of the presence of these two *ts* lesions with a predictable level of attenuation. Virology **101:**242–249.
28. **Massicot, J. G., B. R. Murphy, K. van Wyke, K. Y. Huang, and R. M. Chanock.** 1980. *ts* P1 and P3 genes are responsible for satisfactory level of attenuation of ts-1A2 recombinants bearing H1N1 or H3N2 surface antigens of influenza A virus. Virology **106:**187–190.
29. **McCauley, J. W., and C. R. Penn.** 1990. The critical cut-off temperature of avian influenza viruses. Virus Res. **17:**191–198.
30. **Mukaigawa, J., and D. P. Nayak.** 1991. Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. J. Virol. **65:**245–253.
31. **Murphy, B. R., and R. M. Chanock.** 1981. Genetic approaches to the prevention of influenza A virus infection, p. 601–615. *In* D. P. Nayak (ed.), Genetic variation among influenza viruses. Academic Press, Inc., New York, N.Y.
32. **Murphy, B. R., L. J. Markoff, R. M. Chanock, S. B. Spring, H. F. Maassab, A. P. Kendal, N. J. Cox, M. M. Levine, R. G. Douglas, Jr., R. F. Betts, R. B. Couch, and T. R. Cate, Jr.** 1980. Genetic approaches to attenuation of influenza A viruses for man. Philos. Trans. R. Soc. London Ser. B **288:**401–415.
33. **Murphy, B. R., L. J. Markoff, N. T. Hosier, J. G. Massicot, and R. M. Chanock.** 1982. Production and level of genetic stability of an influenza A virus temperature-sensitive mutant containing two genes with *ts* mutations. Infect. Immun. **37:**235–242.
34. **Murphy, B. R., M. D. Tolpin, J. G. Massicot, H. Y. Kim, R. H. Parrott, and R. M. Chanock.** 1980. Escape of a highly defective influenza A virus mutant from its temperature sensitive phenotype by extragenic suppression and other types of mutation. Ann. N.Y. Acad. Sci. **354:**172–182.
35. **Nichol, K. L., K. L. Margolis, J. Wuorenma, and T. Von Sternberg.** 1994. The efficacy and cost-effectiveness of vaccination against influenza among elderly persons living in the community. N. Engl. J. Med. **331:**778–784.
36. **Parkin, N. T., P. Chiu, and K. L. Coelingh.** 1996. Temperature sensitive mutants of influenza A virus generated by reverse genetics and clustered charged to alanine mutagenesis. Virus Res. **46:**31–44.
37. **Patriarca, P. A., J. A. Weber, R. A. Parker, W. N. Hall, A. P. Kendal, D. J. Bregman, and L. B. Schonberger.** 1985. Efficacy of influenza vaccine in nursing homes. Reduction in illness and complications during an influenza A (H3N2) epidemic. JAMA **253:**1136–1139.
38. **Plotch, S. J., M. Bouloy, I. Ulmanen, and R. M. Krug.** 1981. A unique cap(m^7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. Cell **23:**847–858.
39. **Powers, D. C., L. F. Fries, B. R. Murphy, B. Thumar, and M. L. Clements.** 1991. In elderly persons live attenuated influenza A virus vaccines do not offer an advantage over inactivated virus vaccine in inducing serum or secretory antibodies or local immunologic memory. J. Clin. Microbiol. **29:**498–505.
40. **Powers, D. C., B. R. Murphy, L. F. Fries, W. H. Adler, and M. L. Clements.** 1992. Reduced infectivity of cold-adapted influenza A H1N1 viruses in the elderly: correlation with serum and local antibodies. J. Am. Geriatr. Soc. **40:**163–167.
41. **Shi, L., D. F. Summers, Q. Peng, and J. M. Galarza.** 1995. Influenza A virus RNA polymerase subunit PB2 is the endonuclease which cleaves host cell mRNA and functions only as the trimeric enzyme. Virology **208:**38–47.
42. **Smith, H., and C. Sweet.** 1988. Lessons for human influenza from pathogenicity studies with ferrets. Rev. Infect. Dis. **10:**56–75.
43. **Snyder, M. H., R. F. Betts, D. DeBorde, E. L. Tierney, M. L. Clements, D. Herrington, S. D. Sears, R. Dolin, H. F. Maassab, and B. R. Murphy.** 1988. Four viral genes independently contribute to attenuation of live influenza A/Ann Arbor/6/60 (H2N2) cold-adapted reassortant virus vaccines. J. Virol. **62:**488–495.
44. **St. Angelo, C., G. E. Smith, M. D. Summers, and R. M. Krug.** 1987. Two of the three influenza viral polymerase proteins expressed by using baculovirus vectors form a complex in insect cells. J. Virol. **61:**361–365.
45. **Subbarao, E. K., Y. Kawaoka, and B. R. Murphy.** 1993. Rescue of an influenza A virus wild-type PB2 gene and a mutant derivative bearing a site-specific temperature-sensitive and attenuating mutation. J. Virol. **67:** 7223–7228.
46. **Subbarao, E. K., E. J. Park, C. M. Lawson, A. Y. Chen, and B. R. Murphy.** 1995. 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. J. Virol. **69:**5969–5977.
47. **Tolpin, M. D., M. L. Clements, M. M. Levine, R. E. Black, A. J. Saah, W. C. Anthony, L. Cisneros, R. M. Chanock, and B. R. Murphy.** 1982. Evaluation of a phenotypic revertant of the A/Alaska/77-ts-1A2 reassortant virus in hamsters and in seronegative adult volunteers: further evidence that the temperature-sensitive phenotype is responsible for attenuation of ts-1A2 reassortant viruses. Infect. Immun. **36:**645–650.
48. **Tolpin, M. D., J. G. Massicot, M. G. Mullinix, H. W. Kim, R. H. Parrott, R. M. Chanock, and B. R. Murphy.** 1981. Genetic factors associated with loss of the temperature-sensitive phenotype of the influenza A/Alaska/77-ts-1A2 recombinant during growth in vivo. Virology **112:**505–517.
49. **Treanor, J., M. Perkins, R. Battaglia, and B. R. Murphy.** 1994. Evaluation of the genetic stability of the temperature-sensitive PB2 gene mutation of the influenza A/Ann Arbor/6/60 cold-adapted vaccine virus. J. Virol. **68:**7684–7688.
50. **Ulmanen, I., B. A. Broni, and R. M. Krug.** 1981. Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m^7GpppNm) on RNAs and in initiating viral RNA transcription. Proc. Natl. Acad. Sci. USA **78:** 7355–7359.
51. **Wright, P. F., S. H. Sell, T. Shinozaki, J. Thompson, and D. T. Karzon.** 1975. Safety and antigenicity of influenza A/Hong Kong/68-ts-1 [E] (H3N2) vaccine in young seronegative children. J. Pediatr. **87:**1109–1116.
52. **Yamanaka, K., N. Ogasawara, M. Ueda, H. Yoshikawa, A. Ishihama, and K. Nagata.** 1990. Characterization of a temperature-sensitive mutant in the RNA polymerase PB2 subunit gene of influenza A/WSN/33 virus. Arch. Virol. **114:**65–73.