JOURNAL OF VIROLOGY, Feb. 2004, p. 2029–2036 Vol. 78, No. 4
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.4.2029–2036.2004

Codon Substitution Mutations at Two Positions in the L Polymerase Protein of Human Parainfluenza Virus Type 1 Yield Viruses with a Spectrum of Attenuation In Vivo and Increased Phenotypic Stability In Vitro

Josephine M. McAuliffe, Sonja R. Surman, Jason T. Newman, Jeffrey M. Riggs, Peter L. Collins, Brian R. Murphy, and Mario H. Skiadopoulos*

Respiratory Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

Received 7 July 2003/Accepted 27 October 2003

The Y942H and L992F temperature-sensitive (*ts*) and attenuating amino acid substitution mutations, previously identified in the L polymerase of the HPIV3*cp*45 vaccine candidate, were introduced into homologous positions of the L polymerase of recombinant human parainfluenza virus type 1 (rHPIV1). In rHPIV1, the Y942H mutation specified the *ts* phenotype in vitro and the attenuation (*att*) phenotype in hamsters, whereas the L992F mutation specified neither phenotype. Each of these codon mutations was generated by a single nucleotide substitution and therefore had the potential to readily revert to a codon specifying the wild-type amino acid residue. We introduced alternative amino acid assignments at codon 942 or 992 as a strategy to increase genetic stability and to generate mutants that exhibit a range of attenuation. Twenty-three recombinants with codon substitutions at position 942 or 992 of the L protein were viable. One highly *ts* and *att* mutant, the Y942A virus, which had a difference of three nucleotides from the codon encoding a wild-type tyrosine, also possessed a high level of genetic and phenotypic stability upon serial passage in vitro at restrictive temperatures compared to that of the parent Y942H virus, which possessed a single nucleotide substitution. We obtained mutants with substitutions at position 992 that, in contrast to the L992F virus, possessed the *ts* and *att* phenotypes. These findings identify the use of alternative codon substitution mutations as a method that can be used to generate candidate vaccine viruses with increased genetic stability and/or a modified level of attenuation.

Human parainfluenza virus type 1 (HPIV1), a nonsegmented, single-stranded, negative-sense RNA virus, is a member of the *Paramyxovirinae* subfamily of the *Paramyxoviridae* family of which HPIV types 2, 3, 4A, and 4B (HPIV2, HPIV3, HPIV4A, and HPIV4B, respectively) are also members (3). HPIV1 and its animal counterpart, *Sendai virus*, and HPIV3 and its animal counterpart, bovine PIV3, are classified together in the *Respirovirus* genus. In infants and young children, HPIV1, HPIV2, and HPIV3 cause both mild respiratory tract illness, including rhinitis, pharyngitis, and otitis media, and more severe disease, such as croup, bronchiolitis, and pneumonia (3, 6, 12, 13, 19, 25). HPIV1, HPIV2, and HPIV3 have been identified as etiologic agents responsible for 6.0, 3.2, and 11.5%, respectively, of hospitalizations of infants and young children for respiratory tract disease (3). Together, they account for nearly one-fifth of all pediatric hospitalizations for respiratory disease. The HPIVs are also receiving increasing recognition as causes of respiratory tract disease in adults and in immunocompromised subjects (1, 5, 9, 14, 17, 18).

Licensed vaccines are currently not available for any of the HPIVs, and we are pursuing studies to develop live-attenuated vaccines for HPIV1, HPIV2 and HPIV3 for intranasal immunization (15, 16, 29, 31). The present paper describes initial studies that use reverse genetics to identify attenuation (*att*) mutations that would be useful in a recombinant live-attenuated HPIV1 vaccine. The HPIV1/Washington/20993/1964 (HPIV1/Wash/64) strain, which is 15,600 nucleotides in length and has a genomic organization that is similar to that of other members of the *Respirovirus* genus, has recently been recovered from cDNA (23). The genome of HPIV1 encodes several nucleocapsid-associated proteins, including the nucleocapsid protein (N), the phosphoprotein (P), and the large polymerase (L), and several envelope-associated proteins, including the matrix protein (M) and the major protective antigenic determinants, the fusion glycoprotein (F) and hemagglutinin-neuraminidase glycoprotein (HN). The gene order is N, P/C, M, F, HN, and L, where C is an accessory protein encoded by an alternative open reading frame (ORF) in P that, in part, counteracts the antiviral activity of interferon (10). The availability of the cDNA-based recovery system for HPIV1 makes it possible to molecularly engineer live-attenuated HPIV1 vaccine candidates and to optimize vaccine properties from the inception of this vaccine development project. Previous studies have demonstrated that it is possible to transfer an attenuating mutation present in one paramyxovirus to a homologous site in another member of the group and thereby rapidly achieve attenuation of the antigenically distinct recipient virus (7, 8, 31). Analysis of nucleotide and amino acid sequence alignments has demonstrated that HPIV1 is most closely related to

* Corresponding author. Mailing address: NIH, Building 50, Room 6511, 50 South Dr., MSC 8007, Bethesda, MD 20892-8007. Phone: (301) 594-2271. Fax: (301) 480-1268. E-mail: mskiadopoulos@niaid.nih.gov.

Sendai virus, followed by HPIV3 (23), suggesting that *Sendai virus* and HPIV3 would be good donors of attenuating mutations to HPIV1.

The present study was initiated by the transfer of two of the three independent attenuating amino acid substitutions (Tyr-942 to His, Y942H; Leu-992 to Phe, L992F; Thr-1558 to Ile, T1558I) identified in the L protein of the HPIV3*cp*45 vaccine candidate, Y942H and L992F, to rHPIV1 (30). Attempts to generate recombinants containing all three of the *cp*45 mutations in HPIV1 were problematic, indicating that these three mutations are not compatible when present together in HPIV1. Each of the two codons (Y942H or L992F) present in HPIV3*cp*45 was transferred separately to the homologous position in the HPIV1 such that the resulting HPIV1 L gene differed from the HPIV1 wild-type L gene by one nucleotide and the proteins differed by one amino acid. In the HPIV3 backbone, the Y942H and L992F mutations each specify the temperature-sensitive (*ts*) phenotype in vitro and the *att* phenotype in hamsters (28). One objective in the present study was to determine the effect of each mutation in the HPIV1 backbone. We also were interested in exploring a general strategy to increase the genetic and phenotypic stability of *ts* and *att* amino acid point mutations in live-attenuated respiratory virus vaccines. Previous studies identified a number of *ts* and *att* amino acid substitution mutations in HPIV3, respiratory syncytial virus, and influenza virus that each involved a single nucleotide change (4, 22, 35). The high mutation rate for RNA viruses in general would make single-nucleotide changes susceptible to genetic and phenotypic instability. For example, respiratory viruses bearing two or more *ts* mutations demonstrated the tendency to lose their phenotype following replication in vivo (22, 33, 35). This finding suggests that it would be desirable to make an effort early in the development of a HPIV1 live-attenuated virus vaccine to increase the genetic stability of individual mutations that are to be included in the vaccine. Since one mechanism of loss of the *ts* phenotype is reversion of the nucleotide substitution to the wild-type assignment, we sought to modify the recombinant HPIV1 (rHPIV1) codons such that two or three nucleotide changes would be required to restore the wild-type amino acid coding assignment. This strategy has been employed previously to generate a set of attenuated recombinant Sindbis viruses bearing codon substitution mutations at several sites in the E2 virion protein (24, 27). These recombinant viruses exhibited different levels of attenuation in vivo, but their phenotypic stability in vitro or in vivo was not explored. Thus, we used mutagenesis at rHPIV1 codons 942 and 992 to explore the range of viable amino acid coding assignments at each position and recovered 13 rHPIV1 viruses that each had a different amino acid at position 942 and 10 viruses that each had a substitution at position 992. These codon substitution mutants were assayed for temperature sensitivity of replication in vitro and for their ability to replicate in the respiratory tracts of hamsters. rHPIV1 viruses with increased attenuation or genetic stability were identified in both codons. These mutations will be useful attenuating mutations in an rHPIV1 vaccine candidate.

MATERIALS AND METHODS

Viruses and cells. LLC-MK2 cells (ATCC CCL 7.1) and HEp-2 cells (ATCC CCL 23) were maintained in Opti-MEM I (Gibco-Invitrogen, Inc., Grand Island, N.Y.) supplemented with 5% fetal bovine serum, gentamicin sulfate (50 μg/ml), and 2 mM glutamine (Gibco-Invitrogen, Inc.). rHPIV1 and rHPIV1 mutants were grown in LLC-MK2 cells as described previously (23).

Construction of point mutations in the antigenomic HPIV1 cDNA. The mutations were introduced into the appropriate rHPIV1 subgenomic clones (23) using a modified PCR mutagenesis protocol described elsewhere (20) with the Advantage-HF PCR kit (Clontech Laboratories, Palo Alto, Calif.). The subgenomic clone containing the mutation was then sequenced for the entirety of the region that was PCR amplified by using an ABI 3100 sequencer with the BigDye sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom) to confirm that the subclone contained the introduced mutation but did not contain any adventitious mutations introduced during PCR amplification. Full-length HPIV1 cDNA clones (FLCs) containing the mutations were assembled using standard molecular cloning techniques (23), and the region containing the introduced mutation in each FLC was sequenced as described above to ensure that the FLC contained the introduced mutation.

Recovery of rHPIV1 mutant viruses. Recovery of rHPIV1 mutants was performed as described previously (23). To confirm that viruses contained the appropriate mutations, viral RNA was isolated from infected cell supernatant fluids using the Qiaquick vRNA kit (Qiagen Inc., Valencia, Calif.), and the appropriate region in each was amplified by reverse transcription (RT)-PCR as described previously (23) and analyzed by sequencing. All of the sequence analysis of viral RNA in this study involved direct analysis of uncloned RT-PCR products. Control RT-PCRs were performed in which the RT enzyme was omitted to confirm that the RT-PCR products were generated from RNA rather than contaminating DNA. For FLCs containing a codon substitution mutation, initial virus recovery attempts were made as described above using the pTM(L_1) support plasmid that contained the HPIV1 wild-type L protein sequence (23). In several instances involving the 942 or 992 codon, the virus recovered contained the wild-type L protein coding sequence, indicating that recombination had occurred between the pTM(L_1) support plasmid and the mutant FLC (11). Therefore, subsequent recovery attempts were performed in which the wild-type pTM(L_1) support plasmid was replaced with one containing the appropriate L mutation present in the FLC being rescued. The recovered rHPIV1 viruses were biologically cloned by two successive rounds of terminal dilution by using LLC-MK2 monolayers in 96-well plates (Costar; Corning, Inc., Corning, N.Y.). The presence of the introduced mutation in each biologically cloned virus was confirmed by sequence analysis of the RT-PCR product.

Replication of rHPIV1 mutants in LLC-MK2 cells at permissive and restrictive temperatures. The *ts* phenotype for each of the rHPIV1 mutant viruses was determined by comparing the replication level of each virus to that of rHPIV1 wild-type virus at 32, 35, 36, 37, 38, and 39°C as described previously (32). Virus titer, which is expressed as a mean $\log_{10}$ 50 percent tissue culture infectious dose per milliliter ($\log_{10}$ $TCID_{50}$/ml), was determined in one to three separate experiments. The reduction in titer ($\log_{10}$) at each restrictive temperature, determined by comparison to the titer at permissive temperature of 32°C, was recorded for each experiment, and the mean reduction was calculated. The *ts* phenotype was defined as a 100-fold or greater reduction in titer compared to that of the wild-type virus.

Replication of rHPIV1 mutant viruses in the respiratory tracts of hamsters. Four-week-old Golden Syrian hamsters were inoculated intranasally with 0.1 ml of L-15 (Invitrogen Corp., Grand Island, N.Y.) containing $10^{6.0}$ $TCID_{50}$ of a wild-type or mutant HPIV1. Four days later, the nasal turbinates and lungs were collected as previously described (23). Virus present in the samples was quantified by titration on LLC-MK2 monolayers at 32°C. Infected cells were detected on day 6 postinfection by hemadsorption with guinea pig erythrocytes. The mean titer ($\log_{10}$ $TCID_{50}$/g) was calculated for each group of six hamsters. The *att* phenotype was defined as a 100-fold or greater reduction in virus titer in either or both anatomical locations compared to that for wild type.

Determination of the genetic and phenotypic stability of rHPIV1-Y942H and rHPIV1-Y942A in LLC-MK2 cells by passage at restrictive temperatures. rHPIV1 mutants with the original Y942H mutation or with the Y942A mutation were grown on LLC-MK2 monolayers at 32°C with an input inoculum of approximately 0.01 $TCID_{50}$ per cell until cytopathology was visible (approximately 5 to 7 days). The virus in the supernatant was diluted 1 to 1,000 and was passed again on LLC-MK2 monolayers at 32°C. This process was repeated for a total of 10 passages. Alternatively, the two viruses were also passaged at increasingly restrictive temperatures as follows: two passages (as described above) at 32°C, two at 35°C, two at 36°C, and two at 37°C, after which undiluted supernatant from the second 37°C passage harvest was passed to LLC-MK2 monolayers at 38°C and then passed once at 39°C, for a total of 10 passages.

At each passage level, aliquots were frozen for phenotypic and genotypic analysis. The level of replication and temperature sensitivity of both rHPIV1-

TABLE 1. Recovery of rHPIV1 bearing codon substitution mutations at amino acid position 942 in the L protein

Virus	Codon[a]	No. of nucleotide changes needed to restore wild-type amino acid	rHPIV1 mutant recovered	Adventitious coding mutations in L ORF
rHPIV1 wild type	TAT, TAC	0	+	ND[b]
rHPIV1-Y942H	CAC	1	+	None
rHPIV1-Y942C[c]	TGC	1	+	None
rHPIV1-Y942F	TTT	1	+	None
rHPIV1-Y942N[c]	AAC	1	+	None
rHPIV1-Y942D[c]	GAC	1	+	None
rHPIV1-Y942S	AGC	2	+	None
rHPIV1-Y942W	TGG	2	+	None
rHPIV1-Y942Q	CAG	2	+	S1302N
rHPIV1-Y942K	AAA	2	−[d]	ND
rHPIV1-Y942I	ATC	2	−	ND
rHPIV1-Y942E	GAG	2	−	ND
rHPIV1-Y942M[c]	ATG	3	+	L1367S
rHPIV1-Y942A	GCG	3	+	None
rHPIV1-Y942T[c]	ACA	3	+	None
rHPIV1-Y942G	GGG	3	+	None
rHPIV1-Y942V[c]	GTG	3	+	G1755Q
rHPIV1-Y942L[c]	CTG	3	+	None
rHPIV1-Y942R	CGG	3	−	ND
rHPIV1-Y942P	CCG	3	−	ND

[a] In the case of wild type, the two possible codons yielding the wild-type amino acid for tyrosine (Y) are shown; for each mutant, the codon chosen for introduction into rHPIV1 is shown.

[b] ND, not done. The L gene sequence was not confirmed either because the virus is the previously sequenced wild type or because the virus was not recovered.

[c] Virus was recovered with pTML containing the indicated codon substitution. The other viruses were recovered using wild-type pTML.

[d] −, not recovered.

Y942H and rHPIV1-Y942A were determined and compared to those for rHPIV1 as described above. Sequence analysis of each virus was performed as described above.

RESULTS

Recovery of rHPIV1 bearing codon substitution mutations at amino acid position 942 in the L protein. The Y942H mutation in the L protein of the attenuated HPIV3*cp*45 virus was introduced by reverse genetics into the homologous position in rHPIV1, resulting in a viable virus designated rHPIV1-Y942H (Table 1). This mutation in rHPIV1, as in the original HPIV3*cp*45 virus, involved a single nucleotide substitution (TAC to CAC, substitution underlined). Given the sequences of the possible codons for Tyr (TAT and TAC) and His (CAT and CAC), this amino acid substitution could not be designed to involve more than a single nucleotide change. To evaluate the full range of possible phenotypes involving this amino acid locus, we prepared additional mutant cDNAs in which position 942 was changed to each of the 18 other possible amino acid assignments. Whenever possible, codons were chosen so as to maximize the number of nucleotide differences compared to the two possible codons for the wild-type Tyr assignment (Table 1).

Of these 18 additional codon substitution mutants, 13 were recovered in infectious virus. If the desired rHPIV1 recombinant was not recovered after three to five attempts, we considered the mutant to be nonviable. Each of the recovered viruses was biologically cloned, and the complete L gene was sequenced, confirming in each case the presence of the introduced mutation. Of the 14 recovered rHPIV1 codon substitution mutants, including the original Y942H mutant, three were found to each have one additional adventitious coding mutation in L (Table 1). In our experience, it is not unusual to find adventitious mutations, often phenotypically silent, in cloned biologically derived or recombinant virus when the extra expedient is taken to perform the extensive sequence analysis involved. The possible contribution of these adventitious mutations to phenotypes exhibited by these 3 mutants was not further studied because the 11 mutants lacking adventitious mutations in the L ORF were more than sufficient for analysis for the purposes of this study. Each of the 14 rHPIV1-942 codon substitution mutants replicated efficiently in vitro at 32°C and achieved titers of at least 10^7 $TCID_{50}$/ml (Table 2).

Temperature sensitivity and attenuation phenotype of rHPIV1 codon 942 substitution mutants. The rHPIV1 virus bearing the Y942H mutation transferred from HPIV3*cp*45 was strongly *ts* in vitro, exhibiting a 3.1 $\log_{10}$ reduction in virus yield at 37°C relative to 32°C (Table 2). This finding demonstrated that this imported mutation functioned efficiently as a *ts* mutation in the rHPIV1 backbone. Remarkably, each of the other 13 recovered rHPIV1 mutants was also *ts*, and a spectrum of temperature sensitivity was seen among the mutants. Five rHPIV1 mutants, namely, those with the Y942D, Y942M, Y942A, Y942V, or Y942L mutation, were found to be as *ts* as rHPIV1-Y942H, and the latter four involved codons which would each require three nucleotide changes to restore a Tyr at position 942. Thus, we achieved the goal of deriving a virus with a *ts* phenotype comparable to that of rHPIV1-Y942H but one that would require three nucleotide changes at position 942 to restore the wild-type Tyr residue.

We next compared the ability of the codon substitution viruses to replicate in the upper (nasal turbinates) and lower (lungs) respiratory tract of infected hamsters to that of wild-type rHPIV1 (Table 2). The rHPIV1 bearing the Y942H mutation transferred from HPIV3*cp*45 was highly attenuated in both the upper and lower respiratory tract of hamsters compared to its rHPIV1 parent virus (Table 2). This finding demonstrated that this HPIV3-derived mutation functioned efficiently as an *att* mutation in the HPIV1 backbone. Eleven of the 13 rHPIV1 mutants were attenuated in hamsters, as defined by exhibiting a 100-fold or greater decrease in virus titer in either the upper or lower respiratory tract compared to that for HPIV1 wild type (the underlined values in Table 2). Each of the six rHPIV1 mutants that would require three nucleotide changes in codon 942 to restore the wild-type amino acid (Y942M, Y942A, Y942T, Y942G, Y942V and Y942L) was as attenuated as rHPIV1-Y942H. Thus, we achieved the goal of deriving a virus with an *att* phenotype comparable to that of rHPIV1-Y942H but one that would require three nucleotide changes at position 942 to restore the wild-type Tyr.

Passage of rHPIV1 mutants with the original Y942H mutation or the stabilized Y942A codon at restrictive temperatures. For *ts* viruses, passage of the virus at restrictive temperature has been an effective method to determine the level of stability of the *ts* phenotype (2, 26, 34). Therefore, we employed this

TABLE 2. Temperature sensitivity and attenuation phenotype of rHPIV1 L protein codon 942 substitution mutants

Virus	No. of nucleotide changes needed to restore wild-type amino acid	Virus titer at 32°C (log_{10} $TCID_{50}$/ml)	Mean log_{10} reduction in virus titer at indicated temp (°C)[a]					Replication of virus in hamsters		
								No. of animals	Mean titer of virus (log_{10} $TCID_{50}$/g ± SE)	
			35	36	37	38	39		Nasal turbinates	Lungs
rHPIV1 wild type	0	7.8	0.1	0.4	0.8	1.5	1.8	30	4.2 ± 0.2	3.9 ± 0.4
rHPIV1-Y942H*cp*45	1	8.0		1.4	**3.1**	**5.1**	**5.8**	18	≤1.5 ± 0.0[b]	≤1.5 ± 0.0
rHPIV1-Y942C	1	8.0	1.8	2.1	2.3	**4.8**	**6.5**	6	4.4 ± 0.2	3.4 ± 0.4
rHPIV1-Y942F	1	8.1	0.3	1.3	1.9	3.4	**5.4**	6	3.8 ± 0.1	3.9 ± 0.2
rHPIV1-Y942N	1	9.0	1.0	2.1	**3.7**	**4.3**	**7.0**	6	2.0 ± 0.2	1.6 ± 0.1
rHPIV1-Y942D	1	7.9	**3.3**	**5.3**	**>6.6**	**>6.7**	**>6.9**	6	1.6 ± 0.1	1.8 ± 0.3
rHPIV1-Y942S	2	8.3	0.6	1.2	**3.6**	**5.2**	**>6.4**	6	3.2 ± 0.1	1.8 ± 0.2
rHPIV1-Y942W	2	8.4	0.8	1.5	**3.0**	**4.2**	**>6.4**	12	1.8 ± 0.2	1.6 ± 0.1
rHPIV1-Y942Q	2	7.0	1.4	**3.6**	**>4.7**	**>5.8**	**>5.9**	12	1.5 ± 0.1	1.7 ± 0.2
rHPIV1-Y942M	3	7.3	**3.0**	**5.3**	**>6.0**	**>5.8**	**>6.2**	6	≤1.5 ± 0.0	≤1.5 ± 0.0
rHPIV1-Y942A	3	8.1	**3.5**	**3.5**	**>5.1**	**>6.4**	**>6.8**	6	≤1.5 ± 0.0	≤1.5 ± 0.0
rHPIV1-Y942T	3	8.0	1.8	**3.0**	**>5.0**	**>6.8**	**>6.9**	6	≤1.5 ± 0.0	≤1.5 ± 0.0
rHPIV1-Y942G	3	8.1	1.0	**3.1**	**>4.1**	**>5.8**	**>7.3**	6	≤1.5 ± 0.0	≤1.5 ± 0.0
rHPIV1-Y942V	3	7.0	**2.6**	**5.0**	**>5.7**	**>5.8**	**>6.1**	12	≤1.5 ± 0.0	1.6 ± 0.1
rHPIV1-Y942L	3	7.0	**2.8**	**5.0**	**5.5**	**5.5**	**>5.8**	6	≤1.5 ± 0.0	≤1.5 ± 0.0

[a] Mean titer reduction compared to titer at 32°C. Each values represents the average of at least three experiments. A mutant is considered to be temperature sensitive if its reduction in titer at a nonpermissive temperature is 100-fold greater than the reduction in titer of the wild type HPIV1 tested at that temperature (indicated by values in bold).

[b] Each underlined value represents a greater-than-100-fold reduction compared to that for rHPIV1.

procedure to compare the stability of the *ts* phenotype of the original mutant (rHPIV1-Y942H), containing a single nucleotide substitution at codon 942, with that of rHPIV1-Y942A, containing three nucleotide substitutions in this codon. The two viruses were (i) passaged 10 times at the permissive temperature of 32°C, or (ii) passaged at increasingly restrictive temperatures as follows: twice at 32°C, twice at 35°C, twice at 36°C, twice at 37°C, once at 38°C, and once at 39°C, for a total of 10 passages. Aliquots of virus from various passage levels were analyzed for *ts* phenotype and were subjected to partial or complete sequence analysis of the L gene (Table 3).

The *ts* phenotype of each of the two mutant viruses, rHPIV1-Y942H and rHPIV1-Y942A, was unchanged by 10 passages at 32°C (passage series 1 and 3, respectively, in Table 3), and sequence analysis of each L gene indicated that the respective mutant codon was unchanged. No additional mutations were detected. In contrast, analysis of aliquots from the passage of the rHPIV1-Y942H mutant (passage series 2 in Table 3) at restrictive temperature showed that by passage level p6-36 (36°C) the virus had lost its *ts* phenotype and the consensus sequence at codon 942 had reverted directly back to that of the wild-type assignment of Tyr (CAC to TAC). This single nucleotide change restored the ability of Y942H-p6 to replicate at restrictive temperatures, rendering it indistinguishable from the rHPIV1 wild-type virus in this regard. There were no other mutations in the L gene of this virus, even following additional passage at increasingly restrictive temperatures, as confirmed by sequence analysis of the complete L gene of the virus from passage levels p9-38 and p10-39 (38 and 39°C, respectively, passage series 2, Table 3).

In two independent series of passages, the Y942A mutant did not revert at codon 942 even at the highly restrictive temperatures of 38 or 39°C (Table 3, passage series 4 and 5), i.e., the sequence at codon 942 remained GCG in all passages sequenced. The level of temperature sensitivity of virus from passage levels p8-37 and p9-38 (37 and 38°C, respectively, Table 3, passage series 4) remained highly *ts* at both 38 and 39°C. However, there was a partial loss of the *ts* phenotype, such that the replication of these isolates was increased about 20-fold at 38°C (Table 3, passage series 4) compared to that of the original Y942A mutant. This shift in *ts* phenotype was associated with the acquisition of second-site amino acid point mutations in the L protein, V1016L and N1125D. The relative contribution of these mutations to the loss of temperature sensitivity was not addressed, and since the complete Y942A p9-38 genome was not sequenced, it is also possible that mutations in other parts of the genome (e.g., the N or P genes) could also have contributed. Nonetheless, the p8-37 and p9-38 viruses remained 200-fold more restricted in their replication at 38°C than wild-type rHPIV1, and both viruses failed to replicate at 39°C in the *ts* assay. Similarly, the virus from the p10-39 (39°C) passage level of an independent passage series (Table 3, passage series 5) manifested an intermediate level of temperature sensitivity at 38°C, between that of the wild-type rHPIV1 and the starting Y942A virus, but remained sufficiently *ts* that it failed to replicate at 39°C (Table 3). This partial loss of temperature sensitivity was associated with the development of a third second-site mutation in L, namely, S1328P. However, the entire genome was not sequenced, and mutations outside of the L ORF may also have contributed to the partial loss of temperature sensitivity. Thus, a *ts* mutation involving a single nucleotide substitution readily reverted to the wild-type assignment and rapidly became the predominant viral species during passage at restrictive temperatures. In con-

TABLE 3. Temperature sensitivity and sequence analysis of rHPIV1 mutants with the L protein Y942H mutation (single nucleotide substitution) or the Y942A mutation (three nucleotide substitutions) following passage at restrictive temperatures

Virus	Passage series[a]	Passage level analyzed[b] (°C)	Mean titer (log_{10} $TCID_{50}$/ml) at 32°C	Reduction in virus titer at the indicated temp[c]		Sequence of L gene and protein[d]		
				38°C	39°C	Codon 942 (amino acid assignment)	Extent of sequencing[e]	Second-site mutation
rHPIV1 wild type			8.0	1.2	3.4	TAT (Tyr)	F	None
rHPIV1-Y942H		Unpassaged	6.7	**3.7**[f]	>5.2[g]	CAC (His)	C	None
rHPIV1-Y942H	1	p10-32	7.7	**3.5**	**5.5**	CAC (His)	C	None
rHPIV1-Y942H	2	p4-35	7.5	**3.3**	5.3	CAC (His)	C	None
rHPIV1-Y942H	2	p6-36	7.2	2.0	4.0	TAC (Tyr)	C	None
rHPIV1-Y942H	2	p8-37	7.0	0.3	3.5	TAC (Tyr)	C	None
rHPIV1-Y942H	2	p9-38	6.5	1.0	3.3	TAC (Tyr)	F	None
rHPIV1-Y942H	2	p10-39	5.2	1.0	3.0	TAC (Tyr)	F	None
rHPIV1-Y942A		Unpassaged	6.5	**4.8**	>5.0[g]	GCG (Ala)	P	None
rHPIV1-Y942A	3	p10-32	7.5	**4.0**	4.5	GCG (Ala)	P	None
rHPIV1-Y942A	4	p4-35	7.0	**4.3**	**>5.5**[g]	GCG (Ala)	P	None
rHPIV1-Y942A	4	p6-36	7.0	**4.0**	4.8	GCG (Ala)	P	V1016L
rHPIV1-Y942A	4	p8-37	6.7	**3.5**	>5.2[g]	GCG (Ala)	P	V1016L/N1125D[h]
rHPIV1-Y942A	4	p9-38	6.2	**3.5**	>4.7[g]	GCG (Ala)	F	V1016L/N1125D
rHPIV1-Y942A	5	p10-39	5.2	3.0	>3.7[g]	GCG (Ala)	F	S1328P

[a] Series 1 and 2 involve the Y942H mutant at the permissive and restrictive temperature regimens, respectively (Materials and Methods). Series 3 involves the Y942A mutant at the permissive regimen, while 4 and 5 are independent parallel series at the restrictive regimen.

[b] Samples were analyzed from various passage levels within series 1 to 5: each passage level is identified by its number (p1 to p10) and its temperature (32 to 39, representing 32°C to 39°C).

[c] Data shown are the results of a single experiment. Because of the HPIV1 requirement for the LLC-MK2 cells to be incubated in the presence of trypsin, values at higher temperatures can vary from experiment to experiment.

[d] Consensus sequence determined from uncloned RT-PCR products.

[e] The amount of the L gene sequenced was F (full), the entire L gene ORF; P, partial, nucleotides 10,000 to 13,300; C, codon-proximal, the region immediately surrounding codon 942.

[f] Titer reduction is compared to titer at 32°C. A mutant is considered to be temperature sensitive if its reduction in titer at a nonpermissive temperature is 100-fold greater than the reduction of titer of the wild-type HPIV1 tested at that temperature (indicated by values in bold).

[g] No detectable virus.

[h] Sequencing electropherogram indicated 50% of each nucleotide (A or G) at nucleotide 12,143 in codon 1,125.

trast, reversion was not observed involving a codon that differed from the wild type by three nucleotides, but a partial loss of the *ts* phenotype was observed after eight passages at increasingly restrictive temperatures, and putative second-site intragenic suppressor mutations were detected.

Recovery of rHPIV1 bearing codon substitution mutations at amino acid position 992 in the L protein. A second L protein mutation from HPIV3*cp*45 L, L992F, was introduced into the homologous position in rHPIV1 by reverse genetics, resulting in a viable virus designated rHPIV1-L992F (Table 4). We also prepared additional mutants (Table 4) in which position 992 was changed into 17 of the 18 other possible amino acid assignments (lacking only Ser) (Table 4). Codons were chosen to maximize the number of nucleotide differences in comparison to the possible codons for the wild-type assignment of Leu, but this was made difficult by the existence of six different codons for Leu. Nonetheless, nine of the substitution mutations could be designed to involve two nucleotide differences compared to any Leu codon (Table 4). Of these 17 additional mutations, 10 recombinant viruses bearing the appropriate mutation were recovered and were readily propagated in vitro and biologically cloned. Of the seven remaining rHPIV1 mutants, three (L992R, L992P, and L992Q) were recovered in the transfection harvest but reverted to wild type during biological cloning and two others (L992E and L992D) were recovered in the transfection harvest but replicated very inefficiently and could not be propagated. Two others (L992G and L992T) recombined with the wild type pTM(L_1) and were not further studied. The L gene was sequenced around the site of the mutation in each of the 10 recovered, stable mutant viruses, and the presence of each introduced mutation was confirmed. Each of the 11 recovered codon 992 substitution mutants replicated efficiently in vitro at 32°C and achieved titers of $\geq 10^7$ $TCID_{50}$/ml (Table 5).

Temperature sensitivity and attenuation phenotype of rHPIV1 codon 992 substitution mutants. The rHPIV1 virus bearing the L992F mutation transferred from HPIV3*cp*45 was not *ts* in vitro (Table 5). Seven of the 10 additional rHPIV1 mutants that were recovered were *ts*, and a spectrum of temperature sensitivity was seen among the mutants. One virus, rHPIV1-L992C, exhibited the greatest level of temperature sensitivity, with a 100-fold reduction of replication at 36°C. Thus, we were able to derive a rHPIV1 codon 992 substitution mutant that, in contrast to the original non-*ts* rHPIV1-L992F mutant, was substantially *ts*.

TABLE 4. Recovery of rHPIV1 bearing codon substitution mutations at amino acid position 992 in the L protein

Virus	Codon[a]	No. of nucleotide changes needed to restore wild-type amino acid	rHPIV1 mutant recovered
rHPIV1 wild type	TTA, TTG, CTT, CTC, CTA, CTG	0	+
rHPIV1 L992F*cp*45	TTT	1	+
rHPIV1 L992M	ATG	1	+
rHPIV1 L992H	CAC	1	+
rHPIV1 L992I	ATC	1	+
rHPIV1 L992W	TGG	1	+
rHPIV1 L992R	CGG	1	−[b]
rHPIV1 L992V	GTC	1	+
rHPIV1 L992Q	CAG	1	−[b,c]
rHPIV1 L992E	GAG	1	−[d]
rHPIV1 L992P	CCG	1	−[b]
rHPIV1 L992A	GCG	2	+
rHPIV1 L992Y	TAC	2	+
rHPIV1 L992C	TGC	2	+
rHPIV1 L992N	AAC	2	+
rHPIV1 L992D	GAC	2	−[d]
rHPIV1 L992T	ACC	2	−[c]
rHPIV1 L992G	GGG	2	−[c]
rHPIV1 L992K	AAG	2	+

[a] In the case of wild type, the six possible codons yielding the wild-type amino acid for leucine (L) are shown; for each mutant, the codon chosen for introduction into rHPIV1 is shown. A cDNA bearing an L992S mutation was not constructed.
[b] Mutant was recovered but reverted to wild type during passage at 32°C and was not further studied.
[c] Mutant recombined with wild-type $pTML_1$ and was not further studied.
[d] Virus was recovered from transfection, but titer was low and virus was lost on subsequent passage.

The level of replication of the codon 992 substitution mutants in the upper and lower respiratory tracts of hamsters was compared with that of rHPIV1 (Table 5). The rHPIV1-L992F virus was not attenuated in hamsters: thus, the L992F mutation is attenuating in HPIV3 (28) but not in HPIV1. However, 3 of the other 10 codon 992 substitution mutants (L992V, L992N, and L992C) were reduced in replication 100-fold or more compared to that for wild-type virus. Thus, we were able to derive several rHPIV1 codon 992 substitution mutants with an *att* phenotype. Interestingly, the rHPIV1-L992V mutant was only slightly *ts*, suggesting that the L992V mutation confers a predominantly non-*ts* attenuation phenotype.

DISCUSSION

The ability to recover recombinant negative-strand RNA viruses from cDNA makes it possible to develop live-attenuated virus vaccine candidates by the planned introduction of attenuating mutations to a wild-type virus. This process can be accomplished in a sequential manner in response to ongoing preclinical and clinical evaluation until a desired balance between attenuation and immunogenicity is achieved (4, 21). Genetic stability of the engineered vaccine can be achieved by the accumulation of a sufficient number of attenuating mutations to make loss of the attenuation phenotype unlikely during manufacture and during the brief period of replication of these respiratory viruses in humans (4, 21). In addition, attenuating mutations with improved stability can be designed, such as the deletion of entire genes or, as in the present study, the stabilization of codon substitution mutations. The addition of point mutations that specify the *ts* and *att* phenotype to partially attenuated viruses has been very useful in the incremental attenuation of viruses such as respiratory syncytial virus and HPIV3 (4, 31). However, as indicated above, the *att* phenotype specified by these mutations can be subject to modification by direct reversion or other mutation during replication in vivo

TABLE 5. Temperature sensitivity and attenuation phenotype of rHPIV1 L protein codon-992 substitution mutants

Virus	No. of nucleotide changes needed to restore wild-type amino acid	Virus titer at 32°C (log_{10} $TCID_{50}$/ml)[b]	Mean log_{10} reduction[a] in virus titer at indicated temp (°C)					Replication of virus in hamsters		
								No. of animals	Mean titer of virus (log_{10} $TCID_{50}$/g ± SE)	
			35	36	37	38	39		Nasal turbinates	Lungs
rHPIV1-wild type	0	7.5	0.1	0.1	0.5	0.6	1.2	30	4.2 ± 0.2	3.9 ± 0.4
rHPIV1 L992F*cp*45	1	8.1	0.2	0.3	1.3	0.9	2.4	12	4.4 ± 0.3	3.0 ± 0.2
rHPIV1 L992M	1	8.4	0.2	0.2	0.4	0.7	2.7	6	4.7 ± 0.3	3.2 ± 0.4
rHPIV1 L992I	1	8.2	0.3	0.5	0.5	**2.7**	**4.3**[b]	6	4.1 ± 0.2	2.6 ± 0.3
rHPIV1 L992W	1	8.5	0.0	0.5	0.8	**3.7**	**3.8**	6	4.0 ± 0.2	3.3 ± 0.1
rHPIV1 L992H	1	8.1	0.3	1.3	1.3	1.8	**4.9**	6	3.3 ± 0.2	3.4 ± 0.1
rHPIV1 L992V	1	6.2	0.7	0.5	1.5	2.5	**4.0**	6	<u><1.5 ± 0.0</u>[c]	<u>1.7 ± 0.2</u>
rHPIV1 L992N	2	7.8	0.6	0.3	0.6	2.0	**3.3**	6	2.7 ± 0.2	<u><1.5 ± 0.0</u>
rHPIV1 L992Y	2	8.5	0.0	0.2	0.5	1.4	2.9	6	4.5 ± 0.2	3.3 ± 0.4
rHPIV1 L992K	2	8.6	0.0	1.2	0.2	1.8	3.0	6	4.1 ± 0.3	3.1 ± 0.4
rHPIV1 L992A	2	7.9	0.5	0.3	1.2	**3.0**	**4.5**	6	3.6 ± 0.2	3.2 ± 0.3
rHPIV1 L992C	2	7.3	0.7	**2.2**	**2.9**	**4.0**	**>4.9**	6	3.2 ± 0.2	<u>1.7 ± 0.1</u>

[a] Mean reduction in titer compared to titer at 32°C. Each value represents the average of two or three experiments.
[b] A mutant is considered to be temperature sensitive if its reduction in titer at a nonpermissive temperature is 100-fold greater than the reduction of titer of the wild-type HPIV1 tested at that temperature (indicated by values in bold).
[c] Underlined values represent greater-than-100-fold reduction compared to that for rHPIV1 in the same pulmonary compartment.

(33, 35). Since molecular engineering makes it possible to alter codons that specify the *ts* and *att* phenotypes, we sought in the present study to examine whether the choice of alternative codons at a given locus can be used to enhance the genetic stability of a *ts-att* mutation and to augment the level of attenuation.

In the present study, we took advantage of a mutation at codon 942 in L that occurred during passage of HPIV3 at low temperature and that specified a *ts* phenotype in HPIV3. This previous finding identified this site in L as being susceptible to mutation, and thus it represented a reasonable site to select for examination of the effect of codon substitution mutations on in vitro and in vivo phenotypes. The introduction of the Y942H mutation of the L gene of HPIV3*cp*45 into the homologous position of rHPIV1 yielded a virus that possessed the *ts* and *att* phenotypes. However, these phenotypes were the result of a single nucleotide substitution in codon 942 and thus might be subject to instability. Thirteen additional viable rHPIV1 mutants were generated that contained various alternative amino acid assignments at codon 942, and all were *ts*. We assume that our failure to recover five amino acid substitution mutants indicates that mutations that specify these amino acids in codon 942 are dead-end mutations, i.e., they would be nonviable. Six highly attenuated mutants (those with a Met, Ala, Thr, Gly, Val, or Leu substitution at position 942) were identified that would require three nucleotide changes to occur to generate a codon that specified the wild-type Tyr residue at codon 942. However, 2 other mutants (those with a Cys or Phe at position 942) out of the 13 recovered rHPIV1 mutants replicated in hamsters as efficiently as wild-type HPIV1. Thus, the naturally occurring Tyr-942 assignment was not the only one that conferred a wild-type-like phenotype: Cys-942 and Phe-942 did as well. Therefore, one must choose a 942 assignment that (i) specifies an appropriate level of attenuation, and (ii) differs by two, or preferably three, nucleotides from all possible codons for Tyr, Cys, and Phe. For four of the six highly attenuated viruses (those with a Met, Gly, Val, or Leu substitution), it would take only two nucleotide substitutions in their codons to give rise to a virus with a Tyr, Cys, or Phe at position 942. The remaining two highly attenuated viruses (with Ala or Thr assignments) would take three nucleotide substitutions to generate a codon for Tyr, Cys, or Phe. Thus, in this systematic examination of the *ts* and *att* phenotypes of 13 viable codon 942 substitution mutations, the Y942A and the Y942T mutations were identified as codon 942 substitutions that were highly attenuated and would require three nucleotide changes to occur to yield a virus with a wild-type-like phenotype. Fortunately, both of these viruses were among the 11 mutants that were recovered without adventitious mutations in the L gene, and thus these findings are unambiguous.

The genetic and phenotypic stability of one of these two rHPIV1 viruses, the Y942A virus, was examined following replication at restrictive temperatures. This process was done in parallel with that for the original Y942H virus, which differed from wild type by only a single nucleotide. The Y942H virus readily reverted to the wild-type phenotype after only four passages at 35 to 36°C, whereas the codon-stabilized Y942A virus exhibited only a partial loss of the *ts* phenotype even after eight passages at elevated temperatures. The phenotypic instability of the Y942H virus was accompanied by a direct reversion to the wild-type sequence at codon 942. This change occurred only following replication at elevated temperature, demonstrating the important role that elevated temperature played in selecting for the *ts* rHPIV1-Y942H revertant.

Sequence analysis confirmed that the stabilized Y942A virus retained the GCG Ala codon throughout its passages at elevated temperatures. However, the partial loss of the *ts* phenotype noted above was associated with the acquisition of one or more second-site amino acid substitutions involving three different positions in L. The finding that the stabilized Y942A virus retained a high degree of temperature sensitivity even following passage at highly restrictive temperatures suggests that it would also be more phenotypically stable in vitro and in vivo than the nonstabilized Y942H virus. This analysis of the codon substitutions at residue 942 of the HPIV1 L protein provides an example of how to identify mutations that exhibit a set of properties including attenuation and phenotypic stability desirable for inclusion in a live-attenuated virus vaccine. This particular example turned out to have several advantages that facilitated the selection of a stabilized mutant assignment: (i) the wild-type assignment of Tyr has only two possible codons, and (ii) among the mutants with alternative amino acid assignments, only two were not highly attenuated, and (iii) these two assignments each has only two possible codons.

In a previous study, the introduction of the HPIV3*cp*45 L992F L protein mutation into a wild-type HPIV3 backbone conferred the *ts* phenotype in vitro and the *att* phenotype in hamsters (28), identifying L992F as an independent *ts att* mutation. However, the introduction of this mutation into the homologous position in the HPIV1 background in the present study did not confer either phenotype. When alternative amino acid assignments were introduced at codon 992, only 10 of 17 mutants were recovered, indicating that this site is indeed sensitive to mutation. Three codon substitution mutants were identified, rHPIV1-L992V, rHPIV1-L992N, and rHPIV1-L992C, that were 10- to 100-fold restricted in replication in the upper or lower respiratory tract of hamsters compared to that for rHPIV1 wild-type virus. Thus, these findings exemplify a second use of codon substitution mutations, i.e., to generate mutants with an enhanced level of attenuation in vivo. It was of interest to find that each of the three attenuated recombinants (rHPIV1-L992C, rHPIV1-L992V, and rHPIV1-L992N) was also temperature sensitive. However, there was a dissociation between the level of temperature sensitivity and the level of attenuation. For example, the L992C mutation restricted replication in vitro at 36 to 37°C and was attenuating primarily in the lower respiratory tract, whereas the L992V mutation restricted replication in vitro only at 39°C but was highly attenuating in both the upper and lower respiratory tracts. Thus, the L992V mutation is predominantly of the non-*ts* type.

Previous studies have demonstrated that live-attenuated virus vaccines that contain *ts* attenuating mutations are optimally stabilized by the addition of non-*ts* attenuating mutations (22). Also, *ts* mutations exert their attenuating effect more prominently in the lower, warmer region of the respiratory tract, whereas non-*ts* mutations would attenuate irrespective of this temperature gradient. Hence, a mixture of both types of mutations would be optimal to achieve the dual goals of preventing serious lower respiratory tract disease and reducing upper respiratory tract congestion. The *ts* and non-*ts* codon substitu-

tion mutations identified in this report are now being combined with additional non-*ts* attenuating mutations to generate rHPIV1 vaccines that are satisfactorily attenuated, efficacious, and genetically stable.

REFERENCES

1. **Atmar, R. L., E. Guy, K. K. Guntupalli, J. L. Zimmerman, V. D. Bandi, B. D. Baxter, and S. B. Greenberg.** 1998. Respiratory tract viral infections in inner-city asthmatic adults. Arch. Intern. Med. **158:**2453–2459.
2. **Belshe, R. B., L. S. Richardson, T. J. Schnitzer, D. A. Prevar, E. Camargo, and R. M. Chanock.** 1977. Further characterization of the complementation group B temperature-sensitive mutant of respiratory syncytial virus. J. Virol. **24:**8–12.
3. **Chanock, R. M., B. R. Murphy, and P. L. Collins.** 2001. Parainfluenza viruses, p. 1341–1379. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Strauss (ed.), Fields virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, Pa.
4. **Collins, P. L., and B. R. Murphy.** 2002. Respiratory syncytial virus: reverse genetics and vaccine strategies. Virology **296:**204–211.
5. **Cortez, K. J., D. D. Erdman, T. C. Peret, V. J. Gill, R. Childs, A. J. Barrett, and J. E. Bennett.** 2001. Outbreak of human parainfluenza virus 3 infections in a hematopoietic stem cell transplant population. J. Infect. Dis. **184:**1093–1097.
6. **Counihan, M. E., D. K. Shay, R. C. Holman, S. A. Lowther, and L. J. Anderson.** 2001. Human parainfluenza virus-associated hospitalizations among children less than five years of age in the United States. Pediatr. Infect. Dis. J. **20:**646–653.
7. **Durbin, A. P., J. M. McAuliffe, P. L. Collins, and B. R. Murphy.** 1999. Mutations in the C, D, and V open reading frames of human parainfluenza virus type 3 attenuate replication in rodents and primates. Virology **261:**319–330.
8. **Feller, J. A., S. Smallwood, M. H. Skiadopoulos, B. R. Murphy, and S. A. Moyer.** 2000. Comparison of identical temperature-sensitive mutations in the L polymerase proteins of Sendai and parainfluenza3 viruses. Virology **276:**190–201.
9. **Fiore, A. E., C. Iverson, T. Messmer, D. Erdman, S. M. Lett, D. F. Talkington, L. J. Anderson, B. Fields, G. M. Carlone, R. F. Breiman, and M. S. Cetron.** 1998. Outbreak of pneumonia in a long-term care facility: antecedent human parainfluenza virus 1 infection may predispose to bacterial pneumonia. J. Am. Geriatr. Soc. **46:**1112–1117.
10. **Garcin, D., J. B. Marq, S. Goodbourn, and D. Kolakofsky.** 2003. The amino-terminal extensions of the longer Sendai virus C proteins modulate pY701-Stat1 and bulk Stat1 levels independently of interferon signaling. J. Virol. **77:**2321–2329.
11. **Garcin, D., T. Pelet, P. Calain, L. Roux, J. Curran, and D. Kolakofsky.** 1995. A highly recombinogenic system for the recovery of infectious Sendai paramyxovirus from cDNA: generation of a novel copy-back nondefective interfering virus. EMBO J. **14:**6087–6094.
12. **Heikkinen, T., M. Thint, and T. Chonmaitree.** 1999. Prevalence of various respiratory viruses in the middle ear during acute otitis media. N. Engl. J. Med. **340:**260–264.
13. **Henderson, F. W., A. M. Collier, M. A. Sanyal, J. M. Watkins, D. L. Fairclough, W. A. Clyde, Jr., and F. W. Denny.** 1982. A longitudinal study of respiratory viruses and bacteria in the etiology of acute otitis media with effusion. N. Engl. J. Med. **306:**1377–1383.
14. **Jokinen, C., L. Heiskanen, H. Juvonen, S. Kallinen, M. Kleemola, M. Koskela, M. Leinonen, P. R. Ronnberg, P. Saikku, M. Sten, A. Tarkiainen, H. Tukiainen, K. Pyorala, and P. H. Makela.** 2001. Microbial etiology of community-acquired pneumonia in the adult population of 4 municipalities in eastern Finland. Clin. Infect. Dis. **32:**1141–1154.
15. **Karron, R. A., M. Makhene, K. Gay, M. H. Wilson, M. L. Clements, and B. R. Murphy.** 1996. Evaluation of a live attenuated bovine parainfluenza type 3 vaccine in two- to six-month-old infants. Pediatr. Infect. Dis. J. **15:**650–654.
16. **Karron, R. A., P. F. Wright, F. K. Newman, M. Makhene, J. Thompson, R. Samorodin, M. H. Wilson, E. L. Anderson, M. L. Clements, B. R. Murphy, and R. B. Belshe.** 1995. A live human parainfluenza type 3 virus vaccine is attenuated and immunogenic in healthy infants and children. J. Infect. Dis. **172:**1445–1450.
17. **Lewis, V. A., R. Champlin, J. Englund, R. Couch, J. M. Goodrich, K. Rolston, D. Przepiorka, N. Q. Mirza, H. M. Yousuf, M. Luna, G. P. Bodey, and E. Whimbey.** 1996. Respiratory disease due to parainfluenza virus in adult bone marrow transplant recipients. Clin. Infect. Dis. **23:**1033–1037.
18. **Marx, A., H. E. Gary, Jr., B. J. Marston, D. D. Erdman, R. F. Breiman, T. J. Torok, J. F. Plouffe, T. M. File, Jr., and L. J. Anderson.** 1999. Parainfluenza virus infection among adults hospitalized for lower respiratory tract infection. Clin. Infect. Dis. **29:**134–140.
19. **Marx, A., T. J. Torok, R. C. Holman, M. J. Clarke, and L. J. Anderson.** 1997. Pediatric hospitalizations for croup (laryngotracheobronchitis): biennial increases associated with human parainfluenza virus 1 epidemics. J. Infect. Dis. **176:**1423–1427.
20. **Moeller, K., I. Duffy, P. Duprex, B. Rima, R. Beschorner, S. Fauser, R. Meyermann, S. Niewiesk, V. ter Meulen, and J. Schneider-Schaulies.** 2001. Recombinant measles viruses expressing altered hemagglutinin (H) genes: functional separation of mutations determining H antibody escape from neurovirulence. J. Virol. **75:**7612–7620.
21. **Murphy, B. R., and P. L. Collins.** 2002. Live-attenuated virus vaccines for respiratory syncytial and parainfluenza viruses: applications of reverse genetics. J. Clin. Investig. **110:**21–27.
22. **Murphy, B. R., E. J. Park, P. Gottlieb, and K. Subbarao.** 1997. An influenza A live attenuated reassortant virus possessing three temperature-sensitive mutations in the PB2 polymerase gene rapidly loses temperature sensitivity following replication in hamsters. Vaccine **15:**1372–1378.
23. **Newman, J. T., S. R. Surman, J. M. Riggs, C. T. Hansen, P. L. Collins, B. R. Murphy, and M. H. Skiadopoulos.** 2002. Sequence analysis of the Washington/1964 strain of human parainfluenza virus type 1 (HPIV1) and recovery and characterization of wild-type recombinant HPIV1 produced by reverse genetics. Virus Genes **24:**77–92.
24. **Polo, J. M., and R. E. Johnston.** 1991. Mutational analysis of a virulence locus in the E2 glycoprotein gene of Sindbis virus. J. Virol. **65:**6358–6361.
25. **Reed, G., P. H. Jewett, J. Thompson, S. Tollefson, and P. F. Wright.** 1997. Epidemiology and clinical impact of parainfluenza virus infections in otherwise healthy infants and young children <5 years old. J. Infect. Dis. **175:**807–813.
26. **Richardson, L. S., T. J. Schnitzer, R. B. Belshe, E. Camargo, D. A. Prevar, and R. M. Chanock.** 1977. Isolation and characterization of further defective clones of a temperature sensitive mutant (ts-1) of respiratory syncytial virus. Arch. Virol. **54:**53–60.
27. **Schoepp, R. J., and R. E. Johnston.** 1993. Directed mutagenesis of a Sindbis virus pathogenesis site. Virology **193:**149–159.
28. **Skiadopoulos, M. H., A. P. Durbin, J. M. Tatem, S. L. Wu, M. Paschalis, T. Tao, P. L. Collins, and B. R. Murphy.** 1998. Three amino acid substitutions in the L protein of the human parainfluenza virus type 3 *cp*45 live attenuated vaccine candidate contribute to its temperature-sensitive and attenuation phenotypes. J. Virol. **72:**1762–1768.
29. **Skiadopoulos, M. H., A. C. Schmidt, J. M. Riggs, S. R. Surman, W. R. Elkins, M. St. Claire, P. L. Collins, and B. R. Murphy.** 2003. Determinants of the host range restriction of replication of bovine parainfluenza virus type 3 in rhesus monkeys are polygenic. J. Virol. **77:**1141–1148.
30. **Skiadopoulos, M. H., S. Surman, J. M. Tatem, M. Paschalis, S. L. Wu, S. A. Udem, A. P. Durbin, P. L. Collins, and B. R. Murphy.** 1999. Identification of mutations contributing to the temperature-sensitive, cold-adapted, and attenuation phenotypes of the live-attenuated cold-passage 45 (*cp*45) human parainfluenza virus 3 candidate vaccine. J. Virol. **73:**1374–1381.
31. **Skiadopoulos, M. H., S. R. Surman, M. St. Claire, W. R. Elkins, P. L. Collins, and B. R. Murphy.** 1999. Attenuation of the recombinant human parainfluenza virus type 3 cp45 candidate vaccine virus is augmented by importation of the respiratory syncytial virus cpts530 L polymerase mutation. Virology **260:**125–135.
32. **Skiadopoulos, M. H., T. Tao, S. R. Surman, P. L. Collins, and B. R. Murphy.** 1999. Generation of a parainfluenza virus type 1 vaccine candidate by replacing the HN and F glycoproteins of the live-attenuated PIV3 cp45 vaccine virus with their PIV1 counterparts. Vaccine **18:**503–510.
33. **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.
34. **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.
35. **Wright, P. F., R. A. Karron, R. B. Belshe, J. Thompson, J. E. Crowe, Jr., T. G. Boyce, L. L. Halburnt, G. W. Reed, S. S. Whitehead, E. L. Anderson, A. E. Wittek, R. Casey, M. Eichelberger, B. Thumar, V. B. Randolph, S. A. Udem, R. M. Chanock, and B. R. Murphy.** 2000. Evaluation of a live, cold-passaged, temperature-sensitive, respiratory syncytial virus vaccine candidate in infancy. J. Infect. Dis. **182:**1331–1342.