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Recombinant human parainfluenza virus type 2 vaccine candidates containing a 3' genomic promoter mutation and L polymerase mutations are attenuated and protective in non-human primates

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Abstract

Previously, we identified several attenuating mutations in the L polymerase protein of human parainfluenza virus type 2 (HPIV2) and genetically stabilized those mutations using reverse genetics (Nolan et al., 2005). Here we describe the discovery of an attenuating mutation at nucleotide 15 (15^{T→C}) in the 3' genomic promoter that was also present in the previously characterized mutants. We evaluated the properties of this promoter mutation alone and in various combinations with the L polymerase mutations. Amino acid substitutions at L protein positions 460 (460A or 460P) or 948 (948L), or deletion of amino acids 1724 and 1725 (Δ 1724), each conferred a temperature sensitivity (*ts*) phenotype whereas the 15^{T→C} mutation did not. The 460A and 948L mutations each contributed to restricted replication in the lower respiratory tract of African green monkeys, but the Δ 1724 mutation increased attenuation only in certain combinations with other mutations. We constructed two highly attenuated viruses, rV94(15C)/460A/948L and rV94(15C)/948L/ Δ 1724, that were immunogenic and protective against challenge with wild-type HPIV2 in African green monkeys and, therefore, appear to be suitable for evaluation in humans.

1. Introduction

Human parainfluenza virus type 2 (HPIV2) is a medically important respiratory pathogen that causes croup and bronchiolitis in infants and young children [1-7]. The three major human parainfluenza viruses, HPIV1, HPIV2 and HPIV3, cause approximately 6.0%, 3.2%, and 11.5%, respectively, of pediatric hospitalizations due to viral respiratory disease [8]. The combined disease burden of these three viruses is nearly equivalent to that of respiratory syncytial virus (RSV), the leading cause of pediatric hospitalizations for respiratory disease [8]. Despite their medical impact, there are no licensed vaccines for any of these viruses. Our laboratory is currently developing live attenuated virus vaccines for intranasal immunization against each of these viruses to prevent or reduce the burden of severe lower respiratory tract disease in infants and young children [9-16]. Live attenuated respiratory virus vaccines were

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chosen for development because they can stimulate a broad immunological response, including the induction of serum and mucosal antibodies as well as virus-specific CD4⁺ and CD8⁺ T cells [17]. Furthermore, intranasally administered live attenuated virus vaccines or vaccine candidates, including the influenza A virus vaccine (FluMistTM), the parainfluenza type 3 (PIV3)-cold passage mutant 45 (*cp45*), and the recombinantly-derived RSV rA2cp248/404/1030ΔSH vaccine candidate, have been found to strike an acceptable balance between attenuation and immunogenicity in humans [18-21].

HPIV2 is an enveloped, non-segmented, negative-strand RNA virus belonging to genus *Rubulavirus* of the family *Paramyxoviridae*. The genome is 15,654 nucleotides in length and encodes 7 polypeptides from 6 genes arranged in the order 3'-N-V/P-M-F-HN-L-5'. HPIV2 conforms to the "rule of six", which means that efficient replication requires a genome length that is a multiple of six [22]. Transcription and replication are directed by the viral nucleocapsid protein (N), the phosphoprotein (P), and the large polymerase (L) protein [23]. The L protein is responsible for nucleotide polymerization and mRNA capping and methylation [23-28]. The fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins are associated with the viral envelope, along with the internal matrix protein (M). The HN and F proteins are the major protective antigens of parainfluenza viruses and are the two virus neutralization antigens [8]. The V protein has been identified as an antagonist of the interferon (IFN) response affecting both induction of type I IFN and signaling through the IFN receptor [29-33]. Transcription initiates at a single major promoter that spans the 3' extragenic leader region of the genome and extends into the upstream portion of the N gene [34].

Previously we constructed and characterized several HPIV2 mutants with temperature sensitive (*ts*) and attenuation (*att*) phenotypes conferred by mutations in the large polymerase (L) protein [16]. To generate these HPIV2 mutants, reverse genetics was used to "import" attenuating mutations from the L proteins of RSV, HPIV3, or bovine parainfluenza virus 3 (BPIV3) to homologous sites in the L protein of HPIV2. We increased the genetic and phenotypic stability of the original single nucleotide substitution mutations either by replacement with an alternative codon that would require two or three nucleotide changes to revert to codons encoding the wild type assignment, or by deletion of two codons (to maintain the "rule of six") encompassing the codon in question. Mutations at three sites in L - at amino acid positions 460, 948 and 1724 - generated rHPIV2 mutants that were *ts* for replication in vitro. At the site of the F460L mutation (imported from RSV [35-37]), it was found that alternative codons F460A and F460P were more *ts* than the original F460L mutation and were more *att* in both the upper (URT) and lower respiratory tract (LRT) of hamsters [16]. At the site of the Y948H mutation (imported from HPIV3*cp45* [38-40]), one alternative codon, Y948L, was significantly more *ts* and was 1000-fold more *att* in the URT and LRT of hamsters than the original Y948H mutation [16]. At the site of the S1724I mutation (imported from BPIV3 [12]), the original mutation was stabilized by deletion of codons 1724 and 1725 and is designated Δ1724. Its phenotype was slightly more *ts* and *att* in hamsters than that of the S1724I mutant [16]. All of these previously derived viruses had been evaluated in vivo in hamsters but not in non-human primates.

Upon evaluation of the HPIV2 L polymerase mutants for growth in African green monkeys (AGMs) for the present study, we identified a previously unrecognized attenuating mutation at nucleotide 15 (15^{T→C}) in the HPIV2 genome. This mutation involves a T to C nucleotide substitution in the 3' genomic promoter. It was not recognized previously because it is also present in the preparation of biological virus that was the basis for the construction of the full-length HPIV2 cDNA used in our reverse genetic system. However, in this study, we found that the 15^{T→C} mutation is not present in earlier passage levels of the biological virus and appears to be an adventitious mutation. To study the individual and aggregate contributions of the three L mutations and the newly identified 15^{T→C} mutation to the *ts* and *att* phenotypes of HPIV2,

we created recombinant viruses (four single, four double, and four triple mutants) using reverse genetics and evaluated them for attenuation in AGMs. With this information, we generated two HPIV2 live attenuated vaccine candidates by combining two genetically stabilized *ts* L gene mutations with the non-*ts* 3' promoter mutation. These viruses were evaluated for the temperature sensitivity of their replication in vitro as well as for their attenuation, immunogenicity, and efficacy in AGMs.

2. Materials and Methods

2.1 Cell lines and Viruses

Human HEp-2 (ATCC CCL 23) and rhesus monkey LLC-MK2 (ATCC CCL 7.1) cells were maintained in OptiMEM I (Invitrogen, Grand Island, NY) supplemented with 5% FBS and gentamicin sulfate (50 µg/mL). BSR T7/5 cells, which are baby hamster kidney 21 cells that constitutively express T7 RNA polymerase [41], were kindly supplied by Dr. Ursula Buchholz (NIAID) and were maintained in Glasgow minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 1X MEM amino acids solution (Invitrogen), 10% FBS, and 1 mg/ml Geneticin (Invitrogen). The biologically derived HPIV2 strain V9412-6 (V94) [22] was isolated from a nasal wash specimen obtained from an infected infant and was provided by Dr. Peter Wright of Vanderbilt University. V94 was biologically cloned and amplified on African green monkey Vero cells for a total of nine passages, followed by one passage on LLC-MK2 cells for further amplification; the sequence for this working pool was determined previously (Genbank accession # AF533010). HPIV2 strain V9811-18 (V98) (Genbank accession # AF533011) [22], also provided by Dr. Peter Wright, was isolated in rhesus monkey kidney cells from a nasal wash specimen and was passaged three times on LLC-MK2 cells. The HPIV2 Greer strain (Genbank accession # AF533012) [22] was obtained from the ATCC (VR-92; lot #1450727) and was passaged once on LLC-MK2 cells. The HPIV2 viruses were grown in LLC-MK2 cells. Virus titers were determined at 32°C by serial dilution on LLC-MK2 monolayers in 96-well plates using hemadsorption with guinea pig erythrocytes to detect infected wells [22], [42]. Titers are expressed as log₁₀ TCID₅₀/ml (50% tissue culture infectious dose/ml).

2.2 Introduction of mutations into rV94 cDNA

The HPIV2 recombinants described here are based on the HPIV2 strain V94 sequence (Genbank accession # AF533010). Two versions of the recombinant V94 (rV94) were derived from cDNA, one with and one without an introduced *NotI* restriction site in the upstream non-translated region of the N gene (HPIV2 nucleotide positions 149-156), as described previously [16,22]. As described below, this *NotI* site was phenotypically silent and is present in the rV94 viruses described in this paper except for the rV94(15C) recombinant HPIV2 virus, which was made with and without the *NotI* site (see footnote e in Table 1). Mutations were introduced into the HPIV2/V94 antigenomic sense cDNA using site-directed PCR mutagenesis. The 3' promoter mutation was introduced using a one-step PCR mutagenesis protocol, while L gene mutations were introduced by a two-step PCR mutagenesis protocol using the Advantage-HF PCR kit (Clontech Laboratories, Palo Alto, CA). The regions of the mutated cDNAs that had been subjected to in vitro polymerase reactions were sequenced using a Perkin-Elmer ABI 3730 sequencer with the BigDye sequencing kit v1.1 (Perkin-Elmer Applied Biosystems, Warrington, UK) as previously described [43]. Due to primer error, all mutants with the Δ1724 mutation also had an additional amino acid change at position 1726 except where noted. The wild type amino acid sequence was L₁₇₂₂HSTLT₁₇₂₇, which was mutated to L₁₇₂₂H—FT to create the Δ1724 mutation; where noted, the Δ1724 mutation with the correction at position 1726 has the sequence L₁₇₂₂H—LT.

2.3 Recovery of rHPIV2 L polymerase mutant viruses

Recovery and propagation of each recombinant virus was performed at the permissive temperature of 32°C as described previously [16,22]. Briefly, HEp-2 cells were cotransfected with a full-length HPIV2/V94 cDNA plasmid and three support plasmids that encode the HPIV2 N, P, and L proteins (pTM-N₂, pTM-P₂, and pTM-L₂), and were infected with recombinant vaccinia virus MVA-T7, as described [22]. In some transfections, the MVA-T7 virus and HEp-2 cells were replaced with BSR T7/5 cells [41]. Transfections for virus recovery in BSR T7/5 cells were done using Lipofectamine 2000 (Invitrogen) in OptiMEM without trypsin or serum. Transfected cells were maintained overnight at 37°C, then the Lipofectamine transfection medium was removed and replaced with Glasgow MEM without trypsin or serum, and the transfected cells were moved to 32°C. At two to four days post-transfection, cell supernatant was transferred to LLC-MK2 monolayers and propagated as described previously [16,22].

The rHPIV2 mutants were biologically cloned either by two rounds of plaque purification or by serial terminal dilution on LLC-MK2 monolayer cultures. To confirm that recovered recombinant viruses contained the appropriate mutations, viral RNA (vRNA) was isolated from medium supernatants of infected cells and amplified by RT-PCR using the SuperScript First-Strand Synthesis System (Invitrogen) for the RT step and the Advantage-HF PCR kit (Clontech Laboratories) for the PCR step according to protocols specified by the manufacturer as previously described [22,44]. Reactions lacking reverse transcriptase were included to confirm that the template for the amplified product was indeed RNA. Sequencing was performed on uncloned RT-PCR product. Each virus preparation used in this study was sequenced in its entirety except for the last 95 nucleotides (5' RACE was not performed for all preparations).

2.4 Replication of HPIV2 mutant in LLC-MK2 cells at permissive and restrictive temperatures

The level of temperature sensitivity of replication of each rV94 mutant was determined by comparing its level of replication to that of rV94 at the permissive temperature of 32°C and at elevated temperatures of 36°C to 40°C as previously described [45]. Briefly, each virus was serially 10-fold diluted on 96-well LLC-MK2 monolayer cultures in L15 media (Invitrogen). Replicate plates were incubated at the temperatures indicated above for six to seven days, and virus-infected wells were detected by hemadsorption with guinea pig erythrocytes. The virus titer at each temperature is the mean of two to three separate experiments and is expressed as a mean log₁₀ TCID₅₀/ml reduction in titer compared to the titer at 32°C. The shut-off temperature is defined as the lowest temperature at which the reduction in virus titer at that temperature compared to its titer at 32°C was at least 100-fold greater than that of rV94 at the same temperature compared to that at 32°C.

2.5 Replication of rHPIV2 mutants in the respiratory tract of hamsters

Four to five week-old Golden Syrian hamsters (Charles River Laboratories, NY) in groups of six were inoculated intranasally (IN) with 10^{6.0} TCID₅₀ of biologically derived or recombinant HPIV2 in 0.1 ml of L15 medium. Lungs and nasal turbinates were harvested on day four post-infection, and the titer of virus in tissue homogenates was determined by titration on LLC-MK2 monolayers as described above [22,44]. The virus titer, expressed as the mean log₁₀ TCID₅₀ per gram of tissue, was calculated for each group of six hamsters. For the V94 and rV94 groups, the mean was calculated from three separate experiments.

2.6 Replication of rHPIV2 mutants in the respiratory tract of AGMs

HPIV2 seronegative AGMs were inoculated IN and intratracheally (IT) with 1 ml of L15 medium per site containing 10^{6.0} TCID₅₀ of biologically derived or recombinant HPIV2. Nasopharyngeal (NP) swabs were collected on days 0-10 post-infection, and tracheal lavage

(TL) samples were collected on days 2, 4, 6, 8, and 10 post-infection. The virus titer in the samples was determined by serial dilution on LLC-MK2 monolayers at 32°C as described above. The mean peak virus titer was calculated using the highest titer detected in each animal in the group irrespective of day and was expressed as $\log_{10}$ TCID₅₀/ml. The lower limit of detection was 0.5 $\log_{10}$ TCID₅₀/ml. The total amount of virus shed over the course of the infection was calculated from the sum of daily virus titers on all sampling days for each animal. A mean of this sum was determined and is referred to as the mean sum of daily virus titers. The lower limit of detection was 5.5 $\log_{10}$ TCID₅₀/ml for NP swabs and 2.5 $\log_{10}$ TCID₅₀/ml for TL samples. Twenty-eight days after immunization the monkeys were challenged IN and IT with 1 ml ($10^{6.0}$ TCID₅₀) per site of V94. NP and TL samples were collected on days 2, 4, 6, and 8 post-challenge. The virus titer was determined as described above. Sera were collected from the monkeys on days 0, 28, and 56, and the hemagglutination inhibition (HAI) antibody titer was determined as previously described [46].

2.7 Sequence analysis of virus recovered from infected AGMs

The nucleotide assignment at position 15 of the HPIV2 genome was determined for a subset of TL samples obtained on day 4 from AGMs inoculated with the biologically derived V94. The virus present in these specimens was passaged once on LLC-MK2 monolayers to amplify the virus. vRNA was isolated and cDNA was prepared using 3' RACE, followed by sequencing of the cDNA product as previously described [44]. For analysis of the genetic stability of recombinant mutant viruses following replication in AGMs, the N, P, and L genes of viruses grown from NP and TL samples from the AGMs inoculated with biologically-derived and recombinant V94 viruses were sequenced to determine the stability of the mutations incorporated into rV94 following replication in AGMs. For the indicated animals, NP and TL samples from the last days of viral shedding were passaged once or twice on LLC-MK2 monolayers to amplify the virus. vRNA was isolated, and RT-PCR products spanning the N, P and L genes were prepared and sequenced as described above.

3. Results

3.1 Identification of an attenuating mutation at nucleotide position 15 in the HPIV2 3' genomic promoter region

We previously developed a reverse genetic system for HPIV2 strain V94 based on an antigenomic cDNA whose sequence was identical to the consensus sequence of the biological virus except for a silent C to T substitution at position 6265, located in the coding region of the F gene, and a *NotI* site that was introduced into the upstream non-coding region of the N gene. Recovered recombinant virus, referred to here as rV94(15C), was found to be indistinguishable from its biological parent on the basis of the kinetics and magnitude of replication in vitro and also was found to replicate efficiently in the respiratory tract of hamsters [16,22].

In the present study, we compared rV94(15C) to its biologically derived V94 parent with respect to the efficiency of replication in AGMs (Table 1). Unexpectedly, rV94(15C) was found to be significantly attenuated in the LRT compared to its biologically derived V94 parent (Table 1, see footnote f, and compare rV94(15C) with V94). The presence or absence of the *NotI* site in rV94(15C) did not affect its level of replication (see footnote e in Table 1). Since the only other known difference between rV94(15C) and its biological parent was the above mentioned silent mutation in the F gene, these results suggested that there might be another, unrecognized sequence difference.

Re-sequencing and closer inspection of the genomes of rV94(15C) and its V94 parent revealed that our working stock of the biologically derived V94 virus contained a mixed assignment (C

and T) at nucleotide position 15 in the 3' genomic promoter (Figure 1A, V94 electropherogram), whereas the rV94(15C) recombinant virus had only C at this position (not shown). While the predominant assignment in V94 was C, there was also a significant peak corresponding to an assignment of T, suggesting that our working stock of V94 contained two populations of virus that differed at nucleotide position 15.

We hypothesized that T might be the wild type assignment at nucleotide position 15 and that the C assignment might be attenuating in AGMs. In agreement with this hypothesis, virus shed by all AGMs infected with V94 had a T as the predominant assignment at position 15, whereas the assignment at position 15 in the input V94 virus was predominantly C (Figure 1A). This result indicated that virus with T at position 15 had outgrown virus with C at that position. In contrast, virus isolated from all four monkeys infected with rV94(15C) had a C at nucleotide position 15 (data not shown), matching the input virus and indicating that this is a stable assignment when present as a homogeneous population of virus. These observations suggest that the viruses with T at nucleotide 15 replicated more efficiently in the lower respiratory tract of AGMs than viruses with C.

We sequenced V94 preparations from earlier passages to test the hypothesis that the assignment of C at position 15 had been acquired in cell culture. The V94 clinical isolate had been passaged nine times in Vero cells and once in LLC-MK2 cells. Analysis of V94 virus from Vero passage nine (V94-P9) showed that it had a T at nucleotide 15 (Figure 1B). This supported the idea that the assignment in the original clinical isolate was T and that the substitution with C had occurred in cell culture during the one passage in LLC-MK2 cells. We also examined sequences that were available for two other strains of wild type HPIV2 (Figure 1B) [22]. The V98 strain, a minimally-passaged HPIV2 strain, has T at this position, whereas the multiply-passaged prototype Greer strain has C at position 15 [22]. Taken together, these observations suggest that T indeed is the wild type assignment at position 15 and that substitution to C can occur during passage in vitro, especially in LLC-MK2 cells.

To directly examine whether the T to C substitution at nucleotide 15 was responsible for the *att* phenotype of rV94(15C) in AGMs, a recombinant virus with T at position 15, rV94(15T), was constructed and recovered from cDNA. The recovered and fully sequenced rV94(15T) virus differed from the rV94(15C) virus only by the assignment of T versus C at position 15. When inoculated into AGMs, rV94(15T) replicated to a significantly higher level than rV94(15C) in both the URT and LRT (Table 1, see footnote g). Thus, the T to C substitution (referred to here as the 15^{T→C} mutation), a change that arose during passage of HPIV2 in cell culture, attenuates HPIV2 for replication in the URT and LRT of AGMs. In addition, rV94(15T) replicated as efficiently as the biologically derived V94 virus in the LRT and marginally (although not significantly) more efficiently in the URT. This indicates that rV94(15T) indeed has a wild type phenotype.

We also evaluated the efficiency of replication of the biological V98 and Greer strains (bearing T and C, respectively, at position 15) in AGMs for comparison with the V94 (bearing mixed C/T population), rV94(15T), and rV94(15C) viruses. Those viruses containing T or a mixture of T and C at nucleotide position 15 (namely V94, rV94(15T), and V98) grew to a higher titer in the TL specimens (4.4 to 4.6 log₁₀TCID₅₀/ml) than those with exclusively C at this position (namely rV94(15C) and Greer), which grew to 3.0 to 3.7 log₁₀TCID₅₀/ml (Table 1). However, these differences were not statistically significant, possibly due to the small number of animals tested for two of the five groups as well as the other genetic differences between the V94-based viruses versus the V98 and Greer strains.

Since the T-to-C substitution at position 15 occurred during passage in cell culture, we investigated whether it conferred a growth advantage that might explain its predominance in

the multiply-passaged V94 virus (and the Greer strain). When the rV94(15T) and rV94(15C) viruses were evaluated in multi-step growth studies in LLC-MK2 and Vero cell cultures, the two recombinant viruses exhibited similar replication kinetics (data not shown) and achieved titers of over $8.0 \log_{10}$ TCID₅₀/mL. Therefore, there was no apparent growth advantage for the virus bearing the 15^{T→C} mutation in these assays. However, we noted that rV94(15C) was considerably more cytopathic than rV94(15T) in Vero cells, although the extent of infection of the monolayer seemed comparable (data not shown). Thus, the difference in the nucleotide assignment at position 15 was associated with a subtle phenotypic difference in Vero cells, but the basis and significance of this effect is not known, especially since the 15^{T→C} mutation occurred during passage in LLC-MK2 cells.

3.2 Evaluation of rV94(15T) virus replication in hamsters

Next, we compared rV94(15C), rV94(15T), and V94 with regard to their efficiency of replication in hamsters following IN inoculation with $10^{6.0}$ TCID₅₀ of virus. In the URT of hamsters, the viruses grew to similar levels (Table 2). However, in the LRT, rV94(15C) and V94 were reduced 10-fold in their level of replication compared to rV94(15T). Thus, the 15^{T→C} mutation slightly attenuated HPIV2 for replication in the LRT of hamsters, in addition to attenuating its replication in AGMs.

3.3 Evaluation of rV94(15T) mutants for replication in vitro at permissive and restrictive temperatures

We had previously generated a series of V94 recombinants bearing independent amino acid substitutions in the L polymerase protein (Y948L, F460A, and F460P, the latter two being two different mutant assignments at position 460) with codons chosen to enhance their genetic and phenotypic stability [16]. We also created a deletion mutation, $\Delta 1724$, by deleting codons 1724 and 1725 of the L gene [16]. We previously recovered viruses containing each of these mutations individually in the rV94(15C) background [16]; their designations have been modified here to indicate the presence of the previously unrecognized attenuating 15^{T→C} mutation (Table 3). Since each of these mutations in L conferred temperature sensitivity in vitro and restricted replication in hamsters in the V94(15C) background, they were considered promising mutations for incorporation into HPIV2 vaccine candidates [16]. However, since we now realize that these previously generated viruses in fact each contained two mutations, namely the individual L protein mutation plus the 15^{T→C} mutation, the *ts* and *att* phenotype associated with each L protein mutation on its own remained unknown. Therefore, new rV94 mutants were generated with the individual L mutations in the 15T background (Table 3). In addition, we made additional viruses containing various combinations of one or two L mutations in the 15C background for pre-clinical evaluation as potential vaccine candidates.

We sequenced the genome of each of the viruses constructed in the present study. We also sequenced the genome of each of the viruses used in this study that had been constructed previously and had been only partially sequenced in previous work [16]. The sequences of the viruses were confirmed to be as expected with two exceptions. First, every virus containing the $\Delta 1724$ mutation shown in Table 3 was found to also contain the substitution of Leu to Phe at amino acid position 1726 in the L protein, the position immediately downstream of the deletion. Second, rV94(15C)/ $\Delta 1724$ was found to contain three additional adventitious amino acid substitutions, namely G118V in the P protein, D243S in the M protein, and K141E in the L protein.

These single, double, and triple mutants were evaluated at permissive (32° C) and potentially restrictive (36-40° C) temperatures to determine their level of temperature sensitivity for replication in LLC-MK2 cells (Table 3). rV94(15T) and rV94(15C) were not significantly restricted in replication at 40° C, indicating that the 15^{T→C} mutation is a non-*ts* attenuating

mutation. Each of the mutants containing a single L polymerase mutation was *ts* (shut-off temperature 39-40°C), irrespective of whether position 15 was T or C. The combination of the 460A/948L or 948L/ Δ 1724 mutations conferred a higher level of temperature sensitivity (shutoff temperature 37°C) than that of the single L mutations. Thus, these two combinations of mutations in L were additive for temperature sensitivity. Surprisingly, the mutants containing a combination of Δ 1724 and either 460A or 460P were not more *ts* than the mutants bearing only one of these mutations.

A slight difference in temperature sensitivity was observed between rV94(15T)/ Δ 1724 (40°C shut-off temperature) and rV94(15C)/ Δ 1724 (39°C shut-off temperature). This likely was due to the three adventitious amino acid substitutions present in the latter virus, as already noted. Since *ts* mutations often are attenuating, one or more of these adventitious mutations might be of potential value for vaccine development, although this was not investigated further in the present study. Since all of the mutants bearing the Δ 1724 mutation also had an amino acid substitution (Leu 1726 to Phe) in the L protein, as noted above, we constructed a new version of rV94(15T)/ Δ 1724 in which this L1726F substitution was corrected (data not shown). Complete sequence analysis confirmed the lack of any adventitious mutations in this repaired virus. Repair of the L1726F substitution in rV94(15T)/ Δ 1724 had no effect on the level of restriction of replication in vitro at 39°C (data not shown), confirming that the *ts* phenotype of rV94(15T)/ Δ 1724 was due to the deletion and was not affected by the Leu to Phe substitution at residue 1726. The corrected version was not further evaluated.

3.4 Evaluation of rV94 mutants for replication in the respiratory tract of AGMs

rV94 L polymerase mutants were next evaluated for their level of replication in the respiratory tract of AGMs (Table 4). The level of attenuation of rV94 viruses bearing either the 460A, 948L, or Δ 1724 mutation in the rV94(15T) background were compared to rV94(15T) to determine the individual contribution that each mutation makes to attenuation (compare Groups 3, 4, and 5 to Group 1). Groups of AGMs were infected IN and IT with $10^{6.0}$ TCID₅₀ of each virus. Both the 460A and 948L mutations caused significant attenuation of replication in the LRT of infected animals (approximately 500- and 1000-fold, respectively), while replication in the URT was not significantly restricted (Groups 3 and 4, Table 4). This observation is consistent with the temperature sensitivity data of rV94(15T)/460A and rV94(15T)/948L (Table 3) because AGMs have a high average core temperature of 38.6°C. The rV94(15T)/ Δ 1724 mutant (Group 5) was not attenuated in either the URT or the LRT, indicating that the Δ 1724 mutation was not attenuating on its own.

We then evaluated the 460A, 460P, 948L, and Δ 1724 mutations in the 15C background (Groups 7-10, Table 4) for replication in AGMs and compared them to the viruses described above bearing the same individual mutations in the 15T background (Groups 3-5). This was done to determine if the attenuation conferred by the 15^{T→C} mutation was additive with that of each L mutation. While the rV94(15C)/460A, rV94(15C)/460P, and rV94(15C)/948L viruses were more restricted in replication in the LRT than rV94(15C), they were no more attenuated in the LRT than their counterparts in the 15T background, indicating that the inclusion of the 15^{T→C} mutation did not increase the level of attenuation conferred by these L mutations. rV94(15C)/ Δ 1724 was not significantly more attenuated than rV94(15C) in the LRT; this was consistent with the lack of attenuation associated with Δ 1724 on its own, as was observed above, and indicated that Δ 1724 also did not augment the level of attenuation conferred by the 15^{T→C} mutation.

We then evaluated recombinant viruses containing two L polymerase mutations incorporated into the 15C background for replication in AGMs (Groups 11-14, Table 4). Surprisingly, the combination of the 15^{T→C} mutation and the Δ 1724 mutation with either the 460A or the 460P mutation in L resulted in viruses (Groups 11 and 12, Table 4) that were no longer restricted

for replication in the respiratory tract of AGMs. This indicates that the combination of these three mutations abrogated the *att* phenotype specified by the amino acid substitutions at L protein position 460 as well as that specified by the 15^{T→C} mutation. Therefore, these two rHPIV2 mutants were considered unsatisfactory for further development as an HPIV2 vaccine.

The two other viruses containing two L polymerase mutations incorporated into the 15C background, namely rV94(15C)/948L/Δ1724 and rV94(15C)/460A/948L, contained the 948L and the 15^{T→C} mutations in combination with either the 460A or the Δ1724 mutation in L. The peak mean titer of virus from AGMs infected with rV94(15C)/948L/Δ1724 or with rV94(15C)/460A/948L (Groups 13 and 14, Table 4) was approximately 70 to 700-fold less in the URT and 4000-fold less in the LRT than that from AGMs infected with rV94(15T) wild type HPIV2. These two viruses were the most *ts* in vitro (Table 3) and the most *att* in vivo (Table 4), suggesting that the additive effects of all three mutations defined their phenotype. For example, adding the Δ1724 mutation to the rV94(15C)/948L mutation changed the shut-off temperature of the virus from 39°C to 37°C and resulted in restricted replication in both the URT and LRT (compare Groups 9 and 13, Table 4). Thus, even though the Δ1724 mutation is not attenuating as an individual mutation (Group 5, Table 4), it has a strong effect in the context of the 15C and 948L mutations. Similarly, additivity to the *ts* and *att* phenotypes was seen when the 948L mutation was added to rV94(15C)/460A (compare Groups 7 and 14, Table 4). In summary, rV94(15C)/948L/Δ1724 and rV94(15C)/460A/948L are two highly attenuated rHPIV2 mutants with growth properties in AGMs that make them attractive as vaccine candidates for evaluation in clinical trials.

3.5 Analysis of the genetic stability of mutations in vaccine candidates following their replication in AGMs

To enhance safety, successful vaccine candidates should maintain their attenuating mutations during replication in the immunized host. As a first step in evaluating the genetic stability of our panel of attenuating HPIV2 mutations, we performed partial sequence analysis of viral isolates recovered from AGMs that had been inoculated with rV94(15C), rV94(15C)/460A, rV94(15C)/460P, rV94(15C)/948L, or rV94(15C)/Δ1724 in the experiments summarized in Table 4 (Table 5). These particular mutants were chosen for analysis because they replicated to a higher titer and for a longer duration compared to the more restricted vaccine candidates, namely rV94(15C)/948L/Δ1724 and rV94(15C)/460A/948L, and thus provided a greater opportunity for potential spontaneous mutations to occur and be amplified. The samples that were examined were collected from the URT or LRT of infected monkeys during the last several days of virus shedding (typically day six or seven post-inoculation), which also would provide a greater opportunity for any potential spontaneous mutations to occur and be amplified. For each input virus, isolates were analyzed from one or more days of shedding from between three and thirteen animals, for a total of six to sixteen samples per virus. The isolates were amplified by one or two passages in LLC-MK2 cells and subjected to sequence analysis of the N, P, and L genes. Position 15 was not analyzed because the 3' RACE procedure was cumbersome for this large number of samples in a pre-clinical setting, although this analysis will be performed on subsequent clinical samples.

Each of the *att* mutations was found to be completely intact in each of the samples with the exception of a single isolate from an animal that had been infected with rV94(15C)/460P (Table 5). In this exception, a single nucleotide substitution had occurred in the L protein that changed the amino acid at position 460 from a proline to a leucine. The assignment at this position in the original biological virus was phenylalanine. Previous work demonstrated that the 460L mutation was less attenuating than the 460P mutation [16]. Thus, this constituted a partial phenotypic reversion at this site. In addition, the various isolates contained as many as four adventitious amino acid substitutions in N, P, and L per isolate, with the total number of

mutations per virus group ranging from zero to eight (Table 5). These occurred in both wild type and mutant viruses. The effect of these adventitious mutations on the level of replication of the isolates in vitro or in vivo was not tested.

3.5 Immunogenicity and efficacy of rV94(15C) mutants against challenge with wild type HPIV2 in AGMs

We evaluated the various mutant viruses for immunogenicity and protective efficacy against a challenge with wild type HPIV2. The antibody response to infection with biologically derived or recombinant HPIV2 was assessed by a hemagglutination inhibition assay (HAI). All of the animals were negative for HPIV2 HAI serum antibody at the start of the studies (HAI titer $\leq 1 \log_2$). Following the single initial immunization, each group of immunized animals had at least a three-fold rise in HAI antibody titer, with the exception of the groups that received rV94 (15T)/948L (2.8-fold rise), rV94(15C)/ Δ 1724 (2.2-fold rise), and rV94(15C)/948L/ Δ 1724 (2.3-fold rise) (Groups 4, 10, and 13, respectively, Table 6).

The level of protective efficacy conferred by immunization with rV94 mutants containing two or more *att* mutations was evaluated by challenge with biologically derived V94 virus at four to six weeks after the initial immunization. Protective efficacy was evaluated on the basis of the reduction in the titer of infectious challenge virus in NP and TL samples. Immunization with any of the HPIV2 mutants conferred a high level of protection in the upper and lower respiratory tract of the challenged animals, such that there was at least a 100-fold decrease in the mean peak titer of challenge virus in both the NP and TL samples, and in a number of cases, no challenge virus was detected (Table 6). Even rV94(15C)/460A/948L and rV94(15C)/948L/ Δ 1724, the two vaccine candidates that were highly attenuated, protected immunized AGMs against challenge with wild type HPIV2 (Groups 13 and 14, Table 6). The mean HAI antibody titer for each group of AGMs increased after challenge compared to the post-immunization titer, indicating that the HPIV2 challenge virus replicated to some extent in most of the immunized monkeys, which would be consistent with our expectations. The levels of immunogenicity and protective efficacy achieved with a single immunization of the more highly attenuated mutant viruses make them excellent candidates for clinical evaluation.

4. Discussion

The reverse genetic system for HPIV2 enables the introduction of attenuating mutations into the HPIV2 genome in desired combinations to generate defined, live attenuated vaccine candidates [16,22]. Furthermore, the reverse genetic system has been used to increase the genetic and phenotypic stability of introduced attenuating mutations and to increase the level of temperature sensitivity and attenuation associated with the original mutations [9,16].

In the current study, we uncovered an attenuating, non-*ts* mutation, referred to as the 15^{T→C} mutation, that is present in all of the rHPIV2 wild type and mutant viruses that we had previously generated [16]. Until now, this mutation was unnoticed because it is also the predominant position 15 nucleotide assignment in the preparation of biologically-derived wild type V94 on which the reverse genetic system was based. Although C was the predominant assignment in the working stock of V94 (and hence its inclusion in the reverse genetic system), examination of viral isolates recovered from AGMs that had been inoculated with this preparation showed that the minor population with the 15T assignment outgrew the 15C-containing population. The preferential amplification of the 15T subpopulation in infected AGMs would explain why the preparation of biological V94 virus achieved a higher titer compared to the rV94(15C) virus, which contained only the attenuating C assignment at this position.

Several observations support our hypothesis that T is the wild type assignment at nucleotide 15 and that C is an attenuating assignment that was acquired during passage in vitro. First, when we constructed a recombinant virus, rV94(15T), that was identical to rV94(15C) but for the assignment of T at position 15, we found that this virus replicated significantly more efficiently in AGMs than the rV94(15C) virus. This confirmed that 15^{T→C} is an attenuating mutation. Second, the 15^{T→C} mutation was acquired during passage in cell culture. Analysis of virus from the ninth and final passage of V94 in Vero cells revealed the assignment of T at position 15, indicating that the assignment of C at this position indeed represented a mutation and had arisen during the subsequent, single passage in LLC-MK2 cells. Third, we noted that the V98 strain, which had not been passaged extensively in vitro, had the assignment of T at position 15, whereas the Greer strain, which had been passaged extensively in vitro, had the assignment of C. Mutation of position 15 from T to C during passage may be a common phenomenon for HPIV2.

Thus, we concluded that 15T is the wild type nucleotide assignment at this position, whereas C is an attenuating assignment that was acquired during passage in cell culture. Although the assignment of C at this position was associated with increased cytopathic effect in Vero cells, it did not confer a noticeable advantage in virus yield in either Vero or LLC-MK2 cells. Therefore, whatever advantage the 15^{T→C} mutation provides appears to be subtle, which would explain why the subpopulation bearing C did not rapidly and completely outgrow that bearing T.

Importantly, these findings identified a novel non-*ts att* mutation that has the added advantage of growing well in Vero cells, the cell substrate that would be used for vaccine manufacture. The observation that the 15^{T→C} mutation appears to be favored during replication in vitro indicates probable genetic stability during vaccine manufacture. In addition, the C at position 15 was maintained following replication in vivo in the limited number of animals tested, although genetic stability will be evaluated more extensively during clinical trials. This mutation can therefore be included as an attenuating mutation in a live attenuated HPIV2 vaccine candidate. Similar adventitious mutations that arose spontaneously during passage of wild type virus in cell culture and that are attenuating in vivo are not uncommon and have been documented for diverse families of viruses [14,47,48]. However, because each of the previously derived rHPIV2 viruses bearing attenuating mutations in L actually contained two attenuating mutations, the 15C and the L mutations, it was necessary to reevaluate the relative contribution of each individual L protein mutation to the observed in vitro and in vivo phenotypes of these viruses.

We therefore generated rHPIV2 mutants bearing only single *att* mutations in L (i.e., without the adventitious 15^{T→C} mutation) in order to define the individual contribution of the stabilized L mutations (the 460A, 948L, or Δ1724 mutation) to the in vitro *ts* phenotype as well as to the in vivo *att* phenotype in AGMs. Each of these three rHPIV2 L mutants was found to be temperature sensitive for replication in vitro. The 460A and 948L mutations specified the same level of temperature sensitivity in either the 15T or 15C background, indicating that the level of temperature sensitivity of the previously generated rV94(15C)/460A and rV94(15C)/948L was due solely to the mutation in L. In vivo, the 460A and 948L individual mutations specified *att* phenotypes in AGMs, whereas the Δ1724 mutation was not an independent attenuating mutation in HPIV2. The attenuation conferred by the 15C mutation was apparent in both the URT and the LRT, whereas that specified by 460A and 948L was observed only in the LRT, which was the anticipated result for a *ts* mutant that is restricted in replication in the warmer lower respiratory tract of an animal model with an average body core temperature of 38.6°C.

Upon evaluating the double mutants containing the 15^{T→C} mutation and one of the four L mutations (460A, 460P, 948L, or Δ1724), we found that the attenuation specified by each of

the four L mutations did not add to that specified by the 15^{T→C} mutation. In other words, the level of restriction of replication was not the sum of that specified by two independent attenuating mutations but, instead, reflected the restriction specified by the more attenuating mutation. Each of the double mutants was restricted in replication in both the URT and the LRT, a finding that was expected since each virus contains the 15^{T→C} mutation that attenuates HPIV2 in both sites.

The genetic stability of selected single and double mutants during replication in AGMs was investigated by isolating virus from the URT and LRT of infected animals toward the end of the course of replication. Sequencing of the L, N, and P genes, which are the potential interacting genes in the HPIV2 transcriptase/replicase complex, determined that the L polymerase attenuating mutations were maintained in 43 of the 44 isolates. The one exception was 460P in a single isolate, which mutated to 460L, an assignment that was not the same as the assignment in the biological virus (phenylalanine) but previously had been shown to yield a weakly attenuated phenotype in hamsters [16]. We plan to evaluate the genetic (sequence) and phenotypic (*att* phenotype in hamsters) stability of virus isolates recovered from upcoming clinical trials of two HPIV2 vaccine candidates.

Four rHPIV2 viruses with combinations of three mutations were constructed in an attempt to create highly attenuated vaccine candidates. Two of these, rV94(15C)/460A/Δ1724 and rV94(15C)/460P/Δ1724, were shown to be *ts* in vitro but, unexpectedly, were not attenuated for AGMs. Thus, addition of the Δ1724 mutation to the highly attenuated rV94(15C)/460A or rV94(15C)/460P mutant had the paradoxical effect of increasing virus replication in both the URT and LRT of AGMs. A plausible explanation is that the combination of the 460A or 460P substitution with Δ1724 alters the conformation of the L polymerase and restores wild type activity in the cells of the respiratory tract of AGMs. rV94(15C)/460A/Δ1724 and rV94(15C)/460P/Δ1724 were at least 10,000-fold restricted in replication at 39°C but replicated like wild type rHPIV2 in vivo (in an experimental animal with a body temperature of 38.6°C), suggesting that their *ts* phenotype was host-dependent, i.e., they were *ts* in LLC-MK2 cells in culture but not in vivo in respiratory epithelium. Such mutants have been described for other viruses [49]. This reversal of phenotype was also seen when a pair of *ts att* mutations in the L polymerase (at amino acid residues 992 and 1558) of HPIV3 were combined in one virus. Specifically, when the 992 and 1558 mutations were combined (r992/1558), the result was to augment, rather than further attenuate, HPIV3 replication in vivo [38]. Clearly, the rV94(15C)/460A/Δ1724 and rV94(15C)/460P/Δ1724 mutants are not candidates for a live attenuated HPIV2 vaccine.

In contrast, the two other vaccine candidates containing three *att* mutations, rV94(15C)/460A/948L and rV94(15C)/948L/Δ1724, exhibited properties of an acceptable vaccine candidate. Both viruses were more temperature sensitive than mutants containing the individual component mutations (shut-off temperature of 37°C versus 39-40°C) suggesting an additive effect of the individual mutations on the level of temperature sensitivity in vitro. This level of temperature sensitivity correlates well with the high level of attenuation of these mutants in the LRT of AGMs, and appears to be due to an additive effect of the attenuation conferred by each of the three mutations. It was interesting to find that the Δ1724 mutation, although not attenuating by itself in AGMs, increased the level of temperature sensitivity and attenuation of rV94(15C)/948L, especially attenuation in the URT. Although both triple mutant candidates were highly restricted in replication in the URT and LRT of AGMs, they nonetheless induced a substantial level of protection against HPIV2 wild type virus challenge. This protection was associated with the induction of only a low level of serum HAI antibodies. The decreased immunogenicity of the vaccine candidates compared to that of HPIV2 wild type was not unexpected since attenuated vaccine viruses are typically less immunogenic than their wild type parent virus due primarily to reduced replication and antigen production in vivo.

We plan to evaluate the attenuation and immunogenicity of the highly attenuated vaccine candidates, rV94(15C)/948L/Δ1724 and rV94(15C)/460A/948L, in humans. We predict that these viruses will replicate slightly better in humans due to a lower core body temperature (37°C for humans versus 38.6°C for AGMs) and the fact that HPIV2 is better adapted to humans, its natural host, than to AGMs, in which it causes only an asymptomatic infection. This increased replication in humans should be accompanied by an increase in immunogenicity. We also plan to further explore potentially non-*ts*, *att* mutations in the V protein to develop recombinants deficient in their ability to inhibit the production and signaling of interferon.

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Figure 1.

Nucleotide assignment at position 15 in the 3' genomic promoter region of recombinant and biologically derived HPIV2 strains. A.) Sequence analysis of HPIV2 isolates recovered from AGMs inoculated with biologically derived V94. Electropherograms show the sequence of nucleotides 12-18 for each indicated isolate. The predominant nucleotide at each position is listed, and nucleotide 15 is indicated with a dot. The first electropherogram shows the sequence of the biologically derived V94 HPIV2, the virus used to infect the AGMs. The remaining four electropherograms to the right show the sequences of HPIV2 isolated from the TL samples of four different AGMs on day four after IN and IT inoculation with V94. Compare nucleotide position 15 of the V94 input virus (far left) with that of isolates from AGMs. B.) The sequence of nucleotides 1-30 is shown for the biologically derived viruses V98 and V94 (at the ninth passage in Vero cells and at an additional passage in LLC-MK2 cells), recombinant V94, and the laboratory strain Greer. Bold letters indicate the nucleotide assignment at position 15 for each virus.

Table 1
Replication of biologically derived and recombinant HPIV2 in the upper and lower respiratory tract of AGMs

HPIV2 virus ^a	Nucleotide assignment at position 15	No. of animals ^b	Mean peak virus titer ^c (log ₁₀ TCID ₅₀ /ml ± S.E.)				Mean sum of daily virus titers ^d (log ₁₀ TCID ₅₀ /ml ± S.E.)
			Nasopharyngeal Wash (NP)	Tracheal Lavage (TL)	NP	TL	
rV94(15T)	T	11	3.6 ± 0.3	4.6 ± 0.5	20.9 ± 2.4	11.5 ± 1.4	
rV94(15C) ^e	C	21	2.9 ± 0.3	3.0 ± 0.2^{f,g}	13.4 ± 1.4^g	7.6 ± 0.7^{f,h}	
V94	C/T ⁱ	10	2.7 ± 0.3	4.4 ± 0.3	12.9 ± 1.2^g	13.4 ± 1.3	
V98	T	4	2.6 ± 0.6	4.6 ± 0.5	11.5 ± 2.5	14.5 ± 2.6	
Greer	C	4	2.7 ± 0.6	3.7 ± 0.5	11.8 ± 2.6	12.9 ± 2.1	

^a AGMs in groups of 2-6 were inoculated on day 0 IN and IT with 10^{6.0} TCID₅₀ of the indicated virus at each site. NP swabs were collected on days 1-10 post-infection and TL samples were collected on days 2, 4, 6, 8, and 10 post-infection, and virus titers were determined by limiting dilution on LLC-MK2 cells at 32°C.

^b Total number of animals from several independent experiments.

^c Mean of the peak virus titers for the individual animals in each group irrespective of sampling day. S.E. = standard error. The lower limit of detection was 0.5 log₁₀ TCID₅₀/ml.

^d Mean of the sum of the daily viral titers for each animal in the group taken on all sampling days (a measure of the aggregate viral load over the course of the experiment). The limit of detection was 5.5 log₁₀ TCID₅₀/ml in the NP swabs and 2.5 log₁₀ TCID₅₀/ml in the TL samples.

^e The rV94(15C) group included 13 monkeys immunized with the version of the virus that contains a *NorI* site in the pre-N position and 8 monkeys immunized with the version without the *NorI* site. There was no significant difference in replication between the two viruses in either the upper or lower respiratory tract, and therefore the data were combined.

^f Replication of the indicated virus was reduced compared to V94 using the Tukey-Kramer multiple comparisons test with a p value <0.05.

^g Replication of the indicated viruses was reduced compared to rV94(15T) using the Tukey-Kramer multiple comparisons test with a p value <0.05.

^h Replication of the indicated virus was reduced compared to V98 using the Tukey-Kramer multiple comparisons test with a p value <0.05.

ⁱ The V94 working pool contained a mixed population of virus at nucleotide position 15 with a predominant peak of C and a secondary peak of T (see Fig. 1A).

Table 2
Replication of wild type and recombinant HPIV2 in the upper and lower respiratory tract of hamsters

Virus ^a	No. of animals ^b	Mean virus titer ^c (log ₁₀ TCID ₅₀ /g ± S.E.)	
		Nasal turbinates	Lungs
V94	18	5.0 ± 0.1	5.3 ± 0.4
rV94(15C)	18	5.3 ± 0.1	5.3 ± 0.2 ^d
rV94(15T)	6	5.5 ± 0.1	6.4 ± 0.2 ^d

^aHamsters in groups of 6 were infected IN with 10^{6.0} TCID₅₀ of the indicated virus.

^bData shown were compiled from three separate experiments for V94 and rV94(15C); rV94(15T) was included in one of these experiments.

^cNasal turbinates and lung tissues were harvested on day four. Virus present in the tissue homogenates was quantified by serial dilution on LLC-MK2 monolayers at 32°C and is expressed as the mean virus titer per gram of tissue. S.E. = standard error.

^dThe difference between rV94(15C) and rV94(15T) was significant using the unpaired t test (p <0.003).

Table 3

Replication of wild type and mutant HPIV2 at permissive and restrictive temperatures in LLC-MK2 cells

Virus	Mean titer at 32°C	Mean reduction (log ₁₀) in virus titer at the indicated temperature versus 32°C ^a				
		36°C	37°C	38°C	39°C	40°C
rV94(15T)	7.6	-0.3	-0.2	-0.2	0.2	0.1
rV94(15C) ^b	7.5	-0.1	-0.2	-0.1	0.3	0.5
rV94(15C)/460A ^b	7.0		0.7	1.1	2.1	≥5.0
rV94(15T)/460A	8.0		0.3	0.9	2.2	≥5.8
rV94(15C)/460P ^b	6.8		1.3	1.1	2.2	≥5.1
rV94(15C)/948L ^b	7.1		1.1	1.9	≥5.5	≥5.7
rV94(15T)/948L	7.8		0.3	1.7	4.9	≥6.1
rV94(15C)/Δ1724 ^{bc}	7.3		1.0	1.5	4.0	≥6.2
rV94(15T)/Δ1724 ^c	7.9		0.3	0.3	0.6	3.5
rV94(15C)/460A/Δ1724	7.5		0.4	1.7	4.2	≥4.8
rV94(15C)/460P/Δ1724	7.4		0.6	1.9	≥5.1	≥4.7
rV94(15C)/948L/Δ1724	7.2	0.7	2.1	≥5.5	≥5.2	≥4.8
rV94(15C)/460A/948L	6.2	1.4	3.3	≥5.0	≥5.0	≥5.0

^a Values are the means from at least 2 experiments. Values in bold type indicate temperatures at which the mean log₁₀ reduction versus 32°C was ≥2.0 log₁₀ than that of rV94(15T) at the same temperature versus 32°C. The lowest temperature in bold is the shut-off temperature. The limit of detection was 1.2 log₁₀ TCID₅₀/ml. A virus is designated as *ts* if restricted replication was observed at 35-40°C.

^b Data for viruses bearing the L polymerase mutations in the 15C background were previously reported [Nolan SM, 2005 #16355] and are shown here for comparison with viruses bearing the same L polymerase mutations in the 15T background.

^c The rV94(15C)/Δ1724 virus contained 3 adventitious mutations (G118V in the P protein, D243S in the M protein, and K141E in the L protein) that likely account for its increased temperature sensitivity compared to rV94(15T)/Δ1724.

Table 4

Replication of recombinant wild type and mutant HPIV2 in the upper and lower respiratory tract of AGMs

Group no.	Virus ^a	Shut-off temperature ^b	No. of animals ^c	Mean peak virus titer (log ₁₀ TCID ₅₀ /ml ± S.E.) ^d		Mean of the sum of daily virus titers (log ₁₀ TCID ₅₀ /ml ± S.E.) ^e	
				NP	TL	NP	TL
1	rV94(15T)	>40	11	3.6 ± 0.3	4.6 ± 0.5	20.9 ± 2.4	11.5 ± 1.4
2	V94	>40	10	2.7 ± 0.3	4.4 ± 0.3	12.9 ± 1.2^g	13.4 ± 1.3
3	rV94(15T)/460A	39	4	2.7 ± 0.6	1.9 ± 0.5	12.2 ± 2.1	6.2 ± 1.3
4	rV94(15T)/948L	39	4	2.5 ± 0.4	1.6 ± 0.4	14.1 ± 2.1	4.1 ± 0.7
5	rV94(15T)/Δ1724	40	4	3.4 ± 0.7	3.3 ± 0.1	20.7 ± 4.9	10.1 ± 1.0
6	rV94(15C) ^f	>40	21	2.9 ± 0.3	3.0 ± 0.2	13.4 ± 1.4	7.6 ± 0.7
7	rV94(15C)/460A	40	6	1.5 ± 0.5	1.7 ± 0.4^h	9.2 ± 2.0	5.0 ± 0.8^h
8	rV94(15C)/460P	39	4	1.9 ± 0.6	1.3 ± 0.4^h	10.1 ± 1.7	3.6 ± 0.6^h
9	rV94(15C)/948L	39	6	2.6 ± 0.3	0.8 ± 0.3^h	12.4 ± 1.6	2.8 ± 0.3^h
10	rV94(15C)/Δ1724	39	10	2.4 ± 0.4	2.2 ± 0.1	12.4 ± 1.8	6.6 ± 0.4
11	rV94(15C)/460A/ Δ1724	39	4	3.6 ± 0.2	4.3 ± 0.1	21.5 ± 1.5	14.0 ± 0.5
12	rV94(15C)/460P/ Δ1724	39	4	3.7 ± 0.2	3.0 ± 0.5	24.7 ± 1.3	9.4 ± 1.2
13	rV94(15C)/948L/ Δ1724	37	4	1.8 ± 0.4	1.0 ± 0.3^h	8.8 ± 1.8	3.0 ± 0.3^h
14	rV94(15C)/460A/ 948L	37	6	0.8 ± 0.1^h	0.8 ± 0.3^h	5.9 ± 0.2^h	2.8 ± 0.3^h

^a AGMs in groups of 2-6 were immunized IN and IT on day 0 with 10^{6.0} TCID₅₀ of the indicated virus at each site. NP swabs were collected on days 1-10 post-infection and TL samples were collected on days 2, 4, 6, 8, and 10 post-infection, and virus titers were determined by limiting dilution on LLC-MK2 cells at 32°C.

^b See footnote a in Table 3.

^c Total number of animals from several independent experiments.

^d Mean of the peak virus titers for the individual animals in each group irrespective of sampling day. S.E. = standard error. The lower limit of detection was 0.5 log₁₀ TCID₅₀/ml.

^e Mean of the sum of the daily viral titers for each animal in the group taken on all sampling days. The limit of detection was 5.5 log₁₀ TCID₅₀/ml in the NP swabs and 2.5 log₁₀ TCID₅₀/ml in the TL samples.

^f See footnote e in Table 1.

^g Replication of the underlined viruses was significantly reduced compared to rV94(15T) using the Tukey-Kramer multiple comparisons test with a p value <0.05.

^h Replication of indicated viruses was significantly reduced compared to rV94(15C) using the Tukey-Kramer multiple comparisons test with a p value <0.05.

Table 5
Genetic stability of recombinant HPIV2 mutants following replication in AGMs

Virus administered ^a	Site of isolation ^b	Original mutation ^c	Adventitious mutations in indicated genes ^d			Identities of the coding mutations			
			# tested (# different animals)	# with coding mutation		N	P	L	
				N	P				
V94	NP	n/a	4 (2)	0	1	3	236 D->G 99 F->L 245 M->V 1263 V->G 2220 I->V		
rV94(15C)	TL	n/a	4 (2)	0	0	1			
rV94(15C)/460A	NP	Not done	4 (2)	0	0	0			
	TL	4/4	4 (2)	0	0	0			
rV94(15C)/460P	NP	2/2	2 (1)	0	0	0			
	TL	4/4	4 (2)	1	0	1	350 G->V 1513 I->T		
rV94(15C)/948L	NP	5/5	5 (4)	0	1	3	11 V->A 876 S->F 1513 I->T 2063 G->E 435 F->L 1725 T->P 460 P->L ^e		
	TL	2/3 ^e	3 (2)	1	0	2	246 A->V		
rV94(15C)/Δ1724 ^f	NP	10/10	10 (6)	1	0	5	375 V->I 980 S->P 984 Y->N 1726 L->S 1874 N->D 1917 S->L		
	TL	Not done	Not done						
rV94(15C)/Δ1724 ^f	NP	7/7	7 (5)	1	0	0	396 T->I 159 K->R 318 F->C 2127 A->V		
	TL	9/9	9 (8)	0	8 ^f	3			

^a AGMs were inoculated with the indicated virus on day 0 (see footnote a, Table 4). These samples were from the experiments summarized in Table 4.

^b Virus was isolated from nasopharyngeal wash (NP) or tracheal lavage (TL) specimens that were selected from the last several days of shedding in the experiments in Table 4. Specimens were passaged once on LLC-MK2 cells to amplify virus; five of the TL samples had to be passaged twice. vRNA was purified from virus harvested from the cell culture medium and consensus sequences were determined for the N, P, and L genes.

^c The original mutation, indicated by underlining in the virus name in the “Viruses administered” column, refers to the entire mutated sequence (both silent and coding mutations).

^d This indicates the aggregate number of adventitious mutations in all of animals infected with the indicated virus. In some cases, samples from more than one day were tested for a given animal; if the same mutation was found on more than one day from the same animal, it was only counted once.

^e In one TL isolate of the rV94(15C)/460P virus, the original cloned mutation changed from a proline (CCA) to a leucine (CTA); note that the wild type assignment was phenylalanine.

^fTwo isolates appear to have undergone biased hypermutation in the P gene involving adenine deamination {Murphy, 1991 #12665; Bass, 1997 #12662}. One isolate contained 6 A->G substitutions that yielded 4 coding changes between codons 50 and 75 (T50A, K58R, K66E, and N75D). The other isolate contained 4 T->C substitutions yielding 3 coding changes between codons 111 and 125 (M111T, I116T, and Y125H).

Table 6
Efficacy of recombinant HPIV2 (rV94) mutants against challenge with wild type HPIV2 V94

Group no.	Immunizing virus ^a	No. of animals ^b	Mean peak challenge virus titer (log ₁₀ TCID ₅₀ /ml ± S.E.) ^c		HAI antibody titer (reciprocal mean log ₂ ± S.E.) ^d	
			NP	TL	Day 28 Post-immunization	Day 56 Post-challenge
1	rV94(15T)	3	≤0.5 ± 0.0	0.8 ± 0.3	5.6 ± 0.8 ^e	8.4 ± 0.2
2	V94	10	0.6 ± 0.1	0.8 ± 0.2	3.4 ± 0.9	5.0 ± 0.8
3	rV94(15T)/460A ^f	4	n.d.	n.d.	3.4 ± 0.9	n.d.
4	rV94(15T)/948L ^f	4	n.d.	n.d.	2.8 ± 0.7	n.d.
5	rV94(15T)/Δ1724 ^f	4	n.d.	n.d.	4.8 ± 0.3	n.d.
6	rV94(15C)	19	0.6 ± 0.1	0.7 ± 0.1	4.7 ± 0.6	6.3 ± 0.5
7	rV94(15C)/460A	6	0.8 ± 0.3	1.0 ± 0.3	4.2 ± 1.1	7.3 ± 0.8
8	rV94(15C)/460P	4	≤0.5 ± 0.0	2.0 ± 0.5	6.8 ± 0.8	8.5 ± 0.6
9	rV94(15C)/948L	6	≤0.5 ± 0.0	0.9 ± 0.2	4.3 ± 0.6	6.7 ± 0.8
10	rV94(15C)/Δ1724	10	0.6 ± 0.1	1.1 ± 0.2	2.2 ± 0.5	5.2 ± 0.4
11	rV94(15C)/460A/Δ1724	4	≤0.5 ± 0.0	≤0.5 ± 0.0	4.8 ± 0.8	7.3 ± 0.3
12	rV94(15C)/460P/Δ1724	4	≤0.5 ± 0.0	0.6 ± 0.1	5.0 ± 0.8	7.0 ± 0.4
13	rV94(15C)/948L/Δ1724	4	≤0.5 ± 0.0	≤0.5 ± 0.0	2.3 ± 0.8	4.6 ± 1.1
14	rV94(15C)/460A/948L	6	≤0.5 ± 0.0	1.8 ± 0.5	4.7 ± 0.2	5.3 ± 0.2
15	Control (L15) ^g	6	3.7 ± 0.5	4.6 ± 0.3	≤1.0 ± 0.0	5.1 ± 0.8

^a See footnote a in table 4.

^b Total number of animals from several independent experiments.

^c On day 28 after the initial immunization, the monkeys were challenged IN and IT with 10^{6.0} TCID₅₀/ml of V94 per site. NP swabs and TL samples were collected on days 2, 4, 6, and 8 post-infection and virus titers were determined by serial dilution on LLC-MK2 cells at 32°C. The mean of the peak virus titers for the individual animals in each group irrespective of sampling day is shown. S.E. = standard error. The lower limit of detection was 0.5 log₁₀ TCID₅₀/ml.

^d Mean serum HAI antibody titer from samples collected on day 28 post-immunization and 28 days post-challenge (day 56).

^e The HAI antibody titer for rV94(15T) at day 28 post-immunization was calculated from serum of seven AGMs immunized with the rV94(15T) virus, but only three of these seven received challenge virus.

^f These groups did not receive challenge virus. n.d. = not done.

^g Control animals were challenged with V94 virus without receiving an initial immunization.