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Codon Stabilization Analysis of the “248” Temperature Sensitive Mutation for Increased Phenotypic Stability of Respiratory Syncytial Virus Vaccine Candidates

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Abstract

Human respiratory syncytial virus (RSV) is the most important viral agent of serious pediatric respiratory tract illness worldwide. Presently, the most promising vaccine candidate is a live, attenuated, cDNA-derived virus, RSV rA2cp248/404/1030ΔSH, whose attenuation phenotype is based in large part on a series of point mutations including a glutamine to leucine (Q to L) substitution at amino acid residue 831 of the polymerase protein L, a mutation originally called “248”. This mutation specifies both a temperature sensitive (*ts*) and attenuation phenotype. Reversion of this mutation from leucine back to glutamine was detected in some samples in clinical phase 1 trials. To identify the most genetically stable “attenuating” codon at this position to be included in a more stable RSV vaccine, we sought to create and evaluate recombinant RSVs representing all 20 possible amino acid assignments at this position, as well as small insertions and deletions. The recoverable viruses constituted a panel representing 18 different amino acid assignments, and were evaluated for temperature sensitivity *in vitro* and attenuation in mice. The original leucine mutation was found to be the most attenuating, followed only by phenylalanine. The paucity of highly attenuating assignments limited the possibility of increasing genetic stability. Indeed, it was not possible to find a leucine or phenylalanine codon requiring more than a single nucleotide change to yield a “non-attenuating” codon, as is necessary for the stabilization strategy. Nonetheless, serial passage of the six possible leucine codons *in vitro* at increasing temperatures revealed differences, with slower reversion to non-attenuated phenotypes for a subset of codons. Thus, it should be possible to modestly increase the phenotypic stability of the rA2cp248/404/1030ΔSH vaccine virus by codon modification at the locus of the 248 mutation. In addition to characterizing the phenotypes associated with a particular locus in the RSV L protein, this manuscript provides insight into the problem of the instability of point mutations and the limitations of strategies to stabilize them.

Keywords

Pneumovirus; respiratory syncytial virus vaccine; temperature sensitive

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Introduction

Human respiratory syncytial virus (RSV), a negative-strand RNA virus of genus Pneumovirus, family Paramyxoviridae, is the most important viral agent of serious respiratory tract illness in infants and children worldwide [1–3]. Nearly everyone has been infected by RSV at least once by the age of 2 years. RSV disease ranges from rhinitis to bronchiolitis or pneumonia. In the United States, between 58,000 and 125,000 infants and children are hospitalized annually due to RSV disease [3,4].

In the 1960's, a live-attenuated RSV vaccine candidate was generated from wild type (wt) RSV strain A2, an RSV subgroup A virus, by serial passage at incrementally-decreasing, suboptimal temperatures (cold-passages, *cp*) [5–7]. The resulting *cp*RSV virus was not significantly cold-adapted or temperature-sensitive (*ts*), but was found to be attenuated in chimpanzees and human volunteers. However, while *cp*RSV was restricted for replication in seropositive adults and children, it was not sufficiently attenuated for RSV-seronegative children. Other mutant RSV vaccine candidates developed during this period were either over- or under-attenuated, with evidence of genetic instability [8–10]. In the 1990's, a new panel of vaccine candidates was generated by subjecting *cp*RSV to two rounds of chemical mutagenesis followed by identification of *ts* derivatives ([11,12], reviewed in [13]). One virus, called *cpts*248/404, was satisfactorily attenuated in seronegative young children and became the first live RSV vaccine to be evaluated in 1- to 2-month old infants, the target vaccine population [14]. This virus had promising features of higher infectivity and moderate immunogenicity, but unfortunately it caused brief nasal congestion in young infants and was considered to be under-attenuated [14].

With the development of RSV reverse genetics [15], the individual mutations responsible for the *ts* and attenuation phenotypes of the *cp* and *cpts* mutants were identified by sequence analysis, and the putative attenuating mutations were confirmed by introduction into recombinant virus [16–18]. This showed that the attenuation phenotype of *cp*RSV is based on a set of five amino acid substitutions in the N, F, and L proteins. In the various further-attenuated *cpts* derivatives, a series of amino acid point mutations in the L protein, as well as a nucleotide (nt) point mutation in the gene-start (GS) transcription signal for the M2 gene, were identified and shown to be mutations that independently specified both temperature sensitivity and attenuation. In particular, the *cpts*248/404 mutant described above contained the set of five amino acid substitutions from *cp*RSV combined with the nt substitution in the M2 GS transcription signal (a mutation originally called “404”) and the substitution of glutamine to leucine at amino acid position 831 in the L protein (a mutation originally called “248”). (Note that the original “248” and “404” designations were based on clone number during the original isolation of the mutants rather than on sequence position.)

Additional vaccine viruses were constructed by reverse genetics. One such vaccine candidate, called rA2cp248/404/1030ΔSH [19], is based on a recombinant copy of the *cpts*248/404 virus described above with two further modifications: (i) deletion of the SH gene, encoding a small accessory surface protein, which specifies a weak, but definite, attenuation phenotype in chimpanzees, and (ii) inclusion of an additional *ts* attenuating mutation called “1030”, which was identified in one of the *cpts* viruses where tyrosine is substituted for leucine at amino acid position 1321 in the L protein. The rA2cp248/404/1030ΔSH virus was satisfactorily attenuated in 1- to 2-month infants and also was immunogenic, and thus it represents the most promising live RSV vaccine candidate to date [19]. However, analysis of nasal wash specimens from vaccinees showed that approximately one-third of the isolates contained virus with a partial loss of temperature-sensitivity, suggesting a partial loss of attenuation. Sequence analysis showed that some of the specimens had lost a single *ts* mutation, namely either the “248” Q831L mutation or the “1030” Y1321L mutation [19]. While the loss of either mutation resulted in a

virus that remained highly attenuated, it would be preferable to increase the genetic and phenotypic stability of the rA2cp248/404/1030ΔSH vaccine candidate.

Attenuating amino acid substitutions in biologically-derived attenuated viruses typically result from single-nt substitutions, as is the case with all of the attenuated RSV vaccine candidates mentioned above. Given the high mutation rate of RNA viruses, reversion to a wild-type phenotype is relatively frequent, and outgrowth of mutant virus can readily occur under selective pressure, as was observed for the rA2cp248/404/1030ΔSH virus. (Note: in the interests of simplicity, in this manuscript we will use the term “reversion” for any mutation that restores a wt or a wt-like phenotype.) However, we previously described a strategy for “stabilizing” an attenuating amino acid substitution [20]. This strategy requires that the phenotype associated with each of the possible 20 amino acid assignments at the locus in question be determined. This segregates the assignments into those with phenotypes that are “attenuating”, as in the original mutation, or “non-attenuating”, as in the wt parent, or are “intermediate”. Then, the degenerate genetic code is examined to identify an “attenuating” codon that differs by two or, preferably, three nt from any possible “non-attenuating” codon. This is based on the premise that, if the frequency of substitution involving a single nt position is on the order of 10^{-4} (the approximate mutation frequency of RNA viruses such as RSV), the frequency of substitution involving two or three positions would be 10^{-8} and 10^{-12} , respectively. The codon that is chosen for stability might be an alternative codon for the original mutant amino acid assignment, or might involve a codon for a different “attenuating” amino acid assignment. In addition, it also may be possible to identify amino acid assignments that provide a greater degree of attenuation than the original mutant assignment [20]. If the attenuation phenotype is linked to temperature-sensitivity, one can readily assess phenotypic stability by an *in vitro* “stress test”, in which the virus is subjected to serial passage beginning at a permissive temperature and escalating to non-permissive temperatures. Genetic stability during the passages can be assessed by sequence analysis, and phenotypic stability can be assessed by measuring the level of temperature sensitivity of the passaged virus. In the present study, we applied this stabilization strategy to the “248” Q831L mutation in the RSV L protein, which is an important component of the most promising live RSV vaccine candidate.

Methods

Cells

HEp-2 cells (ATCC CCL23) were maintained in Opti-MEM I (Gibco-Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS) (HyClone, Logan, UT) and 1 mM L-glutamine (Gibco-Invitrogen). BSR T7/5 cells are baby hamster kidney 21 (BHK-21) cells that constitutively express T7 RNA polymerase [21]. These cells are maintained in Glasgow minimal essential medium (GMEM) (Gibco-Invitrogen) supplemented with 2 mM L-glutamine, 2% MEM amino acids (Gibco-Invitrogen), and 10% FBS. Every other passage, the media was supplemented with 2% geneticin (Gibco-Invitrogen) to select for cells that retain the T7 polymerase construct.

Virus growth and titration

RSV strains were propagated in HEp-2 cells at 32°C in media containing 2% FBS, 250 Units/mL of penicillin and 250 µg/mL of streptomycin (Gibco-Invitrogen). Virus stocks were generated by scraping infected cells into media followed by three rounds of freeze-thawing of cell pellets, clarification of the supernatant by centrifugation, and addition of 10x SPG (2.18 M sucrose, 0.038 M KH₂PO₄, 0.072 M K₂HPO₄, 0.06 M L-glutamine at pH 7.1) to a final concentration of 1x. Virus aliquots were snap frozen and stored at -80°C. Virus titers were determined by plaque assay on HEp2 cells under 0.8% methylcellulose overlay. After 5-day

incubation at 32°C, plates were fixed with 80% cold methanol, and plaques were visualized by immunostaining with a cocktail of three HRSV specific monoclonal antibodies [22].

Construction of recombinant RSV mutants

The recombinant RSV mutants were constructed using a reverse genetics system based on strain A2 [15]. The full-length RSV antigenome cDNA was modified previously by deleting a 112-nt region from the downstream noncoding region of the SH gene and silently modifying the last few codons of the SH open reading frame (ORF), resulting in antigenome cDNA D46/61200: these changes were made to improve stability of the cDNA during growth in *E. coli* and had no effect on the efficiency of virus replication *in vitro* or in mice [23]. Although the D46/6120 cDNA contains a deletion, for simplicity the numbering of sequence positions in the present manuscript is based on the complete sequence of biologically-derived strain A2 (Genbank accession number M74568).

A set of full-length cDNAs representing all possible amino acid assignments for codon 831 of the RSV L protein (nt 10,988–10,990) was generated, as well as several full-length cDNAs containing small deletions or insertions at or near position 831 (described in Results). A 1,061 base pair (bp) SpeI-XbaI cDNA fragment of the RSV genome (nt 10,149 to 11,209) that includes the “248” codon (nt 10,988–10,990) [18] was subcloned into pBlueScriptII (Stratagene-Agilent, Santa Clara, CA). Mutations were introduced using QuikChange site-directed mutagenesis (Stratagene-Agilent). The SpeI-XbaI fragment was then completely sequenced to confirm the presence of the desired mutation and the absence of adventitious mutations. Sequence analysis employed an ABI 3730 sequencer and the BigDye sequencing kit (Applied Biosystems, Carlsbad, CA). Full-length RSV cDNA clones containing each mutation were assembled by first cloning the SpeI-XbaI fragment into a shuttle vector containing the partial RSV genome (L ORF and trailer, nt 8,501 to 15,222) and 2,902 bp derived from the D46/6120 cDNA plasmid backbone. Full length cDNA plasmids were generated by ligating a 9,433 bp EagI-BamHI fragment [1,044 bp derived from pBR322, plus nt 1 to 8,500 (leader region to BamHI) of RSV] from the D46/6120 cDNA plasmid to a 9,623 bp EagI-BamHI fragment containing nt 8,501 to 15,222 of the RSV genome, including the “248” codon, plus 2,902 bp of the plasmid backbone. After the generation of endo-free DNA preparations (Qiagen, Valencia, CA), the identity of wt and mutant plasmids were confirmed by sequencing the region containing codon 831 of the L gene.

Generation of recombinant RSV viruses from cDNA

BSR T7/5 cells were grown to 95% confluency in 6-well plates. Before transfection, cells were washed twice with GMEM containing 3% FBS, 1 mM L-glutamine, and 2% MEM amino acids prior to the addition of 2 ml of media per well. Cells were transfected using Lipofectamine 2000 and a plasmid mixture containing 5 µg of full-length plasmid, 2 µg each of pTM1-N and pTM1-P, and 1 µg each of pTM1-M2-1 and pTM1-L [15,21]. Transfected cells were incubated overnight at 37°C. To increase the frequency of virus recovery, some plates were then heat-shocked at 43°C, 3% CO₂ for 3 hours [24]. All plates were incubated at 32°C for at least 24 h. The BSR T7/5 cells were harvested by scraping into media, added to subconfluent monolayers of HEp-2 cells, and incubated at 32°C. Virus was harvested between 11 and 14 days post-transfection and titers were determined by plaque assay. Viruses were passaged twice prior to the isolation of RNA from infected cells and the complete sequence of each viral genome was determined from infected-cell RNA by RT-PCR and direct sequence analysis. The only sequences that were not directly confirmed for each genome were the positions of the outermost primers, namely nt 1–29 and 15,191–15,222.

Evaluation of the *ts* phenotype

The *ts* phenotype for each of the rRSV viruses was evaluated by efficiency of plaque formation at 32, 35, 36, 37, 38, 39, and 40°C. Plaque assays were done on HEp2 cells, in duplicate, and incubated in sealed caskets at various temperatures in temperature controlled waterbaths as previously described [25].

Evaluation of the attenuation phenotype

Virus replication was evaluated in the upper and lower respiratory tracts of mice as described [16]. Briefly, 10-week-old female BALB/c mice in groups of five were inoculated intranasally under methoxyflurane anesthesia on day 0 with 10^6 PFU of rRSV. On day 4, mice were sacrificed by carbon dioxide inhalation. Nasal turbinates and lung tissue were harvested and homogenized separately in L-15 medium containing 1x SPG, 2% L-glutamine, 0.06 mg/ml ciprofloxacin, 0.06 mg/ml clindamycin phosphate, 0.05 mg/ml gentamycin, and 0.0025 mg/ml amphotericin B. Virus titers were determined in duplicate on HEp-2 cells incubated at 32°C.

Results

Recovery and sequence analysis of rRSVs with mutations at amino acid position 831 in the L protein

To evaluate the range of possible phenotypes conferred by substitutions at amino acid position 831 in the L protein, mutant cDNAs were generated in which the assignment was changed from that of wt (glutamine), to the leucine assignment shown to confer attenuation in the original “248” mutant, and to each of the other 18 amino acid assignments. Infectious virus was recovered bearing the original wt glutamine assignment, the original attenuating “248” leucine assignment, and 16 of the other 18 possible amino acid assignments (Table 1). The two viruses containing the assignments 831K or 831P could not be recovered in five independent transfections; they were considered to be nonviable and thus would not contribute to reversion back to wt phenotype or stabilization.

The genome of each of the 18 recovered viruses was analyzed by complete RT-PCR consensus sequencing. For eleven of the 18 viruses with codon-831 substitutions, no additional, adventitious mutations were detected in the entire genome (Table 1). For three other viruses, it was necessary to analyze two (831D) or three (831R and A) independently recovered isolates in order to identify one that was free of adventitious mutations. The remaining four viruses each contained a single adventitious mutation that was deemed acceptable (Table 1). Specifically, the wt 831Q virus contained a single A nt insertion in the downstream noncoding region of the L gene, a change considered to be inconsequential since it did not affect amino acid coding or any known cis-acting signal and exhibited a wild-type phenotype in vivo (see below). Mutants 831G and 831Y each contained an insertion of a single A nt into the oligoA tract in the downstream part of the transcriptional gene-end (GE) signal of the L gene, increasing the length of this tract from five to six nt. Since the RSV strain A2 GE signals naturally end with a tract of four to seven A nt, this was considered inconsequential. Mutant 831E contained a missense mutation (M>K) in the NS1 protein, a protein that functions to counter the host type I interferon (IFN) response. However, the growth of the 831E mutant in IFN-competent HEp-2 cells was indistinguishable from that of wt RSV both with regard to the kinetics of replication and plaque size (not shown). Therefore, there appeared to be no increase in IFN sensitivity, and this adventitious mutation in NS1 was considered to be inconsequential.

Including five additional recovered viruses described below, the genomes of a total of 28 viruses were completely sequenced in this study. This corresponds to a total of 424,648 unique nt (i.e., not including overlapping, repeat, or opposite-direction sequencing). A total of 10

adventitious mutations were identified (including those indicated in Table 1). Six of these mutations involved the insertion of one or, in a single case, two A nt into a run of five or seven A nt, occurring either in a noncoding gene sequence, in a GE signal, or in the G ORF. One virus isolate had two missense mutations involving adjacent codons, and four adventitious mutations were missense mutations that each arose from a single nt substitution. With a total of 10 adventitious mutations identified from 424,648 sequenced nts, this corresponds to a frequency of 2.3×10^{-5} . This is similar to the frequency of isolation of monoclonal antibody-resistant RSV mutants [26], indicating that the process of producing live RSV from cDNA did not appear to augment the frequency of mutations.

We also attempted to recover virus containing more extensive and therefore less reversible mutations, including small deletions or insertions at or near codon 831. The deletions that were engineered involved a single amino acid deletion of 831Q as well as double deletions involving 830A plus 831Q or 831Q plus 832A. However, none of these was recoverable in multiple attempts (not shown), suggesting that the viruses were not viable. The insertions that were engineered involved a single amino acid residue, either an asparagine or threonine residue, or the tetrapeptide ALNT, or the nonapeptide ADYKDDDDKAD, each inserted immediately following 831Q. The choice of threonine or asparagine was made because they seemed most compatible with the local primary sequence with regard to possible secondary structure. Insertion of ALNT was performed because the area seemed to have an alpha helical nature, and these four residues would be equivalent to one helical turn. Finally, the ADYKDDDDKAD insert was introduced because it contains a FLAG affinity tag. However, none of these insertions could be recovered in multiple attempts (not shown). Thus, 18 of 20 possible amino acid substitution viruses were recovered and their temperature sensitivity and attenuation phenotypes were investigated next.

Temperature sensitivity of the codon-831 substitution mutants

All of the 18 recovered viruses were propagated on HEp-2 cells at 32°C, conditions that are fully permissive for growth of the original 831L *ts* mutant. Under these conditions, the final titers of all of the RSV mutants were within a range of 7.2 and 8.1 log₁₀ PFU per ml (data not shown). Temperature sensitivity was evaluated by replica plaque assays that were incubated at different temperatures ranging from 32°C to 40°C, all of which are permissive for wt RSV. Two independent experiments were performed, for logistical reasons, and these were presented separately (note Table 2, column 2).

The shut-off temperature (T_{SH}) is defined as the lowest restrictive temperature at which there is a reduction in virus titer compared to the permissive temperature 32°C that is 100-fold or greater than that of wt RSV at the two temperatures. For RSV, the *ts* phenotype is defined as having a T_{SH} of 40°C or less. The T_{SH} for the virus bearing the original 831L mutation was found to be 38°C (Table 2), consistent with previous results [18]. Among the remaining thirteen viruses in experiment #1, there was a second virus with a T_{SH} of 38°C (831I). Another virus, 831F, had a T_{SH} of 39°C in experiment #1 and 38°C in experiment #2: the latter value was assigned. Among the other viruses, one had a T_{SH} of 39°C (831V), four had a T_{SH} of 40°C (831W, C, G, and Y), and six had a T_{SH} of >40°C (831M, H, T, S, E, and N). Even though the T_{SH} of these last six viruses was the same as that of wt 831Q, 40°C, the first four (831M, H, T, and S) differed from wt in having small or micro plaque phenotype at 40°C, indicative of a small *ts* effect, even if not the defined *ts* phenotype based on plaque number (Table 2).

None of the alternative assignments at position 831 were as attenuating as leucine

The 18 recovered viruses were evaluated for the ability to replicate in the upper (nasal turbinates) and lower (lungs) respiratory tract of mice as a measure of attenuation (Table 2). The results of the *ts* and attenuation phenotype analysis in Table 2 are summarized graphically

in Fig. 1A (experiment 1) and B (experiment 2). There was a highly significant positive correlation between T_{SH} and virus titer in nasal turbinates (Spearman rank correlation test, $\rho = 0.8$; $p = 0.0001$), and a significant correlation between T_{SH} and lung titers ($\rho = 0.5$, $p = 0.03$). Six amino acid assignments (831L, F, V, I, Y, and E) conferred a statistically significant reduction in virus replication in either the nasal turbinates or the lungs in at least one experiment. Each of these attenuating assignments also conferred the *ts* phenotype, with the exception of glutamate (E). Two other assignments (amino acids W and C) were somewhat attenuating, even if the reductions were not statistically significant, and each conferred the *ts* phenotype. The remaining assignments (amino acids Q, G, M, H, T, S, N, R, A, and D) replicated with wt-like efficiency and, with the exception of glycine (G), were not *ts*. Thus, there was a general paucity of highly *ts* and attenuating assignments possible for position 831. With regard to the *ts* phenotype, isoleucine and phenylalanine were the only alternatives that were comparable to the original L assignment, with each having a T_{SH} of 38°C. With regard to the more important attenuation phenotype, phenylalanine was the most attenuating of the alternative assignments, but none of the alternative assignments including phenylalanine was comparable to the original leucine assignment with regard to the magnitude of attenuation.

Predicted outcomes of single nt substitutions in the leucine and phenylalanine codons

Based on the magnitude of restriction of virus replication *in vivo*, only the original leucine assignment or, secondarily, the alternative assignment of phenylalanine would be acceptable at position 831. Therefore, each of the six possible codons for leucine and the two possible codons for phenylalanine were evaluated to predict the coding outcomes of all possible single-nt substitutions at each codon position (Table 3). Three possible outcomes were defined: (i) acceptable assignments were leucine or phenylalanine, since these were the two most attenuating assignments, as well as proline, lysine, or a stop codon, since these would be nonviable and could not contribute to reversion; (ii) “intermediate” assignments (amino acids I, Y, V, W, C or E) were those that conferred a lesser attenuating phenotype and, with one exception (glutamate [E]), were *ts*, and (iii) “wt-like” assignments constituted the remaining assignments (amino acids Q, G, M, H, T, S, N, R, A, and D) and conferred unrestricted or nearly-unrestricted replication *in vitro* and, with one exception (glycine, G), were non-*ts*. For example, with the TTA leucine codon (Table 3), nine of the 12 possible permutations yielded acceptable substitutions, namely leucine (five instances), phenylalanine (two instances), and a stop codon (two instances). Two of the possible permutations were “intermediate”, namely isoleucine (one instance) and valine (one instance). One permutation yielded a wt-like assignment (serine) and would be unacceptable.

Examination of the six possible leucine codons and two possible phenylalanine codons indicated that each could revert to a wt-like assignment by at least one single-nt substitution, as well as to various “intermediate” assignments by one or more single-nt substitution. This indicated that it would not be possible to identify a leucine or phenylalanine codon that would require two or three nt substitutions to yield a wt-like assignment, as is necessary to achieve a large increase in phenotypic stability. However, it remained possible that there might be differences in phenotypic stability following multicycle replication. Therefore, we subjected the leucine codons to *in vitro* “stress tests” by passage at increasing restrictive temperatures.

Passage of 831L mutants at increasingly restrictive temperatures

The 831L virus analyzed above contained the leucine codon CTA at the mutant position (Table 1). Viruses containing each of the other five possible leucine codons at this position were generated, sequenced completely, and were found to be free of adventitious mutations. Two experiments were performed. An initial experiment evaluated three of these codons, namely CTA (the attenuating codon found in the original biologically-derived 248 mutant virus [18]), CTG, and TTG. Two independent aliquots of each virus were serially passaged two times each

at 32°C, 37°C, 38°C, 39°C, and 40°C for a total of ten passages (Fig. 2A, left panel); two independent aliquots were serially passaged 10 times at the permissive temperature of 32°C as controls (Fig. 2A, right panel). Aliquots of each passage level were titrated by plaque assay at 32°C to detect changes in yield that might be indicative of changes in the *ts* phenotype (Fig. 2A).

Titration analysis indicated that infectious virus was no longer detectable in CTA lineage #2 and TTG lineage #2 after the second passage at 40°C (generation 10) (Fig. 2A). This would be the expected result of restriction due to the *ts* phenotype. In contrast, virus was readily detectable at all passage levels at increasing temperature for lineages #1 of CTA and TTG and for both lineages of CTG. This would be the expected result of a loss of the *ts* phenotype. The control viruses passaged at 32°C also contained high viral titers at each passage level, as would be expected at this permissive temperature. Direct sequence analysis confirmed the genetic instability of those viruses that replicated efficiently at elevated temperatures (Fig. 2B). Specifically, for both CTG lineages and for CTA lineage #1, reversion to wt (glutamine, Q) was observed by direct sequencing of RT-PCR products beginning at passage 5 or 6 (38°C) (Fig. 2B). For TTG lineage #1, the reversion was to serine, a wt-like assignment, and was detected beginning at passage 4 (37°C, Fig. 2B) and became the sole detectable assignment by 5 (38°C). For the viruses that remained highly restricted at elevated temperatures, namely lineage #2 of CTA and TTG, the mutant codon remained intact at passage 4 and 5, respectively; subsequent passages contained insufficient material for analysis. For the six virus isolates grown at 32°C, there was no detectable reversion during 10 passages (not shown).

In a second experiment, all six 831L viruses were evaluated, representing all six possible leucine codons. Ten independent flasks of each virus were serially passaged twice at 37°C, followed by two passages at 38°C. These temperatures were chosen because they correspond to passage numbers where reversion was first detected in the experiment in Fig. 2B, and because 38°C is the T_{SH} for 831L. In addition, the passages were done at a dilution of 1:5, rather than 1:1000 used in the first experiment, in order to ensure sufficient progeny for analysis. As controls, two additional independent flasks for each virus were incubated for four passages at 32°C. Aliquots of each passage level were titrated by plaque assay at 32°C as above. All of the samples for all of the viruses contained high viral titers (not shown), indicative of extensive replication at both temperatures. Sequence analysis of viruses from passages 2 (37°C) and 4 (38°C) showed a high rate of mutations at codon 831 by passage 4 (Table 4). All the reversions were to an amino acid associated with a T_{SH} of 40°C (arginine, R) or higher (amino acids Q, H, S, M), and wt-like replication in mice (Fig. 1).

Based on reversion frequency and amino acid usage for 831L mutants in response to increasing temperature, the leucine codons could be divided into two categories. The first category, comprising codons CTC, TTA, and CTG, had a high reversion rate with the majority ($\geq 80\%$) of the cultures having revertant codons present after the second passage at 37°C, and complete reversion to an amino acid associated with a non-*ts*, non-attenuating phenotype after the second passage at 38°C. For each virus in this rapidly-reverting group, all 10 cultures contained the same revertant codon: CTC changed to CAC (histidine, H), TTA changed to TCA (serine, S), and CTG changed to CAG (glutamine, Q). The second category of viruses, comprising codons CTT, CTA, and TTG, showed delayed reversion or the absence of reversion in the majority of replicas following two passages at 37°C, with $\geq 70\%$ of the cultures retaining the input 831-codon free of detectable reversion. Following the second passage at 37°C, the difference between the groups (i.e., CTC, TTA, and CTG versus CTT, CTA, and TTG) with regard to the number of flasks containing revertants versus the number of flasks lacking revertants was statistically significant (Fisher's exact test, $p = 3.875^{-9}$). Following the second passage at 38°C, 80 to 100% of the samples contained revertants, with multiple revertant codons represented rather than a single codon (Table 4). Thus, all of the six codons for leucine exhibited reversion.

Following the second passage at 38°C, the difference between the two groups (CTC, TTA, and CTG versus CTT, CTA, and TTG) with regard to the number of flasks that had completely reverted to a codon of wt phenotype versus those that had not also was significantly different (Fisher's exact test, $p = 0.049$). There was a tendency for leucine codon TTG to revert more slowly, compared to CTA and CTT, with a single culture that had no detectable revertants after two passages at 38°C, but this was not statistically significant.

Discussion

The rA2cp248/404/1030ΔSH virus is the most promising attenuated RSV vaccine candidate available to date [1]. However, in a clinical trial of this virus in young children and infants, one-third of nasal wash specimens contained virus with a partial loss of the *ts* phenotype, and sequence analysis documented nt substitutions and coding changes at the “1030” and “248” loci [19]. The original biologically-derived “248” mutant contains the leucine codon CTA at amino acid position 831 in the L protein, which differs by a single nt (underlined) from the CAA glutamine codon at this position in its wt parent [12]. In the recombinant rA2cp248/404/1030ΔSH virus that was evaluated clinically, the leucine codon at position 831 was engineered to be CTG, which differed by two nt from the original wt assignment of CAA (differences underlined), but differed by only a single nt assignment from the other possible glutamine codon, CAG [19]. Reversion to this CAG glutamine codon indeed was observed in vaccine virus shed during the clinical trial. In a subsequent study, another version of rA2cp248/404/1030ΔSH was made in which stabilization of the “248” mutation was attempted by a different codon choice. This involved the use of the leucine codon TTA, which differed by two or three nt from the two glutamine codons CAA and CAG, respectively, and thus might confer stability. This mutation was called “248s”, and the virus was called rA2cp248s/404/1030ΔSH. However, when serially passaged *in vitro* at increasing temperature, this TTA codon was observed to change by a single nt substitution to CTA, which encodes serine. This change was associated with a partial loss of the *ts* phenotype, but the attenuation phenotype was not investigated [27]. In the present study, we evaluated the phenotype associated with serine at position 831 in the absence of other attenuating mutations and confirmed that it is not *ts* and does not confer attenuation in the respiratory tract of mice.

The preliminary attempts at stabilization in the previous work described above confirmed the idea that, in order to evaluate the possibility of stabilizing an amino acid point mutation, it is necessary to perform a phenotypic analysis of all possible amino acid assignments at the mutation site of interest [20]. Unfortunately, in the case of the “248” mutation in the present study, phenotypic analysis revealed a paucity of highly attenuating assignments possible at amino acid position 831 in the L protein. Therefore, the only possibility for stabilizing the “248” mutation would be to use an alternative leucine codon. However, in an exercise on paper (Table 3), we found that each of the six possible leucine codons could revert to one or more wt-like assignments with a single-nt change. Thus, it would not be possible to identify a codon that would require two or three nt changes for reversion, which is the basis of the stabilization strategy.

Phenotypic analysis of the six possible leucine codons revealed unexpected differences in the kinetics of reversion, and the six codons could be segregated into two groups. The first group was rapidly reverting, with revertants predominant or present in nearly all cultures following passage at 37°C and being the sole detectable species in all cultures following passage at 38°C in the experiment shown in Table 4. This rapid reversion group comprised the CTC codon, the TTA codon that was evaluated in the “stabilized” rA2cp248s/404/1030ΔSH virus but was found to revert during *in vitro* passage [27], and the CTG codon that was present in the rA2cp248/404/1030ΔSH virus that was evaluated clinically [19]. The second group of leucine codons was found to exhibit delayed reversion, with most of the cultures retaining the input

“attenuating” codon following passage at 37°C, and many of the cultures exhibiting incomplete reversion following passage at 38°C in the experiment in Table 4.

An additional difference between the “rapidly reverting” and “delayed reversion” groups is that the members of the former group each reverted to a single alternative nt assignment yielding a single alternative amino acid assignment, while the members of the latter group each reverted to two or three alternatives at 38°C (Table 4). Indeed, one of the cultures for CTA contained a mixture of reversions that each arose from substitutions at two codon positions: a transition (T to A) at codon position two and a mixture of two transitions (A to G or T) at codon position three. Thus, in the “rapidly reverting” group, a single pathway to reversion predominated and alternative pathways were not followed, whereas in the “delayed reversion” group all of the possible wt-like assignments appeared, and indeed an additional one involving two nt substitutions was observed.

It is not understood why the different leucine codons appeared to differ with respect to the frequency of reversion. Inspection of Table 4 did not reveal any obvious patterns of bias with respect to the substituting nt or the resulting amino acid. For example, CTC readily reverted to CAC, yielding histidine, but CTT reverted less readily to the alternative histidine codon of CAT, even though both events involved a T to A transition at the second codon position to yield histidine. The distinction between the “rapidly reverting” and “delayed reversion” groups could not be explained by differences in the number of possible “non-attenuating” mutations obtainable by single-nt changes (Table 3). For example, codon TTA had only a single possibility to revert to a wt or wt-like assignment, and yet reverted readily (to serine, S), whereas all three of the “delayed reversion” codons CTT, CTA, and TTG each had two possibilities to revert. Within the “rapidly reverting” group, the codon with the greatest number of single-nt changes that would yield non-attenuating assignments, namely CTG, appeared to be the slowest in the group to revert. An alternative possibility was that the differences in the ease of reversion might be due to “codon pair bias” [28,29]. Codon pair bias is a species-specific effect in which certain synonymous codon pairs are present more frequently in the genome than statistically expected. Codon pair bias-driven reversion, in this case involving codon pairs 830/831 and 831/832, would have resulted in faster reversion of codon 831 to certain synonymous codons, driven by the neighboring codons 830 and 832. However, there was neither a positive nor a negative correlation of reversion kinetics with the known human codon pair bias (data not shown). Thus, the observed differences in the ease of reversion could not be attributed to codon pair bias. In addition, this is not the only instance of unanticipated differences in reversion frequency involving a mutation in rA2cp248/404/1030ΔSH. Specifically, both in the clinical setting and *in vitro*, reversion at the “1030” locus was several times more common than at the “248” locus, even though both involve a single nt change and both involve “38°C” *ts* mutations with indistinguishable attenuation phenotypes when analyzed separately in a *wt* background.

While it will not be possible to increase the stability of the 831 assignment by achieving a two- or three-nt difference from all possible wt-like codons, it may be possible to achieve a more modest increase in stability. Even codon changes that reduce reversion several-fold each at the “248” and “1030” sites might be sufficient to reduce the frequency of partial phenotypic reversion from one-third (as observed for rA2cp248/404/1030ΔSH in the clinical setting [19]) to a few percent. If the kinetics of reversion and outgrowth *in vivo* can be slowed so that it occurs later in the course of vaccine virus replication, revertants would be more subject to restriction by the developing adaptive immune response. Unfortunately, the CTG codon that was used in the rA2cp248/404/1030ΔSH virus that was evaluated clinically probably was the worst possible choice: it reverts rapidly even at 37°C, and it reverts to glutamine, which is the original wt assignment. Conversely, CTT and TTG represent plausible choices, since they resisted reversion at 37°C and the reversion that occurred at 37–38°C was to arginine or histidine in the case of CTT, and to serine or methionine in the case of TTG. While none of

these assignments was significantly *ts* or attenuating, each conferred small or micro-plaque phenotype at 40°C and small decreases in replication in mice, indicating that they do not confer the full wt phenotype.

The next step to improve the stability of the promising rA2cp248/404/1030ΔSH virus will be to investigate stabilization of the “1030” locus, a study that is already in progress. We will then modify the present rA2cp248/404/1030ΔSH virus by (i) changing the “248” 831L CTG codon to TTG or CTT, and (ii) changing the 1030 locus to whatever alternative(s) are suggested from the results of that ongoing codon stabilization study. The resulting potential improved version (s) of the vaccine candidate would then be analyzed to define the *ts* and attenuation phenotypes, and would be subjected to an *in vitro* stress test to evaluate stability.

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Figure 1. *T_s* and attenuation phenotypes of recombinant RSVs bearing substitutions at amino acid 831 of the L protein

For each virus, the mean viral titer in the nasal turbinates is plotted versus the mean viral titer in the lungs, using the data from Table 2. Viruses are identified by the single letter code corresponding to amino acid 831 of the L protein. The symbol used for each virus indicates its *in vitro* T_{SH} (°C), from the data in Table 2. The hatched lines mark the replication level of *wt* virus (831Q, glutamine) in lungs (vertical line) or nasal turbinates (horizontal lines). Mutants that replicated at a statistically significantly (Kruskal-Wallis test and Dunn's post hoc analysis; $P \leq 0.05$) lower level in the lungs or nasal turbinates compared to *wt* virus (831Q) are underlined. Experiments 1 and 2 from Table 2 are shown in A and B, respectively.

Figure 2. Growth of mutant RSVs *in vitro* at increasing restrictive temperatures and sequence analysis

Duplicate samples (1 and 2) of RSV mutants bearing CTA, CTG, or TTG as the codon at position 831 in the L gene were grown on HEp-2 monolayers at 32°C (non-restrictive temperature) with an input multiplicity of infection (moi) of 0.01 PFU/cell until cytopathology was visible (between 4 to 14 days). Viruses were then serially passed at increasingly restrictive temperature as follows: two passages at 32°C; two at 37°C; two at 38°C; two at 39°C; and two at 40°C, for a total of 10 passages [20] (A, left panels). For the first four passages, each virus-containing supernatant was diluted 1 to 1,000 before the next passage. For passages 5 through 10, 1 ml (out of a total of 5 ml) of the supernatant was used to inoculate the next passage. In

parallel, duplicate samples were passaged for 10 passages at 32°C (non-restrictive temperature), serving as controls (A, right panels). For each passage, aliquots were frozen for titration (A) and sequence analysis (B). (A) Virus titers of the CTA, CTG, and TTG mutants at the different passage levels, with the passage temperatures indicated. Virus titer was determined by plaque assay at the permissive temperature (32°C). Passage number 0 represents the input virus. (B) Sequence analysis of codon 831 of the L ORF from RT-PCR products from genomic RNA. For each phenotype revertant, the first passage number with an identifiable codon change is indicated. Mixtures of nt assignments are indicated in brackets. For virus in which no changes were detected, the passage indicated in parentheses is the last at which there was sufficient material for sequence analysis. The encoded amino acids are indicated.

Table 1

Recovery and sequence analysis of recombinant RSVs bearing substitutions at amino acid 831 of the L protein

Mutation	831 codon	Recovery	Adventitious mutations ^a
831Q (wt)	CAA	+	Insertion of a single A nt in a run of A nt (nt 15,015–19) in the downstream noncoding region of L
831L ^b	CTA	+	
831I	ATT	+	
831F	TCC	+	
831V	GTC	+	
831W	TGG	+	
831C	TGC	+	
831G	GGT	+	Insertion of a single A nt in a run of A nt (nt 15,063–67) in the L GE signal
831Y	TAT	+	Same as 831G, above
831M	ATG	+	
831H	CAT	+	
831T	ACC	+	
831S	TCT	+	
831E	GAG	+	Missense mutation (nt 475) at NS1 codon 126 (ATG>AAG, M>K)
831N	AAC	+	
831R	CGC	+	
831A	GCT	+	
831D	GAC	+	
831K	AAA	No	
831P	CCT	No	

^a Genome positions according to the complete unmodified sequence of biologically-derived RSV strain A2 (Genbank accession number M74568). GE: gene-end transcription signal.

^b Assignment in the original biologically-derived “248” virus [12,18].

Table 2

Characterization of the temperature sensitivity and attenuation phenotypes of recombinant RSV's bearing substitutions at amino acid 831 of the L protein

Virus	Exp ^d	Virus titer (PFU per mL) at indicated temperature (°C) ^a										Replication in mice ^b			
		32	35	36	37	38	39	40	T _{SH} ^e	Nasal turbinates	Lung	Titer (log ₁₀ PFU/g ± SE)	Nasal turbinates	Lung	Mean log ₁₀ reduction ^c
831Q (wt)	1	7.8	7.8	7.8	7.8	7.8	7.9	7.7	> 40	4.7 ± 0.2	5.1 ± 0.2				
831L ^f	1	7.6	7.7	7.6	7.2*	≤ 1 ^e	< 1	< 1	38	3.3 ± 0.4 ^g	3.7 ± 0.2 ^g		1.4		1.4
831H	1	7.7	7.8	7.7	7.7*	≤ 1	< 1	< 1	38	4.1 ± 0.1	4.3 ± 0.1 ^g		0.6		0.8
831F	1	7.6	7.6	7.6	7.5*	6.1 [#]	≤ 1	< 1	39	3.7 ± 0.3	4.0 ± 0.1 ^g		1.0		1.1
831V	1	7.5	7.4	7.3	7.3	6.4*	≤ 1	< 1	39	3.7 ± 0.3	4.5 ± 0.2		1.0		0.6
831W	1	7.6	7.7	7.7	7.7	7.3*	6.2 [#]	≤ 1	40	4.0 ± 0.1	4.7 ± 0.2		0.7		0.4
831C	1	8.1	8.1	8.1	8.0	7.9*	7.0 [#]	≤ 1	40	3.9 ± 0.2	5.0 ± 0.2		0.8		0.1
831G	1	7.7	7.7	7.5	7.7	7.6	7.2*	≤ 1	40	4.6 ± 0.2	4.8 ± 0.0		0.1		0.3
831Y	1	7.9	7.9	7.9	7.9	7.9	7.4*	≤ 1	40	3.6 ± 0.2 ^g	4.8 ± 0.1		1.1		0.3
831M	1	8.1	8.1	8.1	8.1	8.0	7.8*	7.4 [#]	> 40	5.0 ± 0.1	4.8 ± 0.1		-0.3		0.3
831H	1	8.0	8.0	8.0	7.8	7.9	7.7	7.3 [#]	> 40	4.7 ± 0.3	4.6 ± 0.1		0		0.5
831T	1	7.9	7.9	8.0	7.9	7.9	7.6*	6.4*	> 40	4.4 ± 0.3	5.0 ± 0.1		0.3		0.1
831S	1	7.5	7.6	7.6	7.5	7.6	7.6	7.1*	> 40	4.4 ± 0.4	4.9 ± 0.1		0.3		0.2
831E	1	7.3	7.1	7.2	6.9	7.0	7.0	6.6	> 40	4.6 ± 0.1	4.3 ± 0.2 ^g		0.1		0.8
831N	1	7.6	7.6	7.6	7.5	7.5	7.7	7.3	> 40	4.8 ± 0.3	5.2 ± 0.1		-0.1		-0.1
831Q (wt)	2	8.1	8.1	8.1	8.1	8.0	8.0	7.9	> 40	4.8 ± 0.1	4.4 ± 0.2				
831L ^f	2	7.9	7.8	7.7*	7.0 [#]	4.0[#]	< 1	< 1	38	3.9 ± 0.2 ^g	4.1 ± 0.2		0.9		0.3
831F	2	7.8	7.8	7.8*	7.6*	≤ 1	< 1	< 1	38	4.0 ± 0.1 ^g	4.0 ± 0.2		0.8		0.4
831R	2	7.8	7.8	7.8	7.8*	7.7*	6.5*	4.6*	40	4.4 ± 0.4	4.3 ± 0.1		0.4		0.1
831A	2	8.1	8.0	8.0	8.0	8.0	8.0*	7.4*	> 40	4.8 ± 0.2	4.5 ± 0.2		0		-0.1
831D	2	7.9	7.9	7.8	7.7	7.9	7.6*	7.3*	> 40	4.5 ± 0.2	4.3 ± 0.1		0.3		0.1
831N	2	8.1	8.1	8.0	8.0	8.0	7.9	7.7	> 40	4.7 ± 0.1	4.5 ± 0.2		0.1		-0.1

- ^aThe *ts* phenotype for each virus was evaluated by plaque assay on HEp2 cells at the indicated temperatures.
- * small plaques,
- # micro plaques. Values indicating a *ts* phenotype are marked (bold, underlined).
- ^b10-week-old mice in groups of five were inoculated intranasally with 10⁶ PFU of the indicated virus. Nasal turbinates and lungs were harvested on day 4, and virus titers were determined by plaque assay. SE: Standard error.
- ^cReduction in mean titer compared to 831Q (wt) virus of same experiment.
- ^dExperiment number. Viruses 831R, A and D (underlined) were analyzed in experiment 2; viruses 831Q (wt), L, F, and N were analyzed in both.
- ^eShut off temperature (T_{SH}) is defined as the lowest restrictive temperature at which the reduction compared to 32°C is 100-fold or greater than that observed for wt RSV at the two temperatures. The *ts* phenotype is defined as having a shut off temperature of 40°C or less (bold, underlined).
- ^fOriginal "248" amino acid assignment.
- ^gStatistically significant (Kruskal-Wallis test and Dunn's post hoc analysis; $P \leq 0.05$, underlined) difference compared to wt 831Q control virus in same experiment.

Predicted outcomes of all possible single nucleotide substitutions in all possible leucine and phenylalanine codons

Amino acid	Codon	Effect of nt point mutation (N to indicated nt) on amino acid assignment ^{a,b,c}				Number and type of phenotypic reversions	
		T	C	A	G	Intermed ^b	Wt ^c
L	CTC or CTT ^d	NTY ^e	L	I ^b	V	2	2
		CNY	P	H^c	R		
		CTN	L	L	L		
L	TTA	NTA	L	I	V	2	1
		TNA	S	stop	stop		
		TTN	F	L	L		
L	CTG ^f	NTG	L	M	V	1	3
		CNG	P	Q	R		
		CTN	L	L	L		
L	CTA ^g	NTA	L	I	V	2	2
		CNA	P	Q	R		
		CTN	L	L	L		
L	TTG	NTG	L	M	V	2	2
		TNG	S	stop	W		
		TTN	F	L	L		
F	TTC or TTT ^d	NTY ^e	L	I	V	4	1
		TNY	S	Y	C		
		TTN	F	L	L		

^aThe four possible nt assignments, T, C, A, and G, are listed across the top. The resulting amino acids are indicated below by their single letter codes.

^bAmino acids that confer an intermediate attenuation phenotype (see the text) are italicized.

^cAmino acids that confer a wt-like phenotype (see the text) are indicated in bold and are underlined.

^dThe two codons yield the same outcomes.

^eY denotes C or T.

^fCodon present in the rA2cp248/404/1030ΔSH virus that was evaluated in clinical trials [19].

⁸Codon present in the original biologically-derived "248" mutant [12,18].

Table 4
Stability of the various leucine codons encoding amino acid 831 of the L protein during passage at restrictive temperatures^a

Passage 2 (37°C)				Passage 4 (38°C)				
Virus	Type of reversion observed ^b	% cultures with revertants ^c	Revertant codon observed ^d	Amino Acid ^e	% cultures with revertants ^c	Revertant codon observed ^d	% cultures with indicated codon	Amino acid ^e
CTC	rapid	100	C[T/ <u>A</u>]C	H	100	CAC	100	H
TTA	rapid	100	T[T/ <u>C</u>]A	S	100	TCA	100	S
CTG ^f	rapid	80	C[T/ <u>A</u>]G	Q	100	CAG	100	Q
CTT	delayed	0			100	CAT	80	H
CTA ^g	delayed	30	C[T/ <u>A</u>]G	Q	100	C[T/ <u>G</u>]T	20	R
						CAA	70	Q
						C[T/ <u>A</u>]A	10	Q
						C <u>A</u> [A/ <u>G</u> / <u>T</u>]	10	Q: H
TTG	delayed	20	[T/ <u>A</u>]TG	M	90	C[T/ <u>G</u>]A	10	R
						T <u>C</u> G	50	S
						T[T/ <u>C</u>]G	10	S
						ATG	20	M
						[T/ <u>A</u>]TG	10	M

^aTen replicate 25 cm² flasks of HEp-2 cells were infected with the indicated 831L mutant at an MOI of 0.1 PFU/cell at 37°C. Virus was harvested between 5 and 7 days post-infection, serially passaged again at 37°C, and serially passaged twice at 38°C, for a total of four passages, each by transferring 1 ml (out of a total of 5 ml) of supernatant to a fresh 25 cm² flask of HEp-2 cells. In parallel, two control flasks per mutant were passaged four times at the permissive temperature of 32°C. For each passage, aliquots were frozen for titration and genotype analysis. No mutations were detected in the 32°C controls (not shown).

^bThe first three viruses, CTC, TTA, and CTG, constitute a rapidly-reverting group that each reverted to a single assignment, whereas reversion of the last three, CTT, CTA, and TTG, was delayed and involved a greater number of alternative assignments (see Discussion).

^c% of cultures with detectable revertants.

^dObserved codon sequence: mixtures are indicated in bracket. Nt changes are underlined.

^eAmino acid coding: Colon indicates a mixed population of the specified amino acids.

^fCodon present in the rA2cp248/404/1030ASH virus evaluated in clinical trials [19].

^gCodon present in the original biologically-derived “248” mutant [12,18].