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Increased Genetic and Phenotypic Stability of a Promising Live-Attenuated Respiratory Syncytial Virus Vaccine Candidate by Reverse Genetics

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Human respiratory syncytial virus (RSV) is the most important viral cause of serious pediatric respiratory illness worldwide. Currently, the most promising live-attenuated vaccine candidate is a temperature-sensitive (*ts*) cDNA-derived virus named rA2*cp248/404/1030*ΔSH, in reference to its set of attenuating mutations. In a previous clinical study, more than one-third of postvaccination nasal wash isolates exhibited partial loss of the *ts* phenotype. Most of this instability appeared to be due to reversion at a missense point mutation called *1030*. This *1030* mutation is a single-nucleotide tyrosine-to-asparagine substitution at position 1321 (Y1321N) of the polymerase L protein that contributes to the *ts* and attenuation phenotypes of the vaccine candidate. The goals of the present study were to identify a reversion-resistant codon at position 1321 conferring a comparable level of attenuation and to use this to develop a genetically stable version of the vaccine virus. We modified wild-type (wt) RSV to insert each of the 20 possible amino acids at position 1321; 19 viruses were recoverable. We also investigated small deletions at or near this position, but these viruses were not recoverable. Phenotypic analysis identified alternative attenuating amino acids for position 1321. Several of these amino acids were predicted, based on the genetic code, to be refractory to deattenuation. Classical genetics, using temperature stress tests *in vitro* combined with nucleotide sequencing, confirmed this stability but identified a second site with a compensatory mutation at position 1313. It was possible to stabilize the 1313 site as well, providing a stable *1030* mutation. Further stress tests identified additional incidental mutations, but these did not reverse the *ts*/attenuation phenotype. An improved version of the vaccine candidate virus was constructed and validated *in vitro* by temperature stress tests and *in vivo* by evaluation of attenuation in seronegative chimpanzees. In addition to developing an improved version of this promising live-attenuated RSV vaccine candidate, this study demonstrated the propensity of an RNA virus to escape from attenuation but also showed that, through systematic analysis, genetics can be used to cut off the routes of escape.

Human respiratory syncytial virus (RSV), a member of the *Paramyxoviridae* family, infects essentially everyone worldwide early in life and causes at least 33.8 million pediatric lower respiratory tract infections and 199,000 pediatric deaths worldwide each year (1, 26). Despite a well-recognized public health need for RSV vaccines, there is no licensed vaccine or effective antiviral therapy for RSV (9), although infants and young children at high risk for severe RSV disease can be substantially protected by passive immunoprophylaxis with RSV-neutralizing antibody (24, 38). A long-standing goal has been the development of a pediatric live-attenuated intranasal vaccine that is safe and well tolerated yet satisfactorily immunogenic in the target population, young infants under 6 months of age. Several biologically derived vaccine candidates have been tested in clinical trials, beginning in the 1960s, but were found to be unsatisfactorily attenuated and in some cases exhibited genetic instability (2, 19, 20, 35, 37). More recently, reverse genetics has been used both to identify existing and create new attenuating mutations and to make new cDNA-derived vaccine candidates containing desired combinations of mutations (7, 18, 36).

The most promising vaccine candidate to date is a cDNA-derived virus called rA2*cp248/404/1030*ΔSH, a name that summarizes its attenuating mutations (18). This virus contains five independent attenuating elements that were identified in biologically derived viruses or created by reverse genetics and subsequently combined: (i) a set of five amino acid substitutions in the N, F, and L proteins that were identified in a cold-passaged (*cp*) RSV (16, 17, 32), (ii) the substitution of leucine for glutamine at amino acid 831 in the L protein (a mutation originally called *248*) (14), (iii) the nucleotide substitution of C for T (positive sense) at position 9 of the gene start (GS) transcription signal of the M2 gene start (a mutation originally called *404*) (10), (iv) the substitution of asparagine for tyrosine at amino acid 1321 of the L protein (a mutation originally called *1030*) (10, 12), and (v) deletion of the SH gene (5, 29). The original *248*, *1030*, and *404* designations were based on plaque number during the original isolation of the mutants rather than on sequence position. These mutations have been evaluated in some detail in preclinical studies (10, 11, 29, 31, 32). The *248*, *404*, and *1030* mutations each render RSV temperature sensitive (*ts*): individually, they shift the shutoff temperature from >40°C for wild-type (wt) RSV to 38°C [*248* and *1030* (11, 22, 30, 31)] or 37°C [*404* (30)]. The *cp* and ΔSH mutations are also attenuating (29, 32) but do not confer a *ts* phenotype to RSV. The combination of these five independently attenuating elements by reverse genetics resulted in the highly attenuated and highly temperature-sensitive vaccine candidate rA2*cp248/404/1030*ΔSH, with a shutoff temperature of 35°C. Clinical studies showed that this virus

Received 24 May 2012 **Accepted** 19 July 2012

Published ahead of print 25 July 2012

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Supplemental material for this article may be found at http://jvi.asm.org/.

doi:10.1128/JVI.01227-12

was well-tolerated and immunogenic in 1- to 2-month old infants and was protective against a second vaccine dose (18).

When rA2*cp248/404/1030*ΔSH was evaluated in children and infants in the initial clinical study, analysis of specimens recovered from nasal washes in the days following vaccination showed that more than one-third of the isolates exhibited a partial loss of the *ts* phenotype (18, 21). Sequence analysis of a limited number of the recovered isolates identified two types of genetic changes, namely, loss of either the *248* or the *1030* mutation, with 80% of the observed changes involving *1030* (L amino acid 1321) (18, 21). The wt assignment at amino acid 1321, tyrosine (TAT), and the *ts*/attenuating *1030* mutation, asparagine (AAT), differ by a single nucleotide (underlined). In clinical trial specimens, reversion at this position was due to asparagine (AAT) being replaced with the wt assignment of tyrosine (TAT) or, in one case, with histidine (CAT) (18). Reversion to the wt or histidine assignment could account for the partial loss of the *ts* phenotype. Since reversion at the *1030* mutation was the most frequent change observed in the clinical trial samples, it was of interest to investigate whether this mutation could be stabilized.

The rA2*cp248/404/1030*ΔSH virus remains the most promising RSV vaccine candidate. A second, biologically equivalent version of this virus is presently being further evaluated in a phase 1/2 clinical study (ClinicalTrials.gov identifier NCT00767416) under the name Medi-559. The first and second versions will hereinafter be called rA2*cp248/404/1030*ΔSH and Medi-559, respectively. They differ by 37 nucleotide substitution point mutations scattered throughout the genome, none of which affect amino acid coding. The nucleotide differences are due to naturally occurring variability in the wt RSV used as the template for reverse transcription (RT)-PCR and to differences in restriction site markers. All of the attenuating mutations of rA2*cp248/404/1030*ΔSH are present in Medi-559, except that the codon for the 248 mutation (Q831L) is TTA in rA2*cp248/404/1030*ΔSH and CTG in Medi-559. (Note that the nomenclature lists wt amino acid assignments to the left of the sequence position [e.g., Q831] and mutant assignments to the right [e.g., 831L]). Both of these codons readily reverted during a temperature stress test (22) in which virus bearing the mutation of interest is passaged multiple times at progressively increasing temperature to select for revertants. Thus, it is unlikely that there is a significant difference in the genetic stability of rA2*cp248/404/1030*ΔSH and Medi-559. The available data also indicate that these two viruses are indistinguishable with regard to replication in cell culture, temperature sensitivity, and attenuation *in vivo*.

We previously described a strategy to increase the phenotypic and genetic stability of attenuating amino acid substitutions that are based on a single-nucleotide substitution, such as the *248* and *1030* mutations (22, 23). This strategy is based on increasing the number of nucleotides that must be changed in a given mutant codon in order to encode an amino acid assignment conferring deattenuation, which can involve direct reversion to the wt assignment or change to another assignment that confers a wt-like phenotype. Substitution at any single-nucleotide position in RNA viruses can occur at a relatively high rate, $\sim 10^{-4}$, thus providing for relatively frequent deattenuation if only a single nucleotide is involved. However, if deattenuation at the amino acid level requires changes at two or, preferably, three positions within the codon, the frequency will be much less, $\sim 10^{-8}$ and $\sim 10^{-12}$, respectively. As the first step in this analysis, the position of interest is changed systematically to encode each of the 20 possible amino acids on the wt background, resulting in a panel of viruses that are then evaluated to identify the *ts* and attenuation phenotypes associated with each amino acid assignment. Then, based on the genetic code, the sequences of all the possible codons encoding attenuating amino acids are compared on paper to the wt-like codons with the goal of identifying a codon that would require changes at two or three positions in order to revert to a wt-like codon. This strategy can reduce deattenuation at a given codon to below the level of detection, although it is not always possible to identify a reversion-resistant codon (22, 23). In the present study, we used this strategy to increase the genetic and phenotypic stability of the *1030* mutation involving position 1321 in the L protein. This mutation was used to construct an improved version of the Medi-559 vaccine candidate, called cps2, that has increased genetic stability and remains satisfactorily attenuated in seronegative chimpanzees.

MATERIALS AND METHODS

Virus growth and titration. RSV strains were propagated in HEp-2 cells at 32°C in Opti-MEM I (Gibco Life Technologies, Grand Island, NY) containing 2% fetal bovine serum (FBS), 1% L-glutamine, 250 units/ml penicillin, and 250 μg/ml streptomycin (Gibco Life Technologies). Virus stocks were generated by scraping infected cells into the medium, vortexing the cell suspension to release cell-associated virus, clarifying the supernatant by centrifugation, and adding 10× SPG (2.18 M sucrose, 0.038 M KH_2PO_4, 0.072 M K_2HPO_4, 0.06 M L-glutamine at pH 7.1) to a final concentration of 1× SPG to stabilize the virus during freezing. Virus aliquots were snap-frozen and stored at −80°C. Virus titers were determined by plaque assay on HEp-2 cells under an 0.8% methylcellulose overlay. After a 5-day incubation at 32°C, plates were fixed with 80% cold methanol, and plaques were visualized by immunostaining with a mixture of three RSV-specific monoclonal antibodies (25).

Construction of recombinant RSV mutants. Recombinant RSVs (rRSVs) were generated using a reverse genetics system based on strain A2 (6). The full-length RSV antigenome cDNA was modified previously by deleting a 112-nucleotide (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). The size of the RSV antigenome contained in this plasmid (termed D46/6120) is 15,111 nt. These changes were made to improve the stability of the cDNA during growth in *Escherichia coli* and had no effect on the efficiency of virus replication *in vitro* or in mice (4). This cDNA was used to generate the mutants described in this study.

To construct viral mutants, a 4,086-bp XbaI-SalI fragment of the D46/6120 cDNA (containing 4,013 bp of the downstream and L gene and adjacent trailer region, plus 73 bp from the adjacent hepatitis delta virus [HDV] ribozyme sequence) was subcloned into the multiple cloning site of pBluescript. Mutations at codons 1321 (nucleotides 12458 to 12460) and 1313 (nucleotides 12434 to 12436) were introduced by site-directed mutagenesis using the QuikChange mutagenesis kit (Agilent, Santa Clara, CA), following the procedures recommended by the supplier. The sequence of the 4,086-bp fragment and the presence of the desired mutations were confirmed, and the 4,086-bp XbaI-SalI fragment was inserted into an intermediate pBluescript cloning vector containing a 9,623-bp BamHI-EagI fragment of the RSV D46/6120 full-length plasmid (consisting of the downstream 6,722-bp L gene and trailer fragment of the RSV genome, plus an adjacent 2,901-bp fragment of the pBR322 moiety of plasmid D46/6120). To create each of the viral mutants, the 9,623-bp BamHI-EagI fragment with the individual point mutations was transferred from the pBluescript shuttle vector to the RSV D46/6120 antigenome cDNA plasmid.

Nomenclature and sequence numbering convention. When mutations are designated by codon number and amino acid assignment (e.g., Y1321N), the wt amino acid assignment is to the left of the sequence position (e.g., Y1321) and mutant assignments are to the right of the

sequence position (e.g., 1321N). The wt assignment is not always indicated (e.g., 1321N). Codon sequences sometimes are included, in parentheses and always to the right [e.g., K(AAA)].

Sequence positions are always based on the complete sequence of biologically derived strain A2 (GenBank accession number M74568) and are not adjusted for deletions or insertions.

Generation of recombinant RSV from cDNA. BSR T7/5 cells were grown to 95% confluence in 6-well plates. Cells were washed twice with Glasgow minimal essential medium (GMEM), containing 3% FBS, 1 mM L-glutamine, and 2% minimal essential medium (MEM) amino acids. Cells were transfected by adding 500 μl Opti-MEM I medium (Invitrogen) containing a mixture of 10 μl Lipofectamine 2000 and 5 μg full-length plasmid, 2 μg each pTM1-N and pTM1-P, and 1 μg each pTM1-M2-1 and pTM1-L (3, 6). Transfected cells were incubated overnight at 37°C. In some cases, plates were then heat shocked at 43°C, 3% CO_2 for 3 h to increase the efficiency of transfection (33). All plates were incubated at 32°C for at least 24 h. The BSR T7/5 cells were harvested by scraping them into the medium, and the cell suspension was added to subconfluent monolayers of HEp-2 cells in 10 ml Opti-MEM I containing 5% FBS and 1% L-glutamine and incubated at 32°C. Virus was harvested between 11 and 14 days posttransfection, 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 of uncloned PCR products. The only sequences that were not directly confirmed for each genome were the positions of the outermost primers, namely, nucleotides 1 to 29 and 15,191 to 15,222.

Evaluation of the *ts* phenotype. The *ts* phenotype for each of the rRSVs was evaluated by the efficiency of plaque formation at 32, 35, 36, 37, 38, 39, and 40°C. Serial 10-fold dilutions were plated in duplicate on HEp-2 cells in L-15 medium containing 2% FBS, 250 units/ml penicillin, 250 μg/ml streptomycin (Gibco-Life Technologies), and 1.25 μg/ml amphotericin B (Quality Biological, Gaithersburg, MD). The cells were incubated in sealed caskets in temperature-controlled water baths as previously described (13) and processed for plaque enumeration as described above.

Evaluation of the attenuation phenotype in experimental animals. Virus replication was evaluated in the upper and lower respiratory tracts of mice as described previously (30). Briefly, 10-week-old female BALB/c mice in groups of five were inoculated intranasally under methoxyflurane anesthesia with 10^6 PFU of rRSV in a 100-μl volume. 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 1× SPG, 2% L-glutamine, 0.06 mg/ml ciprofloxacin, 0.06 mg/ml clindamycin phosphate, 0.05 mg/ml gentamicin, and 0.0025 mg/ml amphotericin B. Virus titers were determined in duplicate on HEp-2 cells incubated at 32°C.

Juvenile chimpanzees were pair housed in spacious glass isolator suites and maintained as previously described (15). Chimpanzees were used because they are the only known experimental animal that approaches humans with respect to susceptibility to RSV infection, replication, and disease and because they have the same body temperature as humans and thus provide reliable evaluation of *ts* mutants. Selected use of chimpanzees in evaluating RSV vaccines expedites clinical evaluation. Animals were confirmed to be RSV seronegative. Two animals were immunized by intranasal and intratracheal inoculation with 10^6 PFU of Medi-559 per site, and three animals were inoculated with 10^6 PFU of RSV cps2 per site. Animals were monitored twice daily for clinical symptoms. Nasal washes were performed daily for 12 days postinoculation with 3 ml of lactated Ringer's solution per nostril. On days 2, 4, 6, and 8, bronchoalveolar lavages (BAL) were done using 6 ml of phosphate-buffered saline (PBS), and on days 10 and 12, tracheal lavages (TL) were performed with 3 ml of PBS. The procedures were performed under ketamine anesthesia. The virus titers in nasal wash, BAL fluid, and TL aspirates were determined by plaque assay on Vero cells at 32°C. The mouse and chimpanzee studies were approved by the Animal Care and Use Committee of NIAID, NIH. The chimpanzee study was performed prior to the recent recommendation by the Institute of Medicine that chimpanzees not be used for RSV research. This represents the last use of chimpanzees for RSV research, and it will expedite the development of this modified vaccine.

RESULTS

Creating a panel of recombinant RSVs representing the possible amino acid assignments at position 1321 of the RSV L protein. Recombinant RSVs were generated by reverse genetics in which amino acid 1321 of the L protein was changed from the wt assignment of tyrosine (Y, TAT) to asparagine (N, AAT) of the original biologically-derived *1030* mutant, as well as to the other 18 possible amino acid assignments. Nineteen of the 20 amino acid assignments at position 1321 yielded recoverable virus (see Table S1 in the supplemental material). The only one that did not was the 1321R mutant. The 1321R virus was not recovered in five independent transfections involving two independent antigenomic cDNAs. The failure in recovery probably was not due to an adventitious mutation, since the other mutants were successfully recovered using the same antigenomic cDNA backbone and since the L gene of this 1321R mutant cDNA was confirmed by sequencing to be completely correct. Thus, the 1321R mutant was considered nonviable.

The 19 recovered viruses replicated efficiently on HEp-2 cells at the permissive temperature of 32°C, with the exception of the 1321D virus, for which the titers were reduced more than 10-fold. The genomes of the 19 viable viruses were completely sequenced, which confirmed their mutations but identified adventitious mutations in four mutants. Each involved the insertion of one or, in one case, three adenosine residues (positive sense) into gene end signals or nontranslated regions (see Table S1 in the supplemental material). Adventitious mutations in these regions are not unusual during RSV growth in cell culture (22), and these mutations were considered to be insignificant. These results indicated that alternative amino acids could be accommodated at position 1321.

We also attempted to introduce small deletions at or near position 1321, on the premise that a codon deletion is less subject to reversion than a nucleotide substitution. This involved deletion of codon 1321 alone, 1320 alone, 1320 plus 1321, or 1321 plus 1322 (see Table S1 in the supplemental material). However, we were unable to recover infectious virus from any of these mutants in four to six independent attempts each, whereas positive controls were readily recovered (see Table S1). Since the antigenomic cDNA was successful with other mutants and since the L gene of each mutant cDNA was confirmed by sequencing to be correct, we concluded that these codon deletions were not tolerated by RSV.

Temperature sensitivity and attenuation phenotypes of recombinant RSVs with substitutions at amino acid position 1321 of the L protein. The *ts* phenotypes of the various viable mutants were evaluated by replication *in vitro* over a range of increasing temperatures (32°C to 40°C) that are permissive for wt RSV (Table 1). To identify possible attenuation phenotypes, the mutants were evaluated for their ability to replicate in the nasal turbinates and lungs of BALB/c mice (Table 1). The mouse is an *in vivo* model for RSV replication that, like humans, has a 37°C body temperature and thus can be used to evaluate *ts* mutants. Although mice are much less permissive for RSV replication than humans and therefore cannot be used to predict absolute levels of replication, mu-

TABLE 1 Characterization of the temperature sensitivity and attenuation phenotypes of recombinant RSVs with substitutions at amino acid position 1321 of the L protein

Amino acid at position 1321	Virus titer (PFU per ml) at indicated temp (°C)[a]							T_{SH}[d]	Replication in mice[e]			
									Titer (log_{10} PFU/g ± SE)		Mean log_{10} reduction[f]	
	32	35	36	37	38	39	40		Nasal turbinates	Lung	Nasal turbinates	Lung
Y (wt)	7.8	7.8	7.8	7.8	7.8	7.9	7.7	>40	4.6 ± 0.1	4.2 ± 0.1		
N[g]	7.7	7.8	7.6	7.4[b]	**<1**	<1	<1	**38**	3.5 ± 0.1	3.6 ± 0.0	1.1	0.6
D	6.1[b]	**3.4**	3.5	3.3	3.3	3.1	3.2	**35**	ND	ND		
E	7.5	7.2[b]	6.3[b]	**3.5**[b]	<1	<1	<1	**37**	1.9 ± 01	1.7 ± 0.0	2.7	2.5
P	7.7	7.8	7.7	4.5	**<1**	<1	<1	**37**	3.6 ± 0.1	2.8 ± 0.0	1.0	1.4
K	7.5	7.5	7.4	7.3[b]	**<1**	<1	<1	**38**	4.0 ± 0.1	2.7 ± 0.2	0.6	1.5
G	7.5	7.4	7.3	6.9	**<1**	<1	<1	**38**	3.7 ± 0.1	3.5 ± 0.1	0.9	0.7
T	7.6	7.6	7.6	7.5[b]	**<1**	<1	<1	**38**	4.0 ± 0.1	3.8 ± 0.0	0.6	0.4
C	7.9	8.0	8.0	7.8	**<1**	<1	<1	**38**	3.9 ± 0.5	3.9 ± 0.1	0.7	0.3
Q	7.6	7.7	7.6	7.5	**<1**	<1	<1	**38**	4.4 ± 0.1	3.7 ± 0.1	0.2	0.5
V	7.9	7.9	7.9	7.8[b]	**4.9**[c]	<1	<1	**38**	4.4 ± 0.1	3.9 ± 0.1	0.2	0.3
A	7.2	7.3	7.2	7.0	6.1[b]	**<1**	<1	**39**	4.2 ± 0.0	3.7 ± 0.1	0.4	0.5
S	7.9	7.9	7.8	7.7	6.6[b]	**<1**	<1	**39**	4.2 ± 0.1	3.9 ± 0.1	0.4	0.3
I	7.5	7.5	7.5	7.4	7.1[b]	**5.1**[b]	<1	**39**	4.1 ± 0.2	3.8 ± 0.1	0.5	0.4
L	7.5	7.5	7.5	7.4	7.4	7.2[b]	**<1**	**40**	4.4 ± 0.1	4.4 ± 0.0	0.2	None
F	7.5	7.5	7.6	7.4	7.3[b]	6.7[c]	**<1**	**40**	4.3 ± 0.1	4.1 ± 0.1	0.3	0.1
W	7.7	7.7	7.6	7.7	7.5[b]	6.1[c]	**<1**	**40**	4.2 ± 0.1	4.3 ± 0.1	0.4	None
H	7.9	6.9	6.9	6.9	6.7	6.1[b]	**<1**	**40**	4.5 ± 0.1	4.3 ± 0.0	0.1	None
M	7.8	7.9	7.9	7.8	7.7	7.1[c]	6.3[c]	>40	4.7 ± 0.0	4.4 ± 0.1	None	None

[a] Viruses were evaluated for *ts* phenotype by plaque assay on HEp-2 cells at the indicated temperatures. For viruses with a ts phenotype, titers indicating the shutoff temperature (T_{SH}, see footnote *d*) are in boldface.
[b] Small plaques.
[c] Microplaques.
[d] Shutoff temperature (T_{SH}) is defined as the lowest restrictive temperature at which the reduction in titer compared to the titer at 32°C is 100-fold or greater than that observed for wt RSV at the two temperatures (22, 23). The *ts* phenotype is defined as having a T_{SH} of 40°C or less. For viruses with the *ts* phenotype, T_{SH} values are in boldface.
[e] Ten-week-old mice in groups of five were inoculated intranasally with 10^6 PFU of the indicated virus. Nasal turbinates and lungs were harvested on day 4, and virus titers were determined by plaque assay. The limits of detection were 2 log_{10} PFU per g for nasal turbinates and 1.7 log_{10} PFU per g for lungs. Results that are statistically significant different ($P \leq 0.05$) from the results for Y1321 (wt) control virus are underlined. ND, not detectable.
[f] Reduction in mean titer compared to titer of 1321Y (wt) virus.
[g] Original 1030 mutant amino acid assignment.

tants can be ranked with regard to their relative levels of attenuation.

As shown in Table 1, the original 1321N mutation conferred an *in vitro* shutoff temperature (T_{SH}) of 38°C, consistent with previous results (31). It also conferred log_{10} reductions in titer of 1.1 and 0.6 in the nasal turbinates and lungs, respectively, compared to the titer of wt Y1321 virus (Table 1). Of the 17 other mutant viruses tested, 16 had a T_{SH} lower than that of wt virus. For 9 mutants (with mutations 1321D, 1321E, 1321P, 1321K, 1321G, 1321T, 1321C, 1321Q, and 1321V), the level of temperature sensitivity was substantial, with T_{SH} values of 35 to 38°C, compared to 38°C for the original 1321N mutant (Table 1). When evaluated in mice, a number of viruses appeared to be attenuated relative to the replication of wt virus, and five of these viruses, namely, those with mutations 1321E, 1321P, 1321K, 1321G, and 1321T, exhibited a statistically significant reduction in replication. The 1321D virus, the most temperature sensitive of the viruses, could not be recovered from mice and thus was overattenuated. The viruses with mutations 1321L, 1321F, 1321W, and 1321H exhibited only a modest amount of temperature sensitivity and little or no attenuation. The 1321M virus was neither temperature sensitive nor attenuated, although it did form microplaques at 39°C and 40°C, indicative of some impairment. The amino acids conferring attenuation did not have any particular pattern with regard to their chemical structures (not shown).

The *ts* and attenuation phenotypes are compared in Fig. 1. There was a highly significant positive correlation between T_{SH} and virus titer in the nasal turbinates (Spearman rank correlation test, $\rho = 0.68$; $P = 0.001$) and a significant correlation between T_{SH} and lung titers ($\rho = 0.82$; $P = 0.00002$). Thus, each of the significantly attenuating assignments also conferred a substantial reduction in T_{SH}.

In summary, the phenotypic analysis reported in Table 1 iden-

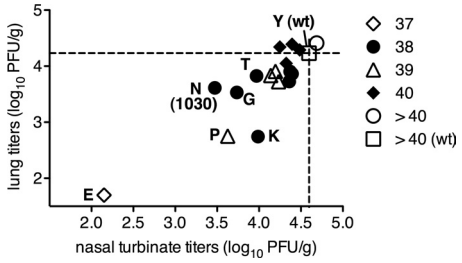

FIG 1 *ts* and attenuation phenotypes of recombinant RSVs bearing substitutions at amino acid 1321 of the L protein. For each virus, the mean viral titer in the nasal turbinates of mice is plotted versus the mean viral titer in the lungs, using the data presented in Table 1. The dashed lines mark the level of replication of wt RSV. Viruses are identified by the single-letter code corresponding to amino acid 1321 of the L protein. The symbol used for each virus indicates its *in vitro* T_{SH} (°C), from the data in presented in Table 1.

TABLE 2 Theoretical outcomes of all possible single-nucleotide substitutions in all possible codons of selected amino acid assignments

Amino acid analyzed	Codon(s)[a]	Amino acid assignment resulting from nucleotide point mutation (n to indicated nucleotide)[b]					No. of changes resulting in reversion to indicated attenuation phenotype	
		Position[c]	T	C	A	G	Intermediate	Wild type
N	AAT[d] or AAC	nAY	Y[e]	H[e]	N	D	2	3
		AnY	*I*	*T*	N	S		
		AAn	N	N	K	K		
P	CCT or CCC	nCY	S	P	*T*	*A*	2	3
		CnY	L	P	H[e]	R		
		CCn	P	P	P	P		
P	CCA or CCG	nCR	S	P	*T*	*A*	3	2
		CnR	L	P	*Q*	R		
		CCn	P	P	P	P		
G	GGG	nGG	W	R	R	G	3	1
		GnG	*V*	*A*	E	G		
		GGn	G	G	G	G		
G	GGT or GGC	nGY	*C*	R	S	G	2	1
		GnY	*V*	*A*	D	G		
		GGn	G	G	G	G		
K	AAG	nAG	Stop	*Q*	K	E	2	1
		AnG	M	*T*	K	R		
		AAn	N	N	K	K		
E	GAA or GAG	nAR	Stop	*Q*	K	E	3	
		GnR	*V*	*A*	E	G		
		GAn	D	D	E	E		
K	AAA	nAA	Stop	*Q*	K	E	3	
		AnA	*I*	*T*	K	R		
		AAn	N	N	K	K		
G	GGA	nGA	Stop	R	R	G	2	
		GnA	*V*	*A*	E	G		
		GGn	G	G	G	G		

[a] Where two alternative codons are shown for an amino acid assignment, they yielded the same outcomes.
[b] Each codon was analyzed for substitutions at codon positions 1, 2, and 3 (nucleotide position in the codon where substitution occurs is indicated with n). The amino acid assignments resulting from T, C, A, and G substitutions at this position are shown. Amino acids that confer an intermediate attenuation phenotype are italicized. Those that confer a wt-like phenotype are underlined.
[c] Y denotes C or T; R denotes G or A.
[d] AAT is the codon present in the rA2*cp248/404/1030*ΔSH virus that was evaluated in clinical trials (18).
[e] Amino acid conferring an upward shift in T_{SH} detected in samples from clinical trials (18).

tified four amino acid assignments (1321E, 1321P, 1321K, and 1321G) that were comparable in attenuation (1321P, 1321K, and 1321G) or even more attenuated (1321E) than the original 1321N mutation, and thus, there are possible alternative assignments. In addition, knowledge of the phenotype associated with each amino acid (Table 1) provides the basis for evaluating whether particular nucleotide changes would confer deattenuation.

Predicted outcomes of single-nucleotide substitutions in codons for 1321E, 1321P, 1321K, and 1321G. Since mutations 1321E 1321P, 1321K, and 1321G were possible replacements for the original 1321N mutation, we evaluated all of the codons for each of these amino acids (plus 1321N for comparison) for genetic stability in a paper exercise. We made all possible single-nucleotide substitutions at each codon position and listed the amino acid outcomes (Table 2). The outcomes were scored as wt-like, attenuated, or intermediate based on the *ts* and attenuation phenotypes shown in Table 1. Specifically, wt-like assignments were defined as the ones for which the T_{SH} was ≥39°C and the titers in both the nasal turbinates and lungs of mice were reduced by ≤0.4 $\log_{10}$ (Y, S, L, F, W, H, and M) (Table 1). These assignments would not be desirable because they would confer a substantial deattenuation. Attenuated assignments were defined as ones for which the viral titers in either the nasal turbinates or the lungs were reduced by ≥0.9 $\log_{10}$ (N, D, E, P, K, and G) or resulted in nonviable virus (R) and, thus, would not yield phenotypic reversion (Table 1). The remaining residues (T, C, Q, V, A, and I) were defined as having intermediate, or partially attenuated, phenotypes. The goal of this exercise was to identify one or more codons for 1321E, 1321P, 1321K, and 1321G that could not be changed by any single-nucleotide substitution into a codon specifying a wt-like assignment

and, preferably, would also have a minimum number of possible changes to a codon specifying an intermediate assignment. Any such codon should be more refractory to deattenuation.

As shown in Table 2, a number of the codons that were examined had one or more outcomes that would yield a wt-like phenotype and, thus, would not be desirable. For example, both possible codons for the original 1321N assignment of the original biologically derived *1030* mutant had three outcomes that would yield a wt-like assignment. The same was true for two of the four possible codons for proline (CCT or CCC), whereas the other two possible proline codons (CCA or CCG) could each yield two wt-like assignments. Several codons, such as the G codons GGG, GGT, or GGC, had only one possible substitution for each codon that would lead to a wt-like assignment and, thus, would be improved alternatives to 1321N. Importantly, four codons were identified in which any possible single-nucleotide substitution would not yield a wt-like assignment, namely, those for E (GAA), E (GAG), K (AAA), and G (GGA). Thus, these four codons represent the most promising alternatives to the 1321N assignment.

Genetic stability of several alternative 1321 mutants at increasingly restrictive temperatures. It was important to confirm that the proposed alternative codons indeed conferred increased phenotypic stability. Therefore, we performed temperature stress tests on viruses containing the original 1321N(AAT) mutation versus viruses containing 1321E(GAA), 1321K(AAA), 1321G(GGT), 1321G(GGA), or 1321P(CCT). Since both 1321E codons had the same predicted reversion outcomes (Table 2), only one (GAA) was evaluated. Similarly, two of the G codons (GGT and GGC) and two of the P codons (CCT and CCC) had the same outcomes (Table 2), and thus, only the first of each pair was assayed. Ten independent aliquots of each virus were serially passaged two times each at 35°C, 36°C, and 37°C, for a total of six passages (Fig. 2). As controls, two independent aliquots of each virus were serially passaged 6 times at the permissive temperature of 32°C (Fig. 2, dotted lines). Aliquots of each passage level were titrated by plaque assay at 32°C to detect changes in virus yield that might be indicative of changes in the *ts* phenotype. Titration analysis indicated that all lineages replicated at 36°C and 37°C (Fig. 2), with no restriction due to the *ts* phenotype. In addition, virus from passage 6 of each passage series was subjected to sequence analysis of a 249-nt region of the L gene (nucleotides 12261 to 12511 in the RSV genome) containing codon 1321 in order to directly investigate the stability of the *ts* mutation (Table 3).

For the 1321N(AAT) virus, representing the original mutation, subpopulations with reversions to the wild type [Y(TAT)] were detected in 9 out of 10 flasks (lineages), and one of these had mixed subpopulations with 1321N, Y1321, and 1321H (Table 3), as was seen in clinical isolates (18). Direct reversion to Y1321 or mutation to 1321H was consistent with predictions (Table 2). In addition, Y1321 and 1321H had been observed in isolates from vaccinees in clinical trials that received an experimental vaccine containing the 1321N *1030* mutation (18). In that study, these changes were associated with a partial loss of the *ts* phenotype, although in the case of 1321H virus, causality was uncertain due to limited sequence analysis (18). However, the analysis presented in Table 1 clearly showed that the 1321H assignment is wt-like, with a T_{SH} of 40°C and little or no attenuation in mice, and thus, is deattenuating. This showed concordance between the predicted outcomes (Table 2), our *in vitro* assay stress test results (Table 3), and the results from clinical trials in infants and children.

In contrast, neither of the two 1321G codons, GGA or GGT, was altered by the stress test, indicative of increased stability at this position. However, a second-site mutation at codon S1313 was detected in 50% and 90% of the flasks (lineages), respectively, resulting in a change from serine to cysteine (S1313C). Another tested virus, with 1321K(AAA), had no detectable change at position 1321 but had the same second-site mutation, 1313C, in 90% of its lineages. The other tested mutants, with E(GAA) and P(CCT), had changes at position 1321 in some lineages. All of the changes at position 1321 in 1321E(GAA) virus involved changes to valine or alanine (Table 3), which had intermediate attenuation phenotypes (Table 1). Thus, the 1321E(GAA) assignment was stable against reversion to wt-like assignments. All of the changes at position 1321 in 1321P(CCT) virus involved changes to leucine or histidine (Table 3), which had wt-like phenotypes (Table 1). Although a number of 1321P(CTT) lineages exhibited deattenuation, this assignment was more stable than the original 1321N(AAT) assignment. However, the passaged 1321E(GAA) and 1321P(CCT) viruses also had the second-site mutation S1313C in nearly all of their lineages. Thus, the stress test evaluation provided evidence of increased genetic stability at position 1321 with the G(GGA), G(GGT), and K(AAA) assignments and, to a lesser extent, with the E(GAA) and P(CCT) assignments. However, the presence of the S1313C mutation in many lineages (which in all cases involved an AGC to TGC missense mutation [the mutation is underlined]) suggested that it might be a compensatory second-site mutation.

S1313C is a compensatory second-site mutation that confers substantial deattenuation of 1321 assignments. To investigate the significance of the S1313C mutation, recombinant viruses were constructed in which wt Y1321 or the attenuating 1321G, 1321E, or 1321K assignments were combined with either wt S1313 or the proposed compensatory mutation 1313C (Fig. 3). In the case of glycine at position 1321, two different codons were evaluated (GGA and GGT). The recovered viruses were amplified and sequenced in their entirety. Except for an inconsequential single A insert in the downstream end of the noncoding region of the L gene in the 1321K(AAA)/1313C mutant (not shown), the viruses were to shown to be free of adventitious mutations.

As shown in Table S2 in the supplemental material, the introduction of the 1313C assignment into the wt background (Y1321/1313C) did not confer temperature sensitivity or attenuation. However, when the 1313C assignment was combined with the attenuating 1321G, 1321E, and 1321K codons, the T_{SH} was increased by 2°C, and each of these viruses replicated in mice with an efficiency equivalent to that of wt RSV (see Table S2). Thus, the S1313C mutation is a compensatory mutation for the *ts* and attenuation phenotypes of mutations at position 1321.

Combined stabilization of positions 1321 and 1313. The ability of the second-site 1313C mutation to cause deattenuation of assignments at position 1321 indicated that it would be necessary to simultaneously stabilize both positions. Therefore, in combination with the attenuating 1321K(AAA) assignment, we changed the existing wt serine AGC codon to TCA, a choice that would require two rather than one nucleotide substitution to yield cysteine (Fig. 4A). This did not address the possibility that the codon could change to encode another amino acid assignment that might also be compensatory. As would be expected, this

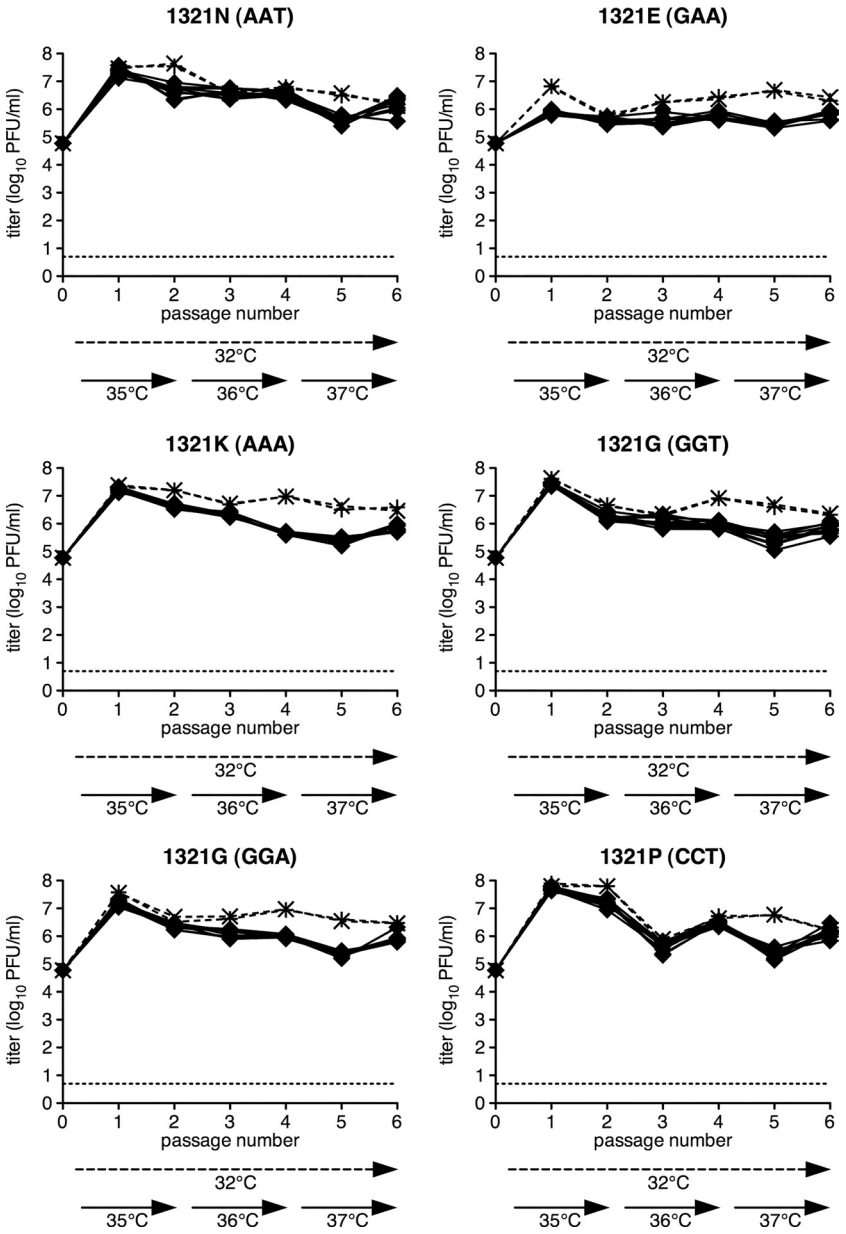

FIG 2 Growth of mutant RSVs *in vitro* at increasingly restrictive temperatures in a temperature stress test designed to assess phenotypic and genetic stability. Ten independent aliquots of each RSV bearing the indicated codon at position 1321 of the L protein in wt RSV were serially passaged at increasingly restrictive temperatures as follows: two passages at 35°C, two at 36°C, and two at 37°C, for a total of six passages (23). In parallel, two independent replicas were passaged six times at 32°C (permissive temperature), serving as controls (dashed lines). For each passage, aliquots were frozen for titration and sequence analysis. Virus titers of the mutants at the different passage levels are shown, and the passage temperatures are indicated. Virus titer was determined by plaque assay at the permissive temperature (32°C). Passage 0 represents the input virus.

silent change did not affect the *ts* phenotype, and the resulting 1321K(AAA)/S1313(TCA) virus retained a T_{SH} of 38°C (see Table S2).

An abbreviated temperature stress test (Fig. 4B) of two passages at 37°C followed by two passages at 38°C [the restrictive temperature for the 1321K(AAA)/S1313(TCA) virus] was done. To confirm that this abbreviated assay provided a stringent test of stability, this analysis also included the 1321E(GAA)/1313C(TGC) mutant virus (Fig. 3). Sequence analysis showed that 80% of the 1321E(GAA)/1313C(TGC) virus lineages exhibited mutations at codon 1321, yielding amino acid substitutions of valine, lysine, and alanine (Table 4), consistent with the predictions in Table 2 and similar to the stress test results in Table 3. This showed that this abbreviated stress test format indeed was sufficient to document genetic instability.

Importantly, the 1321K(AAA)/S1313(TCA) virus retained the *ts* phenotype in this stress test, as evidenced by the substantially reduced titer compared to those of the control cultures passaged at the permissive temperature of 32°C (Fig. 4B). Sequencing of the vicinity of codon 1321 also showed that there was no reversion or

TABLE 3 Observed stability of various codons coding for amino acid 1321 of the L protein during passage at restrictive temperatures, and occurrence of a potential compensatory 1313C mutation[a]

Amino acid (codon)	% of cultures with revertant(s) or compensatory mutation(s)[b]	Codon 1321		Compensatory mutation(s) at 1313 [wt assignment is S(AGC)]	
		Revertant codon(s) observed[c]	Amino acid(s)[d]	Mutant codon(s) observed[c]	Amino acid(s)[d]
N (AAT)[e]	80	[A/T]AT	N:Y		
	10	[A/T/C]AT	N:Y:H		
G (GGA)	90			[A/T]GC	S:C
G (GGT)	50			[A/T]GC	S:C
K (AAA)	90			[A/T]GC	S:C
E (GAA)	40			TGC	C
	40			[A/T]GC	S:C
	10	G[A/T]A	E:V	[A/T]GC	S:C
	10	G[A/T/C]A	E:V:A	[A/T]GC	S:C
P (CCT)	10			[A/T]GC	S:C
	50	C[C/T]T	P:L	[A/T]GC	S:C
	20	C[C/A/T]T	P:L:H	[A/T]GC	S:C
	10	CTT	L	[A/T]GC	S:C
	10	C[A/T]T	L:H		

[a] Ten replicate 25-cm^2 flasks of HEp-2 cells were infected with the indicated 1321 mutants at a multiplicity of infection of 0.1 PFU/cell at 35°C. Virus was harvested between 5 and 7 days postinfection and serially passaged once more at 35°C and twice each at 36°C and 37°C for a total of six passages, each by transferring 1 ml (of a total of 5 ml) of supernatant to a fresh 25-cm^2 flask of HEp-2 cells. In parallel, two control flasks per mutant were passaged six times at the permissive temperature of 32°C. For each passage, aliquots were frozen for titration. Genotype analysis was done after the sixth passage by sequencing of a 249-nt region of the RSV L gene (RSV nucleotides 12261 to 12511; GenBank accession number M74568). No mutations were detected in the 32°C controls (not shown).

[b] Percentage of cultures with detectable revertant mutations at codon 1321 and/or compensatory mutations at codon S1313.

[c] Observed codon sequence(s), shown only for lineages where mutation was detected; mixtures are indicated in brackets. Changes are underlined.

[d] Amino acid(s) coded, shown only for lineages where mutation was detected; colon indicates a mixed population of the specified amino acids. Changes are underlined.

[e] Codon present in the rA2*cp248/404/1030*ΔSH virus evaluated in clinical trials (18).

mutation at position 1313 or 1321 (Table 4), indicating that the 1321 and 1313 codons had both been stabilized.

To search for possible additional compensatory changes, the L gene was completely sequenced from all 12 of the 1321K(AAA)/S1313(TCA) virus lineages at passage 4 of the stress test whose results are shown in Fig. 4B. Seven of the 10 lineages passaged at 38°C had additional mutations in L. Five missense mutations were considered incidental an S420G/D1430E double mutation, Q874H, I1043L, and Y1361F. Each occurred only in single lineages, and thus, they are unlikely to be compensatory changes. A sixth missense mutation, E649D, was found in three lineages (not shown). To evaluate the possibility that E649D and Q874H might be additional compensatory mutations, viruses were constructed in which the E649D or Q874H mutation was placed individually in the backbone of wt RSV or the 1321K(AAA)/S1313(TCA) virus. The mutant viruses were recovered, their complete sequences were confirmed, and their *ts* and attenuation phenotypes were determined (see Table S3 in the supplemental material). When placed individually in the wt RSV backbone, the E649D and Q874H mutations had similar effects: they did not result in a *ts* phenotype, but surprisingly, each conferred substantial ($\leq$1.5 $\log_{10}$) decreases in replication in the upper and lower respiratory tract of mice (see Table S3). More importantly, when placed individually in the attenuated 1321K(AAA)/S1313(TCA) backbone, the E649D and Q874H mutations had no effect on T_{SH} (E649D) or caused a slight increase in T_{SH} (Q874H), and neither mutation affected the attenuation phenotype. Thus, neither E649D or Q874H is a compensatory mutation. On the contrary and unexpectedly, each is a non-*ts* attenuating mutation. The lack of compensatory mutations confirmed the genetic and phenotypic stability of the 1321K(AAA)/S1313(TCA) assignments.

Generation of cps2, a genetically stabilized version of Medi-559. We modified the Medi-559 virus to contain changes at L protein positions 831 (the *248* locus), 1313 (the second-site compensatory mutation affecting position 1321), and 1321 with the purpose of increasing genetic and phenotypic stability. The *248* mutation 831L(TTA) of Medi-559 was replaced by the codon 831L(TTG), which we previously showed conferred a modest increase in genetic stability (22). In addition, based on the results in the present study, the *1030* mutation 1321N(AAT) of Medi-559 was replaced by 1321K(AAA), and the wt assignment S1313(AGC) was replaced by S1313(TCA). The resulting virus was designated cps2. It differs from Medi-559 by five nucleotide substitutions and one amino acid substitution. cps2 was readily recovered and was indistinguishable from rA2*cp248/404/1030*ΔSH and Medi-559 with regard to efficient replication at permissive temperature *in vitro* and *ts* phenotype and in being highly attenuated in mice (data not shown).

The genetic stability of the cps2 virus was evaluated by an *in vitro* stress test, in parallel with the rA2*cp248/404/1030*ΔSH virus, which had exhibited genetic instability in vaccine recipients and during passage *in vitro* (18) (Table 5). The two viruses were each passaged in 10 parallel cultures for two passages each at 33, 34, 35, 36, and 37°C. Note that, for these viruses, temperatures of 36°C and higher are restrictive, and the use of a total of 10 passages constituted a rigorous test of stability. Following the final passage, the genome regions containing the *248* and *1030* mutations were subjected to sequence analysis (Table 5). In the case of the

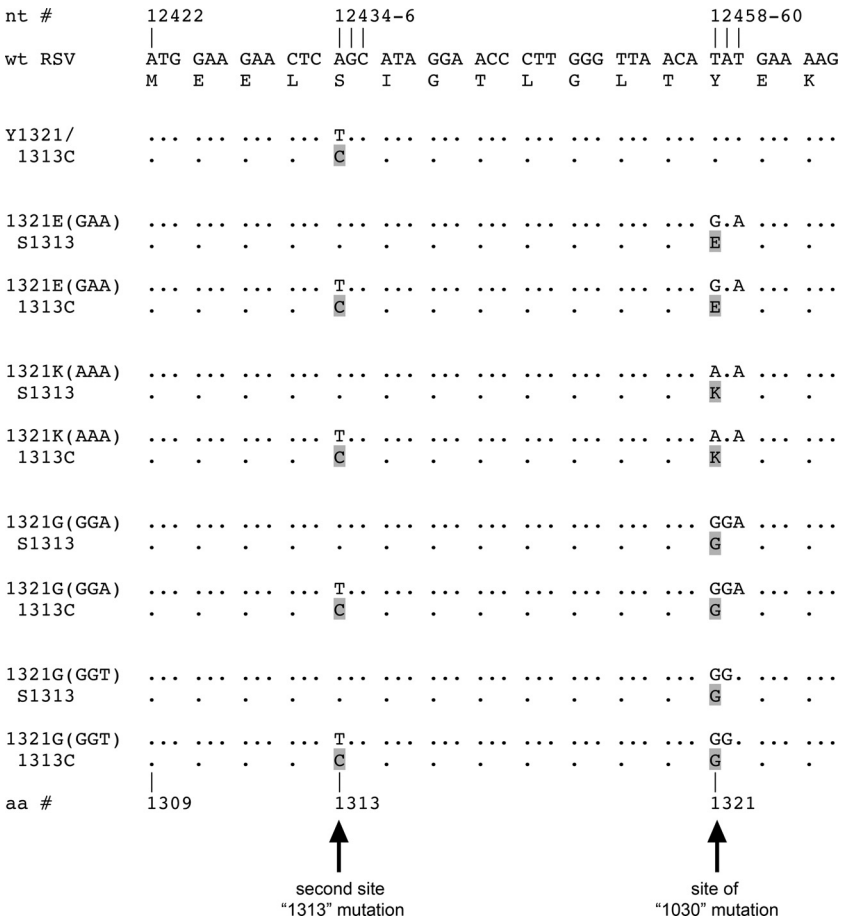

FIG 3 Nucleotide and amino acid changes at L codons 1313 and 1321 designed to evaluate the effects of serine versus cysteine at position 1313. The nucleotide sequence and amino acid coding assignments for the region of the L gene encompassing nucleotides 12422 to 12466 are shown for wt RSV and for mutants with the indicated assignments at codons 1313 and 1321. Amino acid substitutions relative to the wt sequence are highlighted in gray.

rA2*cp248/404/1030*ΔSH virus, the assignment at the 248 locus, 831L(CTG), reverted to the wt assignment of glutamine in 9 out of 10 cultures. In the case of cps2, the *248* assignment, 831L(TTG), changed to serine in 6 out of 10 cultures. The assignment 831S in the L gene specifies a wt phenotype (22) and therefore represents deattenuation. Thus, the frequency of deattenuation at the *248* site was 90% with rA2*cp248/404/1030*ΔSH and 60% with cps2. With regard to the *1030* mutation, the assignment of 1321N(AAT) in the rA2*cp248/404/1030*ΔSH virus reverted to Y1321 (wt assignment) in 9 out of 10 cultures. In addition, the only culture that did not exhibit reversion at codon 1321 had 100% reversion at codon 831, and thus, there was no culture in which the rA2*cp248/404/1030*ΔSH virus did not revert at one or both of these sites. In contrast, 9 of the 10 cultures of cps2 at the restrictive temperatures retained the attenuating assignment 1321K(AAA). In the remaining culture for cps2, 30% of the culture appeared to have the assignment of arginine, which had been shown to be lethal in the wt RSV background (Table 1; also see Discussion). The assignment at position 1313 at cps2 was found to be completely stable during passage, and no other adventitious mutations were observed. Thus, in cps2, the *248* mutation was moderately stabilized and the *1030* mutation was completely stabilized against deattenuation.

Evaluation of the attenuation phenotype of cps2 in chimpanzees. The efficiency of replication of cps2, a measure of attenuation, was evaluated in RSV-seronegative juvenile chimpanzees in parallel with that of Medi-559. Chimpanzees are the most permissive experimental animals for RSV replication and have the same body temperature as humans, important for evaluation of viruses with a *ts* phenotype. Animals were infected by combined intranasal and intratracheal inoculations of 10^6 PFU per site, and virus shedding in the respiratory tract was evaluated by nasal washes daily for 12 days postinfection, bronchoalveolar lavages (BAL) on days 2, 4, 6, and 8, and tracheal lavages on days 10 and 12 (see Tables S4 and S5 in the supplemental material). Virus titers were determined by plaque assay on Vero cells at 32°C. Clinical symptoms were not observed with either virus. Medi-559 replicated at a low level over 8 to 9 days, with virus being detected primarily in the nasal washes. This is consistent with Medi-559 being a highly attenuated virus. Importantly, shedding of cps2 virus was comparable to that of Medi-559, indicating that cps2 was also highly attenuated, comparable to the replication of Medi-559.

DISCUSSION

RSV has long been recognized as a ubiquitous, important cause of respiratory tract disease worldwide, particularly in the very young and in the elderly, and remains a vaccine priority. Subunit vaccines are contraindicated in RSV-naïve recipients, based on the experience in the 1960s with a formalin-inactivated disease that

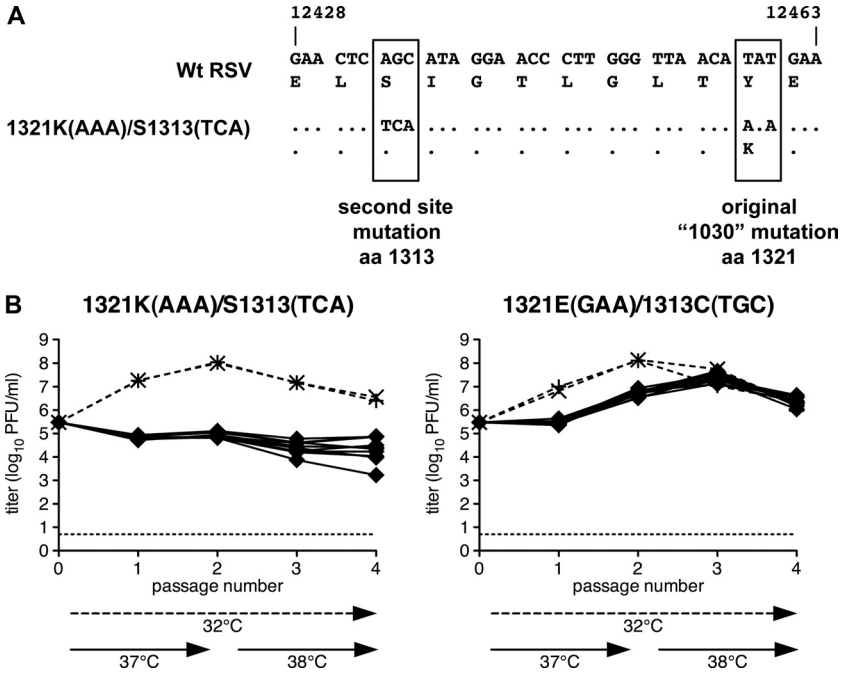

FIG 4 Stabilization of codons 1313 and 1321 in the L ORF. (A) Nucleotide and amino acid coding assignments (nucleotides 12422 to 12466 in the L gene) for wt RSV and the derivative 1321K(AAA)/S1313(TCA) with assignments at codons 1313 and 1321 that were designed for genetic stability. (B) Temperature stress test for 1321K(AAA)/S1313(TCA) and 1321E(GAA)/1313C(TGC) viruses, the latter serving as a control. Ten replicas were passaged twice at 37°C and twice at 38°C for a total of four passages (solid lines). Two replicas were passaged at the permissive temperature of 32°C for four passages as controls (dashed lines). Virus titers of the mutants at different passage levels are shown. The substantial decrease in titers for the 1321K(AAA)/S1313(TCA) virus (left panel) at the restrictive temperatures (solid lines) compared to the titers at the permissive temperature of 32°C (dashed lines) indicates that there was a substantial restriction of growth at the restrictive temperature, consistent with genetic and phenotypic stability.

primed for enhanced RSV disease and on apparently similar effects observed in experimental animals in response to subunit RSV vaccines (8, 25). Live-attenuated vaccines did not prime for disease enhancement in experimental animals or in clinical studies (34) and, thus, are suitable for use in RSV-naïve recipients. However, development of a live-attenuated RSV vaccine has been difficult due to the relatively poor growth of RSV *in vitro*, the filamentous and physically unstable nature of the particle, the relative lack of predictive and permissive animal models, the immunologic immaturity of the young infant, the heightened concerns for vaccine safety in young infants, and the immunosuppressive effects of maternally derived RSV-specific serum antibodies present in this population (35). The first live-attenuated RSV vaccine to be evaluated in infants and young children (cpRSV) was reported more than 40 years ago (20). Initial efforts to produce biologically derived vaccine candidates (10, 11, 14, 19, 35) were superseded by candidates designed by reverse genetics (28–32), and the first vaccine candidate to be well tolerated and immuno-

TABLE 4 Stability of the assignments at L protein amino acid positions 1321 and 1313 in the 1321K(AAA)/1313(TCA) mutant during passage at restrictive temperature[a]

Virus description[b]	% of cultures with codon 1321 revertants[c]	Codon 1321		Codon 1313	
		Revertant codon(s) observed[d]	Amino acid(s)[e]	Codon observed[d]	Amino acid[e]
1321E(GAA)/1313C(TGC)[f]	50	G[A/T]A	E:V	TGC	C
	20	[G/A]AA	E:K	TGC	C
	10	G[A/T/C]A	E:V:A	TGC	C
1321K(AAA)/1313S(TCA)	0			TCA	S

[a] Ten replicate 25-cm^2 flasks of HEp-2 cells were infected with the indicated 831L mutant at a multiplicity of infection of 0.1 PFU/cell at 37°C. Virus was harvested between 5 and 7 days postinfection, serially passaged again at 37°C, and serially passaged twice at 38°C, for a total of four passages, each by transferring 1 ml (of a total of 5 ml) of supernatant to a fresh 25-cm^2 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. Genotype analysis was done after the fourth passage by sequencing of a 249-nt region of the RSV L gene (RSV nucleotides 12261 to 12511; GenBank accession number M74568). No mutations were detected in the 32°C controls (not shown).

[b] Amino acid assignments of codons 1321 and 1313 are indicated in single-letter code. The codon sequence is shown in parentheses.

[c] Percentage of cultures with detectable revertants.

[d] Observed codon sequences; mixtures are indicated in brackets. Changed nucleotides are underlined.

[e] Amino acid(s) coded; colon indicates a mixed population of the specified amino acids. Changed amino acids are underlined.

[f] Control to show that the abbreviated stress test sensitively detects instability.

TABLE 5 Stability of L protein codons 831 and 1321 in rA2*cp248/404/1030*/ΔSH and cps2 during passage at restrictive temperatures[a]

Virus	% of cultures with revertant codons[b]	Codon 831L					Codon 1321				
		Originally present in virus:		Revertant codon observed[c]	Amino acid(s)[d]	Reversion rate (estimated avg % of population ± SD)[e]	Originally present in virus:		Revertant codon observed[c]	Amino acid[d]	Reversion rate (estimated avg % of population)[e]
		Codon	Amino acid				Codon	Amino acid			
rA2*cp248/404/1030*ΔSH[f]	70	CTG	L	C[T/A]G	L:Q	47 ± 15	AAT	N	[A/T]AT	Y	64 ± 22
	10			CAG	Q	100			[A/T]AT	Y	10
	10			CAG	Q	100					0
	10					0			TAT	Y	100
cps2	40	TTG	L	TCG	S	100	AAA	K[g]			0
	10			TCG	S	100			A[A/G]A	R[h]	30
	10			T[T/C]G	L:S	70					0

[a] Ten replicate 25-cm^2 flasks of HEp-2 cells were infected with the indicated virus at a multiplicity of infection of 0.1 PFU/cell at 33°C. Virus was harvested between 5 and 7 days postinfection, serially passaged again at 33°C, and serially passaged twice at 34°C, 35°C, 36°C, and 37°C, for a total of 10 passages, each by transferring 1 ml (of a total of 5 ml) of supernatant to a fresh 25-cm^2 flask of HEp-2 cells. In parallel, two control flasks per mutant were passaged 10 times at the permissive temperature of 32°C. For each passage, aliquots were frozen for titration and genotype analysis. Genotype analysis was done after the 10th passage from a 2,921-bp PCR fragment of the RSV genome (nucleotides 12271 to 15191; GenBank accession number M74568) which was partially sequenced. No mutations were detected in the 32°C controls (not shown).

[b] Percentage of cultures with detectable revertants.

[c] Observed codon sequence(s); mixtures are indicated in brackets. Changed nucleotides are underlined.

[d] Amino acid(s) coded; colon indicates a mixed population of the specified amino acids. Changed amino acids are underlined.

[e] In cultures with mixed populations, percentages of subpopulations with reversions were estimated from sequencing chromatograms. Values from cultures with mixed populations are shown.

[f] As noted in the text, two cDNA versions, rA2*cp248/404/1030*ΔSH and MEDI-559, have been constructed that share the same attenuating mutations (except for a silent codon difference at the *248* mutation) and differ by a number of incidental mutations. The virus used here is version rA2*cp248/404/1030*ΔSH that had previously been analyzed in clinical studies by Karron et al. (18).

[g] The stabilized codon 1321K(AAA) was used together with codon S1313(TCA); the latter site was completely stable (not shown).

[h] This assignment yields nonviable virus, as shown in Table S1 in the supplemental material, and its presence here presumably depends on complementation by nondefective virus during this *in vitro* infection.

genic in young infants, rA2*cp248/404/1030*ΔSH, was described in 2005 (18). The present study describes work to improve the genetic and phenotypic stability of this promising RSV vaccine candidate, specifically, the version called Medi-559 (ClinicalTrials.gov identifier NCT00767416).

When the original rA2*cp248/404/1030*ΔSH virus was evaluated in RSV-naive infants and young children, a substantial frequency of reversion was observed at either of two of the five attenuating features of the virus, namely, the *248* (831L) and *1030* (1321N) mutations (18), which are single-nucleotide amino acid substitutions that each contributes to the *ts* and attenuation phenotypes of the virus (31). Reversion at either or both of these sites was also observed previously during passage *in vitro* (21). Both *in vitro* and *in vivo*, reversion was more frequent for the *1030* mutation. While these revertants sometimes became the predominant species in vaccine recipients, they were not associated with enhanced replication or disease, reflecting the fact that by retaining four of the five original attenuating features, the revertants remained highly attenuated. However, since phenotypic reversions were observed in one-third to one-half of the isolates obtained from vaccines, and since the loss of more than a single attenuating mutation was observed during passage *in vitro* (21), it would be desirable to increase the genetic stability of this vaccine candidate.

In previous work, we attempted to stabilize the *248* Q831L mutation present in rA2*cp248/404/1030*ΔSH (18), using the same strategy as in the present study. It was possible to achieve a modest increase in stability for the *248* Q831L mutation by introducing the leucine codon TTG, which was the most stable codon based on temperature stress tests (18).

In the present work, we investigated stabilization of the *1030* mutation at codon Y1321, which was the most frequent site of reversion in the previous clinical study (18). In this case, we were able to identify several alternative amino acids that were associated with levels of attenuation similar to that of the original *1030* 1321N(AAT) mutant. Specifically, analysis of the possible amino acid assignments for position 1321 identified four (glycine, lysine, glutamic acid, and proline) that conferred a *ts* phenotype (T_{SH} = 38°C) and an attenuation phenotype similar to those of 1321N. Of note, there was a positive correlation between T_{SH} and virus replication attenuation in mice (Fig. 1), and thus, both parameters could be used to assess assignments at position 1321. Incidentally, given the knowledge of the *ts* and attenuation phenotypes associated with each amino acid assignment at position 1321, it also may be possible to incrementally increase or decrease the *ts*/attenuation phenotype of the rA2*cp248/404/1030*ΔSH or Medi-559 vaccine viruses by choosing appropriate amino acid assignments. However, this was outside the scope of the present study, whose goal was increased stability without affecting the *ts*/attenuation phenotypes.

The codon options for the chosen four alternative amino acid assignments (glycine, lysine, glutamic acid, and proline) at position 1321 were examined to predict the outcomes of all possible single-nucleotide substitutions, using the knowledge of the *ts*/attenuation phenotype associated with each amino acid assignment at that position. This theoretical analysis identified four codons that each would require at least two nucleotide changes to yield any possible amino acid assignment specifying a wt-like phenotype. There were no possibilities that would require three nucleotide changes to yield a wt-like assignment and would be the most refractory to reversion, although a previous study with human

parainfluenza virus type 1 showed that sometimes this is possible (23). This analysis suggested 1321G(GGA) as the most phenotypically stable *ts* mutation, followed by mutants 1321K(AAA) and 1321E(GAA)/(GAG). According to this theoretical analysis, any of these four codons would be significantly more stable than 1321N or 1321P or the other possible codons, 1321G and 1321K.

In vitro stress tests showed that 1321G (GGA or GGT) and 1321K (AAA) mutants were stable under conditions in which virus with the original 1321N(AAT) mutant assignment exhibited reversion in every culture. The 1321E(GAA) mutant was somewhat less stable, with deattenuation in 20% of the populations, and virus with the 1321P(CCT) mutation, which was included as coding for an alternative amino acid that was not predicted to be more stable, exhibited deattenuation in 90% of the populations, similar to the 1321N mutant. However, following passage in the stress tests, we frequently detected a serine-to-cysteine missense mutation, S1313C, in the G, K, E, and P viruses. Analysis of recombinant virus in which this 1313C assignment was combined with attenuating 1321 assignments confirmed that this mutation was a compensatory second-site mutation that alleviated the *ts*/attenuation phenotype conferred by the 1321 assignment.

The ability of the S1313C mutation to dramatically compensate for attenuating mutations at position 1321 was unexpected. When placed in the wt RSV background, this S1313C mutation had no detectable phenotypic effect on either temperature sensitivity *in vitro* or attenuation in mice. Thus, serine and cysteine appear to be interchangeable at this position in the wt background. The idea that a serine-to-cysteine substitution would be conservative also is suggested by the structural similarity of these residues: specifically, serine and cysteine are similarly small and nucleophilic, differing in a hydroxyl group (serine) versus a sulfhydryl group (cysteine). However, recent work with beta-lactamase showed that this seemingly conservative substitution can be substantially destabilizing due to alterations in hydrogen bonding and can have a substantial impact on protein folding (27). It also is interesting that, in the present study, the ability of 1313C to cause deattenuation associated with position 1321 was largely independent of the structure of the 1321 assignment: 1313C appeared to be compensatory for glycine, lysine, glutamic acid, and proline (but apparently not asparagine). In general, the effects of substitutions at positions 1313 and 1321 in the RSV L protein were not readily predictable or interpretable by consideration of amino acid structure.

Given the frequent occurrence of this S1313C compensatory mutation, it was necessary to stabilize this position in order to maintain the attenuating effects of the assignment at codon 1321. While the S(AGC) codon present in wt RSV could mutate to encode cysteine by a single-nucleotide substitution, two other serine codons, S(TCA) and S(TCG), would require two substitutions. Therefore, we combined the 1321K(AAA) and S1313(TCA) mutations to create a stabilized version of the 1030 mutation. In the wt RSV background, the 1321K(AAA)/S1313(TCA) combination was indistinguishable from the 1321K(AAA) mutation with regard to *ts* phenotype, and the combination was completely stable in temperature stress tests.

Based on these results, we modified Medi-559 to yield cps2 by the following modifications: (i) codon 831 in the L protein (the *248* mutation) was changed from L(TTA) to L(TTG); codon 1313 (the second-site mutation) was changed from S(AGC) to S(TCA); and (iii) codon 1321 (the *1030* mutation) was changed from N(AAT) to K(AAA). This involved a total of 5 nucleotide substitutions (underlined) and one amino acid substitution. *In vitro* temperature stress tests combined with sequence analysis provided evidence of increased stability: reversion at position 831 was reduced by one-third, and reversion at positions 1313 and 1321 did not occur. One flask had evidence of mutation of position 1321 to arginine. However, this residue appeared to be lethal when we attempted to recover it in the wt background. Its presence in the stress test probably reflects genomes that encoded defective L protein but could be replicated by complementation by nondefective virus present due to the high multiplicity of infection used during the sequential passages, similar to the well-known situation with defective interfering genomes. Thus, this population of virus is probably nonviable and so does not represent deattenuation.

Analysis of virus from the published clinical study of rA2*cp248/404/1030*ΔSH (18, 21) indicated that the 248 mutation (position 831) reverted in 1 of 7 subjects examined who shed partially revertant virus, while the *1030* mutation (position 1321) reverted in 4 of 7 subjects with revertant virus. This suggests that 4 of 5 reversions involve a change in the *1030* mutation. Thus, stabilizing this position would be predicted to reduce the reversion rate by 80%. As noted, the modification at position 831 would be predicted to reduce the reversion rate by a further third. This estimates that the reversion rate will be reduced by ~87%. However, it will be necessary to evaluate stability in a clinical study in seronegative individuals. The cps2 virus was indistinguishable from Medi-559 with regard to both *ts* phenotype and replication in seronegative chimpanzees: this comparability indicates that cps2 can replace Medi-559 as the lead RSV vaccine candidate.

ACKNOWLEDGMENTS

We thank Lijuan Yang for excellent technical assistance, and we thank Anthony Cook and Charlene Shaver, Bioqual, Inc., for performing the chimpanzee study and William R. Elkins, NIAID, NIH, for advice regarding the chimpanzee study.

This research was supported by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases. This research was also supported in part by a Cooperative Research and Development Agreement (CRADA) between NIH and MedImmune, LLC, for the development of RSV vaccines.

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