Table S1: Recovery and sequence analysis of recombinant RSVs with substitutions at amino acid 1321 of the L protein or with small deletions in proximity of amino acid 1321[a].

Mutation	Codon	Recovery [b]	Adventitious mutations
Y1321 [c]	TAT	+ (2/2)	Insertion of a single A residue in a run of A nt at 15019 between the L stop codon and L GE signal
1321N [c]	AAT	+ (6/6)	
1321G	GGT	+ (2/2)	
1321A	GCT	+ (2/2)	
1321S	TCT	+ (2/2)	
1321T	ACT	+ (2/2)	
1321C	TCT	+ (2/2)	Insertion of 3 A residues in a run of A nt (nt 5595) in the G GE signal
1321V	GTT	+ (2/2)	
1321L	TTA	+ (2/2)	
1321I	ATT	+ (2/2)	
1321M	ATG	+ (2/2)	
1321P	CCT	+ (2/2)	
1321F	TTT	+ (2/2)	Insertion of a single A nt into a run of A residues at the end of the leader region (nt 44); insertion of a single A residue in a run of A nt (nt 5595) in the G GE signal
1321W	TGG	+ (2/2)	Insertion of an A residue in a run of A nt (nt 2328) in the N GE signal
1321D	GAT	+ (2/2)	
1321E	GAA	+ (2/2)	
1321Q	CAG	+ (2/2)	

Mutation	Codon	Recovery [b]	Adventitious mutations
1321H	CAT	+ (2/2)	
1321K	AAA	+ (2/2)	
1321R	CGT	- (0/10)	
ΔY1321 [d]	[TAT] [e]	- (0/6)	
ΔTY 1320+1321 [d]	[ACA TAT]	- (0/6)	
ΔYE 1321+1322 [d]	[TAT GAA]	- (0/4)	
ΔT1320 [d]	[ACA]	- (0/4)	

[a] Amino acid position in the RSV L protein (Genbank Accession number M74568).

[b] Efficiency of recovery (number of recoveries/number of recovery attempts)

[c] Y is the wt assignment at position 1321. N is the mutant assignment in the original biologically-derived "1030" virus (11, 14).

[d] Deletion (Δ) of indicated amino acid of the L protein. The deleted codon(s) are shown in rectangular brackets.

Table S2: Demonstration that the S1313C mutation in the RSV L protein is a compensatory mutation, and evaluation of viruses in which the 1313 codon has been silently changed from AGC to TCA.

Virus	Virus titer (PFU per mL) at indicated temperature (°C) [a]									Replication in mice[b]			
										Titer (log_{10} PFU/g ± SE)		Mean log_{10} reduction[c]	
	32	35	36	37	38	39	40	T_{SH}[d]	Δ T_{SH}[e]	Nasal turbinates	Lung	Nasal turbinates	Lung
Wt (Y1321/S1313)[f]	7.8	7.8	7.7	7.6	7.6	7.6	7.2	> 40		4.2 ± 0.1	4.6 ± 0.1		
Y1321/1313C[g]	8.4	8.4	8.3	8.2	8.2	8.1	7.9	> 40	0	4.4 ± 0.1	4.7 ± 0.0		
1321G(GGA)/S1313	7.7	7.7	7.6	7.1	**≤ 1**	< 1	< 1	**38**		n.d.[j]	n.d.	n.d.	n.d.
1321G(GGA)/1313C[g]	8.1	8.1	8.2	8.0	7.7	6.6	**≤ 1**	**40**	+2	4.2 ± 0.1	4.3 ± 0.1		0.3
1321G (GGT)/S1313	7.5	7.5	7.3	6.9	**≤ 1**	< 1	< 1	**38**		3.6 ± 0.1	3.1 ± 0.2*[h]	0.6	1.5
1321G (GGT)/1313C[g]	8.3	8.2	8.1	8.1	7.8	6.4	**≤ 1**	**40**	+2	4.3 ± 0.2	4.5 ± 0.1		0.1
1321K(AAA)/S1313	7.7	7.6	7.5	7.3	**≤ 1**	< 1	< 1	**38**		3.8 ± 0.1	2.8 ± 0.2*[h]	0.4	1.8
1321K(AAA)/1313C[g]	8.2	8.2	8.0	8.1	7.8	7.2	**≤ 1**	**40**	+2	4.2 ± 0.2	4.5 ± 0.1		0.1
1321E(GAA)/S1313	7.6	7.4	6.3	**3.5**	< 1	< 1	< 1	**37**		2.0 ± 0.0*[h]	1.8 ± 0.1***[h]	2.2	2.8
1321E(GAA)/1313C[g]	8.2	8.1	8.1	7.8	7.2	**≤ 1**	< 1	**39**	+2	4.3 ± 0.0	4.2 ± 0.1		0.4
1321K(AAA)/S1313 (TCA)	8.4	8.3	8.2	7.2	**≤ 1**	< 1	< 1	**38**	0	nd[j]	nd[j]		

[a] The *ts* phenotype for each virus was evaluated by plaque assay on HEp-2 cells at the indicated temperatures. For viruses with the *ts* phenotype, values indicating the T_{SH} are marked (bold, underlined). The T_{SH} is defined in footnote d below.

[b] 10-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 limit of detection was 2 $\log_{10}$ PFU per g for nasal turbinates, and 1.7 $\log_{10}$ PFU per g for lungs; SE: Standard error.

[c] Reduction in mean titer compared to the wt virus (Y1321/S1313) of the same experiment.

[d] Shut off temperature (T_{SH}) is defined as the lowest restrictive temperature at which the reduction compared to 32ºC is 100-fold or greater than that observed for wt RSV at the two temperatures. The *ts* phenotype is defined as having a T_{SH} of 40º C or less.

[e] ΔT_{SH}, Difference (°C) in T_{SH} between the indicated 1321 mutant bearing the original S1313 assignment versus the same 1321 mutant bearing the 1313C mutation.

[f] Amino acid assignments at positions 1321 and 1313 in wt RSV. Note that all of the mutants in this Table were constructed in the recombinant wt RSV 6120 backbone (see the Description of Figure 2 for an explanation).

[g] Second site compensatory mutation 1313C.

[h] Statistically significant difference compared to the wt RSV (one way ANOVA, Kruskal-Wallis test with Dunn's post-hoc test, ***, $P \leq 0.001$, *, $P \leq 0.001$ underlined).

[j] n.d., not done

Table S3: Effect of L protein mutations E649 and Q874 on the temperature sensitivity and attenuation phenotypes of wt RSV and the mutant RSV 1321(AAA)/S1313(TCA)

Virus	Exp #[d]	Virus titer (PFU per mL) at indicated temperature (°C)[a]									Replication in mice[b]			
											Titer (log_{10} PFU/g ± SE)		Mean log_{10} reduction[c]	
		32	35	36	37	38	39	40	T_{SH}[e]	DT_{SH}[f]	Nasal turbinates	Lung	Nasal turbinates	Lung
Wt	1	7.7	7.7	7.7	7.6	7.6	7.6	7.1	> 40		4.0 ± 0.1 (10/10)	4.5 ± 0.0 (10/10)		
	2	7.7	7.7	7.6	7.6	7.5	7.6	7.4	> 40					
1321K(AAA)/S1313 (TCA)	2	8.1	8.2	8.1	8.1	7.4#	**5.3#**	3.4#	39		3.1 ± 0.1 (5/5)	3.9 ± 0.1 (5/5)	0.9	0.6
1321K(AAA)/ S1313 (TCA) + E649D	1	7.2	7.1	7.1	6.8	6.1#	**≤ 1**	< 1	39		2.8 ± 0.1 (5/5)	3.0 ± 0.1 (5/5)	1.2	1.5
	2	7.0	7.0	7.0	6.8	6.6	5.7#	**≤ 1**	40					
E649D[g]	1	7.0	7.0	7.0	6.9	6.8	6.8	6.5	> 40	1	2.4 ± 0.2 (5/5)	3.0 ± 0.2 (5/5)	1.6	1.5
1321K(AAA)/S1313 (TCA) + Q874H	1	7.2	7.1	7.0	7.0	6.8	6.4	**4.5#**	40		2.0 ± 0.1 (5/5)[h]	2.3 ± 0.1 (5/5)	2.0	2.3
	2	6.8	6.7	6.8	6.7	6.5	6.3	5.7#	> 40					
Q874H[g]	1	6.4	6.6	6.4	6.3	6.4	6.2	6.0	> 40	0-1	≤ 1.9 (0/5)[h]	1.7 ± 0.0 (1/5)[h]	≥ 2.1	2.8

[a] The *ts* phenotype for each virus was evaluated by plaque assay on HEp-2 cells at the indicated temperatures. For viruses with a *ts* phenotype, the T_{SH} is marked (bold, underlined). See footnote e for the definition of T_{SH}. #, micro plaque phenotype.

[b] 5-week-old mice in groups of five (or ten for wt rA2) 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 limit of detection was 1.9 $\log_{10}$ PFU per g for nasal turbinates, and 1.7 $\log_{10}$ PFU per g for lungs; SE: Standard error. All of the data on replication in mice were from the same experiment (expt. #1).

[c] Reduction in mean titer compared to the wt virus (wt rA2).

[d] T_{SH} experiment number; two independent plaque assay experiments (#1 and #2) were performed to evaluate the *ts* phenotype.

[e] Shut off temperature (T_{SH}) is defined as the lowest restrictive temperature at which the reduction compared to 32ºC is 100-fold or greater than that observed for wt RSV at the two temperatures. The *ts* phenotype is defined as having a T_{SH} of 40ºC or less.

[f] DT_{SH}, Difference (°C) in shutoff temperature between a given viral backbone bearing the original E649 or Q874 assignment versus the 649D or 874H mutation.

[g] In the wt backbone. Note that the mutants in this Table were constructed in the recombinant wt RSV 6120 backbone (see the Description of Figure 2 for an explanation).

[h] Statistically significant difference compared to wt RSV (one way ANOVA, Kruskal-Wallis test with Dunn's post-hoc test, $P \leq 0.001$, underlined).

Table S4: Viral titers of nasal wash samples from chimpanzees inoculated with the RSV vaccine candidate Medi-559 or cps2[a]

RSV Vaccine candidate	Chimp ID	NW virus titer (log_{10}PFU/mL) on indicated days[b]											Duration of shedding[c]	Peak virus titer
		1	2	3	4	5	6	7	8	9	10	12		
Medi-559	A8A007	-	1.5	-	1.8	-	1.8	1.8	2.6	1.5	1.0	-	9	2.6
	A8A008	-	1.9	1.9	2.4	2.7	2.8	2.6	2.9	2.4	-	-	8	2.9
	Mean:												***8.5***	***2.7***
cps2	A8A009	-	1.0	2.3	-	-	2.2	2.0	2.8	2.3	1.0	-	9	2.8
	A9A002	-	-	1.5	2.2	3.3	2.8	3.3	3.3	1.7	1.5	-	8	3.3
	4X0533	-	-	1.0	2.2	3.7	2.4	4.6	2.6	1.6	1.0	-	8	4.6
	Mean:												***8.3***	***3.6***

[a] Chimpanzees were inoculated by the combined intranasal and intratracheal routes with 10^6 PFU of the indicated virus in a 1 mL inoculum per site (total dose: $2x10^6$ PFU per animal). The chimpanzee study was approved by the Animal Care and Use Committee of NIAID, NIH.

[b] Nasal wash was performed with 3 mL of Lactated Ringer's solution per nostril. Virus titrations were performed on Vero cells at 32°C. The lower limit of detection was 1.0 log_{10} PFU/mL of nasal wash solution. Samples with no detectable virus are represented as "-". Peak titers for each animal are underlined.

[c] The period of days from the first to the last day on which virus was detected, including negative days (if any) in between.

Table S5: Viral titers of bronchoalveolar and tracheal lavage samples from chimpanzees inoculated with the RSV vaccine candidate Medi-559 or cps2[a]

RSV vaccine candidate	Chimp ID	Bronchoalveolar/tracheal Lavage virus titer (log_{10}PFU/mL) on indicated days[b]						Duration of shedding[c]	Peak virus titer
		2	4	6	8	10	12		
Medi-559	A8A007	<u>2.7</u>	-	-	-	-	-	1	2.7
	A8A008	-	-	-	-	-	-	0	1.0
	Mean:							***0.5***	***1.8***
cps2	A8A009	-	-	-	-	-	-	0	1.0
	A9A002	-	1.9	<u>3.7</u>	-	-	-	3	3.7
	4X0533	-	1.0	-	<u>1.6</u>	-	-	5	1.6
	Mean:							***4.0***	***2.1***

[a] Chimpanzees were inoculated by the combined intranasal and intratracheal routes with 10^6 PFU of the indicated virus in a 1 mL inoculum per site (total dose: 2 x 10^6 PFU per animal). The chimpanzee study was approved by the Animal Care and Use Committee of NIAID, NIH.

[b] On days 2, 4, 6, and 8, bronchoalveolar lavage was performed with 6 mL of PBS; on days 10 and 12, tracheal lavage was done using 3 mL of PBS per animal. Virus titrations were performed on Vero cells at 32°C. The lower limit of detection was 1.0 log_{10} PFU/mL of lavage solution. Samples with no detectable virus are represented as "-". Peak titers for each animal are underlined.

[c] The period of days from the first to the last day on which virus was detected, including negative days (if any) in between.