Supporting Information

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SI Materials and Methods

Cells. African green monkey (AGM) kidney cells (Vero) and human HEp-2 cells (ATCC CCL23) were grown in Opti-MEM (Gibco-Life technologies) supplemented with 5% (vol/vol) FBS (HyClone) and 1 mM L-glutamine (Gibco-Life technologies). BSR T7/5 cells are baby hamster kidney 21 (BHK-21) cells constitutively expressing T7 RNA polymerase (1). These cells are grown in Glasgow MEM (GMEM) (Gibco-Life technologies) supplemented with 2 mM L-glutamine, 2% (vol/vol) MEM amino acids (Gibco-Life technologies), and 10% FBS. Every other passage, the media was supplemented with 2% (vol/vol) geneticin (Gibco-Life technologies) to select for cells that retain the T7 RNA polymerase construct.

Recoding the Genes of Respiratory Syncytial Virus. Different organisms have evolved different preferences for how to best encode a protein. A highly significant but underappreciated preference is codon-pair bias (CPB) that is specific to different phyla. A given amino acid pair can be encoded by as many as 36 different codon pairs (e.g., the pair Arg-Leu). Based on the known species-specific codon use, there is an expected frequency at which each codon pair should occur. However, pairs of synonymous codons seldom appear in protein-encoding sequences at their expected frequency (i.e., the frequency expected based on the frequency of the individual codons). Instead, certain codon pairs are statistically overrepresented in the ORFeome whereas other pairs are underrepresented: thus, the term codon-pair bias. Every codon pair is assigned a codon pair score (CPS, also referred to as value), which is the natural logarithm of the ratio of the observed frequency of the codon pair to the expected frequency of the codon pair [i.e., CPS = ln (Observed/Expected)] (2). Thus, preferred pairs have positive scores, and unpreferred pairs have negative scores. The codon-pair bias score (or "value" as used in Fig. 1) for a gene is the average sum of all of the codon pair scores for the individual codon pairs. Codon-pair bias between humans and other mammals is very conserved, such that a virus that is codon pair-deoptimized for human cells is also deoptimized for other mammalian species.

Using a heuristic computer algorithm (2), the ORFs of respiratory syncytial virus (RSV) were computationally recoded to contain a large number of codon pairs that are underrepresented in the human host, while retaining wild-type codon use and amino acid sequence. The computer-redesigned viral genome is then chemically synthesized de novo, and whole virus is regenerated by reverse genetics (3). Recoded genes with underrepresented codon pairs [codon pair-"deoptimized" (CPD)] lead to phenotypes as depicted in Fig. 2. In Fig. 1, these RSV CPD genes are referred to as G-Min, L-Min, etc., to signify a gene design with the most negative codon-pair bias ["minimized" (Min)].

Construction of cDNAs Encoding CPD Recombinant RSVs. Recombinant RSVs (rRSVs) were constructed by reverse genetics (4) using the antigenome cDNA D46/6120, a derivative of the rA2 cDNA plasmid with a deletion of a 112-nt fragment of the downstream noncoding region of the SH gene and synonymous codon changes for the last three codons of the SH ORF (5). These changes were made to achieve improved stability during growth in *Escherichia coli* and do not affect the efficiency of virus replication in vitro or in mice (5). For simplicity, the numbering of sequence positions in the present manuscript is based on the complete sequence of biologically derived strain A2 (GenBank accession no. M74568). Four full-length cDNA plasmids were generated, named Min A, Min B, Min L, and Min FLC (Fig. 1*A*). Min A contained the CPD ORFs of NS1, NS2, N, P, M and SH. A 4,508-bp NotI-XhoI fragment of synthetic RSV cDNA with these six CPD ORFs was transferred into the similarly cleaved D46/6120. Min B contained the CPD G and F ORFs that were transferred by cloning a 3,907-bp XhoI-BamHI cDNA fragment containing the synthetic CPD ORFs into the similarly cleaved D46/6120. Min L contained a 6,750-bp BamHI-KasI fragment with the CPD L ORF that was transferred into the similarly cleaved D46/6120. Finally, Min FLC [full-length clone (FLC)] contained CPD ORFs for every gene except M2. Min FLC was generated by successively transferring all three synthetized CPD fragments (NotI-XhoI, XhoI-BamHI, and BamHI-KasI) into the D46/6120. After the generation of endotoxin-free DNA preparations (Qiagen), the sequences of WT and of all four CPD plasmids were confirmed by sequence analysis of the RSV antigenome sequences contained in the cDNA plasmids. The GenBank accession numbers of Min A, Min B, Min L, and Min FLC full-length nucleotide sequences are KJ817798, KJ817799, KJ817800, and KJ817801, respectively.

Generation of rRSVs from cDNA. BSR T7/5 cells grown to 95% confluency in six-well plates were washed twice with GMEM containing 3% FBS, 1 mM L-glutamine, and 2% MEM amino acids and were transfected using Lipofectamine 2000 (Life Technologies) 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 (1, 4). After overnight incubation at 37 °C, transfected cells were harvested by scraping into media, added to subconfluent monolayers of Vero cells, and incubated at 32 °C. The rescued viruses WT rRSV, Min A, Min B, Min L, and Min FLC were harvested between 11 d and 14 d posttransfection.

Virus Growth and Titration. Following rescue, WT and CPD rRSVs were propagated once on Vero cells at 32 °C in media containing 2% FBS. Virus stocks were generated by scraping infected cells into media, followed by vortexing for 30 s, clarification of the supernatant by centrifugation, and addition of 10× SPG [2.18 M sucrose, 0.038 M KH_2PO_4, 0.072 M K_2HPO_4, 0.06 M L-glutamine (pH 7.1)] to a final concentration of 1×. Virus aliquots were snap-frozen and stored at −80 °C. Virus titers were determined by plaque assay on Vero cells with a 0.8% methylcellulose overlay. After a 10- to 12-d 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 (6). Titers were expressed as plaque-forming units (pfu) per mL. Viral RNA was isolated from all virus stocks, and sequence analysis of the viral genomes was performed from overlapping RT-PCR fragments, confirming that the genomic sequences of the recombinant viruses were correct and free of adventitious mutations. The only sequences that were not directly confirmed for each genome were the positions of the outermost primers, namely nucleotides 1–23 and 15,174–15,222.

Evaluation of the Temperature-Sensitive Phenotype. The temperature-sensitive (*ts*) phenotype of each of the rRSV viruses was evaluated by the efficiency of plaque formation at 32 °C, 35 °C, 36 °C, 37 °C, 38 °C, 39 °C, and 40 °C. Plaque assays were performed on Vero and HEp-2 cells in duplicate, and incubated in sealed caskets at various temperatures in temperature-controlled water baths as previously described (7).

Kinetics of CPD rRSV Replication in Vitro. Multicycle and single-cycle replication experiments were performed in confluent monolayers of Vero cells in six-well plates. In multicycle replication experiments, wells were infected in triplicate at a multiplicity of infection (MOI) of 0.01 pfu per cell and incubated at 32 °C or 37 °C. Viruses were harvested daily from day 1–14 (with the exception of Min B, which was assayed from day 1–12 at 32 °C only due to low titer of virus stocks) by scraping infected cells into media followed by vortexing for 30 s and clarification by low-speed centrifugation. Three wells were processed individually for each sample/time point. Virus aliquots were snap-frozen and stored at −80 °C until titrated as described in *Virus Growth and Titration*. After all inoculations, the MOIs were confirmed by titration of the inocula on Vero cells at 32 °C.

In single-cycle replication experiments, wells were mock-infected or infected in duplicate at an MOI of 1 pfu per cell with WT rRSV, Min A, Min L, or Min FLC and incubated at 32 °C or 37 °C. Cultures were washed once after the first 2 h of incubation. Every 4 h [from 4 h to 24 h postinfection (hpi)], a single well for each virus/time point was harvested and processed for infectious virus. A second well was harvested by scraping the cells into the medium. This suspension was divided into two aliquots, and cells were pelleted by centrifugation at 300 × g. One aliquot of cells was used to isolate cell-associated RNA using the RNeasy mini kit (Qiagen), as recommended by the manufacturer and treated with DNase I to remove residual genomic DNA. The other aliquot of cells was lysed using a nondenaturing and nonreducing cell-lysis buffer containing 1× protease inhibitor (Roche) and homogenized using a QIAshredder (Qiagen) for analysis of viral-associated proteins by Western blotting.

Reverse Transcription and Strand-Specific Quantitative PCR. Cell-associated RNA derived from single-cycle replication experiments was used to specifically quantify viral negative sense (genomic) and positive sense (mRNA and antigenomic) RNA. The TaqMan assay for antigenomic/mRNA does not distinguish between these latter two RNA species. However, Northern blot experiments have shown that antigenomic RNA accounts for ∼5% of the total positive-sense RSV RNAs, with the remainder being mRNA (8). The M2 gene was used as a target because it was the only gene that remained unchanged in the WT and CPD rRSVs. Based on a strategy originally developed to study enterovirus replication (9), strand-specific quantitative TaqMan-based RT-PCR (qRT-PCR) assays were designed using strand-specific primers that consisted of an RSV M2 gene-specific 3′-tail sequence that is complementary to RSV genomic RNA (genome-specific assay) or complementary to antigenome and mRNA (antigenomic/mRNA-specific assay). Both of these primers are chimeric because they contain an unrelated 5′-tag of 19 nt (9). One microgram of RNA was reverse transcribed using SuperScript III (Invitrogen) in a 20-μL reaction using one tagged first strand primers, specific either to genome or to antigenomic/mRNA. After a fivefold dilution, each of the cDNAs was amplified with a tag-specific primer, a second M2-specific primer, and a probe. Thus, only cDNAs containing the tagged RT primer sequence were amplified. The probe sequence was RSV M2 gene-specific. To normalize results, 18S rRNA was quantified in parallel using first-strand cDNA generated with random primers, and a standard 18S rRNA TaqMan assay (Applied Biosystems). qPCR results were analyzed using the comparative threshold cycle (ΔCt) method, normalized to 18S rRNA, and then expressed as fold increase over each 4-h time point. Negative controls without first-strand primer were performed for each of the strand-specific qPCRs to demonstrate the absence of nonspecific priming during first-strand cDNA synthesis.

Specific Infectivity of the CPD rRSVs. Viral RNAs derived from 2×10^2 pfu of WT rRSV, Min A, Min L, Min FLC, or Min B were extracted using the viral RNA extraction kit (Qiagen). Four microliters of the 60-μL RNA extraction were subjected to RT using the strand-specific primers described above. Then, 10% of each cDNA reaction was amplified by the genome-specific qPCR described in *Reverse Transcription and Strand-Specific Quantitative PCR*. The results of the quantification of genomic RNA are expressed as fold difference compared with WT rRSV.

Western Blot Analysis. Cell lysates prepared from single-cycle infection experiments described above were separated on NuPAGE 4–12% Bis-Tris SDS/PAGE gels with Mes electrophoresis buffer (Life Technologies) in parallel with an Odyssey Two-Color Protein Molecular Weight Marker (Li-Cor). Proteins were transferred to PVDF membranes (Millipore) in 1× NuPAGE buffer. The membranes were blocked with Odyssey blocking buffer (Li-Cor) and incubated with primary antibody in the presence of 0.1% Tween 20. The primary antibodies and the dilutions used were as follows: rabbit anti-RSV–specific polyclonal antibodies (used at 1:15,000), mouse anti-RSV G monoclonal antibody (1:1,000; Abcam), mouse anti-RSV F monoclonal antibody (1:1,000; Abcam), mouse anti-tubulin monoclonal antibody (1:10,000; Sigma), and rabbit anti-GAPDH polyclonal antibody (1:200; Santa Cruz). The secondary antibodies used at a 1:10,000 dilution were goat anti-rabbit IgG IRDye 800 (Li-Cor) and goat anti-mouse IgG IRDye 680 (Li-Cor). The secondary antibodies used at a 1:15,000 dilution were goat anti-rabbit IgG IRDye 680 (Li-Cor) and goat anti-mouse IRDye 800 (Li-Cor). Membranes were scanned on the Odyssey Infrared Imaging System. Data collected were analyzed using Odyssey software, version 3.0 (Li-Cor). For quantification of identified RSV proteins of interest, background fluorescence was corrected. Values reported indicate the median fluorescence intensity per protein band.

Evaluation of the Replication of CPD rRSVs in Mice. Virus replication was evaluated in the upper and lower respiratory tract (URT and LRT) of mice as described previously (10). Briefly, 6-wk-old female BALB/c mice in groups of 10 were inoculated intranasally under methoxyflurane anesthesia on day 0 with 4.5×10^5 pfu of WT rRSV, Min A, Min L, or Min FLC. On days 4 and 5, 5 mice from each group were killed by carbon dioxide inhalation. Nasal turbinates (NTs) and lung tissue were harvested and homogenized separately in Leibovitz (L)-15 medium containing 1× 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 Vero cells incubated at 32 °C as described in *Virus Growth and Titration*.

Evaluation of Replication and Immunogenicity of CPD rRSVs in African Green Monkeys. RSV-seronegative AGMs in groups of four were inoculated intranasally and intratracheally with 1 mL of L-15 medium per site containing 10^6 pfu of WT rRSV, Min A, Min L, or Min FLC (Exp. 1), RSV cps2 (11) or WT rRSV (Exp. 2), RSV ΔNS2 Δ1313 I1314L (12) or WT rRSV (Exp. 3). Nasopharyngeal (NP) swabs were collected every day from day 0–10 and on day 12 (Exp. 1) or day 14 (Exp. 2 and Exp. 3) postinoculation and were placed in 2 mL of L-15 medium containing 1× SP [2.18 M sucrose, 0.038 M KH_2PO_4, 0.072 M K_2HPO_4 (pH 7.1)] as stabilizer. Tracheal lavage (TL) samples were collected every other day from day 0–12 (Exp. 1) or day 14 (Exp. 2 and Exp. 3). The lavage was done with 3 mL of PBS, and the recovered fluid was mixed with an equal volume of L-15 medium containing 2× SP. Virus titers in the collected NP and TL samples were determined in duplicate on Vero cells incubated at 32 °C as described in *Virus Growth and Titration*. The TL titers were adjusted to correct for the twofold dilution noted above. Sera were collected at day 0 and day 28. The 60% plaque reduction neutralizing antibody

titers ($PRNT_{60}$) were determined on Vero cells using GFP-expressing rRSV as described previously (13). All animal experiments were approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases.

Statistical Analysis. Datasets were assessed for significance using nonparametric Mann–Whitney or Kruskal–Wallis with Dunn's post hoc test. Statistics were performed using Prism 5 (GraphPad Software). Data were only considered significant at $P < 0.05$.

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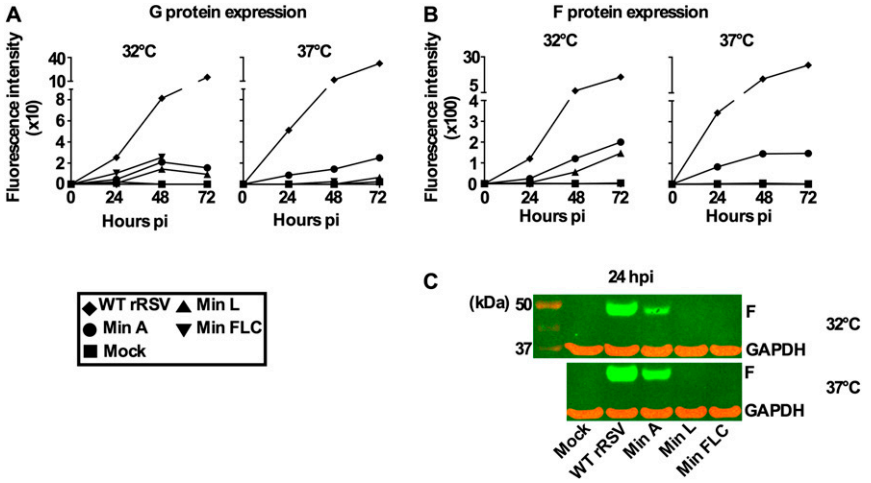

Fig. S1. G and F protein expression of Min A, Min L, Min FLC, and WT rRSV in Vero cells. Replicate wells of Vero cell monolayers were mock-infected or infected at an MOI of 1 pfu per cell with the indicated virus and incubated at 32 °C or 37 °C, as indicated. At 24 hpi, 48 hpi, and 72 hpi (with the exception of Min FLC for the latter time point), one well was harvested and processed for analysis of cell-associated protein by Western blotting. Thirty micrograms of cell lysates were separated on 4–12% SDS/PAGE gels, and proteins were transferred to PVDF membranes. The blots were analyzed with mouse anti–RSV-G (*A*) or -F (*B* and *C*) monoclonal Abs and rabbit polyclonal anti-GAPDH Ab (loading control), followed by secondary antibodies labeled with an infrared fluorophore. Membranes were scanned on the Odyssey Infrared Imaging System. Data were analyzed using Odyssey software. For quantification of RSV G and F proteins (*A* and *B*), background fluorescence was corrected, and values indicate the median fluorescence intensity per protein band.

Table S1. Percent nucleotide identity and number of substitutions between WT and CPD RSV ORFs

ORF	% identity	No. of substitutions
NS1	87.8	65
NS2	88.1	60
N	80.0	241
P	84.4	143
M	83.0	163
SH	92.3	23
G	78.7	197
F	77.8	422
L	79.1	1,378

Table S2. RSV-neutralizing antibody titers in the serum of AGMs inoculated with CPD viruses RSV cps2, RSV ΔNS2 Δ1313 I1314L, or WT rRSV at 28 dpi

Virus	Mean RSV neutralization titer (log_2 of reciprocal)[a]
Exp. 1	
Min A	5.7
Min L	5.3
Min FLC	2.4*
WT rRSV	6.4
Exp. 2	
RSV cps2	2.8*
WT rRSV	9.5
Exp. 3	
RSV ΔNS2 Δ1313 I1314L	6.3
WT rRSV	7.7

Four AGMs were inoculated by the combined intranasal and intratracheal routes as described in Table 2.

[a]Sera were collected at day 28, and the 60% plaque reduction neutralizing antibody titers were determined in a plaque reduction neutralization assay on Vero cells using GFP-expressing rRSV and expressed in log_2. Data were considered significant only at $*P \leq 0.05$.