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(54) Titre : SOUCHES DU VIRUS RESPIRATOIRE SYNCYTIAL RECOMBINANT COMPRENANT DES MUTATIONS
DANS L'ORF DE M2-2 OFFRANT UNE GAMME DE PHENOTYPES ATTENUES
(54) Title: RECOMBINANT RESPIRATORY SYNCYTIAL VIRUS STRAINS WITH MUTATIONS IN THE M2-2 ORF
PROVIDING A RANGE OF ATTENUATION PHENOTYPES

(57) **Abrégé/Abstract:**

Provided herein are novel recombinant respiratory syncytial viruses (RSV) having an attenuated phenotype that contain mutations in the M2-2 open reading frame that interfere with the expression of the M2-2 protein. The M2-2 mutations may be present in combination with mutations at other loci. Using methods described herein, combinations of mutations are provided to achieve desired levels of attenuation. The recombinant RSV strains described here are suitable for use as live- attenuated RSV vaccines. Also provided are polynucleotide sequences of the described viruses, as well as methods for producing and using the viruses.

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(57) **Abstract:** Provided herein are novel recombinant respiratory syncytial viruses (RSV) having an attenuated phenotype that contain mutations in the M2-2 open reading frame that interfere with the expression of the M2-2 protein. The M2-2 mutations may be present in combination with mutations at other loci. Using methods described herein, combinations of mutations are provided to achieve desired levels of attenuation. The recombinant RSV strains described here are suitable for use as live- attenuated RSV vaccines. Also provided are polynucleotide sequences of the described viruses, as well as methods for producing and using the viruses.

RECOMBINANT RESPIRATORY SYNCYTIAL VIRUS STRAINS WITH MUTATIONS IN THE M2-2 ORF PROVIDING A RANGE OF ATTENUATION PHENOTYPES

5 **RELATED APPLICATION**

This application claims priority to U.S. Provisional Application No. 62/266,199, filed December 11, 2015, which is incorporated by reference in its entirety.

FIELD

10 The subject matter disclosed herein relates to respiratory syncytial virus (RSV) and attenuated, mutant strains thereof suitable for use as vaccines.

BACKGROUND

Human respiratory syncytial virus (RSV) infects nearly everyone worldwide early in life and is responsible for considerable mortality and morbidity. In the United States alone, RSV is responsible for 15 75,000-125,000 hospitalizations yearly, and conservative estimates indicate that RSV is responsible worldwide for 64 million pediatric infections and 160,000 or more pediatric deaths each year. Another notable feature of RSV is that severe infection in infancy frequently is followed by lingering airway dysfunction, including a predisposition to airway reactivity, that in some individuals lasts for years and 20 can extend into adolescence and beyond. RSV infection exacerbates asthma and may be involved in initiating asthma.

RSV is a member of the Paramyxoviridae family and, as such, is an enveloped virus that replicates in the cytoplasm and matures by budding at the host cell plasma membrane. The genome of RSV is a single, negative-sense strand of RNA of 15.2 kilobases that is transcribed by the viral 25 polymerase into 10 mRNAs by a sequential stop-start mechanism that initiates at a single viral promoter at the 3' end of the genome. Each mRNA encodes a single major protein, with the exception of the M2 mRNA that has two overlapping open reading frames (ORFs) encoding two separate proteins M2-1 and M2-2. The 11 RSV proteins are: the RNA-binding nucleoprotein (N), the phosphoprotein (P), the large polymerase protein (L), the attachment glycoprotein (G), the fusion protein (F), the small hydrophobic (SH) surface glycoprotein, the internal matrix protein (M), the two nonstructural proteins NS1 and NS2, 30 and the M2-1 and M2-2 proteins. The RSV gene order is: 3'-NS1-NS2-N-P-M-SH-G-F-M2-L. Each gene is flanked by short conserved transcription signals called the gene-start (GS) signal, present on the upstream end of each gene and involved in initiating transcription of the respective gene, and the gene-end (GE) signal, present at the downstream end of each gene and involved in directing synthesis of a 35 polyA tail followed by release of the mRNA.

The development of RSV vaccines has been in progress since the 1960's but has been complicated by a number of factors. For example, immunization of RSV-naïve infants with inactivated RSV has been shown to prime for enhanced disease upon subsequent natural RSV infection, and studies

in experimental animals indicate that disease enhancement also is associated with purified RSV subunit vaccines.

Another obstacle to immune protection is that RSV replicates and causes disease in the superficial cells of the respiratory airway lumen, where immune protection has reduced effectiveness.

5 Thus, immune control of RSV infection is inefficient and often incomplete, and it is important for an RSV vaccine to be as immunogenic as possible. Another obstacle to RSV vaccines is that the magnitude of the protective immune response is roughly proportional to the extent of virus replication (and antigen production). Thus, the attenuation of RSV necessary to make a live vaccine typically is accompanied by a reduction in replication and antigen synthesis, and a concomitant reduction in immunogenicity, and
10 therefore it is beneficial to identify a level of replication that is well tolerated yet satisfactorily immunogenic.

Another obstacle is that RSV grows only to moderate titers in cell culture and is often present in long filaments that are difficult to purify. RSV can readily lose infectivity during handling. Another obstacle is the difficulty in identifying and developing attenuating mutations. Appropriate mutations
15 must be attenuating *in vivo*, but should be minimally restrictive to replication *in vitro*, since this is preferred for efficient vaccine manufacture. Another obstacle is genetic instability that is characteristic of RNA viruses, whereby attenuating mutations can revert to the wild-type (wt) assignment or to an alternative assignment that confers a non-attenuated phenotype. Instability and de-attenuation are particularly problematic for point mutations.

20 Taking these factors together, there is a need for live attenuated RSV strains that replicate efficiently *in vitro*, are maximally immunogenic, are satisfactorily attenuated, and are refractory to de-attenuation.

SUMMARY

25 Disclosed herein are mutations that are useful, either individually or in combinations that may include other known mutations, in producing recombinant strains of human RSV exhibiting a range of attenuation phenotypes. The mutations interfere with the expression of the open reading frame (ORF) encoding the viral M2-2 protein. Also disclosed are recombinant strains of RSV which contain such mutations, either alone or in combination with one or more additional mutations at other loci, which can
30 reduce or, alternatively, increase the magnitude of the attenuation phenotype. Thus, disclosed herein are novel live-attenuated RSV strains with a range of attenuation phenotypes suitable for use as RSV vaccines. In some examples, the disclosed embodiments of recombinant RSV are shown to be infectious, attenuated, and self-replicating, and to elicit a surprisingly high titer of neutralizing antibodies in human subjects.

35 In some embodiments, a recombinant RSV is provided that is attenuated by one or more modifications to the genome of the virus, such as a modification that interferes with the expression of the open reading frame (ORF) encoding the viral M2-2 protein. In some embodiments, the genome of the recombinant RSV comprises one or more modifications comprising a deletion in a M2-2 ORF

corresponding to a deletion of 241 nucleotides at positions 8189-8429 of SEQ ID NO: 1, combined with nucleotide mutations, such as T to C substitutions, at positions corresponding to T8161, T8167 and T8179 of SEQ ID NO: 1 (“ΔM2-2”). The nucleotide mutations disrupt translation start codons at these positions. In some embodiments, the genome of the recombinant RSV comprises one or more
5 modifications comprising a deletion in a M2-2 ORF corresponding to a deletion of 234 nucleotides at positions 8203-8436 of SEQ ID NO: 1, combined with nucleotide mutations T8198A and C8200G at positions corresponding to T8198 and C8200 of SEQ ID NO: 1 (“ΔM2-2-*AcII*”). In some embodiments, the genome of the recombinant RSV comprises one or more modifications comprising a deletion in a M2-2 ORF corresponding to a deletion of 234 nucleotides at positions 8203-8436 of SEQ ID NO: 1,
10 combined with nucleotide mutations T8198A and C8199G at positions corresponding to T8198 and C8199 of SEQ ID NO: 1 (“ΔM2-2-*HindIII*”), wherein the recombinant RSV genome is a D46 genome attenuated by the one or more modifications.

In addition to the modification that interferes with the expression of the open reading frame (ORF) encoding the viral M2-2 protein, the genome of the recombinant RSV can comprise further
15 modifications to increase or decrease viral attenuation, or other properties of the recombinant virus. In some embodiments the one or more modifications to the genome of the recombinant RSV can further comprise a deletion of 112 nucleotides corresponding to positions 4499-4610 of SEQ ID NO: 1, combined with nucleotide mutations C4489T, C4492T, A4495T, A4497G, and G4498A at positions corresponding to C4489, C4492, A4495, A4497, and G4498 of SEQ ID NO: 1 (“6120”). For example,
20 in some embodiments, the recombinant RSV is attenuated by one or more genomic modifications comprising a deletion in a M2-2 ORF corresponding to a deletion of 234 nucleotides at positions 8203-8436 of SEQ ID NO: 1, combined with nucleotide mutations T8198A and C8199G at positions corresponding to T8198 and C8199 of SEQ ID NO: 1 (“ΔM2-2-*HindIII*”), and a deletion of 112 nucleotides corresponding to positions 4499-4610 of SEQ ID NO: 1, combined with nucleotide mutations
25 at positions corresponding to C4489T, C4492T, A4495T, A4497G, and G4498A of SEQ ID NO: 1 (“6120”).

In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise nucleotide mutations encoding amino acid substitutions of V267I in the N protein, E218A and T523I in the F protein, and C319Y and H1690Y in the L protein of the RSV (“cp”). In some
30 embodiments, the one or more modifications to the genome of the recombinant RSV further comprise nucleotide mutations to introduce a Y1321K substitution in the L protein of the RSV, and wherein the L protein comprises a S1313 residue, wherein the codons encoding the Y1321K substitution and the S1313 residue are AAA and TCA codons respectively (“1030s”). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise nucleotide mutations encoding
35 amino acid substitution K51R in the NS2 protein of the RSV (“NS2”). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise nucleotide mutations encoding amino acid substitution T24A in the N protein of the RSV (“N”). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise nucleotide mutations

encoding amino acid substitution K51R in the NS2 protein and T24A in the N protein of the RSV (“NS2/N”). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise a deletion in a SH ORF corresponding to deletion of 419 nucleotides at positions 4198-4616 of SEQ ID NO: 1 (“ΔSH”). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise replacing the nucleotide sequence encoding a G protein of the RSV with a corresponding codon optimized nucleotide sequence encoding a G protein from the clinical isolate A/Maryland/001/11 (such as SEQ ID NO: 8, G001BB). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise replacing the nucleotide sequence encoding a F protein of the RSV with a corresponding codon-optimized nucleotide sequence set forth as SEQ ID NO: 9 (FBB). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise replacing the nucleotide sequence encoding a F protein of the RSV with a corresponding nucleotide sequence set forth as SEQ ID NO: 10 (F001), which encodes the F protein from the clinical isolate A/Maryland/001/11. In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise replacing the nucleotide sequence encoding a F protein of the RSV with a corresponding codon optimized nucleotide sequence encoding the F protein from the clinical isolate A/Maryland/001/11 (such as SEQ ID NO: 11, F001BB). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise nucleotide mutations encoding amino acid substitutions K66E and Q101P in the F protein of the RSV (“HEK”). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise nucleotide mutations encoding amino acid substitutions E218A and T523I in the F protein of the RSV (F cp substitutions). In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise reversing the order of the genes encoding the G and the F proteins in the RSV genome.

In some embodiments, the one or more modifications to the genome of the recombinant RSV further comprise or consist of a combination of mutations selected from any one of: ΔM2-2, cp/ΔM2-2, cp/ΔM2-2/HEK, ΔM2-2/1030s, NS2/N/ΔM2-2, NS2/ΔM2-2, N/ΔM2-2, ΔSH/ΔM2-2, cp/ΔSH/ΔM2-2, 6120/ΔM2-2, 6120/cp/ΔM2-2, 6120/ΔM2-2/1030s, 6120/NS2/N/ΔM2-2, 6120/G001BB/FBB/ΔM2-2, 6120/FBB/G001BB/ΔM2-2, 6120/G001BB/F/ΔM2-2, 6120/G/FBB/ΔM2-2, 6120/G/FBBHEK/ΔM2-2, 6120/G/FBBcpHEK/ΔM2-2, 6120/FBB/G/ΔM2-2, 6120/G001BB/F001BB/ΔM2-2, 6120/NS2/ΔM2-2, or 6120/N/ΔM2-2; or ΔM2-2-*AcII*, cp/ΔM2-2-*AcII*, cp/ΔM2-2-*AcII*/HEK, ΔM2-2-*AcII*/1030s, NS2/N/ΔM2-2-*AcII*, NS2/ΔM2-2-*AcII*, N/ΔM2-2-*AcII*, ΔSH/ΔM2-2-*AcII*, cp/ΔSH/ΔM2-2-*AcII*, 6120/ΔM2-2-*AcII*, 6120/cp/ΔM2-2-*AcII*, 6120/ΔM2-2-*AcII*/1030s, 6120/NS2/N/ΔM2-2-*AcII*, 6120/G001BB/FBB/ΔM2-2-*AcII*, 6120/FBB/G001BB/ΔM2-2-*AcII*, 6120/G001BB/F/ΔM2-2-*AcII*, 6120/G/FBB/ΔM2-2-*AcII*, 6120/G/FBBHEK/ΔM2-2-*AcII*, 6120/G/FBBcpHEK/ΔM2-2-*AcII*, 6120/FBB/G/ΔM2-2-*AcII*, 6120/G001BB/F001BB/ΔM2-2-*AcII*, 6120/NS2/ΔM2-2-*AcII*, or 6120/N/ΔM2-2-*AcII*; or ΔM2-2-*HindIII*; cp/ΔM2-2-*HindIII*, cp/ΔM2-2-*HindIII*/HEK, ΔM2-2-*HindIII*/1030s, NS2/N/ΔM2-2-*HindIII*, NS2/ΔM2-2-*HindIII*, N/ΔM2-2-*HindIII*, ΔSH/ΔM2-2-*HindIII*, cp/ΔSH/ΔM2-2-*HindIII*, 6120/ΔM2-2-*HindIII*, 6120/cp/ΔM2-2-*HindIII*, 6120/ΔM2-2-*HindIII*/1030s, 6120/NS2/N/ΔM2-2-*HindIII*,

6120/G001BB/FBB/ Δ M2-2-*Hind*III, 6120/FBB/G001BB/ Δ M2-2-*Hind*III, 6120/G001BB/F/ Δ M2-2-*Hind*III, 6120/G/FBB/ Δ M2-2-*Hind*III, 6120/G/FBBHEK/ Δ M2-2-*Hind*III, 6120/G/FBBcpHEK/ Δ M2-2-*Hind*III, 6120/FBB/G/ Δ M2-2-*Hind*III, 6120/G001BB/F001BB/ Δ M2-2-*Hind*III, 6120/NS2/ Δ M2-2-*Hind*III, or 6120/N/ Δ M2-2-*Hind*III.

5 In some embodiments, the genome of the recombinant RSV comprises the one or more mutations as discussed above, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at least 95% or at least 99%) identical to SEQ ID NO: 1 (D46 sequence). In some embodiments, the genome of the recombinant RSV is a D46 genome modified with the one or more mutations as discussed above. In some embodiments, the genome of the recombinant RSV comprises the 6120 and
10 Δ M2-2 mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at least 95% or at least 99%) identical to SEQ ID NO: 5 (LID/ Δ M2-2 sequence). In some embodiments, the genome of the recombinant RSV comprises the cp and Δ M2-2 mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at least 95% or at least 99%) identical to SEQ ID NO: 1 (D46 sequence). In some embodiments, the genome of the
15 recombinant RSV comprises the cp and Δ M2-2 mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at least 95% or at least 99%) identical to SEQ ID NO: 15 (D46/cp/ Δ M2-2 sequence). In some embodiments, the genome of the recombinant RSV comprises the 6120, Δ M2-2, and 1030s mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at least 95% or at least 99%) identical to SEQ ID NO: 16 (LID/ Δ M2-
20 2/1030s sequence). In some embodiments, the genome of the recombinant RSV comprises the 6120, cp, and Δ M2-2 mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at least 95% or at least 99%) identical to SEQ ID NO: 17 (LID/cp/ Δ M2-2 sequence). In some embodiments, the genome of the recombinant RSV comprises the NS2, N, Δ M2-2-*Hind*III mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at
25 least 95% or at least 99%) identical to SEQ ID NO: 4 (D46/ Δ M2-2-*Hind*III sequence). In some embodiments, the genome of the recombinant RSV comprises the NS2, N, Δ M2-2-*Hind*III mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at least 95% or at least 99%) identical to SEQ ID NO: 18 (D46/NS2/N/ Δ M2-2-*Hind*III sequence). In some
30 embodiments, the genome of the recombinant RSV comprises the NS2, N, Δ M2-2-*Acc*II mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at least 95% or at least 99%) identical to SEQ ID NO: 3 (D46/ Δ M2-2-*Acc*II sequence). In some embodiments, the genome of the recombinant RSV comprises the NS2, N, and Δ M2-2-*Acc*II mutations, the following nucleotide mutations with positions relative to SEQ ID NO: 1: 404C, 779G, deletion of C1099, 1139A, 1140G, 1182G, 1210G, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T,
35 and 13634A; and a nucleotide sequence corresponding to a positive-sense sequence at least 90% (such as at least 95% or at least 99%) identical to SEQ ID NO: 19 (276 sequence).

In some embodiments, the genome of the recombinant RSV is a D46/cp/ Δ M2-2 genome, a LID/ Δ M2-2/1030s genome, a LID/cp/ Δ M2-2 genome, a D46/NS2/N/ Δ M2-2-*Hind*III genome, a

LID/ Δ M2-2 genome, or a 276 genome. In some embodiments, the genome of the recombinant RSV comprises or consists of a nucleotide sequence corresponding to a positive-sense sequence set forth as any one of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, or SEQ ID NO: 19.

5 In some embodiments, the genome of the recombinant RSV comprises a nucleotide sequence corresponding to a positive-sense sequence set forth as SEQ ID NO: 3 further modified by introduction of the following nucleotide mutations relative to SEQ ID NO: 1: 404C, 779G, 1099T, 1139A, 1140G, 1182G, 1210G, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, and 13634A. In some embodiments, the genome of the recombinant RSV comprises a nucleotide
10 sequence corresponding to a positive-sense sequence set forth as SEQ ID NO: 3 further modified by introduction of the following nucleotide mutations relative to SEQ ID NO: 1: 404C, 779G, deletion of C1099, 1139A, 1140G, 1182G, 1210G, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, and 13634A. In some embodiments, the genome of the recombinant RSV comprises a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID
15 NO: 4, further modified by introduction of one or more of the following nucleotide substitutions with positions relative to SEQ ID NO: 1: 404C, 779G, 1099T, 1139A, 1140G, 1182G, 1210G, 1938A, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, 13634A, 13901T.

The embodiments of recombinant RSV disclosed herein can be subtype A RSV or a subtype B
20 RSV. The embodiments of recombinant RSV disclosed herein are infectious, attenuated, and self-replicating.

Also provided herein are methods and compositions related to the expression of the disclosed viruses. For example, isolated polynucleotide molecules that include a nucleic acid sequence encoding the genome or antigenome of the described viruses are disclosed.

25 Pharmaceutical compositions including the recombinant RSV are also provided. The compositions can further include an adjuvant. Methods of eliciting an immune response in a subject by administering an immunogenically effective amount of a disclosed recombinant RSV to the subject are also disclosed. In some embodiments, the subject is a human subject, for example, a human subject between 1 and 6 months of age, or between 1 and 12 months of age, or between 1 and 18 months of age,
30 or older.

The foregoing and other features and advantages of this disclosure will become more apparent from the following detailed description of several embodiments which proceeds with reference to the accompanying drawings.

35 BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A and 1B. The " Δ M2-2" mutation in the RSV genome silences the M2-2 ORF in the RSV backbone by introduction of a 241-nt deletion and eliminating three potential translational start codons for the M2-2 protein. Sequence numbering is according to the complete sequence of the wt

human RSV strain A2 that is represented by GenBank accession number M74568, which is incorporated by reference herein. (FIG. 1A) Organization of the RSV genome and the overlapping M2-1 and M2-2 ORFs. The three potential ATG translational start codons of the M2-2 ORF are shown. (FIG. 1B) Details of the Δ M2-2 mutation. The upper nucleotide sequence is that of biological wt RSV (M74568) and shows nucleotides corresponding to 8150-8204 and 8247-8440 of SEQ ID NO: 1. The nucleotide numbering between M74568 and SEQ ID NO: 1 is off by one nucleotide because of a single nucleotide insertion at position 1099 of SEQ ID NO: 1 compared to M74568. The amino acid sequence immediately underneath is of the C-terminal end of the M2-1 protein (SEQ ID NO: 12). The next amino acid sequence is the N-terminal residues of the M2-2 protein (SEQ ID NO: 13). The three potential ATG initiation codons for the M2-2 ORF are boxed and in bold. The second nucleotide sequence (for the Δ M2-2 mutation) shows the mutations that silence the M2-2 ORF (nucleotides 8150-8188 and 8430-8440 of SEQ ID NO: 1 are shown, with amino acid sequence SEQ ID NO: 13 shown directly below). For the Δ M2-2 mutation, each of the three potential translational ATG start sites was changed to ACG, and M74568 nucleotides 8188 to 8428 were deleted, removing 241 nucleotides. TER, translation termination codon.

FIG. 2. A depiction of the gene maps of two examples of attenuated recombinant RSVs using the D46 backbone and comprising the Δ M2-2 mutation. These are D46/ Δ M2-2 (comprising the Δ M2-2 mutation) and LID/ Δ M2-2 (comprising the Δ M2-2 mutation and the “6120” mutation, see FIG. 3) viruses. Antigenomic cDNA sequences of these two constructs are denoted by SEQ ID NO: 2 and SEQ ID NO: 5, respectively.

FIGs. 3A and 3B. The “6120” mutation in the RSV genome comprises a 112 nucleotide deletion in the downstream nontranslated region of the SH gene. Sequence numbering is according to the complete sequence of the wt human RSV strain A2 that is represented by GenBank accession number M74568. RSV genome map (FIG. 3A) and downstream end of the SH gene (FIG. 3B) containing the “6120” mutation, which is present in LID/ Δ M2-2. In this disclosure, the use of “6120” in a virus name indicates the presence of the 6120 mutation. (FIG. 3A) RSV genome map. The arrow indicates the location of the 6120 mutation in the downstream nontranslated region of the SH gene. (FIG. 3B) Details of the 6120 mutation. The upper nucleotide sequence is that of biological wt RSV (M74568) and shows nucleotides 4481-4498 and 4611-4632 of SEQ ID NO: 1, which correspond to the downstream end of the SH gene. The last five codons of the SH ORF are shown, with corresponding amino acid sequence (ARVNT, SEQ ID NO: 14) provided, followed by the translation termination codon (TER). This is followed to the right by the downstream nontranslated region of the SH gene (nucleotides 4611-4632 of SEQ ID NO: 1), with the SH gene-end signal underlined. The three dots represent 112 nucleotides of the downstream nontranslated region (M74568 nucleotides 4498-4609) that are deleted in the 6120 mutation. The lower nucleotide sequence (showing nucleotides 4481-4520 of SEQ ID NO: 5, with corresponding amino acid sequence (ARVNT, SEQ ID NO: 14) provided) depicts the 6120 mutation, which includes the 112-nucleotide deletion as well as five silent point mutations (bold) in the downstream three codons

and the termination codon of the SH ORF. Naturally occurring *Xho*I and *Pac*I restriction sites are italicized.

FIG. 4. Schematic diagrams of the genomes of three examples of derivatives of RSV D46/ Δ M2-2 that each contains one or more additional attenuating mutations. The Δ M2-2 mutation is depicted. The other attenuating mutations include the set of “cp” mutations (five amino acid substitutions in the N, F, and L proteins: N (V267I), F (E218A and T523I), and L (C319Y and H1690Y)), and the stabilized 1030 mutation (“1030s”) mutation in the L protein, which includes a Y1321K substitution generated by introducing an AAA codon for L amino acid 1321, and a stabilizing version of the serine codon at L position 1313 (nt 12435-12437 of SEQ ID NO:1; AGC changed to TCA) (1321K(AAA)+S1313(TCA); Luongo, *et al.* 2012. J Virol 86:10792-10804). Further mutations are the HEK changes in the F protein (K66E and Q101P)(Connors, *et al.* 1995. Virology 208:478-484; Whitehead, *et al.* 1998. J Virol 72:4467-4471).

FIG. 5. Schematic diagrams of the genomes of four examples of derivatives of RSV LID/ Δ M2-2 that each contains one or more additional attenuating mutations. The “LID” backbone is a D46-based genome containing the “6120” mutation. The Δ M2-2 mutation and the 6120 mutation are indicated. The other attenuating mutations include the set of “cp” mutations (five amino acid substitutions in the N, F, and L proteins: N (V267I), F (E218A and T523I), and L (C319Y and H1690Y)), deletion of the SH gene (see FIG. 6), and the “1030s” mutation in the L protein. Note that viruses from which the entire SH gene has been deleted (RSV Δ SH/ Δ M2-2 and RSV cp/ Δ SH/ Δ M2-2) are not referred to as “LID” because the SH deletion removes the 6120 mutation.

FIG. 6. Details of the “ Δ SH” mutation, which is a 419 nucleotide deletion that silences the SH gene. Sequence numbering is according to the complete sequence of the wt human RSV strain A2 that is represented by GenBank accession number M74568. In the upper sequence (showing nucleotides 4191-4229 and 4617-4637 of SEQ ID NO: 1), the features from left to right include: the M gene-end signal (underlined), the M/SH intergenic sequence (IGS), the SH gene-start signal (underlined), three dots that indicate the main body of the SH gene (M74568 nucleotides 4229-4615, which are not shown), the SH gene-end signal (underlined), and part of the SH/G IGS. The bottom sequence (nucleotides 4191-4197 and 4617-4637 of SEQ ID NO: 1) illustrates the 419-nucleotide deletion that yields the Δ SH mutation. Note that, although this is operationally called deletion of the SH gene, the deletion actually spans from immediately upstream of the M gene-end signal to immediately upstream of the SH gene-end signal.

FIG. 7. Replication of exemplary recombinant RSV in BALB/c mice. Results for replication of RSV D46 (the recombinant wt parent), RSV LID/ Δ M2-2, RSV Δ SH/ Δ M2-2, RSV LID/ Δ M2-2/1030s, and RSV cp/ Δ SH/ Δ M2-2 in the respiratory tract of BALB/c mice, with replication in the nasal turbinates and lungs evaluated by RT-qPCR, is shown. Mice in groups of 10 (or 12 for RSV D46) were inoculated by the intranasal (IN) route with $5.8 \log_{10}$ PFU of the indicated virus in 0.1 ml. Five mice per virus (or six for RSV D46) were sacrificed on days 4 and 5 (D4, D5), and nasal turbinates and lungs were removed, homogenized, and evaluated by RT-qPCR specific to the RSV M gene, with copy number determined relative to a cloned M cDNA evaluated in parallel. LoD: limit of detection.

FIGs. 8A and 8B. Replication of exemplary RSV recombinant viruses in the respiratory tract of African green monkeys (AGMs). Replication data of RSV LID/ Δ M2-2, RSV Δ SH/ Δ M2-2, RSV LID/ Δ M2-2/1030s, and RSV cp/ Δ SH/ Δ M2-2 in the respiratory tract of AGMs with shedding evaluated by RT-qPCR, is shown. AGMs in groups of 4 were inoculated by the combined IN and intratracheal (IT) routes with (per site) $6.0 \log_{10}$ PFU of the indicated virus in 1.0 ml. Nasopharyngeal (NP) swabs and tracheal lavages from the indicated days were evaluated by RT-qPCR specific to the RSV M gene, with copy number determined based on a cloned M cDNA evaluated in parallel. Graphed results are from the assays described in Example 2, Tables 1 and 2.

FIGs. 9A and 9B. Peak titers of exemplary recombinant RSV in seronegative infants and children. Peak titers of RSV MEDI/ Δ M2-2 and RSV rA2cp248/404/1030 Δ SH (FIG. 9A) or RSV LID/ Δ M2-2 (FIG. 9B) in nasal washes of seronegative infants and children (6-24 months of age) following a single IN inoculation are shown. The results for RSV MEDI/ Δ M2-2 and rA2cp248/404/1030 Δ SH are from Karron, *et al.* 2015. Science Transl Med 2015 7(312):312ra175. Viral titers were determined by plaque titration of nasal wash specimens: specimens from the RSV MEDI/ Δ M2-2 and RSV rA2cp248/404/1030 Δ SH studies were assayed side-by-side, whereas specimens from the RSV LID/ Δ M2-2 study were assayed separately. Symbols indicate peak values for individual subjects. The mean peak titers are shown. Vaccinees had each received a single IN dose of $5.0 \log_{10}$ PFU (RSV MEDI/ Δ M2-2, RSV LID/ Δ M2-2) or $5.3 \log_{10}$ PFU (RSV rA2cp248/404/1030 Δ SH) vaccine virus. The original report of the rA2cp248/404/1030 Δ SH vaccine candidate was Karron, *et al.* 2005. J Infect Dis 191:1093-1104.

FIGs. 10A and 10B. The " Δ M2-2-*AcII*" and " Δ M2-2-*HindIII*" mutations to the RSV genome. The Δ M2-2-*AcII* mutation silences the M2-2 ORF by site directed mutagenesis to delete 234 nucleotides and introduce two point mutations creating an *AcII* restriction site and a translational termination site (TER). The Δ M2-2-*HindIII* mutation silences the M2-2 ORF by site directed mutagenesis to delete 234 nucleotides and introduce two point mutations creating a *HindIII* restriction site and a translational termination site (TER). Sequence numbering is according to the complete sequence of the wt human RSV strain A2 that is represented by GenBank accession number M74568. FIG. 10A shows the organization of the RSV genome and the overlapping M2-1 and M2-2 ORFs. The three potential ATG translational start codons of the M2-2 ORF are shown, but are not modified in Δ M2-2-*AcII* and Δ M2-2-*HindIII* mutations. FIG. 10B shows details of the Δ M2-2-*AcII* and Δ M2-2-*HindIII* mutations. The upper nucleotide sequence (nucleotides 8150-8204 and 8427-8440 of SEQ ID NO: 1) is that of biological wt RSV (M74568). The amino acid sequences immediately underneath is of the C-terminal end of the M2-1 protein (SEQ ID NO: 12). The next amino acid sequence is that of an N-terminal portion of the M2-2 protein (SEQ ID NO: 13). The three potential ATG initiation codons for the M2-2 ORF are boxed and in bold. The second nucleotide sequence (nucleotides 8150-8202 of SEQ ID NO: 3) shows the sequence of the Δ M2-2-*AcII* mutation that results from deletion of nucleotides 8202-8435 and introduction of the point mutations T8197A and C8199G to create an *AcII* site as well as a TAA termination codon at codon 13 in the M2-2 ORF. Amino acid sequences 12 and 13 are shown under the Δ M2-2-*AcII* sequence. The

third nucleotide sequence (nucleotides 8150-8202 of SEQ ID NO: 4) shows the sequence of the Δ M2-2-*HindIII* mutation that results from deletion of nucleotides 8202-8435 and introduction of the point mutations T8197A and C8198G to create a *HindIII* site as well as a TAA termination codon at codon 13 in the M2-2 ORF. As shown, the mutated M2-2 ORF in both Δ M2-2-*AcII* and Δ M2-2-*HindIII* has the potential to encode a 12-amino acid peptide representing the N-terminal end of the M2-2 protein. Amino acid sequences 12 and 13 are shown under the Δ M2-2-*HindIII* sequence.

FIG. 11. Schematic diagrams of RSV D46 and LID genomes bearing the Δ M2-2-*AcII* mutation, termed D46/ Δ M2-2-*AcII* and LID/ Δ M2-2-*AcII*. Antigenomic cDNA sequences of these two constructs are denoted by SEQ ID NO: 3 and SEQ ID NO: 6 respectively.

FIGs. 12A-12C. Schematic diagrams of genomes of examples of Δ M2-2 viruses into which the K51R (NS2 gene) and T24A (N gene) mutations have been introduced. In the viral names, K51R and T24A are abbreviated as “NS2” and “N”, respectively. FIG. 12A shows the introduction of the K51R and T24A mutations together into the D46/ Δ M2-2-*AcII* backbone and the LID/ Δ M2-2-*AcII* backbone. FIGs. 12B and 12C show the introduction of the K51R and T24A mutations into the D46/ Δ M2-2 backbone (FIG. 12B) individually or together, or into the LID/ Δ M2-2 backbone (FIG. 12C) individually or together.

FIG. 13. Schematic diagrams of the genomes of examples of derivatives of RSV D46 and LID bearing the “ Δ M2-2-*HindIII*” mutation, as well as derivatives bearing the Δ M2-2-*HindIII* genome in combination with the K51R and T24A mutations. The introduction of the Δ M2-2-*HindIII* mutation alone into the D46 or LID backbones resulted in the genomes D46/ Δ M2-2-*HindIII* and LID/ Δ M2-2-*HindIII* (the first and third genomes from the top). The introduction of the Δ M2-2-*HindIII* mutation into the D46 or LID backbones in combination with the K51R and T24A mutations resulted in the genomes D46/NS2/N/ Δ M2-2-*HindIII* and LID/NS2/N/ Δ M2-2-*HindIII* (the second and fourth genomes from the top). Sequences of D46/ Δ M2-2-*HindIII* and RSV LID/ Δ M2-2-*HindIII* constructs are denoted by SEQ ID NO: 4 and SEQ ID NO: 7 respectively.

FIGs. 14A and 14B. Schematic diagrams of the genomes of examples of derivatives of RSV LID/ Δ M2-2 (*i.e.*, containing the Δ M2-2 mutation shown in FIG. 1 and the 6120 mutation shown in FIG. 3) containing additional modifications to the F and G genes. For these constructs, the identifier “6120” is used instead of “LID” to refer to the 6120 mutation. G001 and F001 refer to the G and F genes, respectively, of a clinical isolate of a subgroup A strain (not A2) called RSV A/Maryland/001/11. All other genes are from strain A2. “BB” refers to codon-optimized sequence. HEK refers to the two amino acid substitutions in the F protein, K66E and Q101P. In this case, “FBBcpHEK,” refers to a codon optimized F sequence further including the HEK substitutions, and the “cp” substitutions that fall within the F protein, namely E218A and T523I.

FIG. 15. Virus yields in Vero cells for the constructs shown in FIGs. 14A and 14B. The P1 titer is the yield of the first passage (done blindly, *i.e.*, without quantification of the input multiplicity of infection, MOI) following transfection. The P2 titer is the yield of second passage done with an input

MOI of 0.01 PFU/cell; note that one virus is represented by P3, the yield of a third passage at MOI 0.001. Wt LID is D46 containing the 6120 mutation.

FIG. 16. Schematic diagram illustrating the genome of RSV D46/276/ Δ M2-2-AcII.

5

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. The Sequence Listing is submitted as an ASCII text file in the form of the file named "Sequence.txt" (~240 kb), which was created on December 8, 2016, and which is incorporated by reference herein. In the accompanying sequence listing:

10

SEQ ID NO: 1 is the antigenomic cDNA sequence for recombinant RSV strain D46.

SEQ ID NO: 2 is the antigenomic cDNA sequence for recombinant RSV strain D46/ Δ M2-2.

SEQ ID NO: 3 is the antigenomic cDNA sequence for recombinant RSV strain D46/ Δ M2-2-AcII.

15

SEQ ID NO: 4 is the antigenomic cDNA sequence for recombinant RSV strain D46/ Δ M2-2-HindIII.

SEQ ID NO: 5 is the antigenomic cDNA sequence for recombinant RSV strain LID/ Δ M2-2.

SEQ ID NO: 6 is the antigenomic cDNA sequence for recombinant RSV strain LID/ Δ M2-2-AcII.

20

SEQ ID NO: 7 is the antigenomic cDNA sequence for recombinant RSV strain LID/ Δ M2-2-HindIII.

SEQ ID NO: 8 is an exemplary polynucleotide sequence encoding G001BB.

SEQ ID NO: 9 is an exemplary polynucleotide sequence encoding FBB.

SEQ ID NO: 10 is an exemplary polynucleotide sequence encoding F001.

25

SEQ ID NO: 11 is an exemplary polynucleotide sequence encoding F001BB.

SEQ ID NO: 12 is a C-terminal amino acid sequence of the M2-1 protein (SDTNDHAKNNDTT).

SEQ ID NO: 13 is an N-terminal amino acid sequence of the M2-2 protein (MTMPKIMILPDKYPC).

30

SEQ ID NO: 14 is a C-terminal amino acid sequence of the SH protein (ARVNT).

SEQ ID NO: 15 is the antigenomic cDNA sequence for recombinant RSV strain D46/cp/ Δ M2-2.

SEQ ID NO: 16 is the antigenomic cDNA sequence for recombinant RSV strain LID/ Δ M2-2/1030s.

SEQ ID NO: 17 is the antigenomic cDNA sequence for recombinant RSV strain LID/cp/ Δ M2-2.

35

SEQ ID NO: 18 is the antigenomic cDNA sequence for recombinant RSV strain D46/NS2/N/ Δ M2-2-HindIII.

SEQ ID NO: 19 is the antigenomic cDNA sequence for recombinant RSV strain "276".

SEQ ID NOs: 20 and 21 are the nucleotide sequences of gene-start transcription signals (GGGGCAAATA and GGGGCAAACA, respectively).

DETAILED DESCRIPTION

5 Provided herein are recombinant RSV strains suitable for use as attenuated, live vaccines in humans. The RSV strains are produced by introducing mutations that block expression of the M2-2 protein and confer attenuation. Further provided are recombinant RSV strains in which the mutations that block expression of the M2-2 protein are present in combination with one or more additional engineered mutations at one or more other loci that increase or decrease the magnitude of attenuation,
10 providing vaccine candidates with graded degrees of attenuation.

 Mutations that reduce or ablate expression of the M2-2 ORF result in up-regulated expression of the viral genes including those encoding protective antigens (Bermingham and Collins. 1999. Proc Natl Acad Sci USA 96:11259-11264), and have the potential to confer increased immunogenicity. However, increased immunogenicity had not previously been demonstrated and was not evident in experimental
15 animals including chimpanzees (Teng, *et al.* 2000. J Virol 74:9317-9321). Clinical evaluation of the presently disclosed strains which contain the M2-2 mutations described herein demonstrated that these strains exhibit increased immunogenicity. Studies in seronegative infants and children, which represent the primary vaccine target population, showed that the strains disclosed herein were attenuated and generally well-tolerated, and induced a substantial titer of RSV-neutralizing serum antibodies that was
20 significantly greater than that of a previous RSV vaccine candidate (rA2cp248/404/1030ΔSH) that has a different basis of attenuation (i.e., a series of point mutations in several viral genes including the polymerase, which represents a more typical type of live vaccine candidate) and was evaluated in a similar subject population (Karron, *et al.* 2005. J Infect Dis 191:1093-1104).

 Thus, this disclosure provides novel, improved attenuated RSV strains bearing novel M2-2
25 deletion mutations, which possess increased immunogenicity in humans. The use of a large deletion that ablates most of a viral ORF provides genetic stability. Further, this disclosure provides sets of novel attenuated RSV strains bearing the M2-2 deletion mutations in combination with additional mutations that modify the phenotype. This provides viral strains with graded attenuation phenotypes.

 The recombinant RSV strains of the present invention comprise a wild type RSV genome or
30 antigenome containing further modifications or mutations as described in detail below. The wild type RSV virus genome or antigenome encodes the following 11 proteins: the RNA-binding nucleoprotein (N), the phosphoprotein (P), the large polymerase protein (L), the attachment glycoprotein (G), the fusion protein (F), the small hydrophobic (SH) surface glycoprotein, the internal matrix protein (M), the two nonstructural proteins NS1 and NS2, and the M2-1 and M2-2 proteins. The RSV gene order is: 3'-NS1-
35 NS2-N-P-M-SH-G-F-M2-L. The complete amino acid sequences of these proteins are known in the art.

 Given that a variety of RSV strains exist (e.g., RSV A2, RSV B1, RSV Long), those skilled in the art will appreciate that certain strains of RSV may have nucleotide or amino acid insertions or deletions that alter the position of a given residue. For example, if a protein of another RSV strain had,

in comparison with strain A2, two additional amino acids in the upstream end of the protein, this would cause the amino acid numbering of downstream residues relative to strain A2 to increase by an increment of two. However, because these strains share a large degree of sequence identity, those skilled in the art would be able to determine the location of corresponding sequences by simply aligning the nucleotide or amino acid sequence of the A2 reference strain with that of the strain in question. Therefore, it should be understood that the amino acid and nucleotide positions described herein, though specifically enumerated in the context of this disclosure, can correspond to other positions when a sequence shift has occurred or due to sequence variation between virus strains. In the comparison of a protein, or protein segment, or gene, or genome, or genome segment between two or more related viruses, a “corresponding” amino acid or nucleotide residue is one that is thought to be exactly or approximately equivalent in function in the different species.

Unless context indicates otherwise, the numbering used in this disclosure is based on the sequence of the wild-type RSV A2 strain (GenBank accession number M74568) and viral genomic sequences described are in positive-sense.

In some embodiments of the present invention, the recombinant RSV strains were derived from the recombinant version of strain A2 that is called D46. The complete sequence of D46 is shown in US patent 6,790,449 and is provided herein as SEQ ID NO: 1. (In some instances and publications, the parent virus and sequence is called D53 rather than D46, a book-keeping difference that refers to the strain of bacteria used to propagate the antigenomic cDNA and has no other known significance or effect. For the purposes of this invention, D46 and D53 are interchangeable.) SEQ ID NO: 1 (the nucleotide sequence of D46) differs from the sequence of RSV A2 strain M74568 in 25 nucleotide positions, which includes a 1-nt insert at position 1099. Therefore, sequence numbering relative to SEQ ID NO: 1 differs from numbering relative to M74568 by increment of 1 nucleotide, when the nucleotide is located at a position beyond nucleotide 1099.

In some embodiments, the RSV genome or antigenome is modified by a deletion in the M2-2 ORF. The RSV M2-2 protein is encoded by the second, downstream ORF in the M2 mRNA, which slightly overlaps the 5'-proximal, upstream M2-1 ORF (FIG. 1A). There are three potential translation start codons that would give rise to products of 90, 88, and 84 amino acids in length for strain A2 (boxed in FIG. 1B). The M2-2 mutations described herein include deletion of large numbers of nucleotides (typically more than 200 nucleotides each) involving most of the M2-2 ORF, and thus largely or completely ablate expression of the M2-2 protein. The M2-2 mutations therefore are refractory to compensation or reversion that might confer loss of attenuation. This genetic stability was confirmed in a clinical study. Most of the previous RSV vaccine candidates have involved attenuating point mutations, which are prone to reversion or compensation resulting in de-attenuation (e.g., Karron, *et al.* 2005. J Infect Dis 191:1093-1104; Malkin, *et al.* 2013. PLoS One 8:e77104; Karron, Buchholz, Collins. 2013. Curr Top Microbiol Immunol 372:259-284). De-attenuation has the potential for increased virus replication in a vaccinee, which might result in reactogenicity, and also the potential for spread of under-

attenuated derivatives to susceptible contacts. Therefore, the M2-2 mutations described herein obviate a major concern of RSV vaccine development.

In some embodiments, the M2-2 mutation comprises a deletion of 241 nucleotides located at positions 8188-8428 (8189-8429 of SEQ ID NO: 1) and mutations T8160C, T8166C and T8178C (T8161C, T8167C and T8179C of SEQ ID NO: 1) which eliminate the three potential start codons. This mutation is explained in FIG. 1B and is referred herein as the “ Δ M2-2” mutation.

In some embodiments, the M2-2 mutation comprises a deletion of 234 nucleotides located at positions 8202-8435 (8203-8436 of SEQ ID NO: 1), combined with the presence of 8197A and 8199G (8198A and 8200G of SEQ ID NO: 1) corresponding to the presence of an *AcII* restriction enzyme site. This mutation is explained in FIG. 10 and is referred herein as the “ Δ M2-2-*AcII*” mutation.

In some embodiments, the M2-2 deletion comprises a mutation of 234 nucleotides located at positions 8202-8435 (8203-8436 of SEQ ID NO: 1), combined with the presence of 8197A and 8198G (8198A and 8199G of SEQ ID NO: 1) corresponding to the presence of a *HindIII* restriction enzyme site. This mutation is explained in FIG. 10 and referred herein as the “ Δ M2-2-*HindIII*” mutation. In some embodiments the RSV strain used for constructing the strain may be D46 (SEQ ID NO: 1). In that case, the resultant recombinant strain is called D46/ Δ M2-2-*HindIII*.

The presence of the term “ Δ M2-2” in a virus name in this disclosure indicates the presence of the Δ M2-2 mutation shown in FIG. 1 in that virus, except in the case of “MEDI/ Δ M2-2,” which refers to a different mutation that is described in the Examples below. Other mutations are specified by the terms Δ M2-2-*AcII* or Δ M2-2-*HindIII*.

Additional mutations may be further introduced in combination with one of the M2-2 mutations defined above to construct additional viral strains with desired characteristics. For example, the added mutations may specify different magnitudes of attenuation, and thus give incremental increases in attenuation. Thus, candidate vaccine strains can be further attenuated by incorporation of at least one, and preferably two or more different attenuating mutations, for example mutations identified from a panel of known, biologically derived mutant RSV strains. A number of such mutations are discussed here as examples. From this exemplary panel a large “menu” of attenuating mutations can be created, in which each mutation can be combined with any other mutation(s) within the panel for calibrating the level of attenuation and other desirable phenotypes. Additional attenuating mutations may be identified in non-RSV negative stranded RNA viruses and incorporated in RSV mutants of the invention by mapping the mutation to a corresponding, homologous site in the recipient RSV genome or antigenome and mutating the existing sequence in the recipient to the mutant genotype (either by an identical or conservative mutation). Additional useful mutations can be determined empirically by mutational analysis using recombinant minigenome systems and infectious virus as described in the references incorporated herein.

In some embodiments, the disclosed recombinant RSV vaccine strains can be produced using a recombinant DNA-based technique called reverse genetics (Collins, *et al.* 1995. Proc Natl Acad Sci USA 92:11563-11567). This system allows *de novo* recovery of infectious virus entirely from cDNA in a

qualified cell substrate under defined conditions. Reverse genetics provides a means to introduce predetermined mutations into the RSV genome via the cDNA intermediate. Specific attenuating mutations were characterized in preclinical studies and combined to achieve the desired level of attenuation. Derivation of vaccine viruses from cDNA minimizes the risk of contamination with adventitious agents and helps to keep the passage history brief and well documented. Once recovered, the engineered virus strains propagate in the same manner as a biologically derived virus. As a result of passage and amplification, the vaccine viruses do not contain recombinant DNA from the original recovery.

The recombinant virus strains that contain various combinations of mutations discussed herein are for exemplary purposes only and are not meant to limit the scope of the present invention. Other attenuating mutations not described here may also be used in combination with a disclosed M2-2 mutation (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutation).

For example, in some embodiments, the recombinant RSV strains of the present invention further comprise a deletion of the non-translated sequences. In one embodiment, such deletion occurs in the downstream end of the SH gene, resulting in a mutation called the “6120” mutation herein. The “6120” mutation is shown in FIG. 3. It involves deletion of 112 nucleotides of the downstream non-translated region of the SH gene and the introduction of five translationally-silent point mutations in the last three codons and the termination codon of the SH gene (Bukreyev, *et al.* 2001. J Virol 75:12128-12140). Presence of the term “LID” or “6120” in a recombinant virus name indicates that the recombinant virus contains the 6120 mutation.

The 6120 mutation stabilizes the antigenomic cDNA in bacteria so that it could be more easily manipulated and prepared. In wt RSV, this mutation was previously found to confer a 5-fold increase in replication efficiency *in vitro* (Bukreyev, *et al.* 2001. J Virol 75:12128-12140), whereas it was not thought to increase replication efficiency *in vivo*. When RSV LID/ Δ M2-2 was evaluated for the possibility of increased replication associated with the 6120 mutation, a modest but inconsistent increase in growth efficiency was observed.

The 6120 mutation was associated with increased replication in seronegative infants and children. Thus, the 6120 mutation provided another means to shift the level of attenuation. While the use of this strategy is demonstrated herein in conjunction with a Δ M2-2 mutation, it can be applied to other attenuated strains for the same purpose. Also, the deletion of sequence exemplified by the 6120 mutation in the downstream non-translated region of the SH gene, but in principle could involve any comparable genome sequence that does not contain a critical cis-acting signal (Collins and Karron. 2013. Fields Virology 6th Edition, pp 1086-1123). Genome regions that are candidates for deletion include, but are not limited to, non-translated regions in other genes, in the intergenic regions, and in the trailer region.

In some embodiments the recombinant RSV strains may comprise the “cp” mutation. This mutation refers to a set of five amino acid substitutions in three proteins (N (V267I), F (E218A and T523I), and L (C319Y and H1690Y)) that together (on their own) confer an approximate 10-fold

reduction in replication in seronegative chimpanzees, and a reduction in illness (Whitehead, *et al.* 1998. J Virol 72:4467-4471). It was previously shown that the cp mutation is associated with a moderate attenuation phenotype (Whitehead, *et al.* 1999. J Virol 72:4467-4471).

In addition, previous analysis of 6 biological viruses that had been derived by chemical
 5 mutagenesis of cpRSV and selected for the temperature-sensitive (ts) phenotype yielded a total of 6 independent mutations that each conferred a ts attenuation phenotype and could be used in various combinations. Five of these were amino acid substitutions in the L protein, which were named based on virus number rather than sequence position: "955" (N43I), "530" (F521L), "248" (Q831L), "1009" (M1169V), and "1030" (Y1321N) (Juhász, *et al.* 1999. Vaccine 17:1416-1424; Collins, *et al.* 1999. Adv
 10 Virus Res 54:423-451; Firestone, *et al.* 1996. Virology 225:419-422; Whitehead, *et al.* 1999. J Virol 73:871-877). The sixth mutation (called "404") was a single nucleotide change in the gene-start transcription signal of the M2 gene (GGGGCAAATA (SEQ ID NO: 20) to GGGGCAAACA (SEQ ID NO: 21), mRNA-sense) (Whitehead, *et al.* 1998. Virology 247:232-239). Reverse genetics has been used to increase the genetic stability of the 248 and 1030 mutations (Luongo, *et al.* 2009. Vaccine 27:5667-
 15 5676; Luongo, *et al.* 2012. J Virol 86:10792-10804). In addition, a new attenuating mutation was created by deleting codon 1313 in the L protein and combining it with an I1314L substitution to confer increased genetic stability (Luongo, *et al.* 2013. J Virol 87:1985-1996).

In some embodiments, the recombinant strains may comprise one or more changes in the F protein, e.g. the "HEK" mutation, which comprises two amino acid substitutions in the F protein namely
 20 K66E and Q101P (described in Connors, *et al.* 1995. Virology 208:478-484; Whitehead, *et al.* 1998. J Virol 72:4467-4471). The introduction of the HEK amino acid assignments into the strain A2 F sequence of this disclosure results in an F protein amino acid sequence that is identical to that of an early-passage (human embryonic kidney cell passage 7, HEK-7) of the original clinical isolate of strain A2 (Connors, *et al.* 1995. Virology 208:478-484; Whitehead, *et al.* 1998. J Virol 72:4467-4471). It results in
 25 an F protein that is much less fusogenic and is thought to represent the phenotype of the original A2 strain clinical isolate (Liang *et al.* J Virol 2015 89:9499-9510). The HEK F protein also forms a more stable trimer (Liang *et al.* J Virol 2015 89:9499-9510). This may provide a more authentic and immunogenic form of the RSV F protein, possibly enriched for the highly immunogenic pre-fusion conformation (McLellan *et al.* Science 2013 340(6136):1113-7; Science 2013 342(6158):592-8.). Thus,
 30 mutations can be introduced with effects additional to effects on the magnitude of virus replication.

In some embodiments the recombinant strains may comprise one or more changes in the L protein, e.g. the stabilized 1030 or the "1030s" mutation which comprises 1321K(AAA)/1313S(TCA) (Luongo, *et al.* 2012. J Virol 86:10792-10804).

In some embodiments the recombinant strains may comprise deletions of one or more RSV
 35 genes. Deletion of the SH, NS1, and NS2 genes individually and in combination has been shown to yield viruses that retain their ability to replicate in cell culture but are attenuated *in vivo* in the following order of increasing magnitude: SH<NS2<NS1 (Bukreyev, *et al.* 1997. J Virol 71:8973-8982; Whitehead, *et al.* 1999. J Virol 73:3438-3442; Teng, *et al.* 2000. J Virol 74:9317-9321). Therefore, deletion or other

mutations of the SH, NS2, or NS1 genes, or parts of their ORFs, may be combined with a disclosed M2-2 mutation (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutation). For example, in some embodiments, the recombinant strains may comprise one or more changes in the SH protein, including an ablation or elimination of the SH protein. In some embodiments, the viral strains comprise a deletion in the SH gene. For example, in some embodiments, the viral strains comprise a 419 nucleotide deletion at position 4197-4615 (4198-4616 of SEQ ID NO: 1), denoted herein as the “ Δ SH” mutation. This deletion results in the deletion of M gene-end, M/SH intergenic region, and deletion of the SH ORF as shown in FIG. 6. In some embodiments, the recombinant strains may comprise one or more changes in the NS1 or the NS2 protein, which may include an ablation or elimination of the protein. In some embodiments, the mutation may be an amino acid substitution such as K51R in the NS2 protein. In some embodiments the recombinant strains may comprise one or more changes in the N protein, e.g. an amino acid substitution such as T24A.

Various features can be introduced into RSV strains bearing a disclosed M2-2 mutation (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutation) that change the characteristics of the virus in ways other than attenuation. For instance, codon optimization of the ORFs encoding the proteins may be performed. Major protective antigens F and G can result in increased antigen synthesis. The F and/or G protein gene may be shifted upstream (closer to the promoter) to increase expression. However, the present disclosure also describes unexpected limitations to this strategy in the case of Δ M2-2 recombinant virus strains. The F and/or G protein amino acid sequences can be modified to represent currently-circulating strains, which can be may be relevant in the case of the divergent G protein, or to represent early-passage clinical isolates. Deletions or substitutions may be introduced into the G protein to obtain improved immunogenicity or other desired properties. For example, the CX3C fractalkine motif in the G protein might be ablated to improve immunogenicity (Chirkova *et al.* J Virol 2013 87:13466-13479).

For example, in some embodiments, the nucleotide sequence encoding the G protein of the RSV may be replaced with a corresponding nucleotide sequence from the clinical isolate A/Maryland/001/11. In some embodiments, the nucleotide sequence encoding the F protein of the RSV may be replaced with a corresponding nucleotide sequence from the clinical isolate A/Maryland/001/11, e.g. F001 (SEQ ID NO: 10).

In some embodiments, a native or naturally occurring nucleotide sequence encoding a protein of the RSV may be replaced with a codon optimized sequence designed for increased expression in a selected host, in particular the human. For example, in some embodiments, the nucleotide sequence encoding the F protein of the RSV may be replaced with the codon optimized sequence FBB (“FBB”) (SEQ ID NO: 9). In some embodiments, the nucleotide sequence encoding the F protein of the RSV may be replaced with the codon optimized sequence from the clinical isolate A/Maryland/001/11 (“F001BB”) (SEQ ID NO: 11). In some embodiments, the nucleotide sequence encoding the G protein of the RSV may be replaced with the codon optimized nucleotide sequence G001BB (SEQ ID NO: 8) from the clinical isolate A/Maryland/001/11 (“G001BB”).

Yet additional aspects of the invention involve changing the position of a gene or altering gene order to create or modify a M2-2 deletion mutant RSV. For example, the NS1, NS2, SH and G genes may be deleted individually, or the NS1 and NS2 gene may be deleted together, thereby shifting the position of each downstream gene relative to the viral promoter. For example, when NS1 and NS2 are deleted together, N is moved from gene position 3 to gene position 1, P from gene position 4 to gene position 2, and so on. Alternatively, deletion of any other gene within the gene order will affect the position (relative to the promoter) only of those genes which are located further downstream. For example, SH occupies position 6 in Wild type virus, and its deletion does not affect M at position 5 (or any other upstream gene) but moves G from position 7 to 6 relative to the promoter. It should be noted that gene deletion also can occur (rarely) in a biologically-derived mutant virus. For example, a subgroup B RSV that had been passaged extensively in cell culture spontaneously deleted the SH and G genes (Karron *et al.* Proc. Natl. Acad. Sci. USA 94:13961 13966, 1997; incorporated herein by reference).

Gene order shifting modifications (*i.e.*, positional modifications moving one or more genes to a more promoter-proximal or promoter-distal location in the recombinant viral genome) result in viruses with altered biological properties. For example, RSV lacking NS1, NS2, SH, G, NS1 and NS2 together, or SH and G together, have been shown to be attenuated *in vitro*, *in vivo*, or both. In particular, the G and F genes may be shifted, singly and in tandem, to a more promoter-proximal position relative to their wild-type gene order. These two proteins normally occupy positions 7 (G) and 8 (F) in the RSV gene order (NS1-NS2-N-P-M-SH-G-FM2-L). In some embodiments, the order of the nucleotide sequences encoding the G and the F proteins may be reversed relative to the naturally occurring order.

The RSV F and G proteins are known to induce RSV neutralizing antibodies, and are the major protective antigens. The F protein generally is considered to be is a more effective neutralization and protective antigen than the G protein. F also is relatively well-conserved among RSV strains, whereas the G protein can be substantially divergent. The divergence in G is a major factor in segregating RSV strains into two antigenic subgroups, A and B (~53% and ~90% amino acid sequence identity between the two subgroups for G and F, respectively). The tools and methods of the present disclosure focus on RSV strain A2 of subgroup A, but can readily be applied to other strains of either subgroup.

In some embodiments, the recombinant RSV strain comprises a recombinant RSV genome comprising the Δ M2-2, Δ M2-2-*AclI*, or Δ M2-2-*HindIII* mutation in combination with one or more of the mutations described above. In some embodiments, the recombinant RSV strain comprises a recombinant RSV genome comprising a D46 (SEQ ID NO: 1) genome that has been modified with the Δ M2-2, Δ M2-2-*AclI*, or Δ M2-2-*HindIII* mutation, as well as one or more of the above mutations described above.

In some embodiments, the recombinant strain can be a D46-based RSV strain including the “276” mutations, and further including one of the disclosed M2-2 mutations, such as a Δ M2-2, Δ M2-2-*AclI*, or Δ M2-2-*HindIII* mutation. As discussed in Example 8, the “276” mutations include the following nucleotide mutations: 404C, 779G, deletion of C1099, 1138A, 1139G, 1181G, 1209G, 5611A, 5615A, 5639G, 6215C, 6221C, 6386T, 7214C, 7481T, 7559A, 7701G, 10514T, and 13633A (relative to SEQ ID

NO: 1, these mutations are the following: 404C, 779G, deletion of C1099, 1139A, 1140G, 1182G, 1210G, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, and 13634A). An exemplary antigenomic cDNA sequence for a Δ M2-2-*HindIII*-based RSV strain including the “276” mutations is provided as SEQ ID NO: 19.

5 In some embodiments, the recombinant RSV strain comprises a genome comprising a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical (such as at least 95% identical or at least 99% identical) to the antigenomic cDNA sequence set forth as SEQ ID NO: 1, that has been modified to comprise one of the Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutations as described above, alone or in combination with one or more of the attenuating mutations provided herein.

10 In some embodiments, the recombinant RSV strain comprises a genome comprising the cp and Δ M2-2 mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 1 (D46 sequence).

15 In some embodiments, the recombinant RSV strain comprises a genome comprising the Δ M2-2 mutation as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 2 (D46/ Δ M2-2 sequence).

20 In some embodiments, the recombinant RSV strain comprises a genome comprising the Δ M2-2-*AcII* mutation as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 3 (D46/ Δ M2-2-*AcII* sequence).

25 In some embodiments, the recombinant RSV strain comprises a genome comprising the Δ M2-2-*HindIII* mutation as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 4 (D46/ Δ M2-2-*HindIII* sequence).

In some embodiments, the recombinant RSV strain comprises a genome comprising the LID and Δ M2-2 mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 5 (LID/ Δ M2-2 sequence).

30 In some embodiments, the recombinant RSV strain comprises a genome comprising the LID and Δ M2-2-*AcII* mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 6 (LID/ Δ M2-2-*AcII* sequence).

35 In some embodiments, the recombinant RSV strain comprises a genome comprising the LID and Δ M2-2-*HindIII* mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 7 (LID/ Δ M2-2-*HindIII* sequence).

In some embodiments, the recombinant RSV strain comprises a genome comprising the cp and ΔM2-2 mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 15 (D46/cp/ΔM2-2 sequence).

5 In some embodiments, the recombinant RSV strain comprises a genome comprising the 6120, ΔM2-2, and 1030s mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 16 (LID/ΔM2-2/1030s sequence).

10 In some embodiments, the recombinant RSV strain comprises a genome comprising the 6120, cp, and ΔM2-2 mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 17 (LID/cp/ΔM2-2 sequence).

15 In some embodiments, the recombinant RSV strain comprises a genome comprising the NS2, N, ΔM2-2-*HindIII* mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 4 (D46/ΔM2-2-*HindIII* sequence).

20 In some embodiments, the recombinant RSV strain comprises a genome comprising the NS2, N, ΔM2-2-*HindIII* mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 18 (D46/NS2/N/ΔM2-2-*HindIII* sequence).

In some embodiments, the recombinant RSV strain comprises a genome comprising the NS2, N, ΔM2-2-*AcII* mutations as described herein, and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 3 (D46/ΔM2-2-*AcII* sequence).

25 In some embodiments, the recombinant RSV strain comprises a genome comprising the NS2, N, and ΔM2-2-*AcII* mutations as described herein, the following nucleotide mutations with positions relative to SEQ ID NO: 1: 404C, 779G, deletion of C1099, 1139A, 1140G, 1182G, 1210G, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, and 13634A; and a nucleotide sequence corresponding to an antigenomic cDNA sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 19 (276 sequence).

In some embodiments, the recombinant RSV strain comprises a genome comprising a nucleotide sequence corresponding to a positive-sense sequence set forth as any one of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, or SEQ ID NO: 19.

35 In several embodiments, the genome of the recombinant RSV comprises the one or more mutations as discussed herein, and any remaining sequence difference of the genome of the recombinant RSV compared to the genomic sequence of D46 RSV (SEQ ID NO: 1) is biologically insignificant (for example, the remaining sequence differences do not include changes to the wild-type genomic sequence

that modify a known cis-acting signal or change amino acid coding, or measurably affect in vitro replication or plaque size of the virus).

In addition to the above described mutations, infectious M2-2 deletion mutants (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutant) can incorporate heterologous, coding or non-coding nucleotide sequences from any RSV or RSV-like virus, e.g., human, bovine, ovine, murine (pneumonia virus of mice), or avian (turkey rhinotracheitis virus) pneumovirus, or from another enveloped virus, e. g., parainfluenza virus (PIV). Exemplary heterologous sequences include RSV sequences from one human RSV strain combined with sequences from a different human RSV strain. Alternatively, M2-2 deletion mutants (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutant) may incorporate sequences from two or more, wild-type or mutant human RSV subgroups, for example a combination of human RSV subgroup A and subgroup B sequences. In yet additional aspects, one or more human RSV coding or non-coding polynucleotides are substituted with a counterpart sequence from a heterologous RSV or non-RSV virus to yield novel attenuated vaccine strains.

In addition to the recombinant RSVs having the particular mutations, and the combinations of those mutations, described herein, the disclosed viruses may be modified further as would be appreciated by those skilled in the art. For example, the recombinant RSVs may have one or more of its proteins deleted or otherwise mutated or a heterologous gene from a different organism may be added to the genome or antigenome so that the recombinant RSV expresses or incorporates that protein upon infecting a cell and replicating. Furthermore, those skilled in the art will appreciate that other previously defined mutations known to have an effect on RSV may be combined with one or more of any of the mutations described herein to produce a recombinant RSV with desirable attenuation or stability characteristics.

In some embodiments, the mutations described herein, when used either alone or in combination with another mutation, may provide for different levels of virus attenuation, providing the ability to adjust the balance between attenuation and immunogenicity, and provide a more stable genotype than that of the parental virus.

With regard to sequence numbering of nucleotide and amino acid sequence positions for the described viruses, a convention was used whereby each nucleotide or amino acid residue in a given viral sequence retained the sequence position number that it has in the original 15,222-nucleotide biological wt strain A2 virus (GenBank accession number M74568), irrespective of any modifications. Thus, although a number of genomes contain deletions and/or insertions that cause changes in nucleotide length, and in some cases amino acid length, the numbering of all of the other residues (nucleotide or amino acid) in the genome and encoded proteins remains unchanged. It also is recognized that, even without the expedient of this convention, one skilled in the art can readily identify corresponding sequence positions between viral genomes or proteins that might differ in length, guided by sequence alignments as well as the positions of open reading frames, well-known RNA features such as gene-start and gene-end signals, and amino acid sequence features.

Additional representative viruses from those described in this disclosure may be evaluated in cell culture for infectivity, replication kinetics, yield, efficiency of protein expression, and genetic stability

using the methods described herein and illustrated in examples using exemplary recombinant strains. Additional representative strains may be evaluated in rodents and non-human primates for infectivity, replication kinetics, yield, immunogenicity, and genetic stability. While these semi-permissive systems may not reliably detect every difference in replication, substantial differences in particular may be
 5 detected (e.g., as between RSV D46/ Δ M2-2 and LID/ Δ M2-2, Tables 4 and 5). Also recombinant strains may be evaluated directly in seronegative children without the prior steps of evaluation in adults and seropositive children. This may be done, for example, in groups of 10 vaccine recipients and 5 placebo recipients, which is a small number that allows simultaneous evaluation of multiple candidates. Candidates may be evaluated in the period immediately post-immunization for vaccine virus infectivity,
 10 replication kinetics, shedding, tolerability, immunogenicity, and genetic stability, and the vaccinees may be subjected to surveillance during the following RSV season for safety, RSV disease, and changes in RSV-specific serum antibodies, as described in Karron, *et al.* 2015, Science Transl Med 2015 7(312):312ra175, which is incorporated herein in its entirety. Thus, analysis of selected representative viruses may provide for relatively rapid triage to narrow down candidates to identify the most optimal.

15 Reference to a protein or a peptide includes its naturally occurring form, as well as any fragment, domain, or homolog of such protein. As used herein, the term "homolog" is used to refer to a protein or peptide which differs from a naturally occurring protein or peptide (*i.e.*, the "prototype" or "wild-type" protein) by minor modifications to the naturally occurring protein or peptide, but which maintains the basic protein and side chain structure of the naturally occurring form. Such changes include, but are not
 20 limited to: changes in one or a few amino acid side chains; changes in one or a few amino acids, including deletions (e.g., a truncated version of the protein or peptide) insertions and/or substitutions; changes in stereochemistry of one or a few atoms; and/or minor derivatizations, including but not limited to: methylation, glycosylation, phosphorylation, acetylation, myristoylation, prenylation, palmitation, amidation. A homolog can have either enhanced, decreased, or substantially similar properties as
 25 compared to the naturally occurring protein or peptide. A homolog of a given protein may comprise, consist essentially of, or consist of, an amino acid sequence that is at least about 50%, or at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95%, or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% identical (or any percent identity between
 30 45% and 99%, in whole integer increments), to the amino acid sequence of the reference protein.

In one aspect of the invention, a selected gene segment, such as one encoding a selected protein or protein region (e.g., a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.) from one RSV, can be substituted for a counterpart gene segment from the same or different RSV or other source, to yield
 35 novel recombinants having desired phenotypic changes compared to wild-type or parent RSV strains. For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one RSV fused to an ectodomain of another RSV. Other exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions. As

used herein, “counterpart” genes, gene segments, proteins or protein regions, are typically from heterologous sources (e.g., from different RSV genes, or representing the same (*i.e.*, homologous or allelic) gene or gene segment in different RSV strains). Typical counterparts selected in this context share gross structural features, e.g., each counterpart may encode a comparable structural “domain,” such as a cytoplasmic domain, transmembrane domain, ectodomain, binding site or region, epitopic site or region, etc. Counterpart domains and their encoding gene segments embrace an assemblage of species having a range of size and amino acid (or nucleotide) sequence variations, which range is defined by a common biological activity among the domain or gene segment variants. For example, two selected protein domains encoded by counterpart gene segments within the invention may share substantially the same qualitative activity, such as providing a membrane spanning function, a specific binding activity, an immunological recognition site, etc. More typically, a specific biological activity shared between counterparts, e.g., between selected protein segments or proteins, will be substantially similar in quantitative terms, *i.e.*, they will not vary in respective quantitative activity profiles by more than 30%, preferably by no more than 20%, more preferably by no more than 5-10%.

In alternative aspects of the invention, the infectious RSV produced from a cDNA-expressed genome or antigenome can be any of the RSV or RSV-like strains, e.g., human, bovine, murine, etc., or of any pneumovirus or metapneumovirus, e.g., pneumonia virus of mice or avian metapneumovirus. To engender a protective immune response, the RSV strain may be one which is endogenous to the subject being immunized, such as human RSV being used to immunize humans. The genome or antigenome of endogenous RSV can be modified, however, to express RSV genes or gene segments from a combination of different sources, e.g., a combination of genes or gene segments from different RSV species, subgroups, or strains, or from an RSV and another respiratory pathogen such as human parainfluenza virus (PIV) (see, e.g., Hoffman *et al.* J. Virol. 71:4272-4277 (1997); Durbin *et al.* Virology 235(2):323-32 (1997); Murphy *et al.* U.S. Patent Application Ser. No. 60/047,575, filed May 23, 1997, and the following plasmids for producing infectious PIV clones: p3/7(131) (ATCC 97990); p3/7(131)2G(ATCC 97889); and p218(131) (ATCC 97991); each deposited Apr. 18, 1997 under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 10801 University Blvd., Manassas, Va. 20110-2209, USA., and granted the above identified accession numbers.

In certain embodiments of the invention, recombinant RSV are provided wherein individual internal genes of a human RSV are replaced with, e.g., a bovine or other RSV counterpart, or with a counterpart or foreign gene from another respiratory pathogen such as PIV. Substitutions, deletions, etc. of RSV genes or gene segments in this context can include part or all of one or more of the NS1, NS2, N, P, M, SH, and L genes, or the M2-1 open reading frames, or non-immunogenic parts of the G and F genes. Also, human RSV cis-acting sequences, such as promoter or transcription signals, can be replaced with, e.g., their bovine RSV counterpart. Reciprocally, means are provided to generate live attenuated bovine RSV by inserting human attenuating genes or cis-acting sequences into a bovine RSV genome or antigenome background.

Thus, infectious recombinant RSV intended for administration to humans can be a human RSV that has been modified to contain genes from, e.g., a bovine RSV or a PIV, such as for the purpose of attenuation. For example, by inserting a gene or gene segment from PIV, a bivalent vaccine to both PIV and RSV is provided. Alternatively, a heterologous RSV species, subgroup or strain, or a distinct
 5 respiratory pathogen such as PIV, may be modified, e.g., to contain genes that encode epitopes or proteins which elicit protection against human RSV infection. For example, the human RSV glycoprotein genes can be substituted for the bovine glycoprotein genes such that the resulting bovine RSV, which now bears the human RSV surface glycoproteins and would retain a restricted ability to replicate in a human host due to the remaining bovine genetic background, elicits a protective immune
 10 response in humans against human RSV strains.

The ability to analyze and incorporate other types of attenuating mutations into infectious RSV for vaccine development extends to a broad assemblage of targeted changes in RSV clones. For example, any RSV gene which is not essential for growth may be ablated or otherwise modified to yield desired effects on virulence, pathogenesis, immunogenicity and other phenotypic characters. In addition,
 15 a variety of other genetic alterations can be produced in a recombinant RSV genome or antigenome for incorporation into infectious recombinant RSV, alone or together with one or more attenuating point mutations adopted from a biologically derived mutant RSV.

As used herein, "heterologous genes" refers to genes taken from different RSV strains or types or non-RSV sources. These heterologous genes can be inserted in whole or in part, the order of genes
 20 changed, gene overlap removed, the RSV genome promoter replaced with its antigenome counterpart, portions of genes removed or substituted, and even entire genes deleted. Different or additional modifications in the sequence can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions (e.g., a unique *Stu*I site between the G and F genes) or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign
 25 sequences.

Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or gene segments in recombinant RSV of the invention yield highly stable vaccine candidates, which may be relevant in the case of immunosuppressed individuals. Many of these mutations will result in attenuation of resultant vaccine strains, whereas others will specify different types of desired phenotypic
 30 changes. For example, certain viral genes are known which encode proteins that specifically interfere with host immunity (see, e.g., Kato *et al.*, EMBO. J. 16:578-87 (1997). Ablation of such genes in vaccine viruses is expected to reduce virulence and pathogenesis and/or improve immunogenicity.

Other mutations within RSV of the present invention involve replacement of the 3' end of genome with its counterpart from antigenome, which is associated with changes in RNA replication and
 35 transcription. In addition, the intergenic regions (Collins *et al.*, Proc. Natl. Acad. Sci. USA 83:4594-4598 (1986)) can be shortened or lengthened or changed in sequence content, and the naturally-occurring gene overlap (Collins *et al.*, Proc. Natl. Acad. Sci. USA 84:5134-5138 (1987)) can be removed or changed to a different intergenic region by the methods described herein.

In another embodiment, a sequence surrounding a translational start site (preferably including a nucleotide in the -3 position) of a selected RSV gene is modified, alone or in combination with introduction of an upstream start codon, to modulate RSV gene expression by specifying up- or down-regulation of translation.

5 Alternatively, or in combination with other RSV modifications disclosed herein, RSV gene expression can be modulated by altering a transcriptional GS signal of a selected gene(s) of the virus. In one exemplary embodiment, the GS signal of NS2 is modified to include a defined mutation to superimpose a ts restriction on viral replication.

Yet additional RSV clones within the invention incorporate modifications to a transcriptional GE
10 signal. For example, RSV clones are provided which substitute or mutate the GE signal of the NS1 and NS2 genes for that of the N gene, resulting in decreased levels of readthrough mRNAs and increased expression of proteins from downstream genes. The resulting recombinant virus exhibits increased growth kinetics and increased plaque size, providing but one example of alteration of RSV growth properties by modification of a cis-acting regulatory element in the RSV genome.

15 In another aspect, expression of the G protein may be increased by modification of the G mRNA. The G protein is expressed as both a membrane bound and a secreted form, the latter form being expressed by translational initiation at a start site within the G gene translational open reading frame. The secreted form may account for as much as one-half of the expressed G protein. Ablation of the internal start site (e.g., by sequence alteration, deletion, etc.), alone or together with altering the sequence
20 context of the upstream start site yields desired changes in G protein expression. Ablation of the secreted form of the G protein also will improve the quality of the host immune response to exemplary, recombinant RSV, because the soluble form of the G protein is thought to act as a “decoy” to trap neutralizing antibodies. Also, soluble G protein has been implicated in enhanced immunopathology due to its preferential stimulation of a Th2-biased response.

25 In related aspects, levels of RSV gene expression may be modified at the level of transcription. In one aspect, the position of a selected gene in the RSV gene map may be changed to a more promoter-proximal or promoter-distal position, whereby the gene will be expressed more or less efficiently, respectively. According to this aspect, modulation of expression for specific genes can be achieved yielding reductions or increases of gene expression from two-fold, more typically four-fold, up to ten-
30 fold or more compared to wild-type levels. In one example, the NS2 gene (second in order in the RSV gene map) is substituted in position for the SH gene (sixth in order), yielding a predicted decrease in expression of NS2. Increased expression of selected RSV genes due to positional changes can be achieved up to 10-fold, 30-fold, 50-fold, 100-fold or more, often attended by a commensurate decrease in expression levels for reciprocally, positionally substituted genes.

35 In some exemplary embodiments, the F and G genes may be transpositioned singly or together to a more promoter-proximal or promoter-distal site within the (recombinant) RSV gene map to achieve higher or lower levels of gene expression, respectively. These and other transpositioning changes yield novel RSV clones having attenuated phenotypes, for example due to decreased expression of selected

viral proteins involved in RNA replication. In yet other embodiments, RSV useful in a vaccine formulation may be conveniently modified to accommodate antigenic drift in circulating virus. Typically the modification will be in the G and/or F proteins. The entire G or F gene, or the segments encoding particular immunogenic regions thereof, is incorporated into the RSV genome or antigenome cDNA by replacement of the corresponding region in the infectious clone or by adding one or more copies of the gene such that several antigenic forms are represented.

Progeny virus produced from the modified RSV cDNA are then used in vaccination protocols against the emerging strains. Further, inclusion of the G protein gene of RSV subgroup B as a gene addition will broaden the response to cover a wider spectrum of the relatively diverse subgroup A and B strains present in the human population.

An infectious RSV clone of the invention may also be engineered according to the methods and compositions disclosed herein to enhance its immunogenicity and induce a level of protection greater than that provided by infection with a wild-type RSV or an incompletely attenuated parental virus or clone. For example, an immunogenic epitope from a heterologous RSV strain or type, or from a non-RSV source such as PIV, can be added by appropriate nucleotide changes in the polynucleotide sequence encoding the RSV genome or antigenome. Recombinant RSV can also be engineered to identify and ablate (e.g., by amino acid insertion, substitution or deletion) epitopes associated with undesirable immunopathologic reactions. In other embodiments, an additional gene may be inserted into or proximate to the RSV genome or antigenome which is under the control of an independent set of transcription signals. Genes of interest may include, but are not limited to, those encoding cytokines (e.g., IL-2 through IL-15, especially IL-2, IL-6 and IL-12, etc.), gamma-interferon, and include those encoding cytokines (e.g., IL-2 through IL-15, especially IL-2, IL-6 and IL-12, etc.), gamma-interferon, and proteins rich in T helper cell epitopes. The additional protein can be expressed either as a separate protein or as a chimera engineered from a second copy of one of the RSV proteins, such as SH. This provides the ability to modify and improve the immune response against RSV both quantitatively and qualitatively.

In addition to the above described modifications to recombinant RSV, different or additional modifications in RSV clones can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions (e.g., a unique *Stu*I site between the G and F genes) or elsewhere. Nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

Introduction of the foregoing, defined mutations into an infectious RSV clone can be achieved by a variety of well-known methods. By “infectious clone” is meant cDNA or its product, synthetic or otherwise, which can be transcribed into genomic or antigenomic RNA capable of producing an infectious virus. The term “infectious” refers to a virus or viral structure that is capable of replicating in a cultured cell or animal or human host to produce progeny virus or viral structures capable of the same activity. Thus, defined mutations can be introduced by conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of antigenome or genome cDNA

subfragments to assemble a complete antigenome or genome cDNA is well-known by those of ordinary skill in the art and has the advantage that each region can be manipulated separately (smaller cDNAs are easier to manipulate than large ones) and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or any subfragment thereof, can be used as template for
 5 oligonucleotide-directed mutagenesis. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. Mutations can vary from single nucleotide changes to replacement of large cDNA pieces containing one or more genes or genome regions.

Recombinant RSV may be produced by the intracellular coexpression of a cDNA that encodes the RSV genomic RNA, together with those viral proteins necessary to generate a transcribing,
 10 replicating nucleocapsid. Plasmids encoding other RSV proteins may also be included with these essential proteins. Alternatively, RNA may be synthesized in *in vitro* transcription reactions and transfected into cultured cells.

Accordingly, also described herein are isolated polynucleotides that encode the described mutated viruses, make up the described genomes or antigenomes, express the described genomes or
 15 antigenomes, or encode various proteins useful for making recombinant RSV *in vitro*. Polynucleotides comprising the sequences of any of the SEQ ID NOs described herein are included in the present invention. Further included are polynucleotides comprising sequences that consist or consist essentially of any of the aforementioned sequences, sequences that possess at least about 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 percent
 20 identity (or any percent identity in between) to any of the aforementioned SEQ ID NOs, as well as polynucleotides that hybridize to, or are the complements of the aforementioned molecules.

These polynucleotides can be included within or expressed by vectors in order to produce a recombinant RSV. Accordingly, cells transfected with the isolated polynucleotides or vectors are also within the scope of the invention and are exemplified herein.

25 In related aspects of the invention, compositions (e.g., isolated polynucleotides and vectors incorporating an RSV-encoding cDNA) and methods are provided for producing an isolated infectious recombinant RSV bearing an attenuating, M2-2 deletion mutation (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutant). Included within these aspects of the invention are novel, isolated polynucleotide molecules and vectors incorporating such molecules that comprise a RSV genome or
 30 antigenome which is modified as described herein. Also provided is the same or different expression vector comprising one or more isolated polynucleotide molecules encoding the RSV proteins. These proteins also can be expressed directly from the genome or antigenome cDNA. The vector(s) is/ are preferably expressed or coexpressed in a cell or cell-free lysate, thereby producing an infectious M2 ORF2 deletion or knock out mutant RSV particle or subviral particle.

35 In one aspect, a method for producing one or more purified RSV protein(s) is provided which involves infecting a host cell permissive of RSV infection with a recombinant RSV strain under conditions that allow for RSV propagation in the infected cell. After a period of replication in culture, the cells are lysed and recombinant RSV is isolated therefrom. One or more desired RSV protein(s) is

purified after isolation of the virus, yielding one or more RSV protein(s) for vaccine, diagnostic and other uses.

The above methods and compositions for producing M2-2 deletion mutants (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutant) yield infectious viral or subviral particles, or derivatives thereof.

5 An infectious virus is comparable to the authentic RSV virus particle and is infectious as is. It can directly infect fresh cells. An infectious subviral particle typically is a subcomponent of the virus particle which can initiate an infection under appropriate conditions. For example, a nucleocapsid containing the genomic or antigenomic RNA and the N, P, L and M2-1 proteins is an example of a subviral particle which can initiate an infection if introduced into the cytoplasm of cells. Subviral particles provided
10 within the invention include viral particles which lack one or more protein(s), protein segment(s), or other viral component(s) not essential for infectivity.

In other embodiments the invention provides a cell or cell free lysate containing an expression vector which comprises an isolated polynucleotide molecule encoding an M2-2 deletion mutant (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutant) RSV genome or antigenome as described above, and
15 an expression vector (the same or different vector) which comprises one or more isolated polynucleotide molecules encoding the N, P, L and RNA polymerase elongation factor proteins of RSV. One or more of these proteins also can be expressed from the genome or antigenome cDNA. Upon expression the genome or antigenome and N, P, L, and RNA polymerase elongation factor proteins combine to produce an infectious RSV viral or sub-viral particle.

20 The recombinant RSV of the invention are useful in various compositions to generate a desired immune response against RSV in a host susceptible to RSV infection. Attenuated M2-2 deletion mutant (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutant) RSV strains disclosed herein are capable of eliciting a protective immune response in an infected human host, yet are sufficiently attenuated so as to not cause unacceptable symptoms of severe respiratory disease in the immunized host. The attenuated
25 virus or subviral particle may be present in a cell culture supernatant, isolated from the culture, or partially or completely purified. The virus may also be lyophilized, and can be combined with a variety of other components for storage or delivery to a host, as desired.

In another aspect, M2-2 deletion mutants (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutant) may be employed as “vectors” for protective antigens of other pathogens, particularly respiratory
30 tract pathogens such as parainfluenza virus (PIV). For example, recombinant RSV having a M2-2 deletion (such as a Δ M2-2, Δ M2-2-*AcII*, or Δ M2-2-*HindIII* mutant) may be engineered which incorporate sequences that encode protective antigens from PIV to produce infectious, attenuated vaccine virus.

In related aspects, the invention provides a method for stimulating the immune system of an individual to elicit an immune response against RSV in a mammalian subject. The method comprises
35 administering an immunogenic formulation of an immunologically sufficient amount of an attenuated, M2-2 deletion mutant RSV as described herein in a physiologically acceptable carrier and/or adjuvant.

The invention further provides novel vaccines comprising a physiologically acceptable carrier and/or adjuvant and an isolated attenuated M2-2 deletion mutant RSV particle or subviral particle. In

preferred embodiments, the vaccine is comprised of an M2-2 deletion mutant RSV having at least one; and preferably two or more attenuating mutations or other nucleotide modifications as described above to achieve a suitable balance of attenuation and immunogenicity.

To select candidate vaccine viruses from the host of recombinant RSV strains provided herein,
5 the criteria of viability, efficient replication *in vitro*, attenuation *in vivo*, immunogenicity, and phenotypic stability are determined according to well-known methods. Viruses which will be most desired in vaccines of the invention should maintain viability, should replicate sufficiently *in vitro* well under permissive conditions to make vaccine manufacture possible, should have a stable attenuation phenotype, should be well-tolerated, should exhibit replication in an immunized host (albeit at lower levels), and
10 should effectively elicit production of an immune response in a vaccine sufficient to confer protection against serious disease caused by subsequent infection from wild-type virus.

To propagate a RSV virus for vaccine use and other purposes, a number of cell lines which allow for RSV growth may be used. RSV grows in a variety of human and animal cells. Preferred cell lines for propagating attenuated RS virus for vaccine use include DBSFRhL-2, MRC-5, and Vero cells. Highest
15 virus yields are usually achieved with epithelial cell lines such as Vero cells. Cells are typically inoculated with virus at a multiplicity of infection ranging from about 0.001 to 1.0, or more, and are cultivated under conditions permissive for replication of the virus, e.g., at about 30-37°C and for about 3-10 days, or as long as necessary for virus to reach an adequate titer. Temperature-sensitive viruses often are grown using 32°C as the “permissive temperature.” Virus is removed from cell culture and separated
20 from cellular components, typically by well-known clarification procedures, e.g., centrifugation, and may be further purified as desired using procedures well known to those skilled in the art.

RSV which has been attenuated as described herein can be tested in various well known and generally accepted *in vitro* and *in vivo* models to confirm adequate attenuation, resistance to phenotypic reversion, and immunogenicity for vaccine use. In *in vitro* assays, the modified virus, which can be a
25 multiply attenuated, biologically derived or recombinant RSV, is tested for temperature sensitivity of virus replication or “ts phenotype,” and for the small plaque phenotype. Modified viruses are further tested in animal models of RSV infection. A variety of animal models (e.g., murine, cotton rat, and primate) have been described and are known to those skilled in the art.

In accordance with the foregoing description and based on the Examples below, the invention
30 also provides isolated, infectious RSV compositions for vaccine use. The attenuated virus which is a component of a vaccine is in an isolated and typically purified form. By isolated is meant to refer to RSV which is in other than a native environment of a wild-type virus, such as the nasopharynx of an infected individual. More generally, isolated is meant to include the attenuated virus as a component of a cell culture or other artificial medium. For example, attenuated RSV of the invention may be produced
35 by an infected cell culture, separated from the cell culture and added to a stabilizer.

RSV vaccines of the invention contain as an active ingredient an immunogenically effective amount of RSV produced as described herein. Biologically derived or recombinant RSV can be used directly in vaccine formulations. The biologically derived or recombinantly modified virus may be

introduced into a host with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or in frozen form that is thawed prior to use, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, which include, but are not limited to, pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sucrose, magnesium sulfate, phosphate buffers, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, sorbitan monolaurate, and triethanolamine oleate. Acceptable adjuvants include incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum, which are materials well known in the art. Preferred adjuvants also include Stimulon™ QS-21 (Aquila Biopharmaceuticals, Inc., Worcester, Mass.), MPL™ (3-O-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, Mont.), and interleukin-12 (Genetics Institute, Cambridge, Mass.).

Upon immunization with a RSV vaccine composition, the host responds to the vaccine by producing antibodies specific for RSV virus proteins, e.g., F and G glycoproteins. In addition, innate and cell-mediated immune responses are induced, which can provide antiviral effectors as well as regulating the immune response. As a result of the vaccination the host becomes at least partially or completely immune to RSV infection, or resistant to developing moderate or severe RSV disease, particularly of the lower respiratory tract.

The vaccine compositions containing the attenuated RSV of the invention are administered to a subject susceptible to or otherwise at risk of RSV infection in an "immunogenically effective dose" which is sufficient to induce or enhance the individual's immune response capabilities against RSV. An RSV vaccine composition may be administered by any suitable method, including but not limited to, via injection, aerosol delivery, nasal spray, nasal droplets, oral inoculation, or topical application. In the case of human subjects, the attenuated virus of the invention is administered according to well established human RSV vaccine protocols (Karron *et al.* JID 191:1093-104, 2005). Briefly, adults or children are inoculated intranasally via droplet with an immunogenically effective dose of RSV vaccine, typically in a volume of 0.5 ml of a physiologically acceptable diluent or carrier. This has the advantage of simplicity and safety compared to parenteral immunization with a non-replicating vaccine. It also provides direct stimulation of local respiratory tract immunity, which plays a major role in resistance to RSV. Further, this mode of vaccination effectively bypasses the immunosuppressive effects of RSV-specific maternally-derived serum antibodies, which typically are found in the very young. Also, while the parenteral administration of RSV antigens can sometimes be associated with immunopathologic complications, this has not been observed with a live virus.

In some embodiments, the vaccine may be administered intranasally or subcutaneously or intramuscularly. In some embodiments, it may be administered to the upper respiratory tract. This may be performed by any suitable method, including but not limited to, by spray, droplet or aerosol delivery.

Often, the composition will be administered to an individual seronegative for antibodies to RSV or possessing transplacentally acquired maternal antibodies to RSV.

In all subjects, the precise amount of RSV vaccine administered and the timing and repetition of administration will be determined by various factors, including the patient's state of health and weight, the mode of administration, the nature of the formulation, etc. Dosages will generally range from about 3.0 log₁₀ to about 6.0 log₁₀ plaque forming units ("PFU") or more of virus per patient, more commonly from about 4.0 log₁₀ to 5.0 log₁₀ PFU virus per patient. In one embodiment, about 5.0 log₁₀ to 6.0 log₁₀ PFU per patient may be administered during infancy, such as between 1 and 6 months of age, and one or more additional booster doses could be given 2-6 months or more later. In another embodiment, young infants could be given a dose of about 5.0 log₁₀ to 6.0 log₁₀ PFU per patient at approximately 2, 4, and 6 months of age, which is the recommended time of administration of a number of other childhood vaccines. In yet another embodiment, an additional booster dose could be administered at approximately 10-15 months of age. In any event, the vaccine formulations should provide a quantity of attenuated RSV of the invention sufficient to effectively stimulate or induce an anti-RSV immune response (an "effective amount").

In some embodiments, the vaccine may comprise attenuated M2-2 deletion virus that elicits an immune response against a single RSV strain or antigenic subgroup, e.g. A or B, or against multiple RSV strains or subgroups. In this regard, M2-2 deletion mutant RSV can be combined in vaccine formulations with other RSV vaccine strains or subgroups having different immunogenic characteristics for more effective protection against one or multiple RSV strains or subgroups. They may be administered in a vaccine mixture, or administered separately in a coordinated treatment protocol to elicit more effective protection against one RSV strain, or against multiple RSV strains or subgroups.

The resulting immune response can be characterized by a variety of methods. These include taking samples of nasal washes or sera for analysis of RSV-specific antibodies, which can be detected by tests including, but not limited to, complement fixation, plaque neutralization, enzyme-linked immunosorbent assay, luciferase-immunoprecipitation assay, and flow cytometry. In addition, immune responses can be detected by assay of cytokines in nasal washes or sera, ELISPOT of immune cells from either source, quantitative RT-PCR or microarray analysis of nasal wash or serum samples, and restimulation of immune cells from nasal washes or serum by re-exposure to viral antigen *in vitro* and analysis for the production or display of cytokines, surface markers, or other immune correlates measured by flow cytometry or for cytotoxic activity against indicator target cells displaying RSV antigens. In this regard, individuals are also monitored for signs and symptoms of upper respiratory illness.

The level of attenuation of vaccine virus may be determined by, for example, quantifying the amount of virus present in the respiratory tract of an immunized host and comparing the amount to that produced by wild-type RSV or other attenuated RS viruses which have been evaluated as candidate vaccine strains. For example, the attenuated virus of the invention will have a greater degree of restriction of replication in the upper respiratory tract of a highly susceptible host, such as a chimpanzee, compared to the levels of replication of wild-type virus, e.g., 10- to 1000-fold less. In order to further

reduce the development of rhinorrhea, which is associated with the replication of virus in the upper respiratory tract, an ideal vaccine candidate virus should exhibit a restricted level of replication in both the upper and lower respiratory tract. However, the attenuated viruses of the invention should be sufficiently infectious and immunogenic in humans to confer protection in vaccinated individuals.

5 Methods for determining levels of RSV in the nasopharynx of an infected host are well known in the literature. Specimens are obtained by aspiration or washing out of nasopharyngeal secretions and virus quantified in tissue culture or other by laboratory procedure. See, for example, Belshe *et al.*, J. Med. Virology 1:157-162 (1977), Friedewald *et al.*, J. Amer. Med. Assoc. 204:690-694 (1968); Gharpure *et al.*, J. Virol. 3:414-421 (1969); and Wright *et al.*, Arch. Ges. Virusforsch. 41:238-247 (1973). The virus
10 can conveniently be measured in the nasopharynx of host animals, such as chimpanzees.

Additional embodiments

Clause 1. An isolated polynucleotide molecule encoding a recombinant respiratory syncytial virus (RSV) variant having an attenuated phenotype comprising a RSV genome or antigenome sequence,
15 wherein

(a) the RSV genome or antigenome is modified by a deletion in the M2-2 ORF corresponding to a deletion comprising a deletion of 241 nucleotides located at positions 8189-8429 of SEQ ID NO: 1 combined with mutations at positions T8161, T8167 and T8179 of SEQ ID NO: 1; or

(b) the RSV genome or antigenome is modified by a deletion in the M2-2 ORF corresponding to
20 a deletion comprising a deletion of 234 nucleotides located at positions 8203-8436 of SEQ ID NO: 1 combined with the presence of 8198A and 8200G of SEQ ID NO: 1; or

(c) the RSV genome or antigenome has a positive-sense sequence denoted by SEQ ID NO: 1 modified by a deletion in the M2-2 ORF comprising a deletion of 234 nucleotides located at positions 8203-8436 of SEQ ID NO: 1 combined with the presence of 8198A and 8199G of SEQ ID NO: 1.

25 Clause 2. The isolated polynucleotide molecule of clause 1, wherein the RSV genome or antigenome recited in a and b has a positive-sense sequence denoted by a sequence that is at least 90% identical to a sequence denoted by SEQ ID NO: 1.

Clause 3. The isolated polynucleotide molecule of clause 1 or 2, wherein the RSV genome or antigenome is further modified by a deletion of 112 nucleotides located at positions 4499-4610 of SEQ
30 ID NO: 1 combined with the mutations C4489T, C4492T, A4495T, A4497G, and G4498A of SEQ ID NO: 1 ("6120").

Clause 4. An isolated polynucleotide molecule encoding a recombinant respiratory syncytial virus (RSV) variant having an attenuated phenotype, comprising a RSV genome or antigenome

having a positive-sense sequence denoted by a sequence that is at least 90% identical to SEQ ID
35 NO: 1, wherein the RSV genome or antigenome is modified by a deletion in the M2-2 ORF corresponding to a deletion comprising a deletion of 234 nucleotides located at positions 8203-8436 of SEQ ID NO: 1,

wherein the RSV genome or antigenome is further modified by a deletion of 112 nucleotides located at positions 4499-4610 of SEQ ID NO: 1 combined with the mutations C4489T, C4492T, A4495T, A4497G, and G4498A of SEQ ID NO: 1.

Clause 5. The isolated polynucleotide molecule of clause 1, 2, 3 or 4, wherein the RSV genome or antigenome is further modified by introduction of one or more of the following changes to SEQ ID NO: 1:

mutations encoding amino acid substitutions V267I in the N protein, E218A and T523I in the F protein, and C319Y and H1690Y in the L protein of the RSV (“cp”);

mutations encoding amino acid substitutions K66E and Q101P in the F protein of the RSV (“HEK”);

a deletion of 419 nucleotides located at positions 4198-4616 of SEQ ID NO: 1 which encodes a deletion of the SH protein of the RSV (Δ SH);

a mutation encoding amino acid substitution K51R in the NS2 protein of the RSV (“NS2”);

a mutation encoding amino acid substitution T24A in the N protein of the RSV (“N”);

the nucleotide sequence encoding the G protein of the RSV is replaced with a corresponding codon optimized nucleotide sequence encoding the G protein from the clinical isolate A/Maryland/001/11;

the nucleotide sequence encoding the F protein of the RSV is replaced with a corresponding nucleotide sequence encoding the F protein from the clinical isolate A/Maryland/001/11; or a corresponding codon optimized nucleotide sequence encoding the F protein from the clinical isolate A/Maryland/001/11; or the codon optimized sequence FBB (“FBB”); and

the order of the nucleotide sequences encoding the G and the F proteins of the RSV in SEQ ID NO: 1 is reversed.

Clause 6. The isolated polynucleotide molecule of clause 1(a) or 2, comprising a nucleotide sequence of SEQ ID NO: 2.

Clause 7. The isolated polynucleotide molecule of clause 1(b) or 2, comprising a nucleotide sequence of SEQ ID NO: 3.

Clause 8. The isolated polynucleotide molecule of clause 1(c), comprising a nucleotide sequence of SEQ ID NO: 4.

Clause 9. The isolated polynucleotide molecule of clause 3 comprising a nucleotide sequence of SEQ ID NO: 5.

Clause 10. The isolated polynucleotide molecule of clause 3 or 4 comprising a nucleotide sequence of SEQ ID NO: 6.

Clause 11. The isolated polynucleotide molecule of clause 3 or 4 comprising a nucleotide sequence of SEQ ID NO: 7.

Clause 12. The isolated polynucleotide molecule of clause 5, wherein the modified RSV genome or antigenome comprises a combination of mutations selected from the group consisting of:

cp/ Δ M2-2, cp/ Δ M2-2/HEK, Δ M2-2/1030s, NS2/N/ Δ M2-2, NS2/ Δ M2-2, N/ Δ M2-2, NS2/N/ Δ M2-2-*AcII*, Δ SH/ Δ M2-2, cp/ Δ SH/ Δ M2-2, 6120/cp/ Δ M2-2, 6120/ Δ M2-2/1030s, 6120/NS2/N/ Δ M2-2, 6120/G001BB/FBB/ Δ M2-2, 6120/FBB/G001BB/ Δ M2-2, 6120/G001BB/F/ Δ M2-2, 6120/G/FBB/ Δ M2-2, 6120/G/FBB/HEK/ Δ M2-2, 6120/G/FBB/cp/HEK/ Δ M2-2, 6120/FBB/G/ Δ M2-2, 5 6120/G001BB/F001BB/ Δ M2-2, 6120/NS2/ Δ M2-2, 6120/N/ Δ M2-2, 6120/NS2/N/ Δ M2-2-*AcI*-I, NS2/N/ Δ M2-2-*HindIII*, and 6120/ NS2/N/ Δ M2-2-*HindIII*.

Clause 13. The isolated polynucleotide molecule of clause 1, wherein the RSV genome or antigenome comprises a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, further modified by introduction of one or more of the following nucleotide 10 substitutions in SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4: 404C, 779G, 1099T, 1139A, 1140G, 1182G, 1210G, 1939A, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, 13634A, 13901T.

Clause 14. The isolated polynucleotide molecule of any one of clauses 1-12, further comprising a previously characterized RSV mutation or deletion.

15 Clause 15. A vector comprising the isolated polynucleotide molecule of any one of clauses 1-13.

Clause 16. A cell comprising the isolated polynucleotide of any one of clauses 1-13.

Clause 17. A pharmaceutical composition comprising an immunologically effective amount of the recombinant RSV variant encoded by the isolated polynucleotide molecule of any one of clauses 1-13.

20 Clause 18. A method of vaccinating a subject against RSV comprising administering the pharmaceutical composition of clause 16.

Clause 18. The method of clause 17, wherein the pharmaceutical composition is administered intranasally.

25 Clause 20. The method of clause 17, wherein the respiratory syncytial virus is administered via injection, aerosol delivery, nasal spray or nasal droplets.

Exemplary Sequences

Antigenomic cDNA sequence of D46 (SEQ ID NO: 1)

30 ACGGGAAAAAATGCGTACAACAACTTGCATAAACCAAAAAAATGGGGCAAATAAGAATTTGATAAGTACCACTTAA
ATTAACTCCCTTGGTTAGAGATGGGCAGCAATTCATTGAGTATGATAAAAGTTAGATTACAAAATTTGTTTGACAA
TGATGAAGTAGCATTGTTAAAAATAACATGCTATACTGATAAATTAATACATTTAACTAATGCTTTGGCTAAGGCAG
TGATACATACAATCAAATTGAATGGCATTGTGTTTGTGCATGTTATTACAAGTAGTGATATTTGCCCTAATAATAAT
ATTGTAGTAAAATCCAATTTCAACAATGCCAGTACTACAAAATGGAGGTTATATATGGGAAATGATGGAATTAAC
35 ACATTGCTCTCAACCTAATGGTCTACTAGATGACAATTGTGAAATTAAATTTCTCCAAAAAATAAGTGATTCAACAA
TGACCAATTATATGAATCAATTATCTGAATTACTTGGATTTGATCTTAATCCATAAATTATAATTAATATCAACTAG
CAAATCAATGTCACTAACACCATTAGTTAATATAAACTTAACAGAAGACAAAAATGGGGCAAATAAATCAATTCAG
CCAACCCAACCATGGACACAACCCACAATGATAATACACCACAAAGACTGATGATCACAGACATGAGACCGTTGTCA
CTTGAGACCATAATAACATCACTAACCCAGAGACATCATAACACACAAATTTATATACTTGATAAATCATGAATGCAT
40 AGTGAGAAAATTTGATGAAAGACAGGCCACATTTACATTCCTGGTCAACTATGAAATGAACTATTACACAAAGTAG
GAAGCACTAAATATAAAAAATATACTGAATACAACACAAAATATGGCACTTTCCCTATGCCAATATTCATCAATCAT
GATGGGTTCTTAGAATGCATTGGCATTAAAGCCTACAAAGCATACTCCCATAATATACAAGTATGATCTCAATCCATA
AATTTCAACACAATATTCACACAATCTAAAACAACAACCTCTATGCATAACTATACTCCATAGTCCAGATGGAGCCTG
AAAATTATAGTAATTTAAACTTAAGGAGAGATATAAGATAGAAGATGGGGCAAATACAACCATGGCTCTTAGCAAA
45 GTCAAGTTGAATGATACACTCAACAAAGATCAACTTCTGTTCATCCAGCAAATACACCATCCAACGGAGCACAGGAGA
TAGTATTGATACTCCTAATTATGATGTGCAGAAACACATCAATAAGTTATGTGGCATGTTATTAATCACAGAAGATG

CTAATCATAAATTCACCTGGGTTAATAGGTATGTTATATGCGATGTCTAGGTTAGGAAGAGAAGACACCATAAAAATA
 CTCAGAGATGCGGGATATCATGTAAAAGCAAATGGAGTAGATGTAAACAACACATCGTCAAGACATTAATGGAAAAGA
 AATGAAATTTGAAGTGTTAACATTGGCAAGCTTAACAACCTGAAATTCAAATCAACATTGAGATAGAATCTAGAAAAT
 CCTACAAAAAATGCTAAAAGAAATGGGAGAGGTAGCTCCAGAATACAGGCATGACTCTCCTGATTGTGGGATGATA
 5 ATATTATGTATAGCAGCATTAGTAATAACTAAATTAGCAGCAGGGGACAGATCTGGTCTTACAGCCGTGATTAGGAG
 AGCTAATAATGTCTTAAAAAATGAAATGAAACGTTACAAAGGCTTACTACCCAAGGACATAGCCAACAGCTTCTATG
 AAGTGTTTGAAAAACATCCCCACTTTATAGATGTTTTTGTTCATTTTGGTATAGCACAAATCTTCTACCAGAGGTGGC
 AGTAGAGTTGAAGGGATTTTTTGCAGGATTGTTTATGAATGCCTATGGTGCAGGGCAAGTGATGTTACGGTGGGGAGT
 CTTAGCAAAATCGGTTAAAAATATTATGTTAGGACATGCTAGTGTGCAAGCAGAAATGGAACAAGTTGTTGAGGTTT
 10 ATGAATATGCCCAAAAATTGGGTGGTGAAGCAGGATTCTACCATATATTGAACAACCCAAAAGCATCATTATTATCT
 TTGACTCAATTTCTCTCACTTCTCCAGTGTAGTATTAGGCAATGCTGCTGGCCTAGGCATAATGGGAGAGTACAGAGG
 TACACCGAGGAATCAAGATCTATATGATGCAGCAAAGGCATATGCTGAACAACCTCAAAGAAAATGGTGTGATTAAC
 ACAGTGTACTAGACTTGACAGCAGAAGAACTAGAGGCTATCAAACATCAGCTTAATCCAAAAGATAATGATGTAGAG
 CTTTGAGTTAATAAAAAATGGGGCAAATAAATCATCATGGAAAAGTTTGCTCCTGAATTCCATGGAGAAGATGCAAA
 15 CAACAGGGCTACTAAATTCCTAGAATCAATAAAGGGCAAATTCACATCACCCAAAGATCCCAAGAAAAAAGATAGTA
 TCATATCTGTCAACTCAATAGATATAGAAGTAACCAAAGAAAGCCCTATAACATCAAATTCAACTATTATCAACCCA
 ACAATGAGACAGATGATACTGCAGGGAACAAGCCCAATTATCAAAGAAAACCTCTAGTAAGTTTCAAAGAAGACCC
 TACACCAAGTGATAATCCCTTTTTCTAAACTATACAAAGAAACCATAGAAACATTTGATAACAATGAAGAAGAATCCA
 GCTATTCATACGAAGAAATAAATGATCAGACAAACGATAATATAACAGCAAGATTAGATAGGATTGATGAAAAATTA
 20 AGTGAAATACTAGGAATGCTTCACACATTAGTAGTGGAAGTGCAAGGACCTACATCTGCTCGGGATGGTATAAGAGA
 TGCCATGGTTGGTTTAAGAGAAGAAATGATAGAAAAAATCAGAACTGAAGCATTAAATGACCAATGACAGATTAGAAG
 CTATGGCAAGACTCAGGAATGAGGAAAGTGAAAAGATGGCAAAAGACACATCAGATGAAGTGTCTCTCAATCCAACA
 TCAGAGAAATTGAACAACCTATTGGAAGGGAATGATAGTGACAATGATCTATCACTTGAAGATTTCTGATTAGTTAC
 CAATCTTCACATCAACACACAATACCAACAGAAGACCAACAACTAACCAACCCCAATCATCCAACCAACATCCATC
 25 CGCCAATCAGCCAAACAGCCAAACAAAACAACCAGCCAATCCAAAACCTAACCACCCGGAAGAAAATCTATAATATAGTT
 ACAAAAAAAGGAAAGGGTGGGGCAAATATGGAAACATACGTGAACAAGCTTCACGAAGGCTCCACATACACAGCTGC
 TGTTCAATACAATGTCTTAGAAAAAGACGATGACCCTGCATCACTTACAATATGGGTGCCCATGTTCCAATCATCTA
 TGCCAGCAGATTTACTTATAAAAGAACTAGCTAATGTCAACATACTAGTGAAACAAATATCCACACCCCAAGGGACCT
 TCACTAAGAGTCATGATAAACTCAAGAAGTGCAAGTGTAGCACAATGCCCAGCAAATTTACCATATGCGCTAATGT
 30 GTCCTTGATGAAAGAAGCAAACCTAGCATATGATGTAAACCACACCCTGTGAAATCAAGGCATGTAGTCTAACATGCC
 TAAAATCAAAAAATATGTTGACTACAGTTAAAGATCTCACTATGAAGACACTCAACCCTACACATGATATTATTGCT
 TTATGTGAATTTGAAAACATAGTAACATCAAAAAAAGTCATAATACCAACATACCTAAGATCCATCAGTGTGAGAAA
 TAAAGATCTGAACACACTTGAAAATATAACAACCCTGAATTCAAAAATGCTATCACAAATGCAAAAAATCATCCCTT
 ACTCAGGATTACTATTAGTCATCACAGTGAAGTGAACAACAAAGGAGCATTCAAATACATAAAGCCACAAAGTCAATTC
 35 ATAGTAGATCTTGGAGCTTACCTAGAAAAAGAAAGTATATATTATGTTACCACAAATTGGAAGCACACAGCTACACG
 ATTTGCAATCAAACCCATGGAAGATTAACCTTTTTCTCTACATCAGTGTGTTAATTCATACAACTTTCTACCTAC
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55 **Antigenomic cDNA sequence of D46/ Δ M2-2 (SEQ ID NO: 2)**

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Antigenomic cDNA sequence of D46/ Δ M2-2-Ac1I (SEQ ID NO: 3)

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Antigenomic cDNA sequence of D46/ Δ M2-2-HindIII (SEQ ID NO: 4)

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10 **Antigenomic cDNA sequence of LID/ Δ M2-2 (SEQ ID NO: 5)**

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15 **Antigenomic cDNA sequence of LID/ Δ M2-2-AclI (SEQ ID NO: 6)**

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20 **Antigenomic cDNA sequence of LID/ Δ M2-2-HindIII (SEQ ID NO: 7)**

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25 Polynucleotide sequence encoding G001BB (SEQ ID NO: 8)

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Polynucleotide sequence encoding FBB (SEQ ID NO: 9)

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Polynucleotide sequence encoding F001 (SEQ ID NO: 10)

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Polynucleotide sequence encoding F001BB (SEQ ID NO: 11)

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Antigenomic cDNA sequence of D46/cp/ Δ M2-2 (SEQ ID NO: 15)

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60 **Antigenomic cDNA sequence of LID/ Δ M2-2/1030s (SEQ ID NO: 16)**

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Antigenomic cDNA sequence of LID/cp/ Δ M2-2 (SEQ ID NO: 17)

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15 **Antigenomic cDNA sequence of RSV 276 genome (SEQ ID NO: 19)**

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In summary, the materials, information, and methods described in this disclosure provide an
 array of attenuated strains with graded attenuation phenotypes, and provide guidance in selecting suitable
 vaccine candidate strains based on clinical benchmarks. The following examples are provided by way of
 25 illustration, not limitation.

EXAMPLES

1. The recombinant RSV strains exemplified in this disclosure were derived from the recombinant version of wt strain A2 that is called D46 (Collins, *et al.* 1995. Proc Natl Acad Sci USA 30 92:11563-11567). The complete nucleotide sequence of D46 is shown as SEQ ID NO: 1. The RSV MEDI/ΔM2-2 virus is not derived from D46.
2. In the examples below, when a virus name includes the term “LID” or “6120”, this indicates that its backbone contains the “6120” mutation shown in FIG. 3.
3. Viruses are named herein by listing the combination of mutations present in them. The use of 35 the symbol “/” in a virus name (as in RSV D46/cp/ΔM2-2 which denotes RSV D46 comprising the mutations cp and ΔM2-2) has no significance apart from being present to make the name easier to read, particularly when present in text. Hence, RSV D46/cp/ΔM2-2 is the same as RSV D46cpΔM2-2. Also, RSV D46/cp/ΔM2-2 also is the same as RSV D46cpΔM2-2 or RSV D46 cpΔM2-2, etc. Also, a virus name typically begins with RSV, as in RSV D46/cp/ΔM2-2.
- 40 4. As noted previously, the “ΔM2-2” mutation refers to the 241-nucleotide deletion together with the three point mutations as shown in FIG. 1. Other mutations that silence the M2-2 ORF are specified by different names, e.g. ΔM2-2-*AcII* and ΔM2-2-*HindIII* shown in FIG. 10. The presence of “ΔM2-2” in virus names in this disclosure indicates the presence of the “ΔM2-2” mutation.
5. The magnitude of virus replication *in vivo* is used as an indication of virus attenuation:
 45 specifically, decreased replication *in vivo* is used as an indicator of increased attenuation, and vice versa. This reflects the general observation that increased RSV replication is associated with increased illness

both for wt RSV infection (e.g., El Saleeby, *et al.* 2011. J Infect Dis 204:996-1002; DeVincenzo, *et al.* 2010. Am J Respir Crit Care Med 182:1305-1314) and for attenuated RSV candidates in clinical studies (e.g., Karron, *et al.* 1997. J Infect Dis 176:1428-1436; Karron, *et al.* 2005. J Infect Dis 191:1093-1104). These terms are used for descriptive purposes, rather than as a limiting definition.

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Example 1

This example illustrates design and construction of novel RSV variants bearing a deletion in the M2-2 ORF (Δ M2-2), alone and in combination with additional mutations.

Representative viruses were constructed and evaluated pre-clinically. One representative virus of this panel, and a second M2-2 ORF mutant virus from another source, were evaluated in a phase 1 clinical study in seronegative infants and young children, which constitute the primary pediatric RSV vaccine target group. This example provides new vaccine strains together with clinical benchmarks for representative examples in the most relevant human population.

RSV rA2-K5 virus. A RSV strain called RSV rA2-K5 was previously constructed (from the parental wt D46 cDNA-derived virus), in which expression of the M2-2 ORF was silenced by a combination of three types of mutations: (i) introduction of a frame shift midway through the M2-2 ORF, (ii) changing the three potential ATG translational start codons of the M2-2 ORF (see FIG. 1A for a diagram of the RSV genome and overlapping M2-1 and M2-2-ORFs) into ACG codons, and (iii) introducing stop codons into all three registers of the M2-2 sequence shortly after the end of the M2-1 ORF (Bermingham and Collins. 1999. Proc Natl Acad Sci USA 96:11259-11264). This rA2-K5 virus (which is not illustrated in this disclosure) was evaluated for replication in the respiratory tract of seronegative chimpanzees, showing that it was restricted at least 2800-fold in the upper respiratory tract and was not detected in the lower respiratory tract (representing a reduction of at least 55,000-fold) (Teng, *et al.* 2000. J Virol 74:9317-9321).

Creation of RSV D46/ Δ M2-2 and RSV LID/ Δ M2-2. Additional recombinant virus was constructed in which most of the M2-2 ORF was deleted. The wt D46 cDNA was modified so that each of the three potential translational ATG start codons for the M2-2 ORF was changed to ACG, and nucleotides 8188-8428 were deleted (total deletion of 241 nt), removing most of the M2-2 ORF (FIG. 1). Thus, with all of the known potential ATG translational start sites mutated and most of the ORF deleted, expression of complete M2-2 protein should not be possible, and there should be little or no expression of any truncated M2-2 fragments. A gene map of the resulting RSV D46/ Δ M2-2 virus is shown in FIG. 2.

Additional Δ M2-2 mutants were constructed that would have a range of attenuation phenotypes. Because the prototype RSV rA2-K5 virus was highly attenuated in chimpanzees, as noted above (Teng, *et al.* 2000. J Virol 74:9317-9321), it was possible that a virus in which M2-2 was not expressed might be over-attenuated. On the other hand, it also was possible that it might be under-attenuated, particularly in seronegative infants and young children. Therefore, additional viral variants were constructed to identify derivatives with increased as well as decreased replication.

There was no established method for increasing the replication of an RSV strain, and in particular a Δ M2-2 mutant. It was previously reported that moving the G and F genes from being the 6th and 7th genes in the gene order (this was done in a virus in which the SH gene had been deleted, and thus G and F were the 6th and 7th genes rather than their native positions as 7th and 8th) to being the 1st and 2nd genes, respectively, resulted in a ~10-fold increase in replication *in vitro*, although there was not a statistically significant increase in replication in mice (Krempl, *et al.* 2002. J Virol 76:11931-11942) or AGMs. One limitation is that the established pre-clinical assays for evaluating RSV replication and attenuation (e.g., replication in cell lines, rodents, and non-human primates other than chimpanzees) may be relatively semi-permissive and insensitive, making it difficult to demonstrate statistically significant changes in replication efficiency, and therefore any change in replication seems noteworthy even if it is not detected in every assay. Therefore, it was attempted to modify a Δ M2-2/ Δ SH virus to move the G and F genes to the promoter-proximal positions. (The Δ SH deletion had been included in an initial study in the wild type backbone [Krempl, *et al.* 2002. J Virol 76:11931-11942] to avoid instability in this sequence during plasmid amplification in bacteria and was considered incidental, and the Δ SH deletion also was used with the Δ M2-2-backbone.) Several permutations were evaluated, such as in which the G and F genes were placed as the first and second genes, respectively, or as the second and first genes, respectively. However, these modifications reduced virus replication by 100- to 1000-fold, indicating that these particular changes were not well tolerated in infectious virus. It may be that the increase in expression of G and F known to be associated with movement of their genes to the promoter-proximal locations (Krempl, *et al.* 2002. J Virol 76:11931-11942), combined with an increase in protein expression associated with the Δ M2-2 mutation, was not tolerated by RSV, at least in this Δ M2-2/ Δ SH backbone.

Additionally, it is known that changes in genome length can affect the efficiency of replication. Specifically, it has been shown that increasing the length of a paramyxovirus genome can decrease its replication efficiency. For example, increasing the length of the RSV genome by 140 or 160 nucleotides in a fashion that did not perturb gene expression resulted in a 5- to 25-fold restriction for replication in mice (Bukreyev, Murphy, Collins. 2000. J Virol 74:11017-11026). In another study with a related virus, namely human parainfluenza virus type 3 (PIV3), increasing the genome length either by adding additional genes or by increasing the genome length by inserts in non-translated regions (which thus did not change the gene number) retained efficient replication *in vitro* but was attenuating in hamsters (Skiadopoulos, *et al.* 2000. Virology 272:225-234). It is presumed that attenuation associated with increased genome length occurs because of the greater burden of replicating a longer genome. The observation that increasing the length of the genome reduced replication efficiency suggested the converse idea, namely that reducing the genome length might increase replication efficiency. To this end, the RSV D46/ Δ M2-2 virus was modified to contain a mutation called “6120”, resulting in a virus called RSV LID/ Δ M2-2 (genome diagram shown in FIG. 2, bottom diagram). In this document, “LID” in a virus name indicates the presence of the 6120 mutation.

The “6120” mutation (FIG. 3) involves deletion of 112 nucleotides of the downstream non-translated region of the SH gene and the introduction of five translationally-silent point mutations in the

last three codons and the termination codon of the SH gene (Bukreyev, *et al.* 2001. J Virol 75:12128-12140). The main purpose in the original design of this mutation was to stabilize the antigenomic cDNA in bacteria so that it could be more easily manipulated and prepared, which indeed was the case. In wt RSV, this mutation was previously found to confer a 5-fold increase in replication efficiency *in vitro* (Bukreyev, *et al.* 2001. J Virol 75:12128-12140), whereas it did not appear to increase replication efficiency in mice. When RSV LID/ Δ M2-2 was evaluated for the possibility of increased replication *in vitro* associated with the 6120 mutation, a modest increase in growth efficiency was observed in some experiments but not others.

Inclusion of additional mutations in RSV D46/ Δ M2-2 and RSV LID/ Δ M2-2. A series of further derivatives of the RSV D46/ Δ M2-2 and RSV LID/ Δ M2-2 viruses was constructed in which one or more additional mutations were variously inserted into one or both of the viruses, with the goal of achieving a spectrum of further-attenuated viruses.

Examples of derivatives of RSV D46/ Δ M2-2 are shown in FIG. 4. For example, the derivative RSV D46/cp/ Δ M2-2 virus (FIG. 4, top diagram) combines the Δ M2-2 mutation (FIG. 1) with the “cp” mutations, which is a set of five amino acid substitutions in three proteins (N (V267I), F (E218A and T523I), and L (C319Y and H1690Y)) that together (on their own) confer an approximate 10-fold reduction in replication in seronegative chimpanzees, and a reduction in illness (Whitehead, *et al.* 1998. J Virol 72:4467-4471). The availability of phenotypic data from chimpanzees is noteworthy because this experimental animal approaches humans in its permissiveness to RSV replication and disease. Note that the D46/cp/ Δ M2-2 construct had a single adventitious nucleotide change in the D46 backbone, at the DNA level: specifically there was a silent nucleotide change G3878A, present in the M ORF.

Another derivative, the RSV D46/ Δ M2-2/1030s virus (FIG. 4, second diagram from the top), contains the Δ M2-2 mutation in combination with the genetically stabilized 1030 mutation (“1030s”), which consists of 1321K(AAA)/S1313(TCA) (Luongo, *et al.* 2012. J Virol 86:10792-10804). The 1030s mutation conferred a 0.6 and 1.5 mean log₁₀ reduction in RSV replication in the upper and lower respiratory tract, respectively, of mice. It also has been evaluated in seronegative chimpanzees, but only in combination with a number of additional attenuating mutations (Luongo, *et al.* 2012. J Virol 86:10792-10804).

Another derivative, the RSV D46/cp/ Δ M2-2/HEK virus (FIG. 4, bottom diagram), combines the cp and Δ M2-2 mutations with the “HEK” mutations. The HEK mutations consist of two amino acid substitutions in the RSV F protein, K66E and Q101P, that match the sequence at the amino acid level to an early-passage of the same strain (A2) called HEK-7, which was derived by passaging the original strain A2 clinical isolate seven times on human embryonic kidney (HEK) cells (Connors, *et al.* 1995. Virology 208:478-484; Whitehead, *et al.* 1998. J Virol 72:4467-4471), and which is thought to most closely resemble (and likely be identical to) the original strain A2 clinical isolate (Liang, *et al.* 2014. J Virol 88:4237-4250; Liang, *et al.* 2015. J Virol 89:9499-9510). It was previously shown that the HEK mutations stabilized the F protein trimer and conferred a hypofusogenic phenotype that is thought to resemble that of the original clinical isolate (Liang, *et al.* 2014. J Virol 88:4237-4250; Liang, *et al.* 2015.

J Virol 89:9499-9510). In addition to likely being found in the original strain A2 clinical isolate, the HEK assignments are found in nearly all clinical isolates of RSV subgroup A present in GenBank (Liang, *et al.* 2015. J Virol 89:9499-9510). Thus, the HEK mutations may provide a more authentic and immunogenic form of the RSV F protein, possibly enriched for the highly immunogenic pre-fusion conformation (McLellan *et al.*, Science 2013 340(6136):1113-7; Science 2013 342(6158):592-8.). Thus, rather than necessarily being associated *per se* with attenuation, the HEK mutations provide a version of the F protein that more accurately reflects the original strain A2 clinical isolate as well as clinical isolates of other RSV strains.

Examples of derivatives of RSV LID/ Δ M2-2 are shown in FIG. 5. One derivative, the RSV LID/cp/ Δ M2-2 virus (FIG. 5, top diagram), combines the Δ M2-2 and cp mutations. Another, the RSV LID/ Δ M2-2/1030s virus (FIG. 5, second diagram from the top), combines the Δ M2-2 and 1030s mutations. Another, the RSV Δ SH/ Δ M2-2 virus (FIG. 5, third diagram from the top), combines the Δ M2-2 mutation with deletion of the SH gene (see FIG. 6 for the details of the construction of the SH deletion). Deletion of the SH gene was previously shown to result in a 40-fold reduction in RSV replication in seronegative chimpanzees, and a reduction in illness (Whitehead, *et al.* 1999. J Virol 73:3438-3442). Another derivative, the RSV cp/ Δ SH/ Δ M2-2 virus (FIG. 5, bottom diagram), combines the Δ M2-2 and cp mutations with deletion of the SH gene. Note that viruses in which the entire SH gene was deleted are not referred to as “LID” because deletion of the SH gene removes the 6120 mutation.

All of the mutants in FIGs. 2, 4, and 5 were readily recovered by reverse genetics using standard methods. However, contrary to expectations based on previous work (e.g., Bukreyev *et al.*, J Virol 1997 71:8973-8982; Whitehead *et al.* J Virol 73:3438-3442 1999), viruses that contained a Δ SH mutation in the context of a Δ M2-2 mutation replicated approximately 10-fold less efficiently than other Δ M2-2 mutants. This finding illustrates how unanticipated but important effects can emerge when viruses are actually made and evaluated.

Regarding the attenuating mutations noted in FIGs. 4 and 5, previous studies in chimpanzees of the cp, Δ SH, 1030 (the parent of 1030s), and Δ M2-2 mutations indicated that their order of increasing attenuation is: cp $\approx$ Δ SH < 1030s < Δ M2-2 (Whitehead, *et al.* 1999. J Virol 73:3438-3442; Whitehead, *et al.* 1999. J Virol 73:871-877; Teng, *et al.* 2000. J Virol 74:9317-9321). Thus the cp, Δ SH, and 1030s mutations, when combined singly or in combination with a Δ M2-2 mutation, provide a range of increasing levels of added attenuation. These may be evaluated in a clinical study in the relevant vaccine target population (seronegative infants and young children).

Example 2

This example describes preclinical evaluation of LID and D46 Δ M2-2 viruses.

Representative viruses of the disclosure were evaluated for replication in the respiratory tract of BALB/c mice, namely: RSV D46 wt, RSV LID/ Δ M2-2, RSV Δ SH/ Δ M2-2, RSV LID/ Δ M2-2/1030s, and RSV cp/ Δ SH/ Δ M2-2 (FIG. 7). Animals were inoculated intranasally with 5.8 log₁₀ of the indicated virus and sacrificed on days 4 and 5 post-inoculation, and nasal turbinates and lungs were harvested,

homogenized, and evaluated by RT-qPCR, which provides for more sensitive detection than assaying for infectious particles and thus is useful for this semi-permissive experimental animal. This showed that all of the viruses containing attenuating mutations were more restricted than the wt D46 virus. Thus, the further addition of the various attenuating mutations was further attenuating, although the level of replication in this semi-permissive rodent model was so restricted that detailed comparisons were not feasible (FIG. 7).

The same four Δ M2-2-containing viruses were investigated for replication in the respiratory tract of AGMs, namely: RSV LID/ Δ M2-2, RSV Δ SH/ Δ M2-2, RSV LID/ Δ M2-2/1030s, and RSV cp/ Δ SH/ Δ M2-2 (FIGs. 8A and 8B, Tables 1-3). The AGM is a more authentic model for RSV replication than are rodents because of its closer phylogenetic and anatomical relatedness to the natural human host for RSV. In addition, AGMs support somewhat-higher levels of RSV replication compared to rhesus macaques and cynomolgous monkeys, and therefore appear to be the most suitable available monkey model. However, AGMs are nonetheless only semi-permissive for RSV replication, and the level of RSV replication in AGMs is substantially less than in chimpanzees or humans. AGMs in groups of four were inoculated by the combined IN and IT routes with 6 log₁₀ PFU per ml per each of the two sites (IN and IT). NP swabs were taken daily on days 1-10 and 12, and tracheal lavages were taken on days 2, 4, 6, 8, 10, and 12 (Tables 1 and 2). This showed that all three viruses that contained one or more additional attenuating mutations were more attenuated than RSV LID/ Δ M2-2. In particular, the RSV Δ M2-2/1030s virus appeared to be the most attenuated. All three of these viruses induced titers of RSV-neutralizing serum antibodies that were approximately 2-fold (RSV Δ SH/ Δ M2-2 and RSV cp/ Δ SH/ Δ M2-2) or 4-fold (RSV LID/ Δ M2-2/ Δ 1030s) less than that of RSV LID/ Δ M2-2 (Table 3), consistent with the general expectation that a decreased level of replication and resulting decreased antigenic load can be associated with decreased immunogenicity, and thus care should be taken to identify an RSV vaccine candidate that is well-tolerated but is sufficiently immunogenic.

Table 1. Viral titers of nasopharyngeal swab samples from AGMs inoculated with RSV LID/ Δ M2-2, RSV Δ SH/ Δ M2-2, RSV LID/ Δ M2-2/1030s, or RSV cp/ Δ SH/ Δ M2-2^a.

RSV Vaccine candidate	AGM ID	NP virus titer (log ₁₀ PFU/mL) on indicated days ^b												Duration of shedding ^c	Peak virus titer	Sum of daily titers ^d
		1	2	3	4	5	6	7	8	9	10	12				
RSV LID/ ΔM2-2	7806	-	1.4	1.7	2.7	2.6	<u>4.0</u>	3.9	1.4	-	2.7	-	9	4.0	21.4	
	7705	-	-	-	2.7	2.3	<u>3.6</u>	2.4	1.2	-	-	-	5	3.6	14.3	
	7747	-	-	1.3	0.7	-	<u>1.5</u>	1.3	-	-	-	-	5	1.5	7.2	
	7674	-	0.7	-	-	-	<u>2.3</u>	1.8	1.5	-	-	-	7	2.3	8.8	
	Mean:												6.5	2.9	12.9	
RSV ΔSH/ ΔM2-2	7811	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9	
	7796	-	-	-	-	1.4	-	-	-	-	-	-	1	1.4	4.9	
	7789	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9	
	7808	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9	
	Mean:												0.3	0.6	4.1	

RSV LID/ΔM2- 2/ 1030s	8033	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	7720	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	7844	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	7847	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	Mean:												0	0.35	3.9
RSV cp/ ΔSH/ΔM2- 2	8008	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	7741	-	-	-	1.2	1.0	-	0.7	-	1.2	-	-	6	1.2	6.6
	7765	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	7637	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	Mean:												1.5	0.6	4.5

^aAGMs were inoculated by the combined intranasal and intratracheal routes with 6.0 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose: 6.3 log₁₀ PFU per animal).

^bCombined NP swabs were placed in 2 mL of L-15 medium with sucrose phosphate buffer as stabilizer.

Virus titrations were performed on Vero cells at 37°C. The lower limit of detection was 0.7 log₁₀ PFU/mL. Samples with no detectable virus are represented as “-”. Peak titers for each animal are underlined.

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

^dThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). A value of 0.35 was used for samples with no detectable virus.

Table 2. Viral titers of tracheal lavage samples from AGMs inoculated with RSV LID/ΔM2-2, RSV ΔSH/ΔM2-2, RSV LID/ΔM2-2/1030s, or RSV cp/ΔSH/ΔM2-2^a.

RSV vaccine candidate	AGM ID	Tracheal lavage virus titer (log₁₀ PFU/mL) on indicated days^b						Duration of shedding^c	Peak virus titer	Sum of daily titers^d
		2	4	6	8	10	12			
RSV LID/ ΔM2-2	7806	2.5	3.4	<u>4.6</u>	-	-	-	7	<u>4.6</u>	12.6
	7705	1.6	-	<u>3.3</u>	1.5	-	-	9	<u>3.3</u>	8.5
	7747	1.8	1.0	<u>6.0</u>	2.3	-	-	9	<u>6.0</u>	12.5
	7674	-	1.3	<u>2.7</u>	2.3	1.0	-	9	<u>2.7</u>	8.7
	Mean:							9.0	4.2	10.6
RSV ΔSH/ ΔM2-2	7811	-	-	-	-	1.3	-	3	1.3	4.8
	7796	-	-	-	-	-	-	0	0.7	4.2
	7789	-	-	-	-	-	-	0	0.7	4.2
	7808	-	1.6	-	-	-	-	3	1.6	5.1
	Mean:							1.5	1.1	4.5
RSV LID/ΔM2-2/ 1030s	8033	-	-	-	-	-	-	0	0.7	4.2
	7720	-	-	-	-	-	-	0	0.7	4.2
	7844	-	-	-	-	-	-	0	0.7	4.2
	7847	-	-	-	-	-	-	0	0.7	4.2
	Mean:							0	0.7	4.2

RSV cp/ΔSH/ΔM2-2	8008	-	-	-	-	-	-	0	0.7	4.2
	7741	-	-	-	-	-	-	0	0.7	4.2
	7765	-	-	-	-	-	-	0	0.7	4.2
	7637	1.0	-	-	-	-	-	3	1.0	4.5
	Mean:							0.8	0.8	4.3

^aAGMs were inoculated by the combined intranasal and intratracheal routes with 6.0 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose: 6.3 log₁₀ PFU per animal). The AGM study was approved by the Animal Care and Use Committee of NIAID, NIH.

^bOn days 2, 4, 6, 8, 10, and 12, tracheal lavage was performed with 3 mL of PBS. Virus titrations were performed on Vero cells at 37°C. The lower limit of detection was 1.0 log₁₀ PFU/mL of lavage solution. Samples with no detectable virus are represented as “-”. Peak titers for each animal are underlined.

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

^dThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). A value of 0.7 was used for samples with no detectable virus.

Table 3. Neutralizing antibody titers of from AGMs inoculated with RSV LID/ Δ M2-2, RSV Δ SH/ Δ M2-2, RSV LID/ Δ M2-2/1030s, or RSV cp/ Δ SH/ Δ M2-2^a

RSV Vaccine candidate	AGM ID	Neutralizing antibody titers (PRNT ₆₀ , reciprocal log ₂) on indicated days ^b		
		0	21	28
RSV LID/ΔM2-2	7806	<3.3	7.2	7.2
	7705	<3.3	8.8	8.2
	7747	<3.3	8.3	8.4
	7674	<3.3	6.7	6.2
	Mean:	<3.3	7.8	7.5
RSV ΔSH/ΔM2-2	7811	<3.3	6.9	5.9
	7796	<3.3	7.2	7.1
	7789	<3.3	6.5	5.8
	7808	<3.3	7.1	7.2
	Mean:	<3.3	6.9	6.5
RSV LID/ΔM2-2/1030s	8033	<3.3	5.4	6.6
	7720	<3.3	<3.3	<3.3
	7844	<3.3	<3.3	4.3
	7847	<3.3	6.8	6.8
	Mean:	<3.3	4.7	5.2
RSV cp/ΔSH/ΔM2-2	8008	<3.3	6.3	6.8
	7741	<3.3	6.4	5.8
	7765	<3.3	6.0	5.9
	7637	<3.3	6.3	6.3
	Mean:	<3.3	6.3	6.2

^aAGMs were inoculated i.n. and i.t. with 6.0 log₁₀ of the indicated virus in a 1 mL inoculum per site (total dose = 6.3 log₁₀ PFU per animal).

^bOn days 0, 21, and 28 p.i., serum was obtained. Neutralizing antibody titers were determined in a 60% plaque reduction neutralization assay. The lower limit of detection was 3.3 (1:10).

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Another experiment was performed in AGMs to compare RSV LID/ΔM2-2 with the following three viruses: D46/ΔM2-2, RSV D46/cp/ΔM2-2, D46/cp/ΔM2-2/HEK (Tables 4-6). This showed that the RSV LID/ΔM2-2 virus replicated substantially more efficiently in the upper (Table 4) and lower (Table 5) respiratory tracts than RSV D46/ΔM2-2. Importantly, since the only difference between these viruses was the 6120 mutation present in RSV LID/ΔM2-2, this showed that the 6120 mutation conferred increased replication in a primate host. It therefore provides a means to incrementally reduce the level of viral restriction and attenuation. Thus, the LID and D46 backbones provide a substantial difference in replication efficiency, such that the inclusion of additional mutations into either backbone can provide a range of attenuation phenotypes. RSV D46 viruses with additional mutations, namely RSV D46/cp/ΔM2-2/HEK and RSV D46/cp/ΔM2-2, had substantially reduced replication, indicative of increased attenuation. All of the viruses induced substantial titers of RSV-neutralizing serum antibodies (Table 6). RSV LID/ΔM2-2 induced the highest titers; the titers induced by RSV D46/ΔM2-2 and RSV D46/cp/ΔM2-2 were lower by less than 2-fold, and the titer induced by RSV D46/cp/ΔM2-2/HEK was almost 6-fold lower. This indicated that the inclusion of mutations specifying varying degrees of attenuation yielded a range of attenuation phenotypes. It also provided a further indication that reduced replication can result in reduced immunogenicity.

20

Table 4. Viral titers of nasopharyngeal swab samples from AGMs inoculated with D46/cp/ΔM2-2/HEK, D46/cp/ΔM2-2, D46/ΔM2-2, or RSV LID/ΔM2-2^a.

RSV Vaccine candidate	AGM ID	NP virus titer (log ₁₀ PFU/mL) on indicated days ^b											Duration of shedding ^c	Peak virus titer	Sum of daily titers ^d
		1	2	3	4	5	6	7	8	9	10	12			
D46/cp/ ΔM2-2/HEK	8401	-	-	-	-	<u>0.7</u>	-	-	-	-	-	-	1	0.7	4.2
	8195	-	-	-	-	-	-	-	<u>2.0</u>	-	0.7	-	3	2.0	5.8
	7867	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	8392	-	-	-	0.7	<u>1.0</u>	0.7	-	-	-	-	-	3	1.0	5.2
	Mean:											1.8	1.0	4.8	
D46/cp/ ΔM2-2	57413	-	-	-	-	-	-	-	<u>0.7</u>	-	-	-	1	0.7	4.2
	8054	-	-	-	-	<u>1.2</u>	-	-	-	-	-	-	1	1.2	4.7
	8172	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	8445	-	-	-	-	-	-	-	<u>0.7</u>	-	-	-	1	0.7	4.2
	Mean:											0.8	0.7	4.2	
D46/ΔM2-2	8279	-	-	-	-	-	-	<u>1.2</u>	<u>1.2</u>	-	-	-	2	1.2	5.5
	32956	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	8246	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9

	7856	-	-	-	-	-	-	<u>1.9</u>	-	-	-	-	1	1.9	5.4
	Mean:												0.8	1.0	4.7
RSV LID/ ΔM2-2	62403	-	1.2	1.9	3.6	3.6	<u>4.2</u>	3.1	1.7	-	-	-	7	4.2	20.7
	8258	-	1.7	3.0	<u>3.2</u>	2.5	1.5	2.7	2.3	1.4	-	-	8	3.2	19.4
	8232	-	2.2	3.0	<u>3.8</u>	3.1	1.5	2.8	2.4	1.7	-	-	8	3.8	21.7
	582126	0.7	2.0	3.0	3.7	<u>3.8</u>	1.6	2.5	2.1	1.0	-	-	9	3.8	22.4
	Mean:												8	3.7	21.0

^aAGMs were inoculated by the combined intranasal and intratracheal routes with 6.7 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose: 7.0 log₁₀ PFU per animal). The AGM study was approved by the Animal Care and Use Committee of NIAID, NIH.

^bCombined NP swabs were placed in 2 mL of L-15 medium with sucrose phosphate buffer as stabilizer.

5 Virus titrations were performed on Vero cells at 32°C. The lower limit of detection was 0.7 log₁₀ PFU/mL. Samples with no detectable virus are represented as “-”. Peak titers for each animal are underlined.

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

10 ^dThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). A value of 0.35 was used for samples with no detectable virus.

Table 5. Viral titers of tracheal lavage samples from AGMs inoculated with D46/cp/ΔM2-2/HEK, D46/cp/ΔM2-2, D46/ΔM2-2, or RSV LID/ΔM2-2^a.

RSV vaccine candidate	AGM ID	Tracheal lavage virus titer (log ₁₀ PFU/mL) on indicated days ^b						Duration of shedding ^c	Peak virus titer	Sum of daily titers ^d
		2	4	6	8	10	12			
D46/cp/ ΔM2-2/HEK	8401	<u>1.5</u>	-	-	-	-	-	3	1.5	5.0
	8195	1.3	-	-	<u>1.7</u>	-	-	9	1.7	5.8
	7867	-	-	<u>1.0</u>	<u>1.0</u>	-	-	5	1.0	4.8
	8392	<u>1.5</u>	-	1.0	1.3	-	-	9	1.5	5.6
	Mean:							6.5	1.4	5.3
D46/cp/ ΔM2-2	57413	<u>1.0</u>	-	-	-	-	-	3	1.0	4.5
	8054	-	-	<u>1.0</u>	-	-	-	3	1.0	4.5
	8172	<u>1.6</u>	-	-	-	-	-	3	1.6	5.1
	8445	-	1.6	<u>2.2</u>	-	-	-	5	2.2	6.6
	Mean:							3.5	1.5	5.4
D46/ΔM2-2	8279	-	-	-	-	-	-	0	0.7	4.2
	32956	1.7	<u>1.8</u>	-	-	-	-	5	1.8	6.3
	8246	1.0	-	<u>1.6</u>	-	-	-	7	1.6	5.4
	7856	-	-	-	-	<u>1.0</u>	-	3	1.0	4.5
	Mean:							3.8	1.3	5.1
	62403	1.0	3.5	<u>3.9</u>	1.0	-	-	7	3.9	10.5

RSV LID/ ΔM2-2	8258	1.0	1.7	1.0	<u>2.9</u>	1.8	-	9	2.9	8.8
	8232	1.6	<u>4.2</u>	3.1	2.7	-	-	9	4.2	12.9
	582126	1.6	2.4	2.5	<u>2.8</u>	-	-	9	2.8	10.7
	Mean:							8.5	3.5	10.7

^aAGMs were inoculated by the combined intranasal and intratracheal routes with 6.7 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose: 7.0 log₁₀ PFU per animal).

^bOn days 2, 4, 6, 8, 10, and 12, tracheal lavage was performed with 3 mL of PBS. Virus titrations were performed on Vero cells at 32°C. The lower limit of detection was 1.0 log₁₀ PFU/mL of lavage solution. Samples with no detectable virus are represented as “-”. Peak titers for each animal are underlined.

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

^dThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). A value of 0.7 was used for samples with no detectable virus.

Table 6. Neutralizing Antibody Titers of AGMs inoculated with D46/cp/ΔM2-2/HEK, D46/cp/ΔM2-2, D46/ΔM2-2, or RSV LID/ΔM2-2^a.

RSV Vaccine candidate	AGM ID	Neutralizing antibody titers (PRNT ₆₀ , reciprocal log ₂) on indicated days ^b			
		0	14	21	28
D46/cp/ ΔM2-2/HEK	8401	<3.3	<5.3	5.3	6.3
	8195	<3.3	<5.3	6.9	7.1
	7867	<3.3	<5.3	7.1	7.4
	8392	<3.3	<5.3	5.3	5.9
	Mean:	<3.3	<5.3	6.2	6.7
D46/cp/ΔM2-2	57413	<3.3	5.8	6.8	7.9
	8054	<3.3	7.3	9.9	10.6
	8172	<3.3	<5.3	8.0	8.6
	8445	<3.3	6.0	7.7	8.0
	Mean:	<3.3	6.1	8.1	8.8
D46/ΔM2-2	8279	<3.3	<5.3	8.3	7.9
	32956	<3.3	6.1	8.6	8.1
	8246	<3.3	5.8	8.4	8.6
	7856	<3.3	5.6	8.2	9.1
	Mean:	<3.3	5.7	8.4	8.4
RSV LID/ΔM2-2	62403	<3.3	5.9	7.8	8.7
	8258	<3.3	<5.3	7.6	8.8
	8232	<3.3	7.8	8.7	9.0
	582126	<3.3	8.2	9.4	10.2
	Mean:	<3.3	6.8	8.4	9.2

^aAGMs were inoculated i.n. and i.t. with $6.7 \log_{10}$ PFU of the indicated virus in a 1 mL inoculum per site (total dose = $7.0 \log_{10}$ PFU per animal).

^bOn days 0, 21, and 28 p.i., serum was obtained. Neutralizing antibody titers were determined in a 60% plaque reduction neutralization assay. The lower limit of detection was 3.3 (1:10).

5

Example 3

This example describes the clinical evaluation of RSV MEDI/ΔM2-2 virus.

Another RSV mutant with modified M2-2 designated as RSV MEDI/ΔM2-2 was previously described (Jin, *et al.* 2000. J Virol 74:74-82). The RSV MEDI/ΔM2-2 virus was made by introducing
 10 *Hind*III sites at nucleotide positions 8197-8201 and 8431-8436 in the antigenomic cDNA, followed by *Hind*III restriction digestion and ligation to delete the intervening 234 nucleotides from the M2-2 ORF (Jin, *et al.* 2000. J Virol 74:74-82). Thus, the RSV MEDI/ΔM2-2 virus does not contain the “ΔM2-2” mutation as described herein (see, for example, FIG. 1). This virus was not derived from D46 (which was developed from preparations of the RSV A2 strains by Collins and colleagues (Collins *et al.* Proc
 15 Natl Acad Sci USA 1995 92:11563-11567); instead it was derived from a different preparation of the RSV A2 strain (Example 5 below describes the differences between these backbones). Clinical trial material (CTM) of RSV MEDI/ΔM2-2 suitable for human evaluation as a live attenuated intranasal vaccine was manufactured. The nucleotide sequence of the RSV MEDI/ΔM2-2 CTM was determined and was found to be identical to that of its cDNA clone of origin except for dimorphisms (mixtures of
 20 two different nucleotide assignments) at three sequence positions: (i) nucleotide 285, in the NS1 gene, was a mixture of A/G in the CTM compared to A in the cDNA, resulting in a mixture of amino acid assignments S/G in the CTM compared to S in the cDNA; (ii) nucleotide 900, in the NS2 gene, was a mixture of C/T, compared to C in the cDNA, with no effect on amino acid coding; and (iii) nucleotide 4311, in the SH gene, was a mixture of T/G in the CTM compared to T in the cDNA, resulting in a
 25 mixture of amino acid assignments N/K in the CTM, compared to N in the cDNA. It is common to find polymorphisms in an RNA virus and, since the CTM had a high level of infectivity (determined by plaque assay), did not exhibit dimorphism in plaque phenotype, and replicated efficiently *in vitro*, these were deemed likely to be inconsequential. Sequence evaluation of virus shed from experimental animals and clinical subjects may be conducted to determine whether any of these sequence differences was
 30 favored *in vivo*, which will indicate whether any of them are significant. Sequence from one clinical vaccine isolate was obtained, and traces of dimorphisms were still present at all of indicated sites, showing that none of these changes was significant.

RSV MEDI/ΔM2-2 was evaluated as an intranasal vaccine candidate in phase 1 clinical studies successively in adults, RSV seropositive children, and RSV seronegative infants and children 6-24
 35 months of age (ClinicalTrials.gov NCT01459198; Karron, *et al.* 2015. Science Transl Med 2015 7(312):312ra175). The adult study was open-label, and the studies in seropositive and seronegative infants and children were double-blind, randomized, and placebo-controlled. The study was performed at

the Center for Immunization Research (CIR) at the Johns Hopkins University Bloomberg School of Public Health (JHU).

When evaluated in adults and RSV seropositive children, this experimental vaccine was very poorly infectious, very poorly immunogenic, and well-tolerated, as would be expected for an attenuated strain of RSV. In RSV-seronegative infants and young children, 20 vaccinees received a single dose of 5.0 log₁₀ PFU of the RSV MEDI/ΔM2-2 vaccine and 10 subjects received placebo. Rates of fever and cough were similar in vaccinees versus placebo, whereas upper respiratory illness occurred twice as frequently in vaccinees versus placebos (85% versus 44%), although this was not statistically different. There was frequent isolation of various adventitious respiratory viruses from both groups, which likely caused much of the illness and which confounded determination of vaccine tolerability in this particular study. The incidence of infection and disease by adventitious viruses can vary unpredictably between different studies, and in this case the incidence was unusually high and will necessitate further studies to assess the tolerability of RSV MEDI/ΔM2-2. The shedding of vaccine virus in nasal washes was detected by plaque assay in 12/20 recipients and by RT-qPCR in 17/20 recipients. The mean titer of shed virus in those children who shed infectious virus was 1.5 log₁₀ PFU/ml (FIG. 9, left hand side). These findings raised the possibility that the vaccine might be overly restricted in replication, since studies generally aim at >90% shedding based on plaque assay, and a mean titer of infectious shed virus of approximately 2.5 log₁₀ PFU/ml. With regard to antibody responses, 19/20 seronegative children had increases of ≥4-fold in RSV-neutralizing serum antibody titers, with a mean titer of 6.6 log₂ (1:97). This suggested that the RSV MEDI/ΔM2-2 virus was substantially immunogenic. However, the observation that only 12/20 subjects shed infectious virus, combined with the low titers of shed vaccine virus, raised the possibility that the RSV MEDI/ΔM2-2 virus had suboptimal replication, and that a M2-2 mutant virus that replicated somewhat more efficiently might be more effective. This is a relevant issue because immune protection of the superficial epithelium of the respiratory tract, where RSV replicates and causes disease, is inefficient, and therefore it is desirable for an RSV vaccine to be as immunogenic as practicable.

Example 4

This example illustrates the clinical evaluation of RSV LID/ΔM2-2.

As noted above, comparison of RSV D46/ΔM2-2 and RSV LID/ΔM2-2 in AGMs indicated that the presence of the “6120” mutation in RSV LID/ΔM2-2 was associated with increased replication (Tables 4 and 5). Further comparison was made of the replication in AGMs of RSV LID/ΔM2-2 versus the CTM of RSV MEDI/ΔM2-2, in parallel with wt RSV (Tables 7-9). Analysis of the shedding of infectious virus in NP swabs (Table 7) or tracheal lavage specimens (Table 8) showed that both viruses were more attenuated than wt RSV evaluated in parallel. However, there was no evident difference in shedding, and hence replication, between RSV LID/ΔM2-2 and RSV MEDI/ΔM2-2. All three viruses (RSV LID/ΔM2-2, RSV MEDI/ΔM2-2, and wt RSV) induced similar titers of RSV-neutralizing serum antibodies (Table 9).

The RSV LID/ Δ M2-2 virus was evaluated in a clinical study to determine whether it might replicate more efficiently in humans than RSV MEDI/ Δ M2-2 and might be more immunogenic. A lot of CTM for RSV LID/ Δ M2-2 suitable for human administration as an experimental intranasal RSV vaccine was manufactured. Nucleotide sequence analysis showed that the CTM had the same sequence as its
 5 cDNA clone, indicating an absence of detectable adventitious mutations during manufacture. Its replication efficiency in Vero cells (which are used for vaccine manufacture) was essentially the same as RSV MEDI/ Δ M2-2. The RSV LID/ Δ M2-2 CTM was evaluated in parallel with wt RSV for replication and immunogenicity in AGMs (Tables 10-12). Titration of infectious virus from NP swabs (Table 10) and tracheal lavage specimens (Table 11) confirmed the attenuated phenotype of the RSV LID/ Δ M2-2
 10 CTM. Nonetheless, the titer of RSV-neutralizing serum antibodies induced by the CTM was nearly the same as that induced by wt RSV (Table 12), indicating that this experimental vaccine retained much of the immunogenicity of its wt parent.

The RSV LID/ Δ M2-2 CTM was evaluated in RSV-seronegative infants and children of 6-24 months of age in a double-blinded placebo-controlled study that was performed with CIR/JHU
 15 (ClinicalTrials.gov NCT02040831) and with seven clinical sites from the International Maternal Pediatric Adolescent AIDS Clinical Trials Network (IMPAACT, ClinicalTrials.gov NCT02237209). In total, 20 subjects received a single dose of $5.0 \log_{10}$ PFU of vaccine, and nine received placebo. With respect to respiratory illness following vaccination, respiratory illnesses occurred frequently in both vaccinees and placebo recipients. The rates of fever, otitis media, upper respiratory illness, lower respiratory illness,
 20 cough, or any respiratory or febrile illness were essentially the same between the two groups. Adventitious viruses, including rhinovirus, adenovirus, parainfluenza, and coronavirus, were detected frequently in both vaccinees and placebo recipients. A single vaccinee experienced a brief episode of mild lower respiratory tract illness (rhonchi) on day 9 that resolved by day 11 and was coincident with shedding of vaccine virus as well as detection of rhinovirus and enterovirus as adventitious agents. Thus,
 25 causality of this clinical illness remains unclear. Infectious shed vaccine virus was recovered from 19/20 vaccinees, with a mean peak titer of $3.4 \log_{10}$ PFU/ml (FIG. 9B).

Thus, the RSV LID/ Δ M2-2 virus was more infectious than RSV MEDI/ Δ M2-2 in the human host based on the number of individuals shedding infectious virus (19/20 versus 12/20) and on the basis of the mean peak titer ($3.4 \log_{10}$ PFU/ml compared to $1.5 \log_{10}$ PFU/ml, which was significantly different). The
 30 RSV LID/ Δ M2-2 virus also replicated more efficiently than a previous lead candidate called rA2cp248/404/1030 Δ SH that had been evaluated in a previous clinical study (Karron, *et al.* 2005. J Infect Dis 191:1093-1104): a number of specimens from this previous study were analyzed side-by-side with specimens from the MEDI/ Δ M2-2 study, showing that rA2cp248/404/1030 Δ SH had a mean peak titer of $2.5 \log_{10}$ (FIG. 9A, right panel). The RSV LID/ Δ M2-2 virus also induced a higher mean titer of RSV-
 35 neutralizing serum antibodies (1:137) compared to RSV MEDI/ Δ M2-2 (1:97) and rA2cp248/404/1030 Δ SH (1:34) analyzed in parallel (Karron, *et al.* 2015. Science Transl Med 2015 7(312):312ra175).

The clinical study described above showed that the RSV LID/ Δ M2-2 virus was more infectious and replicated more efficiently than RSV MEDI/ Δ M2-2 in the human host. It also was more immunogenic. As noted, a difference in the efficiency of virus replication between these two viruses had not been demonstrated reproducibly in cell lines or in AGMs, and greater immunogenicity for LID/ Δ M2-2 versus MEDI/ Δ M2-2 in AGMs also had not been demonstrated. Thus, contrary to pre-clinical studies, RSV LID/ Δ M2-2 provides a more replication-competent, more immunogenic alternative to RSV MEDI/ Δ M2-2.

Additional derivatives of RSV LID/ Δ M2-2 that possess one or more additional attenuating mutations were designed and constructed. Examples of these strains include: RSV LID/cp/ Δ M2-2 (see Example 8), RSV LID/ Δ SH/ Δ M2-2, RSV LID/cp/ Δ SH/ Δ M2-2, and RSV LID/ Δ M2-2/1030s. Based on the previous evaluation of the cp, Δ SH, 1030s, and Δ M2-2 mutations in seronegative chimpanzees, it is expected that the order of increasing attenuation of the present strains would be: RSV LID/cp/ Δ M2-2 $\approx$ RSV LID/ Δ SH/ Δ M2-2 < RSV LID/cp/ Δ SH/ Δ M2-2 < RSV LID/ Δ M2-2/1030s. Additional strains are also provided, such as ones that include mutations described Examples 5-8.

Table 7. Titers of virus in Nasopharyngeal Swab Samples from AGMs Inoculated with RSV LID/ Δ M2-2 or wt RSV rA2^a.

RSV Vaccine candidate	AGM ID	NP virus titer (log ₁₀ PFU/mL) on indicated days ^b												Duration of shedding ^c	Peak virus titer	Sum of daily titers ^d
		1	2	3	4	5	6	7	8	9	10	12				
RSV LID/ ΔM2-2	7845	-	-	-	-	1.2	<u>1.5</u>	0.7	-	-	-	-	3	1.5	6.2	
	7394	-	-	0.7	-	2.1	2.4	<u>2.6</u>	1.9	-	-	-	6	2.6	11.8	
	7802	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9	
	7832	-	-	-	-	-	<u>2.1</u>	-	-	-	0.7	-	5	2.1	6.0	
	Mean:												3.5	1.6	7.0	
RSV MEDI ΔM2-2	7534	-	-	-	-	-	-	<u>0.7</u>	-	-	-	-	1	0.7	4.2	
	7882	-	-	1.2	0.7	0.7	1.0	1.4	1.7	<u>2.2</u>	1.2	-	8	2.2	11.2	
	7568	-	-	-	-	<u>0.7</u>	-	-	-	-	-	-	1	0.7	4.2	
	7890	-	-	2.2	<u>2.3</u>	1.3	-	1.9	0.7	-	-	-	6	2.3	10.5	
	Mean:												4.0	1.5	7.5	
wt RSV rA2	7822	-	-	1.0	1.3	1.0	2.6	<u>2.7</u>	0.7	-	1.0	-	8	2.7	11.7	
	7894	-	0.7	1.0	0.7	1.4	<u>4.0</u>	3.7	1.7	2.0	1.5	1.0	11	4.0	18.1	
	7622	-	-	2.0	1.4	<u>2.5</u>	2.3	-	1.9	1.3	-	-	7	2.5	13.2	
	7831	-	-	-	1.0	-	-	-	-	<u>2.1</u>	-	-	6	2.1	6.3	
	Mean:												8.0	2.9	12.3	

^aAGMs were inoculated by the combined intranasal and intratracheal routes with 6.0 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose: 6.3 log₁₀ PFU per animal). The AGM study was approved by the Animal Care and Use Committee of NIAID, NIH.

^bCombined NP swabs were placed in 2 mL of L-15 medium with sucrose phosphate buffer as stabilizer. Virus titrations were performed on Vero cells at 37°C. The lower limit of detection was 0.7 log₁₀

PFU/mL. Samples with no detectable virus are represented as “-“. Peak titers for each animal are underlined.

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

5 ^dThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). A value of 0.35 was used for samples with no detectable virus

Table 8. Titers of Virus in Tracheal Lavage Samples from AGMs Inoculated with RSV LID/ΔM2-2 or wt RSV rA2^a.

RSV vaccine candidate	AGM ID	Tracheal lavage virus titer (log ₁₀ PFU/mL) on indicated days ^b						Duration of shedding ^c	Peak virus titer	Sum of daily titers ^d
		2	4	6	8	10	12			
RSV LID/ΔM2-2	7845	1.0	1.9	<u>2.2</u>	-	1.3	-	11	2.2	7.7
	7394	1.6	2.5	<u>2.6</u>	-	-	-	7	2.6	9.0
	7802	2.0	1.5	<u>2.3</u>	-	-	-	7	2.3	7.9
	7832	1.0	2.8	<u>4.1</u>	3.3	2.6	-	11	4.1	14.6
	Mean:							9.0	2.8	9.8
RSV MEDI ΔM2-2	7534	1.3	2.1	<u>2.8</u>	2.1	-	-	9	2.8	9.7
	7882	2.4	1.5	<u>2.9</u>	2.3	-	-	9	2.9	10.5
	7568	-	2.0	<u>2.7</u>	1.6	-	-	7	2.7	8.4
	7890	2.4	<u>2.7</u>	2.0	1.9	1.3	-	11	2.7	10.9
	Mean:							9.0	2.8	9.9
wt RSV rA2	7822	2.5	2.5	<u>4.7</u>	2.6	1.3	-	11	4.7	14.2
	7894	3.3	2.9	<u>4.0</u>	3.5	2.0	-	11	4.0	16.4
	7622	2.3	2.8	<u>4.3</u>	2.0	1.0	1.0	13	4.3	13.5
	7831	2.0	3.8	<u>4.3</u>	4.2	2.5	-	11	4.3	17.4
	Mean:							11.5	4.3	15.4

10 ^aAGMs were inoculated by the combined intranasal and intratracheal routes with 6.0 log₁₀ of the indicated virus in a 1 mL inoculum per site (total dose: 6.3 log₁₀ PFU per animal).

^bOn Days 2, 4, 6, 8, 10, and 12, tracheal lavage was performed with 3 mL of PBS. Virus titrations were performed on Vero cells at 37°C. The lower limit of detection was 1.0 log₁₀ PFU/mL of lavage solution. Samples with no detectable virus are represented as “-“. Peak titers for each animal are underlined.

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

^dThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). A value of 0.7 was used for samples with no detectable virus.

Table 9. Neutralizing Antibody Titers of from AGMs inoculated with RSV LID/ Δ M2-2 or WT RSV rA2^a.

RSV Vaccine candidate	AGM ID	Neutralizing antibody titers (PRNT ₆₀ , reciprocal log ₂) on indicated days ^b		
		0	21	28
RSV LID Δ M2-2	7845	<3.3	3.3	6.7
	7394	<3.3	5.8	6.1
	7802	<3.3	8.9	9.7
	7832	<3.3	6.1	6.2
	Mean:	<3.3	6.0	7.2
RSV MEDI Δ M2-2	7534	<3.3	8.7	8.2
	7882	<3.3	6.9	9.1
	7568	<3.3	7	7.5
	7890	<3.3	7.6	8.8
	Mean:	<3.3	7.6	8.4
wt RSV rA2	7822	<3.3	8	8.4
	7894	<3.3	7.8	7.7
	7622	<3.3	6.9	8
	7831	<3.3	5.7	6.1
	Mean:	<3.3	7.1	7.6

^a AGMs were inoculated i.n. and i.t. with 6.0 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose = 6.3 log₁₀ PFU per animal).

5 ^b On Days 0, 21, and 28 p.i., serum was obtained. Neutralizing antibody titers were determined in a 60% plaque reduction neutralization assay. The lower limit of detection was 3.3 (1:10).

Table 10. Titers of virus in nasopharyngeal swab samples from AGMs inoculated with the CTM RSV LID/ Δ M2-2, or with recombinant wt RSV rA2.

Virus ^a	AGM ID	NP virus titer (log ₁₀ PFU/mL) on indicated days ^b												Peak virus titer	Sum of daily titers ^c
		1	2	3	4	5	6	7	8	9	10	12			
RSV LID/ΔM2-2	7728	-	-	-	-	-	0.7	-	-	-	<u>1.2</u>	-	1.2	5.0	
	7833	-	-	3.6	2.6	3.6	<u>4.3</u>	1.7	-	1.3	-	-	4.3	18.9	
	7706	-	-	-	1.2	2.0	<u>2.7</u>	0.7	-	1.7	-	-	2.7	10.3	
	7767	-	-	-	-	0.7	-	-	-	-	<u>1.0</u>	-	1.0	4.8	
	Mean:												2.3	9.8	
RSV rA2 wt RSV	7877	-	-	3.4	<u>3.5</u>	3.4	1.6	3.4	2.9	1.5	-	-	3.5	21.1	
	7885	-	-	2.3	<u>3.8</u>	3.3	3.1	2.9	2.3	1.7	-	-	3.8	20.9	
	7758	-	0.7	-	0.7	0.7	2.1	2.2	<u>2.7</u>	1.5	1.0	-	2.7	12.6	
	7724	-	1.4	2.2	2.8	2.1	<u>3.4</u>	3.0	2.4	3.1	2.0	-	3.4	23.2	
	Mean:												3.4	19.4	

10 ^aMonkeys were inoculated i.n. and i.t. with 5.9 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose = 6.2 log₁₀ PFU/AGM).

^bVirus titrations were performed on Vero cells at 37°C. The lower limit of detection was 0.7 log₁₀ PFU/mL. Samples with no detectable virus are represented as “-”. Peak titers for each animal are underlined. The results show that RSV LID ΔM2-2 is strongly restricted in the URT of AGMs compared to RSV rA2.

- 5 ^cThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). A value of 0.35 was used for samples with no detectable virus.

Table 11. Titers of virus in tracheal lavage samples from AGMs inoculated with the CTM RSV LID/ΔM2-2, or with recombinant wt RSV rA2.

Virus Test Article ^a	AGM ID	TL virus titer (log ₁₀ PFU/mL) on indicated day ^b						Peak virus titer	Sum of daily titers ^c
		2	4	6	8	10	12		
RSV LID ΔM2-2	7728	1.3	-	<u>2.0</u>	-	-	-	2.0	6.1
	7833	2.2	<u>2.6</u>	2.0	2.1	-	-	2.6	10.2
	7706	-	2.5	<u>2.7</u>	1.7	1.3	-	2.7	9.6
	7767	1.0	-	-	<u>2.6</u>	2.0	-	2.6	7.7
	Mean:							2.5	8.4
RSV rA2 wt RSV	7877	2.4	3.0	<u>3.9</u>	2.8	1.0	-	3.9	13.8
	7885	1.8	2.9	<u>3.5</u>	3.0	-	-	3.5	12.6
	7758	1.9	2.9	3.7	<u>3.9</u>	1.3	-	3.9	14.5
	7724	-	2.3	2.7	3.5	<u>4.1</u>	2.1	4.1	15.4
	Mean:							3.8	14.1

- 10 ^aMonkeys were inoculated i.n. and i.t. with 5.9 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose = 6.2 log₁₀ PFU/AGM).

^bVirus titrations were performed on Vero cells at 32°C. The lower limit of detection was 1.0 log₁₀ PFU/mL. Samples with no detectable virus are represented as “-”. Underlined value indicates maximum titer for each animal. As expected, the highly temperature sensitive virus RSV LID ΔM2-2 did not replicate in the LRT of AGMs (body temperature: 39°C). TL, tracheal lavage.

^cThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). Values of 0.7 are used for samples with no detectable virus.

Table 12. Serum PRNT₆₀ antibody titers in AGMs inoculated with the CTM RSV LID/ΔM2-2 or with recombinant wt RSV rA2.

Virus ^a	AGM ID	RSV Neutralization Titer (Log ₂ of reciprocal) on days		
		0	21	28
RSV LID/ΔM2-2	7728	-	8.1	10.1
	7833	-	7.2	7.4
	7706	-	5.7	6.4
	7767	-	6.4	6.3

	Mean:	-	6.9	7.6
RSV rA2 wt RSV	7877	-	8.6	8.5
	7885	-	8.2	9.1
	7758	-	7.8	7.9
	7724	-	7.5	7.8
	Mean:		8.0	8.3

^aMonkeys were inoculated i.n. and i.t. with 5.9 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose = 6.2 log₁₀ PFU/AGM).

^bThe lower limit of detection of the 60% Plaque Reduction assay is 3.3 (Log₂ of the dilution reciprocal). Samples below the lower limit of detection are recorded as “-”.

5

Example 5

This example illustrates differences in RSV LID/ΔM2-2, RSV D46/ΔM2-2, and RSV MEDI/ΔM2-2 that may affect replication efficiency *in vivo*.

As noted above, the RSV D46/ΔM2-2 and RSV LID/ΔM2-2 viruses are identical by sequence except for the 6120 mutation in the SH gene of RSV LID/ΔM2-2, which removes 112 nucleotides from the downstream non-translated region and makes silent nucleotide changes in the last three codons and stop codon of the SH ORF (FIG. 3). RSV LID/ΔM2-2 replicated significantly more efficiently than RSV D46/ΔM2-2 in the upper (Table 4) and lower (Table 5) respiratory tract of AGMs. Since these viruses are otherwise identical, this showed that the 6120 mutation is associated with increased replication in primates, and the effect was sufficiently great to detect unequivocally in AGMs.

As noted above, the RSV LID/ΔM2-2 and RSV MEDI/ΔM2-2 viruses could not be distinguished with regard to replication efficiency in cell culture and in AGMs, yet the former was significantly more efficient in replication in seronegative infants and children and was more immunogenic. Thus, these viruses also differ in replicative efficiency, but this was only evident in the fully permissive human host. RSV LID/ΔM2-2 has the 6210 mutation while RSV MEDI/ΔM2-2 does not. Yet, in this case the 6120 mutation was not associated with increased replication in semi-permissive AGMs, but was associated with increased replication in seronegative infants and children, the permissive natural host. These observations suggest that, while RSV LID/ΔM2-2 clearly has greater replication efficiency than RSV MEDI/ΔM2-2, this may be somewhat reduced by some other difference between the LID and MEDI viruses. There are two such differences additional to the 6120 mutation:

One of the differences is that the details of the mutations that silence the M2-2 ORF are different between RSV LID/ΔM2-2 and RSV MEDI/ΔM2-2. In RSV LID/ΔM2-2 (and RSV D46/ΔM2-2), the deletion is 241-nt in length and begins after nucleotide 8187 and, in addition, all three ATG translational start codons are changed to ACG, such that there should be little or no translation of any M2-2-derived peptides (FIG. 1). In contrast, in RSV MEDI/ΔM2-2, the deletion mutation involved the insertion of a foreign *Hind*III restriction site beginning at nucleotide 8196, involved a deletion of 234 nt, and would

encode a 12-amino acid peptide representing the N-terminus of the longest version of the M2-2 protein (Jin, *et al.* 2000. J Virol 74:74-82).

The second of the differences is that the RSV MEDI/ΔM2-2 and RSV LID/ΔM2-2 cDNAs differ at 21 additional nucleotide sequence positions scattered through the two backbones (Table 13). Of these, 6 nucleotide differences (including a 1-nucleotide insert at position 1099 of RSV LID/ΔM2-2) are due to restriction sites that were added during the construction of the D46 cDNA clone (Collins, *et al.* 1995. Proc Natl Acad Sci USA 92:11563-11567). These six changes are thought to be phenotypically silent because biological wt RSV and recombinant wt D46 RSV replicate with similar efficiencies in chimpanzees and cause a similar level of disease (e.g. Whitehead, *et al.* 1998. J Virol 72:4467-4471). These changes also are present in a number of vaccine candidates evaluated to date in humans. It therefore seems unlikely that these 6 nucleotides and their associated restriction sites influence replication, although this has not been unequivocally determined. The remaining 15 nucleotide differences between RSV MEDI/ΔM2-2 and RSV LID/ΔM2-2 are point mutations that are thought to reflect adventitious differences present in the two different parent biologic virus stocks of strain A2 from which the two independent reverse genetics systems were derived. It is not uncommon to find numerous nucleotide differences between two preparations of the same RSV strain that have different passage histories. Two of these 15 nucleotide differences result in amino acid differences, one in the NS2 protein (K51R), and the other in the N protein (T24A) (amino acid assignments are given with LID first followed by MEDI). A recent study indicated that neither of these two amino acid differences had an effect on replication efficiency *in vitro* (Lawlor, Schickli, and Tang. 2013. J Gen Virol 94:2627-2635).

Table 13: Differences in genomic sequence (positive sense) between RSV MEDI/ΔM2-2 and RSV LID/ΔM2-2, in addition to the 6120 and ΔM2-2 mutations.

Gene Region	RSV Nucleotide			Amino Acid (Comment)		
	Genomic nucleotide position ¹	<u>RSV MEDI/ΔM2-2</u>	<u>RSV LID/ΔM2-2</u>	Amino Acid Position	<u>RSV MEDI/ΔM2-2</u>	<u>RSV LID/ΔM2-2</u>
		cDNA	cDNA		cDNA	cDNA
NS1	404	C	T	102	N	N
NS2	779	G	A	51	R	K
NS2/N ig	1099	T	C*	ncr ²	n/a	(1-nt insert, creates AflIII restriction site in RSV LID/ΔM2-2)
N	1138	A	C*	ncr ²	n/a	(creates NcoI restriction site in RSV LID/ΔM2-2)
N	1139	G	C*	ncr ²	n/a	
N	1181	G	A	14	K	K
N	1209	G	A	24	A	T
N	1937	A	G	266	S	S
G/F ig	5611	A	G*	ncr ²	n/a	

G/F ig	5615	A	T*	ncr ²	n/a	(creates StuI restriction site in RSV LID/ΔM2-2)
G/F ig	5639	G	A	ncr ²	n/a	n/a
F	6215	C	T	185	V	V
F	6221	C	T	187	V	V
F	6386	T	C	242	G	G
F	7214	C	T	518	A	A
F	7481	T	C	ncr ²	n/a	n/a
F/M2 ig	7559	A	C*	ncr ²	n/a	(creates SphI restriction site in RSV LID/ΔM2-2)
M2	7701	G	C	32	P	P
L	10514	T	C	673	L	L
L	13633	A	C	1712	T	T
L	13900	T	C	1801	S	S

¹Genomic position numbered relative to WT RSV strain A2 (Genbank accession number M74568). All sequences are positive-sense.

²ncr, non-coding region

⁴ig, intergenic region

- 5 *Changes engineered as markers into the original LID antigenomic cDNA clone (Collins *et al.* PNAS 92:11563-7 1995 PMID 8524804). These changes are present in most of the recombinant RSV vaccine candidates that have been evaluated in humans.

Thus, new reagents and information are provided that indicate that:

1. RSV LID/ΔM2-2 replicates substantially more efficiently in AGMs than RSV D46/ΔM2-2.

10 Since these viruses differ only in the presence of the 6120 mutation in RSV LID/ΔM2-2, it is concluded that this mutation confers a phenotype of increased replication in primates, a difference that was detectable even in the semi-permissive AGM model. This substantial difference in replication efficiency between RSV LID/ΔM2-2 and RSV D46/ΔM2-2 provides two backbones that differ considerably in attenuation. Therefore, one can introduce a common set of attenuating mutations (e.g., cp, ΔSH, 1030s) into each backbone and obtain a broad spectrum of attenuation phenotypes that is directly linked to benchmark data in humans (*i.e.*, the clinical study of RSV LID/ΔM2-2).

2. RSV LID/ΔM2-2 did not replicate more efficiently than RSV MEDI/ΔM2-2 in AGMs, but did so in the more permissive natural host, namely seronegative infants and children. This suggests that RSV LID/ΔM2-2 has a replication advantage versus RSV MEDI/ΔM2-2, but less than it has versus RSV D46/ΔM2-2. Thus, the order of replicative efficiency in humans for RSV LID/ΔM2-2 and RSV MEDI/ΔM2-2 is RSV LID/ΔM2-2 > RSV MEDI/ΔM2-2, and the data from AGMs supports the further conclusion that RSV D46/ΔM2-2 is even more attenuated, giving the order of attenuation: RSV LID/ΔM2-2 > RSV MEDI/ΔM2-2 > D46/ΔM2-2. This suggests that one or more difference in RSV MEDI/ΔM2-2 versus the D46/LID backbones is responsible for its intermediate phenotype. The likeliest candidates are the K51R and T24A mutations in NS2 and N, respectively, despite the published data

noted immediately above suggesting that they are phenotypically silent (Lawlor, Schickli, and Tang. 2013. J Gen Virol 94:2627-2635). These two amino acid substitutions are considered to be the most likely candidates because amino acid changes are more likely to affect phenotype – through effects on protein structure and function - than are silent nucleotide signals not contained in a *cis*-acting signal. An alternative or additional possibility is that the difference in the details of the construction of the Δ M2-2 mutations plays a role. A further possibility is that one or more of the 19 other translationally silent nucleotide differences play a role. These possibilities can be distinguished using strains selected from panels described below.

Example 6

This example illustrates additional Δ M2-2 constructs with combinations of features from RSV LID/ Δ M2-2, RSV D46/ Δ M2-2, and RSV MEDI/ Δ M2-2.

Additional Δ M2-2-based viruses were constructed using the above results as guidance to obtain different combinations containing one of several Δ M2-2 mutations, the 6120 mutation, the K51R/T24A mutations, and the other incidental differences between the MEDI and D46/LID backbones.

An additional M2-2 deletion was created that is based on site-directed mutagenesis that deleted 234 nucleotides of the M2-2 ORF (nucleotides 8202-8435) and introduced T8197A and C8199G point mutations that created an *Ac*I site and introduced a termination codon at codon 13 in the M2-2 ORF (FIG. 10). This modification was done to both the RSV D46 and RSV LID backbones, resulting in RSV D46/ Δ M2-2-*Ac*I and RSV LID/ Δ M2-2-*Ac*I (FIG. 11). These viruses combine the D46 or LID backbone (*i.e.*, without and with the 6120 mutation, respectively, but otherwise identical) with a Δ M2-2 mutation (Δ M2-2-*Ac*I) that resembles that of RSV MEDI/ Δ M2-2, including the potential for expression of a peptide representing the 12 N-terminal amino acids of M2-2 (FIG. 11).

The RSV D46/ Δ M2-2-*Ac*I and RSV LID/ Δ M2-2-*Ac*I cDNAs were further modified by inclusion of the K51R mutation in the NS2 protein and the T24A mutation in the N protein (FIG. 12A). Thus, this incorporated into the D46/ Δ M2-2 and LID/ Δ M2-2 backbones the two most prominent differences versus the MEDI backbone.

As further examples, the K51R mutation in the NS2 protein and the T24A mutation in the N protein also were introduced into the D46/ Δ M2-2 backbone individually (FIG. 12B, first and second constructs from the top) and together (third construct). These mutations were also introduced into the LID/ Δ M2-2 backbone (FIG. 12C) individually (fourth (under construction) and fifth constructs) and together (bottom construct).

In addition, the Δ M2-2-*Hind*III mutation (described in FIG. 10) was introduced into D46 to generate the D46/ Δ M2-2-*Hind*III and LID/ Δ M2-2-*Hind*III backbones (FIG. 13, the first and third constructs from the top). Further derivatives included the further additions of the K51R and T24A amino acid substitutions in the NS2 and N proteins (FIG. 13, the second and fourth constructs from the top).

Example 7

This example illustrates additional Δ M2-2 constructs with additional modifications to the F and/or G genes.

RSV LID/ Δ M2-2 (FIG. 2) was modified with further alterations to the F and/or G genes. In general, these modifications were not designed primarily to affect attenuation, but rather to affect other parameters such as the efficiency of antigen expression or the inclusion of genes from another strain. Note that these strains use the terminology “6120” rather than “LID” to indicate the presence of the 6120 mutation. These constructs are as follows:

RSV 6120/G001BB/FBB/ Δ M2-2 (FIG. 14A): contains the codon optimized G gene (G001BB) from a recent (year 2011), low-passage clinical isolate A/Maryland/001/11. This construct also contains a codon-optimized strain A2 F gene (FBB). Note that the native sequence of the G0001 sequence proved to be unstable during cloning in bacteria. The codon optimization, resulting in G001BB, had the effect of conferring stability.

RSV 6120/FBB/G001BB/ Δ M2-2 (FIG. 14A); contains codon-optimized A2 F gene (FBB) and the codon optimized G gene from the recent clinical isolate (G001BB), but their order in the gene map has been reversed, from G-F to F-G, in order to obtain increased expression of the F protein, the major RSV neutralization and protective antigen.

RSV 6120/G001BB/F/ Δ M2-2 (FIG. 14A): contains G001BB and native A2 F gene.

RSV 6120/G/FBB/ Δ M2-2 (FIG. 14A, fourth construct from the top): contains the native A2 G gene and codon-optimized A2 F gene (FBB).

RSV 6120/G/FBBHEK/ Δ M2-2 (FIG. 14B): contains the native A2 G gene and codon-optimized A2 F gene (FBB) that also has the two HEK mutations, K66E and Q101P.

RSV 6120/G/FBBcpHEK/ Δ M2-2 (FIG. 14C): contains the native A2 G gene and codon-optimized A2F gene (FBB) that also has the two HEK mutations, K66E and Q101P and the two cp mutations contained in the F gene, namely E218A and T523I.

RSV 6120/FBB/G/ Δ M2-2 (FIG. 14C): contains codon optimized A2 F (FBB) and the native A2 G gene, but their order in the gene map has been reversed, from G-F to F-G.

RSV 6120/G001BB/F001BB/ Δ M2-2 (FIG. 14C): contains the G001BB gene and the F001 gene that have been codon optimized (G001BB, F001BB).

In brief, the use of G and/or F genes from the recent clinical isolate called A/Maryland/001/11 (which was isolated in 2011 from a health care provider with substantial respiratory illness) was to investigate whether these combinations might yield improved replication and/or immunogenicity. It also would show that a live RSV vaccine could be up-dated readily to contain surface proteins from more recent strains. The use of codon-optimization (BB) was to increase expression of one or both major protective antigens. The change in gene order of G and F from G-F to F-G was designed to increase antigen expression, and was done knowing that moving F and G all the way to the promoter-proximal positions in the gene map in the context of a Δ M2-2 mutation resulted in viruses that unexpectedly exhibited a reduced level of replication *in vitro*, as described in Example 1. The use of HEK mutations,

with or without the two F cp mutations, was done to obtain a more stable F protein, which might have superior immunogenicity due to the preservation of neutralization epitopes. This is based on the idea that the meta-stable nature of the RSV F protein might contribute to immune evasion by presenting denatured antigen (Sakurai, *et al.* 1999. J Virol 73:2956-2962; Collins and Graham, 2008. J Virol 82:2040-2055),
 5 and thus providing a more stable form might induce a qualitatively superior immune response.

Each of the viruses shown in FIGs. 14A and 14B was readily recovered from cDNA. Passage P1 is the first passage following transfection, and is performed without titering the inoculum (blind passage). The resulting P1 yields of the constructs in FIGs. 14A and 14B were compared to wt RSV containing the 6120 mutation (wt LID), and to RSV LID/ Δ M2-2, which was the parent of the constructs (FIG. 15). This
 10 showed that the P1 titers of all of the viruses compared favorably with the two controls, with the sole exception that the P1 titer of RSV 6120/G001BB/F001BB/ Δ M2-2 was approximately 3.0 log₁₀ reduced. However, this titer rebounded during the P2 passage to levels consistent with the other constructs (FIG. 15). In general, this showed that all of the modifications were well tolerated, including the novel genes, the change in gene order, the codon optimization, and the introduction of HEK and/or cp mutations.

Example 8

This example illustrates evaluation of additional RSV Δ M2-2 constructs.

As described above, RSV D46/cp/ Δ M2-2 (FIG. 4, top genome), was found to replicate efficiently in Vero cells, necessary for vaccine manufacture, and to be highly attenuated yet highly immunogenic in
 20 AGMs (Tables 4-6). Therefore, vaccine seed virus was prepared and used to manufacture clinical trial material (CTM) of D46/cp/ Δ M2-2. As noted, this construct had a single adventitious nucleotide change in the D46 backbone, at the DNA level: specifically, G3878A, which is present in the M ORF and is silent at the amino acid level. Automated sequence analysis showed that the sequence of the CTM was identical to that of the cDNA. Analysis of the replication and immunogenicity of this CTM in AGMs
 25 confirmed that it is highly attenuated (Tables 14 and 15) yet highly immunogenic (Table 16). This vaccine candidate was evaluated in a double-blind placebo-controlled study in 15 RSV seropositive children 12 to 59 months of age (10 vaccine recipients, 5 placebo recipients), performed at CIR/JHU (ClinicalTrials.gov identifier NCT02601612). Following intranasal administration at a dose of 10⁶ PFU, vaccine shedding was undetectable, and D46/cp/ Δ M2-2 was poorly immunogenic in seropositive children.
 30 This indicates that the vaccine is highly restricted and attenuated, predicting that it will be safe and appropriate for evaluation in seronegative infants and children. Evaluation in RSV seronegative infants and children 6-24 months of age is currently ongoing. This will provide information on a promising vaccine candidate, indicating whether it is suitable for expanded studies. This information also will provide a further benchmark linking preclinical and clinical studies.

In addition, the LID counterpart of this virus, LID/cp/ Δ M2-2 (FIG. 5, top genome) was constructed. It was found to replicate efficiently in Vero cells, and analysis of the replication and immunogenicity of this virus in AGMs showed that it is highly attenuated (Tables 17 and 18) yet highly immunogenic (Table 19). This showed that the addition of the cp mutations to LID/ Δ M2-2, which was

incompletely attenuated in seronegative infants and children (FIG. 9B), to create LID/cp/ Δ M2-2, resulted in increased attenuation in AGMs (i.e., compare data for LID/ Δ M2-2, Tables 1 and 2, 4 and 5, 7 and 8, and 10 and 11, to that for LID/cp/ Δ M2-2, Tables 17 and 18). This suggests that LID/cp/ Δ M2-2 should have increased attenuation in seronegative humans, although clinical evaluation is needed to confirm this, to determine the magnitude of the increased attenuation, and to confirm safety.

Clinical trial material was then manufactured for LID/cp/ Δ M2-2 using the antigenomic cDNA whose sequence is shown in SEQ ID NO: 17. The sequence of the clinical trial material (LIDcp Δ M2-2, Lot RSV#009B) was confirmed by consensus sequence analysis to match that of the cDNA from which the recombinant virus was derived except for a C-to-T point mutation at nucleotide position 9,972 (note that all sequences are reported in positive, or antigenomic sense). This mutation is silent at the amino acid level and was also present in the seed virus used to generate LIDcp Δ M2-2, Lot RSV#009B. Adventitious mutations can appear during passage of RSV, as with most RNA viruses, due to the high error rate of the RNA-dependent RNA polymerase. When such changes do not involve a known cis-acting signal or change amino acid coding, and if they do not measurably affect in vitro replication and plaque size of the virus, they are considered likely to be biologically inconsequential. The silent C9972T point mutation in the LIDcp Δ M2-2 clinical trial material likely is inconsequential, but will be monitored.

The LID/cp/ Δ M2-2 vaccine virus is being evaluated in seronegative children 6-24 months of age in a double-blind placebo-controlled clinical trial. At least 5 subjects have received the vaccine, with no evidence of reactogenicity during the period when the vaccine virus would be anticipated to be shedding, suggesting that this vaccine is well-tolerated.

As already noted, a non-clinical experimental lot of the LID/ Δ M2-2/1030s virus (FIG. 5, second genome from the top) was prepared, and was found to replicate efficiently in Vero cells. It was evaluated in AGMs and shown to be highly attenuated (Tables 1 and 2) and yet highly immunogenic (Table 3).

A preparation of CTM was manufactured was prepared for LID/ Δ M2-2/1030s using the antigenomic cDNA shown in SEQ ID NO: 16, and automated sequence analysis showed that the sequence of the clinical trial material (CTM) was identical to that of the cDNA. This vaccine was evaluated in seronegative children 6-24 months of age in a double-blind placebo-controlled trial. A total of 33 subjects were enrolled, with an anticipated vaccinee:placebo ratio of 2:1. Nasal washes from 30 participants were evaluated by plaque assay (viral culture) as well as by quantitative RT-PCR (qPCR) for shedding of vaccine virus LID/ Δ M2-2/1030s, as a measure of attenuation. This showed that 17 of the subjects had apparent vaccine virus shedding (it is anticipated that a total of 20 subjects received vaccine). 15/30 subjects in the LID/ Δ M2-2/1030s trial were positive by plaque assay, and that all 15 of these plus two additional subjects were positive by qPCR, which is a more sensitive assay. It is anticipated that subjects that shed virus during the 14-18 days following administration of the vaccine will be found to be vaccinee recipients, and hence these data can be used as a presumptive assessment of vaccine virus shedding and hence attenuation. In comparison, a similar shedding analysis for RSV LID/ Δ M2-2 in a comparable cohort of seronegative children 6-24 months of age, ClinicalTrials.gov NCT02040831, found viral shedding in 19 of 20 vaccine recipients by plaque assay and qRT-PCR. In

the LID/ Δ M2-2/1030s trial, the presumptive mean peak titers were: 5.1 log₁₀ copies/ml by PCR, and 2.9 log₁₀ PFU/ml by culture, whereas for the LID/ Δ M2-2 trial, the mean peak titers were 5.9 log₁₀ copies/ml by PCR, and 3.4 log₁₀ PFU/ml by culture. Thus, the LID/ Δ M2-2/1030s virus appeared to be more attenuated than the LID/ Δ M2-2 virus based on the rate of infection and the titers of shed virus. With the LID/ Δ M2-2/1030s virus, the three highest individual peak titers were 4.7, 4.5, and 4.1 log₁₀ PFU/ml, compared to 5.4, 5.3, and 5.1 log₁₀ PFU/ml for LID/ Δ M2-2. In addition, for the LID/ Δ M2-2/1030s virus, six subjects shed infectious virus for only a single day, compared to two for the LID/ Δ M2-2 virus. Thus, by each of these measures, the insertion of the 1030s mutation into LID/ Δ M2-2 provided a measurable, consistent decrease in viral shedding in seronegative children, who are the vaccine target.

The RSV LID/ Δ M2-2/1030s virus was evaluated for the temperature-sensitive phenotype, since the 1030s mutation is a temperature sensitivity mutation (Table 20). This analysis showed that RSV LID/ Δ M2-2/1030s has a shut-off temperature (T_{SH}) of 40°C and a small plaque temperature (T_{SP}) of 38°C, whereas wild type RSV, LID/ Δ M2-2, MEDI/ Δ M2-2, LID/cp/ Δ M2-2, and D46/cp/ Δ M2-2 have T_{SH} and T_{SP} of >40° C. Thus, RSV LID/ Δ M2-2/1030s, but not these other wild type and Δ M2-2-based viruses, has a temperature sensitivity phenotype. Two other known temperature-sensitive viruses, RSV Δ NS2/ Δ 1313/I1314L and RSV cps2, were included as positive controls, and confirmed that the assay was accurate. Thus, introduction of the 1030s mutation into RSV LID/ Δ M2-2 conferred the temperature-sensitive phenotype. This is significant because the temperature-sensitive phenotype is thought to preferentially restrict replication in the warmer lower respiratory tract compared to the cooler upper respiratory tract, and thus confers additional safety against reactogenicity. This is thought to be the case even if the T_{SH} and/or T_{SP} are higher than physiological temperature.

Thus, this provides a spectrum of attenuated viruses with increasingly reduced replication: LID/ Δ M2-2>LID/cp/ Δ M2-2>LID/ Δ M2-2/1030s, having varying balances of attenuation and immunogenicity.

Table 14: Viral titers of nasopharyngeal swab samples from AGMs inoculated with D46/cp/ Δ M2-2.

RSV Vaccine candidate ^a	AGM ID	NP virus titer (log ₁₀ PFU/mL) on indicated days ^b											Peak virus titer
		1	2	3	4	5	6	7	8	9	10	14	
D46/cp/ Δ M2-2	8573	-	-	0.7	-	-	-	-	-	0.7	-	-	0.7
	N1327	-	-	0.7	-	-	-	1.0	-	-	-	-	1.0
	8555	-	-	-	-	-	-	-	-	-	-	-	0.35
	8577	-	-	-	-	-	-	0.7	-	-	-	-	0.7
	Mean:	-	-	0.5	-	-	-	0.6	-	0.4	-	-	0.7
L-15	8551	-	-	-	-	-	-	-	-	-	-	-	-
	8417	-	-	-	-	-	-	-	-	-	-	-	-
	8489	-	-	-	-	-	-	-	-	-	-	-	-
	8565	-	-	-	-	-	-	-	-	-	-	-	-
	Mean	-	-	-	-	-	-	-	-	-	-	-	-

^aMonkeys were inoculated i.n. and i.t. with 6 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose =6.3 log₁₀ PFU/AGM).

^bCombined NP swabs were placed in 2 mL of L-15 medium with sucrose phosphate buffer as stabilizer.

Virus titrations were performed on Vero cells at 32°C. The lower limit of detection was 0.7 log₁₀ PFU/mL. Samples with no detectable virus are represented as “-“. A value of 0.35 was used for samples with no detectable virus. The results show that D46/c/pΔM2-2 is strongly restricted in the URT of AGMs.

Table 15. Viral titers of tracheal lavage samples from AGMs inoculated with D46/cp/ΔM2-2.

RSV Vaccine candidate ^a	AGM ID	TL virus titer (log ₁₀ PFU/mL) on indicated day ^b						Peak virus titer
		2	4	6	8	10	14	
D46/cp/ΔM2-2	8573	1.0	-	1.9	1.3	-	-	1.9
	N1327	-	1.0	1.8	1.0	-	-	1.8
	8555	-	-	-	-	-	-	0.7
	8577	-	-	-	-	-	-	0.7
	Mean:	0.8	0.8	1.3	0.9	-	-	1.9
L-15	8551	-	-	-	-	-	-	-
	8417	-	-	-	-	-	-	-
	8489	-	-	-	-	-	-	-
	8565	-	-	-	-	-	-	-
	Mean:	-	-	-	-	-	-	-

^aMonkeys were inoculated i.n. and i.t. with 6 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose =6.3 log₁₀ PFU/AGM).

^bOn days 2, 4, 6, 8, 10, and 14, tracheal lavage (TL) was performed with 3 mL of PBS Virus. Titrations were performed on Vero cells at 32°C. The lower limit of detection was 1.0 log₁₀ PFU/mL. Samples with no detectable virus are represented as “-“. A value of 0.7 was used for samples with no detectable virus. D46/cp/ΔM2-2 is strongly restricted in the LRT of AGMs.

Table 16. Serum PRNT₆₀ titers from AGMs inoculated with D46/cp/ΔM2-2.

RSV Vaccine candidate	AGM ID	Neutralizing antibody titers (PRNT ₆₀ , reciprocal log ₂) on indicated days ^b		
		0	21	29
D46/cp/ΔM2-2^a	8573	-	8.4	8.4
	N1327	-	8.4	9.1
	8555	-	6.6	6.1
	8577	-	7.2	6.9
	Mean:	-	7.7	7.6
L-15	8551	-	-	-
	8417	-	-	-
	8489	-	-	-

	8565	-	-	-
	Mean:	-	-	-

^aAGMs were inoculated i.n. and i.t. with 6.0 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose = 6.3 log₁₀ PFU per animal).

^bOn days 0, 21, and 29 p.i., serum was obtained. Neutralizing antibody titers were determined in a 60% plaque reduction neutralization assay. The lower limit of detection was 3.3 (1:10).

5

Table 17: Viral titers of nasopharyngeal swab samples from AGMs inoculated with LID/cp/ΔM2-2.

RSV Vaccine candidate ^a	AGM ID	NP virus titer (log ₁₀ PFU/mL) on indicated days ^b											Peak virus titer
		1	2	3	4	5	6	7	8	9	10	12	
LID/cp/ΔM2-2	8120	-	-	-	-	-	-	-	-	-	-	-	0.35
	8528	-	-	-	-	-	-	-	-	-	-	-	0.35
	8336	-	-	-	0.7	-	-	-	-	-	-	-	0.7
	Mean:	-	-	-	0.5	-	-	-	-	-	-	-	0.5

^aMonkeys were inoculated i.n. and i.t. with 6 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose = 6.3 log₁₀ PFU/AGM). A value of 0.35 was used for samples with no detectable virus.

^bCombined NP swabs were placed in 2 mL of L-15 medium with sucrose phosphate buffer as stabilizer.

10 Virus titrations were performed on Vero cells at 32°C. The lower limit of detection was 0.7 log₁₀ PFU/mL. Samples with no detectable virus are represented as “-”. A value of 0.35 was used for samples with no detectable virus. The results show that LID/cp/ΔM2-2 is strongly restricted in the URT of AGMs.

15 **Table 18:** Viral titers of tracheal lavage samples from AGMs inoculated with LID/cp/ΔM2-2.

RSV Vaccine candidate ^a	AGM ID	TL virus titer (log ₁₀ PFU/mL) on indicated day ^b						Peak virus titer
		2	4	6	8	10	12	
LID/cp/ΔM2-2	8120	2.1	-	-	-	-	-	2.1
	8528	1.0	-	-	-	-	-	1.0
	8336	-	1.0	-	-	-	-	1.0
	Mean:	1.3	0.8	-	-	-	-	1.4

^aAGMs were inoculated by the combined intranasal and intratracheal routes with 6 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose: 6.3 log₁₀ PFU per animal).

^bOn days 2, 4, 6, 8, 10, and 12, tracheal lavage was performed with 3 mL of PBS. Virus titrations were performed on Vero cells at 32°C. The lower limit of detection was 1.0 log₁₀ PFU/mL of lavage solution. Samples with no detectable virus are represented as “-”. A value of 0.7 was used for samples with no detectable virus. LID/cp/ΔM2-2 is strongly restricted in the LRT of AGMs.

20

Table 19: Serum PRNT₆₀ titers from AGMs inoculated with LID/cp/ΔM2-2.

RSV Vaccine candidate	AGM ID	Neutralizing antibody titers (PRNT ₆₀ , reciprocal log ₂) on indicated days ^b		
		0	21	29
LID/cp/ΔM2-2 ^a	8120	-	10.0	10.5
	8528	-	10.1	9.8
	8336	-	8.1	7.7
	Mean:	-	9.4	9.3

^aAGMs were inoculated i.n. and i.t. with 6.0 log₁₀ PFU of the indicated virus in a 1 mL inoculum per site (total dose = 6.3 log₁₀ PFU per animal).

^bOn days 0, 21, and 29 p.i., serum was obtained. Neutralizing antibody titers were determined in a 60% plaque reduction neutralization assay. The lower limit of detection was 3.3 (1:10).

Table 20: Temperature sensitivity of RSV LID/ΔM2-2/1030s and related viruses

Virus	Virus titer (log ₁₀ PFU per mL) at indicated temperature (°C) ^a								
	32	35	36	37	38	39	40	T _{SH} ^b	T _{SP} ^c
RSV A2	7.3	7.2	7.2	7.2	7.3	7.2	7.0	> 40	> 40
D46 6120	7.7	7.7	7.6	7.6	7.6	7.4	7.4	> 40	> 40
LID ΔM2-2	5.9	5.9	5.8	5.7	5.7	5.6	5.4	> 40	> 40
Medi ΔM2-2	7.0	7.0	6.9	7.0	7.0	6.9	6.8	> 40	> 40
LID cp ΔM2-2	4.5	4.5	4.4	4.2	4.1	3.9	3.5	> 40	> 40
LID ΔM2-2 1030s	7.1	7.0	7.0	7.0	6.8*	6.1	<u>1.7</u>	40	38
D46 cp ΔM2-2	6.2	6.2	6.1	6.0	5.9	5.7	5.5	> 40	> 40
RSV ΔNS2 Δ1313 I1314L ^{xx}	7.1	6.9	6.8*	6.4	6.4 ^d	<u>≤ 2</u>	< 2	39	36
RSV cps2 ^{xx}	6.4	5.8*	4.7 ^d	<u>≤ 2</u>	< 2	< 2	< 2	37	35

^aThe ts phenotype for each virus was evaluated by plaque assay on Vero cells at the indicated temperatures. For viruses with ts phenotype, titers at shut-off temperatures (T_{SH}) are marked (bold, underlined). See footnote b for the definition of T_{SH}.

^bT_{SH} (bold, underlined) is defined as the lowest restrictive temperature at which the reduction compared to 32°C is 100-fold or greater than that observed for wt RSV at the two temperatures. The ts phenotype is defined as having a T_{SH} of 40° C or less.

^cT_{SP}, Small plaque temperature is defined as the lowest restrictive temperature at which the small-plaque phenotype is observed. Titers at lowest restrictive temperature are marked with an asterisk.

^dItalics: micro plaque temperature is defined as the lowest restrictive temperature at which the small-plaque phenotype is observed. Titers at lowest restrictive temperature for the microplaque phenotype are marked with an asterisk.

^{xx}Control ts viruses

Example 9

This example describes the construction of RSV D46/276/ Δ M2-2-AclI, and its comparison in African green monkeys with RSV D46/NS2/N/ Δ M2-2-HindIII and selected control viruses.

An additional M2-2 mutant virus was constructed to represent a further combination of features from the D46/ Δ M2-2 and MEDI/ Δ M2-2 backbones, yielding a virus called RSV D46/276/ Δ M2-2-AclI, which is also referred to herein as “RSV 276” or “276”.

The structure of RSV 276 is summarized in FIG. 16 and in Table 21 and is provided in SEQ ID NO: 19. Compared to D46 (i.e., the complete wild type antigenomic cDNA, SEQ ID NO: 1), RSV 276 differed by a total of 21 nucleotide differences (including the deletion of a single nt at position 1099) plus the deletion of nucleotides 8202-8435 inclusive, yielding a deletion of 234 nucleotides). The nucleotide changes removed four non-native restriction sites (AflII, NcoI, StuI, and SphI) that had been intentionally inserted into D46 during its original construction (Collins, et al. 1995 Proc Natl Acad Sci USA 92:11563-11567), and in addition inserted an AclI site spanning the M2-2 deletion (thus, the Δ M2-2-AclI deletion is that same as described in FIG. 10). These changes in restriction sites involved eight nucleotides. Most of the other 13 changes introduced selected assignments from RSV MEDI/ Δ M2-2 into the D46-derived backbone of the new RSV 276 virus.

Table 21: Comparison of genomic sequences (positive sense) of wt RSV D46 (cDNA, SEQ ID NO: 1) and RSV 276 (cDNA and CTM Lot RSV#014A, SEQ ID NO: 19)

Gene Region	RSV Nucleotide (cDNA)				Encoded Amino Acid Residue		
	Genomic nt position ¹	SEQ ID NO: 1 position	WT D46 RSV	RSV 276	Amino Acid Position ¹	WT D46 RSV	RSV 276
NS1	404	404	T	C	102	N	N
NS2	779	779	A	G	51	K	R ³
NS2/Nig ⁴	1099	1099	C*	-	ncr ²	n/a	n/a
N	1138	1139	C*	A	ncr ²	n/a	n/a
N	1139	1140	C*	G	ncr ²	n/a	n/a
N	1181	1182	A	G	14	K	K
N	1209	1210	A	G	24	T	A ³
G/F ig ⁴	5611	5612	G*	A	ncr ²	n/a	n/a
G/F ig ⁴	5615	5616	T*	A	ncr ²	n/a	n/a
G/F ig ⁴	5639	5640	A	G	ncr ²	n/a	n/a
F	6215	6216	T	C	185	V	V
F	6221	6222	T	C	187	V	V
F	6386	6387	C	T	242	G	G
F	7214	7215	T	C	518	A	A
F	7481	7482	C	T	ncr ²	n/a	n/a
F/M2 ig ⁴	7559	7560	C*	A	ncr ²	n/a	n/a
M2-1	7701	7702	C	G	32	P	P
M2-2	8197	8198	T	A**	13	Y	stop codon

M2-2	8199	8200	C	G**	15	C	nontranslated
M2-2 deletion				8202-8435 (234 nt)			aa 13-90 ³ : M2-2 deletion
L	10514	10515	C	T	673	L	L
L	13633	13634	C	A	1712	T	T

¹In table 21, the numbering of the nucleotide and amino acid sequences is relative to biological wt RSV strain A2 (GenBank accession number M74568), which was the first complete sequence of RSV strain A2. That genome is 15,222 nt in length. Thus, deletions or insertions in viruses do not change the
5 *sequence numbering* of the remaining nucleotides (or amino acids). Nucleotide and amino acid *sequence assignments* are relative to RSV D46 WT (SEQ ID NO:1) unless otherwise indicated. D46 is a second, recombinantly-derived version of strain A2 that differs in nucleotide length due to a single nucleotide insert at position 1099 (as indicated), resulting in a genome nucleotide length of 15,223.

This insertion was removed in RSV 276 and the assignment at that position became T.

10 ²ncr, non-coding region.

³Amino acids in RSV 276 that differ from RSV D46 are shaded in grey.

⁴ig, intergenic region

*Changes engineered into D46 to create four restriction site markers (Collins et al PNAS 92:11563-7 1995 PMID 8524804). These were removed in RSV 276.

15 **Nucleotide changes that create an AclI site in RSV 276.

The 276 virus was constructed using the D46 antigenomic cDNA (SEQ ID NO: 1) in combination with synthetic cDNA fragments. Specifically, a cDNA was synthesized that spanned from a unique NotI site in the plasmid vector upstream of the leader region to the unique AvrII site at positions
20 2129-2134 in the N gene in D46. A second cDNA was synthesized spanning from the unique XhoI site in D46 (positions 4481-4486) to the unique BamHI site (positions 8499-8505). This latter piece also contained the ΔM2-2-AclI mutation, except that the desired AclI site was HindIII (a restriction site that differs by inversion of the order of two nucleotides, not shown). These two pieces were substituted into D46 by conventional molecular cloning techniques, thereby achieving most of the desired nucleotide
25 changes shown in FIG. 16 and Table 21. Then, three site-directed mutagenesis steps were performed: the HindIII site was changed to the desired AclI site (involving changing two adjacent nucleotides), and the C10514T and C13633A substitutions in L were made. This resulted in the antigenomic cDNA for RSV 276 (SEQ ID NO: 19).

RSV 276 virus was readily recovered as experimental lots, and was confirmed to replicate
30 efficiently in Vero cells. In addition, a lot of RSV 276 virus clinical trial material was recovered and manufactured under conditions suitable for human use, in preparation for a clinical trial. Its sequence was confirmed to be free of adventitious mutations.

In addition, a lot of clinical trial material was made for the virus D46/NS2/N/ΔM2-2-HindIII (see FIG. 13, the second virus from the top). The sequence of the D46/NS2/N/ΔM2-2-HindIII antigenomic

cDNA is shown in SEQ ID NO: 18. The sequence of the clinical trial material of D46/NS2/N/ Δ M2-2-HindIII (Lot RSV#011B) matched that of the cDNA plasmid from which the recombinant virus was derived, except for three polymorphisms: (1) G2485A (~20-40% A); codon: GAT to AAT; amino acid: D47N in the P ORF; (2) a single-nucleotide thymidine insertion in a poly-thymidine stretch (nt 4537-39) in the 3' noncoding region of the SH gene (+ 1 nt; present in a subpopulation of about 30%); (3) a two-nucleotide adenosine insertion in a poly-adenosine stretch (nt 14,830-35) of the L gene end signal (+1A in about 30% of the population, + 2A in about 70% of the population). These polymorphisms are considered biologically inconsequential.

In a series of studies, an experimental lot of RSV 276 and three different lots of RSV D46/NS2/N/ Δ M2-2-HindIII were assayed for replication and immunogenicity in African green monkeys. Each preparation was evaluated separately due to constraints of timing and animal availability. The results are compared together in Tables 22, 23, and 24 in parallel with data for two comparators (LID/ Δ M2-2 and LID/ Δ M2-2/1030s) taken from Table 1. Virus replication was evaluated by quantitation of viral shedding sampled by NP swab (Table 22) and tracheal lavage (Table 23), quantified by plaque assay. This showed that, in the upper respiratory tract (by NP swab) two of the three lots of D46/NS2/N/ Δ M2-2-HindIII (studies 2 and 3) shed detectably (mean peak titers of 1.1-1.6 log₁₀ PFU/ml) over a period of 5.5 - 7.3 days, whereas shedding for the third lot (study 1) was minimal. In the lower respiratory tract (tracheal lavage), the results for the three lots were very similar, with moderate levels of shedding (2.2 – 2.6 log₁₀ PFU/ml) over 7.8 – 9.2 days. In comparison, shedding for RSV 276 was very similar to that of D46/NS2/N/ Δ M2-2-HindIII studies 2 and 3 in the upper respiratory tract, and was very similar to all three studies for shedding in the lower respiratory tract. In comparison, shedding by LID/ Δ M2-2 in both anatomical compartments was substantially greater, while shedding by LID/ Δ M2-2/1030s was substantially less. The 60% PRNT titer of these viruses at day 28 was 8.3 and 8.5 recip. log₂ for two of the lots of D46/NS2/N/ Δ M2-2-HindIII, and 6.3 recip. log₂ for the third lot, and 8.5 recip. log₂ for RSV 276. These titers generally equaled or exceeded those shown for LID/ Δ M2-2 and LID/ Δ M2-2/1030s (Table 24). Thus, these viruses provide a further spectrum of attenuation phenotypes based on Δ M2-2 backbones.

Table 22: Viral titers of nasopharyngeal swab samples from AGMs inoculated with LID Δ M2-2, LID Δ M2-2 1030s, D46/NS2/N/ Δ M2-2-HindIII, or RSV 276^a

RSV Vaccine candidate	AGM ID	NP virus titer (log ₁₀ PFU/mL) on indicated days ^b												Duration of shedding ^c	Peak virus titer	Sum of daily titers ^d
		1	2	3	4	5	6	7	8	9	10	12				
LID ΔM2-2	7806	-	1.4	1.7	2.7	2.6	<u>4.0</u>	3.9	1.4	-	2.7	-	9	4.0	21.4	
	7705	-	-	-	2.7	2.3	<u>3.6</u>	2.4	1.2	-	-	-	5	3.6	14.3	
	7747	-	-	1.3	0.7	-	<u>1.5</u>	1.3	-	-	-	-	5	1.5	7.2	
	7674	-	0.7	-	-	-	<u>2.3</u>	1.8	1.5	-	-	-	7	2.3	8.8	
	Mean:												6.5	2.9	12.9	
LID ΔM2-2 1030s	8033	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9	
	7720	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9	
	7844	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9	

	7847	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	<i>Mean:</i>												0	0.35	3.9
D46/NS2/N/ ΔM2-2- HindIII (study 1)	8417	<u>0.7</u>	-	-	-	-	-	-	-	-	-	-	1	0.7	4.2
	8489	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	8515	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	8574	-	-	-	-	-	-	-	<u>1.2</u>	-	-	-	8	1.2	4.7
	<i>Mean:</i>												2.3	0.7	4.2
D46/NS2/N/ ΔM2-2- HindIII (study 2)	N1330	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	N1326	-	-	-	1.0	0.7	-	<u>1.8</u>	-	1.0	-	-	6	1.8	6.9
	8566	-	-	-	<u>1.0</u>	0.7	0.7	-	0.7	0.7	-	-	6	1.0	5.9
	8551	-	-	-	<u>1.0</u>	0.7	<u>1.0</u>	-	-	-	-	-	3	1.0	5.5
	<i>Mean:</i>												3.8	1.1	5.5
D46/NS2/N/ ΔM2-2- HindIII (study 3)	9041	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	8938	-	-	-	-	1.3	1.3	1.3	<u>1.9</u>	1.0	-	-	5	1.9	8.9
	8926	-	-	-	-	-	0.7	-	<u>1.4</u>	<u>1.4</u>	-	-	4	1.4	6.3
	8911	-	-	-	0.7	1.0	1.0	2.2	<u>2.3</u>	1.0	-	-	6	2.3	10.0
	<i>Mean:</i>												3.8	1.6	7.3
RSV 276	8918	-	-	-	-	-	-	-	-	-	-	-	0	0.35	3.9
	8902	-	1.5	2.0	2.9	2.5	3.0	<u>3.1</u>	2.5	1.4	1.3	-	9	3.1	21.0
	8913	-	-	-	-	-	0.7	<u>1.4</u>	1.0	-	-	-	3	1.4	6.0
	8952	-	-	-	-	0.7	0.7	<u>1.5</u>	-	-	1.2	-	6	1.5	6.5
	<i>Mean:</i>												4.5	1.6	9.3

^aAGMs were inoculated by the combined nasopharyngeal and intratracheal routes with 10^6 PFU of the indicated virus in a 1 mL inoculum per site (total dose: 2×10^6 PFU per animal). AGM studies were approved by the Animal Care and Use Committee of NIAID, NIH. Results from a previous study of LID ΔM2-2 and LID ΔM2-2 1030s are shown for comparison.

^bCombined NP swabs were placed in 2 mL of L-15 medium with sucrose phosphate buffer as stabilizer. Virus titrations were performed on Vero cells at 37°C. The lower limit of detection was $0.7 \log_{10}$ PFU/mL. Samples with no detectable virus are represented as “-”. Peak titers for each animal are underlined.

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

^dThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). A value of 0.35 was used for samples with no detectable virus.

Table 23. Viral titers of tracheal lavage samples from AGMs inoculated with LID ΔM2-2, LID ΔM2-2 1030s, D46/NS2/N/ΔM2-2-HindIII, or RSV 276^a

RSV vaccine candidate	AGM ID	Tracheal lavage virus titer ($\log_{10}$ PFU/mL) on indicated days ^b						Duration of shedding ^c	Peak virus titer	Sum of daily titers ^d
		2	4	6	8	10	12			

LID ΔM2-2	7806	2.5	3.4	<u>4.6</u>	-	-	-	7	4.6	12.6
	7705	1.6	-	<u>3.3</u>	1.5	-	-	9	3.3	8.5
	7747	1.8	1.0	<u>6.0</u>	2.3	-	-	9	6.0	12.5
	7674	-	1.3	<u>2.7</u>	2.3	1.0	-	9	2.7	8.7
	Mean:							9	4.2	10.6
LID ΔM2-2 1030s	8033	-	-	-	-	-	-	0	0.7	4.2
	7720	-	-	-	-	-	-	0	0.7	4.2
	7844	-	-	-	-	-	-	0	0.7	4.2
	7847	-	-	-	-	-	-	0	0.7	4.2
	Mean:							0	0.7	4.2
D46/NS2/N/ ΔM2-2-HindIII (study 1)	8417	-	2.3	<u>2.5</u>	1.6	1.3	-	7	2.5	9.1
	8489	1.0	2.7	<u>3.2</u>	3.2	-	-	9	3.2	11.5
	8515	1.3	<u>2.0</u>	-	1.7	-	-	9	2.0	7.1
	8574	1.8	2.1	<u>2.2</u>	1.7	-	-	9	2.2	9.1
	Mean:							9	2.5	9.2
D46/NS2/N/ ΔM2-2-HindIII (study 2)	N1330	0.7	<u>1.0</u>	0.7	0.7	-	-	3	1.0	4.5
	N1326	1.7	1.7	1.8	<u>2.5</u>	-	-	9	2.5	9.1
	8566	1.8	0.7	<u>3.2</u>	2.4	-	-	9	3.2	9.5
	8551	1.9	<u>2.3</u>	1.7	0.7	-	-	7	2.3	8.0
	Mean:							7	2.2	7.8
D46/NS2/N/ ΔM2-2-HindIII (study 3)	9041	2.0	1.9	<u>2.3</u>	0.7	-	-	9	2.3	8.3
	8938	1.0	1.6	<u>2.5</u>	2.5	-	-	9	2.5	9.0
	8926	0.7	0.7	<u>2.6</u>	1.7	-	-	9	2.6	7.1
	8911	2.2	2.6	0.7	<u>3.0</u>	-	-	9	3.0	9.8
	Mean:							9	2.6	8.6
RSV 276	8918	0.7	1.7	<u>2.3</u>	1.6	1.0	-	9	2.3	8.0
	8902	2.2	1.6	<u>2.9</u>	2.1	0.7	-	9	2.9	10.3
	8913	0.7	0.7	<u>3.2</u>	1.3	0.7	-	5	3.2	7.3
	8952	1.8	<u>2.1</u>	1.6	1.8	0.7	-	9	2.1	8.8
	Mean:							8.0	2.6	8.6

^aAGMs were inoculated by the combined nasopharyngeal and IT routes with 10⁶ PFU of the indicated virus in a 1 mL inoculum per site (total dose: 2 x 10⁶ PFU per animal). Results from a previous study of LID ΔM2-2 and LID ΔM2-2 1030s are shown for comparison.

5 ^bOn days 2, 4, 6, 8, 10, and 12, tracheal lavage was performed with 3 mL of PBS. Virus titrations were performed on Vero cells at 37°C. The lower limit of detection was 1.0 log₁₀ PFU/mL of lavage solution. Samples with no detectable virus are represented as “-”. Peak titers for each animal are underlined.

10 ^cThe period of days from the first to the last day on which virus was detected, including negative days (if any) in between.

^dThe sum of daily titers is used as an estimate for the magnitude of shedding (area under the curve). A value of 0.7 was used for samples with no detectable virus.

Table 24. Neutralizing antibody titers of AGMs inoculated with LID ΔM2-2, LID ΔM2-2 1030s, D46/NS2/N/ΔM2-2-HindIII, or RSV 276^a

RSV Vaccine candidate	AGM ID	Neutralizing antibody titers (PRNT ₆₀ , reciprocal log ₂) on indicated days ^b		
		0	21	28
LID ΔM2-2	7806	<3.3	7.2	7.2
	7705	<3.3	8.8	8.2
	7747	<3.3	8.3	8.4
	7674	<3.3	6.7	6.2
	Mean:	<3.3	7.8	7.5
LID ΔM2-2 1030s	8033	<3.3	5.4	6.6
	7720	<3.3	<3.3	<3.3
	7844	<3.3	<3.3	4.3
	7847	<3.3	6.8	6.8
	Mean:	<3.3	4.7	5.2
D46/NS2/N/ΔM2-2-HindIII (study 1)	8417	<3.3	8.8	10.4
	8489	<3.3	6.6	8
	8515	<3.3	6.1	6.4
	8574	<3.3	9.4	8.2
	Mean:	<3.3	7.7	8.3
D46/NS2/N/ΔM2-2-HindIII (study 2)	N1330	<3.3	8.2	8.8
	N1326	<3.3	8.3	9.1
	8566	<3.3	7	7.3
	8551	<3.3	8.9	8.7
	Mean:	<3.3	8.1	8.5
D46/NS2/N/ΔM2-2-HindIII (study 3)	9041	<3.3	6.9	6.4
	8938	<3.3	7.1	7.2
	8926	<3.3	6.7	5.5
	8911	<3.3	7.2	5.9
	Mean:	<3.3	7.0	6.3
RSV 276	8918	<3.3	6.1	6.2
	8902	<3.3	8.4	8.8
	8913	<3.3	8.8	8.9
	8952	<3.3	9.7	9.9
	Mean:	<3.3	8.3	8.5

^aAGMs were inoculated i.n. and i.t. with 10⁶ PFU of the indicated virus in a 1 mL inoculum per site (total dose = 10^{6.3} PFU per animal). Results from a previous study of LID ΔM2-2 and LID ΔM2-2 1030s are shown for comparison.

^bOn days 0, 21, and 28 p.i., serum was obtained. Neutralizing antibody titers were determined in a 60% plaque reduction neutralization assay. The lower limit of detection was 3.3 (1:10).

It will be apparent that the precise details of the methods or compositions described may be varied or modified without departing from the spirit of the described embodiments. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

Claims:

1. A recombinant respiratory syncytial virus (RSV) attenuated by one or more modifications to a recombinant RSV genome, wherein the one or more modifications comprise:
 - (a) a deletion in a M2-2 open reading frame (ORF) corresponding to a deletion of 241 nucleotides at positions 8189-8429 of SEQ ID NO: 1, combined with nucleotide mutations at positions corresponding to T8161, T8167 and T8179 of SEQ ID NO: 1 (“ΔM2-2”);
 - (b) a deletion in a M2-2 ORF corresponding to a deletion of 234 nucleotides at positions 8203-8436 of SEQ ID NO: 1, combined with nucleotide mutations at positions corresponding to 8198A and 8200G of SEQ ID NO: 1 (“ΔM2-2-*AcII*”); or
 - (c) a deletion in a M2-2 ORF corresponding to a deletion of 234 nucleotides at positions 8203-8436 of SEQ ID NO: 1, combined with nucleotide mutations at positions corresponding to 8198A and 8199G of SEQ ID NO: 1 (“ΔM2-2-*HindIII*”), wherein the recombinant RSV genome is a D46 genome attenuated by the one or more modifications.
2. The recombinant RSV of claim 1, wherein the one or more modifications further comprise a deletion of 112 nucleotides corresponding to positions 4499-4610 of SEQ ID NO: 1, combined with nucleotide mutations at positions corresponding to C4489T, C4492T, A4495T, A4497G, and G4498A of SEQ ID NO: 1 (“6120”).
3. A recombinant respiratory syncytial virus (RSV) attenuated by one or more modifications to a recombinant RSV genome, wherein the one or more modifications comprise:
 - a deletion in a M2-2 ORF corresponding to a deletion of 234 nucleotides at positions 8203-8436 of SEQ ID NO: 1, combined with nucleotide mutations at positions corresponding to 8198A and 8199G of SEQ ID NO: 1 (“ΔM2-2-*HindIII*”), and
 - a deletion of 112 nucleotides corresponding to positions 4499-4610 of SEQ ID NO: 1, combined with nucleotide mutations at positions corresponding to C4489T, C4492T, A4495T, A4497G, and G4498A of SEQ ID NO: 1 (“6120”).
4. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise nucleotide mutations encoding amino acid substitutions of V267I in the N protein, E218A and T523I in the F protein, and C319Y and H1690Y in the L protein of the RSV (“cp”).
5. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise nucleotide mutations to introduce a Y1321K substitution in the L protein of the RSV, and wherein the L protein comprises a S1313 residue, wherein the codons

encoding Y1321K substitution and the S1313 residue are AAA and TCA codons respectively (“1030s”).

6. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise nucleotide mutations encoding amino acid substitution K51R in the NS2 protein of the RSV (“NS2”).

7. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise nucleotide mutations encoding amino acid substitution T24A in the N protein of the RSV (“N”).

8. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise nucleotide mutations encoding amino acid substitution K51R in the NS2 protein and T24A in the N protein of the RSV (“NS2/N”).

9. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise a deletion in a SH ORF corresponding to deletion of 419 nucleotides at positions 4198-4616 of SEQ ID NO: 1 (“ΔSH”).

10. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise replacing the nucleotide sequence encoding a G protein of the RSV with a corresponding codon optimized nucleotide sequence encoding a G protein from the clinical isolate A/Maryland/001/11.

11. The recombinant RSV of claim 10, wherein the codon optimized nucleotide sequence encoding the G protein from the clinical isolate A/Maryland/001/11 comprises a nucleotide sequence corresponding to SEQ ID NO: 8 (G001BB).

12. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise replacing the nucleotide sequence encoding a F protein of the RSV with a corresponding codon-optimized nucleotide sequence set forth as SEQ ID NO: 9 (FBB).

13. The recombinant RSV of any of claims 1-11, wherein the one or more modifications further comprise replacing the nucleotide sequence encoding a F protein of the RSV with a corresponding nucleotide sequence set forth as SEQ ID NO: 10 (F001), which encodes the F protein from the clinical isolate A/Maryland/001/11.

14. The recombinant RSV of any of claims 1-11, wherein the one or more modifications further comprise replacing the nucleotide sequence encoding a F protein of the RSV with a corresponding codon optimized nucleotide sequence encoding the F protein from the clinical isolate A/Maryland/001/11.

15. The recombinant RSV of claim 14, wherein the codon optimized nucleotide sequence encoding the F protein from the clinical isolate A/Maryland/001/11 comprises a nucleotide sequence corresponding to SEQ ID NO: 11 (F001BB).

16. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise nucleotide mutations encoding amino acid substitutions K66E and Q101P in the F protein of the RSV ("HEK").

17. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprise nucleotide mutations encoding amino acid substitutions E218A and T523I in the F protein of the RSV (F cp substitutions).

18. The recombinant RSV of any of the prior claims, wherein the one or more modifications further comprises reversing the order of the genes encoding the G and the F proteins in the RSV genome.

19. The recombinant RSV of claim 1, wherein the one or more modifications comprise or consist of a combination of mutations selected from any one of:

Δ M2-2, cp/ Δ M2-2, cp/ Δ M2-2/HEK, Δ M2-2/1030s, NS2/N/ Δ M2-2, NS2/ Δ M2-2, N/ Δ M2-2, Δ SH/ Δ M2-2, cp/ Δ SH/ Δ M2-2, 6120/ Δ M2-2, 6120/cp/ Δ M2-2, 6120/ Δ M2-2/1030s, 6120/NS2/N/ Δ M2-2, 6120/G001BB/FBB/ Δ M2-2, 6120/FBB/G001BB/ Δ M2-2, 6120/G001BB/F/ Δ M2-2, 6120/G/FBB/ Δ M2-2, 6120/G/FBBHEK/ Δ M2-2, 6120/G/FBBcpHEK/ Δ M2-2, 6120/FBB/G/ Δ M2-2, 6120/G001BB/F001BB/ Δ M2-2, 6120/NS2/ Δ M2-2, or 6120/N/ Δ M2-2; or

Δ M2-2-*AcII*, cp/ Δ M2-2-*AcII*, cp/ Δ M2-2-*AcII*/HEK, Δ M2-2-*AcII*/1030s, NS2/N/ Δ M2-2-*AcII*, NS2/ Δ M2-2-*AcII*, N/ Δ M2-2-*AcII*, Δ SH/ Δ M2-2-*AcII*, cp/ Δ SH/ Δ M2-2-*AcII*, 6120/ Δ M2-2-*AcII*, 6120/cp/ Δ M2-2-*AcII*, 6120/ Δ M2-2-*AcII*/1030s, 6120/NS2/N/ Δ M2-2-*AcII*, 6120/G001BB/FBB/ Δ M2-2-*AcII*, 6120/FBB/G001BB/ Δ M2-2-*AcII*, 6120/G001BB/F/ Δ M2-2-*AcII*, 6120/G/FBB/ Δ M2-2-*AcII*, 6120/G/FBBHEK/ Δ M2-2-*AcII*, 6120/G/FBBcpHEK/ Δ M2-2-*AcII*, 6120/FBB/G/ Δ M2-2-*AcII*, 6120/G001BB/F001BB/ Δ M2-2-*AcII*, 6120/NS2/ Δ M2-2-*AcII*, or 6120/N/ Δ M2-2-*AcII*; or

Δ M2-2-*HindIII*, cp/ Δ M2-2-*HindIII*, cp/ Δ M2-2-*HindIII*/HEK, Δ M2-2-*HindIII*/1030s, NS2/N/ Δ M2-2-*HindIII*, NS2/ Δ M2-2-*HindIII*, N/ Δ M2-2-*HindIII*, Δ SH/ Δ M2-2-*HindIII*, cp/ Δ SH/ Δ M2-2-*HindIII*, 6120/ Δ M2-2-*HindIII*, 6120/cp/ Δ M2-2-*HindIII*, 6120/ Δ M2-2-*HindIII*/1030s,

6120/NS2/N/ Δ M2-2-*Hind*III, 6120/G001BB/FBB/ Δ M2-2-*Hind*III, 6120/FBB/G001BB/ Δ M2-2-*Hind*III, 6120/G001BB/F/ Δ M2-2-*Hind*III, 6120/G/FBB/ Δ M2-2-*Hind*III, 6120/G/FBBHEK/ Δ M2-2-*Hind*III, 6120/G/FBBcpHEK/ Δ M2-2-*Hind*III, 6120/FBB/G/ Δ M2-2-*Hind*III, 6120/G001BB/F001BB/ Δ M2-2-*Hind*III, 6120/NS2/ Δ M2-2-*Hind*III, or 6120/N/ Δ M2-2-*Hind*III.

20. The recombinant RSV of any of the prior claims, wherein the recombinant RSV genome comprises the one or more modifications, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical to SEQ ID NO: 1.

21. The recombinant RSV of any of the prior claims, wherein the recombinant RSV genome comprises the one or more modifications, and a nucleotide sequence corresponding to a positive-sense sequence at least 95% identical to SEQ ID NO: 1.

22. The recombinant RSV of any of the prior claims, wherein the recombinant RSV genome comprises the one or more modifications, and the remaining nucleotides of the RSV genome comprise a nucleotide sequence corresponding to a positive-sense sequence at least 99% identical to SEQ ID NO: 1.

23. The recombinant RSV of any of the prior claims, wherein the recombinant RSV genome is a D46 genome comprising the one or more modifications.

24. The recombinant RSV of claim 1, wherein the recombinant RSV genome comprises one of:

(a) 6120 and Δ M2-2 mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 5 (LID/ Δ M2-2 sequence);

(b) cp and Δ M2-2 mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 1 (D46 sequence);

(c) cp and Δ M2-2 mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 15 (D46/cp/ Δ M2-2 sequence);

(d) 6120, Δ M2-2, and 1030s mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 16 (LID/ Δ M2-2/1030s sequence);

(e) 6120, cp, and Δ M2-2 mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 17 (LID/cp/ Δ M2-2 sequence);

(f) NS2, N, Δ M2-2-*Hind*III mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 4 (D46/ Δ M2-2-*Hind*III sequence);

(g) NS2, N, Δ M2-2-*Hind*III mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 18 (D46/NS2/N/ Δ M2-2-*Hind*III sequence);

(h) NS2, N, Δ M2-2-*Ac*II mutations, and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 3 (D46/ Δ M2-2-*Ac*II sequence); or

(i) NS2, N, and Δ M2-2-*Ac*II mutations, the following nucleotide mutations with positions relative to SEQ ID NO: 1: 404C, 779G, deletion of C1099, 1139A, 1140G, 1182G, 1210G, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, and 13634A; and a nucleotide sequence corresponding to a positive-sense sequence at least 90% identical, at least 95% identical, and/or at least 99% identical to SEQ ID NO: 19 (276 sequence).

25. The recombinant RSV of claim 1, wherein the recombinant RSV genome is one of:

- a D46/cp/ Δ M2-2 genome;
- a LID/ Δ M2-2/1030s genome;
- a LID/cp/ Δ M2-2 genome;
- a D46/NS2/N/ Δ M2-2-*Hind*III genome;
- a LID/ Δ M2-2 genome; or
- a 276 genome.

26. The recombinant RSV of claim 1, wherein the nucleotide sequence of the recombinant RSV genome comprises or consists of a nucleotide sequence corresponding to a positive-sense sequence set forth as any one of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, or SEQ ID NO: 19.

27. The recombinant RSV of claim 1, wherein the recombinant RSV genome comprises a nucleotide sequence corresponding to a positive-sense sequence set forth as SEQ ID NO: 3 further modified by introduction of the following nucleotide mutations relative to SEQ ID NO: 1: 404C,

779G, 1099T, 1139A, 1140G, 1182G, 1210G, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, and 13634A.

28. The recombinant RSV of claim 1, wherein the recombinant RSV genome comprises a nucleotide sequence corresponding to a positive-sense sequence set forth as SEQ ID NO: 3 further modified by introduction of the following nucleotide mutations relative to SEQ ID NO: 1: 404C, 779G, deletion of C1099, 1139A, 1140G, 1182G, 1210G, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, and 13634A.

29. The recombinant RSV of claim 1, wherein the recombinant RSV genome or antigenome comprises a sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4, further modified by introduction of one or more of the following nucleotide substitutions with positions relative to SEQ ID NO: 1: 404C, 779G, 1099T, 1139A, 1140G, 1182G, 1210G, 1938A, 5612A, 5616A, 5640G, 6216C, 6222C, 6387T, 7215C, 7482T, 7560A, 7702G, 10515T, 13634A, 13901T.

30. The recombinant RSV of any of the prior claims, wherein the RSV is a subtype A RSV.

31. The recombinant RSV of any of claims 1-19, wherein the RSV is a subtype B RSV.

32. The recombinant RSV of any of the prior claims, wherein the RSV is infectious, attenuated, and self-replicating.

33. An isolated polynucleotide molecule comprising the nucleotide sequence of the recombinant RSV genome of any of claims 1-32, or an antigenomic cDNA or RNA sequence of the RSV genome.

34. A vector comprising the polynucleotide molecule of claim 33.

35. A cell comprising the polynucleotide molecule or vector of claim 33 or claim 34.

36. A method of producing a recombinant RSV, comprising:
transfecting a permissive cell culture with the vector of claim 34;
incubating the cell culture for a sufficient period of time to allow for viral replication; and
purifying the replicated recombinant RSV.

37. A recombinant RSV produced by the method of claim 36.
38. A pharmaceutical composition comprising the recombinant RSV of any of claims 1-32 or 37.
39. A method of eliciting an immune response to RSV in a subject comprising administering an immunogenically effective amount of the pharmaceutical composition of claim 38 to the subject.
40. The method of claim 39, wherein the immune response is a protective immune response.
41. The method of claim 39 or claim 40, wherein the pharmaceutical composition is administered intranasally.
42. The method of claim 39 or claim 40, wherein the RSV is administered via injection, aerosol delivery, nasal spray or nasal droplets.
43. The method of any of claims 39-42, wherein the subject is a human.
44. The method of any of claims 39-43, wherein the subject is between 1 and 6 months of age.
45. The method of any of claims 39-44, wherein the subject is seronegative for RSV.
46. Use of the recombinant RSV of any of claims 1-32 or claim 37 to elicit an immune response to RSV in a subject.

FIG. 1A

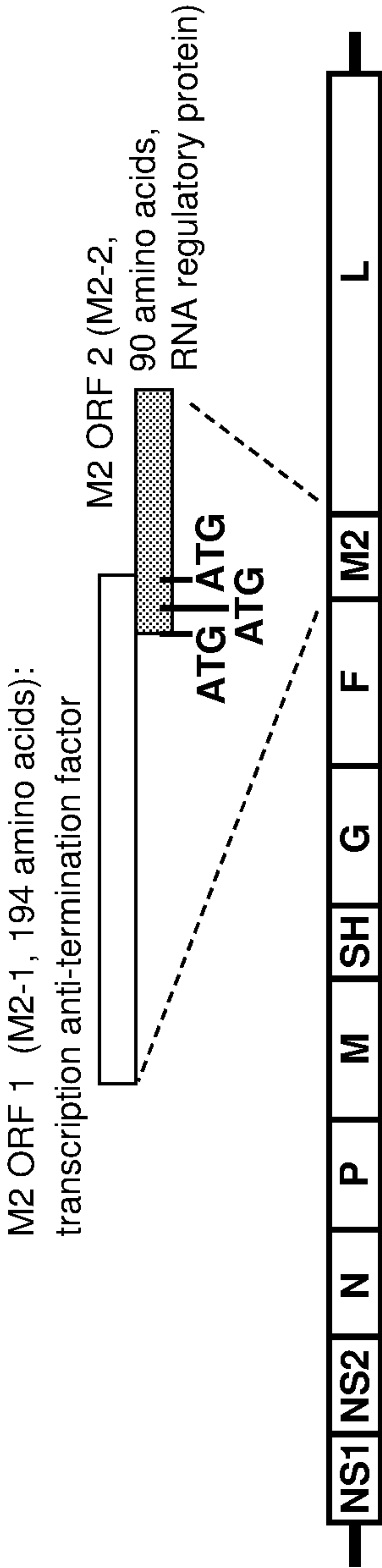


FIG. 1B

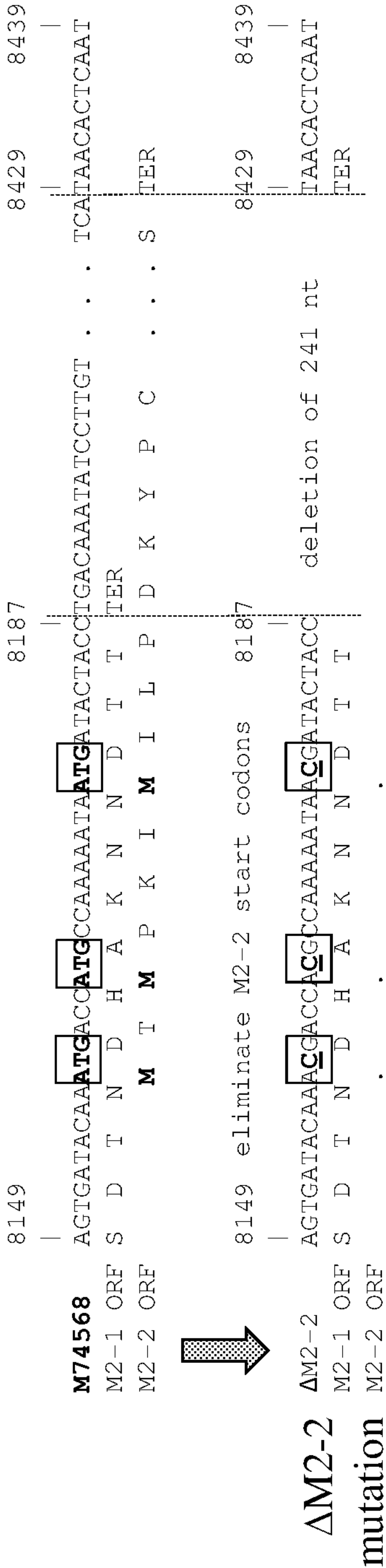


FIG. 2

RSV D46/ Δ M2-2 and RSV LID/ Δ M2-2

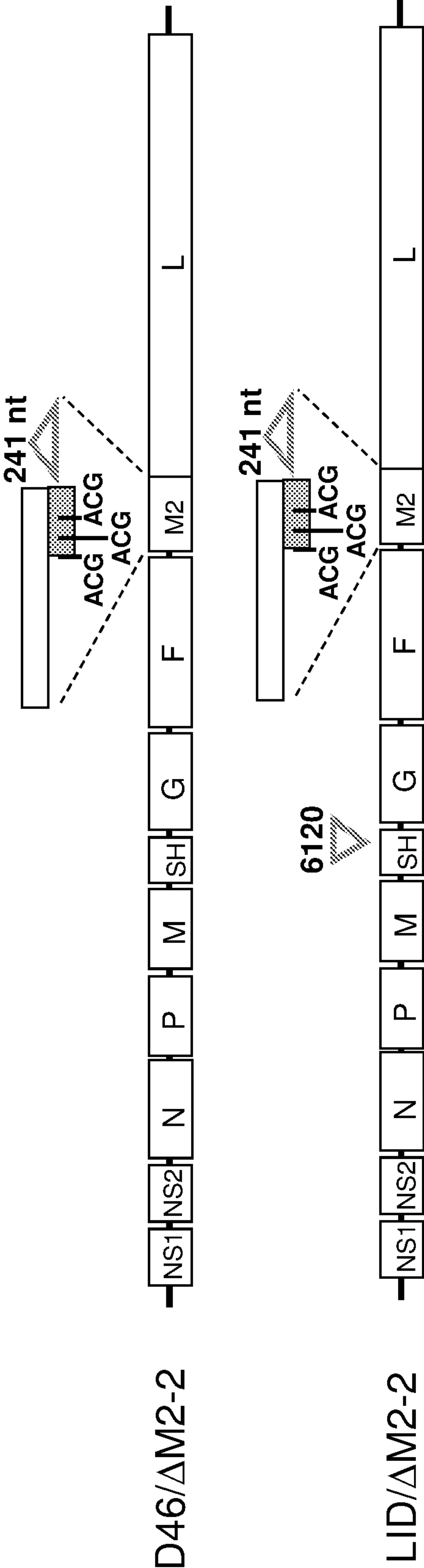


FIG. 3A

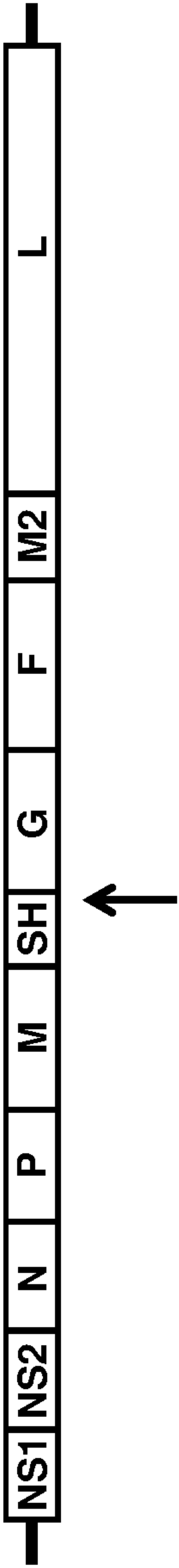


FIG. 3B

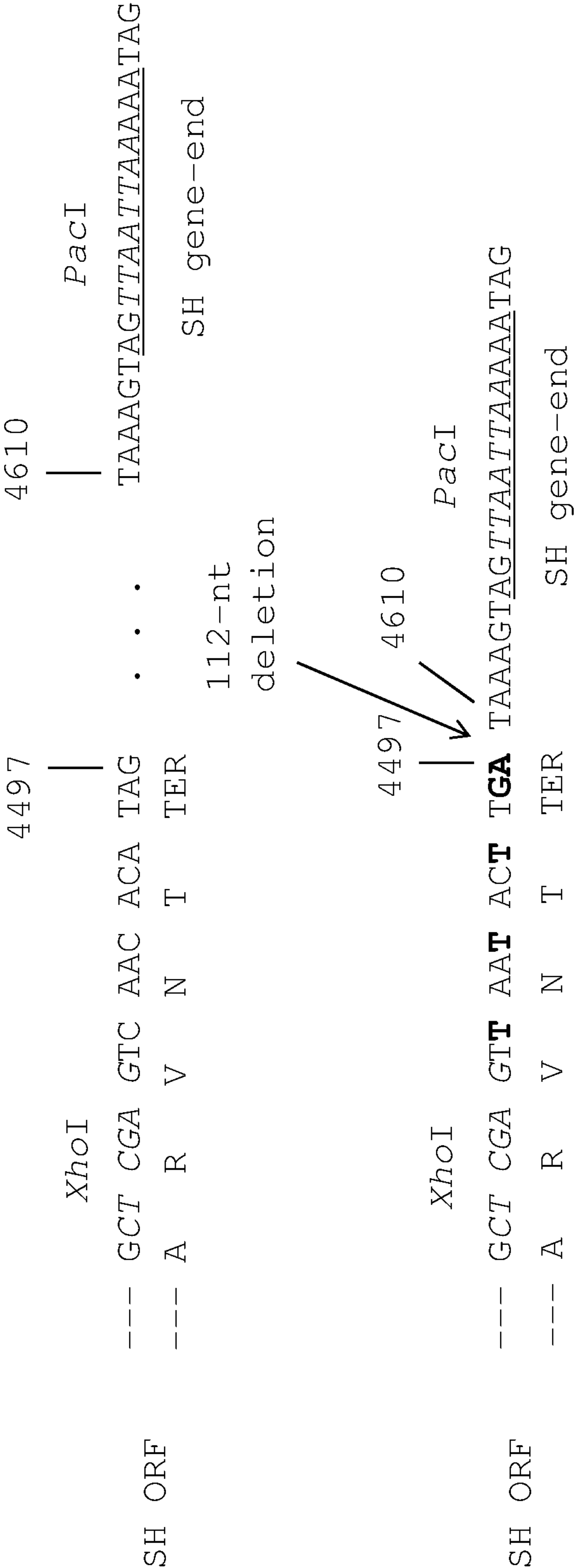


FIG. 4

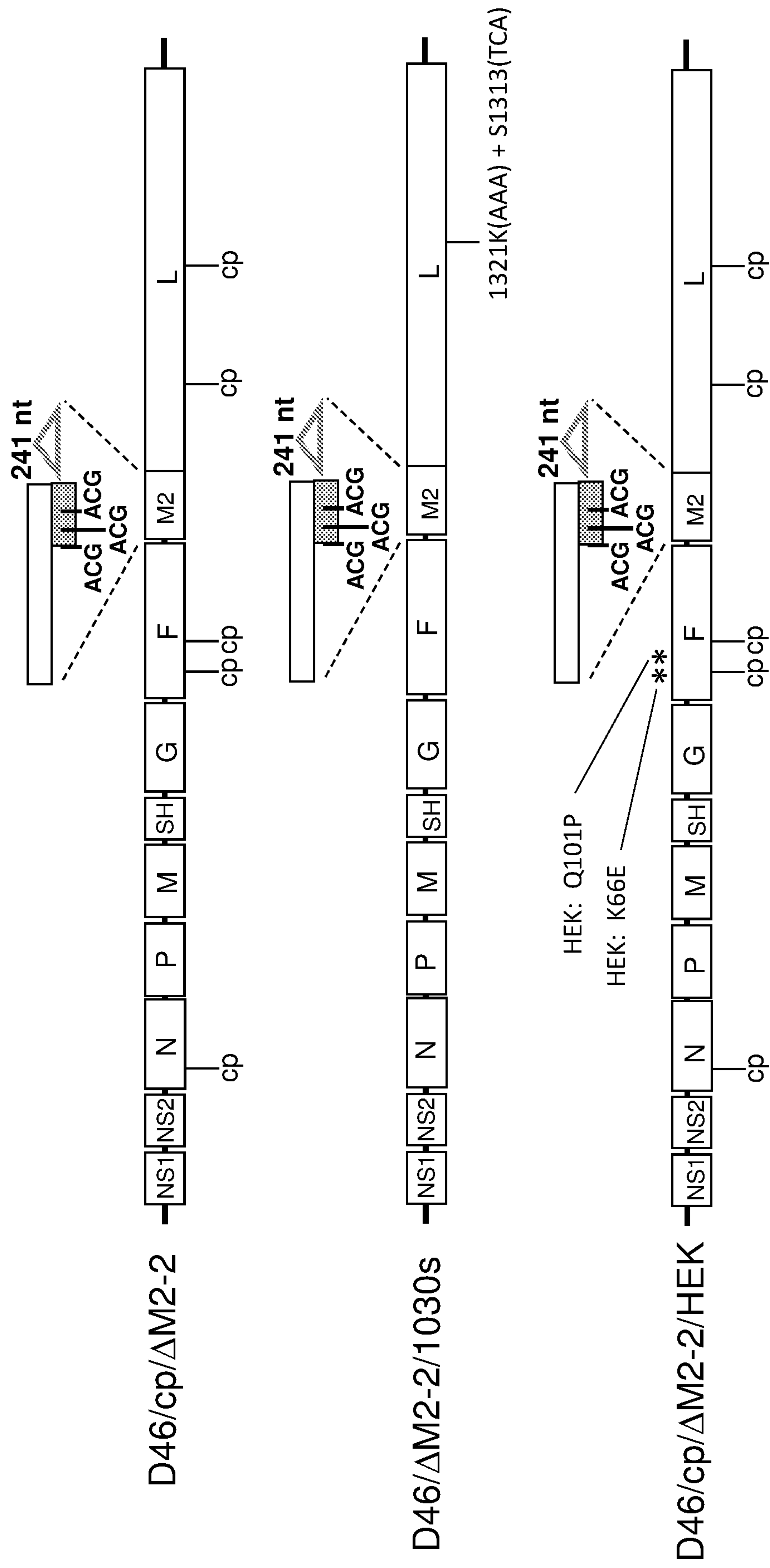


FIG. 5

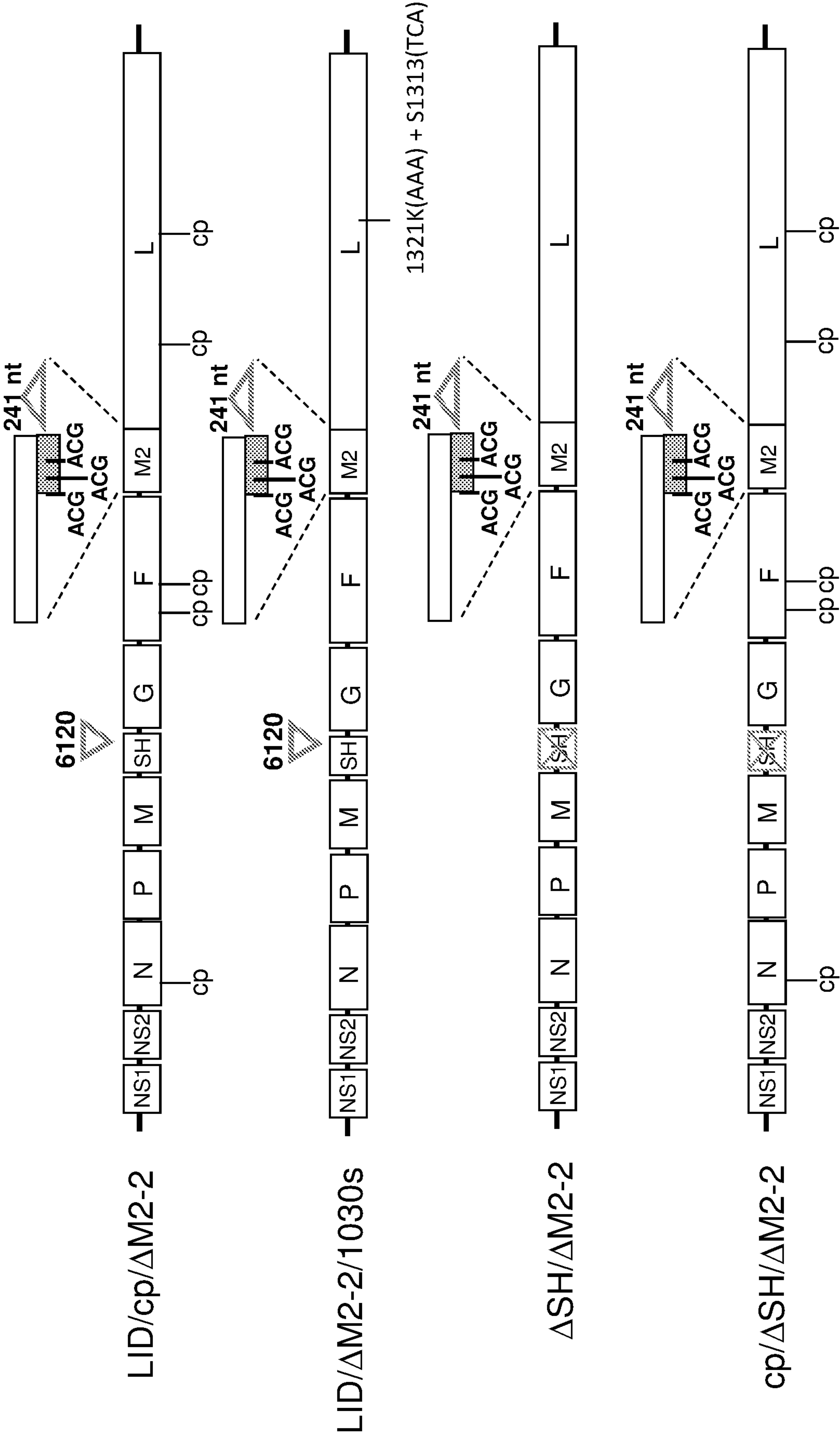


FIG. 7
Replication of selected ΔM2-2 mutant viruses in BALB/c mice

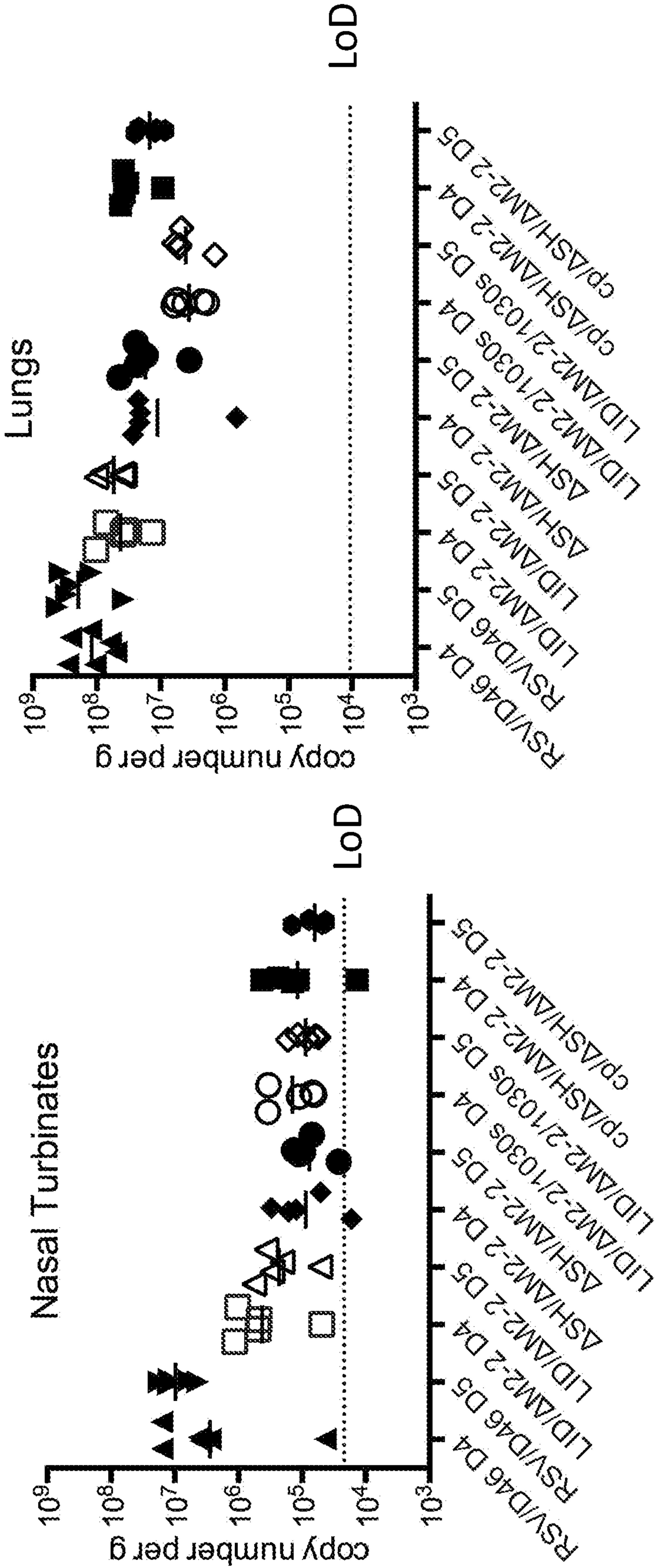


FIG. 8A

Replication of selected ΔM2-2 mutant viruses in African Green Monkeys

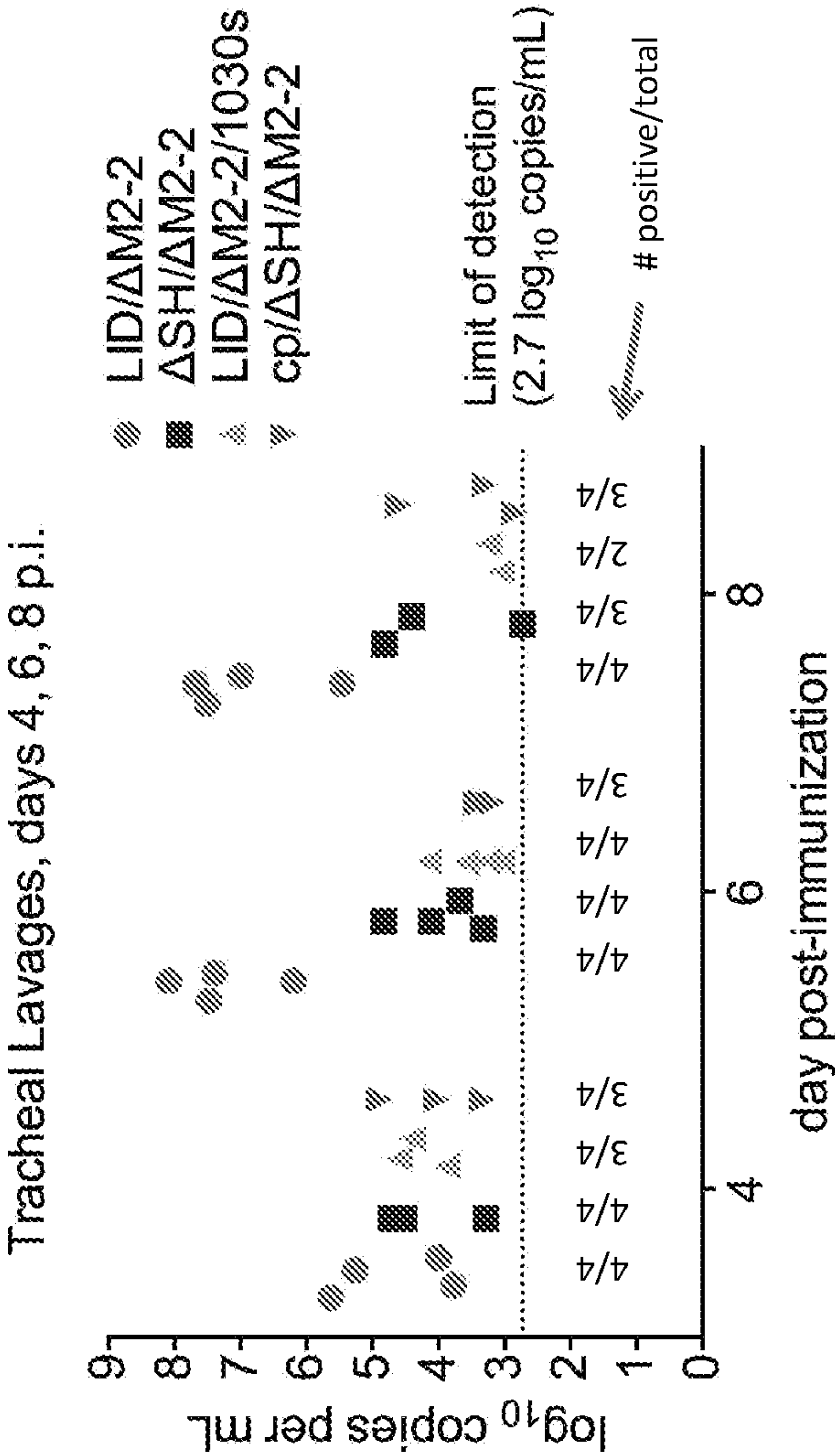


FIG. 8B

Replication of selected ΔM2-2 mutant viruses in African Green Monkeys

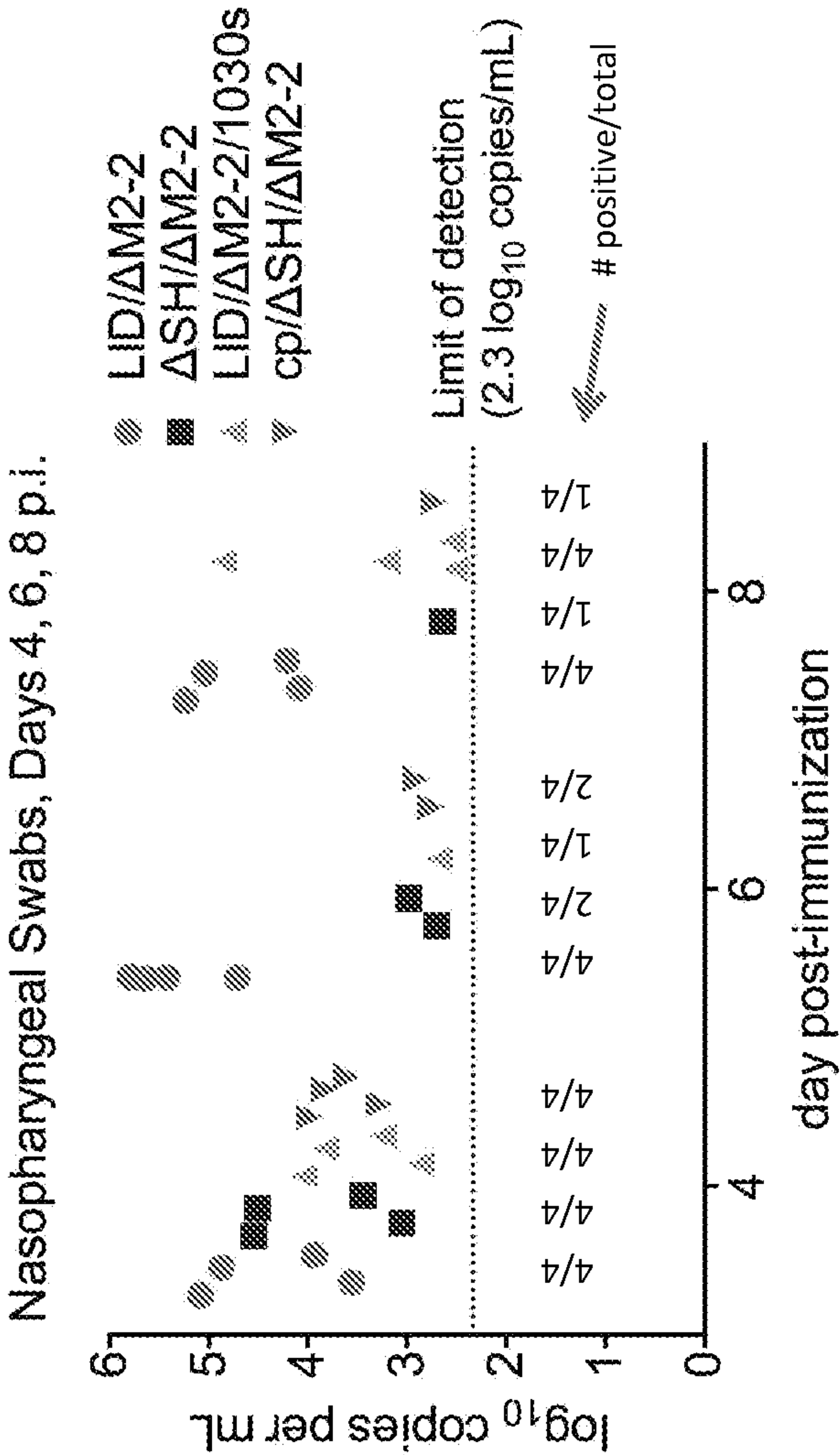


FIG. 9B
Nasal wash titer of LID/ Δ M2-2

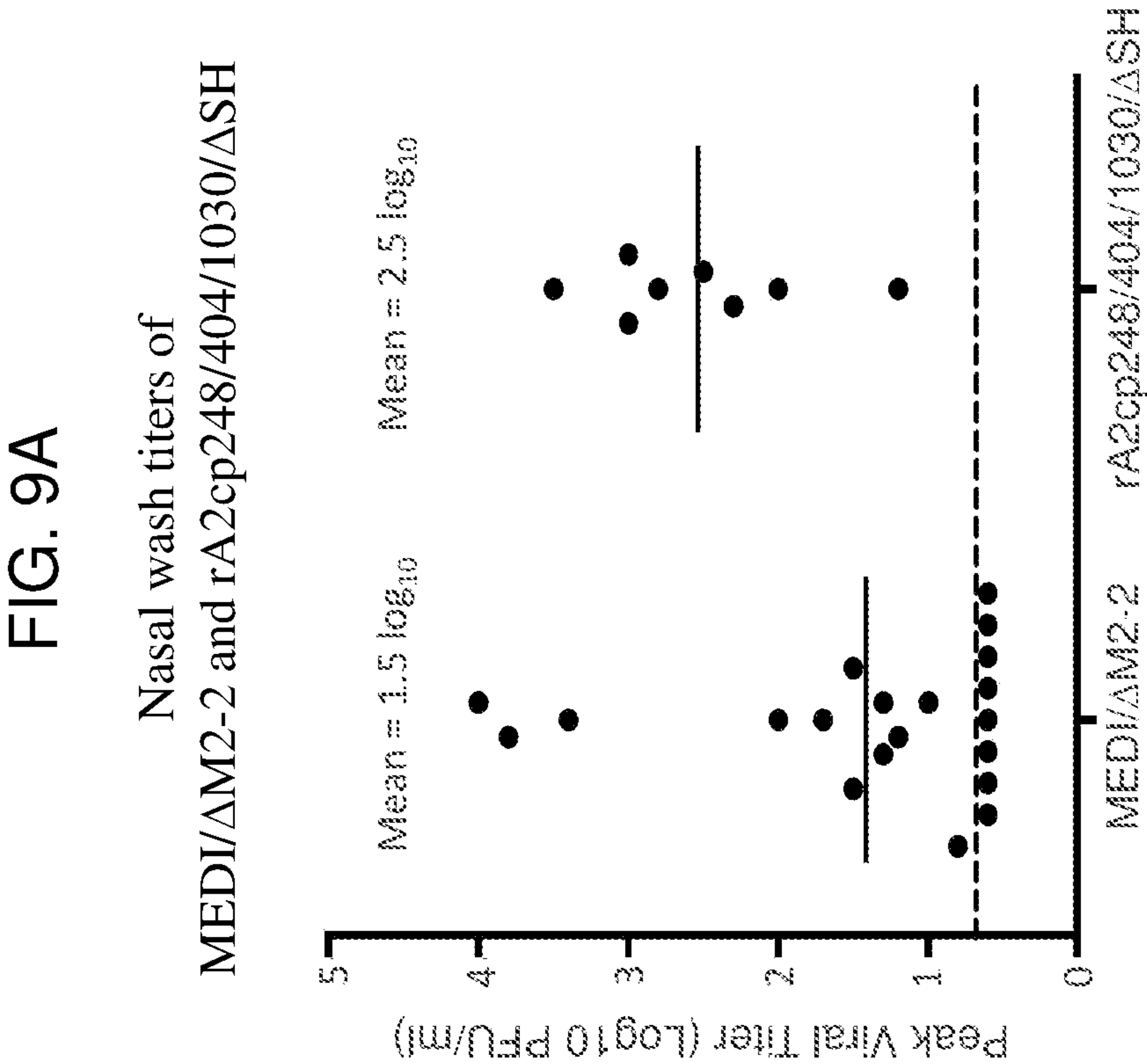
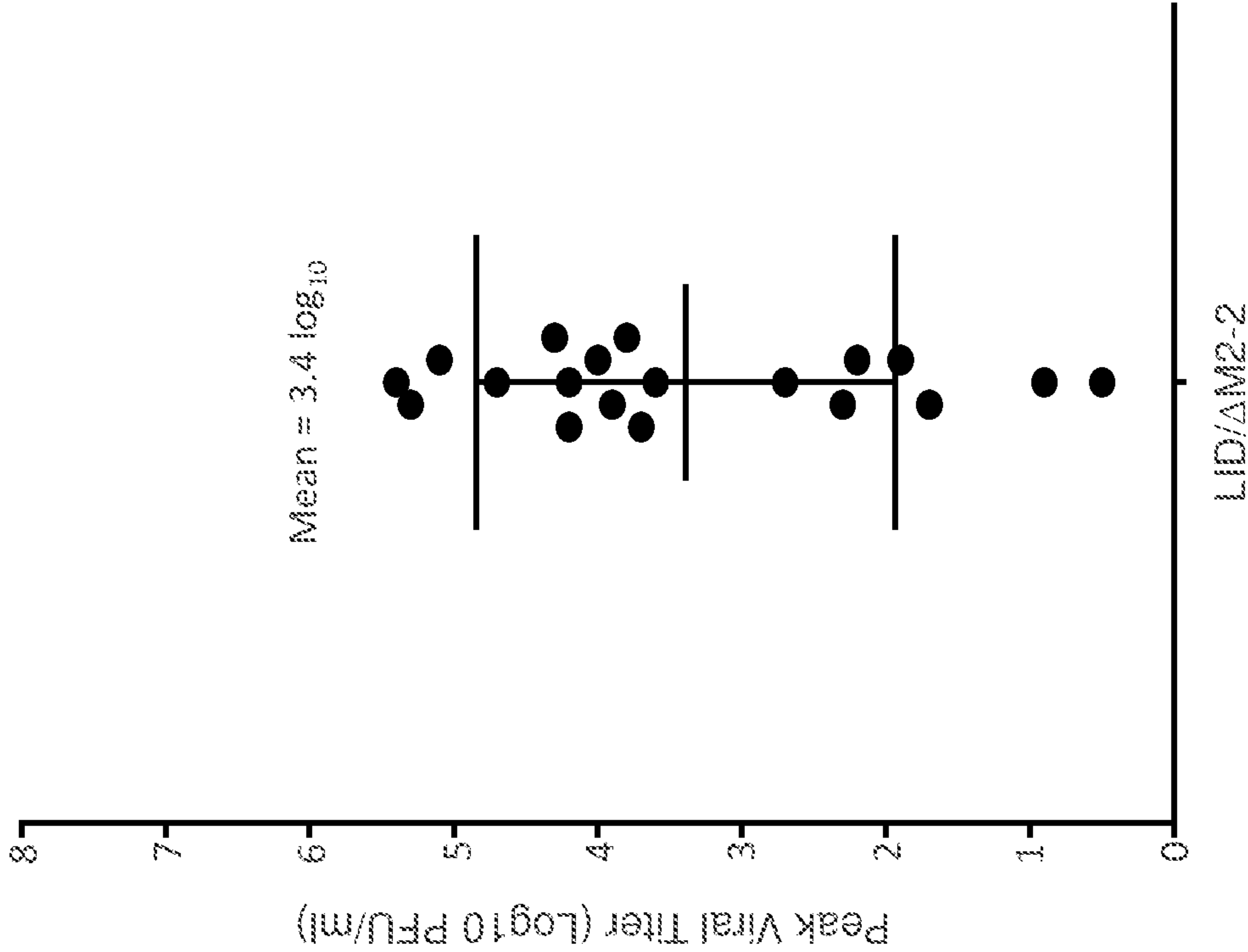


FIG. 10A

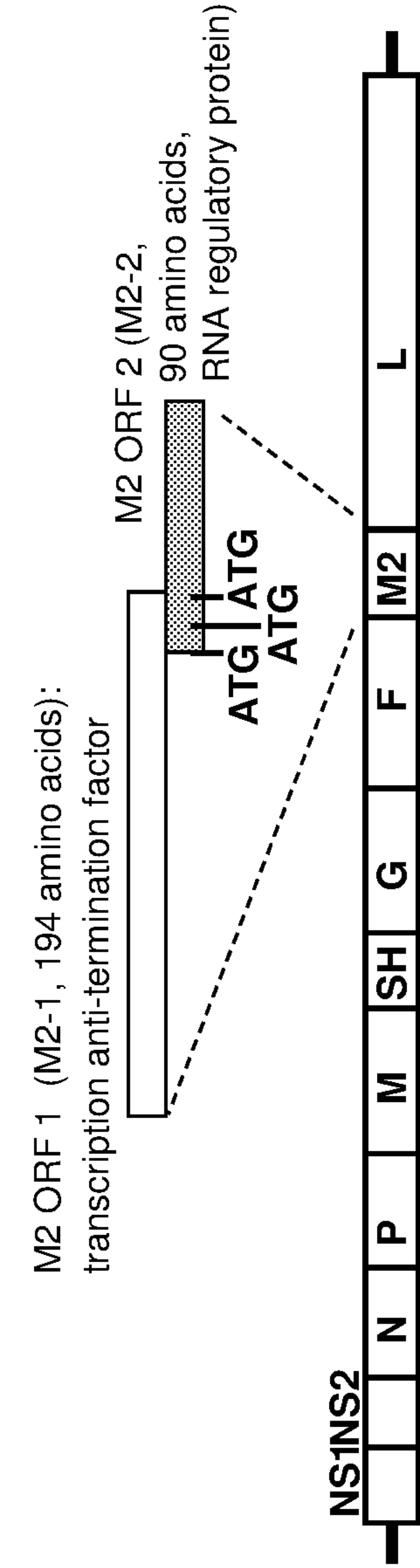


FIG. 10B

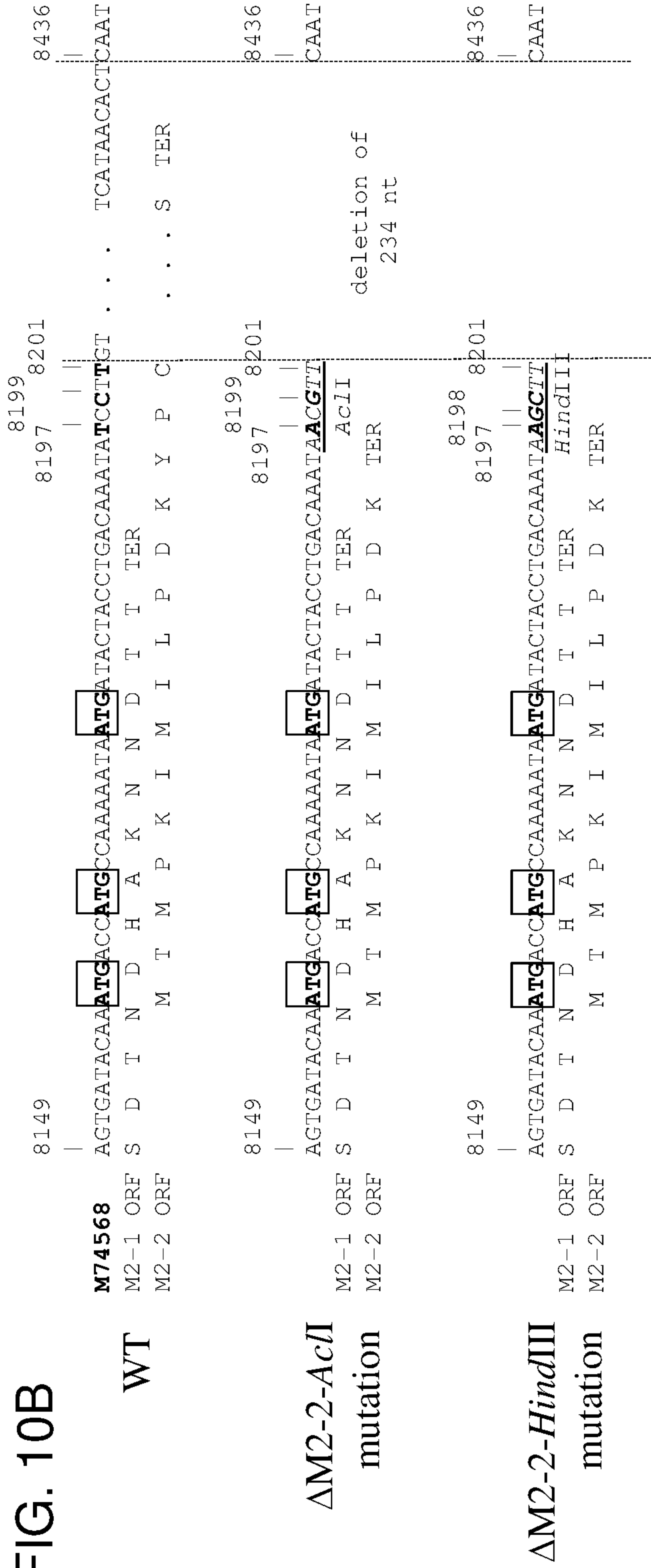


FIG. 11
D46 and LID viruses bearing the ΔM2-2-Ac/I mutation

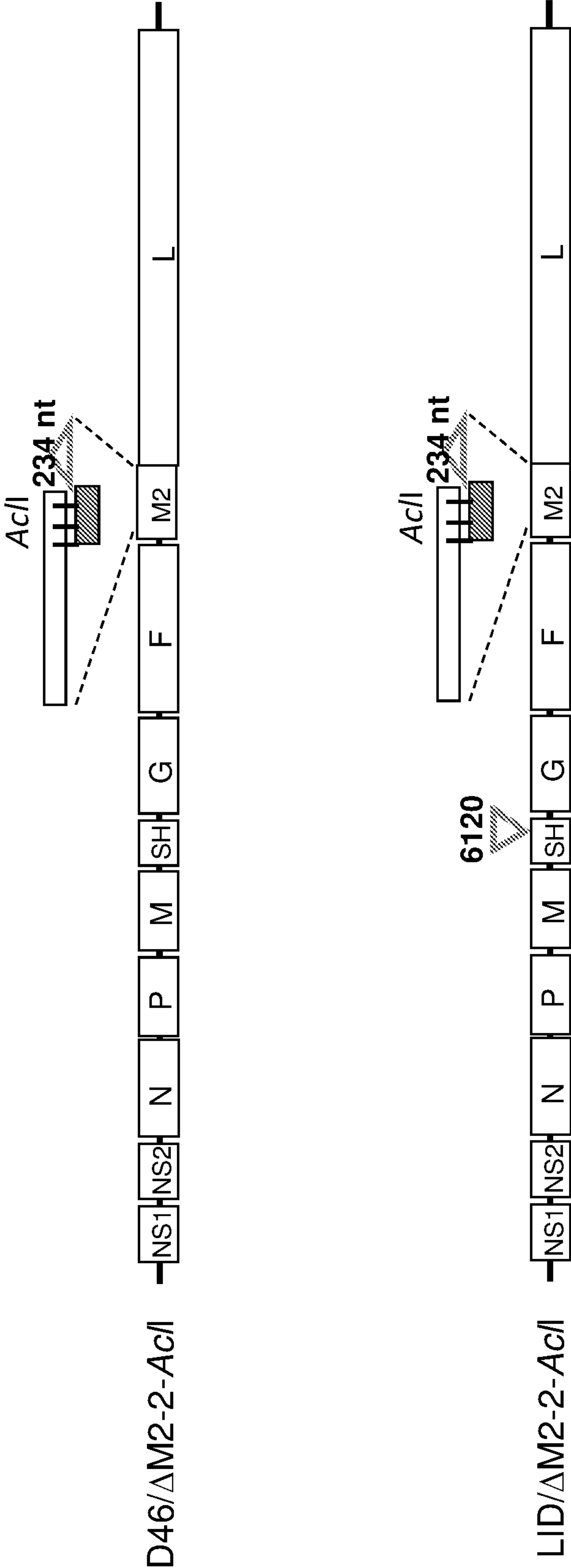


FIG. 12A

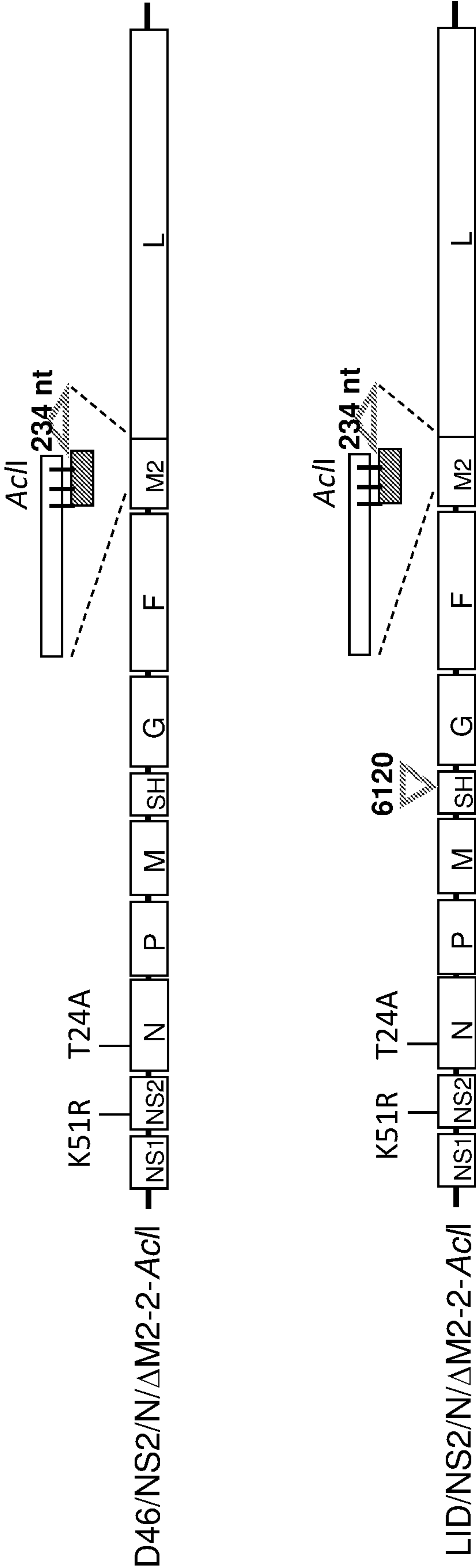


FIG. 12B

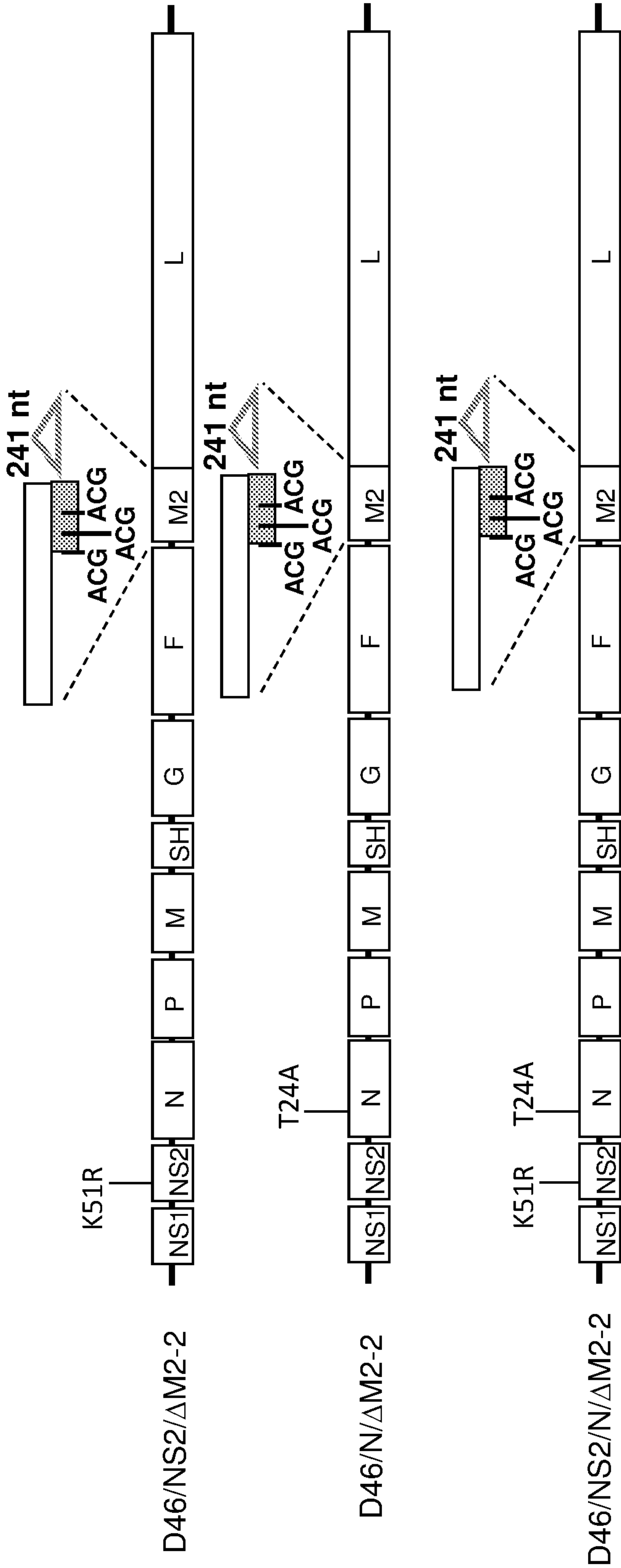


FIG. 12C

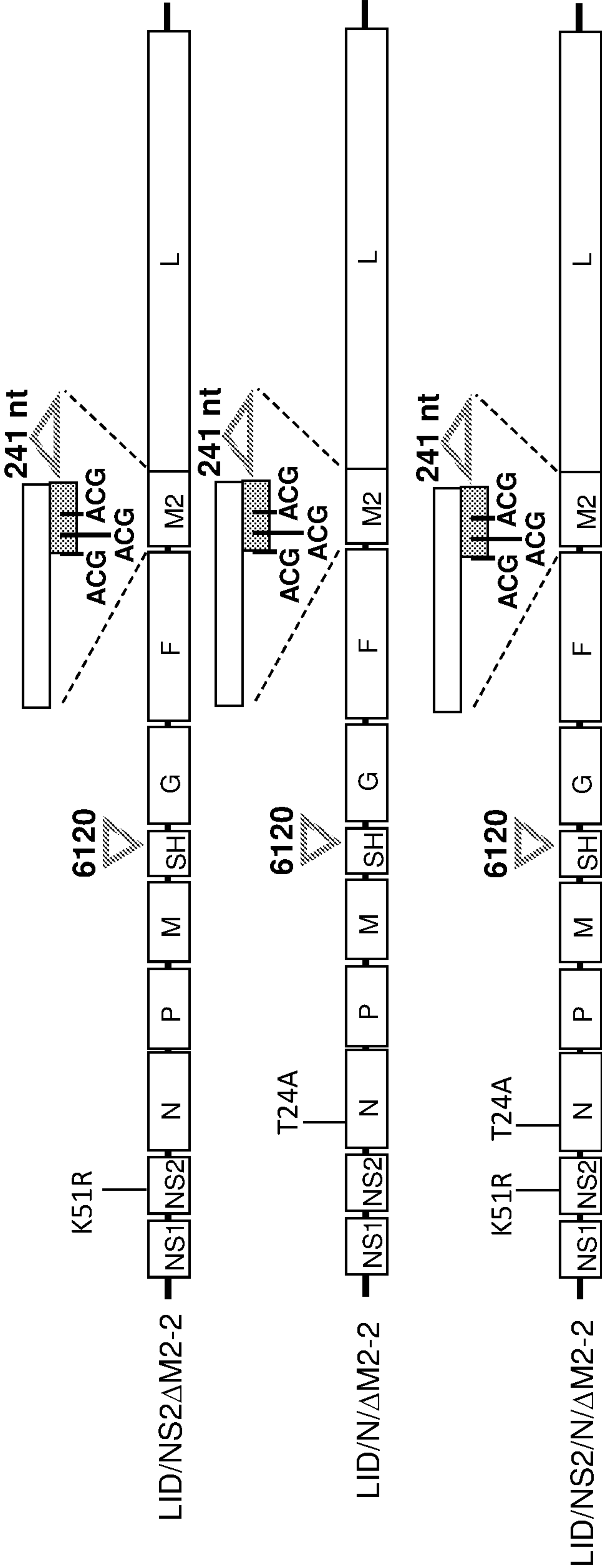


FIG. 13

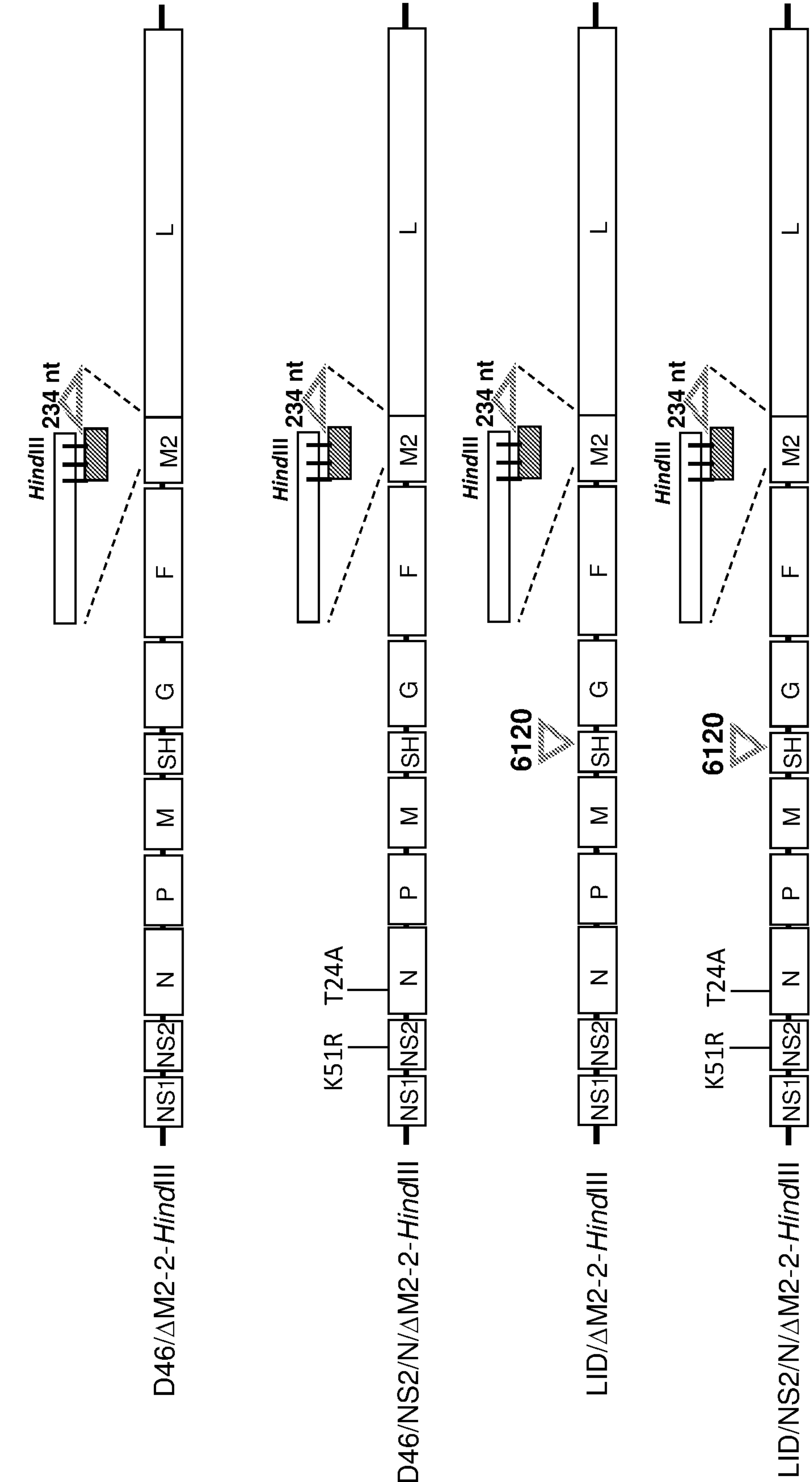


FIG. 14A

ΔM2-2 viruses with additional modifications to the F and G genes

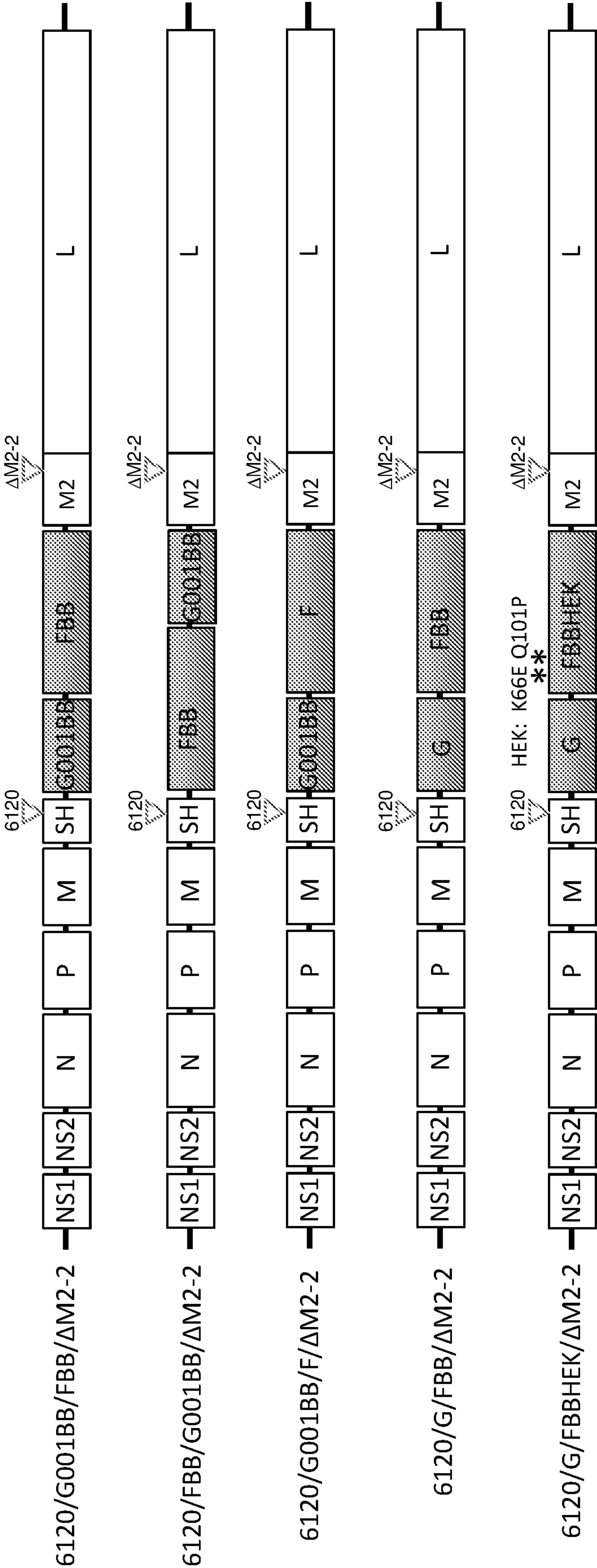


FIG. 14B

ΔM2-2 viruses with additional modifications to the F and G genes

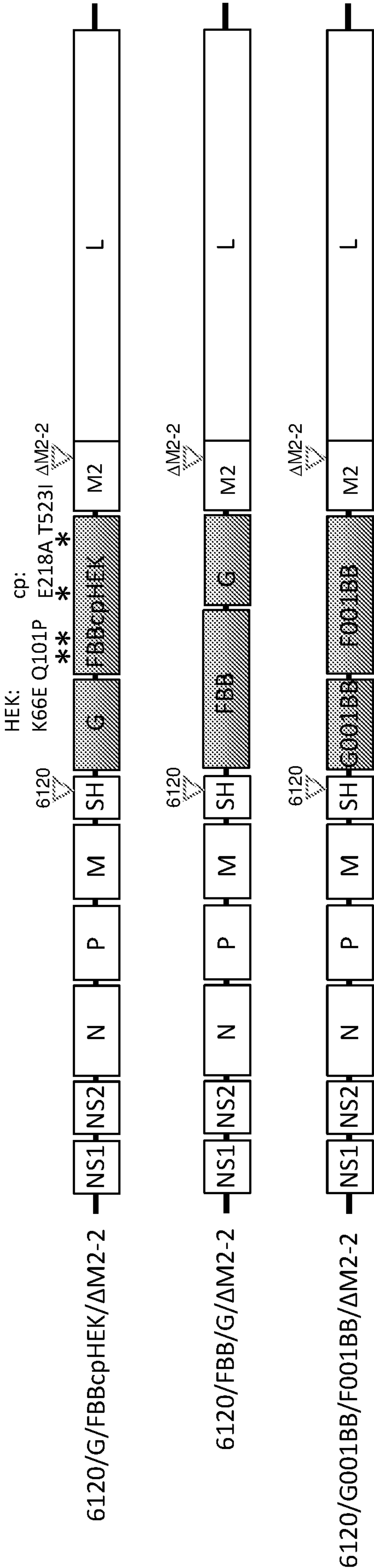


FIG. 15

Comparison of P1 virus titers at 32°C, and P2 virus titers at 32°C and 37°C

Virus	P1 Titer pfu/ml 32°C	P2 Titer pfu/ml 32°C (MOI ~0.01)	P2 Titer pfu/ml 37°C (MOI ~0.01)
6120/G001BB/FBB/ΔM2-2	1.7*10 ⁷	1.95*10 ⁷	2.35*10 ⁷
6120/FBB/G001BB/ΔM2-2	3.25*10 ⁷	3.1*10 ⁷	3.3*10 ⁷
6120/G001BB/F/ΔM2-2	6.66*10 ⁶	4.8*10 ⁷	3.65*10 ⁷
6120/G/FBB/ΔM2-2	1.8*10 ⁷	1.05*10 ⁷	1.1*10 ⁷
6120/G/FBBHEK/ΔM2-2	1.55*10 ⁶	7.5*10 ⁶	6.05*10 ⁶
6120/G/FBBcpHEK/ΔM2-2	5.2*10 ⁶	1.0*10 ⁷	1.4*10 ⁷
6120/FBB/G/ΔM2-2	2.5*10 ⁷	6.5*10 ⁶	6*10 ⁶
6120/G001BB/F001BB/ΔM2-2	5.0*10 ³	1.1*10 ⁷ (P3)	1*10 ⁷ (P3)
wt LID	2.1*10 ⁷	5*10 ⁶	1.4*10 ⁷
LID/ΔM2-2	4.6*10 ⁶	2.35*10 ⁷	2*10 ⁷

Virus yields for ΔM2-2 viruses with additional modifications to the F and G genes

FIG. 16

D46/276/ Δ M2-2-AcII
(mutations relative to D46 with genomic positioning)

