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(54) Title: LIVE, ATTENTUATED RESPIRATORY SYNCYTIAL VIRUS

(57) Abstract: The present invention features live, attenuated respiratory syncytial viruses (RSV) useful as vaccines against RSV infection and/or the development of severe RSV-associated illnesses. The disclosed viruses are attenuated to the extent of being nonpathogenic when administered to a subject but substantially retain the antigenic and immunogenic properties of wild-type RSV.

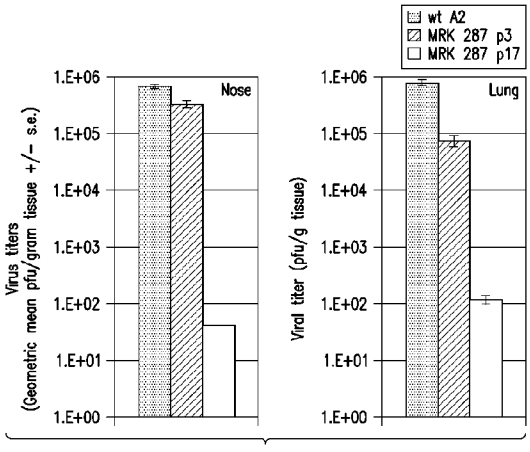

FIG.5

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TITLE OF THE INVENTION

LIVE, ATTENUATED RESPIRATORY SYNCYTIAL VIRUS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Application No. 61/198,327, filed November 5, 2008, hereby incorporated by reference herein.

BACKGROUND OF THE INVENTION

Human respiratory syncytial virus (RSV) is a negative-sense, single-stranded RNA virus and a member of the genus Pneumovirus, family Paramyxoviradae. RSV is the leading cause of viral pneumonia and bronchiolitis in infants under one and is a major cause of hospitalization and fatal respiratory tract disease in these infants. Serious disease can also develop in children with certain underlying illnesses (e.g., immunodeficiencies, congenital heart disease, and bronchopulmonary dysplasia). Virtually all children are infected by age two, and re-infection is common in older children and adults. (Chanock et al., in Viral Infections of Humans, 3rd ed., A. S. Evans, ed., Plenum Press, N.Y. (1989)) In healthy adults, most infections are asymptomatic and are generally confined to mild, upper respiratory tract disease; however, elderly patients and immunocompromised individuals are more likely to have severe and possibly life-threatening infections.

Two major, antigenic subgroups of RSV have been identified, A and B, as well as several different genotypes within each subgroup (Anderson et al., 1985, *J. Infect. Dis.* 151:626-633; Mufson et al., 1985, *J. Gen. Virol.* 66:2111-2124). The two antigenic subgroups are approximately 25% antigenically related by reciprocal cross-neutralization analysis. Multiple variants of each subgroup have been found to co-circulate in epidemics which occur annually during late fall, winter, and spring months in temperate climates (Anderson et al., 1991, *J. Infect. Dis.* 163:687-692). There is evidence that children infected with one of the two major RSV subgroups may be protected against re-infection by the homologous subgroup (Mufson et al., 1987, *J. Clin. Microbiol.* 26:1595-1597). This, along with evidence that protective immunity will accumulate following repeated infections, suggests that it is feasible to develop an RSV vaccination regiment for infants and young children which would provide sufficient immunity to protect against serious disease and death.

A native RSV genome typically comprises a negative-sense polynucleotide molecule which, through complementary viral mRNAs, encodes eleven species of viral proteins, i.e., the nonstructural species NS1 and NS2, N, P, matrix (M), small hydrophobic (SH), glycoprotein (G), fusion (F), M2(ORF1), M2(ORF2), and L, substantially as described in Mink et al., 1991, *Virology* 185:615-624; Stec et al., 1991, *Virology* 183:273-287; and Connors et al., 1995, *Virology*. 208:478-484.

While an immune prophylaxis, Synagis®, is currently marketed for prevention of RSV-associated diseases in premature birth, high-risk infants, despite decades of research, there is no safe and effective vaccine to combat RSV infection and the associated clinical diseases. Secretory antibodies appear to be most important in protecting the upper respiratory tract, whereas high levels of serum antibodies are thought to have a major role in resistance to RSV infection in the lower respiratory tract. However, purified human immunoglobulin (Ig) preparations suffer from the possibility of transmitting blood-borne viruses, while recombinant Ig preparations are expensive to manufacture.

Early attempts (1966) to vaccinate young children used a parenterally-administered, formalin-inactivated RSV vaccine. Unfortunately, administration of this vaccine in several field trials was shown to be specifically associated with the development of a significantly exacerbated illness following subsequent natural infection with RSV (Kapikian et al, 1968, *Am. J Epidemiol.* 89:405-421; Kim et al, 1969, *Am. J Epidemiol.* 89:422-434; Fulginiti et al, 1969, *Am. J Epidemiol.* 89:435-448; Chin et al; 1969, *Am. J Epidemiol.* 89:449-463). The reasons why this vaccine enhanced RSV disease are not clear. It has been suggested that this exposure to RSV antigens elicited an abnormal or unbalanced immune response which led to an immunopathological potentiation of natural disease (Kim et al, 1976, *Pediatr. Res.*10:75-78; Prince et al, 1986, *J. Virol.* 57:721-728).

The use of a live-attenuated or live-vectored virus vaccine has several advantages over subunit or inactivated virus vaccines. Live, attenuated virus vaccines can mimic natural viral infection, efficiently triggering the host's immune system, and are more likely than a subunit or inactivated vaccine to give a robust immunity comprising both humoral and cellular components.

A first aspect of the invention provides for a live, attenuated respiratory syncytial virus (RSV) comprising a viral genome, wherein the viral genome encodes proteins that comprise a glutamic acid at position 204 of the protein encoded by the G gene; a glutamic acid at position 205 of the protein encoded by the G gene; an alanine at position 211 of the protein encoded by the G gene; a glutamic acid at position 213 of the protein encoded by the G gene; a glycine at position 221 of the protein encoded by the G gene; a glutamic acid at position 223 of the protein encoded by the G gene; a glycine at position 232 of the protein encoded by the G gene; a glycine at position 486 of the protein encoded by the F gene; and an alanine at position 148 of the protein encoded by the L gene

A second aspect of the invention provides for an immunogenic composition comprising the live, attenuated RSV of the first aspect of the invention and a pharmaceutically acceptable carrier.

A third aspect of the invention provides for a nucleic acid molecule comprising a genomic or antigenomic sequence encoding the live, attenuated RSV of the first aspect of the invention.

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A fourth aspect of the invention provides for a live, attenuated respiratory syncytial virus (RSV) population that comprises the attenuated RSV of the first aspect of the invention.

A fifth aspect of the invention provides for an immunogenic composition comprising the live, attenuated RSV population of the fourth aspect of the invention and a pharmaceutically acceptable carrier.

A sixth aspect of the invention provides for a method of producing a protective immune response in a subject against RSV infection comprising the step of administering to said subject an immunologically effective amount of one or more of the following:

(a) the attenuated RSV of the first aspect of the invention;

(b) the attenuated RSV population of the fourth aspect of the invention;

(c) the immunogenic composition of the second aspect of the invention; or,

(d) the immunogenic composition of the fifth aspect of the invention.

An seventh aspect of the invention provides for use of an immunologically effective amount one or more of the following:

(a) the attenuated RSV of any one of the first aspect of the invention;

(b) the attenuated RSV population of the fourth aspect of the invention;

(c) the immunogenic composition of the second aspect of the invention; or,

(d) the immunogenic composition of the fifth aspect of the invention.

in the manufacture of a medicament for inducing a protective immune response in a patient against RSV infection.

Different aspects of the present invention are directed to a live, attenuated RSV comprising: (1) one or more nucleotide and/or amino acid mutations identified herein; (2) a viral genome which comprises one or more of the nucleotide mutations and/or encodes one or more of the amino acid mutations identified in the attenuated RSV disclosed herein; and/or (3) a nucleic

acid comprising a nucleotide sequence either having one or more of the nucleotide mutations described herein or encoding one or more amino acid mutations described herein.

Reference to "mutation" refers to the presence of a different amino acid or nucleotide at a specified amino acid or nucleotide position within a protein sequence or a gene and/or genomic sequence, respectively, than provided in a reference or wild-type sequence. The resulting mutation can be directly introduced into the reference or wild-type sequence or can be provided by a sequence other than the reference or wild-type sequence, as long as the end result provides for the indicated mutation.

A live, attenuated RSV of the present invention is immunogenic and protective against either RSV infection or the development of severe respiratory illness associated with RSV infection. In a preferred embodiment, a live, attenuated RSV is a human RSV suitable for immunization against wild-type RSV infection of subgroup A and/or B. Also disclosed herein are pharmaceutical compositions comprising said live, attenuated RSV and methods of treating a subject, preferably a human, against RSV infection by administering said compositions.

Examples of nucleic acids comprising a sequence either encoding one or more amino acid mutations as described herein or having one or more nucleotide mutations as described herein include: (1) full-length RSV genomic sequences based on a particular full-length RSV reference sequence; (2) different RSV nucleic acid regions based on particular RSV nucleic acid sequences described herein; (3) partial genomic sequences comprising one or more particular RSV nucleic acid sequences described herein; and, (4) full-length genomic sequences containing one or more particular RSV nucleic acid sequences described herein. Reference to a particular RSV nucleic acid sequence includes a specified sequence and/or the indicated identity to a specified sequence.

The term "treating" or "treatment" refers to both therapeutic and/or prophylactic treatment, including prevention or reduction of infection or reinfection, or the reduction or elimination of symptoms, diseases, or conditions associated with RSV infection. Those in need of treatment include the general population and/or patients infected with RSV. Examples of those in need include those already with an RSV-associated disorder(s), those prone to develop such a disorder, and/or those in which such a disorder is to be prevented.

A "disorder" is any condition resulting in whole or in part from RSV infection, including but not limited to pneumonia and bronchiolitis. Encompassed by the term "disorder" are chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

The term "protect" or "protection," when used in the context of an attenuated virus or treatment method of the present invention, means reducing the likelihood of RSV infection, reinfection, a disorder(s) resulting from RSV infection, as well as reducing the severity of the infection, reinfection and/or a disorder(s) resulting from such infection.

The term "effective amount" means sufficient amount that, when introduced to a vertebrate host, preferably a human host, results in a protective immune response. One skilled in the art recognizes that this level may vary.

The live, attenuated RSV described herein can be derived from any RSV subgroup (A or B) or strain (e.g., human, bovine, murine), preferably derived from human. 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 term "wild-type" or "wt," in reference to a RSV strain, refers both to viruses having a genomic sequence that is wild-type at known markers for attenuation and/or viruses that are phenotypically wild-type (i.e., not attenuated), as shown in either an *in vivo* (e.g., in a human or other suitable animal model) or *in vitro* model of attenuation. The term "wild-type," in reference to a RSV protein or nucleic acid (e.g., gene, genomic) sequence refers to a sequence that does not contain markers known to contribute to an attenuated phenotype.

"Live, attenuated virus," in reference to a RSV disclosed herein, means a virus that is both genotypically different from a wild-type RSV (i.e., comprising differences in the genomic sequence) and phenotypically different from said wild-type virus (i.e., as evidenced by an attenuated phenotype). An attenuated virus is phenotypically different by displaying reduced virulence whilst still viable (or "live"); thus, viruses can display different degrees of attenuation. Those live, attenuated RSV of the present invention are attenuated, making them nonpathogenic in a subject host. An incompletely-attenuated virus displays a reduction in pathogenesis but is still capable of generating a disease response in a subject host. A live, attenuated RSV of the present invention can be naturally derived (e.g., via passaging a wild-type or incompletely-attenuated strain) or recombinantly produced. If recombinantly produced, recombinant RSV encompasses a RSV or RSV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus or subviral particles produced there from. The live, attenuated RSV disclosed herein is in an isolated and typically purified form.

"Substantially similar" means that a given nucleic acid or amino acid sequence shares at least 80%, preferably at least 85%, more preferably at least 90%, and even more preferably at least 95% or at least 99% identity with a reference sequence, as dictated by the context of the text. Sequence identity to a reference sequence is determined by aligning a sequence with the reference sequence and determining the number of identical nucleotides or amino acids in the corresponding regions. This number is divided by the total number of amino acids in the reference sequence, multiplied by 100, and then rounded to the nearest whole number. Sequence identity can be determined by a number of art-recognized sequence comparison algorithms or by visual inspection (see generally Ausubel, F M, et al., Current Protocols in Molecular Biology, 4, John Wiley & Sons, Inc., Brooklyn, N.Y., A.1E.1-A.1F.11, 1996-2004). A substantially similar nucleic acid or amino acid sequence of the present

invention, if it encodes or consists of the NS2, G, L or F protein, will maintain one or more of the nucleotide or amino acid mutations identified within p17, described in detail *infra*.

Reference to "isolated" indicates a different form than found in nature or in other than the native environment of wild-type virus, such as the nasopharynx of an infected individual. Thus, an isolated virus can be a heterologous component of a cell culture or other system. The different form can be, for example, a different purity than found in nature and/or a structure that is not found in nature.

Reference to open-ended terms such as "comprises" allows for additional elements or steps. Occasionally, phrases such as "one or more" are used with or without open-ended terms to highlight the possibility of additional elements or steps.

Unless explicitly stated, reference to terms such as "a," "an," and "the" is not limited to one and include the plural reference unless the context clearly dictates otherwise. For example, "a cell" does not exclude "cells." Occasionally, phrases such as one or more are used to highlight the possible presence of a plurality.

The term "mammalian" refers to any mammal, including a human.

The abbreviation "Kb" refers to kilobases.

The abbreviation "pfu" refers to plaque forming units.

The abbreviation "ORF" refers to the open reading frame of a gene.

Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples. The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows an alignment of the amino acid sequences of the NS2 protein from a wild-type RSV strain, hRSV S2 (SEQ ID NO: 2; see NCBI GenBank Accession no. U39662; Tolley et al., 1996, *Vaccine* 14:1637-1646), Merck strain 287 (SEQ ID NO: 4) and p17 (SEQ ID NO: 6). The single-underlined amino acids indicate differences in the NS2 protein between the hRSV S2 strain and both the Merck 287 and p17 strains. The NS1 amino acid sequence of Merck strain p17_pp is the same as for p17 (SEQ ID NO: 6).

FIGURE 2 shows an alignment of the amino acid sequences of the G protein from hRSV S2 (SEQ ID NO: 8), Merck strain 287 (SEQ ID NO: 10) and p17 (SEQ ID NO: 12). The single-underlined amino acids indicate differences in the G protein between the hRSV S2 strain and both the Merck 287 and p17 strains. The double-underlined amino acids indicate differences between the p17 strain and both the hRSV S2 and Merck 287 strains. The G amino acid sequence of Merck strain p17_pp is the same as for p17 (SEQ ID NO: 12).

FIGURES 3A and B show an alignment of the amino acid sequences of the F protein from hRSV S2 (SEQ ID NO: 14), Merck strain 287 (SEQ ID NO: 16) and p17 (SEQ ID NO: 18). The single-underlined amino acids indicate differences in the F protein between the hRSV S2 strain and both the Merck 287 and p17 strains. The double-underlined amino acids indicate differences between the p17 strain and both the hRSV S2 and Merck 287 strains. Amino acid position 294 of SEQ ID NO: 18 is designated as an "X," representing either a Glu or Lys residue. Amino acid position 486 of SEQ ID NO: 18 is also designated as an "X," representing either an Asp or Gly residue.

FIGURES 4A-G show an alignment of the amino acid sequences of the L protein from hRSV S2 (SEQ ID NO: 20), Merck strain 287 (SEQ ID NO: 22) and p17 (SEQ ID NO: 24). The single-underlined amino acids indicate differences in the L protein between the hRSV S2 strain and both the Merck 287 and p17 strains. The double-underlined amino acids indicate differences between the p17 strain and both the hRSV S2 and Merck 287 strains. Amino acid position 148 of SEQ ID NO: 24 is designated as an "X," representing either an Asp or Ala residue. Amino acid position 2054 is also designated as an "X," representing either a Leu or Phe residue.

FIGURE 5 compares viral titers (pfu/gram of tissue) of the wild-type hRSV A2 strain (Huang and Wetz, 1982, *J. Virol.* 43:150), passage 3 of Merck strain 287 ("p3"), and passage 17 of Merck strain 287 ("p17") in both nose and lung samples from cotton rats intranasally inoculated with said viruses.

FIGURE 6 compares viral titers (pfu/gram of tissue) of the wild-type hRSV A2 strain, passage 3 of Merck strain 287 ("p3"), and passage 17 of Merck strain 287 ("p17") in both nose and lung samples from African green monkeys inoculated with said viruses by combined intranasal or intratracheal routes.

FIGURE 7 compares viral titers (pfu/gram of tissue) of passages 3 ("p3"), 5 ("p5"), 10 ("p10") and 15 ("p15") of Merck strain 287 in both nose and lung samples from cotton rats intranasally inoculated with said viruses.

FIGURE 8 compares the seroneutralizing (SN) antibody titers against wild-type hRSV A2 following immunization with wt hRSV A2, passage 3 of Merck strain 287 ("p3") or passage 17 of Merck strain 287 ("p17") at days 14, 28 and 56 post immunization in cotton rats. The rats were immunized intramuscularly with the viruses.

FIGURE 9 compares the viral titers (pfu/gram of tissue) in nose and lung samples of cotton rats first immunized intramuscularly with either wild-type hRSV A2, Merck strain 287 p3 or Merck strain 287 p17, or no virus, and then challenged intranasally with $10^{5.5}$ pfu of wt A2 virus.

FIGURE 10 compares the seroneutralizing (SN) antibody titers against wild-type hRSV A2 in African green monkeys twenty-eight days after immunization with passage 17 of

Merck strain 287 ("p17") or no virus ("none") in the nose and lung. The monkeys were immunized intramuscularly.

FIGURE 11 compares the mean viral titers (pfu/ml) in nose and lung samples of African green monkeys first immunized intramuscularly with either Merck strain 287 p17 or no virus, and then challenged by intranasal and intratracheal inoculation with $10^{5.5}$ pfu of wt A2 virus.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a live, attenuated respiratory syncytial virus (RSV) for use as a vaccine against RSV infection and/or the development of RSV-associated illnesses. The virus disclosed herein is attenuated such that it is nonpathogenic when administered to a subject, preferably a human, but substantially retains the antigenic and immunogenic properties of wild-type RSV. As such, the attenuated viruses disclosed herein are useful for stimulating a protective immune response in an inoculated mammal without producing the respiratory symptoms caused by wild-type virus.

I. Live, Attenuated RSV

The live, attenuated RSV viruses described herein were identified by serial passaging a hRSV A type strain, hereinafter called "Merck strain 287," "strain 287," or "p0." Merck strain 287 was derived from a RSV strain that was initially amplified two times in GMK cells. After this initial amplification, the virus underwent five additional passages in the human diploid cell line, WI-38. This passage 5 material, renamed Merck strain 287, was sequenced and shown to be both genetically wild-type at genetic markers known for attenuation and phenotypically wild-type (see Examples, *infra*).

Merck strain 287 was passaged in VERO CCL-81 monkey kidney cells at a fixed multiplicity. Virus titers remained constant over passaging, and the cytopathic effect of the virus in culture was also consistent. Attenuation of the passaged viruses was monitored by measuring a reduction in viral shedding in *in vivo* rodent and primate models. A marked attenuation was seen between passage 3 (p3) and passage 17 (p17) in both the cotton rat model and African green monkeys, showing a 2-4 log reduction in viral load. Sequence comparison of the genomic DNA isolated from the p17 virus population with the original Merck strain 287 identified nucleotide mutations within the p17 genome, the majority of which are located within the genes encoding the G, F, and L proteins (hereinafter referred to as the G, F and L genes, respectively). P17 contains a total of 21 nucleotide differences and 11 amino acid differences when compared with Merck strain 287. A plaque-purified virus derived from p17 (p17_pp) contains an additional nucleotide mutation when compared with Merck strain 287. Further sequence analysis was performed on passage 5 (p5), passage 10 (p10) and passage 15 (p15) to monitor the genetic changes over the passaging process, as well as on passage 18 (p18) and passage 22 (p22) to

measure the genetic stability of the induced mutations. Immunogenicity and protection studies were also performed. These studies show that the p17 strain disclosed herein is genetically-stable, immunogenic and protective, and avirulent. Thus, a live, attenuated RSV of the present invention can be used as a vaccine to protect against disease caused by infection with RSV.

A detailed summary of the sequence comparison between Merck strain 287 (passage 0 or "p0") and various passaged strains thereof can be found in Examples 2 and 4, *infra*. Briefly, the full-length viral genome for p0, p17, and p17_pp (a plaque purified virus derived from p17) were sequenced and compared, as well as targeted sequences of passages 5, 10, 15, and 18. The genomic sequence of Merck strain 287 is set forth herein as SEQ ID NO: 25. The genomic sequences of p17 and the p17_pp strain are set forth herein as SEQ ID NO: 26 and SEQ ID NO: 91, respectively. The location of the 21 nucleotide differences between the viral genomes of Merck strain 287 and p17, as well as the specific nucleotide mutations, are listed in Table 5 (*infra*) (e.g., an alanine (A) to guanine (G) mutation at position 5295). The genomic sequence of the p17_pp strain is set forth herein as SEQ ID NO: 91. The genomic sequence of p17_pp is the same as that of p17 with the exception of an additional, silent nucleotide mutation within the gene encoding the NS1 protein (at nucleotide position 162 of the NS1 gene). Table 8 (*infra*) compares the nucleotide differences between Merck strain 287, p17 and p17_pp. Tables 6 and/or 9 (*infra*) also lists the location of the nucleotide mutations within the p17 and p17_pp genomic sequences, as well as the corresponding location of each mutation within a RSV gene open reading frame (ORF). For example, the mutation at position 5295 of the p17 viral genome corresponds to a mutation at nucleotide position 610 of the G gene ORF. Tables 6 and 9 additionally list whether or not a particular point mutation, or combination of point mutations, generates an amino substitution, as well as the amino acid position of said substitution. For example, the mutation at nucleotide position 5295 of the p17 viral genome leads to an amino acid substitution from lysine (LYS) to glutamic acid (GLU) at amino acid residue position 204 of the G protein. Of the 21 nucleotide differences between p17 and Merck strain 287 (and the 22 nucleotide differences between p17_pp and Merck strain 287), one nucleotide difference is located within an untranslated region of the viral genome (i.e., nucleotide 15046 within the 5' untranslated region).

Of the 21 nucleotide differences identified in the p17 viral genome, compared to the parental p0 strain (Merck strain 287), four of the nucleotide differences may represent polymorphisms within the virus population comprised within passage 17, located at nucleotide positions 6538, 7115, 8937 and 14656 of the RSV viral genome (see Example 2, *infra*). These positions are designated with an "n" in SEQ ID NO: 26. The "n" at position 6538 can be either a G or A; the "n" at position 7115 can be either an A or G; the "n" at position 8937 can be either an A or C; and, the "n" at position 14656 can be either a G or T. These nucleotide polymorphisms are also marked with an "n" in the gene sequences corresponding to the F and L genes (SEQ ID NOs: 17 and 23, respectively) and with an "x" in the protein sequences corresponding to the F

and L proteins (SEQ ID NOs: 18 and 24, respectively). Polymorphisms represent the existence of two (or more) forms of a gene within a population, wherein each form is too common to be due merely to a new, minor mutation. Two of these potential polymorphisms are located within the coding region for the F protein, and two are located within the L protein coding region. These polymorphisms are evident on sequencing chromatograms, represented by double peaks corresponding to at least two nucleotides. The polymorphisms indicate that the population of viruses within the p17 pool of viruses from which DNA was extracted for sequence analysis is not a homogenous population of viruses. Each of these four nucleotide polymorphisms induces an amino acid substitution (see Example 2, *infra*).

The p17 RSV strain was plaque purified to a clonal population, p17_pp virus, and sequenced (see Example 4, *infra*). In addition to identifying a silent mutation at nucleotide 162 of the gene encoding the NS1 protein within the p17_pp genome, the polymorphisms identified within the genes encoding the L and F proteins of p17 were resolved (see Tables 8 and 9, *infra*). Thus, the genes encoding the F and L proteins of the p17_pp virus are set forth herein as SEQ ID NO: 92 and SEQ ID NO: 94, respectively, and the encoded proteins are SEQ ID NO: 93 and SEQ ID NO: 95.

Table 7 (within Example 2, *infra*) shows the progression of mutations induced over the passaging of Merck strain 287 described herein. For example, only one nucleotide mutation is seen in passage 5 (p5), located at nucleotide position 954 of the RSV genome. The number of nucleotide mutations increases over the passage process, and by p15, at least 20 of the 21 nucleotide mutations within p17 are present. While the twenty-first nucleotide difference seen in p17, located at nucleotide 15046 of the viral genome within the 5' untranslated region, may be present in p15, the gene segment that contains that portion of the p15 viral genome was not sequenced.

The results in Figure 7 highlight the relationship between viral passage and the degree of attenuation, comparing the in vivo replication of p3, p5, p10, and p15 (see also Example 3, *infra*). Passage 5 (p5) already begins to show reduced replication compared to p3. Passages 10 (p10) and 15 (p15) display an even further reduction in replication, with a marked decrease in replication between p10 and p15. The p15 virus appears to be as attenuated as the p17 virus described above (see Figure 5). When comparing the array of mutations present within p10 and p15, p15 has only two additional nucleotide differences, located within the F and L protein coding regions. Thus, and without being bound by any particular theory, it is possible that the mutations within the F and/or L protein coding regions are particularly important for attenuating RSV. A study of the attenuation of the plaque purified derivative of p17, p17_pp, shows that the virus is as attenuated as the parent p17 strain (see Example 4, *infra*).

Table 1 provides a summary of the Sequence Identification Numbers (SEQ ID NOs) for the NS1, NS2, G, F, and L gene open reading frames and encoded proteins for Merck

strain 287 (MRK287), p17, p17_pp, and a published wild-type strain, hRSV S2 (disclosed in NCBI GenBank Accession no. U39662; Tolley et al., 1996, *Vaccine* 14:1637-1646).

Table 1:

	NS1 SEQ ID NO:		NS2 SEQ ID NO:		G SEQ ID NO:		F SEQ ID NO:		L SEQ ID NO:	
RSV strain	**gene**	**prot.**	**gene**	**prot.**	**gene**	**prot.**	**gene**	**prot.**	**gene**	**prot.**
Merck 287 (SEQ ID NO:25)	89	88	3	4	9	10	15	16	21	22
p17 (SEQ ID NO:26)	89	88	5	6	11	12	17	18	23	24
p17_pp (SEQ ID NO: 91)	90	88	5	6	11	12	92	93	94	95
S2	87	88	1	2	7	8	13	14	19	20

RSV is a negative-sense, nonsegmented, single-stranded RNA genome. As such, reassortment of genomic segments does not occur. However, a dependence on RNA polymerase lacking RNA proofreading and editing ability results in the RSV genome being quite mutable. Sequence studies of various RSV genes have confirmed the division of human RSV into two major groups (A and B), while also identifying many variants or lineages within each group (see, e.g., Peret et al., 1998, *J. Gen. Virol.* 79:2221-2229). Between the two antigenic subgroups of human RSV, amino acid sequence identity ranges from 96% (for the N protein) to 53% (for the G protein) (Johnson et al., 1987, *Proc. Natl. Acad. Sci. USA* 84:5625-5629). Within group A and group B strains, the G protein shows up to 20% and 9% amino acid diversity, respectively (Cane et al., 1991, *J. Gen. Virol.* 72:649-357; Sullender et al., 1991, *J. Virol.* 65:5425-5434).

As examples of this genetic heterogeneity, alignments of the NS2, G, F, and L proteins from Merck strain 287, p17, and hRSV S2 can be found in Figures 1, 2, 3 and 4, respectively. As shown in the alignment of the NS2 proteins in Figure 1, while there are no amino acid differences between Merck strain 287 and the p17 passaged strain described herein, there are two amino acid differences between the wild-type hRSV S2 strain and both Merck strain 287 and p17 (underlined in Figure 1). It is important to note, however, that both Merck strain 287 and hRSV S2 are phenotypically wild-type; and, thus, these amino acid differences between hRSV S2 and both Merck strain 287 and p17 neither induces nor contributes to the attenuation of p17 or p17_pp.

between hRSV S2 and both Merck strain 287 and p17 neither induces nor contributes to the attenuation of p17 or p17_pp.

Similarly, there are amino acid differences between the wild-type hRSV S2 strain and both Merck strain 287 and p17 in the G, F, and L protein sequences (see the single-underlined amino acids in Figures 2, 3, and 4, respectively). However, there are also amino acid differences between the G, F, and L protein sequences of Merck strain 287 and p17 (see the double-underlined amino acids in Figures 2, 3, and 4, respectively). It is likely that one or more of these double-underlined amino acid differences contributes to the attenuated phenotype demonstrated for p17 and p17_pp.

As such, the present invention relates to live, attenuated RSV having a modified viral genome comprising one or more of the nucleotide mutations as described in p17 and/or p17_pp and/or encoding one or more of the amino acid mutations as described in p17 herein, as compared to Merck strain 287. In one embodiment, the viral genome of said attenuated RSV may contain, for example, additional nucleotide differences and/or encode additional amino acid differences, as compared to the Merck strain 287 genomic sequence as set forth in SEQ ID NO: 25 or another phenotypically wild-type RSV strain (e.g., hRSV S2), wherein said additional nucleotide and/or amino acid mutations do not substantially contribute to the attenuation phenotype.

In another embodiment, a live, attenuated RSV of the present invention may comprise one or more of the nucleotide and/or amino acid mutations as described in p17 and/or p17_pp in addition to further mutations at genetic markers known for attenuation (see, e.g., Conners et al., 1995, Virology, 208:478-484; Crowe et al., 1996, Virus Genes 13:269-273; Firestone et al., 1996, Virology 225:419-422; Whitehead et al., 1998, J. Virol. 72:4467-4471).

II. Examples of Different Embodiments

In a first embodiment, the present invention relates to a live, attenuated respiratory syncytial virus (RSV) comprising a viral genome, wherein the viral genome encodes proteins comprising one or more amino acids selected from the group consisting of: a glutamic acid at position 204 of the protein encoded by the G gene; a glutamic acid at position 205 of the protein encoded by the G gene; an alanine at position 211 of the protein encoded by the G gene; a glutamic acid at position 213 of the protein encoded by the G gene; a glycine at position 221 of the protein encoded by the G gene; a glycine at position 223 of the protein encoded by the G gene; a glycine at position

232 of the protein encoded by the G gene; a lysine at position 294 of the protein encoded by the F gene; a glycine at position 486 of the protein encoded by the F gene; an alanine at position 148 of the protein encoded by the L gene; and, a phenylalanine at position 2054 of the protein encoded by the L gene. Thus, in this embodiment, a live, attenuated RSV of the present invention comprises one, all or a subset of the amino acid residues referenced in this paragraph. The location of the recited amino acid residues are referenced as per their

In a further embodiment, a live, attenuated RSV of the present invention comprises a viral genome comprising one or more nucleotides selected from the group consisting of: an adenine at nucleotide position 162 of the NS1 gene; an adenine at nucleotide position 327 of the NS2 gene; a guanine at nucleotide position 610 of the G gene; a guanine at nucleotide position 613 of the G gene; a guanine at nucleotide position 630 of the G gene; a guanine at nucleotide position 631 of the G gene; a guanine at nucleotide position 637 of the G gene; a guanine at nucleotide position 639 of the G gene; a guanine at nucleotide position 654 of the G gene; a guanine at nucleotide position 661 of the G gene; a guanine at nucleotide position 662 of the G gene; a guanine at nucleotide position 666 of the G gene; a guanine at nucleotide position 667 of the G gene; a guanine at nucleotide position 668 of the G gene; a guanine at nucleotide position 675 of the G gene; a guanine at nucleotide position 695 of the G gene; a guanine at nucleotide position 696 of the G gene; an adenine at nucleotide position 880 of the F gene; a guanine at nucleotide position 1457 of the F gene; a cytosine at nucleotide position 443 of the L gene; and, a thymine at nucleotide position 6162 of the L gene. The viral genome may comprise one, all or a subset of the specified nucleotides.

One, all or a subset of the specified amino acid residues and/or nucleotides can be comprised with a live, attenuated RSV of the present invention. The amino acid residues and nucleotides recited above correspond to the location of specific amino acid residues and nucleotides present in p17 and p17_pp described herein (see Tables 5, 6, 8 and 9, *infra*). For example, a live, attenuated RSV within this embodiment may comprise a lysine at position 294 of the protein encoded by the F gene, a glycine at position 486 of the protein encoded by the F gene, an alanine at position 148 of the protein encoded by the L gene, and a phenylalanine at position 2054 of the protein encoded by the L gene. As described *supra*, the results presented herein suggest that recited amino acid residues within the F and L proteins substantially contribute to the attenuated phenotype of p17 and p17_pp.

In a second embodiment, the present invention relates to a live, attenuated RSV comprising a viral genome which comprises one or more nucleotide mutations compared to a non-attenuated RSV viral genome, wherein one or more of the nucleotide mutations result in one or more amino acid mutations, and wherein the one or more amino acid mutations are located at amino acid positions selected from the group consisting of: positions 204, 205, 211, 213, 221, 223, and 232, each of the protein encoded by the G gene; positions 294 and 486, each of the protein encoded by the F gene; and, positions 148 and 2054, each of the protein encoded by the L gene. The non-attenuated RSV strain can be a genetically and/or phenotypically wild-type RSV and provides reference sequences. The location of the amino acid mutations are referenced as per their position within a particular amino acid sequence encoded by a gene within the viral genome. The G, F and L proteins are encoded by the G, F and L genes, respectively. The amino acid mutations recited above correspond to the location of amino acid substitutions present in

mutations recited above correspond to the location of amino acid substitutions present in p17 and p17_pp described herein when compared to a genetically wild-type (RSV S2 strain) and a phenotypically wild-type (Merck strain 287) RSV strain (see Tables 6 and 9, infra).

In a further embodiment, an amino acid mutation at position 204 of the protein encoded by the G gene is to a glutamic acid, an amino acid mutation at position 205 of the protein encoded by the G gene is to a glutamic acid, an amino acid mutation at position 211 of the protein encoded by the G gene is to an alanine, an amino acid mutation at position 213 of the protein encoded by the G gene is to a glutamic acid, an amino acid mutation at position 221 of the protein encoded by the G gene is to a glycine, an amino acid mutation at position 223 of the protein encoded by the G gene is to a glycine, an amino acid mutation at position 232 of the protein encoded by the G gene is to a glycine, an amino acid mutation at position 294 of the protein encoded by the F gene is to a lysine, an amino acid mutation at position 486 of the protein encoded by the F gene is to a glycine, an amino acid mutation at position 148 of the protein encoded by the L gene is to an alanine, and an amino acid mutation at position 2054 of the protein encoded by the L gene is to a phenylalanine.

In a still further embodiment, an amino mutation at position 204 of the protein encoded by the G gene is Lys204Glu, an amino acid mutation at position 205 of the protein encoded by the G gene is Lys205Glu, an amino acid mutation at position 211 of the protein encoded by the G gene is Thr211Ala, an amino acid mutation at position 213 of the protein encoded by the G gene is Lys213Glu, an amino acid mutation at position 221 of the protein encoded by the G gene is Lys221Gly, an amino acid mutation at position 223 of the protein encoded by the G gene is Lys223Gly, an amino acid mutation at position 232 of the protein encoded by the G gene is Glu232Gly, an amino acid mutation at position 294 of the protein encoded by the F gene is Glu294Lys, an amino acid mutation at position 486 of the protein encoded by the F gene is Asp486Gly, an amino acid mutation at position 148 of the protein encoded by the L gene Asp148Ala, and an amino acid mutation at position 2054 of the protein encoded by the L gene is Leu2054Phe. As an example, the term "Lys204Glu," used in reference to the G protein, indicates that the lysine (Lys) amino acid residue at amino acid position 204 of the G protein is substituted with a glutamic acid (Glu) amino acid residue. The amino acid residue abbreviations are recited infra. The amino acid mutations recited above correspond to the amino acid substitutions present in p17 and p17_pp described herein (see Table 6 and Table 9, infra).

In another embodiment, the viral genome comprises nucleotide mutations that result in either all or a subset of the recited amino acid mutations. For example, the viral genome of an attenuated RSV within this embodiment may comprise nucleotide mutations resulting in amino acid mutations at one or all of the recited amino acid positions within the proteins encoded by the F and L genes. As described supra, the results presented herein suggest that mutations within the F and L proteins substantially contribute to the attenuated phenotype of p17 and p17_pp.

In a further embodiment, the one or more nucleotide mutations within the viral genome of the attenuated RSV that result in the one or more amino acid mutations recited above are located at a nucleotide position selected from the group consisting of: positions 610, 613, 631, 637, 639, 661, 662, 667, 668, 695 and 696 of the G gene; positions 880 and 1457 of the F gene; and, positions 443 and 6162 of the L gene. In this embodiment, two nucleotide positions may need to be mutated to generate an amino acid substitution. The location of the nucleotide mutations are referenced as per their position within a particular ORF contained within the viral genome. In an alternative, the location of nucleotide mutations can be reference as per their position within the viral genome itself. For example, nucleotide position 610 of the G gene corresponds to nucleotide position 5295 of the RSV genome. The relationship between a mutation at a specific position within an ORF described herein and its corresponding position within the RSV genome can be found in Table 6 and Table 9, *infra*.

In a still further embodiment, the viral genome further comprises one or more silent nucleotide mutations located at nucleotide positions selected from the group consisting of position 327 of the NS2 gene, position 630 of the G gene, position 654 of the G gene, position 666 of the G gene, and position 675 of the G gene. The viral genome may further comprise a silent mutation at nucleotide position 162 of the NS1 gene. The viral genome may comprise all or a subset of these silent nucleotide mutations. In another embodiment, the viral genome comprises all of the nucleotide mutations (silent and non-silent) recited above.

In another embodiment, the viral genome comprises one or more nucleotide mutations as compared to the viral genome of either a non-attenuated or an incompletely-attenuated RSV strain, wherein said one or more mutations are located at nucleotide positions selected from the group consisting of position 162 of the NS1 gene, position 327 of the NS2 gene, positions 610, 613, 630, 631, 637, 639, 654, 661, 662, 666, 667, 668, 675, 695, and 696 of the G gene, positions 880 and 1457 of the F gene, and, positions 443 and 6162 of the L gene. Said non-attenuated strain can be a genetically and/or phenotypically wild-type RSV. In a further embodiment, said viral genome comprises mutations at all of the recited nucleotide positions.

In a further embodiment, a nucleotide mutation at nucleotide position 162 of the NS1 gene is T260A, a nucleotide mutation at nucleotide position 327 of the NS2 gene is G327A, a nucleotide mutation at nucleotide position 610 of the G gene is A610G, a nucleotide mutation at nucleotide position 613 of the G gene is A613G, a nucleotide mutation at nucleotide position 630 of the G gene is A630G, a nucleotide mutation at nucleotide position 631 of the G gene is A631G, a nucleotide mutation at nucleotide position 637 of the G gene is A637G, a nucleotide mutation at nucleotide position 639 of the G gene is A639G, a nucleotide mutation at nucleotide position 654 of the G gene is A654G, a nucleotide mutation at nucleotide position 661 of the G gene is A661G, a nucleotide mutation at nucleotide position 662 of the G gene is A662G, a nucleotide mutation at nucleotide position 666 of the G gene is A666G, a nucleotide mutation at nucleotide position 667 of the G gene is A667G, a nucleotide mutation at nucleotide position 668

of the G gene is A668G, a nucleotide mutation at nucleotide position 675 of the G gene is A675G, a nucleotide mutation at nucleotide position 695 of the G gene is A695G, a nucleotide mutation at nucleotide position 696 of the G gene is A696G, a nucleotide mutation at nucleotide position 880 of the F gene is G880A, a nucleotide mutation at nucleotide position 1457 of the F gene is A1457G, a nucleotide mutation at nucleotide position 443 of the L gene is A443C, and a nucleotide mutation at nucleotide position 6162 of the L gene is G6162T. As an example the term "G327A," when used in reference to the NS2 gene of an attenuated RSV of this embodiment, indicates that the guanine nucleotide at this position is mutated to an adenine (A) nucleotide. The nucleotide abbreviations for thymine and cytosine are "T" and "C," respectively. The viral genome may comprise nucleotides mutations at all or a subset of the recited nucleotide positions.

In a further embodiment, the present invention also relates to a live, attenuated respiratory syncytial virus (RSV) comprising a viral genome which comprises one or more nucleotide mutations as compared to the viral genome of either a non-attenuated or an incompletely-attenuated RSV strain, wherein said one or more mutations are located at nucleotide positions selected from the group consisting of position 260, 954, 5295, 5298, 5315, 5316, 5322, 5324, 5339, 5346, 5347, 5351, 5352, 5353, 5360, 5380, 5381, 6538, 7115, 8937 and 14656 of the RSV genome. Each of the nucleotide mutations recited above is present within the RSV p17 and/or p17_pp strain described herein. In a further embodiment, said modified viral genome comprises mutations at all of the recited nucleotide positions.

When referencing a particular location within either an open reading frame encoding a RSV protein, the entire RSV genomic sequence, or an amino acid sequence of a RSV protein, it is appreciated that precise locations may vary slightly between RSV strains within and/or between antigenic subgroups (A versus B) and viral species (e.g., human versus bovine). As such, comparative nucleotide and/or amino acid analysis, whereby multiple reference sequences are aligned, can be used to identify the exact nucleotide and/or amino acid position that corresponds to a specifically-recited position described herein. Thus, encompassed within the prior embodiments, and those embodiments hereafter which recite specific amino acid or nucleotide positions, are live, attenuated RSV comprising a viral genome comprising one or more of the nucleotide and/or amino acid residues and/or one or more of the nucleotide mutations and/or amino acid mutations at positions that correspond to those specifically-recited positions as determined by sequence alignment and analysis of and with reference sequences.

In a third embodiment the viral genome of a live, attenuated RSV of the present invention comprises a nucleotide sequence encoding a NS1, NS2, G, F and/or L protein that is related to a wild-type NS1, NS2, G, F and/or L protein (e.g., SEQ ID NO: 88, 2, 8, 14 and 20, respectively, which correspond to the RSV S2 NS1, NS2, G, F and/or L protein sequences), wherein the NS1, NS2, G, F and/or L protein sequence contains one or a combination of the amino acids, or amino acid mutations, identified herein to differ between p17 and/or p17_pp and

wild-type amino acid sequences of the specific viral protein, and wherein said related sequence has at least 95%, preferably 99%, sequence identity to said wild-type RSV protein sequence in the region outside of the one or combination of differing amino acids or amino acid mutations.

In a further embodiment the viral genome of a live, attenuated RSV of the present invention comprises a nucleotide sequence of a NS1, NS2, G, F and/or L gene that is related to a wild-type NS1, NS2, G, F and/or L gene sequence (e.g., SEQ ID NO: 87, 1, 7, 13 and 19, respectively, which correspond to the RSV S2 NS1, NS2, G, F and/or L gene sequences), wherein the NS1, NS2, G, F and/or L gene sequence contains one or a combination of the nucleotides, or nucleotide mutations, identified herein to differ between p17 and/or p17_pp and wild-type nucleotide sequences of the specific genes, and wherein said related sequence has at least 95%, preferably 99%, sequence identity to said wild-type RSV gene sequence in the region outside of the one or combination of differing nucleotides or nucleotide mutations.

In a fourth embodiment, the viral genome of a live, attenuated RSV encompassed by the present invention comprises an open reading frame encoding a G, F and/or L protein, wherein said protein(s) consist of the amino acid sequences as set forth in SEQ ID NO: 12, SEQ ID NO: 18 or SEQ ID NO: 93, and SEQ ID NO: 24 or SEQ ID NO: 95, respectively. SEQ ID NOs: 12, 18 and 24 represent the amino acid sequences of the G, F and L proteins within p17 described herein (see also Figures 2, 3 and 4, respectively). The polymorphisms within the F and L proteins are indicated in the corresponding recited sequences (SEQ ID NOs: 18 and 24). Thus, in a further embodiment, position 294 of the F protein of p17 (SEQ ID NO: 18) is a Lys residue, and/or position 486 of the F protein of p17 is a Gly residue. In a still further embodiment, position 148 of the L protein of p17 (SEQ ID NO: 24) is an Ala residue, and/or position 2054 of the L protein of p17 is a Phe residue. SEQ ID NO: 93 and SEQ ID NO: 95 represent the amino acid sequences of the F and L proteins within the plaque purified derivative of p17, p17_pp. The polymorphisms within these sequences have been resolved in the plaque purified virus and are reflected in SEQ ID NOs: 93 and 95.

In a further embodiment, the viral genome of a live, attenuated RSV comprises an open reading frame encoding a G, F and/or L protein, wherein the protein(s) consist of an amino acid sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to the amino acid sequences set forth in SEQ ID NO: 12, SEQ ID NO: 18 or SEQ ID NO: 93, and SEQ ID NO: 24 or SEQ ID NO: 95, respectively, and wherein one or a combination of the specific amino acids within said G, F and/or L proteins identified herein to differ between p17 and/or p17_pp and wild-type amino acid sequences of the specific viral proteins are present.

In a fifth embodiment, the viral genome of a live, attenuated RSV described herein comprises an open reading frame encoding an NS1, NS2, G, F and/or L protein, wherein one or more of said open reading frames consist of the nucleotide sequences as set forth in SEQ ID NO: 89 or SEQ ID NO: 90, SEQ ID NO: 5, SEQ ID NO: 11, SEQ ID NO: 17 or SEQ ID NO: 92, and/or SEQ ID NO: 23 or SEQ ID NO: 94, respectively. SEQ ID NOs: 89, 5, 11, 17 and 23

represent the nucleotide sequences encoding the NS1, NS2, G, F and L proteins, respectively, within p17 described herein. The nucleotide sequences encoding the F and L proteins of p17 contain the nucleotide polymorphism described *supra*. Thus, in a further embodiment, position 880 of the F gene of p17 (SEQ ID NO: 17) is an adenine nucleotide, and/or position 1457 of the F gene of p17 is a guanine nucleotide. In a still further embodiment, position 443 of the L gene of p17 (SEQ ID NO: 23) is a cytosine nucleotide, and/or position 6162 of the L gene of p17 is a thymine nucleotide. SEQ ID NOs: 90, 5, 11, 92 and 94 represent the nucleotide sequences encoding the NS1, NS2, G, F and L proteins, respectively within p17_pp. The polymorphisms within the nucleotide sequences encoding the F and L proteins of p17 have been resolved in the plaque purified virus and are reflected in SEQ ID NOs: 92 and 94.

In a further embodiment, the viral genome of a live, attenuated RSV comprises open reading frame corresponding to an NS1, NS2, G, F and/or L gene that is at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to a nucleotide sequence set forth in SEQ ID NO: 90, SEQ ID NO: 5, SEQ ID NO: 11, SEQ ID NO: 17 or SEQ ID NO: 92, and/or SEQ ID NO: 23 or SEQ ID NO: 94, respectively, wherein one or a combination of the specific nucleotides within said NS1, NS2, G, F and/or L genes identified herein to differ between p17 and/or p17_pp and wild-type nucleotide sequences of the specific viral genes are present.

In a sixth embodiment, the viral genome of a live, attenuated RSV described in the prior embodiments further contains either a cytosine at position 15046, a mutation at position 15046, a mutation at position 15046 to a cytosine, or a mutation of A15046C, within the viral genome. Nucleotide position 15046 is located within the 5' untranslated region of the RSV genome.

In a seventh embodiment, the viral genome of a live, attenuated RSV described in the prior embodiments contains either further nucleotide and/or amino acid mutations at genetic markers known for attenuation of RSV or further nucleotides and/or amino acids known to confer an attenuated phenotype to RSV, preferably human RSV. This embodiment encompasses all of the live, attenuated RSV described in the prior embodiments, including strains derived there from that are further attenuated by, for example, chemical mutagenesis, cold adaptation, or genetic recombination (e.g. site-directed mutagenesis). Thus, incompletely-attenuated RSV mutants known in the art may be further attenuated (e.g., more completely attenuated) by introduction of the nucleotides and/or amino acids described herein to contribute to the attenuation of p17 and/or p17_pp.

In an eighth embodiment, a live, attenuated RSV described in the prior embodiments belong to either antigenic subgroup A or B of RSV. In a further embodiment, the live, attenuated RSV is a human RSV.

In a ninth embodiment, the viral genome of a live, attenuated RSV of the present invention comprises the nucleotide sequence as set forth in SEQ ID NO: 26 or SEQ ID NO: 91. SEQ ID NO: 26 represents the nucleotide sequence of the viral genome of p17 described herein.

Nucleotide positions 6538, 7115, 8937 and 14656 of SEQ ID NO: 26 contain possible polymorphisms. Thus, the nucleotides at those positions within SEQ ID NO: 26 represent either G or A (for position 6538), A or G (for position 7115), A or C (for position 8937), and G or T (for position 14656). In a further embodiment, position 6538 is an adenine nucleotide; position 7115 is a guanine nucleotide; position 8937 is a cytosine nucleotide; and/or position 14656 is a thymine nucleotide. SEQ ID NO: 91 represents the nucleotide sequence of the viral genome of p17_pp described herein. In a further embodiment, a live, attenuated RSV comprises a nucleotide sequence that is at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical, across the genome, to the nucleotide sequence set forth in SEQ ID NO: 26 or SEQ ID NO: 91, wherein one or a combination of the specific nucleotides identified herein to differ between p17 and/or p17_pp and wild-type nucleotide sequences of the specific viral genes are present.

In a tenth embodiment, a live, attenuated RSV of the present invention comprises a variant protein and/or nucleic acid sequence that is substantially similar to those sequences comprised within the attenuated RSV described in the prior embodiments corresponding to said attenuated RSV, having both physical/structural and functional properties that are substantially the same. In one embodiment, said live, attenuated RSV comprises a substantially similar protein encoded by a nucleic acid molecule that hybridizes under stringent conditions to the complement of a nucleic acid molecule encoding one or more of the modified proteins within the p17 or p17_pp virus. In a further embodiment, said live, attenuated RSV comprises nucleic acid molecules that hybridize to one or more of the modified nucleic acid molecules, or regions thereof (either coding or non-coding), within the p17 or p17_pp virus under stringent conditions.

In an eleventh embodiment, a live, attenuated RSV described in the prior embodiments is comprised within a population of live, attenuated RSV. A population of attenuated RSV means that said population of viruses is not necessarily homogenous for a single virus. For example, as described herein, polymorphisms have been found in the later passages of the RSV strains disclosed herein. There are four polymorphic sites within the p17 genome described herein. As such, a population of viruses, as represented by p17, may consist of different attenuated viruses having a genome comprising the fixed nucleotide mutations (i.e., non-polymorphic) described herein, as well as one of two possible nucleotides at the four polymorphic positions, 6538, 7115, 8937 and 14656. A RSV population can be generated by serially passaging either a non-attenuated or an incompletely-attenuated RSV strain in cell culture as described herein (see Example 1, *infra*), including but not limited to serially passaging Merck strain 287 having a viral genome as set forth in SEQ ID NO: 25. Virus populations can be purified to homogeneity or substantial homogeneity by passaging on suitable cell types or by performing a series of one or more cloning steps.

A further embodiment of the present invention encompasses a live, attenuated RSV, or population thereof, as described in the previous embodiments, wherein said virus is

produced by a process comprising incorporating, by recombinant methods, one or more of the nucleotides and/or amino acids identified herein to differ between p17 or p17_pp and wild-type sequences of the specific viral proteins or genes (described in detail *supra* and *infra*) into either a non-attenuated or incompletely-attenuated RSV strain. In a further embodiment of the present invention, a live, attenuated RSV, or population thereof, as described in the prior embodiments is produced by a process comprising serially passaging Merck strain 287 (having the genomic sequence as set forth in SEQ ID NO: 25) as per the method described in Example 1, *infra*. Thus, said embodiment encompasses passaging a non-attenuated RSV strain comprising a viral genome as set forth in SEQ ID NO: 25 in a cell line (including, but not limited to African Green Monkey kidney cell line, Vero CCL-81) at a fixed multiplicity of infection (MOI) (e.g., between 1:100 and 1:1000).

A further embodiment of the present invention encompasses a method of attenuating a RSV comprising incorporating, by recombinant methods, one or more of the nucleotides and/or amino acids identified herein to differ between p17 or p17_pp and wild-type nucleotide or amino acid sequences of the specific viral genes or proteins (described in detail *supra* and *infra*) into either a non-attenuated or incompletely-attenuated RSV strain. A further embodiment of the present invention encompasses a method of attenuating a RSV comprising serially passaging Merck strain 287 (having the genomic sequence as set forth in SEQ ID NO: 25) as per the method described in Example 1, *infra*.

The present invention also includes one or more of the live, attenuated RSV described in the prior embodiments, a population thereof, or a vaccine comprising said attenuated viruses (i) for use in, (ii) for use as a medicament for, or (iii) for use in the preparation of a medicament for: (a) therapy (e.g., of the human body); (b) medicine; (c) inhibition of RSV replication; (d) treatment or prophylaxis of infection by RSV; or, (e) treatment, prophylaxis of, or delay in the onset or progression of RSV-associated disease(s). In these uses, the attenuated virus and/or vaccine can optionally be employed in combination with one or more anti-viral agents.

III. Pharmaceutical Compositions

A pharmaceutical composition comprising a live, attenuated RSV, or a virus population comprising said live, attenuated RSV, as described herein, and a pharmaceutically acceptable carrier is also provided by this invention.

Thus, a live, attenuated RSV described herein can be formulated with pharmaceutically acceptable carriers to help retain biological activity while also promoting increased stability during storage within an acceptable temperature range. As used herein, the term "pharmaceutically acceptable carrier" encompasses any suitable pharmaceutical carriers well known in the art and described in a variety of texts, such as Remington's Pharmaceutical Sciences. Potential carriers include, but are not limited to, physiologically balanced culture

medium, phosphate buffer saline solution, water, emulsions (e.g., oil/water or water/oil emulsions), various types of wetting agents, cryoprotective additives or stabilizers such as proteins, peptides or hydrolysates (e.g., albumin, gelatin), sugars (e.g., sucrose, lactose, sorbitol), amino acids (e.g., sodium glutamate), or other protective agents. The resulting aqueous solutions may be packaged for use as is or lyophilized. Lyophilized preparations are combined with a sterile solution prior to administration for either single or multiple dosing. Formulated compositions, especially liquid formulations, may contain a bacteriostat to prevent or minimize degradation during storage, including but not limited to effective concentrations (usually ≤1% w/v) of benzyl alcohol, phenol, m-cresol, chlorobutanol, methylparaben, and/or propylparaben. A bacteriostat may be contraindicated for some patients; therefore, a lyophilized formulation may be reconstituted in a solution either containing or not containing such a component.

The pharmaceutical composition may optionally include an adjuvant to enhance the immune response of the host. Suitable adjuvants are, for example, toll-like receptor agonists, alum, AlPO4, alhydrogel, Lipid-A and derivatives or variants thereof, oil-emulsions, saponins, neutral liposomes, liposomes containing the vaccine and cytokines, non-ionic block copolymers, and chemokines. Non-ionic block polymers containing polyoxyethylene (POE) and polyxylpropylene (POP), such as POE-POP-POE block copolymers may be used as an adjuvant (Newman et al., 1998, *Critical Reviews in Therapeutic Drug Carrier Systems* 15:89-142). These adjuvants have the advantage in that they help to stimulate the immune system in a non-specific way, thus enhancing the immune response to a pharmaceutical product.

A live, attenuated RSV described herein, derived from one particular RSV subgroup or strain, can be combined with other attenuated RSV derived from a different subgroup or strain. The different viruses can be in an admixture and administered simultaneously, or administered separately. Due to the phenomenon of cross-protection among certain strains of RSV, immunization with one strain may protect against several different strains of the same or different subgroup. Thus, an isolated, attenuated RSV described herein may be combined with other non-naturally occurring RSV or exist within a population of attenuated RSV.

In some instances it may be desirable to combine a live, attenuated RSV described herein, or a composition thereof, with other pharmaceutical products (e.g., vaccines) which induce protective responses to other agents, particularly those causing other childhood illnesses. For example, an attenuated RSV composition described herein can be administered simultaneously (typically separately) or sequentially with other vaccines recommended by the Advisory Committee on Immunization Practices (ACIP; http://www.cdc.gov/vaccines/recs/ACIP/default.htm) for the targeted age group (e.g., infants from approximately one to six months of age). These additional vaccines include, but are not limited to, other parenterally-administered vaccines. As such, a live, attenuated RSV composition described herein may be administered simultaneously or sequentially with vaccines

against, for example, hepatitis B (HepB), diphtheria, tetanus and pertussis (DTaP), pneumococcal bacteria (PCV), *Haemophilus influenzae* type b (Hib), polio, influenza and rotavirus.

IV. Methods of Use

Pharmaceutical compositions comprising a live, attenuated RSV, or a virus population comprising said live, attenuated RSV, are useful to vaccinate a subject to treat RSV infection and illnesses associated therewith. The scope of this invention is meant to include maternal immunization.

Thus, the present invention further provides a method of vaccinating a subject against RSV infection by administering to the subject an effective amount of a pharmaceutical composition described hereinabove. This subject may be an animal, for example, a mammal, such as a chimp or a human. Pharmaceutical compositions containing a live, attenuated RSV described herein are administered to a subject susceptible to or otherwise at risk of RSV infection or the development of severe illnesses associated with RSV infection (including re-infection), enhancing the subject's own immune response capabilities. In particular, due to the potentially serious consequences of RSV infection in neonates, seronegative and seropositive infants and young children, and the elderly, these individuals will benefit from immunization with the disclosed live, attenuated RSV compositions. Thus, particularly suitable candidates for immunization with the described pharmaceutical products are infants, children, the elderly, and adult candidates for immunosuppressive therapies. Seronegative individuals are those who exhibit no immunologic evidence of previous infection with a subgroup A or B RSV. Seropositive individuals are those who have acquired detectable RSV antibodies, either passively from the mother or as a result of past RSV infection.

Pharmaceutical compositions comprising the live, attenuated RSV described herein elicit the production of an immune response that is protective against serious lower respiratory tract disease, such as pneumonia and bronchiolitis, when the subject is subsequently infected or re-infected with a wild-type RSV. While the naturally circulating virus is still capable of causing infection, particularly in the upper respiratory tract, there is a much reduced possibility of rhinitis as a result of the vaccination and a possible boosting of resistance by subsequent infection by wild-type virus. Following vaccination, there are detectable levels of host engendered serum and secretory antibodies which are capable of neutralizing homologous (of the same subgroup) wild-type virus *in vitro* and *in vivo*. In many instances the host antibodies will also neutralize wild-type virus of a different, non-vaccine subgroup. To achieve higher levels of cross-protection, for example, against heterologous strains of another subgroup, subjects can be vaccinated with a live, attenuated RSV described herein from at least one predominant strain of both subgroups A and B. As such, a live, attenuated RSV described herein can belong to either antigenic subgroup A or B, and virus from both subgroups may be combined in vaccine formulations for more comprehensive coverage against prevalent RSV infections.

The live, attenuated RSV described herein, and pharmaceutical compositions thereof, are provided in an effective amount to induce or enhance an effective immune response against RSV in a subject, preferably a human. An effective amount will allow some growth and proliferation of the virus, in order to produce the desired immune response, but will not produce RSV-associated symptoms or illnesses. Based on the guidance provided herein, persons skilled in the art will readily be able to determine the proper amount of virus to use in the live vaccine. The precise amounts will depend on several factors, for example, the subject's state of health and weight, the mode of administration, the degree of attenuation of the virus, the nature of the formulation, and whether the immune system of the subject is compromised. In one embodiment, a general range of virus administration is about 10^3 to about 10^7 plaque forming units (PFU) or more of virus per human subject, including about 10^4 to about 10^5 PFU virus per human subject.

Administration may be in a form found to be effective at stimulating a protective immune response, choices which may include parenterally, intravenously, orally, or topically applied to a mucosal surface. In most instances, the live, attenuated RSV described herein is administered parenterally.

Single or multiple administrations of the live, attenuated RSV described herein, and pharmaceutical compositions thereof, can be carried out. In neonates and infants, multiple administration may be required to elicit sufficient levels of immunity. Administration may begin within the first months of life and continue at intervals throughout childhood, as necessary to maintain sufficient levels of protection against native (wild-type) RSV infection. For example, one possible vaccine schedule may consist of an initial administration at one month, and subsequent administrations at two months, six months, one year and/or two years. Vaccination frequency may be modified to be consistent with current guidelines for other concomitant use vaccines. Levels of induced immunity can be monitored by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection.

In any event, the vaccine formulations, effective amounts thereof for administration, and specific mode of administration should provide a quantity of a live, attenuated RSV described herein sufficient to effectively stimulate, induce or boost an anti-RSV immune response, e.g., as can be determined by complement fixation, plaque neutralization, and/or enzyme-linked immunosorbent assay, among other methods (e.g., correlating responses with monitoring subjects for protection from signs and symptoms of upper and lower respiratory illness).

V. Generation of Live, Attenuated RSV

The live, attenuated RSV described herein may be biologically derived or recombinantly generated. A "biologically-derived RSV" means any RSV not produced by

recombinant means (e.g., through passaging). A "recombinantly-generated RSV" means any RSV generated via viral cloning techniques (e.g., reverse genetics). The live, attenuated RSV comprise genomic variations compared to a reference RSV sequence, wherein said reference RSV sequence may be derived from a wild-type or an incompletely-attenuated RSV.

Accordingly, the RSV genome into which the specific nucleotides and/or amino acid residues described herein are introduced can be from a genetically and/or phenotypically wild-type virus or a derivative of wild-type virus, such as a virus already partially attenuated, in which case the newly incorporated nucleotides and/or amino acid residues act to further attenuate the strain (e.g., to a desired level of restricted replication in a mammalian host while retaining sufficient immunogenicity to confer protection in a vaccinated subject). Either a biologically-derived or a recombinantly-generated subgroup A or B RSV can be partially attenuated by, for example, cold-passaging, chemical mutagenesis, or site-directed mutagenesis. Attenuating a virus by cold-passaging involves subjecting the virus to passage in cell culture at progressively lower temperatures. For example, whereas wild-type virus is typically cultivated at about 34-37°C, a partially-attenuated virus can be produced by passage in cell cultures (e.g., primary bovine kidney cells) at sub-optimal temperatures, e.g., 20-26°C. Attenuating a virus by chemical mutagenesis involves, for example, replication of the virus in the presence of a mutagen such as 5-fluorouridine or 5-fluorouracil at a concentration of about 10^{-3} to 10^{-5} M, preferably about 10^{-4} M, or exposure of the virus to nitrosoguanidine at a concentration of about 100 µg/ml, according to general procedures described in, e.g., Gharpure et al., 1969, *J. Virol.* 3:414-421 and Richardson et al., 1978, *J. Med. Virol.* 3:91-100. Other chemical mutagens can also be used. Partially-attenuated RSV can also be generated recombinantly by incorporating attenuating mutations (albeit, incomplete attenuating mutations) into the genome of the wild-type RSV. Various selection techniques may be combined to produce partially-attenuated mutants from non-attenuated subgroup A or B strains which are useful for further derivatization as described herein.

The live, attenuated RSV described herein can be produced by recombinant methods, e.g., from cDNA. Nucleotide and/or amino acid changes, either alone or in combination, can be incorporated into the genome of a wild-type or partially-attenuated RSV. These changes will specify the desired, phenotypic characteristics of the biologically-derived, attenuated RSV described in the Examples, *infra*. Infectious RSV can be produced by the intracellular co-expression in mammalian cells of a cDNA that encodes the genome or antigenome RNA of the live, attenuated RSV, together with those viral proteins necessary to generate a transcribing, replicating nucleocapsid containing associated proteins and genomic RNA (see, e.g., Palese, 1995, *Trends in Microbiology*, 3:123-125; Lawson et al., 1995, *Proc. Natl. Acad. Sci. USA* 92:4477-4481; Schnell et al, 1994, *EMBO J.* 13:4195-4203). An RSV antigenome means an isolated, positive-sense polynucleotide molecule which serves as the template for the synthesis of a progeny, negative-sense RSV genome. Thus, methods for producing a live, attenuated RSV having one or more of the nucleotide and/or amino acid

described herein from one or more isolated polynucleotides, e.g., one or more cDNAs, are also encompassed by the present invention.

Introduction of the foregoing, defined nucleotides/amino acid residues (alone or in combination) 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 serving as template to produce the genome of an infectious virus or subviral particle. Defined mutations can be introduced by conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of a RSV genome or antigenome. The use of antigenomic or genomic cDNA subfragments to assemble a complete antigenomic or genomic cDNA 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. A mutated subfragment, for example, can then be substituted for its counterpart subfragment from a genomic or antigenomic sequence from either a wild-type or incompletely attenuated RSV. Counterpart subfragments share substantial sequence identity with the selected, mutated subfragment. Thus, the complete antigenomic or genomic cDNA, or any subfragment thereof, can be used as template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a single-stranded phagemid form, such as using the Muta-gene® kit of Bio-Rad Laboratories (Richmond, Calif.), a method using the double-stranded plasmid directly as template such as the Chameleon mutagenesis kit of Stratagene (La Jolla, Calif.), or by polymerase chain reaction employing either an oligonucleotide primer or template which contains the mutation(s) of interest. The RSV genome or antigenome may also be constructed by, e.g., assembling the cloned cDNA segments, representing in aggregate a complete antigenome, by polymerase chain reaction (PCR Protocols: A Guide to Methods and Applications, Innis et al., eds., Academic Press, San Diego (1990), incorporated herein by reference) of reverse-transcribed copies of RSV mRNA or genomic RNA. Alternatively, antigenomic or genomic RNA can be synthesized *in vitro*.

To produce infectious RSV from a cDNA-expressed genome or antigenome, the genome or antigenome is co-expressed with those RSV accessory proteins necessary to (i) produce a nucleocapsid capable of RNA replication, and (ii) render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other RSV proteins and initiates a productive infection. Alternatively, additional RSV proteins needed for a productive infection can be supplied by co-expression.

For example, the infectious RSV clone of the live, attenuated virus genome described herein can be first incorporated into a plasmid vector. Plasmid vectors suitable for subsequent transfections and expression in a mammalian host cell are commercially available. The plasmid vector containing a cDNA copy can then be transfected into a host cell that expresses bacteriophage T7 RNA polymerase. Additional plasmid vectors that express the RSV major nucleocapsid (N) protein, nucleocapsid phosphoprotein (P), large (L) polymerase protein,

and, optionally, the transcriptional elongation factor M2 ORF1 protein, can be co-transfected into the host cell. The cDNA is transcribed to produce full-length, negative-sense (genomic) RNA. Expression of the N, L and P proteins, and optionally the M2(ORF1) protein, facilitates synthesis of progeny virus. The virions are then isolated. For this method, it is preferable that a cDNA is constructed which is a positive-sense version of the RSV genome, corresponding to the replicative intermediate RNA, or antigenome, so as to minimize the possibility of hybridizing with positive-sense transcripts of the complementing sequences that encode the proteins necessary to generate a transcribing, replicating nucleocapsid (e.g., sequences that encode the N, P, L and M2(ORF1) proteins). When using a RSV minigenome system, the genome and antigenome are equally active in rescue, whether complemented by RSV or by plasmids, indicating that either genome or antigenome can be used depending on methodologic or other grounds.

The N, P, L and, optionally, M2(ORF1) proteins can be encoded by one or more expression vectors which can be the same or separate from that which encodes the genome or antigenome, and various combinations thereof. Additional proteins may be included as desired, encoded by separate vectors or by a vector encoding a N, P, L, or M2(ORF1) protein or the complete genome or antigenome. Expression of the genome or antigenome and proteins from transfected plasmids can be achieved, for example, by each cDNA being under the control of a promoter for T7 RNA polymerase, which in turn is supplied by infection, transfection or transduction with an expression system for the T7 RNA polymerase, e.g., a vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase (Wyatt et al., 1995, *Virology*, 210:202-205).

Isolated polynucleotides (e.g., cDNA) encoding the genome or antigenome and, separately, the N, P, L and, optionally, M2(ORF1) proteins, can be inserted by transfection, electroporation, mechanical insertion, transduction or the like, into cells which are capable of supporting a productive RSV infection, e.g., HEp-2, FRhL-DBS2, MRC, and Vero cells. Transfection of isolated polynucleotide sequences may be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler et al., 1978, *Cell* 14:725; Corsaro & Pearson, 1981, *Somatic Cell Genetics* 7:603; Graham & Van der Eb, 1973, *Virology* 52:456), electroporation (Neumann et al., 1982, *EMBO J.* 1:841-845), DEAE-dextran mediated transfection (Ausubel et al., (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY (1987)), cationic lipid-mediated transfection (Hawley-Nelson et al., 1993, *Focus* 15:73-79) or a commercially available transfection regent, e.g., LipofectACE® (Life Technologies). The viral proteins, and/or T7 RNA polymerase, can also be provided from transformed mammalian cells, or by transfection of preformed mRNA or protein.

Once a live, attenuated RSV of the present invention has been generated (e.g., as per the cloning and rescue experiments described above), the attenuated virus may be propagated in a number of cell lines which allow for RSV growth. RSV grows in a variety of human and

animal cells. Examples of cell lines for propagation of the live, attenuated RSV include DBS-FRhL-2, MRC-5, and Vero cells. Highest 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 about 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 3-5 days, or as long as necessary for virus to reach an adequate titer. Virus is removed from cell culture and separated 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 *in vivo* and/or *in vitro* models to confirm adequate attenuation, genetic stability, and immunogenicity. The level of attenuation of a genetically-modified RSV may be determined by, for example, quantifying the amount of virus present in the respiratory tract of an infected subject and comparing the amount to that produced by a phenotypically wild-type RSV (e.g., RSV A2, Merck strain 287). Attenuated viruses can be tested a variety of animal models of RSV infection, described and summarized in Meignier et al., eds., Animal Models of Respiratory Syncytial Virus Infection, Merieux Foundation Publication, (1991). For example, the cotton rat model of RSV infection has been long held to be predictive of attenuation and efficacy in humans (U.S. Pat. No. 4,800,078; Prince et al., 1985, *Virus Res.* 3:193-206; Prince et al., 1987, *J. Virol.* 61:1851-1854). Other rodents, including mice, could also be similarly useful as these animals are permissive for RSV replication and have a core temperature more like that of humans (Wight et al., 1970, *J. Infect. Dis.* 122:501-512; Byrd and Prince, 1997, *Clin. Infect. Dis.* 25:1363-1368). In particular, primate models are genetically and immunologically relevant host systems in which to study RSV infection (McArthur-Vaughan and Gershwin, 2002, *J. Med. Primatol.* 31:61-73). For example, a primate model of RSV infection using the chimpanzee is predictive of attenuation and efficacy in humans (see, e.g., Richardson et al., 1978, *J. Med. Virol.* 3:91-100; Wright et al.,1982, *Infect. Immun.* 37:397-400; Crowe et al., 1993, *Vaccine* 11:1395-1404. African green monkeys have also been used as a model of RSV infection (Cheng et al., 2001, *Virology* 283:59-68; Kakuk et al., 1993, *J. Infect. Dis.* 167:553-61; Weiss et al., 2003, *J. Med. Primatol.* 32:82-88). Additionally, *in vitro* analysis of attenuation may include assessing growth of the virus in human airway epithelial cells (Wright et al., 2005, *J. Virol.* 79:8651-8654).

The live, attenuated RSV described herein will have a greater degree of restricted replication in both the upper and lower respiratory tracts of highly susceptible hosts, such as a monkeys and cotton rat, compared to the levels of replication of wild-type virus, e.g., over 1000-fold less. Methods for determining levels of RSV in the nasopharynx of an infected host are well known in the literature. Briefly, specimens can be obtained by aspiration or washing out of nasopharyngeal secretions and virus quantified in tissue culture or by other laboratory procedure. See, for example, Belshe et al., 1977, *J. Med. Virology* 1:157-162; Friedewald et al., 1968, *J.*

Amer. Med. Assoc. 204:690-694; Gharpure et al., 1969, *J. Virol.* 3:414-421; and, Wright et al., 1973, *Arch. Ges. Virusforsch.* 41:238-247.

In addition to the criteria of viability, attenuation and immunogenicity, the properties of the live, attenuated RSV described herein must also be as stable as possible so that the desired attributes are maintained. Ideally, a virus which is useful as part of a pharmaceutical product (e.g., vaccine) must maintain its viability, its property of attenuation, its ability to replicate in the immunized host (albeit at lower levels), and its ability to effectively elicit the production of an immune response in a vaccinated subject that is sufficient to confer protection against serious disease caused by subsequent infection by wild-type virus.

VI. Recombinant Nucleic Acids

Nucleic acid molecules comprising either a full-length or partial attenuated RSV genome or antigenome described herein, a nucleotide sequence that encodes a modified viral protein described herein or a portion thereof, or a nucleotide sequence of a modified viral gene described herein or a portion thereof (see, e.g., the description of nucleic acids comprised within live, attenuated RSV of the present invention within Section II, *supra*), are also within the scope of the present invention. These nucleic acids may be deoxyribonucleic acid (DNA) molecules, complementary DNA molecules (cDNA), or ribonucleic acid (RNA) molecules.

A "triplet" codon of four possible nucleotide bases can exist in over 60 variant forms. Because these codons provide the message for only 20 different amino acids (as well as transcription initiation and termination), some amino acids can be coded for by more than one codon, a phenomenon known as codon redundancy. Thus, due to this degeneracy of the genetic code, a large number of different encoding nucleic acid sequences can be used to code for a particular protein. Amino acids are encoded by the following RNA codons:

A=Ala=Alanine: codons GCA, GCC, GCG, GCU
C=Cys=Cysteine: codons UGC, UGU
D=Asp=Aspartic acid: codons GAC, GAU
E=Glu=Glutamic acid: codons GAA, GAG
F=Phe=Phenylalanine: codons UUC, UUU
G=Gly=Glycine: codons GGA, GGC, GGG, GGU
H=His=Histidine: codons CAC, CAU
I=Ile=Isoleucine: codons AUA, AUC, AUU
K=Lys=Lysine: codons AAA, AAG
L=Leu=Leucine: codons UUA, UUG, CUA, CUC, CUG, CUU
M=Met=Methionine: codon AUG
N=Asn=Asparagine: codons AAC, AAU
P=Pro=Proline: codons CCA, CCC, CCG, CCU
Q=Gln=Glutamine: codons CAA, CAG

R=Arg=Arginine: codons AGA, AGG, CGA, CGC, CGG, CGU
S=Ser=Serine: codons AGC, AGU, UCA, UCC, UCG, UCU
T=Thr=Threonine: codons ACA, ACC, ACG, ACU
V=Val=Valine: codons GUA, GUC, GUG, GUU
W=Trp=Tryptophan: codon UGG
Y=Tyr=Tyrosine: codons UAC, UAU

Thus, the present invention includes nucleic acids that differ from the disclosed nucleic acid molecules but which encode the same protein sequence.

Also encompassed by the present invention are nucleic acid molecules comprising non-coding sequences (i.e., untranslated sequences) of the RSV of the present invention. These non-coding regions include 5' non-coding regions, 3' non-coding regions, intergenic sequences, and other non-coding regions of the viral genome, including but not limited to, transcriptional, translational, and other regulatory regions. These nucleic acid molecules also may be DNA molecules, cDNA molecules or RNA molecules.

The present invention further includes substantially similar nucleic acid molecules to those nucleic acid molecules which encode one or more proteins of the live, attenuated RSV of the present invention, including but not limited to the NS1, NS2, G, F and/or L genes of the live, attenuated RSV described herein. When incorporated into a RSV viral genome, said NS1, NS2, G, F and/or L genes will produce the same attenuated, phenotypic effect. The present invention also encompasses substantially similar nucleic acid molecules characterized by changes in non-coding regions that do not alter the phenotype of the resulting virus produced there from.

Accordingly, included within the scope of this invention are nucleic acid molecules comprising either nucleotide sequences that encode variants of the proteins comprised within the live, attenuated RSV described herein, or nucleic acid molecules comprising variants of the non-coding nucleotide sequences comprised within the live, attenuated RSV described herein.

Included within the scope of the present invention are nucleic acid molecules containing one or more of the indicated nucleotides that hybridize to the complement of the nucleic acid molecules of the subject invention under stringent conditions. By way of example, and not limitation, a procedure using conditions of high stringency is described. Prehybridization of filters containing DNA is carried out for about 2 hours to overnight at about 65°C in buffer composed of 6x SSC, 5x Denhardt's solution, and 100 μg/ml denatured salmon sperm DNA. Filters are hybridized for about 12 to 48 hrs at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 x 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for about 1 hour in a solution containing 2x SSC, 0.1% SDS. This is followed by a wash in 0.1x SSC, 0.1% SDS at 50°C for 45 minutes before autoradiography. Other procedures using conditions of high stringency would include either a hybridization step carried out in 5x SSC, 5x Denhardt's solution, 50% formamide at about 42°C for about 12 to 48

hours or a washing step carried out in 0.2x SSPE, 0.2% SDS at about 65°C for about 30 to 60 minutes. Reagents mentioned in the foregoing procedures for carrying out high stringency hybridization are well known in the art. Details of the composition of these reagents can be found in Sambrook et al., Molecular Cloning: A Laboratory Manual 2nd Edition; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, (1989) or Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Edition. Cold Spring Harbor Laboratory Press, Plainview, NY (2001). In addition to the foregoing, other conditions of high stringency which may be used are also well known in the art.

Nucleic acid molecules of this invention may be operatively linked to a promoter of RNA transcription, as well as other regulatory sequences. As used herein, the term "operatively linked" means positioned in such a manner that the promoter will direct the transcription of RNA off of the nucleic acid molecule. An example of a promoter is the T7 promoter. Vectors which contain both a promoter and a cloning site to which an inserted piece of nucleic acid is operatively linked to the promoter, are well known in the art. These vectors may be capable of transcribing nucleic acid *in vitro* and *in vivo*. Examples of such vectors include those comprising nucleic acids derived from viral genomes of other RSV types (including both other attenuated subtypes and wild-type strains) that have been modified by substituting the nucleic acid regions encoding the polypeptides and/or the non-coding regions of the live, attenuated RSV described herein for the nucleic acids of said other RSV types. Further provided are recombinant virus encoded by the nucleic acid molecules of this invention.

Examples are provided below further illustrating different features of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

EXAMPLE 1

Passaging of MRK RSV strain 287

Cells and virus -African Green Monkey kidney cell line Vero CCL-81 was utilized both for virus growth studies and plaque assay to monitor viral output. Parent virus utilized was Merck 287 strain hRSV which was stored lyophilized at -20C. The parent Merck 287 virus had undergone two passages in GMK cells, then five additional passages in WI-38 cells (Bunyak et al., 1978, *Proc. Soc. Exp. Biol. Med.* 157:636-642; Bunyak et al., 1979, *Proc. Soc. Exp. Biol. Med.* 160:272-277; Belshe et al., 1982, *J. Infect. Dis.* 145:311-319). By sequence analysis, it was confirmed to retain a wild-type genotype at all known positions associated with attenuation.

Virus passaging and virus stock preparation - Parent virus was used to infect week-old confluent Vero monolayers at a 1:100 or 1:1000 MOI with a 1 hr attachment at 32°C; overlay post attachment with maintenance medium comprised of WM's Medium E (Gibco), 1.6% recombinant human albumin (Delta biotechnologies), 2 mM L-glutamine (Gibco), and 50 μg/ml

Neomycin (Sigma). Cultures were observed for maximal cytopathic effect (CPE) >90%, and harvested and re-fed for additional harvests at 24 hr intervals until CPE was too advanced for optimal viral output.

Plaque assay - Harvested virus samples were titered at serial tenfold dilutions via plaque assay and used to inoculate confluent (48-72 hr) VERO monolayers in 12-well assay format (Costar), 100 µl/well, in triplicate per dilution of serial tenfold dilutions, four dilutions per plate total. Plates incubate at 35°C ±1°C plus 5.0% CO_2 for one hour, overlay with maintenance medium comprised of WM's Medium E (Gibco), 1.6% recombinant human albumin (Delta biotechnologies), 2 mM L-glutamine (Gibco), and 50 µg/ml Neomycin (Sigma), and 0.5% final concentration of SeaPlaque® agarose. Plaques develop for 6-8 days at 35°C ±1°C plus 5.0% CO_2. Plaques were visualized by adding 250 µl/well of 5 mg/mL MTT; Thialzolyl Blue Tetrazolium Bromide (Sigma-Aldrich®) in PBS and waiting 2-6 hours for cells to metabolize the stain. Titers were obtained on all harvests between passages not only to monitor viral yield, but to accurately measure titers for calculating the appropriate dilution for the MOI for the subsequent passage of virus.

Results - Merck HRSV strain 287 is derived from an isolated hRSV strain that was first amplified twice in GMK cells. After the initial amplification, the virus underwent five additional passages in the human diploid cell line WI-38. This passage 5 material was utilized in human clinical trials for vaccine use via a parenteral administration route. Merck strain 287, hRSV A type, showed a wild-type (WT) phenotype both in sequence analysis and *in vivo* in animal shedding experiments (see *infra*). Merck strain 287 was serially passaged 22 times (passage 1 (p1) - passage 22 (p22)) at a fixed multiplicity in VERO-CCL-81 monolayers. Virus titers remained constant over passaging and the cytopathic effect of the virus in culture also remained consistent. Virus yields were in the range of 10^6-10^7 pfu/ml.

EXAMPLE 2

Sequencing of MRK RSV strain 287 and Passaged Virus Stocks

RSV samples - Materials were supplied in 500-1000 µl aliquots of harvested viral supernatants from infected VERO cells. Full-length sequences were generated for: 1) MRK RSV Strain 287; 2) MRK RSV Strain 287, passage 17 ("p17"); and, 3) MRK RSV Strain 287, passage 22 ("p22"). Partial (targeted) sequences were generated for: 1) MRK RSV Strain 287, passage 5 ("p5"); 2) MRK RSV Strain 287, passage 10 ("p10"); 3) MRK RSV Strain 287, passage 15 ("p15"); and, 4) MRK RSV Strain 287, passage 18 ("p18").

RNA extraction - RNA was extracted from virus culture supernatants using the QiaAMP Viral RNA Extraction Kit (Qiagen, cat. no. 52904) as per the manufacturer's protocol. In short, 140 µl of virus culture supernatants were added to lysis buffer, loaded onto a binding filter, washed and eluted in 60 µl of RNase-free water. Extracted RNA samples were stored at minus 20°C. Extracted RNA samples were used as templates for amplification of genome into

double-stranded DNA fragments by reverse-transcriptase polymerase chain reaction (RT-PCR). Multiple extractions of each sample were performed to obtain sufficient materials for the RT-PCR amplifications.

Amplification of RSV genome by RT-PCR - The RSV genome of samples was amplified into double-stranded DNA using the Qiagen OneStep RT-PCR kit (Qiagen, cat. no. 210212). For full-length sequencing (MRK RSV 287 and passages P17 and P22), thirty (30) amplifications of 1000 base pair (bp) fragments were conducted, with each fragment overlapping adjacent fragments by approximately 500 bp. Amplification reactions were numbered 1 to 30 (for the primer pair) and carried out as listed below. For partial-length sequencing (MRK RSV 287 passages P5, P10, P15 and P18) seven (7) amplifications of 1000 base pair (bp) fragments were conducted, using primer pairs 2, 10, 11, 13, 14, 18, and 29. Amplification reactions were numbered accordingly (for the primer pair) and carried out as listed below.

Master Mix (per reaction): 10 µl 5X QIAGEN OneStep RT-PCR buffer; 2 µl dNTP mix; 3 µl primer A (forward primer) – 10 µM stock, 600nM final; 3 µl primer B (reverse primer) - 10 µM stock, 600nM final; 2 µl QIAGEN OneStep RT-PCR Enzyme Mix; 5 µl RNA template; 25 µl molecular biology grade water.

Amplification reactions were carried out in a Biometra thermocycler with the following conditions: 30 minutes, 50°C - reverse transcription step; 15 min 95°C - initial PCR activation step (DNA polymerase activation) 3-step cycling (repeat for 40 cycles total); 1 min, 94°C - denaturation; 1 minute, 55°C - annealing; 1 minute, 72°C - extension; 10 min 72°C - final extension.

Primers used for the RT-PCR amplifications are listed in Tables 2 and 3.

Table 2: RT-PCR amplification (sequencing) primers, pairs 1-15

Primer Pair	Primer	Primer Sequence (5' → 3')	SEQ ID NO	5' binding position	Product Length
1	FWD	ACGGGAAAAAATGCGTACAACAAAC	27	1	1056
	REV	GTGTAGTCATGCATAGAGTTGTTGTT TTAGATTGTGTGAA	28	1056	
2	FWD	TATCAACTAGCTAATCAATGTCACTA ACACC	29	530	1043
	REV	CCTCTCCCATTTCTTTTAGCATTTT	30	1572	
3	FWD	TTCACACAATCTAAAACAACAACTCT ATGCATGACTACAC	31	1017	1000
	REV	TTTGGGCATATTCATAAACCTCAAC	32	2016	

4	FWD	AATCCCACAAAAAAATGCTAAAAGA AATGGGAGAGGTAGC	33	1537	1018
	REV	CTGTCTCATTTGTTGGGTTGATAATA GTTGA	34	2554	
5	FWD	TATGAATATGCCCAAAAATTGGGTGG TGAAGCA	35	2001	1194
	REV	TGGCTGGTTGTTTTGTTGGCTGCTT	36	3194	
6	FWD	GCCCTATAACATCAAATTCAACTATT ATCAACCC	37	2507	1028
	REV	CGCTTATGGTAAATTTGCTGGGCAT	38	3534	
7	FWD	ACATCAGAGAAATTGAACAACCTGT	39	3001	1002
	REV	TTGTAGCTGTGTGCTTCCAATTTGT	40	4002	
8	FWD	CTAGCACAAATGCCCAGCAAATTTAC CATAAGCG	41	3501	1097
	REV	GTGGTTTGCATGGTGGGACGTTGAT	42	4597	
9	FWD	AGATTTGCAATCAAACCTATGGAAG	43	4002	1002
	REV	TTCAGACGGATTAGAGGGACTGATT	44	5003	
10	FWD	CATCAATCCAACAGCTCAAAACAGT	45	4501	1002
	REV	TACCTTCGGAGGAAGTTGAGTGGAA	46	5502	
11	FWD	GAAATTACATCAACAAATCACCACC A	47	5001	1000
	REV	AAACCTTGGTAGTTCTCTTCTGGCT	48	6000	
12	FWD	TAATCCAAGCCCTTCTCAAGTCTCC	49	5501	1114
	REV	GATGTGTGTAATTTCCAACAAGGTG	50	6614	
13	FWD	CACTCAACAATGCCAAAAAAACCAA TGTAAC	51	6011	1117
	REV	GCATCAAATTCACCAGAGGGGAATA	52	7127	
14	FWD	TAGACAGCAAAGTTACTCTATCATG	53	6501	1001
	REV	GATGGTTTATAGATGAGAGTTTCGA	54	7501	
15	FWD	ACACTGTGTCTGTAGGCAACACATTA TATTATG	55	7001	1067
	REV	GTCTGCTGGCAATCTTTTTAACAGAT GGATAGTTTGTTTA	56	8067	

Table 3: RT-PCR amplification (sequencing) primers, pairs 16-30

Primer Pair	Primer	Primer Sequence (5'-→ 3')	SEQ ID NO	5' binding position	Product Length
16	FWD	TGCCAGATTAACTTACTATCTGAAAA ATGAAAACTGGGG	57	7558	1020
	REV	CACTCTGAGAAAGAGATAACACCTTT TAAATAACTATCGG	58	8577	
17	FWD	TAAACAAACTATCCATCTGTTAAAAA GATTGCCAGCAGAC	59	8028	1000
	REV	GAGTGATTTTTGCCTGCTTTAAGAT	60	9027	
18	FWD	GGAAATTCTGCTAATGTTTATCTAAC CG	61	8513	1046
	REV	CGTTTTCTGAATTGATCTTCTTCTG	62	9558	
19	FWD	TCATCTTAAAGCAGGCAAAAATCACT CTACA	63	9001	1053
	REV	CGTAGTCCTGATAACACAATCAAATC TCTTTCTGTAAGTT	64	10053	
20	FWD	CAGAAGAAGATCAATTCAGAAAACG	65	9534	1010
	REV	GCGATTTGATTTGTTACTTATTCCTGC	66	10543	
21	FWD	CCTTCTTTGTTGGAACTTACAGAAA	67	10001	1000
	REV	GCAAATAATCTGCTTGAGCATGAGT	68	11000	
22	FWD	GAAAGCAGGAATAAGTAACAAATCA AATCGC	69	10513	1136
	REV	CGTTAGGGTTTTTGTCAAACGTGATT ATGCATGTTAAGAA	70	11648	
23	FWD	ATAGCCTTAAATTACTGTATAAAGAG TATGCAGGCATAGG	71	11010	1000
	REV	TTTTTCCTCATCATCTCAGTGGCTC	72	12009	
24	FWD	GACTTCCTCACAGAGGCTATAGTTC	73	11501	1016
	REV	GTAAGTCGATGCAAATAGTTGACAC	74	12516	
25	FWD	ACTTTGCTTATAAGGATACTTCCATT GG	75	12014	1041
	REV	CTCTCCCCAATCTTTTTCAAAAATAC CCTTAGAATCTTTC	76	13054	

26	FWD	ATTTGCATCGACTTACAGTCAGTAG	77	12501	1131
	REV	GGGTTTCTGGTGTAGGATGATATAATT	78	13631	
27	FWD	GAAAGATTCTAAGGGTATTTTTGAAAAAGATTGGGGAGAG	79	13015	1044
	REV	CCTTCACCTATGAATGCATAACAATTGGGATCTTTA	80	14058	
28	FWD	TTACAACAAATTATATCATCCTACACCAGAAACCCTAGAG	81	13597	1025
	REV	CAGCTTTCTTAGGCATGATGAAATTTTTGGTTCTTGATAG	82	14621	
29	FWD	ATTAAAGATCCCAATTGTATAGCATTCATAGGTGAAGGAG	83	14021	909
	REV	GGTTGTCAAGCTGTTTAACAATTCA	84	14929	
30	FWD	TCGGAGGTTTACTTAGTCATCACAA	85	14501	537
	REV	GGTAGTGTATAGCTATGGGAATCTTTAT	86	15037	

Amplified RT-PCR products were purified using the QIAGEN QIAquick PCR Purification kit (QIAGEN, cat. no. 28104), as per the manufactures protocol. Purified RT-PCR products were numbered 1 to 30 (based on primer pair), stored at -20°C and shipped for sequencing to GeneWiz (South Plainfield, NJ).

Sequencing of amplified RT-PCR products - Purified RT-PCR products were submitted to GeneWiz (South Plainfield, NJ) for dye-termination sequencing. Sequencing was conducted using the RT-PCR amplification primers, which were submitted together with the RT-PCR products. For each RT-PCR products two (2) sequences were generated, one using the forward primer and one using the reverse primer.

RT-PCR product sequence analysis and RSV genome assembly - Generated RT-PCR fragment sequences were imported into Sequencher™ sequence analysis software (GeneCodes, Ann Arbor, Michigan) and sequences were assembled by performing "contig" assemblies. This consists of the importation of all 60 RT-PCR product sequences (2 sequences for each of 30 products) and the assembly of a full-length sequence using the 500 bp overlap regions as scaffolds. Sequences were edited for extraneous and missed peaks using commonly accepted practices. Finalized sequences were exported as fasta-formatted files and imported into VectorNTI™ for comparisons and sequences analyses. For targeted sequencing of p5, p10, p15, and p18, individual fragment assemblies were made (i.e., fragments 2, 10, 11, etc.) rather than a

full-length genome assembly. Final sequences were imported into VectorNTI™ and sequence comparisons and analyses were performed.

Results - Full-length genomic sequences were generated for the original source material (MRK 287), as well as passage 17 (p17) and passage 22 (p22). Targeted sequences of additional passage levels were obtained for passages 5 (p5), 10 (p10), 15 (p15) and 18 (p18). Viral RNA was extracted from culture supernatant samples; double-stranded DNA fragments of the genome were generated from the RNA by reverse-transcriptase polymerase chain reaction (RT-PCR); RT-PCR products were purified and sequenced; and, generated sequences were assembled into a full-length viral genome.

The following comparisons and analyses were conducted:

1) Homology "blast" analysis of MRK RSV 287 strain sequence was conducted against known sequencing in common databases (Genbank, EMBL) to determine sequence-relatedness of MRK RSV 287 against other RSV sequences.

2) Sequence alignment of MRK RSV 287 strain against MRK RSV p17 to determine sequence differences between original MRK RSV strain 287 and p17.

3) Amino acid analyses of any sequence differences between original MRK RSV strain 287 and p17 to determine amino acid sequence differences, if any, associated with nucleic acid sequence differences between the 2 samples.

4) Sequence alignment of MRK RSV 287 strain p17 against MRK RSV p22 to determine sequence differences between MRK RSV strain 287 p17 and p22, in order to determine whether additional point mutations are acquired elsewhere in the viral genome.

5) Comparison of gene segments 2, 10, 11, 13, 14, 18 and 29 from MRK RSV strain 287 and p5, p10, p15, p17, p18, and p22 to determine sequence differences between original MRK RSV strain 287 and associated passaged materials, in order to understand the acquisition of point mutations with increasing passage level and the possible markers of attenuation.

The results of sequence analyses are summarized below:

1) MRK RSV Strain 287 sequence analysis - Comparisons of MRK RSV strain 287 with known wild-type and vaccine strains of RSV (listed in Table 4) indicate that MRK RSV 287 does not contain point mutations previously identified as attenuation markers (data not shown). MRK RSV strain 287 yielded a sequence of 15,205 nucleotides. Passages 17 and 22 yielded 15,000 nucleotides, with the extreme 5' end missing. MRK RSV Strain 287 was similar to known RSV subgroup A strains, with greater than 95% homology scores.

Table 4: Genbank accession information on selected RSV sequences

Genbank Accession Number	Sequence Description
U50362.1	Human RSV, mutant cp-RSV, complete genome
U50363.1	Human RSV, mutant cpts-248, complete genome
U63644.1	Human RSV, mutant cpts-248/404, complete genome
AF035006.1	Human RSV, recombinant mutant rA2cp, complete genome

2) Comparison of MRK RSV Strain 287 and Passage 17 sequences - Comparisons of genomic sequences from MRK RSV strain 287 and p17 indicates that there are 21 sequence differences (point mutations) between the 2 sequences. A summary of sequence differences is listed in Table 5.

Table 5: Sequence differences between MRK RSV Strain 287 and p17

Nucleotide Position	MRK RSV Strain 287	p17
954	G	A
5295	A	G
5298	A	G
5315	A	G
5316	A	G
5322	A	G
5324	A	G
5339	A	G
5346	A	G
5347	A	G
5351	A	G
5352	A	G
5353	A	G
5360	A	G
5380	A	G
5381	A	G
6538	G	A (R?) *
7115	A	G (R?) *
8937	A	C (M?) *
14656	G	T (K?) *
15046	A	C

* possible polymorphisms; R, M and K represent IUPAC codes for polymorphisms: R=A or G; K=G or T; M=A or C

3) Comparison of MRK RSV Strain 287 and Passage 17 Amino Acid Sequences - The gene location of each identified point mutation in p17 compared to the original MRK RSV Strain 287 sequence was mapped and an amino acid sequence was generated for each gene containing point mutations. Of the 21 point mutations, 1 is in the 5' untranslated region (3' to the viral L gene) and 20 are within open-reading frames (viral genes). Of the 20 open-reading frame (ORF) mutations, five (5) are silent and the remaining fifteen (15) affect eleven (11) amino acids in three (3) genes. The 3 affected ORFs encode the G (Glycoprotein), F (Fusion protein) and L (Large protein, RNA dependent RNA polymerase) proteins. One silent mutation occurs with the NS2 gene ORF, and four (4) silent mutations with the G gene ORF. A summary of sequence differences is listed in Table 6.

Table 6: Amino acid sequence comparison between MRK RSV 287 and Passage 17

Nucleotide (nt) Position	**RSV gene - nt position**	**Gene amino acid position**	**Note**	**MRK RSV 287 amino acid**	**MRK RSV p17 amino acid**
954	NS2-327	109	Silent mutation	LYS	LYS
5295	G-610	204		LYS	GLU
5298	G-613	205		LYS	GLU
5315	G-630	210	Silent mutation	THR	THR
5316	G-631	211		THR	ALA
5322	G-637	213		LYS	GLU
5324	G-639				
5339	G-654	219	Silent mutation	GLN	GLN
5346	G-661	221		LYS	GLY
5347	G-662				
5351	G-666	222	Silent mutation	SER	SER
5352	G-667	223		LYS	GLY
5353	G-668				
5360	G-675	226	Silent mutation	VAL	VAL
5380	G-695	232		GLU	GLY
5381	G-696				

6538	F-880	294		GLU	LYS*[1]
7115	F-1457	486		ASP	GLY*[2]
8937	L-443	148		ASP	ALA*[3]
14656	L-6162	2054		LEU	PHE*[4]
15046	NTR	NA	5' untranslated region	NA	NA

* possible polymorphisms: 1=GLU/LYS, 2=ASP/GLY, 3=ASP/ALA, 4=LEU/PHE

4) Sequence alignment of MRK RSV p17 against MRK RSV p22 - Sequence comparisons between p17 and p22 found the two sequences to be identical. There was a possible difference in the polymorphism level at nucleotide position number 14656, but this was not been quantified.

5) Comparison of gene segments 2, 10, 11, 13, 14, 18, and 29 from MRK RSV strain 287 and passages 5, 10, 15, 17, 18 and 22 - Sequences were generated from selected gene segments of MRK RSV strain 287 passages p5, p10, p15 and p18. These sequences were compared to MRK RSV strain 287, as well as to MRK RSV Strain passage 17 and passage 22. Table 7 is a comparison of the sequences at the previously identified sites of the RSV p17 point mutations. It is evident from the data presented that the silent NS2 mutation was acquired by the 5th passage, while the G gene mutations were present by the 10th passage. Mutations in the F and L genes were beginning to appear by passage 10, and evidence of all point mutations is present at passage 17. The polymorphism at position 14656 is still evident, while the presence of other polymorphisms is in question. Table 7 summarizes the comparisons of the various passage levels analyzed.

Table 7:

Nucleotide Position	Strain 287	p5	p10	p15	p17	p18	p22
954	G	**A**	**A**	**A**	**A**	**A**	**A**
5295	A	A	**G**	**G**	**G**	**G**	**G**
5298	A	A	**G**	**G**	**G**	**G**	**G**
5315	A	A	**G**	**G**	**G**	**G**	**G**
5316	A	A	**G**	**G**	**G**	**G**	**G**
5322	A	A	**G**	**G**	**G**	**G**	**G**
5324	A	A	**G**	**G**	**G**	**G**	**G**
5339	A	A	**G**	**G**	**G**	**G**	**G**
5346	A	A	**G**	**G**	**G**	**G**	**G**
5347	A	A	**G**	**G**	**G**	**G**	**G**
5351	A	A	**G**	**G**	**G**	**G**	**G**
5352	A	A	**G**	**G**	**G**	**G**	**G**
5353	A	A	**G**	**G**	**G**	**G**	**G**
5360	A	A	**G**	**G**	**G**	**G**	**G**
5380	A	A	**G**	**G**	**G**	**G**	**G**
5381	A	A	**G**	**G**	**G**	**G**	**G**
6538	G	G	G	**A (R?)***	**A (R?)***	**A (R?)***	**A (R?)***
7115	A	A	**G (R?)***	**G (R?)***	**G (R?)***	**G (R?)***	**G (R?)***

8937	A	A	C (M?)*	C (M?)*	C (M?)*	C (M?)*	C (M?)*
14656	G	G	G	T (K?)*	T (K?)*	T (K?)*	T (K?)*
15046	A	no data	no data	no data	C	no data	C

* possible polymorphisms; R, M and K represent IUPAC codes for polymorphisms: R=A or G; K=G or T; M=A or C

EXAMPLE 3

Attenuation of MRK RSV 287 Passaged Strains

African Green monkey challenge study - All animals were prescreened for seroneutralizing antibody titers. Only those with titers ≤ 4 were used in the current studies. The monkeys were anesthetized using 10 mg/kg ketamine, intramuscularly, and challenged with two doses each of $10^{5.5}$ pfu of virus. The virus was administered by combined intranasal and intratracheal inoculation, 1 ml at each site per dose. Following challenge, nasopharyngeal swabs were collected daily from each monkey for 12 consecutive days, and bronchoalveolar lavage were collected at days 4, 5, 7, and 10. The nasopharyngeal samples were collected by gently rubbing 2-3 areas of the oropharynx region using a Darcon swab and placing the tips in a solution containing Hanks balanced salt solution (HBSS) containing 0.2 M Sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2PO_4 and 4.4 mM monosodium glutamate (SPG), and 0.1% gelatin. For bronchoalveolar lavage, approximately 5-7 ml HBSS was infused directly into the lung and aspirated via a sterile French catheter and syringe. Recovered samples were supplemented with 1/10 volume of 10x SPG and 1/10 volume of 1% gelatin, aliquoted and immediately stored frozen at -70°C.

Cotton rat challenge study - Four to 8-week old, female, cotton rats (*Sigmodon hispidus*) were inoculated intranasally with $10^{5.5}$ pfu of virus in 0.1 ml volume at day 0. Lung (left lobes) and nasal turbinates were removed four days post inoculation, homogenized in 10 volumes of Hanks Balanced Salt Solution (Walkersville, MD) containing SPG on ice. Samples were clarified by centrifugation at 2000 rpm for 10 minutes, aliquoted and immediately stored frozen at -70°C.

Viral titration - Virus titers were determined on Hep-2 cells. Briefly, test sample were made with serial dilutions. 0.1 ml of the sample was added to a well of 24-well plates containing confluent Hep-2 cells. Cells were incubated at 37°C for 1 hour. After incubation, the cells were washed once with PBS and overlaid with 0.5 ml of 1% agarose in MEM per well and incubated at the 37°C for 4 days. After incubation, the agarose overlays were removed and cells were stained with crystal violet and viral plaques were counted. Viral titers were expressed as the plaque forming units (pfu) per gram tissue.

Viral neutralization assay - For viral neutralization assay, all sera were heat-inactivated at 56°C for 30 minutes. Test sera were prepared in serial 2-fold dilutions in Eagle's Minimum Essential Medium (EMEM) and incubated. Wild-type A2 virus was as the target virus and was diluted in EMEM to the final titer of 10^3 pfu/ml. Equal volume of serum and the virus

were mixed and incubated at 37°C for 1 hour. After incubation, 0.1 ml of the virus was transferred to a well of 24-well plates, which was followed by the viral plaque assay as described above. The neutralizing titers were defined as the highest dilution which induced > 50% reduction of the viral plaques.

Results –

1) MRK 287 p17 is highly attenuated in cotton rats and African green monkeys - To evaluate *in vivo* replication properties, we compared p17 virus with its parental p3 virus and wt A2 strain in cotton rat and African green challenge models. The data from cotton rats and the non-human primate studies collectively suggest that p17 virus is highly attenuated.

In the cotton rat study, 6 animals per group were inoculated intranasally with $10^{5.5}$ pfu of a test virus; 4 days after challenge, nose and lung samples were collected and used for virus titration (Figure 5). The parental p3 virus was found to be fully replication competent, which yielded approximately 5 logs of the virus in both nose and lung tissues. The titers in the nose were comparable to that of wt A2 strain, while that in the lungs were approximately 1 log lower. In contrast, the replication of p17 virus was restricted. No virus was recovered in the nose based on the lower assay detection limit, 40 pfu per gram tissue, and lung virus was detected only in one out of the four animals. The viral titer of this animal was also low, i.e., 200 pfu per gram tissue. Compared with the parental p3 virus, the p17 had at least 4- and 2.5-log lower titers in the nose and lungs, respectively.

In the African green monkey study, four animals per group were inoculated with a total of 2 x $10^{5.5}$ pfu of virus, of which half was administered by intranasal inoculation and the other half by intratracheal inoculation. At various time points post challenge, nasopharyngeal swabs and lung lavage were collected and used for virus titration. The results are shown in Figure 6. Consistent with that in cotton rats, the parental p3 virus showed significant viral replication both in the nose and lungs, where the peak titers were around 10^4 and 10^5 pfu/ml in nose and lungs, respectively. In addition, its overall viral shedding profiles were rather similar to that of wt A2 virus, in that the titers peaked between days 4 and 7 and lasted around 10 days. In contrast, the p17 virus was only detected sporadically and with low titers. For example, nose virus was detected only in two monkeys on day 2 and in one monkey on day 8, and lung virus was detected in 1, 3 and 1 monkeys on days 2, 3 and 7, respectively. Among all the positive samples, the highest titer, which was from one of the day 2 nose samples, was 400 pfu per gram tissue.

To better understand the relationship between viral passage and *in vivo* replication, we also tested MRK 287 viruses at passages preceding p17 in the cotton rat model. Six animals per group were challenged with 10^5 pfu of virus and samples were harvested on day 4 post challenge. As shown in Figure 7, the virus at passage 5 (p5) already begins to show reduced replication, and those in passages 10 (p10) and 15 (p15) show further reduction in replication. The p15 virus seems to be as attenuated as the p17 virus described above (Figure 5).

No p17 virus was recovered in the lungs and while it was detected in the nasal samples, the titers were > 3 logs lower than that of the parental p3 virus.

2) Single intramuscular immunization with MRK287 p17 vaccine virus induces strong seroneutralizing (SN) antibody responses and confers significant protection against wt virus challenge in cotton rats and African green monkeys - Immunogenicity and protection studies were conducted in both cotton rats and African green monkeys. The data demonstrates that single systemic immunization with the attenuated vaccine strain MRK287 p17 is able to induce significant SN titers and confer protection against wt virus challenge in the animal models.

For the cotton rat study, animals of 4 per group were immunized intramuscularly with $10^{4.5}$ pfu of wt A2, MRK287 p3, or MRK287 p17 virus. Blood samples were collected at days 14, 28, and 56 and used for seroneutralizing (SN) antibody determination against wt A2 virus. At day 56, both the immunized animals and a group of naïve animals were challenged intranasally with $10^{5.5}$ pfu of wt A2 virus, and nose and lung tissues were collected at 4 days post challenge. Single intramuscular immunization with all three viruses induced significant SN titers, which reached peak level around day 28 and persisted till day 56 when the animals were challenged (Figure 8). The animals receiving the A2 virus exhibited higher SN titers than those receiving MRK 287 viruses. This could be due to the fact the SN was determined against A2 strain. The SN titers of the animals receiving the parent MRK287 p3 virus and those receiving p17 virus were comparable, although there was trend that the latter induced higher SN titers at days 28 and 56. Following challenge, there was no detectable virus from the lung tissues of all three immunized groups (Figure 9), indicating the vaccination provided a complete protection in the lower respiratory tract. With regard to nasal samples, animals receiving wt A2 had no detectable virus, and animals receiving either MRK287 p3 or p17 had very low titers, approximately 3 logs lower than that of the naïve animals.

The African green monkey study involved two groups, one experimental group and one naïve control group, with 4 animals each. For the experimental group, animals were immunized intramuscularly with $10^{5.5}$ pfu of MRK287 p17 virus at day 0. Both groups were challenged with 2 x $10^{5.5}$ pfu of wt A2 virus by intranasal and intratracheal inoculation at day 28. Nasal swabs and lung lavage were collected at day 7 post challenge for virus titration. Figure 10 shows the SN titers at day 28. The animals receiving the vaccine developed approximately mean SN titers of 1:128 (7 log2). Following challenge, native animals had approximately 3.5- and 5-log pfu/ml virus at day 7 in the nasal and lung samples, respectively (Figure 11). The animals that received the vaccine had about 1 log lower viral titers in the nose and 3 logs lower titers in the lungs than that in the naïve animals.

EXAMPLE 4

Plaque Purified p17

Plaque purification - The p17 virus passage was titrated for the purpose of isolating and harvesting individual plaques with the intent of identifying single (clonal), genetic populations of the passage 17 virus. The virus was titrated to target ≤10 plaques per well. The starting titer of the virus stock was $1.7x10^6$ pfu/mL, and the range of final dilutions run for assay were 1:10,000, 1:20,000, and 1:40,000. On day 7 post inoculation, plates were visualized both macroscopically and microscopically for singular plaques on the monolayer. Passage 17 virus produced two, distinct plaque morphologies, large (approximately 2mM in diameter) and small (≤ 1mM in diameter). For the initial isolation, ten small morphology (#1-10), and ten large morphology plaques (#11-20) were isolated. Isolation was performed by placing a 1mL sterile serological pipet through the agarose and circling the perimeter of the plaque to be isolated. By drawing up the area with suction, the material was then transferred to 1mL of RSV maintenance medium, dispersed and aliquotted 5x200uL. Plaque #1 and #11 were chosen for titration and immediate amplification in Vero tissue culture plates. The remainder of aliquots were frozen via liquid nitrogen and stored at -70°C. Secondary amplification consisted of titering plaques at 1:20, 1:200, and 1:2000 in Vero plates. Some plates received agarose overlay for creating a secondary population of plaques from the parent plaque, while others received liquid RSV maintenance medium overlay to create stocks of parent plaques #1 and #11 for subsequent sequence analysis. Out of secondary plaque assay of plaques #1 and #11, two plaques were isolated. These plaques were chosen due to their segregation from other plaques on the plate (thus reducing the likelihood of obtaining a mixed population) and their plaque morphology being similar to the parent. Two plaques each were isolated from parent plaque and labeled #1-1 and #1-2 (e.g., plaques isolated from plaque #11 received nomenclature of #11-1 and #11-2). Plaque #1-2 was chosen to titrate and amplify in a third round of plaque purification: 1x200ul of plaque #1-2 was titrated simultaneously for plaque purification and amplification to create additional stocks of #1-2 parent. Plaques isolated from #1-2 were labeled #1-2.1, #1-2.2, #1-2.3, #1-2.4, #1.2-5, and #1-2.6. Sequence analysis performed on isolates halted after analysis of #1-2.1 showed a clonal population.

Amplification of plaque #1-2.1 for virus stocks - Growth of virus stocks followed standard procedure, outlined previously. 200ul of original plaque was amplified in Vero cell, 12-well cultures. Stock from first amplification (pp1) were used to inoculate Vero T150 cultures; harvested material (pp2) was amplified one additional time to create a large volume (about 1L) of material for experimental use. Aliquots of #1-2.1 taken from each scale up were provided for sequence analysis. PP3 material was utilized for use in cotton rat studies.

Cotton rat challenge study - Four, 4-8 week old, female, cotton rats (*Sigmodon hispidus*) were inoculated intranasally, under isoflurane anesthesia with 10^5 pfu of virus in 0.1ml volume at day 0. Lung (left lobes) and nasal turbinates were removed four days post inoculation,

homogenized in 10 volumes of Hanks Balanced Salt Solution (Walkersville, MD) containing SPG on ice. Samples were clarified by centrifugation at 2000 rpm for 10 minutes, aliquoted and immediately stored frozen at -70°C. The viruses include: (1) MRK287 p22, (2) MRK287 p17, (3) MRK287 p17 plaque purified, (4) MRK287 p15, (5) MRK287 p10, (6) MRK287 p5, (7) MRK287 p3, and (8) RSV A2 wild-type. Nose and lung homogenates were titrated in Vero cells and were expressed as plaque forming units (pfu) per gram tissue.

Results – MRK287 passage 17 (p17) was plaque purified to a clonal population ("p17_pp") and sequenced as described in Example 2. One additional nucleotide mutation was seen in the plaque purified virus, located at nucleotide position 260 within the RSV genome, corresponding to nucleotide position 162 within the gene coding for the NS1 protein. This is a silent mutation (*i.e.*, does not result in an amino acid mutation). SEQ ID NO: 87 corresponds to the wild-type gene sequence encoding NS1 (e.g., S2 strain). SEQ ID NO: 89 corresponds to the nucleotide sequence encoding NS1 within MRK287 and p17. SEQ ID NO: 90 corresponds to the nucleotide sequence encoding NS1 within p17_pp. Tables 8 and 9 compare the mutations in MRK287 p17 and the plaque purified version (p17_pp). SEQ ID NO: 88 corresponds to the amino acid sequence of NS1 (which is the same for the wt S2 virus, MRK287, MRK287 p17 and MRK287 p17_pp).

Table 8: Sequence differences between MRK RSV Strain 287, p17 and p17_pp

Nucleotide Position	MRK RSV 287	p17	p17_pp
260	T	T	A
954	G	A	A
5295	A	G	G
5298	A	G	G
5315	A	G	G
5316	A	G	G
5322	A	G	G
5324	A	G	G
5339	A	G	G
5346	A	G	G
5347	A	G	G
5351	A	G	G
5352	A	G	G
5353	A	G	G
5360	A	G	G
5380	A	G	G
5381	A	G	G
6538	G	A (R?) *	A

14656	L-6162	2054		LEU	PHE*[4]	PHE
15046	NTR	NA	5' untranslated region	NA	NA	NA

* possible polymorphisms: 1=GLU/LYS, 2=ASP/GLY, 3=ASP/ALA, 4=LEU/PHE

The mean titers of the 4 individual animals in the cotton rat challenge study, and the lower and upper confidence intervals (CI), for each animal inoculated with (1) MRK287 p22, (2) MRK287 p17, (3) MRK287 p17 plaque purified, (4) MRK287 p15, (5) MRK287 p10, (6) MRK287 p5, (7) MRK287 p3, or (8) RSV A2 wild-type are shown in Table 10. The virus titers of RSV A2 wild-type and MRK287 p3 have above 4 log pfu/g tissue. MRK287 p5 lung samples still have approximately 4 logs virus pfu/g. In contrast, the virus at and after passage 10, including the plaque purified MRK287 p17, show a reduction by more than 2 logs from the wild -type strains, in both lung and nose samples. Furthermore, these data confirm that plaque purified MRK287 p17 is as attenuated as MRK287 p17.

Table 10: Viral titers (log pfu/gram tissue)

Virus	**Nose***			**Lung***		
		Lower	Upper		Lower	Upper
	Mean	95% CI	95% CI	Mean	95% CI	95% CI
RSV A2 wt	**4.77**	4.72	4.82	**4.65**	4.45	4.84
MRK287 P3	**4.09**	3.52	4.66	**4.19**	3.64	4.73
MRK287 P5	***nd***	-	-	**4.08**	3.90	4.26
MRK287 P10	**2.51**	2.07	2.94	**2.19**	1.65	2.73
MRK287 P15	**1.60**	1.60	1.60	**2.00**	2.00	2.00
MRK287 P17	**1.60**	1.60	1.60	**2.08**	1.87	2.28
MRK287 P17 plaque purified	**2.11**	1.56	2.66	**2.32**	1.43	3.21
MRK287 P22	**1.90**	1.31	2.49	**2.91**	2.01	3.81

* Detection limit was 1.6 pfu/g for nose and 2.0 pfu/g for lung samples

7115	A	G (R?) *	G
8937	A	C (M?) *	C
14656	G	T (K?) *	T
15046	A	C	TBD

* possible polymorphisms; R, M and K represent IUPAC codes for polymorphisms: R=A or G; K=G or T; M=A or C

Table 9: Amino acid sequence comparison between MRK RSV 287, p17 and p17_pp

Nucleotide (nt) Position	**RSV gene - nt position**	**Gene amino acid position**	**Note**	**MRK RSV 287 amino acid**	**p17 amino acid**	**p17_pp amino acid**
260	NS1-162	54	Silent mutation	ILE	ILE	ILE
954	NS2-327	109	Silent mutation	LYS	LYS	LYS
5295	G-610	204		LYS	GLU	GLU
5298	G-613	205		LYS	GLU	GLU
5315	G-630	210	Silent mutation	THR	THR	THR
5316	G-631	211		THR	ALA	ALA
5322	G-637	213		LYS	GLU	GLU
5324	G-639					
5339	G-654	219	Silent mutation	GLN	GLN	GLN
5346	G-661	221		LYS	GLY	GLY
5347	G-662					
5351	G-666	222	Silent mutation	SER	SER	SER
5352	G-667	223		LYS	GLY	GLY
5353	G-668					

5360	G-675	226	Silent mutation	VAL	VAL	VAL
5380	G-695	232		GLU	GLY	GLY
5381	G-696					
6538	F-880	294		GLU	LYS*[1]	LYS
7115	F-1457	486		ASP	GLY*[2]	GLY
8937	L-443	148		ASP	ALA*[3]	ALA

14656	L-6162	2054		LEU	PHE*[4]	PHE
15046	NTR	NA	5' untranslated region	NA	NA	NA

* possible polymorphisms: 1=GLU/LYS, 2=ASP/GLY, 3=ASP/ALA, 4=LEU/PHE

The mean titers of the 4 individual animals in the cotton rat challenge study, and the lower and upper confidence intervals (CI), for each animal inoculated with (1) MRK287 p22, (2) MRK287 p17, (3) MRK287 p17 plaque purified, (4) MRK287 p15, (5) MRK287 p10, (6) MRK287 p5, (7) MRK287 p3, or (8) RSV A2 wild-type are shown in Table 10. The virus titers of RSV A2 wild-type and MRK287 p3 have above 4 log pfu/g tissue. MRK287 p5 lung samples still have approximately 4 logs virus pfu/g. In contrast, the virus at and after passage 10, including the plaque purified MRK287 p17, show a reduction by more than 2 logs from the wild -type strains, in both lung and nose samples. Furthermore, these data confirm that plaque purified MRK287 p17 is as attenuated as MRK287 p17.

Table 10: Viral titers (log pfu/gram tissue)

Virus	**Nose***			**Lung***		
		Lower	Upper		Lower	Upper
	Mean	95% CI	95% CI	Mean	95% CI	95% CI
RSV A2 wt	**4.77**	4.72	4.82	**4.65**	4.45	4.84
MRK287 P3	**4.09**	3.52	4.66	**4.19**	3.64	4.73
MRK287 P5	***nd***	-	-	**4.08**	3.90	4.26
MRK287 P10	**2.51**	2.07	2.94	**2.19**	1.65	2.73
MRK287 P15	**1.60**	1.60	1.60	**2.00**	2.00	2.00
MRK287 P17	**1.60**	1.60	1.60	**2.08**	1.87	2.28
MRK287 P17 plaque purified	**2.11**	1.56	2.66	**2.32**	1.43	3.21
MRK287 P22	**1.90**	1.31	2.49	**2.91**	2.01	3.81

* Detection limit was 1.6 pfu/g for nose and 2.0 pfu/g for lung samples

Claims:

1. A live, attenuated respiratory syncytial virus (RSV) comprising a viral genome, wherein the viral genome encodes proteins that comprise a glutamic acid at position 204 of the protein encoded by the G gene; a glutamic acid at position 205 of the protein encoded by the G gene; an alanine at position 211 of the protein encoded by the G gene; a glutamic acid at position 213 of the protein encoded by the G gene; a glycine at position 221 of the protein encoded by the G gene; a glycine at position 223 of the protein encoded by the G gene; a glycine at position 232 of the protein encoded by the G gene; a glycine at position 486 of the protein encoded by the F gene; and an alanine at position 148 of the protein encoded by the L gene.

2. The attenuated RSV of claim 1, wherein the viral genome encodes proteins that further comprise a lysine at position 294 of the protein encoded by the F gene and a phenylalanine at position 2054 of the protein encoded by the L gene.

3. The attenuated RSV of claim 1 to 2, wherein the proteins encoded by the G, F and L genes comprise amino acid sequences that are at least 95% identical to the is amino acid sequences as set forth in SEQ ID NO: 12, SEQ ID NO: 18 and SEQ ID NO: 24, respectively.

4. The attenuated RSV of claim 3, wherein the proteins encoded by the G, F, and L genes consist of the amino acid sequences as set forth in SEQ ID NO: 12, SEQ ID NO: 18 and SEQ ID NO: 24, respectively.

5. The attenuated RSV of any one of the preceding claims, wherein the viral genome further comprises one or more nucleotides selected from the group consisting an adenine at nucleotide position 162 of the NSI gene, an adenine at nucleotide position 327 of the NS2 gene, a guanine at nucleotide position 630 of the G gene, a guanine at nucleotide position 654 of the G gene, a guanine at nucleotide position 666 of 25 the G gene, and a guanine at nucleotide position 675 of the G gene.

6. The attenuated RSV of claim 5, wherein the viral genome comprises an adenine at nucleotide position 162 of the NS1 gene, an adenine at nucleotide position 327 of the NS2 gene, a guanine at nucleotide position 630 of the G gene, a guanine at nucleotide position 654 of the G gene, a guanine at nucleotide position 666 of the G gene, 30 and a guanine at nucleotide position 675 of the G gene.

7. The attenuated RSV of claim 6, wherein the viral genome comprises NS1, NS2, G, F and L genes that are at least 95% identical to the nucleotide sequences as set forth in SEQ ID NO: 90, SEQ ID NO: 5, SEQ ID NO: 11, SEQ ID NO: 17, and SEQ ID NO: 23, respectively.

8. The attenuated RSV of claim 7, wherein the viral genome comprises NS1, NS2, G, F, and L genes consisting of the nucleotide sequences as set forth in SEQ ID NO: 90, SEQ ID NO: 5, SEQ ID NO: 11, SEQ ID NO: 17, and SEQ ID NO: 23, respectively.

9. An immunogenic composition comprising the live, attenuated RSV of any one of claims 1 to 8 and a pharmaceutically acceptable carrier.

10. A nucleic acid molecule comprising a genomic or antigenomic sequence encoding the live, attenuated RSV of any one of claims 1 to 8.

11. The nucleic acid molecule of claim 10, wherein said nucleic acid molecule is an expression vector.

12. A recombinant cell comprising the expression vector of claim 11.

13. A live, attenuated respiratory syncytial virus (RSV) population that comprises the attenuated RSV of any one of claims 1 to 8.

14. An immunogenic composition comprising the live, attenuated RSV population of claim 13 and a pharmaceutically acceptable carrier.

15. A method of producing a protective immune response in a subject against RSV infection comprising the step of administering to said subject an immunologically effective amount of one or more of the following:

(a) the attenuated RSV of any one of claims 1 to 8;

(b) the attenuated RSV population of claim 13;

(c) the immunogenic composition of claim 9; or,

(d) the immunogenic composition of claim 14.

16. The method of claim 15, wherein said subject is a human.

17. Use of an immunologically effective amount one or more of the following:

(a) the attenuated RSV of any one of claims 1 to 8;

(b) the attenuated RSV population of claim 13;

(c) the immunogenic composition of claim 9; or,

(d) the immunogenic composition of claim 14.

in the manufacture of a medicament for inducing a protective immune response in a patient against RSV infection.

18. The use of claim 17, wherein the patient is a human.

19. A live, attenuated respiratory virus as defined in claim 1 and substantially as hereinbefore described with reference to any one of the Examples.

Dated 17 January 2013
Merck Sharp & Dohme Corp.

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

```
                  1                                                  40
     S2_NS2   (1) MDTTHNDTTPQRLMITDMRPLSLETIIISLTRDIITHRFI
MRK_287_NS2   (1) MDTTHNDTTPQRLMITDMRPLSLETIITSLTRDIITHKFI
     P17_NS2  (1) MDTTHNDTTPQRLMITDMRPLSLETIITSLTRDIITHKFI

                  41                                                 80
     S2_NS2  (41) YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK
MRK_287_NS2  (41) YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK
     P17_NS2  (41) YLINHECIVRKLDERQATFTFLVNYEMKLLHKVGSTKYKK

                  81                                                120
     S2_NS2  (81) YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY
MRK_287_NS2  (81) YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY
     P17_NS2  (81) YTEYNTKYGTFPMPIFINHDGFLECIGIKPTKHTPIIYKY

                  121
     S2_NS2 (121) DLNP- (SEQ ID NO: 2)
MRK_287_NS2 (121) DLNP- (SEQ ID NO: 4)
     P17_NS2 (121) DLNP- (SEQ ID NO: 6)
```

FIG.1

```
                 1                                                  40
     S2_G   (1) MSKNKDQRTAKTLEKTWDTLNHLLFISSCLYKLNLKSIAQ
MRK_287_G   (1) MSKNKDQRTAKTLERTWDTLNHLLFISSCLYKLNLKSVAQ
    P17_G   (1) MSKNKDQRTAKTLERTWDTLNHLLFISSCLYKLNLKSVAQ

                41                                                  80
     S2_G  (41) ITLSILAMIISTSLIIAAIIFIASANHKVTLTTAIIQDAT
MRK_287_G  (41) ITLSILAMIISTSLIIAAIIFIASANHKVTSTTTIIQDAT
    P17_G  (41) ITLSILAMIISTSLIIAAIIFIASANHKVTSTTTIIQDAT

                81                                                 120
     S2_G  (81) SQIKNTTPTYLTQNPQLGISFSNLSETTSQTTTILASTTP
MRK_287_G  (81) SQIKNTTPTYLTQSPQLGISPSNPSEITSQITTILASTTP
    P17_G  (81) SQIKNTTPTYLTQSPQLGISPSNPSEITSQITTILASTTP

               121                                                 160
     S2_G (121) SVKSTLQSTTVKTKNTTTTKIQPSKPTTKQRQNKPPNKPN
MRK_287_G (121) GVKSTLQSTTVGTKNTTTTQAQPSKPTTKQRQNKPPSKPN
    P17_G (121) GVKSTLQSTTVGTKNTTTTQAQPSKPTTKQRQNKPPSKPN

               161                                                 200
     S2_G (161) NDFHFEVFNFVPCSICSNNPTCWAICKRIPNKKPGKKTTT
MRK_287_G (161) NDFHFEVFNFVPCSICSNNPTCWAICKRIPNKKPGKKTTT
    P17_G (161) NDFHFEVFNFVPCSICSNNPTCWAICKRIPNKKPGKKTTT

               201                                                 240
     S2_G (201) KPTKKPTIKTTKKDLKPQTTKPKEVPTTKPTEKPTINTTK
MRK_287_G (201) KPTKKPTFKTTKKDPKPQTTKSKEVPTTKPTEEPTINTTK
    P17_G (201) KPTEEPTFKTAKEDPKPQTTGSGEVPTTKPTGEPTINTTK

               241                                                 280
     S2_G (241) TNIRTTLLTNNTTGNPEHTSQKGTLHSTSSDGNPSPSQVY
MRK_287_G (241) TNITTTLLTSNTTRNPELTSQMETFHSTSSEGNPSPSQVS
    P17_G (241) TNITTTLLTSNTTRNPELTSQMETFHSTSSEGNPSPSQVS

               281             299
     S2_G (281) TTSEYLSQPPSPSNTTNQ- (SEQ ID NO: 8)
MRK_287_G (281) ITSEYLSQPSSPPNTPR-  (SEQ ID NO: 10)
    P17_G (281) ITSEYLSQPSSPPNTPR-- (SEQ ID NO: 12)
```

FIG.2

```
                    1                                                  40
     S2_F   (1) MELPILKTNAITAILAAVTLCFASSQNITEEFYQSTCSAV
MRK_287_F   (1) MELPILKANAITTILTAVTFCFASSQNITEEFYQSTCSAV
    P17_F   (1) MELPILKANAITTILTAVTFCFASSQNITEEFYQSTCSAV

                   41                                                  80
     S2_F  (41) SKGYLSALRTGWYTSVITIELSNIKENKCNGTDAKVKLIK
MRK_287_F  (41) SKGYLSALRTGWYTSVITIELSNIKENKCNGTDAKVKLIK
    P17_F  (41) SKGYLSALRTGWYTSVITIELSNIKENKCNGTDAKVKLIK

                   81                                                 120
     S2_F  (81) QELDKYKSAVTELQLLMQSTPATNNRARRELPRFMNYTLN
MRK_287_F  (81) QELDKYKNAVTELQLLMQSTPAANNRARRELPRFMNYTLN
    P17_F  (81) QELDKYKNAVTELQLLMQSTPAANNRARRELPRFMNYTLN

                  121                                                 160
     S2_F (121) NTKNTNVTLSKKRKRRFLGFLLGVGSAIASGIAVSKVLHL
MRK_287_F (121) NAKKTNVTLSKKRKRRFLGFLLGVGSAIASGIAVSKVLHL
    P17_F (121) NAKKTNVTLSKKRKRRFLGFLLGVGSAIASGIAVSKVLHL

                  161                                                 200
     S2_F (161) EGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYID
MRK_287_F (161) EGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYID
    P17_F (161) EGEVNKIKSALLSTNKAVVSLSNGVSVLTSKVLDLKNYID

                  201                                                 240
     S2_F (201) KQLLPIVNKQSCSISNIETVIEFQQKNNRLLEITREFSVN
MRK_287_F (201) KQLLPIVNKQSCSISNIETVIEFQQKNNRLLEITREFSVN
    P17_F (201) KQLLPIVNKQSCSISNIETVIEFQQKNNRLLEITREFSVN

                  241                                                 280
     S2_F (241) AGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI
MRK_287_F (241) AGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI
    P17_F (241) AGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQI

                  281                                                 320
     S2_F (281) VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSP
MRK_287_F (281) VRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKLHTSP
    P17_F (281) VRQQSYSIMSIIKXEVLAYVVQLPLYGVIDTPCWKLHTSP
```

FIG.3A

```
                 321                                                 360
     S2_F (321) LCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPLAETCKV
MRK_287_F (321) LCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKV
    P17_F (321) LCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKV

                 361                                                 400
     S2_F (361) QSNRVFCDTMNSLTLPSEVNLCNIDIFNPKYDCKIMTSKT
MRK_287_F (361) QSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKT
    P17_F (361) QSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKT

                 401                                                 440
     S2_F (401) DVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCD
MRK_287_F (401) DVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCD
    P17_F (401) DVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCD

                 441                                                 480
     S2_F (441) YVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDP
MRK_287_F (441) YVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDP
    P17_F (441) YVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDP

                 481                                                 520
     S2_F (481) LVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGK
MRK_287_F (481) LVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGK
    P17_F (481) LVFPSXEFDASISQVNEKINQSLAFIRKSDELLHNVNAGK

                 521                                                 560
     S2_F (521) STTNIMITTIIIVIIVILLSLIAVGLLLYCKARSTPVTLS
MRK_287_F (521) STTNIMITAIIIVIVVILLSLIAVGLLLYCKARSTPVTLS
    P17_F (521) STTNIMITAIIIVIVVILLSLIAVGLLLYCKARSTPVTLS

                 561         575
     S2_F (561) KDQLSGINNIAFSN- (SEQ ID NO: 14)
MRK_287_F (561) KDQLSGINNIAFSN- (SEQ ID NO: 16)
    P17_F (561) KDQLSGINNIAFSN- (SEQ ID NO: 18)
```

FIG.3B

```
                    1                                                  40
     S2_L    (1) MDPIINGNSANVYLTDSYLKGVISFSECNALGSYIFNGPY
MRK_287_L    (1) MDPIINGNSANVYLTDSYLKGVISFSECNALGSYIFNGPY
     P17_L   (1) MDPIINGNSANVYLTDSYLKGVISFSECNALGSYIFNGPY

                   41                                                  80
     S2_L   (41) LKNDYTNLISRQNPLIEHINLKKLNITQSLISKYHKGEIK
MRK_287_L   (41) LKNDYTNLISRQNPLIEHMNLKKLNITQSLISKYHKGEIK
     P17_L  (41) LKNDYTNLISRQNPLIEHMNLKKLNITQSLISKYHKGEIK

                   81                                                 120
     S2_L   (81) IEEPTYFQSLLMTYKSMTSLEQITTTNLLKKIIRRAIEIS
MRK_287_L   (81) LEEPTYFQSLLMTYKSMTSSEQIATTNLLKKIIRRAIEIS
     P17_L  (81) LEEPTYFQSLLMTYKSMTSSEQIATTNLLKKIIRRAIEIS

                  121                                                 160
     S2_L  (121) DVKVYAILNKLGLKEKDKIKSNNGQDEDNSVITTIIKDDI
MRK_287_L  (121) DVKVYAILNKLGLKEKDKIKSNNGQDEDNSVITTIIKDDI
     P17_L (121) DVKVYAILNKLGLKEKDKIKSNNGQDEXNSVITTIIKDDI

                  161                                                 200
     S2_L  (161) LLAVKDNQSHLKAVKNHSTKQKDTIKTTLLKKLMCSMQHP
MRK_287_L  (161) LSAVKDNQSHLKAGKNHSTKQKDTIKTTLLKKLMCSMQHP
     P17_L (161) LSAVKDNQSHLKAGKNHSTKQKDTIKTTLLKKLMCSMQHP

                  201                                                 240
     S2_L  (201) PSWLIHWFNLYTKLNNILTQYRSSEVKNHGFILIDNHTLN
MRK_287_L  (201) PSWLIHWFNLYTKLNNILTQYRSNEVKNHGFTLIDNQTLS
     P17_L (201) PSWLIHWFNLYTKLNNILTQYRSNEVKNHGFTLIDNQTLS

                  241                                                 280
     S2_L  (241) GFQFILNQYGCIVYHKELKRITVTTYNQFLTWKDISLSRL
MRK_287_L  (241) GFQFILNQYGCIVYNKELKRITVTTYNQFLTWKDISLSRL
     P17_L (241) GFQFILNQYGCIVYNKELKRITVTTYNQFLTWKDISLSRL

                  281                                                 320
     S2_L  (281) NVCLITWISNCLNTLNKSLGLRCGFNNVILTQLFLYGDCI
MRK_287_L  (281) NVCLITWISNCLNTLNKSLGLRCGFNNVILTQLFLYGDCI
     P17_L (281) NVCLITWISNCLNTLNKSLGLRCGFNNVILTQLFLYGDCI
```

FIG.4A

```
                  321                                                 360
     S2_L (321) LKLFHNEGFYIIKEVEGFIMSLILNITEEDQFRKRFYNSM
MRK_287_L (321) LKLFHNEGFYIIKEVEGFIMSLILNITEEDQFRKRFYNSM
    P17_L (321) LKLFHNEGFYIIKEVEGFIMSLILNITEEDQFRKRFYNSM

                  361                                                 400
     S2_L (361) LNNITDAANKAQKSLLSRVCHTLLDKTVSDNIINGRWIIL
MRK_287_L (361) LNNITDAANKAQKNLLSRVCHTLLDKTVSDNIINGRWIIL
    P17_L (361) LNNITDAANKAQKNLLSRVCHTLLDKTVSDNIINGRWIIL

                  401                                                 440
     S2_L (401) LSKFLKLIKLAGDNNLNNLSELYFLFRIFGHPMVDERQAM
MRK_287_L (401) LSKFLKLIKLAGDNNLNNLSELYFLFRIFGHPMVDERQAM
    P17_L (401) LSKFLKLIKLAGDNNLNNLSELYFLFRIFGHPMVDERQAM

                  441                                                 480
     S2_L (441) DAVKVNCNETKFYLLSSLSMLRGAFIYRIIKGFVNNYNRW
MRK_287_L (441) DAVKINCNETKFYLLSSLSMLRGAFIYRIIKGFVNNYNRW
    P17_L (441) DAVKINCNETKFYLLSSLSMLRGAFIYRIIKGFVNNYNRW

                  481                                                 520
     S2_L (481) PTLRNAIVLPLRWLTYYKLNTYPSLLELTERDLIVLSGLR
MRK_287_L (481) PTLRNAIVLPLRWLTYYKLNTYPSLLELTERDLIVLSGLR
    P17_L (481) PTLRNAIVLPLRWLTYYKLNTYPSLLELTERDLIVLSGLR

                  521                                                 560
     S2_L (521) FYREFRLPKKVDLEMIINDKAISPPKNLIWTSFPRNYMPS
MRK_287_L (521) FYREFRLPKKVDLEMIINDKAISPPKNLIWTSFPRNYMPS
    P17_L (521) FYREFRLPKKVDLEMIINDKAISPPKNLIWTSFPRNYMPS

                  561                                                 600
     S2_L (561) HIQNYIEHEKLKFSESDKSRRVLEYYLRDNKFNECDLYNC
MRK_287_L (561) HIQNYIEHEKLKFSESDKSRRVLEYYLRDNKFNECDLYNC
    P17_L (561) HIQNYIEHEKLKFSESDKSRRVLEYYLRDNKFNECDLYNC

                  601                                                 640
     S2_L (601) VVNQSYLNNPNHVVSLTGKERELSVGRMFAMQPGMFRQVQ
MRK_287_L (601) VVDQSYLNNPNHVVSLTGKERELSVGRMFAMQPGMFRQVQ
    P17_L (601) VVDQSYLNNPNHVVSLTGKERELSVGRMFAMQPGMFRQVQ
```

FIG.4B

```
                641                                                 680
     S2_L (641) ILAEKMIAENILQFFPESLTRYGDLELQKILELKAGISNK
MRK_287_L (641) ILAEKMIAENILQFFPESLTRYGDLELQKILELKAGISNK
    P17_L (641) ILAEKMIAENILQFFPESLTRYGDLELQKILELKAGISNK

                681                                                 720
     S2_L (681) SNRYNDNYNNYISKCSIITDLSKFNQAFRYETSCICSDVL
MRK_287_L (681) SNRYNDNYNNYISKCSIITDLSKFNQAFRYETSCICSDVL
    P17_L (681) SNRYNDNYNNYISKCSIITDLSKFNQAFRYETSCICSDVL

                721                                                 760
     S2_L (721) DELHGVQSLFSWLHLTIPHVTIICTYRHAPPYIRDHIVDL
MRK_287_L (721) DELHGVQSLFSWLHLTIPHVTIICTYRHAPPYIGDHIVDL
    P17_L (721) DELHGVQSLFSWLHLTIPHVTIICTYRHAPPYIGDHIVDL

                761                                                 800
     S2_L (761) NNVDEQSGLYRYHMGGIEGWCQKLWTIEAISLLDLISLKG
MRK_287_L (761) NNVDEQSGLYRYHMGGIEGWCQKLWTIEAISLLDLISLKG
    P17_L (761) NNVDEQSGLYRYHMGGIEGWCQKLWTIEAISLLDLISLKG

                801                                                 840
     S2_L (801) KFSITALINGDNQSIDISKPVRLMEGQTHAQADYLLALNS
MRK_287_L (801) KFSITALINGDNQSIDISKPIRLMEGQTHAQADYLLALNS
    P17_L (801) KFSITALINGDNQSIDISKPIRLMEGQTHAQADYLLALNS

                841                                                 880
     S2_L (841) LKLLYKEYAGIGHKLKGTETYISRDMQFMSKTIQHNGVYY
MRK_287_L (841) LKLLYKEYAGIGHKLKGTETYISRDMQFMSKTIQHNGVYY
    P17_L (841) LKLLYKEYAGIGHKLKGTETYISRDMQFMSKTIQHNGVYY

                881                                                 920
     S2_L (881) PASIKKVLRVGPWINTILDDFKVSLESIGSLTQELEYRGE
MRK_287_L (881) PASIKKVLRVGPWINTILDDFKVSLESIGSLTQELEYRGE
    P17_L (881) PASIKKVLRVGPWINTILDDFKVSLESIGSLTQELEYRGE

                921                                                 960
     S2_L (921) SLLCSLIFRNVWLYNQIALQLKNHALCNNKLYLDILKVLK
MRK_287_L (921) SLLCSLIFRNVWLYNQIALQLKNHALCNNKLYLDILKVLK
    P17_L (921) SLLCSLIFRNVWLYNQIALQLKNHALCNNKLYLDILKVLK
```

FIG.4C

```
                961                                                1000
S2_L      (961) HLKTFFNLDNIDTALTLYMNLPMLFGGGDPNLLYRSFYRR
MRK_287_L (961) HLKTFFNLDNIDTALTLYMNLPMLFGGGDPNLLYRSFYRR
P17_L     (961) HLKTFFNLDNIDTALTLYMNLPMLFGGGDPNLLYRSFYRR

               1001                                               1040
S2_L     (1001) TPDFLTEAIVHSVFILSYYTNHDLKDKLQDLSDDRLNKFL
MRK_287_L (1001) TPDFLTEAIVHSVFILSYYTNHDLKDKLQDLSDDRLNKFL
P17_L    (1001) TPDFLTEAIVHSVFILSYYTNHDLKDKLQDLSDDRLNKFL

               1041                                               1080
S2_L     (1041) TCIITFDKNPNAEFVTLMRDPQALGSERQAKITSEINRLA
MRK_287_L (1041) TCIITFDKNPNAEFVTLMRDPQALGSERQAKITSEINRLA
P17_L    (1041) TCIITFDKNPNAEFVTLMRDPQALGSERQAKITSEINRLA

               1081                                               1120
S2_L     (1081) VTEVLSTAPNKIFSKSAQHYTTTEIDLNDIMQNIEPTYPH
MRK_287_L (1081) VTEVLSTAPNKIFSKSAQHYTTTEIDLNDIMQNIEPTYPH
P17_L    (1081) VTEVLSTAPNKIFSKSAQHYTTTEIDLNDIMQNIEPTYPH

               1121                                               1160
S2_L     (1121) GLRVVYESLPFYKAEKIVNLISGTKSITNILEKTSAIDLT
MRK_287_L (1121) GLRVVYESLPFYKAEKIVNLISGTKSITNILEKTSAIDLT
P17_L    (1121) GLRVVYESLPFYKAEKIVNLISGTKSITNILEKTSAIDLT

               1161                                               1200
S2_L     (1161) DIDRATEMMRKNITLLIRIFPLDCNRDKREILSMENLSIT
MRK_287_L (1161) DIDRATEMMRKNITLLIRILPLDCNRDKREILSMENLSIT
P17_L    (1161) DIDRATEMMRKNITLLIRILPLDCNRDKREILSMENLSIT

               1201                                               1240
S2_L     (1201) ELSKYVRERSWSLSNIVGVTSPSIMYTMDIKYTTSTIASG
MRK_287_L (1201) ELSKYVRERSWSLFNIVGVTSPSIMYTMDIKYTTSTIASG
P17_L    (1201) ELSKYVRERSWSLFNIVGVTSPSIMYTMDIKYTTSTIASG

               1241                                               1280
S2_L     (1241) IIIEKYNVNSLTRGERGPTKPWVGSSTQEKKTMPVYNRQV
MRK_287_L (1241) IIIEKYNVNSLTRGERGPTKPWVGSSTQEKKTMPVYNRQV
P17_L    (1241) IIIEKYNVNSLTRGERGPTKPWVGSSTQEKKTMPVYNRQV
```

FIG.4D

```
                  1281                                                1320
     S2_L (1281) LTKKQRDQIDLLAKLDWVYASIDNKDEFMEELSIGTLGLT
MRK_287_L (1281) LTKKQRDQIDLLAKLDWVYASIDNKDEFMEELSIGTLGLT
    P17_L (1281) LTKKQRDQIDLLAKLDWVYASIDNKDEFMEELSIGTLGLT

                  1321                                                1360
     S2_L (1321) YEKAKKLFPQYLSVNYLHRLTVSSRPCEFPASIPAYRTTN
MRK_287_L (1321) YEKAKKLFPQYLSVNYLHRLTVSSRPCEFPASIPAYRTTN
    P17_L (1321) YEKAKKLFPQYLSVNYLHRLTVSSRPCEFPASIPAYRTTN

                  1361                                                1400
     S2_L (1361) YHFDTSPINRILTEKYGDEDIDIVFQNCISFGLSLMSVVE
MRK_287_L (1361) YHFDTSPINRILTEKYGDEDIDIVFQNCISFGLSLMSVVE
    P17_L (1361) YHFDTSPINRILTEKYGDEDIDIVFQNCISFGLSLMSVVE

                  1401                                                1440
     S2_L (1401) QFTNVCPNRIILIPKLNEIHLMKPPIFTGDVDIHKLKQVI
MRK_287_L (1401) QFTNVCPNRIILIPKLNEIHLMKPPIFTGDVDIHKLKQVI
    P17_L (1401) QFTNVCPNRIILIPKLNEIHLMKPPIFTGDVDIHKLKQVI

                  1441                                                1480
     S2_L (1441) QKQHMFLPDKISLTQYVELFLSNKTLKSGSHVNSNLILAH
MRK_287_L (1441) QKQHMFLPDKISLTQYVELFLSNKTLKSGSHVNSNLILAH
    P17_L (1441) QKQHMFLPDKISLTQYVELFLSNKTLKSGSHVNSNLILAH

                  1481                                                1520
     S2_L (1481) KISDYFHNTYILSTNLAGHWILIIQLMKDSKGIFEKDWGE
MRK_287_L (1481) KISDYFHNTYILSTNLAGHWILIIQLMKDSKGIFEKDWGE
    P17_L (1481) KISDYFHNTYILSTNLAGHWILIIQLMKDSKGIFEKDWGE

                  1521                                                1560
     S2_L (1521) GYITDHMFINLKVFFNAYKTYLLCFHKGYGRAKLECDMNT
MRK_287_L (1521) GYITDHMFINLKVFFNAYKTYLLCFHKGYGKAKLECDMNT
    P17_L (1521) GYITDHMFINLKVFFNAYKTYLLCFHKGYGKAKLECDMNT

                  1561                                                1600
     S2_L (1561) SDLLCVLELIDSSYWKSMSKVFLEQKVIKYILSQDASLHR
MRK_287_L (1561) SDLLCVLELIDSSYWKSMSKVFLEQKVIKYILSQDASLHR
    P17_L (1561) SDLLCVLELIDSSYWKSMSKVFLEQKVIKYILSQDASLHR
```

FIG.4E

```
                1601                                                1640
    S2_L (1601) VKGCHSFKLWFLKRLNVAEFTVCPWVVNIDYHPTHMKAIL
MRK_287_L (1601) VKGCHSFKLWFLKRLNVAEFTVCPWVVNIDYHPTHMKAIL
    P17_L (1601) VKGCHSFKLWFLKRLNVAEFTVCPWVVNIDYHPTHMKAIL

                1641                                                1680
    S2_L (1641) TYIDLVRMGLINIDKIYIKNKHKFNDEFYTSNLFYINYNF
MRK_287_L (1641) TYIDLVRMGLINIDRIHIKNKHKFNDEFYTSNLFYINYNF
    P17_L (1641) TYIDLVRMGLINIDRIHIKNKHKFNDEFYTSNLFYINYNF

                1681                                                1720
    S2_L (1681) SDNTHLLTKHIRIANSELENNYNKLYHPTPETLENILTNP
MRK_287_L (1681) SDNTHLLTKHIRIANSELENNYNKLYHPTPETLENILANP
    P17_L (1681) SDNTHLLTKHIRIANSELENNYNKLYHPTPETLENILANP

                1721                                                1760
    S2_L (1721) VKCNDKKTLNDYCIGKNVDSIMLPLLSNKKLIKSSTMIRT
MRK_287_L (1721) IKSNDKKTLNEYCIGKNVDSIMLPLLSNKKLIKSSAMIRT
    P17_L (1721) IKSNDKKTLNEYCIGKNVDSIMLPLLSNKKLIKSSAMIRT

                1761                                                1800
    S2_L (1761) NYSKQDLYNLFPTVVIDKIIDHSGNTAKSNQLYTTTSHQI
MRK_287_L (1761) NYSKQDLYNLFPMVVIDRIIDHSGNTAKSNQLYTTTSHQI
    P17_L (1761) NYSKQDLYNLFPMVVIDRIIDHSGNTAKSNQLYTTTSHQI

                1801                                                1840
    S2_L (1801) PLVHNSTSLYCMLPWHHINRFNFVFSSTGCKISIEYILKD
MRK_287_L (1801) SLVHNSTSLYCMLPWHHINRFNFVFSSTGCKISIEYILKD
    P17_L (1801) SLVHNSTSLYCMLPWHHINRFNFVFSSTGCKISIEYILKD

                1841                                                1880
    S2_L (1841) LKIKDPNCIAFIGEGAGNLLLRTVVELHPDIRYIYRSLKD
MRK_287_L (1841) LKIKDPNCIAFIGEGAGNLLLRTVVELHPDIRYIYRSLKD
    P17_L (1841) LKIKDPNCIAFIGEGAGNLLLRTVVELHPDIRYIYRSLKD

                1881                                                1920
    S2_L (1881) CNDHSLPIEFLRLYNGHINIDYGENLTIPATDATNNIHWS
MRK_287_L (1881) CNDHSLPIEFLRLYNGHINIDYGENLTIPATDATNNIHWS
    P17_L (1881) CNDHSLPIEFLRLYNGHINIDYGENLTIPATDATNNIHWS
```

FIG.4F

```
                1921                                                1960
    S2_L (1921) YLHIKFAEPISLFVCDAELPVTVNWSKIIIEWSKHVRKCK
MRK_287_L (1921) YLHIKFAEPISLFVCDAELPVTVNWSKIIIEWSKHVRKCK
    P17_L (1921) YLHIKFAEPISLFVCDAELPVTVNWSKIIIEWSKHVRKCK

                1961                                                2000
    S2_L (1961) YCSSVNKCTLIVKYHAQDDIDFKLDNITILKTYVCLGSKL
MRK_287_L (1961) YCSSVNKCMLIVKYHAQDDIDFKLDNITILKTYVCLGSKL
    P17_L (1961) YCSSVNKCMLIVKYHAQDDIDFKLDNITILKTYVCLGSKL

                2001                                                2040
    S2_L (2001) KGSEVYLVLTIGPANVFPVFNVVQNAKLILSRTKNFIMPK
MRK_287_L (2001) KGSEVYLVITIGPANIFPAFNVVQNAKLILSRTKNFIMPK
    P17_L (2001) KGSEVYLVITIGPANIFPAFNVVQNAKLILSRTKNFIMPK

                2041                                                2080
    S2_L (2041) KADKESIDANIKSLIPFLCYPITKKGINTALSKLKSVVSG
MRK_287_L (2041) KADKESIDANIKSLIPFLCYPITKKGINTALSKLKSVVSG
    P17_L (2041) KADKESIDANIKSXIPFLCYPITKKGINTALSKLKSVVSG

                2081                                                2120
    S2_L (2081) DILSYSIAGRNEVFSNKLINHKHMNILKWFNHVLNFRSTE
MRK_287_L (2081) DILSYSIAGRNEVFSNKLINHKHMNILKWFNHVLNFRSTE
    P17_L (2081) DILSYSIAGRNEVFSNKLINHKHMNILKWFNHVLNFRSTE

                2121                                                2160
    S2_L (2121) LNYNHLYMVESTYPYLSELLNSLTTNELKKLIKITGSLLY
MRK_287_L (2121) LNYNHLYMVESTYPYLSELLNSLTTNELKKLIKITGSLLY
    P17_L (2121) LNYNHLYMVESTYPYLSELLNSLTTNELKKLIKITGSLLY

                2161
    S2_L (2161) NFHNE--- (SEQ ID NO: 20)
MRK_287_L (2161) NFHNE--I (SEQ ID NO: 22)
    P17_L (2161) NFHNE--I (SEQ ID NO: 24)
```

FIG.4G

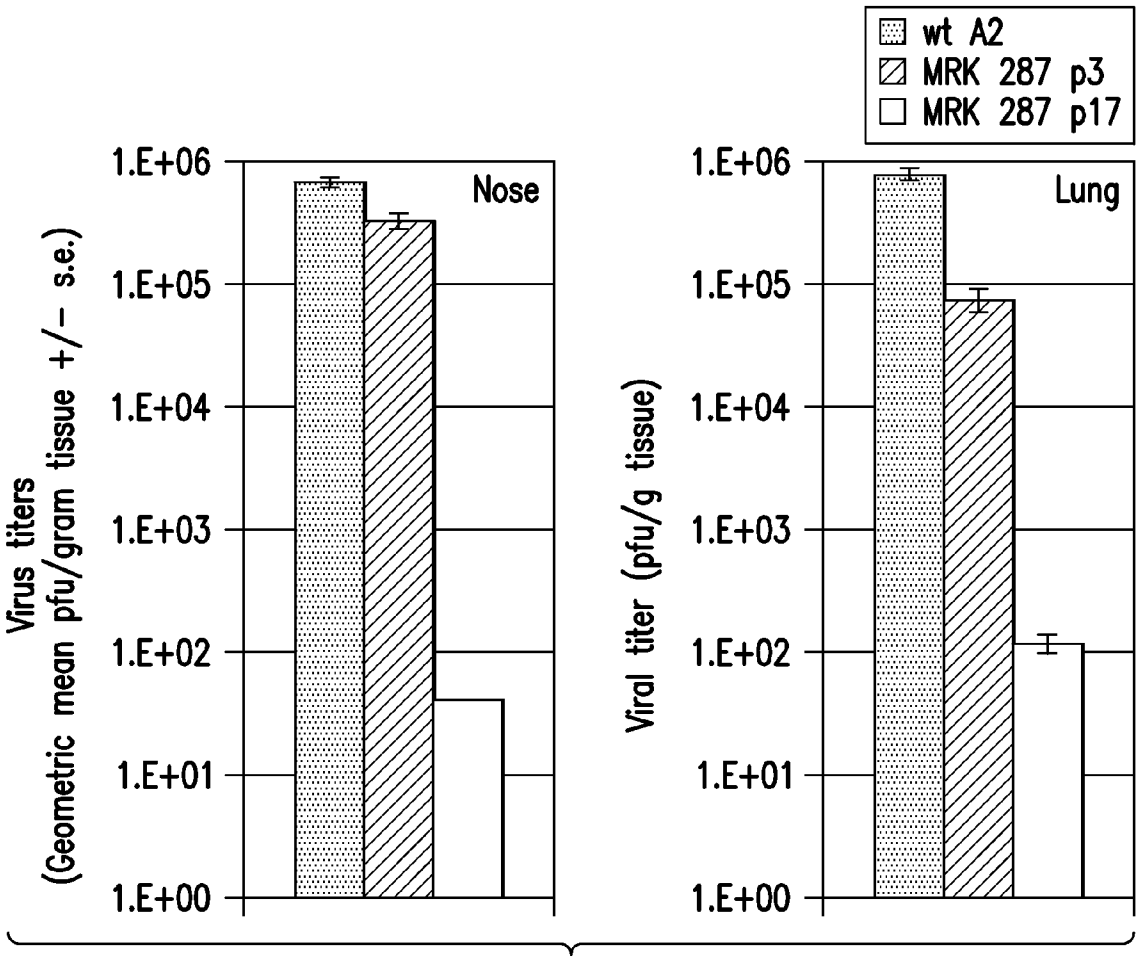
wt A2
MRK 287 p3
MRK 287 p17
Nose
Lung
Virus titers
(Geometric mean pfu/gram tissue +/- s.e.)
Viral titer (pfu/g tissue)
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00

FIG.5

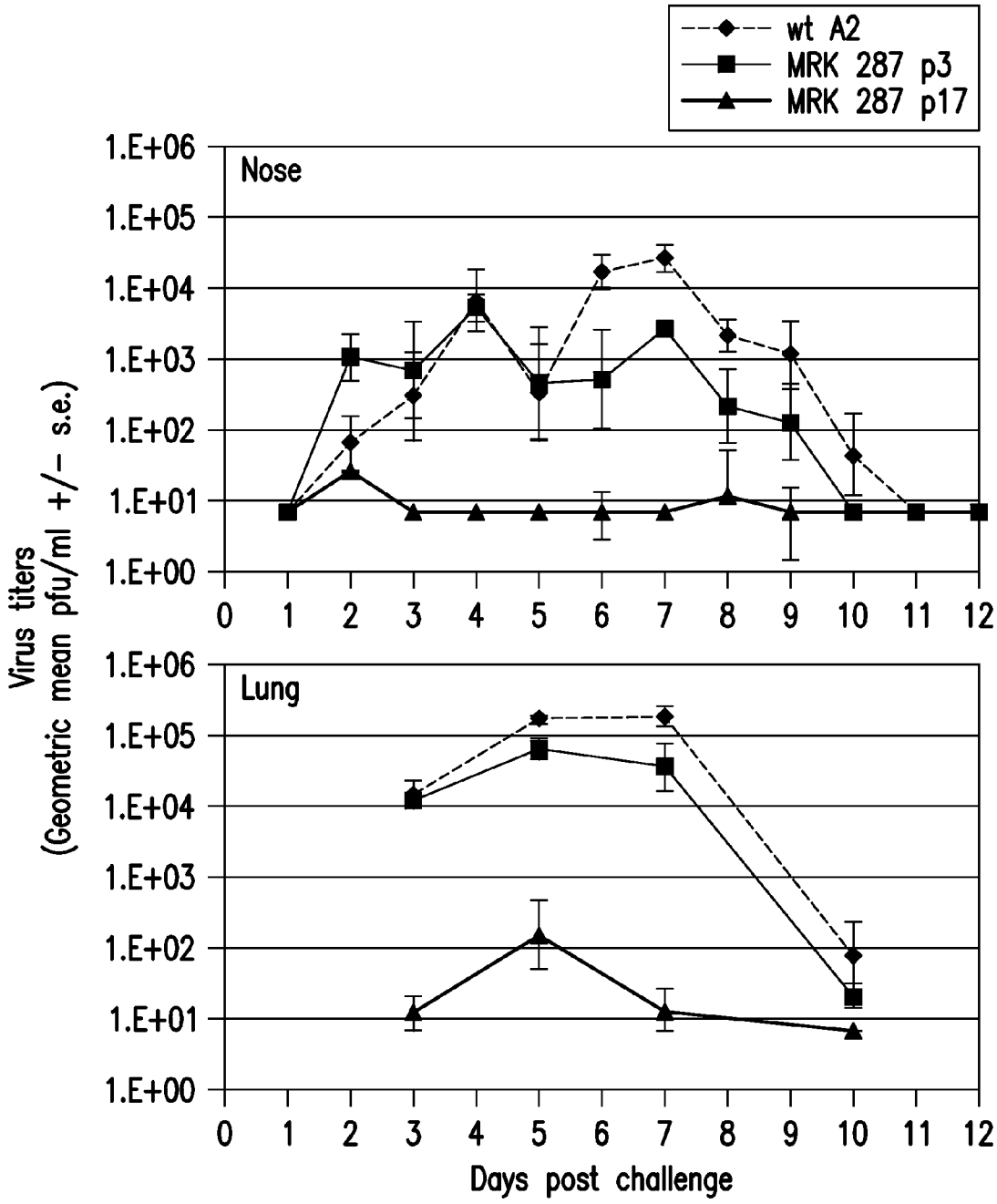
wt A2
MRK 287 p3
MRK 287 p17
Nose
Lung
Virus titers
(Geometric mean pfu/ml +/- s.e.)
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
0 1 2 3 4 5 6 7 8 9 10 11 12
Days post challenge

FIG.6

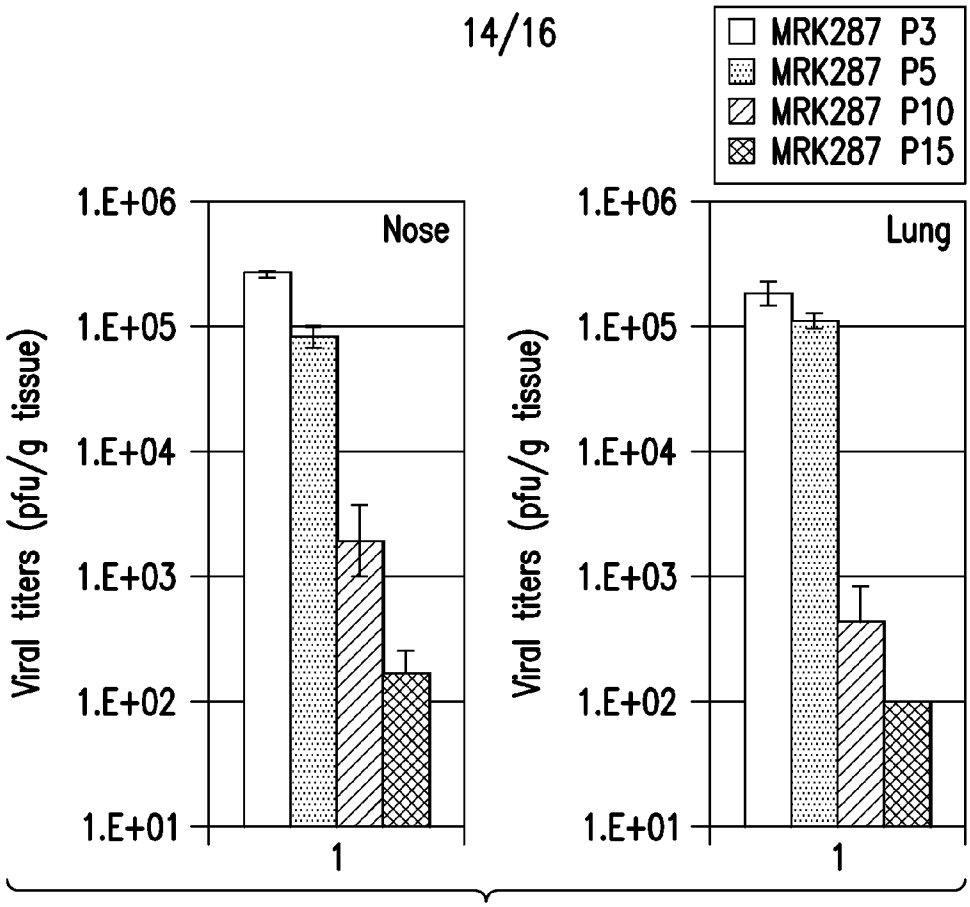
MRK287 P3
MRK287 P5
MRK287 P10
MRK287 P15
Nose
Lung
Viral titers (pfu/g tissue)
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1

FIG.7

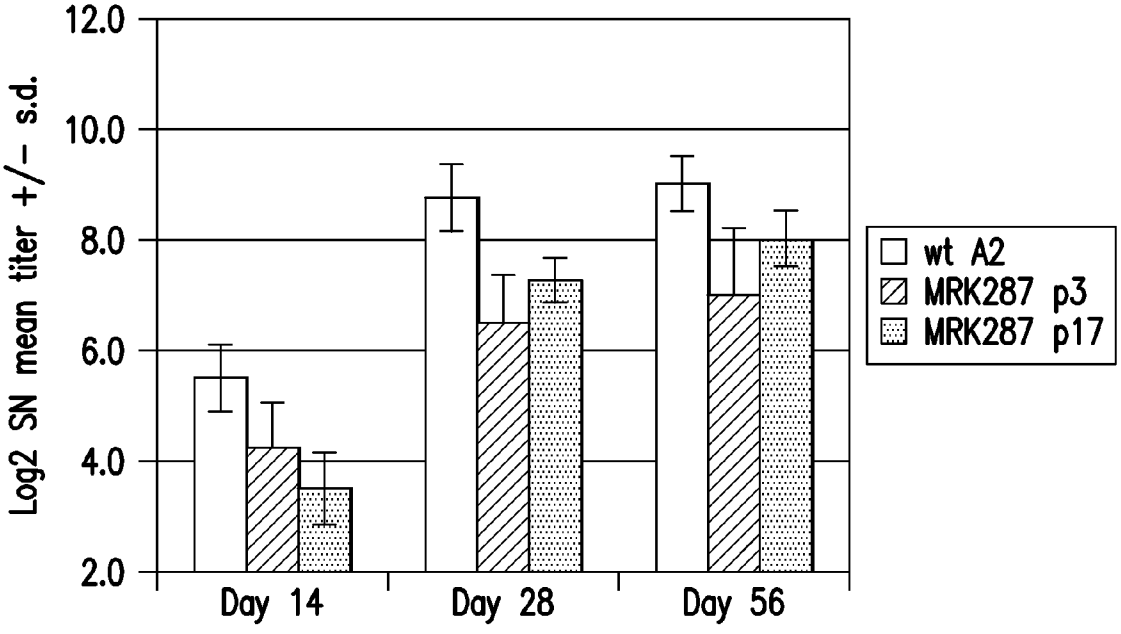
Log2 SN mean titer +/- s.d.
12.0
10.0
8.0
6.0
4.0
2.0
Day 14
Day 28
Day 56
wt A2
MRK287 p3
MRK287 p17

FIG.8

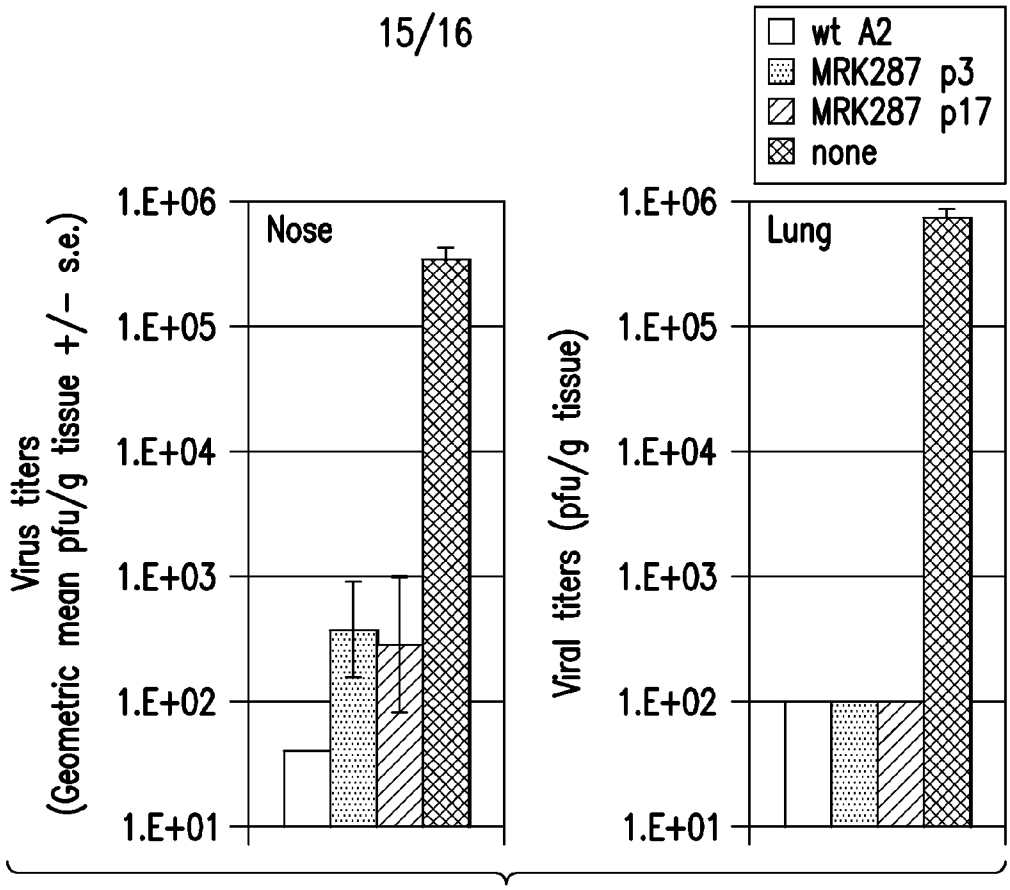
wt A2
MRK287 p3
MRK287 p17
none
Nose
Lung
Virus titers
(Geometric mean pfu/g tissue +/- s.e.)
Viral titers (pfu/g tissue)
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01

FIG.9

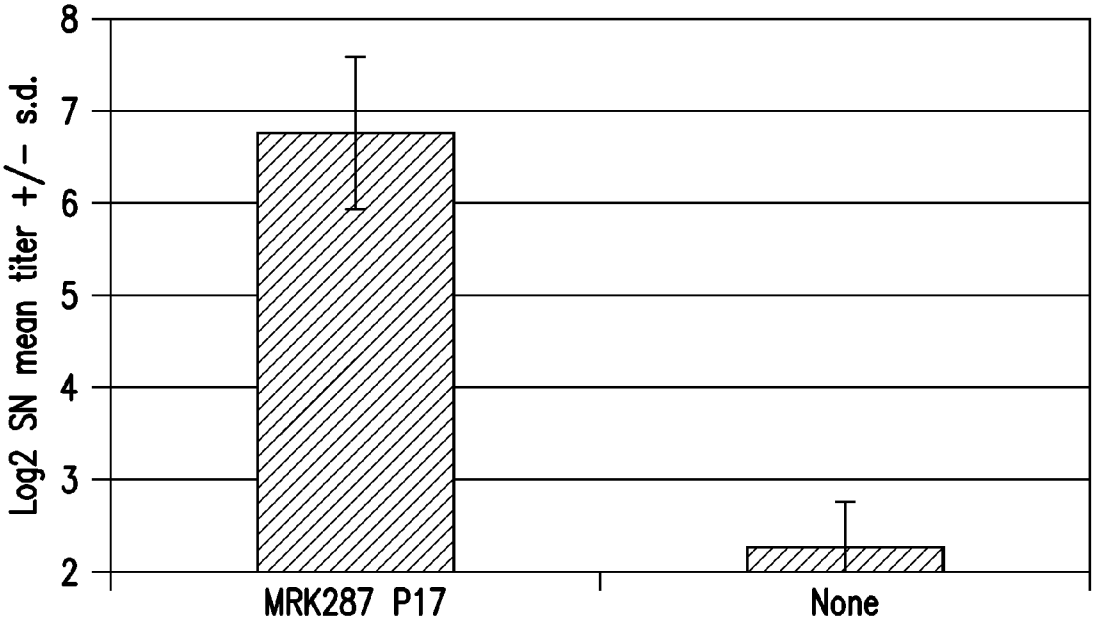
Log2 SN mean titer +/- s.d.
8
7
6
5
4
3
2
MRK287 P17
None

FIG.10

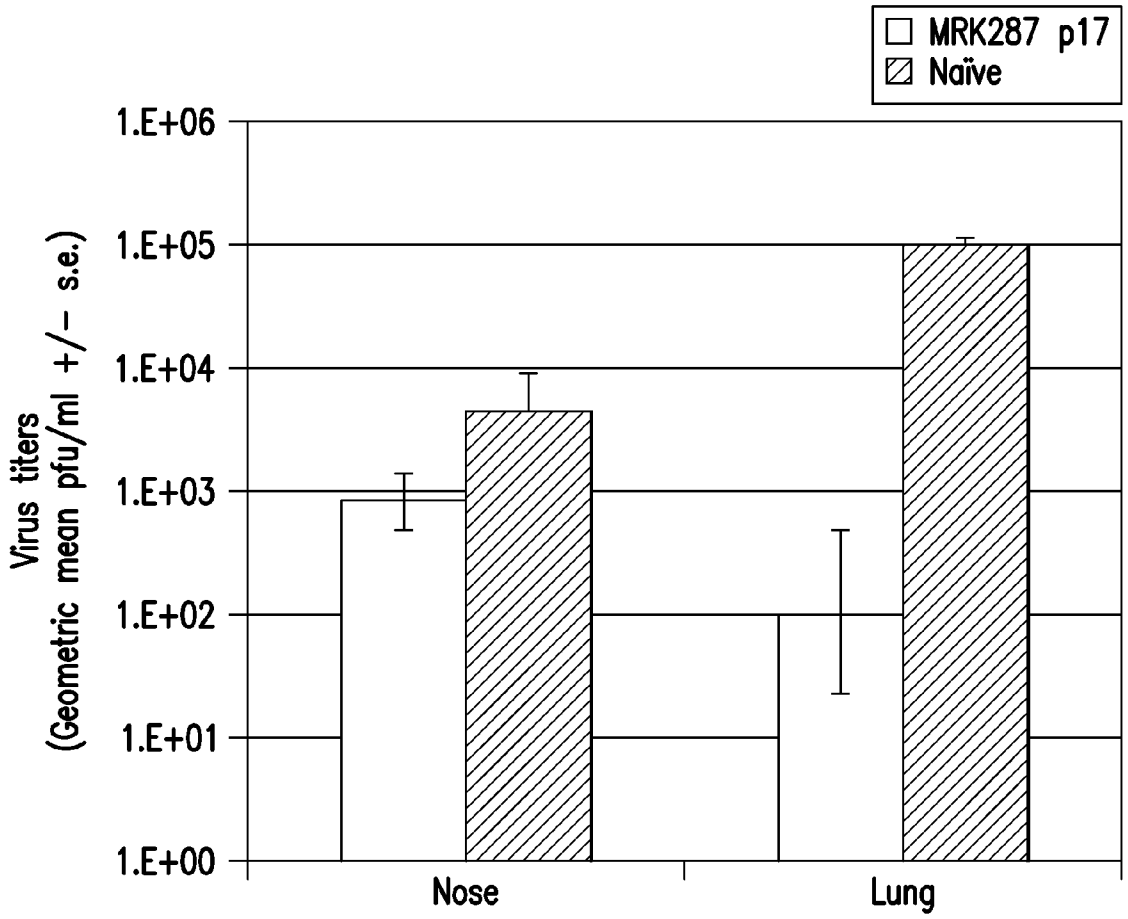
MRK287 p17
Naïve
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
Virus titers
(Geometric mean pfu/ml +/- s.e.)
Nose
Lung

FIG.11

The Sequence Listing for this application has been submitted to WIPO in electronic format and can be obtained upon request from the International Bureau or found on the WIPO website at http://www.wipo.int/pctdb/en/sequences/