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(71) Applicant(s)
Wyeth Holdings Corporation

(72) Inventor(s)
Parks, Christopher L.

(74) Agent/Attorney
Spruson & Ferguson, Level 35 St Martins Tower 31 Market Street, Sydney, NSW, 2000

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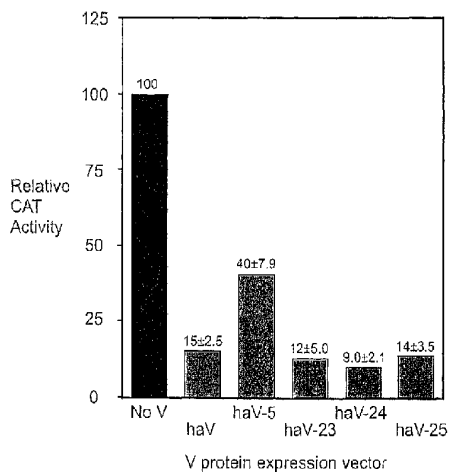
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- (71) Applicant (for all designated States except US): AMERICAN CYANAMID COMPANY [US/US]; Five Giralda Farms, Madison, NJ 07940 (US).
- (72) Inventor; and
- (75) Inventor/Applicant (for US only): PARKS, Christopher, L. [US/US]; 118 South Terrace, Boonton, NJ 07005 (US).
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(54) Title: MODIFIED MORBILLIVIRUS V PROTEINS



(57) Abstract: Modified *Morbilliviruses* having at least one mutation in the region corresponding to amino acids 112-134 of the measles virus V protein are described wherein one or both of amino acids 113 or 114 is mutated. Such modified *Morbilliviruses* exhibit reduced repression of gene expression. Additional mutations or deletions in other regions of the genome may be included, including in the carboxy-terminal region.

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MODIFIED MORBILLIVIRUS V PROTEINS

Field of the Invention

5 This invention relates to isolated,
recombinantly-generated, negative-sense, single-
stranded RNA viruses of the genus *Morbillivirus* having
one or more mutations and/or deletions which reduce the
repression normally caused by the V protein.

10

Background of the Invention

Enveloped, negative-sense, single-stranded
RNA viruses are uniquely organized and expressed. The
15 genomic RNA of negative-sense, single-stranded viruses
serves two template functions in the context of a
nucleocapsid: as a template for the synthesis of
messenger RNAs (mRNAs) and as a template for the
synthesis of the antigenome (+) strand. Viral
20 replication occurs after synthesis of the mRNAs and
requires the continuous synthesis of viral proteins.
The newly synthesized antigenome (+) strand serves as
the template for generating further copies of the (-)
strand genomic RNA.

25

The RNA-dependent RNA polymerase complex
actuates and achieves transcription and replication by
engaging the cis-acting signals at the 3' end of the
genome, in particular, the promoter region. Viral
genes are then transcribed from the genome template
30 unidirectionally from its 3' to its 5' end.

30

Based on the revised reclassification in 1993
by the International Committee on the Taxonomy of
Viruses, an Order, designated Mononegavirales, has been
established. This Order contains three families of
35 enveloped viruses with single-stranded, nonsegmented
RNA genomes of minus polarity (negative-sense). These

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families are the Paramyxoviridae, Rhabdoviridae and
Filoviridae. The family Paramyxoviridae has been
further divided into two subfamilies, Paramyxovirinae
and Pneumovirinae. The subfamily Paramyxovirinae
5 contains three genera, *Respirovirus* (formerly
Paramyxovirus), *Rubulavirus* and *Morbillivirus*. The
subfamily Pneumovirinae contains the genus *Pneumovirus*.
The new classification is based upon morphological
criteria, the organization of the viral genome,
10 biological activities and the sequence relatedness of
the genes and gene products. The current taxonomical
classification of the *Morbilliviruses* is as follows:

Order Mononegavirales
15 Family Paramyxoviridae
Subfamily Paramyxovirinae
Genus *Morbillivirus*
Measles virus
Dolphin morbillivirus
20 Canine distemper virus
Peste-des-petits-ruminants virus
Phocine distemper virus
Rinderpest virus

25 For many of these viruses, no vaccines of any
kind are available. Thus, there is a need to develop
vaccines against such human and animal pathogens. Such
vaccines would have to elicit a protective immune
response in the recipient. The qualitative and
30 quantitative features of such a favorable response are
extrapolated from those seen in survivors of natural
virus infection, who, in general, are protected from
reinfection by the same or highly related viruses for
some significant duration thereafter.

35 A variety of approaches can be considered in
seeking to develop such vaccines, including the use of:

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(1) purified individual viral protein vaccines (subunit vaccines); (2) inactivated whole virus preparations; and (3) live, attenuated viruses.

5 Subunit vaccines have the desirable feature of being pure, definable and relatively easily produced in abundance by various means, including recombinant DNA expression methods. To date, with the notable exception of hepatitis B surface antigen, viral subunit vaccines have generally only elicited short-lived and/or inadequate immunity, particularly in naive recipients.

10 Formalin inactivated whole virus preparations of polio (IPV) and hepatitis A have proven safe and efficacious. In contrast, immunization with similarly inactivated whole viruses such as respiratory syncytial virus and measles virus vaccines elicited unfavorable immune responses and/or response profiles which predisposed vaccinees to exaggerated or aberrant disease when subsequently confronted with the natural or "wild-type" virus.

20 Appropriately attenuated live derivatives of wild-type viruses offer a distinct advantage as vaccine candidates. As live, replicating agents, they initiate infection in recipients during which viral gene products are expressed, processed and presented in the context of the vaccinee's specific MHC class I and II molecules, eliciting humoral and cell-mediated immune responses, as well as the coordinate cytokine and chemokine patterns, which parallel the protective immune profile of survivors of natural infection.

25 This favorable immune response pattern is contrasted with the delimited responses elicited by inactivated or subunit vaccines, which typically are largely restricted to the humoral immune surveillance arm. Further, the immune response profile elicited by some formalin inactivated whole virus vaccines, e.g.,

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measles and respiratory syncytial virus vaccines developed in the 1960's, have not only failed to provide sustained protection, but in fact have led to a predisposition to aberrant, exaggerated, and even fatal illness, when the vaccine recipient later confronted the wild-type virus.

While live, attenuated viruses have highly desirable characteristics as vaccine candidates, they have proven to be difficult to develop. The crux of the difficulty lies in the need to isolate a derivative of the wild-type virus which has lost its disease-producing potential (i.e., virulence), while retaining sufficient replication competence to infect the recipient and elicit the desired immune response profile in adequate abundance.

Historically, this delicate balance between virulence and attenuation has been achieved by serial passage of a wild-type viral isolate through different host tissues or cells under varying growth conditions (such as temperature). This process presumably favors the growth of viral variants (mutants), some of which have the favorable characteristic of attenuation. Occasionally, further attenuation is achieved through chemical mutagenesis as well.

This propagation/passage scheme typically leads to the emergence of virus derivatives which are temperature sensitive, cold-adapted and/or altered in their host range -- one or all of which are changes from the wild-type, disease-causing viruses -- i.e., changes that may be associated with attenuation.

Several live virus vaccines, including those for the prevention of measles and mumps (which are paramyxoviruses), and for protection against polio and rubella (which are positive strand RNA viruses), have been generated by this approach and provide the

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mainstay of current childhood immunization regimens throughout the world.

Nevertheless, this means for generating attenuated live virus vaccine candidates is lengthy and, at best, unpredictable, relying largely on the selective outgrowth of those randomly occurring genomic mutants with desirable attenuation characteristics. The resulting viruses may have the desired phenotype *in vitro*, and even appear to be attenuated in animal models. However, all too often they remain either under- or overattenuated in the human or animal host for whom they are intended as vaccine candidates.

Even as to current vaccines in use, there is still a need for more efficacious vaccines. For example, the current measles vaccines provide reasonably good protection. However, recent measles epidemics suggest deficiencies in the efficacy of current vaccines. Despite maternal immunization, high rates of acute measles infection have occurred in children under age one, reflecting the vaccines' inability to induce anti-measles antibody levels comparable to those developed following wild-type measles infection (Bibliography entries 1,2,3). As a result, vaccine-immunized mothers are less able to provide their infants with sufficient transplacentally-derived passive antibodies to protect the newborns beyond the first few months of life.

Acute measles infections in previously immunized adolescents and young adults point to an additional problem. These secondary vaccine failures indicate limitations in the current vaccines' ability to induce and maintain antiviral protection that is both abundant and long-lived (4,5,6). Recently, yet another potential problem was revealed. The hemagglutinin protein of wild-type measles isolated over the past 15 years has shown a progressively

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increasing distance from the vaccine strains (7). This "antigenic drift" raises legitimate concerns that the vaccine strains may not contain the ideal antigenic repertoire needed to provide optimal protection. Thus, there is a need for improved vaccines.

Rational vaccine design would be assisted by a better understanding of these viruses, in particular, by the identification of the virally encoded determinants of virulence as well as those genomic changes which are responsible for attenuation.

Because of its significance as a major cause of human morbidity and mortality, measles virus has been quite extensively studied. Measles virus is a large, relatively spherical, enveloped particle composed of two compartments, a lipoprotein membrane and a ribonucleoprotein particle core, each having distinct biological functions (8). The virion envelope is a host cell-derived plasma membrane modified by three virus-specified proteins: The hemagglutinin (H; approximately 80 kilodaltons (kD)) and fusion (F_{1,2}; approximately 60 kD) glycoproteins project on the virion surface and confer host cell attachment and entry capacities to the viral particle (9). Antibodies to H and/or F are considered protective since they neutralize the virus' ability to initiate infection (10,11,12). The matrix (M; approximately 37 kD) protein is the amphipathic protein lining the membrane's inner surface, which is thought to orchestrate virion morphogenesis and thus consummate virus reproduction (13). The virion core contains the 15,894 nucleotide long genomic RNA upon which template activity is conferred by its intimate association with approximately 2600 molecules of the approximately 60 kD nucleocapsid (N) protein (14,15,16). Loosely associated with this approximately one micron long helical ribonucleoprotein particle are enzymatic levels

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of the viral RNA-dependent RNA polymerase (L; approximately 240 kD) which in concert with the polymerase cofactor (P; approximately 70 kD), and perhaps yet other virus-specified as well as host-encoded proteins, transcribes and replicates the measles virus genome sequences (17).

The six virion structural proteins of measles virus are encoded by six contiguous, non-overlapping genes which are arrayed as follows: 3'-N-P-M-F-H-L-5'. Two additional measles virus gene products of as yet uncertain function have also been identified. These two nonstructural proteins, known as C (approximately 20 kD) and V (approximately 45 kD), are both encoded by the P gene. The C protein is encoded by a second reading frame within the P mRNA. The V protein is encoded by a cotranscriptionally edited P gene-derived mRNA which encodes a hybrid protein having the amino terminal sequences of P and a zinc finger-like cysteine-rich carboxy terminal domain which is lacking in the P protein (9).

All *Morbilliviruses* produce a V protein (18), including measles virus, rinderpest virus, canine distemper virus and phocine distemper virus (19). Measles virus V protein is a nonstructural protein encoded by the P gene (8). Like most paramyxoviruses, measles virus encodes multiple proteins from the P gene including V protein, P protein, and C protein (9). Translation of both P and V proteins initiates at the same methionine codon resulting in polypeptides that are identical for the first 230 amino acids. The carboxy-terminus (C-terminus) of V protein differs from P protein because RNA editing occurs in some P gene mRNAs causing a frameshift that results in translation of a shorter, unique V protein C-terminus (18). The C protein amino acid sequence is unrelated to V and P protein because it is translated entirely from a

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different reading frame that begins at a downstream translation initiation codon (20).

5 The P and V mRNAs of measles virus share the same start codon and the first 230 amino acids of the P and V proteins are identical. The V mRNA contains a "G" residue insertion that expands the sequence "GGG" at nucleotides 2496 to 2498 to include a fourth "G" residue. Editing takes place during transcription when an extra non-template-directed "G" residue is inserted between nucleotides 2495 and 2499, causing a shift in the reading frame, whereby the carboxy-terminal 276 amino acids of the P protein are replaced with a 68 amino acid cysteine-rich carboxy-terminus of the V protein.

15 The function of V protein is not well understood, but all *Morbilliviruses* encode a V protein. This indicates that V protein performs beneficial functions that have made it advantageous for *Morbilliviruses* to conserve the capacity to synthesize V protein. It is known that V protein expression is not essential for viral replication in cultured cells (19,21-25), but in animal model systems expression of V protein seems to influence the severity of infection. For example, Sendai virus (a non-*Morbillivirus* paramyxovirus) normally produces pneumonia in mouse model systems but is less virulent if the infection is performed with a recombinant virus that is defective for V protein expression (22,26). Recombinant human parainfluenza virus type 3 (another non-*Morbillivirus* paramyxovirus) also exhibits an attenuated phenotype in rodents and monkeys if a defect in D protein expression is combined with a defect in the V protein open reading frame (23).

30 Similarly, results from studies with animal model systems used for measles virus also suggest a role for V protein in pathogenicity. Infection of

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cotton rat lungs by recombinant measles virus generates less progeny virus if the infecting virus was defective for V protein expression (27). Also, human thymocyte survival in tissue transplanted in SCID mice was less susceptible to infection with measles virus if the infecting virus did not express V protein (28).
5 Finally, CD46 transgenic mice inoculated intracranially with measles virus had greater rates of survival if the virus did not express V protein (29). The conclusion
10 that measles virus V protein plays a role in pathogenicity also is supported by sequence analyses that have found V protein coding region mutations in less pathogenic variants or vaccine strains (30,31). Taken together, these results support the hypothesis
15 that V protein plays an important role in determining the virulence of measles virus and several other paramyxoviruses.

Although it seems clear that V protein can influence the course of infection, the mechanism behind
20 this phenomenon is not known. Results from a number of studies have begun to assign potential functions to V protein. For example, it has been shown that amino acid sequences shared by V protein and P protein mediate interaction with the viral nucleocapsid (N)
25 protein (27,32-39). This interaction between V protein and N protein seems to affect the cellular distribution N protein (27,40,41) and probably has some additional unidentified functions. Some V proteins also have been found to interact with cellular proteins (42,43), and
30 in the case of simian virus 5 (SV5), it is possible that interaction with a cellular protein is responsible for inhibition of the interferon signaling pathway during infection (44). In addition to the protein-protein interactions that involve V protein, several
35 studies have linked V protein to control mechanisms that regulate viral gene expression and replication.

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Sendai virus V protein expression in a transient expression system inhibits defective-interfering (DI) particle replication (45) and similarly inhibits DI particle replication in an *in vitro* transcription reaction (35). Consistent with these observations relating V protein with repression, several viruses defective for V protein expression have been observed to produce elevated levels of genome RNA, mRNA, and viral proteins during infection (21,26,27).

In addition to the properties just described, all of the viral V proteins contain a cysteine-rich C-terminus. The paramyxovirus V proteins do not share a high degree of amino acid similarity, but they all contain seven identically positioned cysteine residues (46). This striking feature has led to speculation (47) that V proteins may actually be zinc-finger proteins or at least form some type of zinc-coordinated secondary structure (48,49,50), and in fact, several V proteins have been found to bind zinc (51,52,53). The possibility that V protein forms a zinc-coordinated structure generates considerable interest because these types of structures often form protein domains that are involved in nucleic acid interaction or protein-protein interaction (48,49,50). It is also noteworthy that a recombinant Sendai virus that expresses a truncated V protein lacking the unique C-terminal region also is less pathogenic, suggesting that the role of V protein in pathogenicity requires this domain (24).

In addition to the sequences encoding the virus-specified proteins, the measles virus genome contains distinctive non-protein coding domains resembling those directing the transcriptional and replicative pathways of related viruses (9,54). These regulatory signals lie at the 3' and 5' ends of the measles virus genome and in short internal regions spanning each intercistronic boundary. The former

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5 encode the putative promoter and/or regulatory sequence
elements directing genomic transcription, genome and
antigenome encapsidation, and replication. The latter
signal transcription termination and polyadenylation of
each monocistronic viral mRNA and then reinitiation of
transcription of the next gene. In general, the
measles virus polymerase complex appears to respond to
these signals much as the RNA-dependent RNA polymerases
of other non-segmented negative strand RNA viruses
10 (9,54,55,56). Transcription initiates at or near the
3' end of the measles virus genome and then proceeds in
a 5' direction producing monocistronic mRNAs
(16,54,57).

15 Measles virus appears to have extended its
terminal regulatory domains beyond the confines of
leader and trailer encoding sequences (54). For
measles, these regions encompass the 107 3' genomic
nucleotides (the "3' genomic promoter region", also
referred to as the "extended promoter", which comprises
20 52 nucleotides encoding the leader region, followed by
three intergenic nucleotides, and 52 nucleotides
encoding the 5' untranslated region of N mRNA) and the
109 5' end nucleotides (69 encoding the 3' untranslated
region of L mRNA, the intergenic trinucleotide and 37
25 nucleotides encoding the trailer). Within these 3'
terminal approximately 100 nucleotides of both the
genome and antigenome are two short regions of shared
nucleotide sequence: 14 of 16 nucleotides at the
absolute 3' ends of the genome and antigenome are
30 identical. Internal to those termini, an additional
region of 12 nucleotides of absolute sequence identity
have been located. Their position at and near the
sites at which the transcription of the measles virus
genome must initiate and replication of the antigenome
35 must begin, suggests that these short unique sequence
domains encompass an extended promoter region.

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5 These discrete sequence elements may dictate
alternative sites of transcription initiation -- the
internal domain mandating transcription initiation at
the N gene start site, and the 3' terminal domain
10 directing antigenome production (54,58,59). In
addition to their regulatory role as cis-acting
determinants of transcription and replication, these 3'
extended genomic and antigenomic promoter regions
15 encode the nascent 5' ends of antigenome and genome
RNAs, respectively. Within these nascent RNAs reside
as yet unidentified signals for N protein nucleation,
another key regulatory element required for
nucleocapsid template formation and consequently for
20 amplification of transcription and replication.

15 In all *Morbilliviruses*, the cis-acting
signals required for essential viral functions,
including replication, transcription and encapsidation
are contained in the non-coding genomic termini. The
obligatory trans-acting elements for functionality are
20 contained in the N, P and L genes. Additional
transacting factors, such as the V and C proteins, may
modulate functionality. Mutations in any of these
regions may result in alteration of vital functions,
including attenuation of viral transcription/-
25 replication efficiency.

 The apparent connection between V protein
expression and pathogenicity, and continuing interest
in vaccine attenuation (30,60) has led to a need to
examine measles virus V protein function in more
30 detail. In particular, there is a need to utilize
transient expression systems to study several V protein
properties including V protein repression activity, the
interaction of V protein with N protein, and the
ability of V protein to bind RNA.
35

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Summary of the Invention

Accordingly, it is an object of this invention to identify regions of the genomes of Morbilliviruses responsible for the repression of gene expression by the V protein of these viruses. It is a further object of this invention to generate mutant versions of the V protein of Morbilliviruses in which the repression of gene expression is reduced. It is a still further object of this invention to generate recombinantly-generated Morbilliviruses containing one or more of such mutations. It is yet another object of this invention to formulate vaccines or immunogenic compositions containing such recombinantly-generated Morbilliviruses. It is an aim of the invention to satisfy at least one of the above objects. In one embodiment of the invention, the V protein is from measles virus.

These and other objects of the invention as discussed below are achieved for Morbilliviruses by modifying the region corresponding to amino acids 112- 134 (conserved region 2; see Figure 2) of the V protein of these Morbilliviruses, wherein one or both of amino acids 113 of the measles virus V protein or the amino acids corresponding thereto (a tyrosine) and 114 of the measles V protein or the amino acid corresponding thereto (aspartic acid) is mutated. In one embodiment of the invention, these amino acids are mutated to alanine.

A further modification of the V protein may be made by mutating or deleting at least a portion of the carboxy-terminal (C-terminal) region of the Morbillivirus V protein, corresponding to amino acids 231-299 of the V protein of measles virus, canine distemper virus and dolphin morbillivirus, and to amino acids 231-303 of the rinderpest virus.

These modifications have the effect of reducing the repression of gene expression by the V protein in a minireplicon system. The results are extended readily to the recovery of full-length

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infectious *Morbilliviruses* by the use of the "rescue" system known in the art and described below.

Measles virus minireplicon with chloramphenicol acetyltransferase (CAT) reporter gene expression in transient assays was strongly repressed by V protein. Repression activity was diminished by amino acid substitution in a region located in the amino terminal third of the protein between amino acids 112-134, as well as by mutating or deleting at least a portion of the cysteine-rich C-terminal region of V protein (amino acids 231-299).

In the case of measles virus, the mutations described above may be further combined with mutations which are attenuating, as follows:

(1) at least one attenuating mutation in the 3' genomic promoter region selected from the group consisting of nucleotide 26 (A → T), nucleotide 42 (A → T or A → C) and nucleotide 96 (G → A), where these nucleotides are presented in positive strand, antigenomic, message sense;

(2) at least one attenuating mutation in the RNA polymerase gene selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 331 (isoleucine → threonine), 1409 (alanine → threonine), 1624 (threonine → alanine), 1649 (arginine → methionine), 1717 (aspartic acid → alanine), 1936 (histidine → tyrosine), 2074 (glutamine → arginine) and 2114 (arginine → lysine);

(3) for the N gene, at least one attenuating mutation selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 129

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(glutamine → lysine), 148 (glutamic acid → glycine) and 479 (serine → threonine);

5 (4) for the P gene, at least one attenuating mutation selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 225 (glutamic acid → glycine), 275 (cysteine → tyrosine) and 439 (leucine → proline);

10 (5) for the C gene, at least one attenuating mutation selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 73 (alanine → valine), 104 (methionine → threonine) and 134 (serine → tyrosine); and

15 (6) for the F gene-end signal (the cis-acting transcription termination signal), the change at nucleotide 7243 (T → C), where these nucleotides are presented in positive strand, antigenomic, that is, message (coding) sense.

20 In another embodiment of this invention, these mutant *Morbilliviruses* are used to prepare vaccines or immunogenic compositions which elicit a protective immune response against the wild-type form of each virus.

25 In a further embodiment of this invention, there is described a method for reducing the repression caused by a V protein of *Morbilliviruses* which comprises inserting at least one mutation in the region corresponding to amino acids 112-134 of a *Morbillivirus* V protein, wherein the mutation in the region
30 corresponding to amino acids 112-134 of a *Morbillivirus* V protein is selected from the group consisting of the mutation of amino acids 113 and 114.

In still another embodiment of this invention, there is described an isolated nucleotide sequence encoding a Morbilliviruses V protein which has been modified by inserting at least one mutation in the region corresponding to amino acids 112-134 of a Morbillivirus V protein, wherein the mutation in the region corresponding to amino acids 112-134 of a Morbillivirus V protein is selected from the group consisting of the mutation of amino acids 113 and 114.

In yet another embodiment of this invention, there is provided a composition which comprises a transcription vector comprising an isolated nucleic acid molecule encoding a genome or antigenome of a Morbillivirus, wherein the portion of the isolated nucleic acid molecule encoding the V protein has been modified so as to insert at least one mutation in the region corresponding to amino acids 112-134 of a Morbillivirus V protein, wherein the mutation in the region corresponding to amino acids 112-134 of a Morbillivirus V protein is selected from the group consisting of the mutation of amino acids 113 and 114, together with at least one expression vector which comprises at least one isolated nucleic acid molecule encoding the trans-acting proteins N, P and L necessary for encapsidation, transcription and replication, whereby host cells are transformed, infected or transfected with these vectors and cultured under conditions which permit the co-expression of these vectors so as to produce the desired Morbillivirus. Each such virus is then used to prepare vaccines or immunogenic compositions which elicit a protective immune response against the wild-type form of each virus.

In another embodiment there is provided a use of the composition of the invention in the preparation of a composition for immunizing an individual against a nonsegmented, negative-sense, single-stranded RNA virus of the genus Morbillivirus.

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Brief Description of the Figures

Figure 1A depicts the four plasmid expression vectors used for transfections. T7 RNA polymerase-dependent expression vectors (61) were prepared to direct expression of Edmonston wild-type N, P, L, or V genes in cells infected with MVA/T7 (62).

Figure 1B depicts the minireplicon which was derived from pMV107-CAT (63). The Edmonston measles virus vaccine leader sequence in pMV107-CAT was converted to the wild-type sequence (60). Transcription of the minireplicon plasmid DNA by T7 RNA polymerase generated a negative-sense RNA minireplicon copy in transfected cells.

Figure 1C depicts a CAT assay that demonstrated the effect of V protein expression on minireplicon activity in a transient expression assay. Lane 1 was the positive control obtained from cells that were transfected with all plasmid vectors (N, P and L) necessary to drive minireplicon expression. Lane 2 was identical to lane 1, except that the cells were transfected with all plasmids except the L polymerase vector. In lanes 3 through 7, increasing amounts of V protein expression vector was included in the transfection. The total mass of transfected DNA was held constant by including the appropriate amount of vector DNA without an insert. Relative CAT activity was calculated based on 100% activity in lane 1.

Figure 2 depicts a comparison of the amino acid sequences of V proteins from four different *Morbilliviruses*. Edmonston wild-type measles virus V protein amino acid sequence (Gene Bank accession number AF266288) (SEQ ID NO:1) was compared to canine distemper virus (AF014953) (SEQ ID NO:4), rinderpest virus (Z30697) (SEQ ID NO:2), and dolphin morbillivirus (Z47758) (SEQ ID NO:3). Regions containing higher

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levels of identity are overlined with a cross-hatched bar and designated as conserved regions (CR1 through CR6). Additional notable sequences are underlined with a black bar. These include regions involved in the V (and P) protein interaction with N protein (34), a region involved in the cellular localization of the V-N protein complex (27,64), and the cysteine-rich region that contains the zinc-binding domain (51). Other sequences of interest include a basic region between 229-234, a measles virus vaccine amino acid substitution (glutamic acid to glycine) at position 225 (30), and a leucine repeat region reminiscent of a leucine zipper between positions 93-107 (65,66). The region common to V and P protein extends from amino acid 1-230 and the unique V protein sequences (bold amino acid letters) extend from 231-299. The amino acid positions given in the Figure along the top of the aligned sequences correspond to those of the measles virus V protein.

Figure 3 depicts a map of mutant measles virus V protein expression vectors. The epitope-tagged V protein expression vectors used in these studies are shown diagrammatically. Mutant V protein vectors were generated in a vector plasmid backbone that expressed V protein with an influenza virus HA tag (67) in place of the V protein initiator methionine. Sequence highlights are shown on a V protein map at the top of the Figure and are described more fully with respect to the description of Figure 2 above. Wild-type (haV-wt) and mutant proteins (haV1-11) are illustrated below the V protein map. The HA tag is drawn as a crosshatched box at the amino-terminus and the remaining V protein sequence is drawn as an open box. Amino acid substitutions are indicated as black dots along with amino acid positions and amino acid changes. Deletion or truncation mutants are drawn with an interrupted V protein map.

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Figure 4A depicts minireplicon repression by mutant measles virus V proteins, in the form of a CAT assay showing the results of a transient expression experiment testing the activity of V protein mutants (haV-1 to haV-8; see description for Figure 3). The amount of V protein vector (200 or 400ng) and the identity of the mutants are shown in Figure 4A below the CAT assay. Relative activity is calculated as a percentage of the lane 2 that was derived from a transfection performed without any V protein expression vector. Lane 1 was a negative control performed without L protein.

Figure 4B depicts a Western blot performed to monitor V protein expression in transient expression experiments. Lanes 1 and 2 are negative controls derived from cells transfected in the absence of a V protein vector (lane 1) or transfected with a V protein vector that expresses a V protein without a tag. The Western blot was probed with anti-HA antibody.

Figure 4C depicts an analysis of minireplicon repression by haV-1 using increasing amounts (100 ng to 1 μ g) of the expression vector.

Figure 5 depicts the RNA binding activity associated with measles virus V protein. Crude cytoplasmic extracts prepared from transfected cells by NP40 lysis were analyzed for RNA binding activity using agarose beads linked to polyribonucleotide homopolymers. A flow diagram illustrating the procedure is shown in Figure 5A. Figure 5B depicts Western blots used to examine proteins captured on the polyribonucleotide resins. Cells were transfected using the same conditions as used for minireplicon experiments (lanes 1-8), except that in some transfections the cells were transfected only with haV expression vector (lanes 9-12). Lanes 1-4 depict an analysis of N protein binding to the four different

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polynucleotide resins. Lanes 5-12 depict an analysis of the bound fraction for haV protein. Similarly, lanes 9-16 depict an analysis of V protein that bound to poly(G) in the presence of EGTA (EG), EDTA (ED), or yeast RNA (RNA).

Figure 6 depicts RNA binding by mutant measles virus V proteins. The polyribonucleotide homopolymer binding assay described in Figure 5 was used for analysis of mutant measles virus V proteins (haV 1-11, lanes 3-13; see description for Figure 3). In this experiment, the cells were transfected with only V expression vectors. The cell extract examined in lane 1 was a negative control that contained V protein that did not contain an epitope tag. Poly(G) was used to test all of the mutant proteins.

Figure 7 depicts a comparison of the amino acid sequences in CR2 (amino acids 100-140) for wild-type measles virus V protein, designated haV (SEQ ID NO:7); a mutant where the tyrosine at amino acid 113 and the aspartic acid at amino acid 114 are substituted with alanines, designated haV-5 (SEQ ID NO:8); a mutant where amino acids 112-134 are deleted, designated haV-23 (SEQ ID NO:9); a mutant where the aspartic acid at amino acid 114 and the histidine at amino acid 115 are substituted with alanines, designated haV-24 (SEQ ID NO:10); and a mutant where the tyrosine at amino acid 113, the aspartic acid at amino acid 114 and the histidine at amino acid 115 are substituted with alanines, designated haV-25 (SEQ ID NO:11).

Figure 8 depicts the effect of the CR2 mutations described in Figure 7 in a CAT assay that demonstrated the effect of V protein expression on minireplicon activity in a transient expression assay. Bar 1 was the positive control obtained from cells that were transfected with all plasmid vectors (N, P and L) necessary to drive minireplicon expression. Bar 2 was

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identical to bar 1, except that the cells were also transfected with an expression vector encoding haV; in bars 3-6, the cells were transfected with expression vectors encoding the indicated CR2 mutations. Relative CAT activity was calculated based on 100% activity in bar 1.

Figure 9 depicts a comparison of the amino acid sequences in the C-terminus (amino acids 220-299) for wild-type measles virus V protein, designated haV (SEQ ID NO:12); a mutant where amino acids 232-299 are deleted, designated haV-1 (SEQ ID NO:13); a mutant where the cysteines at amino acids 251 and 255 are substituted with alanines, designated haV-12 (SEQ ID NO:14); a mutant where the cysteines at amino acids 269 and 272 are substituted with alanines, designated haV-13 (SEQ ID NO:15); a mutant where amino acids 279-299 are deleted, designated haV-14 (SEQ ID NO:16); a mutant where amino acids 267-299 are deleted, designated haV-15 (SEQ ID NO:17); a mutant where amino acids 250-299 are deleted, designated haV-16 (SEQ ID NO:18); a mutant where amino acids 243-299 are deleted, designated haV-17 (SEQ ID NO:19); a mutant where amino acids 236-299 are deleted, designated haV-18 (SEQ ID NO:20); a mutant where amino acids 229-299 are deleted, designated haV-19 (SEQ ID NO:21); a mutant where the arginines at amino acids 233 and 234 are substituted with alanines, designated haV-20 (SEQ ID NO:22); a mutant where the arginines at amino acids 233 and 234 are each substituted with aspartic acid, designated haV-21 (SEQ ID NO:23); and a mutant where amino acids 229-237 are deleted, designated haV-22 (SEQ ID NO:24).

Figure 10 depicts the effect of the C-terminal mutations described in Figure 9 in a CAT assay that demonstrated the effect of V protein expression on minireplicon activity in a transient expression assay. Bar 1 was the positive control

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obtained from cells that were transfected with all
plasmid vectors (N, P and L) necessary to drive
minireplicon expression. Bar 2 was identical to bar 1,
except that the cells were also transfected with an
5 expression vector encoding haV; in bars 3-14, the cells
were transfected with expression vectors encoding the
indicated C-terminal mutations. Relative CAT activity
was calculated based on 100% activity in bar 1.

10

Detailed Description of the Invention

Although exemplified with measles virus, the
invention is also applicable to other *Morbilliviruses*,
15 including but not limited to canine distemper virus and
rinderpest virus.

A consideration of the potential connection
between measles virus attenuation and mechanisms
involved in the control of gene expression and
20 replication (30,60) led to the analysis of the V
protein. V protein expression has been linked to viral
pathogenicity (22,23,26-29) and also to control of gene
expression and replication (21,26,27,35,45). The goal
was to further analyze the effect of V protein on
25 measles virus gene expression.

Before constructing mutant V protein
expression vectors, the amino acid similarity between V
proteins from several different *Morbilliviruses* was
examined (Figure 2). Regions of high amino acid
30 identity may contain important functional domains, and
that one or more of these conserved regions (CR) may
participate in minireplicon repression. Alignment of V
proteins from Edmonston wild-type measles virus,
rinderpest virus, dolphin morbillivirus, and canine
35 distemper virus revealed several conserved regions of
notable sequence identity designated CR1-6 (shown in

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Figure 2), in addition to confirming earlier observations (46) that the C-terminus contained seven cysteine residues spaced identically among the *Morbilliviruses*. Several of the CRs were targeted while generating mutant V protein vectors.

In addition to identifying regions containing high levels of identity, computer analysis was used to search for potential functional motifs and the literature was examined for additional clues concerning the possible location of potential measles virus V protein functional domains. The results of these analyses are illustrated on the alignment in Figure 2. Several studies have located regions of V and P protein that influence the interaction with N protein. The extreme amino terminus (N-terminus) of V and P protein, located within CR1, contains sequences that mediate interaction with N protein (34) (Figure 2) in a two-hybrid assay. Additional sequences involved in the V-N protein-protein interaction have been located between amino acids 204 to 230 (27,64) which encompasses CR4. The measles virus V protein zinc-binding domain (51) is in the C-terminus and probably requires at least several of the cysteine residues found in CR5 and CR6. At the N-terminal end of the cysteine-rich domain there is a well-conserved region containing basic amino acids (229-234) that is part of CR5. Near the same region (amino acid 225) in the measles virus V protein is an amino acid substitution found in Edmonston vaccine strains (wild-type glutamic acid to glycine (30)). Finally, in measles virus V protein there was a leucine repeat (amino acids 93-107) that was reminiscent of a leucine zipper motif (65,66). A number of these domains and motifs were attractive candidates for further study using the minireplicon system and V protein mutant expression vectors.

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In addition to preparing a V protein expression vector (Figure 1A), T7 expression plasmids were prepared for the three basic replication apparatus components including Edmonston wild-type N, P and L proteins. To eliminate any potential confusion due to C protein expression from the downstream translation initiation codon in the P and V protein vectors, the C protein open reading frame was modified to prevent translation of C protein. The C protein ATG codon was converted to ACG and the second codon in the C protein open reading frame was converted to a stop codon (TCA to TAA). These modifications were silent with respect to P and V proteins.

Indications that V protein may be involved in regulating measles virus mRNA transcription and genome replication (21,26,27,35,45) suggested an experiment to test whether a minireplicon system would respond to V protein expression. The minireplicon system was set up with Edmonston wild-type components so that V gene mutations that affect wild-type V protein function could be assessed, and potentially apply these findings to future genetic studies of virus attenuation using recombinant wild-type virus. The Edmonston wild-type measles virus-minireplicon (Figure 1B, pMVwt107-CAT) was derived from the p107MV-CAT minireplicon (63) by converting the vaccine leader sequence in pMV107-CAT to the Edmonston wild-type leader sequence (60).

To determine if the wild-type components were capable of driving detectable minireplicon expression, HEP2 cells were transfected with minireplicon DNA and N, P and L protein expression vectors while simultaneously infecting with MVA/T7 (62) to provide T7 RNA polymerase. At 48 hours after transfection, cells were harvested and cell extracts were analyzed for CAT activity. The Edmonston wild-type minireplicon system readily produced CAT activity over background levels

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produced in negative controls that were transfected with all DNAs except the L polymerase protein expression vector (Figure 1C, lanes 1 and 2, and data not shown). This indicated that the CAT activity produced during the transient expression assay was specific and was dependent upon an intact measles virus replication apparatus.

The minireplicon system was then used to determine whether expression of V protein had a detectable effect on minireplicon expression in a transient expression assay. Minireplicon assay transfections were performed with increasing amounts of V protein expression vector. The overall mass of DNA transfected was held constant by including the appropriate amount of expression vector lacking an insert. The effect of increasing the amount of V protein expression vector from 0 to 400 ng is shown in Figure 1C. The positive control in lane 1 showed the amount of CAT activity obtained in the absence of V protein expression (Figure 1C, lane 1). Lane 2 was a negative control that showed that CAT activity was undetectable when the L protein expression vector was omitted. Lanes 3-7 illustrate the negative effect of increasing amounts of V protein expression. Repression of CAT activity was evident at even the lowest amounts of V protein expression vector (lanes 3 and 4) and was very strong at higher amounts, virtually eliminating detectable minireplicon expression when 400 ng of V protein expression plasmid was transfected (lane 7). The two-fold increases in V protein expression vector used in lanes 3-7 also correlated well with observed decreases in relative CAT activity. These results clearly show that one aspect of V protein function can be observed with a minireplicon assay. This readily-detectable negative effect of V protein expression in a minireplicon assay provided a convenient format to

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further examine the minireplicon repression phenotype of V protein mutants.

To begin analyzing V protein mutants, the V protein expression vector (Figure 1A) was modified to include an epitope tag at the amino-terminus of V protein (pMV-haV-wt; Figure 3). This was done to facilitate Western blot detection of protein in lysates from transfected cells and to allow a relative comparison of the stability and steady-state levels of mutant V proteins. The initiator methionine codon of the wild-type V protein expression vector was replaced with the influenza hemagglutinin (HA) epitope tag (67). This modified V protein vector (pMV-haV-wt) also retained the base substitutions that prevent expression of C protein. Testing pMV-haV-wt in minireplicon experiments revealed that the presence of the N-terminal HA tag had no detectable effect on the ability of V protein to repress minireplicon activity (Figure 4, lanes 1-4, and data not shown).

The first series of mutations introduced into pMV-haV-wt was directed at some of the sequence motifs illustrated in Figure 2. One of these mutations resulted in a truncated V protein that lacked the unique V protein C-terminus containing the cysteine residues (amino acids 231-299 were deleted) (Figure 3, pMV-haV-1). Plasmid pMV-haV-2 converted the wild-type V coding sequence to the Edmonston vaccine sequence (amino acid codon 225; glutamic acid to glycine). The remaining six mutations (pMV-haV numbers 3-8) were amino acid substitutions directed at some of the CRs. Initially, amino acid substitutions were introduced in an attempt to alter the function of specific domains in V protein without grossly altering the protein structure. Using a strategy suggested by the charge-to-alanine approach (68,69,70), mutations were introduced that primarily targeted two consecutive

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amino acid codons that specified charged or polar amino acid residues and converted them to codons encoding alanine.

5 Although this invention is exemplified by
mutants having alanines rather than the wild-type
residues, other nonconservative mutations of the wild-
type residues are also within the scope of this
invention. For example, aspartic acid is an acidic
(negatively charged) molecule. Therefore, a
10 nonconservative mutation will be one in which a
substitution is made to an amino acid other than
glutamic acid, which is also an acidic molecule.
Suitable alternative amino acids include the amino
acids lysine, histidine and arginine which are basic
15 (positively charged) molecules. Suitable alternative
amino acids further include the amino acids with
nonpolar functional groups such as alanine, isoleucine,
leucine, methionine, phenylalanine, proline, tryptophan
and valine, and the amino acids with uncharged polar
20 functional groups such as asparagine, cysteine,
glutamine, glycine, serine, threonine and tyrosine.

 Similarly, tyrosine is an uncharged polar
molecule. Therefore, a nonconservative mutation will
be one in which a substitution is made to an amino acid
25 other than asparagine, cysteine, glutamine, glycine,
serine and threonine, which are also uncharged polar
molecules. Suitable alternative amino acids include
the amino acids lysine, histidine and arginine which
are basic (positively charged) molecules. Suitable
30 alternative amino acids further include the amino acids
with nonpolar functional groups such as alanine,
isoleucine, leucine, methionine, phenylalanine,
proline, tryptophan and valine, and the amino acids
with acidic (negatively charged) functional groups such
35 as aspartic acid and glutamic acid.

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Analysis of the mutant V protein vectors 1 to 8 in a minireplicon assay is shown in Figure 4A. The results indicate that two vectors (pMV-haV-1 and pMV-haV-5) had diminished ability to repress minireplicon activity. One mutant (haV-1) was a deletion mutant that lacked the unique C-terminus of V protein (amino acids 231-299); the second mutant (haV-5) contained substitution mutations at amino acids 113 and 114 (located in CR2; see Figure 2). This suggested that this function could be partially mediated independently by CR2 and the C-terminus. Like the C-terminal truncation mutant, the CR2 mutant also retained residual repression activity.

Lanes 1 and 2 on Figure 4A contained control samples. The transfection analyzed in Lane 1 lacked the L protein expression vector and revealed a low level of background CAT activity. The positive control in lane 2 showed the maximal activity observed in the absence of V protein. Repression mediated by 200 and 400 ng of wild-type V protein vector containing the HA tag (pMV-haV-wt) is shown in lanes 3 and 4. As described above, the N-terminal HA tag had little effect on the ability of V protein to repress the minireplicon, and in this experiment, 200 and 400 ng of haV-wt vector repressed minireplicon activity by about 7 and 16 fold, respectively. Most of the mutants repressed CAT activity nearly as effectively as the haV-wt vector, by reducing CAT activity to 20% or less (lanes 3, 7, 9, 11, 15, 17, 19) of the control activity (lane 2), when 200 ng of V protein vector was transfected. Two V protein vectors (pMV-haV-1, lanes 5 and 6; pMV-haV-5, lanes 13 and 14) were noticeably less effective at repressing minireplicon activity in this and repeat experiments. Plasmid pMV-haV-1 lacked the cysteine-rich V protein C-terminus, while pMV-haV-5

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contained a double alanine substitution in CR2 (amino acids 113-114).

Both domains implicated in repression have interesting features. The C-terminus includes the zinc-binding domain and also contains an RNA binding domain (Figure 5). The primary amino acid sequence of CR2 is noteworthy because computer models predict that a small cluster of charged residues may be positioned along one face of a proposed alpha helix (residues 114 D, 118 E, 121 K, 125 D). Thus, CR2 is potentially an amphipathic alpha helix similar to sequences that have been observed in some transcription factors (71).

The repression activity associated with CR2 also is interesting because this amino acid sequence is present in both the V and P proteins. The essential roles played by P protein as polymerase subunit and nucleocapsid assembly factor (72) are quite distinct from the nonessential role of V protein in repression. Yet, these two proteins share a significant amount of amino acid sequence. CR2 may be a site of protein-protein interaction that functions differently in the context of V and P. Without being bound by theory, it may also be possible that the unique C-terminus of V protein imparts a tertiary structure to V protein that exposes CR2, in turn allowing it to function as a repressor domain, while in P protein this domain is sequestered.

To determine if the reduction in repression caused by mutant haV-1 or haV-5 simply correlated with poor protein expression, a Western blot was performed using anti-HA antibody to estimate the relative abundance of V protein in transfected cells (Figure 4B). Cells for this experiment were transfected exactly as performed during minireplicon experiments, so that the cells contained N, P and L proteins as well as V protein. Tagged V proteins were detectable in all

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transfected-cell extracts except for two negative controls. No V protein vector was transfected in cells used to make extract analyzed in lane 1, and in lane 2, the cells were transfected with the vector encoding the V protein that does not include an epitope tag. The protein detected in lane 3 was the HA-tagged wild-type V protein. The protein in lane 4 was the truncation mutant that lacked the C-terminus and its mobility was noticeably altered. The remaining proteins were mutants (as described above) that contained amino acid substitutions (Figure 4B, lanes 5-11). Several of these proteins had small but noticeable mobility changes in this 12% polyacrylamide gel (for example, lanes 10 and 11). However, this was not unexpected, since charged and polar residues were replaced by alanines in these proteins. The relative abundance of all the proteins was judged to be quite similar, except that haV-1 (lane 4) was lower in this experiment and this was reproducible in repeat experiments. This suggested that the reduced repression activity of haV-1 may result from lower proteins levels, while the reduced repression activity of haV-5 was not caused by an unstable protein.

To further examine the possibility that haV-1 repressed poorly simply because it was unstable, a minireplicon assay was performed with increasing amounts of V protein vector to help supplement the intracellular quantity of haV-1 protein (Figure 4C). This minireplicon experiment was performed as described above, except that the maximum amount of V protein vector was increased from 400ng to 1 μ g. The mass of DNA was held constant in all transfections by including the appropriate quantity of vector backbone DNA (lacking insert). Figure 4C, lane 1, was the positive control that was performed without added V protein vector. Lanes 2 and 3 show the effect of adding wild-

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type ha-V vector at 100 (lane 2) and 400 (lane 3) ng, which reduced activity in this experiment to 58% and 26%, respectively. Addition of increasing amounts of haV-1 had little effect on the activity of MV-CAT and no obvious trend was observed for correlating increasing mass of haV-1 vector with greater levels of repression. This suggested that the diminished repression activity of haV-1 was not simply due to reduced intracellular protein levels and was instead an effect of the loss of the C-terminus.

A region containing a high content of basic amino acids in the N-terminal third of the SV5 V protein mediates RNA binding (73). A homologous region is not apparent in measles virus V protein (73), but the possibility was examined that measles virus V protein may also bind RNA through the zinc finger-like sequences in the C-terminus (Figure 5).

To assess the RNA binding activity of V protein, a polyribonucleotide homopolymer binding assay was used (Figure 5A) that has been used successfully to study several cellular RNA binding proteins (74-77). A number of RNA binding proteins display characteristic affinity for polyribonucleotide homopolymers. For example, the cellular hnRNP U protein binds with relatively high affinity to poly(G) and poly(U,) but binds little if at all to poly(A) or poly(C) (75). This concept was used to determine if V protein in transfected cell extracts could be captured on homopolymer resins. Cells were transfected with all of the expression vectors (N, P, L, V and minireplicon) used in the minireplicon transfection and were then infected with MVA/T7. Cytoplasmic cell lysates were prepared 48 hours later and incubated for 30-60 minutes at 4°C with agarose beads linked to one of the four polyribonucleotide homopolymers. The beads were collected by centrifugation, then resuspended and

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washed three times in cell lysis buffer. The bound proteins were eluted from the beads by boiling in SDS-gel loading buffer.

5 Initially, to test the assay, the ability to capture a known RNA binding protein expressed from a transfected plasmid was examined. Accordingly, proteins eluted from the four different resins were analyzed by Western Blot using monoclonal antibody specific for N protein (Figure 5B). N protein was detected readily in samples bound to poly(G) and poly(U) (lanes 2 and 4). The amount of N protein bound to poly(G) was generally greater in this assay and several repeat experiments. Little or no N protein was captured with poly(A) or poly(C).

15 The experiment was repeated as described above to analyze the ability of haV protein to interact with the homopolymer resins (Figure 5B, lanes 5-8). Analysis of a Western blot with anti-HA antibody revealed results that were similar to the results obtained after analysis of N protein (Figure 5B, lanes 1-4). The poly(G) and poly(U) resins both displayed affinity for haV (lanes 6 and 8). V protein bound best to poly(G) and more weakly to poly(U), while binding poorly to poly(A) or poly(C). In some experiments, a very weak band was seen, indicating a low affinity interaction with poly(A); however, usually this interaction is undetectable.

20 The fact that V protein bound readily to poly(G) and poly(U) and not well to poly(A) and poly(C) indicated that the assay was not simply measuring affinity for polyanion resins, and also showed that V protein demonstrated a preference for two polynucleotides. Both V protein and N protein preferred poly(G) and poly(U) (Figure 5); this is a feature displayed by a number of cellular RNA binding proteins (74,76,77).

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The fact that the binding profile of haV protein was quite similar to N protein raised the possibility that V protein was captured indirectly on poly(G) and poly(U) resins through interaction with N protein, rather than a direct interaction with RNA. To examine this possibility, cells were transfected only with the haV protein expression vector and cell extracts were prepared and treated as described above. Analysis by Western blot showed that haV protein (Figure 5B, lanes 9-12) was again bound to poly(G) and poly(U). This indicated that the interaction of V protein with homopolymer resins required no other viral protein. In fact, in the absence of other viral proteins, greater quantities of V protein were usually bound to poly(G) and poly(U) (compare lanes 6 to 10 and 8 to 12). This suggested that one of the other viral proteins may slightly interfere with the interaction between V protein and the polyribonucleotides.

A limited analysis of conditions that influence the binding of haV to poly(G) was also performed (Figure 5B, lanes 13-16). Addition of 10 mM EGTA or EDTA (lanes 14 and 15) to the binding reaction reduced the quantity of V protein bound to poly(G). Addition of yeast RNA competitor (lane 16) to the binding reaction (25 μ g per ml) had little effect on binding. However, this was not especially surprising, because the cytoplasmic extracts already contained significant quantities of cellular RNA and addition of yeast RNA likely had little impact. These results suggest that binding of V protein to poly(G) is stimulated by divalent cations and is a relatively specific interaction, because addition of a nonspecific RNA (yeast RNA) did not seem to diminish the amount of V protein bound to the resin.

To determine if RNA binding could be correlated with the ability to repress minireplicon

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activity, the poly(G) binding assay was next used to analyze the RNA binding activity of the haV protein mutants. The haV protein mutants (Figure 3) were expressed in cells without any other viral proteins. Crude cytoplasmic extracts were prepared and bound to poly(G) resins as described above, and bound proteins were analyzed by Western blotting (Figure 6). As shown earlier, haV was captured by poly(G) and detected by anti-HA antibody on a Western blot (lane 2), while a sample containing V protein without an HA-tag (lane 1) generated no background signal. Analysis of the mutant proteins revealed similar levels of poly(G) binding activity for most of the V proteins (lanes 4-13). The exception was mutant haV-1. Very low levels of this mutant protein bound to poly(G). This clearly indicated that the protein was defective for binding (lane 3). The low but detectable levels of binding by haV-1 indicated that RNA binding activity was substantially reduced, but not entirely eliminated. These results demonstrated that most of the mutants retained RNA binding activity nearly equivalent to wild-type haV. One of the two mutants that was defective for minireplicon repression (haV-5) bound poly(G) as well as wild-type haV, while the other mutant (haV-1) exhibited significantly reduced poly(G) binding activity. This suggested that there may be a correlation between minireplicon repression and RNA binding activity mediated by the unique C-terminus of V protein. In turn, this suggested that V protein RNA binding activity may play a regulatory role in measles virus gene expression and replication.

A potential connection between RNA binding activity associated with V protein and minireplicon repression could be drawn from the results that showed that a V protein mutant lacking the C-terminus

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cysteine-rich region bound RNA poorly and was also a less effective minireplicon repressor.

In a separate experiment, minireplicon repression was shown not to correlate with the ability of V protein to bind N protein. Formation of a V-N protein complex was not necessary for V protein-mediated minireplicon repression; V protein mutants haV-9 and haV-11 (Figure 3) failed to interact with N protein, but retained full repression activity (data not shown).

Analysis of V protein mutants suggested that the C-terminus was required for high affinity RNA binding. Deletion of this region that includes the zinc-binding domain (51) greatly reduced the amount of protein that could be collected on poly(G) resins, but did not entirely eliminate all binding. This may suggest that there is a second weak RNA binding domain in V protein. Without being bound by the following, an attractive possibility is that the zinc finger-like domain in the C-terminus forms one important component of a nucleic acid binding domain, and a second domain in V protein cooperates with the C-terminus to create a higher affinity binding site.

These studies of RNA binding also showed that V protein could interact with RNA without any additional viral proteins present in crude cell extracts. This suggests that V protein directly bound to RNA. This conclusion is not absolute, because it remains possible that V protein interacts with a cellular protein that in turn is responsible for binding to RNA. If this model were true, the data indicates that the C-terminus may mediate the protein-protein interaction responsible for interaction with a cellular factor. Purified recombinant V protein will be important to further examine whether V protein directly binds RNA.

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Although the mutant haV-5 protein was defective for minireplicon repression (Figure 4), it bound to RNA (Figure 6) as well as the wild-type protein. Thus, the minireplicon repression activity associated with CR2 did not readily correlate with the other two activities analyzed (RNA binding and interaction with N protein). For CR2 to function effectively as a repressing motif, it may require an intact RNA binding domain in the C-terminus of V protein. V protein may be similar to some RNA polymerase II transcription factors and it has a modular structure (78).

To more fully test the possibility that CR2 was directly involved in repression, this domain (amino acids 112-134) was deleted to generate haV-23 (Figure 7; SEQ ID NO:9). Surprisingly, this deletion did not greatly affect repression activity (Figure 8). The fact that CR2 could be deleted without affecting repression argues that CR2 does not contain a domain that actively participates in repression. Instead, it appears that the defect caused by the substitution of amino acids 113 plus 114 found in haV-5 (Figures 7 and 8) generated a dominant-negative effect (previous Western blot analysis indicated that the defect in repression was not caused by haV-5 instability).

Without being bound by theory, the YD to AA substitution in haV-5 generated a subtle alteration in the tertiary structure of V protein that partially blocks repression activity. Alternatively, there may be interaction between V protein and other viral proteins or cellular proteins. The double alanine substitution may favor a protein-protein interaction that inhibits repression activity or sequesters V protein in an inactive protein complex.

The concept that the weak repression phenotype was caused by a dominant-negative effect

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created by the double alanine substitution was tested further by making additional amino acid substitutions that were very similar but distinct from the mutations found in haV-5 (Figure 7). The original YD to AA substitution in haV-5 was engineered in a well-conserved motif consisting of VYDH located at the amino boundary of CR2 (Figure 2). This motif was identical in V protein sequences from measles virus, canine distemper virus, rinderpest virus and dolphin morbillivirus. In mutant haV-24 (SEQ ID NO:10), the VYDH sequence was converted to VYAA, and in mutant haV-25 (SEQ ID NO:11), VYDH was converted to VAAA. Both of these mutants repressed MV minireplicon gene expression as well as the wild-type haV protein (Figure 8). This implies that the haV-5 defect is very specific for the VYDH to VAAH substitution.

The possibility that a C-terminal zinc-finger-like domain plays a role in repression was examined further by introducing amino acid substitutions in pairs of cysteine residues in the C-terminus (Figure 9). Mutant haV-12 (SEQ ID NO:14) was generated with cysteine residues 1 and 2 (amino acids 251 and 255) converted to alanines. Similarly, mutant haV-13 (SEQ ID NO:15) was generated with C-terminal cysteine residues 4 and 5 (amino acids 269 and 272) replaced with alanines. Cysteine residues were mutated in pairs expecting that this would significantly alter a zinc-finger-like structure in the C-terminus and produce a readily detectable alteration in repression activity.

Surprisingly, CAT expression studies of these mutants (Figure 10) revealed that they were only mildly affected by the cysteine substitutions; both haV-12 and haV-13 retained a significant proportion of wild-type haV protein repression activity (about 65% of wild-type). This result was somewhat unexpected; it was

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5 predicted that substitution of zinc-coordinating
cysteine residues would abolish repression. Possibly,
substitution of only two out of seven cysteine residues
did not completely disrupt the putative zinc-finger
nucleic acid binding structure. Alternatively, it is
possible that the putative zinc-finger motif plays a
less prominent role in repression than previously
predicted. Thus, two additional series of mutations
were prepared to further examine the role of the C-
10 terminus in repression.

In one set of mutations, small incremental
deletions were made starting from the C-terminus (haV-
14 through haV-19; SEQ ID NOS:16-21). The smallest
deletion (haV-14) removed 21 amino acids from the C-
15 terminus, whereas the largest deletion (haV-19) removed
71 amino acid residues. Mutant haV-19 was constructed
even though it is nearly identical to haV-1 (Figure 9;
SEQ ID NO:13), because the deletion in haV-19 removed
several additional amino acids that were part of the
20 small basic motif located between amino acids 229-234,
and the phenotype of haV-19 could be viewed as
verification of the mutant phenotype displayed by haV-
1.

25 Analysis of the C-terminal mutants in the
minireplicon assay revealed that the large deletion
mutants (haV-1 and haV-19) behaved similarly; they
repressed minireplicon gene expression only about two
fold or less instead of the 5-8 fold repression induced
by wild-type haV (Figure 10). Interestingly, the
30 diminished repression activity displayed by the large
deletions was reproduced by all of the smaller
truncation mutants. Even the smallest C-terminal
deletion (haV-14) resulted in a V protein that
repressed only about two fold. This result implied
35 that the integrity of the extreme C-terminus is
essential for full repression activity.

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In addition to analyzing the effect of the C-terminal deletion mutations, several additional mutations were generated to examine the possibility that the basic motif (KKGHRR; amino acids 229-237, SEQ ID NO:12) at the beginning of the unique V protein C-terminus played some role in repression. Conversion of the two arginine residues to alanine (haV-20; SEQ ID NO:22) or aspartic acid (haV-21; SEQ ID NO:23) had an effect that was similar to the C-terminal deletion mutations. The two substitution mutants repressed minireplicon expression by an average of only 1.5 to 3 fold whereas wild-type V protein repression normally ranged from 5-8 fold (Figure 10). These results implied that perturbation of the basic motif also disrupted normal V protein repression function.

This result was examined further by deleting the basic motif (Figure 9, haV-22; SEQ ID NO:24), while leaving the remainder of the C-terminus intact. Surprisingly, one preliminary experiment has indicated that deletion of the basic motif had no discernable effect on repression activity; haV-22 repressed CAT activity by about nine-fold (Figure 10). Taken together, these results suggest that amino acid substitutions in the basic motif produce a dominant-negative effect on repression activity possibly by causing an unfavorable change in protein tertiary structure. On the other hand, deletion of the basic motif produced little change in the ability of V protein to repress minireplicon gene expression.

The mechanism of measles virus V protein minireplicon repression is unknown. The results suggest that the mechanism does not require interaction with N protein, but does involve interaction with RNA. RNA binding could result in reduced CAT levels in a minireplicon assay through a number of different mechanisms. For example, RNA binding activity could

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repress translation if V protein binds viral mRNAs. V protein could also influence rates of encapsidation if it binds nascent viral RNAs and prevents association of RNA with N protein. Conversely, it could somehow stimulate nucleocapsid assembly, thereby forcing the minireplicon system to overproduce genomic-length RNA at the expense of mRNA. V protein could also be envisioned as analogous to a transcription factor that binds at or near the promoter and represses transcription. If V protein is analogous to a transcription factor, it could be a regulator of the switch that balances genome synthesis and mRNA synthesis.

Dissection of V protein functions provides a basis for introducing attenuating mutations in candidate *Morbillivirus* strains for use in immunogenic compositions. Published studies have shown that elimination of V protein expression results in attenuated viral replication (19,22,23,26-29). Varying degrees of attenuation are introduced by targeting amino acid substitutions to specific domains of V protein, rather than eliminating expression. For example, partial loss of V protein repression function is achieved by mutation of one or more cysteine residues in the unique C-terminus. Substitutions in a region shared by P and V are suitable, such as the alanine substitutions in CR2, if they have an effect on V protein function without much effect on P protein. These mutations are evaluated using the reverse genetic systems for "rescue", as described in the art (19,79,80,81).

The reverse genetics system must be used to generate an infectious *Morbillivirus* containing the mutations of this invention, because naked genomic RNA cannot serve as a template for transcription and replication. Instead, these genomic sequences are

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recognized only when they are entirely encapsidated by the N protein into the nucleocapsid structure. It is only in that context that the genomic and antigenomic terminal promoter sequences are recognized to initiate the transcription or replication pathways.

Transcription and replication of negative-sense, single-stranded RNA viral genomes are achieved through the enzymatic activity of a multimeric protein acting on the ribonucleoprotein core (nucleocapsid). All *Morbilliviruses* require the three viral proteins, N, P and L, for these pathways to proceed.

The mutations described herein are introduced into *Morbillivirus* strains by using site-directed mutagenesis. One or more mutations as defined herein are introduced into a *Morbillivirus* strain. Cumulative effects of different combinations of mutations can be assessed.

This invention is exemplified with the minireplicon system. The changes in nucleotide sequences encoding the mutations to the CR2 and the C-terminus of the V protein of *Morbilliviruses* (as well as to the promoter region and L protein, the N, P and/or C proteins and/or the F gene-end signal of measles virus) can be inserted readily into full length viruses using techniques known in the art. The mutations are introduced by standard recombinant DNA methods into a DNA copy of the viral genome. This may be a wild-type or a modified viral genome background, thereby generating a new virus. Infectious clones or particles containing these mutations are generated using the cDNA rescue system, which has been applied to a variety of negative-sense RNA viruses, including Sendai virus (82); measles virus (83,88); respiratory syncytial virus (84); PIV-3 (85); rabies (86); vesicular stomatitis virus (VSV) (87); and rinderpest

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virus (89); these references are hereby incorporated by reference.

Briefly, all *Morbillivirus* rescue systems can be summarized as follows: Each requires a cloned DNA equivalent of the entire viral genome placed between a suitable DNA-dependent RNA polymerase promoter (e.g., the T7 RNA polymerase promoter) and a self-cleaving ribozyme sequence (e.g., the hepatitis delta ribozyme) which is inserted into a propagatable bacterial plasmid. This transcription vector provides the readily manipulable DNA template from which the RNA polymerase (e.g., T7 RNA polymerase) can faithfully transcribe a single-stranded RNA copy of the viral antigenome (or genome) with the precise, or nearly precise, 5' and 3' termini. The orientation of the viral genomic DNA copy and the flanking promoter and ribozyme sequences determine whether antigenome or genome RNA equivalents are transcribed. Also required for rescue of new virus progeny are the virus-specific trans-acting proteins needed to encapsidate the naked, single-stranded viral antigenome or genome RNA transcripts into functional nucleocapsid templates: the viral nucleocapsid (N) protein, the polymerase-associated phosphoprotein (P) and the polymerase (L) protein. These proteins comprise the active viral RNA-dependent RNA polymerase which must engage this nucleocapsid template to achieve transcription and replication.

Typically, these viral trans-acting proteins are generated from one or more plasmid expression vectors encoding the required proteins, although some or all of the required trans-acting proteins may be produced within mammalian cells engineered to contain and express these virus-specific genes and gene products as stable transformants.

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The typical (although not necessarily exclusive) circumstances for rescue include an appropriate mammalian cell milieu in which T7 polymerase is present to drive transcription of the antigenomic (or genomic) single-stranded RNA from the viral genomic cDNA-containing transcription vector. Either cotranscriptionally or shortly thereafter, this viral antigenome (or genome) RNA transcript is encapsidated into functional templates by the nucleocapsid protein and engaged by the required polymerase components produced concurrently from co-transfected expression plasmids encoding the required virus-specific trans-acting proteins. These events and processes lead to the prerequisite transcription of viral mRNAs, the replication and amplification of new genomes and, thereby, the production of novel viral progeny, i.e., rescue.

For the rescue of the non-Morbilliviruses rabies, VSV and Sendai, T7 polymerase is provided by recombinant vaccinia virus VTF7-3. This system, however, requires that the rescued virus be separated from the vaccinia virus by physical or biochemical means or by repeated passaging in cells or tissues that are not a good host for poxvirus. For measles virus cDNA rescue (and presumably for other Morbilliviruses), this requirement is avoided by creating a cell line that expresses T7 polymerase, as well as viral N and P proteins. Rescue is achieved by transfecting the genome expression vector and the L gene expression vector into the helper cell line. Advantages of the host-range mutant of the vaccinia virus, MVA-T7, which expresses the T7 RNA polymerase, but produces little or no infectious progeny in mammalian cells, are exploited to rescue measles virus and other Morbilliviruses. After simultaneous expression of the necessary encapsidating proteins, synthetic full length

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antigenomic viral RNA are encapsidated, replicated and transcribed by viral polymerase proteins and replicated genomes are packaged into infectious virions. In addition to such antigenomes, genome analogs have now
5 been successfully rescued for Sendai and PIV-3 (85,90).

The rescue system thus provides a composition which comprises a transcription vector comprising an isolated nucleic acid molecule encoding a genome or antigenome of a *Morbillivirus*. The nucleic acid
10 molecule contains at least one mutation in the region corresponding to amino acids 112-134 of the measles virus V protein (and, optionally, other mutations described herein), together with at least one expression vector which comprises at least one isolated
15 nucleic acid molecule encoding the trans-acting proteins necessary for encapsidation, transcription and replication (e.g., N, P and L for a *Morbillivirus*). Host cells are then transformed, infected or transfected with the at least two expression vectors
20 just described. The host cells are cultured under conditions which permit the co-expression of these vectors so as to produce the infectious modified virus.

The rescued infectious virus is then tested for its desired phenotype (reduced repression by V
25 protein, temperature sensitivity, cold adaptation, plaque morphology, and transcription and replication attenuation), first by *in vitro* means. The mutations in the N, P or C genes or the F gene-end signal of measles virus are also tested using the minireplicon system
30 where the required trans-acting encapsidation and polymerase activities are provided by wild-type or vaccine helper viruses, or by plasmids expressing the N, P and different L genes harboring gene-specific attenuating mutations (63,83).

35 If the attenuated phenotype of the rescued virus is present, challenge experiments are conducted

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with an appropriate animal model. Non-human primates provide the preferred animal model for the pathogenesis of human disease. These primates are first immunized with the attenuated, recombinantly-generated virus, then challenged with the wild-type form of the virus. Monkeys are infected by various routes, including but not limited to intranasal, intratracheal or subcutaneous routes of inoculation (91).

Experimentally infected rhesus and cynomolgus macaques have also served as animal models for studies of vaccine-induced protection against measles (92).

Protection is measured by such criteria as disease signs and symptoms, survival, virus shedding and antibody titers. If the desired criteria are met, the recombinantly-generated virus is considered a viable candidate vaccine or immunogenic composition for testing in humans. The "rescued" virus is considered to be "recombinantly-generated", as are the progeny and later generations of the virus, which also incorporate the mutations.

Even if a "rescued" virus is underattenuated or overattenuated relative to optimum levels for vaccine use, this is information which is valuable for developing such optimum strains.

Optimally, a codon containing a point mutation may be stabilized by introducing a second or a second plus a third mutation in the codon without changing the amino acid encoded by the codon bearing only the point mutation. Infectious virus clones containing the stabilizing mutations are also generated using the cDNA "rescue" system described above.

Previously, in published International patent application WO98/13501 (93), which is hereby incorporated by reference, the generation and isolation of recombinantly-generated, attenuated, nonsegmented, negative-sense, single-stranded RNA viruses of the

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Order Mononegavirales (such as measles virus) having at least one attenuating mutation in the 3' genomic promoter region and having at least one attenuating mutation in the RNA polymerase gene was disclosed.

5

Specifically, these mutations comprised:

(1) at least one attenuating mutation in the 3' genomic promoter region selected from the group consisting of nucleotide 26 (A → T), nucleotide 42 (A → T or A → C) and nucleotide 96 (G → A), where these nucleotides are presented in positive strand, antigenomic, message sense; and

10

(2) at least one attenuating mutation in the RNA polymerase gene selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 331 (isoleucine → threonine), 1409 (alanine → threonine), 1524 (threonine → alanine), 1649 (arginine → methionine), 1717 (aspartic acid → alanine), 1936 (histidine → tyrosine), 2074 (glutamine → arginine) and 2114 (arginine → lysine).

20

Furthermore, in in published International patent application WO99/49017 (94), which is hereby incorporated by reference, the generation and isolation of recombinantly-generated, attenuated, measles viruses having at least one attenuating mutation in the N, P or C genes or in the F gene-end signal, was disclosed.

25

Specifically, these mutations comprised:

(1) for the N gene, at least one attenuating mutation selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 129 (glutamine → lysine), 148 (glutamic acid → glycine) and 479 (serine → threonine);

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(2) for the P gene, at least one attenuating mutation selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 225 (glutamic acid → glycine), 275 (cysteine → tyrosine) and 439 (leucine → proline);

(3) for the C gene, at least one attenuating mutation selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 73 (alanine → valine), 104 (methionine → threonine) and 134 (serine → tyrosine); and

(4) for the F gene-end signal (the cis-acting transcription termination signal), the change at nucleotide 7243 (T → C), where these nucleotides are presented in positive strand, antigenomic, that is, message (coding) sense.

Individual or combinations of attenuating mutations from either or both of these sets of mutations can be incorporated into the *Morbilliviruses* of this invention, including specifically those with at least one mutation in the region corresponding to amino acids 112-134 of the V protein, as well as to those containing both such a mutation in amino acids 112-134 and a mutation in or deletion of at least a portion of the C-terminal region beginning at amino acid 231.

The viruses of this invention are used to formulate a vaccine or immunogenic composition. To do so, the virus is adjusted to an appropriate concentration and formulated with any suitable adjuvant, diluent or carrier. Physiologically acceptable media may be used as carriers and/or diluents. These include, but are not limited to: water, an appropriate isotonic medium, glycerol, ethanol and other conventional solvents, phosphate

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buffered saline and the like. Suitable adjuvants include, but are not limited to aluminum phosphate, aluminum hydroxide, MPL™ (3-O-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, MT, now Corixa), synthetic lipid A analogs such as 529 (Corixa), Stimulon™ QS-21 (Aquila Biopharmaceuticals, Framingham, MA) and IL-12 (Genetics Institute, Cambridge, MA).

In one embodiment of this invention, the formulation including the *Morbilivirus* is intended for use as a vaccine or immunogenic composition. The virus may be mixed with cryoprotective additives or stabilizers such as proteins (e.g., albumin, gelatin), sugars (e.g., sucrose, lactose, sorbitol), amino acids (e.g., sodium glutamate), saline, or other protective agents. This mixture is maintained in a liquid state, or is then desiccated or lyophilized for transport and storage and mixed with water immediately prior to administration.

Formulations comprising the *Morbiliviruses* of this invention are useful to immunize a human or other vertebrate subject to induce protection against infection by the wild-type counterpart of the virus. Thus, this invention further provides a method of immunizing a subject to induce protection against infection by a *Morbilivirus* by administering to the subject an effective immunizing amount of a formulation of the vaccine or immunogenic composition incorporating a version of that virus generated as described hereinabove.

A sufficient amount of the vaccine or immunogenic composition in an appropriate number of doses is administered to the subject to elicit an immune response. Persons skilled in the art will readily be able to determine such amounts and dosages. Administration may be by any conventional effective

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form, such as intranasally, parenterally, orally, or topically applied to any mucosal surface such as intranasal, oral, eye, lung, vaginal or rectal surface, such as by an aerosol spray. The preferred means of administration is by intranasal administration.

All patents and publications cited herein are hereby incorporated by reference.

In order that this invention may be better understood, the following examples are set forth. The examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention.

Examples

Example 1

Cells and Virus

HEp2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Chicken embryo fibroblasts (CEFs; SPAFAS, Inc) were maintained in the same media. The attenuated strain of vaccinia virus that expresses phage T7 RNA polymerase (MVA/T7; 62) was grown in CEFs. Plaque assays were performed also on CEFs.

Example 2

Recombinant DNA

The measles virus N, P and L protein expression clones (Figure 1A) were each prepared from infected-cell total RNA by reverse transcription and PCR amplification (RT/PCR) with gene-specific primers, followed by cloning into an appropriate T7 RNA polymerase-dependent expression vector (61). Vero cells were infected with the Edmonston wild-type strain

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of measles virus, and when about 70% or more of the cells exhibited a cytopathic effect, RNA was prepared by the guanidinium-phenol extraction method (95). RT/PCR was performed with avian myoblastosis virus RT and Pwo polymerase contained in the one-tube Titan amplification kit (Roche Molecular Biology). The RT step was performed for 30-60 minutes at 47°C, followed by 30-35 cycles of PCR amplification. Amplified DNA fragments were cloned into a T7 expression plasmid (Figure 1; (61,83)) with the translation initiation codon placed in the NcoI site of the vector. Cloned DNAs were checked by cycle-sequencing (96) and nucleotide substitution errors were corrected by oligonucleotide mutagenesis using the Morph kit (5prime-3prime, Inc.) or by replacing subfragments with newly-amplified DNA fragments as described previously (96).

The initial V protein expression clone was prepared by PCR amplification from an Edmonston wild-type full-length cDNA clone using primers flanking the V protein coding region. The amplified DNA was cloned into the T7 expression vector and the additional G nucleotide residue required to generate the V gene frameshift was added at the editing site (18) by oligonucleotide-directed mutagenesis. Wild-type and mutant V protein expression vectors were also prepared with an influenza virus hemmagglutinin epitope tag (HA tag; (67)) at the amino terminus. The T7 vector plasmid was modified to include a sequence that includes an initiation codon and encodes the HA epitope tag (CC ATG GCT TAT CCT TAT GAC GTG CCT GAC TAT GCC) (SEQ ID NO:5), followed by a polylinker (plasmid pT7/HA). The V protein coding region was cloned with the HA tag at the amino terminus. This served to replace the V protein initiator methionine codon, resulting in the generation of a plasmid designated

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pmV-haV-wt. V protein mutants were prepared in the pmV-haV-wt backbone by oligonucleotide-directed or deletion mutagenesis.

5 The primer designed to amplify the 5' end of the P and V coding regions (CGGCCATGGCAGAAGAGACAGGCACGCCACCGTAAAAACGGAC) (SEQ ID NO:6) contained two base changes (underlined) to disrupt the downstream C protein open reading frame. These changes were silent with respect to the P and V open reading frames. The same nucleotide changes were carried over to the pT7MV-haV constructs.

10 For all protein expression constructs, the cDNA insert was cloned 3' of an internal ribosome entry site (IRES) to facilitate translation of the T7 RNA polymerase transcript. A stretch of 50 adenosine residues was located at the 3' end, followed by a T7 RNA polymerase terminator. Both the P and V expression vectors contained base substitutions designed to disrupt translation initiation from the downstream C protein open reading frame.

15 The measles virus minireplicon (pmVwt107-CAT, Figure 1B) was a derivative of pmV107-CAT (63). Plasmid pmV107-CAT contained the leader sequence found in vaccine strains of measles virus (60) and was converted to plasmid pmV107wt-CAT (which contained the wild-type leader) using oligonucleotide-directed mutagenesis.

30 Example 3

Transient Expression Experiments

35 Analysis of transient minireplicon expression was performed essentially as described previously (96) using varying amounts of viral protein expression vectors (Figure 1B) and a measles virus minireplicon containing the CAT reporter gene (Figure 1B). The

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measles virus minireplicon was a derivative of pMV107-CAT (63) containing the CAT reporter gene and the measles leader sequence from the Edmonston wild-type strain of measles virus (60). HEP2 cells in six-well plates were used for transfection when the cells were about 70-90% confluent. Transfection mixes were prepared by combining minireplicon DNA (50-200ng pMVwt107-CAT) and expression plasmids (400 ng pMVwt-N, 300 ng pMVwt-P[C], 100 ng pMVwt-L) in 200 μ l of serum-free OptiMEM. V protein expression plasmids were included in this mix according to the amounts ranging from 25-400ng, as specified in Figure 1C. Lipofectace (12-15 μ l; Invitrogen/Life Technologies) was added to the DNA-medium mixture and incubated for 20 to 30 minutes at room temperature. A separate MVA/T7 mixture was prepared in sufficient quantity to provide 0.8 ml of serum-free OptiMEM containing enough MVA/T7 to infect each well of cells to be transfected with about 2 pfu per cell. Before initiating transfection, the DNA-medium-Lipofectace transfection mix was combined with 800 μ l of the MVA/T7-media mix and mixed gently by pipetting. Next, the culture media was removed from the cell monolayers and the combined 1ml transfection mixture was added to the cells.

After overnight incubation, the transfection mixture and media were replaced with DMEM supplemented with 10% FBS and the cells were incubated an additional day. About 48 hours after the start of transfection, the cells were harvested and extracts prepared for analysis of CAT activity as described previously (96). The expression of CAT is shown in Figure 1C. In some experiments, proteins in crude cell extracts were analyzed by Western blotting to monitor protein expression (97). Transfected cells were lysed using TN buffer (50 mM Tris [pH 7.4], 150 mM NaCl) supplemented with 0.2% NP40. The cell extracts were cleared by

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centrifugation to remove nuclei and an equal volume of Laemmli sample buffer (62.5 mM Tris [pH 6.8], 25% glycerol, 2% SDS, 0.01% bromophenol blue) was added to the cytoplasmic extract. The samples were adjusted to contain approximately 2.5% β -mercaptoethanol and then boiled. SDS polyacrylamide gel electrophoresis and electroblotting were performed using standard protocols (97). Epitope-tagged V protein was detected using either mouse monoclonal antibody 12CA5 (Roche Molecular Biology) or rat monoclonal antibody 3F10 (Roche Molecular Biology). Detection was performed with a peroxidase-conjugated secondary antibody (Sigma) and chemiluminescence reagents (Roche Molecular Biology or New England Nuclear).

Example 4

Repression of Minireplicon Expression by Mutant Measles Virus V Proteins

The transient minireplicon expression CAT assay described in Example 3 was repeated using varying amounts of the measles virus V protein mutants haV-1 to haV-8, which have the following differences from the wild-type sequence (haV-wt) (see Figure 3):

- haV-1 Deletion of amino acids 231-299
- haV-2 Mutation of glutamic acid at amino acid 225 to glycine
- haV-3 Mutations of lysines at amino acids 229 and 230 to alanines
- haV-4 Mutations of lysine and threonine at amino acids 204 and 209 to alanines
- haV-5 Mutations of tyrosine and aspartic acid at amino acids 113 and 114 to alanines
- haV-6 Mutations of leucine and glutamine at amino acids 100 and 101 to alanines

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haV-7 Mutations of glutamic acid and
cysteine at amino acids 14 and 15 to
alanines

haV-8 Mutations of glutamic acid at amino
acids 3 and 4 to alanines

5
10
15
Either 200 or 400ng of each V plasmid
(encoding wild-type or haV-1 through haV-8 V protein)
was used and the relative CAT activity was measured as
a percentage of the activity resulting from a
transfection performed without any V protein expression
vector (lane 2). The results are depicted in Figure
4A. A lower percentage correlates with a higher degree
of repression of CAT expression. The expression of V
protein was monitored by a Western blot which was
analyzed with anti-HA antibody (Figure 4B).

The CAT assay was repeated using increasing
amounts (100ng to 1 μ g) of the V plasmid encoding haV-1
and the relative activities are depicted in Figure 4C.

20 Example 5

RNA Binding Assays

25
30
35
RNA binding assays (74,76,77) were performed
to evaluate the binding of measles virus V protein to
RNA, using agarose resins couple with
polyribonucleotides (Sigma). Transfected cells were
lysed as described above using TN buffer supplemented
with 0.5% NP40, 5% glycerol, 1mM MgCl₂, 1mM ZnCl₂ and
protease inhibitor cocktail (Roche Molecular Biology).
Agarose resins containing polyribonucleotides
(approximately 25-50 μ l of settled volume of beads)
were added to the cleared cell lysate and incubated 30-
60 minutes at 4°C with rocking. After incubation, the
resins were washed three times to remove unbound
proteins. Proteins were eluted from the resins by
boiling in Laemmli buffer supplemented with 2.5% β -

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mercaptoethanol. The procedure is summarized by the flow diagram of Figure 5A. Proteins captured by the polynucleotide resins were analyzed by Western blotting as described above. The assay was carried out first with wild-type V protein, and then with haV-1 through haV-11. The mutants haV-1 through haV-8 are as described above; the mutants haV-9 through haV-11 have the following differences from the wild-type sequence (haV-wt) (see Figure 3):

- 10 haV-9 Deletion of amino acids 1-20
- haV-10 Deletion of amino acids 208-230
- haV-11 Deletion of amino acids 1-20 and 208-230

15 The results are depicted in Figures 5B (wild-type) and 6 (mutants).

Example 6

Repression of Minireplicon Expression by Additional CR2 Mutant Measles Virus V Proteins

20 The transient minireplicon expression CAT assay described in Examples 3 and 4 was repeated using the measles virus V protein CR2 mutants haV-5 and haV-23 to haV-25, which have the following differences from the wild-type sequence (haV) (SEQ ID NO:7) in CR2 (amino acids 100-140; see Figure 7):

- 25 haV-5 Mutations of tyrosine and aspartic acid at amino acids 113 and 114 to alanines (SEQ ID NO:8)
- 30 haV-23 Deletion of amino acids 112 to 134 (SEQ ID NO:9)
- haV-24 Mutations of aspartic acid and histidine at amino acids 114 and 115 to alanines (SEQ ID NO:10)
- 35 haV-25 Mutations of tyrosine, aspartic acid and histidine at amino acids 113 to

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155 (SEQ ID NO:11)

5 Four hundred nanograms of each V plasmid
(encoding wild-type, haV-5 or haV-23 through haV-25 V
protein) was used and the relative CAT activity was
measured as a percentage of the activity resulting from
a transfection performed without any V protein
expression vector (bar 1). The results are depicted in
10 Figure 8. A lower percentage correlates with a higher
degree of repression of CAT expression.

Example 7

Repression of Minireplicon Expression by Additional
C-Terminal Mutant Measles Virus V Proteins

15 The transient minireplicon expression CAT
assay described in Examples 3 and 4 was repeated using
the measles virus V protein mutants haV-1 and haV-12 to
haV-22, which have the following differences from the
20 wild-type sequence (haV) (SEQ ID NO:7) in the C-terminus
(amino acids 220-299; see Figure 9):

- haV-1 Deletion of amino acids 232 to 299
(SEQ ID NO:13)
- 25 haV-12 Mutations of cysteines at amino acids
251 and 255 to alanines (SEQ ID NO:14)
- haV-13 Mutations of cysteines at amino acids
269 and 272 to alanines (SEQ ID NO:15)
- haV-14 Deletion of amino acids 279 to 299
(SEQ ID NO:16)
- 30 haV-15 Deletion of amino acids 267 to 299
(SEQ ID NO:17)
- haV-16 Deletion of amino acids 250 to 299
(SEQ ID NO:18)
- haV-17 Deletion of amino acids 243 to 299
35 (SEQ ID NO:19)
- haV-18 Deletion of amino acids 236 to 299

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- (SEQ ID NO:20)
- haV-19 Deletion of amino acids 229 to 299
(SEQ ID NO:21)
- 5 haV-20 Mutations of arginines at amino acids
233 and 234 to alanines (SEQ ID NO:22)
- haV-21 Mutations of arginines at amino acids
233 and 234 to aspartic acid (SEQ ID
NO:23)
- 10 haV-22 Deletion of amino acids 229 to 237
(SEQ ID NO:24)

15 Four hundred nanograms of each V plasmid
(encoding wild-type, haV-1 or haV-12 through haV-22 V
protein) was used and the relative CAT activity was
measured as a percentage of the activity resulting from
a transfection performed without any V protein
expression vector (bar 1). The results are depicted in
20 Figure 10. A lower percentage correlates with a higher
degree of repression of CAT expression.

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The Claims defining the invention are as follows:

1. An isolated recombinantly-generated, nonsegmented, negative-sense, single-stranded RNA virus of the genus *Morbillivirus*, wherein said virus comprises at least one mutation in its *Morbillivirus* V protein characterized in that at least one mutation is selected from the group consisting of mutations of amino acids 113 and 114 of the measles virus V protein or of the amino acids of a *Morbillivirus* V protein corresponding thereto.
2. The *Morbillivirus* of claim 1, wherein the virus is selected from the group consisting of measles virus, canine distemper virus, rinderpest virus, peste-des-petits ruminants virus, dolphin morbillivirus and phocine distemper virus.
3. The *Morbillivirus* of claim 1, wherein the mutation is at amino acid 113 of the measles virus V protein or the amino acid corresponding thereto.
4. The *Morbillivirus* of claim 3, wherein the mutation at amino acid 113 of the measles virus V protein or the amino acid corresponding thereto is from tyrosine to alanine.
5. The *Morbillivirus* of claim 1, wherein the mutation is at amino acid 114 of the measles virus V protein or the amino acid corresponding thereto.
6. The *Morbillivirus* of claim 5, wherein the mutation at amino acid 114 of the measles virus V protein or the amino acid corresponding thereto is from aspartic acid to alanine.
7. The *Morbillivirus* of claim 1, wherein there is a mutation at both amino acids 113 and 114 of the measles virus V protein or both amino acids corresponding thereto.
8. The *Morbillivirus* of claim 7, wherein the mutation at amino acid 113 of the measles virus V protein or the amino acid corresponding thereto is from tyrosine to alanine and the mutation at amino acid 114 of the measles virus V protein or the amino acid corresponding thereto is from aspartic acid to alanine.
9. The *Morbillivirus* of claim 2, wherein the virus is measles virus.
10. The measles virus of claim 9, which further comprises a mutation in or deletion of at least a portion of the carboxy-terminal (C-terminal) region comprising or corresponding to amino acids 231-299 of the measles virus V protein.
11. The measles virus of claim 10, wherein the mutation in the C-terminal region is at each of amino acids 233 and 234.
12. The measles virus of claim 11, wherein the mutation at each of amino acids 233 and 234 is from arginine to alanine.

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13. The measles virus of claim 11, wherein the mutation at each of amino acids 233 and 234 is from arginine to aspartic acid.
14. The measles virus of claim 10, wherein the deletion is selected from the group consisting of the deletion of amino acids 232 to 299, 279 to 299, 267 to 299, 250 to 299, 243 to 299 and 236 to 299.
15. The measles virus of claim 14, wherein the deletion is from amino acids 232 to 299.
16. The measles virus of claim 15, wherein the deletion extends upstream from the C-terminal region and is from amino acids 229 to 299.
17. The measles virus of claim 9, which further comprises:
- (a) at least one attenuating mutation in the 3' genomic promoter region selected from the group consisting of nucleotide 26 (A → T), nucleotide 42 (A → T or A → C) and nucleotide 96 (G → A), where these nucleotides are presented in positive strand, antigenomic, message sense; and
- (b) at least one attenuating mutation in the RNA polymerase gene selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 331 (isoleucine → threonine), 1409 (alanine threonine), 1624 (threonine → alanine), 1649 (arginine → methionine), 1717 (aspartic acid → alanine), 1936 (histidine → tyrosine), 2074 (glutamine → arginine) and 2114 (arginine → lysine).
18. The measles virus of claim 9, which further comprises at least one attenuating mutation selected from the group consisting of:
- (a) for the N gene, nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 129 (glutamine → lysine), 148 (glutamic acid → glycine) and 479 (serine → threonine);
- (b) for the P gene nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 225 (glutamine acid → glycine), 275 (cysteine → tyrosine) and 439 (leucine → proline);
- (c) for the C gene nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 73 (alanine → valine), 104 (methionine → threonine) and 134 (serine tyrosine); and
- (d) for the F gene-end signal, the change at nucleotide 7243 (T → C), where these nucleotides are presented in positive strand, antigenomic, message sense.
19. The measles virus of claim 10, which further comprises:

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- a) at least one attenuating mutation in the 3' genomic promoter region selected from the group consisting of nucleotide 26 (A → T), nucleotide 42 (A → T or A → C) and nucleotide 96 (G → A), where these nucleotides are presented in positive strand, antigenomic, message sense; and
- 5 b) at least one attenuating mutation in the RNA polymerase gene selected from the group consisting of nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 331 (isoleucine → threonine), 1409 (alanine threonine), 1624 (threonine → alanine), 1649 (arginine → methionine), 1717 (aspartic acid → alanine), 1936 (histidine → tyrosine), 2074 (glutamine → arginine) and 2114
- 10 (arginine → lysine).
20. The measles virus of claim 10, which further comprises at least one attenuating mutation selected from the group consisting of:
- (a) for the N gene, nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 129 (glutamine → lysine), 148 (glutamic
- 15 acid → glycine) and 479 (serine → threonine);
- (b) for the P gene, nucleotide changes which produce changes in an amino acid selected from the group consisting of residues 225 (glutamic acid → glycine), 275 (cysteine → tyrosine) and 439 (leucine → proline);
- (c) for the C gene nucleotide changes which produce changes in an amino acid selected
- 20 from the group consisting of residues 73 (alanine → valine), 104 (methionine → threonine) and 134 (serine tyrosine); and
- (d) for the F gene-end signal, the change at nucleotide 7243 (T → C), where these nucleotides are presented in positive strand, antigenomic, message sense.
21. The *Morbillivirus* of claim 2, wherein the virus is canine distemper virus.
- 25 22. The canine distemper virus of claim 21, which further comprises a mutation in or deletion of at least a portion of the C-terminal region corresponding to amino acids 231-299 of the canine distemper virus V protein.
23. The *Morbillivirus* of claim 2, wherein the virus is rinderpest virus.
24. The rinderpest virus of claim 23 which further comprises a mutation in or
- 30 deletion of at least a portion of the C-terminal region corresponding to amino acids 231 - 299 of the rinderpest virus V protein.
25. The *Morbillivirus* of claim 2, wherein the virus is dolphin morbillivirus.
26. The dolphin morbillivirus of claim 25 which further comprises a mutation in or deletion of at least a portion of the C-terminal region corresponding to amino acids 231
- 35 -303 of the dolphin morbillivirus V protein.

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27. An immunogenic composition comprising an isolated virus according to claim

1.

28. Use of a composition according to claim 27 in the preparation of a composition for immunizing an individual against a nonsegmented, negative-sense, single stranded RNA virus of the genus *Morbillivirus*.

29. The immunogenic composition of claim 27 which further comprises an adjuvant.

30. Use of a composition according to claim 29 in the preparation of a composition for immunizing an individual against a nonsegmented, negative-sense, single-stranded RNA virus of the genus *Morbillivirus*.

31. A method for reducing the repression caused by a V protein of the genus *Morbillivirus* which comprises inserting at least one mutation into the *Morbillivirus* V protein, wherein said mutation is selected from the group consisting of mutation of amino acids 113 and 114 of the measles virus V protein or of the amino acids of a *Morbillivirus* V protein corresponding thereto.

32. An isolated nucleotide sequence encoding a *Morbillivirus* V protein which has been modified by inserting at least one mutation into the *Morbillivirus* V protein, wherein said mutation is selected from the group consisting of mutation of amino acids 113 and 114 of the measles virus V protein or of the amino acids of a *Morbillivirus* V protein corresponding thereto.

33. A composition which comprises:

(i) a transcription vector comprising an isolated nucleic acid molecule encoding a genome or antigenome of a *Morbillivirus*, wherein the portion of the isolated nucleic acid molecule encoding the V protein is the nucleotide sequence of claim 32, and

(ii) at least one expression vector which comprises at least one isolated nucleic acid molecule encoding the trans-acting proteins N, P and L necessary for encapsidation, transcription and replication, whereby upon expression an infectious *Morbillivirus* is produced.

34. A method for producing an infectious *Morbillivirus* which comprises transforming, infecting or transfecting host cells with the at least two vectors of claim 33 and culturing the host cells under conditions which permit the co-expression of these vectors so as to produce the infectious *Morbillivirus*.

35. A method of immunizing an individual to induce protection against a nonsegmented, negative-sense, single-stranded RNA virus of the genus *Morbillivirus*

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which comprises administering to the individual the immunogenic composition of Claim 27 or Claim 29.

36. An isolated, recombinantly-generated, non-segmented, negative-sense, single-stranded RNA virus of the genus *Morbillivirus* according to claim 1, substantially as hereinbefore described with reference to any one of the examples.

Dated 26 February, 2007
Wyeth Holdings Corporation

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

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FIGURE 1

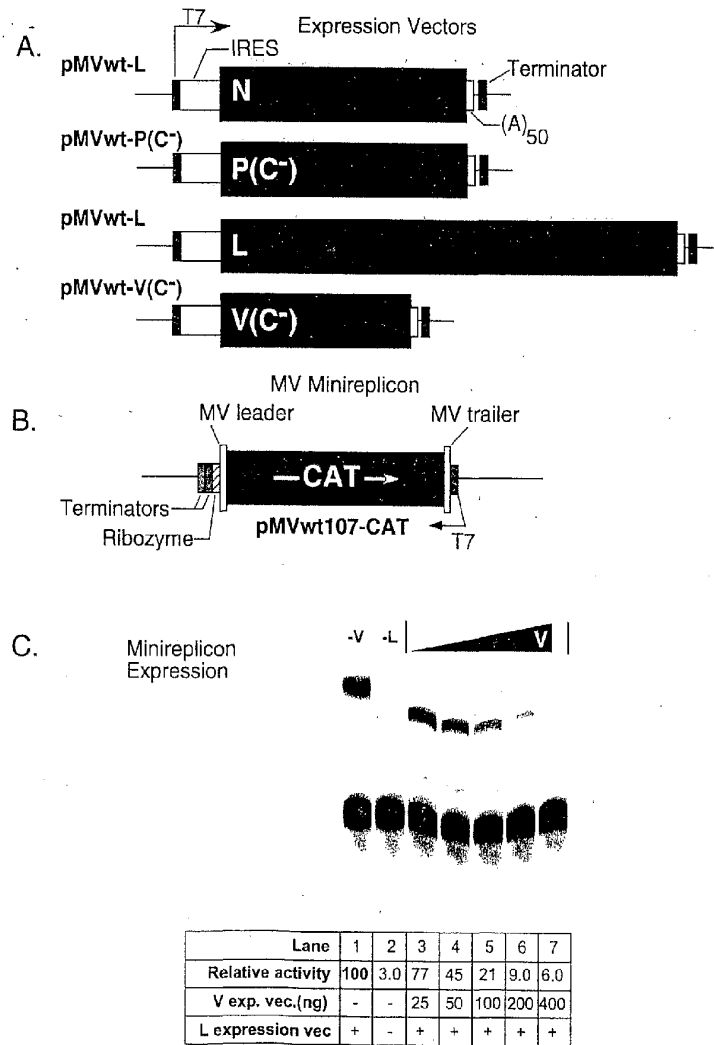


FIGURE 2

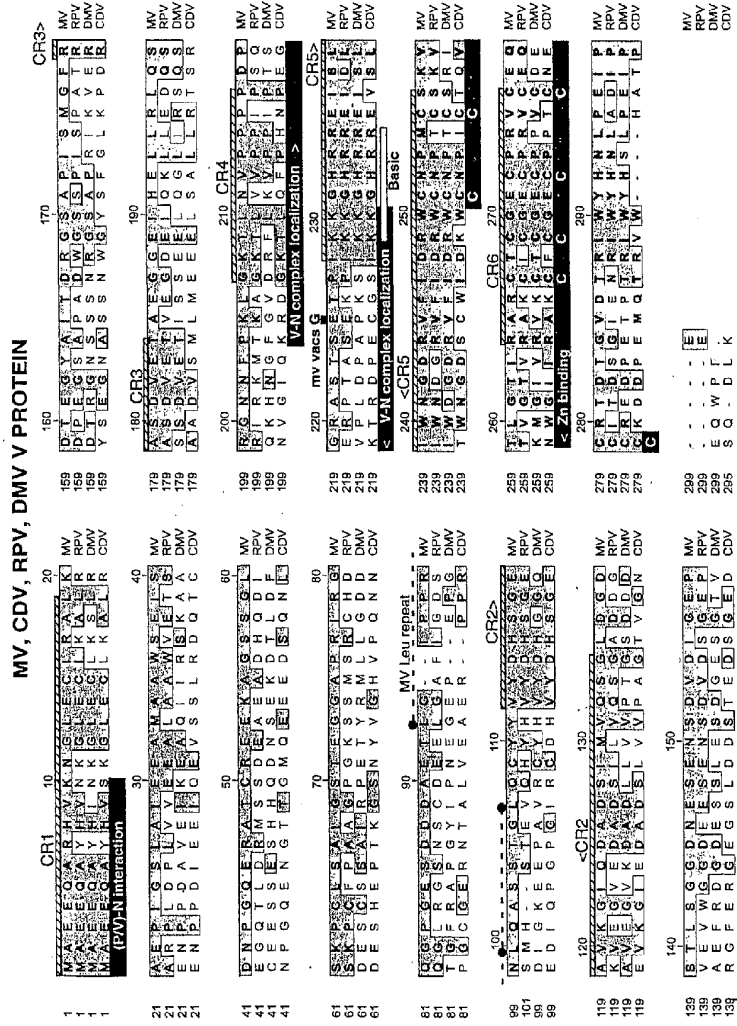


FIGURE 3

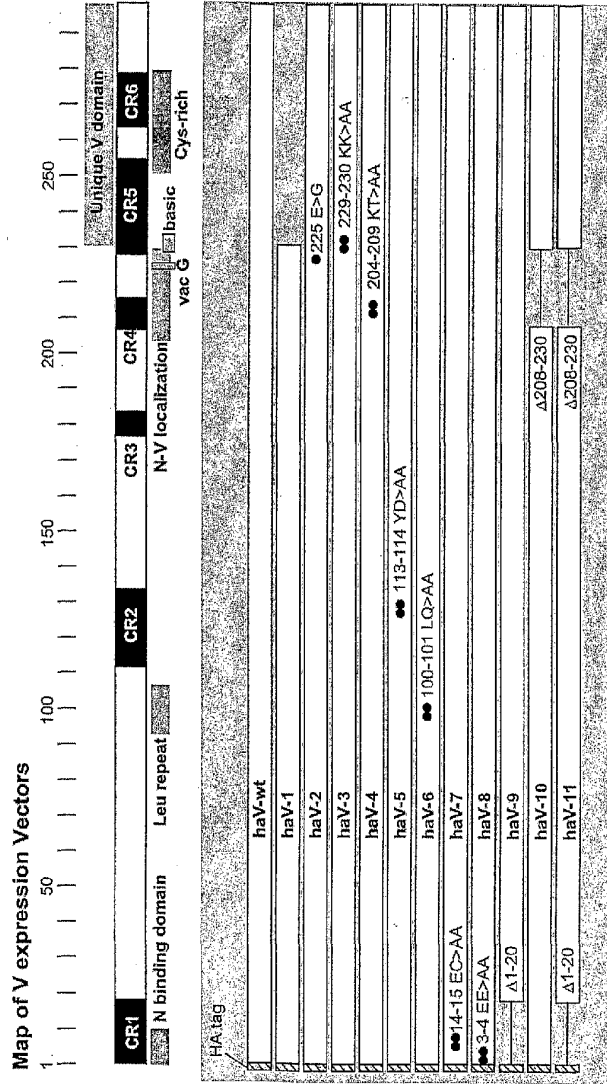
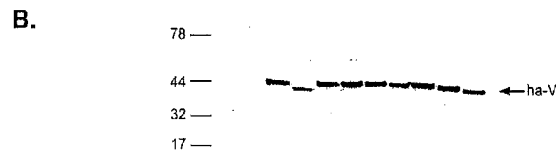


FIGURE 4



#	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
V plasmid	-	-	haV-wt	haV-1	haV-2	haV-3	haV-4	haV-5	haV-6	haV-7	haV-8									
ng V plasmid	0	0	200	400	200	400	200	400	200	400	200	400	200	400	200	400	200	400	200	400
L plasmid	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rel. Act.	-	100	14	6.7	50	37	18	3.0	11	3.3	7.1	2.6	53	48	10	2.2	2.0	2.5	14	3.2



#	1	2	3	4	5	6	7	8	9	10	11
V vector	-	V wt	haV wt	haV 1	haV 2	haV 3	haV 4	haV 5	haV 6	haV 7	haV 8



#	1	2	3	4	5	6	7	8	9
Rel. Act.	100	58	26	143	75	103	74	118	-
L vec	+	+	+	+	+	+	+	+	+
ng V vec	0	100	400	100	400	600	800	1000	0
V vec	-	haV wt				haV 1			-

FIGURE 5

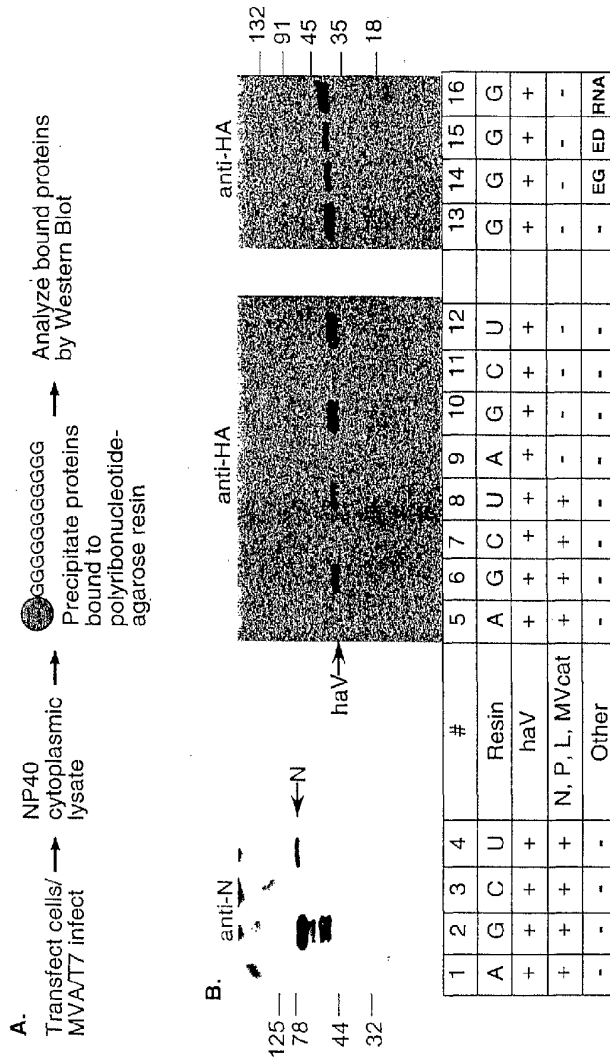


FIGURE 6

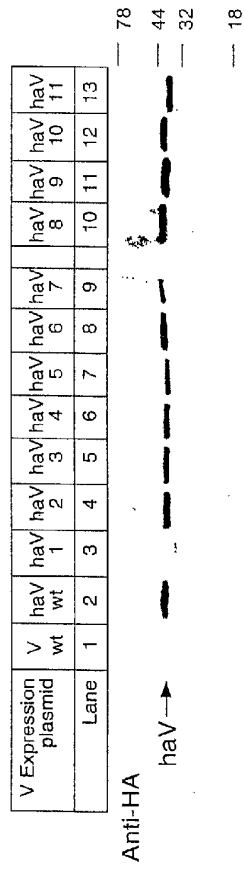


FIGURE 7



FIGURE 8

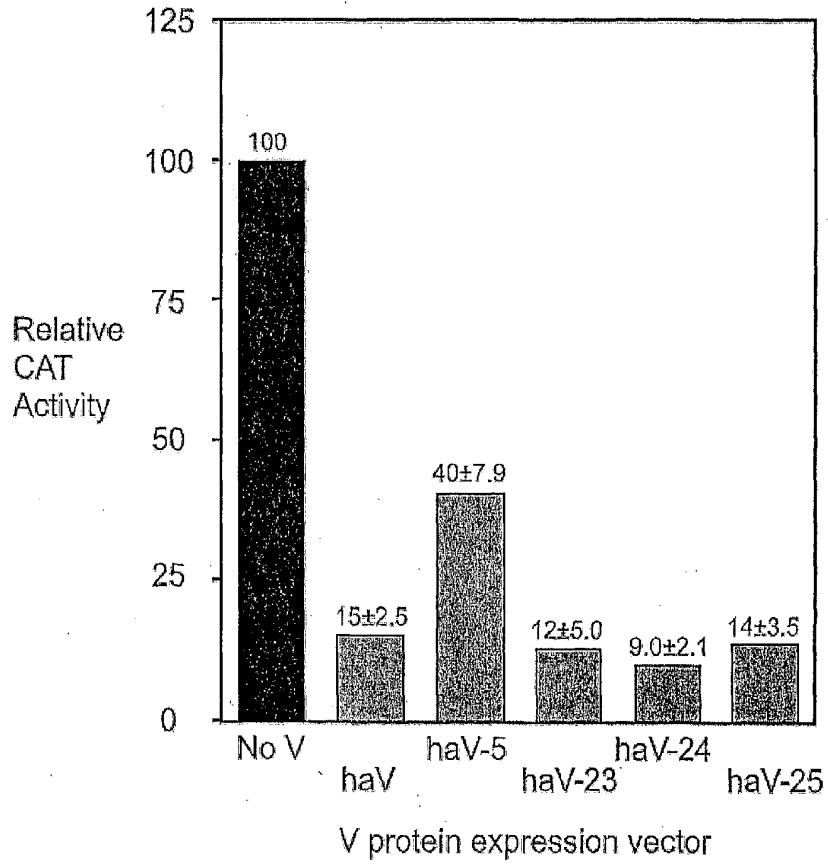


FIGURE 9

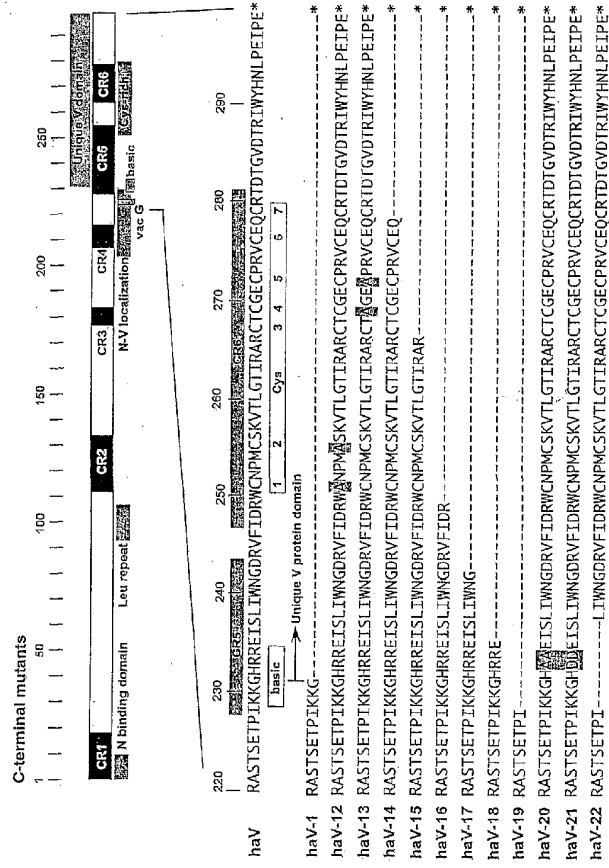
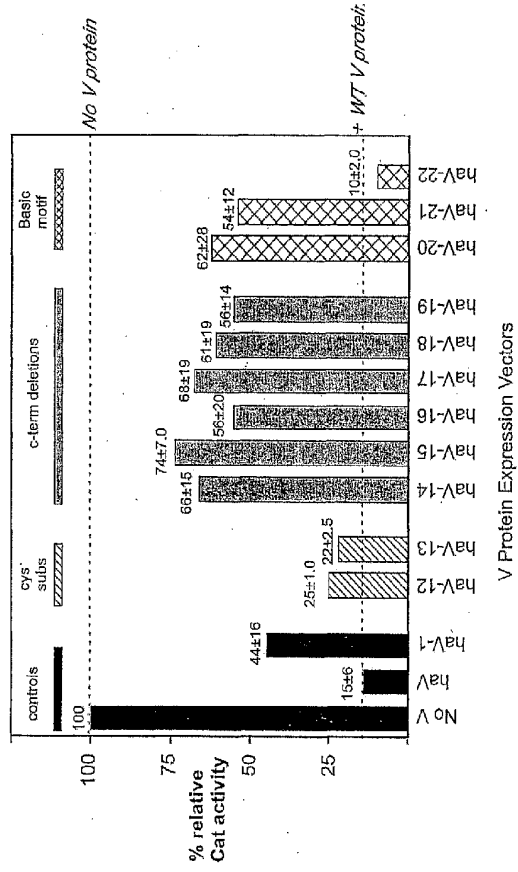


FIGURE 10



EDITORIAL NOTE

APPLICATION NUMBER - 2001267014

The following Sequence Listing pages **1** to **11** are part of the description. The claims pages follow on pages **64** to **68**.

SEQUENCE LISTING

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 35 40 45
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 50 55 60
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 65 70 75 80
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 85 90 95
 Pro Arg Asn Leu Gln Ala Ser Ser Thr Gly Leu Gln Cys Tyr Tyr Val
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 Tyr Asp His Ser Gly Glu Ala Val Lys Gly Ile Gln Asp Ala Asp Ser
 115 120 125
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 145 150 155 160
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 Phe Leu Lys Val Pro Pro Ile Pro Thr Ser Val Pro Leu Asp Pro Ala
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Ser Thr

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 <213> Measles virus

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Gln Ser Gly Leu Asp Gly Asp Ser Thr
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 35 40

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20 25 30
Asn Pro Met Cys Ser Lys Val Thr Leu Gly Thr Ile Arg Ala Arg Cys
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Thr Ala Gly Glu Ala Pro Arg Val Cys Glu Gln Cys Arg Thr Asp Thr
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Gly Val Asp Thr Arg Ile Trp Tyr His Asn Leu Pro Glu Ile Pro Glu
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Ile Ser Leu Ile Trp Asn Gly Asp Arg Val Phe Ile Asp Arg Trp Cys
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Asn Pro Met Cys Ser Lys Val Thr Leu Gly Thr Ile Arg Ala Arg
35 40 45

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<211> 30
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<400> 18
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 20 25 30
 Asn Pro Met Cys Ser Lys Val Thr Leu Gly Thr Ile Arg Ala Arg Cys
 35 40 45
 Thr Cys Gly Glu Cys Pro Arg Val Cys Glu Gln Cys Arg Thr Asp Thr
 50 55 60
 Gly Val Asp Thr Arg Ile Trp Tyr His Asn Leu Pro Glu Ile Pro Glu
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 35 40 45
 Thr Cys Gly Glu Cys Pro Arg Val Cys Glu Gln Cys Arg Thr Asp Thr
 50 55 60
 Gly Val Asp Thr Arg Ile Trp Tyr His Asn Leu Pro Glu Ile Pro Glu
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 20 25 30
 Gly Thr Ile Arg Ala Arg Cys Thr Cys Gly Glu Cys Pro Arg Val Cys
 35 40 45
 Glu Gln Cys Arg Thr Asp Thr Gly Val Asp Thr Arg Ile Trp Tyr His
 50 55 60
 Asn Leu Pro Glu Ile Pro Glu

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