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(54) Title: COMPOSITIONS AND METHODS FOR SCREENING ANTIVIRAL AGENTS

(57) Abstract: The invention includes the fields of viruses and methods for screening compounds for antiviral activity. In particular, the invention includes methods for measuring replication in a non-host cell-culture system.

Compositions And Methods For Screening Antiviral Agents

Cross-Reference to Related Applications

This application claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional Application No. 61/583,493, filed January 5, 2012, the content of which is hereby incorporated by reference in its entirety.

Field of the Invention

The invention includes the fields of viruses and methods for screening compounds for antiviral activity. In particular, the invention includes methods for measuring replication in a non-host cell-culture system.

Cross-Reference to Sequence Listing

The Sequence Listing created on January 2, 2013, and identified as "VTC0003-00WO_ST25.txt" (1000 bytes), is hereby incorporated by reference.

Background of the Invention

Use of attenuated virus is often the most effective way of vaccinating. If a commercial attenuated vaccine is to be produced by treating an infectious virus, a commercial quantity of infectious virus must be produced for treatment. An impediment to that production often exists since an infective virus will kill its host cell before commercial quantities can be obtained. Accordingly, an alternative means for production of commercial quantities of an infectious or attenuated virus are needed.

Baculoviruses represent a family of large, rod-shaped enveloped viruses with a double stranded DNA genome size of from 80-180 Kb. Baculoviruses are considered to be species- specific among invertebrates with over 600 host species described, but they are not known to infect mammalian or other vertebrate animal cells. In the 1940's they were used widely as biopesticides and since the 1990's they have been used for producing complex eukaryotic proteins in insect culture cells *(e.g.,* Sf9) or insect larvae *(e.g.,* lepidopteran larvae). The most widely studied baculovirus is *Autographa californica multicapsid nucleopolyhedrovirus* (AcMNPV), a 134 Kb genome virus with 154 open reading frames.

Detailed Description of the Invention

The present invention includes and utilizes a method for replicating an RNA viral genome in a facilitating host cell comprising introducing into a facilitating host cell a facilitating DNA virus comprising a promoter operably linked to a DNA sequence that codes for an RNA viral genome culturing said facilitating host cell under conditions suitable for transcription of said RNA viral genome; and replicating said transcribed RNA viral genome in said facilitating host cell culture. Cells that are otherwise non-permissive to a virus by natural route of infection, non-host cells, can be transfected, transduced, or transfected and transduced using a DNA virus-mediated infection, transduction, or infection and transduction and the replication of the RNA virus can be monitored by strand-specific real-time PCR.

In another aspect, the present invention includes and utilizes a facilitating virus capable of infecting a facilitating host comprising a positive strand promoter flanking each nucleic acid sequence that codes for a functional genomic segment of a mono-, bi-, tri-, or multi- segmented positive-sense single-stranded RNA ("(+)sense RNA") vims that is not capable of infecting the facilitating host. The present invention includes a virus capable of infecting a facilitating host that codes for a mono-, bi-, tri-, or multi- segmented positive-sense single stranded RNA ("(+)sense RNA") viral genome. In an aspect, a positive-sense RNA virus replicates via a negative-sense RNA intermediate template, and the levels of the negative-sense strand serve as an indicator of replication of positive-sense RNA viruses in the non-host cells.

In another aspect, the present invention includes and utilizes a facilitating virus capable of infecting a facilitating host comprising a positive strand promoter and a negative stand promoter flanking each nucleic acid sequence that codes for a genomic segment of a mono-, bi-, tri-, or multi- segmented double-stranded RNA ("dsRNA") virus that is not capable of infecting the facilitating host, wherein the negative strand promoter has a corresponding terminator before the positive strand promoter. The present invention includes a virus capable of infecting a facilitating host that codes for a mono-, bi-, tri-, or multi- segmented double stranded RNA ("(+)sense RNA") viral genome. In an aspect, viruses containing negative-sense RNA genome replicate via a positive-sense RNA intermediate template and the levels of the positive-sense RNA in facilitating or non-host cell serves as an indicator of replication of negative-sense RNA viruses in the non-host cell

culture system.

For viruses containing a double- stranded RNA genome (dsRNA), transcriptional initiation by viral RNA-dependent-RNA -polymerase (RdRp) can lead to the production of positive-sense RNA which is translated thereby producing virally encoded proteins. The positive-sense RNA and the viral proteins are assembled into immature virions. The positive- sense RNA is further transcribed into dsRNA in virions by viral RdRp. In an aspect, measuring the levels of the negative-sense serves as an indicator of replication of dsRNA viruses in the non-host cells.

Upon transfected, transduced, or transfected and transduced of facilitating cells with a facilitating virus containing the genome of positive-, or negative-sense RNA or dsRNA virus, one or more antiviral agents can be added in the cell culture media, optionally in combination with other agents, and the levels of replication of the intermediate form of the RNA virus can be measured by a strand-specific real-time PCR. This provides a rapid means of screening antiviral drugs using non-host cell culture systems. Agents that can be used in combination with anti-viral drugs include inhibitors of facilitating host cell transcription.

A plurality of virions according to the present invention may include a mixture of virions with positive and negative strands of said RNA viral genome, wherein each said virion has either only positive strands of said RNA viral genome or only negative strands of said RNA viral genome. The mixture can be any ratio of positive to negative strands, including without limitation, less than 5, 10, or 25% negative strand of RNA viral genome or less than 5, 10, or 25% positive strand of RNA viral genome. In another aspect, there may be only one strand type of RNA viral genome in a plurality of virions of the present invention, and no mixture.

One skilled in the art may refer to general reference texts for detailed descriptions of known techniques discussed herein or equivalent techniques, such as, but not Limited to cell culture or real-time PCR. These texts include Ausubel *et al, Current Protocols in Molecular Biology,* John Wiley and Sons, Inc. (1995); Sambrook *etal., Molecular Cloning, A Laboratory Manual* (2d ed.), Cold Spring Harbor Press, Cold Spring Harbor, New York (1989); Birren *et al., Genome Analysis: A Laboratory Manual,* volumes 1 through 4, Cold Spring Harbor Press, Cold Spring Harbor, New York (1997-1999); R. K. Upadhyay, Advances in microbial control of

insect pests, Springer; 1 edition (2003); Klein, Donald W.; Prescott, Lansing M.; Harley. John (1993). *Microbiology.* Dubuque, Iowa: Wm. C. Brown. ISBN 0-697-01372-3; Baculovirus and Insect Cell Expression Protocol (Methods in Molecular Biology, 2007, Second Edition, Ed. David W. Murhammer, Humana Press. These texts can, of course, also be referred to in making or using an aspect of the invention.

A promoter can be any promoter. Promoters include cis-acting transcriptional control elements and regulatory sequences that are involved in regulating or modulating the timing and/or rate of transcription of a gene or genes. A promoter can be operably linked to a coding sequence for RNA polymerase to initiate transcription at the promoter and transcribe the coding sequence into RNA. In an aspect, a promoter can be pantropic, cell type-specific, tissue-specific, or a promoter functional in an insect cell, a mammalian cell, or a human cell. In a preferred aspect, the promoter is a pantropic promoter. In another aspect, a promoter is *CMV,pol, plO, polH, gp64, TK, P2, Pll* and *P61* of *IHHNV, or iel* of white spot syndrome virus or *P2, PI 1* and *P48* of HPV. *See* Dhar, A. K., Kaizer, K. N., Lakshman, D. K. 2010. Transcriptional analysis of *Penaeus stylirostris* densovims genes, Virology, 402:112-120; Dhar, A.K., Lakshman, D. K., Natarajan, S., Allnutt, F. C. T, and van Beek, N. A.M., 2007. Functional characterization of putative promoter elements from infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimp and in insect and fish cell lines. Virus Res. 127: 1-8; Liu, W-J., Chang, Y- S., Wang, C-H., Kou, G-H., Lo, C-F. 2005. Microarray and RT-PCR screening for white spot syndrome virus immediate-early genes in cyclohexamide-treated shrimp. Virology 334: 327-341 ; Sukhumsirichart, W, Attasart, P., Boonsaeng, V., and Panyirn, S., 2006. Complete nucleotide sequence and genome organization of hepatopancreatic parvovirus (HPV) of *Penaeus monodon.* Virology 346: 266-277, all hereby incorporated by reference in their entirety. In one aspect, phylogenetic analysis reports that HPV is closely related to mosquito densoviruses, *Aedes aegypti* densovims *(AaeDNV)* and *Aedes albopictus* DNV *(AalDNV)* and IHHNV. Based on the genome organization and phylogenetic relationship, it has been proposed that HPV should be a new member of the sub-family *Densovirinae,* family *Parvoviridae* (Sukhumsirchart et al., 2006).

A positive strand promoter can be any promoter that is operably linked to a nucleic acid sequence that codes for a viral genomic segment and controls the transcription of a

nucleic acid sequence that codes for a positive strand of an RNA virus. In an aspect, a positive strand promoter is *CMV, pol, plO, polH, gp64, TK, P2, Pll* and *P61* of *IHHNV,* or *iel* of white spot syndrome virus or *P2, Pll* and *P48* of HPV. A positive strand promoter flanking a nucleic acid sequence that codes for a genomic segment results in the transcription of (+)strand RNA A negative strand promoter can be any promoter that is operably linked to a nucleic acid sequence that codes for a viral genome segment and controls the transcription of a nucleic acid sequence that codes for a negative strand of an RNA virus. A negative strand promoter flanking a nucleic acid sequence that codes for a genomic segment results in the transcription of (-)strand RNA. In an aspect, a negative strand promoter is a promoter recognized by a single chain DNA-dependent RNA polymerase. In a preferred aspect, a negative strand promoter is a T7 DNA-dependent RNA polymerase promoter, a T3 DNA-dependent RNA polymerase promoter, or an SP6 DNA-dependent RNA polymerase promoter.

In one aspect, a promoter operably linked to a segment is under the control of a promoter that is heterologous to the non-host cell and heterologous to the RNA viral genome. The term "heterologous" is used hereinafter for any combination of nucleic acid sequences that is not normally found intimately associated with a mono-segmented, bi-segmented, tri-segmented, or multi-segmented (-)sense, single-stranded RNA virus, a mono-segmented, bi-segmented, tri- segmented, or multi-segmented (+)sense single stranded RNA virus, a mono-segmented, bi-segmented, tri-segmented, or multi-segmented double stranded RNA virus, double stranded RNA virus or mono-, bi-, tri-, or multi-segmented (-)sense, single-stranded RNA virus host in nature, a mono-segmented, bi-segmented, tri-segmented, or multi-segmented (+)sense single stranded RNA virus host in natureor a mono-segmented, bi-segmented, tri-segmented, or multi- segmented double stranded RNA virus, double stranded RNA virus host in nature. In an aspect, chimeric promoter domains are heterologous promoters. Issues of codon usage, specific and unique regulatory sequences, and post-translational modifications all need to be considered when using heterologous production systems.

A segment is the genetic element of many viruses consisting of one copy of a nucleic acid (DNA or RNA) molecule, and its nucleic acid sequence codes for a single functional RNA transcript or polypeptide. For example, the genome of hepatitis C virus (HCV) consists of a single RNA molecule that contains one cistron which codes for a large

polypeptide. The viral genome in the case of HCV is represented by a single segmented RNA (mono-segmented) and the RNA is mono-cistronic. A cistron is a nucleic acid sequence that is equivalent to a gene and codes for a single functional polypeptide. There are also viruses, the genome of which consists of a single RNA molecule (mono-segmented) that contain a single cistron which codes for more than a single functional polypeptide. A second polypeptide may be produced from a second open reading frame or may be generated by endoproteolytic cleavage of a single precursor polypeptide.

An RNA viral genome that has a single segment is a mono-segmented vims. Negative-sense, single-stranded, linear non-segmented RNA viruses are classified in the Order *Mononegavirales,* families *Bornaviridae* (Genus: *Bornavirus),* family *Rhabdoviridae* (Genus: *Vesiculovirus, Lyssavirus, Ephemerovirus, Novirhabdovirus, Cytorhabdovirus, Nucleorhabdovirus),* family *Filoviridae* (Genus: Marburgvirus and Ebola virus), and family *Paramyxoviridae* (Genus: *Rubalavirus, Avulavirus, Respirovirus, Henipavirus, Morbillivirus, Pneumovirus,* and *Metapneumovirus*). These viruses contain negative sense, single-stranded, linear, non-segmented RNA, 8.9-19.0 kb in size and encodes envelope glycoprotein(s), a matrix protein, a major RNA binding protein, nucleocapsid associated protein(s), and a large polymerase protein. The 5'- and the 3' terminal ends of the viruses contain inverse complementarity and conserved motives (Pringle, C. R. Order: Mononegavirales, In: Virus Taxonomy, Eight Report of the International Committee on Taxonomy ofViruses. (Eds.) C. M. Faquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball, International Union of Microbiological Societies, pp609-614).

Such viruses belonging to the above listed families infect plants, invertebrates and vertebrates, cause a wide range of diseases and sometimes cause fatal infection. For example, *Rabies virus* (RABV), the type species in the genus *Lyssavirus,* is the etiological agent of rabies encephalitis in mammals including humans. *Infectious hematopoietic necrosis virus* (IHNV), the type species of the genus *Novirhabdovirus,* is an important viral pathogen of salmonid fish, and isprevalent in Europe, North America and Asia. *Lettuce necrotic yellow virus* (LNYV), the type species of the genus *Cytorhabdovirus,* is an important plant virus that infects lettuce. *Marburg virus* (MARY), and *Ebolavirus (EBOV),* members of the family *Filoviridae,* are the two deadliest viral pathogens in humans. *Mump virus* (MuV), the type species of the genus *Rubulavirus* infects humans. *Newcastle disease virus (NDV),* the type species of the genus *Aulavirus,* infects chickens.

Human respiratory syncytial virus (HRSV), the type species of the genus *Pneumovirus* infects humans. *Citrus psorosis virus* (CPsV), the type species of the genus *Ophiovirus,* causes diseases in citrus.

There are also (-) strand RNA viruses, the genome of which consists of more than one RNA segment. These viruses include (-)strand RNA viruses containing bi-segmented genomes such as those belonging to the family *Arenaviridae* (2 +/- segments); (-)strand RNA viruses containing 3/4/5-segmented genome such as those belonging to the family *Bunyaviridae* (3 or +/- segments), family *Orthomyxoviridae* (7-8 segments), and the unassigned genera of *Varicosavirus* and Ophiovirus (3-4 segments).

Examples of single-stranded negative-sense RNA viruses containing tri-segmented genomes include viruses in each of the five genera *Orthobunyavirus, Hantavirus, Nairovirus, Phelbovirus and Topsovirus* in the family *Bunyaviridae.* Virions representing these genera are generally spherical or pleomorphic, 80-120 nm in diameter with glycoprotein projections that are embedded in the lipid bilayered envelope of the virus. The genome of these viruses consists of three negative or ambisense, single-stranded RNA molecules designated as L (large), M (medium) and S (small), and the terminal nucleotide sequences of each genomic fragment are base-paired forming non-covalently closed circular RNAs. The nucleotide lengths of the three RNAs vary from about 6.5 to 12.2 kb for L, about 3.2 to 4.9 kb for M, and about 0.96 to 2.9 kb for S. The L segment codes for a viral RNA polymerase, the M segment codes for envelop glycoproteins and non-structural proteins, and the S segment codes a nucleocapsid and non-structural proteins (Nichol, S.T., Beaty, B.J., Elliott, R.M., Goldbach, R., Plyusnin, A., Schmaljohn,C. S., and Tesh, AR.B. 2005. Family: *Bunyaviridae,* In: Virus Taxonomy, Eight Report of the International Committee on Taxonomy of Viruses. (Eds.) C.M.Faquet, M.A.Mayo, J.Maniloff,U. Desselberger, L.A. Ball, International Union of Microbiological Societies, pp 695-716).

There are also positive sense, single-stranded, linear non-segmented RNA viruses, the genome of which consists of a single RNA molecule (mono-segmented) that contain more than one cistron, e.g. Taura syndrome virus (TSV) of shrimp. TSV genome consists of a single RNA molecule that contains two cistrons. One cistron codes for a polypeptide that represents non-structural proteins, whereas the second cistron codes for a polypeptide that represents the structural proteins. The viral genome in this case is represented by a single segmented of RNA and the RNA is bi-cistronic.

Then there are other viruses for which the genome contains more than one piece of DNA or RNA. Thus when the viral genome contains two pieces of nucleic acid (DNA or RNA), the genome is bi-segmented, when the genome contains three copies of nucleic acid (DNA or RNA), the genome is tri-segmented, and so forth. For example, the genome of cowpea mosaic virus (CPMV) (family *Comoviridae*, genus *Comovirus)* contains two genomic segments (or bi-segmented genome), RNA-1 and RNA2, and each RNA contains one cistron. RNA1 codes for a polymerase, a genome-linked protein (VPg) and a protein containing a nucleotide binding site, whereas RNA2 codes for the two capsid proteins, VP37 and VP23, and a movement protein.

Carnation ring spot virus (CRSV) (Family: *Tombusviridae,* Genus: *Dianthovirus)* genome is also bi-segmented, like CPMV genome. However, RNAI of CRSV is bi-cistronic, one cistron codes for non-structural gene and the second cistron codes for structural gene. RNA2 contains one cistron and codes for a non-structural gene. Thus, CRSV genome is bi-segmented and one segment is bi-cistronic while the other segment is mono-cistronic.

In an aspect, a bisegmented virus is a multi-segmented virus. In such an aspect, the methods of the present invention used to amplify, replicate, or amplify and replicate a bi-segmented (+)sense RNA virus can be used for all multi-segmented viruses. Viruses for which the genome is multi-segmented include beet necrotic yellow vein virus (BNYVV, *Benyvirus).* The BNYVV viral genome consists of five pieces of positive-sense RNA and is thus a multi-segmented genome.

In an aspect of the present invention, each segment of a (+)sense RNA viral genome is operably linked to only a single promoter sequence. As used herein, linked means physically linked , operably linked, or physically and operably linked. As used herein, physically linked means: that the physically linked nucleic acid sequences are located on the same nucleic acid molecule, for example a facilitating viral genome can be physically linked to a bisegment, tri-segment, or multi-segment (+)sense viral genome as part of a single nucleic acid molecule. In a preferred aspect, only one promoter is operably linked to each DNA sequence that codes for a (+)sense RNA viral genome segment of the present invention such that the single promoter transcribes a single (+)sense RNA viral genome segment. In another aspect, a DNA sequence that codes for a bi-segment, tri-segment, or multi-segment (+)sense RNA viral genome of the present invention is not operably linked to any promoter derived

from a host of the (+)sense RNA virus.

A promoter in a facilitating or non-host cell can be operably linked to a DNA sequence coding for a double-stranded RNA viral genome. Double-stranded RNA viruses are classified in the families *Cystoviridae, Reoviridae (Genera Orthoreovirus, Orbivirus, Rotavirus, Coltivirus, Seadornavirus, Aquareovirus, Idnoreovirus, Cypovirus, Fijivirus, Phytoreovirus, Oryzavirus, and Mycoreovirus), Birnaviridae (Genera Aquabirnavirus, Avibirnavirus and Entompbirnavirus), Totiviridae (Genera Totivirus, Giardiavirus and Leishmaniavirus), Partitiviridae (Genera Partitivirus, Alphacryptovirus and Betacryptovirus, Chlysoviridae (Genus Chlysovirus), Hypoviridae (Genus Hypovirus)and a monotypic genus, Endornavirus.*

Genomes of these viruses include either non-segmented *(e.g. Endornaviral genome* which contains a linear dsRNA of about 14-17.6 kbp in length, *Cryphonectria hypovirus 1 in the family Hypoviridae the* genome of which contains a linear dsRNA of about 9-13 kbp in size; *Totivirus, Giardiavirus and Leishmaniavirus* of the family Totiviridae the genome of which contains a single molecule of linear uncapped dsRNA of 4.6-7.0 kb in size) or bi-segmented *(e.g. White clover Clyptic virus 1* in the genus *Alphacryptovirus* which contains two dsRNA of about 1.7 and 2.0 kbp; *White clover cryptic virus 2* in the genus *Betacryptovirus* which contains two dsRNA of about 2.1 and 2.25 kbp; *Atkinsonella hypoxylon virus* in the genus *Partitivirus* the genome of which contains two dsRNA of 1.4 to 2.2 kbp; *Aquabirnavirus, Avibirnavirus* and *Entompbirnavirus* in the family *Birnaviridae* the genome of which contains two dsRNA of about 3.2 kbp and about 2.7 to 2.9 kbp); tri-segmented *(e.g. Cystovirus* in the family *Cystoviridae* with three dsRNA of about6.3 kbp, about 4.1 kbp, and about2.9 kbp) and multi-segmented *(e.g Chrysovirus* where the genome contains four linear dsRNA of 2.4 to 3.6 kbp in size; and 10, 11 or 12 segmented genome of the members of the family *Reoviridae).* (In: Virus Taxonomy, Eight Report of the International Committee on Taxonomy of Viruses. (Eds.) C. M. Faquet, M. A Mayo, J. Maniloff, U. Desselberger, L. A Ball, International Union of Microbiological Societies, pp443-605).

In an aspect, a bi-segmented virus is a multi-segmented virus. In such an aspect, the methods of the present invention used to amplify, replicate, or amplify and replicate a bi- segmented (-)sense RNA virus can be used for all multi-segmented viruses.

Viruses for which the genome is multi-segmented include members of the family *"Orthomyxoviridae"* (among others) and include members of the genus Influenzavirus A. The Influenzavirus viral genome consists of eight pieces of negative-sense RNA and is thus a multi-segmented genome.

Throughout the detailed description, unless specifically indicated, an RNA viral genome means an RNA genome from any one of the following viruses: refers to a mono-segmented (+)sense single stranded RNA virus, a bi-segmented (+)sense single stranded RNA virus, a tri-segmented (+)sense single stranded RNA virus, a multi-segmented (+)sense single stranded RNA virus, a mono-segmented (-)sense single stranded RNA virus, a bi-segmented (-)sense single stranded RNA virus, a tri-segmented (-)sense single stranded RNA virus, a multi- segmented (-)sense single stranded RNA virus, a mono-segmented double stranded RNA virus, a hi-segmented double stranded RNA virus, a tri-segmented double stranded RNA virus, and a multi-segmented double stranded RNA virus. In a preferred aspect, the RNA viral genome is the genome of a mono-segmented (+)sense single stranded RNA virus. In another preferred aspect, the RNA viral genome is the genome of hepatitis C virus (HCV), a mono-segmented (+)sense single stranded RNA virus.

In an aspect of the present invention, each segment of an RNA viral genome is operably linked to at least one promoter sequence, preferably two if it is a (-)sense RNA viral genome. As used herein, linked means physically linked, operably linked, or physically and operably linked. As used herein, physically linked means that the physically linked nucleic acid sequences are located on the same nucleic acid molecule, for example a facilitating viral genome can be physically linked to a mono-, bi-, tri-, or multi- segmented viral genome as part of a single nucleic acid molecule. In a preferred aspect, two promoters are operably linked to each DNA sequence that codes for an RNA viral genome segment of the present invention such that a single promoter transcribes a single strand of an RNA viral genome segment and a second promoter transcribes the reverse complementary strand of an RNA viral genome segment. Promoter elements, motifs, boxes, or regions can be used alone, in combination with each other, or in combination with other promoters as described in WO201 1/130125.

In another preferred aspect, a transcription terminator sequence is present 3' to a promoter and the functional RNA viral genome segment. In an aspect, a DNA sequence

that codes for a mono-, bi-, tri-, or multi- segmented RNA viral genome of the present invention is not operably linked to any promoter derived from a host of the RNA virus. A transcription terminator sequence is a nucleic acid sequence present in a DNA template that causes the termination of transcription leading to the formation of the 3' terminus of an RNA transcript. A transcription terminator sequence is strand-specific and terminates transcription of a DNA- dependent RNA polymerase initiated at a promoter located on a DNA nucleic acid sequence 5' of the terminator. In an aspect of the invention, a transcription terminator sequence is present 3' (e.g. downstream) to a promoter and the functional RNA viral genome segment. In .another aspect, the transcription terminator sequence terminates transcription of the transcript initiated from a negative strand promoter. In a preferred aspect of the invention, the negative strand terminator sequence is present before the positive strand promoter. In an aspect of the invention, a transcription terminator can be a viral terminator. In another aspect, a transcription terminator can be a bacteriophage transcription terminator. In another aspect, the transcription terminator sequence can be a T7 transcription terminator, a T3 transcription terminator or an SP6 transcription terminator.

In an aspect, a promoter or any number of promoters operably linked to a DNA sequence that codes for an RNA viral genomic segment or complement thereof is a promoter in an infectious hypodermal and hematopoetic virus (IHHNV) of a shrimp, or has more than about 95%, 90%, 80% or 70% sequence identity to a IHHNV promoter. Percent identity can include freely available, or subscription-based algorithms including BLAST, TBLASTN, GOTOH, CLUSTAL, TBLASTX, MOTIF, or other nucleotide and/or protein sequence alignments based on the Needleman-Wunsch algorithm and/or the Smith-Waterman algorithm. In an aspect, the Needleman-Wunsch algorithm is preferred. In another aspect, a promoter or any number of promoters operably linked to a DNA sequence that codes for an RNA viral genomic segment or complement thereof is heterologous to a host cell of the RNA virus.

In an aspect, an RNA viral genome can be the genetic material of a virus whose genetic information consists of a single strand of RNA that is the sense (or positive) strand of the corresponding naturally occurring virus which encodes messenger RNA (mRNA) and protein in its host cell. Replication of a (-)sense RNA virus in its host cell is via a positive-strand intermediate template and the levels of the positive-sense RNA in

facilitating or non-host cell serves as an indicator of replication of negative-sense RNA viruses in the non-host cell culture system. Replication of an (-)sense RNA virus in its host cell requires an RNA-dependant RNA Polymerase (RdRP) to transcribe the (-)sense RNA viral genome before translation. A functional (-)sense RNA viral genome can encode a single protein which is modified by host and viral proteins to fomlthe various proteins needed for replication and infection. One of these proteins is RNA-dependent RNA polymerase, which copies the viral RNA to form a double-stranded replicative form, which in turn directs the formation of new virions. A positive-sense RNA virus replicates via a negative-sense RNA intermediate template, and the levels of the negative- sense strand serve as an indicator of replication of positive-sense RNA viruses. For viruses containing a dsRNA, measuring the levels of the negative-sense can serve as an indicator of replication of dsRNA viruses.

In another aspect, an RNA viral genome is inserted into a facilitating viral genome as a complimentary DNA copy of an RNA viral genome. In an aspect, an RNA viral genome is from a natural isolate or from an attenuated modification thereof In an aspect, an RNA viral genome can be the equivalent amount, about 75%, 75%, about 85%, 85% about 90%, 90%, about 95%, 95% about 97%, 97%, about 98%, 98%, or about 99%, 90%-95%, 80%-95%, 99%, 90%-95%, 80%-95% of an entire naturally occurring RNA viral genome. In another aspect, an RNA viral genome can be sufficient to produce a functional RNA virus. In another aspect, an RNA viral genome can be the open reading frame only. The identity can be over contiguous or noncontiguous nucleotides. In this aspect, an entire naturally occurring RNA viral genome can be identified on GenBank on the NCBI website.

As used herein, the percent identity is preferably determined using the "Best Fit" or "Gap" program of the Sequence Analysis Software Package™ (Genetics Computer Group, Inc., University of Wisconsin Biotechnology Center, Madison, WI). "Gap" utilizes the algorithm of Needleman and Wunsch to find the alignment of two sequences that maximizes the number of matches and minimizes the number of gaps. Percent identity can include freely available, or subscription-based algorithms including BLAST, TBLASTN, GOTOH, CLUSTAL, TBLASTX, MOTIF, or other nucleotide and/or protein sequence alignments based on the Needleman-Wunsch algorithm and/or the Smith-Waterman algorithm. "BestFit" performs an optimal alignment of the best segment of similarity between two sequences and inserts gaps to maximize the number

of matches using the local homology algorithm of Smith and Waterman. The percent identity calculations may also be performed using the Megalign program of the LASERGENE bioinformatics computing suite (default parameters, DNASTAR Inc., Madison, Wisconsin). The percent identity is most preferably determined using the "Best Fit" program using default parameters.

In an aspect, an RNA virus can consist of a segment, a mono- segmented, hi-segmented, a tri-segmented, or a multi-segmented genome. In this aspect, a viral genome for two or more transcripts of an RNA virus can be operably controlled by multiple promoters. As the number of segments increase, the choice of promoter is a more critical aspect so that a mono-, bi-, tri-, or multi- segmented RNA virus having two or more transcripts will package correctly for proper infection in its host cell.

An RNA virus, coded for by a facilitating host virus, is not capable of infecting the facilitating host or replicating independently of the facilitating viral genome inside a facilitating host cell. A functional mono-, bi-, tri-, or multi-segmented RNA virus can infect and replicate within its native host cell. A functional RNA virus includes structural and non-structural viral genes sufficient for infection in a mono-, bi-, tri-, or multi- segmented RNA virus host cell. Structural and non- structural viral genes are defined in the art and are specific to a mono-, bi-, tri-, or multi- segmented RNA virus. As used herein, the term "gene" refers to a nucleic acid sequence that can be translated to produce a polypeptide chain, or regulate gene transcription, reproduction or stability. In an aspect, genes include regions preceding and following the coding region, such as leader and trailer, promoters and enhancers, as well as, where applicable, intervening sequences (introns) between individual coding segments (exons).

Structural genes, non- structural genes, or a combination of structural and non-structural genes can be incorporated in the same facilitating virus operably linked to the same or a different promoter with a same or different promoter sequence than a promoter operably linked to an entire segment of a mono-, bi-, tri-, or multi-segmented RNA viral genome. A structural viral gene, non- structural viral gene, or both operably linked to at least one separate promoter may improve production of a mono-, bi-, tri-, or multi- segmented RNA virus or replication of its RNA viral genome in a facilitating host or its own host. Examples of structural genes in mono-, bi-, tri-, or

multi- segmented RNA viruses are envelope proteins or proteases. In an aspect, a facilitating viral genome includes a second, third, or greater DNA sequence that codes for a protease gene of the mono-, bi-, tri-, or multi- segmented RNA virus.

An RNA virus host can be any cell type or organism, cell culture, or larvae having a cell type that a mono-, bi-, tri-, or multi- segmented RNA virus can infect. As used herein, infection is characterized by RNA viral entry and viral protein expression. In an aspect, a mono-, bi-, tri-, or multi- segmented RNA virus host cell is a mammalian cell, a human cell, a hepatoma cell, a vertebrate cell, an animal cell, a pig cell, a monkey cell, a canine cell, a mouse cell, or a eukaryotic cell. In this aspect, a mono-, bi-, tri-, or multi- segmented RNA virus host cell can be a cell line including but not limited to Huh-7 cells, Chinese Hamster Ovary (CHO) cells, HeLa cells, Hep2G cells, primary hepatocyte cells of human or other mammalian origin, and others. In another aspect, an RNA virus host cell can be a primary culture including but not limited to cells derived from hepatocytes. In another aspect, a mono-, bi-, tri-, or multi- segmented RNA virus host is a human cell. In a further aspect, a mono-, bi-, tri-, or multi- segmented RNA virus host cell is a human patient in need of gene therapy. In an aspect, there is more than one host cell for a mono-, bi-, tri-, or multi-segmented RNA virus, such as a primary and secondary host cell, where a mono-, bi-, tri-, or multi- segmented RNA virus kills a culture of primary host cells more quickly than the same number of secondary host cells in the same culture conditions. In this aspect, a secondary host could be vaccinated with a mono-, bi-, tri-, or multi-segmented RNA virus native in a primary host cell, such as with cowpox.

A facilitating host can be any organism or cell that is not infected by the mono-, bi-, tri-, or multi- segmented RNA virus, which genome is coded for by the facilitating viral genome. In an aspect, a facilitating host can be any cell type or organism, cell culture, or larvae having a cell type where the facilitating virus can replicate and infect another cell and the mono-, bi-, tri-, or multi- segmented RNA virus cannot infect the same cell. In an aspect, a facilitating host can also be any whole organism-based production system, such as a crustacean or shrimp, a plant, algae, a cell from the Phyla Arthropoda, an invertebrate cell. In this aspect, a facilitating host is an insect cell, such as *S. frugiperda* cell or Sf9 cell or any Lepidopteran insect species. In another aspect, a facilitating host can be a mammalian cell when a mono-segmented, bi-segmented, bi-

segmented, tri-segmented, multi-segmented RNA virus does not infect and replicate in a mammalian cell. In another aspect of the invention, the facilitating host can be yeast including, but not limited to, *Saccharomyces cerevisiae, Hansenula polymorpha,* and *Pichia pastoris. Saccharomyces* has the advantages of being a well-established transformation system with many integrating and non-integrating plasmids and many selectable markers that can be used for the selection of transformation and expression of genes in a well-defined system. *Pichia* has several additional advantages including high yields of expression of foreign genes, use of simple culture media, ease of fermentation to high cell densities, and genetic stability and scale-up without loss of yield.

In one aspect of the present invention, nucleic acid sequences that code for one or more functional segments of a mono-, bi-, tri-, or multi- segmented RNA viral genome may undergo replication within said facilitating host cell. In an aspect, replication of the RNA viral genome is the synthesis of a complimentary copy of the viral genome using RNA as a template and an RNA-dependent RNA polymerase. In an aspect, for an RNA virus, the RNA template may be the negative strand of the RNA genome or may be the positive strand of the RNA genome. In some aspects, the replication of the RNA genome may require the synthesis of proteins from the RNA genome itself In another aspect, the proteins required for the replication of the RNA genome may be provided by the facilitating host cell or by a helper virus. For a (-)strand RNA genome, replication may produce a complementary (+)strand RNA genome synthesized by an RNA-dependent RNA polymerase (RdRP). In this aspect, the resultant (+)strand RNA copy may then serve as a template for the production of one or more complementary (-)strand RNA genome copies. In an aspect, mono-, bi-, tri-, or multi- segmented(-) sense, single stranded RNA viral genomes produced by either transcription or replication may be assembled into a mature virion in a facilitating host or its host.

In an aspect, a facilitating virus is a double-stranded DNA virus. A facilitating virus can include a virus from a family of virus such as *Ascoviridae, Ascoviruses, Baculoviridae, Iridoviridae, Parvoviridae, Polydnaviridae,* and *Poxviridae.* In an aspect, a facilitating virus is a baculovirus. In an aspect, a recombinant baculovirus can contain a mono-, bi-, tri-, or multi-segmented RNA viral genome. The baculoviral genome can be based on a commercial vector system or other known baculovirus vectors such as, but not limited to, Sapphire™ Baculovirus, pBAC5, pBAC-6, BestBac *(e.g.* v-cath/chit

deleted), AcNPV Baculovirus, and pFastBacDual with a mono-, bi-, tri-, or multi-segmented RNA viral genome included. Any virus with a sufficiently large genome or packaging flexibility can be used as a facilitating virus. In an aspect, a facilitating virus is capable of transducing a mono-, bi-, tri-, or multi- segmented RNA viral genome into a host cell of the mono-, bi-, tri-, or multi- segmented RNA virus. As used herein, transducing refers to a facilitating virus mediating delivery of a dsDNA copy of an RNA viral genome into a host cell of the mono-, bi-, tri-, or multi- segmented RNA virus. A facilitating virus may or may not induce an immune response in the host of the mono-, bi-, tri-, or multi- segmented RNA virus. For additional description of an adjuvant having insect cells or proteins, *see* US Patent No. 6,224,882 (hereby expressly incorporated in its entirety). In an aspect, the present invention includes a DNA molecule comprising a first DNA sequence coding for a baculovirus and a second DNA sequence coding for a functional mono-, bi-, tri-, or multi- segmented RNA viral genome that does not infect insect cells, where the second DNA sequence is operably linked to only one promoter.

The term "immune response" means the reaction of the immune system when a foreign substance or microorganism enters the organism. By definition, the immune response is divided into a specific and an non-specific reaction although both are closely related. The non-specific immune response is the immediate defense against a wide variety of foreign substances and infectious agents. The specific immune response is the defense raised after a lag phase, when the organism is challenged with a substance for the first time. The specific immune response is highly efficient and is responsible for the fact that an individual who recovers from a specific infection is protected against this specific infection. Thus, a second infection with the same or a very similar infectious agent causes much milder symptoms or no symptoms at all, since there is already a "pre-existing immunity" to this agent. Such immunity and immunological memory persist for a long time, in some cases even lifelong. Accordingly, an attenuated virus of the present invention can be advantageous as a way to induce an inlmunological memory by vaccination. In an aspect, attenuation of a virus by treatment of a mono-, bi-, tri-, or multi- segmented (-)sense RNA virus which would otherwise have the replication and infection properties of a naturally occurring version of the same strain may be the best way to induce immunological memory.

In an aspect, a mono-, bi-, tri-, or multi- segmented RNA virus is produced by a facilitating host cell. Following infection of a facilitating host cell with a facilitating virus,

such as a baculovirus, a heterologous viral genome, such as a mono-, bi-, tri-, or multi-segmented RNA viral genome, is transcribed in parallel with the facilitating viral genome as part of its lifecycle. In an aspect, mature virions of the facilitating virus and those of a mono-, bi-, tri-, or multi- segmented RNA virus will be produced as translated and processed viral coat proteins combine with RNA transcribed from a complementary DNA copy of an entire mono-, bi-, tri-, or multi- segmented (-)sense RNA viral genome embedded in the facilitating viral genome, such as a recombinant baculovirus vector. In this aspect, facilitating virions, (-)sense RNA virions, or facilitating virions and (-)sense RNA virions can be obtained from the facilitating host cell. Many (-)sense RNA virions can be produced in the facilitating host regardless of how toxic the(-)sense RNA virus would be in its host cell since only the facilitating virus and not the (-)sense RNA virus will be able to infect new facilitating host cells. In an aspect, a recombinant baculovirus is able to produce 10 -50 times, 50-100 times, 100-1000 times, 1000-10^4 times, 10^4-10^5 times, 10^5-10^6 times, 10^6-10^7 times, and as much as 10^{10} times as many (-) sense RNA virions in insect cell culture as in an equivalent mono-, bi-, tri-, or multi- segmented (-) sense RNA virus host cell culture.

In an aspect, the amplification of a mono-, bi-, tri-, or multi- segmented RNA viral genome can be measured by real-time (RT-)PCR. For example, Sf9 cells are infected with recombinant baculovirus containing a mono-, bi-, tri-, or multi- segmented (-)sense RNA viral genome at 100 MOI. Total RNA can be isolated at 0, 24,48, 72, 96 and 120 hours post-infection. SYBR® Green Real-time RT-PCR is performed using primers designed based on the genome sequence of the (-)sense RNA virus and following published protocols. *See* Dhar, A. K., Bowers, R A, Licon, K. S., Veaze, G., and Reads, B. 2009. Validation of reference genes for quantitative measurement of immune gene expression in shrimp. *Molecular Immunology,* 46:1688-95; Dhar, A. K.,Raux,M. and Klimpel, K. R 2001. Detection and quantification of infectious hypodermal and hematopoietic necrosis virus. (IHHNV) and white spot virus (WSV) of shrimp by real time quantitative PCR using SYBR® Green chemistry. *Journal of Clinical Microbiology* 39: 2835-2845, hereby incorporated by reference in their entirety. Copy number of an RNA virus at different times post-infection (at 24, 48, 72, 96 and 120 hours) can be calculated and normalized to the viral copy number at 0 hr to determine the amplification of the (-)sense RNA viral genome over time. Copy number

of a mono-, bi-, tri-, or multi- segmented (-)sense RNA viral genome can also be expressed with respect to the total genomic content of a single Sf9 cell since the number of Sf9 cells seeded before infecting with the recombinant baculovirus is known.

In an aspect of the present invention, a baculovirus that infects a facilitating host has a DNA sequence that codes for a functional mono-, bi-, tri-, or multi- segmented RNA viral genome or complement thereof that does not infect the facilitating host, but is packaged. In a further aspect, a promoter operably linked to the mono-, bi-, tri-, or multi- segmented RNA viral genome or complement thereof is not derived from a host cell of the mono-, bi-, tri-, or multi- segmented RNA virus. In another aspect, the present invention includes a composition comprising a first virus capable of infecting a facilitating host comprising a first DNA sequence that codes for a functional mono-, bi-, tri-, or multi- segmented RNA viral genome or complement thereof that is not capable of infecting the facilitating host and a second DNA sequence coding for a baculoviral genome capable of infecting the facilitating host comprising a third DNA sequence that codes for a structural gene of the mono-, bi-, tri-, or multi- segmented RNA virus, where the expression of the first DNA sequence is under the control of a single promoter. Optionally in such an aspect, a second DNA sequence further comprises a fourth DNA sequence that codes for a non-structural gene of the RNA virus, where the fourth DNA sequence is under the control of one or more promoters.

In an aspect of the present invention, a functional genomic segment of a mono-, bi-, tri-, or multi- segmented RNA virus is a nucleic acid sequence that when expressed as an RNA is capable of at least being packaged into an RNA viral particle. In another aspect of the present invention, a functional genomic segment capable of being packaged into an RNA virus particle has an attenuating, inactivating or other mutation that affects the function of the virus particle. In another aspect of the invention, an RNA virus particle comprising a functional genomic segment of a mono-, bi-, tri-, or multi-segmented RNA virus can be a functional RNA virus particle. In another aspect of the invention, an RNA virus particle comprising a functional genomic segment of a mono-, bi-, tri-, or multi- segmented RNA virus can be a non-functional RNA virus particle.

In an aspect of the present invention, a functional mono-, bi-, tri-, or multi-segmented RNA virus can be attenuated or killed for use as a vaccine. In an aspect of the present invention, a vaccine may cause an antibody-mediated immune response, cell-

mediated immunity, or both in the mono-, bi-, tri-, or multi- segmented RNA virus host. The present invention includes a vaccine containing a baculovirus capable of infecting a facilitating host comprising a DNA sequence that codes for a mono-, bi-, tri-, or multi-segmented RNA virus that is not capable of infecting the facilitating host, where the expression of mono-, bi-, tri-, or multi- segmented RNA viral genome is under the control of a single promoter. In another aspect, a vaccine included in the present invention has a mono-, bi-, tri-, or multi- segmented RNA virus, which can be in an attenuated form, that is not capable of infecting a facilitating host and has a viral envelope comprising proteins from the facilitating host. The origin of the envelope proteins can be determined based on comparison of glycosylation patterns and fatty acid profiles of the different host cells. The present invention also includes a vaccine containing a DNA molecule that codes for a baculovirus capable of infecting a facilitating host and codes for a mono-, bi-, tri-, or multi-segmented RNA viral genome or complement thereof that is not capable of infecting the facilitating host, where the expression of RNA viral genome is under the control of a single promoter. In an aspect, a DNA vaccine of the present invention has a DNA molecule comprising a first DNA sequence that codes for a facilitating host cell virus comprising structural and non-structural genes sufficient for infection in a facilitating host cell and a second DNA sequence that codes for a mono-, bi-, tri-, or multi- segmented RNA viral genome or complement thereof that comprises structural and non-structural genes sufficient for infection of a host cell of an RNA virus, but not sufficient for infection of a facilitating host cell, where the expression of the mono-, bi-, tri-, or multi- segmented RNA viral genome or complement thereof is under the control of a single promoter.

In another aspect of the present invention, a mono-, bi-, tri-, or multi- segmented RNA virus can be used directly or after purification from the facilitating host cell, host cell proteins, or both. In an RNA virus with one or more glycosylated envelope protein, one or more envelope proteins can be glycosylated from the facilitating host cell, rather than the mono-, bi-, tri-, or multi- segmented RNA virus host cell or a native host cell. In an aspect, a vaccine containing such a virus would have the envelope coat proteins from a facilitating host cell or yeast cell, such as an insect cell. This could be an advantage due to an increase in stimulating an immune response in a host cell. *See* US Patent No. 6,224,882, hereby explicitly incorporated in its entirety by reference. In an aspect a vaccine may include one or more pharmaceutically acceptable and/or approved carriers, additives, antibiotics,

preservatives, adjuvants, diluents and/or stabilizers. Such auxiliary substances can be water, saline, glycerol, ethanol, wetting or emulsifying agents, pH buffering substances, or the like. Suitable carriers are typically large, slowly metabolized molecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates, or the like.

For the preparation of vaccines, an attenuated or killed mono-, bi-, tri-, or multi- segmented RNA virus of the present invention is converted into a physiologically acceptable form. This can be done based on experience in the preparation of poxvirus vaccines used for vaccination against smallpox (as described by Stickl, H. *et al.* [1974] Dtsch. med. Wschr. 99, 2386-2392). For example, the 45 purified virus is stored at -80° C. with a titer of $5x10^8$ TCID/ml formulated in about 10 rnM Tris, 140 mM NaCl , pH 7.4. For the preparation of vaccine shots, *e.g.,* 10^2-10^8 particles of the virus are lyophilized in 100 ml of phosphate- buffered saline (PBS) in the presence of 2% peptone and 1% 50 human albumin in an ampoule, preferably a glass ampoule. Alternatively, the vaccine shots can be produced by stepwise, freeze-drying of the virus in a formulation. This formulation can contain additional additives such as mannitol, dextran, sugar, glycine, lactose, polyvinylpyrrolidone, or other additives, such as antioxidants or inert gas, stabilizers or recombinant proteins *(e.g.* human serum albumin) suitable for in vivo administration. The glass ampoule is then sealed and can be stored between 4° C and room temperature for several months. However, as long as no need exists the ampoule is stored preferably at temperatures below -20° C.

For vaccination or therapy, the lyophilisate can be dissolved in 0.1 to 0.5 ml of an aqueous solution, preferably physiological saline or Tris buffer, and administered either systemically or locally, *i.e.,* by parenteral, intramuscular, or any other path of administration know to a skilled practitioner. The mode of administration, dose, and number of administrations can be optimized by those skilled in the art in a known manner.

In another aspect, the present invention includes a vaccine against a (-)sense RNA virus comprising a mono-, bi-, tri-, or multi- segmented (-)sense RNA viral genome or complement thereof comprising a signature (non-native) nucleic acid sequence having a length of 5-200, about 100, 50-60, 25-200, 50-100 nucleotides at the 5' end, 3' end, or 5' and 3' ends of the viral genome. The signature nucleic acid sequence is an addition of

nucleic acid sequence at the 5' or 3' end of the mono-, bi-, tri-, or multi- segmented (-) sense RNA viral genome of the present invention relative to a naturally occurring mono-, bi-, tri-, or multi- segmented (-)sense RNA viral genome of the same strain. In a particular aspect, the nucleic acid sequence of the signature is the complement of a portion of a facilitating host promoter DNA sequence. Even more specifically, the dangling bit of nucleic acid sequence is a transcription initiation site or the sequence 20-30 nucleotides downstream of the TATA box from a facilitating host promoter DNA sequence or complements thereof.

In another aspect, a mono-, bi-, tri-, or multi- segmented RNA virus may have one or more mono-, bi-, tri-, or multi- segmented RNA viral genome segments missing up to 200, about 200, 100-200 nucleotides at the 5' end and up to 500 nucleotides, about 500, 200-500 nucleotides on the 3' end of at least one said RNA viral genomic segment relative to a naturally occurring mono-, bi-, tri-, or multi- segmented RNA viral genomic segment of the same strain. The missing untranscribed nucleotides can result in a replication deficient, inactivated, or attenuated mono-, bi-, tri-, or multi- segmented RNA virus. In an aspect, the at least one segment missing at least 200 nucleotides on the 5' end encodes RdRP. In another aspect, the missing 5' untranslated region can increase expression of that segment by reducing the distance between the start of the segment and its initiation start codon as well as optionally removing secondary structure from the untranslated region.

In another aspect, an RNA virus can have envelope proteins from a RNA virus host cell, even if it is a secondary host, such as a CHO cell line for example. Viruses that have envelopes are known in the art, such as influenza and HCV. In an aspect, a mono-, bi-, tri-, or multi- segmented RNA virus from a facilitating host cell can infect a mono-, bi-, tri-, or multi- segmented RNA virus host cell and the RNA viral genome will be replicated and packaged into new RNA virus. In an aspect, a functional or nonfunctional RNA virus is attenuated, inactivated/killed after obtaining it from an RNA virus host cell or facilitating host cell. Methods of attenuating a virus are known in the art as are methods of killing, such as by treatment with formalin. In an aspect, an RNA virus is attenuated if it has reduced virulence relative to the viral genome of a naturally occurring RNA virus of the same strain. For example, the rate of infection or replication or both with an attenuated RNA virus is reduced compared to a naturally occurring RNA virus of the same strain. The growth behavior or amplification/replication of a virus can be

expressed by the ratio of virus produced from an infected cell (Output) to the amount originally used to infect the cell in the first place (Input) ("amplification ratio"). A ratio of "1" between Output and Input defines an amplification status where the amount of virus produced from the infected cells is the same as the amount initially used to infect the cells. Such a ratio is understood to mean that the infected cells are permissive for virus infection and virus reproduction. An amplification ratio of less than 1, *i.e.,* a decrease of the amplification below input level, indicates a lack of reproductive replication. In a particular aspect of attenuated viruses, a mono-, bi-, tri-, or multi- segmented RNA viral genome or complement thereof has a codon bias different than the codon bias of its native RNA virus host cell.

Also provided and included in the present invention is a method for preparing an altered RNA virus capable of infecting a non-host cell of a naturally occurring RNA virus that shares over half of the genome of said altered RNA virus comprising introducing into a facilitating host cell a facilitating DNA virus comprising a promoter operably linked to a DNA sequence that codes for said genome of said altered RNA virus; culturing said facilitating host cell under conditions suitable for transcription of said RNA viral genome; and producing said altered RNA virus in said facilitating host cell. In an aspect of the present invention, non-host cells that are otherwise non-permissive to a RNA virus by a natural route of infection, can be infected if the RNA virus is altered, for example by expression of a recombinant viral coat protein sequence ofan altered viral coat protein due to expression of the RNA virion in a non-host cell. Accordingly, an altered RNA virus can be capable of infecting a cell that would otherwise not be a host cell ofa naturally occurring corresponding RNA virus. In an aspect, a naturally occurring corresponding RNA virus is a naturally occurring RNA virus that shares over half of the genome of an altered RNA virus or a RNA virus otherwise expressed by a method of the present invention.

In another aspect, vaccines produced using the instant invention can be delivered to vertebrates, including human, by subcutaneous injection, or via technologies know in the art for mucosal delivery of vaccines such as, but not limited to, oral or nasal delivery. In an aspect, a vaccine can be used in a vaccination program. "Vaccination" means that a mono-, bi-, tri-, or multi- segmented (-)sense RNA virus host is challenged with (-)sense RNA virus of the present invention, *e.g.,* an attenuated or inactivated form of a (-)sense

RNA virus, to induce a specific immunity. A specific immune response against a mono-, bi-, tri-, or multi- segmented (-)sense RNA virus may be induced. The (-)sense RNA virus host, thus, is immunized, or has immunity, against the (-)sense RNA virus.

In a preferred aspect, the facilitating host cells are insect cells. The present invention includes a method of making a baculovirus vector containing a nucleic acid sequence of a mono-, bi-, tri-, or multi- segmented RNA virus or the complement thereof by obtaining a nucleic acid sequence of a mono-, bi-, tri-, or multi- segmented RNA virus; and cloning that sequence into a baculovirus vector. Another aspect of the present invention includes a method of amplifying a mono-, bi-, tri-, or multi- segmented RNA viral genome by transfecting an insect cell with a DNA molecule comprising a nucleic acid sequence coding for an insect viral genome and a nucleic acid sequence coding for a mono-, bi-, tri-, or multi- segmented RNA viral genome or complement thereof; where the nucleic acid sequence coding for a mono-, bi-, tri-, or multi- segmented RNA viral genome or complement thereof is operably linked to a heterologous promoter; obtaining supernatant, cell lysate, or supernatant and cell lysate of progeny of the transfected insect cell; and isolation of the mono-, bi-, tri-, or multi- segmented RNA virus.

In a further aspect, a method of the present invention includes producing a mono-, bi-, tri- or multi- segmented RNA virus by expressing in a facilitating host cell a DNA molecule with a nucleic acid sequence coding for a portion of a virus of the facilitating host cell and a nucleic acid sequence coding for a mono-, bi-, tri-, or multi- segmented RNA viral genome or complement thereof that does not infect the facilitating host cell, obtaining a mono-, bi-, tri-, or multi- segmented RNA virus that does not infect the facilitating host cell. Optionally, each segment has a different promoter.

In a further aspect, a method of the present invention includes producing a vaccine by expressing in a facilitating host cell a baculovirus comprising a DNA sequence that encodes a mono-, bi-, tri-, or multi- segmented viral genome or complement thereof; and amplification of the mono-, bi-, tri-, or multi- segmented viral genome or complement thereof inside the facilitating host cell. The mono-, bi-, tri-, or multi- segmented viral genome or complement thereof can serve as the vaccine or, in another aspect, the facilitating host cell can package the viral genome or complement thereof for production of an RNA virus and that virus can be inactivated or attenuated.

Having now generally described the invention, the same will be more readily

understood through reference to the following examples that are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Each periodical, patent, and other document or reference cited herein is herein incorporated by reference in its entirety.

EXAMPLES

Example 1. Infection of Sf9 cells with a baculovirus containing the genome of an RNA virus and strand- specific real-time RT-PCR to measure the levels of positive and negative-sense RNA.

Example la: Infection ofSJ9 cells

Recombinant baculovirus carrying the genome of the RNA virus, hepatitis C virus (HCV), as described in the WO201 1/130125, is generated using a Bac-to-Bac system (Invitrogen, Inc.). Recombinant baculovirus is used to infect Sf9 cells (2 x 10^6 cells/ml) at 0.1 to 100 MOI in several experimental treatments. Beginning at 6 hours post-infection, cells and cell supernatants are collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs and at 120 hrs, and the levels of the positive- and negative-sense RNA of the RNA virus is measured by real-time RT-PCR following published protocols (Dhar et al., 2002; Komurian-Pradel et al., 2004; Carriere et al., 2007).

Example l b: Quantitation of positive- and negative-sense RNA

Total RNA is isolated from infected cells using TRI reagent and following manufacturer's protocol (Molecular Research Center, Cincinnati, Ohio). RNA samples are treated with DNase I (Ambion, Inc., Austin, TX) and the amount of RNA quantified by measuring the absorbance at 260 nm using a spectrophotometer (SmartSpec™ 3000, Bio-Rad). First-strand cDNA synthesis is carried out in a 40 111 reaction volume containing 1.0 */lg* total RNA, IX RT-PCR buffer, 1 mM dNTPs, RC1 (5'-GGC CGT CAT GGT GGC GAA TAA GTC TAG CCA TGG CGT TAG TA-3' (SEQ ID NO: 1), Komurian-Pradel et al., 2004) or RC21 (5'-CTC CCG GGG CAC TCG CAA GC-3' (SEQ ID NO:2), Besnard and Andre, 1994) primer, 4 U of RNase inhibitor, and 5 U of MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) at 42°C for 1 h. The cDNA is diluted 1:100 using DNase; RNase free molecular biology grade water and 5 μL of the diluted

cDNA is used for each real-time RT-PCR.

Real-time RT-PCR amplifications is performed in a Bio-Rad iCycler iQ™ (Bio-Rad Laboratories, Inc., Richmond, CA) following published protocols (Dhar et al., 2002). Briefly, the reaction mixture contains 12.5 μL of 2X SYBR Green Supermix (iQ SYBR Green Supermix), 300 nM each of forward (104: 5'-AGA GCC ATA GTG GTC TGC GG-3'; SEQ ID NO:3) and reverse (197R: 5'-CTT TCG CGA CCC AAC ACT AC-3'; SEQ ID NO:4) primers and 5 μL of cDNA in a 25 μL reaction volume. The amplifications are carried out in a 96 well plate and each sample is measured in triplicate. The RNA quality of the samples is checked by amplifying shrimp elongation factor-la gene as an internal control (Dhar et al., 2002). The thermal profile for SYBR Green real-time RT-PCR is 95 °C for 10 minutes, followed by 40 cycles at 95 °C of 10 seconds and 60 °C for 1 minute. At the end of the PCR run, the specificity of the amplicon is determined by running a melting curve analysis.

Example lc: Calculations and quantitation ofHCV positive- and negative- sense RNA copy number

To calculate the copy number of HCV positive and negative-sense RNA, a standard curve is generated using cDNA derived from *in vitro* transcribed RNA from a HCV full-length clone (as described in the WO201 1/130125). *In vitro* transcription is performed using an *in vitro* transcription kit MEGAscript® T7 transcription kit (Ambion, Inc., Austin, TX) followed by tailing of the *in vitro* transcribed RNA using a A-Plus™ Poly(A) Polymerase Tailing Kit and manufacturer's protocol (EPICENTRE® Biotechnologies, Madison, WI). Real-time RT-PCR is performed using a serial dilution of cDNA containing about 10^7 to about 10^1 copies of the template to generate a standard curve.

Following the SYBR Green PCR run, data acquisition and subsequent data analyses are performed using the iCycler iQ Real-Time PCR Detection System (Biorad iQ Software Version 1.3). The copy number of HCV positive- and negative-sense RNA is determined by extrapolating the Ct values from the standard curve.

Example 2. Transduction of mammalian cells with a baculovirus containing HCV replicon and strand-specific real-time RT-PCR to measure the levels of positive and negative-sense RNA.

Recombinant baculovirus carrying the genome ofRNA virus hepatitis C (HCV), as described for example in WO201 1/130125, is generated using a Bac-to-Bac system (Invitrogen, Inc.). Recombinant baculovirus is used to infect mammalian primary hepatocytes, (2 x 10^6 cells/ml) at 0.1 to 100 MOI in several experimental treatments. At 6 hours post-infection, cells and cell supernatants are collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs and at 120 hrs, and the levels of the positive- and negative-sense HCV RNA is measured by real-time RT-PCR as described in the Example 1.

Example 3. Screening of HCV antiviral drug using a cell culture system.

Example 3a: Screening of NS3 protease inhibitor VX-950 (Telaprevir; Vertex)

To screen for drugs exhibiting HCV antiviral activities, insect cells are infected with a recombinant baculovirus carrying HCV genome, and at 12 hours post-infection, HCV antiviral drug, VX-950 (Telaprevir; Vertex) an NS3 inhibitor, is added into the cell culture media. Samples of cells are removed at 6, 12, 24, 36, 48, 60, 72 and 96 hours following the addition of drug, and strand-specific RT-PCR performed to determining the ratio of HCV positive- sense to HCV negative-sense RNA following methods described in Example 1.

The efficacy ofVX-950 is also determined by western blot analysis using aliquots of cell culture samples collected at 6, 12, 24, 36, 48, 60, 72 and 96 hours following the addition of drug.

Example 3b: Screening of NS3 protease inhibitor Boceprevir (SCH503034; Schering-Plough)

To screen for drugs exhibiting HCV antiviral activities, insect cells are infected with a recombinant baculovirus carrying HCV genome, and at 12 hours post-infection, HCV antiviral drug, Boceprevir (SCH503034; Schering-Plough) an NS3 inhibitor, is added into the cell culture media. Samples of cells are removed at 6, 12, 24, 36, 48, 60, 72 and 96 hours following the addition of drug, and strand-specific RT-PCR performed to determining the ratio of HCV positive-sense to HCV negative-sense RNA following methods described in Example 1.

The efficacy of Boceprevir is also determined by western blot analysis using

aliquots of cell culture samples collected at 6, 12, 24, 36, 48, 60, 72 and 96 hours following the addition of drug.

Example 3c: Screening of NS5B polymerase inhibitor R7128 (Roche /Pharmasset)

To screen for drugs exhibiting HCV antiviral activities, insect cells are infected with a recombinant baculovirus carrying HCV genome, and at 12 hours post-infection, HCV antiviral drug, R7128 (Roche / Pharmasset) an NS5B polymerase inhibitor, is added into the cell culture media. Samples of cells are removed at 6, 12, 24, 36, 48, 60, 72 and 96 hours following the addition of drug, and strand-specific RT-PCR performed to determining the ratio of HCV positive-sense to HCV negative-sense RNA following methods described in Example 1.

The efficacy of R7128 is also determined by western blot analysis of the NS5B polymerase using aliquots of cell culture samples collected at 6, 12, 24, 36, 48, 60, 72 and 96 hours following the addition of drug.

Example 3d: Screening of NS5B polymerase inhibitor Valopicitabine (NM-283,-Idenix/Novartis)

To screen for drugs exhibiting HCV antiviral activities, insect cells are infected with a recombinant baculovirus carrying HCV genome, and at 12 hours post-infection, HCV antiviral drug, Valopicitabine (NM-283; Idenix/Novartis) an NS5B polymerase inhibitor, is added into the cell culture media. Samples of cells are removed at 6, 12, 24, 36,48, 60, 72 and 96 hours following the addition of drug, and strand-specific RT-PCR performed to determining the ratio of HCV positive-sense to HCV negative-sense RNA following methods described in Example 1.

The efficacy of Valopicitabine is also determined by western blot analysis of the NS5B polymerase using aliquots of cell culture samples collected at 6, 12, 24, 36, 48, 60, 72 and 96 hours following the addition of drug.

Example 4. Construction of an insect cell line containing a stably-integrated HCV genome.

Plasmid DNA, containing a full-length HCV genome cDNA cloned downstream (3') from a heterologous promoter as described in EP 11769353, and containing the

antibiotic resistance gene *Sh ble* gene, are transfected into insect cells. Transformed cells are selected by growth of the transfected cells in insect cell defined medium containing Zeocin™.

Integration of the plasmid DNA is confirmed by Southern Blot Hybridization and expression of the HCV genome and virus as performed as described in EP 11769353.

Example 5. Screening ofHCV antiviral drug using a transgenic an insect cell line expressing HCV.

Example 5a: Screening ofNS3 protease inhibitor VX-950 (Telaprevir; Vertex) in transgenic insect cells

Insect cells prepared as described in Example 4 above are cultured in insect cell defined medium to 2 x 10^ cells/ml and the HCV antiviral drug, VX:-950 (Telaprevir; Vertex) an NS3 inhibitor, is added into the cell culture media. Beginning at 6 hours post-addition, cells and cell supernatants are collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs and at 120 hrs, and the levels of the positive- and negative-sense RNA of the RNA virus is measured by real-time RT-PCR following published protocols (Dhar et al., 2002; Komurian-Pradel et al., 2004; Carriere et al., 2007) as described in Example 1.

The efficacy ofVX-950 is also determined by western blot analysis using aliquots of cell culture samples collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs and at 120 hrs and anti-NS3 protease/helicase antibodies are used for western blot analysis.

Example 5b: Screening of NS5B polymerase inhibitor R7128 (Roche / Pharmasset) in transgenic insect cells

Insect cells prepared as described in Example 4 above are cultured in insect cell defined medium to 2 x 10^ cells/ml and the HCV antiviral drug, R7128 (Roche / Pharmasset) an NS5B polymerase inhibitor, is added into the cell culture media. Beginning at 6 hours post-addition, cells and cell supernatants are collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs and at 120 hrs, and the levels of the positive- and negative-sense RNA of the RNA virus is measured by real- time RT-PCR following published

protocols (Dhar et al., 2002; Komurian-Pradel et al., 2004; Carriere et al., 2007) as described in Example 1.

The efficacy of VX-950 is also determined by western blot analysis using aliquots of cell culture samples collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 96 hrs and at 120 hrs and anti-NS5B antibodies are used for western blot analysis.

Example 6. Preparation of a yeast cell line containing an inducible plasmid expressing HCV.

A yeast autonomously-replicating double stranded DNA plasmid (YRp) carrying the selectable marker *ura3* (allows growth of uracil auxotrophs on minimal media) is prepared using a cDNA complement of the genome of the RNA virus HCV such as described in EPl 1769353, that is inserted into the yeast plasmid and operationally linked to the constitutive promoter *pykl* . A culture of a *Saccharomyces cerevisiae* that is auxotrophic for uracil (either self-prepared or commercially available such as *ura3-52* from Clonetech, Mountain View CA, USA) is then transformed with the plasmid and maintained on minimal media. Expression of the YRp at high copy numbers will result in the generation of the positive- and negative-sense RNA corresponding to the HCV genome which will form into inactive viral like particles (VLPs) as well as active infectious virions. VLPs and infectious virions are then prepared by breakage of the facilitating host yeast cells and separation of VLPs and virions by density gradient centrifugations. Purified virions and/or VLPs can then be rendered non-infective by treatment with formaldehyde and used directly as a vaccine.

Example 7. Construction of a yeast cell line containing a stably-integrated HCV genome.

A yeast double stranded Integrative DNA plasmid (Yip) carrying the selectable marker HIS4 *(Pichia* Expression Kit from Invitrogen/Life Technologies Carlsbad, CA, USA) is prepared using a cDNA complement of the genome of the RNA virus HCV such as described in EPl 1769353, that is inserted into the Yip and operationally linked to the AOX1 promoter. The commercially available plasmids from Invitrogen are designed to integrate at the AOX1 locus in the methanotrophic yeast *Pichia pastoris* by a single crossover event at any of the three AOX1 regions on the vector. This results in the insertion of one or more copies of the vector upstream or downstream of the AOX1 gene. The phenotype of this recombinant is His^+ (now capable of growing on minimal media) and either Mut^+ (grows well on methanol) or Mut^s

(grows slowly on methanol). The prepared Yip is used to transform *Pichia pastoris* GS115 (a host *Pichia* strain available from Invitrogen that has a mutation in the histidinol dehydrogenase gene (his4) that prevents them from synthesizing histadine and rendering them unable to grow on minimal media; i.e., His^-). Transformed yeasts are selected on minimal media containing methanol. Transformed cells will express both positive- and negative- sense copies of the HCV genome.

Expression of the HCV genome at high copy numbers in the facilitating *Pichia* cells will result in the formation of inactive viral like particles (VLPs) as well as active infectious virus within the facilitating host cells. VLPs and infectious virions are then prepared by breakage of the facilitating host yeast cells and separation of VLPs and virions by density gradient centrifugations. Purified virions and/or VLPs can then be rendered non-infective by treatment with formaldehyde and used directly as a vaccine.

Example 8. Screening of HCV antiviral drug using a transgenic yeast cell line expressing HCV.

Example 8a: Screening ofNS3 protease inhibitor MK-5172 (Merck & Co) in transgenic yeast cells.

Yeast cells prepared as described in Example 7 above are cultured in yeast cell minimal medium with methanol to at least 25 x 10^6 cells/ml and the HCV antiviral drug, MK-5172 (Merck & Co) an NS3 inhibitor, is added to the cell culture media. Beginning at 6 hours post-addition, cells and cell supernatants are collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 9 hrs, and 120 hrs, and the levels of the positive- and negative-sense RNA or the RNA virus is measured by real-time RT-PCR following published protocols (Dhar et al., 2002;Konurian-Pradel et al., 2004; Carriere et al., 2007) as described in Example 1.

The efficacy of MK-5172 is also determined by Western blot analysis using aliquots of cell culture samples collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 9 hrs, and 120 hrs and anti-NS3 protease/helicase antibodies are used for Western blot analysis.

Example 8b: Screening ofNS5B polymerase inhibitor R7128 (Roche/Pharmasset) in transgenic yeast cells.

Yeast cells prepared as described in Example 7 above are cultured in yeast cell minimal

medium containing methanol to at least 25 x 10^6 cells/ml and the HCV antiviral drug, R7128 (Roche/Pharmasset) an NS5B polymerase inhibitor, is added to the cell culture media. Beginning at 6 hours post-addition, cells and cell supernatants are collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 9 hrs, and 120 hrs, and the levels of the positive- and negative-sense RNA or the RNA virus is measured by real-time RT-PCR following published protocols (Dhar et al., 2002;Konurian-Pradel et al., 2004; Carriere et al., 2007) as described in Example 1.

The efficacy of SN5B is also determined by Western blot analysis using aliquots of cell culture samples collected at 12 hrs, 24 hrs, 48 hrs, 72 hrs, 9 hrs, and 120 hrs and anti-NS3 protease/helicase antibodies are used for Western blot analysis.

What is claimed is:

1. A method for replicating an RNA viral genome in a facilitating host cell comprising:

a) introducing into a facilitating host cell a facilitating DNA virus comprising a promoter operably linked to a DNA sequence that codes for an RNA viral genome;

b) culturing said facilitating host cell under conditions suitable for transcription of said RNA viral genome; and

c) replicating said transcribed RNA viral genome in said facilitating host cell culture.

2. The method of Claim 1, further comprising determining an amount of said replicated RNA viral genome in said facilitating host cell culture.

3. The method of claim 2, further comprising

d) treating said facilitating host cell culture with a composition; and

e) determining a second amount of RNA viral replication in said facilitating host cell culture.

4. The method of Claim 3, wherein said treating comprises more than one composition, preferably comprising an inhibitor of facilitating host cell transcription.

5. The method of Claim 1, wherein said RNA viral genome naturally occurs in an RNA virus that does not naturally infect said facilitating host cell.

6. The method of Claim 1, wherein said RNA viral genome is functional to package as an RNA virus.

7. The method of Claim 6, wherein said RNA virus is capable of inducing an immune response from a host.

8. The method of Claim 6, wherein said RNA virus is selected from the group consisting of a double- stranded RNA virus, a plus-strand RNA virus, and a minus-strand RNA virus.

9. The method of Claim 6, wherein said RNA virus is selected from the group consisting of

a mono- segmented (+)sense single stranded RNA virus,

a bi- segmented (+) sense single stranded RNA virus,

a tri-segmented (+)sense single stranded RNA virus,

a multi- segmented (+)sense single stranded RNA virus,
a mono- segmented (-)sense single stranded RNA virus,
a bi-segmented (-) sense single stranded RNA virus,
a tri-segmented (-)sense single stranded RNA virus,
a multi- segmented (-) sense single stranded RNA virus,
a mono- segmented double stranded RNA virus,
a bi-segmented double stranded RNA virus,
a tri-segmented double stranded RNA virus, and
a multi- segmented double stranded RNA virus.

10. The method of Claim 6, wherein said RNA virus is selected from the group consisting of a mono- segmented (+)sense single stranded RNA virus, a bi-segmented (+)sense single stranded RNA virus, a tri-segmented (+)sense single stranded RNA virus, a multi-segmented (+)sense single stranded RNA virus, a mono- segmented (-)sense single stranded RNA virus, a bi-segmented (-)sense single stranded RNA virus, a tri-segmented (-)sense single stranded RNA virus, a multi- segmented (-)sense single stranded RNA virus.

11. The method of Claim 9, wherein said RNA virus is a mono- segmented (+)sense single stranded RNA virus.

12. The method of Claim 11, wherein said RNA virus is a HCV virus.

13. The method of Claim 1, wherein said facilitating DNA virus is a recombinant baculo virus.

14. The method Claim 13, wherein said recombinant baculovirus is introduced by the method selected from the group consisting of infection, transduction, transfection, and cell fusion with recombinant baculovirus containing cell.

15. The method of Claim 1, wherein said replicated RNA viral genome is packaged into a virion.

16. A plurality of virions according to the method of Claim 15.

17. The plurality of virions according to Claim 16, wherein said plurality comprises a mixture of virions with positive and negative strands of said RNA viral genome, wherein each said virion has either only positive strands of said RNA viral genome or only negative

strands of said RNA viral genome.

18. The plurality of virions according to Claim 16, wherein said plurality does not comprise a mixture of positive strands and negative strands of said RNA viral genome.

19. The method of Claim 15, wherein said virion can infect a host cell.

20. The method of Claim 15, wherein said virion is not capable of infecting a host cell.

21. The method of Claim 1, wherein said RNA viral genome is replication defective.

22. The method of Claim 21, wherein said facilitating cell provides a component of the replication machinery *in trans* to complement said replication defective RNA viral genome.

23. The method of Claim 1, wherein said promoter is selected from the group consisting of a constitutive promoter, a cell-type specific promoter, and an inducible promoter.

24. The method of Claim 1, wherein said facilitating host cell is an insect cell, a mammalian cell, or a yeast cell.

25. A non-host cell comprising a genome having a stably integrated promoter operably linked to a DNA sequence that codes for an RNA viral genome.

26. The non-host cell of Claim 25, wherein said stably integrated DNA sequence codes for a functional RNA viral genome.

27. The non-host cell of Claim 25, wherein said stably integrated DNA sequence codes for a RNA viral genome that does not package into a virion.

28. The non-host cell of Claim 25, wherein said stably integrated DNA sequence codes for a complete RNA viral genome relative to the number of genes in a similar naturally occurring RNA virus.

29. The non-host cell of Claim 28, wherein said naturally occurring RNA virus is obtained from a virus selected from the group consisting of

a mono-segmented (+)sense single stranded RNA virus,
a bi-segmented(+)sense single stranded RNA virus,
a tri-segmented (+)sense single stranded RNA virus,
a multi- segmented (+)sense single stranded RNA virus,
a mono- segmented (-)sense single stranded RNA virus,

a bi-segmented (-)sense single stranded RNA virus,
a tri-segmented (-)sense single stranded RNA virus,
a multi-segmented (-)sense single stranded RNA virus,
a mono-segmented double stranded RNA virus,
a bi-segmented double stranded RNA virus,
a tri-segmented double stranded RNA virus, and
a multi-segmented double stranded RNA virus.

30. The non-host cell of Claim 29, wherein said non-host cell genome comprises a positive strand promoter and a negative stand promoter flanking each segment of said DNA sequence that codes for an RNA viral genome of a mono-segmented, bi-segmented, tri-segmented, or multi-segmented negative-sense single-stranded RNA ("(-)sense RNA") virus, wherein said RNA virus comprising said RNA viral genome is not capable of infecting said non-host cell and said negative strand promoter has a corresponding terminator between said positive strand promoter and each segment of said RNA viral genome.

31. The non-host cell of Claim 30, wherein at least one of said each segment is under the control of a promoter heterologous to said non-host cell and heterologous to said RNA viral genome.

32. The non-host cell of Claim 30, wherein said multi-segmented virus comprises 3, 4, 5, 6, 7, or 8 segments.

33. The non-host cell of Claim 32, wherein said multi-segmented virus comprises an influenza viral genome.

34. The non-host cell of Claim 29, wherein said non-host cell genome comprises a DNA sequence that codes for an RNA viral genome of a mono-segmented, bi-segmented, tri-segmented, or multi-segmented positive-sense single-stranded RNA ("(+)sense RNA") virus that is not capable of infecting said non-host cell.

35. The non-host cell of Claim 34, wherein said DNA sequence is under the control of at least one promoter heterologous to said non-host cell and heterologous to said RNA viral genome.

36. The non-host cell of Claim 34, wherein said RNA viral genome occurs naturally in a

Hepatitis C virus.

37. The non-host cell of Claim 34, wherein said multi-segmented virus comprises 3, 4, 5, 6, 7, or 8 segments.

38. A non-host cell of Claim 25 that has been clonally selected.

39. The non-host cell of Claim 29, wherein said DNA sequence is expressed as an RNA molecule in said non-host cell.

40. The non-host cell of Claim 39, wherein said transcribed RNA molecule is packaged into a virion.

41 A virion obtained from the non-host cell of Claim 40.

42. A plurality of virions according to Claim 41.

43. The plurality of virions according to Claim 40, wherein said plurality comprises a mixture of virions containing genomes selected the group consisting of:
a mono-segmented (+)sense single stranded RNA genome and a complementary strand of a mono-segmented (+)sense single stranded RNA genome;
a bi-segmented (+)sense single stranded RNA genome and a complementary strand of a bi-segmented (+)sense single stranded RNA genome, and a combination virion containing a (+)sense single stranded RNA segment and a complementary strand of a (+)sense single stranded RNA segment;
a tri-segmented (+)sense single stranded RNA genome and a negative strand of a tri-segmented (+)sense single stranded RNA genome and a combination virion containing a (+)sense single stranded RNA segment of tri-segmented (+)sense single stranded RNA genome and at least one complementary stranded (+)sense single stranded segment of tri-segmented (+)sense single stranded RNA genome;
a mono-segmented (-)sense single stranded RNA genome and a complementary strand of a mono-segmented (-)sense single stranded RNA genome;
a bi-segmented (-)sense single stranded RNA genome and a complementary strand of a bi-segmented (-)sense single stranded RNA genome, and a combination virion containing a (-)sense single stranded RNA segment and a complementary strand of a (-)sense single stranded RNA;
a tri-segmented (-)sense single stranded RNA genome and a negative strand of a tri-

segmented (-)sense single stranded RNA genome and a combination virion containing a (-) sense single stranded RNA segment of tri-segmented (-)sense single stranded RNA genome and at least one complementary stranded (-)sense single stranded segment of tri-segmented (-) sense single stranded RNA genome.

44. The non-host cell of Claim 25 which is an insect cell, a mammalian cell, or a yeast cell.

45. A method for preparing an altered RNA virus capable of infecting a non-host cell of a naturally occurring RNA virus that shares over half of the genome of said altered RNA virus comprising:

a) introducing into a facilitating host cell a facilitating DNA virus comprising a promoter operably linked to a DNA sequence that codes for said genome of said altered RNA virus;

b) culturing said facilitating host cell under conditions suitable for transcription of said RNA viral genome; and

c) producing said altered RNA virus in said facilitating host cell.

46. The method of Claim 45, wherein said RNA viral genome comprises a recombinant viral coat protein sequence.

47. The method of Claim 45, wherein said altered RNA virus comprises an altered viral surface coat.

48. The method of Claim 47, wherein said altered viral surface coat has a mutation from a naturally occurring viral surface coat selected from the group consisting of altered O-glycosylation of a viral coat protein, altered N-glycosylation of a viral coat protein, a recombinant viral coat protein, and a combination thereof

49. The method of Claim 48, wherein said recombinant viral coat protein has a mutation from a naturally occurring coat protein selected from the group consisting of an altered protein glycosylation site, the addition of a protein epitope, the removal of a protein epitope, the replacement of a protein domain, and the removal of a protein domain.

50. A virion prepared by the method of Claim 45.

51. The method of Claim 45, wherein the facilitating host cell is an insect cell, a mammalian cell, or a yeast cell.

52. A method of infecting a cell with an RNA virus that is not infected in nature by said RNA virus comprising expressing in a non-host cell a transgene for a receptor for said RNA virus selected from the group consisting of a double- stranded RNA virus, a plus-strand RNA virus, and a minus-strand RNA virus.

53. The method of Claim 52, wherein said transgenic cell expresses said receptor transgene on the cell surface.

54. The method of Claim 52, further comprising expressing a co-receptor of said RNA virus on the surface of said transgenic cell.

55. The method of Claim 52, wherein said non-host cell is an insect cell, a mammalian cell, or a yeast cell.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/020301

A. CLASSIFICATION OF SUBJECT MATTER

C12Q l/68(2006.01)i, C12N 15/11(2006.01)1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12Q 1/68; C12N 15/51; C12N 5/06; C12N 5/10; C12N 1/15; C12N 15/866; A61K 39/00; A61K 39/29; C12Q 1/70; A61K 39/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords:replication, RNA virus, DNA virus, screening antiviral agent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	W0 2011-130125 Al (VIRACINE THERAPEUTICS CORPORATION et al.) 20 October 2011 See claims 33, 37; pages 6-8, 11-17.	1-44 45-55
A	US 7148006 B2 (KATZE et al.) 12 December 2006 See the whole document.	1-55
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A	US 6251654 Bl (GORDON et al.) 26 June 2001 See the whole document.	1-55

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 April 2013 (05.04.2013)	**08 April 2013 (08.04.2013)**

Name and mailing address of the ISA/KR

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

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