Titre: PARAMYOXYVIRUSES HAVING MODIFIED TRANSCRIPTION INITIATION SEQUENCE

Abstract:
Virus vectors of the family Paramyxoviridae in which the transcription initiation sequence has been modified and thus the expression of genes located downstream thereof has been modified; a process for producing the same; and use of the same. By measuring the transcription initiation efficiency of each gene carried by Sendai viruses, it is clarified that the transcription initiation sequence of F gene has a significantly lower ability to promote the transcription than the three other transcription initiation sequences. When the transcription initiation sequence of the F gene of wild type Sendai virus is substituted by the transcription initiation sequence of the P/MHN genotype showing a high efficiency, the F gene of the resultant Sendai virus mutant and genes located downstream thereof show elevated expression doses. It is also found out that this mutant proliferates more quickly than the wild type. The above-described vectors are useful in producing medicinal compositions and vaccines.
ABSTRACT

The present invention provides virus vectors of the family Paramyxoviridae in which the transcription start (S) sequence has been modified so as to modify the expression of genes located downstream thereof, a method for producing the vectors, and uses thereof. By measuring the transcription initiation efficiency of the S sequence of each gene carried by Sendai viruses (SeV), it was clarified that the S sequence of F gene has a significantly lower ability to promote transcription than the other three S sequences. When the S sequence of the F gene of wild type Sendai virus was substituted by the S sequence of the P/M/HN gene-type showing a high transcription initiation efficiency, the F gene of the resultant Sendai virus mutant and genes located downstream thereof show elevated expression levels. It was also revealed that this mutant proliferates more quickly than the wild type. The vectors of this invention are useful in elevating the expression of foreign genes and producing pharmaceutical compositions and vaccines.
DESCRIPTION

PARAMYXOVIRUSES COMPRISING MODIFIED TRANSCRIPTION START SEQUENCE

5 Technical Field
The present invention relates to recombinant viruses of Paramyxoviridae comprising a modified transcription start sequence.

Background Art

Paramyxoviruses have a non-segmented negative strand RNA as the genome. Six genes are coded in the genome, and a short sequence (E-IG-S signal) is commonly linked to each gene. These signal sequences are highly conserved especially within a genus and within a family, and is extremely high among genes of a given virus species (Feldmann, H. E. et al., 1992, Virus Res. 24:1-19).

Sendai virus (SeV), classified into Respirovirus in the family Paramyxoviridae, is an enveloped, non-segmented negative-strand RNA virus that is considered to be the prototype for the subfamily Paramyxovirinae. The SeV genome is 15,384 bases in size, starting with a short 3' leader region, followed by six genes encoding the N (nucleocapsid), P (phospho), M (matrix), F (fusion), HN (hemagglutinin-neuraminidase) and L (large) proteins, and ending with a short 5' trailer region. In addition to the P protein, the second gene expresses the accessory V and C proteins by a process known as co-transcriptional editing that inserts a G residue not comprised in the template (Park, K.H. and M. Krystal, 1992, J. Virol. 66:7033-7039; Paterson, R.G., and R.A. Lamb, 1990, J. Virol. 64:4137-4145; Thomas, S.M. et al., 1988, Cell, 54:891-902; Vidal, S. et al., 1990, J. Virol. 64:239-246) and by alternative translational initiations, respectively (Gupta, K. C., and E. Ono, 1997, Biochem. J. 321:811-818; Kuronati, A. et al., 1998, Genes Cells 3:111-124). The genome is tightly associated with the N protein, forming a helical ribonucleoprotein (RNP) complex. This RNP, but not the naked RNA, is the template for both transcription and replication (Lamb, R.A., and D. Kolakofsky, 1996, Paramyxoviridae: The viruses and their replication. pp.1177-1204. In Fields Virology, 3rd edn.

After the translation of the mRNAs and accumulation of translation products, genome replication takes place. Here, the same viral RNA polymerase conducts replication using the same RNP template, but now somehow ignores the respective E sequence and S sequence of each mRNA and generates a full length antigenic positive sense (+)RNP (Lamb, R.A., and D. Kolakofsky, 1996, Paramyxoviridae: The viruses and their replication. pp.1177-1204. In Fields Virology, 3rd edn. Fields, B. N., D. M. Knipe, and P. M. Howley et al. (ed.), Raven Press, New York, N. Y.). The polymerase enters the promoter at the 3' end of (+)RNP to generate genomic (-)RNP, which serves as the template for the next round of transcription and replication.

The E sequence (3'-AUUCUUUUUUU-5' in the genomic negative sense) is completely conserved among the six genes in the SeV genome. The five U residues in the latter half are thought to allow the polymerase slippage-generating poly(A). In contrast, the S sequences are
slightly varied and are generalized as 3'-UCCCWVUWUC-5' (Gupta, K. C., and D. W. Kingsbury, 1984, Nucleic Acids Res. 12:3829-3841). Specifically, the S sequence is UCCCACUUC for P, M and HN genes, UCCCAGUUUC for N gene, UCCCuaUUUC for F gene, and UCCCACUUAc for L gene. Identical differences are seen in all SeV strains sequenced to date, regardless of differences in isolation procedure, passage history, and virulence for the natural host such as mice, suggesting that the variations are locus-specific. It is possible that these differences arise as a result of nucleotide accumulation in sites that are unaffected by variations in the S sequence. Another possibility is that these differences arise due to nucleotide substitutions at important sites of the signal and the selection of viruses that have acquired the ability to regulate the expression of each gene during viral evolution.

trans-acting proteins masks the subtle effects of mutations by, for example, posttranscriptional modifications by capping enzymes encoded by vaccinia viruses. In addition, transfection efficiencies might not be equal throughout the whole experiment (Bukreyev, A. et al., 1996, J. Virol. 70:6634-6641; He, B. et al., 1997, Virology 237:249-260). Namely, effects of nucleotide substitutions in the S sequence on transcription initiation cannot be accurately examined in model template systems. Thus, to comprehensively evaluate the roles of S sequence and E sequence, it was necessary to introduce mutations into the full-length viral genome.

Disclosure of the Invention

An objective of the present invention is to provide virus vectors of Paramyxoviridae in which the S sequence has been modified so as to modify the expression of genes located downstream thereof, a method for producing the vectors as well as uses thereof.

The present inventors have already succeeded in constructing a system to produce infectious SeV by manipulating their genomes using recombinant DNA techniques. The use of this system enables the regeneration of negative strand RNA viruses based on their corresponding DNA, and to perform reverse genetics of SeV by manipulating various genes of the infectious virus (Kato, A. et al., 1997, EMBO J. 16: 578-587; Kato, A. et al., 1997, J. Virol. 71: 7266-7272; Kuo, L. et al., 1996, J. Virol. 70: 6892-6901; Nagai, Y., 1999, Rev. Medical. Virol. 9: 83-99; Sakaguchi, T. et al., 1997, Virology 235: 360-366). Using this system, the present inventors have attempted to elucidate the significance of heterogeneity found in the S sequences of SeV.

Newly synthesized E sequence and S sequence were ligated to the upstream of the firefly luciferase gene, and this was inserted to the downstream of the noncoding region of the N gene. The S sequences were designed to have same sequence as the four naturally-occurring variations described above. In the constructed recombinant virus, the N mRNA transcription starts by its own S sequence and stops by the synthetic E sequence within the inserted reporter (luciferase) gene. The reporter gene expression, which is driven by each of the
different S sequences, was quantitated and compared.

The results obtained here clearly showed that the natural S sequence for the F gene had a significantly lower reinitiation activity than the other three S sequences. When de novo protein synthesis is blocked and genome replication is inhibited, only transcription occurs, and replication does not. By conducting experiments under such conditions, it was confirmed that the reduced luciferase gene expression by the F specific signal was indeed caused primarily at the transcriptional level, and was not a secondary result of replication (Fig. 4). This experiment further showed that the reinitiation activity driven by the S sequence of F gene was approximately one forth of that of the other three.

The reinitiation capacity of different S sequences was then assessed by replacing the natural S sequence of the F gene with that of P/M/HN genes having a higher reinitiation efficiency and by examining replication capability of the recovered virus (SeV/mSF) in cultured cells, in ovo, and in mice. As a result, the inventors found that the replaced S sequence enhances not only F gene expression, but also the expression of downstream genes, again at the transcriptional level (Figs. 7 and 9).

That is, the present inventors found that the reinitiation activity of S sequence of each gene of viruses belonging to Paramyxoviridae varies from the S sequence. It was also revealed that the substitution of S sequence of a particular gene by another S sequence having a different reinitiation activity enables the modification of expression of not only the gene right after the sequence, but also genes located further downstream of the gene at the transcriptional level, to complete the invention.

This invention relates to virus vectors of Paramyxoviridae in which a S sequence has been modified so as to modify expression levels of genes located downstream of the S sequence, a method for producing such vectors and the use thereof, more specifically to relates to: (1) a virus vector DNA, wherein the transcription start (S) sequence of at least one gene on the genome of a virus belonging to Paramyxoviridae has been modified so as to modify the expression level of said gene and genes located downstream thereof within the host,
(2) the virus vector DNA according to (1), wherein said modification of the transcription start sequence comprises the substitution of said sequence by the transcription start sequence of another gene of a virus belonging to Paramyxoviridae,

(3) the virus vector DNA according to (1), wherein said modification of transcription start sequence comprises the substitution of the transcription start sequence of F gene by the transcription start sequence of another gene,

(4) the virus vector DNA according to (3), wherein said transcription start sequence of another gene comprises that of a P/M/HN gene type,

(5) a virus vector DNA according to any one of (1) to (4), wherein said virus vector DNA is defective in F gene and/or HN gene,

(6) a virus vector DNA according to any one of (1) to (5), wherein a foreign gene has been inserted into said virus vector DNA,

(7) a virus vector of Paramyxoviridae comprising a transcription product from a virus vector DNA according to any one of (1) to (6) within virus particles,

(8) the vector according to (7), wherein said vector is a Sendai virus (SeV) vector,

(9) the vector according to (7) or (8), wherein the proliferation capability in the host is elevated compared to that of the wild type virus,

(10) a method for producing a virus vector of Paramyxoviridae, wherein said method comprises the steps of transferring a virus vector DNA according to any one of (1) to (6) into the host, and expressing the virus protein in said host, and

(11) the method according to (10), wherein said virus of Paramyxoviridae used to produce the vector is Sendai virus.

Herein, a “virus vector of Paramyxoviridae” is defined as a vector (or carrier) that is derived from a virus of Paramyxoviridae, and which can transfer a gene to a host cell. The virus vector of Paramyxoviridae of the present invention may be a ribonucleoprotein (RNP) or a virus particle having infectivity. Here, “infectivity” is defined as the ability of the virus vector to transfer, through its cell adhesion and membrane fusion abilities, the virus genome contained in the virus particles to cells, and to express it.
The virus vector of Paramyxoviridae may have a replication capability, or may be a defective vector without the replication capability. Herein, "have a replication capability" is defined as the ability of virus vectors to replicate and produce infective virus particles in host cells infected with the virus vectors.

The virus vector of Paramyxoviridae of this invention can carry a foreign gene in an expressible manner. Such virus vectors can be prepared as recombinant virus vectors of Paramyxoviridae. Herein, a "recombinant" virus vector of Paramyxoviridae is defined as one constructed by genetic engineering, or its amplified products. For instance, recombinant virus vectors of Paramyxoviridae can be generated from a recombinant virus cDNA of Paramyxoviridae.

Herein, a virus of Paramyxoviridae is defined as a virus belonging to the family Paramyxoviridae, or a derivative thereof. The present invention can be applied to, for example, a virus of Paramyxoviridae such as the Sendai virus, Newcastle disease virus, Mumps virus, Measles virus, Respiratory syncytial virus, rinderpest virus, Canine distemper virus, simian parainfluenza virus (SV5), and type I, II, and III human parainfluenza virus. The virus vector and vector DNA of the present invention are preferably derived from a virus of the genus Paramyxovirus or a derivative thereof. Viruses of the genus Paramyxovirus to which the present invention is applicable include type I parainfluenza viruses including Sendai virus and human HA2, type II parainfluenza viruses including simian SV5 and SV41 and human CA, type III parainfluenza viruses including bovine SF and human HA1, type IV parainfluenza viruses including subtype A and subtype B, Mumps virus, Newcastle disease virus, and many other viruses of the genus Paramyxovirus. Most preferably, the virus vector and vector DNA of the invention are derived from the Sendai virus. These viruses may be wild-type strains, mutant strains, laboratory-passaged strains, artificially constructed strains, and so on. Incomplete viruses such as the DI particle (Willenbrink W. and Neubert W. J., J. Virol., 1994, 68, 8413–8417), synthesized oligonucleotides, and so on, may also be utilized as material for generating the virus vector of the present invention.

Herein, "virus vector DNA" means DNA comprising a nucleotide
sequence encoding the genome of a virus vector. "DNA" herein includes single-stranded DNA and double-stranded DNA.

Here, the "N, P, M, F, HN, and L genes" of the viruses of Paramyxoviridae represent those encoding the nucleocapsid protein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase, and large protein, respectively. Genes of each virus of the subfamily Paramyxovirinae are described generally as follows. In general, N gene may also be indicated as "NP gene".

- **Respirovirus** N P/C/V M F HN - L
- **Rublaviruse** N P/V M F HN (SH) L
- **Morbillivirus** N P/C/V M F H - L

For instance, the accession numbers in the nucleotide sequence database of each gene of the Sendai virus, are M29343, M30202, M30203, M30204, M51331, M55565, M69046, and X17218 for N gene; M30202, M30203, M30204, M55565, M69046, X00583, X17007, and X17008 for P gene; D11446, K02742, M30202, M30203, M30204, M69046, U31956, X00584, X53056 for M gene; D00152, D11446, D17334, D17335, M30202, M30203, M30204, M69046, X00152, and X02131 for F gene; D26475, M12397, M30202, M30203, M30204, M69046, X00586, X02808, X56131 for HN gene; and D00053, M30202, M30203, M30204, M69040, X00587, and X58886 for L gene.

This invention provides virus vector DNAs in which the S sequence of at least one gene on the genome of a virus belonging to Paramyxoviridae has been modified so as to modify the expression levels of the gene and genes located downstream thereof in the host. Virus vector DNAs of this invention are capable of modifying transcription levels of not only a gene right after the S sequence but also gene(s) downstream thereof, by modifying the S sequence.

"Modification of a transcription start (S) sequence" in this invention refers to carrying out the substitution, deletion, addition and/or insertion of one or more nucleotides in the S sequence of a gene on the genome of a virus belonging to Paramyxoviridae or the substitution of the S sequence of a gene by that of another gene of a virus belonging to Paramyxoviridae.

The modification of the S sequence to obtain a sequence having a desired reinitiation activity may be carried out by designing a
variety of S sequences, and detecting the reinitiation activity using the luciferase assay and such as described in Example 1 to select a sequence having the desired activity. S sequences may be modified by using known genetic engineering techniques. For example, as described in Example 3, any desired mutation can be introduced into the S sequence of the F gene on the genome of a virus belonging to Paramyxoviridae using site-specific mutagenesis.

Virus vectors of Paramyxoviridae according to this invention include those in which a S sequence has been modified so that the expression level of, for example, the F gene is significantly elevated as compared with the wild type virus. Significant elevation refers to an elevation in expression levels, for example, by 20% or more, preferably 40% or more, more preferably 2-fold or more, even more preferably 3-fold or more as compared with the expression of the wild type F gene. Such vectors can be produced, for example, by substituting the S sequence of the F gene by that of P, M, HN, N or L gene. Virus vectors of Paramyxoviridae according to this invention include those in which the expression level of any of P, M, HN, N or L gene, or any combinations thereof is significantly reduced as compared with the expression of the wild type. Significant reduction means a reduction in expression, for example, by 20% or more, preferably 30% or more, more preferably 40% or more, and even more preferably 60% or more as compared with that of the wild type. Such vectors can be produced, for example, by substituting the S sequence of P, M, HN, N and/or L gene by that of F gene. Expression levels of genes can be measured, for example, through the detection of mRNA (transcription product) or proteins (translation product). The gene expression level is measured preferably under conditions that minimize the effect of virus replication rate. For example, as shown in Fig. 3 of Example 1, gene expression level can be measured under conditions in which only one replication cycle takes place, alternatively, as represented in Fig. 4, by specifically estimating the primary transcription through the detection of RNAs or proteins. These measurements can be carried out, for example, by the methods described in Example 1 or 2.

The present inventors examined the reinitiation activity of 4
different S sequences found in a virus of Paramyxoviridae (Sendai virus), and discovered that the activity was different in each of them and that while the reinitiation activities of the S sequences of L gene (AGGTTGAAT), P/M/HN gene (AGGGTGAAA) and N gene (AGGGTCAAA) showed a high value, the reinitiation activity of the S sequence of F gene (AGGGATAAAA) was low. Therefore, when a high reinitiation activity is desired, S sequences of L gene, P/M/HN gene or N gene may be used, while when a low reinitiation activity is preferred, the S sequence of F gene may be used. For example, the substitution of S sequence of F gene by that of P/M/HN gene having a high reinitiation activity can lead to the elevation of transcription levels of F gene and genes located downstream thereof.

There are a variety of advantages of modifying transcription levels of virus genes of Paramyxoviridae. For example, with a virus in which the S sequence of F gene has been substituted by one having a higher reinitiation activity, the viral proliferation capability can be elevated. In addition, by exchanging the S sequence of F gene and that of L gene, it can be expected that only expression levels of F and HN genes would be elevated, leaving the viral proliferation capability unaffected. Furthermore, in the case of a protein whose high expression is undesirable, the expression level of the protein can be restricted by linking the gene thereof to the downstream of the S sequence with a low reinitiation activity, such as that of F gene.

In a viral genome comprising a S sequence modified to have a higher transcription reinitiation activity, the expression level of mRNA encoded by the gene downstream of the modified S sequence is increased compared with the original wild type genome. Accordingly, when a desired foreign gene is located downstream of the modified S sequence, the gene product level is also expected to elevate. Therefore, virus vectors having such genomes are advantageous in that the production efficiency of gene product(s) has been improved. In addition, a virus having such a genome has the advantage of yielding a large amount of viruses in a short time, when collecting recombinant virus particles or virus-like particles as pharmaceutical compositions or vaccines. For example, it has been known that virus
particles incubated at 37°C for 2 days form complexes among them and undergo an aging phenomenon in which their original morphology changes. (Kim, J. et al., Virology 95: 523-535 (1979)). Observation of these under an electron microscope has revealed that the nucleocapsid structure is tightly folded in de novo synthesized viral particles, but unfolds and becomes loose with aging. When utilizing viral particles or virus-like particles as pharmaceutical compositions and vaccines, it is important to obtain homogeneous materials. Therefore, it is necessary to recover viruses from a culture as short as possible. As shown in Examples, the present invention may allow the preparation of modified virus having a titer as high as 100-folds as compared with the wild type virus (Fig. 5).

The virus vector of Paramyxoviridae of the present invention includes, for example, vectors that have the replication capability and those that are capable of autonomous proliferation. In general, the genome of the wild type paramyxovirus contains a short 3' leader region followed by six genes encoding N, P, M, F, HN, and L proteins, and has a short 5' trailer region on the other terminus. The vector of the present invention that is able to replicate autonomously can be obtained by designing a genome having a similar structure to that described above. The virus vector of Paramyxoviridae of the invention may have an altered alignment of virus genes, compared with wild type virus.

The virus vector of Paramyxoviridae of the present invention may be defective in any of the genes that are contained in the wild type virus. For instance, in the case of the reconstitution of the Sendai virus vector, proteins encoded by N, P/C, and L genes are thought to be required in trans, but the genes may not be a component of the virus vector. In one embodiment, an expression vector carrying genes encoding the proteins may be co-transfected into host cells with another expression vector encoding the vector genome to reconstitute a virus vector. Alternatively, an expression vector encoding the virus genome is transfected into host cells carrying genes encoding the proteins, and thus a virus vector can be reconstituted by using the proteins provided by the host cell. The amino acid sequence of these proteins may not be identical to those
derived from the original virus as long as it has an equivalent or higher activity in nucleic acid transfer, and may be mutated or substituted with that of a homologous gene of another virus.

Proteins encoded by M, F, and HN genes are thought to be essential for cell-to-cell propagation of almost all viruses of Paramyxoviridae. However, these proteins are not required when the virus vector of Paramyxoviridae is prepared as RNP. If genes M, F, and HN are components of the genome contained in RNP, products of these genes are produced when introduced into host cells, and virus particles having infectivity are generated.

RNP can be introduced into cells as a complex formed with lipofectamine, polycationic liposome, and the like. Specifically, a variety of transfection reagents can be used, for instance, DOTMA (Boehringer), SuperFect (QIAGEN #301305), DOTAP, DOPE, DOSPER (Boehringer #1811169). Chloroquine may be added to prevent degradation in the endosome (Calos M. P., Proc. Natl. Acad. Sci. USA, 1983, 80, 3015). In the case of replicative viruses, the produced viruses can be amplified or passaged by re-infecting into cultured cells, embryonating hen eggs, or animals (e.g. mammalian such as mice).

Contrastingly, the virus vector of Paramyxoviridae of the present invention may be those lacking the M, F, and/or HN genes. These vectors can be reconstituted by providing deficient gene products exogenously. Such vectors can still adhere to host cells and induce cell fusion as the wild type could. However, daughter virus particles do not have the same infectivity as the original ones because the vector genome introduced into cells lacks one of the above genes. Therefore, these vectors can be safer virus vectors that are capable of only a single gene transfer. For instance, genes deleted from the genome may be F and/or HN genes. Virus vectors defective in F gene can be reconstituted by co-transfection of an expression plasmid encoding the genome of a recombinant virus vector of Paramyxoviridae lacking the F gene (containing virus vector DNA), an expression vector for the F protein, and that for N, P/C, and L proteins into host cells (PCT/JP00/03194 and PCT/JP00/03195). Alternatively, host cells in which the F gene is integrated into the chromosome may be used. The
amino acid sequence of these proteins provided exogenously may not be identical to those of the wild type and may be mutated or replaced by a homologous protein of another virus as long as they provide equivalent or higher gene transfer activity.

The envelope proteins of the virus vector of Paramyxoviridae of the present invention may comprise a protein other than the envelope protein of the original vector genome. There is no limitation on such proteins. These may include envelope proteins of other viruses such as the G protein of the vesicular stomatitis virus (VSV-G). Thus, the virus vector of the invention includes a pseudo type virus vector that has an envelope protein derived from a virus different from the original virus.

Any desired foreign gene, which may or may not encode proteins, can be inserted into the virus vector DNAs of this invention. For example, the foreign gene may encode functional RNA such as a ribozyme or anti-sense RNA. Foreign genes can comprise either naturally-occurring or artificially-designed sequences. For example, in gene therapy and such, a gene for treating an objective disorder is inserted into the virus vector DNA. When virus vector DNAs of this invention are used for manufacturing gene therapy vectors, it is desirable to delete F, HN and/or M genes from the virus vector DNAs so as to suppress their toxicity within the host. In the case of introducing a foreign gene into the DNA of virus vector, for example, in that of Sendai virus vector, it is desirable to insert a sequence comprising a multiple of six nucleotides of the foreign gene between the E sequence and S sequence of the virus vector DNA (J. Virol., Vol. 67, No. 8, 1993, p. 4822-4830), etc. A foreign gene can be inserted before and/or after the respective viral genes (N, P, M, F, HN or L genes). E-I-S sequence (transcription end sequence-intervening sequence-transcription start sequence) or portion thereof is appropriately inserted before or after a foreign gene so as not to interfere with the expression of genes before or after the foreign gene. Expression level of an inserted foreign gene can be regulated by the type of S sequence added to the 5' side (head) of the foreign gene as well as the site of gene insertion and nucleotide sequences before and after the gene. For example, in SeV, it has been
known that the nearer the insertion site to the N gene, the higher the expression level of the inserted gene.

Generally, the closer to the 3'–terminus of the negative strand RNA of the virus genome (the closer to N gene in the gene arrangement on the wild type virus genome) the insertion position is, the higher the expression level of the inserted gene will be. To achieve a high expression of a foreign gene, it is preferably inserted into the region near the 3' terminus of the negative stranded genome such as the upstream of the N gene (3' flanking sequence on the minus strand), or between N and P genes. Conversely, the closer to the 5'–terminus of the negative strand RNA (the closer to L gene in the gene arrangement on the wild type virus genome) the insertion position is, the lower the expression level of the inserted gene will be. To reduce the expression of a foreign gene, it may be inserted into the most 5' position on the negative strand, that is, downstream of the L gene in the wild type virus genome (5' flanking region of the L gene on the negative strand) or upstream of the L gene (3' flanking region of L gene on the negative strand). Thus, the insertion position of a foreign gene can be properly adjusted so as to obtain a desired expression level of the gene or optimize the combination of the insert with the virus genes surrounding it. To help the easy insertion of a foreign gene, a cloning site may be designed at the position of insertion. For example, the cloning site may be the recognition sequence of restriction enzymes. The restriction sites in the virus vector DNA can be used to insert a foreign gene. The cloning site may be a multicloning site that contains recognition sequences for multiple restriction enzymes. The vector DNA of the present invention may have other foreign genes at positions other than that used for above insertion.

Recombinant SeV vectors comprising a foreign gene can be constructed as follows according to, for example, the description in "Kato, A. et al., 1997, EMBO J. 16: 578–587" and "Yu, D. et al., 1997, Genes Cells 2: 457–466".

First, a DNA sample comprising the cDNA nucleotide sequence of a desired foreign gene is prepared. It is preferable that the DNA sample can be electrophoretically identified as a single plasmid at
concentrations of 25 ng/μl or more. Below, a case where a foreign gene is inserted to DNA encoding viral genome utilizing NotI site will be described as an example. When NotI recognition site is included in the objective cDNA nucleotide sequence, it is preferable to delete the NotI site beforehand by modifying the nucleotide sequence using site-specific mutagenesis and such method so as not to alter the amino acid sequence encoded by the cDNA. From this DNA sample, the desired gene fragment is amplified and recovered by PCR. To have NotI sites on the both ends of amplified DNA fragment and further add a copy of E-I-S sequence of SeV to one end, a forward side synthetic DNA sequence and reverse side synthetic DNA sequence (antisense strand) are prepared as a pair of primers containing NotI restriction enzyme cleavage site sequence, E-I-S sequence and a partial sequence of the objective gene.

For example, to secure cleavage by NotI, the forward side synthetic DNA sequence is arranged in a form in which any two or more nucleotides (preferably 4 nucleotides excluding GCG and GCC, sequences originating in NotI recognition site, more preferably ACTT) are selected on the 5′-side of the synthetic DNA, NotI recognition site “gcggccgc” is added to its 3′-side, and to the 3′-side thereof, any desired 9 nucleotides or nucleotides of 9 plus a multiple of 6 nucleotides are added as the spacer sequence, and to the 3′-side thereof, about 25 nucleotide-equivalent ORF including the initiation codon ATG of the desired cDNA is added. It is preferable to select about 25 nucleotides from the desired cDNA as the forward side synthetic DNA sequence so as to have G or C as the final nucleotide on its 3′-end.

In the reverse side synthetic DNA sequence, any two or more nucleotides (preferably 4 nucleotides excluding GCG and GCC, sequences originating in the NotI recognition site, more preferably ACTT) are selected from the 5′-side of the synthetic DNA, NotI recognition site “gcggccgc” is added to its 3′-side, and to its further 3′-side, an oligo DNA is added as the insertion fragment to adjust the length. This oligo DNA is designed so that the total nucleotide number including the NotI recognition site “gcggccgc”, complementary sequence of cDNA and EIS nucleotide sequence of SeV genome originating
in the virus described below becomes a multiple of six (so-called "rule of six"); Kolakofski, D. et al., J. Virol. 72: 891-899, 1998). Further to the 3'-side of inserted fragment, a sequence complementary to S sequence of Sendai virus, preferably 5'-CTTTTCCTCCCT-3', I sequence, preferably 5'-AAG-3', and a sequence complementary to E sequence, preferably 5'-TTTTTCTTTACTACGG-3', is added, and further to the 3'-side thereof, about 25 nucleotide-equivalent complementary sequence counted in the reverse direction from the termination codon of the desired cDNA sequence the length of which is adjusted to have G or C as the final nucleotide, is selected and added as the 3'-end of the reverse side synthetic DNA.

PCR can be done according to the usual method with, for example, ExTaq polymerase (Takara Shuzo). Preferably, PCR is performed using Vent polymerase (NEB), and desired fragments thus amplified are digested with NotI, then inserted to NotI site of the plasmid vector pBluescript. Nucleotide sequences of PCR products thus obtained are confirmed with a sequencer to select a plasmid having the right sequence. The inserted fragment is excised from the plasmid using NotI, and cloned to the NotI site of the plasmid carrying the genomic cDNA. Alternatively, it is also possible to obtain the recombinant Sendai virus cDNA by directly inserting the fragment to the NotI site without the mediation of the plasmid vector pBluescript.

By transferring a virus vector DNA of this invention into host cells to express it therein, it is possible to prepare a virus vector comprising a transcription product from the virus vector DNA within virus particles. Specifically, a virus vector DNA of this invention may be transferred into host cells to express a viral protein within the host cells. Transfer of the virus vector DNA into host cells may precede the expression of viral proteins inside the host cells or vice versa, or these processes may be simultaneously carried out. Viral proteins can be expressed inside host cells by transferring, for example, expression vectors encoding the viral proteins to the host. When a virus vector DNA is made defective in F, HN and/or M genes, infectious virus particles are not formed with such a defective vector. However, it is possible to form infectious virus particles by separately transferring these defective genes, genes encoding
other viral envelope proteins, and such, to host cells and expressing them therein.

Methods for transferring virus vector DNA into cells include the following: 1) the method of preparing DNA precipitates that can be taken up by objective cells; 2) the method of preparing a DNA comprising complex which is suitable for being taken up by objective cells and which is also not very cytotoxic and has a positive charge, and 3) the method of instantaneously boring on the objective cellular membrane pores wide enough to allow DNA molecules to pass through by electric pulse.

In Method 2), a variety of transfection reagents can be utilized, examples being DOTMA (Boehringer), SuperFect (QIAGEN #301305), DOTAP, DOPE, DOSPER (Boehringer #1811169), etc. An example of Method 1) is a transfection method using calcium phosphate, in which DNA that entered cells are incorporated into phagosomes, and a sufficient amount is incorporated into the nuclei as well (Graham, F. L. and Van Der Eb, AJ., 1973, Virology 52: 456; Wigler, M. and Silverstein, S., 1977, Cell 11: 223). Chen and Okayama have investigated the optimization of the transfer technique, reporting that suitable DNA precipitates can be obtained under the conditions where 1) cells are incubated with DNA in an atmosphere of 2 to 4% CO₂ at 35°C for 15 to 24 h, 2) circular DNA with a higher precipitate-forming activity than linear DNA is used, and 3) DNA concentration in the precipitate mixture is 20 to 30 μg/ml (Chen, C. and Okayama, H., 1987, Mol. Cell. Biol. 7: 2745). Method 2) is suitable for a transient transfection. An old method is known in the art in which a DEAE-dextran (Sigma #D-9885, M.W. 5 x 10⁵) mixture is prepared in a desired DNA concentration ratio to perform the transfection. Since most of the complexes are decomposed inside endosomes, chloroquine may be added to enhance transfection effects (Calos, M. P., 1983, Proc. Natl. Acad. Sci. USA 80: 3015). Method 3) is referred to as electroporation, and is more versatile compared to methods 1) and 2) because it doesn’t have cell selectivity. Method 3) is the to be efficient under optimal conditions for pulse electric current duration, pulse shape, electric field potency (gap between electrodes, voltage), conductivity of buffers, DNA concentration, and cell density.
Among the above-described three categories, transfection reagents (method 2)) are suitable in this invention, because method 2) is easily operable, and facilitates the examining of many test samples using a large amount of cells. Preferably, SuperFect (QIAGEN #301305) or DOSPER (Boehringer #1811169) is used.


For example, simian kidney-derived LLC-MK2 cells are cultured in 24-well to 6-well plastic culture plates or 100 mm diameter culture dish using a minimum essential medium (MEM) containing 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin G and 100 µg/ml streptomycin) to 70 to 80% confluency, and infected, for example, with recombinant vaccinia virus vTF7-3 expressing T7 polymerase at 2 PFU/cell. This virus can be inactivated by a UV irradiation treatment for 20 min in the presence of 1 µg/ml psoralen (Fuerst, T. R. et al., Proc. Natl. Acad. Sci. USA 83: 8122-8126, 1986; Kato, A. et al., Genes Cells 1: 569-579, 1996). Amount of psoralen added and UV irradiation time can be appropriately adjusted. One hour after the virus adsorption, the cells are transfected with 2 to 60 µg, more preferably 3 to 5 µg, of the above-described recombinant SeV cDNA by the lipofection method and such using plasmids (24 to 0.5 µg of pGEM-N, 12 to 0.25 µg of pGEM-P and 24 to 0.5 µg of pGEM-L, more preferably 1 µg of pGEM-N, 0.5 µg of pGEM-P and 1 µg of pGEM-L) (Kato, A. et al., Genes Cells 1: 569-579, 1996) expressing trans-acting viral proteins required for the production of full-length SeV genome together with SuperFect (QIAGEN). The transfected cells are cultured
in a serum-free MEM containing 100 µg/ml each of rifampicin (Sigma) and cytosine arabinoside (AraC) if desired, more preferably only containing 40 µg/ml of cytosine arabinoside (AraC) (Sigma), and concentrations of reagents are set at optima so as to minimize cytotoxicity due to the vaccinia virus and maximize the reconstitution rate of the virus (Kato, A. et al., 1996, Genes Cells 1, 569-579). After culturing for about 48 to 72 h following the transfection, the cells are reconstituted, disrupted by repeating three cycles of freezing and thawing, transfected to LCMK2 cells, and cultured.

After culturing the cells for 3 to 7 days, the culture solution is collected. Virus vectors defective in the envelope protein-encoding gene without replication capability can be reconstituted by using LCMK2 cells expressing envelope proteins for transfection, or transfecting together with an envelope-expressing plasmid. Defective virus vectors can be amplified by culturing the transfected cells overlaid on LCMK2 cells expressing envelope proteins (PCT/JP00/03194 and PCT/JP00/03195). Virus titer contained in the culture supernatant can be determined by measuring the hemagglutination activity (HA), which can be assayed by "endo-point dilution method" (Kato, A. et al., 1996, Genes Cells 1, 569-579). Virus stock thus obtained can be stored at -80°C without the aging.

The type of host cells used for virus reconstitution is not particularly limited, so long as virus vector can be reconstituted therein. For example, in the reconstitution of SeV vector and such, culture cells such as simian kidney-derived CVI cells and LCMK2 cells, hamster kidney-derived BHK cells, human-derived cells, and so on can be used. To obtain SeV vector in a large quantity, the vector can be amplified by infecting virus vector obtained from the above-described host cells into embryonated hen eggs. Methods for manufacturing virus using hen eggs have been already developed (Nakanishi, et al. (eds.), 1993, "Shinkei-kagaku Kenkyu-no Sentan-gijutu Protocol III (High Technology Protocol III of Neuroscience Research), Molecular Neurocyte Physiology, Koseisha, Osaka, pp.153-172). Specifically, for example, fertilized eggs are placed in an incubator and incubated for 9 to 12 days at 37 to 38°C to grow embryos. Virus vector is inoculated into chorioallantoic
cavity of eggs, and cultured for several days to proliferate the virus. Conditions such as culture duration may be varied depending on the type of recombinant virus used. Subsequently, chorioallantoic fluid comprising the virus is recovered. Separation and purification of SeV vector can be performed according to the standard methods (Tashiro, M., "Virus Experiment Protocols", Nagai and Ishihama (eds.), Medicalview, pp. 68-73 (1995)).

Also, the virus vector of the invention may have on the surface of its envelope adhesion molecules, ligands, receptors, or fragments thereof so as to adhere to specific cells. If vectors comprising a chimeric protein having these proteins in its extracellular domain and a polypeptide derived from the virus envelope protein in its intracellular domain, and such are prepared, it enables the production of a vector targeting a particular tissue. These factors may be encoded by the virus genome itself, or supplied at the time of virus reconstitution through expression of genes other than virus genome (for example, another expression vector or host cell chromosome).

The virus genes contained in the recombinant virus vector may be modified, for example, to reduce antigenicity or enhance RNA transcription efficiency or replication efficiency. Specifically, it is possible to modify at least one of the N, P/C, and L genes, which are genes of replication factors, to enhance transcription or replication. It is also possible to modify the HN protein, a structural protein having hemagglutinin activity and neuraminidase activity, to enhance the virus stability in blood by weakening the former activity and to regulate infectivity by modifying the latter activity. It is also possible to modify the F protein, which is implicated in membrane fusion, to regulate the fusion ability of membrane-fused liposomes. Furthermore, it is possible to generate a virus vector of Paramyxoviridae that is engineered to have weak antigenicity through analyzing the antigen presenting epitopes and such of possible antigenic molecules on the cell surface such as the F protein and HN protein.

In preparing defective virus vectors, two different virus vectors defective in a different envelope gene may be transfected into the same cell. In this case, each defective envelope protein
is supplied through expression from the other complex, and this mutual complementation permits the generation of infective virus particles, which can replicate and propagate. Thus, two or more of the virus vectors of the present invention may be simultaneously inoculated in a combination that complement each other, thereby producing a mixture of each envelope defective virus vector at a low cost and in a large scale. Because such viruses lacking an envelope gene have a smaller genome, they can allow the insertion of a long foreign gene. In addition, it is difficult for these viruses, which are intrinsically non-infective, to keep the status of co-infection after being diluted outside cells, and thus they are sterilized and less harmful to the environment.

In applying a virus vector thus obtained to gene therapy, it is possible to express a foreign gene with which treatment effects are expected, or an endogenous gene the supply of which is insufficient in a patient's body, by either direct or indirect (ex vivo) administration of the virus vector. There is no particular limitation in the type of the foreign gene, which may be, in addition to nucleic acids encoding proteins, nucleic acids that do not encode a protein such as an antisense or ribozyme. There is no particular limitation on the type of proteins encoded by foreign genes, and examples of natural proteins are hormones, cytokines, growth factors, receptors, enzymes, peptides, etc. These proteins can be secretory proteins, membrane proteins, cytoplasmic proteins, nucleoproteins, etc. Examples of artificial proteins are fusion proteins such as chimeric toxins, dominant negative proteins (including soluble molecules of receptors or membrane-binding dominant negative receptors), deletion-type cell adhesion molecules and cell surface molecules. These proteins may be those to whom a secretion signal, membrane localization signal, nuclear localization signal, etc., has been added. It is also possible to suppress functions of undesirable genes expressed in kidney cells by expressing an antisense RNA molecule or RNA-cleaving ribozyme, etc. Objects of gene therapy to which administrable vectors of this invention can be applied may include cancer therapy achieved by expressing, for example, a gene causing cell death such as a suicide gene (HSV tk, etc.) which exhibits
toxicity to infected cells. Another example is preventive therapy for coronary artery restenosis due to arterial sclerosis. In addition, the application of a virus vector of this invention in gene therapy that aims at maintaining cell survival may include the supplementation of gene products of genes such as adenosine deaminase gene (ADA), cystic fibrosis transmembrane conductance regulator gene (CFTR), and so on, which have been known to be deleted or defective in monogenic disorders, etc.

Regardless of whether the aim of gene therapy is to cause cell death or maintain cell survival, vectors of this invention comprising RNA as the genome can be applied to a wide range of disorders, because they are not converted into DNA during transcription and self-replication processes thereof, and also because they are unlikely to be incorporated into chromosomes of reproductive cells, and such, to affect genes of the succeeding generations. That is, vectors of this invention can be applied to disorders caused by many genes, such as hypertension, diabetes mellitus, asthma, ischemic heart disease, and so on, treatments and prevention for many healthy subjects, such as vaccines, and vaccination to prevent various infectious diseases such as AIDS, malaria, influenza, etc.

The virus vector of the present invention can be made as a composition together with a desired, pharmaceutically acceptable carrier. Herein, a “pharmaceutically acceptable carrier” is defined as those materials that can be administered with a vector, but does not inhibit gene transfer by the vector. For instance, the virus vector of this invention may be appropriately diluted with physiological saline, phosphate buffered saline (PBS), and so on to make a composition. If the virus vector of the invention is propagated in hen eggs, and such, the composition may contain a chorioallantoic fluid. Also, the composition may contain carriers such as deionized water or a 5% dextrose aqueous solution. It may further contain stabilizers, antibiotics, or the like. The virus vector-containing composition of the invention can be administered to any mammals including humans, monkeys, mice, rats, rabbits, sheep, cattle, dogs, etc.
Brief Description of the Drawings

Figure 1 shows construction of the plasmid pSeV18c(+) and insertion of the luciferase gene into the downstream region of N ORF. An 18 nucleotide-fragment designed to contain a NotI site was inserted between 1698 and 1699 nucleotides from the 3' end of SeV genome in pSeV(+) by site-directed mutagenesis (Shioda, T. et al., 1983, Nucleic Acids Res. 11:7317-7330). The resulting plasmid encoding the SeV antigenome with the 18 nucleotides-insertion was named pSeV18c(+). The ORF of the luciferase gene was PCR-amplified with 4 sets of NotI-tagged primers (ESn/NotLr, ESP/NotLr, ESf/NotLr and ES1/NotLr) from the template plasmid, pHvLuc-RT4 (Kato, A. et al., 1996, Genes to Cells 1: 569-579) to generate the fragments containing each of the different natural S sequences placed at the head of the luciferase gene. These amplified fragments were digested with NotI, and introduced into the same site of pSeV18c(+). The resulting plasmids, named pSeV(+)+SnLuc, pSeV(+)+SpLuc, pSeV(+)+SfLuc and pSeV(+)+S1Luc, were used to recover the recombinant SeV/SnLuc, SeV/SpLuc, SeV/SfLuc and SeV/S1Luc, respectively.

Figure 2 shows gene construction of SeV.

Figure 3 shows luciferase expression of SeV/SpLuc, SeV/SnLuc, SeV/SfLuc and SeV/S1Luc. The recombinant viruses were inoculated to CV1 cells at an moi of 10 (pfu/cell). The luciferase activities were measured at the times (hr) indicated.

Figure 4 is a photograph and graph showing luciferase expression of recombinant SeV. The recombinant viruses were inoculated to CV1 cells at an moi of 100 (pfu/cell). The cells were cultured in the presence of cycloheximide for 12 hr. Portions of cells were harvested to prepare RNA and probed with the luciferase probe (top). The remaining of cells was additionally incubated for 0, 2 and 4 hrs without cycloheximide to allow the protein synthesis and luciferase activity was measured (bottom).

Figure 5 shows growth kinetics of SeV/mSf. The titers of the wild-type SeV and mutant SeV/mSf were measured at the time points indicated under single-cycle conditions. Open bars and filled bars represent hemagglutination units (HAU) of wild-type and mutant viruses, respectively. Lines with open and filled circles represent
pfu per ml of the wild-type and mutant viruses, respectively.

Figure 6 is a photograph showing cytopathogenicity of SeV/mSf. CV1 cells were infected with the wild-type or SeV/mSf virus at an moi of 20 (pfu/cell) in the presence (+) and absence (−) of trypsin. The pictures were taken 48 hr post infection.

Figure 7 is a photograph showing intracellular expression of viral genes. CV1 cells infected with the wild-type SeV or SeV/mSf virus were analyzed by Northern hybridization with the viral N, P, F or L gene probes at various times (hrs) post infection. The positions of mRNAs and genomic/antigenomic RNA (vRNA) are marked.

Figure 8 is a photograph showing intracellular expression of viral genes. Intracellular expression of viral genes in CV1 cells was analyzed on Western blotting with anti-SeV antibody at various times (hrs) indicated at the top of each lane.

Figure 9 is a photograph showing intracellular expression of viral genes. CV1 cells were infected with wild-type SeV and SeV/mSf at moi of 100 pfu in the presence of cycloheximide. RNAs were extracted after 12 hr inoculation and analyzed by Northern hybridization. The specific bands obtained were analyzed using the BAS 2000 Image Analyzer.

Figure 10 is a photograph showing competition assays of the wild-type SeV and SeV/mSf in serial copassages. (A) The specific primer sets (left) to detect either of viral RNAs (right). (B) Each passage was initiated with input doses of $10^4$ (SeV/mSf) and $10^4$ (wild-type SeV) pfu/egg or $10^4$ (SeV/mSf) and $10^2$ (wild-type SeV) pfu/egg. The chorioallantoic fluids were harvested every 3 days, diluted to $10^{-6}$ and co-inoculated into new eggs serially up to 10 passages. Viral RNAs were extracted and analyzed by one-step RT-PCR method using the specific primer sets. Passage number is shown on the top of each lane. "Wild-type" and "SeV/mSf" represent DNA fragments amplified by using specific primer sets for respective sequences.

Figure 11 shows body weight gain of normal BALB/c and thymus deficient BALB/c (nu/nu) mice infected with the wild-type SeV and SeV/mSf viruses. Five mice were inoculated intranasally with various doses of viruses ($10^4$ to $10^7$ pfu per mouse). The weight gain of mice
were measured in grams every day up to 14 days post inoculation. Dead mice are marked by †.

Figure 12 shows pulmonary lesions and viral loads in the lungs of BALB/c and BALB/c (nu/nu) mice. Each mouse was intranasally inoculated with $10^4$ pfu of the viruses. These mice were sacrificed at 0, 1, 2, 3, 5, 7 and 9 days post inoculation to grade lesion scores (top) and to determine virus titers in the lungs (bottom). All these values are individually shown for each mouse.

Best Mode for Carrying out the Invention

The present invention will be explained in detail below with reference to examples, but it is not to be construed as being limited thereto.

[Example 1] Construction of recombinant viruses and luciferase assay

The nine nucleotides of the SeV E sequence are conserved exactly among all genes. On the other hand, there are minor differences in the nine nucleotides of S sequence. While S sequence of three (P, M and HN) of six genes are 3′-UCCCAUUU-5′, that of N, F and L gene are 3′-UCCCAgUUU-5′, 3′-UCCCuUUU-5′, and 3′-UCCCAUUa-5′, respectively (Fig. 2). These minor differences are completely conserved in all strains of SeV regardless of the passage history, virulence and isolation strategy. To examine the role of these minor differences of S, the inventors created the four recombinant SeVs named SeV/SpLuc, SeV/SnLuc, SeV/SfLuc and SeV/SiLuc expressing the luciferase under the control of synthetic S sequence.

1-1. Creation of an insertion site after the N ORF

The plasmid pSeV(+) contained the cDNA copy of full-length SeV antigenome (Kato, A. et al., 1996, Genes to Cells 1: 569-579) was used as the starting material for plasmid construction. In order to insert a luciferase gene having synthetic E sequence and S sequence, a unique NotI site was created at downstream of N ORF in N gene. Eighteen nucleotides (5′-gagggcccgcggccgga-3′/SEQ ID NO: 1) containing NotI restriction site was inserted between 1698 and 1699 nucleotides from the 3′ end of SeV genome which was located within the 5′ non-coding (in negative sense) region of N gene as shown in
Fig. 1 (Shioda, T. et al., 1983, Nucleic Acids Res. 11:7317-7330). For the insertion, the inventors used site-directed mutagenesis by a PCR-mediated overlap primer extension method (Ho, S. N. et al., 1989, Gene 77:51-59) essentially according to the previous paper (Hasan, M. K. et al., 1997, J. Gen. Virol. 78:2813-2820). Briefly, two primers (NmF; 5'–gaggggcccgcggcgcgca1699TACGAGGTTCAGGTACCT1718–3'/SEQ ID NO:2 and NmR; 5’–tcggccgcccggccggccctc1698TGATCCTAGCTTTCCTTCTAC1670-3'/SEQ ID NO: 3) with overlapping 18 nucleotides ends, and two outer primers (OP1, 5’-61CAAAGTATCCACCCCTGAGGACCAGTTCCAGACCCCTTGTGCTTGCC165-3'/SEQ ID NO: 4 and OP2, 5’-2467TTAAGTTGGTGAVGTAGCTCT2449-3'/SEQ ID NO: 5) were synthesized. First PCRs were performed with the OP1/NmF primer pairs and the OP2/NmF primer pairs using the pSeV(+) as a template to gave rise to 1.6 Kb- and 0.8 Kb-fragments, respectively. Second PCR was then performed with OP1/OP2 primer pairs using the purified 1.6 Kb- and 0.8 Kb-fragments as the template to generate the single 2.4 Kb-fragment with the 18 nucleotides. The 2.4 Kb-fragment was purified and digested with SphI and SalI. The plasmid pSeV(+) was cut at the positions of 610 and 2070 on the SeV genome by these enzymes. The sequence of the resulting 1.47 Kb-fragment was verified by sequencing using an AFLII automated DNA sequencer (Pharmacia, Uppsala) and replaced with the corresponding fragment of parental pSeV(+), thus generating pSeV18c(+) containing an unique (sole) restriction site after the N ORF.

Like parental plasmid pSeV(+), recombinant viruses can be reconstituted from thus obtained plasmid having an 18-nucleotide insert containing an NotI restriction site. The infectivity and replication capability of the generated viruses were also similar to those of the parental pSeV(+).

1-2. Insertion of luciferase gene regulated by various S sequences into vector

The luciferase gene from the firefly (Photinus pyralis) derived from the pHVlucRT4(-) (Kato, A. et al., 1996, Genes to Cells 1: 569-579) was amplified by PCR with the following four primer pairs corresponding to the four different S sequences; four forward primers (ESP;
5'-TTgcgggccgCTGAAGAAAAACTTTAGGTGAAAGTTTCACTTCACGATGGAAGACGGCAAAAA
CAT-3'/SEQ ID NO: 6, ESn;
5'-TTgcgggccgCTGAAGAAAAACTTTAGGTGTAaAAGTTTCACTTCACGATGGAAGACGGCAAAAA
CAT-3'/SEQ ID NO: 7, ESf;
5'-TTgcgggccgCTGAAGAAAAACTTTAGGTGAAaGTTCACCTTCACGATGGAAGACGGCAAAAA
CAT-3'/SEQ ID NO: 8, and ES1;
5'-TTgcgggccgCTGAAGAAAAACTTTAGGTGAAATGTTCACCTTCACGATGGAAGACGGCAAAAA
CAT-3'/SEQ ID NO: 9) and one common reverse primer (NotLr;
5'-TCgcggccgcTATTACAATTTGGACTTTCCG-3'/SEQ ID NO: 10). Underlined
are a new set of SeV E sequence and S sequence connected with the
conserved intergenic trinucleotide and the lower case letters without
underline represent the NotI restriction site. The lower case
letters with underline represent each of the unique nucleotides in
the primers. The 1.7 Kb-fragments amplified with the primer pairs
of ESP/NotLr, ESN/NotLr, ESf/NotLr and ES1/NotLr were purified,
digested with NotI and directly introduced into the NotI site of
pSeV18c(+) (Fig. 1). The final constructs were named pSeV(+)-SpLuc,
pSeV(+)-SnLuc, pSeV(+)-SfLuc and pSeV(+)S1Luc, respectively,
according to the S sequence used.

1-3. Virus recovery from cDNAs

Viruses were recovered from cDNAs essentially according to the
previously described procedures (Kato, A. et al., 1996, Genes to Cells
1: 569-579). Briefly, 2 x 10^6 of LLCMK2 cells in 6 cm diameter plate
were infected with vaccinia virus (VV), vTF7-3, a gift of Dr. B. Moss
at moi of 2 PFU/cell. Then, 10 µg of the parental or mutated pSeV(+)
and the plasmids encoding trans-acting proteins, pGEM-N (4 µg), pGEM-P
(2 µg) and pGEM-L (4 µg) (Kato, A. et al., 1996, Genes to Cells 1:
569-579) were transfected simultaneously with the aid of the
lipofection reagent DOTAP (Boehringer-Mannheim, Mannheim). The
cells were maintained in serum free MEM in the presence of 40 µg/ml
araC (1-β-D-arabinofuranosylcytosine) and 100 µg/ml rifampicin to
minimize VV cytopathogenicity and thereby maximize the recovery rate.
Forty hours after transfection, cells were harvested, disrupted by
three cycles of freezing and thawing and inoculated into 10-day-old
embryonated hen eggs. After 3 days of incubation, the
chorioallantoic fluid was harvested. The titers of recovered viruses were expressed in hemagglutination units (HAU) and PFU/ml as described previously (Kato, A. et al., 1996, Genes to Cells 1: 569-579). The helper VV contaminating the chorioallantoic fluid of the eggs, containing $10^8$ to $10^9$ pfu/ml of the recovered SeVs, was eliminated by the second propagation in eggs at a dilution of $10^{-7}$. This second passaged fluids, stored at -80°C, were used as the seed virus for all the experiments.

1-4. Cell cultures and virus infection

Monkey kidney-derived cell lines LLCMK2 and CV1, were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum at 37°C. Monolayer cultures of these cells were infected with the mutant viruses recovered from cDNAs at an input moi of 10 PFU/cell, and maintained in serum-free MEM. The wild-type SeV (Z strain) recovered from the cDNA (Kato, A. et al., 1996, Genes to Cells 1: 569-579) was used as a control.

It was found that the four recombinant viruses had replicated more slowly than the wild type in CV1 cells probably because of accommodating an extra gene as long as 1,728 nucleotides (Hasan, M. K. et al., 1997, J. Gen. Virol. 78:2813-2820). Among the four recombinants, SeV/SfLuc has replicated most slowly.

1-5. Luciferase assay

Luciferase activities expressed from the recombinant SeVs were compared with each other. The expression of luciferase activity from SeV was studied in 5x $10^5$ cells/well of CV1 cells in 6-well plates at various input multiplicities from 1 to 300 pfu per cell. Under the single-cycle growth conditions, cells were harvested at 0, 6, 14, 20 and 26 hrs post infection (p.i.). The luciferase activity of harvested cells was measured by a luciferase assay kit (Promega, Madison) with a luminometer (Luminos CT-9000D, Dia-Iatron, Tokyo) as described before (Hasan, M. K. et al., 1997, J. Gen. Virol. 78:2813-2820; Kato, A. et al., 1996, Genes to Cells 1: 569-579).

The luciferase activities expressed from SeVs increased in accordance with the infection time and infective dose in all recombinants. Figure 3 shows changes of luciferase activity when the viruses were infected at moi 10 to CV1 cells.
These cells were collected, and Northern hybridization was performed by using luciferase cDNA as probe. Northern hybridization was conducted as follows. RNAs were extracted from the cells using TRIzol (Gibco BRL, N.Y.). The RNAs were ethanol precipitated, dissolved in formamide/formaldehyde solution, then electrophoresed in 0.9% agarose-formamide/MOPS gels, and capillary transferred onto Hibond-N filters (Amersham, Buckinghamshire). The filters were probed with $^{32}$P-labeled probes made by the multi-prime labeling kit (Amersham, Buckinghamshire). For the luciferase probe, the NarI/HincII (1270 bp) fragment was purified from pHvlucRT4 (Kato, A. et al., 1996, Genes to Cells 1: 569-579). It was verified that the luciferase mRNAs are synthesized as monocistronoc mRNAs.

These data unequivocally demonstrated that the synthetic E sequence and S sequence inserted just before the luciferase ORF are correctly recognized by the viral RNA polymerase. However, there were differences in luciferase activities in the cells infected with these four viruses even under same condition. The highest activity was obtained with SeV/S1Luc and the lowest activity with SeV/SfLuc at 26 hr p.i. (Fig. 3). SeV/SpLuc and SeV/SnLuc were slightly lower than SeV/S1Luc at 26 hr p.i. However, this was not seen at 14 and 20 hrs p.i. Thus, the reinitiation capacities of Sp, Sn and S1 were regarded to be comparable.

[Example 2] Comparison of primary transcription amounts from recombinant viruses

To see whether or not the differences of the expression amounts among four recombinant SeVs observed in Example 1 were primarily brought about at the level of transcription, but not in the replication process, CV1 cells infected with the recombinants were incubated in the presence of cycloheximide, which inhibits protein synthesis and hence, blocks viral replication requiring de novo viral protein synthesis. Under these conditions, only the viral primary transcription catalyzed by the virion-associated RNA polymerase is allowed.

In a similar manner as in Example 1, CV1 cells were infected with the recombinant viruses at an m.o.i. of 100, and the infected
cells were incubated in the presence of 100 μg/ml cycloheximide (Sigma, St. Louis) for 12 h. The RNA in infected cells was prepared as described above, and the Northern hybridization was performed using the luciferase cDNA as the probe (Fig. 4, top). A different batch of cells was incubated to synthesize proteins in the absence of cycloheximide for 0, 2 and 4 h, and the luciferase activity was measured.

As a result, in all cells infected with any recombinant virus, it was found that the longer the incubation period was after cycloheximide removal, the higher the luciferase activity was. However, the luciferase expression of SeV/SfLuc-infected cells was again significantly lower than the other three (Fig. 4, bottom). The amounts of luciferase mRNA in each of the virus-infected cells correlated well with the activities of luciferase. The luciferase activities at 4 hr incubation were normalized by the count of SeV/SpLuc, as this type of S sequence is shared with three of the six genes. Luciferase activities in SeV/SnLuc- and SeV/SlLuc-infected cells were 0.86 and 1.19, respectively, and thus nearly comparable to that in SeV/SpLuc. In contrast, the value of SeV/SfLuc-infected cells reached only 0.24 of the control.

These results strongly suggested that the signal used for F gene expression possesses a lower reinitiation potential than the other S sequences.

[Example 3] SeV mutant comprising a modified S sequence for the F gene

The results described above suggested that there is a down-regulation of transcription at the F gene in the natural genome context of SeV. To investigate this, the inventors next created mutant SeV, SeV/mSf, whose S sequence of the F gene was replaced with that of the P/M/HN gene, as described below and compared its replication with that of the wild-type. 3-1. Mutagenesis to modify the S sequence of F gene in full-length SeV cDNA

Two nucleotides substitutions were performed on the S sequence of F gene as follows. First, pSeV(+) was cleaved by BanIII at the
SeV potions of 2088 and 5333 in SeV genome, and the resulting 3.4 Kb-fragment was recloned into the same restriction site of pBluescript KS(+) (Stratagene, La Jolla) to make a pB/BanIII. Then, site-directed mutagenesis by a PCR-mediated overlap primer extension method (Ho, S. N. et al., 1989, Gene 77:51-59) was performed as described above using synthesized two primers (mGS1F; 5'–CTAGGGTGAAAGTCCCTTG5'–4830/SEQ ID NO: 11 and mGS1R; 5'–ACAAGGACTTTCAACCCTAAG5'–4810/SEQ ID NO: 12) and two outer primers (M1F, 5'–TACCCATAGGTGCGCAGAA5'–3951/SEQ ID NO: 13 and T7, 5'–TAATACGACTCACTATAGGC–3' /SEQ ID NO: 14). Underlined letters are the mutagenized points. The first PCRs performed with M1F/mGS1R primer pairs and T7/mGS1F primer pairs using the pB/BanIII as a template yielded 0.9 Kb- and 0.6 Kb-fragment, respectively. These two fragments were purified, and the second PCR was then performed with M1F/T7 primer pairs using the purified fragments as the templates, generating a single 1.5 Kb-fragment with the two nucleotides mutations. This fragment was purified and digested with BanIII and recloned into the same restriction site of pSeV(+) to make a pSeV(+)mSf. The cloned sequence was verified by nucleotide sequencing. Viruses were reconstituted from the cDNA by the same procedures as Example 1.

The proliferation of this virus was examined using CV1 cells. The SeV/mSf was found to grow faster than the wild-type SeV in CV1 cells (Fig. 5). In the absence of trypsin, round cells and detached cells were observed. In the presence of exogenous trypsin to proteolytically activate the F glycoprotein, fused cells were observed more for the SeV/mSf than for the wild-type (Fig. 6).

3-2. Expression of SeV/mSf genes

The mRNA levels in CV1 cells infected with the wild-type and SeV/mSf at moi = 10 were analyzed by Northern blotting like Example 1 at various hours p.i. For the Sendai virus N probe, the PstI/PvuI (1189 bp) fragment was purified from the pGEM-N and used. For P probe, 792 bp of SmaI/SmaI fragment was purified from the pGEM-P and used. For M, F, HN and L probes, the NdeI/NdeI (878 bp), BamHI/BamHI (902 bp), ScaI/ScaI (1108 bp) and BamHI/BamHI (1654 bp) fragments were purified from pSeV(+) and used, respectively.

As shown in Fig. 7, the F and L transcripts from SeV/mSf were
detected earlier and reached remarkably higher levels, compared with
the wild-type infection. The P and N transcripts were also detected
earlier in SeV/mSf infection, although the peak levels were comparable
to the wild-type.

In order to confirm viral protein expression in infected cells,
Western blotting was performed by using anti-SeV antibody. CV1 cells
(2 x 10^5) grown in 6-well plates were infected at a moi of 10 with
the wild-type or SeV/mSf and harvested various hrs post infection.
The cells were centrifuged, and the cell pellets were lysed and run
in 12.5% SDS-PAGE (Laemmli, U.K., 1970, Nature 227:680-685) and
analyzed by Western blotting with anti SeV rabbit serum as described
(Kato, A. et al., 1995, Virology 209:480-488; Kato, A. et al., 1996,
Genes to Cells 1: 569-579). As a result, the levels of F0 protein
in the SeV/mSf-infected cells were significantly higher than in the
wild-type (Fig. 8) at any time point throughout infection. The
downstream gene products, HN and L, were not well resolved in this
experiment.

To compare the level of transcription directly, after the cells
infected with either wild-type SeV or SeV/mSf were treated with
cycloheximide to block de novo protein synthesis, RNAs were extracted
from the cells and analyzed by Northern hybridization as above. The
radioactivities of viral genomic RNA contained in hybridized bands
were analyzed by using the BAS 2000 Image Analyzer (Fujifilm, Tokyo).
Enhanced expression of the F and L genes, but not of the N and P gene,
was also clearly seen in mutant SeV (Fig. 9). These results again
unequivocally demonstrated that the S sequence naturally occurring
for the F gene transcription possesses a lower reinitiation activity
and hence down-regulates the expression of F and downstream genes.
Therefore, it was also shown that transcription level of not only
F gene but also downstream genes thereof can be elevated by replacing
the S sequence of the F gene with one having high efficiency. Probably
because of enhanced L gene expression in the SeV/mSf, the virion (v)
RNA levels were higher for the mutant than for the wild-type throughout
infection (Fig. 7). Earlier detection of mRNAs in the mutant SeV
infected cells as demonstrated in Fig. 7 might be also due to the
increased L gene expression.
[Example 4] Successive co-passages of the wild-type SeV and SeV/mSf in embryonated hen eggs

Although the wild-type SeV replicated slower than SeV/mSf in CV1 cells under single-cycle conditions as shown in Fig. 5, the possibility still remained that, when cultured slowly at multiple-cycle conditions, the naturally occurring down-regulation of transcription for the F and downstream genes would be more advantageous than the artificially introduced up-regulation. The inventors thus examined whether either the wild-type SeV or SeV/mSf would compete out the other under the multiple-cycle conditions of successive co-passages of the two viruses in eggs.

The SeV/mSf and wild-type SeV were co-inoculated into two embryonated hen eggs with the respective doses of both $10^4$ pfu/egg (10^4:10^4 inoculation), and in another experiment, $10^4$ and $10^2$ pfu/egg (10^4:10^2 inoculation). Every three days post inoculation, the chorioallantoic fluids were harvested and after dilution to $10^{-6}$, 0.1 ml of this was reinoculated into new eggs. These reinoculations were successively repeated 10 times. Viral RNAs were extracted from each chorioallantoic fluid by using TRIzol/LS (Gibco BRL, N.Y.) as Example 1, and amplified by one-step RT-PCR with two sets of specific primers. The viruses grown in the chorioallantoic fluids were semi-quantitatively measured by RT-PCR with specific primer pairs. One primer pair was designed to amplify only fragments having wild-type S sequence for the F gene (AGGGatAAAG), and the other mutant sequence (AGGGtgAAAG) (Fig. 10A). Specifically, the RNA was extracted from 25 μl of each chorioallantoic fluid, and reverse transcribed with HvM primer (5'--TCTCTCAGTGTTACAGCCAGAG--3'/SEQ ID NO: 15) at 50°C for 30 min using Superscript II (Gibco BRL, N.Y.), and were heat denatured at 94°C for 2 min. The cDNAs were amplified by PCR with HvM and GS2WR (5'--GCACGACTCAAGAGTTT--3'/SEQ ID NO: 16) primers for SeV/mSf and with HvM and GS2MR (5'--GCACGACTCAAGAGTTT--3'/SEQ ID NO: 17) primers for wild-type SeV as described previous (Kato, A. et al., 1997, EMBO J. 16:578-587; Kuronati, A. et al., 1998, Genes Cells 3:111-124). The lower case letters represent the mutated dinucleotides. The
respective specific products were analyzed by electrophoresis in agarose gel as described.

It was found that the wild-type genome had disappeared by the eighth passage in the case of $10^4:10^4$ inoculation and by fifth passages following $10^4:10^2$ inoculation (Fig. 10B). In control experiments, each virus was individually passaged and the genome sequences were determined. The results indicated that both of the viral genomes were stably maintained during 10 successive passages without any nucleotide change in the regions sequenced. These data indicated that the naturally occurring F gene S sequence conferred no replication advantage on SeV at least in ovo under the multiple-cycle conditions.

[Example 5] Virulence of SeV/mSf in mice

Highly complicated conditions are required for exhibiting virulence of SeV in natural host mice at individual level, compared with cultured cells or eggs. Whether the mutant SeV/mSf replicates earlier than the wild-type and shows stronger virulence in mice was examined.

Specific pathogen-free (SPF), 3-week-old of mice BALB/c and 4-week old of nude mice BALB/c (nu/nu) were purchased from Charles-River, Japan and used for virus infection experiments. These mice were infected intranasaly with $10^4$, $10^5$, $10^6$, $10^7$ or $10^8$ pfu/mouse of the wild-type or SeV/mSf under mild anesthetization with ether (Kiyotani, K. et al., 1990, Virology 177:65-74). Their body weights were individually measured every day up to 14 days. At 0, 1, 3, 5, 7 and 9 days post infection, three mice in each group were sacrificed and the virus titers in the lungs were measured for BALB/c and nude mice inoculated with $10^4$ pfu. Pulmonary lesions were scored at the same time (Kato, A. et al., 1997, EMBO J. 16:578-587). The results are shown in Fig. 11.

The mouse body weight gain was strongly disturbed by $10^7$ pfu of both virus inoculations. All mice were killed by either virus at similar days p.i. At $10^6$ pfu significant differences were found between the two viruses. SeV/mSf more strongly affected the body weight gain compared with the wild-type. The former killed all mice
while the latter killed only one and allowed the remaining mice to gain the weight again. At $10^5$ pfu, all mice infected with the wild-type showed a pattern of weight gain nearly comparable to that of the mock infected mice, and survived, while those infected with the mutant SeV/mSf did not and half of the mice died. Thus, SeV/mSf was clearly more virulent than the wild-type. The difference in virulence was quantitated by 50% lethal dose ($LD_{50}$); the $LD_{50}$ was $1.78 \times 10^6$ pfu for the wild-type and $7.94 \times 10^4$ pfu for the mutant (Table 1). The mutant virus was thus 22 times more virulent than the wild-type for BALB/c strain.

<table>
<thead>
<tr>
<th>Inoculation</th>
<th>BALB/C</th>
<th>$LD_{50}$</th>
<th>BALB/C(nu/nu)</th>
<th>$LD_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^8$</td>
<td>5/5*</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^7$</td>
<td>5/5</td>
<td>3/5</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>$10^6$</td>
<td>1/4</td>
<td>$1.78 \times 10^6$</td>
<td>2/5</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>0/5</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^8$</td>
<td>5/5</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^7$</td>
<td>5/5</td>
<td>5/5</td>
<td></td>
</tr>
<tr>
<td>SeV mS/f</td>
<td>$10^6$</td>
<td>5/5</td>
<td>$7.94 \times 10^4$</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>3/5</td>
<td>3/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
</tbody>
</table>

* Dead individuals/Inoculated individuals

Cytotoxic T lymphocytes (CTL) modulate SeV pathogenesis in two different ways. They contribute to eliminating or clearing the virus from body on one hand, and on the other, accelerate disease progression by immunopathological processes. That is, experimental results in BALB/c mice indicate the possibility of indirect exacerbation due to an enhanced immuno response induced by the mutant SeV, rather than direct effects resulting from a high reproducibility of the mutant SeV in the mouse body. Therefore, in an attempt to deny the possibility of aggravated pathogenicity resulting from an induced immunity, pathogenicities of the wild type and mutant viruses were
compared in thymus-deficient nude mice (Fig. 11). The LD₅₀ values of each virus were comparable for nude mice and for the parental normal mice, and a similar difference (to 40 fold) between the two viruses was found for the nude mice (Table 1). These results suggested that

CTL did not play a major role in pathogenesis of both wild-type and mutant viruses during the observation period (14 days) at least on the bases of LD₅₀. However, both the wild-type and mutant viruses persisted in the lungs of nude mice throughout, while cleared in the parental mice (Fig. 12). These results suggest that CTL and other thymus-dependent responses play at least partial roles in the virus pathology.

From the above-described results, it has been indicated that the natural S sequence of the F gene partially suppresses the replication of SeV so as to allow infected mice to survive for a longer time.

**Industrial Applicability**

The present invention provides virus vectors of Paramyxoviridae S sequences of which have been modified. In the virus vectors of this invention, S sequences have been modified so that transcription levels of genes on the genome have been modified compared to the wild type virus. These viruses are useful for elevating the virus proliferation capability and expression of a desired foreign gene. Such virus vectors are advantageous in improving the production efficiency of gene products. In contrast, in the case of proteins too high expressions of which are undesirable, it is possible to suppress expression levels of genes encoding the proteins by linking the genes to the downstream of the S sequence with the reduced reinitiation activity, such as the S sequence of F gene. In addition, when recombinant virus particles or virus-like particles are recovered as pharmaceutical compositions or vaccines, viruses having a genome in which the S sequence has been modified to elevate the proliferation capability are advantageous in being capable of yielding a large amount of viruses in a short time.
CLAIMS

1. A virus vector DNA, wherein the transcription start (S) sequence of at least one gene on the genome of a virus belonging to Paramyxoviridae has been modified so as to modify the expression level of said gene and genes located downstream thereof within the host.

2. The virus vector DNA according to claim 1, wherein said modification of the transcription start sequence comprises the substitution of said sequence by the transcription start sequence of another gene of a virus belonging to Paramyxoviridae.

3. The virus vector DNA according to claim 1, wherein said modification of transcription start sequence comprises the substitution of the transcription start sequence of F gene by the transcription start sequence of another gene.

4. The virus vector DNA according to claim 3, wherein said transcription start sequence of another gene comprises that of a P/M/HN gene type.

5. A virus vector DNA according to any one of claims 1 to 4, wherein said virus vector DNA is defective in F gene and/or HN gene.

6. A virus vector DNA according to any one of claims 1 to 5, wherein a foreign gene has been inserted into said virus vector DNA.

7. A virus vector of Paramyxoviridae comprising a transcription product from a virus vector DNA according to any one of claims 1 to 6 within virus particles.

8. The vector according to claim 7, wherein said vector is a Sendai virus (SeV) vector.

9. The vector according to claim 7 or 8, wherein the proliferation capability in the host is elevated compared to that of the wild type virus.

10. A method for producing a virus vector of Paramyxoviridae, wherein said method comprises the steps of transferring a virus vector DNA according to any one of claims 1 to 6 into the host, and expressing the virus protein in said host.

11. The method according to claim 10, wherein said virus of Paramyxoviridae used to produce the vector is Sendai virus.
Application number/ Numéro de demande: JP 00/06051

Documents of poor quality scanned
(request original documents in File Prep. Section on the 10th floor)

Documents de piètre qualité numérisés
(Pour obtenir les documents originaux, veuillez vous adresser à la Section de préparation des dossiers, située au 10e étage)
Figure 4

Luciferase mRNA

Luciferase activity (x10^3 cps)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>0</th>
<th>2</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/M/HN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1.0</td>
<td>0.86</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.19</td>
</tr>
<tr>
<td>L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

hrs
Figure 6

<table>
<thead>
<tr>
<th>Trypsin</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mock

Wild-type

SeV/mSf
### Figure 7

<table>
<thead>
<tr>
<th>Probe</th>
<th>Wild-type</th>
<th>SeV/mSf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0  6  14  20</td>
<td>0  6  14  20</td>
</tr>
<tr>
<td>L</td>
<td><img src="image1" alt="vRNA" /> <img src="image2" alt="mRNA" /></td>
<td><img src="image3" alt="vRNA" /> <img src="image4" alt="mRNA" /></td>
</tr>
<tr>
<td>F</td>
<td><img src="image5" alt="vRNA" /> <img src="image6" alt="mRNA" /></td>
<td><img src="image7" alt="vRNA" /> <img src="image8" alt="mRNA" /></td>
</tr>
<tr>
<td>P</td>
<td><img src="image9" alt="vRNA" /> <img src="image10" alt="mRNA" /></td>
<td><img src="image11" alt="vRNA" /> <img src="image12" alt="mRNA" /></td>
</tr>
<tr>
<td>N</td>
<td><img src="image13" alt="vRNA" /> <img src="image14" alt="mRNA" /></td>
<td><img src="image15" alt="vRNA" /> <img src="image16" alt="mRNA" /></td>
</tr>
</tbody>
</table>
Figure 8

<table>
<thead>
<tr>
<th>Wild-type</th>
<th>SeV/mSf</th>
</tr>
</thead>
<tbody>
<tr>
<td>0  6  14  20  26  38</td>
<td>0  6  14  20  26  38</td>
</tr>
</tbody>
</table>

Legend:
- P
- F0
- N0
Figure 9

Wild-type  SeV/mSf

vRNA

N mRNA

P mRNA

F mRNA

L mRNA
Figure 11