

Recombinant Mononegaviral Virus Vectors

This invention relates to a recombinant Mononegavirales virus vector harboring an additional transcription unit comprising a foreign gene operatively linked with an upstream Mononegavirales virus gene start (GS) sequence and a downstream Mononegavirales virus gene end (GE) sequence as well as with a vaccine comprising such a recombinant Mononegavirales virus vector.

Live viruses that are able to replicate in an infected host induce a strong and long-lasting immune response against their expressed antigens. They are effective in eliciting both humoral- and cell-mediated immune responses, as well as stimulating cytokine and chemokine pathways. Therefore, live, attenuated viruses offer distinct advantages over vaccine compositions based on either inactivated or subunit immunogens which typically largely only stimulate the humoral arm of the immune system.

Over the last decade recombinant DNA technology has revolutionized the field of genetic engineering of the genomes of both DNA and RNA viruses. In particular, it is now possible to introduce foreign genes into the genome of a virus such that upon replication of the new vector virus in a host animal a foreign protein is expressed that can exert biological effects in the host animal. As such recombinant vector viruses have been exploited not only for the control and prevention of microbial infections, but also for devising target therapies for non-microbial diseases such as malignancies and in gene therapy.

The generation of non-segmented, negative stranded RNA viruses (viruses of the order Mononegavirales) entirely from cloned cDNA by a technique designated as "reverse genetics", first reported in 1994 (Schnell et al., EMBO J., 13, 4195-4203, 1994), has made it possible to use also viruses of the order Mononegavirales (MV) as vectors. Since then studies have been published that describe the use of many viruses of the order MV as viral vectors to express foreign antigens derived from a pathogen aiming at developing vaccines against that pathogen.

The order of Mononegavirales is classified into four main families: Paramyxoviridae, Rhabdoviridae, Filoviridae and Bornaviridae. Viruses belonging to these families have genomes that are represented by a single, negative (-) sense RNA molecule, i.e. the polarity of the RNA genome is opposite to the polarity of messenger RNA (mRNA) that is designated as plus (+) sense. The classification of the main human and veterinary MV viruses is presented in the table below:

Table 1: Classification of the viruses within the order of Mononegavirales

Family	Genus	Species
Rhabdoviridae	Lyssavirus	Rabies virus (RV)
	Vesiculovirus	Vesicular stomatitis virus (VSV)
	Novirhabdovirus	Infectious hematopoietic necrosis virus (IHNV)
Paramyxoviridae	Respirovirus	Sendai virus (SeV)
		Human parainfluenza virus type 1 and type 3 (hPIV 1/3)
		Bovine parainfluenza virus type 3 (bPIV 3)
	Morbillivirus	Measles virus (MV)
		Rinderpest virus
		Canine distemper virus (CDV)
	Rubulavirus	Simian virus 5 (SV-5)
		Human parainfluenza virus type 2 (hPIV 2)
		Mumps virus
	Avulavirus	Newcastle disease virus (NDV)
		Human respiratory syncytial virus (hRSV)
	Pneumovirus	Bovine respiratory syncytial virus (bRSV)
Filoviridae	Ebola-like virus	Ebolavirus
		Marburg virus

The genomic organization and details of the life cycle of viruses of the order MV is well understood these days and is reviewed by various authors (Neumann et al., *J. Gen. Virology* **83**, 2635-2662, 2002; Whelan et al., *Curr. Top. Microbiol. Immunol.* **203**, 63-119, 2004; Conzelmann, K.; *Curr. Top. Microbiol. Immunol.* **203**, 1-41, 2004). Although Mononegavirales viruses have different hosts and distinct morphological and biological properties, they have many features in common, such as genomic organization and the elements essential for their typical mode of replication and gene expression, illustrating that they have originated from a common ancestor. They are enveloped viruses that replicate in the cytoplasm of the cell and produce mRNAs that are not spliced.

A Mononegavirales virus consists of two major functional units, a ribonucleoprotein (RNP) complex and an envelope. The complete genome sequences for representative viruses of the genera of all the families mentioned above have been determined. The genomes range in size from about 9,000 nucleotides to about 19,000 and they contain from 5 to 10 genes. The structure and the organization of the genomes of the MV viruses are very similar and are governed by their particular mode of gene expression. All of the MV virus genomes comprise three core genes encoding: a nucleoprotein (N or NP), a phosphoprotein (P) and a RNA-dependent RNA polymerase (L). The viral envelope is composed of a matrix (M) protein and one or more transmembrane glycoproteins (e.g. G, HN and F proteins) that play a role in virus assembly/budding as well as in the cell attachment and/or entry of the virus. Depending on the genus, the protein repertoire is extended by accessory proteins that display certain specific regulatory functions in transcription and virus replication or that are involved in virus host reactions (e.g. C, V and NS proteins). The gene order of MV viruses is highly conserved with the core genes N and P, at or near the 3' terminus and with the large (L) gene at the 5' distal position. The M, the surface glycoprotein genes, as well as the other accessory genes, are located between the N, P and L genes.

In the RNP complex, the genomic or antigenomic RNA is tightly encapsidated with the N protein and is associated with the RNA-dependent RNA polymerase that consists of the L and P protein. After infection of a cell, the RNP complex, but not the naked RNA genome, serves as a template for two distinct RNA synthesis functions, i.e. transcription of subgenomic mRNAs and replication of full length genomic RNA.

All of the tandemly arranged genes are separated by so called "gene junction" structures. A gene junction comprises a conserved "gene end" (GE) sequence, a non-transcribed "intergenic region" (IGR) and a conserved "gene start" (GS) sequence. These sequences are both sufficient and necessary for gene transcription. During transcription each gene is sequentially transcribed into mRNA by the viral RNA-dependent RNA polymerase that starts the transcription process at the 3' end of the genomic RNA at the first GS sequence. At each gene junction transcription is interrupted as a result of the disengagement of the RNA polymerase at the GE sequence. Re-initiation of transcription occurs at the subsequent GS sequence, although with a reduced efficiency. As a result of this interrupted process, also designated as a "stop-start" process, attenuation of transcription occurs at each gene junction as a result of which the 3' proximal genes on a MV virus genome are transcribed more abundantly than successive down stream genes. The modular form of transcription of MV virus genes in which each gene is part of a separate cistron or transcription unit makes these viruses extremely suited for the insertion and expression

of foreign genes. Each transcription unit in a MV virus genome comprises the following elements: 3'-GS—open reading frame (ORF)—GE-5'.

At the 3'- and 5'-genomic termini all of the MV virus genomes have a short non-transcribed region called "leader" (about 40-50 nt) and "trailer" (about 20-600 nt), respectively. The leader and trailer sequences are essential sequences that control the replication of genomic RNA, viral encapsidation and -packaging.

The reverse genetics technology and the rescue of infectious MV virus have made it possible to manipulate its RNA genome through its cDNA copy. The minimal replication initiation complex required to synthesize viral RNA is the RNP complex. Infectious MV virus can be rescued by intracellular co-expression of (anti)genomic RNAs and the appropriate support proteins from (T7) RNA polymerase driven plasmids. Since the initial report in 1994 by Schnell et al., 1994 (*supra*), reliable recovery of many MV virus species has been achieved based on the original protocol (or slight variations thereof).

Newcastle disease and avian influenza are important diseases of poultry, which can cause severe economic losses in the poultry industry worldwide. Newcastle disease virus is a non-segmented, negative stranded RNA virus within the order of MV. The genome, which is about 15 kb in length, contains six genes which encode the nucleoprotein (NP), phosphoprotein and V protein (P/V), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein and RNA-dependent RNA polymerase or large (L) protein. The NDV genes are arranged sequentially in the order 3'-NP-P-M-F-HN-L-5', and are separated by intergenic regions of different length. All genes are preceded by a gene start (GS) sequence which is followed by a noncoding region, the open reading frame encoding the NDV proteins, a second noncoding region and the gene end (GE) sequence. The length of the NDV genome is a multiple of six, which has to be considered for the introduction of foreign genes.

Avian influenza (AI) is a disease of poultry characterized by mild respiratory signs to severe disease with high mortality. The causative agent is an avian influenza A virus (AIV) belonging to the family Orthomyxoviridae. AIV contains eight genomic RNA segments of negative polarity which encode 10 proteins. Based on the antigenicity of the surface glycoproteins hemagglutinin (HA) and neuraminidase (N), AI viruses were subtyped. Up to now, 16 hemagglutinin (H 1 – H 16) and nine neuraminidase (N 1 – N 9) subtypes are known. Antibodies to H and N are important in humoral immune response and inhibit infection or prevent disease.

Avian influenza and Newcastle disease viruses can be grouped into two distinct pathotypes according to their virulence. Symptoms caused by low pathogenic AIV (LPAI) or lentogenic NDV are considered of less relevance. In contrast, highly pathogenic avian influenza (HPAI) and Newcastle disease caused by high virulent viruses (NDV: mesogenic and velogenic strains) are notifiable diseases.

Whereas routine vaccination against NDV with lentogenic NDV strains is performed to protect chicken against highly virulent NDV strains, vaccination against HPAI is not performed in most countries, since HPAI is controlled by an eradication strategy. However, vaccination may be used as a strategy to minimize losses and to reduce the incidence of disease. Immunity induced by vaccines is subtype specific, which means that a subtype H5 vaccine can protect against H5 AIV but not against the other H subtypes. Normally, influenza virus replication is restricted to the lungs because hemagglutinin of LPAI viruses can be cleaved only by trypsin, a serine protease restricted to the lungs. So far, all HPAI viruses have been of H5 and H7 subtype. These HPAI viruses contain multiple basic amino acids at the H cleavage site so that it can be cleaved by ubiquitous furin and subtilisin-like enzymes into the subunits HA1 and HA2. Such viruses can therefore grow in other organs.

Subtype H5 and H7 vaccines can provide protection of chickens and turkeys against clinical signs and death following infection with HPAI. In addition to conventional inactivated oil-based whole AIV, vector virus, subunit protein and DNA vaccines have been shown experimentally to be effective for immunization against AI. Since the advent of reverse genetics for different viruses the generation of recombinant viruses for use as vaccine vectors is an important application. Different recombinant negative-strand RNA viruses expressing foreign proteins have been constructed. Also, the hemagglutinin of AIV was inserted into different vector viruses like the infectious laryngotracheitis virus (ILTV) (Luschow et al., *Vaccine* **19**, 4249-59, 2001), Rinderpest virus (Walsh et al., *J. Virol.* **74**, 10165-75, 2000) and vesicular stomatitis virus (VSV) (Roberts et al., *J. Virol.* **247**, 4704-11, 1998).

Rinderpestvirus was also used as vector virus for the expression of the foot- and mouth-disease virus VP1 capsid protein (Baron et al., 1999, *J. of Gen. Virol.*, vol. 80, p. 2031-2039).

Tao et al. (1998, *J. of Virol.*, vol. 72, p. 2955-2961) describe the construction of a chimeric human parainfluenza virus (hPIV) type 3, wherein the HN and F genes from hPIV type 1 were used for a replacement of (not an addition to) the endogenous hPIV type 3 HN and F genes.

Also NDV was used for the expression of AIV hemagglutinin. The hemagglutinin gene of influenza A/WSN/33 was inserted between P and M genes of NDV strain Hitchner B1. This recombinant protected mice against lethal infection although there was a detectable weight loss in mice which recovered fully within 10 days Nakaya et al. (J. Virol. 75, 11868-73, 2001). A further recombinant NDV with the same insertion site for the foreign gene expressed the H7 of a LPAI but only 40 % of the vaccinated chicken were protected from both velogenic NDV and HPAI (Swayne et al., Avian Dis. 47, 1047-50, 2003).

These publications however do not disclose any advantageous effect of the so-called non-coding regions of endogenous MV genes on the expression of additional foreign genes inserted into the genome of an MV vector.

It is an object of the embodiments disclosed herein to provide a recombinant MV virus vector that displays a higher expression level of a protein encoded by a foreign gene inserted into the genome of the vector virus and/or that shows a stronger immunogenicity than existing MV virus vectors.

The present inventors have found that this object can be met by a recombinant Mononegavirales virus vector according to the disclosure. Therefore, the disclosure, in a first aspect, provides a recombinant Mononegavirales virus vector harboring an additional transcription unit comprising a foreign gene operatively linked with an upstream Mononegavirales virus gene start (GS) sequence and a downstream Mononegavirales virus gene end (GE) sequence, characterized in that between the GS sequence and a start codon of the foreign gene and between a stop codon of the foreign gene and the GE sequence, a 3' non-coding region- and a 5' non-coding region (genome sense) of a Mononegavirales virus gene are located, respectively.

In a second aspect, there is provided a vaccine against a microbial pathogen, characterized in that it comprises a recombinant Mononegavirales virus vector as defined above in a live or inactivated form, and a pharmaceutically acceptable carrier or diluent.

It is noted that the indications of the polarity of the nucleic acid strands here and in the rest of the text are given in the genome (-) sense, except in the context of mRNA and cDNA sequences.

It has been found that the presence of the 3'- and 5' non-coding regions of a MV

virus gene in a transcription unit comprising a foreign gene inserted into the genome of a MV virus has a positive effect on the transcription and/or expression of the foreign gene. It is shown in Figure 3 that engineering the non-coding regions of a MV virus gene between a GS sequence and an avian influenza virus (AIV) hemagglutinin (HA) gene and between the AIV HA gene and a GE sequence increases the amount of HA mRNA synthesized by a MV virus vector harboring that AIV HA gene. A positive effect is also observed on the protein expression level: comparison of the MV virus vectors

only showed an intense immunological staining with AIV HA-specific antiserum in case the MV virus vector harboring the foreign AIV HA gene was flanked by the non-coding regions (Figure 4). Therefore, it has been found that, although the inventors do not want to be bound to any theory or model that might explain these observations, the presence of MV virus non-coding regions flanking a foreign gene, has a positive effect on the performance of the resulting MV virus vector.

A foreign gene is a polynucleotide molecule that encodes a polypeptide or protein and that is not present in nature in the genome of the recipient MV virus.

As already outlined in detail above, a common characteristic of the genomic organization of MV viruses is their modular form of transcription wherein tandemly arranged transcription units are successively transcribed. In a wild-type MV virus the transcribed genes are flanked (i) at their 3' end with a GS sequence and a nucleotide sequence designated in the art as "non-coding region", and (ii) at their 5' end with a nucleotide sequence also designated as "non-coding region" and a GE sequence. Therefore, the term (3' or 5') "non-coding region" as used herein defines a nucleotide sequence that is located upstream (3') or downstream (5') of a natural gene of a MV virus and that spans the region between the GS sequence and the start codon (ATG) of the MV virus gene and the region between the stop codon (TAA, TAG or TGA) of the MV virus gene and the GE sequence, respectively. The non-coding regions used herein are derived from a gene of the same virus as the vector virus (i.e. the non-coding regions are homologous to the MV virus vector).

Detailed information of the genomic organization of MV viruses is known in the art, including the nucleotide sequences of the various MV virus genes and their transcription control (GS and GE) sequences and non-coding sequences that flank the genes. Such information is, for example, available from the database of the National Center for Biotechnology Information (NCBI), e.g. via their web-page on the internet; see Tables 2 and 3).

The non-coding sequences that are preferably used in this invention are derived from natural MV virus genes, but substitution of one or more nucleotides in a natural non-coding region is also considered to be within the invention. In particular, nucleotide substitutions are contemplated that are located immediately up- or downstream of the start/stop codon of the foreign gene, respectively, and result from the introduction of artificial restriction enzyme cleavage sites that allow the genetic manipulation of these regions.

In a preferred recombinant MV virus vector according to the present invention the non-coding regions are of a gene encoding a MV virus envelope protein, in particular a M, G, F or HN protein, or a RNP protein, in particular, a N, P or L protein.

In a particularly preferred recombinant MV virus according to the invention the non-coding regions are of a gene encoding a F or HN protein.

Specific nucleotide sequences of non-coding regions to be used in a recombinant MV virus vector according to the inventions are presented in the Table 3.

Table 2: Genome- and vector information on Mononegavirales viruses

MV virus	Genome information: NCBI accession no.	Recombinant virus vector reference
RV	NC_001542	Mebatsion et al., PNAS <u>93</u> , 7310-14, 1996
VSV	NC_001560	Kretzschmar et al., J. Virol. <u>71</u> , 5982-89, 1997
IHN	NC_001652	-
SeV	NC_001552	Sakai et al., FEBS Lett. <u>456</u> , 221-6, 1999
hPIV 1	NC_003461	-
H PIV 3	NC_001796	Tao et al., J. Virol. <u>74</u> , 6448-58, 2000
bPIV 3	NC_002161	Tang et al., Vaccine <u>23</u> , 1657-67, 2005
Measles virus	NC_001498	Singh et al., J. Virol. <u>73</u> , 4823-28, 1999
Rinderpest virus	NC_006296	Walsh et al., J. Gen. Virol. <u>81</u> , 709-18, 2000
CDV	NC_001921	Plattet et al., Virus Res. <u>101</u> , 147-53, 2004
SV 50	NC_006430	He et al., Virology <u>237</u> , 249-60, 1997
NDV	NC_002617	Zhao et al., J. Gen. Virol. <u>84</u> , 781-88, 2003
Mumps virus	NC_002200	-
hPIV 2	NC_003443	-
hRSV	NC_001781	Bukreyev et al., PNAS <u>96</u> , 2367-72, 1999
bRSV	NC_001989	Stope et al., J. Virol., <u>75</u> , 9367-77, 2001
Ebola virus	NC_006432	-
Marburg virus	NC_001608	-

The GS and GE sequences to be used in this invention as the transcriptional control sequences are preferably those derived from the natural genes of the MV viruses. It is believed that these sequences modulate the activity of the RNA polymerase during the transcription process, in particular in the process of transcription initiation and mRNA 5' end modification and in control of transcription 3' end polyadenylation and termination. For each MV virus, the beginning of each gene is marked by a sequence of about 10 nucleotides. Whereas in some MV virus species the GS sequences are the same for each gene, the GS sequences in the genome of other MV virus species may display subtle differences.

In view of their common function in mRNA 3' end poly-A tail formation and transcription termination, the GE sequences in MV viruses share common sequence

features. A typical GE sequence comprises a U-tract of between 4-8 nucleotides in length. Furthermore, there is a strong conservation of a C-residue directly upstream of the U-tract and which is preceded by a stretch of nucleotides that is A/U rich. In various GE sequences the 4 nucleotides directly upstream of the U-tract is composed of 3'-AUUC-5'.

The transcriptional control sequences that define the gene borders or gene junctions of MV virus genes have been identified for many MV virus genes by comparing the nucleotide sequences in the genomic template and the nucleotide sequences present at the mRNA termini. In addition many studies have identified GS and GE consensus sequences that are required for efficient gene expression. General features and specific examples of GS and GE sequences can be derived from the information in the NCBI sequence database (see Table 2 for NCBI accession no.'s) and are also reviewed by Neumann et al. (J. Virol. **83**, 2635-2662, 2002), and Whelan et al. (Current Topics Microbiol. Immunol. **203**, 63-119, 2004).

Specifically preferred GS and GE sequences to be used in a recombinant MV virus vector according to the invention are listed in Table 3, although it is recognized that a precise border between GS-, NCR- and GE sequences can not always be determined. This sequence information is disclosed in the NCBI database (see accession no. in Table 2). For NDV, reference is made to EMBL accession no. Y18898, for RV to GenBank accession no. M31046.

Table 3: Gene junction sequence information of various MV genes (+ sense)

Virus	GS	NCR (nt) between GS and start codon	ORF	NCR (nt) between stop codon and GE	GE	IGR (between GE and GS)
RV	aacacccct	3	N	50	tgaaaaaaaa	ct
	aacacccct	20	P	59	tgaaaaaaaa	caggc
	aacaccact	6	M	171	tgaaaaaaaa	ctatt
	aacatccct	18	G	459	agaaaaaaaa	cattagatcagaag aacaactggc
	aacacttct	21	L	52	tgaaaaaaaa	
IHNV	ggcacttcagttg	100	NP	70	tagaaaaaaaa	t
	ggcactatagtc	25	P	32	agaaaaaaaa	t
	ggcacgcaagtgt	41	M2	90	agacagaaaaaaaa	t
	ggcactttgtgc	39	G	28	agacagaaaaaaaa	c
	ggcacatttgtgc	14	NV	-6	agatagaaaaaaaa	t
	ggcactttgtgc	64	L	41	agatacaaaaaaaaa	
Sendai	agggtcaaag	54	NP	34	taagaaaaa	ctt

virus						
	agggtgaaag	93	P	74	taagaaaaa	ctt
	agggtgaaag	22	M	85	taagaaaaa	ctt
	agggataaag	43	F	61	taagaaaaa	ctt
	agggtgaaag	46	HN	95	taagaaaaa	ccc
	agggtgaatg	18	L	76	taagaaaaa	
hPIV1	agggtaaag	54	N	31	aagtaagaaaaa	ctt
	agggtgaatg	93	P	71	aattaagaaaaa	ctt
	agggtcaaag	22	M	82	aaataagaaaaa	cgt
	agggacaaag	265	F	76	aagtaagaaaaa	ctt
	agggttaaag	46	HN	98	gaataagaaaaa	ctt
	agggttaatg	18	L	88	tagtaagaaaaa	
bPIV3	aggattaaag	45	N	34	taagaaaaa	ctt
	aggattaatg	69	P	116	taagaaaaa	ctt
	aggatgaaag	22	M	51	aaacaaaaa	ctt
	aggatcaaag	101	F	26	tacaaaaaa	ctt
	aggaacaaag	63	HN	86	taataaaaaa	ctt
	aggagaaaag	12	L	62	taagaaaaa	
hPIV3	aggattaaag	45	NP	34	taagaaaaa	ctt
	aggattaaag	69	P	53	taagaaaaa	ctt
	aggattaaag	22	M	51	taatcaaaaa	ctt
	aggacaaaag	183	F	28	ttataaaaaa	ctt
	aggagtaaag	63	HN	81	tataaaaaa	ctt
	aggagcaaag	12	L	62	taataaaaaa	
Measles virus	aggattcaaag	42	N	49	ttataaaaaa	ctt
	aggaaccagg	50	P	62	ttataaaaaa	ctt
	aggagcaaag	22	M	417	taaacaaaa	ctt
	agggccaaag	574	F	128	taaltaaaa	ctt
	agggtgcaag	10	H	74	ttaagaaaaa	cgt
	agggtccaag	12	L	59	ttaaagaaaa	
Rinder-pest virus	aggattcaag	42	N	49	ttataaaaaa	ctt
	aggaccagg	49	P	62	ttataaaaaa	ctt
	aggagcaaag	22	M	408	taccaaacaaaa	ctt
	agggtcaaag	579	F	125	tataaacaaaaa	ctt
	aggatgcaag	10	H	98	ttataaaaaa	cgt
	agggtccaag	12	L	60	taaagaaaa	
CDV	agggtcaatg	42	N	49	ttataaaaaa	ctt
	aggaccagg	49	P	62	ttataaaaaa	ctt
	aggacacaag	22	M	394	taattaatcaaaa	ctt
	agggtccagg	16	F	122	ttaaagaaaa	ctt
	agggtccagg	10	H	100	ttataaaaaa	cta
	aggatccaag	12	L	53	tacgaaaaaaaa	
SV5	agggtccggaacct	83	NP	92	tttaagaaaaaaaa	t
	aggcccggaagggt	47	P	54	tttagaaaaaa	cgattaacgataa ta
	agcccgaacact	20	M	193	ttcaagaaaa	caatcatattaagac

						tatccta
	agcacgaacccat	15	F	25	ttttaagaaaaaaa	cgat
	aggaccgaacct	67	SH	64	ttttaagaaaaaaa	ta
	aggcccgaacact	54	HN	99	ttttaagaaaaa	ccaagagaacaat
	aggccagaatg	-3	L	22	Tttaagaaaaaaa	
VSV	aacagtaatc	3	NP	42	tgaaaaaaa	ct
	aacagatc	0	NS	-3	tgaaaaaaa	gt
	aacagatc	31	M	98	tgaaaaaaa	ct
	aacagagatc	19	G	98	tgaaaaaaa	ct
	aacagcaatc	0	L	31	tgaaaaaaa	
NDV	acgggtagaa	56	NP	200	ttagaaaaaa	gt
	acgggtagaa	73	P	169	ttaagaaaaaa	t
	acgggtagaa	24	M	102	ttagaaaaaa	c
	acgggtagaa	36	F	73	ttaagaaaaa	ctaccggtttagat gaccaaaggacga tat
	acgggtagaa	81	HN	166	ttaagaaaaaa	tgtaagtggcaatg agatacaaggcaa aacagctcatggta aataat
	acgggtagga	1	L	67	ttagaaaaaa	

In a preferred recombinant MV virus vector of the invention the GS-, GE sequence and non-coding regions are derived from the same MV virus gene.

Methods for the preparation of a recombinant MV virus vector harboring an additional transcription unit comprising a foreign gene are well known in the art. For example, Table 2 refers to documents that describe the preparation of such recombinant vector viruses for various MV virus species. In principle, the method used in the present invention is the same as that in the prior art, except that the foreign gene to be inserted into the MV virus genome is flanked by the appropriate 3'- and 5'- non-coding regions, as defined above.

In a general method according to the invention the recombinant MV virus vector is prepared by inserting an isolated nucleic acid molecule comprising (i) a foreign gene flanked by the 3'- and 5'- non-coding regions as defined above and (ii) the appropriate transcriptional control sequences, into the genome of the MV virus, such that in the resulting MV virus vector the foreign gene is both preceded and followed by a MV virus gene junction, in particular by a genomic nucleotide sequence fragment comprising GE-IGR-GS elements. The presence of such upstream and downstream elements guarantee the appropriate transcription not only of the inserted foreign genes, but also

of the homologous MV virus genes that are located up- and downstream of the inserted foreign gene.

More in particular, in this method the isolated nucleic acid molecule and the genome of the MV virus are used in their cDNA form (+ sense). This allows easy manipulation and insertion of the desired nucleic acid molecules into the viral genome.

In general, various parts of the genome could be used for the insertion of the foreign gene, between two genes, i.e. in intergenic regions (IGR), 3' or 5' non-coding regions of a gene as well as 3' promoter-proximal (before the N/NP genes) or 5' distal end (after the L genes) of a genome.

The foreign gene could advantageously be inserted before the N/NP gene, between NP-P, P-M, M-G/F, G/F-HN, HN-L and after L gene.

The simplest way is to use an already existing restriction enzyme (RE) recognition sequence at one of these sites by cutting with the enzyme and introducing an appropriate transcription cassette. Since naturally existing restriction enzyme recognition sequences are not always located at the desired location, RE recognition sites could be introduced into the genome conventionally by site directed- or PCR mutagenesis. Examples of suitable IGRs for insertion of the foreign gene can be found in Table 3.

The composition of the transcription cassette to be inserted depends on the site of insertion. For example, in case a transcription cassette is inserted into an IGR the cassette may comprise the following elements: 3' RE recognition site-GS-non coding region-ORF (of foreign gene)-non coding region-GE-RE recognition site 5'.

Alternatively, in case a transcription cassette is introduced into a 5' non-coding region of a natural MV virus gene the cassette may be composed of: 3' RE recognition site-GE-IGR-GS-non coding region-ORF (of foreign gene)-non coding region-RE recognition site 5'.

Similarly, in case a transcription cassette is to be introduced into a 3' non-coding region of a natural MV virus gene the cassette may be composed of: 3' RE recognition site-non coding region-ORF (of foreign gene)-non coding region-GE-IGR-GS-RE recognition site 5'.

The preparation of such transcription cassettes and the insertion thereof into a MV virus genome only involve routine molecular biologic techniques, such as exemplified in the literature references listed in Table 2 and in the present Examples. In particular, techniques such as site-directed- and PCR mutagenesis can be used for this purpose (Peeters et al., 1999, *supra*; Current Protocols in Molecular Biology, eds.: F. M. Ausubel et al., Wiley N.Y., 1995 edition, pages 8.5.1.-8.5.9; Kunkel et al., Methods in Enzymology Vol. 154, 376-382, 1987).

More in particular, a recombinant MV virus vector according to the present invention can be prepared by means of the well established "reverse genetics" method that enables the genetic modification of non-segmented, negative stranded RNA viruses of the order MV (reviewed for example by Conzelmann, K.K., *Current Topics Microbiol. Immunol.* 203, 1-41, 2004; and Walpita et al., *FEMS Microbiol. Letters* 244, 9-18, 2005).

In this method, an appropriate cell is co-transfected by a vector comprising a cDNA molecule comprising a nucleotide sequence that encodes a full length genome, or, preferably, an antigenome (positive sense) of a MV virus, and one or more vectors comprising the cDNA molecules comprising nucleotide sequences that encode the required support proteins, under conditions sufficient to permit transcription and co-expression of the MV (anti)genome and support proteins and the production of a recombinant MV vector. In this method, the said nucleic acid molecule encoding the full length MV virus (anti)genome comprises an additional transcription unit as defined above.

With vector is meant a replicon, such as a plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached DNA segment and its transcription and/or expression in a cell transfected with this vector.

Preferably, the vector for the transcription of the full length genome is a plasmid that comprises a cDNA sequence encoding the (anti)genome of the MV virus, flanked by a T7 polymerase promoter at its 5' end and a (hepatitis delta) ribozyme sequence at its 3' end, although a T3 or SP6 RNA polymerase promoter can also be used.

For the intra-cellular expression of the appropriate support proteins use is made, preferably, of plasmids comprising the cDNA sequence encoding these proteins, under the control of appropriate expression control sequences, e.g. a T7 polymerase promoter.

In a particularly preferred method for the preparation of a recombinant MV virus vector according to the present invention, expression plasmids are used that encode the N (or NP), P and L proteins of the MV virus.

The amounts or ratios of transfected support plasmids to be used in this reverse genetic technology cover a broad range. Ratios for the support plasmids N:P:L may range from about 20:10:1 to 1:1:2 and efficient transfection protocols for each virus are known in the art.

An exact copy of the genomic RNA is made in a transfected cell by the combined action of the T7 RNA polymerase promoter and the ribozyme sequence, and this RNA

is subsequently packaged and replicated by the viral support proteins supplied by the co-transfected expression plasmids.

It is preferred that a T7 polymerase enzyme is provided by a recombinant vaccinia virus that infects the transfected cell, in particular by the vaccinia virus vTF7-3, yet also other recombinant pox vectors, such as fowl pox virus, e.g. fpEFLT7pol, or other viral vectors may be used for the expression of T7 RNA polymerase.

Separation of the rescued virus from vaccinia virus can easily be accomplished by simple physical techniques, such as filtration. For rescue of Sendai virus or NDV, rescue can be achieved by inoculation of the supernatant of transfected cells in embryonated eggs.

In an even more preferred embodiment cell lines are used for the transfection of the transcription- and expression vectors that constitutively express the (T7) RNA polymerase and/or one or more of the required support proteins.

For example, rescue of Measles virus can be achieved in a human embryo kidney cell line, 293-3-46, that expresses both T7 RNA polymerase and Measles virus support proteins N and P (Radecke et al., EMBO J. 14, 5773-5784, 1995). Another very useful cell line that can be used advantageously in the present invention is based on BSR cells expressing the T7 RNA polymerase, i.e. cell line BSR-T7/5 (Buchholz et al., J. Virol. 73, 251-259, 1999).

Furthermore, more detailed information concerning the reverse genetics technology to be used herein for the preparation of a MV virus according to the present invention is disclosed in the review by Conzelmann, K.K. (supra) and Example 1.

The ability of recombinant MV virus vectors to stably express foreign genes has resulted in the development of vectors for both prophylactic and therapeutic applications.

In a recombinant MV virus vector according to the present invention the foreign gene can vary depending on the specific MV virus vector species and the application of the vector virus.

The foreign gene may encode an antigen of an (other) microbial pathogen (e.g. a virus, bacterium or parasite), especially the foreign gene encodes an antigen of a pathogen that is able to elicit a protective immune response.

For example, heterologous gene sequences that can be inserted into the virus vectors of the invention include, but are not limited to influenza virus glycoprotein genes, in particular, H5 and H7 hemagglutinin genes of avian influenza virus, genes derived from Infectious Bursal Disease Virus (IBDV), specifically VP2 of (IBDV), genes derived from Infectious Bronchitis Virus (IBV), feline leukemia virus, canine distemper

virus, equine infectious anemia virus, rabies virus, ehrlichia organism, in particular *Ehrlichia canis*, respiratory syncytial viruses, parainfluenza viruses, human metapneumoviruses and measles virus.

Alternatively, the foreign gene may encode a polypeptide immune-modulator that is able to enhance or modulate the immune response to the virus infection, for example by co-expressing a cytokine such as an interleukin (e.g. IL-2, IL-12, IFN- γ , TNF- α or GM-CSF).

The order of MV includes both viruses that are able to replicate in humans and animals, or in both (e.g. rabies virus and Newcastle disease virus). Therefore, the foreign gene can be selected from a wide variety of human and veterinary microbial pathogens.

Although all MV viruses can be used as a vector virus in the present invention, in a preferred embodiment of the invention the recombinant MV virus vector is a virus of the family Rhabdoviridae, preferably of the genus *Lyssavirus* or *Novirhabdovirus*, more preferably of the species rabies virus or IHNV, respectively.

In an also preferred embodiment the recombinant MV virus is a virus of the family Paramyxoviridae, preferably of the genera *Respovirus*, in particular the species hPIV3 or bPIV3, *Morbillivirus*, in particular the species CDV, *Pneumovirus*, in particular the species RSV and *Avulavirus*, in particular the species NDV.

In a particularly preferred embodiment of the present invention a recombinant MV virus vector is provided wherein the virus is Newcastle disease virus (NDV). As NDV is able to replicate in both humans and animals, in particular poultry, more in particular chickens, a recombinant NDV vector according to the invention may comprise a foreign gene that encodes an antigen of a pathogen, in particular of a respiratory pathogen, or an immune-modulator that is capable of eliciting an appropriate immune response in humans or any of these animals.

Reverse genetics methods for the genetic manipulation of NDV have been disclosed specifically for NDV by Peeters et al. (*J. Virology* 73, 5001-5009, 1999), Römer-Oberdörfer et al. (*J. Gen. Virol.* 80, 2987-2995, 1999), and in the review by Conzelmann, K.K. (*supra*). Furthermore, it is also known that NDV can be used as a vector for the expression of foreign genes, for example, for the eliciting of an immune response in animals infected with the NDV vector (Nakaya et al., 2001, *supra*) and Swayne et al., *Avian Dis.* 47, 1047-50, 2003).

A foreign gene can advantageously be introduced into a NDV genome at various positions as outlined in general for MV viruses above. In particular, in a recombinant NDV vector according to the invention, a foreign gene (as part of an appropriate

transcription unit) can be inserted between the following NDV genes: NP-P, P-M, M-F, F-HN, HN-L and at the 3' proximal- and 5' distal locus (Zhao et al., 2003, supra; Nakaya et al., 2001, supra), preferably in the 3' proximal, P-M, M-F and F-HN regions, the F-HN region being most preferred.

Furthermore, in a recombinant NDV vector according to the present invention the non-coding regions that flank the foreign gene can be derived from all naturally occurring NDV genes, in particular from the N, P, M, F or HN genes, the HN gene being preferred.

In a particular embodiment of the invention a recombinant NDV vector is provided wherein the additional transcription unit is located between the F-HN genes and the inserted foreign gene is flanked by the non-coding regions of the NDV HN gene.

More specifically, a NDV vector is provided wherein the 3'- and 5' NCR (and optionally the GS and GE sequence) have a nucleotide sequence as shown in SEQ ID. No. 1 and 2 or 3 and 4.

A recombinant NDV vector according to the present can advantageously be used to induce an immune response in poultry, in particular chickens, against other pathogens. Therefore, the recombinant NDV vector, preferably comprises a foreign gene that encodes a protective antigen of an avian pathogen, in particular of influenza virus, marek's disease virus (MDV), infectious laryngotracheitis virus (ILTV), infectious bronchitis virus (IBV), infectious bursal disease virus (IBDV), chicken anemia virus (CAV), reo virus, avian retro virus, fowl adeno virus, turkey rhinotracheitis virus (TRTV), *E. coli*, *Eimeria* species, *Cryptosporidia*, *Mycoplasmas*, such as *M. gallinarum*, *M. synoviae* and *M. meleagridis*, *Salmonella*-, *Campylobacter*-, *Ornithobacterium* (ORT) or *Pasteurella* sp.

More preferably, the recombinant NDV vector comprises a foreign gene that encodes an antigen of AIV, MDV, ILTV, IBV, TRTV, *E. coli*, ORT or *Mycoplasma*.

In particular, the recombinant NDV vector mutant comprises a hemagglutinin (HA) gene of an influenza virus, preferably of an avian influenza virus (AIV), more preferably of a highly pathogenic H5 or H7 AIV.

In principle, the HA gene of all (avian) influenza strains can be used in this invention. The nucleotide sequences of many HA gene have been disclosed in the art and can be retrieved from nucleic acid sequence databases, such as GenBank or the EMBL database.

The hemagglutinin (HA) gene of the recently isolated, highly pathogenic H5N2 subtype AIV A/chicken/Italy/8/98 can advantageously be used as a foreign gene in the

present invention as outlined above. The gene is reverse transcribed, cloned in the eukaryotic expression vector pcDNA3 (Invitrogen), and sequenced (Lüschow et al., Vaccine, vol. 19, p. 4249-4259, 2001, and GenBank Accession No. AJ305306). From the obtained expression plasmid pCD-HA5 the HA gene can be obtained by amplification by using specific primers that generate artificial RE recognition sites that allow insertion of the HA gene in NDV genomic sequences.

In a further embodiment, the HA gene of the highly pathogenic H7N1 subtype AIV A/chicken/Italy/445/99 can be used as a foreign gene in the present invention as outlined above. The HA gene is reverse transcribed, and amplified by PCR. The 1711 bp product is cloned in the SmaI-digested vector pUC18 (Amersham) and sequenced (Veits et al., J. Gen. Virol. 84, 3343-3352, 2003; and GenBank Accession No. AJ580353).

In a particularly advantageous recombinant MV virus vector according to the present invention, the MV vector virus is attenuated, that is to say: the vector virus is not pathogenic for the target animal or exhibits a substantial reduction of virulence compared to the wild-type virus. Many MV viruses used herein as virus vectors have a long safety record as live attenuated vaccines such as the measles virus and NDV, whereas other viruses, such as SeV and VSV are considered non-pathogenic to humans. In addition, conventional techniques exist to obtain and screen for attenuated viruses that show a limited replication or infectivity potential. Such techniques include serial (cold) passaging the virus in a heterologous substrate and chemical mutagenesis.

A recombinant NDV vector according to the invention can be derived from any conventional ND vaccine strain. Examples of such suitable NDV strains present in commercially available ND vaccines are: Clone-30[®], La Sota, Hitchner B1, NDW, C2 and AV4, Clone-30[®] being the preferred strain.

It has also been found by the present inventors that a recombinant MV virus vector according to the present invention is able to induce a protective immune response in animals.

Therefore, in another embodiment of this invention a vaccine against a microbial pathogen is provided that comprises a recombinant MV virus vector as defined above in a live or inactivated form, and a pharmaceutically acceptable carrier or diluent.

A vaccine according to the invention can be prepared by conventional methods such as those commonly used for the commercially available live- and inactivated MV virus vaccines.

Briefly, a susceptible substrate is inoculated with the recombinant MV virus vector and propagated until the virus has replicated to a desired titre after which the virus containing material is harvested. Subsequently, the harvested material is formulated into a pharmaceutical preparation with immunizing properties.

Every substrate which is able to support the replication of the recombinant MV virus vector can be used in the present invention. As a substrate host cells can be used from both prokaryotic- and eukaryotic origin, depending on the MV virus. Appropriate host cells may be vertebrate, e.g. primate cells. Suitable examples are; the human cell lines HEK, WI-38, MRC-5 or H-239, the simian cell line Vero, the rodent cell line CHO, BHK, the canine cell line MDCK or avian CEF or CEK cells.

A particularly suitable substrate on which a recombinant NDV vector according to the present invention can be propagated are SPF embryonated eggs. Embryonated eggs can be inoculated with, for example 0.2 ml NDV containing allantoic fluid comprising at least $10^{2.0}$ EID₅₀ per egg. Preferably, 9- to 11-day old embryonated eggs are inoculated with about $10^{5.0}$ EID₅₀ and subsequently incubated at 37 °C for 2-4 days. After 2-4 days the ND virus product can be harvested preferably by collecting the allantoic fluid. The fluid can be centrifuged thereafter for 10 min. at 2500 g followed by filtering the supernatant through a filter (100 µm).

The vaccine according to the invention comprises the recombinant MV virus vector together with a pharmaceutically acceptable carrier or diluent customarily used for such compositions.

The vaccine containing the live virus can be prepared and marketed in the form of a suspension or in a lyophilized form. Carriers include stabilizers, preservatives, and buffers. Diluents include water, aqueous buffer and polyols.

In another aspect of the present invention a vaccine is provided comprising the recombinant MV virus vector in an inactivated form. The major advantages of an inactivated vaccine are its safety and the high levels of protective antibodies of long duration that can be induced.

The aim of inactivation of the viruses harvested after the propagation step is to eliminate reproduction of the viruses. In general this can be achieved by well known chemical or physical means.

If desired, the vaccine according to the invention may contain an adjuvant. Examples of suitable compounds and compositions with adjuvant activity for this purpose are aluminum hydroxide, -phosphate or -oxide, oil-in-water or water-in-oil emulsions based on, for example a mineral oil, such as Bayol F® or Marcol 52® or a vegetable oil such as vitamin E acetate, and saponins.

The administration of a vaccine according to the invention may be by any of the well known effective forms and may depend on the type of MV virus vector. Suitable modes of administration include, parenteral-, intranasal, oral and spray vaccination.

A NDV vector vaccine according to the invention is preferably administered by the inexpensive mass application techniques commonly used for NDV vaccination. For NDV vaccination these techniques include drinking water and spray vaccination.

A vaccine according to the invention comprises an effective dosage of the recombinant MV virus vector as the active component, i.e. an amount of immunizing MV virus material that will induce immunity in the vaccinated birds against challenge by a virulent microbial organism. Immunity is defined herein as the induction of a significantly higher level of protection in a population of humans or animals against mortality and clinical symptoms after vaccination, compared to an unvaccinated group. In particular, the vaccine according to the invention protects a large proportion of vaccinated humans or animals against the occurrence of clinical symptoms of the disease and mortality.

Typically, the live vaccine can be administered in a dose of $10^{2.0}$ - $10^{8.0}$ tissue culture/embryo infectious dose (TC/EID₅₀), preferably in a dose ranging from $10^{4.0}$ - $10^{7.0}$ TC/EID₅₀. Inactivated vaccines may contain the antigenic equivalent of $10^{4.0}$ - $10^{9.0}$ TC/EID₅₀.

The invention also includes combination vaccines comprising, in addition to the recombinant MV virus vector according to the invention, a vaccine strain capable of inducing protection against a further pathogen.

EXAMPLES

Example 1: Generation of a recombinant MV virus vector expressing an avian influenza virus HA gene (NDV/AIVH5)

▪ Viruses and cells

Rescued recombinant NDV and the influenza virus isolate A/chicken/Italy/8/98 were propagated in specific pathogen free (SPF) 10-day-old embryonated chicken eggs. The velogenic NDV strain Herts 33/56 and the NDV Clone 30 vaccine (Nobilis®) were used.

BSR-T7/5 cells stably expressing phage T7 RNA polymerase were used to recover infectious NDV from cDNA.

▪ Construction of cDNA encoding NDV antigenomic RNA containing the AIV H5 gene

The numbering in brackets as used herein to identify nucleotide positions on the NDV genome and amino acid residues in the NDV proteins is as described by Römer-Oberdörfer et al. (J. Gen. Virol. 80, 2987-2995, 1999, EMBL accession no. Y18898). The plasmid pflNDV, expressing the full-length antigenomic RNA of Clone 30 (Römer-Oberdörfer et al., supra) was used to introduce the AIV H5 gene which had been amplified from plasmid pCD-HA5 (Luschow et al., supra) by specific primers with artificial MluI restriction sites (PH5F1: 5'- cta aac gcg taa aat gga gaa aat agt gc -3' (SEQ ID NO: 5) and PH5R1: 5'- tgc gac gcg ttt aaa tgc aaa ttc tgc act g -3' (SEQ ID NO: 6), MluI sites are underlined) for rNDV/AIVH5-A and with NcoI or AflII sites (PH5F2: 5'-cct tcc atg gag aaa ata gtg ctt c -3' (SEQ ID NO: 7) and PH5R2: 5'- cct cct taa gta taa ttg act caa tta aat gca aat tct gca ctg caa tga tcc -3' (SEQ ID NO: 8), restriction sites are underlined) for rNDV/AIVH5-B. The introduction of H5 into the Clone 30 antigenome (Fig. 1 A) was done using the MluI sites as previously described for the GFP insertion (Engel-Herbert et al., J. Virol. Methods 108, 19-28, 2003). Briefly, for construction of the full-length plasmid pflNDV/AIVH5-A the H5 ORF was amplified with primers containing artificial MluI sites (see above) which were used to insert the H5 ORF into the minimal gene cassette between the F and HN gene of NDV (Fig. 1 A). Construction of the full-length plasmid containing the AIV H5 gene for generation of rNDV/AIVH5-B is given in Figure 1 B. Mutagenesis reactions were done using the Quik Change® II XL site directed mutagenesis kit (Stratagene). To this end, a pUC 18 plasmid (pUCNDV1) containing the NotI/BsiWI-fragment (nt 4953 - 8852) of Clone 30 genome and following primers were used: MP1 (5'- gac aac agt cct caa cca tgg acc

gcg cgc -3') (SEQ ID NO: 9) and MP2 (5'- ctg gct agt tga gtc aat tct taa gga gtt gga aag atg gc -3') (SEQ ID NO: 10) for mutagenesis A (Fig. 1 B) resulting in the plasmid pUCNDV1a with newly created NcoI and AflII sites (restriction sites in primers are underlined). After digestion with NcoI and AflII the HN ORF of Clone 30 was substituted by the amplified AIV H5 ORF. For mutagenesis B to create SgfI- and SnaBI-sites in the intergenic region in front of the L gene of pUCNDVH5 resulting in pUCNDV/AIVH5-1b (Fig. 1 B) primers MP3 (5'- caa aac agc tca tgg tac gta ata cgg gta gga cat gg -3') (SEQ ID NO: 11) and MP4 (5'- gta agt ggc aat gcg atc gca ggc aaa aca gct cat gg -3') (SEQ ID NO: 12) were used. Mutagenesis C was done with MP3 and MP5 (5'- gaa aaa act acc ggc gat cgc tga cca aag gac gat ata cgg g -3') (SEQ ID NO: 13) resulting in plasmid pUCNDV1c to obtain SgfI- and SnaBI-sites which were used for the introduction of the Clone 30 HN gene into the intergenic region in front of the L gene in plasmid pUCNDVH5_1b. Finally, the NotI/BsiWI-fragment of pflNDV-1 was substituted by the NotI/BsiWI-fragment of pUCNDVH5_1c (Fig. 1 B). The lengths of the new generated full-length genomes represent a multiple of six (16938 nt for rNDV/AIVH5-A and 17196 nt for rNDV/AIVH5-B).

▪ Transfection and virus propagation

Transfection experiments, virus propagation and confirmation of the recovery of infectious virus were carried out as described previously (Römer-Oberdörfer et al., supra; Engel-Herbert et al., supra). The only difference was that a total amount of DNA of 20 µg (10 µg full-length genome containing plasmid, 6 µg pCiteNP, 2 µg pCiteP and 2 µg pCiteL) was used for transfection.

▪ Results

The AIV H5 open reading frame was inserted between the F and HN genes of the previously described plasmid pflNDV (Römer-Oberdörfer et al., supra). To this end, the AIV H5 ORF of the AIV isolate A/chicken/Italy/8/98 (H5N2) was amplified from plasmid pCD-HA5 (Luschow et al., supra) by specific primers with *Mlu*I restriction sites which were used for insertion of the AIV H5 ORF into the singular *Mlu*I restriction site of pflNDV1; (Engel-Herbert et al., supra) resulting in plasmid pflNDV/AIVH5-A (Fig. 1 A). In this construct the AIV H5 ORF was flanked by artificial gene start (GS) and gene end (GE) sequences in the intergenic region between the F and HN genes of NDV. For construction of full-length plasmid pflNDV/AIVH5-B the HN ORF of plasmid pUCNDV1a was substituted by the amplified H5 ORF as a *Nco*I/*Afl*II-fragment (Fig. 1 B). In the resulting plasmid pUCNDVH5 *Sgf*I and *Sna*BI restriction sites were created in the intergenic region downstream of the H5 gene resulting in plasmid

pUCNDVH5_1b (Fig. 1 B). The created *SgfI* and *SnaBI* restriction sites were used to introduce the HN gene from plasmid pUCNDV1c, in which the HN gene was also flanked by *SgfI* and *SnaBI* restriction sites (Fig. 1 B). Finally, the resulted plasmid pUCNDVH5_1c was used to replace the *NotI/BsWI*-fragment of pflNDV (Fig. 1 B). The constructed pflNDV/AIVH5-B differs from plasmid pflNDV/AIVH5-A since the noncoding regions of NDV HN were additionally inserted between the transcriptional control elements (GS, GE) and the H5 ORF.

NDV recombinants rNDV/AIVH5-A and rNDV/AIVH5-B were recovered from BSR-T7/5 cells transfected with the respective full-length cDNA and support plasmids as previously described (Römer-Oberdörfer et al., supra; Engel-Herbert et al., supra). For virus recovery the transfection supernatants were injected into the allantoic cavities of 10-day-old embryonated chicken eggs and incubated for 5 days. The allantoic fluid was harvested and analyzed for presence of virus by hemagglutination test or indirect immunofluorescence (IF). Virus-containing allantoic fluids were used for a second egg passage for further virus propagation. The presence of the inserted H5 gene in the viral genomes of rNDV/AIVH5-A and rNDV/AIVH5-B was confirmed by reverse transcription-PCR and sequencing (data not shown). Figure 2A and 2B show the nucleotide sequence of regions flanking the HN ORF in NDV and of the H5 ORF in the NDV vector.

Example 2: In vitro characterization of the NDV/AIVH5 vector

RNA analyses

CEF cells were infected with NDV Clone 30, rNDV/AIVH5-A, rNDV/AIVH5-B and AIV A/chicken/Italy/8/98 (H5N2) at a multiplicity of infection (MOI) of 10 per cell and incubated for 8 h at 37°C. Total RNA of infected and uninfected cells was prepared, separated in denaturing agarose gels and hybridized with radiolabeled cRNA's. The plasmids pCD-HA5 and pCD-NDVHN which contain the open reading frame of AIV A/chicken/Italy/8/98 (H5N2) H5 and NDV Clone 30 HN, respectively, were used for in vitro transcription of ³²P-labeled cRNA (SP6/T7 Transcription kit, Roche).

To verify transcription of the inserted AIV H5 gene in rNDV/AIVH5-A and -B Northern blot analyses were performed with total RNA of NDV/AIVH5 recombinant infected primary chicken embryo fibroblasts. RNA preparation of NDV Clone 30 and AIV A/chicken/Italy/8/98 (H5N2) infected cells were used as controls. Transcription of the inserted AIV H5 gene was detected for rNDV/AIVH5-A as well as for rNDV/AIVH5-B

with gene-specific antisense cRNA (Fig. 3). It can be observed that the AIV-H5B transcript is extended by about 81 nt and is more abundantly present than the AIV-H5A transcript.

▪ Western blot analyses

CEK cells were infected with NDV Clone 30, rNDV/AIVH5-A, rNDV/AIVH5-B and AIV A/chicken/Italy/8/98 (H5N2) and incubated for 20 h at 37°C. Lysates of infected and of uninfected control cells were separated by SDS-PAGE (ca. 10^4 cells per lane), and transferred to nitrocellulose filters (Trans-Blot® SD cell, Bio-Rad). Blots were incubated with a polyclonal rabbit antiserum against NDV, or a polyclonal chicken antiserum against AIV of the subtype H5 at dilutions of 1 : 20000 and 1 : 2500, respectively. Binding of peroxidase-conjugated species-specific secondary antibodies was detected by chemiluminescence using SuperSignal® West Pico Chemiluminescent Substrate (Pierce) on X-ray films (Hyperfilm® MP, Amersham).

In Western blot analyses the H5 protein was detectable only in rNDV/AIVH5-B infected cells. The AIV subtype H5-specific antiserum detected two prominent proteins of approximately 70 and 50 kDa and a barely visible protein of ca. 25 kDa, which were not found in NDV Clone 30 infected cells (Fig. 4).

▪ Indirect immunofluorescence (IF) tests

For indirect IF tests CEF cells were infected at low MOI with NDV Clone 30, rNDV/AIVH5-A, rNDV/AIVH5-B and AIV A/chicken/Italy/8/98 (H5N2) for 20 h. After fixation with methanol and acetone (1:1) the cells were subsequently incubated with either a polyclonal rabbit antiserum against NDV or a polyclonal chicken antiserum against AIV of subtype H5 at dilutions of 1 : 3000 and 1 : 100 respectively. After incubation with F(ab)₂ fragment of anti rabbit IgG and fluorescein-conjugated anti chicken IgG antibodies samples were analyzed by conventional fluorescence microscopy.

H5 expression was examined by indirect IF test of infected CEF cells. After incubation with a NDV-specific antiserum pronounced fluorescence was detectable in cells infected with NDV Clone 30, rNDV/AIVH5-A and rNDV/AIVH5-B, but not in cells infected with AIV A/chicken/Italy/8/98 (H5N2) or in non infected cells (Fig. 5; right panel). Incubation with an AIV subtype H5-specific antiserum showed a marked fluorescence in AIV infected cells. Comparing the two recombinants, rNDV/AIVH5-B

showed a more intense H5-specific fluorescence than rNDV/AIVH5-A indicating a higher expression level of the H5 protein (Fig. 5, left panel).

▪ Immunoelectron microscopy

The viral particles were adsorbed to formvar coated copper grids for 7 min. The grids were washed four times with PBS containing 0.5 % bovine serum albumin and subsequently incubated with a NDV-specific or a AIV subtype H5-specific antiserum for 45 min. After several washings with PB, grids were incubated for further 45 min with protein A gold (10 nm, PAG 10, Biocell International) or rabbit anti-chicken gold (10 nm, RCHL 10, Biocell International). After final washings with PB the virus particles were contrasted with phosphotungstic acid (PTA, pH 7.2) and examined with an electron microscope.

When virions of NDV Clone 30 or rNDV/AIVH5-A were examined, staining was observed only with NDV-specific antiserum. In contrast, for rNDV/AIVH5-B staining was observed using antisera against NDV and also by using antisera against AIV, demonstrating that virions of rNDV/AIVH5-B contain hemagglutinin H5. Gold particles were found predominantly along the surface of rNDV/AIVH5-B virions, indicating that the hemagglutinin was anchored in the viral membrane.

Example 3: In vivo characterization of the NDV/AIVH5 vector

▪ Evaluation of protection by recombinant rNDV/AIVH5-A and rNDV/AIVH5-B:

One-day-old chickens were randomly assigned to two groups and vaccinated oculonasally with 10^6 EID₅₀ of rNDV/AIVH5-A or with commercial NDV Clone 30 vaccine (Nobilis®, Intervet, NL) via spray. At 28 days of age a second immunization was administrated the same way. On day 12 after the second immunization blood was collected for assessing presence of NDV and AIVH5 antibodies by HI test. Two weeks after the second vaccination the immunized groups were divided and one part of each group was challenged oculonasally with 10^8 EID₅₀ of the highly pathogenic AIV isolate A/chicken/Italy/8/98 (H5N2). The remaining chickens were used to evaluate the protective efficacy of the vaccines against velogenic NDV. Therefore the birds and additional control animals received $10^{5.3}$ EID₅₀ of NDV strain Herts 33/56 intramuscularly.

After immunization and challenge infection all birds were observed daily for a period of 10 days for clinical signs and classified as healthy (0), ill (1; one of the following signs: respiratory signs, depression, diarrhea, cyanosis, oedema, nervous

signs), severely ill (2; more than one of the following signs: respiratory signs, depression, diarrhea, cyanosis, oedema, nervous signs), or dead (3). A clinical score was calculated which represents the mean value of all chickens per group for this period. Finally, three weeks after challenge blood samples were taken from all surviving animals in order to evaluate antibody titers against AIV and NDV.

The NDV recombinant rNDV/AIVH5-B was tested in a separate animal trial of nearly identical experimental design. The only differences were that immunizations with 10^6 EID₅₀ of either rNDV/AIVH5-B or NDV Clone 30 vaccine were administered in both groups oculonasally and no NDV challenge infection was performed.

All data of these experiments are summarized in Table 4 and Figure 6. Sera of the immunized chickens were analyzed by HI test three weeks after vaccination, but no HA-specific serum antibodies could be detected in both cases. All animals of both groups developed NDV-specific antibodies at high levels already after the first immunization (mean HI-titers of 2^6 - 2^7) the animals were fully protected against an infection with velogenic NDV, whereas all control animals died within 4 days exhibiting typical signs of ND. As expected, the AIV challenge infection caused severe disease in NDV Clone 30 immunized chickens with a mortality rate of 90 %. Animals of the rNDV/AIVH5-A immunized group survived a lethal dose of highly pathogenic AIV, but all chickens exhibited varying signs of avian influenza with a clinical score of 0.64 indicating significant yet partial protection. However, chickens immunized with rNDV/AIVH5-B were completely protected against any signs of disease after infection with a highly pathogenic avian influenza A virus.

Table 4: Summary of animal experiments rNDV/AIVH5-A and rNDV/AIVH5-B

1. Immunization	Time scale ¹⁾	rNDV/AIV H5-A oculon.	Clone 30 spray	control	Time scale ¹⁾	rNDV/AIV H5-B oculon.	Clone 30 oculon.
EID ₅₀ /animal		10 ^{5.7}	10 ^{6.0}	-		10 ^{6.0}	10 ^{6.0}
Mortality	1.-10. d pi	0/24	0/34	-	1.-10. d pi	0/5	0/5
Morbidity clinical score ²⁾	1.-10. d pi	6/24 0.034	5/34 0.016	-	1.-10. d pi	0/5 0	0/5 0
NDV-specific Ab Ø HI-titer	17 d pi	22/22 2 ^{5.6}	34/34 2 ^{7.2}	-	21 d pi	5/5 2 ^{5.6}	5/5 2 ^{7.6}
HA-specific Ab Ø HI-titer	17 d pi	22/22 0	34/34 0	-	21 d pi	5/5 0	5/5 0

2. Immunization	28 d pi	rNDV/AIV H5-A oculon.	Clone 30 spray	control	28 d pi	rNDV/AIV H5-B oculon.	Clone 30 oculon.
EID ₅₀ /animal		10 ^{6.0}	10 ^{6.0}	-		10 ^{6.0}	10 ^{6.0}
Mortality	1-10. d pb	0/22	0/24	-	1-10. d pb	0/5	0/5
Morbidity clinical score ²⁾	1-10. d pb	4/22 0.023	3/24 0.017	-	1-10. d pb	0/5 0	0/5 0
NDV-specific Ab Ø HI-titer	12 d pb	21/21 2 ^{7.3}	24/24 2 ^{6.8}	5/5 0	12 d pb	5/5 2 ^{6.0}	5/5 2 ^{7.8}
HA-specific Ab Ø HI-titer	12 d pb	2/21 2 ³	24/24 0	5/5 0	12 d pb	5/5 0	5/5 0

Challenge EID ₅₀ /animal	14 d pb	NDV Herts 33/56 10 ^{5.3}					
Mortality	3.-4. d pc	0/10	0/10	5/5			
Morbidity clinical score ²⁾	1.-10. d pc	4/10 0.04	5/10 0.05	5/5 2.4			
NDV-specific Ab Ø HI-titer	20 d pc	10/10 2 ^{8.9}	10/10 2 ^{9.2}	†			

Challenge EID ₅₀ /animal	14 d pb	AIV A/ch/Italy/8/98 (H5N2) 10 ^{8.0}			14 d pb	AIV A/ch/Italy/8/ (H5N2) 10 ^{7.7}	
Mortality	3.-6. d pc	0/10	9/10		3.-4. d pc	0/5	4/5
Morbidity clinical score ²⁾	1.-10. d pc	10/10 0.64	10/10 2.57		1.-10. d pc	0/5 0	5/5 2.2
AIV-specific Ab Ø HI-titer	20 d pc	10/10 2 ^{6.5}	1/1 2 ^{9.0}		24 d pc	5/5 2 ^{8.0}	1/1 2 ¹¹

Example 4: Construction of an NDV/AIV-H5 vector with flanking ncr from the NDV F-gene.

Using essentially the same methods and materials as described above (Engel-Herbert et al., supra), an NDV vector construct was made carrying the H5 AIV gene in between the F and HN genes of the previously described plasmid pflNDV (Römer-Oberdörfer et al., supra), but now the H5 insert was flanked with non-coding regions from the NDV F-gene.

Briefly the various steps and materials used were:

A pUC plasmid with the 1,6 kb NotI-PstI fragment of NDVH5 (pUCIRA) was mutated to create an MluI restriction enzyme site, using mutagenesis primers: pMPMLUIGRFHNF (5'- ggt tgt aga tga cca aag gac ggc tta **cgg gta gaa** cgg taa gag agg -3'; SEQ ID NO: 14) and pMPMLUIGRFHNR (5'- cct ctc tta ccg ttc tac ccg taa cgc gtc ctt tgg tca tct aca acc -3'; SEQ ID NO: 15) (MluI site underlined, with GS sequence in bold), resulting in plasmid pUCIRAMLU.

Two oligo's were annealed: OFVOF: 5'- agg acg cgt tac ggg tag aag att ctg gat ccc ggt tgg cgc cct cca ggt gca gca cca tgg ag -3' (SEQ ID NO: 16, MluI site underlined) and OFVOR: 5'- ctc cat ggt gct gca cct gga ggg cgc caa ccg gga tcc aga atc ttc tac ccg taa cgc gtc ct -3' (SEQ ID NO: 17, MluI site underlined). Next these were digested with MluI and NcoI.

Digestion of the plasmid pUCIRAMLU with MluI and NcoI.

Ligation of the approximately 4,3 kb MluI - NcoI fragment of pUCIRAMLU with the MluI-NcoI digested OFVOF/OHVOR oligohybrid. The resulting plasmid was named pUCIRA2.

A pUC plasmid (pUCAROK) with the NotI-BsiWI fragment of NDV with the H5 orf instead of the HN orf and pUCIRA2 were digested with NcoI and SgfI and the NotI-NcoI fragment of pUCAROK was substituted by that of pUCIRA2, resulting in the plasmid pUCAROK2.

HF-PCR (Roche) was done to amplify the ncr of the NDV F gene behind the inserted F gene, using primers: PNCRFHIF: 5'- ata ctt aag ttc cct aat agt aat ttg tgt -3' (SEQ ID NO: 18, AfII site underlined), and PNCRFHIR: 5'- cac gca atc gca ttg cca ctg tac att ttt tct taa ctc tct gaa ctg aca gac tac c -3' (SEQ ID NO: 19, SgfI site underlined), and plasmid pUCAROA (pUC with NotI-SpeI fragment of NDV). The resulting ~ 100 bp fragment was ligated into pGEMTeasy vector to give pGEMFncrhi plasmid.

pUCAROK2 and pGEMFncrhi plasmids were digested with AflIII and SgfI to substitute the HN ncr behind H5 of plasmid pUCAROK2 by that of F from pGEMFncrhi. The resulting plasmid was named pUCAROK4.

In the last step the NotI-SgfI fragment of the NDVH5 plasmid was substituted by that of pUCAROK4 resulting in the new full-length plasmid E18C, comprising the AIV H5 gene inserted in the NDV vector between F and HN, and flanked by F-gene ncr's.

With the new constructs transfection experiments, rec virus propagation and confirmation of the recovery of infectious virus were carried out as described above. Next the virus was characterized biochemically and biologically.

Example 5: Generation of other NDV vector constructs and rec viruses:

Using similar techniques several other inserts were made in the NDV vector, using different inserted genes, and different insertion sites.

With the details already provided herein these are all within easy reach of the skilled persons' capabilities, therefore it suffices to present these in table form:

Table 5: Other NDV vector constructs according to the invention:

Inserted gene	NDV insertion region	NDV flanking ncr region
AIV H5	P - M	HN
AIV H5	M - F	HN
AIV H7	F - HN	HN
AIV N1	F - HN	HN
AIV H5	Before NP	HN

Example 6: Generation of a recombinant Rabies virus vector expressing an EIAV envelope gene

To demonstrate that the advantageous effects of the invention (the use of MV gene non-coding regions to increase the expression and/or presentation of foreign proteins in rec MV virions) expands beyond the family *Paramyxoviridae*, rabies virus -a member of the family *Rhabdoviridae*- was used as a vector to express an envelope protein derived from an unrelated virus, equine infectious anemia virus (EIAV).

In this example the construction is described of a rec rabies vector virus comprising the EIAV envelope protein, inserted between the rabies genes G and L, whereby the env gene is flanked by the ncr's from the rabies G-protein gene.

Subclones of rabies viruses were prepared in pBluescript® SK+ phagemid using the SAD-D29 full-length clone (Mebatsion, 2001, J. Virol., vol. 75, p. 11496-11502), which is referred here as ORA-D. To prepare a cloning vector, a pSK vector was first digested with SacI, blunted with Klenow enzyme, followed by digestion with HindIII and gel purification of the ~3Kb fragment. The insert was prepared by digesting ORA-D with StuI and HindIII, purifying the resultant 1.3 kb fragment, and ligating into the prepared pSK vector to generate plasmid pNCR-b.

The pNCR-b plasmid was digested with BstXI and HindIII and the ~4.0 kb fragment was purified and used for ligation with the oligo's BSSNH+ and BSSNH- (see Table 6) to create plasmid pSSNsc containing a minimum transcription cassette and ligation with oligos RABGNCR1-4 (Table 6) to generate the construct GNCR-b, containing non-coding regions in addition to the minimum transcription unit (Figure 7).

The EIAV strain Wyoming env gene was obtained by amplification from a 2052 nt synthetic gene which had been codon-optimized, RNA splice sites removed (Cook, et al. 2005, Vet. Micro., vol. 108, p. 23-37), and truncated by 134 amino acids at the 3' coding region of the original gene.

Table 6: Oligo sequences used during the construction of rec rabies viruses

Oligo-name	SEQ ID NO:	Oligo sequence (5'→ 3')
BSSNH+	20	CTGGTGAAAAAACTAACACCCCTGCTAGCA
BSSNH-	21	CGTTGACCACTTTTTTTGATTGTGGGGACGA-TCGTTTCA
RABGNCRoligo1 (*)	22	CTGGTGAAAAAACTATTAACATCCCTCAAAA-GACTCAAGGATACGTACT
RABGNCRoligo2	23	GTATCCTTGAGTCTTTTGAGGGATGTTAATAG-TTTTTTACCAGTTGC
RABGNCRoligo3	24	GGCCGTCCCTTTCAACGATCCAAGTCCTGAAGA-TCACCTCCCCTTGGGGGA
RABGNCRoligo4	25	AGCTTCCCCCAAGGGGAGGTGATCTTCAGGAC-TTGGATCGTTGAAAGGACGGCCAGTAC
EIAsynCDF	26	ATGGTGTCCATCGCCTTCTA
EIAsynCDstopR	27	TCAGTGTATGTTGTGTTGGGC

(*) 3 nt changed from ORA-D original : GGAAAG GGACTGG,
to: GGATAC GTACTGG

The amplified env gene was kinased and inserted into subclones as follows: a ~2.0 Kb amplicon, engineered to represent the entire truncated EIAV envelope protein, was generated using the primer set EIAsynCDF + EIAsynCDstopR (see Table 6). The amplicon was inserted into subclone GNCR-b which had been predigested with SnaBI and dephosphorylated to generate the recombinant plasmid pGNCR-b:envG. The ~2.0 Kb fragment was also inserted into the SSNsc subclone which had been predigested with NheI, blunt-ended and dephosphorylated to generate the recombinant plasmid pSSNsc:env.

Each recombinant construct was digested with SphI and HindIII and ligated into the SSNsc subclone, which was SphI/HindIII predigested and dephosphorylated with CIAP. After returning to the SSNsc subclone, the modified inserts were returned to the ORA-D backbone by digestion with SphI and MluI to generate the recombinant rabies viruses RV-env and RV-envG (Figure 8). The 5' and 3' ends of the constructs were verified by sequence analysis using Big-Dye® Terminator Cycle Sequencing chemistry (Applied Biosystems) and analyzed using an Applied Biosystems 3100-Avant Capillary Electrophoresis Sequencer.

The construction of a full length cDNA clone based on the modified SAD rabies strain ORA-D and generation of a recombinant rabies virus has been described (Schnell et al., 1994, EMBO J., vol. 13, p. 4195-4203; Mebatsion, 2001, supra). Each recombinant rabies virus was transfected into BSR cells as previously described (Schnell, et al., 1994, supra) using Mirus Trans-IT-LT1. Three days post-transfection, cells and supernatant were harvested and passed. Subsequent passages were by supernatant only, using an estimated moi of 0.5. Each recombinant virus was passaged a minimum of five times to verify stability.

BSR cells infected with recombinant rabies were fixed ~40 hours post-infection. These were analyzed by direct immunofluorescence using FITC Anti-Rabies Monoclonal Globulin (FDI Diagnostics Inc.) or using anti-EIAV polyclonal horse sera. In 10-fold serial dilutions, the ratio of infected cells producing rabies virus were also compared to infected cells expressing EIAV env to monitor virus stability and recombinant antigen expression.

Recombinant rabies viruses from pass five were inoculated into T-75 flasks with fresh BSR cells at an moi of 0.01. 24-hours post infection, media was changed to serum-free. Supernatant was collected and clarified by centrifugation (10,000 x g) at 72 hours post-infection. Viral supernatants were purified by sucrose gradients to analyze purified virions.

Purified virions and total infected cellular protein were combined with 2x Laemmli-reducing sample buffer and placed in boiling water for 5-10 minutes. Samples were loaded onto a 10% Tris-HCL acrylamide gel (Bio-Rad) in 1x SDS-PAGE Running Buffer. SDS-PAGE gels were run at 20 mA until the dye-front was near or at the bottom of the gel. The separated proteins were transferred onto Immobilon-P (PVDF) membrane (IPVH10100, Immobilon) by blotting at 225 mA for 45 minutes. Blots were incubated in blocking buffer (PBS-Tween 20 + 1% dry non-fat milk) for one hour at room temperature and then rinsed 3 times for 5 minutes in PBS-Tween 20. Rabies expression was detected using rabbit anti-rabies polyclonal sera directed against rabies glycoprotein and nucleoprotein diluted 1:20000 and 1:2000, respectively, in blocking buffer.

Simultaneously, EIAV env expression was detected using anti-EIAV polyclonal horse sera diluted 1:500 in blocking buffer. Blots were incubated for one hour at room temperature and then rinsed 3 times for 5 minutes in PBS-Tween20. Blots were placed in HRP labeled conjugate goat anti-rabbit IgG (H+L) (KPL) and

HRP labeled conjugate goat anti-horse IgG (H+L) (Bethyl Labs), each diluted 1:2000 in blocking buffer and incubated for one hour at room temperature. Blots were then rinsed 3 x 5 minutes in PBS-Tween20. Blots were incubated in TMB membrane peroxidase substrate (KPL) until development was complete, approximately 1-3 minutes. Blots were placed in distilled water to stop the reaction.

A typical result of such a Western blot experiment is shown in Figure 9, and demonstrates the protein composition of recombinant rabies viruses expressing EIAV envelope protein.

Lane 1: Broad-range MW ladder (Bio-Rad);

Lane 2: ORA-D backbone virus;

Lane 3: RV-env, comprising the EIAV-env gene, inserted between the rabies G and L genes, without flanking ncr's

Lane 4: RV-envG, comprising the EIAV-env gene, flanked by the ncr regions of the rabies virus G-protein.

Both recombinant rabies viruses yielded comparable infectious titer and stably expressed the EIAV-env gene insert after multiple passage *in vitro*.

However, despite subjecting comparable amounts of virions to the Western blot, recombinant RV-env, constructed in the customary way (thus without flanking ncr's) exhibited only a very weak band corresponding to the EIAV-env protein; whereas the recombinant virus RV-envG, that had the G-protein non-coding regions flanking the inserted EIAV-env protein, was expressed at a much higher rate: Figure 9, compare the band for the env protein in lanes 3 - 4.

As the constructs and inserts of RV-envG and RV-env are otherwise identical, this is strong proof that the non-coding regions have a positive effect in promoting high level of foreign protein production and immune-presentation.

CLAIMS

1. A recombinant Mononegavirales virus vector harboring an additional transcription unit comprising a foreign gene operatively linked with an upstream Mononegavirales virus gene start (GS) sequence and a downstream Mononegavirales virus gene end (GE) sequence, characterized in that between the GS sequence and a start codon of the foreign gene and between a stop codon of the foreign gene and the GE sequence, a 3' non-coding region- and a 5' non-coding region (genome sense) of a Mononegavirales virus gene are located, respectively.
2. A recombinant Mononegavirales virus vector according to claim 1, characterized in that the 3' and 5' non-coding regions are of a gene encoding an envelope protein of a Mononegavirales virus, in particular of a M, G, F or HN gene.
3. A recombinant Mononegavirales virus vector according to claim 1, characterized in that the 3' and 5' non-coding regions are of a gene encoding a RNP protein of a Mononegavirales virus.
4. A recombinant Mononegavirales virus vector according to claim 1-3 characterized in that the foreign gene encodes an antigen of a pathogen.
5. A recombinant Mononegavirales virus vector according to claim 1-3, characterized in that the foreign gene encodes an immune-modulator.
6. A recombinant Mononegavirales virus vector according to claim 1-5, characterized in that the Mononegavirales virus vector is a virus of the family Rhabdoviridae.
7. A recombinant Mononegavirales virus vector according to claim 6, characterized in that the Mononegavirales virus vector is a rabies virus.
8. A recombinant Mononegavirales virus vector according to claim 6, characterized in that the Mononegavirales virus vector is an infectious hematopoietic necrosis virus.

9. A recombinant Mononegavirales virus vector according to claim 6-8, characterized in that the the 3' and 5' non-coding regions are of a N, P, M or G gene.
10. A recombinant Mononegavirales virus vector according to claim 6-9, characterized in that the additional transcription unit is located at a 3' proximal position or between P-M, M-G or G-L genes.
11. A recombinant Mononegavirales virus vector according to claim 1-5, characterized in that the Mononegavirales virus vector is a virus of the family Paramyxoviridae.
12. A recombinant Mononegavirales virus vector according to claim 11, characterized in that the Mononegavirales virus vector is a Newcastle disease virus, canine distemper virus or (bovine) parainfluenza virus.
13. A recombinant Mononegavirales virus vector according to claim 11-12, characterized in that the the 3' and 5' non-coding regions are of a NP, P, M, F or HN gene.
14. A recombinant Mononegavirales virus vector according to claim 11-13, characterized in that the additional transcription unit is located at a 3' proximal position or between P-M, M-F, F-HN or HN-L genes.
15. A recombinant Mononegavirales virus vector according to claim 11-14, characterized in that the Mononegavirales virus vector is Newcastle disease virus.
16. A recombinant Mononegavirales virus vector according to claim 15, characterized in that the additional transcription unit is located between F-HN genes.
17. A recombinant Mononegavirales virus vector according to claim 15-16, characterized in that the non-coding regions are of a HN gene.
18. A recombinant Mononegavirales virus vector according to claim 15-17, characterized in that the foreign gene encodes an antigen of an avian pathogen.

19. A recombinant Mononegavirales virus vector according to claim 15-18, characterized in that the foreign gene encodes a hemagglutinin (HA) of an influenza virus, preferably a H5 or H7 hemagglutinin.
20. A recombinant Mononegavirales virus vector according to claims 1-19, characterized in that the Mononegavirales virus vector is an attenuated virus.
21. A vaccine against a microbial pathogen, characterized in that it comprises a recombinant Mononegavirales virus vector according to claims 1-20 in a live or inactivated form, and a pharmaceutically acceptable carrier or diluent.
22. A vaccine according to claim 21, characterized in that it additionally comprises an adjuvant.
23. A vaccine according to claims 21-22, characterized in that it comprises an additional vaccine strain.