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MINIREVIEW

# Recovery of Negative-Strand RNA Viruses from Plasmid DNAs: A Positive Approach Revitalizes a Negative Field

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Viruses with completely or predominantly negative-sense RNA genomes span six viral families: the nonsegmented *Rhabdoviridae, Paramyxoviridae,* and *Filoviridae* and the segmented *Orthomyxoviridae* (8 segments), *Bunyaviridae* (3 segments), and *Arenaviridae* (2 segments). These virus families include some of the world's most notorious pathogens, and methods for engineering attenuated variants or recombinant viruses for vaccine purposes have long been needed. In addition, many of these viruses serve as important models for basic research on all aspects of viral replication. Thus the recovery of complete negative-stranded RNA viruses from cloned cDNAs is among the most exciting breakthroughs in RNA virology in the 1990s since it has opened the door to directed engineering of the viral genomes and a detailed understanding of the function of the viral genes and their products.

Investigators studying influenza virus were able to recover viruses incorporating engineered RNA segments as early as 1989 and 1990 (Luytjes *et al*., 1989; Enami *et al*., 1990). Although the method applied to influenza allowed the first site-directed mutagenesis in negative-stranded RNA viruses, complete recovery of influenza virus from cDNAs has still not been achieved. Engineering of influenza virus was accomplished with an ingenious though cumbersome method involving reconstitution *in vitro* of ribonucleocapsids (RNPs) containing a single negative-sense RNA segment (derived by *in vitro* transcription from a DNA clone), the nucleoprotein, and the three polymerase subunits. This RNP complex was then transfected into cells infected with influenza helper virus defective in the gene product corresponding to the RNA segment provided. This system has proven effective in generating influenza viruses that contain mutations in or substitutions of six of the eight influenza A virus segments and one of the influenza B virus segments (reviewed in Palese *et al*., 1996). This system has not been successful in the rescue of nonsegmented, negative stranded (NNS) RNA viruses since reproducible reconstitution of infectious RNPs containing full-length genomes, all of which are greater than 11 kb in length, has not yet been achieved.

Schnell *et al.* (1994) found a key to recovery of NNS RNA viruses that had eluded the field for several years. The method they described is as follows. Plasmids encoding the viral nucleocapsid protein (N) and the polymerase proteins (L and P) under the control of T7 promoters were transfected into cells previously infected with recombinant vaccinia virus expressing the T7 polymerase protein (vTF7–3). In addition to these plasmids, a plasmid encoding a full-length *antigenomic* viral RNA under the control of a T7 promoter at the 5′ end and a self-cleaving ribozyme at the 3′ end was also transfected into the cells (Fig. 1). After transcription of RNAs from the T7 promoters and translation of the encoded proteins, nucleocapsid proteins assemble around the antigenomic RNAs, and polymerase proteins then replicate these RNPs to form RNPs containing genomic RNAs. After transcription of mRNA from the genomic RNP and translation, infectious virus is assembled. This procedure led to successful recovery of recombinant rabies virus, but only from approximately 1 of $10^7$ transfected cells.

The use of the positive sense antigenome RNA, rather than the negative sense genome, was critical because of an antisense problem. If the negative sense genome is used instead, mRNAs encoding viral proteins can hybridize to the naked genomic RNA and prevent the critical assembly of the genome into the RNP, the template for transcription and replication (M. Whitt and J. Rose, unpublished data). The negative-strand viruses always keep their genome in RNP form, probably in part to avoid this antisense problem. When one starts with the posi-

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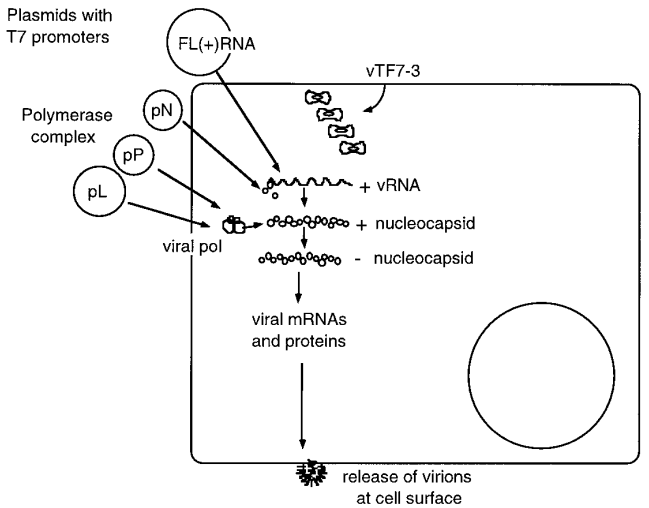


FIG. 1 Recovery of negative stranded RNA viruses from plasmid DNAs. A typical procedure for recovery is shown. Cells are infected with a vaccinia virus recombinant encoding the T7 RNA polymerase and then transfected with plasmids encoding the full-length viral antigenome and three plasmids encoding the viral nucleocapsid (N) and polymerase proteins (P and L). The antigenome assembles into a nucleocapsid that is replicated to a genomic nucleocapsid. Subsequent transcription, translation, and virus assembly lead to release of infectious virus particles.

tive-strand antigenome, this RNA can form an RNP without any interference from the mRNAs. Once in RNP form, the positive strand can then be replicated to form full-length minus strand RNPs that are wrapped into RNPs as nascent RNA chains and thus immune to interference from mRNAs.

The recovery of rabies by Schnell *et al.*, reported in the September, 1994 *EMBO Journal*, was also a key presentation at the Ninth International Conference of Negative Stranded RNA Viruses in Estoril, Portugal in October, 1994. Many researchers returned to their labs from this meeting sensing that they too might obtain recoveries in their own system by starting with the antigenomic RNA. The initial success in rabies virus was indeed followed by several successful recoveries in other NNS viral systems (Fig. 2) and by one application of the methodology in the segmented, negative sense RNA viruses. In 1995 using a very similar strategy, Lawson *et al.* reported the first successful recoveries of vesicular stomatitis virus (VSV) from cDNAs. This paper described recoveries that were obtained independently in the laboratories of J. Rose and M. Whitt. Three months later a third group reported recovery of VSV from cDNAs using the same system (Whelan *et al*., 1995).

Application of the system within viruses of the *Rhabdoviridae* family was just the beginning. In December 1995 there were three reports of recovery of viruses from the *Paramyxoviridae* family of viruses. Radecke *et al.* reported recovery of measles virus (a morbillivirus); Collins *et al.* reported successful application to human respiratory syncytial virus (RSV, a pneumovirus); and Garcin *et al.* reported success with Sendai virus (a paramyxovirus). Although variations within the recovery systems were employed, each group found successful recovery of progeny viruses dependent upon expression of the viral positive-sense, antigenomic RNAs. Radecke *et al.* employed a vaccinia-free system in which the measles virus (MV) N and P proteins along with the T7 RNA polymerase were stably coexpressed in a 293 cell line. Upon transfection of this cell line with plasmids encoding the MV L protein and MV antigenomic RNA, progeny MV were recovered at rates similar to those reported by Schnell *et al.* and Lawson *et al.* Collins *et al.* found coexpression of an additional RSV protein, M2(ORF1), a transcriptional elongation factor, necessary for recovery of RSV in a vaccinia MVA-T7 system.

In June of 1996, Kato *et al.* provided the second report of recovery of Sendai virus from cDNAs. In this report, virus was recovered from both negative and positive sense RNAs transcribed *in vivo* in the vaccinia T7 system, or transfected into the system after being transcribed *in vitro*. The efficiency of recoveries from antigenomic RNA constructs was much higher than reported by Garcin *et al*. The authors attribute this to: (1) truncation of the T7 promoter (by removing the guanosine triplet) thus providing a precise 5′ end to the viral RNA transcripts, (2) optimization of the NP, P, and L plasmid ratios, and (3) inhibition of vaccinia cytopathic effect by incubating in the presence of both ara C and rifampicin. Although the

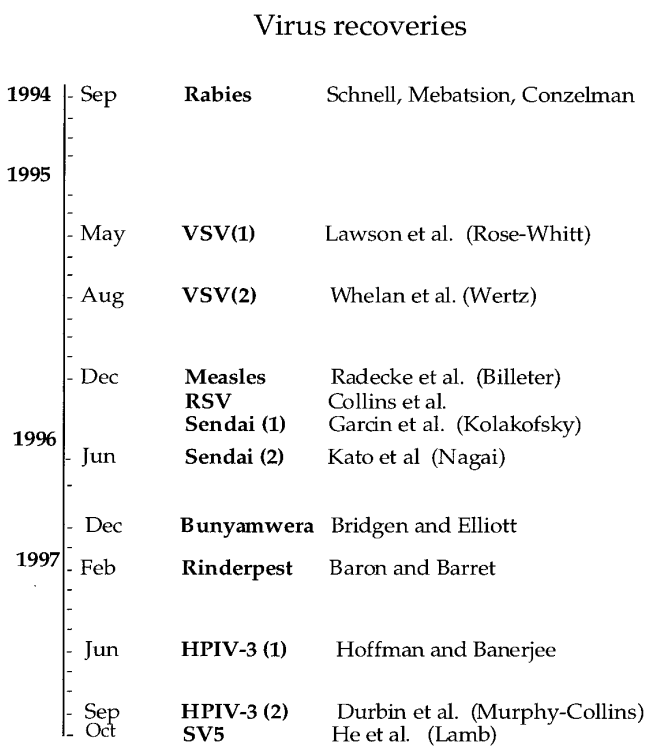


FIG. 2. Chronology of recoveries for complete, infectious, negative-strand RNA viruses from plasmid DNAs. Numbers in parentheses indicate the first or second reports of recoveries for the same virus.

investigators were successful in recovering virus from genomic RNA constructs, the efficiency of recoveries was considerably lower (at least 100-fold) than recoveries from the positive RNA constructs.

Although 1996 proved to be a slower year in recovery of new NNS RNA viruses, another success story emerged. Bridgen and Elliott (1996) reported the first recovery of a segmented RNA virus, the Bunyamwera virus, solely from cDNAs. In contrast to the influenza system, which required helper virus, a helper-free system based on the NNS RNA virus recovery systems was employed. Bunyamwera virus, the prototype of the *Bunyaviridae* viruses, has a trisegmented genome. Therefore, Bridgen and Elliott transfected three plasmids expressing the three antigenomic viral segments (L, M, and S) along with three T7-plasmids expressing the viral mRNAs encoding all the viral proteins (N, NSs, G1, G2, NSm, and L) into cells infected with the recombinant vaccinia T7 virus. Each antigenomic RNA construct was expressed from a T7 promoter and had the self-cleaving HDV ribozyme at the 3′ end. Each antigenome transcript contained two extra nonviral guanosine residues at the 5′ end. Bridgen and Elliott also reported a low efficiency of recovery as might be expected from such a complex system.

In 1997 three more viruses from the *Paramyxoviridae* family were recovered from cDNA clones using either the original vaccinia T7 expression system or the MVA-T7 system. Use of MVA, a vaccinia virus deficient in replication in many mammalian cells, alleviates the need for purification of recovered viruses from contaminating vaccinia viruses when the recoveries are passaged in mammalian cells. In February 1997, Baron and Barrett reported the successful recovery of another morbillivirus, rinderpest virus (RPV) using the MVA-T7 system. In June 1997, Hoffman and Banerjee reported the recovery of human parainfluenza virus type 3 (HPIV-3), another paramyxovirus, and in September an independent group also reported recovery of HPIV-3 (Durbin *et al*., 1997). Hoffman and Banerjee used the vaccinia T7 system first described by Schnell *et al.,* whereas Durbin *et al.* used the MVA-T7 system. Durbin *et al.* reported recovery of HPIV-3 from both genomic and antigenome constructs and thus is the second group to report successful recovery from the genomic constructs. In October, the first successful recovery of a rubulavirus, simian virus 5 (SV5), from cDNAs was reported using the MVA-T7 system (He *et al.*, 1997).

Although a complete recovery of influenza virus from DNA has still not been achieved, a plasmid-based system for the expression of influenza RNPs was reported (Pleschka *et al*., 1996). The plasmid-based system was modified to allow the nuclear expression of the influenza RNAs. Expression of the RNA segment of interest was under the control of a truncated pol I promoter at the 5′ end and a self-cleaving ribozyme sequence at the 3′ end. The RNP protein components (PB1, PB2, PA, and NP) were encoded on plasmids under the control of pol II promoters (Pleschka *et al*., 1996). These plasmids were transfected into cells and the RNPs assembled intracellularly. No RNA transcript purification, no protein purification, and no *in vitro* reconstitution of biologically active RNPs were necessary. Although this system still necessitates the use of influenza helper virus, it is a step in the direction of a less cumbersome recovery system for recombinant influenza viruses.

The earliest application of reverse genetics for VSV demonstrated the ability to engineer recombinant viruses substituting the glycoprotein gene from a different serotype (New Jersey) for the endogenous glycoprotein gene (Indiana) (Lawson *et al*., 1995). In 1996, subsequent reports demonstrated that additional genes could be added to the viral genomes. The bacterial chloramphenicol acetyl transferase (CAT) gene was expressed as an extra gene in VSV (Schnell *et al*., 1996b) and in rabies virus (Mebatsion *et al*., 1996). CAT gene expression in recombinant VSVs was shown to be dependent only on insertion of additional transcriptional start and stop signals, which are present within the nearly conserved 23 nucleotide sequence found at each VSV gene junction. CAT expression was quite stable because it was maintained in cells infected with recombinant VSV-CAT even after many low multiplicity passages. Subsequent reports described CAT expression in recombinant RSV (Bukreyev *et al*., 1996), luciferase expression in recombinant Sendai virus (Hasan *et al*., 1997), and expression of the green fluorescent protein (GFP) in recombinant SV5 (He *et al*., 1997).

In the NNS RNA viruses, the construction of viral genomes expressing foreign genes is dependent upon the addition of the foreign gene and the appropriate transcription start and stop signals recognized by the viral polymerase. The level of expression of the foreign gene is dependent upon the location within the genome; the closer the gene is to the initial transcription start at the 3′ end, the greater the expression. The expression level of the bacterial CAT gene as a function of position is illustrated in Fig. 3. The levels of expression obtained are consistent with a well-documented transcription attenuation of ~30% occurring at each gene junction (Iverson and Rose, 1981).

In recombinant VSVs it has been shown that the addition of foreign genes results in bullet-shaped progeny virions longer than the wild-type VSV virions (Schnell *et al*., 1996a; Kretzschmar *et al*., 1997). The additional length of the particles reflects the additional genome length contributed by the insert. Multiple genes, adding up to 4.2 kb of RNA, have been introduced into recombinant VSVs and the helical nucleocapsids grow longer to accommodate the 38% increase in RNA length (J. Forman, K. Haglund, and J. Rose, unpublished data). In some recombinants, the viral titers and budding efficiencies

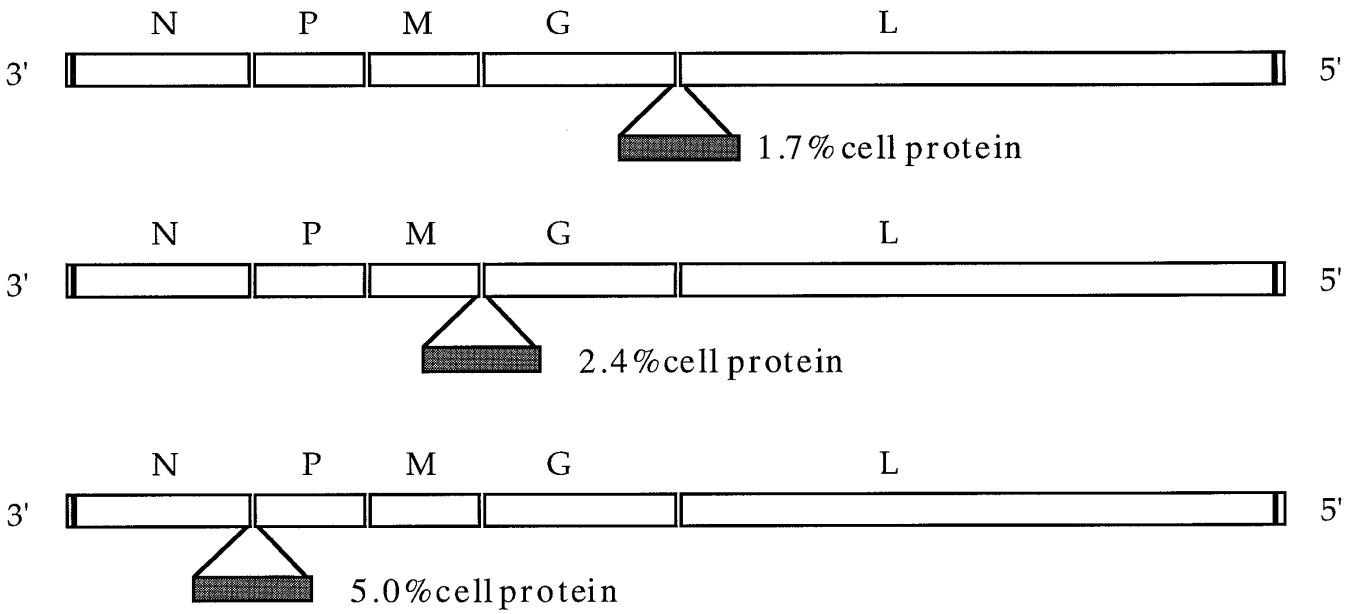


FIG. 3. Gene expression levels at different sites in the VSV genome. VSV vectors allowing foreign gene expression at the indicated sites in the genome were constructed by introducing the appropriate transcription start-stop signals flanking sites for insertion of foreign genes. The expression sites were introduced in the noncoding regions of the indicated intergenic junctions. The levels of CAT protein expression as percent of total cell protein at 7 h after infection are indicated (Schnell *et al.*, 1996b; M. Schnell, L. Buonocore and J. Rose, unpublished data). The levels of protein expression obtained for different genes vary above and below those obtained for CAT depending on the gene being expressed.

are reduced when compared with wild-type VSV. The reduced titers appear to be due to a variety of factors including the length of the insert, the level of the foreign protein expression, and inhibition of the viral life cycle by some foreign proteins. Although an upper limit for the size of recombinant VSV genomes has not yet been reached, one would expect the efficiency of replication and budding to decrease with longer genomes to the point where recovery will not be achieved.

CAT, luciferase, and GFP recombinants demonstrated the ability to express foreign genes in NNS RNA virus genomes. It has also been possible to generate recombinants expressing foreign membrane proteins, which are incorporated into progeny virions. Indeed, the incorporation of foreign plasma membrane proteins into VSV is remarkably permissive. Our group has shown expression and incorporation of the influenza A virus hemagglutinin (HA) and neuraminidase (NA) proteins (Kretzschmar *et al.*, 1997), the HIV-1 envelope protein (gp160) with a VSV-G cytoplasmic tail (Johnson *et al.*, 1997), the MV fusion (F) and hemagglutinin (H) proteins (Schnell *et al.*, 1996a), the RSV glycoprotein (G) and fusion (F) protein (J. Kahn, M. Schnell, L. Buonocore, and J. Rose, unpublished data), and the cellular proteins CD4, CXCR4, and CCR5 (Schnell *et al.*, 1996a, 1997; E. Boritz, M. Schnell, L. Buonocore, and J. Rose, unpublished data). In all cases examined to date except for HIV envelope protein, incorporation of the foreign proteins into the VSV envelope does not require addition of the VSV G cytoplasmic tail. The potential of such recombinants in vaccine applications include the recent demonstration of the recombinant VSV-HA as a highly effective intranasal vaccine. After a single dose VSV-HA completely protects mice from lethal influenza virus challenge (Roberts *et al.*, 1998).

Genes encoding foreign membrane glycoproteins can either be incorporated as extra genes in VSV or can be incorporated in place of the VSV G gene (Schnell *et al.,* 1996a, 1997). By swapping the endogenous VSV G gene for genes encoding foreign glycoproteins, it is possible to obtain viruses containing the foreign proteins in their envelopes. These viruses lack the normally broad tropism conferred by VSV G and can be targeted to specific cells. We use the term "surrogate viruses" for such novel viruses. This term distinguishes them from pseudotypes, which do not encode their foreign envelope proteins but instead incorporate proteins expressed in *trans.* For example, VSV recombinants expressing the HIV receptor and a coreceptor in place of G incorporate both foreign proteins and are targeted specifically to cells infected with HIV-1 which display the HIV-1 envelope proteins on their surface (Schnell *et al.*, 1997). Further applications of surrogate viruses in which VSV G is eliminated (ΔG) may permit reuse of the VSVΔG vector for multiple vaccinations with multiple antigens because the neutralizing antibody response to VSV G will be eliminated.

Other applications of reverse genetics to NNS RNA viruses have included the elimination of alternative gene products encoded in the P genes (C and V proteins) to evaluate their role in viral replication and pathogenesis. Recombinant viruses deleting the VSV C and C′ proteins (Kretzschmar *et al.*, 1996), Sendai virus V protein (Kato *et*

*al.*, 1997), Sendai virus C/C′ proteins and C/C′/Y1/Y2 proteins (Kurotani *et al.*, 1998), MV V protein (Schneider *et al.*, 1997), or MV C protein (Radecke *et al.*, 1996) have been generated. Little or no effect on viral replication was observed with the VSV or measles mutants in tissue culture although MV C− recombinants did have a reduced plaque size. In contrast to VSV and MV mutants, the Sendai virus mutants did have significant effects on viral replication. The Sendai virus V− mutants were either unaffected in viral replication in tissue culture or potentiated when compared with wild-type Sendai virus. However, the Sendai virus V− recombinants were greatly attenuated in pathogenicity in a mouse model. Although the V− recombinant initially replicated at levels comparable with the wild-type virus in mice, it was cleared more rapidly. The Sendai virus C/C′ recombinants were significantly attenuated in viral replication in tissue culture and were less pathogenic in mice than the V− recombinant. The Sendai virus C/C′/Y1/Y2 recombinants were further attenuated in tissue culture growing to even lower titers than the C/C′− recombinants.

Plasmid-based systems for generating recombinants in the *Rhabdoviridae, Paramyxoviridae,* and *Bunyaviridae* have unlocked the great potential of these systems and applications are just beginning. It is feasible that intracellular plasmid-based systems can be applied to the remaining families of the segmented and nonsegmented negative-stranded RNA viruses (*Filoviridae, Orthomyxoviridae,* and *Arenaviridae*). The expression of influenza virus genomic RNAs from plasmids would need modification for nuclear expression as described by Pleshka *et al.* (1996) leaving still the challenge of efficient transfection of 12 plasmids (eight RNA segments and four RNP components) into a single cell for a single recovery event. Further challenges may also remain within viruses that contain ambisense coding strategies (e.g., arenaviruses, hantaviruses, and tospoviruses) before successful recoveries in these viral systems become a reality.

Reverse genetic systems have opened the door to the negative-strand RNA viruses and the technology is moving the field in many new directions. Viral attenuation through specific mutations has obvious practical significance in vaccine development. Such attenuating mutations include those eliminating gene products that are nonessential for replication in tissue culture, those rearranging gene order, and those deleting the cytoplasmic tails of viral glycoproteins (Jin *et al.*, 1996; Kato *et al.*, 1997; Kurotani *et al.*, 1998; Roberts *et al.*, 1998; Wertz *et al.,* 1998). Deletion mutants generally cannot revert, thus permanent attenuation should be possible in such recombinants. Continuing genetic manipulations of the NNS RNA viral genomes will allow determination of the roles of numerous gene products whose functions were not previously accessible.

Now that we can insert foreign genes into NNS viruses and completely change the viral envelope, we can address the potential of surrogate viruses as multiuse vaccines and antiviral vectors. Applications of targeted NNS RNA viruses to specific killing of cancer cells should be possible by incorporating the appropriate antibodies and membrane fusion proteins into the viral envelope. However, controlling the host immune response to the vector itself will be a major obstacle to practical application. Surrogate viruses will also likely serve as tools for the identification of new viral receptors for those viruses that cannot be safely or efficiently grown in tissue culture. Such potential is illustrated by a VSV lacking the G gene pseudotyped with the Ebola virus glycoprotein, VSVΔG/Ebola-G pseudotypes (Takada *et al.*, 1997).

We still need a clearer understanding of the interactions determining efficient budding of the NNS RNA viruses. In VSV, specific interactions of glycoprotein tails with internal virion components have only small effects on virus budding (Schnell *et al.*, 1998), yet the VSV G protein dramatically enhances virus budding. Identification of a minimal VSV G domain enhancing budding could be very important to future design of targeted VSV vectors that bud more efficiently. Detailed exploration of complex problems such as assembly are now possible with the advent of modern genetics to the negative-strand virus field.

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