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Short communication

A single-plasmid reverse genetics system for the rescue of non-segmented negative-strand RNA viruses from cloned full-length cDNA

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ABSTRACT

Reverse genetics systems for non-segmented negative-strand RNA viruses rely on co-transfection of a plasmid containing the full-length viral cDNA and helper plasmids encoding essential viral replication proteins. Here, a system is presented in which virus can be rescued from a single plasmid without the need for helper plasmids in cells infected with a host-restricted recombinant poxvirus that expresses T7 RNA polymerase. This approach relies on the insertion of T7 promoter sequences in the viral cDNA at positions that allow transcription of sub-genomic RNAs encoding essential viral replication proteins.

Reverse genetics of RNA viruses entails the rescue of infectious virus entirely from cloned full-length viral cDNA. During the last few decades, reverse genetics systems have been developed for almost all classes of positive-strand and negative-strand RNA viruses. Because they allow the genetic modification of RNA viruses, reverse genetics systems are very powerful tools for fundamental virus research and for the generation of recombinant viruses that can be used for vaccination or therapeutic purposes.

Due to fundamental differences in the genome replication strategies of positive-strand RNA viruses (PSVs) and negative-strand RNA viruses (NSVs), reverse genetics systems are generally more complicated for NSVs than for PSVs. All PSVs contain a single genome segment and the viral RNA acts both as template for genome replication and as mRNA. Thus, a single plasmid containing the viral cDNA under control of a suitable cellular or viral promoter is sufficient for PSV reverse genetics (Pushko et al., 2016; Stobart and Moore, 2014).

NSVs contain either a segmented genome (sNSVs: *Arenaviridae, Bunyaviridae, Orthomyxoviridae*) or a non-segmented genome (nsNSVs; *Bornaviridae, Nyamaviridae, Rhabdoviridae, Paramyxoviridae*). In both cases the template for replication is a ribonucleoprotein complex consisting of the viral RNA associated with the viral nucleoprotein.

Rescue of nsNSVs from cloned full-length cDNA requires the expression of a number of essential viral replication proteins in addition to the viral RNA template. This is accomplished by co-transfection of 4 or 5 plasmids, one of which is a transcription plasmid that generates full-length viral RNA. The other plasmids are necessary for the expression of the viral replication proteins, i.e. the polymerase protein (L), the polymerase co-factor (or phosphoprotein, P), the nucleocapsid protein (N or NP), and in the case of pneumovirinae also the matrix protein M2-1 (Conzelmann, 1996, 2004; Pfaller et al., 2015; Walpita and Flick, 2005).

The first reverse genetics systems for nsNSVs were developed in the early 1990's (Lawson et al., 1995; Schnell et al., 1994; Whelan et al., 1995), and most currently used systems still more or less follow the original design. To ensure the production of authentic viral RNA, the T7 polymerase promoter sequence is added to the 5′ end of the anti-genomic (plus strand) cDNA and an autocatalytic ribozyme sequence is added to the 3′ end. Transcription by T7 RNA polymerase results in the generation of anti-genomic RNA containing functional 5′ and 3′ terminal ends. In addition, the genes encoding the NP, P, and L replication proteins are cloned behind a suitable promoter in expression plasmids (helper plasmids). Co-transfection of the helper plasmids and the plasmid containing the full-length cDNA into cells that produce T7 RNA polymerase (either constitutively, or after infection with a recombinant poxvirus that expresses T7 RNA polymerase), results in reconstitution of the ribonucleoprotein complex that can be used for transcription and replication by the viral polymerase complex consisting of the P and L proteins, ultimately resulting in the rescue of infectious virus.

Previously, we developed a reverse genetics system for Newcastle Disease virus (NDV) in which we used the above described approach, and in which a Fowlpox-T7 recombinant virus (FPV-T7) (Britton et al., 1996) was used as a helper virus for the provision of T7 RNA polymerase (Peeters et al., 1999). Meanwhile, this system has been used to rescue a number of different NDV strains from different pathotypes (de

Abbreviations: PSVs, positive-strand RNA viruses; NSVs, negative-strand RNA viruses; nsNSVs, non-segmented negative-strand RNA viruses; sNSVs, segmented negative-strand RNA viruses; NDV, Newcastle disease virus

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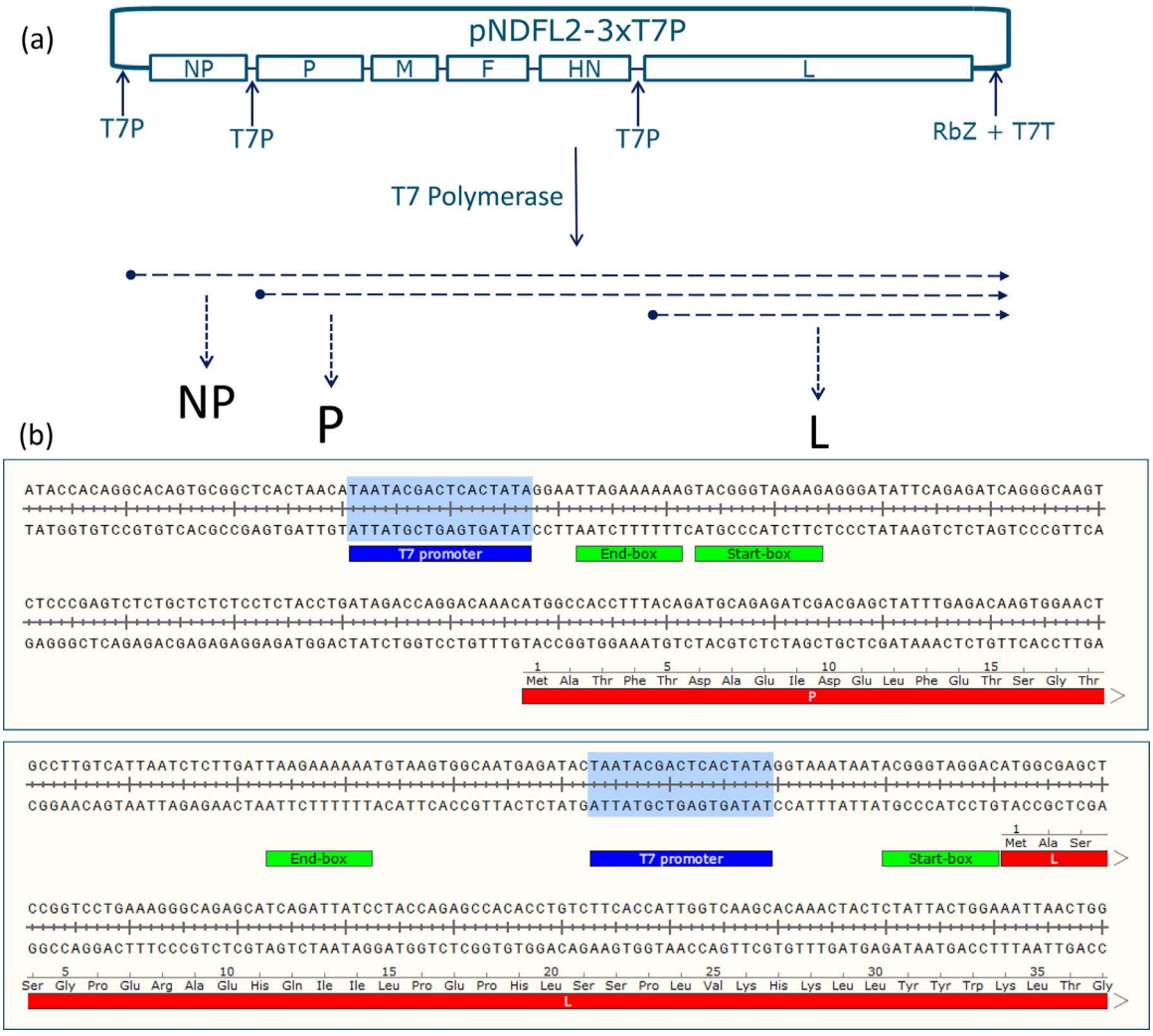

Fig. 1. Positions of T7 promoter sequences within the NDV genome.
(a) Schematic presentation of the full-length NDV LaSota cDNA plasmid pNDFL2-3xT7P showing the positions of the T7 promoter sequences (T7P). RbZ: ribozyme sequence; T7T: T7 transcription termination signal.
The RNAs generated by T7 RNA polymerase (T7Pol) are indicated by dashed lines and the proteins expressed from these RNAs are indicated.
(b) Part of the NDV sequence showing the positions of the T7 promoter sequences (blue boxes) before the P-gene (upper panel) and L-gene (lower panel) in pNDFL2-3xT7P. The positions of the End-box and Start-box (green boxes) and the amino acid sequences of the N-terminal parts of the P- and L-proteins (red boxes) are also indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Leeuw et al., 2005; Dortmans et al., 2009; Peeters et al., 1999) (Peeters & de Leeuw, unpublished results) and in our hands the system is robust and efficient. However, for each NDV strain, apart from the full-length cDNA, also the corresponding strain-specific helper plasmids must be constructed since the use of heterologous helper plasmids may result in chimeric viruses due to plasmid recombination during transfection. The latter is due to the presence of recombination enzymes expressed by the FPV-T7 helper virus. Recombination is apparently quite efficient since infectious NDV could still be rescued when using a helper plasmid containing a single-nucleotide deletion that caused a lethal frame-shift in the L-gene coding sequence (Peeters & de Leeuw, unpublished observation).

By serendipity, it was observed that the helper plasmid encoding the NP protein, but not the P or L protein, could be left out of the helper plasmid mix without affecting the efficiency of virus rescue (Dortmans et al., 2009). The most probable explanation is that this was due to expression of the NP protein from the full-length anti-genomic (plus strand) RNA generated by T7 RNA polymerase. A proportion of this anti-genomic RNA might be capped and perhaps poly-adenylated by the action of the FPV-encoded capping enzyme and poly-A polymerase, both of which are expressed in the cytoplasm of poxvirus infected cells (Condit et al., 2006). In that case one may expect that, due to the monocistronic nature of eukaryotic mRNAs, only the first gene (i.e. NP) will be translated from this RNA species.

If this is indeed the case, this phenomenon could perhaps be used to also express the P and L proteins by inclusion of the minimal T7 promoter sequence in the full-length cDNA at positions before the P- and L-genes. Transcription by T7 RNA polymerase would then result in the generation of 3 RNA species, i.e. the authentic full-length anti-genomic RNA (starting at the T7 promoter before the 5′-end of the NDV cDNA) and two sub-genomic RNA species; one starting before the P-gene and the other starting before the L-gene (Fig. 1a).

To test this 17 nt from the original NDV LaSota sequence (GenBank accession number AF077761) in front of the P-gene (nt 1771–1787) and the L-gene (nt 8343–8359) were replaced by the T7 promoter sequence TAATACGACTCACTATA (Fig. 1b). To this end, two synthetic DNA restriction fragments (SalI-ApaI, nt 1644–2289 and SpeI-BsiWI, nt 8095–8852; GenScript USA Inc) containing the T7 promoter sequences at the indicated positions, were used to sequentially replace the corresponding fragments in the full-length NDV LaSota cDNA plasmid pNDFL2 (Kortekaas et al., 2010; Peeters et al., 1999). In each case the T7 sequence was positioned before two G-residues, based on the