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# Identification and mapping of a region on the mRNA of *Morbillivirus* nucleoprotein susceptible to RNA interference

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ABSTRACT

The *morbillivirus* genus includes important pathogens such as measles virus (MV), peste des petits ruminants virus (PPRV), and rinderpest virus (RPV) and forms a group of antigenically related viruses. The viral nucleoprotein (N) is a well-conserved protein among the genus and plays a central role in the replication of the virus. Using a comprehensive approach for siRNA screening of the conserved sequences of the N gene, including sequence analysis and functional *in vitro* tests, we have identified a region for the design of siRNA effective for the control of PPRV, RPV, and MV replication. Silencing of the N mRNA efficiently shuts down the production of N transcripts, the expression of N protein, and the indirect inhibition of matrix protein, resulting in the inhibition of PPRV progeny by 10,000-fold.

These results suggest that siRNA against this region should be further explored as a therapeutic strategy for morbillivirus infections.

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## 1. Introduction

The *morbillivirus* genus within the Paramyxoviridae family includes highly contagious pathogens that have major importance in human and veterinary medicine, with strong health and socio-economic impacts. The common characteristics of morbillivirus infection are high infectivity, morbidity, and mortality rates. Moreover, no effective or specific treatments exist for infected animals or humans. Well-characterized members of these enveloped negative-stranded RNA viruses are measles virus (MV), peste des petits ruminants virus (PPRV), rinderpest virus (RPV), canine distemper virus (CDV), phocine distemper virus (PDV) and dolphin morbillivirus (DMV). Measles claims about half a million lives annually, principally among young children of the less developed countries (Griffin, 2001). Despite extensive vaccination campaigns against MV, the disease has not been eradicated and, furthermore, outbreaks occur within vaccinated populations (Zandotti et al., 2004). Peste des petits ruminants affects sheep, goats, and small wild ruminants, while rinderpest affects even-toed ungulates, mostly cattle and buffalo. For both diseases, morbidity and mortality rates are variable but can reach 100% (Taylor, 1986; Taylor et al., 1990; Lefèvre and Diallo, 1990). RPV infections have been controlled by mass vaccination with a live-attenuated vaccine (Plowright and Ferris, 1962) that has confined the virus to only one area in Eastern Africa. RPV is currently targeted for global eradication by 2010. In contrast, more than 1 billion small ruminants are at risk for PPRV, and although the current live-attenuated PPRV vaccine (Diallo et al., 1989) is very effective at preventing the disease, there is no clear prospect of global control of PPRV infections. The genome of morbilliviruses contains six tandemly arranged transcription units encoding eight proteins, the nucleoprotein (N), the polymerase-associated phosphoprotein (P) protein, the matrix (M), the surface glycoproteins F and H, and the large (L) protein that, in association with the P, forms the ribonucleoprotein (RNA)-dependent RNA polymerase (RdRP). The gene order is 3′N-P-M-F-H-L5′, as determined by transcriptional mapping (Dowling et al., 1986). N, P, and L genes form the ribonucleoprotein complex, which is the complex essential for the replication of morbilliviruses (Kingsbury, 1990). The nucleoprotein encapsidates the viral genome to form a helical nucleocapsid and plays a central role in transcription and replication. The encapsidated genome is replicated into full-length antigenomes serving as template for the synthesis of genomes, which are then incorporated into progeny virions.

Besides the vaccine used to prevent the disease or the antibiotic treatment applied to avoid secondary bacterial infection, we believe that RNA interference (RNAi) could become a complementary tool for the control of *Morbillivirus* infections. RNAi is a natural biological process first discovered in plants that represses gene expression by mediating sequence-specific mRNA degradation (Matzke et al., 1989). This post-transcriptional gene

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silencing (PTGS) is guided in plants and animals by small double-stranded (ds) RNAs called small interfering RNA (siRNA) (Fire et al., 1998; Hammond et al., 2000, 2001). Elbashir et al. (2001) demonstrated for mammalian cells that synthetic siRNAs of 21–23 nucleotides were able to cleave and degrade the complementary mRNA sequences targeted through the RNA-induced silencing complex (RISC). Application of RNAi has also proved efficient in antiviral strategies of a variety of viruses: HIV (Capodici et al., 2002; Jacque et al., 2002), poliovirus (Gitlin et al., 2002), hepatitis C virus (Kapadia et al., 2003; Randall et al., 2003), influenza virus (Ge et al., 2003), respiratory syncytial virus (Barik, 2004), and foot-and-mouth disease virus (Chen et al., 2004; Liu et al., 2005). Recently, Barik (2004), Otaki et al. (2006), and Reuter et al. (2006) demonstrated in cell cultures that siRNA can interfere with the replication of several non-segmented negative-stranded RNA viruses.

In this study, we hypothesized that siRNA against viral mRNA encoding proteins that constitute the replication complex could have a strong inhibitory effect on virus replication. Therefore, we developed quantitative cell-biology approaches to identify conserved sequences on the nucleoprotein gene of morbilliviruses that can be targeted by siRNA. Using synthetic siRNAs, we have identified a common position on the N gene, that can be efficiently targeted to prevent the replication of three morbilliviruses (MV, PPRV, and RPV).

## 2. Materials and methods

### 2.1. Cell and virus stocks

Vero cells were purchased from the European Collection of Cell Culture (ECACC, France) and maintained in Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS, Eurobio, Courtaboeuf, France) and 2 mM L-glutamin (Gibco, Life technology, UK). Three live-attenuated morbillivirus vaccine strains, the Nigeria 75/1 vaccine strain of PPRV (SK/1, BK/1 and Vero/55; Diallo et al., 1989), the RBOK vaccine strain of rinderpest (BK98, Vero/2, Plowright and Ferris, 1962), and the Schwarz-attenuated vaccine strain of MV (Schwarz, 1962) were prepared by infecting Vero cells at a multiplicity of infection (MOI) of about 0.1 $CCID_{50}$/cell. Virus stocks were prepared by collecting the infected cell supernatant when cytopathic effect (CPE) was almost complete, and it was stored in aliquots at −80 °C. Virus titers were estimated by the method of Reed and Muench (1938) and expressed in $CCID_{50}$/ml.

### 2.2. siRNA selection and preparation

#### 2.2.1. Multiple alignments of the N genes of morbilliviruses

The nucleotide sequences of the N gene of the six viruses PPRV, RPV, MV, CDV, PDV, and DMV were obtained from GenBank and aligned using the Clustal W program (Vector NTI, Informax Inc.) to identify the most conserved regions for selection and synthesis of the siRNAs. The conservation was also assessed on successive stretches of 20 nucleotides covering the full sequence in multiple alignments of 210 N genes of MV. The minimum and maximum percentages of identities were 86% and 100%, respectively. In the rest of the study, the selection of conserved segments for further siRNA design was based on the requirement of a minimum conservation of 93%.

#### 2.2.2. Synthetic siRNA

Conserved regions on the N gene of the PPRV 75-1 vaccine strain (GenBank/EMBL accession number X74443; Diallo et al., 1994) were run through Cenix software provided by Ambion Co. to design synthetic siRNAs. From the N PPR gene sequence, the position and homology of four potential siRNA sequences targeting this gene were deduced and were named siRNA-NPPR1 (position 480–498, 100% homology), siRNA-NPPR2 (position 1318–1336, maximum 63% homology), siRNA-NPPR3 (position 850–868, 95% homology), and siRNA-10 (position 850–868, 100% homology). Since siRNA-NPPR1 was the most active, overlapping siRNAs with one residue frame shift were synthesized and tested to delineate the active region within position 477–500 (Fig. 1). A total of 13 siRNAs of 21

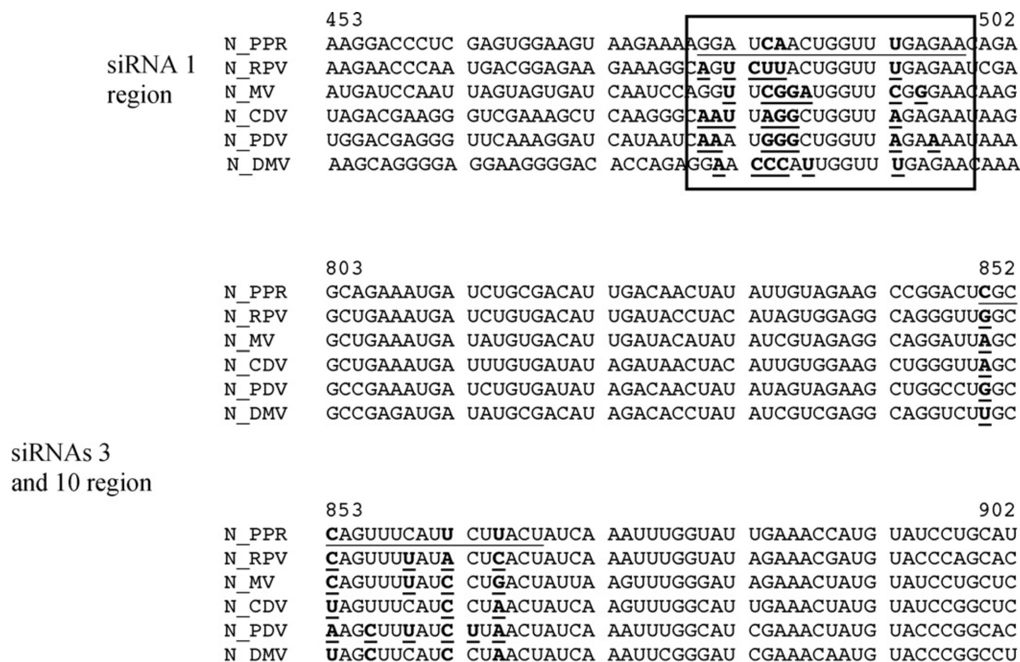


**Fig. 1.** The sequence data for PPRV 75-1, RPV RBOK, MV Schwarz, CDV, PDV, and DMV for the multiple alignments of morbillivirus nucleoprotein were obtained from GenBank with accession numbers X74443 (Diallo et al., 1994), Z30697 (Baron and Barrett, 1995), MVU03668 (Rota et al., 1994) AF014953 (Sidhu et al., 1993), X75717 (Blixenkrone-Möller et al., 1992), and X75961 (Blixenkrone-Möller et al., 1994). The block delimits the position of the locus.

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