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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.

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

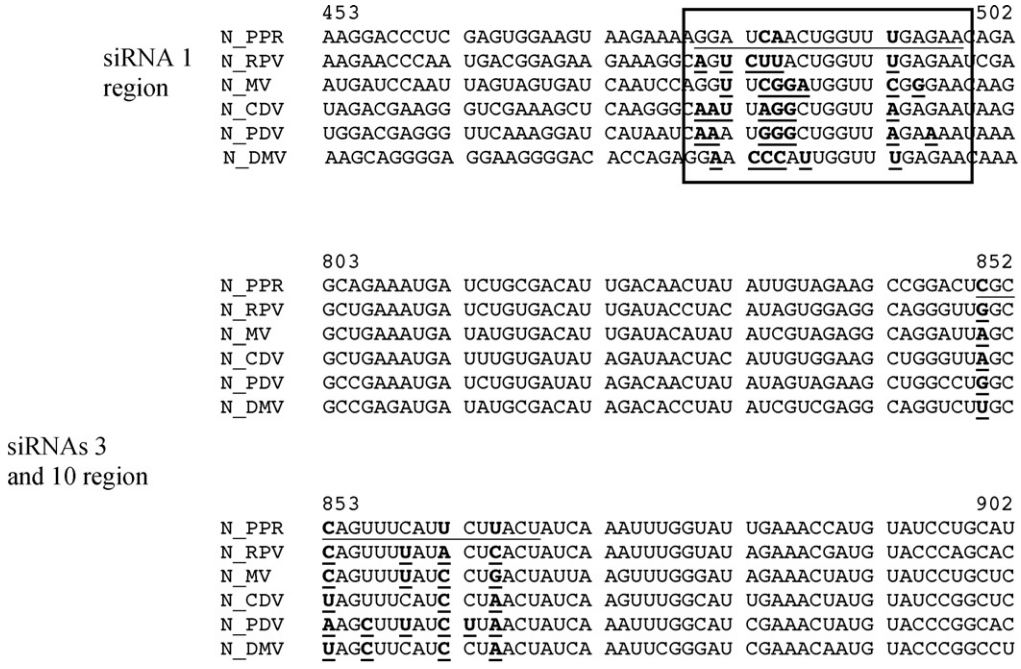

Fig. 1. The sequence data for PPRV 75-1, RPV RBOK, MV Schwarz, CDV, PDV, and DMV for the multiple alignments of morbillivirus nucleoprotein were obtained from GenBank with accession numbers X74443 (Diallo et al., 1994), Z30697 (Baron and Barrett, 1995), MVU03668 (Rota et al., 1994) AF014953 (Sidhu et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

Table 1
Nucleotide sequence of siRNA and primers

Identification	Sequence	Target
NPPR1 – 3	Sense: 5′-AAAGGAUCAACUGGUUUGAtt-3′; antisense: 3′-ctUUUCCUAGUUGACCAAACU-5′	PPRV nucleoprotein
NPPR1 – 2	Sense: 5′-AAGGAUCAACUGGUUUGAGtt-3′; antisense: 3′-ttUUCCUAGUUGACCAAACUG-5′	
NPPR1 – 1	Sense: 5′-AGGAUCAACUGGUUUGAGAtt-3′; antisense: 3′-ttUCCUAGUUGACCAAACUCU-5′	
NPPR1	Sense: 5′-GGAUCAACUGGUUUGAGAAtt-3′; antisense: 3′-ttCCUAGUUGACCAAACUCUU-5′	
NPPR1 + 1	Sense: 5′-GAUCAACUGGUUUGAGAACtt-3′; antisense: 3′-ttCUAGUUGACCAAACUCUUG-5′	
NPPR1 + 2	Sense: 5′-AUCAACUGGUUUGAGAACAtt-3′; antisense: 3′-ctUAGUUGACCAAACUCUUGU-5′	
NPPR2	Sense: 5′-GCUCACCUUCAUUCUUUUCtt-3′; antisense: 3′-ctCGAGUGGAAGUAAGAAAAG-5′	
NPPR3	Sense: 5′-GGCCAGUUUCAUUCUUACUtt-3′; antisense: 3′-ctCCGGUCAAAGUAAGAAUGA-5′	
NPPR10	Sense: 5′-CGCCAGUUUCAUUCUUACUtt-3′; antisense: 3′-ttGCGGUCAAAGUAAGAAUGA-5′	
NRP1 – 2	Sense: 5′-GCAGUCUUACUGGUUUGAGtt-3′; antisense: 3′-ttCGUCAGAAUGACCAAACUC-5′	RPV nucleoprotein
NRP1 – 1	Sense: 5′-CAGUCUUACUGGUUUGAGAtt-3′; antisense: 3′-ctGUCAGAAUGACCAAACUCU-5′	
NRP1	Sense: 5′-AGUCUUACUGGUUUGAGAAtt-3′; antisense: 3′-ctUCAGAAUGACCAAACUCUU-5′	
NRP1 + 1	Sense: 5′-GUCUUACUGGUUUGAGAAUtt-3′; antisense: 3′-ttCAGAAUGACCAAACUCUUA-5′	
MV1 – 2	Sense: 5′-CAGGUUCGGAUGGUUCGGGtt-3′; antisense: 3′-ttGUCCAAGCCUACCAAGCCC-5′	MV nucleoprotein
MV1 – 1	Sense: 5′-AGGUUCGGAUGGUUCGGGAtt-3′; antisense: 3′-ttUCCAAGCCUACCAAGCCCU-5′	
MV1	Sense: 5′-GGUUCGGAUGGUUCGGGAAtt-3′; antisense: 3′-ttCCAAGCCUACCAAGCCCUU-5′	
MV1 + 1	Sense: 5′-GUUCGGAUGGUUCGGGAACtt-3′; antisense: 3′-ttCAAGCCUACCAAGCCCUUG-5′	
GAPDH	Sense: 5′-AAGGUCAUCCAUGACAACUtt-3′; antisense: 3′-ttUUCCAGUAGGUACUGUUGA-5′	GAPDH
qRT-PCR primers		
NP3bisf	Forward: 5′-GTCTCGGAAATCGCCTCACAG-3′	
NP4bisr	Reverse: 5′-CCTCCTCCTGGTCCTCCAGAA-3′	
18Sf	Forward: 5′-TCAAGAACGAAAGTCGGAGG-3′	
18Sr	Reverse: 5′-GGACATCTAAGGGCATCACA-3′	

nucleotides covering this region were designed (Table 1). Five siRNAs homologous with NPPRV were named siRNA-NPPR1 – 3 (position 477–495), siRNA-NPPR1 – 2 (position 478–496), siRNA-NPPR1 – 1 (position 479–497), siRNA-NPPR1 + 1 (position 481–499), and siRNA-NPPR1 + 2 (position 482–500). Four siRNAs homologous with the N gene of RPV (GenBank/EMBL accession number Z30697) were named siRNA-NRP1 – 2 (position 478–496), siRNA-NRP1 – 1 (position 479–497), siRNA-NRP1 (position 480–498), and siRNA-NRP1 + 1 (position 481–499). Four siRNAs homologous with the N gene of MV (GenBank/EMBL NMV Schwarz accession number MVU03668) were named siRNA MV1 – 2 (position 478–496), siRNA MV1 – 1 (position 479–497), siRNA MV1 (position 480–498), and siRNA MV1 + 1 (position 481–499).

The alignments of PPRV, RPV, and MV N genes were analyzed for this region (sequences published in GenBank). In total, 5, 13, and 210 N sequences from vaccine or wild strains of PPRV, RPV, and MV were included (Table 2).

To confirm the specificity of the inhibition, irrelevant siRNA, siRNA-GAPDH (Ambion, Silencer™ GAPDH siRNA Human Control #4605#), was included in the study (Table 1). All siRNA sequences designed were submitted to a blast search against GenBank database in order to check their specificity for morbilliviruses. The siRNA-GAPDH had a maximum 72% of homology with NPPR gene.

2.3. siRNA transfection assays

Vero cells maintained in supplemented MEM were trypsinized (Trypsin–EDTA, Sigma–Aldrich, Lyon France), appropriately diluted, and transferred into 24-well plates to achieve 70–80% cell confluence after 24 h of culture at 37 °C with 5% CO_2. After the medium was removed, cells were incubated 30 min with 1 ml MEM without serum. This medium was removed again, and aliquots (200 μl) of Lipofectamine™ 2000 reagent (Invitrogen) (500 ng) in Opti-MEM I® (Invitrogen) containing siRNA (6.5–12.5–25–50–100 nM) were transiently transfected into Vero cells and incubated at 37 °C, 5% CO_2. New MEM medium with 5% FBS was added 3 h post-transfection, and cells were incubated for an additional period of 24 h. The cells were then infected at a MOI of 0.1 $CCID_{50}$/cell with PPRV, RPV, or MV in medium without FBS. One hour post-infection, cells were washed twice, and MEM supplemented with 5% FBS was added. The efficacy of siRNA-MV1 and siRNA-MV1 – 1 (100 nM) on MV was tested either before (1, 48, 72, 96, and 120 h) or after viral infection (1 and 24 h), using in this latter case additional MOIs between 0.01 and 0.0001 $CCID_{50}$/cell. The cells were checked daily, and the cytopathic effects detected under an inverted microscope by two observers (CPE, scored from 0 for 0% to 3 for 70% CPE according to the severity) were averaged and gated on a scale from 0% to 100% according to the formula: [CPE score/3] × 100. Cells and supernatants were harvested and processed for the quantitative measure by virus infectivity assay and flow cytometry (see below). Controls consisted of non-transfected infected or uninfected cells. Cells and supernatants were titrated using 10-fold serial dilutions from 10^{-1} inoculated on semi-confluent monolayers of Vero cells. The virus titer was expressed in $CCID_{50}$/ml according to the method of Reed and Muench (1938).

2.4. Flow cytometry

The expression of N and M genes was used as indicator of virus propagation through the use of specific monoclonal antibodies, secondary fluorescent antibody, and FACS analysis. Protein detections were done with Mab 38-4, IVB2-4, 19-6, and MAB8906 (Chemicon International, Belgium) specific for the N protein of PPRV, the N of RPV, the matrix protein of both PPRV and RPV (Libeau and Lefèvre, 1990; Libeau et al., 1997), and the N protein of MV antibody, respectively. Mab 11/295/33 (anti-swine CD8; Saalmüller et al., 1994, kindly supplied by Bernard Charley, INRA) was used as a negative isotype control. The fluorescein-conjugated secondary antibody was an anti-mouse IgG (H + L) (Bio-Rad, France). In brief, adherent cells were removed, washed in PBS 0.1% azide, 5% horse serum, and 0.0062% saponin (w/v), and enumerated. Immunolabeling of 10^6 cells was achieved in a two-step procedure: (I) incubation

Table 2
Base variations in the cDNA of the locus 1 of PPRV, RPV, and MV N genes

	siRNA-NPPR1 locus in DNA																				Sequence accession no. (in GenBank)
PPRV N gene alignments																					
	A	G	G	A	T	C	A	A	C	T	G	G	T	T	T	G	A	G	A	A	C	X74443 (lineage II)
Translation sense and locus																						AJ563705 (lineage IV) AJ849636 (lineage) MBLNUC (lineage) AY560591 (lineage IV)
	siRNA-NRP1 – 1 locus in DNA																				Sequence accession no. (in GenBank)
RPV N gene alignments																					
	C	A	G	T	C	T	T	A	C	T	G	G	T	T	T	G	A	G	A	A	T	Z30697 (lineage I)
Translation sense and locus																						X68311 (lineage I) X98291 (vaccine strain) RVU02679 (wild strain)
		G							T													EF186057 (lineage I)
					T				T												C	EF186058 (lineage II)
									T													EF186062 (lineage II)
									T							C					C	EF186059 (lineage II)
Base variations									T												C	EF186060 (lineage II)
									T													EF186061 (lineage II)
									T												C	AF515676 (lineage II)
									T												C	AY899330 (lineage II)
									T												C	Z34262 (lineage II)
	siRNA-NMV1 locus in DNA																					Sequence number analyzed
MV N gene alignments Translation sense and locus	A	G	G	T	T		C	G	G	A	T	G	G	T	T	C	G	G	G	A	A	C	5
																		A					93
					C													A					45
										G						T		A					44
																T		A					13
Base					C		T											A					3
vari-			A		C		T											A					2
a-			A															A					1
tions			A															A				T	1
			A		C													A					1
					C													A			G		1
										G								A					1
Percentage (%) of base variations			2.4		24.7		2.4			21						27		2.4			0.4	0.4	

for 30 min at 4 °C with 100 μl of appropriate dilutions of anti-N, anti-M, and isotype control monoclonal antibodies and (II) staining for 30 min at 4 °C with 50 μl of FITC–conjugate diluted 1:80. Between each incubation step, cells were washed twice. After the final wash, cells were fixed for 15 min at room temperature in PBS 1% PFA and resuspended in Facsflow PBS before acquisition on a FACScan flow cytometer (Becton Dickinson, USA). At least 20,000 cells were examined. Analysis of cell subsets was done with the Lysis II software program of CELLQuest™ software (BD Bioscience).

2.5. Real-time PCR

Cells and culture supernatants were harvested 96 h post-infection and frozen. Total RNA was extracted from 100 μl of sample suspension mixed with lysis solution of the RNeasy Mini kit (QIAGEN) according to the Manufacturer's instructions. RNA was eluted in 30 μl of ultra pure water, and the RNA solution was frozen at −70 °C until use.

Two quantitative RT-PCR assays were performed in duplicate using Mx3000P Instruments (Stratagene, Amsterdam, The Netherlands), the one-step qRT-PCR (Brillant SYBR Green QRT-PCR Master Mix Kit, 1 step, Stratagene) and the primers indicated in Table 1. NP3bis forward and NP4bis reverse PPR-specific primers were adapted from the publication of Couacy-Hymann et al. (2002). NP3bis was modified by addition of one nucleotide (G) in the 5′ position and four deletions in the 3′ position, whereas NP4bis was shortened by three bases in the 3′ position. These primers amplify a fragment of 352 bp. We used 18S forward and 18S reverse primers, designed for specific amplification of a 400-bp nucleotide fragment of ribosomal 18S sub-unit (Plumet and Gerlier, 2005), to quantify total RNA content from each Vero cell preparation. The mix contained 12.5 μl SYBR Green Master Mix (2×), 2.5 μl of 1 μM forward primer (NPPR or RNA 18S), 2.5 μl of 1 μM reverse (NPPR or RNA 18S), 0.0625 μl of StrataScript Reverse Transcriptase RT, and 2.4375 μl of water. Then 5 μl of extracted RNA was added to each tube for duplex qRT-PCR. The thermocycling program consisted of a reverse transcription at 50 °C for 30 min, a denaturation step at 95 °C for 10 min, and then 35 cycles (95 °C for 30 s, 55 °C for 1 min and 72 °C for 30 s). PCR amplifications were followed by one cycle of dissociation at 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. To determine the N PPRV copy number, serial 10-fold dilutions of a known quantity of a pBluescript KS+ containing the N gene (Diallo et al., 1994) were amplified in parallel to serve as standards.

3. Results

3.1. Design and synthesis of a panel of siRNAs targeting the nucleoprotein gene

Our strategy for siRNA design was based on the use of conserved regions of the morbillivirus nucleoprotein gene. We first carried out multiple alignments of morbillivirus N gene, including sequences from MV, PPRV, RPV, canine and phocine distemper viruses, and dolphin morbillivirus (Fig. 1). The most conserved regions were identified, and corresponding sequences on the PPR genome were submitted for siRNA design using the Cenix software and then chemically synthesized by Ambion (Europe, Cambridgeshire, UK). In a first round, four siRNAs were designed and tested.

3.2. Decrease in PPR virus replication and release of virus progeny by N-specific siRNAs

To test whether NPPRV-specific siRNA exerted antiviral effects, Vero cells were transfected with the four siRNAs, named NPPR1, 2, 3, and 10 and one control consisting of siRNA-GAPDH. Concentrations of these siRNAs were between 6.25 and 100 nM, in order to assess the dose/effect relationship. Twenty-four hours later, transfected cells were infected by PPRV at MOI 0.1 $CCID_{50}$/cell. The inhibition of the virus cytopathic effect was assessed at 4 days post-infection (dpi) on an inverted microscope by two independent investigators. CPE was scored on a scale of four grades: [(0, 1/3, 2/3, and 1) × 100]. The average score of the two observers is shown in Table 3. At the highest dose (100 nM), siRNA-NPPR1 (position 480–498) completely inhibited CPE of PPRV. At the lowest dose (6.5 nM), siRNA inhibited half of the CPE. siRNA-NPPR3 (position 850–868), whose sequence differs only by one nucleotide from the target gene sequence, inhibited CPE, but to a lesser extent than siRNA-NPPR1. The maximum inhibition obtained with this siRNA at 100 nM was 50%. At 25 nM, siRNA-NPPR10 (position 850–868), which differs from siRNA-NPPR3 by one nucleotide but exactly matched the sequence of the N gene, was more potent (66%) but still less efficient than siRNA-NPPR1. As expected, no antiviral effects could be detected with siRNA-GAPDH and siRNA-NPPR2, which had only 63% of homology with the NPPR gene at position 1318–1336.

Table 3
Inhibition of PPRV cytopathic effect (CPE) and virus progeny by interfering RNA measured by microscopic observation and viral titration million

Samples	siRNA dose (nM)				
	6.25	12.5	25	50	100
Percentage (%) of PPRV cytopathic effect (CPE) inhibition					
Negative control	100	100	100	100	100
Positive control	0	0	0	0	0
siRNA-NPPR1	50	66	66	66	100
siRNA-NPPR2	0	0	0	0	0
siRNA-NPPR3	17	33	33	50	50
siRNA-NPPR10	50	66	66	50	33
siRNA-GAPDH	0	0	0	0	0
Viral progeny inhibition (titer: number log > inoculum titer)					
Positive control	4.1	4.1	4.1	4.1	4.1
siRNA-NPPR1	1.3	0.7	0.1	0.1	<Inoculum
siRNA-NPPR2	3	3	2.9	3.1	2.9
siRNA-NPPR3	2.1	2.7	2.1	2.3	2.3
siRNA-NPPR10	2.7	1.7	1.9	1.9	2.7
siRNA-GAPDH	5.7	5.5	4.1	3.1	4.1

The antiviral effects of siRNA-NPPR1 were similarly reflected on the production of infectious virus progeny, as verified by virus titration (Table 3). At 4 dpi, PPR virus titers were reduced by 4 log by siRNA-NPPR1 whatever the concentration between 6.25 and 100 nM (Table 3). The best result obtained with siRNA-NPPR3 was a reduction of 2 log of the virus titer. We therefore concluded that among the tested siRNAs, siRNA-NPPR1 gave the strongest and most consistent inhibition of virus replication within the concentration range of 6.25–100 nM.

3.3. Decrease in PPRV RNA production by N-specific siRNAs

To further demonstrate that the inhibitory effect of siRNA occurred through NPPR RNA degradation, we performed real-time quantitative RT-PCR using primers specific for the viral NPPR gene. Viral NPPR RNA was extracted from Vero cells transfected with different concentrations of siRNA-NPPR1, siRNA-NPPR2, siRNA-NPPR3, and siRNA-GAPDH and then infected by PPRV (MOI = 0.1 $CCID_{50}$/cell). In infected cells, viral replication was confirmed by a 10,000-fold increase in the NPPR RNA level compared to

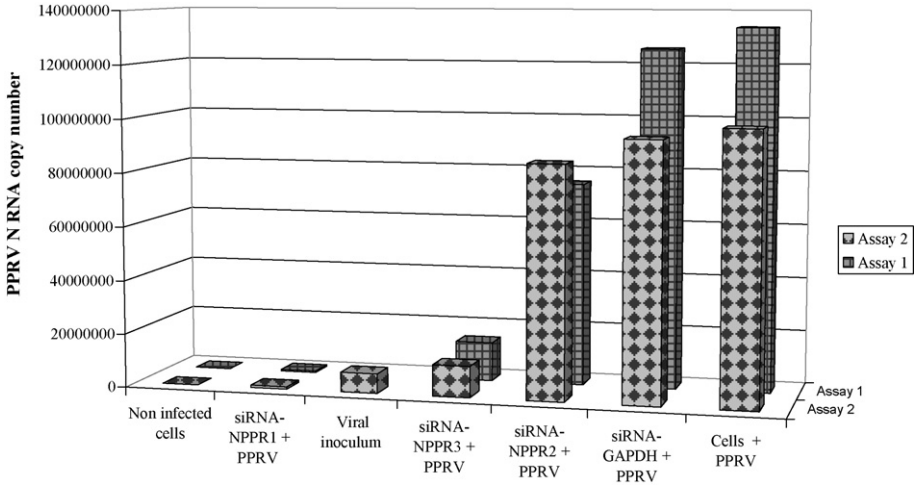

Fig. 2. siRNA-NPPR1 is the most efficient in down-regulating the transcript levels of PPRV nucleoprotein. Vero cells were transfected with siRNA-NPPR1, siRNA-NPPR2, and siRNA-NPPR3 along with siRNA-GAPDH as irrelevant negative control. Cells were then infected with PPRV at MOI 0.1 $CCID_{50}$/cell. Total RNA was extracted at 4 days post-infection, and NPPR copy number was determined by a real-time quantitative PCR. Bars from left to right are uninfected cells, cells transfected with siRNA-NPPR1 and infected with PPRV, viral inoculum of Vero cells, cells transfected with siRNA-NPPR3 and infected with PPRV, cells transfected with siRNA-NPPR2 cells and infected with PPRV, cells transfected with siRNA-GAPDH and infected with PPRV, and Vero cells infected only with PPRV. Two independent tests are shown. Amounts of total RNA extracted were comparable between the different experimental conditions as shown by identical Ct values for real-time PCR amplification of the 18S ribosomal RNA (data not shown).

Table 4
Inhibition of PPRV and RPV nucleoprotein and matrix protein expressions measured by flow cytometry

siRNA (optimal dose)	% Inhibition			
	NPPRV	MPPRV	NRPV	MRPV
siRNANPPR1 (100 nM)	90 ± 2	89 ± 2	0	0
siRNANPPR2 (100 nM)	25 ± 5	11 ± 2.3	nt[a]	nt
siRNANPPR3 (100 nM)	40 ± 3.8	30 ± 2.9	nt	nt
siRNANPPR10 (25 nM)	60 ± 2.5	55 ± 2.7	nt	nt
siRNA-NRP1 – 2 (100 nM)	0	nt	35 ± 3	31 ± 3.4
siRNA-NRP1 – 1 (100 nM)	0	nt	97 ± 2	92 ± 1.7
siRNA-GAPDH (100 nM)	0	5 ± 1.9	13 ± 3.8	5 ± 2.7

Results are representative means for 15 repetitions.
[a] Not tested.

the viral inoculum. Transfection with siRNA-NPPR2 and GAPDH had no significant effect on the viral RNA production. In cells transfected with siRNA-NPPR3, the RNA level decreased by 10-fold. In contrast, siRNA-NPPR1 completely inhibited the production of NPPR RNA (Fig. 2).

3.4. Decrease in N and M viral protein expression by N-specific siRNAs

We finally checked that anti-NPPR siRNAs inhibited the N protein expression in cell culture. Since N protein is an essential component of the viral ribonucleoprotein complex responsible for the production of all viral RNA, we also evaluated the effect of NPPR siRNA on the expression of the virus matrix (M) protein. To this end, Vero cells were siRNA-transfected and PPRV-infected as previously described. Relative expression of N and M proteins was quantified by flow cytometry, using specific monoclonal antibodies against these proteins and a secondary fluorescent antibody. As shown in Table 4, we observed a remarkable inhibition, up to 90%, of the PPRV N protein expression in the infected cells previously transfected with siRNA-NPPR1. This inhibition was subsequently shown to be dose-dependent (Fig. 3). In contrast, N protein expression profile in cells transfected with siRNA-NPPR2 was almost identical to that of the positive control (non-transfected and infected cells). Owing to the central role of the N protein in the viral life cycle, we verified whether the most potent siRNA-NPPR1 also abrogated the expression of PPR M protein, as shown in Table 4. Later on, co-inhibition of RPV N and M proteins was shown using siRNAs specifically designed against the N gene of RPV (see next section and Table 4). As for PPRV, the expression of N and M proteins or RPV was inhibited by at least 90%. Interestingly, there was no cross-reactivity of siRNA-NPPR with RPV or of siRNA-NRP with PPRV. The activity of functional siRNAs on N protein expression lasted for 1–7 days after cell infection for four doses tested (results not shown).

3.5. Identification of an active region common to morbilliviruses

Taken together, the above results indicated that siRNA-NPPR1 could serve as a potent inhibitor of PPRV gene expression. Alignment of the siRNA-NPPR1 sequences of five different PPRV isolates showed no base variation (Table 2), indicating a low rate of mutation in the target sequence among the four PPRV lineages identified so far from phylogenetic studies. However, when siRNA-NPPR1 was evaluated on two other morbilliviruses, measles virus and rinderpest virus, no inhibition was observed because mismatching between the siRNA and MV or RPV N gene represented six and five nucleotides, respectively (Fig. 1). Therefore, we decided to synthesize siRNAs targeting the exact sequence of the vaccine strains of PPRV, RPV, and MV and to delineate the active siRNA region by a mapping approach on each virus. The siRNA region mapping consisted in the synthesis and testing of 21 base-long siRNA overlapping by 1 base upstream or downstream. A total of 14 siRNAs covering this region were designed: 6 siRNAs homologous to the N gene of PPRV, 4 siRNAs homologous to the N gene of RPV, and 4 homologous to the N gene of MV (Table 1). To compare the efficacy of these new siRNAs in inhibiting N protein expression, the same concentrations of siRNA (6.25, 12.5, 25, 50, and 100 nM) were used to transfect Vero cells. The data showed that siRNA targeting the position 480–498 was efficient for the three tested morbilliviruses

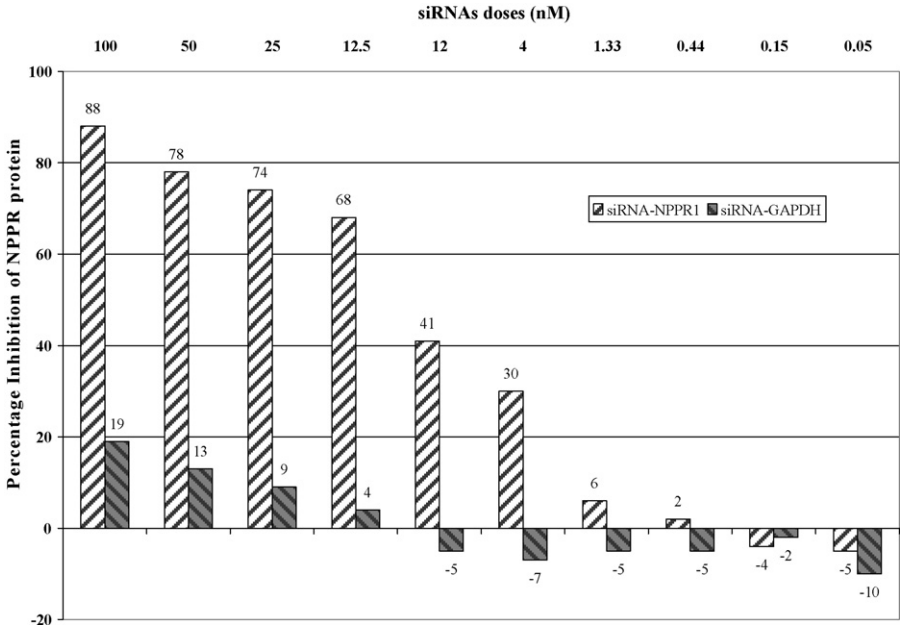

Fig. 3. siRNA-NPPR dose-dependent effect on PPRV nucleoprotein expression. Expression of PPRV N protein was quantified by flow cytometry at 4 days post-infection in Vero cells transfected with twofold then threefold dilution of 100 nM of siRNA-NPPR1 or siRNA-GAPDH. Expression of N protein using a specific monoclonal antibody anti-NPPR (Mab 38-4) and an irrelevant monoclonal antibody, anti-swine CD8 antibody, served as isotype control. siRNA-NPPR1 dose-dependent effect compared to the irrelevant GAPDH siRNA.

Table 5
Mapping of siRNA locus 1 on the nucleoprotein gene of morbilliviruses

	Positions					
	477–495	478–496	479–497	480–498	481–499	482–500
Time of infection = +24 h						
PPR siRNA	NPPR1 − 3	NPPR1 − 2	NPPR1 − 1	NPPR1	NPPR1 + 1	NPPR1 + 2
PI value	7% ± 1.7	15% ± 2.5	78% ± 3.3	90% ± 2	24% ± 3.3	28% ± 3.2
RP siRNA	nt[a]	NRP1 − 2	NRP1 − 1	NRP1	NRP1 + 1	nt
PI value	nt	35% ± 3	97% ± 2	70% ± 3.6	45% ± 3	nt
MV siRNA	nt	MV1 − 2	MV1 − 1	MV1	MV1 + 1	nt
PI value	nt	35% ± 2.8	85% ± 2.1	90% ± 2.4	28% ± 3	nt

siRNAs overlapping by one nucleotide were tested on the *in vitro* replication of peste des petits ruminants virus (PPRV), rinderpest virus (RPV), and measles virus (MV). Results are representative means of eight tests and are expressed as percentages of inhibition (PI) of nucleoprotein expression as measured by flow cytometry.

[a] Not tested.

Table 6
Efficacy of siRNA-MV1 and siRNA-MV1 − 1 (100 nM) on MV when transfected either before or after the viral infection

Time of infection (h)	MOI	Effect duration (h)	MV siRNA (PI)	
			MV1 − 1[a]	MV1[b]
−24	0.001	144	86	96
	0.0001	144	80	99
−24	0.1	72	26	34
	0.01	72	78	84
	0.001	72	96	96
	0.0001	72	95	96
−1	0.1	72	90	93
+1	0.1	72	89	94
+24	0.1	72	85	90
+48	0.1	72	79	85
+72	0.1	72	36	75
+96	0.1	72	31	58
+120	0.1	72	21	28

Antiviral lasting effect (72 or 144 h) of the two active siRNAs are measured against different MOI. Results are expressed as percentages of inhibition (PI) of nucleoprotein expression as measured by flow cytometry.

[a] Position 479–497.

[b] Position 480–498.

(Table 5), although maximum efficacy for RPV required shifting of a single base upstream. If infection occurred 1 day before transfection, an effect was still observed. With MV, almost complete reduction of the MV production as measured by flow cytometry was observed from 72 to 144 h, with siRNA-MV1 and with the neighboring siRNA, MV1 − 1, but MOIs had to be reduced by a factor of 10–100 (Table 6). For infections occurring on transfected cells, different post-transfection periods were tested. Silencing effect remained very high during 3 days post-transfection as evidenced by the reduction of the MV N protein expression and the inhibition of the development of cytopathic effects of MV, which are extremely strong for this virus (Table 6 and Fig. 4). Sequence alignment of the N gene of the different isolates among morbilliviruses showed that the region was conserved for all sequenced wild-type PPRV. In contrast, for RPV, 4 strains from lineage I and 8 strains from lineage II indicated that 16 out of 20 nucleotides of the target region of the active siRNA-NRP1 − 1 were identical to those present in the vaccine strain sequence. Multiple alignments of 210 MV strain N genes (Table 2) showed maximum nucleotide substitutions at position 4 (24.7%), position 8 (21%), and position 14 (27%). Seven percent of the other strains accumulated mutations at positions 2, 5, and 16.

4. Discussion

By quantitative and functional assays, we have identified three active siRNAs targeting the N gene of peste des petits ruminants virus and inhibiting the virus replication *in vitro*. One of these siRNAs, siRNA-NPPR1, could suppress almost the whole expression of N and M genes in infected cells. Repression of the M gene and probably all the other virus genes resulted from the indirect inhibition of the viral RNA-dependent RNA polymerase and transcription complex consisting of the N, P, and L proteins. The RNA interference was further confirmed by the N mRNA inhibition and finally by the reduction by up to 10,000-fold of PPRV progeny. The inhibition was specific since irrelevant siRNAs were inactive and RNA ribosomal 18S transcription levels were not affected (data not shown). This strong effect was previously described by Bitko and Barik (2001) for respiratory syncytial virus, the first negative strand virus inhibited by interfering molecules. Two other studies have shown efficient control of MV replication by two strategies to produce or deliver siRNA into cells: (i) siRNA direct delivery and (ii) vector-mediated delivery. Reuter et al. (2006) generated multiple gene-specific siRNA preparations against the N, P, and L of MV from long double-stranded RNAs using RNase III. In this paper, the siRNAs against N, P, and L were all effective in inhibiting the virus replication, but the N siRNAs were slightly more active than the L siRNAs in down-regulating the expression of P and M mRNA (ninefold reduction for N siRNAs versus five- to ninefold reduction for L siRNAs). In our laboratory, a preliminary study was unsuccessful in attempting to use multiple siRNAs produced by enzymatic digestion (RNase III and DICER) (data not shown). In contrast, synthetic siRNAs targeting N gene mediated antiviral effects against PPRV for 2–7 days post-infection with optimized cell transfection conditions. These siRNAs were able to inhibit 100% of the N mRNA production, corresponding at least to a 100-fold reduction of the mRNA normally produced by the untreated virus. In the study of Ferreira et al. (2007), a 300-fold reduction of N and F mRNA production was observed in cells infected by an avian metapneumovirus and transfected with chemically synthesized N-siRNAs. Otaki et al. (2006) used chemically synthesized siRNA and DNA-based mammalian vectors to produce siRNA intracellularly, which inhibited the expression of the MV L gene. When using anti-L siRNAs, these authors reported a reduction of almost 100% of the MV production as measured by the virus titration in the cell supernatants. Inhibition was also observed if infection preceded transfection within 12 h. In our study, we had an identical reduction of 100% of the production of the three morbilliviruses by using an N-siRNA, and we showed with MV used as a model that we could lengthen the 12 h lag time between infection and transfection only if MOI was reduced by a factor of 10–100-fold.

Since the N gene regions targeted for siRNA design were conserved among morbilliviruses (see later), we looked for a possible interference of siRNA-NPPR1 on MV and RPV. However, when used against RPV or MV, the siRNA-NPPR1 was not active. The five to six bases of difference between the corresponding sequences are

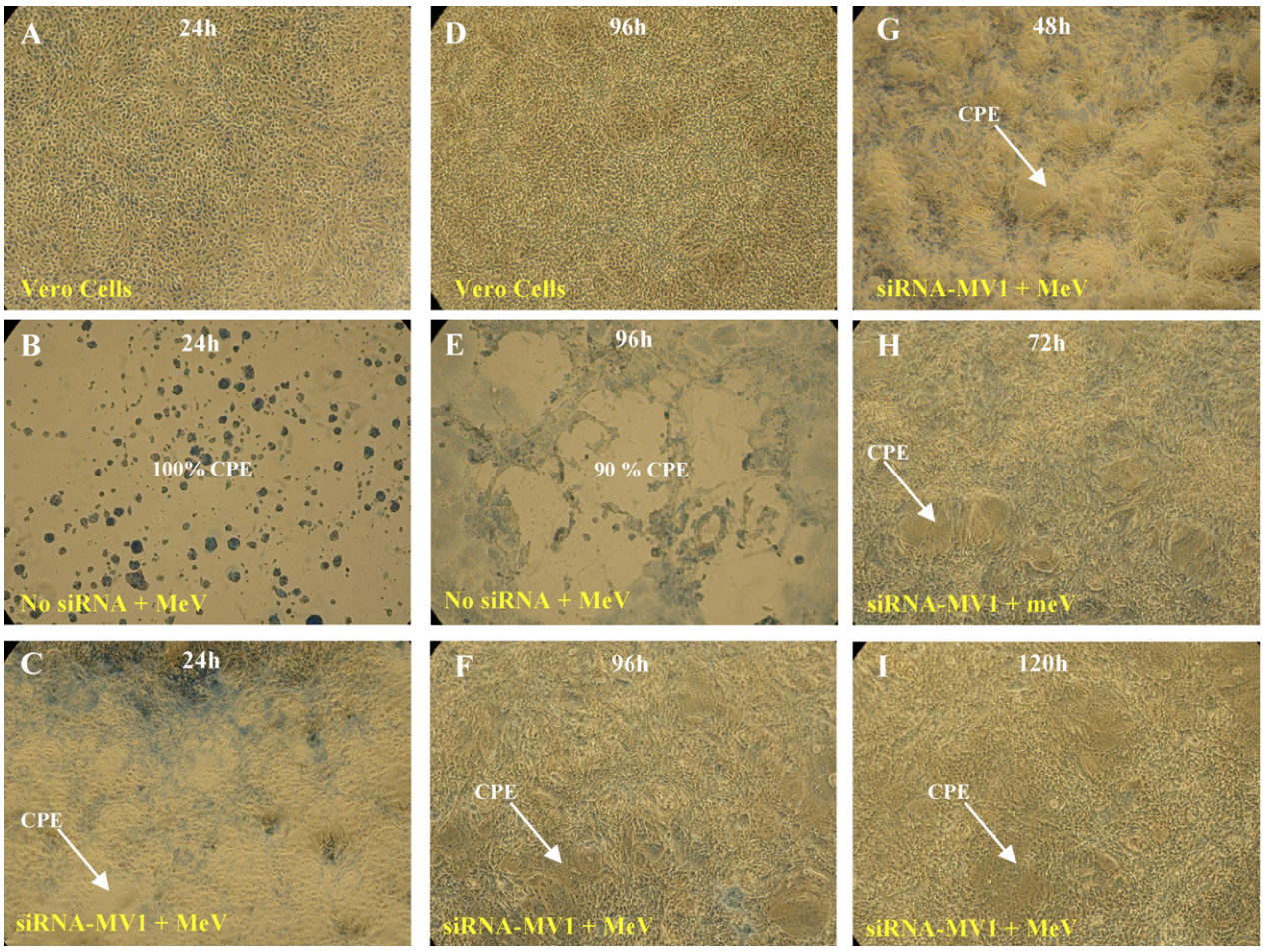

Fig. 4. Effect of siRNA-NMV1 on MV cytopathic effect. Vero cells were transfected with 100 nM of siRNA and infected 24 h (C), 48 h (G), 72 h (H), 96 h (F) or 120 h (I) post-transfection, with the Schwarz strain at MOI 0.1 $CCID_{50}$/cell. At 3 days post-infection, monolayers were examined under the microscope at a 10× magnitude and photographed after trypan blue staining. In the control experiment corresponding to time 24 and 96 h, cytopathic effect with cell fusion and syncytium formation were present in positive control wells (B) and (E) and monolayers appearing without cytopathic effect corresponded to the negative controls (A) and (D).

responsible for this loss of efficacy, confirming the specificity of RNA interference. In contrast, siRNAs 100% homologous with RPV and MV strains and targeting the same region were active. A common region for MV, RPV, and PPRV was thus identified at position 480–498. For RPV, increased efficacy required an adjustment of the siRNA sequence with a shift of one base upstream (5′-end). The region common to the three viruses suggests that the corresponding mRNA sites are exposed efficiently to siRNA annealing and RNA-induced silencing complex attachment. This may result from conserved mRNA structures within the morbillivirus genus. Indeed, the structure of mRNA plays an important role in siRNA activity (Kawasaki et al., 2003; Westerhout et al., 2005; Heale et al., 2005).

There is no specific treatment against morbillivirus infection. Therefore, siRNA interference might serve as important antiviral strategy in the future. To suppress virus replication durably and to reduce the risk of virus escape by sequence mutations, we initially targeted conserved regions of the viral genome for three different morbilliviruses. Indeed, viruses, especially RNA viruses, have a high capacity of mutations that can make them able to escape siRNA control (Barik, 2004; Colbère-Garapin et al., 2005; Gitlin et al., 2005). The conservation was assessed on successive stretches of 20 nucleotides covering the full N gene sequence. The selection of conserved segments for further siRNA design was based on a minimum sequence conservation of 93%. The sequence of the region is conserved among five sequenced PPRV isolates, although they have been divided into four distinct geographical groups according to their fusion (Shaila et al., 1996) and nucleoprotein genes (Kwiatek et al., 2007). The analysis of 210 sequences of the MV N gene retrieved from GenBank showed a percentage of conservation of 96% in the region. The percentage of identity in the region of RPV was 95% (with only 13 sequences available for this virus). In our strategy, we considered that a common conserved region between distinct viral genes may have an important role in morbillivirus biology, either through the nature of the sequence, its position in the mRNA, or its role in mRNA 2D folding. For RPV, nucleotide substitutions among lineages I and II, however, were predominantly present at position 9 of the target region, in the cleavage domain where no mismatches are tolerated. Previous work has demonstrated that transfection with siRNA containing mismatches in the middle of the target sequence reduces the efficiency of gene silencing (Amarzguioui et al., 2003; Brummelkamp et al., 2002; Gitlin et al., 2002). The use of a pool of two siRNAs, covering the C/T mismatch possibility at position 9, may circumvent this risk and allow all RPV strains to be neutralized. For MV, the feature is even more complicated since the main mutations were found at three positions (4, 8, and 14) of the target region. By associating the three siRNA sequences, GGTTCGGATGGTTCGAGAA, GGTCCGGATGGTTCGAGAA, and GGTTCGGGTGGTTTGAGAA, we would theoretically neutralize 182/210 isolates retrieved from GenBank. Taken together, our data allow the delineation of a region common to the three morbilliviruses for efficient siRNA inhibition: this region has as sequence

5′-RRWYYDRNUGGUUYGRG-3′ where R is A or G, W is A or U, Y is C or U, D is G, A or U and N is any of the four bases. Interestingly, the important zone within this sequence that covers the nine most central nucleotides (Amarzguioui et al., 2003) is the most conserved between MV isolates. Only two substitutions were found in this zone for 210 strains, whereas four mutations were observed outside this zone. This finding can result from a critical sequence motif for the N protein, which cannot be easily modified without a lethal effect on the virus. Indeed, translated amino acid motifs for the three morbilliviruses are conserved, especially in the central part. For PPRV, the sequence is R-I-N-W-F -E-N, for RPV, (R/Q)-(S/F)-Y-W-F -E-N, and for MV, R-(S/F)-G-W-F -(E/G)-(N/S), where boxes indicate the amino acid encoded by the nine most central nucleotides. To further prevent the risk of escape mutants, multiple targets can be used for siRNA therapy (Ji et al., 2003; ter Brake et al., 2006). In this context, we have recently identified new targets on the N gene of MV, RPV, and PPRV. In total, 46 siRNAs have been selected and only 12 of them were active and matched three distinct regions on the N gene (Servan de Almeida et al., submitted). One of these regions is described in this work. The two other regions are expected to be used in association with the first region to limit the problem of escape mutants (Servan de Almeida et al., 2007).

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