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Short communication

Avian metapneumovirus phosphoprotein targeted RNA interference silences the expression of viral proteins and inhibits virus replication

Shirin Munir*, Kuljeet Kaur, Vivek Kapur

Department of Microbiology and Biomedical Genomics Center, University of Minnesota, St. Paul, MN 55108, USA

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Abstract

Avian metapneumovirus (aMPV) is one of the major causes of serious respiratory infections of poultry and leads to considerable economic losses to food animal production worldwide. Here, we show that double stranded short interfering RNA (siRNA) molecules corresponding to aMPV phosphoprotein (P) gene silence P RNA and protein expression. These siRNAs broadly reduced the expression of other viral proteins in addition to P, but did not have a discernable effect on cellular protein expression. The exposure of cells to P-specific siRNAs also led to inhibition of virus replication as evidenced by marked reduction in the progeny virion titers. Taken together, the findings suggest that exogenous P silencing siRNAs can inhibit aMPV replication with potential implications in the design of novel siRNA based prophylactics.

Keywords: Avian metapneumovirus; aMPV; RNA interference; siRNA; Phosphoprotein; Post-transcriptional gene silencing

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism activated in the cell in response to endogenously synthesized or exogenously applied short interfering RNA (siRNA) (Agrawal et al., 2003; Hannon, 2002; Zamore, 2002; Zamore et al., 2000). Introduction of siRNA results in degradation of siRNA specific transcripts thus reducing the expression of their protein product. RNAi represents a new antiviral approach and is being increasingly utilized to inhibit the replication of viral pathogens, such as HIV-1 (Novina et al., 2002), hepatitis C (Sen et al., 2003), polio (Gitlin et al., 2002), foot-and-mouth disease (Chen et al., 2004), influenza (Ge et al., 2004, 2003), severe acute respiratory syndrome (Wu et al., 2005) and hepatitis B viruses (McCaffrey et al., 2003).

Avian metapneumovirus (aMPV) is the causative agent of an acute respiratory infection of poultry species that inflicts high morbidity and mortality on infected flocks (Cook, 2000). The virus is endemic in many parts of the world and is a source of significant economic losses to food animal production (Cook, 2000). aMPV is an enveloped, single-stranded, negative sense RNA virus with ~13 kbp long genome. aMPV is a member of the genus *metapneumovirus*, subfamily *pneumovirinae* and family *Paramyxoviridae*. There are four types (A–D) of aMPV prevalent in different parts of the world (Bayon-Auboyer et al., 2000; Dar et al., 2001; Juhasz and Easton, 1994) of which type C is endemic to the United States. The viral genome encodes at least nine proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), matrix (M2-1 and M2-2), small hydrophobic (SH), attachment glycoprotein (G) and large (L) RNA dependent RNA polymerase (RdRP) (Easton et al., 2004). The functional polymerase complex is composed of L, P and N proteins, which are all required for transcription and replication (Barik, 2004; Dupuy et al., 1999; Easton et al., 2004). The RdRP holoenzyme L is a major component of the polymerase complex and binds to the promoter/leader sequence at the 3′-end of the genomic RNA. However, L protein is unable to initiate transcription or replication in the absence of P protein, which serves as an elongation factor for L and facilitates promoter clearance (Dupuy et al., 1999). Keeping in view the significance of P protein in viral transcription and replication, we investigated whether siRNAs targeting aMPV P protein transcripts are able to silence aMPV protein expression and impact replication. We here report the identification of siRNA target regions on aMPV P gene transcript that are able to silence P protein expression and inhibit replication of aMPV type C.

Vero cells (African green monkey kidney cells; ATCC) were maintained in Eagle's Minimal Essential Medium (MEM) supplemented with 8% heat inactivated fetal bovine serum, 0.5% Edamin S, 1 mM sodium pyruvate, 100 IU of penicillin G/ml

* Corresponding author. Tel.: +1 612 625 9277; fax: +1 612 624 6264.
E-mail address: munir001@umn.edu (S. Munir).

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and 100 μg of dihydrostreptomycin/ml. Vero cells lack interferon genes due to spontaneous gene deletions (Desmyter et al., 1968; Mosca and Pitha, 1986). Vero cells were used for all experiments described herein so that any antiviral effects observed on siRNA treatment could be attributed only to P gene silencing and loss of P functions by RNAi and not to the antiviral effects of interferon. A set of four 21-nt siRNAs corresponding to aMPV P gene (GenBank accession number AF176591) were obtained from Qiagen (Valencia, CA) and subjected to BLAST analysis to ensure lack of homology to sequences other than the target gene. siRNAs included siP1 [sense, r(GGACACCUCUGCUCUUAGU)dTdT], siP2 [sense, r(GGGAAGGCAGCCGAGAUGA)dTdT], siP3 [sense, r(AGCCAGGCUGGAAUCUAUUUU] and siP4 [sense, r(GCCAAGGAACUGAACAAAAUU]. A double stranded siRNA [sense, r(UUCUCCGAACGUGUCACGU)dTdT] that does not share identity with any known sequence was also obtained from Qiagen and used as non-silencing control.

Vero cells in six-well tissue culture plates were transfected with 2 μg of siP1, siP2, siP3 or siP4 using 10 μl lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 2.5 ml Opti-MEM I medium (Invitrogen). Cells transfected with control siRNA and those treated with the transfection reagent alone were included as controls. After overnight incubation, complexes were removed followed by inoculation with aMPV type C (Colorado strain) at moi of 0.1. The inhibition of viral antigen accumulation was determined by immunofluorescence staining. Cells were fixed with methanol for 20 min, blocked with 0.1% bovine serum albumin, incubated with 1:200 dilution of turkey anti-aMPV hyperimmune serum (kind gift from Sagar M. Goyal, Veterinary Diagnostic Laboratory, University of Minnesota) for 1 h, washed with PBS–0.05% Tween-20, treated with 1:150 dilution of goat anti-turkey IgG FITC conjugate (KPL, Gaithersburg, MD) for 1 h, washed and observed with an Olympus BX60 fluorescent microscope. Marked reduction was noticed in the expression of aMPV proteins in cells transfected with siP1 and siP3 (Fig. 1a) compared with control siRNA treated cells.

To further quantitate the number of antigen positive cells in all treatment groups, FACS analysis was performed. Cells were trypsinized to single cell suspension, washed once with wash buffer (PBS containing 1% goat serum), treated with turkey anti-aMPV hyperimmune serum, washed once and stained with goat anti-turkey IgG FITC conjugate (KPL). Cells were analyzed on FACSCalibur and data were acquired and analysis performed with CellQuest software (Becton–Dickinson, Franklin Lakes, NJ). FACS showed ~2-fold reduction in aMPV positive cells in siP1 and siP3 relative to control siRNA treated cells (Fig. 1b). siP2 and siP4 had a relatively weak silencing effect compared to siP1 and siP3 by both immunostaining and FACS analyses (Fig. 1a and b). No silencing effect was observed in control siRNA treated cells where the number of aMPV positive cells was comparable to that in siRNA negative cells in both immunoassays (Fig. 1a and b).

Next, in order to determine if the treatment with P specific siRNAs indeed led to down regulation of P protein, lysates of cells were prepared at 48 h p.i. and subjected to western immunoblotting. Cell lysates prepared in 1× sample buffer were separated by electrophoresis on 15% SDS polyacrylamide gels and transferred onto PVDF membranes (Bio-Rad, Hercules, CA). For the detection of aMPV and host cell specific proteins, blots were probed with 1:1500 dilution of rabbit anti-aMPV polyclonal serum (gift from Kakambi V. Nagaraja, Department of Veterinary and Biomedical Sciences, University of Minnesota) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (Ambion, Austin, TX), respectively, followed by horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (KPL) and anti-mouse HRP conjugate (KPL), respectively. Blots were developed with LumiGLO chemiluminiscence substrate (KPL) and exposed to light X-ray film (Kodak, Rochester, NY). Developed blots were scanned by BioChemi System (UVP Bioimaging Systems, Upland, CA) and the relative intensity of P protein bands was determined after normalization against GAPDH expression. Except L, G, F and M2-2 proteins that have predicted $M(r)$ of 229, 58.7, 58 and 8.2 kDa, respectively, the rest of aMPV proteins including P were detected on the blot (Fig. 1c). The aMPV P protein is comprised of 294 amino acids and has a predicted $M(r)$ of 32.4 kDa correlating well with the size of P protein observed on the blot. The P protein percent reduction was determined by densitometric scanning of two immunoblots and calculation of the mean relative P expression value. The P protein percent reduction among treatment groups was statistically compared by one-way analysis of variance (ANOVA) using Tukey–Kramer multiple comparisons test and p-values of less than 0.05 were considered as statistically significant. Reduction of P protein was 94% ($p < 0.001$) and 91% ($p < 0.001$) complete by siP1 and siP3, respectively (Fig. 1d), and was statistically significant as compared to the control siRNA. It is noteworthy that the expression of a non-targeted host protein, GAPDH, remained unaffected by all siRNA duplexes (Fig. 1c) suggesting that siRNAs induce sequence specific degradation of homologous RNA. In addition to P silencing, siP1 and siP3 transfected cells showed reduction in the amount of other viral proteins as well. Consistent with the immunofluorescence and FACS data, siP2 and siP4 induced a weaker inhibition of P and other aMPV proteins. Although, siP2 and siP4 induced only 46 and 25% inhibition of P protein, respectively, the reduction was statistically significant as compared to the control siRNA with $p < 0.001$ and <0.01, respectively. As shown in Fig. 1c, control siRNA did not alter the expression of P or other aMPV proteins and had protein profile comparable to cells not treated with siRNA (Fig. 1c).

With the aim to investigate the basis of reduction in the amounts of P protein, the relative quantities of P gene transcripts as well as viral genomic RNA were examined by SYBR Green based real time RT-PCR detection system (Applied Biosystems, Foster City, CA). Total RNA was extracted with Trizol reagent (Invitrogen), further purified with RNeasy minikit (Qiagen) and quality and quantity analyzed with Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). The sequences of the aMPV P gene (GenBank accession number AF176591) corresponding forward and reverse primers were 5′GTTACCACACCCCCTGAAAG and 5′ATCCCGAATGCCGTCTCT, respectively. Real time RT-PCR was also performed for β-actin mRNA as endogenous

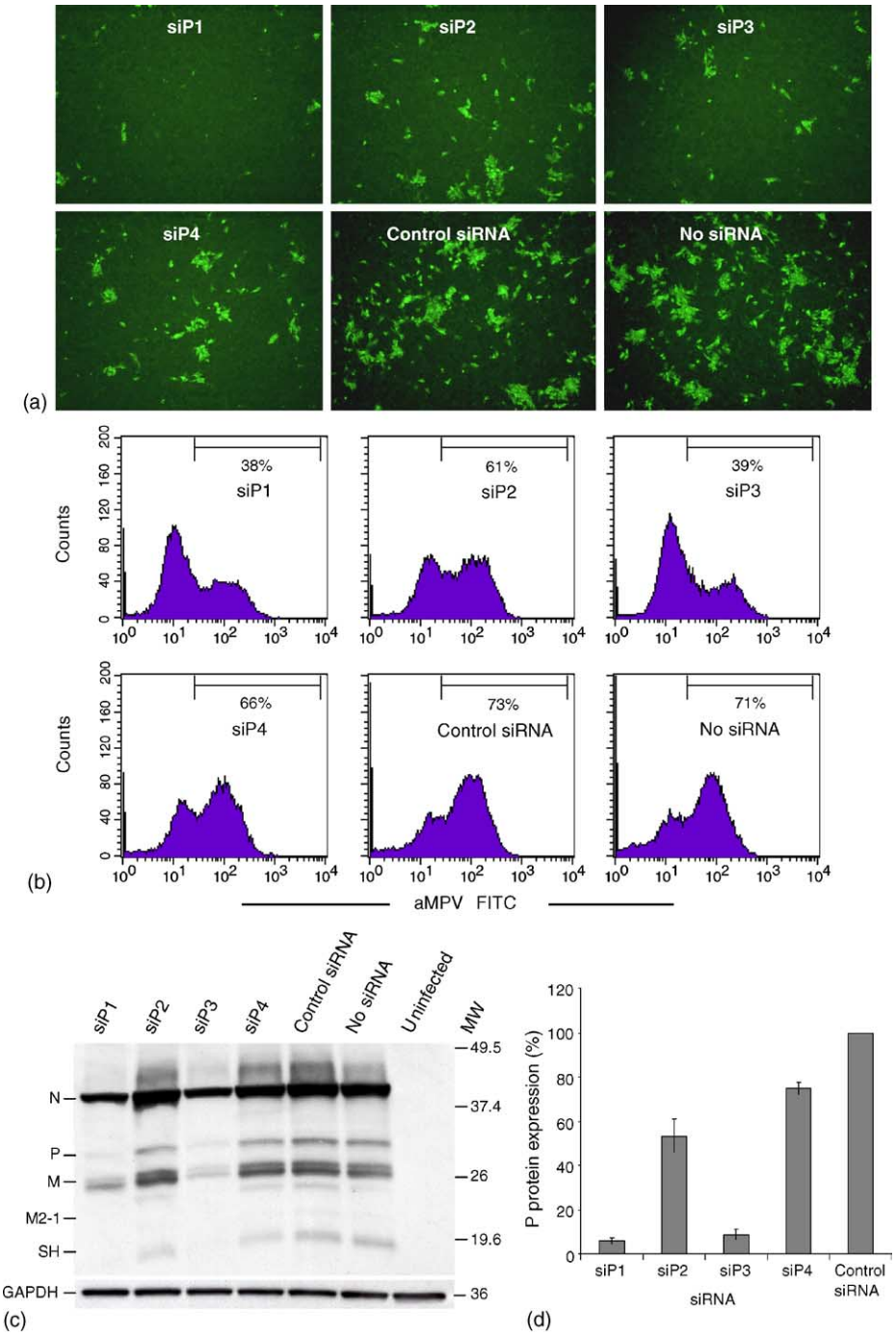

Fig. 1. Silencing of aMPV protein expression by P gene targeted siRNA. Vero cells were transfected and incubated with aMPV P specific siRNAs (siP1, siP2, siP3 and siP4) as well as control or no siRNA followed by inoculation with aMPV subtype C. (a) Immunofluorescence based detection of aMPV proteins at 24 h PI in cells treated with P specific siRNAs or control siRNA. (b) Flow cytometric analysis of aMPV protein expression at 48 h PI. The values shown in each panel indicate the percentage of gated aMPV positive cells in total population. (c) Western immunoblot depicting the expression level of aMPV proteins and GAPDH in Vero cells subjected to transfection with indicated siRNAs. Western blot analysis was performed with total cell lysates and membrane probed with aMPV hyperimmune serum. GAPDH served as the loading control and was detected by GAPDH monoclonal antibody (Ambion). MW, molecular weight markers (kDa) are indicated to the right of the blot. Immunoblotting was repeated twice and the representative blot is shown. (d) Relative intensity of P protein expression. The bar diagram shows the relative expression of P protein that was determined by densitometric analysis of P protein on the immunoblots and normalization against the endogenous reference, GAPDH. Diagram represents means of the relative P expression values obtained from two immunoblots ± standard deviations.

reference and the C_T values obtained were used for data normalization and calculation of relative quantities and fold changes in P gene transcripts and genomic RNA using comparative C_T ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001). The sequences of β-actin mRNA (GenBank accession number AB004047) specific forward and reverse primers were 5′GCGCGGCTACAGCTTCACCAC and 5′GGGCGCCAGGGCAGTAATCTC, respectively. Oligonucleotide primers corresponding to aMPV

genomic RNA (GenBank accession number AY590688) leader sequence (forward primer 5′GCATATAAGACAACTTCCAAAC) and nucleocapsid gene (reverse primer: 5′GCCCTTCCCAAGAGAGTATGTC) were also used in real-time RT-PCR assays to determine siRNA induced reduction in the accumulation of viral genomic RNA. All reactions were run in triplicate on an ABI PRISM 7900 sequence detection system (Applied Biosystems). siRNAs induced seven- and eight-fold (siP1), 4.5- and 3.7-fold (siP3) reduction in P gene transcripts at 24 and 58 h p.i., respectively, as compared with control siRNA (Fig. 2a). Consistent with the P protein data, P RNA analysis by RT-PCR also revealed a relatively weak silencing effect of siP2 and siP4 with siP2 inducing 2.5- and 2-fold and siP4 resulting in 2- and 1.8-fold reduction in P RNA levels at 24 and 58 h p.i., respectively. The P transcript profile in control siRNA treated cells was similar to those not treated with siRNA suggesting that control siRNA did not trigger non-specific degradation of P gene transcripts. aMPV genomic RNA targeted real-time RT-PCR also showed a reduction in genomic RNA accumulation by 3.4- and 2.1-fold in siP1 and siP3 treated cells, respectively (Fig. 2b). Thus the determination of relative P transcript and genomic RNA levels suggested that the suppression of P protein expression observed on immunoblotting was indeed due to reduced stability and degradation of P gene mRNA and reduction in genomic RNA accumulation.

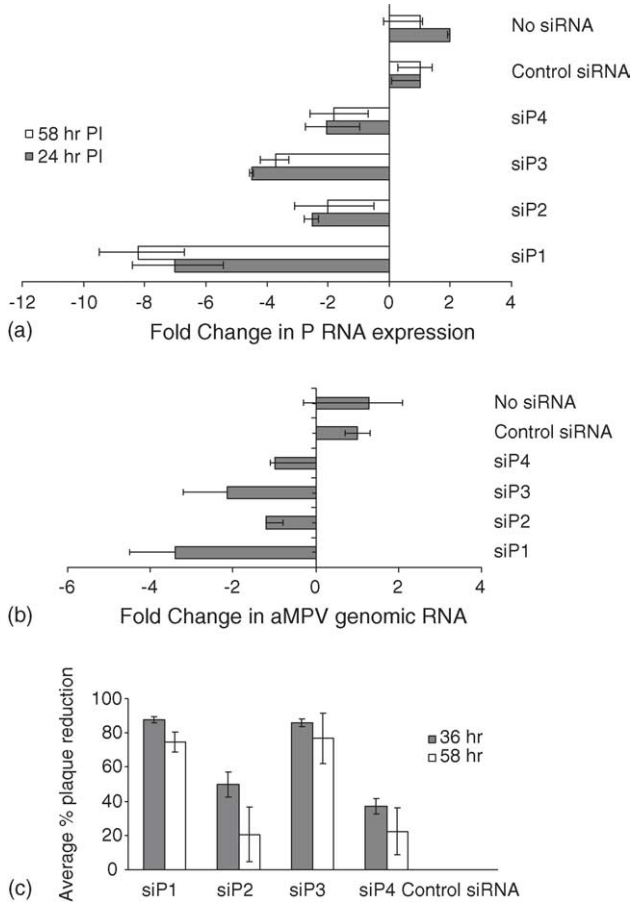

Fig. 2. Attenuation of aMPV replication by P specific siRNA. Real-time RT-PCR analysis for relative quantitation of (a) P RNA expression and (b) aMPV genomic RNA. For both (a) and (b) reactions were run in triplicate using SYBR Green dye based sequence detection system. Fold changes in P RNA expression and genomic RNA levels were calculated by relative quantitation of P RNA and genomic RNA in P siRNA as compared with control siRNA treated cells by comparative C_T ($\Delta\Delta C_T$) method. Real-time RT-PCR for β-actin gene transcripts was included in the assays as endogenous reference and used for normalization of P RNA and genomic RNA fold changes. The results presented are the mean fold changes ($2^{-\Delta\Delta C_T}$) ± standard deviations. (c) Effect of siRNAs on aMPV progeny virus production expressed by plaque reduction assay. Vero cells were transfected with P specific and control siRNAs followed by inoculation with aMPV type C (Colorado strain). Progeny virions in the culture supernatants were titrated by plaque assay. The effect of P siRNAs on progeny virus titers is depicted as percent reduction in plaque forming units as compared to control siRNA (with 0% reduction). Virus titration was performed in two independent experiments with each sample titrated in duplicate. Values shown in the graph are the means ± standard deviations.

We further investigated the effect of P silencing siRNAs on aMPV replication and release of progeny virion particles. Supernatants were collected from infected cultures at 36 and 58 h p.i. and virus titration was performed in two independent experiments by plaque assay as described (O'Mahony et al., 2000). Briefly, Vero cells in six-well tissue culture plates were inoculated with 100 μl of serially diluted virus in duplicates, allowed to adsorb for 1 h, and overlaid with 3 ml agar (1:1 ratio of 1.5% Tragacanth gum and 2× MEM complete growth medium). After 6 days, gum overlay was removed, cells were stained with 0.1% crystal violet for 2 min and plaque counts were determined. Percent plaque reduction by various siRNA molecules was statistically compared with the control siRNA treated cells by one-way ANOVA using Tukey–Kramer multiple comparisons test and *p*-values of less than 0.05 were considered as statistically significant. As shown in Fig. 2c, the P siRNAs were able to effectively inhibit the production of progeny virions. In agreement with their ability to silence P protein expression, siP1 and siP3 were found to be the strongest inhibitors of virus propagation and reduced the infectious viral titers by 88% ($p < 0.001$) and 86% ($p < 0.001$), respectively, at 36 h and by 75% ($p < 0.01$) and 76% ($p < 0.01$), respectively, at 58 h p.i. Likewise, in concordance with the P protein silencing profile, siP2 was relatively less effective and induced up to 50% ($p < 0.001$) and 21% ($p > 0.05$) reduction in virus plaque formation at 36 and 58 h p.i., respectively, while siP4 had a minimal effect on aMPV replication (Fig. 2c). Vero cells transfected with siP1 or siP3 also showed fewer syncytia suggesting limited viral spread to the neighboring cells (data not shown).

The present report describes that siP1 and siP3 are the most potent of the four P siRNAs tested in P gene silencing and inhibition of virus replication. Along with L and N proteins, P protein is packaged in the assembled virions which together initiate transcription and replication during initial stages of infection. However, owing to the critical roles of P protein as RdRP complex subunit (Dupuy et al., 1999; Easton et al., 2004), active synthesis of new P protein is crucial to efficient replication without which blockage of further transcription and genomic RNA replication is imminent. Furthermore, continuous production of new P protein is also required as it is packaged within new virions egressing from the cell. Hence, broad inhibition of viral proteins as well as reduced viral titers may be attributed to the

RNAi induced reduction in the intracellular amount of P protein and the subsequent lack of P protein function.

Of the four P siRNAs used, two siRNAs strongly interfered with P expression and hence aMPV yield. Although all four siRNAs had sequences homologous to the P ORF, there was marked difference in their efficacy to establish P silencing. Such variation has been extensively reported (Ge et al., 2003; Kapadia et al., 2003; Orba et al., 2004; Wang et al., 2004) and is postulated to be due to: (i) secondary and tertiary RNA structure of the target site disabling recognition by siRNA (Elbashir et al., 2001), (ii) limited accessibility of the siRNA target sequence due to bound proteins, such as at the 5′- and 3′-ends of the ORF and (iii) error prone nature of the RdRP generates escape variants during the course of virus replication that possess mutations in their genome as well as the mRNA coded thereof (Gitlin et al., 2002). A single nucleotide mismatch is known to abolish siRNA silencing effect (Bitko and Barik, 2001; Chi et al., 2003; Elbashir et al., 2001; Gitlin et al., 2002; Jacque et al., 2002; Semizarov et al., 2003) and thus siRNAs corresponding to variable regions fail to induce silencing. Thus, it appears that when targeting viral genes for RNAi, it may often be necessary to select several sites and preferably conserved regions in the gene of interest to identify potent siRNA sequences strongly inhibiting virus replication.

RNAi mediated viral gene silencing and replication block offers great potential for novel nucleic acid based antiviral approaches, provided efficient means for in vivo siRNA delivery could be developed. RNAi can be induced in eukaryotic cells by siRNA duplexes that are synthesized by chemical methods, in vitro transcription (Yu et al., 2002; Zamore et al., 2000) or expressed in the form of a double stranded hairpin structure by a DNA (Sui et al., 2002) or viral (Barton and Medzhitov, 2002; Devroe and Silver, 2004; Rubinson et al., 2003) vectors. The approach of expressing hairpin siRNAs by viral vectors may be the most practical and cost-effective delivery vehicle for RNAi based nucleic acid immunization of farm animals. In addition to serving as a fascinating addition to the existing tools for the biotechnological advancement of molecular vaccines, it offers the exciting opportunity to develop virus resistant transgenic animals genomically encoding a set of siRNAs making them immune to a variety of economically significant diseases (Saksela, 2003). Hence, the application of RNAi to generate phenotypic mutation of aMPV described here holds great potential for the development of novel RNAi based antiviral strategies.

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