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RNA interference of avian influenza virus H5N1 by inhibiting viral mRNA with siRNA expression plasmids

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ABSTRACT

Avian influenza virus H5N1 causes widespread infection in the birds and human respiratory tract, but existing vaccines and drug therapy are of limited value. Here we show that small interfering RNAs (siRNAs) specific for conserved regions of the viral genome can potently inhibit influenza virus production in cell lines, embryonated chicken eggs and BALB/c mice. siRNA expression plasmid pBabe-Super was chosen in the study, which directed the synthesis of small interfering RNAs in cells. The inhibition depended on the presence of a functional antisense strand in the small interfering RNA duplex, suggesting that viral mRNA is the target of RNA interference (RNAi). Among the three small interfering RNA expression plasmids we designed, we found that small interfering RNA for nucleocapsid protein (NP) had a specific effect in inhibiting the accumulation of RNAs in infected cells because of a critical requirement for newly synthesized nucleocapsid proteins in avian influenza viral RNA transcription and replication. The findings reveal that newly synthesized nucleocapsid, polymerase A (PA) and polymerase B1 (PB1) proteins are required for avian influenza virus transcription and replication and provide a basis for the development of small interfering RNAs as prophylaxis and therapy for avian influenza infection in birds and humans.

1. Introduction

Avian influenza virus (AIV) was one of the first viral diseases described in poultry. Clinically, AIV was probably first described by Perroncito in 1878, and the disease was shown to be caused by a filterable agent by several groups in 1901 (Alexander, 1986). Since 1996 when highly pathogenic H5N1 virus is isolated from a farmed goose in Guangdong Province, China, H5N1 has become one of the most devastating bird and human's catastrophes in history. Especially in 1997, human infections with avian influenza H5N1 are first reported in Hong Kong. Altogether, 18 cases (6 fatal) are reported in the first known instance of human infection with this virus (WHO, 2007).

The threat of a new avian influenza pandemic persists because, despite intensive efforts, existing vaccines and therapy for avian influenza infection have only a limited value. Current vaccines, consisting of either killed virus or recombinant surface glycoproteins, induce only a weak IgG response. In addition, the existing vaccines have to be reformulated almost every year because the viral antigens [hemagglutinin (HA) and neuraminidase (NA)] that elicit protective antibodies usually undergo changes, rendering the previous year's vaccine ineffective against any new virus subtype. So, the use of drugs for avian influenza is limited because of severe side effects and the possible emergence of resistant viruses (Ge et al., 2003).

RNA interference (RNAi) is a process by which double stranded RNA (dsRNA) directs sequence-specific degradation of messenger RNA (mRNA) (Vaucheret et al., 2003; Sharp, 2001; Brantl, 2002). This phenomenon was initially observed in *Caenorhabditis elegans* (Fire et al., 1998), in plants (Vaucheret et al., 2003; Baulcombe, 2002), and in mammalian cells (Elbashir et al., 2001). In plants, it is an evolutionarily conserved response to virus infection. Naturally occurring RNAi is initiated by the dsRNA-specific endonuclease Dicer-RDE-1, which processively cleaves long dsRNA into double-stranded fragments between 21 and 25 nt in length, termed short interfering RNA (Elbashir et al., 2001). Small interfering RNAs (siRNAs) are then incorporated into a protein complex that recognizes and cleaves target mRNAs. Studies have shown that in mammalian cells, RNAi can be triggered by introducing synthetic 21-nt siRNA duplexes into the cells (McManus and Sharp, 2002), bypassing the requirement for Dicer-RDE-1-mediated processing of long dsRNA. Although 21-nt siRNAs are too short to induce an IFN

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response in mammalian cells (McManus and Sharp, 2002; Kumar and Carmichael, 1998), still with the ability to confer transient interference of gene expression in a sequence-specific manner, they represent a previously unrecognized class of molecules that may have significant medical applications.

To circumvent the high cost of synthetic siRNA and to establish stable gene knock-down cell lines by siRNA, several plasmid vector systems were designed to produce siRNA inside cells driven by RNA polymerase III-dependent promoters such as U6 and H1-RNA gene promoters (Brummelkamp, 2002; Paddison et al., 2002; Sui et al., 2002; Miyagishi and Taira, 2002; Paul et al., 2002). With these plasmid vectors, the phenotypes of gene silencing could be observed by stable transfection of cells (Brummelkamp, 2002). Nevertheless, transient siRNA expression, with low and variable transfection efficiency, remains the problems for chemically synthesized and vector derived siRNA. Recently, several virus vectors have been developed for efficient delivery of siRNA into mammalian cells (Devroe and Silver, 2002; Barton and Medzhitov, 2002; Xia et al., 2002). Retroviral vectors were designed to produce siRNA driven by either U6 or H1-RNA promoter for efficient, uniform delivery and immediate selection of stable knock-down cells (Devroe and Silver, 2002; Barton and Medzhitov, 2002).

In this study, we describe a DNA vector-based approach utilizing the well-defined polymerase III H1-RNA promoter to drive efficient expression of siRNA in MDCK cells, embryonated chicken eggs and BALB/c mice. Our results demonstrate efficient and specific knock-down of NP gene in different cell lines and indicate a promising application of this adenovirus system in avian influenza virus inhibition.

2. Materials and methods

2.1. Viruses and assays

Avian influenza viruses (A/chicken/Qinghaihu/726/2005), subtypes H5N1, were stored in National Research Center for Wildlife Born Diseases, Institute of Zoology. The viruses were grown in the allantoic cavity of 10-day-old embryonated chicken eggs (Chinese Academy of Agriculture, Beijing, China) at 37 °C. Allantoic fluid was harvested 48 h after virus inoculation and stored at −80 °C. Virus titer was measured by hemagglutination assays. The hemagglutination assay was carried out in V-bottom 96-well plates. Serial 2-fold dilutions of virus samples were mixed with an equal volume of a 0.5% suspension (vol/vol) of chicken erythrocytes (Chinese Academy of Agriculture, Beijing, China) and incubated on ice for 1 h. Wells containing an adherent, homogeneous layer of erythrocytes were scored as positive.

2.2. Plasmid constructs

The RNA polymerase III H1-RNA gene promoter was used in our study. H1-RNA promoter from pBabe-Puro was cloned into the promoterless shuttle vector pSuper (Oligoengine) to get a new shuttle vector designated pBabe-Super (Institute of Zoology, Beijing, China) which can drive the expression of siRNA in recombinant adenovirus. The 60 nt oligonucleotides encoding avian influenza virus NP, polymerase A (PA) and polymerase B1 (PB1) specific siRNAs were:

Oligo-NP:
5′-GATCCCCGCAATGGACTCCAACACTCTTCAAGAGAGAGTGTTGGAGTCCATTGCTTTTTA-3′
3′-GGGCGTTACCTGAGGTTGTGAGAAGTTCTCTCTCACAACCTCAGGTAACGAAAAATTCGA-5′

Oligo-PA:
5′-GATCCCCCAAGTTTCAAGGCCCATGTTTCAAGAGAACATGGGCCTTGAAACTTGTTTTTA-3′
5′-AGCTTAAAAACAAGTTTCAAGGCCCATGTTCTCTTGAAACATGGGCCTTGAAACTTGGGG-3′

Oligo-PB1:
5′-GATCCCCGGAATGAGAAGAAGGCTAATTCAAGAGATTAGCCTTCTTCTCATTCCTTTTTA-3′
5′-AGCTTAAAAAGGAATGAGAAGAAGGCTAATCTCTTGAATTAGCCTTCTTCTCATTCCGGG-3′

These oligonucleotides were annealed and ligated to the BglII and HindIII sites of pBabe-Super to get plasmids pBabe-NP, pBabe-PA, pBabe-PB1 and confirmed by EcoRI digestion. The inserted sequences were confirmed by DNA sequencing (Takara, Japan).

2.3. Cell culture and virus infection

MDCK cells were grown in DMEM containing 10% heat-inactivated FCS, at 37 °C under a 5% CO_2/95% air atmosphere. For siRNA introduction, logarithmic-phase MDCK cells were trypsinized, washed, and resuspended in serum-free DMEM medium at 2×10^7 cells/ml. Cells (0.5 ml) were mixed with siRNAs and transformed by using SuperFect Transfection Reagent (Qiagen, Canada). Transformed cells were divided into three wells of a 6 well plate and cultured in DMEM for 24 h, and selected with puromycin for positive cell clones in 2 weeks. Then the culture medium (six-well) was then removed and 100 μl of H5N1 virus in infection medium, consisting of DMEM, 0.3% BSA (Sigma, USA), was added to each well. After incubation for 1 h at room temperature, 2 ml of infection medium containing 4 μg/ml trypsin was added to each well and the cells were cultured at 37 °C under 5% CO_2. At different times after infection, supernatants were harvested from infected cultures and the virus titer was determined.

2.4. Virus and siRNA inoculation in chicken embryos

For each inoculation, 30 μl of Oligofectamine (Invitrogen, USA) was diluted with 30 μl of DMEM (GIBCO). SiRNA expression plasmid (10 μg) was mixed with 30 μl of DMEM and added to dilute Oligofectamine, and the mixture was incubated at room temperature for 30 min. The mixture was then combined with 100 μl of H5N1 virus [5000 plaque-forming units (pfu)/ml] and immediately injected into the allantoic cavity of a 10-day-embryonated chicken egg. The eggs were incubated at 37 °C for 17 h and allantoic fluid was harvested to measure virus titer.

2.5. RNA extraction, reverse transcription (RT), and real-time PCR

MDCK cells (1×10^7) were transfected with or without siRNA expression plasmids, and were infected 8 h later with H5N1 virus. One, two, and three hours after infection, culture medium was removed and the cells were lysed by using Trizol reagent (GIBCO, USA). RNA was isolated by following the manufacturer's protocol. RT was carried out by using an ImProm-II™ Reverse Transcriptase (Promega, USA) in a 20 μl reaction mixture, containing 200 ng of total RNA and specific primers, at 37 °C for 1 h. One microliter of RT reaction mixture was then used for real-time PCR by using gene-specific primers, SYBR green PCR Master Mix (Applied Biosystems), and SYBR green I dsDNA binding dye. Before the PCR, the mixture was incubated at 50 °C for 2 min and 95 °C for 10 min. The reaction was then performed at 94 °C for 15 s and 60 °C for 30 s for 40 cycles. All reactions were done in duplicate. The levels of PCR products were monitored with a Rotor Gene 3000 (Corbet Research)

sequence detection system. Cycle times were analyzed at a reading of 0.2 fluorescence unit. Cycle times that varied by >1.0 unit between duplicates were discarded.

2.6. Western blot

Cells were harvested at the indicated time points after virus infection, washed once with cold PBS and lysed in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH7.4, 2 mM EDTA, 1% NP-40) containing protease inhibitors (Promega). Total proteins were resolved on a 10% SDS–polyacrylamide gel and transferred onto a nylon membrane and incubated with avian influenza positive antigens (Harbin Veterinary Research Institute, PRC) as primary antibody, followed by incubation with HRP conjugated rabbit anti-chicken IgG secondary antibody (Sigma). The bands were visualized expose to X-ray films with SuperSignal West Pico Trial Kit (Pierce, Rockford, IL, USA).

2.7. Viral challenge assay in mice

Groups of 6-week-old female BALB/c mice (two groups of eight each) were injected with siRNA expression plasmid pBabe-NP and pBabe-Negative as control. Mice were infected H5N1 (100 LD_{50}) virus, and recorded for body weight and mortality until all animals were dead or were recovering 18 h later.

3. Results

3.1. Design of siRNA expression plasmids for avian influenza virus

Avian influenza virus has a segmented RNA genome. Three of the eight RNA segments encode three components of the RNA transcriptase (PA, PB1, and PB2) and one encodes nucleocapsid protein (NP). Among Avian influenza viruses, 16 HA subtypes and 9 NA subtypes are known. Extensive differences in nucleotide sequences are also present in other genes among virus isolates from humans and different species. To design siRNAs that remain effective despite antigenic drifts and antigenic shifts, we focused on regions of the viral genome that are conserved among different subtypes and strains of virus from chicken, duck and swine. Besides having no more than 1 mismatch in the 21 nucleotides among different virus subtypes and strains, the siRNAs designed did not share identity with any known human gene. We designed and tested a total of 3 siRNA expression plasmids for NP, PA, PB1 genes. No siRNA for HA and NA was designed because they contain no stretch of conserved 21 nucleotides, a result of extensive variations in these genes among different virus isolates from humans and various other species.

3.2. Inhibition of influenza virus production in a cell line

To test whether the siRNAs from plasmids inhibited avian influenza virus production, we first examined their effects in MDCK cells. SiRNAs expression plasmids (10 μg) were introduced into MDCK cells (1×10^7) by SuperFect Transfection Reagent (Qiagen), and 8 h later the cells were infected with H5N1 virus at a multiplicity of infection (moi) of 0.001, 0.01, or 0.1. At different times after infection, culture supernatants were harvested, serially diluted, and assayed to determine the virus titer by using an HA assay. As a control, pBabe-Negative was similarly introduced into GFP-expressing MDCK cells, followed by virus infection. Virus titer was assayed as above and GFP expression was assayed by flow cytometry. As shown in Fig. 1, in mock transfection (no siRNA expression plasmid), virus titers in the infected cultures increased over time, reaching peak values between 48 and 60 h. Transfection of GFP control vector did not affect virus production at any time point but significantly inhibited GFP expression (data not shown), indicating that siRNA does not interfere nonspecifically with avian influenza virus production. Transfection of siRNA expression plasmids specific for avian influenza virus generated three types of results. First, the siRNAs from two kinds of siRNA expression plasmids (pBabe-PA and pBabe-PB1) had no discernable effects on the virus titer, indicating that they were not effective in interfering with avian influenza virus production in MDCK cells. Second, the real-time PCR result showed that siRNAs from pBabe-NP significantly inhibited about 70% of virus production (Fig. 2). Third, western blot results showed that only inhibition of pBabe-NP was pronounced while the results of the other two vectors (pBabe-PA and pBabe-PB1) were not significant (Fig. 3).

Together, these results show that (i) some siRNAs can potently inhibit influenza virus production in MDCK cells; (ii) influenza virus production can be inhibited by siRNAs specific for different viral genes, including those encoding NP, PA, and PB1.

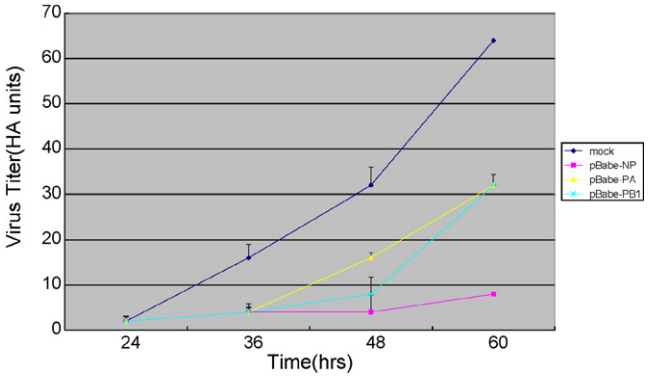

Fig. 1. SiRNAs interfere with avian influenza virus production in MDCK cells. Inhibition of avian influenza virus production in MDCK cells after transfection with selected siRNA expression plasmids. MDCK cells were first transformed with siRNA expression plasmids and selected with puromycin in 2 weeks for positive clones, and then infected with H5N1 virus at a moi of 0.01. Viral titers in the culture supernatants at different times after infection were measured by HA assay. HA units are arithmetic means based on titer endpoints of arithmetic dilutions. Virus titers (HA units) from five siRNA-treated cultures are shown over time. pBabe-NP, etc., are siRNA expression plasmids specific for different viral genes. For example, pBabe-NP indicates a siRNA specific vector for NP sequence of AIV genome. Data represent the means ± S.D. ($n = 3$).

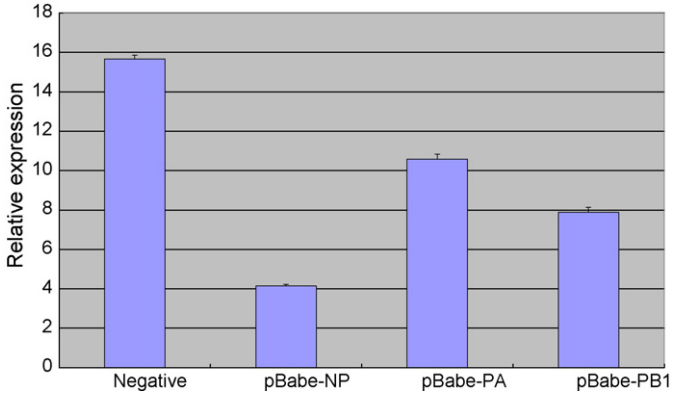

Fig. 2. SiRNAs interfere with avian influenza virus production in MDCK cells with statistical diagram of real-time PCR. MDCK cells (1×10^7) were transformed with or without siRNA expression plasmids, and were infected 8 h later with H5N1 virus. The cells were harvested for RNA purification and real-time PCR. Data are meaning from one out of two representative experiments and dealt with SPSS 10. Data represent the means ± S.D. ($n = 3$).

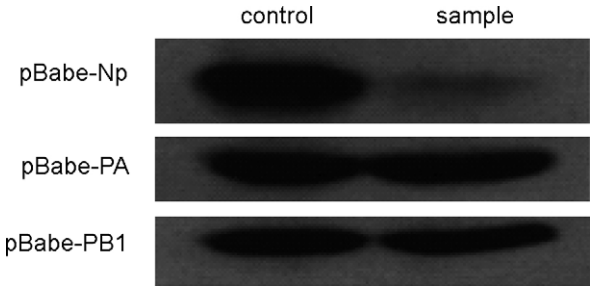

Fig. 3. Western blotting analysis for siRNA expression plasmids in MDCK cells with infection of H5N1 virus. Cells were harvested after virus infection, and proteins were collected. Total proteins were resolved on a 10% SDS–polyacrylamide gel and transferred onto a nylon membrane and incubated with avian influenza positive antigens (Harbin Veterinary Research Institute) as primary antibody, followed by incubation with HRP conjugated rabbit anti-chicken IgG secondary antibody (Sigma). The bands were visualized expose to X-ray films with SuperSignal West Pico Trial Kit (Pierce).

3.3. Inhibition of virus production in embryonated chicken eggs

To extend the results in MDCK cells, we tested the ability of siRNAs to inhibit avian influenza virus production in developing chicken embryos, a widely used in vivo model of influenza virus infection. For siRNA transfection in the embryos, we used Oligofectamine, a lipid-based carrier that has been shown to facilitate intracellular uptake of DNA oligonucleotides (Pederoso et al., 2001). H5N1 virus alone or virus plus siRNA were injected with Oligofectamine into the allantoic cavity of 10-day-old embryonated chicken eggs. Allantoic fluids were collected 17 h later for measurement of virus titers. When virus was injected alone (in the presence of Oligofectamine), high virus titers were detected (Fig. 4). Coinjection of siRNA expression plasmids for avian influenza virus, however, reduced virus titers. The results were concordant with those in MDCK cells. The same siRNA expression plasmid (pBabe-NP) that potently inhibited avian influenza virus production in MDCK cells also inhibited virus production in chicken embryos, whereas siRNA expression plasmids (pBabe-PA, and pBabe-PB1) that were less effective in MDCK cells were ineffective in chicken embryos. No significant reduction of virus titer was effectively observed when Oligofectamine was omitted. Thus, siRNAs also interfered with avian influenza virus production in embryonated chicken eggs.

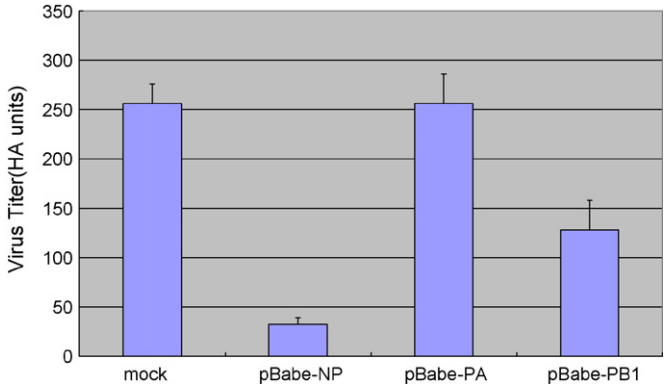

Fig. 4. SiRNA expression plasmids interfere with avian influenza virus production in embryonated chicken eggs. A mixture of siRNAs (10 μg), Oligofectamine, and H5N1 virus (500 pfu) was injected into the allantoic cavity of 10-day-old embryonated chicken eggs. Allantoic fluid was collected 17 h later and assayed for virus titers as in Fig. 2. Data represent the means ± S.D. ($n = 3$).

Table 1
Avian influenza virus specific siRNA treatments significantly decreased lung virus titers in mice infected with H5N1 virus

Treatment	Mean lung virus titer[a] (log $TCID_{50}$)
Negative	6.32 ± 0.34
pBabe-NP	5.12 ± 0.22

BALB/c mice ($n = 4$) were treated as indicated and challenged with 1×10^6 $TCID_{50}$ of H5N1 virus. Two days later, animals were sacrificed and lungs were collected for virus titers. Data represent the means ± S.D. ($n = 3$).

[a] Expressed as log10 $TCID_{50}$.

3.4. Treatment with siRNA expressing plasmids rapidly induced an antiviral response in BALB/c mice

To further assess whether siRNA could inhibit influenza virus replication in vivo, a murine model was established with avian influenza virus infection. SiRNA expression plasmid pBabe-NP was chosen and injected into BALB/c mice. In this assay, 200 μl of the mixture containing 100 μg of DNA (pBabe-NP) was injected into 6-week-old female BALB/c mice by vena caudalis. The mice were administered intranasally under anesthesia with H5N1 viruses 18 h later. The mice were sacrificed 24 h postinfection and lung homogenates were assayed for virus titers.

Virus titers were significantly reduced in lungs of the mice given pBabe-NP compared with that of pBabe-Negative treatment (Table 1). The results indicated the designed siRNAs targeting with NP gene were effective in inhibiting replication of different subtypes of H5N1 virus in the lungs. Furthermore, NP specific siRNA protected two of the eight mice (2/8) challenged with a lethal dose (100 LD_{50}) of H5N1 virus that killed all of the control mice (Fig. 5). The results suggested that pBabe-NP could effectively alleviate the morbidity of the H5N1 virus infected mice and could partially protect the mice challenged with lethal dose avian influenza virus H5N1.

In conclusion, we developed a simple siRNA delivery strategy by combination of well-defined H1-RNA promoter and conventional pBabe-Super adenovirus system. Our results demonstrate significant downregulation of virus production in MDCK cells, embryonated chicken eggs and BALB/c mice. With availability of high titers of adenoviruses and uniform and rapid infection, this technology will have a foreseeable wide application both in experimental biology and molecular medicine.

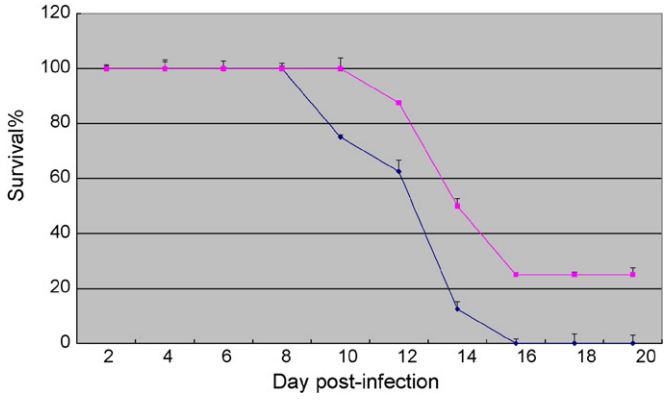

Fig. 5. Avian influenza virus specific siRNA treatments were partially protected mice against lethal challenge with H5N1 virus. BALB/c mice (eight per group) were treated with pBabe-Negative (red) or pBabe-NP (blue) and challenged with H5N1 virus. The percent of survival rate post-challenge were shown. Data represent the means ± S.D. ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

In this study we developed a simple siRNA delivery strategy by combination of well-defined H1-RNA promoter and conventional pBabe-Super adenovirus system. Our results demonstrate siRNA expression plasmids can potently inhibit avian influenza virus production in MDCK cells, embryonated chicken eggs and BALB/c mice.

Avian influenza virus infection is considered as a very dangerous bird's disease because of easy transmission, antigenic shift and drift of the virus, and the limited efficacy of current vaccines and therapy. We showed that siRNA expression plasmids potently inhibited avian influenza virus production in cell lines, embryonated chicken eggs and BALB/c mice. Among three siRNA expression plasmids tested, those that target NP and a component of the RNA transcriptase are especially effective, working at picomolar range and after virus infection has occurred. These results provide a basis for further development of siRNA expression plasmids for prophylaxis and therapy of avian influenza virus infection in birds and humans. Avian influenza virus naturally infects epithelial cells in the upper respiratory tract and lungs in birds. SiRNA expression plasmids could be conveniently administrated via intranasal or pulmonary routes. Considering that the number of virions is probably small at the beginning of a natural infection, sufficient amounts of siRNA may be taken up by epithelial cells in the upper airways and lungs to inhibit virus replication, thus, potentially achieving preventive or therapeutic effects.

The number of NP protein molecules in infected cells has been hypothesized to regulate the levels of mRNA synthesis versus genome RNA (vRNA and cRNA) replication (Lamb and Krug, 2001). Using a temperature-sensitive mutation in the NP protein, previous studies have shown that cRNA, but not mRNA, synthesis was temperature-sensitive both in vitro and in vivo (Medcalf et al., 1999; Shapiro and Krug, 1988). NP protein was also shown to be required for elongation and antitermination of nascent cRNA and vRNA transcripts (Shapiro and Krug, 1988; Beaton and Krug, 1986). We found that pBabe-NP vector has a specific effect in inhibiting the accumulation of RNAs in infected cells. Probably, in the presence of NP-specific siRNA, the newly transcribed NP mRNA is degraded, resulting in inhibition of NP protein synthesis. Without newly synthesized NP, further viral transcription and replication are blocked, so is new virion production.

These findings demonstrate a critical requirement for newly synthesized NP proteins in avian influenza viral RNA transcription and replication. The broad inhibition of all viral RNA accumulation by pBabe-NP siRNA expression plasmid probably occurs because the RNAs are not transcribed. Both the targeted mRNA degradation and the resulting broad inhibition of other viral RNA transcription make the pBabe-NP siRNA expression plasmids especially potent inhibitors of influenza virus infection.

Acknowledgements

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References

Alexander, D.J., 1986. Avian influenza-historical aspects. In: Easterday, B.C. (Ed.), Proceedings of the Second Annual International Symposium on Avian Influenza. United States Animal Health Association, Madison, WI, pp. 4–13.
Barton, G.M., Medzhitov, R., 2002. Retroviral delivery of small interfering RNA into primary cells. Proc. Natl. Acad. Sci. U.S.A. 99 (23), 14943–14945.
Baulcombe, D., 2002. RNA silencing. Curr. Biol. 12, 82–84.
Beaton, A.R., Krug, R.M., 1986. Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 50 capped end. Proc. Natl. Acad. Sci. U.S.A. 83, 6282–6286.
Brantl, S., 2002. Antisense-RNA regulation and RNA interference. Biochem. Biophys. Acta 1575, 15–25.
Brummelkamp, T.R., Bernards, R., Agami, R., 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550–553.
Devroe, E., Silver, P.A., 2002. Retrovirus-delivered siRNA. BMC Biotechnol. 2 (1), 15–19.
Elbashir, S., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001. Nature 411, 494–498.
Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806–811.
Ge, Q., McManus, M., Nguyen, T., Shen, C.-H., Sharp, P.A., Eisen, H.N., Chen, J., 2003. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc. Natl. Acad. Sci. U.S.A. 100, 2718–2723.
Kumar, M., Carmichael, G.G., 1998. Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. Microbiol. Mol. Biol. Rev. 62, 1415–1434.
Lamb, R.A., Krug, R.M., 2001. Orthomyxoviridae: the viruses and their replication. In: Knipe, D.M., Howley, P.M. (Eds.), Fundamental Virology, pp. 725–770.
McManus, M.T., Sharp, P.A., 2002. Gene silencing in mammals by small interfering RNAs. Nat. Rev. Genet. 3, 737–747.
Medcalf, L., Poole, E., Elton, D., Digard, P., 1999. Temperature-sensitive lesions in two influenza A viruses defective for replicative transcription disrupt RNA binding by the nucleoprotein. J. Virol. 73, 7349–7356.
Miyagishi, M., Taira, K., 2002. Development and application of siRNA expression vector. Nucleic Acids Research Supplement. Nat. Biotechnol. 20, 497–500.
Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., Conklin, D.S., 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. 16, 948–958.
Paul, C.P., Good, P.D., Winer, I., Engelke, D.R., 2002. Effective expression of small interfering RNA in human cells. Nat. Biotechnol. 20, 505–508.
Pederoso, M.C., Simoes, S., Pires, P., Faneca, H., Duzgunes, N., 2001. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. Adv. Drug Deliv. Rev. 47 (3), 277–294.
Shapiro, G.I., Krug, R.M., 1988. Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer. J. Virol. 62, 2285–2290.
Sharp, P.A., 2001. RNA interference-2001. Genes Dev. 15, 485–490.
Sui, G., Soohoo, C., Affarel, B., Gay, F., Shi, Y., Forrester, W.C., Shi, Y.A., 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. U.S.A. 99, 5515–5520.
Vaucheret, H., Beclin, C., Fagard, M., 2003. Post-transcriptional gene silencing in plants. J. Cell Sci. 114, 3083–3091.
WHO, 2007. H5N1 avian influenza: timeline of major events.
Xia, H., Mao, Q., Paulson, H.L., Davidson, B.L., 2002. siRNA-mediated gene silencing in vitro and in vivo. Nat. Biotechnol. 20, 1006–1010.