culture was centrifuged and the cell-free medium was used for HIV-1 gag p24 antigen quantification and viral RNA extraction, while the pellet was used for cell-viability counts and FACS analysis of EGFP expression as a marker of transgene expression.

Flow Cytometry Analysis of Long-term EGFP Expression—Half of the culture volume was harvested at 7 and 28 days (vif-shRNA), and 63, 70 and 98 days (DIS-shRNA) post-challenge, pelleted, washed twice in PBS and resuspended in 1% formaldehyde. FACS analysis was performed using the FACS Calibur and *CELLQUEST* software (BD Sciences, San Jose, CA, USA).

Fluorescence Microscopy—Fluorescence microscopy was used to investigate the efficiency of EGFP expression as an index of transgene expression in the SupT1 cells. For intracellular fluorescence studies, SupT1 cells were fixed with 3.7% formaldehyde on alternate days. Fluorescence-activated cells were examined under fluorescence microscopy (Biozero BZ-8000; KEYENCE, Osaka, Japan) at an excitation wavelength of 488 nm using a 10× objective lens. Images were acquired at a 512 × 512 pixel resolution.

Generation of Viruses—To generate HIV-1 viruses, the HIV-1 infectious molecular cloned plasmid vector (HIV-1pNL4-3) was transfected with 3 μg DNA into 5×10^5 seeded HeLa $CD4^+$ cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The culture was incubated at 37°C for 72 h and harvested. The cells were then pelleted by centrifugation to produce a cell-free supernatant yielding the HIV-1_{NL4-3} virus, which was aliquoted and stored at −80°C. HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G\text{-}C)}$ virus was generated from the experiment illustrated in Fig. 4B. To summarize, SupT1 cells were transduced with lentivirus-mediated DIS- or vif-shRNAs at an MOI of 20 in the presence of 4 μg/ml polybrene. After incubation at 37°C for 4 h, the cells were washed three times with PBS and resuspended in growth medium. The cells were then challenged with HIV-1_{NL4-3} virus at an MOI of 0.1 and cultured for 98 d at 37°C. At 21 days, harvested supernatant showing a vif mutation virus, HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G\text{-}C)}$ (siRNA vif target 5049-CAGATG GCAGGTGATGATTGTGT-5569; 5049-CACATGGCAGG TGATGATTGTG-5569: 1 nt substitution), was titred and stored at −80°C until use.

Genotypic Sequence Analysis of the DIS- and vif-siRNA Target Regions of HIV-1_{NL4-3} and Re-challenge of Cells Expressing DIS- and vif-shRNA—Viral RNA from HIV-1_{NL4-3}-challenged CS-DIS or CS-vif shRNA-transduced cultures was analyzed for siRNA-mediated mutations in the vif-shRNA target region at weeks 2 through 14. Viral RNA was isolated from the cell-free culture supernatant using the QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Viral RNA (5 μl) was used in a reverse transcription–PCR containing Powerscript reverse transcriptase (Clontech, Mountain View, CA, USA), 1 mM each of the deoxynucleotide triphosphates, 1× first-strand buffer (Clontech), 200 ng random hexamer (Promega, Madison, WI, USA) and 10 U RNasin (Promega). Reverse transcription was performed at 42°C for 1 h, followed by heat inactivation of the reverse transcriptase enzyme at 70°C for 15 min. cDNA (2 μl) was added to a 48 μl PCR mixture containing 1× Qiagen Taq PCR buffer, 1.5 mM $MgCl_2$, 20 pmol sense primer DIS F (5′-GTGT GGAAAATCTCTAGCAGTGGGG-3′), antisense primer DIS R (5′-CCTCAATAAAGCTTGCCTTGAGTGCT C-3′), sense primer vif F (5′-CGGGTTTATTACAGGGACA GCAGAGA-3′), antisense primer vif R (5′-AAAGGTGAAG GGGCAGTAGTAATACAAGAT-3′), 1 mM each of the deoxynucleotide triphosphates, and 2.5 U Taq polymerase (Qiagen). PCR was performed using a gradient PCR thermal cycler (Astec, Fukuoka, Japan) with the following thermal program: 1 cycle (95°C for 1 min), 35 cycles (95°C for 15 s, 58°C for 30 s, 72°C for 30) and 1 cycle (72°C for 5 min). The PCR product was fractionated, analysed on a 1% SeaKem gel, and purified using a QIAEX II gel extraction kit (Qiagen). Nucleotide cycle sequencing was performed using dye-labeled terminator chemistry.

SupT1 cells stably expressing DIS-shRNA, vif-shRNA, LacZ-shRNA or the control U6-ter were challenged with either wild-type virus HIV-1_{NL4-3} or mutant virus HIV-$1_{NL4-3\text{-}vif\text{-}mut\ (G\text{-}C)}$. SupT1 cells ($1 \times 10^6$) were infected with 50 ng of gag p24 antigen from each virus. Following infection, the cells were washed three times with PBS and resuspended in growth medium. The time-course of the infection was monitored over an 8-day period by HIV-1 gag p24 enzyme-linked immunosorbent assay.

RESULTS

Long-term Inhibition of HIV-1 Replication by Lentiviral Vector-mediated DIS and vif-shRNA—Our major goal in these studies was to introduce both DIS- and vif-shRNAs into a lentiviral construct to achieve their stable expression in transduced cells. Lentiviral vectors offer advantages over conventional retroviral vector systems because they can transduce both dividing and non-dividing cells, and are less prone to transgene silencing (*36–38*). We previously reported the design of lentiviral vectors expressing shRNA against the following regions of HIV-1: 246–266 nt of the DIS region, containing a stem–loop structure with six self-complementary nucleotides at the top (*39*) and 5049–5069 nt of the vif region (*40*).

The transfer vector pCS-U6-shRNA-EGFP contained short-hairpin type DIS, vif, and LacZ-siRNAs driven by a Pol-III U6 promoter. Downstream, the reporter gene EGFP was driven by a cytomegalovirus promoter. The plasmid vector, pCS-U6-ter-EGFP, lacked a short hairpin siRNA (Fig. 1A).

SupT1 cells transduced with the control vectors CS-U6-LacZ-shRNA and CS-U6-ter, or the CS-U6-DIS- and vif-shRNA vectors, showed 98%, 99%, 97% and 92% EGFP expression, respectively, as measured by FACS analysis. This indicates high transduction efficiency (Fig. 1B).

To determine whether HIV-1 was down-regulated by the shRNA in the DIS-shRNA construct, the transduced cells were tested for HIV-1 gag p24 antigen. At 70 days, the HIV-1 gag p24 antigen levels were significantly reduced in DIS-shRNA transduced cells compared to cells transduced with the control vectors

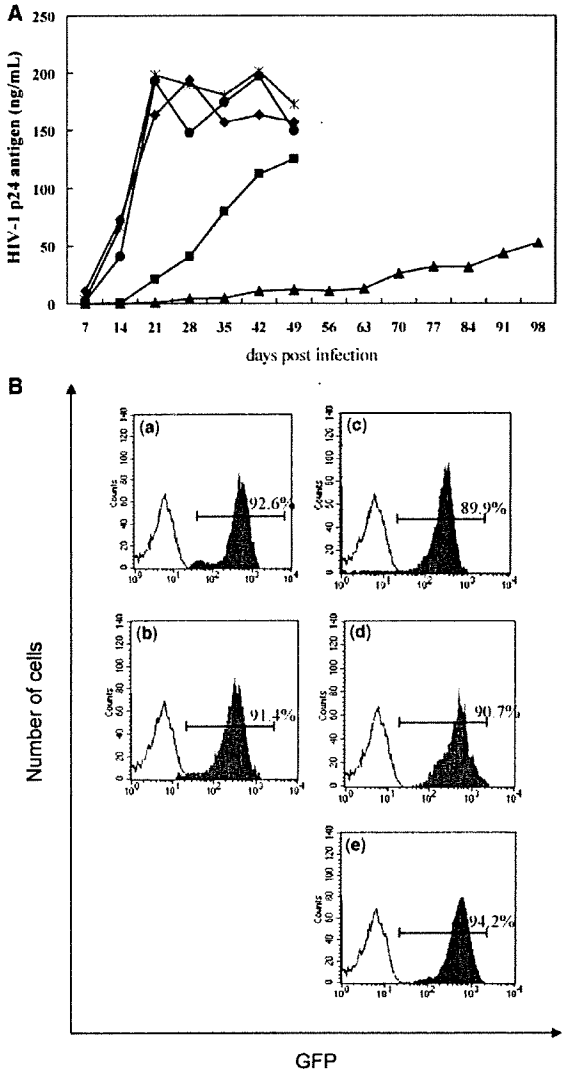

Fig. 2. **Long-term activity of lentivirus-mediated DIS- and vif-shRNA.** (A) Long-term inhibition of HIV-1 replication in SupT1 cells. HIV-1 gag p24 antigen was measured during the 98-day culture of SupT1 cells transduced with the indicated CS-lentiviruses (MOI 20), and challenged with HIV-1_{NL4-3} (MOI 0.1). Negative control, asterisk; vif-shRNA, solid square; LacZ-shRNA, solid diamond; DIS-shRNA, solid triangle; U6-ter, solid circle. (B) Long-term expression of transgenic EGFP in Sup-T1 cells expressing vector transgenes was examined by FACS analysis using *CELLQUEST* software. a, vif-shRNA at 7 days; b, vif-shRNA at 28 days; c, DIS-shRNA at 63 days; d, DIS-shRNA at 70 days; e, DIS-shRNA at 98 days.

CS-U6-LacZ-shRNA and CS-U6-ter (Fig. 2A). Furthermore, SupT1 cells transduced with the CS-U6-DIS-shRNA vector showed ~94% EGFP expression at 98 days (Fig. 2B-e). A steady increase in viral expression was observed, however, after day 70 (Fig. 2A). The anti-HIV-1 activity of vif-shRNA was similarly evaluated. Viral expression steadily increased from 21 days post-infection in the transduced SupT1 cells (Fig. 2A), whereas EGFP reached 91% expression at 28 days (Fig. 2B-b). These results demonstrate that DIS-shRNA results in the generation of resistant viruses 70 days later than vif-shRNA.

RNAi-resistant HIV-1 Variants—Long-term cultures stably expressing siRNAs targeting HIV genes eventually give rise to escape mutants (*20–22*). We therefore investigated the sudden increase in viral replication in cultures expressing vif- and DIS-shRNAs. Sequence analyses showed resistance against their respective shRNAs (Fig. 3A and B). DIS-shRNA showed a potential inhibition of HIV-1 replication compared with vif-shRNA at 77 days post-infection. Most surprising was the emergence of RNAi-resistant viruses containing just a single nucleotide substitution (A256T) on the DIS-shRNA target sequence (Fig. 3A). Notably, hyper-reactivity of the last adenine in the DIS loop (A256), which is the hallmark of *in vitro* RNA dimerization (*41*, *42*), was observed in infected cells (*43*). We also observed the emergence of RNAi-escape viruses with resistance against vif-shRNA that contained nucleotide substitutions or deletions in or near the vif-shRNA target sequence (Fig. 3B).

To determine the inhibitory effects of vif-shRNA and DIS-shRNA on HIV-$1_{NL4-3-vif-mut\ (G-C)}$ and wild-type HIV-1_{NL4-3}, SupT1 cells stably expressing either vif-shRNA or DIS-shRNA were infected with HIV-$1_{NL4-3-vif-mut\ (G-C)}$ and HIV-1_{NL4-3}. When control cells were infected with the wild-type virus and escape virus, both viruses showed a similar replication curve (Fig. 4A). HIV-1_{NL4-3} and HIV-$1_{NL4-3-vif-mut\ (G-C)}$ both caused a potential inhibition of HIV-1 replication at 8 days in SupT1 cells expressing the DIS-shRNA (Fig. 4B). In SupT1 cells expressing vif-shRNA, the RNAi-resistant virus (HIV-$1_{NL4-3-vif-mut\ (G-C)}$) replicated at the same rate as in control cells (Fig. 4C). These results demonstrate that the efficiency of siRNA binding to target RNA can be diminished by nucleotide substitutions or deletions in the target sequence.

Multi-targeting with both DIS- and vif-shRNAs Results in Long Lasting Inhibition of HIV-1 Genes—To test the long-term ability of vif- and DIS-shRNAs to inhibit target gene expression in SupT1 cells, the cells were transduced with either CS-U6-vif-shRNA alone, or with a combination of CS-U6-vif and DIS-shRNAs. In those cells transduced with CS-U6-vif-shRNA alone, a potential inhibition of HIV-1 replication was observed 14 days post-infection. This inhibition was gradually reduced to 65% by 34 days. Other vif-shRNA-expressing cells challenged with HIV-1_{NL-4-3} were further challenged with CS-U6-DIS-shRNA at 21 days. The HIV-1 gag p24 antigen levels were significantly reduced in CS-U6-vif-shRNA/CS-U6-DIS-shRNA transduced cells compared to cells transduced with CS-U6-vif-shRNA alone for 34 days (Fig. 5A). SupT1 cells transduced with CS-U6-vif-shRNA vectors, however, expressed EGFP at 28 days (Fig. 5B-b).

DISCUSSION

In contrast to acute viral infections, chronic infections with viruses such as HIV-1, hepatitis C virus and herpes simplex virus should be targeted with long-term RNAi treatment. In the case of HIV-1, virus-resistant

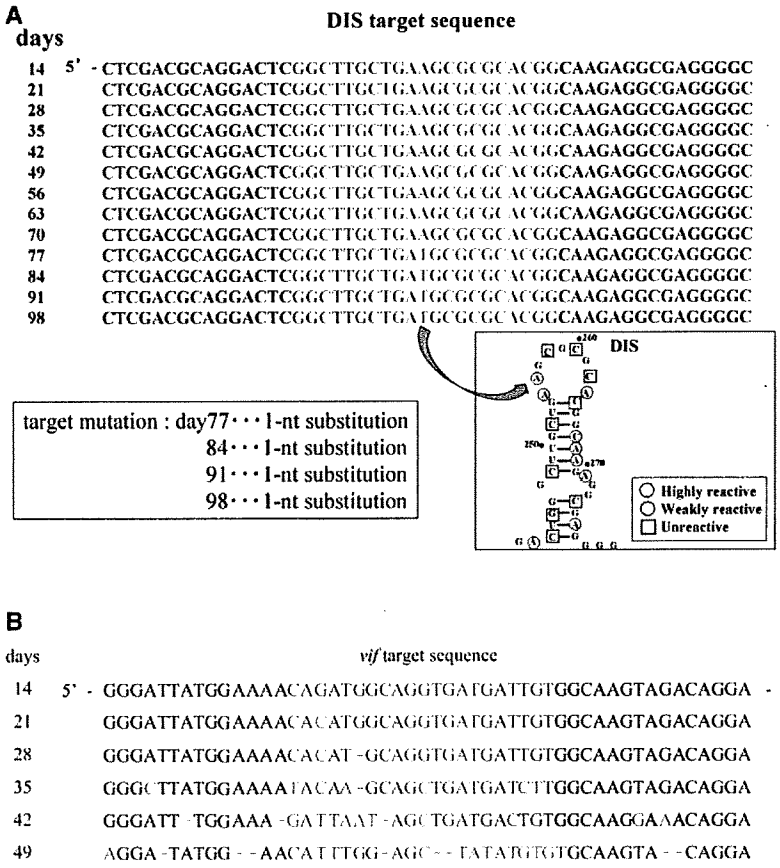

Fig. 3. **HIV sequence variation following shRNA-escape.** (A) Genotype sequence analysis revealed siRNA-mediated mutations in the DIS-shRNA target site (nucleotides 246–266; blue) of HIV-1_{NL4-3} in RNA extracted from DIS-shRNA-expressing culture supernatants. Day of sequencing is indicated. Substitutions are indicated in red. (B) Genotype sequence analysis revealed siRNA-mediated mutations at the vif-shRNA target site (nucleotides 5049–5069, blue) of HIV-1_{NL4-3} in RNA extracted from vif-shRNA-expressing culture supernatants. Day of sequencing is indicated. Deletions are indicated by dashes; substitutions are indicated in red.

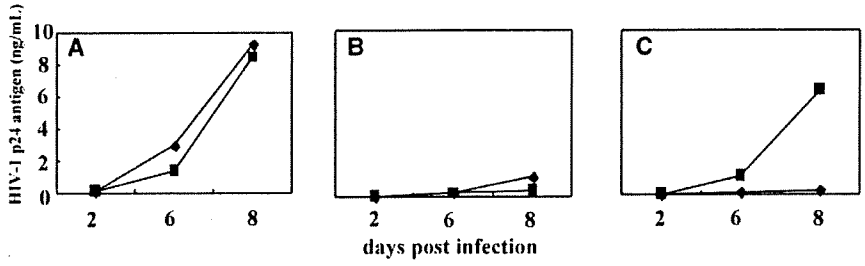

Fig. 4. **Inhibitory effects of vif-shRNA and DIS-shRNA on wild-type or vif-shRNA resistant virus (HIV-$1_{NL4-3-vif-mut\ (G-C)}$).** (A) Control cells were infected with the vif mutant virus HIV-$1_{NL4-3-vif-mut\ (G-C)}$ (solid square) and the wild-type HIV-1_{NL4-3} virus (solid diamond). (B) DIS-shRNA cells were infected with HIV-$1_{NL4-3-vif-mut\ (G-C)}$ (solid square) and HIV-1_{NL4-3} virus (solid diamond). The vif mutant virus HIV-$1_{NL4-3-vif-mut\ (G-C)}$ was strongly inhibited by DIS-shRNA (solid square). (C) vif-shRNA cells were infected with HIV-$1_{NL4-3-vif-mut\ (G-C)}$ (solid square) and HIV-1_{NL4-3} virus (solid diamond). The vif mutant virus HIV-$1_{NL4-3-vif-mut\ (G-C)}$ was resistant to DIS-shRNA (solid square). Replication was monitored by measuring levels of the p24 antigen up to 8 days post-infection.

$CD4^+$ T cells can be produced by *ex vivo* transduction of blood stem cells to express the anti-HIV-1 RNAi trigger before re-introducing these cells to the patient (*44*). Lentiviral vectors are used to stably transduce cells with shRNA expression constructs, resulting in strong inhibition of virus replication (*37*, *38*). Prolonged culturing of these cells, however, results in the selection of escape variants resistant to the expressed siRNA; thus,

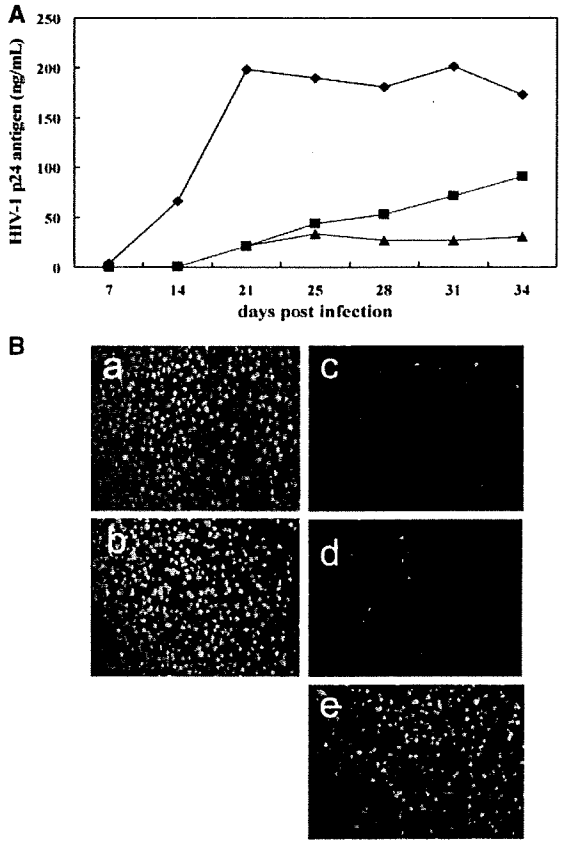

Fig. 5. **Multi-targeting with both DIS- and vif-shRNAs results in longer-lasting inhibition of HIV-1 genes.** (A) vif-shRNA-expressing cells were challenged with HIV-1_{NL-4-3} and the cells were cultured. On day 21, the cells were further challenged with CS-U6-DIS. The levels of HIV-1 gag p24 antigen were reduced significantly in DIS-shRNA-expressing cells compared to vif-shRNA cells alone for 34 days. vif-shRNA, solid square; negative control, solid diamond; vif-shRNA/DIS-shRNA, solid triangle. (B) Expression of transgenic EGFP in Sup-T1 cells expressing vector transgenes was examined by fluorescence microscopy. a, vif-shRNA at 7 days; b, vif-shRNA at 21 days; c, vif-shRNA/DIS-shRNA at 21 days; d, vif-shRNA/DIS-shRNA at 28 days; e, vif-shRNA/DIS-shRNA at 34 days.

the RNAi-induced block of HIV-1 replication is not durable. Nucleotide substitutions or deletions within the siRNA-target sequence of resistant variants may affect important regulatory sequences and/or essential amino acids, resulting in reduced viral fitness (*19*, *20*).

In this study, we described a slower rate of emergence of RNAi-resistant mutants by targeting siRNA to the well-conserved DIS of HIV-1 (*28–30*). The DIS is a stem–loop structure with six self-complementary nucleotides at the top, located between the primer binding site and the splice donor site at the end of a long terminal repeat (*45*). The DIS is involved in the dimerization of the HIV-1 genome, packaging and proviral synthesis (*30*, *46–48*). There are two major motifs in HIV-1: GUGC AC in subtypes A and C, and GCGCGC in subtypes B and D (*49*, *50*). The subtype A motif is a good target for siRNA in an *in vitro* cell-free system where HIV-1 genome dimerization is successfully inhibited by a 9-mer DIS-targeting siRNA, while the corresponding DNA oligonucleotide does not affect dimerization (*51*, *52*).

To determine whether HIV-1 is down-regulated by the expressed shRNA in the CS-U6-DIS-shRNA construct, the transduced cells were tested for HIV-1 gag p24 antigen expression. During the first 70 days post-infection, HIV-1 replication was significantly reduced in CS-U6-DIS-shRNA transduced cells (Fig. 2A). By 77 days, however, p24 antigen levels had increased, indicating the loss of the DIS-shRNA-mediated anti-viral activity (Fig. 2A). Flow cytometry analysis revealed continued intracellular production of the reporter gene EGFP (Fig. 2B). Similarly, viral expression dramatically decreased in CS-U6-vif-shRNA transduced cells for 14 days, followed by an increase beginning at 21 days (Fig. 2A). DIS-shRNA expression in the cells results in the generation of escape mutants 70 days slower than vif-shRNA.

To determine whether HIV-1 escape from RNAi was due to the emergence of mutations within the DIS and *vif* genes, viral RNA was extracted from sequential samples of culture supernatant and the DIS and vif-shRNA target regions were amplified by PCR and sequenced. Any single-nucleotide mismatch between the siRNA and the DIS target sequence is capable of reducing the silencing effect. In particular, a single nucleotide change at the center of the target sequence (A256T), with the exception of position 11, was very effective in allowing the virus to escape siRNA inhibition (Fig. 3A). Notably, hyper-reactivity of the last adenine in the DIS loop (A256), which is the hallmark of *in vitro* RNA dimerization (*41*, *42*), was observed in infected cells (*43*). More recently, Jones *et al.* (*53*) reported that the mutations in a non-coding region of the HIV-1 RNA genome affect the ability of the virus to synthesize viral cDNA in a cell type-dependent manner, illustrating the importance of virus–host cell interactions via an RNA-trigger. Most surprising, there was an emergence of RNAi-resistant viruses that contained nucleotide substitutions or deletions in or near the vif-shRNA target sequence (Fig. 3B). Other studies have suggested that nucleotide polymorphisms at the 3′- and 5′-end of the target sequence do not strongly affect recognition by siRNA (*21*, *54*). Furthermore, Du *et al.* (*55*) reported that target sequences containing mutations at position 12 are well tolerated by the silencing machinery. In addition, HIV-1 silencing is affected by a nucleotide change at residue 12 in a *gag* siRNA target sequence (*27*). Our results show that the efficacy of HIV-1 silencing can be reduced by a single-nucleotide polymorphism at the target sites (Fig. 3A). We also found that DIS-shRNA-expressing cells can slow the generation of escape mutants by about 2 months compared with vif-shRNA.

Lee *et al.* (*27*) reported that targeting conserved sequences, such as the *vif* sequence of HIV-1, delays the emergence of virus escape mutants. We, however, observed nucleotide substitutions or deletions in or near the vif sequence targeted by vif-shRNA. These discrepancies highlight the possible relevance of a specific target sequence in siRNA recognition and escape

from RNAi. Furthermore, not all siRNAs are equally effective, and it is difficult to predict the potential value of a particular siRNA (*56*).

To test the long-term ability of DIS- and vif-shRNAs to inhibit target gene expression, cells were transduced with either vif-shRNAs alone or with a combination of DIS- and vif-shRNAs, and challenged with wild-type HIV-1_{NL-4-3}. In the vif-shRNA transduced SupT1 cells, an increase in viral expression was observed from 21 days (Fig. 5A). Other vif-shRNA-expressing cells challenged with HIV-1_{NL-4-3} were further challenged with CS-U6-DIS-shRNA beginning at day 21. The levels of HIV-1 gag p24 antigen were reduced significantly in these cells compared to the cells transduced with CS-U6-vif-shRNA alone for 34 days (Fig. 5A). These results suggest that HIV-1 may escape the anti-viral activity of RNAi by specific mutations in the targeted sequence, and that targeting conserved sequences and the simultaneous use of multiple siRNAs may be useful strategies in maintaining inhibition of viral replication. Second generation siRNAs that recognize the mutated target sites may be used to prevent the emergence of resistant virus (*54*).

In conclusion, we highlighted specific target loci within the 5′ long-terminal repeat of HIV-1 that are susceptible to shRNA targeting, and may prove advantageous over other RNAi target sites within HIV-1. Although shRNAs require further manipulation to improve their overall efficacy in generating multiple functioning siRNAs, they may prove to be a useful tool in the multifaceted approach to treating HIV-1 infection. In fact, RNAi might be combined effectively with any number of anti-viral therapeutics, including dominant negative antiviral proteins. Future work is needed to develop these principles to identify anti-viral strategies that provide effective and long-term suppression of viral replication and pathogenesis.

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CONFLICT OF INTEREST

None declared.

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Structure and real-time monitoring of the enzymatic reaction of APOBEC3G which is involved in anti-HIV activity

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ABSTRACT

Human apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) is known to play a role in intrinsic cellular immunity against human immunodeficiency virus type 1 (HIV-1). The antiretroviral activity of APOBEC3G is associated with hypermutation of viral DNA through cytidine deamination. APOBEC3G contains two cytidine deaminase domains that are characterized by a highly conserved zinc-coordinating motif. It is known that only the C-terminal domain of APOBEC3G (c-APOBEC3G) is involved in the catalytic activity. Here, we present the solution structure and the interaction with single-stranded DNA of c-APOBEC3G. Furthermore, we have succeeded for the first time in monitoring the deamination reaction of c-APOBEC3G in real-time using NMR signals. The monitoring has demonstrated that the deamination reaction occurs in a strict 3'→5' order along DNA.

INTRODUCTION

Human APOBEC3G has been identified as an anti-HIV-1 host factor[1]. APOBEC3G is a member of a large family of cellular cytidine deaminases that includes APOBEC1, APOBEC2 and activation-induced cytidine deaminase (AID). All APOBEC proteins have one or two cytidine deaminase domains that contain a single zinc-coordinating motif consisting of the conserved $HXE(X)_{23-28}CXXC$[2]. APOBEC3G has two zinc-coordinating motifs, but only the C-terminal domain (c-APOBEC3G) is involved in the catalytic activity[3]. The c-APOBEC3G induces cytidine deamination in the minus-strand of viral DNA thereby resulting in abortive infection. APOBEC3G preferentially deaminates the cytidine residue of the CCC sequence in single-strand DNA (ssDNA)[4]. In an effort to shed light on the molecular mechanism of action by which APOBEC3G inactivates HIV-1, we determined the solution structure of c-APOBEC3G, residues 193-384, and examined its interaction with ssDNA by NMR. Based on the obtained results, we propose the model of c-APOBEC3G in complex with ssDNA. Furthermore, we succeeded for the first time in monitoring the deamination reaction in real-time using NMR signals. This method enabled us to analyze the deamination reaction with high spatial resolution. Deamination in a very strict 3'→5' order was detected for consecutive cytidine residues of ssDNA.

RESULTS AND DISCUSSION

The structure of wild-type c-APOBEC3G as determined in solution is composed of five β-strands, β1-β5, and six α-helices, α0-5, which are arranged in the order of α0-β1-β2/β2'-α1-β3-α2-β4-α3-β5-α4-α5[5]. The second β-strand is interrupted and divided into two short β-strands, β2 and β2' (Fig. 1). The solution structure of mutant c-APOBEC3G and the crystal structure of c-APOBEC3G were reported[6,7]. The differences were noted among the three structures in α0 and β2.

APOBEC3G is a ssDNA cytidine deaminase. In order to determine the mode of the interaction of c-APOBEC3G

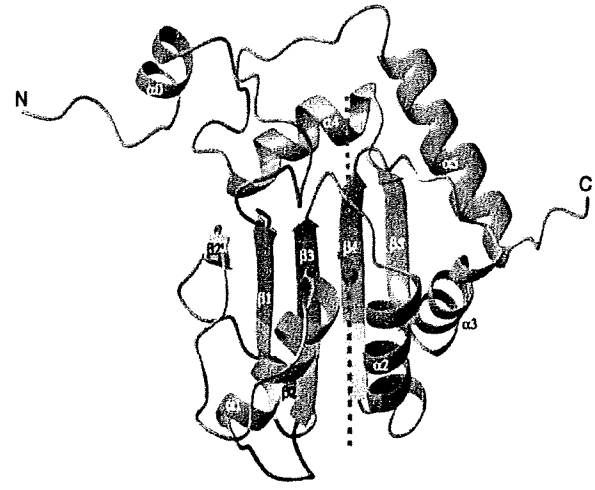

Fig. 1 The structure of c-APOBEC3G. The proposed position of ssDNA is indicated by a dotted line.

with ssDNA, we conducted NMR chemical shift perturbation analysis of c-APOBEC3G with the ssDNA, d(ATTCCCAATT). It had been reported that minus strand deamination by APOBEC3G occurs preferentially at the CCCA sequence[4]. The results of our chemical shift perturbation analysis suggested that in the complex with the wild-type c-APOBEC3G, ssDNA may parallel α1 and α2 and that one end of the main chain of ssDNA is located close to the area composed of the N-terminal regions of β3 and β4 and the C-terminal regions of α1 and α2 (Fig. 1).

Next, we analyzed the deamination reaction of c-APOBEC3G with the same 10-mer ssDNA, d(A1T2T3C4C5C6A7A8T9T10). It was previously reported that deamination occurs preferentially at a C6 residue of the 5'-C4C5C6-3' motif[4]. But it is possible that deamination may also occur at C4 and C5 residues. So as to determine the deamination mechanism of APOBEC3G, we tried for the first time to monitor the deamination reaction in real-time by NMR signals. First, the NMR spectra revealed that the second and third cytidine residues, C5 and C6, were fully deaminated and converted to the uridine residues, U5 and U6, 24 hours after addition of the wild-type c-APOBEC3G whereas the first cytidine residue, C4, was not deaminated at all (Fig. 2a). Second, the time course of the deamination reaction was monitored and chased in real-time using the NMR signal of the generated uridine residue, U6, that occurred at a very early stage of the time course (within 30 minutes) after the start of the reaction. In contrast, we found that the deamination of the second cytidine residue, C5, and the resultant appearance of the NMR signal of the generated uridine residue, U5, occurred at a very late stage of the time course (Fig. 2b). Consequently, it appears as if the deamination of the C5 residue started after deamination of the C6 residue was accomplished. The monitoring demonstrated that the deamination reaction occurs in a strict 3'→5' order.

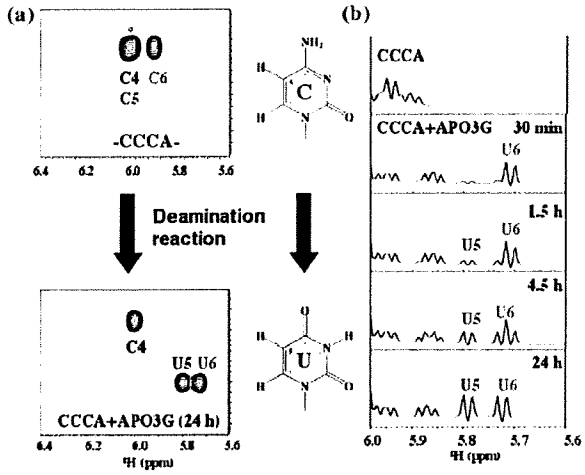

Fig. 2 Deamination in an NMR tube. (a) ^{1}H-^{13}C HSQC spectra. (b) Time chase with ^{1}H NMR spectra.

CONCLUSION

We present the solution structure of wild-type C-terminal cytidine deaminase domain, residues 193-384. The results of the chemical shift perturbation analysis of wild-type APOBEC3G-C in solution enable us to propose the model of interaction. We succeeded for the first time in monitoring the deamination reaction in real-time using NMR signals. This monitoring demonstrated that the deamination reaction occurs in a strict 3'→5' order along ssDNA.

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ORIGINAL ARTICLE

Baculovirus-Mediated Bispecific Short-Hairpin Small-Interfering RNAs Have Remarkable Ability to Cope With Both Influenza Viruses A and B

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Influenza viruses A and B cause widespread infections of the human respiratory tract; however, existing vaccines and drug therapy are of limited value for their treatment. Here, we show that bispecific short-hairpin small-interfering RNA constructs containing an 8-nucleotide intervening spacer, targeted against influenza virus A or influenza virus B, can inhibit the production of both types of virus in infected cell lines. This multiple vector showed remarkable ability to cope with both influenza viruses A and B. Furthermore, the *Autographa californica* multiple nuclear polyhedrosis virus can infect a range of mammalian cells, facilitating its use as a baculovirus vector for gene delivery into cells. In this study, baculovirus-mediated bispecific short-hairpin RNA expression markedly inhibited both influenza viruses A and B production.

Introduction

INFLUENZA IS CAUSED BY enveloped, single-stranded RNA viruses of the *Orthomyxoviridae* family (Lamb and Krug, 2001). These are classified as influenza virus type A, B, or C based on differences in their nucleoproteins and matrix proteins, with the influenza A virus being the most pathogenic. The influenza A virus genome contains 8 RNA segments with negative polarity (Lamb and Krug, 2001). Three of these segments encode the 3 components of the RNA-dependent RNA-transcriptase complex (PA, PB1, and PB2). Three of the other RNA segments code for the major surface glycoprotein hemagglutinin (HA), the major surface glycoprotein neuraminidase (NA), and the scaffolding and RNA-binding nucleocapsid protein (NP), respectively. Each of the remaining 2 RNA segments encodes 2 proteins, M1 and M2 or NS1 and NS2, respectively, which function either as viral structural proteins or as nonstructural proteins in the viral life cycle. The potential for the emergence of a highly virulent strain of influenza virus, either naturally occurring or deliberately constructed, was clearly demonstrated by the sudden appearance of a lethal form of the virus in Hong Kong in 1997, which killed 6 out of 18 infected individuals (Claas et al., 1998; Yuen et al., 1998). Mortality due to influenza virus infection generally affects infants, adults over 65 years of age, and immunocompromised individuals (Kemble and Greenberg, 2003). Thus, as the populations age in industrialized countries, influenza virus infection and its associated complications and mortality are likely to become an even greater public-health problem.

Small-interfering RNAs (siRNAs) that are 21–25 base pairs (bp) in length with 3′ overhangs of 2 nucleotides can cause RNA interference (RNAi; Elbashir et al., 2001). siRNAs are a powerful tool for sequence-specific post-transcriptional gene silencing, and have potential therapeutic and prophylactic applications against cancer, as well as infectious diseases (Haasnoot and Berkhout, 2006). siRNAs have also been used recently to interfere with the replication of an influenza virus (Ge et al., 2003, 2004; Tompkins et al., 2004). Baculoviruses have previously been employed to deliver genes into mammalian cells (Matsuura et al., 1987). The *Autographa californica nuclear polyhedrosis virus* (AcNPV) has long been used as a biopesticide and as a tool for efficient recombinant protein production in insect cells (Matsuura et al., 1987; Luckow and Summers, 1988). The host specificity of this baculovirus was originally thought to be restricted to cells derived from arthropods; however, with an appropriate eukaryotic promoter, it can express foreign genes in several types of mammalian cell (Boyce and Bucher, 1996; Barsoum et al., 1997; Shoji et al., 1997; Condreay et al., 1999; Sarkis et al., 2000). The baculovirus may also be applied in conjunction AQ1

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with other viral vector to escape either pre-existing or therapy-induced anti-viral immunity.

Here, we report on the identification of baculovirus vector-mediated bispecific short-hairpin siRNA expression vectors (Sioud and Leirdal, 2002; Anderson et al., 2003) targeting the NP gene sequence of influenza virus A (Ge et al., 2003) and influenza virus B. This multiple vector showed remarkable ability to cope with either influenza virus A or B. The baculovirus vector is unable to replicate and do not have a cytopathic effect (CPE) in mammalian cells, even at a high multiplicity of infection (moi), this vector is now recognized as a useful viral vector, not only for the expression of foreign proteins in insect cells but also for gene delivery to mammalian cells.

Materials and Methods

Cell lines

Madin-Darby canine kidney (MDCK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; low glucose, Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Influenza virus strains

Influenza virus A/Puerto Rico/8/34 (A/PR/8/34) and influenza virus B/Ibaraki/2/85 (B/Ibaraki) were propagated following previously published procedures (Abe et al., 2003).

Plasmid construction

The pSV2neo-U6 plasmid vectors and pVL1393-based baculovirus vectors (Invitrogen, Carlsbad, CA) were used in this study. The NP genes of influenza viruses A and B were used as targets, and the shRNA expression plasmid vectors were constructed under the control of the human U6 Pol III promoter. We searched for siRNAs targeting the sequences in the influenza B virus NP gene. The selected sequences were used in a BLAST search against the human genome sequence to ensure that the latter was not targeted. To construct the shRNA expression cassette, the following DNA oligonucleotides were synthesized (here the lowercase letters at the 5′ end represent the template plasmid pSVneo-hU6 *Kpn*I site sequence, the uppercase letters represent the NP sequence, which includes the sense and antisense sequences of a particular NP gene region, the lowercase letters indicate the linker (9 nt), the stretch of 5 thymidines at the 3′ end (Pol III terminator) is underlined, and the lowercase letters at the 3′ end represent the template plasmid pSVneo-hU6 *Bam*HI site sequence): BNP-52, 5′-cCCAGAAGAAACAAC TTCTGttcaagagaCAGAAGTTGTTTCTTCTGGTTTTTg-3′ (sense) and 3′-catggGGTCTTCTTTGTTGAAGACaagttct ctGTCTTCAACAAAGAAGACCAAAAAcctag-5′ (antisense); BNP-273, 5′-cCCAGATGATGGTCAAAGCTttcaagaga AGCTTTGACCATCATCTGGTTTTTg-3′ (sense) and 3′-catggGGTCTACTACCAGTTTCGAaagttctctTCGAAAC TGGTAGACCAAAAAcctag-5′(antisense); BNP-824, 5′-cCGG CCTATGAAAAGATTCTttcaagagaAGAATCTTTTCAT AGGCCGTTTTTg-3′ (sense) and 3′-catggGCCGG ATACTTTTCTAAGAaagttctctTCTTAGAAAAGTA TCCGGCAAAAAcctag-5′ (antisense); BNP-1178, 5′-cCCTATGAAGACCTAAGAGTttcaagagaACTCTT AGGTCTTCATAGGTTTTTg-3′ (sense) and 3′-catggGG ATACTTCTGGATTCACAaagttctctTGAGAATCCAGA AGTATCCAAAAAcctag-5′ (antisense); BNP-1237, 5′-cGCATTAAAGTGCAAAGGTTttcaagagaAACC TTTGCACTTTAATGCTTTTTg-3′ (sense) and 3′-catg gCGTAATTTCACGTTTCCAAaagttctctTTGG AAACGTGAAATTACGAAAAAcctag-5′ (antisense); BNP-1492, 5′-cGGAAATCTACTCAAGATGAttcaagaga TCATCTTGAGTAGATTTCCTTTTTg-3′ (sense) and 3′-catg gCCITTAGATGAGTTCTACTaagttctctAGTAGAACTC ATCTAAAGGAAAAAcctag-5′ (antisense); ANP-1496, 5′-cGGATCTTATTTCTTCGGAGttcaagagaCTCCGAAG AAATAAGATCCTTTTTg-3′ (sense) and 3′-catggCCT AGAATAAAGAAGCCTCaagttctctGAGGCTTCTTTA TTCTAGGAAAAAcctag-5′ (antisense) (Ge et al., 2003); ran-shRNA, 5′-cACGCGCACACGCTCGCTCTCTttcaaga gaAGAGAGCGAGCGTGTGCGCGTTTTTTg-3′ (sense) and 3′-catggTGCGCGTGTGCGAGCGAGAGAaagttctctTCT CTCGCTCGCACACGCGCAAAAAAcctag-5′ (antisense). The top-strand and the bottom-strand DNA oligonucleotides were annealed at 95°C for 5 minutes, and incubated at room temperature for 10 minutes. The annealed oligonucleotides were cloned into the *Kpn*I and *Bam*HI cloning sites in the pSV2neo-U6 vector driven by the human U6 promoter. The *Eco*RI sites upstream of the U6 promoter and the *Bam*HI site downstream of the terminating sequence were digested, and subcloned into the *Eco*RI-*Bam*HI site in the baculovirus transfer vector (pVL1392) to generate the recombinant baculoviruses.

Design of bispecific short-hairpin siRNA constructs

The bispecific short-hairpin siRNA constructs contained 2 shRNAs, separated by a spacer sequence. We experimented with 2 different spacer sequences: UUCAACUU (Anderson et al., 2003) and UUCAAGAGA (Anderson et al., 2003). The shRNA constructs were targeted against sequences in the influenza A virus NP gene (ANP-1496) and the influenza B virus NP gene (BNP-52). To construct the bispecific short-hairpin siRNA expression cassette, the following DNA oligonucleotides were synthesized: AB1sh, 5′-cGGATCTTATTTCTTCGGAGttcaacttCTCCGA AGAAATAAGATCCTTCAACTTCCAGAAGAAACAAC TTCTGttcaacttCAGAAGTTGTTTCTTCTGGTTTTTg-3′ (sense) and 3′-catggCCTAGAATAAAGAAGCCTCaac ttgaaGAGGCTTCTTTATTCTAGGAAGGTCTTGG TCTTCTTTGTTGAAGACaacttgaaGTCTTCAACAA AGAAGACCAAAAAcctag-5′ (antisense); AB2sh, 5′-cGGATCTTATTTCTTCGGAGttcaagagaCTCCGA AGAAATAAGATCCTTCAAGAGACCAGAAGAAACAA CTTCTGttcaagagaCAGAAGTTGTTTCTTCTGGTTTTTg-3′ (sense) and 3′-catggCCTAGAATAAAGAAGCCTCaagttctc tGAGGCTTCTTTATTCTAGGAAGGTCTTGGTCTTCTT TGTTGAAGACaagttctctGTCTTCAACAAAGAAGACC AAAAAcctag-5′ (antisense). The DNA oligonucleotides were annealed at 95°C for 5 minutes and incubated at room temperature for 10 minutes. The annealed oligonucleotides were cloned into the *Kpn*I and *Bam*HI cloning sites in the pSV2neo-U6 vector driven by the human U6 promoter.

Inhibition of influenza virus replication by shRNA-expressing plasmid

MDCK cells were plated at 2.5×10^5 cells/well in a 6-well plate and cultured for 24 hours. Each shRNA expressing plasmid (1 μg) was transfected with the FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 8 hours, the MDCK cells were washed with phosphate-buffered saline (PBS) and infected with A/PR/8/34 or B/Ibaraki at a multiplicity of infection (moi) of 0.01 at 37°C for 1 hours. After infection, the cells were washed with PBS and cultured in MEM containing 0.5% heat-inactivated FBS and 4 μg/mL trypsin (Invitrogen) for 3 days. The culture supernatants were harvested, and the virus titers were determined by a plaque assay.

Preparation of baculoviruses

The baculovirus transfer vector, pVL1392-shRNAs, was constructed by inserting the shRNA expression cassettes into the cloning site of the baculovirus transfer vector pVL1392. The recombinant baculovirus containing shRNA of the NP genes of the influenza viruses A and B (rBV-shRNAs) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD Biosciences) following previously published procedures (Abe et al., 2003).

Inhibition of influenza virus replication by baculovirus-mediated bispecific short-hairpin siRNA

MDCK cells were plated at 2.5×10^5 cells/well in a 6-well plate and cultured for 24 h. The MDCK cells were washed with PBS and infected with A/PR/8/34 or B/Ibaraki (moi = 0.01) at 37°C for 60 minutes. After 6-h incubation, MDCK cells were washed with PBS and infected with shRNA-expressing baculovirus (moi = 50 or 100) at 37°C for 60 minutes. After infection, the cells were washed with PBS and cultured in DMEM containing 0.5% BSA and 4 μg/mL trypsin (Invitrogen) for 4 days. The supernatants were harvested and the titers of the viruses were measured by a plaque assay.

Western blotting analysis

The cells were washed with PBS and lysed on ice with cell lysis buffer (containing 0.2% NP-40 in PBS and protease inhibitors). Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Equivalent amounts of cell lysate were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The NP was detected and subsequently incubated with anti-influenza A NP mouse monoclonal (QED Bioscience, Inc. San Diego, CA) or anti-influenza B NP mouse monoclonal antibodies (Japan Millipore, Tokyo, Japan). After washing and subsequent incubation with horseradish peroxidase coupled to anti-mouse immunoglobulin G (IgG), the immunoreactive proteins were visualized by an ECL detection kit (Amersham Biosciences, Piscataway, NJ).

Northern blot analysis

Total RNA was extracted from AB-1-, BNP-52-, and ANP-1496-shRNA-expressing MDCK cells after using Trizol reagent according to the manufacturer's instructions (Invitrogen). Small RNAs <200 nt were separated concentrated using PureLink™miRNA Isolation Kit according to the manufacturer's instructions (Invitrogen). Small RNAs 5 μg samples were loaded onto a 15 % (w/v) polyacrylamide/7 M urea gel. After transfer to a Hybond-N™ nylon membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), synthetic LNA/DNA oligonucleotides (ANP-1496: 5′-GAATCTTATTTCTTCGGAG-Biotin-3′ and BNP-52: 5′-CCAGAAGAAACAACTTCTG-Biotin-3′) complementary to the antisense strand of the AB-1-shRNA were used as probes. The membranes were prehybridized for 1 hours in North2South hybridization buffer (Pierce) at 55°C and hybridized overnight to the 3′-biotin labeled LNA/DNA probe (30 ng/mL of hybridization buffer). Four posthybridization washes were done at 20 minutes each at 65°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate–0.1% sodium dodecyl sulfate (SDS). Detection of LNA/DNA/RNA hybrids was carried out using the North2South chemiluminescent detection system (Pierce).

PCR and analysis of amplified products

Total DNA was purified from MDCK cells and the presence of AcMNPV (moi = 50) or rBV-ANP-1496, BNP-52, AB1sh, and AB2sn (moi = 50) was detected by polymerase chain reaction (PCR) amplification of the viral genes vp39 and gp64. The sequences of the specific primers were as follows: vp39, 5′-TATGGCGCCGCGACAAATGAGAGTT-3′ (sense) and 5′-TTCGCCTGCAACACCAGGCGCAGG-3′(antisense);gp64, 5′-GGGTCCGTACAAGATTAAAACTTGGACAT-3′ (sense) and 5′-CGCTCAGACTGGTGCCGACGCCGCCAAAT-3′ (antisense); G3PDH, 5′-TCCACCACCCTGTTGCTGTA-3′ (sense) and 5′-ACCACAGTCCATGCCATCAC-3′ (antisense).

Results and Discussion

Construction of influenza B NP gene-targeted mono- and bispecific short-hairpin siRNA–expressing vectors

The NP protein is required for elongation and antitermination of nascent complementary RNA (cRNA) and vRNA transcripts (Beaton and Krug, 1986; Shapiro and Krug, 1988). Without newly synthesized NP, further viral transcription and replication are blocked, as is virion production. Therefore, we designed siRNA specific for the NP gene. To determine whether influenza B virus NP gene expression could be inhibited by this siRNA, we designed six 21-nucleotide (nt) siRNA sequences for DNA from different regions of the NP gene, according to the criteria used in influenza A NP gene-targeted siRNA (ANP-1496), and cloned them under the control of the U6 promoter (Lee et al., 2002) into a pSV2-neo vector. The resultant plasmids (pSV2neoU6-BNP-52, pSV2neoU6-BNP-273, pSV2neoU6-BNP-873, pSV2neoU6-BNP-1178, pSV2neoU6-BNP-1237, pSV2neoU6-BNP-1492, pSV2neoU6-ANP-1496, and pSV2neoU6-ran-shRNA) synthesized siRNA under the control of the U6 promoter (Lee et al., 2002; Suzuki et al., 2008), and directed the synthesis of a Pol III–specific RNA transcript, starting with guanosine at position one, as required for efficient transcription initiation from the U6 promoter. The 5 thymidines on the plasmid

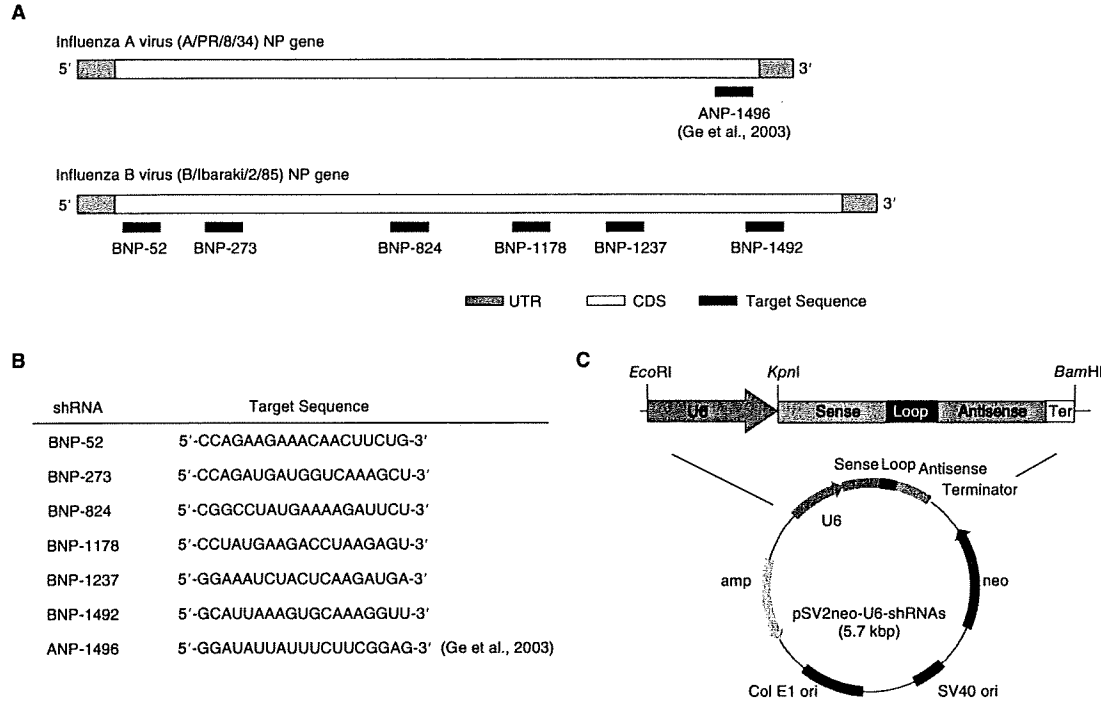
DNA that functioned as a termination signal for Pol III were added at the 3′ end. The resulting RNAs had a guanosine residue at the 5′ end, and 2 complementary 19-nt sequence motifs in an inverted orientation, separated by an 8-nt loop of nonhomologous sequence (5′-UUCAAGAGA-3′; Anderson et al., 2003). This RNA was predicted to fold back to form an shRNA with a 3′ overhang of 2 uridines and a 9-nt loop, and, when cleaved with endonuclease was expected to generate an siRNA of 21 bp with a guanosine at the 5′ end and 2 uridines overhanging at the 3′ end (Fig. 1A and 1B).

Inhibition of influenza B NP gene expression by shRNA

To determine whether the influenza B NP gene-targeted siRNAs could suppress the production of NP protein, MDCK cells were transfected with the shRNA-expressing vector (pSV2neoU6-BNP-shRNAs) using FuGENE 6, followed by B/Ibaraki virus infection, and the virus titers in the culture supernatants were measured. High virus titers were detected in cultures with the control vectors pSV2neoU6-ran-shRNA and pSV2neoU6-ter. Although inhibition of NP protein production occurred with all 6 constructs, the greatest inhibitory effect occurred with the siRNA targeting the 52–71 nt region of the influenza virus B NP gene (65%; Fig. 1D). We also examined the effect of the siRNA targeting the 1,496–1,515 nt region of the influenza virus A NP gene. As expected, low virus titers were detected in cultures transfected with pSV2neoU6-ANP-1496 (Fig. 1E). By contrast, the control vectors (pSV2neoU6-ran-shRNA and pSV2neoU6-ter) had no inhibitory effect on influenza A/PR/8/34 virus. These results point to a sequence-specific inhibitory effect of shRNA.

Downregulation of influenza viruses A and B by bispecific short-hairpin siRNAs

Next, we designed bispecific short-hairpin siRNA constructs against influenza NP genes to inhibit the expression of the corresponding viral protein. Bispecific short-hairpin siRNA constructs, containing an 8-nt intervening spacer (Anderson et al., 2003), targeted against either B/Ibaraki NP or A/PR/8/34 NP, were expressed by pSV2neoU6-ANP-1496/BNP-52 (AB1sh and AB2sh). Intracellular processing of bispecific short-hairpin siRNA constructs was reported previously (Sioud and Leirdal, 2002; Anderson et al., 2003). More recently, Gupta et al. reported that chimeric siRNA-riboxyme interfered strongly with the expression of target gene expression both RNA and protein levels (Gupta et al., 2008). The designs of the bispecific short-hairpin siRNA constructs are shown in Figure 2A. The resultant plasmids (pSV2neoU6-AB1sh and pSV2neoU6-AB2sh) synthesized shRNA under the control of the U6 promoter. To investigate whether influenza A and B virus NP gene targeting bispecific short-hairpin RNAs can be digested to monospecific products of expected size, monomeric siRNAs were performed by Northern blot analysis in AB1sh expressing



shRNA	Target Sequence
BNP-52	5′-CCAGAAGAAACAACUUCUG-3′
BNP-273	5′-CCAGAUGAUGGUCAAAGCU-3′
BNP-824	5′-CGGCCUAUGAAAAGAUUCU-3′
BNP-1178	5′-CCUAUGAAGACCUAAGAGU-3′
BNP-1237	5′-GGAAAUCUACUCAAGAUGA-3′
BNP-1492	5′-GCAUUAAAGUGCAAAGGUU-3′
ANP-1496	5′-GGAUAUUAUUUCUUCGGAG-3′ (Ge et al., 2003)

FIG. 1. siRNA interference with the production of influenza viruses A and B in MDCK cells. (**A**) Genomic profile of A/PR/8/34 and B/Ibaraki showing NP genes. (**B**) Influenza A and B virus NP region target sites and sequences used for the design of shRNAs. (**C**) Construction and schematic representation of transfer vector expressing NP-shRNAs. (*Continued*)

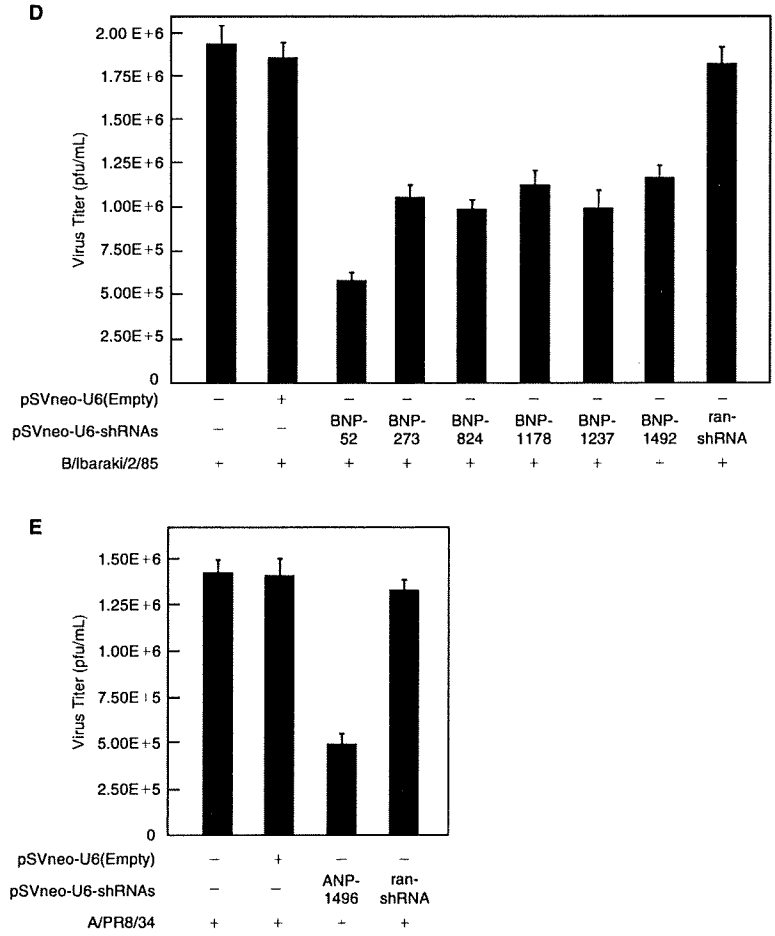


FIG. 1. (*Continued*) (D and E) Effects of each siRNA in influenza A and B virus NP genome. MDCK cells were transfected with each shRNA and ran-shRNA expression plasmid using FuGENE6, and then infected 8 h later with A/PR/8/34 or B/Ibaraki (moi = 0.01). After 3 days, culture supernatants were harvested and the virus titers were determined by a plaque assay. Data shown are from triplicate experiments.

MDCK cells. After 48 h, bispecific-short-hairpin siRNAs yielded products ~20 nt, the expected size of monomeric siRNAs [Fig. 2B, i (oligonucleotide probe, ANP-1496, lane 1: ANP-1496; lane 2: AB1sh); ii (oligonucleotide probe, BNP-52, lane 1: BNP-52; lane 2: AB1sh)].

Next, to determine whether bispecific short-hairpin siRNAs targeting influenza A and B virus NP genes could suppress the expression of both influenza A and B virus NP proteins, MDCK cells were cotransfected with plasmids expressing the shRNA, bispecific short-hairpin siRNA using FuGENE 6, followed by A/PR/8/34 virus or B/Ibaraki virus infection, and the virus titers in the culture supernatants were measured. Figure 2C shows that AB1sh and AB2sh, and ANP-1496 inhibited virus production in the A/PR/8/34 virus-infected MDCK cells, but not BNP-52. By contrast, the control vectors (pSV2neoU6-ran-shRNA and pSV2neoU6-ter) had no inhibitory effect of virus production in the A/PR/8/34 virus-infected MDCK cells. Furthermore, we examined the inhibition of virus production in the B/Ibaraki-infected MDCK cells. The NP gene targeting U6-shRNAs showed the sequence-specific inhibitory effects of the B/Ibaraki virus, but not ANP-1496 (Fig. 2D). By contrast, the control vectors (pSV2neoU6-ran-shRNA and pSV2neoU6-ter) had no inhibitory effect of virus production in the B/Ibaraki virus. These results suggest that the bispecific short-hairpin siRNA induced the sequence-specific inhibition of either influenza A or B virus. We also tested the 2 different spacer sequences, and found no significant differences between them (Fig. 2C and 2D). Thus, our results confirmed the ability of the bispecific constructs to effectively downregulate both the influenza viruses A and B.

Downregulation of influenza viruses A and B by baculovirus-mediated short-hairpin siRNA

To characterize the efficiency of baculovirus as a vehicle for gene therapy, we selected the NP gene of influenza viruses A and B as the target site (Fig. 3A), and constructed a recombinant baculovirus vector expressing the shRNAs and bispecific short-hairpin siRNAs. Figure 3A shows the baculovirus transfer vectors used in this study. The baculovirus transfer vectors pVL1393-ANP-1496 and pVL1393-BNP-52 (monospecific-shRNAs), and pVL1393-ANP-1496/BNP-52 (AB1sh and AB2sh) carried NP-shRNA under the control of the Pol III,

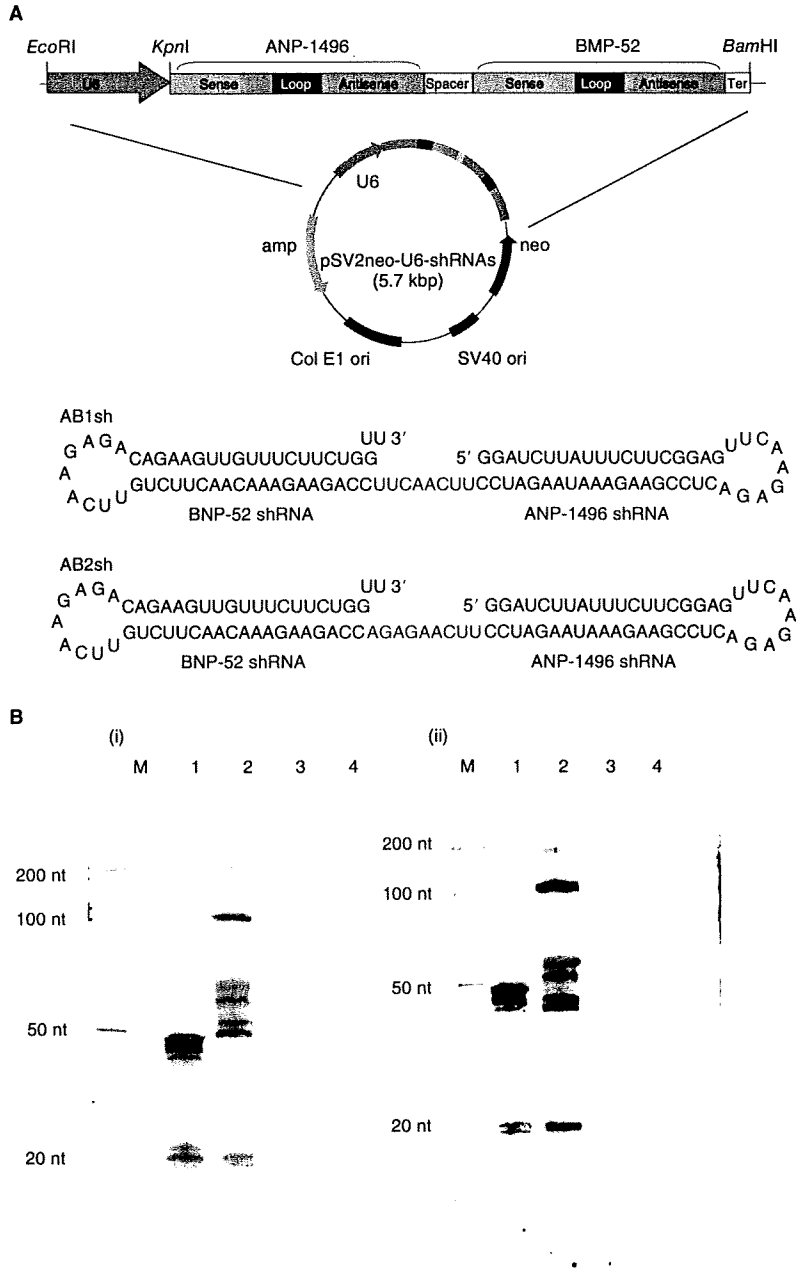


FIG. 2. Effects of bispecific short-hairpin siRNA on influenza A and B virus production in MDCK cells. (**A**) Bispecific construct targeted to the influenza A and B virus NP region and schematic representation of transfer vector expressing bispecific NP-shRNAs. (**B**) Cellular processing of bispecific short-hairpin siRNAs into monomers. To demonstrate the intracellular cleavage of the bispecific construct into the respective monomers, MDCK cells were cotransfected with plasmids expressing the shRNA or bispecific short-hairpin siRNA using FuGENE 6. The mixture was run on a 15% polyacrylamide TBE urea gel after 48 hours. (i) NLA/DNA oligonucleotide probe (ANP-1496: 5′-GAATCTTATTTCTTCGGAG-Biotin-3′); M, Size marker 20-bp ladder; 1, ANP-1496; 2, AB1sh; 3, U6-ter 4, mock; (ii) LNA/DNA oligonucleotide probe (BNP-52: 5′-CCAGAAGAAACAACTTCTG-Biotin-3′); M, Size marker 20-bp ladder; 1, BNP-52; 2, AB1sh; 3, U6-ter 4, mock. (*Continued*)

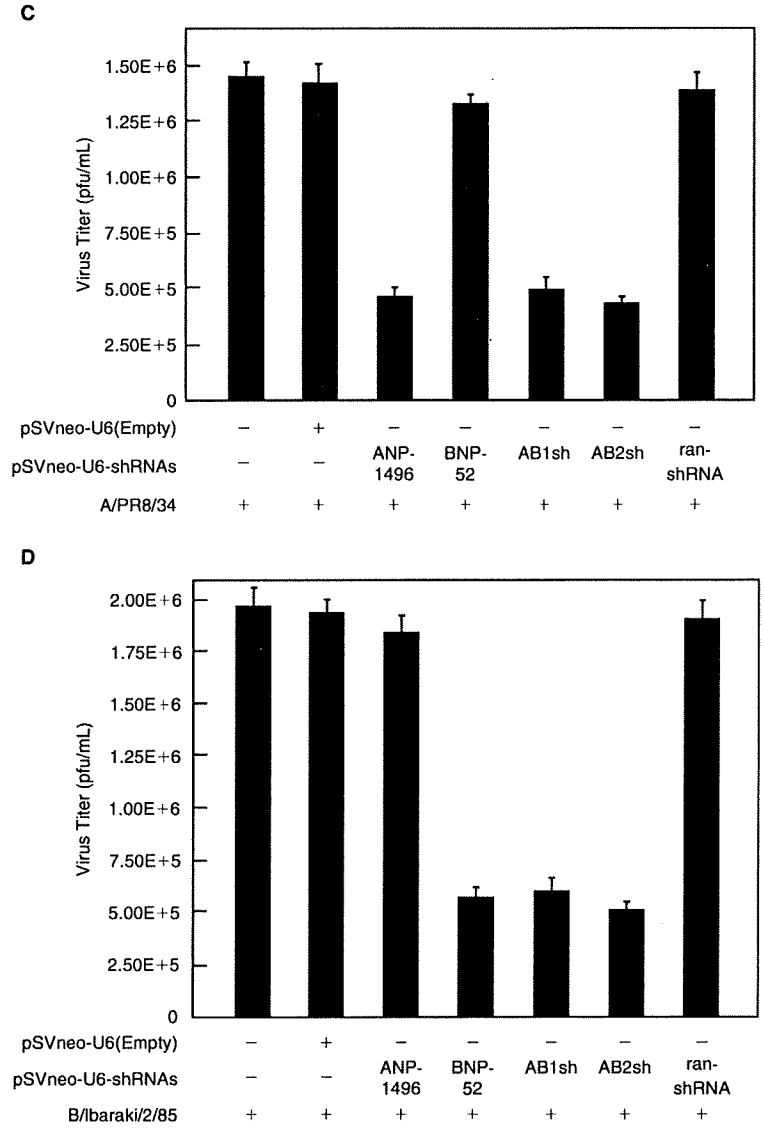


FIG. 2. (*Continued*) (C and D) MDCK cells were cotransfected with plasmids expressing the shRNA, bispecific short-hairpin siRNA, and ran-shRNA using FuGENE 6, followed by A/PR/8/34 virus or B/Ibaraki virus infection, and the virus titers in the culture supernatants were measured. Data shown are from triplicate experiments.

U6 promoter. The recombinant baculovirus containing the shRNA genome (Ac-shRNA) was propagated following previously published procedures (Abe et al., 2003). The viruses were produced at high titers, ranging from 1.5×10^8 to 1.2×10^9 pfu/mL, and the expression of the gp64 and vp39 genes was detected in the baculovirus vector–infected MDCK cells by reverse-transcription (RT)-PCR analysis (Fig. 3B).

To determine whether bispecific short-hairpin siRNAs targeting influenza A and B virus NP genes can suppress the production of these viruses, MDCK cells were infected with A/PR/8/34 or B/Ibaraki (moi = 0.01). After 6 hours, the influenza virus–infected MDCK cells were infected with shRNA or bispecific short-hairpin siRNA expression baculovirus (moi = 50 or 100). At 4 days postinfection, the culture supernatants were harvested and assayed to determine the virus titers by plaque assay. Figure 4A showed that rBV-AB1sh and AB2sh, and ANP-1496 strongly inhibited virus production in the A/PR/8/34 virus-infected MDCK cell, whereas high virus titer was detected with rBV-BNP-52 infected cells. The influenza B virus production was significantly reduced by infection with rBV-BNP-52, rBV-AB1sh, and rBV-AB2sh, but not with rBV-ANP-1496 (Fig. 4C). These results indicate that the NP-targeting bispecific short-hairpin siRNA significantly inhibited the production of both influenza viruses A and B, whereas the empty baculovirus vector alone has no such effect. Furthermore, there was a direct correlation between the level of the virus and NP protein production (Fig. 4B and 4D).

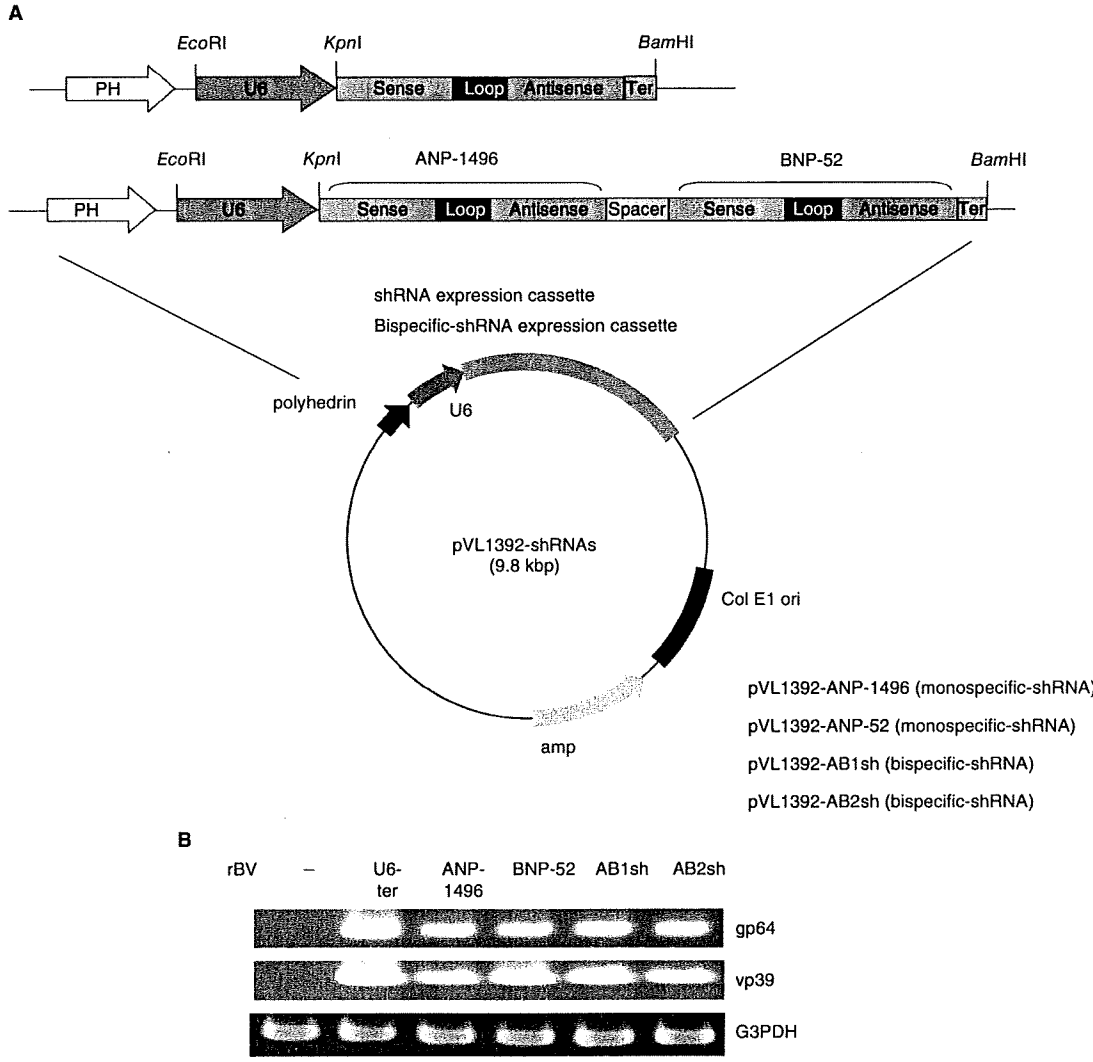


FIG. 3. (**A**) Construction and schematic representation of baculovirus transfer vector expressing influenza A and B virus NP-shRNAs. (**B**) *In vitro* AcMNPV infectivity. MDCK cells were infected with rBV-ANP-1496, BNP-52, AB1sh, and AB2sh, and control, rBV-U6-ter. After 48 hours, cells were harvested and total DNA was isolated. The AcMNPV genome was PCR-amplified using AcMNPV-gp64 and vp39 specific primers.

In conclusion, we have demonstrated the inhibition of influenza viruses A and B production by baculovirus-mediated bispecific short-hairpin siRNA expression vectors. This multiple vector showed remarkable ability to cope with both influenza virus A and B. Baculovirus-mediated bispecific short-hairpin siRNAs targeting the NP proteins of influenza viruses A and B have been shown to be particularly potent in inhibiting influenza virus production in cell lines, whereas that of the empty baculovirus vector showed no such effect. Now baculovirus can be used as an efficient vector for gene delivery mammalian cells for a wide variety of applications.

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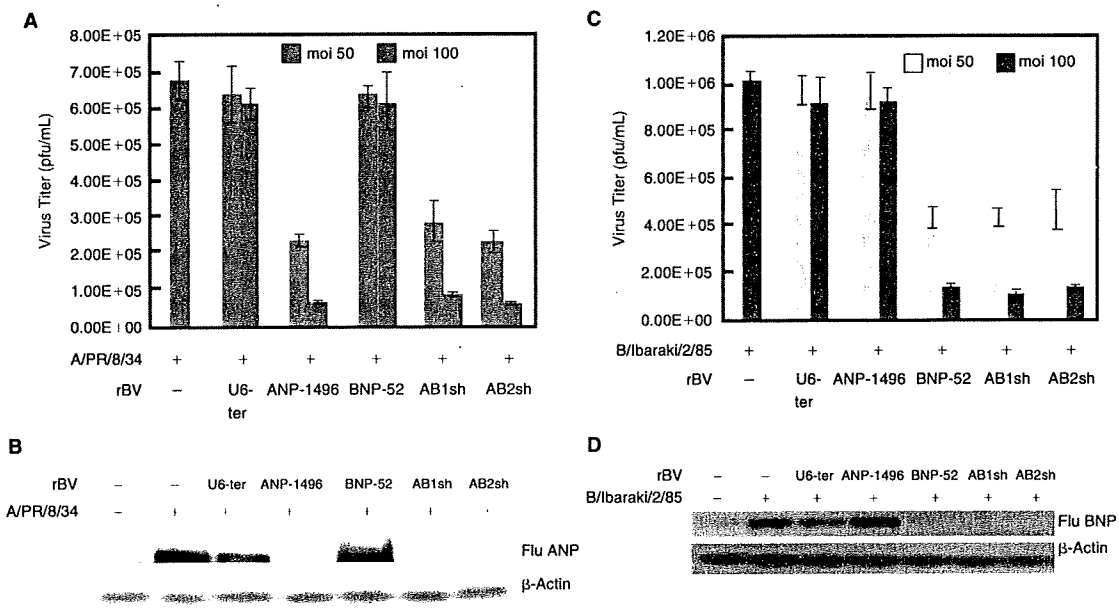

FIG. 4. Baculovirus-mediated bispecific short-hairpin siRNAs inhibit the accumulation of influenza A and influenza B virus NP mRNA. (**A** and **C**) MDCK cells were infected with A/PR8/34 or B/Ibaraki (moi = 0.01). After 6 hours, influenza virus-infected MDCK cells were infected with shRNA or bispecific short-hairpin siRNA–expressing baculovirus (moi = 50 or 100). At 4 days postinfection, the culture supernatants were harvested and assayed to determine the virus titers by plaque assay. Data shown are from triplicate experiments. (**B** and **D**) Western blotting analysis of NP protein production using the anti-influenza A and B antibodies at rBV-shRNAs (moi = 100).

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Baculovirus activates murine dendritic cells and induces non-specific NK cell and T cell immune responses

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ABSTRACT

We previously reported that the baculovirus induced a strong host immune response against infections and malignancies. Among the immune cells, the dendritic cells were most strongly infected and activated by the baculovirus, although the exact mechanism remained unclear. Here, we evaluated the non-specific immune responses of bone marrow-derived dendritic cells (BMDCs) after infection by a wild-type baculovirus. MHC class I and II molecules and co-stimulation molecules (CD40, CD80, and CD86) on BMDCs were up-regulated by baculovirus infection. At the same time, the BMDCs produced pre-inflammatory cytokines (IL-6, IL12p70, and TNF-α) and IFN-α. NK cells showed IFN-γ production, CD69 up-regulation, and enhanced cytotoxicity when they were co-cultured with baculovirus-infected BMDCs. T cells showed IFN-γ production, CD69 up-regulation, and cell proliferation. *Ex vivo* analysis performed *in vitro* produced similar results. These findings suggested that baculovirus-infected dendritic cells induce non-specific immune responses and can be used as an immunotherapeutic agent against viral infections and malignancies, together with present therapeutic drug regimens.

1. Introduction

The *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) is an enveloped insect virus (baculovirus) with a circular double-stranded DNA genome of approximately 130 kbp in length [1]. Baculoviruses have been used extensively in recombinant protein-expression systems [2,3] and as biopesticides [4,5]. Recently, the use of baculoviruses as vectors in gene therapy has been investigated [6,7]. Baculoviruses infect a range of mammalian cell types, but do not replicate [8] and can express foreign genes under the control of mammalian specific promoters [9]. When these cells are infected by baculoviruses, the viral envelope protein gp64 binds to phosphatidyl inositol on the cell surface, and the baculovirus is incorporated into the intracellular compartment by endocytosis [10]. GFP, luciferase, and other reporter genes inserted into recombinant baculoviruses can be expressed *in vitro* in mammalian cells as well as *in vivo* in animals [11]. Several previous articles have reported on baculoviruses that have been developed as vaccines against the influenza virus [12,13], the avian influenza virus [14], malaria [15], and other diseases [16]. Facciabene et al. reported that HCV E2 protein expressing recombinant baculovirus vector induced antigen specific $CD8^+$ T cell in mice [17]. Shan et al. [18] reported that a recombinant baculovirus containing the Epstein–Barr virus nuclear antigen-1 (EBNA-1) gene could express foreign genes for long periods of time in mammalian cells. Recently, Abe et al. [19] reported baculovirus-induced host-cell activation through Toll-like receptor 9 (TLR9). TLR9 was shown to recognize unmethylated CpG sequences, comprising single-stranded DNAs of approximately 20 bp in length, which are abundant within the baculovirus genome. Dendritic cells (DCs) are the most potent antigen-presenting cells of the immune system, and play pivotal roles in the initiation and maintenance of immune responses against viruses [20]. NK cells are the primary effector cells of the innate immune system, and are important for the immunological control of virus infection and tumors via the production of cytokines, mainly IFN-γ, and the potent lysis of transformed or infected cells without prestimulation [21]. Here, we demonstrated that baculovirus infects DCs more potently than other immune cells, and induced anti-tumor effects via immune activation [22]. The relationships among the immune responses of DCs and other immune cells are not fully understood. We therefore, investigated the non-specific immune responses of baculovirus-infected DCs, and the relationship between activated DCs, $CD4^+$ and $CD8^+$ T cells, and NK cells.

Abbreviations: BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; ELISA, enzyme-linked immunosorbeny assay; iDC, immature dendritic cell; MOI, multiplicity of infection.

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