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Screening Efficient siRNAs in vitro as the Candidate Genes for Chicken Anti-Avian Influenza Virus H5N1 Breeding[1]

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Abstract—The frequent disease outbreaks caused by avian influenza virus (AIV) not only affect the poultry industry but also pose a threat to human safety. To address the problem, RNA interference (RNAi) has recently been widely used as a potential antiviral approach. Transgenesis, in combination with RNAi to specifically inhibit AIV gene expression, has been proposed to make chickens resistant to avian influenza. For the transgenic breeding, screening the efficient siRNAs in vitro as the candidate genes is one of the most important tasks. Here, we combined an online search tool and a series of bioinformatics programs with a set of rules for designing the siRNAs targeting different mRNA regions of AIV H5N1 subtype. By this method we chose five rational siRNAs, constructed five U6 promoter-driven shRNA expression plasmids contained the siRNA genes, and used these to produce stably transfected Madin-Darby canine kidney (MDCK) cells. Data from virus titration, IFA, PUI-stained flow cytometry, real-time quantitative RT-PCR and DAS-ELISA analyses showed that all five stably transfected cell lines were effectively resistant to viral replication when exposed to 100 $CCID_{50}$ of AIV, and we finally chose the most effective plasmids (pSi-604i and pSi-1597i) as the candidates for making the transgenic chickens. These findings provide baseline information for breeding transgenic chickens resistant to AIV in combination with RNAi.

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Key words: RNAi, SiRNA, Avian influenza virus, MDCK cells, transgenic breeding

INTRODUCTION

Avian influenza virus (AIV) belongs to the family Orthomyxoviridae. Its genome consists of single-stranded, negative-sense RNA composed of eight segments that encode 10 or 11 proteins [1]. The virulence of AIV results in part from its ability to escape from protective immunity by antigenic drifts [2] and shifts [3]; consequently, existing vaccines have only a limited value. And although many antiviral drugs have been approved for treatment and or prophylaxis of influenza, their use is limited because of potentially severe side effects and the possible emergence of resistant viruses [4]. Development of a new rapid-acting and effective antiviral strategy against AIV infection is therefore warranted.

RNA interference (RNAi) has been used experimentally to interfere with the replication of many animal viruses [5–8]. Recent studies have also demonstrated the utility of vector-based siRNA expression driven by RNA polymerase III promoters such as U6 [9–11].

Transgenesis, in combination with RNAi to specifically inhibit AIV gene expression, has been proposed to make chickens resistant to avian influenza. However, the efficiency of gene silencing varies significantly between siRNAs targeted to different positions of a gene [12, 13]. Until now, no consensus on choosing the siRNA sequence has evolved, and it remains difficult to predict the best or most effective target site.

In this study, based on the mRNA sequences of AIV H5N1 subtype proteins, we designed five siRNAs by an online search tool combined with a series of bioinformatics programs. Their abilities to inhibit AIV replication have been investigated in stably transfected MDCK cells at the gene, protein and cellular morphology levels. Our results indicated that they could be considered as powerful candidate genes for breeding transgenic chickens.

EXPERIMENTAL

Targets selection and plasmids construction. 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 H5N1 subtypes, with target regions having no more than 1 mismatch in regions of 21 nucleotides within all virus subtypes. Accordingly, the genes of A/duck/Fujian/13/2002(H5N1) NP, PA, PB1 and

[1] The article is published in the original.

Properties of siRNAs targeted to the AIV genes

siRNA	Target gene	Position[a]	Target sequence	% of GC	H-b index
604i	NP	604–624	AATGATCGGAATTTCTGGAGA	38.1	31.8
1442i	PA	1442–1462	AGCTGATTCCGATGATAAGCA	42.8	34.6
142i	NS1	142–162	AGCACCCTTGGTCTGGACATC;	57.1	46.7
740i	PB1	740–760	AGATCAGAGGGTTCGTGTACT	47.6	34.8
1597i	PB1	1597–1617	ATATGATAAACAATGACCTGG	33.3	23.6
0i	Scrambled[b]		AAGGGAAAGATTTGATAACCC	38.1	

[a] Nucleotide positions of siRNA according to the genomic RNA of AIV FJ13.
[b] Scrambled shRNA with no significant sequence similarity to the targeted genes of AIV.

NS1 proteins (GenBank accession nos. AY585421, AY585463, AY585484, and AY585442) were selected to provide the targets for the siRNAs.

First, siRNAs were sought using the online *Target Finder and Design Tool* (http://www.ambion.com/techlib/misc/siRNA_finder.html) according to the following criteria: the sequence should be 50–100 bp downstream of the start codon; avoid stretches of 4 or more polypurines or polypyrimidines; and with a GC content of 30–60%.

Second, to avoid potential off-target silencing effects, a BLAST search was conducted against chicken and dog (for MDCK cells) genome sequences, and only candidate siRNAs with more than 4 nt mismatches against unrelated sequences were selected.

Third, using the *RNA structure 4.2* software (http://rna.urmc.rochester.edu) to predict the mRNA secondary structures of the AIV proteins, siRNAs were selected with target regions having no hairpin structure [12]. Hairpin-like structures within siRNA can reduce the effective concentration and silencing potential of the siRNA, so the siRNA sequence cannot contain internal sequences or palindromic sequences that may form fold-back structures. The relative stability and propensity to form internal hairpins can be estimated by *Primer Premier* 5.0 (Primer Biosoft International, Canada).

The final step in design was to calculate the hydrogen bond (H-b) index of each siRNA, as described by [14], and to select those that had the lowest H-b index. The siRNAs sequences finally chosen are listed in table. psiSTRIKE (Promega) was used as the parental vector for constructing the U6-driven shRNA expression plasmids. The shRNA expression vectors were generated by inserting annealed oligonucleotides (synthesized by Sangon) which consisted of two complementary sequences placed in opposition to each other with the insertion of a 9-mer spacer sequence.

Cell culture, transfection and stable selection. MDCK cells were grown in DMEM medium (Gibco) with 10% fetal bovine serum (FBS; HyClone), 100 U/ml penicillin, and 100 μg/ml streptomycin (pH 7.4) at 37°C in 5% CO_2. The cells were transfected with the six shRNA-expression plasmids respectively, using Lipofectamine 2000 reagent (Invitrogen). The transfected cells were selected with 800 μg/ml G418 (Merck) for 3 weeks with medium changes twice one week. Individual colonies were picked and amplified in the same medium with 400 μg/ml G418. We detected the stability of the transfectants by PCR and northern blot, selected the transfectants which stably expressed the shRNAs for at least 5 continual passages, and maintained them as cell lines, stored every batches in liquid nitrogen for the subsequent experiments.

For the PCR detection, the genomes of the colonies were extracted using Genomic DNA Extraction Kit (TransGen Biotech) and the following primers were used to detect shRNA gene insertion: 5'-CGATACAAGGCTGTTAGAGA-3' (sense) and 5'-CAGGCTTTACACTTTATGCT-3' (antisense). To detect the expression of shRNAs, total RNA was extracted from all transfectants with Trizol reagent (Invitrogen) and 20 μg aliquots were fractionated on 15% denaturing polyacrylamide-7M urea gels, then transferred onto positively charged nylon membranes (Amersham). After UV crosslinking, the membranes were probed with ^{32}P-labeled DNA probe complementary to the antisense strand of the shRNA. Probed membranes were washed and exposed to autoradiographic film (Kodak).

Viruses stock and titration. Influenza A virus strains A/duck/Fujian/13/2002 (FJ13), H5N1 subtype, was kindly provided by Professor Hongwei Gao (Academy of Military Medical Sciences, Changchun). The virus was grown in MDCK cells, and virus titers were measured by a standard 50% cell culture infective dose ($CCID_{50}$) assay [15].

The stable transfectants and control (untreated) cells were added to 24-well culture plates at a concentration of 2×10^4 cells/well. Twelve hours later, all cells were infected with 100 $CCID_{50}$ (50% cell culture infectious doses) of FJ13. To detect the off-target effect, all cells were infected with 100 $CCID_{50}$ of CPV-2 (stored by our laboratory), in parallel experiments. Supernatants were collected at 60h post inoculation

(p.i.), and the virus titers were determined as $CCID_{50}$ on normal cultured MDCK cells.

Confocal laser scanning microscopy and immunofluorescence assay (IFA). Stable transfectants and control cells were grown on glass coverslips in 6-well plates. At 72 h p.i., the FJ13 coverslips were fixed with 4% paraformaldehyde for 15 min and permeabilised with 0.2% Triton X-100 (in PBS) for 10 min at room temperature. Cells were then incubated with mouse anti-HA mAb (kindly provided by Dr. M. Sun) for 30 min at 37°C. After additional washes, the cells were incubated with FITC-labeled goat anti-mouse IgG (Sigma) for 1 h at 37°C. For nuclear staining, coverslips were incubated with 100 μg/ml propidium iodide (PI) for 5 min at room temperature, washed, and mounted. Fluorescence was observed with a confocal laser scanning microscope (Nikon TE2000-E).

Apoptosis assay by flow cytometry. At 24 h, 48h and 72h p.i. with 100 $CCID_{50}$ of FJ13, the stable transfectants and control cells were trypsinized, washed and fixed in 70% ethanol at 4°C for 18 hours. Cells were then harvested and sequentially incubated with RNase A (100 μg/ml) at 37°C for 30 minutes and PI (50 μg/ml) at 4°C for 20 minutes. Cell samples were analysed in a flow cytometer (XL, Coulter), with the apoptotic cell population being identified by Pi-stained DNA content. The percentage of apoptotic cells was calculated using MCYCLE 2 software (Phoenix, Germany).

Real-time quantitative RT-PCR (QRT-PCR) analysis. For quantitation of AIV replication in the stable transfectants and control cells from 24 h to 120 h (sampling every 24 h) following infection with 100 $CCID_{50}$ FJ13, total RNA was extracted from 250 μl cell lysate supernatants using Trizol reagent (Invitrogen), and QRT-PCR was performed using SYBR® PrimerScript™ RT-PCR Kit II (TaKaRa) and a DNA Engine Opticon™ 2 Real-Time PCR Detector (MJ Research Inc., Waltham, MA). The specific primers used for identification of the HA gene were: 5'-GCCATTCCACAACATACACCCTC-3' (sense) and 5'-TTCCCTGCCATCCTCCCTC-3' (antisense).

The β-actin gene of MDCK cells was quantified as an internal RNA control. All measurements were normalized to expression of the p-actin gene, and the copies of HA gene mRNAs were quantified by comparison with a standard curve derived from known amounts of plasmids containing the HA gene (pEASY-HA; pEASY vector provided by TransGen Biotech).

Double-antibody sandwich indirect ELISA (DAS-ELISA). To compare the differences in HA protein expression of AIV between the stable transfectants and control cells, supernatants obtained from 24 h to 120 h p.i. (sampling every 24 h) were analysed by DAS-ELISA. For the DAS-ELISA, the plates were coated with HA-specific mAb (1:1000 dilution) at 4°C. After washing and blocking with PBST containing 1% gelatin (Sigma) for 1 h at 37°C, assay samples (100 μl/well) were added and incubated for 1 h at 37°C. The plates were then washed, and chicken anti-FJ13 serum diluted 1 : 300 with antibody diluent was added into each well. The plates were then incubated for 1 h at 37°C followed by three washes, and goat anti-chicken IgG HRP diluted 1 : 2000 (Sigma) in antibody diluent was then added to each well. After incubation for 1 h at 37°C, the plates were washed, then incubated with TMB (3,3',5,5'-tetramethylbenzidine, substrate of HRP). The reaction was stopped with 2 M H_2SO_4, and colour development measured in a kinetic microplate reader (Bio-Rad model 550, USA) with a 450-nm filter. For standardization, known amounts of FJ13 (tested by $CCID_{50}$) were serially diluted twofold to 1 : 128 and assayed by DAS-ELISA as above.

Statistical analysis. All samples in the above experiments were run in triplicate, and results are presented as mean ± standard deviation. The significance of variability among the experimental groups was determined by one-way or two-way ANOVA using GraphPad Prism^R Version 4.0 software (San Diego, CA). Differences among experimental groups were considered statistically significant if $P < 0.05$.

RESULTS

Targeting Positions Determination and Plasmids Charaterization

Using ambion's online tool, we obtained 83 candidate siRNAs. After our local screening, we finally selected 5 rational ones for the subsequent experiments. The secondary structures of the mRNA at the siRNA target regions were shown in Fig. 1a, and constructed five shRNA-expression plasmids (pSi-604i, pSi-1442i, pSi-142i, pSi-740i and pSi-1597i). As a control, pSi-0i containing scrambled shRNA with no sequence similarity to the targeted genes was also constructed (Fig. 1b) and used to transfect MDCK cells.

Characterization of Stable Transfectants

By the selection of G418, thirty cell colonies (5 colonies for each plasmid) were isolated and amplified. After screened by PCR and northern blot, six stable transfectants (1 for each plasmid) were selected and maintained as cell lines (named NP-604, PA-1442, NS1-142, PB1-740, PB1-1597 and SCRM). PCR analysis showed that each cell line incorporated the sequence that contained the complete U6 promoter and shRNA gene into its genome (Fig. 1c). The result of northern blot dedicated that all the inserted genes could successfully transcript the shRNAs (Fig. 1d).

Inhibitory Effects of AIV Replication by Stably Expressed shRNAs

The efficacy of shRNAs to inhibit AIV replication in stably tranfected cells was analyzed. For SCRM and

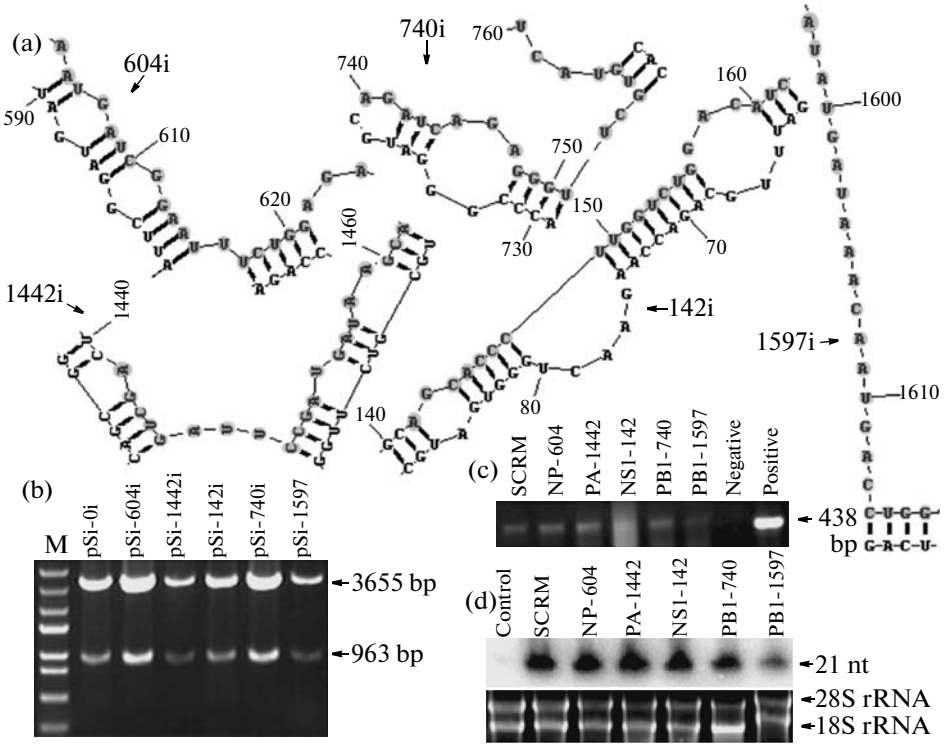

Fig. 1. Targeting positions, plasmids charaterization and characterization of stable transfectants. (a) The local secondary structures of AIV mRNA at the regions targeted by the siRNAs. The nucleotides targeted by siRNA are colored in red. The RNA structures were generated using the *RNA structure 4.2* software. (b) Detection of hairpin inserts by *Pst* I digestion. Lanes 1-6 of this 1% agarose gel shows the expected size fragments for successfully ligated hairpin inserts, and Lane M is a DNA makerlll (TransGen Biotech). (c) Detection of shRNA gene insertion by PCR, using the genome of the stable transfectants. Positive control (using a constructed U6 promoter-driven shRNA plasmid as the template) and negative control (using the normal MDCK cell genome as the template). (d) Northern blot analysis of shRNAs expression under control of U6 promoters. Each lane was loaded with equal amount of total RNA (20 μg/lane) extracted from stable transfected MDCK cells.

control (untreated) cells, cytopathic effects (CPE) were evident even at 24 h p.i. with 100 CCID50 FJ13. In contrast, all stable transfectants obviously reduced susceptibility to AIV infection. Of the 5, NP-604, PA-1442 and PB1-1597 were the most effective, delaying the appearance of CPE until 72 h p.i., NS1-142 and PB1-740, however, showed weaker inhibition, but still significant compared to the controls. CPE at 60 h p.i. is shown in Fig. 2a. IFA analysis at 60 h p.i. showed that all 5 transfectants exhibited only a weakly positive response compared to SCRM and control cells, with NP-604 and PB1-1597 showing least viral antigen (Fig. 2b).

The virus titers of supernatant samples collected at 60 h p.i. were determined. While titers in NP-604, PA-1442, PB1-740, PB1-1597 and NS1-142 cells were markedly reduced compared to controls, no significant inhibition was observed in SCRM. No significant differences were observed among the virus titers of supernatants obtained from each well of CPV-infected cells, indicating that there was no off-target inhibition of other virus' replication in MDCK cells by the shRNAs chosen here (Fig. 3a).

The apoptotic index indicated that significant differences were exhibited between the stable transfectants and control cells at all time points examined ($P < 0.001$), while there was no significant difference between SCRM and control cells ($P > 0.05$) (Fig. 3b).

QRT-PCR analysis indicated that the mRNA levels of the HA gene in NP-604 and PB1-1597 cells decreased markedly compared to controls at all time points p.i. ($P < 0.001$); PA-1442, PB1-740 and NS1-142 showed relatively weaker inhibition effects after 72 h p.i. ($P < 0.05$). No significant differences were exhibited between SCRM and control cells (Fig. 4a).

The results of DAS-ELISA revealed that the HA protein level in all specific targeting transfectants decreased significantly compared to the control cells ($P < 0.001$), with NP-604, PB1-1597 being the most effective, they showed markedly inhibition effects until 120 h p.i. PA-1442, PB1-740 and NS1-142 showed relatively weaker inhibition effects after 96 h p.i. No significant differences were observed between SCRM and control cells. (Fig. 4b).

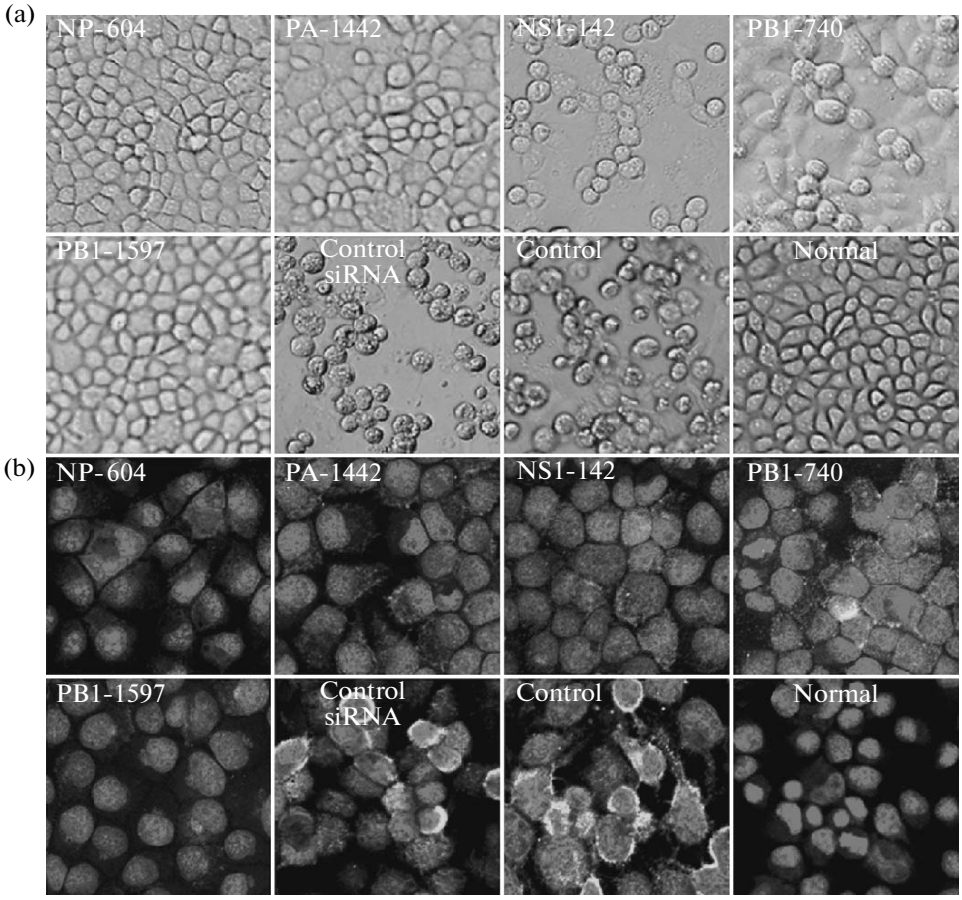

Fig. 2. AIV-induced CPE comparison and IFA analysis, all stable transfectants and control cells were infected with 100 $CCID_{50}$ of FJ13. (a) CPE was examined at 60 h p.i. (b) At the same time, the stable transfectants and control cells were fixed and subjected to IFA using a monoclonal antibody against AIV H5N1 subtype HA protein. All 5 transfectants exhibited only a weakly positive response compared to SCRM and control cells. The green fluorescence values calculated by EZ-C1 3.00 FreeViewer software showed all 5 transfectants exhibited only a weakly positive response compared to SCRM and control cells ($P < 0.001$) (data not shown).

DISCUSSION

Although a set of guidelines for the selection of potential siRNA duplexes has been proposed [16–18], there are no reliable methods for selecting the effective target regions without practical test. It is well known that different siRNA duplexes induce different levels of RNAi activity [5, 12]. Thus, it is important to screen appropriate target regions conferring a strong RNAi activity within the AIV genome. Among avian influenza viruses, extensive differences in nucleotide sequences are present in different subtypes. To design shRNAs that remain effective despite antigenic drifts and antigenic shifts, therefore, we focused on regions of the viral genome that are conserved among different H5N1 subtypes. Influenza NP, PA and PB1 are essential early proteins during viral replication, providing ideal targets for RNAi [4]. NS1 protein has been reported to have anti-RNAi activity [19], and therefore inhibition of NS1 protein may suppress this activity. Consequently, we chose NP, PA, PB1 and NS1 as our target proteins. No siRNAs for HA and NA were designed due to the extensive variations in these genes among different virus strains.

It is known that the target position, the secondary structure of mRNA at the target site and the off-target effect are major considerations in the design process. There are many online selection methods, but most are rather unselective as to candidate genes. Here, we combined an online search tool with a series of bioinformatics programs, and set a series of parameters for designing the siRNAs, thereby considerably narrowing selection of candidate siRNAs and permitting easy choice of the most rational ones.

In this study, we dedicated to make the stable transfectants for the siRNAs' inhibitory detection, because if the percentage of transient transfection was lower than 30%, it would be difficult to detect inhibitory effection [20]. Another aim for us to make the stable transfectants is that we wanted to detect psiSTRIKE vector's recombine ability with the host-cells' genome. Because we wanted to use them for making transgenic

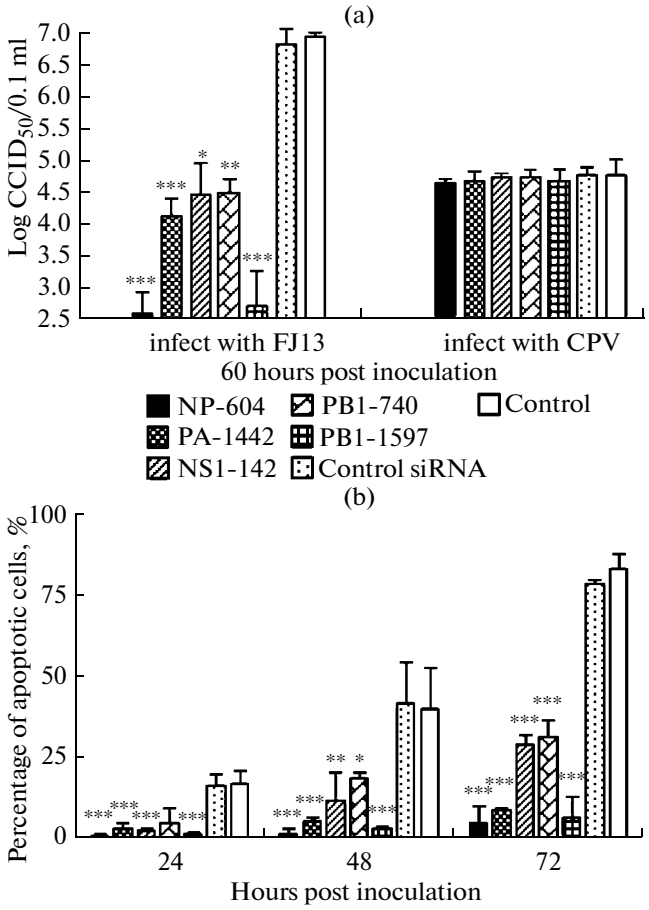

Fig. 3. All cells were infected with 100 $CCID_{50}$ of FJ13, the supernatant samples were collected for viral titration, and cells were trypsinized and fixed with 70% ethanol for apoptosis analysis. (a) Virus titers of supernatant samples collected at 60 h p.i. were determined as $CCID_{50}$ on normal cultured MDCK cells. (b) Apoptosis assay by Pi-staining flow cytometer, at 24 h, 48 h and 72 h p.i. All cell samples were stained with PI and analysed on a flow cytometer. The percentage of apoptotic cells was calculated by comparing the number of cells in the apoptotic population with the total number of cells counted, using MCYCLE 2 software. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

chickens, we must firstly demonstrate whether they could recombine with the host-cells' genome or not.

In summary, our experiments have shown that all siRNAs selected by our design method were capable of effectively inhibiting AIV replication in vitro, all transfectants except SCRM markedly inhibited AIV replication at any time points post-infection and delayed the appearance of CPE. In particular, transfectants NP-604 and PB1-1597 displayed a marked resistance to AIV replication, they showed markedly inhibition effects for about 120 hours. If the transgenic chickens which contained these genes also have the same resistance to AIV replication, the acute-mortality induced by highly pathogenic AIV infection would be remarkably decreased. And, this resistance could earn time for the early immunity's response.

In our experiments, we also infected the transfectants by another subtype (H9N7) of AIV. They showed inhibitory effects, but not very markedly (data not shown), probably by the presence of some mismatches between the siRNAs and viruses' genome.

RNAi has been used experimentally to interfere with the replication of many animal viruses, but the challenge now is how to apply them conveniently, effectively and economically in poultry industry. Since siRNAs are negatively charged and do not readily cross cell membranes, local injection, such as using chemically synthesized siRNA or shRNA-expressed plasmids may not be effective and economical for industrialized usage. So, using shRNA-expression plasmids to create transgenic chickens would be more practicable, we have demonstrated that psiSTRIKE U6-shRNA plasmids can be integrated with the chicken genome (data not shown), and experiments using the direct intra-ovarian injection method [21] are in progress.

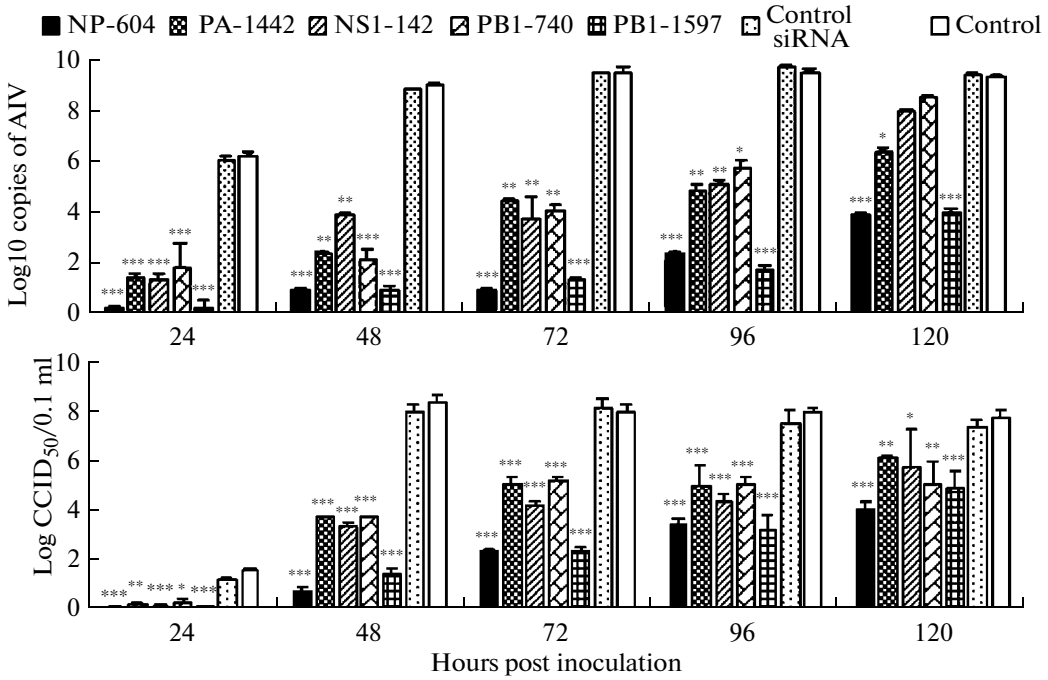

Fig. 4. All cells (2×10^4 cells/well) were infected with 100 $CCID_{50}$ of FJ13, supernatants were collected from 24 h to 120 h p.i. (sampling every 24 h) for QRT-PCR and DAS-ELISA analysis. (a) The mRNA levels of AIV FJ13 were analyzed by QRT-PCR, all measurements were normalized to expression of the β-actin genes, and the copies of HA gene mRNAs were quantified by comparison with a standard curve derived from known amounts of pEASY-HA. (b) Differences in HA protein expression of AIV between the stable transfectants and control cells were detected by DAS-ELISA. For standardization, known amounts of FJ13 (tested by $CCID_{50}$) were serially diluted twofold to 1 : 128 and assayed as above. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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