Antiviral Research 80 (2008) 143–149

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Inhibition of chicken anaemia virus replication using multiple short-hairpin RNAs

Tracey M. Hinton[a,b,*], Timothy J. Doran[a,b]

[a] CSIRO Livestock Industries, Australian Animal Health Laboratory, Private Bag 24, Geelong, Victoria 3220, Australia
[b] Cooperative Research Centre for the Australian Poultry Industry, Armidale, NSW 2315, Australia

ARTICLE INFO

Article history:
Received 30 January 2008
Accepted 20 May 2008

Keywords:
CAV
RNA interference
Short-hairpin RNA
Chicken U6 promoter

ABSTRACT

RNA interference is becoming a powerful tool in gene-specific silencing. New generation vaccines against many pathogens will attempt to incorporate these molecules. Here we report the efficient silencing of chicken anaemia virus (CAV) genes *in vitro* using short-hairpin RNAs (shRNAs) targeting the region of the CAV transcript encoding either viral protein (VP) 1, or overlapping sections of VP2/3 and VP1/2. The shRNAs were first validated against a EGFP–CAV fusion transcript reporter system and then against CAV grown in MDCC-MSB1 cells. The decrease in CAV replication was shown with a flow cytometry assay specific for VP3. Overall the results showed efficient silencing of CAV replication in tissue culture using shRNAs. It was also shown that the combination of three shRNAs being expressed from a single plasmid is less effective at silencing CAV replication than the most active shRNA alone.

1. Introduction

Chicken anaemia virus (CAV) is an important worldwide problem in the poultry industry, causing acute anaemia in young chicks or subclinical infections in adult birds (Adair, 2000). The subclinical infection results in immunosuppression which is particularly troubling as this leaves chickens with enhanced susceptibility to other avian pathogens and decreases the effectiveness of vaccines thereby reducing production efficiency. The current CAV vaccination strategy involves the delivery of attenuated virus in drinking water. Though effective, reversion to virulence of the vaccination strain has been demonstrated, resulting in clinical symptoms in flocks. More robust CAV control strategies are required (Von Bulow and Schat, 1997).

CAV is a member of the *gyrovirus* genus in the Circoviridae family of viruses that are characterized by their small, single-stranded, circular DNA genome. The genome is 2.3 kb from which a single 2.0-kb mRNA transcript encoding the three viral proteins (VP) 1, 2 and 3 in overlapping reading frames is transcribed (Kato et al., 1995; Noteborn et al., 1991). Splice variants of this transcript have recently been identified, however translation of these transcripts has not been demonstrated (Kamada et al., 2006). VP1 is a 52-kDa structural capsid protein; VP3 is a 13.8-kDa virulence factor known to induce apoptosis in transformed cell lines (Noteborn et al., 1994); and VP2, is a 28-kDa dual-specificity protein phosphatase (DSP) (Peters et al., 2002). Mutations in either VP1 or VP2 have been associated with CAV strains exhibiting reduced or inhibited capacity for replication *in vitro* (Peters et al., 2005; Yamaguchi et al., 2001). CAV is difficult to grow in tissue culture and will only grow in transformed lymphoblastoid cell lines such as MDCC-MSB1 cells (Marek's disease virus transformed chicken T cell) (Yuasa, 1983; Yuasa et al., 1983).

RNA interference (RNAi) is a naturally occurring mechanism found in both plants and animals that uses short RNA molecules (21–23 nt) to degrade or sequester mRNA resulting in specific gene suppression (Hannon, 2002; Fire et al., 1998). This mechanism has been taken advantage of by artificially introducing short interfering RNAs (siRNAs) and short-hairpin RNAs (shRNAs) into cells to suppress genes of interest. Introduction of siRNAs and shRNAs targeting viral mRNA has been demonstrated to be effective at silencing several human and animal viruses *in vitro* and *in vivo* including HIV-1 (Coburn and Cullen, 2002), Hepatitis B (Ying et al., 2007), Influenza A (Ge et al., 2003), foot and mouth disease virus (Liu et al., 2005) and bovine viral diarrhea virus (Lambeth et al., 2007).

This study describes the inhibition of a EGFP–CAV mRNA transcript in DF1 cells by using single or multiple shRNAs targeted to various regions of the CAV mRNA. Active shRNAs were then used to reduce CAV replication in MDCC-MSB1 cells.

* Corresponding author at: CSIRO Livestock Industries, Australian Animal Health Laboratory, Private Bag 24, Geelong, Victoria 3220, Australia.
Tel.: +61 3 5227 5746; fax: +61 3 5227 5555.
E-mail address: Tracey.Hinton@csiro.au (T.M. Hinton).

0166-3542/$ – see front matter.
doi:10.1016/j.antiviral.2008.05.009

Table 1
Oligonucleotides used to produce chicken U6 promoter vectors

Primer name	Primer sequence
chU6-4F	TTTGCATGCGTACCTCCTTCTCGCAG
chU6-4R	TTT**GTCGAC**AT**AAGCTT**AT**GTTTAAAC**CCCAGTGTCTCTCG
chU6-1F	CATGCATGCAAACGCTAAGCAGGCACCTAAAG
chU6-1R	TTT**CATATG**AT**ACTAGT**AT**ACCTGCGCAT**GAATATCTCTACCTCCTAGGCGG
chU6-MW1F	TTTGCATGCGTACCTCCTTCTCGCAG
chU6-MW1R	TTT**GTCGAC**AT**AAGCTT**AT**GTTTAAAC**CCCAGTGTCTCTCG
chU6-MW2F	TATGTCGACAAACTCCAGGAGGTGCATGTTTG
chU6-MW2R	TTT**CTCGAG**AT**GAATTC**AT***CCATGG*GACTAAGAGCATCGAGAC
chU6-MW3F	CATCTCGAGAAACGCTAAGCAGGCACCTAAAG
chU6-MW3R	TTT**CATATG**AT**ACTAGT**AT**ACCTGCGCAT**GAATATCTCTACCTCCTAGGCGG

Letters in bold indicate restriction sites for ligation of shRNA annealed oligonucleotides; letters underlined indicate restriction sites for cloning promoter vectors; letters in italics indicate plasmid requires nuclease treatment after digestion.

2. Materials and methods

2.1. Cell culture and virus growth

Chicken lymphoblastoid cells, MDCC-MSB1, transformed by Marek's disease virus (Akiyama and Kato, 1974), were cultured at 37 °C with 5% CO_2 in a 100-mm dish with RPMI1640 medium supplemented with 10% fetal bovine serum (IBL), 2 mM glutamine, 0.01% penicillin and 0.01% streptomycin. Transformed chicken embryo fibroblast cells (DF1, ATCC no. CRL-12203) were grown in DMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.01% penicillin and 0.01% streptomycin at 37 °C with 5% CO_2. Both cell types were subcultured twice weekly.

The Australian isolate CAV269/7 was obtained from G. Browning (School of Veterinary Science, The University of Melbourne, Australia) and grown in MDCC-MSB1 cells. Virus was prepared by freeze/thawing the culture three times and then clarifying by centrifugation at 6000 × *g* for 10 min. Virus was stored at −80 °C.

2.2. pEGFP–CAV and chicken U6 promoter plasmid construction

The pCAV269/7 plasmid was digested with PstI and BamHI (Brown et al., 2000). The 935 bp CAV fragment was gel purified and ligated into the similarly digested pEGFP–C (a generous gift from David Cummins, CSIRO Livestock Industries, Australia) to produce pEGFP–CAV. Single chicken U6 promoters chU6-1, chU6-3 and chU6-4 were amplified by PCR from previously reported plasmids (Wise et al., 2007) and ligated into pGEMTeasy (Promega, Madison, WI). pchU6-4 and pchU6-3 were ligated via SphI and SalI sites on primers and plasmid, whilst chU6-1 was ligated with SphI and NdeI. The reverse primers also contained two cloning sites for insertion of the shRNA. A third restriction site was included for removal of parental vector before transforming *E. coli* cells. Oligonucleotides used are shown in Table 1. All oligonucleotides were synthesised by Geneworks (Australia) and all restriction enzymes were obtained from either Promega or New England Biolabs (Ipswich, MA).

For construction of the multi-promoter vector (pMP), three rounds of overlapping PCR was performed on the three U6 chicken promoter vectors (pchU6-4, pchU6-3 and pchU6-1). Oligonucleotides are shown in Table 1. The first round of PCR amplified the promoter sequences of pchU6-1, pchU6-3 and pchU6-4 individually. The second round combined the pchU6-3 and pchU6-4 PCR DNA templates. A third round of PCR combined the pchU6-3/4 template from round 2 with the individual pchU6-1 PCR product from round 1 to produce a PCR product containing all three promoters. The final product was digested with SphI and NdeI and ligated into a similarly digested pGEMTeasy to produce pMP. Each reverse primer contained restriction sites for ligation of the shRNAs. All PCRs were performed with Platinum TaqHiFi as per manufacturer's instructions (Invitrogen, Carisbad, CA). All plasmids in this study were sequenced by Micromon DNA sequencing facility (Monash University, Australia).

2.3. shRNA design and plasmid construction

siRNAs were designed based on the CAV 269/7 strain sequence (Genebank Accession No. AF227982) using the Dharmacon siRNA Design tool (http://www.dharmacon.com). Sequences were selected to be in the coding sequence of the CAV genome and contain a GC content between 30% and 64%. The first three criteria of the Taxman algorithm (Taxman et al., 2006) were then applied to the first 40 siRNAs predicted from the Dharmacon tool. Six siRNA sequences that had a Taxman score of 3 or 4 and a central duplex closest to a $\Delta G > -12.9$ kcal/mol calculated using the free-energy parameters of the central six bases for predictions of RNA duplex stability as published by Freier et al. (1986) were chosen. Complementary DNA oligonucleotides containing the siRNA followed by

Table 2
Oligonucleotides used to produce shRNA expression vectors

Primer name	Primer sequence
VP2/3-1F	*ATTC*GGAATTACAGTCACTCTATTTCAAGAGAATAGAGTGACTGTAATTCC**TTTTTTGGAA**
VP2/3-1R	*TA***TTCCAAAAAA**GGAATTACAGTCACTCTATTCTCTTGAAATAGAGTGACTGTAATTCC
VP2/3-2F	CAACTGCGGACAATTCAGATTCAAGAGATCTGAATTGTCCGCAGTTG**TTTTTTGGAA**
VP2/3-2R	*TCGA***TTCCAAAAAA**CAACTGCGGACAATTCAGATCTCTTGAATCTGAATTGTCCGCAGTTG
VP2/3-3F	GAAGGTGTATAAGACTGTATTCAAGAGATACAGTCTTATACACCTTC**TTTTTTGGAA**
VP2/3-3R	*TCGA***TTCCAAAAAA**GAAGGTGTATAAGACTGTATCTCTTGAATACAGTCTTATACACCTTC
VP1/2-1F	CAAGCGACTTCGACGAAGATTCAAGAGATCTTCGTCGAAGTCGCTTG**TTTTTTGGAA**
VP1/2-1R	*TCGA***TTCCAAAAAA**CAAGCGACTTCGACGAAGATCTCTTGAATCTTCGTCGAAGTCGCTTG
VP1-1F	*ATTC*GAAGGACTCATTCTACCTATTCAAGAGATAGGTAGAATGAGTCCTTC**TTTTTTGGAA**
VP1-1R	*TA***TTCCAAAAAA**GAAGGACTCATTCTACCTATCTCTTGAATAGGTAGAATGAGTCCTTC
VP1-2F	CATCAATGAACCTGACATATTCAAGAGATATGTCAGGTTCATTGATG**TTTTTTGGAA**
VP1-2R	*TCGA***TTCCAAAAAA**CATCAATGAACCTGACATATCTCTTGAATATGTCAGGTTCATTGATG

Letters in bold indicate PolIII promoter termination signal; letters in italics indicate restriction site overhangs for ligation; letters underlined indicate loop sequence.

the loop sequence (TTCAAGAGA), then the antisense of the siRNA, followed by a PolIII termination sequence were chemically synthesized and annealed at 90 °C for 3 min and 30 °C for 1 h. A 5′ blunt end and 3′ overhang compatible to SalI digested DNA were included for ligation into PmeI and SalI digested pchU6-4. A 5′ overhang compatible to BfuAI and 3′ overhang compatible to NdeI digested DNA were included to ligate into BfuAI and NdeI digested pchU6-1. A non-silencing (pshNS) control and a positive control shRNA targeting GFP (pshGFP) were also used. The sequences have been published previously (Lambeth et al., 2007; Wise et al., 2007). All oligonucleotide sequences used to produce CAV shRNAs are shown in Table 2. The final plasmids are referred to as pshVP2/3-1, pshVP2/3-2, pshVP2/3-3, pshVP1/2, pshVP1-1 and pshVP1-2. VP2/3 and VP1/2 indicate the shRNA sequence occurs where the VP2 and VP3 genes sequences or VP1 and VP2 gene sequences overlap, respectively. The expression of shRNAs from pshVP2/3-2, pshVP2/3-3, pshVP1/2 and pshVP1-2 was under the control of the chU6-4 promoter, whilst pshVP2/3-1 and pshVP1-1 shRNA expression was under the control of the chU6-1 promoter.

To produce the multiple shRNA expression vector, annealed shRNA oligonucleotides were ligated into either PmeI and SalI digested pMP to be transcribed by chU6-4, NcoI (nuclease treated to produce a blunt end) and XhoI digested pMP to be transcribed by chU6-3, or BfuAI and NdeI digested pMP to be transcribed by chU6-1 producing pCAV-shMW.

2.4. Transfection of pEGFP–CAV and shRNA plasmids into DF1 cells

DF1 cells were seeded at 1.5×10^5 cells in 24-well tissue culture plates and grown overnight at 37 °C with 5% CO_2. Plasmids were transfected into the DF1 cells using Lipofectamine 2000 as per manufacturer's instructions (Invitrogen, Carisbad, CA). Briefly, 1 μg of pEGFP–CAV and 1 μg of the relevant shRNA diluted in 100 μl OPTI-MEM (Invitrogen, Carisbad, CA) was mixed with 2 μl of Lipofectamine 2000 diluted in 100 μl OPTI-MEM and incubated at room temperature for 20 min. The DNA:lipofectamine mix was added to cells and incubated for 4 h. Cell media was changed to normal growth media and incubated for 72 h. Cells were then washed twice with PBS, trypsinised and washed twice with FACS wash (PBS with 1% FBS). Cells were subjected to flow cytometry and analysed as a percentage of the non-silencing shRNA mean FITC fluorescence.

2.5. Electroporation of pEGFP–N1 and shRNA plasmids into MDCC-MSB1 cells

MDCC-MSB1 cells were washed twice in RPMI1640 medium (Sigma–Aldrich, St Louis MD), and 4×10^6 cells were resuspended in 350 μl RPMI1640 containing 10 μg of pEGFP–N1 and 10 μg of the relevant shRNA plasmid in a microfuge tube. Transfection was performed using a 0.4-cm gap electroporation cuvette in a Gene Pulser apparatus (Bio-Rad, Hercules, CA) set at 400 V, 900 μF, α resistance and extension capacitance. The cells were incubated at room temperature for 5 min, then resuspended in 1 ml warm growth medium and incubated for 4 h before infection with CAV.

2.6. Infection of transfected MDCC-MSB1 cells

Transfected cells were counted and 1×10^6 cells for each transfection were pelleted and infected with an MOI of 2 CAV269/7 for 1 h at 37 °C or mock infected with 200 μl of growth medium. Cells were then transferred to 3 ml warm growth medium and incubated for 72 or 96 h.

2.7. Detection of EGFP and CAV VP3 by flow cytometry

Cells for experiments with EGFP alone were washed three times in FACS wash (PBS with 1% FBS) and analysed by flow cytometry at 72 and 96 h. At 72 or 96 h 1×10^6 transfected and infected cells were pelleted and fixed in 1 ml BD permeabilisation solution 2 (Becton Dickinson) for 20 min at room temperature. Cells were then washed in PBS with 0.01% Tween 20 and stained with 1:1000 mouse anti-CAV VP3 (Tropbio, Australia) washed three times and stained with 1:500 goat anti mouse IgG1 APC conjugated antibody (Invitrogen, Carisbad, CA) then analysed by flow cytometry for EGFP and APC fluorescence. Dot plots of the cells comparing FITC and APC channels were analysed and histograms of APC fluorescence of EGFP-positive cells were produced. Mean fluorescence of the histograms were taken and analysed as a percentage of the non-silencing shRNA mean fluorescence.

3. Results

3.1. shRNA design and plasmid constructs

The single-stranded DNA genome of CAV is transcribed into a single long mRNA molecule encoding the three overlapping CAV genes (Kato et al., 1995; Noteborn et al., 1991). Therefore targeting any region of the coding sequence should silence the expression of all three genes. The Australian CAV269/7 strain used in the study has been shown to have 95% sequence identity to seven other sequenced isolates (Brown et al., 2000), whilst the seven other isolates had 98–99% identity to each other. Six shRNAs were therefore designed against regions of the genome that were identical in all seven isolates, to give cross-strain specificity. As VP2 is overlapped by the entirety of VP3 and a portion of VP1 (Fig. 1A) most of the shRNAs designed targeted two of the genes (indicated in the plasmid names), although two shRNAs targeted VP1 alone. Each shRNA was inserted into an expression vector individually producing pshVP2/3-2, pshVP2/3-3, pshVP1/2, and pshVP1-2 under the control of the chicken U6-4 promoter and pshVP2/3-1 and pshVP1-1 under the control of the chicken U6-1 promoter (Fig. 1B).

pEGFP–CAV contains 935 bp of the CAV genome at the 3′ end of the EGFP gene. This region contains the target sequence for all six of the CAV shRNAs (Fig. 1A) and is expressed as a single mRNA, therefore an active shRNA will result in the loss of EGFP expression.

3.2. Silencing of EGFP–CAV fusion transcript by single or multiple shRNAs

The six single shRNA expression vectors (pshVP2/3-1, pshVP2/3-2, pshVP2/3-3, pshVP1/2, pshVP1-1 and pshVP1-2) were first tested against the EGFP–CAV fusion mRNA in DF1 cells. All of the shRNAs had some activity, the least active (pshVP2/3-2, Fig. 2 column 5) resulting in 30% knockdown of EGFP expression, measured as a percentage of mean FITC fluorescence of the non-silencing control. shRNA VP2/3-1 appeared to be extremely effective with over 80% knockdown of EGFP–CAV expression, considerably more than the shEGFP-positive control. This indicated that efficient inhibition of CAV replication should be possible. The three most active shRNAs; shVP2/3-1, shVP1-2 and shVP2/3-3 were cloned into the multi-promoter vector, pMP to produce pCAV-shMW (Fig. 1C) to determine whether individual or multiple shRNAs work most effectively. The multi-promoter vector contains three individual transcription units driven by either chicken U6 promoters (chU6) 1, 2 or 3, and has previously been shown to express three different shRNA molecules targeted against influenza by RNase protection assay, indicating the plasmid is functional (unpublished data).

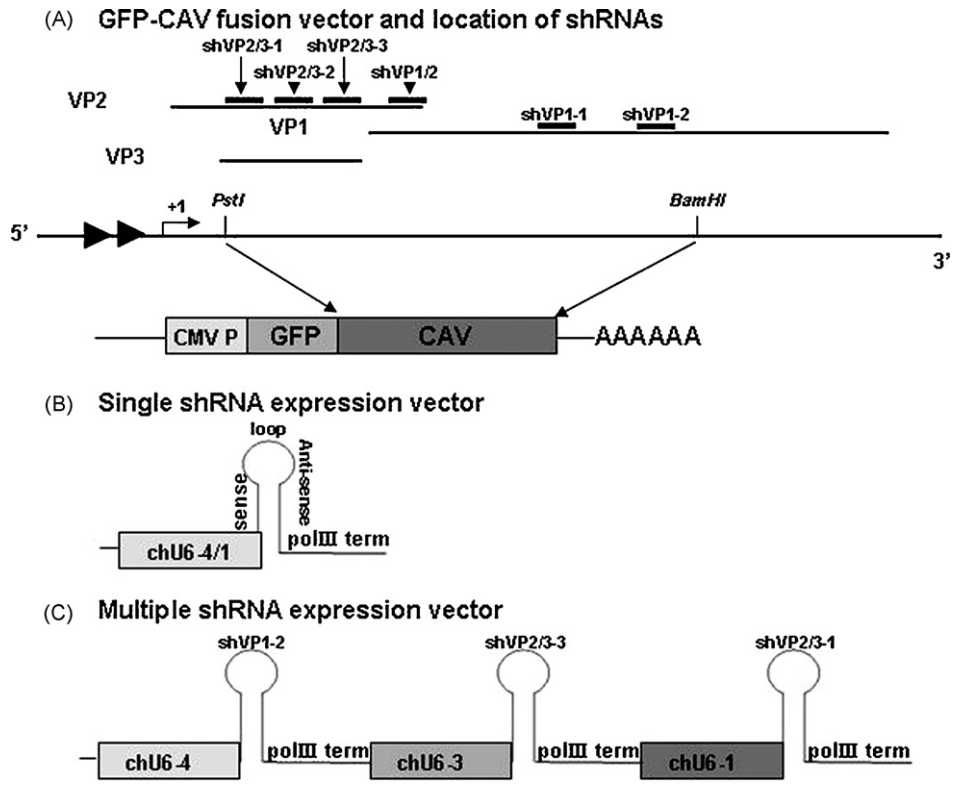

Fig. 1. Schematic representation of vectors. (A) Schematic representation of the linearised CAV genome. Sequences encoding open reading frames for the three known CAV proteins are indicated. The transcriptional start site is indicated by an arrow and +1. The location of the promoter/enhancer repeat region is shown by two arrow heads. Location of the CAV targeting shRNAs is indicated by short lines. Not to scale. The region of the CAV genome included in the fusion plasmid is shown. (B) Schematic representation of the shRNA expression vectors. One vector contains either the ChU6-4 or ChU6-1 promoter. (C) Schematic representation of pCAV-shMW vector.

pCAV-shMW contains shVP1-2 under the control of the chU6-4 promoter, shVPVP2/3-3 under the control of the chU6-3 promoter and shVPVP2/3-1 under the control of the chU6-1 promoter. This construct was examined for its ability to inhibit EGFP–CAV mRNA expression compared to the individual shRNAs. pCAV-shMW was less efficient at silencing EGFP–CAV expression than the single most active shRNA, VP2/3-1 (Fig. 3A column 4 and 6). However the knockdown was still considerable at over 70%. When the three individual shRNAs were co-transfected together with pEGFP–CAV there was no difference in silencing to pCAV-MW (Fig. 3B). No non-specific silencing was observed with the negative control pshNS (Figs. 2 and 3 columns 1 and 2).

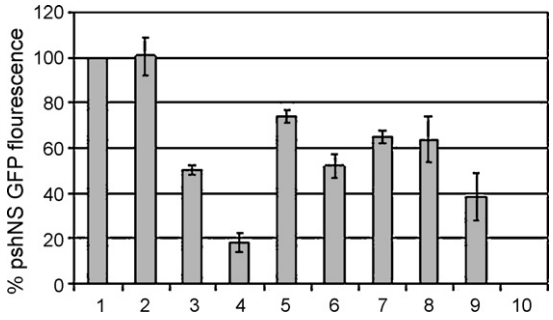

Fig. 2. shRNAs targeting the CAV genome silence the GFP–CAV fusion mRNA in DF1 cells. DF1 cells were co-transfected with 1 μg of the relevant vectors for 72 h. Column 1: pshNS control; column 2: pEGFP–CAV alone; column 3: pshGFP control; column 4: pshVP2/3-1; column 5: pshVP2/3-2; column 6: pshVP2/3-3; column 7: pshVP1/2; column 8: pshVP1-1; column 9: pshVP1-2; column 10: untransfected. Columns 1 and 3–9 were co-transfected with 1 μg of pEGFP–CAV. Cells were then assayed by flow cytometry and analysed in Microsoft Excel. Values are shown as percentages of the negative control shRNA (shNS, column 1), as the mean of three separate experiments in duplicate ± standard deviation.

3.3. MDCC-MSB1 cells have the RNAi pathway

As the transformed MDCC-MSB1 cells have not previously been used in RNAi work it was necessary to determine whether the cells had retained the RNAi pathway. Therefore the EGFP expression vector was electroporated into MDCC-MSB1 cells either alone or with a non-silencing shRNA (pshNS) or a previously published, highly active shRNA targeting GFP (pshGFP) as controls (Lambeth et al., 2007; Wise et al., 2007). Results from both fluorescent microscopy and flow cytometry indicated MDCC-MSB1 cells do have RNAi capacity (Fig. 4A and B). As expected there was minimal non-specific silencing of EGFP mRNA expression from the non-silencing control (Fig. 4B column 1 and 2), whilst 70% knockdown of EGFP expression was observed with pshGFP (Fig. 4B columns 2 and 3). The results also indicated that the highest silencing was observed at the longer 96 h post-transfection time point. This was therefore used for future experiments.

3.4. Silencing of CAV replication using shRNAs

It has been shown that some siRNAs that are highly effective in the EGFP-fusion reporter assay are not effective against the target virus (Lambeth et al., 2007). This is most likely due to different folding configurations of the native viral mRNA compared to the EGFP-fusion mRNA. Therefore to determine the effectiveness of the chosen shRNAs at inhibiting CAV replication, an assay to determine silencing was required. Although it has been shown that MDCC-MSB1 cells have retained the RNAi pathway, the highest

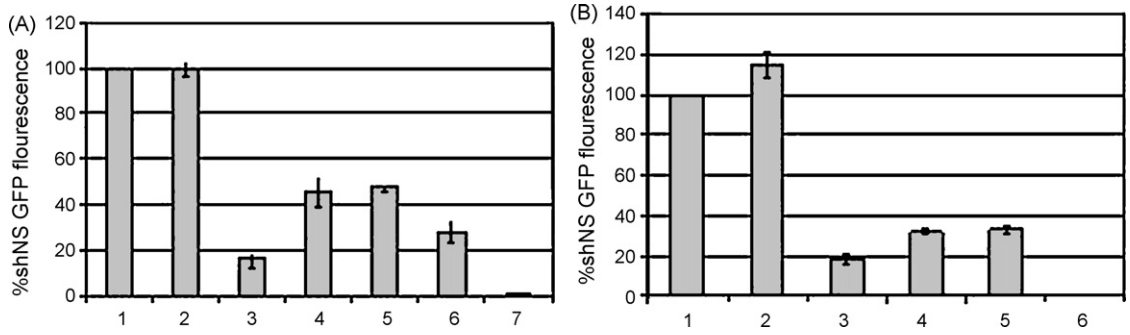

Fig. 3. Expression of multiple shRNAs targeting the CAV genome from a single plasmid silence the GFP–CAV fusion mRNA in DF1 cells. (A) DF1 cells were co-transfected with 1 μg of the relevant vectors for 72 h. Column 1: pshNS control; column 2: pEGFP–CAV alone; column 3: pshVP2/3-1; column 4: pshVP2/3-3; column 5: pshVP1-2; column 6: pCAV-shMW; column 7: untransfected. Columns 1 and 3–6 were co-transfected with 1 μg of pEGFP–CAV. Cells were then assayed by flow cytometry and analysed in Microsoft Excel. Values are shown as percentages of the negative control shRNA (shNS, column 1), as the mean of three separate experiments in duplicate ± standard deviation. (B) DF1 cells were co-transfected with a total of 1 μg of the relevant vectors for 72 h. Column 1: pshNS control; column 2: pEGFP–CAV alone; column 3: pshVP2/3-1; column 4: pCAV-shMW; column 5: pshVP2/3-1, pshVP2/3-3, and pshVP1-2; column 6: untransfected. Columns 1 and 3–5 were co-transfected with 1 μg of pEGFP–CAV. Cells were then assayed by flow cytometry and analysed in Microsoft Excel. Values are shown as percentages of the negative control shRNA (shNS, column 1), as the mean of three separate experiments in duplicate ± standard deviation.

transfection efficiency that has been obtained was approximately 50% of cells (data not shown). This was deemed insufficient to determine efficient CAV silencing as all cells are infected with CAV. Therefore a way to distinguish infected cells from infected and transfected cells was required. This was accomplished by co-transfecting the shRNA with pEGFP–N1 (Clontech). Co-transfection has been reported to be close to 100% efficient (Cullen, 2006) therefore every cell that contains EGFP will also contain the shRNA. A method to detect CAV infection by flow cytometry has recently been elucidated by the use of a monoclonal antibody targeting CAV VP3 and a secondary anti-mouse antibody conjugated with APC (P. Guo, personal communication). Therefore both EGFP transfected and infected cells can be distinguished by flow cytometry by acquiring fluorescence of the two different flourophores. If a higher APC mean fluorescence was detected in EGFP-positive cells it indicates that more of those cells have VP3 expression therefore those cells have a productive CAV infection and CAV is not silenced. Alternatively a lower APC mean fluorescence detected in EGFP-positive cells indicates VP3 expression is less and CAV protein expression is inhibited.

When this assay was used with pCAV-shMW and the three individual shRNAs contained within, a decrease in virus protein expression was observed with all constructs (Fig. 5). Interestingly the most active was shRNA VP2/3-3 with a 60% knockdown of CAV VP3 expression, whilst this was not the most active in the EGFP-fusion reporter system. pCAV-shMW was again slightly less active than the best single shRNA, however a knockdown of over 50% was obtained. All results are shown as a percentage of the non-silencing shRNA negative control.

4. Discussion

CAV is a major problem for the world-wide poultry industry due to its ability to produce sub-clinical infection and immuno-

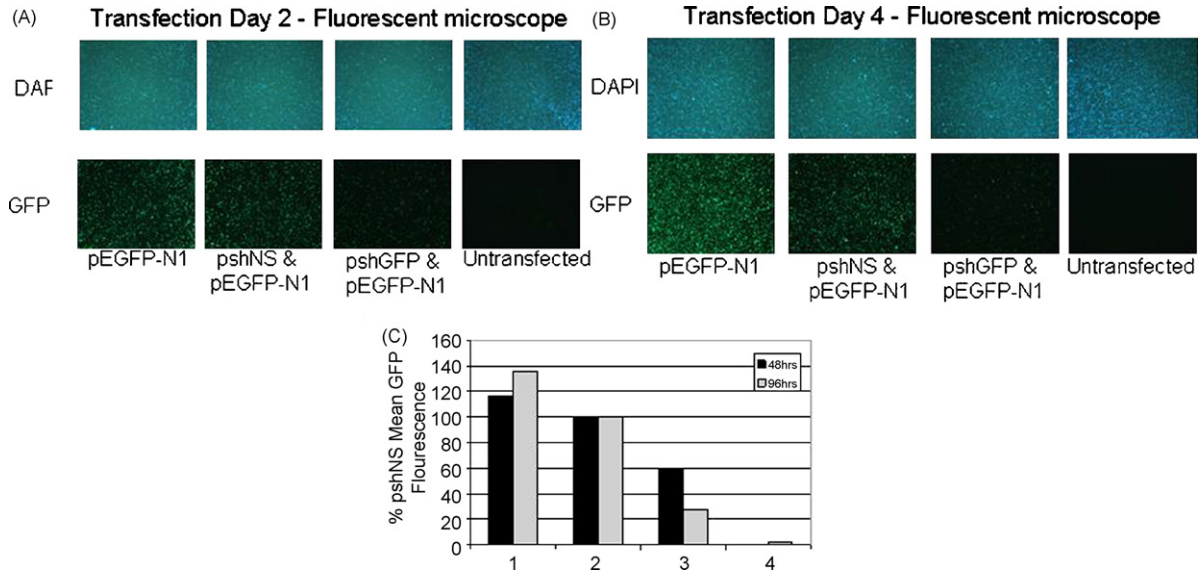

Fig. 4. MSB-1 cells have the RNA silencing pathway. MDCC-MSB1 cells were electroporated with 10 μg of the relevant vectors and incubated for 48 h. (A) Aliquots of cells were taken, spun and resuspended in PBS with DAPI, then allowed to settle onto a microscope slide, images were taken by a fluorescent microscope. The remainder was incubated for a further 48 h. (B) Aliquots of cells were taken at 96 h, spun and resuspended in PBS with DAPI, then allowed to settle onto a microscope slide, images were taken by a fluorescent microscope. (C) At 48 and 96 h aliquots of 1×10^6 cells were removed, washed twice with FACS wash and then analysed for GFP by flow cytometry. Mean fluorescent intensity were obtained and analysed by Microsoft Excel. Column 1: pEGFP–N1; column 2: pshNS; column 3: pshGFP; column 4: untransfected cells, columns 1 and 3 were co-transfected with pEGFP–N1. Values are representative of three experiments and are shown as percentages of the negative control shRNA (pshNS, column 1).

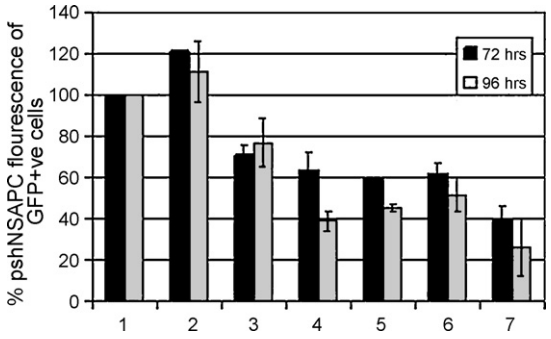

Fig. 5. shRNAs targeting the CAV genome can inhibit expression of CAV VP3. MDCC-MSB1 cells were electroporated with 10 μg vector and incubated for 4 h. 1×10^6 cells were then infected for 1 h with CAV269/7 at an MOI of 2. Cells were added to growth media and incubated for 72 or 96 h. 1×10^6 cells were then fixed, stained for CAV VP3 using anti-VP3 and anti-mouse APC and detected by flow cytometry. Cell quest was used to obtain the results. The mean APC fluorescent intensity of GFP-positive cells was then analysed in Microsoft Excel. Column 1: pshNS; column 2: pEGFP–N1; column 3: pshVP2/3-1; column 4: pshVP2/3-3; column 5: pshVP1-2; column 6: pCAV-shMW; column 7: untransfected/uninfected. Values are shown as percentages of the negative control shRNA (shNS, column 1), as the mean of three separate experiments in duplicate ± standard error of the mean.

suppression in vaccinated mature birds (Adair, 2000). The use of RNAi to silence virus replication has been shown to be highly effective against many human and animal viruses both *in vitro* and *in vivo* (Coburn and Cullen, 2002; Ge et al., 2003; Liu et al., 2005). This study demonstrates the ability to silence CAV gene expression up to 60% in MDCC-MSB1 cells with single shRNAs targeting various regions of the genome. However, the use of a single shRNA, particularly against highly variable RNA viruses such as poliovirus and HIV, has allowed for the production of escape mutants (Das et al., 2004; Gitlin et al., 2005). Whilst this may not be such a significant problem in the small highly conserved CAV DNA genome, expression of multiple shRNAs is preferable.

Several methods to express multiple shRNAs from a single vector have been explored including expression from multiple cassettes using the same promoter (ter Brake et al., 2006), from single vectors containing multiple promoters (Gou et al., 2007) or as extended or long hairpin RNAs containing two siRNA sequences (Liu et al., 2007). However, the most effective of these methods has not been determined. This study utilised a plasmid construct directing the expression of three shRNA molecules from three different chicken U6 promoters, chU6-1, chU6-3 and chU6-4. These promoters have been shown to be highly active in chicken cells and therefore are appropriate for use against an avian pathogen (Wise et al., 2007). The vector pCAV-shMW, was shown to reduce viral protein production by 50%, this is a considerable decrease as the virus dose in this assay would be higher than during a normal environmental exposure. Interestingly expression of the three different shRNAs from pCAV-shMW showed less inhibition than the most active individual shRNA, this has been observed previously when multiple siRNAs or shRNAs are used (O'Brien, 2007; Henry et al., 2006). Expression of the combined three shRNAs from individual expression vectors resulted in the same silencing as from one vector.

Recently Castanotto et al. (2007) demonstrated that expression of multiple shRNAs in cells results in competition for proteins involved in the RNAi pathway. This includes Exportin-5, responsible for transporting the shRNA out of the nucleus, and for incorporation into the RNA-induced silencing complex (RISC) which is responsible for the recognition and degradation of the target mRNA (Yi et al., 2003; Hammond et al., 2000). This competition was shown to be partially sequence specific and to correlate with the activity of the shRNA. Therefore the decrease in silencing observed with pCAV-shMW and the combination of the three individual vectors may be attributed to competition between VP2/3-3 and the less active shRNA sequences VP2/3-1 and VP1-2. Expression of shRNAs have also been shown to out-compete endogenous micro (mi)RNAs, however when the siRNAs were incorporated into a miRNA backbone no competition was observed (Castanotto et al., 2007). This indicates that the competition effect may be overcome by incorporating siRNA sequences into a miRNA backbone or by combining shRNAs with sequences that do not compete with one another.

As polIII promoters are constitutively active the use of promoters that express lower amounts of the shRNAs may mitigate the potential for competition with endogenous miRNAs or among combinations of applied shRNAs. The most active shRNA against pEGFP–CAV was under the control of the chicken U6-1 promoter (Fig. 2). Previous work has shown that the chicken U6-1 promoter is weaker than the chicken U6-4 promoter (Wise et al., 2007). Therefore with an efficient shRNA the use of a weaker promoter is possible. As the delivery of multiple shRNAs will be required for any human or animal viral therapeutic treatment to avoid resistant escape mutants, more work is clearly required on the best delivery method for multiple shRNAs.

5. Conclusion

This report is the first time a method to detect both infected and transfected MDCC-MSB1 cells by flow cytometry has been utilised. It is also the first study that demonstrates silencing of CAV mRNA expression is possible with single or multiple shRNA molecules. As the CAV genome sequence is highly conserved amongst strains, developing shRNAs targeting this virus should be highly efficient at cross-strain protection. It will now be essential to determine whether these shRNAs are able to protect chickens from a productive CAV infection.

Acknowledgements

This work was partly conducted within the Australian Poultry co-operative research centre, established and supported under the Australian Government's co-operative research centres program and CSIRO-Livestock Industries.

References

Adair, B.M., 2000. Immunopathogenesis of chicken anemia virus infection. Dev. Comp. Immunol. 24, 247–255.
Akiyama, Y., Kato, S., 1974. Two cell lines from lymphomas of Marek's disease. Biken J. 17, 105–116.
Brown, H.K., Browning, G.F., Scott, P.C., Crabb, B.S., 2000. Full-length infectious clone of a pathogenic Australian isolate of chicken anaemia virus. Aust. Vet. J. 78, 637–640.
Castanotto, D., Sakurai, K., Lingeman, R., Li, H., Shively, L., Aagaard, L., Soifer, H., Gatignol, A., Riggs, A., Rossi, J.J., 2007. Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. Nucleic Acids Res. 35, 5154–5164.
Coburn, G.A., Cullen, B.R., 2002. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Virol. 76, 9225–9231.
Cullen, B.R., 2006. Induction of stable RNA interference in mammalian cells. Gene Ther. 13, 503–508.
Das, A.T., Brummelkamp, T.R., Westerhout, E.M., Vink, M., Madiredjo, M., Bernards, R., Berkhout, B., 2004. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J. Virol. 78, 2601–2605.
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.
Freier, S.M., Kierzek, R., Jaeger, J.A., Sugimoto, N., Caruthers, M.H., Neilson, T., Turner, D.H., 1986. Improved free-energy parameters for predictions of RNA duplex stability. Proc. Natl. Acad. Sci. U.S.A. 83, 9373–9377.
Ge, Q., McManus, M.T., 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.
Gitlin, L., Stone, J.K., Andino, R., 2005. Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. J. Virol. 79, 1027–1035.
Gou, D., Weng, T., Wang, Y., Wang, Z., Zhang, H., Gao, L., Chen, Z., Wang, P., Liu, L., 2007. A novel approach for the construction of multiple shRNA expression vectors. J. Gene Med. 9, 751–763.
Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404, 293–296.
Hannon, G.J., 2002. RNA interference. Nature 418, 244–251.
Henry, S.D., van der Wegen, P., Metselaar, H.J., Tilanus, H.W., Scholte, B.J., van der Laan, L.J., 2006. Simultaneous targeting of HCV replication and viral binding with a single lentiviral vector containing multiple RNA interference expression cassettes. Mol. Ther. 14, 485–493.
Kamada, K., Kuroishi, A., Kamahora, T., Kabat, P., Yamaguchi, S., Hino, S., 2006. Spliced mRNAs detected during the life cycle of Chicken anemia virus. J. Gen. Virol. 87, 2227–2233.
Kato, A., Fujino, M., Nakamura, T., Ishihama, A., Otaki, Y., 1995. Gene organization of chicken anemia virus. Virology 209, 480–488.
Lambeth, L.S., Moore, R.J., Muralitharan, M.S., Doran, T.J., 2007. Suppression of bovine viral diarrhea virus replication by small interfering RNA and short hairpin RNA-mediated RNA interference. Vet. Microbiol. 119, 132–143.
Liu, M., Chen, W., Ni, Z., Yan, W., Fei, L., Jiao, Y., Zhang, J., Du, Q., Wei, X., Chen, J., Liu, Y., Zheng, Z., 2005. Cross-inhibition to heterologous foot-and-mouth disease virus infection induced by RNA interference targeting the conserved regions of viral genome. Virology 336, 51–59.
Liu, Y.P., Haasnoot, J., Berkhout, B., 2007. Design of extended short hairpin RNAs for HIV-1 inhibition. Nucleic Acids Res., 1–11 (epublished).
Noteborn, M.H., de Boer, G.F., van Roozelaar, D.J., Karreman, C., Kranenburg, O., Vos, J.G., Jeurissen, S.H., Hoeben, R.C., Zantema, A., Koch, G., et al., 1991. Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. J. Virol. 65, 3131–3139.
Noteborn, M.H., Todd, D., Verschueren, C.A., de Gauw, H.W., Curran, W.L., Veldkamp, S., Douglas, A.J., McNulty, M.S., van der, E.B.A.J., Koch, G., 1994. A single chicken anemia virus protein induces apoptosis. J. Virol. 68, 346–351.
O'Brien, L., 2007. Inhibition of multiple strains of Venezuelan equine encephalitis virus by a pool of four short interfering RNAs. Antiviral Res. 75, 20–29.
Peters, M.A., Jackson, D.C., Crabb, B.S., Browning, G.F., 2002. Chicken anemia virus VP2 is a novel dual specificity protein phosphatase. J. Biol. Chem. 277, 39566–39573.
Peters, M.A., Jackson, D.C., Crabb, B.S., Browning, G.F., 2005. Mutation of chicken anemia virus VP2 differentially affects serine/threonine and tyrosine protein phosphatase activities. J. Gen. Virol. 86, 623–630.
Taxman, D.J., Livingstone, L.R., Zhang, J., Conti, B.J., Iocca, H.A., Williams, K.L., Lich, J.D., Ting, J.P., Reed, W., 2006. Criteria for effective design, construction, and gene knockdown by shRNA vectors. BMC Biotechnol. 6, 7.
ter Brake, O., Konstantinova, P., Ceylan, M., Berkhout, B., 2006. Silencing of HIV-1 with RNA interference: a multiple shRNA approach. Mol. Ther. 14, 883–892.
Von Bulow, V., Schat, K.A., 1997. Infectious Anaemia, 10th ed. Iowa State University Press, Aimes, IA, pp. 690–699.
Wise, T.G., Schafer, D.J., Lambeth, L.S., Tyack, S.G., Bruce, M.P., Moore, R.J., Doran, T.J., 2007. Characterization and comparison of chicken U6 promoters for the expression of short hairpin RNAs. Anim. Biotechnol. 18, 153–162.
Yamaguchi, S., Imada, T., Kaji, N., Mase, M., Tsukamoto, K., Tanimura, N., Yuasa, N., 2001. Identification of a genetic determinant of pathogenicity in chicken anaemia virus. J. Gen. Virol. 82, 1233–1238.
Yi, R., Qin, Y., Macara, I.G., Cullen, B.R., 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 17, 3011–3016.
Ying, R.S., Zhu, C., Fan, X.G., Li, N., Tian, X.F., Liu, H.B., Zhang, B.X., 2007. Hepatitis B virus is inhibited by RNA interference in cell culture and in mice. Antiviral Res. 73, 24–30.
Yuasa, N., 1983. Propagation and infectivity titration of the Gifu-1 strain of chicken anemia agent in a cell line (MDCC-MSB1) derived from Marek's disease lymphoma. Natl. Inst. Anim. Health Q (Tokyo) 23, 13–20.
Yuasa, N., Taniguchi, T., Goda, M., Shibatani, M., Imada, T., Hihara, H., 1983. Isolation of chicken anemia agent with MDCC-MSB1 cells from chickens in the field. Natl. Inst. Anim. Health Q (Tokyo) 23, 75–77.