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Inhibition of Anatid Herpes Virus-1 replication by small interfering RNAs in cell culture system

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Abstract

RNA interference (RNAi) mediated by double stranded small interfering RNA (siRNA) is a novel mechanism of post-transcriptional gene silencing. It is projected as a potential tool to inhibit viral replication. In the present paper, we demonstrate the suppression of replication of an avian herpes virus (Anatid Herpes Virus-1, AHV-1) by siRNA mediated gene silencing in avian cells. The UL-6 gene of AHV-1 that codes for a protein involved in viral packaging was targeted. Both cocktail and unique siRNAs were attempted to evaluate the inhibitory potential of AHV-1 replication in duck embryo fibroblast (DEF) cell line. DEF cells were chemically transfected with different siRNAs in separate experiments followed by viral infection. The observed reduction in virus replication was evaluated by cytopathic effect, viral titration and quantitative real-time PCR (QRT-PCR). Among the three siRNA targets used the unique siRNA UL-B sequence was found to be more potent in antiviral activity than the cocktail and UL6-A-siRNA sequences.

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1. Introduction

The RNA interference phenomenon is known to exist in diverse organisms ranging from plants to man. Application of this phenomenon is wide open, from endogenous gene knock down to inhibition of viral replication in cells. RNA interference has widely been proven as an effective mechanism to suppress the replication of different viruses viz. Poliovirus, human corona virus, human immunodeficiency virus-1 (HIV-1), Hepatitis-B virus, Hepatitis-C virus, foot and mouth disease virus (FMDV), etc. (Griffin et al., 2002; Wang et al., 2004; Lee et al., 2002; McCaffrey et al., 2003; Wilson et al., 2003; Chen et al., 2004). The phenomenon operates at the post transcription level, thereby, hindering the expression of some essential proteins required for viral replication. RNAs is presumed to be an evolutionarily conserved host defense mechanism against viruses. Evidences also suggest that viruses also have evolved proteins that suppress the RNA silencing pathway as an adaptive mechanism (Mourrain et al., 2000; Dalmay et al., 2001; Griffin and Andino, 2003). The mechanism has been demonstrated for suppression of viral replication in a wide range of organisms starting from plants, insects, mammals, etc. (Waterhouse et al., 2001; Adelman et al., 2002; Griffin and Andino, 2003). The present report demonstrates the amenability of AHV-1 virus to siRNA mediated viral suppression which may have relevance in developing alternate strategies for control of this virus in ducks and water fowls.

AHV-1 infects birds of the group Anseriformes and causes severe epidemic called duck virus enteritis alternatively known as duck plague (Dardiri, 1975; Kaleta, 1990; Foulon, 1992). The virus belongs to the family Herpesviridae and has not been placed in any of the subfamilies of Herpesviridae (Roizman and Pellett, 2001). The genome of AHV-1 is a linear double stranded DNA (dsDNA) molecule of approximately 180 kb with high G+C content. The genome organization is similar to known alpha herpes viruses (Fukuchi et al., 1984;...
The virus is transmitted by contact of susceptible birds and epidemic may occur within a week. Like other herpes viruses, AHV-1 can also establish latency after primary infection and survivors may act as carriers, shedding other herpes viruses, AHV-1 can also establish latency after susceptible birds and epidemic may occur within a week. Like Gardner et al., 1993). The virus is transmitted by contact of 

AHV-UL6-BsiRNA 5′-AGATCAAGGGTCAAGGTTAGCCTC-3′ 708-1032 325

AHV-UL6-AsiRNA 5′-UAACUCUCCUCUGGACCCUU-3′ 751

T7 promoter appended UL-6 primers

| Name          | Primer sequence | Location | Length |
|---------------|-----------------|----------|--------|
| AHV-UL-6B-F   | 5′-ATAATCAGGGTCAAGGTTAGCCTC-3′ | 708-1032 | 325    |
| AHV-UL-6B-R   | 5′-GTTTCATATCCATCTTCCGCAAATC-3′ | 708-1032 | 325    |
| T7 promoter appended UL-6 primers | 5′-TAATACGAGCTACATTATGGGAGGTAATACGAGCTACTC-3′ | 751 |
| AHV-UL6-BsiRNA | 5′-GTTTCATATCCATCTTCCGCAAATC-3′ | 708-1032 | 325    |
| AHV-UL6-AsiRNA | 5′-UUAAGAAGAAGUUCGAAUCU-3′ | 395 |
| AHV-UL6-BsiRNA | 5′-UUAAGAAGAAGUUCGAAUCU-3′ | 395 |
| AHV-UL6-AsiRNA | 5′-UUAAGAAGAAGUUCGAAUCU-3′ | 395 |

* For the T7 promoter appended UL-6 primers, primer sequences are marked in italics, the purine sequences highlighted and the primer sequences underlined.

The siRNAs, both cocktail and unique, were introduced into DEF cells using the transfection agent siPORT Amine® (Ambion Inc, USA) as per manufacturers recommended protocol. Essentially, oligomers synthesized were annealed with T7 promoter primers and extended by Klenow enzyme to get a 48 bp DNA oligonucleotide template. In vitro transcription was carried out from this template to generate sense and anti-sense siRNA strands that were annealed to produce double-stranded siRNAs. All the primer sequences and the oligonucleotide sequences required for the experiment were got synthesized from Invitrogen®.

2.3. Transient transfection of DEF cells with siRNAs

The siRNAs, both cocktail and unique, were introduced into DEF cells using the transfection agent siPORT Amine® (Ambion Inc, USA). 75 nM final concentration of each siRNA was used for transfecting 70–80% confluent DEF cells. The cells were incubated for 6 h with siRNA-transfection agent complex. After removing the siRNA-transfection agent complex and washing with serum free EMEM, cells were re-incubated in normal growth medium (EMEM with 10% FCS) for another 12 h. The cells were infected with one multiplicity of infection (m.o.i.) final concentration of AHV-1 in maintenance medium (EMEM with 2% FCS). Controls were treated in the same way except for the addition of siRNA. The cells were analyzed for CPE and viral load, 48 h post-infection. Images of cells in control and experimental groups were collected with an Olympus® CK40 microscope fitted with Olympus® SC35 video camera at a magnification of 100×.
2.4. Titration of AHV-1

The chick embryo adapted vaccine strain of AHV-1 was adapted in DEF cells and was titrated in confluent DEF monolayer. Transfection plates were harvested 48 h post-infection and freeze thawed three times. Ten fold dilutions of the sample were used for titration and reading was taken 48 h post-infection. The TCID₅₀ was calculated using Reed and Muench (1938) method.

2.5. Quantitative Real Time-PCR for evaluation of virus load

The standardization of the Real Time-PCR for AHV-1 was carried out using a pair of primers for amplifying a 325 bp long sequence from the UL-6 gene (Table 1). Real Time-PCR was performed from the viral genomic DNA isolated from experimental and control cells using Brilliant SYBR® Green Q-PCR master mix (Stratagene®, USA). Real Time-PCR was performed in an Mx3000p™ instrument from Stratagene®. The reaction was performed in 25 μl volume containing 12.5 μl of Brilliant SYBR® Green Q-PCR master mix, 2.5 μl of genomic DNA isolated from cell culture supernatants and 0.5 μM each of forward and reverse primers. The PCR conditions included 30 cycles, with denaturation at 94 °C for 30 s, primer annealing at 57 °C for 1 min and primer extension at 72 °C for 1 min. An initial denaturation at 94 °C for 10 min and a final extension at 72 °C for 10 min were also included.

3. Results

3.1. Silencing of AHV-1 by cocktail siRNAs

The objective of the present study was to demonstrate the utility of RNAi mechanism to control an avian herpes virus, AHV-1. The chick embryo adapted AHV-1 readily adapted to DEF. The susceptibility of DEF cells for transfection with nucleic acid was also assessed using a functional β-galactosidase construct pSV β-galactosidase (data not shown).

Two approaches, both targeting the UL-6 gene of AHV-1, which is endowed with the function of packaging of mature viral particles (Plummo et al., 1998), were used. In the first instance, to evaluate the amenability of the target gene selected for RNAi, a cocktail siRNA approach spanning 325 bp long region of the genomic sequence was used. This region contained 21 potential
siRNA target sequences as identified using a target finder programme of M/s. Ambion. The cocktail siRNA generated for this region as per the method described earlier was used for transfection of DEF followed by viral infection. The CPE observed at 48 h post-infection in control and experimental group is shown in Fig. 1. Apparently, there is a marked reduction in the CPE, especially in vacuolation and rounding of cells. This was further corroborated by titration of the virus yield between the experimental and control groups. There was 0.88 log difference, which is equivalent to 7.5 fold difference of virions in the cocktail siRNA transfected group in comparison to the control group (average of four individual wells).

3.2. Silencing of AHV-1 by unique siRNAs

3.2.1. CPE and virus titration

After observing the existence of RNAi mechanism directly by reduction in CPE and virus titre using the cocktail approach, two unique siRNA sequences were also synthesized (Table 1), targeting the same UL-6 gene of AHV-1. siRNAs were generated for this region using a different approach as described in the materials and methods. Transfection of 75 nM of individual siRNAs per well followed by infection with ten m.o.i produced marked reduction in CPE, after 48 h of infection. The reduction in CPE between the control and experimental groups were more marked than the cocktail siRNA transfection experiments (Fig. 1). Titration of the virus load, also brought out clear reductions in the experimental groups compared to control groups, confirming the interferences achieved with these siRNAs. The AHV-UL6-AsiRNA brought out a log difference of 1.21 with that of control whereas AHV-UL6-BsiRNA resulted in a log difference of 1.33 (Average value of four independent observations). The log difference brought out by AHV-UL6-AsiRNA and AHV-UL6-BsiRNA corresponds to 16.2 and 21.3 fold differences with control, respectively. This indicates the superiority of AHV-UL6-AsiRNA in eliciting a higher interference effect.

3.2.2. Real Time-PCR

The effect of RNAi using unique siRNAs was also proved by estimating quantitatively the virus DNA concentration present in the experimental and control groups, 48 h post-infection, using Real Time-PCR. SYBR® Green based Q-PCR method was used for this purpose. The threshold cycle \( C_t \), which indicates the significant increase in fluorescent level, is compared between the control and experimental groups. The difference in the threshold cycle \( \Delta C_t \) between the groups will characterize the difference in the initial virus load in the samples. The AHV-UL6-AsiRNA transfected sample attained threshold at 16.70th cycle (average of four independent PCR reactions), and control group attained \( C_t \) at 12.44th cycle (average of four independent PCR reactions). \( \Delta C_t \) between the two was found to be 4.26 cycles, which is equivalent to a reduction of 19 fold difference in virus concentration, 48 h post-infection, in the presence of AHV-UL6-AsiRNA (Fig. 2A). On the other hand, AHV-UL6-BsiRNA transfected sample attained \( C_t \) at 18.28th cycle (average of four independent PCR reactions), and control group attained \( C_t \) at 22.79th cycle (average of four independent PCR reactions), yielding \( \Delta C_t \) of 4.51 cycles (Fig. 2B). This is equivalent to about 23 fold difference in reduction in the virus load in the AHV-UL6-BsiRNA transfected cells, 48 h post-infection. Comparison of \( \Delta C_t \) between AHV-UL6-AsiRNA and AHV-UL6-BsiRNA reveals that the latter produced 3.5 folds more reduction in virus titre in comparison to the former. Thus AHV-UL6-BsiRNA appears to be the choicest siRNA for controlling AHV-1 replication.

4. Discussion

Post-transcriptional gene silencing (PTGS) mediated by siRNA has been a well-proven method for controlling replication of different viruses of plant and animal origin. Even though, initially there were apprehensions about siRNA mediated interference mechanism operating in mammalian systems owing to dsRNA induced global shutdown, Elbashir et al. (2001) had proven that direct introduction of siRNAs can induce RNAi in mammalian cells also. Consequently, many mammalian viruses were demonstrated to be amenable to siRNA mediated suppression both in cell culture system as well as in vivo system (Chen et al., 2004).

Duck virus enteritis, alternatively called as duck plague, caused by an Herpesvirus, is a major problem for the duck rearing regions of the world, as well as for free ranging waterfowl
Consequently, two unique siRNA targets were also identified in the UL-6 genome. These siRNAs, i.e. AHV-UL6-AsiRNA and AHV-UL6-BsiRNA were synthesized by an alternative method using Silencer® siRNA construction kit of Ambion®. Both the siRNAs were administered to DEF cells in separate experiments followed by infection with 1 m.o.i. AHV-1. More predominate reduction in CPE and virus titre was observed than the cocktail siRNAs. This could be due to the differences in the effective concentration of intracellular siRNAs as RNase-III used in the Ambion kit need not essentially generate siRNAs of 21 bp. It is also known that RNase-III generates predominantly siRNAs of 13–15 bp (Byrom et al., 2003). However, it is known that siRNAs of 21 bp is most suited for binding to RNA induced silencing complex (RISC) that eventually affects the degradation of specific mRNA. Further, the targeted region for cocktail siRNA contained many overlapping sequences of potential siRNAs. All these factors could have considerably reduced the effective molar concentration of 21 bp long siRNAs within the cocktail digest in comparison to the unique siRNAs used. However, the cocktail siRNA approach still remains one of the most suited, simple exploratory tool for identifying target sequence for gene silencing. Substitution of RNase-III with Dicer enzyme for generating cocktail would have been more effective, but not tested in the present study.

The present experiment was primarily intended to prove the amenability of siRNA as a method of control against AHV-1. Hence, quantitative technique like Real Time-PCR was also used to estimate the virus load following RNAi. Wang et al. (2004) have already used the same technique for monitoring the effect of siRNA in SARS replication. SYBR® Green based Q-PCR was effective in differentiating the viral DNA concentration in different experimental and control groups. The differences in threshold cycles (ΔCt) while using same volume of template material indicates a direct difference in the initial concentrations of template molecule in the different samples tested. One of the problems of this evaluation method is that viral DNA can get into culture supernatants as infectious virions, non-infectious, physically or genetically defective virions, or as free DNA released from lysed cells that may affect the the ΔCt. Hence the real time data may indicate directly the difference in amount of viral genomic DNA rather than the real infectious particle. On the other hand the viral titration directly indicate the difference in infectious particles. But since the same method is used for both experimental and control groups the ΔCt should indicate a difference in the initial viral concentration between the two. Further the same difference was estimated by virus titration as well to confirm the results. The difference in threshold cycle (ΔCt) between control and AHV-UL6-BsiRNA was 4.51 cycles, whereas between control and AHV-UL6-AsiRNA ΔCt was 4.26 cycles, clearly indicating the superiority of AHV-UL6-BsiRNA in eliciting RNAi (Fig. 2A and B). This finding had been supported by viral titration data also. We propose AHV-UL6-BsiRNA based plasmid vector or Adeno virus/Retro virus based vector for developing an effective prophylactics against AHV-1.
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