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Silencing shrimp white spot syndrome virus (WSSV) genes by siRNA

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Abstract

White spot syndrome virus (WSSV) is a major shrimp pathogen causing large economic losses all over the world. So far, however, there is no efficient approach to control this virus. RNA interference (RNAi), which has been applied to silence virus genes in eukaryotic organisms. In this investigation, a specific 21 bp short interfering RNA (vp28-siRNA) targeting a major envelope protein gene (vp28) of WSSV was used to induce gene silencing in vivo in *Penaeus japonicus* shrimp. It was found that the transcription and expression of vp28 gene were silenced by the sequence-specific vp28-siRNA. However, the RNAi effect disappeared or significantly weakened even if one-nucleotide mutation existed in the vp28-siRNA. As revealed by quantitative PCR, the vp28-siRNA caused a significant reduction in viral DNA production of WSSV-infected shrimp. When treated with the vp28-siRNA, WSSV-infected shrimp had a reduced mortality rate. After three injections of the vp28-siRNA, the virus was completely eradicated from WSSV-infected shrimp. These findings suggest that RNAi is capable of silencing sequence-specific genes of WSSV and might constitute a new therapeutic strategy for WSSV infection in shrimp.

Keywords: WSSV; RNAi; Gene silencing; Antivirus

1. Introduction

White spot syndrome is a major shrimp disease worldwide, which is caused by white spot syndrome virus (WSSV). Except for shrimp, WSSV has also been found in other species of crustaceans, such as crab and crayfish (Chen et al., 1997; Chou et al., 1995; Lo et al., 1996). The virus can cause 100% cumulative mortality within 2–10 days in farmed shrimp, and there is an increasing concern over the possible introduction of this virus into the wild shrimp population. Therefore, WSSV has attracted much attention from a research viewpoint in the past decade. However, there is (as yet) no efficient strategy to control the virus.

The virus contains a double-stranded circular DNA of about 300 kb, which has been completely sequenced on three WSSV isolates (Chen et al., 2002; van Hulten et al., 2001; Yang et al., 2001). The complete genome of WSSV has the capacity to approximately encode 180 open reading frames (ORFs) of 50 amino acids or more (van Hulten et al., 2001; Yang et al., 2001). It is known that, of the viral structural proteins, the envelope proteins play very important roles in WSSV infection in shrimp, such as recognition and attachment to receptors at the host cell surface (Wu et al., 2005; Zhang et al., 2004). In this context, it may be an alternative strategy in the antiviral study of shrimp to inhibit the expression of genes encoding major envelop proteins of WSSV. To this end, RNA interference (RNAi) is one of the most efficient approaches for suppression of WSSV gene expressions, which can mediate gene silencing of the virus and thus generate an antiviral response (Giladi et al., 2003; Gitlin et al., 2002; Lu et al., 2005).

RNAi is a natural process by which small double-stranded RNA (approximately 21–23 nt) directs sequence-specific silencing of homologous genes. This process is evolutionarily conserved and has been found in a wide range of eukaryotic organisms (Hammond et al., 2001; McManus and Sharp, 2002). Sequence-specific inhibition of cellular mRNA by RNAi can be triggered with the introduction of synthetic 21–23-nucleotide duplexes of RNA (Elbashir et al., 2001; Zamore et al., 2000) or, alternatively, by transcription of an expression construct (Brummelkamp et al., 2002), opening up possibilities for controlling replicative processes of pathogenic organisms. RNAi has been used to specifically inhibit gene expression and repli-

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cation of infectious viruses. The replication of a growing number of human pathogenic viruses has been shown to be inhibited by RNAi, including poliovirus, HIV-1, HCV, influenza virus and hepatitis B virus (Randall et al., 2003). RNAi has been revealed to function as an adaptive antiviral immune mechanism (Lu et al., 2005; Wilkins et al., 2005). Recently the in vivo roles of double-stranded RNA (dsRNA) and RNAi in shrimp antiviral immunity were demonstrated (Robalino et al., 2004, 2005; Westenberg et al., 2005). In the marine shrimp *Litopenaeus vannamei*, the antiviral response can be induced by sequence-independent or sequence-specific dsRNA which may activate RNAi-like mechanisms (Robalino et al., 2004, 2005). It has also been reported that siRNA could suppress the gene expression and replication of WSSV in a sequence-independent manner (Westenberg et al., 2005).

In an attempt to characterize the antiviral response in shrimp, the RNAi strategy was followed by using a specific 21 bp short interfering RNA (vp28-siRNA) targeting the vp28 gene of WSSV. The VP28 protein, a major envelop protein of WSSV, is involved in the attachment and penetration into shrimp cells (Wu et al., 2005; Yi et al., 2004). The results showed that the vp28-siRNA was capable of silencing the vp28 gene. This gene silencing was sequence-specific, which was different from the previous report in shrimp (Westenberg et al., 2005). When treated with vp28-siRNA, the expression of vp28 gene and the replication of viral DNA were significantly delayed or inhibited by siRNA, resulting in low mortality of WSSV-infected shrimp. These findings demonstrated the potentials for the gene function research and therapeutic treatment of WSSV by RNAi.

2. Materials and methods

2.1. Shrimp culture and white spot syndrome virus (WSSV)

Cultures of *P. japonicus* shrimp, approximately 10 g and 10–12 cm each, were performed keeping in groups of 20 individuals in 80 l aquariums at 20 °C. They were fed with commercial diet at 5% of body weight before and during the experiments. Hemolymph and gill tissues from *P. japonicus* shrimp were subjected at random to PCR detection with WSSV-specific primers to ensure that they were WSSV-free before experimental infection. The infected tissues from *P. monodan* shrimp with a pathologically confirmed infection were homogenized in TN buffer (20 mM Tris–HCl, 400 mM NaCl, PH 7.4) at 0.1 g/ml. After centrifugation at 2000 × *g* for 10 min, the supernatant was diluted to 1:100 with 0.9% NaCl and filtered through a 0.45 μm filter. Then 0.1 ml of filtrate was injected intramuscularly into healthy *P. japonicus* shrimp in the lateral area of the fourth abdominal segment using a syringe with a 29-gauge needle. Several days later, the WSSV-infected moribund shrimp were collected and stored at −70 °C as WSSV stock.

2.2. Synthesis of siRNAs

The siRNAs used in this study consisted of 21-nucleotide double-stranded RNAs, each strand of which contained a 19-nucleotide target sequence and a two-uracil (U) overhang at the 3′ end. According to the design rule for RNAi (Elbashir et al., 2002), a fragment of vp28 gene from WSSV was predicted to have RNAi capacity. This fragment (5′-GACCATCGAAACCCACACA-3′) was used as the target siRNA (vp28-siRNA). As controls, the sequence of vp28-siRNA was rearranged at random and mutated at one nucleotide, respectively, resulting in the corresponding random-siRNA (5′-CAGA CCTCACGACACAACA-3′) and mutation-siRNA (5′-GACCA*G*CGAAACCCACACA-3′). A fragment of vp28 gene, which had no RNAi capacity as predicted by the design rule (Elbashir et al., 2002), was also used as a control termed as non-siRNA (5′-ATGGATCTT TCTTTCACTC-3′).

siRNAs were synthesized in *vitro* using a commercially available kit according to the Manufacturer's instructions (TaKaRa, Japan). The formation of double-stranded RNAs was monitored by determining the size shift in agarose gel electrophoresis. The synthesized siRNAs were dissolved in siRNA buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl) and quantified by spectrophotometry.

2.3. RNAi assay in shrimp in vivo

WSSV and siRNA (vp28-siRNA, random-siRNA, mutation-siRNA and non-siRNA, respectively) were delivered into shrimp by simultaneous injection into the lateral area of the fourth abdominal segment at 0.1 ml/shrimp using a syringe with a 29-gauge needle (Wu et al., 2005). At the same time, each of four siRNAs only, as well as a negative control (0.9% NaCl) and a positive control (WSSV only), were included in the injections. For each treatment, 20 shrimp were used. After injections, the shrimp mortality and infection by WSSV were monitored daily. The shrimp gills were collected at 24, 48, 72 and 96 h post-infection and immediately stored at −70 °C until used. Three shrimp specimens from each group, selected at random, were subjected to Northern blot, Western blot and PCR analysis. All assays were carried out in triplicate.

2.4. WSSV detection and quantitative analysis by PCR

Twenty milligrams of gills were collected from shrimp and homogenized in 500 μl of guanidine lysis buffer (50 mM Tris–HCl, pH 8.0, 25 mM EDTA, 4 M guanidinium thiocyanate, 0.5% *N*-lauroylarcosine) at room temperature. After centrifugation at 15,000 × *g* for 3 min, 20 μl of silica were added to the supernatant for DNA absorption. Subsequently the mixture was rotated for 5 min, followed by centrifugation at 15,000 × *g* for 30 s. The pellet was rinsed twice with 70% ethanol and resuspended in 20 μl distilled water. Then it was centrifuged at 15,000 × *g* for 2 min. The supernatant was used as PCR template (Yang et al., 1997).

PCR was performed with two WSSV-specific primers (forward primer 5′-TATTGTCTCTCCTGACGTAC-3′ and reverse primer 5′-CACATTCTTCACGAGTCTAC-3′). The conditions for PCR amplification were as follows: 5 min at 94 °C, 40 cycles at 94 °C for 45 s and 68 °C for 1 min and extension at 68 °C for 5 min. For quantitative analysis of viral DNA, a competitive PCR

was conducted (Wang et al., 1997). The DNA template and equal volume of internal standard plasmid serially diluted in 10 folds or less were added to PCR reaction solution (Wang et al., 1997), followed by PCR as described above.

2.5. Northern blot analysis

Total RNAs were extracted from gill tissues of shrimp at different time (24, 48, 72 and 96 h post-infection), using 1 ml of Trizol reagent (Promega, America) according to the Manufacturer's instructions. After treatment with RNase-free DNase I (TakaRa, Japan) for 30 min at 37 °C, RNAs were separated by electrophoresis on a 2% agrose gel in 1× TBE buffer (90 mM Tris–boric acid; 2 mM EDTA, pH 8.0) and transferred to a nitrocellulose membrane (Amersham Biosciences, San Francisco, CA). The blots were probed with DIG-labeled vp28 gene and DIG-labeled shrimp actin gene, respectively. The DIG labeling and detection were performed following the protocol of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany). To detect the vp28-siRNA in shrimp gills, the DIG-labeled DNA fragment corresponding to vp28-siRNA was used in Northern blot.

2.6. Western blot analysis

The shrimp gill samples were analyzed in a 12% SDS-PAGE gel. Then the proteins, visualized using Coommassie brilliant Blue staining, were transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA) in electroblotting buffer (25 mM Tris, 190 mM glycine, 20% methanol) at 70 V for 2 h. The membrane was immersed in blocking buffer (3% BSA, 20 mM Tris, 0.9% NaCl, 0.1% Tween20, pH 7.2) at 4 °C overnight, followed by incubation with the antibody against the VP28 protein of WSSV for 2 h (Zhang et al., 2002). Subsequently, the membrane was incubated in HRP-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) for 1 h and detected with substrate solution (4-chloro-1-naphthol, Sigma).

2.7. Antiviral experiment in shrimp by RNAi

The vp28-siRNA (8 μg corresponding to 6 μM) and WSSV (10^4 copies/ml) were simultaneously injected into *P. japonicus* shrimp in the lateral area of the fourth abdominal segment at 0.1 ml/shrimp using a syringe with 29-gauge needle. The vp28-siRNA only, a negative control (0.9% NaCl) and a positive control (WSSV only) were also included in the injections. In antiviral assays, 30 shrimp were used for each treatment. Everyday, the shrimp mortality was monitored and three shrimp specimens from each group, selected at random, were subjected to PCR analysis. To obtain efficient antiviral effects, after injection with WSSV (10^4 copies/ml), the vp28-siRNA (8 μg) was intramuscularly injected into the same shrimp once (0 h p.i.), twice (0 and 24 h p.i.) or thrice (0, 24 and 48 h p.i.), respectively. Three shrimp were randomly selected and subjected to quantitative PCR analysis. All assays described above were carried out in triplicate.

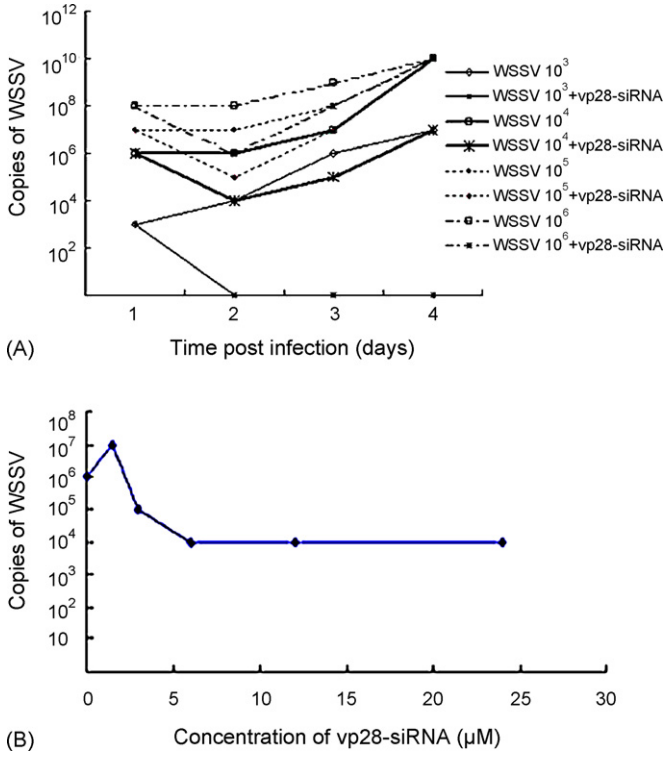

Fig. 1. Dose-dependent assays. (A) Dosage of WSSV copies for RNAi. Days post-infection were shown on the abscissa and accumulated virus copies on the ordinate. The solutions used for injection were indicated on the right. The concentration of vp28-siRNA was 24 μM. (B) The effects of vp28-siRNA concentrations on WSSV infection. The WSSV inoculum was 10^4 copies/ml. Each point represented the mean of triplicate assays within ±1% standard deviation.

3. Results

3.1. Dose-dependent inhibition of WSSV replication in shrimp by RNAi

To examine the ability of RNAi to suppress WSSV gene expression, the vp28 gene encoding a major envelop protein involved in virus infection was selected as the interfering target gene. The RNAi assay was performed in *P. japonicus* shrimp *in vivo* by injections with the vp28-siRNA (32 μg) and different copies of WSSV. The results of quantitative PCR detection revealed that the virus replication in all groups was inhibited by the vp28-siRNA, when compared with the corresponding controls (Fig. 1A). However, the inhibitory effect decreased with the increasing number of WSSV copies. It could be found that the vp28-siRNA induced stable interference effects up to 96 h post-infection (p.i.) for the 10^4 WSSV inoculum group. In the group of 10^3 WSSV inoculum, the virus was not detected at 48, 72 and 96 h p.i. when treated with the vp28-siRNA (Fig. 1A). The above experiments were carried out in triplicate yielding essentially the same results. In the subsequent assays, therefore, the WSSV inoculum with 10^4 copies was used. It was expected to produce the optimal response to siRNA in terms of mortality.

The dose response of siRNA was conducted by injections of 10^4 virus copies with different concentrations of vp28-siRNA. As indicated by quantitative PCR, the effects of RNAi were

equivalent at 8, 16 and 32 μg of vp28-siRNA (Fig. 1B). When the dose of vp28-siRNA was less than 8 μg, the inhibitory effect declined rapidly, suggesting that the optimum concentration of vp28-siRNA was 8 μg (corresponding to 6 μM).

3.2. Sequence-specific gene silencing of WSSV mediated by siRNA

In order to identify whether RNAi in WSSV was sequence-specific, shrimp were simultaneously injected with WSSV (10^4) and vp28-siRNA, random-siRNA, mutation-siRNA and non-siRNA, respectively, as well as WSSV only as a positive control. The Northern blots showed that no transcript or very slight transcript of vp28 gene was observed at 48 and 72 h p.i., respectively, for vp28-siRNA, whereas the vp28 gene was normally transcribed for random-siRNA and non-siRNA by comparison with the positive control (WSSV only) (Fig. 2) (data not shown for 48 h p.i.). The data revealed that the siRNA from a rearranged sequence of vp28-siRNA or a sequence having no RNAi capacity as predicted could not induce gene silencing of WSSV. Compared with the transcript of positive control, the mRNA level of vp28 gene was low for the mutation-siRNA (Fig. 2), indicating that one-nucleotide mutation could decrease the RNAi effect. At 96 h p.i., the transcription of vp28 was markedly reduced upon treatment of vp28-siRNA. These results suggested that the inhibition of WSSV gene transcription by siRNA was sequence-specific.

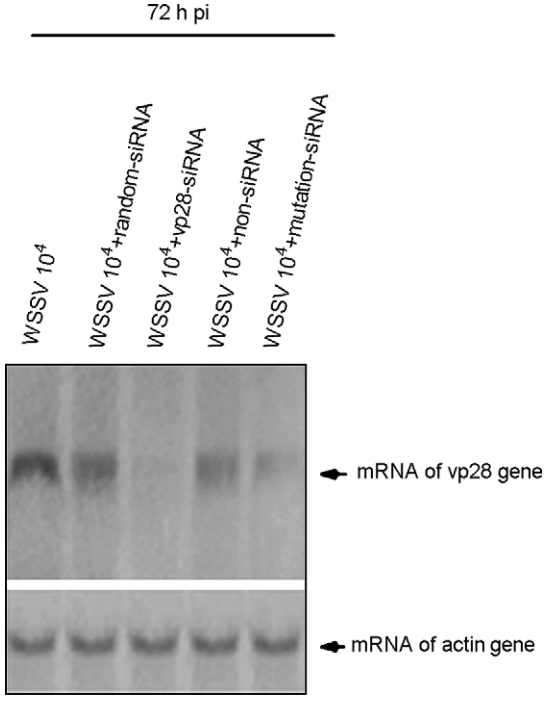

Fig. 2. Effects of siRNAs with various sequences on the transcription of vp28 gene. Shrimp were simultaneously injected with WSSV (10^4) and vp28-siRNA, random-siRNA, mutation-siRNA and non-siRNA, respectively, as well as WSSV only as a positive control. Northern blots were performed using the total RNAs extracted from shrimp gills at 72 h p.i. Shrimp actin gene was used as control.

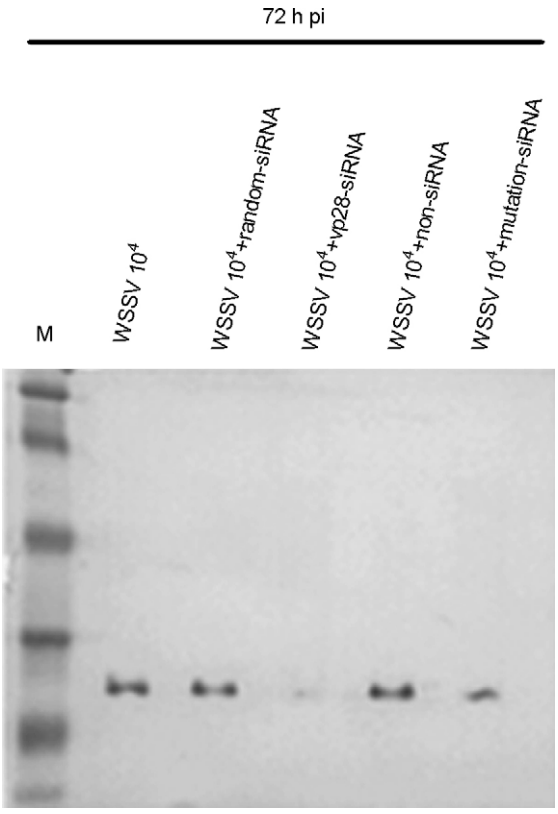

Fig. 3. Western blot analyses to detect the VP28 protein using shrimp gills. The solutions used for injections (WSSV 10^4 only, WSSV 10^4 + random-siRNA, WSSV 10^4 + vp28-siRNA, WSSV 10^4 + non-siRNA or WSSV 10^4 + mutation-siRNA) and time post-infection (72 h) were shown on the top. M: Protein marker.

The gills of shrimp co-infected by WSSV and siRNAs at 48, 72 and 96 h p.i. were transferred onto a nitrocellulose membrane for the detection of VP28 protein from WSSV with Western blot. The results showed that the expression of vp28 gene was almost suppressed up to 72 h p.i. when treated with vp28-siRNA, whereas the vp28 gene was expressed for random-siRNA, non-siRNA and mutation-siRNA, yielding the same results as those with Northern blot (Fig. 3) (data not shown for 48 and 96 h p.i.).

To confirm the results as revealed by Northern and Western bolts, quantitative PCR was used to detect WSSV in gills of *P. japonicus* at various infection stages (48, 72 and 96 h p.i.). By comparison with the positive control, the virus replication up to 96 h p.i. was significantly delayed at 10^2 copies or more for treatment of vp28-siRNA, a representative of which is shown in Fig. 4. However, the WSSV replication was not suppressed by treatment of random-siRNA and non-siRNA, and partly inhibited by treatment of mutation-siRNA (Fig. 4) (data not shown for 48 and 96 h p.i.). Based on the above data as revealed by Northern blot, Western blot and quantitative PCR, it could be inferred that RNAi was able to induce sequence-specific gene silencing of WSSV in shrimp.

3.3. The application of RNAi in shrimp antivirus

In an attempt to test the application of RNAi in shrimp, the vp28-siRNA and WSSV were co-injected into *P. japon-*

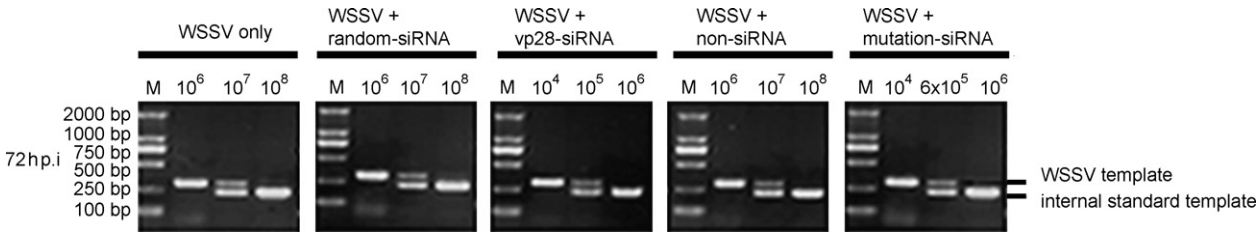

Fig. 4. Quantitative PCR detection of WSSV in shrimp gills at 72 h p.i. Numbers indicated WSSV copies. The solutions used for injections were shown on the top. They were WSSV (10^4) only, WSSV (10^4) + random-siRNA, WSSV (10^4) + vp28-siRNA, WSSV (10^4) + non-siRNA and WSSV (10^4) + mutation-siRNA, respectively. The PCR templates are represented on the right. M: DNA marker.

icus. At the same time, the vp28-siRNA only, as well as a negative control (0.9% NaCl) and a positive control (WSSV only) were included in the injections. The results revealed that the shrimp mortalities were very low for the negative control and vp28-siRNA only, whereas the shrimp from the positive control displayed 93% mortality at 12 days post-infection (Fig. 5). This suggested that the vp28-siRNA had no toxicity to shrimp. When the shrimp were co-infected with vp28-siRNA and WSSV, the shrimp mortality was significantly delayed ($P < 0.01$) (Fig. 5), indicating that the infection of WSSV could be inhibited by vp28-siRNA. The vp28-siRNA could be detected by Northern blot in shrimp gills from 24 to 96 h after injection (Fig. 6).

In the antiviral RNAi assays, the vp28-siRNA was continuously injected into WSSV-infected shrimp everyday up to three times. As revealed by quantitative PCR, the WSSV copies decreased very rapidly with the increasing number of injections of vp28-siRNA (data not shown). After three injections of vp28-siRNA, no virus was found, suggesting that WSSV had been completely eradicated by vp28-siRNA.

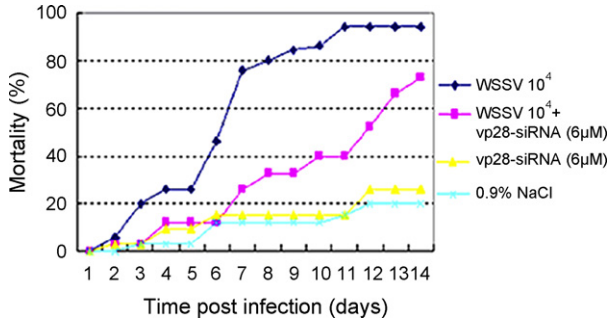

Fig. 5. Antiviral activity assays in *P. japonicus* by vp28-siRNA. The solutions used for injection were shown on the right. Each point represented the mean of triplicate assays within ±1% standard deviation.

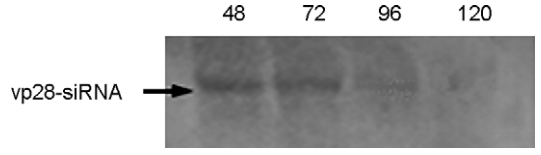

Fig. 6. Detection of vp28-siRNA in shrimp gills with DIG-labeled DNA fragment corresponding to vp28-siRNA. Numbers indicated time in hours after injection.

4. Discussion

RNAi mediated by small interfering RNA (siRNA) can induce the sequence- specific posttranscriptional silencing of a corresponding gene in many eukaryotes. It has been promoted as a powerful tool for functional genomics, but to date the successful stories have principally been in model organisms. Although initial studies of RNAi focused on cellular mRNA targets, present evidence suggests that it can also target sequence-specific viral RNAs, which have been well documented in antiviral systems of many organisms (Randall et al., 2003). However, it has been reported that siRNA, as well as long dsRNA, can induce sequence-independent silencing of viral genes based on the mortality of WSSV-infected shrimp (Robalino et al., 2004; Westenberg et al., 2005). In the present investigation, we have shown that the vp28-siRNA targeting the vp28 gene of WSSV is capable of mediating sequence-specific gene silencing, which is inconsistent with the previous reports (Robalino et al., 2004; Westenberg et al., 2005). This discrepancy might come from the differences of WSSV genes and gene fragments used in RNAi assays. In our study, four different siRNAs from WSSV vp28 gene were used. However, only the vp28-siRNA consistent with the design rule for RNAi could produce positive results.

Our study indicated that the suppression of transcription and expression of vp28 gene by vp28-siRNA resulted in the inhibition or delay of viral DNA replication, suggesting that the VP28 protein was involved in WSSV infection of shrimp, although there might be the contribution of innate immunity in this interfering effect. This was consistent with the previous studies (Wu et al., 2005; Yi et al., 2004). Up to date, it is very difficult to conduct functional studies of shrimp virus genes due to no available cell line for culturing shrimp virus. In this context, RNAi may be a potential tool for knocking out virus genes in shrimp, as well as endogenous genes of shrimp.

RNAi has been linked to viral resistance in eukaryotes raising the possibility that this phenomenon represents a form of sequence-directed immunity, which holds considerable promise as a therapeutic approach to silence disease-causing genes of viruses (Robalino et al., 2005; Soutschek et al., 2004). As indicated in the present study, the mortality of WSSV-infected shrimp treated by vp28-siRNA was significantly lower than that of the positive control (WSSV only) ($P < 0.01$), showing that siRNAs targeting genes involved in virus infection might be an efficient strategy for shrimp virus control. Moreover WSSV could be completely eradicated by three successive injections of

vp28-siRNA everyday. In order to use RNAi to protect shrimp cells against viral infection, therefore, it would be essential to enhance the stability of siRNA in shrimp. To achieve continuous and prolonged expression of siRNA, some effective approaches have been reported, such as the use of plasmids that endogenously express siRNA, virus-based vectors to introduce siRNA, conjugation of siRNA with cholesterol, and short hairpin RNA (Hemann et al., 2003; Qin et al., 2003). The stability of siRNA in shrimp merits further study, which will be very useful in shrimp virus control.

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