Mar Biotechnol (2009) 11:1–9
DOI 10.1007/s10126-008-9155-0

REVIEW

Application of Nucleic-acid-based Therapeutics for Viral Infections in Shrimp Aquaculture

Mudagandur S. Shekhar · Yuanan Lu

Received: 7 March 2008 / Accepted: 2 October 2008 / Published online: 22 October 2008

Abstract Viral infections are one of the major reasons for the huge economic losses in shrimp farming. The control of viral diseases in shrimp remains a serious challenge for the shrimp aquacultural industry, with major pathogens, such as the white spot syndrome virus, yellow head virus, Taura syndrome virus, hepatopancreatic parvovirus, and baculoviruses, being geographically widespread. In the absence of a true adaptive immune response system in invertebrates such as shrimp, one of the alternative and more specific approaches to counteract viral infections in shrimp could be the use of molecular-based gene transfer technologies, such as RNA interference (RNAi). The RNAi mechanism is initiated by double-stranded RNAs (dsRNAs), which are fragmented into shorter 21–23 nucleotides of short interfering RNAs (siRNAs) by a type III endonuclease, the Dicer. RNAi, which is mediated by small interfering RNA (siRNA), results in the sequence-specific post-transcriptional silencing of a target gene. This gene-silencing mechanism is universally conserved and is a well-known phenomenon that exists in many eukaryotes, including invertebrates. It has been recently extended to shrimp as an important potential tool in viral disease prevention. RNAi technology shows considerable promise as a therapeutic approach and efficient strategy for shrimp virus control in the aquaculture industry. Further progress in understanding the mechanism of siRNAs at the molecular level, as well as strategies to achieve their tightly regulated, stable, prolonged and tissue-specific expression in an effective manner, will definitely revolutionize therapeutic approaches for counteracting viral diseases of shrimp. In the present review, the recent development and potential use of RNAi in combating shrimp viral infections is discussed.

Keywords Double-stranded RNA (dsRNA) · Small interfering RNA (siRNA) · Shrimp aquaculture · Viral infection

M. S. Shekhar · Y. Lu (✉)
Department of Public Health Sciences, University of Hawaii, Honolulu, HI 96822, USA
e-mail: ylu@pbrc.hawaii.ed

M. S. Shekhar
Central Institute of Brackishwater Aquaculture, R.A. Puram, Chennai 600028, India

Introduction

In shrimp farming, specifically, viral disease outbreaks in economically important species, such as *Penaeus monodon*, *Litopenaeus vannamei*, and *L. stylirostris*, among others, has led to significant losses in production across different geographical locations since the early 1990s. Viral disease outbreaks are one of the major constraints to aquaculture production and trade, affecting socio-economic development in this sector worldwide. The risk of disease losses is likely to increase as the shrimp sector continues to grow, with newer viral pathogens being identified every year. The economic loss due to disease outbreaks in the Asian region was estimated to be at least $1.4 billion USD in 1990. In China alone, approximately $1 billion USD in production costs was lost in 1993. The loss in 1996 due to the Yellow head virus (YHV) and White spot syndrome virus (WSSV) was estimated to be valued over $500 million USD in Thailand. Based on farm-level surveys of 16 Asian countries, the annual loss in aquaculture production due to disease and environment-related problems was estimated to be more than $3 billion USD. Major economic losses have also been estimated in other parts of the world. For example, in 1993, Ecuador lost 28,000 tons of shrimp due

to an epizootic outbreak of Taura syndrome virus (TSV; Walker and Subasinghe 2000). Moreover, the incidence of viral infection in the wild brood stock, on which many shrimp aquaculture industries heavily rely, is of major concern and can impede progress in this sector. Prevalence of WSSV has been reported in shrimp captured from their natural environment in the coastal waters of Taiwan (Lo et al. 1997) and along the Atlantic coast (Chapman et al. 2004).

At present, there is no any cure for viral infections in crustaceans and the best management practice for shrimp aquaculture industry worldwide is to prevent the occurrence of viral disease to some extent. As an alternative approach to avoid incidences of viral disease, the shrimp aquaculture industry is currently placing a very high priority on obtaining specific pathogen-free (SPF) stocks of shrimp. Novel strategies, such as the use of recombinant vaccines and immunostimulants to control the occurrence viral diseases, would be highly desirable. However, invertebrates such as shrimp, which are devoid of a true adaptive immune response system, respond only by non-specific innate immune mechanisms (Chang et al. 2003). Shrimp viral defense mechanisms are not well understood. Nonetheless, recent reports indicate that studies are being aimed at the identification and characterization of immune response genes in order to have a better understanding of the mechanism involved in host–pathogen interactions and the innate immune system of shrimp. For example, an antiviral gene, *Pm*AV (Luo et al. 2003), as well as hemocyanin (Zhang et al. 2004) and interferon-like protein (IntlP; He et al. 2005) have been identified in penaeid shrimp. More recently, the identification and up-regulation of the *Rab* (*PjRab*) gene in virus-resistant shrimp indicates a novel Rab-dependent signaling complex, triggering a phagocytic defense against viruses in crustaceans (Wu et al. 2008). However, these studies are constrained by the lack of in vitro studies in cell lines of shrimp origin and absence of shrimp genome information.

Recently, the potential of RNAi as a powerful tool for functional genomics has been realized and is now being explored as disease prevention strategies for the therapeutic treatment of viral infections (Lu et al. 2005; Tan and Yin 2004). In the present review, we discuss the application of RNAi technology to shrimp, which will elucidate an improved understanding of the immune mechanisms present in these invertebrates, as well as effective therapeutic control strategies of viral diseases in shrimp.

RNA Interference

RNAi is a cellular mechanism that is initiated by double-stranded RNA (dsRNA) and results in the sequence-specific degradation of target RNA (Fire et al. 1998). The dsRNA is cleaved by an RNase III family enzyme, the Dicer, into shorter fragments of ~21-mer short interfering RNAs (siRNAs; Hammond et al. 2000). These siRNAs are incorporated into the RNA-induced silencing complex (RISC), which is a multi-component and multi-turnover nuclease enzyme complex (Hammond et al. 2000; Hammond et al. 2001; Martinez et al. 2002; Haley and Zamore 2004). RISC, upon incorporation of a siRNA duplex, is activated by ATP and leads to the unwinding and eventual strand separation of the siRNA duplex. The unwound antisense siRNA strand is then used by RISC to find the target mRNA to induce its endonucleolytic cleavage (Wadhwa et al. 2004). This gene-silencing mechanism, which is universally conserved, is a well-known phenomenon that exists in many eukaryotes, including invertebrates (Brown et al. 2003; Denli and Hannon 2003). The sequence-specific inhibition of viral nucleic acids can be achieved either by introducing synthetic 21–23-nucleotide duplexes of RNA (Elbashir et al. 2001a) or by transcription of an expression construct (Brummelkamp et al. 2002), resulting in the silencing of the viral pathogens, thereby inducing an antiviral response at the molecular level.

The inhibition of replication of human pathogenic viruses, such as polio virus (Gitlin et al. 2002), HIV-1 (Novina et al. 2002), HCV (Randall et al. 2003), and hepatitis B virus (Giladi et al. 2003) by this RNAi mechanism is well documented, proving its effectiveness to suppress these viral infections. This suggests the possibility of using RNAi as an antiviral tool for viral disease prevention in shrimp aquaculture. In the shrimp aquaculture industry, white spot syndrome caused by the white spot syndrome virus (WSSV), is one of the most serious diseases. The outbreak of this viral disease is reported to result in 100% mortality within 7–10 days in commercial shrimp farms, leading to huge economic losses worldwide (Cai et al. 1995; Lightner 1996; Flegel 1997). Presently, in the absence of any efficient therapeutic mean to control this or any other viral infections in aquaculture, the use of RNAi technology in shrimp appears very promising. Although initial studies of RNAi focused on cellular mRNA targets, the present evidence and success achieved in targeting sequence-specific viral RNAs in many organisms (Randall et al. 2003) has initiated research in targeting viruses that affect aquatic animals. In shrimp, the use of both exogenously synthetic long dsRNAs or siRNAs have been reported to induce an antiviral response (Robalino et al. 2004, 2005; Tirasophon et al. 2005; Westenberg et al. 2005; Xu et al. 2007). The various gene constructs used in shrimp RNAi technology that result in RNA interference are shown in Table 1.

Table 1 Gene constructs used in shrimp RNAi technology resulting in RNA interference

Shrimp/cells	Gene construct	Protection studies against (virus)	Gene	Reference
Sequence-specific gene constructs				
L. vannamei	dsRNA	WSSV	DNA pol	Robalino et al. 2005
	dsRNA	WSSV	Rr2	
	dsRNA	WSSV	ORF252	
	dsRNA	WSSV	vp28	
	dsRNA	WSSV	vp19	
P. monodon	siRNA	WSSV	vp28	Westenberg et al. 2005
	siRNA	WSSV	vp15	
P. monodon	dsRNA	YHV	Protease	Yodmuang et al. 2006
M. japonicus	siRNA	WSSV	vp28	Xu et al. 2007
L. vannamei	siRNA	WSSV	DNA pol	Wu et al. 2007
	siRNA	WSSV	Rr2	
	siRNA	WSSV	Tk–tmk	
	siRNA	WSSV	vp24	
	siRNA	WSSV	vp28	
P. chinensis	dsRNA	WSSV	vp28	Kim et al. 2007
	dsRNA	WSSV	vp281	
	dsRNA	WSSV	Protein kinase	
P. monodon	dsRNA	YHV	Protease	Tirasophon et al. 2007
P. monodon	dsRNA	WSSV	Vp28	Sarathi et al. 2008a
P. monodon	dsRNA	WSSV	Vp28	Sarathi et al. 2008b
Non-specific gene constructs				
L. vannamei	dsRNA	WSSV	Duck Immunoglobulin	Robalino et al. 2004
	dsRNA	WSSV	Genomic non Coding region of catfish	
	dsRNA	WSSV	Pig immunoglobulin	
	dsRNA	WSSV	Vector pBeloBAC11	
	dsRNA analogue	WSSV	Poly (C–G)	
P. monodon	siRNA	WSSV	Gfp	Westenberg et al. 2005
P. monodon	dsRNA	YHV	Gfp	Yodmuang et al. 2006
P. chinensis	dsRNA	WSSV	Gfp	Kim et al. 2007
Cell culture				
Oka	dsRNA	YHV	Protease	Tirasophon et al. 2005
	dsRNA	YHV	Polymerase	
	dsRNA	YHV	Helicase	
	dsRNA	YHV	gp65	
	dsRNA	YHV	gp116	
Sf21	siRNA	WSSV	vp28	Westenberg et al. 2005
SISK	dsRNA	WSSV	vp28	Sarathi et al. 2008b
Endogenous genes				
L. vannamei	dsRNA		Hemocyanin	Robalino et al. 2005
	dsRNA		CDP	

rr2 Ribonucleotide reductase small subunit, *tk–tmk* thymidine kinase–thymidylate kinase, *gfp* green fluorescent protein; DNA pol = DNA polymerase, *CDP* CUB (complement subcomponents C1r-C1s/sea urchin protein Uegf/bone morphogenetic protein 1) domain protein

Strategy for Target Sequence Selection

siRNAs suppress gene expression by a highly regulated enzyme-mediated process involving multiple RNA–protein interactions. These interactions can lead to biased strand selection during siRNA–RISC complex formation and activation, which may contribute to the overall efficiency of RNAi. RNAi adapts a highly sequence-specific interference of gene expression, making it convenient to design siRNA-targeting specific viral genes, thereby, preventing undesired off-target effects that commonly occur with other pharmaco-therapeutic approaches. Hence, a very important and critical factor for effective siRNA design is the appropriate and effective selection of target sequences on

the gene of interest, which leads to the identification of potent siRNA. The in silico analysis for target identification is an essential step for designing the siRNA. Currently, there are quite a few of on-line software available that assist in predicting the target site for a given gene. The position of the cleavage sites in the target RNA is defined by the 5′ end of the guide siRNA, rather than its 3′ end. There are now a set of rules that are taken into consideration when using computational approaches for designing siRNA and algorithms that incorporate the findings of Elbashir et al. (2001b) and Reynolds et al. (2004). For example, low G/C content, lack of inverted repeats and other features highlight the utility and the most accepted method of rational designing to enhance the gene-silencing effect of siRNA. Based on the findings from examining siRNA in *Drosophila melanogaster* embryo lysate for their requirements regarding length, structure, chemical composition and sequence in order to mediate efficient RNAi (Elbashir et al. 2001c), the general guidelines to be followed for effective siRNA designing are: (1) duplexes of 21 nt siRNAs with 2 nt 3′ overhangs are the most efficient triggers of sequence-specific mRNA degradation; (2) Beginning with the AUG start codon of the transcript, AA dinucleotide sequences are scanned and the 3′-adjacent 19 nucleotides are considered as potential siRNA target sites; (3) An empirical target site (5′-AAN(19)UU) in the region of mRNA with 30–50% GC content is more active than those with a higher G/C content; (iv) siRNA duplexes with an antisense siRNA 3′ overhang of AA, CC or GG are less active when compared with UU; (v) Stretches of more than 4 T's or A's in the target sequence when designing sequences to be expressed from an RNA pol III promoter should be avoided as a 4–6 nucleotide poly(T) tract acts as a termination signal for RNA pol III, creating RNA molecules with a short poly(U) tail; (vi) If the target mRNA does not contain 5′-AAN(19)UU, an alternative target site, 5′-AAN(21) or 5′-NAN(21), is chosen; and (vii) The potential target sites are compared to the appropriate genome database to eliminate the target sequences having homology to other coding sequences.

dsRNA-mediated Gene Silencing in Shrimp

dsRNA-mediated viral infections in vertebrates lead to the recognition of dsRNA by Toll-like receptor 3 and RNA-dependent protein kinases for mounting antiviral responses (Matsumoto et al. 2004; Williams 2001) by the activation of the interferon system, initiation of apoptosis, and inhibition of cellular protein synthesis. This phenomenon of innate immune activation by dsRNA was thought to be restricted to vertebrates, as the genes encoding homologues of interferons and their receptors were not found in invertebrate genomes. Recently, however, the Toll pathway has also been identified as a vital part of the *Drosophila* antiviral response (Zambon et al. 2005). The relatively close relationship between insects and crustaceans, in addition to the recent finding of a toll receptor in shrimp, such as *Litopenaeus vannamei* (Yang et al. 2007), *Penaeus monodon* (Arts et al. 2007), and *Marsupenaeus japonicus* (Mekata et al. 2008), indicates a possible role of the Toll pathway in shrimp antiviral immunity and needs further investigation. Recent studies indicate possible uses of dsRNA for effectively blocking viral disease progression in shrimp against at least three unrelated viruses: WSSV (Robalino et al. 2005), TSV (Robalino et al. 2004), and YHV (Tirasophon et al. 2005; Yodmuang et al. 2006). Several reports suggest that the dsRNA induction of antiviral immunity in shrimp is through two pathways that utilize sequence-independent and sequence-dependent dsRNAs, as described below. These two pathways may most likely be cooperatively functioned in a systemic antiviral defense in shrimp (Yodmuang et al. 2006).

One of the first reports using dsRNA in shrimp demonstrated that the exposure of a marine shrimp (*Litopenaeus vannamei*) to dsRNA induces a general innate antiviral immunity in a sequence-independent manner. Those results further demonstrated that the antiviral immune system of shrimp against WSSV and TSV responded to dsRNA, regardless of its sequence and base composition. For example, each of the sequences derived from the vertebrate immunoglobulin genes of ducks and pigs, fish non-coding genomic DNA, and a bacterial vector sequence (pBeloBAC11 vector), induced protection against WSSV infection (Robalino et al. 2004). An unrelated long dsRNA corresponding to the green fluorescence protein (GFP) gene is also reported to provide higher survival rates against WSSV (Kim et al. 2007). Protection using unrelated dsRNAs such as GFP and TSV-polymerase was also achieved, though partially resulting in the inhibition of YHV by 50% and 17%, respectively (Yodmuang et al. 2006). However, the innate antiviral protection offered by dsRNA in shrimp was overcome when the virus was delivered at a high dose shortly after dsRNA injection (Robalino et al. 2004). Similarly, it was observed that in the case of YHV inhibition by non-specific dsRNA of GFP, there was a greater effect at 48 h than at 72 h post-infection, indicating that non-specific inhibition diminished relatively quickly due to the degradation of long dsRNA and there was a longer effect of the specific dsRNA than the non-specific one (Tirasophon et al. 2005).

It was observed that viral sequence-specific dsRNAs generated by in vitro transcription of sequences representing portions of the ribonucleotide reductase small subunit (rr2), DNA polymerase (dnapol), vp28 and vp19 from WSSV, affords significant and potent antiviral immunity in vivo when challenged with high doses of virus adminis-

tered by injection and oral routes, as compared to a non-specific dsRNA. The degree of protection afforded by specific dsRNAs varies for different viral target genes, supporting the fact that target selection is an important factor for successful suppression of viral diseases by dsRNA (Robalino et al. 2005). For example, shrimp injected with dsRNAs corresponding to WSSV vp28 and protein kinase showed higher survival rates than those injected with dsRNAs corresponding to the WSSV vp281 and non-specific GFP gene (Kim et al. 2007). In case of YHV, dsRNA targeting the non-structural genes (protease, polymerase, and helicase) inhibited YHV replication in an effective manner, whereas dsRNA targeting the structural genes (gp116 and gp64) were the least effective in shrimp primary cell cultures, suggesting an important role of the copy number of the target sequences in viral inhibition (Tirasophon et al. 2005). Thus, these data indicate that not all viral-derived dsRNAs are effective in inhibiting viral replication via the RNAi pathway. Some of the non-structural genes are early genes that are essential, but are expressed at low levels during viral replication. Therefore, knocking down an early gene would be a more efficient strategy to inhibit viral infection by the RNAi mechanism (Yodmuang et al. 2006). The potency of the dsRNA on virus suppression is a dose-dependent phenomenon (Robalino et al. 2005). As reported with WSSV vp19 dsRNA, 1 μg of protease dsRNA was shown to efficiently inhibit YHV, while viral inhibition was drastically reduced in the presence of 0.2 μg (Tirasophon et al. 2005). Efficient prevention of viral propagation and protection of shrimp from mortality has been reported by the pre-administration of *P. monodon* with 25 μg of YHV-specific dsRNA-protease 24 h prior to YHV inoculation (Yodmuang et al. 2006). The dose-dependent effect of dsRNA has also been reported for YHV inhibition by Yodmuang et al. (2006). They showed that using 25 μg of virus-specific dsRNA (protease) was effective for complete inhibition of YHV replication. However, decreasing the dsRNA (protease) concentration to 5 or 1 μg resulted in the diminished efficiency of YHV inhibition to approximately 80% and 40%, respectively. The effect of dsRNA was observed to last for at least 5 days when shrimp were administered with YHV-specific dsRNA (protease) prior to YHV challenge, resulting in complete protection (Yodmuang et al. 2006).

The length of dsRNA also affects the RNAi efficiency, as observed in YHV suppression in shrimp cells, where a long dsRNA could generate more effective siRNAs than shorter ones (Tirasophon et al. 2005). These data imply that the involvement of RNAi-like mechanisms in the antiviral response of shrimp have stronger innate (non-specific) immune responses using a dose-dependent, sequence-specific dsRNA, rather than sequence-independent dsRNA (Robalino et al. 2005). The possible role of RNAi-like responses in *L. vannamei* was further tested using long dsRNA specific for two endogenous genes, hemocyanin and a novel CDP gene and checking for their expression patterns within hepatopancreas and gill cells, respectively. This revealed a substantial and specific depletion of the cognate mRNAs (Robalino et al. 2005) and suggested that the mechanism for transporting dsRNA into the cells (Feinberg and Hunter 2003) and the existence of cell surface receptors in shrimp mediate the uptake of dsRNA, as reported in *Drosophila melanogaster* (Ulvila et al. 2006).

For the first time, the therapeutic effect of dsRNA was reported in shrimp by curing YHV-infected shrimps. Viral replication could be prevented within 12 h, whereas shrimp mortality could be prevented using 25 μg of YHV-specific dsRNA-protease at 3 h post-YHV infection. Interestingly, non-specific dsRNA-GFP was unable to inhibit YHV propagation in a therapeutic mode (Tirasophon et al. 2007).

siRNA-mediated Gene Silencing in Shrimp

There are discrepancies in the results obtained using viral genes for siRNA constructs. This could be due to differences in the viral genes and gene fragments used in the RNAi assays. It is also emphasized that positive results are obtained only if the siRNAs are consistent with the design rules of RNAi (Xu et al. 2007). For example, WSSV vp19-siRNA delivered into shrimp was not successful in gene silencing and was a poor inducer of sequence-specific antiviral responses when compared to long dsRNA. This was attributed to improper design of siRNA, inadequate delivery into cells, limited half-life of siRNA in the extracellular environment of the shrimp, or existence of different mechanisms for uptake of siRNA (Robalino et al. 2005). It has been observed that even a one-nucleotide mutation in the siRNA gene construct results in a significant loss of the RNAi effect in shrimp (Xu et al. 2007). It is interesting to note that silencing of the non-structural viral protein genes of WSSV DNA *pol*, *rr2* and thymidine kinase–thymidylate kinase (*tk–tmk*) increased the shrimp survival rate more than the silencing of the structural protein genes (vp24 and vp28; Wu et al. 2007). However, several studies on RNAi in shrimp suggested that the effective time of RNAi in vivo is limited (Wu et al. 2007; Robalino et al. 2004; Westenberg et al. 2005). This may be due to the short half-life of siRNA constructs, which prevents them from completing the process of viral replication inhibition in an infected cell, indicating the necessity for repetitive siRNA injections into the shrimp. For example, the complete eradication of virus from WSSV-infected shrimp was achieved by three injections of the vp28-siRNA. The antiviral response induced by siRNAs in shrimp is concentration dependent. The

optimum concentration of vp28-siRNA was determined to be 8 μg in order to achieve an inhibitory effect of siRNA against virus replication (Xu et al. 2007). Whereas, Westenberg et al. (2005) reported that 10 μM of WSSV vp28-siRNA, when injected into shrimp, resulted in significant protection of *P. monodon*. However, this effect was reduced when shrimp were inoculated with an amount of siRNA ten times lower. In vitro studies showed that 5 μg of WSSV vp28 siRNA, when transfected into Sf21 cells, were able to specifically and efficiently silence the homologous gene (Westenberg et al. 2005).

Interestingly, as observed with large dsRNA molecules that can induce antiviral immunity in a sequence-independent manner (Robalino et al. 2004), similar results were obtained using sequence-independent siRNA in shrimp. For example, it was reported that shrimp injected with WSSV specific vp28 and vp15 siRNA showed a relative survival rate of 50% and 42%, respectively, which was not significantly different than the 33% relative survival rate observed in shrimp injected with non-specific GFP siRNA. This sequence-independent reaction is suggested to be due to poor intake of siRNAs by shrimp cells, resulting in their circulation in the hemolymph, thereby inducing a general defense response (Westenberg et al. 2005).

Non-specific Response Induced by dsRNA in Shrimp

siRNA and dsRNA are have been observed to induce silencing in numerous mammalian cell lines (Elbashir et al. 2001a; Paddison et al. 2002). The protein kinase R (PKR) and RNase L pathways are known to be activated by long dsRNAs in mammalian cells (Gitlin and Andino 2003). Presently, however, the mechanism of this innate immunity phenomenon in shrimp and other invertebrate taxa remains unknown. It is well known that the recognition of dsRNA by another pathway that involves RNAi is widely distributed among invertebrates as an important mechanism of the invertebrate antiviral response. Therefore, dsRNA can probably engage both innate immune pathways and an RNAi-like mechanism to stimulate potent antiviral immunity in a marine shrimp, as suggested by Robalino et al. (2005).

Higher survival rates of shrimp injected with long non-specific dsRNAs such as GFP (Kim et al. 2007), sequences derived from vertebrate immunoglobulin genes, fish non-coding genomic DNA, and bacterial vector sequence (Robalino et al. 2004) against viral infection indicate that long dsRNA can enhance the shrimp's antiviral immunity in a sequence-independent manner. However, it has been reported that virus-specific dsRNAs offer higher protection than a non-specific GFP dsRNA and specific viral genes play a key role in offering higher degree of protection (Kim et al. 2007). These results indicate that dsRNA is recognized by the shrimp antiviral immune system as a molecular pattern irrespective of its sequence and base composition (Robalino et al. 2004). As mentioned earlier, it is well known that mammalian cells respond to the presence of dsRNA with a general, non-specific shutdown of translation, mediated primarily by PKR and RNase L. Presently, the pathway involved in the enhancement of antiviral immunity induced by long dsRNA is not understood and remains to be explored in shrimp. It is possible that there may be some evolutionary link between vertebrates and invertebrates leading to the recognition of dsRNA processes and innate antiviral immunity phenomena. For example, in mammals, the Toll-like receptor (TLR) is involved in the recognition of various microbial components and dsRNA (Alexopoulou et al. 2001). This leads to the regulation of gene expression of the immune system by signal transduction. The recent findings of a Toll receptor in shrimp (Yang et al. 2007; Arts et al. 2007; Mekata et al. 2008) and other shrimp genes such as ubiquitin protein, interferon-like protein, transcription factor AP-1, protein kinase raf and the peptides sharing certain similarities with the components of the complement and cytokines system (He et al. 2005), suggest the possibility of the existence of a similar antiviral immunity in response in invertebrates.

Counter Defense Mechanism to RNAi by Shrimp Viruses

Direct evidence of inhibition of the gene-silencing mechanism due to virus-induced phenomenon has been reported in shrimp. WSSV-mediated silencing of hemocyanin and homologue of the Signal Transducer and Activator of Transcription (STAT) was found to be tissue specific in hepatopancreas. This indicates that the shrimp viruses have the ability to impair the RNAi mechanism within the host (Robalino et al. 2007). The use of high doses of virus in the challenge has also been observed to result in a diminished RNAi-dependent protection mechanism offered by dsRNA in shrimp (Robalino et al. 2004). The loss of dsRNA-protease potency results in increased mortality rates and was observed at 24 h post-infection in YHV-infected shrimp (Tirasophon et al. 2007). These data suggest that the RNAi is overwhelmed by the increased virus level in vivo due to rapid replication of the virus or by accumulation of certain viral proteins that suppress the RNAi mechanism. For example, the suppression of RNA silencing by the B2 protein encoded by the flock house virus (Li et al. 2002) and Nodamura virus (Sullivan and Ganem 2005), and the core protein encoded by the hepatitis C virus (Wang et al. 2006), clearly demonstrates a conserved counter defense mechanism of RNAi by many

viruses. It remains to be seen if the shrimp viruses also encode B2 proteins, as well as their role as suppressors of RNA silencing.

Limitations and Future Prospects of RNAi Technology in Shrimp

RNAi is currently considered as a very promising strategy for viral disease control in shrimp, however, the delivery of dsRNA/siRNA into target cells and tissues remains a big challenge, at present. Even though most of the reports indicate that the delivery of dsRNA into shrimp by intramuscular injection (Robalino et al. 2004; Wu et al. 2007; Xu et al. 2007) is possible, injection of dsRNA is practically impossible in shrimp farming at the field level. Hence, there is an urgent need for further investigations to develop simple and effective routes for dsRNA/siRNA delivery into shrimp. One of the alternative approaches would be to use edible dsRNA producing bacteria, as used in the case of RNAi application in *C. elegans*. This method made use of bacteria that were engineered to be deficient in RNase III. This, when fed continuously to *C. elegans*, successfully resulted in obtaining RNAi phenotypes that could be maintained over the course of several generations (Timmons et al. 2001). Similarly, ingestion of bacterially expressed double-stranded RNA was shown to inhibit gene expression involved in regenerative processes in planarians (Newmark et al. 2003). A very similar approach has been recently demonstrated in shrimp. Sixty-eight percent and 37% survival was observed in shrimp, fed with pellet feed coated with inactivated vp28-dsRNA-induced bacteria and a vp28-dsRNA–chitosan nanoparticle complex, which were used as carriers to deliver the WSSV-vp28-dsRNA, respectively (Sarathi et al. 2008a). Chitosan is a natural polysaccharide derived from crustacean shells. Previous reports demonstrated that it has successfully been used in oral delivery of a construct expressing the β-galactosidase reporter gene into fish by encapsulating the DNA and incorporating it into fish feeds (Ramos et al. 2005).

To make siRNAs effective in inducing gene silencing, it is essential that siRNAs reach the cytoplasm of the target cell. Since the naked RNA cannot penetrate cellular lipid membranes (Li et al. 2006), it is therefore imperative that delivery of siRNA require carriers such as viral and non-viral vectors for effective transportation into the targeted cells, under in vitro and in vivo conditions. The use of viral systems result in high transfection efficiencies, however, their therapeutic applications is greatly limited due to safety concerns and high cost. In contrast, non-viral transfection agents such as cationic liposomes and polymer particles are simple, cost effective and safe. For example, polyethylenimines (PEI), which are a series of synthetic polymers, bear a high cationic charge density, providing the compactness to carry the DNA into complexes. PEI has been shown to have a high transfection efficiency in shrimp. The evaluation of gene transfer methods by microinjection, electroporation, and transfection reagent into shrimp zygotes revealed that the use of transfection reagent jetPEI had a higher efficiency of gene transfer, hatching rates, and gene expression. This transfection reagent could effectively transfect DNA into shrimp eggs, even in the presence of the shrimp jelly layer (Sun et al. 2005). This data indicates the probable use of transfection reagents having the capacity to perform large scale shrimp egg transfections with dsRNA or siRNA vectors as a means for gene delivery in the future.

A permanent shrimp cell line will greatly facilitate the work of gene transfer technology. In addition, it will lead to the understanding of the host–pathogen interaction at the molecular level, which is very critical in designing strategies, such as RNAi, for ensuring shrimp survival against viral infections. One of the earliest attempts on in vitro culture of shrimp cells was initiated in Taiwan (Chen et al. 1986). However, not much progress has been made and most of the research on shrimp cell culture is limited to the standardization of culture tissue and medium formulation (Nadala et al. 1993; Hsu et al. 1995; Tong and Miao 1996; Shimizu et al. 2001). These efforts have led to some successes, but a permanent shrimp cell line has yet to be established (Rinkevich 2005). At present, the techniques for introducing foreign genes into crustacean cells are underdeveloped and remain to be explored. Also the transfection of plasmid DNA into shrimp cells has been reported to be difficult (Shike et al. 2000). These are some of the reasons that impede the design of gene transfer strategies to create cell lines from shrimp. A modified retroviral vector has been successfully used to achieve foreign gene expression in cell cultures derived from Oka organs and the ovaries of the *P. stylirostris*. However, heterologous promoters of the Moloney murine leukemia virus and Rous sarcoma virus were used in this study to mediate reporter gene expression. Also, both Oka- and ovary-derived primary cell cultures were short lived to test the stability of transgene expression (Shike et al. 2000). The shrimp beta-actin promoter from *P. vannamei* has been used to construct an expression vector. This may find use in developing transgenic shrimps for expression of siRNA (Sun et al. 2002).

In conclusion, RNAi has been reported to be associated with viral resistance in shrimp indicating the possibility that this mechanism represents a type of sequence-directed immunity. The protection offered by specific dsRNA and siRNAs as a dose- and sequence-dependent phenomenon in shrimp indicates that the RNAi mechanism does exist in shrimp to counteract viral diseases. Therefore, it has considerable promise as a therapeutic approach and

efficient strategy for shrimp virus control in the aquaculture industry. Presently, however, the use of RNAi technology in shrimp has indicated that the effective time of RNAi in vivo is limited due to the very short half-life of synthetic RNA duplexes. It is likely that siRNAs will not be present for complete elimination of virus replication in an infected tissue. In order to use RNAi technology in an effective manner to protect shrimp against viral diseases, it would be essential that future studies focus on increasing the stability of siRNA in shrimp. In addition, obtaining continuous and prolonged expression of siRNA by use of high-expression plasmids, virus-based vectors and non-viral transfection agents to introduce siRNA into shrimp remains to be explored. Moreover, a permanent shrimp cell line would be highly desirable, leading to a better understanding of the RNAi mechanism in shrimp.

Acknowledgment The overseas associateship provided to M.S. Shekhar by the Department of Biotechnology, India, during the study period is greatly acknowledged.

References

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413:732–738

Arts JAJ, Cornelissen FHJ, Cijsouw T, Hermsen T, Savelkoul HFJ, Stet RJM (2007) Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, *Penaeus monodon*. Fish Shellfish Immunol 23:504–513

Brown AE, Crisanti A, Catteruccia F (2003) Comparative analysis of DNA vectors at mediating RNAi in *Anopheles* mosquito cells and larvae. J Exp Biol 206:1817–1823

Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. Science 296:550–553

Cai S, Huang J, Wang C, Song X, Sun X, Yu J, Zhang Y, Yang C (1995) Epidemiological studies on the explosive epidemic disease of prawn in 1993–1994. J Fish China 19:112–117

Chang CF, Su MS, Chen HY, Lia IC (2003) Dietarybeta1,3-glucan effectively improves immunity and survival of *Penaeus monodon* challenged with white spot syndrome virus. Fish Shellfish Immunol 15:297–310

Chapman R, Browdy C, Savin S, Prior S, Wenner E (2004) Sampling and evaluation of white spot syndrome virus in commercially important Atlantic penaeid shrimp stocks. Dis Aquat Org 59:179–185

Chen SN, Chi SC, Kou GH, Liao IC (1986) Cell culture from tissues of grass prawn, *Penaeus monodon*. Fish Pathol 21:161–166

Denli AM, Hannon GJ (2003) RNAi: an ever-growing puzzle. Trends Biochem Sci 28:196–201

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001a) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411:494–498

Elbashir SM, Lendeckel W, Tuschl T (2001b) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15:188–200

Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T (2001c) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J 20:6877–6888

Feinberg EH, Hunter CP (2003) Transport of dsRNA into cells by the transmembrane protein SID-1. Science 301:1545–1547

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806–811

Flegel TW (1997) Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. World J Microbiol Biotechnol 13:433–442

Giladi H, Ketzinel-Gilad M, Rivkin L, Felig Y, Nussbaum O, Galun E (2003) Small interfering RNA inhibits hepatitis B virus replication in mice. Mol Ther 8:769–776

Gitlin L, Andino R (2003) Nucleic acid-based immune system: the antiviral potential of mammalian RNA silencing. J Virol 77:7159–7165

Gitlin L, Karelsky S, Andino R (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. Nature 418:430–434

Haley B, Zamore PD (2004) Kinetic analysis of the RNAi enzyme complex. Nat Struct Mol Biol 11:599–606

Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404:293–296

Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 293:1146–1150

He NH, Qin QW, Xu X (2005) Differential profile of genes expressed in hemocytes of White Spot Syndrome Virus-resistant shrimp (*Penaeus japonicus*) by combining suppression subtractive hybridization and differential hybridization. Antiviral Res 66:39–45

Hsu YL, Yang YH, Chen YC, Tung MC, Wu JL, Engelking MH, Leong JC (1995) Development of an in vitro subculture system for the oka organ (lymphoid tissue) of *Penaeus monodon*. Aquaculture 136:43–55

Kim CS, Kosuke Z, Nam YK, Kim SK, Kim KH (2007) Protection of shrimp (*Penaeus chinensis*) against white spot syndrome virus (WSSV) challenge by double-stranded RNA. Fish Shellfish Immunol 23:242–246

Li H, Li WX, Ding SW (2002) Induction and suppression of RNA silencing by an animal virus. Science 296:1319–1321

Li CX, Parker A, Menocal E, Xiang S, Borodyansky L, Fruehauf JH (2006) Delivery of RNA interference. Cell Cycle 5:2103–2109

Lightner DV (1996) A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. World Aquaculture Society, Baton Rouge, LA

Lo CF, Ho CH, Chen CH, Liu KF, Chiu YL, Yeh PY, Peng SE, Hsu HC, Liu HC, Chang CF, Su MS, Wang CH, Kou GH (1997) Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon*, with a special emphasis on reproductive organs. Dis Aquat Org 30:53–72

Lu R, Maduro M, Li F, Li HW, Broitman-Maduro G, Li WX, Ding SW (2005) Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*. Nature 436:1040–1043

Luo T, Zhang X, Shao Z, Xu X (2003) PmAV, a novel gene involved in virus resistance of shrimp *Penaeus monodon*. FEBS Lett 551:53–57

Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 110:563–574

Matsumoto M, Funami K, Oshiumi H, Seya T (2004) Toll-like receptor 3: a link between Toll-like receptor, interferon and viruses. Microbiol Immunol 48:147–154

Mekata T, Kono T, Yoshida T, Sakai M, Itami T (2008) Identification of cDNA encoding Toll receptor, MjToll gene from kuruma

shrimp, *Marsupenaeus japonicus*. Fish Shellfish Immunol 24:122–133

Nadala ECB, Lu Y, Loh PC (1993) Primary culture of lymphoid, nerve, and ovary cells from *Penaeus stylirostris* and *Penaeus vannamei*. In Vitro Cell Dev Biol 29A:620–622

Newmark PA, Reddien PW, Cebria F, Alvarado AS (2003) Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. Proc Natl Acad Sci USA 100:11861–11865

Novina CD, Murray MF, Dykxhoorn DM, Beresford PJ, Riess J, Lee SK, Collman RG, Lieberman J, Shankar P, Sharp PA (2002) siRNA-directed inhibition of HIV-1 infection. Nat Med 8:681–686

Paddison PJ, Caudy AA, Hannon GJ (2002) Stable suppression of gene expression by RNAi in mammalian cells. Proc Natl Acad Sci USA 99:1443–1448

Ramos EA, Relucio JV, Villanueva C (2005) Gene expression in tilapia following oral delivery of chitosan-encapsulated plasmid DNA incorporated into fish feeds. Mar Biotechnol 7:89–94

Randall G, Grakoui A, Rice CM (2003) Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. Proc Natl Acad Sci USA 100:235–240

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004) Rational siRNA design for RNA interference. Nat Biotechnol 22:326–330

Rinkevich B (2005) Marine invertebrate cell cultures: new millennium trends. Mar Biotechnol 7:429–439

Robalino J, Browdy CL, Prior S, Metz A, Parnell P, Gross P, Warr G (2004) Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J Virol 78:10442–10448

Robalino J, Bartlett T, Shepard E, Prior S, Jaramillo G, Scura E (2005) Double-stranded RNA induces sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp: Convergence of RNA interference and innate immunity in the invertebrate antiviral response? J Virol 79:13561–13571

Robalino J, Bartlett T, Chapman RW, Gross P, Browdy C, Warr G (2007) Double-stranded RNA and antiviral immunity in a marine shrimp: inducible host mechanism and evidence for the evolution of viral counter-responses. Dev Comp Immunol 31:539–547

Sarathi M, Simon MC, Venkatesan C, Sahul Hameed AS (2008a) Oral administration of bacterially expressed VP28dsRNA to protect *Penaeus monodon* from White Spot Syndrome Virus. Mar Biotechnol 10:242–249

Sarathi M, Simon MC, Ahmed V, Kumar S, Sahul Hameed AS (2008b) Silencing VP28 gene of White Spot Syndrome Virus of shrimp by bacterially expressed dsRNA. Mar Biotechnol 10:198–206

Shike H, Shimizu C, Klimpel KS, Burns JC (2000) Expression of foreign genes in primary cultured cells of the blue shrimp *Penaeus stylirostris*. Mar Biol 137:605–611

Shimizu C, Klimpel KR, Burns JC (2001) Hemolyph analysis and evaluation of newly formulated media for culture of shrimp cells (*Penaeus stylirostris*). In Vitro Cell Dev Biol-Animal 37:322–329

Sullivan CS, Ganem D (2005) A virus-encoded inhibitor that blocks RNA interference in mammalian cells. J Virol 79:7371–7379

Sun PS, Arakaki KL, Sun SM (2002) Nucleotide sequences of the shrimp beta-action and actin promoters and their use in genetic transformation technology (PCT No. PCT/US02/38523)

Sun PS, Venzon NC, Calderon FRO, Esaki DM (2005) Evaluation of methods for DNA delivery into shrimp zygotes of *Penaeus* (*Litopenaeus*) *vannamei*. Aquaculture 243:19–26

Tan FL, Yin JQ (2004) RNAi, a new therapeutic strategy against viral infection. Cell Res 14:460–466

Timmons L, Court DL, Fire A (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. Gene 263:103–112

Tirasophon W, Roshorm Y, Panyim S (2005) Silencing of yellow head virus replication in penaeid shrimp cells by dsRNA. Biochem Biophys Res Commun 334:102–107

Tirasophon W, Yodmuanga S, Chinnirunvong W, Plongthongkum N, Panyim S (2007) Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-protease dsRNA. Antiviral Res 74:150–155

Tong SL, Miao HZ (1996) Attempts to initiate cell cultures from *Penaeus chinensis* tissues. Aquaculture 147:151–157

Ulvila J, Parikka M, Kleino A, Sormunen R, Ezekowitz RA, Kocks C (2006) Double stranded RNA is internalized by scavenger receptor mediated endocytosis in *Drosophila melanogaster* S2 cells. J Biol Chem 281:14370–14375

Wadhwa R, Kaul SC, Miyagishia M, Taira K (2004) Know-how of RNA interference and its applications in research and therapy. Mutation Res 567:71–84

Walker P, Subasinghe R (2000) DNA-based molecular diagnostic techniques: research needs for standardization and validation of the detection of aquatic animal pathogens and diseases. FAO Fisheries Technical Paper, 395

Wang Y, Kato N, Jazag A, Dharel N, Otsuka M, Tanguchi H, Kawabe T, Omata M (2006) Hepatitis C virus core protein is a potent inhibitor of RNA silencing-based antiviral response. Gastroenterol 130:883–892

Westenberg M, Heinhuis B, Zuidema D, Vlak JM (2005) siRNA injection induces sequence independent protection in *Penaeus monodon* against white spot syndrome virus (WSSV). Virus Res 114:133–139

Williams BR (2001) Signal integration via PKR. Sci STKE, 89, RE2

Wu Y, Lu L, Yang L, Weng S, Chan S, He J (2007) Inhibition of white spot syndrome virus in *Litopenaeus vannamei* shrimp by sequence-specific siRNA. Aquaculture 271:21–30

Wu W, Zong R, Xu J, Zhang X (2008) Antiviral phagocytosis is regulated by a novel Rab-dependent complex in shrimp *Penaeus japonicus*. J Proteome Res 7:123–129

Xu J, Han F, Zhang X (2007) Silencing shrimp white spot syndrome virus (WSSV) genes by siRNA. Antiviral Res 73:126–131

Yang L, Yin Z, Liao J, Huang X, Guo C, Weng S, Chan S, Yu X, He J (2007) A toll receptor in shrimp. Mol Immunol 44:1999–2008

Yodmuang S, Tirasophon W, Roshorm Y, Chinnirunvong W, Panyim S (2006) YHV-protease dsRNA inhibits YHV replication in *Penaeus monodon* and prevents mortality. Biochem Biophys Res Commun 341:351–356

Zambon RA, Nandakumar M, Vakharia VN, Wu LP (2005) The Toll pathway is important for an antiviral response in *Drosophila*. Proc Natl Acad Sci USA 102:7257–7262

Zhang XB, Huang CH, Qin QW (2004) Antiviral properties of hemocyanin isolated from shrimp *Penaeus monodon*. Antiviral Res 61:93–99