RESEARCH PAPER

Leader RNA regulates snakehead vesiculovirus replication via interacting with viral nucleoprotein

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ABSTRACT

Leader RNA, a kind of virus-derived small noncoding RNA, has been proposed to play an important role in regulating virus replication, but the underlying mechanism remains elusive. In this study, snakehead vesiculovirus (SHVV), a kind of fish rhabdovirus causing high mortality to the cultured snakehead fish in China, was used to unveil the molecular function of leader RNA. High-throughput small RNA sequencing of SHVV-infected cells showed that SHVV produced two groups of leader RNAs (named le_{group1} and le_{group2}) during infection. Overexpression and knockout experiments reveal that le_{group1}, but not le_{group2}, affects SHVV replication. Mechanistically, le_{group1}-mediated regulation of SHVV replication was associated with its interaction with the viral nucleoprotein (N). Moreover, the nucleotides 6–10 of le_{group1} were identified as the critical region for its interaction with the N protein, and the amino acids 1–45 of N protein were proved to confer its interaction with the le_{group1}. Taken together, we identified two groups of SHVV leader RNAs and revealed a role in virus replication for one of the two types of leader RNAs. This study will help understand the role of leader RNA in regulating the replication of negative-stranded RNA viruses.

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Introduction

Snakehead vesiculovirus (SHVV), isolated from diseased hybrid snakehead fish (*Channa maculata* ♀ x *Channa argus* ♂) in China, is a new member of the family *Rhabdoviridae* [1]. The genome of SHVV is a single-stranded and negative-sense RNA molecule, which consists of 3' leader region, five transcriptional units encoding nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (L), and 5' trailer region [2]. Because of the lack of effective treatments against SHVV, it is urgently needed to investigate the molecular details on SHVV replication and identify novel anti-SHVV targets. The small noncoding RNA (sncRNA), either derived from virus or host, has been reported to play an important role in virus replication [3,4]. Our previous studies have identified miR-214, a kind of host sncRNA, as an anti-SHVV factor [5–8]. However, little is known about SHVV-derived sncRNA.

Leader RNA is a kind of viral sncRNA transcribed from the 3' leader region of viral genomic RNA [9]. As shown for vesicular stomatitis virus (VSV), leader RNA is synthesized prior to viral mRNAs and neither capped at its 5' end nor polyadenylated at its 3' end [9,10]. Since the first discovery in VSV in 1970s [9–11], leader RNA has been identified in many

viruses including sendai virus [12], rabies virus (RV) [13], newcastle disease virus [14], sonchus yellow net virus [15], measles virus [16,17], chandipura virus (CPV) [18], rinderpest virus (RPV) [19], respiratory syncytial virus (RSV) [20] and borna disease virus [21]. Leader RNA plays an important role in virus replication, especially in regulating the transcription and replication of viral genomic RNA, via interacting with either viral or host proteins [18,19,22–24]. For different viruses, leader RNA interacts with different viral proteins. The VSV and RV leader RNAs interact with viral N protein [22,23], while RPV and CPV leader RNAs interact with viral P protein [18,19]. Viral N protein preferentially binds leader RNA relative to other RNA species, and the P protein confers the specificity of N protein binding to leader RNA [25,26]. Phosphorylation status of viral N/P proteins affects their interaction with leader RNA [18,19,23]. In addition to viral proteins, leader RNA can also interact with host proteins. The cellular La protein is a well-known protein that interacts with leader RNA of RV [13], VSV [27,28], RPV [24] and RSV [20]. La protein shuttles between nucleus and cytosol, and its interaction with leader RNA affects virus replication through shielding leader RNA from RIG-I, a known activator of interferon expression [20]. Moreover, leader RNA can interact with other host factors, such as heat shock cognate 70 kDa

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protein [29] and heterogeneous nuclear ribonucleoprotein U [30].

The aim of this study is to identify the existence of SHVV leader RNA, investigate the role of leader RNA in virus replication, and unveil the underlying mechanism. To this end, we applied high-throughput small RNA sequencing and identified two groups of SHVV leader RNAs (le_{group1} and le_{group2}) from SHVV-infected cells. Through overexpression of leader RNAs in cells and generation of leader RNA-knockout mutants using reverse genetics technology, we found that le_{group1} , but not le_{group2} , affects SHVV replication. Co-Immunoprecipitation-reverse transcription-PCR (Co-IP-RT-PCR) and electrophoretic mobility shift assay (EMSA) revealed that le_{group1} interacted with viral N protein, and the le_{group1} -N protein interaction was critical for SHVV replication.

Results

Identification of SHVV leader RNA

To determine whether SHVV produces leader RNA during infection, high-throughput small RNA sequencing was performed on the RNAs extracted from SHVV-infected or non-infected cells including channel catfish ovary (CCO) cells and striped snakehead (SSN-1) cells. The resulting reads were aligned with the sequence of SHVV genome, and hundreds

of thousands of reads from SHVV-infected cells, but not from non-infected cells, fully matched the 3' leader sequence of SHVV genome. These results indicate that SHVV can produce leader RNA during infection. We then analysed the leader RNAs identified in both SHVV-infected CCO and SSN-1 cells and found two common features (Fig. 1A and S1). First, the SHVV leader RNAs can be artificially clustered into two groups (named le_{group1} and le_{group2}) based on their transcriptional start and end positions. The le_{group1} RNAs are transcribed starting at nt 1 and ending at nt 22 or 30, while le_{group2} RNAs are transcribed starting at nt 38–42 and ending at nt 58–67. Second, the number of le_{group2} is much higher than that of le_{group1} .

In order to verify the results of Fig. 1A, the transcriptional start and end positions of the two groups of leader RNAs were determined using an independent RT-PCR method (Fig. S2). Sequencing of 20 cDNA clones each for le_{group1} and le_{group2} showed that le_{group1} was transcribed starting at position 1 and terminating at positions 22 or 29–31, while le_{group2} was transcribed starting at positions 39–40 and terminating at positions 60–62 (Fig. S2). These results were basically consistent with the results obtained from the high-throughput small RNA sequencing (Fig. 1A).

To further evaluate the expression levels of the two groups of leader RNAs during SHVV infection, qRT-PCR was performed on the RNAs extracted from SHVV-infected CCO cells at 3, 6, 12 and 24 hours post-infection (hpi). The

Figure 1. Identification of SHVV leader RNA. (A) Schematic illustration of SHVV genome organization, showing the 3' leader sequence (72 nt) of SHVV genome. High-throughput small RNA sequencing was performed on the RNAs extracted from SHVV-infected CCO and SSN-1 cells. Upon aligning with the sequence of SHVV genome, the reads that were completely complementary to the 3' leader sequence were identified as leader RNAs. The identified leader RNAs existed in both SHVV-infected CCO and SSN-1 cells were presented (le_{group1} and le_{group2}). (B) CCO cells were infected with SHVV, and the cells were harvested at 3, 6, 12 and 24 hpi. The levels of the two groups of leader RNAs were determined by qRT-PCR, *U6* RNA was used as the internal control. (C) Predicted secondary structures of the two groups of leader RNAs using RNAfold webserver.

le_{group1} and le_{group2} levels steadily increased at 6, 12 and 24 hpi compared to 3 hpi, and the expression level of le_{group2} was higher than that of le_{group1} at all time points (Fig. 1B). We further detected le_{group1} using Northern blotting but failed. It is probably because le_{group1} is the under detection limitation of Northern blotting (data not shown). In addition to the sequence characteristics, RNAfold (<http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi>) predicted that le_{group2} RNAs form a hairpin structure, while le_{group1} RNAs lack any secondary structure (Fig. 1C).

Le_{group1}, but not le_{group2}, affects SHVV replication

To investigate whether the two groups of leader RNAs are involved in SHVV replication, we synthesized two leader RNAs (le_{1-22} for le_{group1} , le_{41-65} for le_{group2}) and a negative control (NC). The leader RNAs and NC were, respectively, transfected into CCO cells, followed by SHVV infection. At 24 hpi, the viral G mRNA levels in infected cells were quantified by qRT-PCR and viral titres in supernatants by determination of the 50% tissue culture infectious dose (TCID₅₀). The results showed that overexpression of le_{1-22} , but not le_{41-65} , significantly increased the levels of G mRNA and viral titre compared to those of NC (Fig. 2A and B), indicating that overexpression of le_{1-22} promotes SHVV replication. In addition, we found that overexpression of the synthesized le_{1-30} , another member in le_{group1} (Fig. 1A), but not le_{38-61} , another member in le_{group2} (Fig. 1A), also significantly promoted SHVV replication compared to NC (Fig. S3 and S4). These results suggest that le_{group1} , but not le_{group2} , affects SHVV replication.

To understand the underlying mechanism on how le_{group1} promoted SHVV replication, CCO cells were transfected with NC or le_{1-22} , followed by SHVV infection. At 3, 12 and 24 hpi, the SHVV genomic RNA (vRNA) and G mRNA were detected by qRT-PCR. The results showed that overexpression of le_{1-22} did not cause any significant differences for vRNA or G mRNA at 3 hpi compared with NC (Fig. 2C). However, overexpression of le_{1-22} significantly increased vRNA but not G mRNA levels at 12 hpi, while significantly increased G mRNA, but barely vRNA levels, at 24 hpi (Fig. 2C). These data suggested that overexpression of le_{1-22} promoted SHVV

replication via regulating the transcription and replication of SHVV genomic RNA.

To fully understand the role of the two groups of leader RNAs in SHVV replication, reverse genetics technology of SHVV [2] was used to generate leader RNA-knockout mutants. At first, we tried to generate a mutant rSHVV-mut₃₈₋₄₂, in which the transcriptional start positions of le_{group2} (positions 38–42 of SHVV genome) were mutated to cytosine. The success in generation of the mutant rSHVV-mut₃₈₋₄₂ could be inferred from three criteria. First, cytopathic effect (CPE) was observed in 293 T and CCO cells for rSHVV-mut₃₈₋₄₂, but not for the control (Fig. 3A). Second, the SHVV G protein was detected in CCO cells for rSHVV-mut₃₈₋₄₂ (Fig. 3B). Third, the mutations at the positions 38–42 of the rSHVV-mut₃₈₋₄₂ genome were verified by RT-PCR amplification and sequencing (data not shown). We then compared the production of leader RNAs for wild-type SHVV and the mutant rSHVV-mut₃₈₋₄₂. The results showed that wild-type SHVV produced two groups of leader RNAs, while rSHVV-mut₃₈₋₄₂ produced le_{group1} , but not le_{group2} (Fig. 3C). Finally, we compared the growth ability of the mutant rSHVV-mut₃₈₋₄₂ with rSHVV. No significant differences were observed in the growth ability between rSHVV-mut₃₈₋₄₂ and rSHVV (Fig. 3D and E), indicating that le_{group2} is not essential for SHVV replication.

In order to generate an SHVV mutant without le_{group1} production, we tried several mutants (rSHVV-mut₁₋₅, rSHVV-mut₆₋₁₂, rSHVV-mut₁₃₋₁₇ and rSHVV-mut₁₈₋₂₂) by mutating the indicated nucleotides of SHVV genome to cytosine. As shown in Fig. S5, the CPE was observed in 293 T and CCO cells for rSHVV, but not for any of the mutants. Also, the G protein could only be detected in CCO cells for rSHVV, indicating that these mutants were not successfully generated.

Le_{group1} affects SHVV replication via interacting with viral N protein

Leader RNA has been proposed to regulate virus replication via interacting with viral N or P protein [18,19,22,23,31]. To identify which protein of SHVV interact with le_{group1} , Co-IP-RT-PCR was performed on SHVV-infected CCO cells using rabbit antibodies against the N, P, M, G or L protein of

Figure 2. Effect of leader RNAs on SHVV replication. (A–B) CCO cells were transfected with synthesized NC, le_{1-22} or le_{41-65} , followed by SHVV infection. At 24 hpi, the cells and supernatants were harvested. The G mRNA in cells (A) and viral titre in supernatants (B) were determined by qRT-PCR or TCID₅₀. For G mRNA quantification, β -actin was used as the internal control. (C) CCO cells were transfected with synthesized NC or le_{1-22} , followed by SHVV infection. At 3, 12 and 24 hpi, the cells were harvested to detect vRNA and G mRNA by qRT-PCR. β -actin was used as the internal control. The * and ** respectively indicate statistically significant differences (*, $p < 0.05$; **, $p < 0.01$).

Figure 3. Generation of rSHVV-mut₃₈₋₄₂ using reverse genetics technology. (A) 293 T cells were transfected with plasmids pCDNA-SHV-mut₃₈₋₄₂, pCDNA-N, pCDNA-P and pCDNA-L (right) or pCDNA-SHV-mut₃₈₋₄₂ alone (control, left) for 3 days. The cells and supernatants were collected and then freeze-thawed for three times, followed by centrifugation at $10,000 \times g$ for 5 min. The supernatants were subsequently incubated with CCO cells, and the cytopathic effect (CPE) was observed at 24 h post infection. (B) The SHV G protein was detected in the CCO cells collected from (A) by Western blotting, M: protein marker; lane 1: CCO cells without SHV infection were used as negative control; lane 2: sample from the left of (A); lane 3: sample from the right of (A); lane 4: CCO cells with SHV infection were used as positive control. (C) The two groups of leader RNAs were detected from rSHVV-mut₃₈₋₄₂-infected, SHV-infected, or non-infected CCO cells. M: DNA marker; lane 1, 3, 5, 7: cDNA from non-infected cells; lane 2, 4: cDNA from rSHVV-mut₃₈₋₄₂-infected cells; lane 6, 8: cDNA from SHV-infected cells; lane 1, 2, 5, 6: detection of *le_{group1}*; lane 3, 4, 7, 8: detection of *le_{group2}*. (D-E) CCO cells were infected with rSHVV or rSHVV-mut₃₈₋₄₂. At 12 and 24 hpi, the cells and supernatants were collected to detect G mRNA and G protein in cells (D) and viral titre in supernatants (E) by qRT-PCR, Western blotting or TCID₅₀. β -actin was used as the internal control for G mRNA and G protein.

SHVV, the rabbit IgG was used as control. The results showed that viral N and P proteins, but not other viral proteins, were co-immunoprecipitated with *le_{group1}* (Fig. 4A). In order to verify that the N and P proteins interacted with *le_{group1}* directly or indirectly, the GST, GST-N and GST-P proteins were recombinantly expressed and purified (Fig. S6A). The EMSA assay showed that GST-N, but not GST-P or GST, resulted in the shift of *le₁₋₂₂* (Fig. 4B), indicating that SHV N protein, but not P protein, interacts directly with *le_{group1}*.

To further determine the effects of overexpression of *le_{group1}* and/or N protein on SHVV replication, CCO cells were transfected with RNAs and plasmids as indicated in Fig. 5A, followed by SHVV infection. We found that simultaneous transfection of *le₁₋₂₂* and a plasmid overexpressing the N protein promoted SHVV replication somewhat stronger than *le₁₋₂₂* alone (Fig. 5A and B). These results indicate that the co-presence of *le_{group1}* and N protein enhances SHVV replication more significantly than *le_{group1}* alone. Furthermore, we investigated the effect of the *le_{group1}*-N interaction on the transcription and replication of SHVV genomic RNA. The results showed that overexpression of both *le₁₋₂₂* and N protein increased vRNA levels most strongly (~twofold) at 12 hpi, and increased G mRNA levels roughly twofold at 12 and 24 hpi relative to the NC

+ pCDNA3.1 control; the increments were stronger than for transfection of *le₁₋₂₂* alone (Fig. 5C and D). *le₁₋₂₂* and N together increased G mRNA levels roughly twofold at 12 h and 24 h compared to *le₁₋₂₂* alone (Fig. 5D). These results therefore suggest that the *le_{group1}*-N interaction affects SHVV propagation on the level of transcription and replication of viral genomic RNA.

Mapping the critical region on SHVV *le_{group1}* for *le_{group1}*-N interaction

To delineate the critical region on SHVV *le_{group1}* for *le_{group1}*-N interaction, we synthesized biotin-labelled *le₁₋₂₂* with several nucleotides deleted (*le₁₋₂₂/Δ1-5*, *le₁₋₂₂/Δ6-10*, *le₁₋₂₂/Δ11-15* and *le₁₋₂₂/Δ16-22*). These deletion mutants are depicted in Fig. 6A. EMSA assay revealed that *le₁₋₂₂/Δ1-5*, *le₁₋₂₂/Δ11-15* and *le₁₋₂₂/Δ16-22*, but not *le₁₋₂₂/Δ6-10*, could interact with the N protein (Fig. 6B). These results indicate that the nucleotides 6–10 of SHVV *le_{group1}* are the critical region for *le_{group1}*-N interaction. Furthermore, we evaluated the effect of *le₁₋₂₂* lacking the N-binding region on SHVV replication. The results showed that *le₁₋₂₂* lacking the nucleotides 6–10 failed to promote SHVV replication (Fig. 6C–D).

Figure 4. *le_{group1}* interacts with SHVV N protein directly. (A) CCO cells were infected with SHVV, and the whole proteins from the cells were collected at 24 hpi for Co-IP using rabbit IgG and rabbit antibodies against the five SHVV proteins. The co-immunoprecipitates were further analysed for the presence of *le_{group1}* by RT-PCR. M: DNA marker; lane 1, 3, 5, 7, 9: using rabbit IgG; lane 2: using rabbit anti-N antibody; lane 4: using rabbit anti-P antibody; lane 6: using rabbit anti-M antibody; lane 8: using rabbit anti-G antibody; lane 10: using rabbit anti-L antibody. (B) EMSA was performed to determine the interaction between *le₁₋₂₂* and GST, GST-N or GST-P using Chemiluminescent EMSA kit (Thermo Scientific).

Mapping the critical domain on SHVV N protein for *le_{group1}*-N interaction

To identify the critical domain on SHVV N protein for *le_{group1}*-N interaction, we at first generated two plasmids to recombinantly express and purify GST-N₁₋₁₄₃ and GST-N₁₄₄₋₄₃₀ (Fig. 7A and S6B). Using EMSA assay, we found that GST-N₁₋₁₄₃, but not GST-N₁₄₄₋₄₃₀, interacted with *le₁₋₂₂* (Fig. 7B). To more precisely map the region of SHVV N that interacts with *le_{group1}*, we constructed three additional plasmids expressing GST-N₁₋₄₅, GST-N₄₆₋₁₀₀ and GST-N₁₀₁₋₁₄₃ (Fig. 7A and S6B). We found that GST-N₁₋₄₅, but not GST-N₄₆₋₁₀₀ or GST-N₁₀₁₋₁₄₃, interacted with *le₁₋₂₂* (Fig. 7B). These results indicate that residues within the region of amino acids 1–45 of the SHVV N protein are critical for *le_{group1}*-N interaction.

To further verify that the amino acids 1–45 of SHVV N protein are the critical region for *le_{group1}*-N interaction, we assessed the *le_{group1}*-N₁₋₄₅ interaction *in vivo*. CCO cells were transfected with a plasmid expressing flag-tagged N₁₋₄₅, followed by SHVV infection. Co-IP-RT-PCR revealed that N₁₋₄₅ interacted with *le_{group1}* in cells (Fig. 8A). These *in vitro* and *in vivo* results indicate that the *le_{group1}*-N interaction site resides within amino acids 1–45 of the SHVV N protein.

Considering that the N₁₋₄₅ can interact with *le_{group1}* in SHVV-infected cells, it is speculated that N₁₋₄₅ might

competitively disrupt *le_{group1}*-N interaction, thus inhibiting SHVV replication. To this end, we evaluated the effect of N₁₋₄₅ on SHVV replication. We found that overexpression of N₁₋₄₅ significantly reduced the levels of G mRNA, G protein and viral titre at 6 and/or 12 hpi, but not at 24 hpi (Fig. 8B and C). These results indicate that N₁₋₄₅ inhibits SHVV replication only at early stage of infection. Moreover, we assessed whether the inhibition of SHVV replication at 6 hpi could be restored by adding *le₁₋₂₂*. Our results demonstrated that N₁₋₄₅-mediated inhibition of SHVV replication at an early stage of infection could be restored by adding *le₁₋₂₂* (Fig. 8D and E).

Discussion

In this study, we identified two groups of leader RNAs (*le_{group1}* and *le_{group2}*) for a fish rhabdovirus SHVV. To our knowledge, this is the first observation that a virus produces more than one group of leader RNAs. Of the two groups of SHVV leader RNAs, *le_{group1}* shared two common features with the leader RNAs of other viruses. First, the SHVV *le_{group1}* was transcribed starting from position 1 of SHVV genome but ending at variable positions. Similarly, the RV leader RNAs were transcribed starting from position 1 and ending at positions 55–58 [13], the newcastle disease virus leader RNAs were transcribed starting from position 1 and ending at positions 47–53 [14], and the sonchus yellow net virus leader RNAs were transcribed starting from position 1

Figure 5. le_{1-22} -N interaction is important for SHVV replication. (A-B) CCO cells were transfected as indicated, followed by SHVV infection. pCDNA3.1 = empty vector, pCDNA-N = vector overexpressing N. At 24 hpi, the cells and supernatants were harvested to detect G mRNA and G protein in cells (A) and viral titre in supernatants (B) by qRT-PCR, Western blotting or TCID₅₀. β -actin was used as the internal control for G mRNA and G protein. The integrated optical densities of the protein bands were measured using Image-Pro Plus 6.0. The values of viral G protein bands were normalized to that of β -actin. The value of the viral G protein band in NC +pCDNA3.1 transfected cells was set as 100. (C-D) CCO cells were transfected as indicated, followed by SHVV infection. At 3, 12 and 24 hpi, the cells were harvested. The vRNA (C) and G mRNA (D) in cells were determined by qRT-PCR. β -actin was used as the internal control.

and ending at positions 139–144 [15]. These data revealed that viral leader RNAs consisted of a panel of small RNAs with different lengths due to a variable 3' terminus, for which the possible reason might be that the transcriptional termination signal of leader RNAs was not precisely fixed [11]. Second, the SHVV le_{group1} was rich in adenylate (A) (~50%). This feature has also been observed in leader RNAs of VSV [10], RV [13], newcastle disease virus [14] and sonchus yellow net virus [15]. Although the precise function of this A-rich sequence in leader RNAs is not known, it deserves further studies, especially in signal recognition by viral proteins.

Leader RNAs can affect viral or host functions. Previous studies have revealed that RV leader RNA is not only involved in virus replication [29] but also activates dendritic cells [32]. In this study, we only investigated the role of SHVV leader RNAs in virus replication, and we found that SHVV le_{group1} , but not le_{group2} , affected SHVV replication. In order to generate SHVV mutants without le_{group1} production, we mutated the nucleotides 1–5, 6–12, 13–17 or 18–22 of SHVV genome, but were unable to obtain functional viruses. The failure was probably because this region of SHVV genome was also the binding region for virus polymerase complex L/P proteins, and mutations at this region might disrupt the binding of L/P proteins to SHVV genome, thus disrupting the transcription and replication of SHVV genome. In addition to virus

replication, the SHVV leader RNAs, especially those of le_{group2} that do not affect virus replication, might play a role in modulating the biological functions of host cells.

Leader RNA has been proposed to be involved in virus replication, especially in the transcription and replication of viral genomic RNA, via interacting with either viral or host proteins [18,22–24]. For different viruses, leader RNAs were found to bind to different viral proteins. The VSV and RV leader RNAs interact with viral N protein [22,23], while RPV and CPV leader RNAs interact with viral P protein [18,19]. In this study, the interaction between le_{group1} and the five SHVV proteins was analysed, and we found that SHVV le_{group1} interacted directly with viral N protein, but not other SHVV proteins. Moreover, we found that overexpression of both le_{group1} and N protein promotes SHVV replication, as well as the transcription and replication of SHVV genome, higher than that of le_{group1} alone, suggesting a role for the le_{group1} -N interaction in regulating SHVV replication. In addition to viral proteins, host factors might also regulate SHVV replication via interacting with le_{group1} , which deserves further investigation.

Leader RNA is preferentially bound by viral N protein relative to other RNA species, and this preference is not due to the RNA size, but sequence-dependent [25,26], which suggests that a specific sequence acting as initiation signal for N protein encapsidation exists in leader RNA [22]. Nucleotides 1–14 of

Figure 6. Mapping the critical region on SHV le_{group1} for le_{group1}-N interaction. (A) Schematic illustration of SHV le₁₋₂₂ and its deletion mutants. (B) EMSA was performed to determine the interaction between GST-N and le_{1-22/Δ1-5}, le_{1-22/Δ6-10}, le_{1-22/Δ11-15} or le_{1-22/Δ16-22} using Chemiluminescent EMSA kit (Thermo Scientific). (C-D) CCO cells were transfected with synthesized NC, le₁₋₂₂, or le_{1-22/Δ6-10}, followed by SHV infection. At 24 hpi, the cells and supernatants were harvested. The G mRNA in cells (C) and viral titre in supernatants (D) were determined by qRT-PCR or TCID₅₀. For G mRNA quantification, β-actin was used as the internal control.

VSV leader RNA and nucleotides 20–30 of RV leader RNA have been identified as the initiation signals for N protein encapsidation, and these specific sequences are rich in A residues [25,26]. In this study, we identified that the nucleotides 6–10 of SHV le_{group1} were critical for le_{group1}-N interaction because the

le_{group1} variant lacking nt 6–10 lost the ability to bind to N protein. Consistent with the initiation signals of VSV and RV leader RNAs, nt 6–10 of SHV le_{group1} are also rich in A residues, suggesting that they may constitute the initiation signal for N protein encapsidation in SHV.

The critical domain on N protein for encapsidation of leader RNA has previously been mapped for VSV and RV. The C-terminal 10 amino acids of the VSV N protein are critical for leader RNA encapsidation [25], while both the N- and C-terminal 45 amino acids of the RV N protein are critical for the same function [26]. In this study, we identified amino acids 1–45 of the SHV N protein as the critical region for le_{group1}-N interaction/encapsidation *in vitro* and *in vivo*. In an attempt to evaluate the anti-SHV role of N₁₋₄₅, we found that SHV replication was significantly inhibited by N₁₋₄₅ early in infection, but restored at later infection stages. Probably, N₁₋₄₅ competed with N for interaction with le_{group1} RNAs early in infection when the level of cellular N protein was quite low, but competitive inhibition was mitigated at later infection stages when the level of N protein increased and possibly exceeded that of N₁₋₄₅. Therefore, an SHV N₁₋₄₅ peptide is unlikely to be effective as an inhibitor in attempts to combat SHV infections.

Figure 7. Mapping the critical domain on SHV N protein for le_{group1}-N interaction. (A) Schematic illustration of SHV N protein and its domains. (B) EMSA was performed to determine the interaction between le₁₋₂₂ and GST, GST-N, GST-N₁₋₁₄₃, GST-N₁₄₄₋₄₃₀, GST-N₁₋₄₅, GST-N₄₆₋₁₀₀ or GST-N₁₀₁₋₁₄₃ using Chemiluminescent EMSA kit (Thermo Scientific).

Materials and Methods

Cells and viruses

Striped snakehead (SSN-1) cells were cultured at 28°C in L-15 medium (Gibco) supplemented with 10% foetal bovine serum (FBS) (Gibco). Channel catfish ovary (CCO) cells were cultured at 25°C in minimum essential medium (MEM) (Gibco) supplemented with 10% FBS. Human 293 T cells were cultured at 37°C and 5% CO₂ atmosphere in Dulbecco's modified eagle medium (DMEM) (Gibco) supplemented with 10%

Figure 8. The anti-SHVV effect of the N₁₋₄₅. (A) CCO cells were transfected with plasmid p3XFLAG-CMV-14 (empty vector control) or p3XFLAG-N₁₋₄₅, followed by SHVV infection. At 24 hpi, the cells were harvested for Co-IP using mouse anti-flag antibody, the eluted samples were used to detect le_{group1} by RT-PCR. M: DNA marker; lane 1 and 2: detection of le_{group1}. (B-C) CCO cells were transfected with plasmid p3XFLAG-CMV-14 or p3XFLAG-N₁₋₄₅, followed by SHVV infection. At 3, 6, 12 and 24 hpi, the G mRNA, G protein in cells (B) and the viral titre in supernatants (C) were determined by qRT-PCR, Western blotting or TCID₅₀. β -actin was used as the internal control for G mRNA and G protein. (D-E) CCO cells were transfected as indicated, followed by SHVV infection. At 6 hpi, the cells and supernatants were harvested to detect G mRNA in cells (D) and viral titre in supernatants (E) by qRT-PCR or TCID₅₀. β -actin was used as the internal control for G mRNA.

FBS. SHVV (GenBank: KP876483.1) was isolated from diseased hybrid snakehead fish in a farm from Guangdong province, China.

Plasmids

The plasmids pCDNA-N, pCDNA-P, pCDNA-M, pCDNA-G and pCDNA-L were constructed by cloning the PCR-amplified products of SHVV genes into vector pCDNA3.1 (+) with primers listed in Table S1. The plasmids pGST-P, pGST-N, pGST-N₁₋₁₄₃, pGST-N₁₄₄₋₄₃₀, pGST-N₁₋₄₅, pGST-N₄₆₋₁₀₀ and pGST-N₁₀₁₋₁₄₃ were constructed by cloning the PCR-amplified products of SHVV genes or gene regions into vector PGEX-4 T-1 with primers listed in Table S1. The plasmid p3XFLAG-N₁₋₄₅ was constructed by cloning the PCR-amplified product of the amino acids 1–45 of SHVV N protein into vector p3XFLAG-CMV-14 with primers listed in Table S1.

Reagents and antibodies

Leader RNAs (le₁₋₂₂ is 5'-OH-ACGAGAAAAAAGAAACCAUA-3', le₁₋₃₀ is 5'-OH-ACGAGAAAAAAGAAACCAUAUACAGAUU-3', le₄₁₋₆₅ is 5'-OH-UUGGAUUCUUUUUCUCGACAUUCA-3') and negative control (NC, the sequence is 5'-OH-UUGUACUACACAAAAGUACUG-3') were synthesized from GenePharma Co., LTD. (Shanghai, China). The rabbit

anti-SHVV-N, -P, -M, -G and -L polyclonal antibodies were made by Abiotech (Jinan, China). The mouse anti-flag antibody, rabbit anti- β -actin antibody and rabbit IgG were purchased from Abclonal Technology (Wuhan, China). The IRDye800CW conjugated donkey anti-rabbit IgG and anti-mouse IgG antibodies were purchased from Gene Co., LTD. (Shanghai, China).

Transfection

Cells in 6-well plates were transfected with RNA or plasmid using TransIntro™ EL Transfection Reagent (TransGen Biotech, China) in 500 μ l Opti-MEM medium (Invitrogen). At 6 h post transfection, the Opti-MEM medium was replaced by 1 ml MEM or DMEM medium containing 10% FBS and the cells were cultured for further 18 h.

Virus infection and titration

CCO cells were infected with SHVV at a multiplicity of infection (MOI) of 0.1. After 2 h adsorption at 25°C, the inoculum was removed and the cells were washed twice with PBS, followed by adding MEM with 5% FBS. At 3, 6, 12 and 24 hpi, the supernatants were collected for virus titration by TCID₅₀, and the cells were harvested for the detection of viral proteins by Western blotting, viral mRNA and vRNA by qRT-PCR with primers listed in Table S1.

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNAs were extracted from cells with TRIzol reagent (Invitrogen) according to manufacturer's instructions. For the detection of viral mRNA and vRNA, 1 µg of total RNA was mixed with 1 µl random primer, 4 µl 4× gDNA wiper Mix, and RNase free H₂O to a total volume of 16 µl. After incubated at 42°C for 2 min, 4 µl 5× select qRT supermix II (Vazyme Biotech, China) was added and incubated at 50°C for 15 min and 85°C for 2 min. The quantitative PCR reactions were conducted in 20 µl volumes containing 10 µl AceQ qPCR SYBR Green Master mix (Vazyme Biotech, China), 1 µl cDNA template, 0.4 µl forward primer (10 µM), 0.4 µl reverse primer (10 µM) (Table S1) and 8.2 µl ddH₂O with the following cycling conditions: 95°C for 5 min, 45 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 15 s, and ended with a 95°C at 5°C/s calefactive velocity to make the melt curve. Data were normalized to the level of β -actin in each sample using the $2^{-\Delta\Delta C_t}$ method.

For the detection of leader RNA, All-in-One miRNA qRT-PCR Detection System kit (GeneCopoeia, China) was used. Two micrograms of total RNA was used to synthesize cDNA according to manufacturer's instructions. The cDNA was then used to detect the level of leader RNA. The reaction mix (20 µl) composed of 2 µl cDNA, 10 µl All-in-One qPCR mixture, 2 µl forward primer (2 µM) as listed in Table S1, 2 µl Universal Adaptor PCR primer (2 µM) as reverse primer, and 4 µl ddH₂O. The thermal cycling parameters were 10 min at 95°C, followed by 40 cycles of 10 s at 95°C, 20 s at 55°C, and 10 s at 72°C. The expression level of leader RNA was calculated using $2^{-\Delta\Delta C_t}$ method after normalization to U6 RNA.

Purification of the GST fusion proteins

The plasmids pGST-P and pGST-N were transformed into Escherichia coli BL21, and protein expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 12 h at 16°C. After the incubation, cells were centrifuged for 10 min at 5,000 rpm and lysed, followed by centrifuging for 40 min at 12,000 rpm and 4°C. The protein in the supernatant was purified by GST sepharose resin column (Sangon biotech, China). All of the other truncations of N were expressed and purified using the same protocol.

Western blotting

Proteins from SHVV-infected cells were extracted with cell lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), heated at 100°C for 10 min, and separated by 12% SDS-PAGE gel, followed by transferring onto nitrocellulose membranes (Biosharp, China). Membranes were blocked with 5% skim milk in tris-buffered saline with 0.1% tween 20 (TBST) at 4°C overnight, followed by the incubation with anti-N (1:1000), anti-P (1:1000), anti-M (1:1000), anti-G (1:1000), anti-L (1:1000), anti-flag (1:3000) or anti- β -actin (1:5000) antibody for 2 h at room temperature. The membranes were then washed three times with TBST and then

incubated with IRDye800CW conjugated donkey anti-rabbit IgG (1:10,000) or anti-mouse IgG (1:10,000) for 1 h at room temperature. The signal intensity was then determined using Odyssey CLx (LI-COR).

Co-Immunoprecipitation-reverse transcription-PCR (Co-IP-RT-PCR)

CCO cells were collected at 24 h post of SHVV infection and subjected to co-Immunoprecipitation using rabbit anti-SHVV -N, -P, -M, -G and -L polyclonal antibodies or mouse anti-flag monoclonal antibody according to the manufacturer's instructions of Pierce Co-IP Kit (ThermoFisher). The eluted samples were then used for RNA isolation, followed by the detection of leader RNA through RT-PCR.

Electrophoretic mobility shift assay (EMSA)

The EMSA assay was performed using Chemiluminescent EMSA kit according to the manufacturer's instructions (Thermo Scientific). The biotin-labelled le₁₋₂₂ or its deletion mutants (25 pmol) were incubated with GST, GST-N, GST-P, GST-N₁₋₁₄₃, GST-N₁₄₄₋₄₃₀, GST-N₁₋₄₅, GST-N₄₆₋₁₀₀ or GST-N₁₀₁₋₁₄₃ (50 µg) for 30 min at room temperature in binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT pH 7.5). Electrophoresis was carried out on 8% w/v non-denaturing PAGE gel in buffer (40 mM Tris pH 8.0, 2.5 mM glycine), followed by transferring onto a nylon membrane. The RNA on membrane was cross-linked by a transilluminator for 15 min with the membrane facing down on a transilluminator equipped with 312 nm bulbs. The membrane was blocked with Blocking Buffer for 15 min, incubated in conjugate/blocking buffer for 15 min, washed with 1X wash solution for 4 times, and then moved into Substrate Equilibration Buffer for 5 min. After that, the membrane was poured into the Substrate Working Buffer and exposed in an equipped CCD camera.

Statistical analysis

All statistical analyses were performed using Graphpad Prism 5.0 (GraphPad Software, San Diego, CA). The statistical significance of the data was determined by the two-tailed unpaired Student *t* test, and *p* < 0.05 was considered statistically significant. All data are representative of at least two independent experiments, with each determination performed in triplicate (mean $\pm$ SD). The * and **, respectively, indicate statistically significant differences (*, *p* < 0.05; **, *p* < 0.01).

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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