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Finding and Characterization of Viral Nonstructural Small Protein in Prospect Hill Virus Infected Cell**

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=Abstract=

Prospect Hill Virus (PHV) is the well known serotype of hantavirus, a newly established genus in family *Bunyaviridae*. Extensive studies have upheld the original view of PHV genetics with three genes such as nucleocapsid (N) protein, envelope proteins (G1, G2) and RNA dependent RNA polymerase. In this study, we report the existence of additional gene that is encoded in an overlapping reading frame of the N protein gene within S genome segment of PHV. This gene is expected to encode a nonstructural small (NSs) protein and it seems to be only found in PHV infected cell. The presence and synthesis of NSs protein could be demonstrated in the cell infected with PHV using anti-peptide sera specific to the predicted amino acid sequence deduced from the second open reading frame. Ribosomal synthesis of this protein appears to occur at AUG codon at the 83rd base of S genome segment, downstream of N protein initiation codon. This protein is small in size (10.4 KDa) and highly basic in nature. The expression strategy of NSs protein appears that a signal mRNA is used to translate both N and NSs protein in PHV infected cell. 10 KDa protein in virus infected cell lysates can bind to mimic dsRNA. This fact strongly suggests that NSs protein may be involved in virus replication on late phase of viral life cycle.

Key Words: Prospect Hill Virus, Nonstructural small (NSs) protein, Synthetic peptide, Overlapping open reading frame, UV-cross linking assay, In vitro translation

INTRODUCTION

In recent years, extensive studies on negative sense RNA viruses have shown that they can use a number of diverse mechanisms to increase the coding potential of their relatively small genome. Some of negative sense RNA viruses use a mRNA processing and modification strategies, while other groups use an alternative translation mechanism. The use of overlapping open reading frames (ORFs) to generate more than one unique protein from a single mRNA has also been reported [4]. The synthesis of vesicular stomatitis virus (VSV) small protein using P gene mRNA and influenza B virus NA and NB glycoproteins are among the well defined examples.

Genus Hantavirus was established in 1982s since the initial isolation of Hantaan (HTN)

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virus as the causative agent of Korean hemorrhagic fever (KHF) in 1976 [15,20,29,30,33]. Since the discovery of HTN virus, the researches have been accelerated to reveal other members of hantavirus: Seoul (SEO) virus [14], Puumala (PUU) virus [2], Prospect Hill virus (PHV) [17] and other hantaviruses including Sin Nombre (SN) virus which cause Hantavirus pulmonary syndrome (HPS) virus [11,23]. Extensive studies resulted in the establishment of hantaviruses as a distinct genus within the family *Bunyaviridae* [33]. Like other members of the family, hantaviruses have negative strand large (L), middle (M), and small (S) genomic RNA segments of about 6,600, 3,600, 1,700 nucleotides in length [29] which encode the viral polymerase, two surface glycoproteins G1 and G2, and nucleocapsid (N) protein, respectively [6]. The three RNA segments have highly conserved 3' and 5' termini of genomic RNA which are complementary to each other, and which result in the formation of panhandle structure [29,31].

In *Bunyaviridae*, bunyaviruses were known to have an actual second ORF in N protein gene using overlapping reading frame [4] and phleboviruses have a coding strategy to encode the second ORF protein by ambisense way [1, 25]. The first coding strategy elucidated for is that of the S genome segment of the bunyavirus snowshoe hare (SSH) [4]. Encoding of the N protein (26.5 KDa) and also of a nonstructural small (NSs) protein (7.4 KDa) by the viral S genome segment has been established by in vitro translation of hybrid selected mRNA [4]. Similar results were obtained from other bunyaviruses, consequently. Like bunyaviruses, phleboviruses encode both of N protein and NSs protein in their S genome segment, but they use a different strategy [25]. In addition to N protein mRNA, they use a viral S genomic RNA (S vRNA) as a NSs mRNA, which is located at the 5' end of S vRNA. Therefore, they produce a much larger NSs protein, 29~37 KD [1,25].

However the presence of such a "second ORF product" in hantaviruses has been neglected so far, because Hantaan and Seoul viruses have no potential ORF for NSs protein [31]. Thereafter, it has been known that hantavirus genus apparently does not encode a NSs protein. Recently, the nucleotide sequence analysis of other members of hantaviruses such as Prospect Hill, Puumala and Sin Nombre viruses led to the prediction that an additional ORF may exist within N protein gene of S genome segment using overlapping reading frame [26,32]. Interestingly, they have a common properties of deduced NSs polypeptide sequences. They also share similar hydrophilicity, highly basic arginine rich nature and small size. And the third base change rate of overlapped region in their N protein gene is significantly lower than other region supports the functionality of NSs gene. Therefore, it had to be clarified whether NSs protein is expressed in PHV infected cells or not.

In this study, we report a direct evidence of the existence of NSs protein in Prospect Hill virus infected cell and suggest some aspects on its function. Anti-NSs protein antisera were prepared against predicted NSs protein by immunization of experimental animal with peptides which were synthesized as the deduced amino acid sequence of NSs protein. Immunoprecipitation using the antisera to PHV infected cell lysates revealed a protein band NSs sized at 7 days postinfection and it became stronger at 14 days postinfection. This suggests that NSs protein is expressed in PHV infected cell at late phase of viral infection cycle. The experiments to elucidate an expression strategy of NSs protein demonstrated that a signal mRNA appears to be used to translate both N and NSs protein in PHV infected cell. Considering the highly basic, arginine rich properties and small in size of NSs protein, RNA binding property was also tested. PHV genomic RNA sequences binding to the NSs protein was tested by UV-cross linking assay. The result shows that 10 KDa

protein of PHV infected cell lysates can bind to mimic dsRNA. This strongly suggests that NSs protein may be involved in virus replication on late phase of viral life cycle. To elucidate the effects of NSs protein on PHV replication cycle, the study constructs a stable cell line which can constantly express the NSs protein is now under way. Through PHV infection to this cell line, a role of NSs protein in PHV replication cycle can be revealed.

MATERIALS AND METHODS

1. Viruses and cells

Vero-E6 cells (CRL 1586) (ATCC, Rockville, MD) were grown as monolayer culture in Dulbeccos Modified Eagle's medium (DMEM) high glucose formulation containing 5% fetal bovine serum (FBS). Prospect Hill virus (PHV/PH-1 strain) was grown in the cell for 1 to 14 days. After infection of virus, the culture medium was saved for later use.

2. Indirect immunofluorescence assay

Virus infected cells were scrapped and resuspended in phosphate buffered saline (PBS), plated on spot slide and fixed with acetone. After drying the fixed cell, rat anti-PHV antiserum was overlayed and incubated at 37℃ for 30 min. Specific interaction between antisera and infected viral antigen was detected by addition of fluorescein isothiocyanate (FITC) conjugated anti-rat IgG antibody (Cappel, USA) [8].

3. Preparation of anti-NSs antisera

The synthetic peptides were:

1) MSSRLSLPGRSSRKLNGR (NS-1); a 18 mer representing amino acids 1 to 18,

2) TQMTLTKVHCKAGGQQCQH (NS-2); a 19mer representing amino acids 22 to 40,

3) QSSRGSLHMSSHVRR (NS-3); a 15mer representing amino acids 46 to 60,

4) LTTILRRGQASNMEMSLM (NS-4); a 18 mer representing amino acids 73 to 90 of the predicted second open reading frame (ORF) of PHV S genome segment.

The peptides were coupled to cationized bovine serum albumin (cBSA) or keyhole limpet hemocyanin (KLH) with Inject Supercarrier EDC (1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) system (Pierce, USA). Antipeptide antisera were raised in rats and rabbits, and final bleedings were taken at 5 weeks for rat, 13 weeks for rabbits postimmunization.

4. Affinity purification of anti-peptide antibodies

The synthetic pepetides were covalently coupled to 2 ml of CNBr activated agarose beads, Affigel-10 (Bio-rad, USA), in 0.1 M MOPS (3-[N-morpholino] propanesurfonic acid) buffer at 4℃ for 2 hrs. The coupled beads were packed and then stripped with 7 M urea containing 1 M NaCl. PBS diluted antisera (1:50) were applied to column and washed with 10 bed volumes of PBS. Bound antibody was eluted with 0.1 N HCl and pH of the elute was adjusted to 7.3 by addition of 0.1 volume 1 M Tris-Cl (pH 9.0) [28].

5. Enzyme linked immunosorbent assay (ELISA)

The synthetic peptides and BSA as a control were dissolved in barbital buffer (50 mM sodium barbital, 10 mM barbital) and placed on 96 well ELISA plate for 12 hrs. Unbound peptides were removed and 1:16 to 1:4096 diluted antibodies were added to each wells serially. After incubation, unbound antibodies were removed and horse radish peroxidase (HRPO) conjugated goat anti-rat antibody was added with 1:1000 dilution. Signals were detected by addition of peroxidase substrates ABTS and 0.1% peroxide. Colorization was read at 405 nm visible light using Spectra max 250 spectrophotometer (Molecular Devices, USA) [28].

6. Immunoprecipitation

Prospect Hill virus infected cells were harve-

sted into lysis buffer containing 50 mM sodium borate, Kanton CG, 150 mM NaCl, 0.1 mg/ml PMSF (phenylmethylsulfonyl fluoride), 1 mg/ml Aprotinin, 1 mg/ml Leupeptidin, 1% Nonidet P-40, 0.5% Sodium deoxycholate. Harvested cells were sonicated at 3 pulses for 30sec each on ice and centrifuged at 12000 g for 10 min to obtain supernatant. Biotin-7-NHS was added to supernatant for labelling of all proteins of infected cell lysates. Precleaning was performed with preimmune sera-protein G sepharose (Boehringer Mannheim, Germany). Precleaned cell lysates were added to the purified antipeptide antibody-protein G sepharose complex for 3 hrs at 4℃. The beads were washed 3 times with wash buffer-1 (50 mM Tris, 150 mM NaCl, 0.1% NP-40), 3 times with wash buffer-2 (50 mM Tris, 500 mM NaCl, 0.1% NP-40) and twice with wash buffer-3 (10 mM Tris). Washed complexes were boiled in 2X SDS sample buffer for 5 min. Proteins were analysed on Tricine-SDS polyacylamide gels, as opposed to glycine-SDS gels, because of their superior ability to separate low molecular weight proteins. Electrophoresed proteins were transferred onto Hybond-C-extra (Amersham) nitrocellulose membrane and the blots were incubated in blocking solution (3% skim milk, 100 mM Tris-Cl pH 7.5, 0.9% (w/v) NaCl) for 1 hr and added streptavidin-AP (akaline phosphatase). Signals were visualized using BCIP/NBT solution [28].

7. Immunoblotting

Proteins were separated on SDS-polyacrylamide gels (10~20% polyacrylamide gradient). Following electrophoresis, the proteins were transferred to Hybond-c-extra nitrocellulose membrane by electroblotting. The blots were preincubated for 1 hr in blocking buffer (3% skim milk, 100 mM Tris-Cl pH 7.5, 0.9% NaCl (w/v), 0.1% Tween 20), which subsequently was replaced with antisera diluted 1:100 in blocking buffer and further incubated for 30 min at room temperature. The blots were washed with wash buffer (blocking buffer without skim milk) and incubated with goat anti-rat immunoglobulin G conjugated to alkaline-phosphatase (KPL, USA) diluted in blocking buffer 1:1000. Protein bands were visualized in BCIP/NBT solution (KPL. USA) [28].

8. RNA extraction from PHV infected cells

Vero-E6 cells were infected with PHV and maintained for 14 days until harvest. Harvested cells were lysed in 4 M guanidium isothiocyanate (GIT) solution and continued with standard guanidium hot phenol protocol [5].

9. RT-PCR amplification

The first strand cDNA synthesis of NSs ORF region was made with oligonucleotide primers, 5'-TTAAGCTTGCCATGAGCAGCAGATTG-3' (containing HindIII site), 5'-TTTCTAGACC-AATCAGCTGTCTGGCC-3' (containing XbaI site) at 42℃ for 1 hr. For PCR amplification of the NSs ORF region, same forward and reverse primers were used with Taq polymerase (Finnzymes, Finland). Reaction was performed for 3 min at 95℃ to denature templates and continued 30 cycles for amplification. Each cycle consisted of 40sec at 94℃, 40sec at 53℃ and 1min at 72℃. Amplification of the whole S genome segment was performed with same reverse transcription condition as above using primer set representing both ends sequence of S segment. 5'-GATTATGCTGATATCCCTAGT-AGTAGACTTCGTAAAGAG-3' (containing T7 promoter sequence) and 5'-TAGTAGTATACT-CCTTGAAAAGC-3'. Cycling reaction condition consisted of 40sec at 95℃, 1 min 20sec at 43℃ and 2 min at 72℃).

10. Cloning of NSs ORF region

Amplified mixture was separated on 2% agarose gel and 320 bp sized band was recovered with Geneclean II kit (Bio 101, USA) using protocol described by manufacture. Recovered DNA was ligated into pT7Blue T vector (Novagen, USA) and transformed to JM109

E. coli at 1800 V, 15 mF and 150 W in Electrophorator II (Invitrogen, Netherlands). Recombinant clones were collected by standard plasmid minipreparation procedure. Collected clones were sequenced with Sequenase version 2.0 kit (Amersham, USA) using supplier manual.

11. In vitro translation

S mRNA was transcribed from amplified S genome segment which contains T7 promoter using T7 Megascript system (Ambion, USA). Transcribed mRNA was translated in rabbit reticulocyte lysates (Boehringer Mannheim, Germany) using 35S methionine (1000 Ci/mmol, Amersham, USA) at 30℃ for 1 hr. Produced proteins were analyzed on SDS polyacrylamide gels and visualized by autoradiography using supplier manual.

12. Northern blotting

Total RNA was prepared from PHV infected cells 7 and 14 days post infection using guanidium hot phenol method. Extracted RNAs were loaded on 1% formaldehyde denaturation gel. Separated RNAs were transferred to Hybond N+ nylon membrane (Amersham, USA) by capillary transfer method. Prehybridization was performed in 6X SSC, 0.1% SDS, with salmon sperm DNA for 12 hrs. Negative and positive sense RNA probes were prepared from pT7NSs plasmids, each has an opposite orientation. Hybridization was done in same prehybridization buffer at 50℃ for 12 hrs. After hybridization, the blot was washed twice in 1X SSC, 0.1% SDS at 55℃ and twice in 0.1X SSC, 0.1% SDS at 55℃. The washed blot was exposed to Hyperfilm-ECL (Amersham, USA) for 16 hrs at -70℃ using supplier manual.

13. Preparation of conserved end sequences of PHV genomes

T7 promoter primer and oligonucleotides which correspond to end sequences of PHV genomes were annealed by heating and slow cooling. Annealed structures were used as a template for in vitro transcription using ^{32}P-UTP (3000 Ci/mmol) (Amersham, USA). Separately transcribed small RNA fragments were purified with push column (Stratagene, USA) and reannealed by heating and slow cooling.

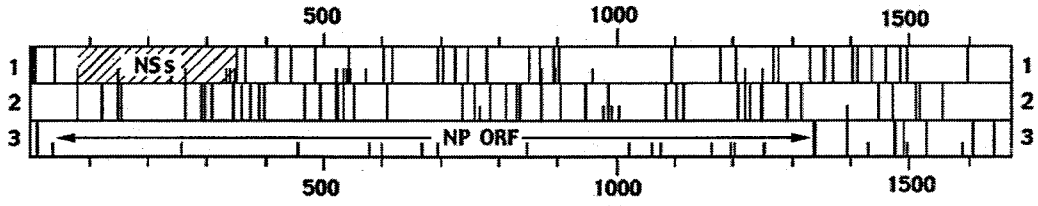
14. UV-cross linking assay

Prepared double strand RNA fragment was incubated with PHV infected cell lysates in RSB-100 binding buffer (10 mM Tris, 100 mM NaCl, 2.5 mM $MgCl_2 \cdot 6H_2O$, pH 7.4). Incubated mixture was exposed to UV-irradiation (1200 mJ/cm^2) for 8 min. Cross-linked mixture was treated with RNase cocktail (50 mM $CaCl_2$, 5 mg/ml RNase A, micrococcal nuclease) for 10 min at room temperature. Treated mixture was loaded on 15% SDS- polyacrylamide gel. Electrophoresed gel was dried and exposed to Hyperfilm-ECL (Amersham) for 5hrs at -70℃.

RESULTS

1. The existence of nonstructural small (NSs) protein ORF

Bases 43 to 1344 of S genome segment of Prospect Hill virus encodes a nucleocapsid (N) protein in the first open reading frame (ORF). Close to the 5' end of N protein gene, a potential nonstructural small (NSs) protein ORF is placed in the region from nucleotide 83 (first initiation codon downstream of N protein initiation codon) to 355 (Figure 1A, 1B) [19]. This 273 bp sized second ORF is overlapped with N protein ORF and has potential to encode a protein of 90 amino acid in length. It is unclear how this ORF could be utilized for given Kozak's rules for ribosomal scanning. These rules state that leaky scanning through an upstream AUG can occur only when the upstream AUG is in weak context. However, in this case, the upstream AUG for N protein gene is in a strong context (G at position -3, A at +4). Downstream AUG for predicted NSs protein also has a strong context of Kozak's rule (G at position -3, A at +4) (Figure 1B).

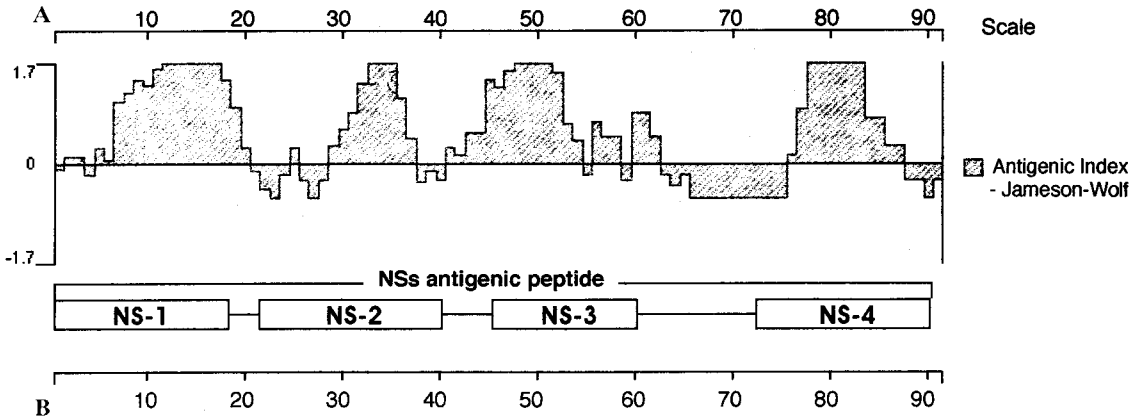


A

5'-TAGTAGTAGA CTCCTTGAAA AGCTACTACT ACAAGTGCTG GGATGAGCCA
ACTCAGGGAA ATACAGGAAG AGATCACTCG CCATGAGCAG CAGCTTGTCA
TTGCCCGGCA GAAGCTCAAG GAAGCTGAAC GGACGGTGGA GGTGGACCCA
GATGACGTTA ACAAAAGTAC ACTGCAAAGC AGGCGGTCAG CAGTGTCAAC
ATTGGAGGAC AAATTGGCAG AGTTCAAGAG GCAGCTTGCA GATGTCATCT
CACGTCAGAA GATGGATGAG AAACCTGTGG ATCCAACTGG TATTGAGCTT
GACGACCATC TTAAGGAGAG GTCAAGCCTC CGATATGGAA ATGTCCTTGA

B

Figure 1. The existence of nonstructural small (NSs) protein ORF in PHV S genome segment. **A:** ORF mapping of PHV S genome segment. Slashed region represents the NSs ORF. **B:** 5' sequence of PHV S genome segment. The initiation codons are thicken and the first and second ATGs are initiation codons of ORF of nucleocapsid (N) protein and nonstructural small (NSs) protein respectively. Another thicken TGA indicates the stop codon of NSs ORF. Kozak's context is underlined.



NS-1 (1~18) : MSSRLSLPGRSSRKLNGR
NS-2 (22~40) : TQMTLTKVHCKAGGQQCQH
NS-3 (46~60) : QSSRGSLHMSSHVRR
NS-4 (73~90) : LTTILRRGQASNMEMSLM

Figure 2. Predicted properties of NSs protein and antigenic peptides. **A:** Analyzed antigenicities of NSs protein. Selected region is shown. **B:** Selected peptide sequences.

2. Cloning of NSs ORF

320 bp PCR product was amplified from extracted RNAs of PHV infected cells. This product was cloned to pT7Blue T vector and sequenced (data not shown). For later uses, the clones with both orientated insert were saved.

3. Preparation of antisera against NSs protein

Four regions were selected from deduced amino acid sequence of NSs protein by hydropathy plot (Kite and Doolitle), antigenicity index (Jameson and Wolf) and surface probability (Emini) prediction using DNA Star program

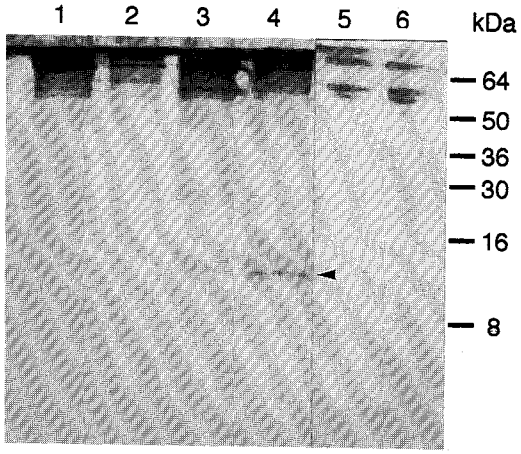


Figure 3. Immunoprecipitation of NSs protein expression in PHV infected cells with time course. Lane 1: Mock infected cells. Lane 2: 1 day post infection. Lane 3: 7 days post infection. Lane 4: 14 days post infection. Lanes 5, 6: Immunoprecipitation in same cells as 4 with pre-immune sera. NSs proteins (◄ marked) are detectable on 7 and 14 days post infection.

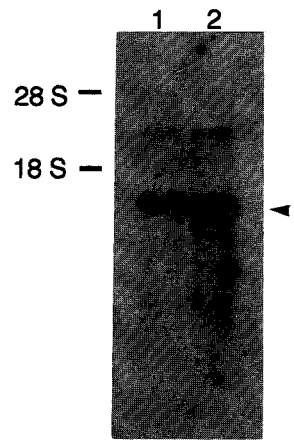


Figure 4. Detection of mRNA (◄) of PHV S genome segment. Northern analysis with PHV infected cells 7 days (lane 1) and 14 days (lane 2) post infection.

(Figure 2). Selected peptide sequences (NS-1, 2, 3 and 4) were synthesized at moderate purity (60~80%). Synthesized peptides were injected into rats and rabbits three times at intervals of 1~2 weeks. After third injection, bloods were collected. Three kinds of reactive antibodies were affinity purified using synthetic peptides column. To test the purified antibodies for the presence of antibodies against each peptides, ELISA titration was performed. The titers of antibodies were concluded to be high as 1: 1024 except for NS-4 (data not shown).

4. Detection of NSs protein in PHV infected cells

In order to determine the actual product of NSs ORF during the PHV infection and establish the expression stage of NSs protein during PHV infection cycle, PHV infected cells were collected 1, 7 and 14 days post infection. Collected cells were lysed and precleaned with preimmune sera and immunoprecipitated with purified anti-NS-3 antibody. The result showed that no signals were detected from uninfected cells and preimmune serum control lane and 1 day postinfection lane. However, NSs protein sized band was observed at 7 days postinfection and it became stronger at 14 days postinfection (Figure 3). This result suggests that NSs protein is expressed in PHV infected cell at late phase of viral infection cycle.

5. Coding strategy of the S genome segments of PHV

To explain the transcriptional and translational relationship between N and NSs genes which are overlapped in same S genome segment, total RNAs were prepared from PHV infected cells 7 and 14 days post infection. Northern blot analysis was performed with negative sensed RNA probe which transcribed from cloned NSs gene. Result indicated that only one S segment mRNA species was detected for both 7 and 14 days post infection (Figure 4). This suggests that a signal mRNA appears to be used to translate both N and NSs protein in PHV infected cell (Figure 5).

6. RNA binding ability of NSs protein

PHV genomic RNA sequences binding to the NSs protein were detected by UV-cross link-

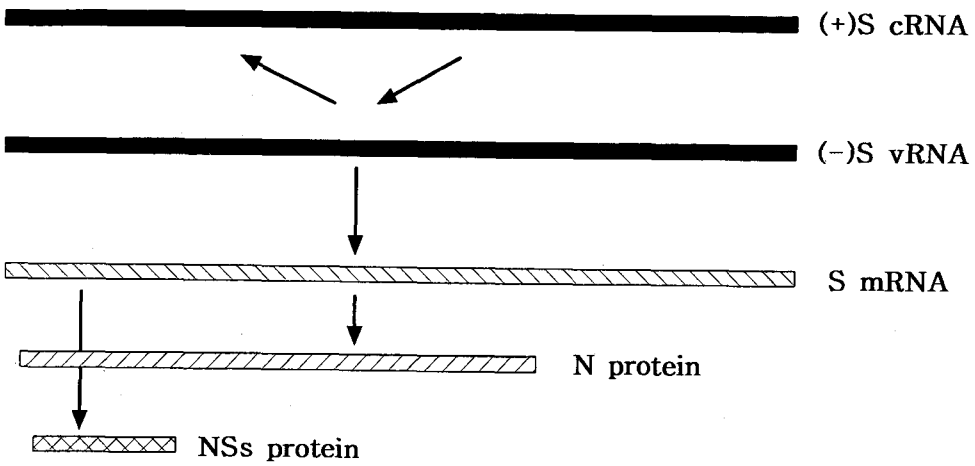


Figure 5. Suggested coding strategy of PHV S genome segment. A signal mRNA appears to be used to translate both N and NSs protein in PHV infected cell.

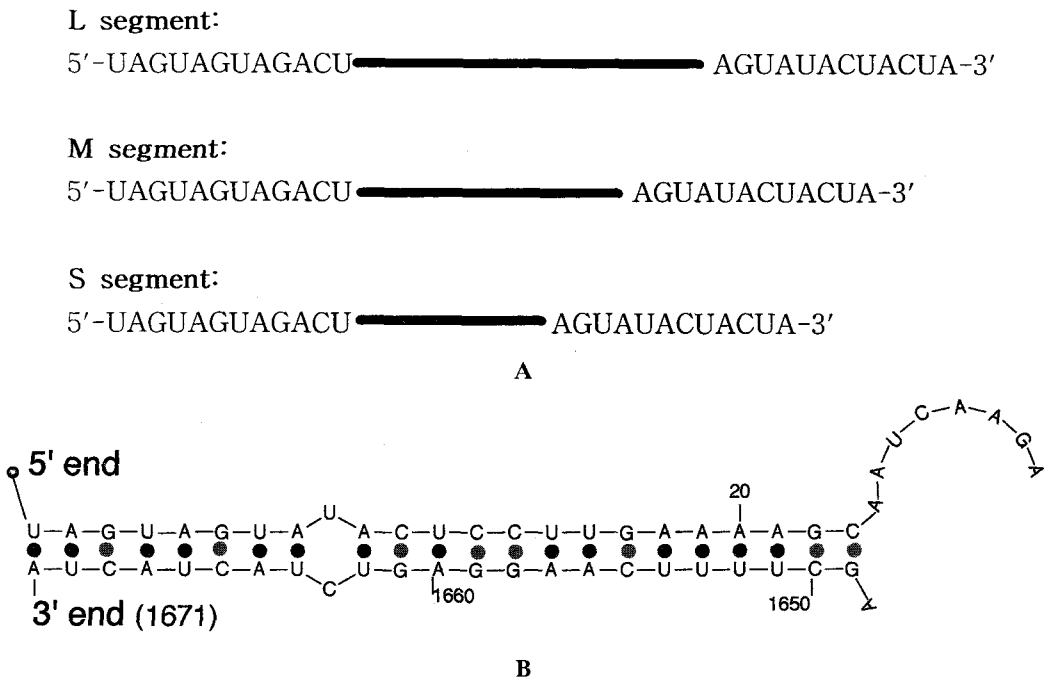


Figure 6. A. Conserved RNA sequences (L, M, S) and **B.** Predicted structure of PHV S genome's end (viral sense).

ing assay. PHV genomic RNA sequence was selected by comparison of three segments (L, M, S) of PHV genomes. Like most bunyaviruses, PHV has a conserved sequences at the both ends of each genome 5'-UAG UAG UAG ACU-3' and 5'-AUG CUA CUA CUA-3' (Figure 6A). These sequences at both ends are complimentary each other, so it can make a panhandle structure of each RNA genomes (Figure 6B). Thus, a mimic structure of conserved handle

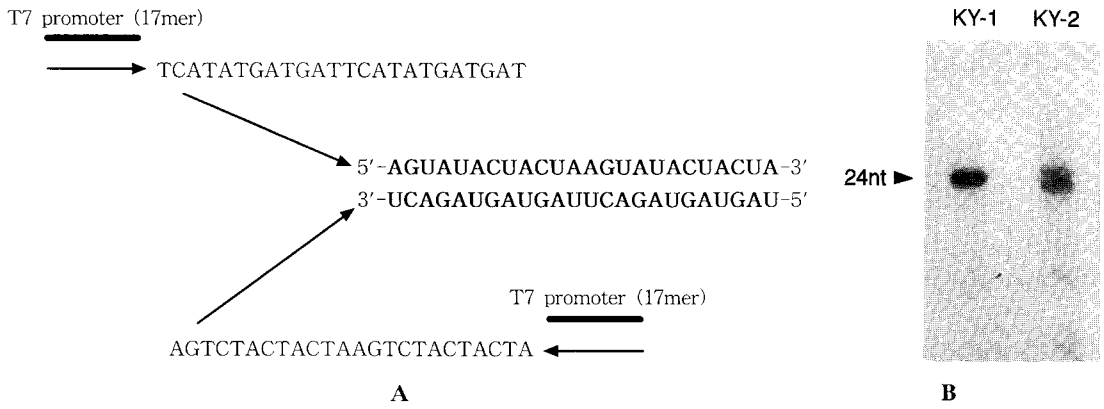


Figure 7. Mimic structure of panhandle region of PHV genomes. **A:** Strategy to construct a mimic structure. **B:** in vitro transcribed RNA fragments 1: 5' end of (−) RNA genome. 2: 3' end of (−) RNA genome.

Figure 8. UV-cross linking (◄) between NSs protein and PHV genome mimics. Lane 1: Binding reaction with uninfected cell lysates. Lanes 2, 3: Binding with PHV infected cell lysates 7days and 14days post infection.

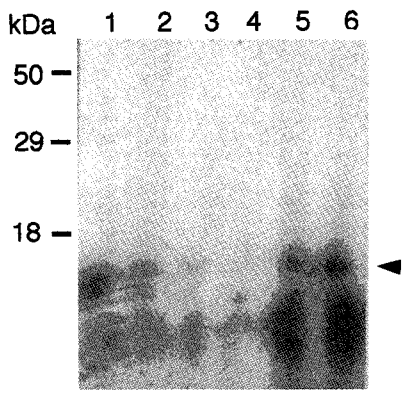


Figure 9. Competitive RNA-protein binding (◄) assay. Lanes 1-4: Increasing of cold competitor amount 1:1 to 1:4 (hot: cold). Lanes 5, 6: Addition of yeast tRNA as a nonspecific RNA. 1:2 and 1:4 (hot: tRNA).

region was made by reannealing of tandem repeats of conserved RNA sequences which were transcribed separately (Figure 7A). Two small RNA fragments were analyzed by TBE-polyacrylamide gel electrophoresis (Figure 7B). Binding reaction between annealed dsRNA and NSs protein from PHV infected cell lysates was performed in low ionic strength condition and bound RNA-protein complex was cross-linked by UV irradiation. Normal Vero-E6 cell lysate was used as binding control. This experiment showed that 10 KDa protein of PHV infected cell can bind to the conserved handle structure of PHV S genomes. No binding was detected in control reaction (Figure 8). To prove the specific interaction between dsRNA and 10 KDa protein in PHV infected cells, a competitive binding assay was carried out using yeast tRNA as a nonspecific competitor and the same dsRNA without ^{32}P-UTP incorporations as a cold competitor. Binding signals became weaker gradually by addition of increasing amount of cold dsRNA (Figure 9, Lane 1-4). However, there was no change on RNA-protein binding

strength by addition of yeast tRNA (Figure 9, Lane 5, 6).

DISCUSSION

Unlike other bunyaviruses, hantaviruses were known that they apparently do not encode a nonstructural protein [31]. The mRNA encoding Hantaan N protein is nearly equivalent in size to genomic S RNA, and no subgenomic messages were detected in infected cells. RNA transcripts of cDNA, which corresponded to Hantaan virus S mRNA, were able to program a cell free translation system to yield N protein, indicating that the single, continuous ORF in the Hantaan virus S segment encode N protein [31]. However, in recent years, numerous hantavirus isolates have now been cloned and sequenced. Analysis of the sequences indicates that some of them, such as Prospect Hill, Puumala, Hantavirus Pulmonary Syndrome virus, do have a second ORF in their S genome segment with different reading frame of N protein [26,32].

In case of PHV, it has a potential overlapping second ORF with initiation codon at position 83 (first initiation codon downstream of N protein initiation codon) and with potential to encode a protein of 90 amino acid in length. If the second ORF were functional, then evolutionary constraint to maintain amino acid sequence in both frames would be expected to lower the third base substitution frequency in this region. Statistical analysis of the third base substitution frequency among the potential second ORF region of Hantavirus pulmonary syndrome virus, five Puumala virus and Prospect Hill virus reveals a significant decrease (c^2 test, p=0.001) in this region (45/64 substitutions per third base position) relative to the rest of the S genome segment (290/343 substitutions per third base position). This results strongly suggest that the second ORF of these viruses may be functional. NSs proteins of other members of *Bunyaviridae* show low level of primary

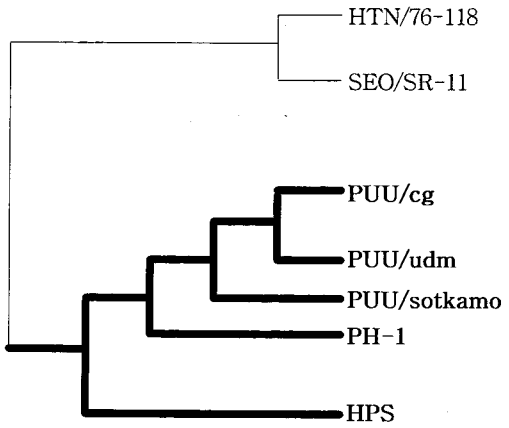


Figure 10. Phylogenetic analysis of hantaviruses using S genome sequence. Members which contain NSs ORF is shown in bold.

amino acid sequence similarity. This is also true of the predicted NSs proteins of Puumala, Prospect Hill and hantavirus pulmonary syndrome viruses. However, they share the overall properties of being small, highly basic (pI=10.5-12.8), arginine-rich proteins with similar hydropathy patterns, suggestive of conserved function. If prediction of the NSs protein in hantavirus is correct, one subgroup of hantavirus which containing NSs ORF has a separate evolutionary trait from other group (HTN, SEO) which does not contain potential ORF of NSs proteins. Actually, phylogenetic analyses of S genome segment sequence of hantaviruses show that HTN, SEO virus group is apparently divided from PH, PUU, HPS virus group (Figure 10). In addition, same analyses on other genomic segments (L, M) indicate the same result as S genome segment analysis results. Second ORF of S genome segment of viruses of PHV group has a conserved Kozak sequence (-3 G and +1 A/G), which is the same as that N gene. If leaky scanning through the first strong AUG is also the case here, then the first AUG after N protein initiation codon will serve to initiate translation.

Pilot immunoblotting analysis of PHV infected cells using anti-synthetic NSs peptides

antisera was shown that specific interaction between antisera and 10 KDa protein was detected in 14 days post infected cells. Time course immunoprecipitation indicates that NSs protein is expressed on relatively late phase of viral infection cycle. It is essential aspect for negative sense RNA viruses that they have to transcribe mRNA at initial stage and translated products act on early infection machinery. After early events of infection cycle, viral structural proteins are translated and genomic RNAs are replicated from cRNA. Therefore, lately expressed NSs protein may be involved in viral replication or translation. In case of phlebovirus, ribosomal association of NSs protein was detected in viral infected cells. However, any informations about functionality of NSs protein have not been shown in *Bunyaviridae*. If NSs protein is involved in PHV replication machinery, it can have an interaction with viral genomic RNAs. Replication form of genomic RNAs were proposed by many scientist, which form a pan-handle structure with their conserved complimentary sequences at both genomic ends. RNA-protein UV-cross linking experiment between mimic structure of pan-handle region of PHV genomes and PHV infected cell lysates shows that 10 KDa protein of PHV infected cell lysates can bind to mimic dsRNA. The specificity of interaction was verified by competition assay with yeast tRNA and cold mimics. This result strongly suggest that NSs protein may be involved in PHV replication on late phase of viral life cycle. This suggestion can be confirmed by early expression of NSs protein during PHV infection. Thus, we constructed a stable cell line which can express NSs protein constantly. Infection of PHV to this cell line and time course northern blotting analysis will show the role of NSs protein in PHV infection cycle.

SUMMARY

In this study, we report an evidence of the existence of NSs protein in Prospect Hill virus (PHV) infected cell and suggest some aspects on its function. To evident this fact, the existence of additional gene that is encoded in an overlapping reading frame of the N protein gene within S genome segment of PHV has been clarified. This gene is expected to encode a nonstructural small (NSs) protein which is found in PHV but not in Hantaan or Seoul virus infected cell. The presence and synthesis of NSs protein could be demonstrated in the cell infected with PHV by immunoprecipitation using anti-peptide sera specific to the predicted amino acid sequence deduced from the second open reading frame. NSs protein was detected at 7 days postinfection and it became stronger at 14 days postinfection. This suggests that NSs protein is expressed in PHV infected cell at late phase of viral infection cycle. Ribosomal synthesis of this protein appears to occur at AUG codon at the 83rd base of S genome segment, downstream of N protein initiation codon. This protein is small in size (10.4 KDa) and highly basic in nature. The experiments to elucidate an expression strategy of NSs protein in PHV infected cell suggested that a signal mRNA appears to be used to translate both N and NSs protein in PHV infected cell. Considering the highly basic, arginine rich properties and small in size of NSs protein, RNA binding property was also tested. PHV genomic RNA sequences binding to the NSs protein were detected by UV-cross linking assay. The result shows that 10 KDa protein of PHV infected cell lysates can bind to mimic dsRNA. This strongly suggests that NSs protein may be involved in PHV replication on late phase of viral life cycle. To elucidate the effects of NSs protein on PHV replication cycle, the study constructs a stable cell line which can constantly express the NSs protein is now under way. Through PHV infection to this cell line, a role of NSs protein in PHV replication cycle can be revealed.

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