Establishment of a Bacterial Expression System and Immunoassay Platform for the Major Capsid Protein of HcRNAV, a Dinoflagellate-Infecting RNA Virus

KEI WADA¹, KEI KIMURA², AKIFUMI HASEGAWA¹, KEICHI FUKUYAMA¹, and KEIZO NAGASAKI²*†

¹Department of Biological Sciences, Graduate School of Science, Osaka University, Toyonaka, Osaka 560–0043, Japan; and ²National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, 2–17–5 Maruishi, Hatsukaichi, Hiroshima 739–0452, Japan

(Received February 22, 2012—Accepted June 17, 2012—Published online October 5, 2012)

HcRNAV is a small icosahedral virus that infects the shellfish-killing marine dinoflagellate Heterocapsa circularisquama, which harbors a dicistronic linear single-stranded RNA (ssRNA) genome ca. 4.4 kb in length. Its major capsid protein (MCP) gene sequence is not expressed by various strains of Escherichia coli, possibly because of a codon usage problem. To solve this problem, a chemically modified (i.e., de novo synthesized) gene was designed and cloned into the pCold-GST expression vector, and transformed into E. coli strain C41 (DE3), in which codon usage was universally optimized to efficiently express the polypeptide having the viral MCP amino acid sequence. The bacterially expressed protein, which was purified after a procedure involving denaturation and refolding, successfully formed virus-like particles that significantly resembled native HcRNAV particles. The purified, denatured protein was used as an antigen to immunize rabbits, and the resulting antiserum was shown to be strongly reactive to not only the bacterially expressed recombinant protein, but also to native HcRNAV MCP by Western blotting and dot immunoassays, respectively. These results indicate that an antiserum recognizing native HcRNAV MCP was successfully obtained using bacterially expressed HcRNAV MCP as the antigen.

Key words: bacterial expression, Heterocapsa circularisquama RNA virus, major capsid protein, polyclonal antibody

To date, eight RNA viruses infecting marine eukaryotic microorganisms have been isolated and characterized to a different extent. Among them, seven are single-stranded RNA (ssRNA) viruses: HaRNAV infects the noxious bloom-forming raphidophyte Heterosigma akashiwo (Raphidophyceae) (10, 28); RsetRNAV (17, 26), CtenRNAV (27), CsfR RNAV (32) and AglaRNAV (33) are diatom (Raphidophyceae) (10, 28); RsetRNAV (17, 26), CtenRNAV (27), CsfrRNAV (32) and AglaRNAV (33) are diatom-infecting viruses that infect diatom viruses. This study was designed to establish a bacterial expression system targeting it by using the expressed MCP as an antigen, which infests the adsorption and infection of their specific host. HcRNAV, which infects the noxious bloom-forming alga H. circularisquama, is the first and only dinoflagellate-infesting RNA virus grown in culture. The HcRNAV genome is a linear ssRNA, ca. 4.4 kb in length, and harbors two open reading frames (ORFs) (16); the upstream ORF (ORF1) encodes a replication polyprotein and the downstream ORF (ORF2) codes for a single major capsid protein (MCP). A recent cryo-electron microscopy study on HcRNAV particles revealed that the virus has a diameter of 34 nm and T=3 symmetry, and consists of 180 quasi-equivalent monomers (13). For dsDNA algal viruses, some notable studies on the entry process of the virus have been reported, including studies on the entry process of Chlorella virus (5, 12, 19, 24) and a phaeovirus (Ectocarpus fasciculatus virus) (11). In contrast, little is known about the mechanisms underlying the adsorption and infection of ssRNA algal viruses. This study was designed to establish a bacterial expression system for HcRNAV MCP and to prepare a polyclonal antibody targeting it by using the expressed MCP as an antigen, which is expected to be a promising tool to analyze the host-virus crosstalk process. Here, we have described the procedure to establish a bacterial expression system and an immunoassay platform for HcRNAV MCP as well as its promising availability.

Materials and Methods

Virion purification

The virus was collected from the HcRNAV-infected H. circularisquama culture using the method described by Tomaru et al. (31). Briefly, 450 mL of exponentially growing H. circularisquama strain HU9433-P was inoculated with 3 mL fresh suspension of HcRNAV strain 34 (16, 31) (HcRNAV34; ca. 10⁷
infectious units mL$^{-1}$. The host cells were lysed and then sequentially filtered through 8.0-, 0.8-, and 0.2-μm filters to remove cell debris. Polyethylene glycol was added to the filtrate to obtain a final concentration of 10% (w/v), and the mixture was stirred in the dark overnight at 4°C; the suspension was centrifuged at 57,000×g for 90 min. The viral pellet was then washed with phosphate buffer (10 mM Na$_2$HPO$_4$ and 10 mM KH$_2$PO$_4$ in distilled water) and centrifuged again at 217,000×g for 4 h at 4°C. The collected virus particles were resuspended in 500 μL phosphate buffer.

**Extraction of HcRNAV genomic ssRNA and cloning of the authentic MCP cDNA**

Genomic ssRNA was purified from HcRNAV34 particles using the High Pure Viral RNA kit (Roche Applied Science, Penzberg, Germany) and reverse-transcribed with random oligo primers and PrimeScript Reverse Transcriptase (Takara, Otsu, Japan). The procedures for ssRNA isolation and reverse transcription (RT)-PCR were in accordance with the manufacturers’ protocols. A pair of the following primers was designed on the basis of the previously reported HcRNAV34 genomic sequence (NCBI Reference Sequence: NC_007518.1): 5'-CAT ATG ACC CGT CCC CTA GCT TGC TGG C-3', which were used to amplify the MCP gene (ORF2) by PCR. PCR amplification was performed using PrimeSTAR Max DNA Polymerase (Takara). The PCR cycle was as follows: 1 cycle by PCR. PCR amplification was performed using PrimeSTAR Max DNA Polymerase (Takara). The PCR cycle was as follows: 1 cycle of denaturation at 98°C (20 s), 30 cycles of (denaturation at 98°C [10 s], annealing at 55°C [5 s], and extension at 72°C [65 s]), followed by an additional extension at 72°C (65 s). The PCR product was initially subcloned into pTA2 (TOYOBO, Osaka, Japan), and the sequence was confirmed by sequencing. For expression in *E. coli*, the gene fragment encoding authentic ORF2 was ligated into the pET-15b and pET-21a expression vectors (Merck Biosciences, Darmstadt, Germany). For the expression in yeast, the gene fragment encoding N-terminal His-tagged authentic ORF2 derived from the pET-15b-orf2 vector was cloned into pHL-D2 and pPIC9 vectors (Invitrogen, San Diego, CA, USA), and the vector constructs were integrated into the genome of *Pichia pastoris* (Medical & Biological Laboratories) as a substrate. The results were monitored spectrophotometrically at an optical density of 80 kV.

**Preparation of polyclonal antibody for MCP**

The purified recombinant MCP in SDS-PAGE running buffer was mixed with Freund’s complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and intradermally injected into rabbits at a dose of 0.1 mg protein per rabbit. Two weeks after the first inoculation, hypodermic inoculations of 0.1 mg protein per rabbit with Freund’s incomplete adjuvant were performed weekly for 6 weeks, and then the rabbits were exsanguinated to collect the antisera. Finally, the polyclonal antibody (designated as “ABH-1”) was stored at −80°C or at −30°C until use.

**ELISA**

TBS (20 mM Tris-HCl [pH 7.4], 2.7 mM KCl, and 100 mM NaCl) containing 1 μg purified recombinant MCP was placed in each well of a 96-well microtiter plate and incubated for 18 h at 4°C. After removing the antigen fluid, the wells were blocked with TBS containing 2% BSA for 2 h at room temperature. After washing the wells three times with TBS containing 0.05% Tween-20, the serial dilution solution (1/50n) of the polyclonal antibody was added to each well, followed by peroxidase-conjugated goat anti-rabbit IgG antibody (Medical & Biological Laboratories, Nagoya, Japan) for 2 h at room temperature. Finally, the peroxidase activity was determined using 3,3',5,5'-tetramethylbenzidine (TMB) (Medical & Biological Laboratories) as a substrate. The results were monitored spectrophotometrically at an optical density of 450 nm.

**Dot blot analysis**

Protein solutions (33 μg mL$^{-1}$) were spotted onto an Immobilon-P Transfer Membrane (Millipore, Bedford, MA, USA) at 2 μL dot$^{-1}$, and the membrane was blocked with 5% (w/v) skim milk in TBST (50 mM Tris-HCl [pH 7.8], 0.1% [v/v] Tween 20, and 150 mM NaCl) for 1 h; subsequently, it was washed with TBST. The anti-MCP polyclonal antibody was diluted 1:40,000 in TBST, and the membrane was incubated with diluted anti-MCP antibody for 1 h. The membrane was then washed with TBST, incubated for 1 h
with anti-rabbit IgG [H+L] (Goat) conjugated to horseradish peroxidase (Nacalai Tesque) and diluted to 1:40,000 in TBST, washed again, developed with an ECL Plus Western Blotting Detection System (GE Healthcare), and then, the resultant membrane was exposed to Black & White Instant Film (Fujifilm, Tokyo, Japan) using an ECL Mini-camera (GE Healthcare).

**Western blotting**

Uninfected and HeRNAV34-infected cells of *H. circularisquama* (2 d post inoculation [d pi]) were collected by centrifugation at 750×g for 10 min. HeRNAV34 particles were collected by the methods described above. The sample pellets were washed in SDS sample buffer (50 mM Tris hydroxymethyl aminomethane-HCl, 2% [w/w] SDS, 10% [v/v] glycerol, 6% [v/v] 2-mercaptoethanol, 0.002% [w/v] bromophenol blue, pH 6.8) using a micro-homogenizer and boiled for 5 min. The samples were then electrophoresed at 200 V for 30 min using a Mini-PROTEAN Cell with a Mini-PROTEAN TGX Precast Gel 12% (Bio-Rad) according to the method of Laemmli (9), and the gel was stained with Coomassie Brilliant Blue R-250 (CBB). The molecular mass was estimated using Precision Standards (Bio-Rad). The gels were blotted onto polyvinylidene difluoride (PVDF) membranes (Sequen-Blot PVDF Membrane, Bio-Rad) using the Mini-TRANSBLOT System (Bio-Rad). The membrane was stained with CBB to capture the protein staining pattern. The membrane was decolorized with 100% methanol, washed in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) several times, and then immunostained with the polyclonal antibody ABH-1 (diluted 1:10,000 in modified TBST containing 0.002% [w/v] NaN₃) (2.5% [w/v] nonfat milk, 5% [w/v] normal goat serum, and 0.05% [w/v] NaN₃ in PBS) for 20 min, and then rinsed with PBS several times. Samples were incubated in blocking solution (2.5% [w/v] nonfat milk, 5% [w/v] normal goat serum, and 0.05% [w/v] NaN₃ in PBS) for 30 min at 37°C. After blocking, they were incubated at 20°C overnight with the polyclonal antibody ABH-1 diluted 1:1,000 in modified TBST containing 0.05% [v/v] Tween-20) using the ProtoBlot II AP Systems with anti-rabbit IgG [H+L] (Goat) conjugated to horseradish peroxidase (Nacalai Tesque) and diluted to 1:40,000 in TBST, washed again, developed with an ECL Plus Western Blotting Detection System (GE Healthcare), and then, the resultant membrane was exposed to Black & White Instant Film (Fujifilm, Tokyo, Japan) using an ECL Mini-camera (GE Healthcare).

**Immunofluorescence microscopy**

Immunofluorescence observation was performed according to the method of Motomura and Hishinuma (14). To fix the intact *H. circularisquama* cells and virus-infected cells (2 d pi), each cell suspension was mixed with an equal volume of fixation buffer (2% [w/v] glutaraldehyde and 3% [w/v] paraformaldehyde in PHEM suspension was mixed with an equal volume of fixation buffer (2% circularisquama TBST served as the control. The immunostaining pattern of the manufacturer's protocol. Another PVDF membrane treated with Stabilized Substrate (Promega, Madison, USA), according to the methods described above. The sample pellets were washed in SDS sample buffer (50 mM Tris hydroxymethyl aminomethane-HCl, 2% [w/w] SDS, 10% [v/v] glycerol, 6% [v/v] 2-mercaptoethanol, 0.002% [w/v] bromophenol blue, pH 6.8) using a micro-homogenizer and boiled for 5 min. The samples were then electrophoresed at 200 V for 30 min using a Mini-PROTEAN Cell with a Mini-PROTEAN TGX Precast Gel 12% (Bio-Rad) according to the method of Laemmli (9), and the gel was stained with Coomassie Brilliant Blue R-250 (CBB). The molecular mass was estimated using Precision Standards (Bio-Rad). The gels were blotted onto polyvinylidene difluoride (PVDF) membranes (Sequen-Blot PVDF Membrane, Bio-Rad) using the Mini-TRANSBLOT System (Bio-Rad). The membrane was stained with CBB to capture the protein staining pattern. The membrane was decolorized with 100% methanol, washed in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) several times, and then immunostained with the polyclonal antibody ABH-1 (diluted 1:10,000 in modified TBST containing 0.002% [w/v] NaN₃) (2.5% [w/v] nonfat milk, 5% [w/v] normal goat serum, and 0.05% [w/v] NaN₃ in PBS) for 20 min, and then rinsed with PBS several times. Samples were incubated in blocking solution (2.5% [w/v] nonfat milk, 5% [w/v] normal goat serum, and 0.05% [w/v] NaN₃ in PBS) for 30 min at 37°C. After blocking, they were incubated at 20°C overnight with the polyclonal antibody ABH-1 diluted 1:1,000 in modified TBST containing 0.05% [v/v] Tween-20) using the ProtoBlot II AP Systems with anti-rabbit IgG [H+L] (Goat) conjugated to horseradish peroxidase (Nacalai Tesque) and diluted to 1:40,000 in TBST, washed again, developed with an ECL Plus Western Blotting Detection System (GE Healthcare), and then, the resultant membrane was exposed to Black & White Instant Film (Fujifilm, Tokyo, Japan) using an ECL Mini-camera (GE Healthcare).

**Results and Discussion**

**Overproduction and purification of recombinant MCP**

Although we intended to establish an overproduction system of recombinant HeRNAV MCP using the authentic nucleotide sequence in *E. coli*, yeast and insect cells, all initial trials were unsuccessful (Fig. S1). Generally, the proportion of each codon used for a given amino acid varies widely between organisms; 15%–20% of the codons were revealed to correspond to rare codons that confound heterologous protein expression. Here, the genetic code used in the host dinoflagellate cell was assumed to be “universal”, because the results of amino acid sequencing due to Edman degradation well coincided in the previous analysis of the HeRNAV genome (16). Hence, an artificial sequence to optimize codon usage for the expression in *E. coli* cells was designed on the basis of the deduced amino acid sequence of HeRNAV MCP, and synthesized de novo (Fig. S2). Consequently, only when the pCold-Hs₆-GST-ORF2 expression vector was transformed into the *E. coli* strain C41 (DE3) was His-GST-MCP was successfully overexpressed in the insoluble fraction. The expressed His-GST-MCP was solubilized with the denaturant and purified with nickel-affinity resin under denaturing conditions (Fig. 1). The purified His-GST-MCP was successfully refolded by the dilution method, and we successfully obtained His-GST-MCP in the soluble fraction. His-GST-MCP was cleaved by HRV3C protease; the subsequent SDS-PAGE gel showed CBB-stained major bands estimated to be at ~35 kDa and ~30 kDa (Fig. 1), which were consistent with the molecular masses of MCP and His-GST, respectively, calculated on the basis of their amino acid sequence. To collect pure MCP separated from the His-GST region, a variety of purification methods such as ion-exchange chromatography, hydrophobic interaction chromatography, and size-exclusion chromatography were applied; however, the MCP and His-GST region were eluted in the same fractions for all these chromatography methods described above. The sample pellets were washed in SDS sample buffer (50 mM Tris hydroxymethyl aminomethane-HCl, 2% [w/v] SDS, 10% [v/v] glycerol, 6% [v/v] 2-mercaptoethanol, 0.002% [w/v] bromophenol blue, pH 6.8) using a micro-homogenizer and boiled for 5 min. The samples were then electrophoresed at 200 V for 30 min using a Mini-PROTEAN Cell with a Mini-PROTEAN TGX Precast Gel 12% (Bio-Rad) according to the method of Laemmli (9), and the gel was stained with Coomassie Brilliant Blue R-250 (CBB). The molecular mass was estimated using Precision Standards (Bio-Rad). The gels were blotted onto polyvinylidene difluoride (PVDF) membranes (Sequen-Blot PVDF Membrane, Bio-Rad) using the Mini-TRANSBLOT System (Bio-Rad). The membrane was stained with CBB to capture the protein staining pattern. The membrane was decolorized with 100% methanol, washed in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) several times, and then immunostained with the polyclonal antibody ABH-1 (diluted 1:10,000 in modified TBST containing 0.002% [w/v] NaN₃) (2.5% [w/v] nonfat milk, 5% [w/v] normal goat serum, and 0.05% [w/v] NaN₃ in PBS) for 30 min at 37°C. After blocking, they were incubated at 20°C overnight with the polyclonal antibody ABH-1 diluted 1:1,000 in modified TBST containing 0.05% [v/v] Tween-20) using the ProtoBlot II AP Systems with anti-rabbit IgG [H+L] (Goat) conjugated to horseradish peroxidase (Nacalai Tesque) and diluted to 1:40,000 in TBST, washed again, developed with an ECL Plus Western Blotting Detection System (GE Healthcare), and then, the resultant membrane was exposed to Black & White Instant Film (Fujifilm, Tokyo, Japan) using an ECL Mini-camera (GE Healthcare).
methods, suggesting the possibility of aggregate formation. The mixture had significantly low migration in a native-PAGE gel and showed only a smeared band near the top of the gel (data not shown), which was consistent with aggregate formation. Hence, we decided to purify MCP by using gel extraction under denaturing conditions; MCP was separated from His-GST by SDS-PAGE and was electrically extracted from the gel. As a result, the gel extraction method provided a target protein with high purity (Fig. 1); a sufficient amount (~2 mg) of recombinant HcRNAV MCP, separated from the His-GST region, was successfully obtained by repetitive extractions. This protein was then used as an immunogen for the production of antiserum.

Self-assembly of recombinant MCP

In further experiments, reconstitution of the icosahedral architecture by using purified recombinant MCP was attempted to evaluate the quality of the recombinant MCP. Under the present experimental conditions, the main hindrance to inhibition of reconstitution was removal of the SDS bound to MCP, because the recombinant MCP was purified by extraction from SDS-PAGE gel. Some amphipathic solvents have been reported to be useful to remove tightly bound SDS from SDS-denatured proteins (8). Hence, we tried combining the buffer used in the dilution-refolding method (FoldIt Screen kit) with amphipathic solvents. When 2-propanol was used as the amphipathic solvent in the refolding reagent, the chromatogram from the subsequent size-exclusion chromatography column showed a peak corresponding to macromolecular formation (i.e., formation of virus-like particles [VLPs]), although the several peaks in the small molecular size region were also detected (Fig. S3). Addition of other amphipathic solvents was not successful. The morphology of the particles in the peak fraction was examined by electron microscopy; spherical VLPs were clearly observed (Fig. 2). The VLPs and native HcRNAV particles were almost indistinguishable in size and shape: VLPs were icosahedral and ca. 30 nm in diameter, similar to native HcRNAV (Fig. 2). The formation of VLPs strongly suggested that the MCP protein expressed in this study, which was designed by the universal codon usage, has a similar nature to authentic MCP protein; however, the reconstruction efficiency of the VLPs was so low (<1%; efficiency was estimated based on the total protein amount) that further characterization is still needed to elucidate the functional features or VLPs.

Reactivity of anti-MCP polyclonal antibody

Initially, we validated the reactivity of the anti-MCP polyclonal antibody (ABH-1) by ELISA. ABH-1 effectively recognized recombinant MCP, while serum from an unimmunized rabbit was scarcely reactive (Fig. 3A). The anti-MCP antibody produced in this study had a high titer against recombinant MCP; its titer (50% binding titer) was estimated to be 1:50,000. By dot blot analysis using intact HcRNAV particles as the target antigen, ABH-1 was shown to successfully recognize intact HcRNAV under non-denaturing conditions (Fig. 3B). Based on the protein concentration of the original antigen samples and the dilution rate at which positive reactivity was detected, the detection limit of ABH-1 for recombinant MCP and intact HcRNAV particles was estimated at ~13 pg and ~66 pg, respectively. Considering that ABH-1 was obtained using completely denatured MCP as an immunogen, it may be reasonable that ABH-1 is less reactive with HcRNAV retaining native MCP.
Immunoanalyses of Dinoflagellate Virus

Specificity of ABH-1

To check the specificity of ABH-1, Western blotting analysis was performed. A clear band of ~35-kDa molecular mass (Fig. 4, lane 2, arrowhead) was observed in the protein sample of virus-infected H. circularisquama cells, but it was not observed in uninfected cells (Fig. 4, lane 1). The molecular mass coincided with that of the single major protein of purified HcRNAV (Fig. 3, lane 3). By Western blotting, ABH-1 clearly recognized the ~35-kDa protein of virus-infected H. circularisquama cells (Fig. 4, lane 5 and 6). In addition, ~70- and ~100-kDa bands were also weakly recognized (Fig. 4, lane 5 and 6); these may correspond to a dimer and trimer of intact HcRNAV MCP as no recognition signal by ABH-1 was detected in uninfected H. circularisquama cells (Fig. 4, lane 4). Based on these results, ABH-1 was considered suitable for use in further experiments on HcRNAV because of its high specificity and high reactivity.

Detection of virus-infected cells by immunofluorescence microscopy

To confirm the ability of ABH-1 to detect HcRNAV-infected cells, immunofluorescence microscopic analysis was conducted. In these experiments, H. circularisquama cells treated with anti-alpha-tubulin antibody (Clone DM1A) served as the positive control, which specifically recognizes alpha-tubulin found in a wide range of organisms (3). Microtubule filaments (skeletal microtubules) (25) and transversal flagella prepared from microtubules were positively probed with the FITC-conjugated secondary antibody. Hence, this immuno-cytological method was shown to be appropriate to estimate the reactivity of ABH-1 to H. circularisquama cells. As a result, in the virus-inoculated H. circularisquama culture, a small portion of cells was probed with ABH-1, which showed green fluorescence of the FITC-labeled antibody using an indirect immunofluorescence technique (Fig. 5A, B). Fluorescence was dispersed throughout the entire cytoplasm, and positively probed cells were spherical. In contrast, we did not detect any cells showing green fluorescence in the uninfected culture (Fig. 5C, D), and the cells were typically spindle-shaped, as reported previously (6). The reason why only a small portion (ca. 2%) of cells was stained is unknown. One possible explanation is that HcRNAV propagation occurred only in some of the cells even in a clonal population despite the possibly of high multiplicity of infection, as was observed in a previous study on the virus resistance of H. circularisquama (34).

Another question is whether ABH-1 is able to recognize intracellular HcRNAV MCP. Based on the epifluorescence photograph shown in Fig. 5A, almost all the cytoplasmic area appears positively immunostained, but with some non-uniformity. On the other hand, virus particles are usually observed as forming patches in a cell (e.g. Tomaru et al. 2004). Considering these two possibilities, it is conceivable that the intracellular region with mature virion assembly was densely stained and that with soluble-form MCP molecules was weakly stained (Fig. 5A). To verify this hypothesis and reach a rational interpretation, establishment of an immunoelectron microscopy system for HcRNAV within its host cell is now underway, which should exhibit the distribution of MCP in the host cell.
Codon usage in *H. circularisquama* genes

In this study, codon usage for *H. circularisquama* is an important issue. The MCP gene was designed by following the deduced amino acid sequence of HeRNAV34 MCP under the assumption that translation is performed following the universal codon usage pattern of dinoflagellates; however, only a limited amount of data concerning the codon-usage pattern of dinoflagellates has been accumulated to date. According to some EST studies of several dinoflagellates (2, 7, 21, 22), their codon usage is predicted to be universal. In addition, in the case of HeRNAV study, the amino acid sequence of the N-terminal region, independently determined by the Edman degradation method (16) and the universal deduction method coincided well with each other. Furthermore, VLPs were successfully formed by the self-assembly of recombinant MCP in this study, suggesting it had the proper 3D/4D configuration. Based on these results, we conclude that the VLPs obtained in this study were structurally equivalent to native HeRNAV, but lacked genomic ssRNA, and also the codon usage for *H. circularisquama* is probably “universal”.

Conclusion and future perspectives

In this study, we developed a polyclonal antibody against MCP. The obtained antiserum was strongly reactive not only with bacterially expressed MCP, but also with native HeRNAV MCP. In addition, immunofluorescence analysis revealed that HeRNAV in the host cells could be detected using the antiserum. Our results demonstrate the feasibility and usefulness of the polyclonal antibody ABH1-1; it would be useful to estimate the progression rate of infection in order to elucidate the HeRNAV infection process, establish an immunochemochemical enumeration method for HeRNAV, enumerate infection prevalence, and to identify the receptor protein to which HeRNAV adsorbs at the initial stage of infection.

Acknowledgements

We are grateful to Dr. I. Imai (Hokkaido University, Japan) for providing the *H. circularisquama* HU9433-P. This work was supported by Grants-in-Aid for Scientific Research 20247002 (KW, EF, and KN) and 23657076 (KW) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of the Japanese Government.

References

1. Attou, H., F. Mohd Jaafar, M. Belhouchet, P. de Micco, X. de Lamballerie, and C.P. Brussaard. 2006. *Micromonas pusilla* reovirus: a new member of the family Reoviridae assigned to a novel proposed genus (Mimoreovirus). J. Gen. Virol. 87:1375–1383.
2. Bachvarov, T.R., G.T. Conception, C.R. Rogers, E.M. Herman, and C.F. Delwiche. 2004. Dinoflagellate expressed sequence tag data indicate massive transfer of chloroplast genes to the nuclear genome. Protist 155:65–78.
3. Biggrove, S.R., and D.L. Kropf. 2004. Cytokinesis in brown algae: studies of asymmetric division in fucoid zygotes. Protoplasma 223:163–173.
4. Brussaard, C.P., A.A. Noordeooos, R.A. Sandaa, M. Heldal, and G. Brathak. 2004. Discovery of a dsRNA virus infecting the marine photosynthetic protist *Micromonas pusilla*. Virology 319:280–291.
5. Cherrier, M.V., V.A. Kostychenko, C. Xiao, et al. 2009. An icosahedral algal virus has a complex unique vertex decorated by a spike. Proc. Natl. Acad. Sci. U.S.A. 106:11085–11089.
6. Horiguchi, T. 1995. *Heterocapsa circularisquama* sp. nov. (Peridiniales, Dinofyceae): A new marine dinoflagellate causing mass mortality of bivalves in Japan. Phycol. Res. 43:129–136.
7. McEwan, M., R. Humayun, C.H. Slamon, C. Raimondo, and P.J. Keeling. 2008. Nuclear genome sequence survey of the Dinoflagellate *Heterocapsa triquetra*. J. Eukaryot. Microbiol. 55:530–535.
8. Michaux, C., N.C. Pomroy, and G.G. Privé. 2008. Refolding SDS-denatured proteins by the addition of amphiphatic cosolvents. J. Mol. Biol. 375:1477–1488.
9. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
10. Lang, A. S., A.I. Culley, and C.A. Suttle. 2004. Genome sequence and characterization of a virus (HaRNAV) related to picorna-like viruses that infects the marine toxic bloom-forming alga *Heterosigma akashiwo*. Virology 320:206–217.
11. Maier, I., D.G. Müller, and C. Katsaros. 2002. Entry of the DNA virus, *Ectococcus fasciculatus* virus type 1 (Phycodnaviridae), into host cell cytosol and nucleus. Phycol. Res. 50:227–231.
12. Meints, R.H., D.E. Barbun, J.L. Van Etten, and D.T. Lamport. 1988. Properties of the *Chlorella* receptor for the virus PBCV-1. Virology 164:15–21.
13. Miller, J.L., J. Woodward, S. Chen, et al. 2011. Three-dimensional reconstruction of *Heterocapsa circularisquama* RNA virus by electron cryo-microscopy. J. Gen. Virol. 92:1960–1970.
14. Motomura, T., and T. Hishinuma. 1997. Methods for immunofluorescence observation on the algal cytokinetic. Jpn. J. Phycol. 45:175–181.
15. Nagasaki, K. 2008. Dinoflagellates, diatoms, and their viruses. J. Microbiol. 46:235–243.
16. Nagasaki, K., Y. Shirai, Y. Takao, H. Mizumoto, K. Nishida, and Y. Tomaru. 2005. Comparison of genome sequences of single-stranded RNA viruses infecting the bivalve-killing dinoflagellate *Heterocapsa circularisquama*. Appl. Environ. Microbiol. 71:8888–8894.
17. Nagasaki, K., Y. Tomaru, N. Katanozaka, Y. Shirai, K. Nishida, S. Itakura, and M. Yamaguchi. 2004. Isolation and characterization of a novel single-stranded RNA virus infecting the bloom-forming diatom *Rhizosolenia setigera*. Appl. Environ. Microbiol. 70:704–711.
18. Nagasaki, K., Y. Tomaru, K. Nakanishi, N. Hata, N. Katanozaka, and M. Yamaguchi. 2004. Dynamics of *Heterocapsa circularisquama* (Dinofyceae) and its viruses in Ago Bay, Japan. Aquat. Microb. Ecol. 34:219–216.
19. Onimatsu, H., K. Suganuma, S. Uenoayama, and T. Yamada. 2006. C-terminal repetitive motifs in Vp130 present at the unique vertex of the Chlorovirus capsid are essential for binding to the host *Chlorella* cell wall. Virology 353:343–442.
20. Ortez, M.L., M. Calero, C. Fernandez Patron, C.F. Patron, L. Castellanos, and E. Mendez. 1992. Imidazole-SDS-Zn reverse staining of proteins in gels containing or not SDS and microsequence of individual unmodified electrobotted proteins. FEBS Lett. 296:300–304.
21. Patron, N.J., R.F. Waller, and P.J. Keeling. 2006. A tertiary plastid uses genes from two endosymbionts. J. Mol. Biol. 357:1373–1382.
22. Patron, N.J., R.F. Waller, J.M. Archibald, and P.J. Keeling. 2005. Complex protein targeting to dinoflagellate plastids. J. Mol. Biol. 348:1015–1024.
23. Raab, D., M. Graf, F. Notka, T. Schidöl, and R. Wagner. 2008. The GeneOptimizer Algorithm: using a sliding window approach to cope with the vast sequence space in multiparameter DNA sequence optimization. Syst. Synth. Biol. 4:215–225.
24. Reisser, W., and E. Kapaun. 1991. Entry of a *Chlorella*-virus into its host-cell. J. Phycol. 27:609–613.
25. Roberts, K.R., J.E. Lemonte, R.M. Schneider, and M.A. Farmer. 1988. The microtubular cytokinetic of three dinoflagellates: an immunofluorescence study. Protoplasma 144:68–71.
26. Shirai, Y., Y. Takao, H. Mizumoto, Y. Tomaru, D. Honda, and K. Nagasaki. 2006. Genomic and phylogenetic analysis of a single-stranded RNA virus infecting *Rhizosolenia setigera* (Stramenopiles: Bacillariophyceae). J. Mar. Biol. Ass. U.K. 86:475–483.
27. Shirai, Y., Y. Tomaru, Y. Takao, H. Suzuki, T. Nagumo, and K. Nagasaki. 2008. Isolation and characterization of a single-stranded RNA virus infecting the marine planktonic diatom *Chaetoceros tenuissimus* Meunier. Appl. Environ. Microbiol. 74:4022–4027.
28. Tai, V., J.E. Lawrence, A.S. Lang, A.M. Chan, A.I. Culley, and C.A. Suttle. 2003. Characterization of HaRNAV, a single-stranded RNA virus causing lysis of Heterosigma akashiwo (Raphidophyceae). J. Phycol. 39:343–352.

29. Takao, Y., K. Mise, K. Nagasaki, T. Okuno, and D. Honda. 2006. Complete nucleotide sequence and genome organization of a single-stranded RNA virus infecting the marine fungoid protist Schizochytrium sp. J. Gen. Virol. 87:723–733.

30. Takao, Y., K. Nagasaki, K. Mise, T. Okuno, and D. Honda. 2005. Isolation and characterization of a novel single-stranded RNA virus infectious to a marine fungoid protist, Schizochytrium sp. (Thraustochytriaceae, Labyrinthulea). Appl. Environ. Microbiol. 71:4516–4522.

31. Tomaru, Y., N. Katanozaka, K. Nishida, Y. Shirai, K. Tarutani, M. Yamaguchi, and K. Nagasaki. 2004. Isolation and characterization of two distinct types of HeRNAV, a single-stranded RNA virus infecting the bivalve-killing microalga Heterocapsa circularisquama. Aquat. Microb. Ecol. 34:207–218.

32. Tomaru, Y., Y. Takao, H. Suzuki, T. Nagumo, and K. Nagasaki. 2009. Isolation and characterization of a single-stranded RNA virus infecting the bloom-forming diatom Chaetoceros socialis. Appl. Environ. Microbiol. 75:2375–2381.

33. Tomaru, Y., K. Toyoda, K. Kimura, N. Hata, M. Yoshida, and K. Nagasaki. 2012. First evidence for the existence of pennate diatom viruses. ISME J. doi:10.1038/ismej.2011.207.

34. Tomaru, Y., H. Mizumoto, and K. Nagasaki. 2009. Virus resistance of the bloom-forming dinoflagellate Heterocapsa circularisquama to its single-stranded RNA virus infection. Environ. Microbiol. 11:2915–2923.