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cDNA Cloning and Expression of Human Rotavirus Outer Capsid Protein VP7 in Insect Cells

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Abstract Rotavirus is a major cause of severe gastroenteritis in young children and animals throughout the world. The VP7 of rotavirus is thought to induce the synthesis of neutralizing antibodies and to be responsible for determining viral serotypes. The cDNA coding for the VP7 capsid protein of human rotavirus, obtained from Korean patients (HRV-Y14), was cloned and its nucleotide sequence was determined. Comparative analysis of the nucleotide sequences between VP7 of Y14 and that of other foreign isolates showed 92.7%~95.2% homology to G1 serotypes (RV-4, KU, K8, WA), 74.2% homolgy to G2 serotype HU-5, 76.4% homology to G3 serotype SA-11, and 77.6% homology to G4 serotype A01321. These data suggest that HRV-Y14 can be classified as a G1 serotype. cDNA coding for VP7 of HRV-Y14 was subcloned into the baculovirus vector and the VP7 glycoprotein was expressed in insect cells. The expressed proteins in Sf9 cell extract and tissue culture fluid were separated on SDS-PAGE, and Western blot analysis with monoclonal antibody raised against the synthetic peptide containing 21 amino acids within the VP7 conserved region was performed. The molecular weight of recombinant VP7 was estimated to be 36 kDa which is about the same size as the native VP7. Addition of tunicamycin in the culture media caused a reduction of the molecular weight of the recombinant VP7 indicating that the expressed protein was glycosylated.

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Rotaviruses are a major cause of acute gastroenteritis in young children and animals throughout the world [13]. It is estimated that, in the U.S.A., over 1 million infants and young children under 5 years old experience a severe rotavirus diarrheal episode, which results in over 20,000 hospitalizations and 150 deaths annually [10]. However, in developing countries, rotaviruses cause an estimated 873,000 deaths annually, which is equivalent to 25% of all diarrheal deaths and 6% of all deaths in infants and young children under 5 years old [6]. In Seoul, Korea, a study using a polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) revealed that rotavirus was the most common enteropathogen identified in the stools of children with diarrhea, accounting for 47% of cases [15]. Studies for electropherotyping and serotyping of the human rotavirus circulating in the Seoul area [16] indicated that G1 serotype was the most frequent cause.

The genome of rotaviruses consists of 11 segments of double-stranded RNA [5]. The dsRNA is enclosed in the viral capsid, which consists of three layers of proteins. The rotavirus outer layer is composed of the glycoprotein VP7 and dimeric spikes of VP4. Both outer capsid proteins induce neutralizing antibodies and are important in serotype determination [4]. VP7, encoded by genome segment 7, 8, or 9 depending on the viral strain, is the second most abundant capsid protein, and induces neutralizing antibodies which specify the G serotype [8].

The VP7 gene contains an open reading frame of 326 amino acids carrying two potential glycosylation sites at Asn-69 and Asn-238. After cleavage of the signal peptide, the mature protein contains 276 amino acids with a molecular weight of approximately 37 kDa. Although part of the cDNA coding for VP7 of human rotavirus obtained from Korean patients [18] was reported, the complete nucleotide sequence of this cDNA has not been determined yet.

Several strategies for vaccine development have been explored with various degrees of success, including using live attenuated strains of rotavirus and subunit vaccines composed of either synthetic peptides or recombinant proteins. Recent trials in the United States, using the two most developed rotavirus vaccine candidates, involve the tetravalent rhesus-bovine reassortant vaccine (RRV-TV) and the quadrivalent human-bovine reassortant vaccines. The tetravalent vaccine consists of a mixture of four viruses that together include the four G serotypes of the most commonly found rotavirus in the United States [14]. The quadrivalent vaccine is a mixture of four human-bovine reassortants that include three of the major G types and one human P serotype [2]. Both vaccine give 50%~60% protection against all rotavirus diseases.

An alternate approach would be the development of subunit or recombinant vaccines by expressing one or more rotavirus proteins which retain the neutralizing epitopes necessary for effective recognition by the host cell. Both VP4 and VP7, the two protein components of the outer capsid, react with neutralizing antibodies and monoclonal antibodies which are directed at either of these proteins and are capable of neutralizing the rotavirus.

In this study, the cDNA coding for VP7 (G1 serotype) of the human rotavirus obtained from Korean patients was cloned and its nucleotide sequence was determined. The VP7 glycoprotein expressed in insect cells using the baculovirus expression system was separated on SDS-PAGE and Western-blot analysis was performed.

MATERIALS AND METHODS

Stool Specimens

Stool specimens were kindly provided by Yonsei University Hospital. The Slidex Rota-Kit2 or Rotadex positive stool specimens were collected from infant patients who were admitted for acute gastroenteritis during 1993-1994. The specimen showing a strong rotavirus RNA migration pattern was selected and used for cDNA synthesis.

Viral RNA Extraction

Rotavirus genomic RNA was extracted from stool specimens as described previoulsy [7]. Briefly, fecal specimen diluted with 10 ml of phosphate buffered saline was centrifuged at 3,000 rpm (IEC clinical centrifuge) for 5 min. The supernatant fluid was vigorously vortexed and centrifuged at 12,000×g for 2 min and the supernatant was adjusted to the final concentration of 1% sodium dodecyl sulfate and incubated for 30 min at 37°C. The nucleic acids in suspension were extracted twice with phenol-chloroform (1:1) followed by treatment with chloroform to remove residual phenol. The aqueous phase containing RNA was added with one tenth volume of 3 M sodium acetate (pH 5.2) and the RNA was precipitated by adding 2 volumes of ethanol at -20°C overnight. The precipitated RNA was vacuum dried and resuspended in deionized water (DIW).

cDNA Synthesis

The template RNA and 20 pmol of antisense primer were heated in the presence of 5% DMSO at 95°C for 5 min and cooled rapidly on ice. After adjustment of the mixture to a volume of 20 μl containing reverse transcriptase buffer, 4 units of RNasin (Promega, Madison, U.S.A.), 1.25 mM each of deoxynucleotide triphosphate (dNTPs), and 20 units of avian myeloblastosis virus reverse transcriptase (Promega, Madison, U.S.A.). It was incubated at 42°C for 1 h. The cDNA was extracted with phenol-chloroform (1:1) and one-tenth volume of 3 M sodium acetate (pH 7.0) was added. The cDNA was precipitated by adding 2 volumes of ethanol, vacuum dried, and resuspended in 20 μl of DIW.

Polymerase Chain Reaction (PCR)

PCR was performed as described in Taniguchi *et al.* [23] with slight modification. The single stranded cDNA was added to the reaction mixture consisting of PCR buffer, 0.2 mM dNTPs, 20 pmol of each sense and antisense primers, and 1 unit of *Taq* DNA polymerase and the final volume was adjusted to 100 μl with DIW. The first cycle was initiated by heating at 94°C for 5 min, followed by 42°C for 3 min and 72°C for 3 min. The next 29 cycles were initiated by denaturation at 94°C for 1 min followed by primer annealing for 2 min at 42°C and extension for 7 min at 72°C. 10 μl portions of each PCR product was separated on 0.8% agarose gel and the DNA bands were visualized by a UV transilluminator.

Table 1. Sequence and location of primers used for PCR.

Primers	Sequences (5'-3')	Size (nt)	Positions[a]
Sense strand	GGCTTTAAAAGAGAGAATTTCC	22	1-22
Antisense strand	GGTCACATCGGACAATTCT	19	1044-1062

[a]Numbers indicate the positions of 5' and 3' end of each primer.

Sequencing and Analysis of Nucleotide Sequences

Nucleotide sequences were determined by the dideoxy chain termination method [22]. Sequenase® Version 2.0 DNA sequencing kit (USB, Ohio, U.S.A.) was used according to the manufacturer's instructions. The DNASIS™, PROSIS™ (Pharmacia, Uppsala, Sweden) and PC/Gene (IntelliGenetics Inc., Oxford, England) programs were used to analyze nucleotide sequences and deduced amino acid sequences.

Viruses and Cell

Autographa californica nuclear polyhedrosis virus (AcNPV) and recombinant baculovirus containing the VP7 gene were propagated in *Spodoptera frugiperda* (Sf9) cells. Cells were cultured at 27°C in Grace's medium (Gibco BRL, Grand Island, U.S.A.) supplemented with 10% fetal bovine serum and antibiotics (Gibco BRL, Grand Island, U.S.A.).

Construction of Recombinant AcNPV

The VP7 cDNA cloned within pMos*Blue* T-vector (Amersham, Buckinghamshire, England) was digested with *Sma*I and *Pst*I. The gel slice of 1,100 bp *Pst*I/*Sma*I fragment was purified by Gene Clean kit II (Bio101, Inc., Vista, U.S.A.). Baculovirus transfer vector pBlueBacIII (Invitrogen, San Diego, U.S.A.) was digested with *Hin*dIII in multiple cloning sites, filled with Klenow (TaKaRa, Shiga, Japan) at 37°C for 30 min in Klenow buffer (10 mM Tris-HCl, pH 7.5, 7 mM $MgCl_2$, 0.1 mM DTT, 0.25 mM each dNTP), digested with *Pst*I in multiple cloning sites and ligated to the purified insert DNA at 30°C for 3 h. After transformation, the recombinant plasmid was screened by restriction fragment mapping and polymerase chain reaction (PCR). The transfer vector containing the VP7 gene was designated pBBIIIVP7. Sf9 cells were co-transfected with 1 μg of AcNPV genomic DNA and 2 μg transfer vector (pBBIIIVP7) as described previously [17]. After 5 days of incubation at 27°C, the cell culture medium containing extracellular virus (ECV) was harvested and then the recombinant virus was purified by the plaque assay.

Expression of VP7 in Insect Cells

A volume of 2 ml of Sf9 cells at a density of 1×10^6 cells/ml in 6-well plates were infected with recombinant virus (rAcVP7-211) at multiplicity of infection (MOI) 10. At each time point, cells were harvested with a scraper and centrifuged at 800×g for 10 min at 4°C. Each cell pellet was washed with phosphate-buffered saline (PBS, 100 mM sodium phosphate, 0.9% NaCl, pH 7.3) and then centrifuged at 800×g for 10 min at 4°C. For tunicamycin treatment, Sf9 cells infected with rAcVP7-211 were cultured in Grace's medium containing 10 μg/ml tunicamycin.

SDS-PAGE

After the rAcVP7-211 infected Sf9 cells were harvested at a given time point, the pellet was resuspended in 50 μl PBS. The resuspended pellet was mixed in equal volume of 2× sample buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 6.8) containing 100 μg/ml PMSF. Each sample disrupted by freezing and thawing was denatured by boiling for 10 min and centrifuged at 12,000×g for 5 min. Proteins in the supernatant were separated on 10% SDS-polyacrylamide gel and visualized by staining with Coomassie blue.

Western-blot Analysis

Proteins resolved by SDS-PAGE were transferred to nitrocellulose membrane (Schleider & Schuell, Keene, U.S.A.) using a semi-dry transfer blotter (Owl, Partsmouth, U.S.A.) at 300 mA for 1 h [24]. The membrane was blocked by 6% skim milk in Tris-buffered saline/Tween-20 (TTBS, 100 mM Tris-HCl, 0.9% NaCl, 0.5% Tween-20, pH 7.5) for 2 h or overnight. The blot was treated with a monoclonal anti-VP7 antibody (1:10 dilution in the blocking buffer) for 3 h and then washed three times with TTBS. The blot was treated with goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma, St. Louis, U.S.A., 1:5,000 dilution) for 1 h and washed three times with TTBS. To initiate colour development of the membrane, bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Boehringer Mannheim, Mannheim, Germany) solution was added in an alkaline phosphatase buffer (100 mM Tris-HCl and 5 mM $MgCl_2$, pH 9.5), and the reaction was terminated by adding stop solution (20 mM EDTA in TBS) to the membrane.

Synthetic Peptide and Monoclonal Antibody

To produce an antibody which can detect the VP7, a peptide corresponding to highly conserved region of VP7 (amino acid sequence 275–295) was synthesized (Table 2) using the solid-phase method. The synthetic peptide was linked to BSA or OVA as a carrier by the glutaldehyde method (amine-amine linking) for priming the immune system of mice. The peptide-BSA conjugate (30 μg/mouse) was mixed with an equal volume of complete Freund's adjuvant by sonication, and a total volume of 0.3 ml was injected into the intraperitoneal cavity of each female BALB/c mouse (68 weeks old). The first injection was followed by three booster injections at 3–4 week intervals. The final injection was carried out without adjuvant. The fusion experiments were carried out as described previously [1]. In brief, spleen cells (released by tearing the removed spleen with fine forceps) and SP2/o-Ag-14 mouse myeloma cells were combined and added with 1 ml of 50% polyethylene glycol. After 1 min, a total of 20 ml of DME was added slowly to the tube for a period of 10 min and the tube was centrifuged

Table 2. Comparison of 21 amino acid sequences of VP7 synthetic peptide within the corresponding region of different rotavirus serotypes.

Serotype	Rotaviruses	Amino acid sequence (275–295)																				
1	Y14	P	T	T	N	P	Q	I	E	R	M	M	R	V	N	W	K	R	W	W	Q	V
1	WA							T										K				
2	DS-1				V			V	Q		I			I								
3	SA11				A			T						I				K				
4	A01321				S			T										K				
6	BRV				A			T						I				K				

for 10 min at 500×g. The cells were resuspended in 60 ml of hypoxanthine-aminopterin-thymidine (HAT) medium, and two drops of cell suspension were transferred into each well of five 96-well plates. About 2 weeks after the fusion, culture supernatants were collected and first screened by immunodot blot analysis. One microliter of the peptide-ovalbumin conjugate (200 μg/ml) was applied to each well of blotting apparatus, air-dried, and incubated for 1 h in 5% non-fat dry milk in PBS. Each well was washed briefly with PBS and treated for 1 h with the culture supernatant (50 μl/well). Then, the blotting apparatus was disassembled and the blot was washed 3 times with PBS and processed by the procedures described in Western blotting.

RESULTS

Cloning and Nucleotide Sequence Determination of VP7 Coding RNA

Human rotavirus RNA extracted from stool specimens was used as a template for RT-PCR. The cDNA amplified by PCR was cloned into the pMOS*Blue* T-vector kit (Amersham, Buckinghamshire, England) and the nucleotide sequence was determined. The nucleotide and its deduced amino acid sequence is shown in Fig. 1. The cDNA sequence was compared with the VP7-coding RNA segment of HRV strains RV-4, K8, KU, Wa, HU-5, SA-11, and A01321. 95.2%, 93.8%, 93.4%, and 92.7% sequence homology were observed with segment 9 of G1 specific strains RV-4, K8, KU, and Wa. 74.2%, 76.4%, and 77.6% sequence homology were observed with segment 9 of G2-specific HU-5, G3-specific SA-11, and G4-specific A01321, respectively. These data indicated that HRV-Y14 most probably belongs to the G1 serotype, which is a dominant HRV serotype circulating in the Seoul area [16].

In the amino acid sequence analyses, the VP7 of Y14 showed over 94.5% homology to that of G1 serotyes and 73% to 80% homology to that of G2, G3, and G4 serotypes. Two potential glycosylation sites located at Asn-69 and Asn-238, and a potential calcium-binding site located between helix (amino acid 140–144) and helix (amino acid 149–164), was also observed (Fig. 1).

Construction of Recombinant AcNPV Containing VP7 Gene

The baculovirus expression system has been reported to be able to produce large quantities of various foreign proteins under the control of a strong polyhedrin promoter. It undergoes eukaryotic post-translational modifications that make the expressed protein functional and has biological characteristics similar to the native protein [21]. Since glycosylation of VP7 is critical for immunogenicity and for the development of a rotavirus oral subunit vaccine, we tried to express VP7 in insect cells by using a baculovirus expression system.

To generate a recombinant virus carrying the VP7 gene, the cDNA coding for the HRV-Y14 VP7 was inserted next to the polyhedrin promoter of transfer vector, pBlueBacIII, which was digested with *Pst*I and *Hin*dIII. The sticky end of the *Hin*dIII site was filled-in to make a blunt end. pBlueBacIII has a *lacZ* gene under the control of the ETL promoter from which β-galactosidase could be expressed and makes recombinant virus-derived plaques blue. The cloned plasmid was confirmed by analyzing the size of DNA fragments (10.4 kb + 0.9 kb) generated by digesting with *Bam*HI.

The recombinant transfer vector, pBBIIIVP7, was co-transfected with wild type AcNPV DNA into Sf9 cells. Tissue culture fluid was plaque assayed and polyhedrin-negative plaques with a blue color were selected as putative recombinant virus. The presence of the polyhedrin gene resulted in the occlusion-positive phenotype which is characterized by the presence of multiple, large polyhedra in the nuclei (Fig. 2A), while replacement of the polyhedrin gene with VP7 gene resulted in the production of recombinant viruses, rAcVP7-211 and rAcVP7-B1, with occlusion-negative phenotype (Fig. 2B).

To confirm the insertion of VP7 cDNA into the recombinant virus, viral DNA extracted by PEG precipitation was analyzed by PCR with primers listed in Table 1. Figure 3 shows that the 1,100-bp long VP7 cDNA was amplified when each of rAcVP7-211 (lane 4), or rAcVP7-B1 (lane 5), or pBBIIIVP7 DNA (lane 6) was used as a template, while no DNA was amplified when Sf9 genomic DNA (lane 2) or AcNPV DNA (lane 3) was used as the template. Southern-blot analysis also indicated that the recombinant virus carries the VP7 gene.

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   1 GGCTTTAAAAGAGAGAATTTCCGTCTGGCTAACGGTTAGCTCCTTTTA

  49 ATG TAT GGT ATT GAA TAT ACC ACA ATT CTA ATC TTT CTG ATA TCA ATC ATT CTA CTC AAC TAT ATA
   1  M  Y  G  I  E  Y  T  T  I  L  I  F  L  I  S  I  I  L  L  N  Y  I

 115 TTA AAA TCA GTG ACT CAA ATA ATG GAC TAC ATT ATA TAT AGA TTT TTG TTA ATT TCT GTA GCA TTA
  23  L  K  S  V  T  Q  I  M  D  Y  I  I  Y  R  F  L  L  I  S  V  A  L

 181 TTT GCC TTG ACT AAA GCT CAG AAC TAT GGA CTT AAT ATA CCA ATA ACA GGA TCA ATG GAT ACT GTA
  45  F  A  L  T  K  A  Q  N  Y  G  L  N  I  P  I  T  G  S  M  D  T  V

 247 TAC TCC AAC TCT ACT CAA GAA GGA ATA TTT CTA ACA TCC ACA TTA TGT TTG TAT TAT CCA ACT GAA
  67  Y  S  N  S  T  Q  E  G  I  F  L  T  S  T  L  C  L  Y  Y  P  T  E

 313 GCA AGT ACT CAA ATC AGT GAT GGT GAA TGG AAA GAC TCA TTA TCG CAA ATG TTT CTT ACA AAA GGT
  89  A  S  T  Q  I  S  D  G  E  W  K  D  S  L  S  Q  M  F  L  T  K  G

 379 TGG CCA ACA GGA TCA GTC TAT TTT AAA GAG TAC TCA AAT ATT GTT GAT TTT TCC GTT GAC CCA CAA
 111  W  P  T  G  S  V  Y  F  K  E  Y  S  N  I  V  D  F  S  V  D  P  Q

 445 TTA TAT TGT GAT TAT AAC TTA GTA CTA ATG AAG TAT GAT CAA AAT CTT GAA TTA GAT ATG TCA GAA
 133  L  Y  C  D  Y  N  L  V  L  M  K  Y  D  Q  N  L  E  L  D  M  S  E

 511 TTA GCT GAT TTG ATA TTG AAT GAA TGG TTA TGT AAT CCA ATG GAT ATA ATA TTA TAT TAT TAC CAA
 155  L  A  D  L  I  L  N  E  W  L  C  N  P  M  D  I  I  L  Y  Y  Y  Q

 577 CAA TCG GGA GAA TCA AAT AAG TGG ATA TCA ATG GGA TCA TCA TGT ACT GTG AAA GTG TGT CCA CTG
 177  Q  S  G  E  S  N  K  W  I  S  M  G  S  S  C  T  V  K  V  C  P  L

 643 AAT ACA CAA ACG TTG GGA ATA GGT TGT CAA ACA ACG AAT GTA GAC TCA TTT GAA ATA GTT GCT GAG
 199  N  T  Q  T  L  G  I  G  C  Q  T  T  N  V  D  S  F  E  I  V  A  E

 709 AAT GAA AAA TTA GCT ATA GTG GAT GTC GTT GAT GGG ATA AAT CAT AAA ATA AAT TTG ACA ACT ACG
 221  N  E  K  L  A  I  V  D  V  V  D  G  I  N  K  K  I  N  L  T  T  T

 775 ACA TGT ACT ATT CGA AAT TGT AAG AAG TTA GGT CCA AGA GAG AAT GTA GCT GTA ATA CAA GTT GGT
 243  T  C  T  I  R  N  C  K  K  L  G  P  R  E  N  V  A  V  I  Q  V  G

 841 GGC TCT AAT ATA TTA GAT ATA ACA GCG GAT CCA ACG ACT AAT CCA CAA ATT GAG AGA ATG ATG AGA
 265  G  S  N  I  L  D  I  T  A  D  P  T  T  N  P  Q  I  E  R  M  M  R

 907 GTG AAT TGG AAA AGA TGG TGG CAA GTA TTT TAT ACT ATA GTA GAT TAT ATT AAT CAG ATT GTA CAG
 287  V  N  W  K  R  W  W  Q  V  F  Y  T  I  V  D  Y  I  N  Q  I  V  Q

 973 GTA ATG TCC AAA AGA TCA AGA TCA TTA AAT TCT GCT GCG TTT TAT TAT AGA GTA TAG ATATATCTTAG
 309  V  M  S  K  R  S  R  S  L  N  S  A  A  F  Y  Y  R  V  *

1041 GTTAGAATTGTCCGATGTGACC
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Fig. 1. Nucleotide and deduced amino acid sequences of VP7 cDNA of HRV isolated from Korean patients (HRV-Y14).

Nucleotides are numbered from the 5' end of the cDNA and amino acid residues are numbered from the first Met (M) of the polypeptide. Two initiation codons are underlined and potential glycosylation sites located at Asn-69 and Asn-238 are indicated by bold letters. The sequence data have been submitted to the GenBank nucleotide sequence database under accession number Bankit 216009 AF083617.

One of the recombinant viruses, rAcVP7-211, was used to prepare high-titer stock (HTS) virus for future use.

Generation of Monoclonal Antibodies (mAbs) to Synthetic Peptide

Since it was difficult to obtain enough viral proteins to immunize and screen for the production of mAbs, an oligopeptide corresponding to amino acids 275–295 within VP7 was synthesized. A 21-amino acid conserved region was chosen because this region has been reported to be the most immunogenic [9]. Table 2 shows the location and the amino acid sequences of this region. To increase immunogenecity of the synthetic peptide, bovine serum albumin or ovalbunim was linked to the N-terminal or C-terminal end of the synthetic peptide. To reduce the efforts of screening the mAbs against carrier proteins, the mice were immunized with the peptide-BSA conjugate and screened with the peptide-ovalbumin conjugates.

From the fusion experiment, a total of 3 hybridomas, which secrete monoclonal antibodies reacting with the peptide-ovalbumin conjugate, was first screened by immunodot blot analysis and propagated for further study. To determine the specificity of the mAbs, total viral proteins were separated by SDS-PAGE and immunoblotted. Among the 3 mAbs, mAb 3-15 showed strong reactivities with the authentic viral protein in Western blot, whereas the rest reacted weakly with the recombinant protein.

Expression of VP7 in Insect Cells

In order to determine the conditions for maximal expression, HTS of rAcVP7-211, titered to 5×10^7 PFU/ml, was infected in Sf9 cells at MOI of 10. Analysis of cell extract by SDS-PAGE followed by Coomassie blue

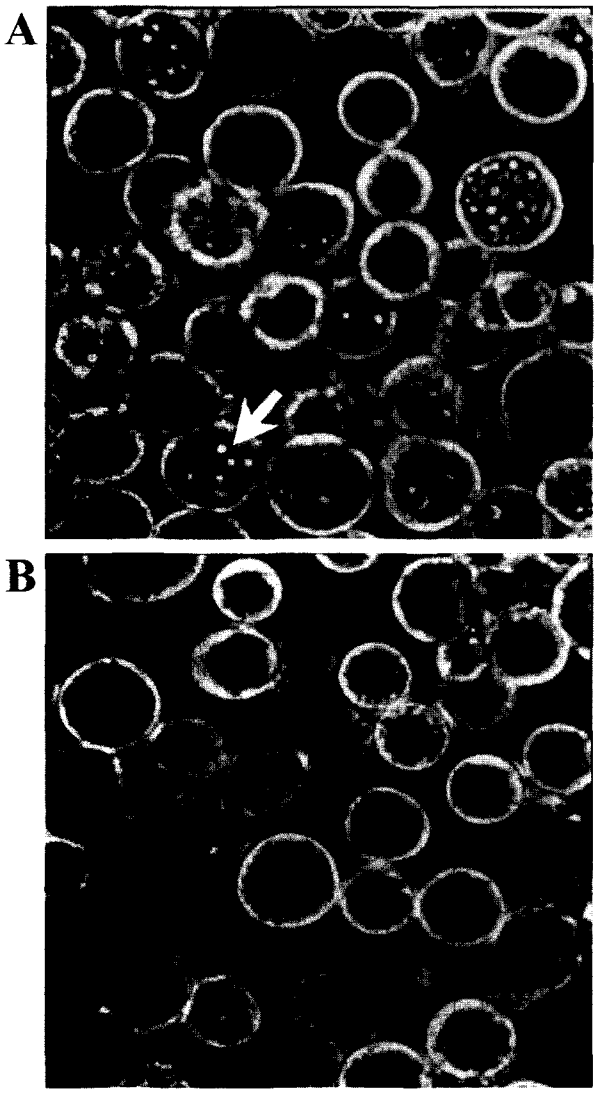

Fig. 2. Photomicrographs of Sf9 cells infected with wild type AcNPV (A) and recombinant rAcVP7-211 (B).

staining showed a new band with about 36 kDa in Sf9 cells infected with rAcVP7-211 (Fig. 4A, lane 5). This new band was not observed in the sample prepared from the mock-infected and wild type AcNPV-infected cells (Fig. 4A, lanes 2 and 3). The polyhedrin protein with 29 kDa was only seen in AcNPV-infected cells (Fig. 4A, lane 3).

To further elucidate the expression of VP7, Sf9 cell extracts and tissue culture fluids were analyzed by Western-blot with monoclonal antibody (mAb 3-15) against a synthetic peptide containing 21 amino acids within the VP7 conserved region. Figure 4B, lanes 5 to 10, show that about 36-kDa proteins were expressed in Sf9 cells infected with rAcVP7-211 immunoreacted with mAb 3-15. No recombinant VP7 was expressed in Sf9 cells or Sf9 cells infected with wild type AcNPV

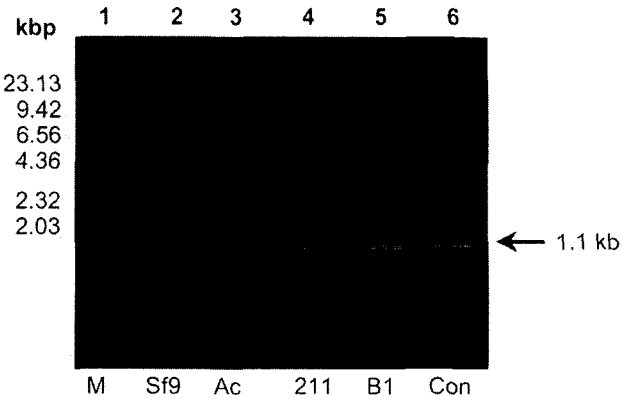

Fig. 3. Agarose gel electrophoresis of the PCR products.
The recombinant viruses were screened for the presence of VP7 gene by PCR. Arrow indicates the 1,100 bp DNA amplified by PCR. Lane 1: λ-DNA digested with *Hin*dIII; lanes 2-6: PCR product generated by using Sf9 DNA as a template (2), AcNPV DNA as a template (3), rAcVP7-211 DNA as a template (4), rAcVP7-B1 DNA as a template (5), and pBBIIIVP7 DNA as a template (6).

(Fig. 4B, lanes 2 and 3). The earliest expression of the recombinant VP7 was observed at 16 h post-infection (Fig. 4B, lane 5). Expression reached the maximum level at 72~96 h post-infection (Fig. 4B, lane 8) and stayed at maximum until 120 h post-infection (Fig. 4B, lanes 9 and 10).

Since the cell density and MOI are reported to be critical for the highest expression of human thrombopoietin [19], Sf9 cells with various densities were infected with rAcVP7-211 at various MOI to determine the maximal conditions for expression of VP7. There were no considerable differences in terms of expression levels in the range of MOI 5 to 20 and at the cell density of 1.0×10^6~3.0×10^6 cells/in 6-well plates.

Glycosylation of Recombinant VP7

The VP7 of Y14 has two potential glycosylation sites at Asn-69 and Asn-238 (Fig. 1). To investigate if the recombinant VP7 expressed in Sf9 cells was glycosylated, the cells infected with rAcVP7-211 were cultured in the presence of tunicamycin which blocks N-linked glycosylation. Cell extracts and tissue culture fluids were separated on SDS-PAGE and Western-blot analysis was performed. As shown in lanes 5, 6, and 7 of Fig. 5, the migration pattern of the recombinant VP7 expressed in Sf9 cells cultured in the presence of tunicamycin has been altered. The fast migrated recombinant VP7 most likely resulted from the reduction of molecular size. This observation implies that tunicamycin inhibits glycosylation resulting in the reduction of the molecular weight of the recombinant VP 7. It remains to be elucidated if the recombinant VP7 expressed in insect cells is glycosylated as it is in mammalian cells.

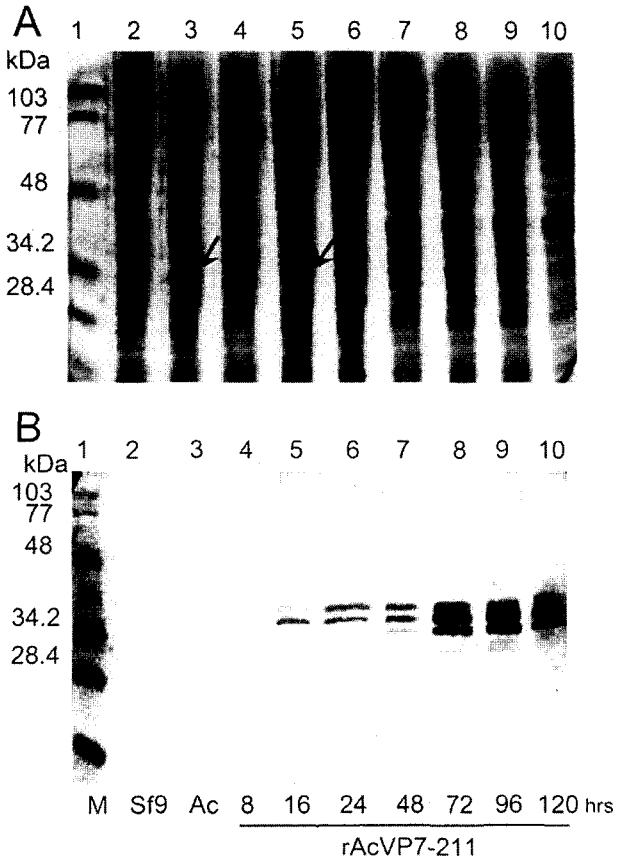

Fig. 4. Time course expression of VP7 in rAcVP7-211 infected Sf9 cells.

A density of 2×10^6 cells per 6-well plate of Sf9 cells was infected with rAcVP7-211 multiplicity of infection (MOI) 10. The cell extracts were subjected to 10% SDS-PAGE (A) and immunoblot (B) analysis was performed with anti-VP7 peptide (275-295) monoclonal antibody. Arrows in lanes 3 and 5 indicate the 32-kDa polyhedrin protein and the 36-kDa recombinant VP7, respectively. Lane 1, prestained molecular weight markers; lane 2, uninfected Sf9 cell lysates; lane 3, Sf9 cell lysates infected with AcNPV; lanes 4–10, Sf9 cell lysates infected with rAcVP7-211 and harvested at 8, 16, 24, 48, 72, 96, 120 h post-infection, respectively.

DISCUSSION

Rotaviruses are a leading cause of severe diarrhea in infants and young children. In developing countries, rotavirus infection is associated with high infant mortality and the cost of rotavirus to the health care system is at least $ 1 billion per year in the United States [3]. In Seoul, Korea, rotavirus was found in 47% of the cases in the stool of children with diarrhea, identifying the most common entero-pathogen [15]. Kim *et al.* [16] reported that the G1 serotype was the most frequently (89%) found in rotavirus-infected patients in the Seoul area. Therefore, development of an effective vaccine to prevent rotavirus infection seems to be urgent. The nucleotide sequence of cDNA coding for VP7 synthesized from Korean patients had 92.7%~95.2% homology to the G1 serotype. We have expressed the outer capsid glycoprotein VP7 using a baculovirus expression system.

The molecular weight of the recombinant VP7 expressed in insect cells was similar to that of native VP 7. It retained the ability to react with both monoclonal antibody raised against the synthetic peptide spanning amino acids from 275 to 295 of VP7 (Fig. 4B) and rotavirus positive anti-serum (data not shown). The region between amino acids 275 to 295 of VP7 is hydrophilic, and highly conserved among most of the human and animal rotavirus isolates of which DNA nucleotide sequences have been determined to-date [11, 12]. The hydrophilic nature of the region suggests that this region is most likely to be exposed on the virion's surface and is an important immunological epitope of VP7. Gunn *et al.* [9] demonstrated that anti-VP7 (275–295) peptide antisera recognized the respective native viral protein in immuno-blot assay.

The recombinant VP7 was expressed in insect cells as two different forms, as shown in Fig. 4B. The lower band with low molecular weight in the figure may represent the mature form that is generated from the native form after cleavage of the signal peptide. Tunicamycin treatment indicated that different intermediate glycosylation forms of VP7 were expressed in Sf9 cells. These results suggest that the larger one may be the fully glycosylated form and the smaller one may be the nonglycosylated form (Fig. 5, lanes 5, 6, and 7).

The VP7 (Y14 strain) has two glycosylation sites. One is a conserved region N-glycosylation site (Asn-69) and the other is a potential N-glycosylation site (Asn-238), which might be used to direct the nascent protein's translocation into the endoplasmic reticulum (ER). In many studies, glycosylation of baculovirus-produced proteins has been characterized by comparison of protein size with tunicamycin treated cells and endoglycosaminidaseH/endoF digestion [21]. Since tunicamycin treatment inhibits the first step of N-glycosylation, the molecular size of the glycoprotein expressed in the presence of tunicamycin would be reduced. The molecular size of VP7, expressed from Sf9 cells infected with recombinant virus (rAcVP7-211) and cultured in the presence of tunicamycin, was reduced (Fig. 5)

EndoH cleaves the majority of high mannose sugars but does not cleave complex N-linked sugars. VP7 expressed in Sf9 cells digested by endoH (data not shown) suggesting that VP7 was in a high mannose form rather than a complex form as reported before [21]. In most reported cases, the N-glycoproteins are initially sensitive to endoH, but become resistant with time [20].

In this study, the outer capsid glycoprotein VP7 of human rotavirus obtained from Korean patients was

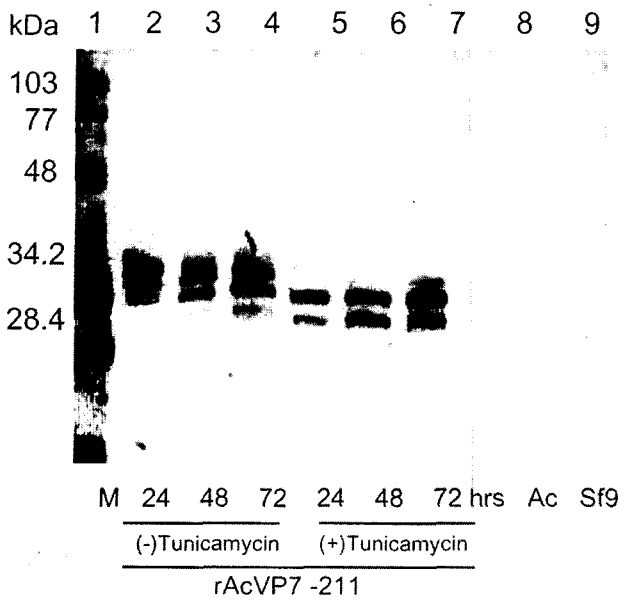

Fig. 5. Immunoblot analysis of recombinant VP7 expressed in rAcVP7-211 infected Sf9 cells cultured in the presence of tunicamycin.

Sf9 cells infected with rAcVP7-211 were cultured in Grace's Media containing 10 μg/ml tunicamycin. The cell extracts harvested at 24, 48, 72 h post-infection were analyzed on 10% SDS-PAGE and subjected to immunoblot analysis. Lane 1, prestained molecular weight markers; lanes 2, 3, and 4, Sf9 cells infected with rAcVP7-211 harvested at 24, 48, 72 h post-infection, respectively; lanes 5, 6, and 7, rAcVP7-211 infected Sf9 cells treated with 10 g/ml of tunicamycin and harvested at 24, 48, 72 h post-infection, respectively.

expressed in insect cells. The recombinant protein retained the ability to react with rota-positive serum. The present data indicate that the recombinant protein may be useful for developing a subunit vaccine and that other G and P serotypes will need to be incorporated into the vaccine to provide broad spectrum protection.

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