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# Expression of Rotavirus Capsid Proteins VP6 and VP7 in Mammalian Cells Using Semliki Forest Virus-Based Expression System

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**Abstract** Rotaviruses are the world-wide leading causative agents of severe dehydrating gastroenteritis in young children and animals. The outer capsid glycoprotein VP7 and inner capsid glycoprotein VP6 of rotaviruses are highly antigenic and immunogenic. An SFV-based expression system has recently emerged as a useful tool for heterologous protein production in mammalian cells, exhibiting a much more efficient performance compared to other gene expression systems. Accordingly, the current study adopted an SFV-based expression system to express the VP7 of a group A human rotavirus from a Korean isolate, and the VP6 of a group B bovine rotavirus from a Korean isolate, in mammalian cells. The genes of the VP6 and VP7 were inserted into the SFV expression vector pSFV-1. The RNA was transcribed *in vitro* from pSFV-VP6 and pSFV-VP7 using SP6 polymerase. Each RNA was then electroporated into BHK-21 cells along with pSFV-helper RNA containing the structural protein gene without the packaging signal. The expression of VP6 and VP7 in the cytoplasm was then detected by immunocytochemistry. The recombinant virus was harvested by ultracentrifugation and examined under electron microscopy. After infecting BHK-21 cells with the defective viruses, the expressed proteins were separated by SDS-PAGE and analyzed by a Western blot. The results indicate that an SFV-based expression system for the VP6 and VP7 of rotaviruses is an efficient tool for developing a diagnostic kit and/or preventive vaccine.

**Key words:** Rotavirus, capsid proteins VP6 and VP7, Semliki Forest virus, expression

Rotaviruses are members of the *Reoviridae* family and are the major causative agent of viral gastroenteritis in young children and animals throughout the world [12]. A rotavirus is a nonenveloped icosahedral virus that consists of three concentric layers of protein that enclose 11 segments of double-stranded RNA [7, 23]. The core of the virion contains VP1, VP2, VP3, and 11 RNA segments. The intermediate layer is formed by VP6, a hydrophobic protein that possesses the intrinsic property of forming a trimer on the virion [1] and infected cells [20, 26]. The outer layer of a rotavirus is composed of the glycoprotein VP7 and dimeric spikes of VP4, which define the virus serotypes G and P, respectively.

The development of a rotavirus vaccine has taken two major approaches. The first is to construct live apathogenic rotavirus strains [27], attenuated animal strains [25], or rhesus-human reassortant strains [28], etc., whereas the second is to develop a subunit or recombinant vaccine [6, 22] by expressing one or more rotavirus proteins that retain immunogenic epitopes for effective recognition by the host cells. The major capsid protein VP6 (43 kDa) includes more than 51% of the virion proteins and contains conserved epitopes as the potential target for eliciting protective immunity against different serotypes within the same group of rotavirus as a subgroup antigen [14, 18]. Although VP6 lacks the elicitation of a neutralizing antibody, previous studies with IgA monoclonal antibodies to VP6 suggest that IgA-mediated intracellular neutralization can occur with a rotavirus [3]. Those particles devoid of a VP6 layer have been found to be transcriptionally inactive [2]. It has also been shown that certain antibodies directed against VP6 can inhibit transcription by single- and double-layered particles [9, 13]. The VP7 of the major outer capsid protein induces neutralization, and is a protective antibody [21], and is primarily responsible for determining the viral serotype [8]. As such, these capsid proteins have been studied as a primary candidate for an effective vaccine.

In the current study, we cloned the cDNA coding for the VP7 and VP6 from a human rotavirus isolated from Korean patients and a bovine rotavirus, respectively. A Semliki Forest Virus (SFV)-based expression system [1, 10, 11, 15, 16, 17, 20] was used to express the capsid proteins of the

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rotavirus in mammalian cells, and the intrinsic properties of the expressed proteins were then confirmed. In addition, SFV particles containing the rotovirus capsid genes were produced for large-scale infection of various mammalian cells.

## MATERIALS AND METHODS

### cDNA Synthesis and Analysis of Nucleotide Sequences

The rotavirus RNA was extracted from eight human rotavirus isolates which were propagated in rhesus monkey kidney cells (MA 104 cells) using standard methods [5]. The VP7 cDNA coding was amplified by a reverse transcription-polymerase chain reaction (RT-PCR, Superscript II, Gibco BRL) using an antisense primer (H1R). For the PCR amplification, the primers H1 (5'-GAGAGAATTTCCGA-CTGGCT-3') and H1R (5'-GGTCACATCATACAGTTCTA-3') were used. The PCR-amplified VP7 gene was cloned in pTZ19R (Pharmacia) and the nucleotide sequences were analyzed by Sanger's dideoxy-mediated chain termination method [29].

### Construction of SFV Recombinants

The gene coding for the bovine rotavirus VP6 and human rotavirus VP7 were PCR-amplified. Oligonucleotide primers with *Bam*HI recognition sites were designed. For VP6, the sense primer (5'-CGCGGATCCGAATTCATGGATGTC-CTGTACTCCTTG-3') and antisense primer (5'-CGCGG-ATCCTCATTTGACAAGCATGCTTCT-3') were used. For VP7, the sense primer (5'-CGCGGA TCCATGTATGGT-ATTGAATAT-3') and antisense primer (5'-CGCGGATCCTA ATCTAAGATATATCTA-3') were used. The amplified fragments were digested with *Bam*HI and ligated into the cloning site of an SFV expression vector (pSFV). *Escherichia coli* DH5 cells were transformed with the ligated DNA mixture. Those cells harboring pSFV-1 and VP6 (pSFV-VP6) or pSFV-1 and VP7 (pSFV-VP7) were cloned.

### *In vitro* Transcription and Transfection

The plasmids, pSFV-VP6 and pSFV-VP7, and a pSFV-helper were linearized with *Spe*I, and the linear DNA (1.5 μg) was then mixed in a total volume of 50 μl solution containing 5 μl 10× SP6 buffer, 10 mM m7G(5')ppp(5')G 5 μl (BMS, Germany), 2.5 μl 100 mM DTT, 5 μl dNTP mix (BMS), 3 μl RNasin (50 units) (Takara), and 2 μl SP6 RNA polymerase (Takara). Capped RNA was synthesized *in vitro* at 37°C for 1 h. Baby hamster kidney (BHK-21) cells ($10^7$ cells/ml) and the synthesized RNAs were transferred to a 0.4 cm cuvette (BioRad, U.S.A.). The electroporation was performed at room temperature using two consecutive pulses of 850 V/25 μF. The electro-transfected cells were diluted 1:20 in a BHK medium (Gibco BRL) containing 5% fetal calf serum and then seeded in tissue culture dishes.

### Electron Microscopy and Immunocytochemistry

The recombinant SFVs (rSFV-VP7, rSFV-VP6) contained in the medium were harvested after 24 to 48 h of transfection by ultracentrifugation at 100,000 ×g for 2 h. The virus pellets were then stored in a TNE buffer (100 mM NaCl, 0.5 mM EDTA, and 20 mM Tris-Cl, pH 7.4) at −70°C until use. For electron microscopy, the virus pellets were resuspended in 100 μl of water. The suspension was then transferred to grids for negative staining with potassium phosphotungstate (pH 6.0). The virus particles on the grids were examined under an electron microscope. After 48 h of transfection, the cells were washed twice with PBS and fixed on a slide glass by treatment with ice-cold methanol for 15 min. The fixed cells were then blocked by 1% gelatin in PBS for 1 h and incubated with the primary antibody for 2 h at room temperature. After three washes with PBS, the cells were incubated with the biotinylated secondary antibody for 1 h at 37°C. After another three washings, the cells were incubated in an avidin-biotin reaction mixture (Vector Laboratories, U.S.A.) for 1 h at 37°C. The antigen-antibody reaction was detected by color development after the addition of DAB (3,3'-diamino benzidine) solution (Vector Laboratories).

### Infection of Recombinant Virus into BHK Cells and Western Blot Analysis

The virus suspension in a TNE buffer was added to a 1/4 volume of 10 mM $CaCl_2$ and 1/20 volume of a chymotrypsin stock solution, and the mixture was incubated on ice for 30 min. The mixture was then inactivated by the addition of a 0.5 volume of aprotinin. The prepared virus was then resuspended and infected into BHK cells for 1 h. The cells were incubated for 48 h and harvested. The cells were incubated in 300 μl of a cell lysis buffer (1% NP40, 150 mM NaCl, 2 mM EDTA, 1 μg/ml PMSF, 50 mM Tris-Cl pH 7.6) on ice for 30 min and centrifuged at 12,000 ×g for 10 min. The cell lysates infected with rSFV-VP7 were mixed in an equal volume of a 2× sample buffer (125 mM Tris-HCl, 4% SDS, 20% glycerol, 0.2 M DTT, 0.02% bromophenol, pH 6.8), denatured by boiling for 10 min, and centrifuged at 12,000 ×g for 5 min. The proteins in the supernatant were separated by 12% SDS-PAGE. The cell lysates infected with rSFV-VP6 were divided into two parts. One part was mixed with a 5× sample buffer under reducing conditions and boiled for 10 min. The other part was treated with the sample buffer under nonreducing conditions without SDS, mercaptoethanol, or heating. The proteins resolved by SDS-PAGE were transferred to a polyvinylidene difluoride (PVDF) membrane. The transferred membrane was then blocked by 5% skim milk in Tris-buffered saline for 1 h and then treated with the primary

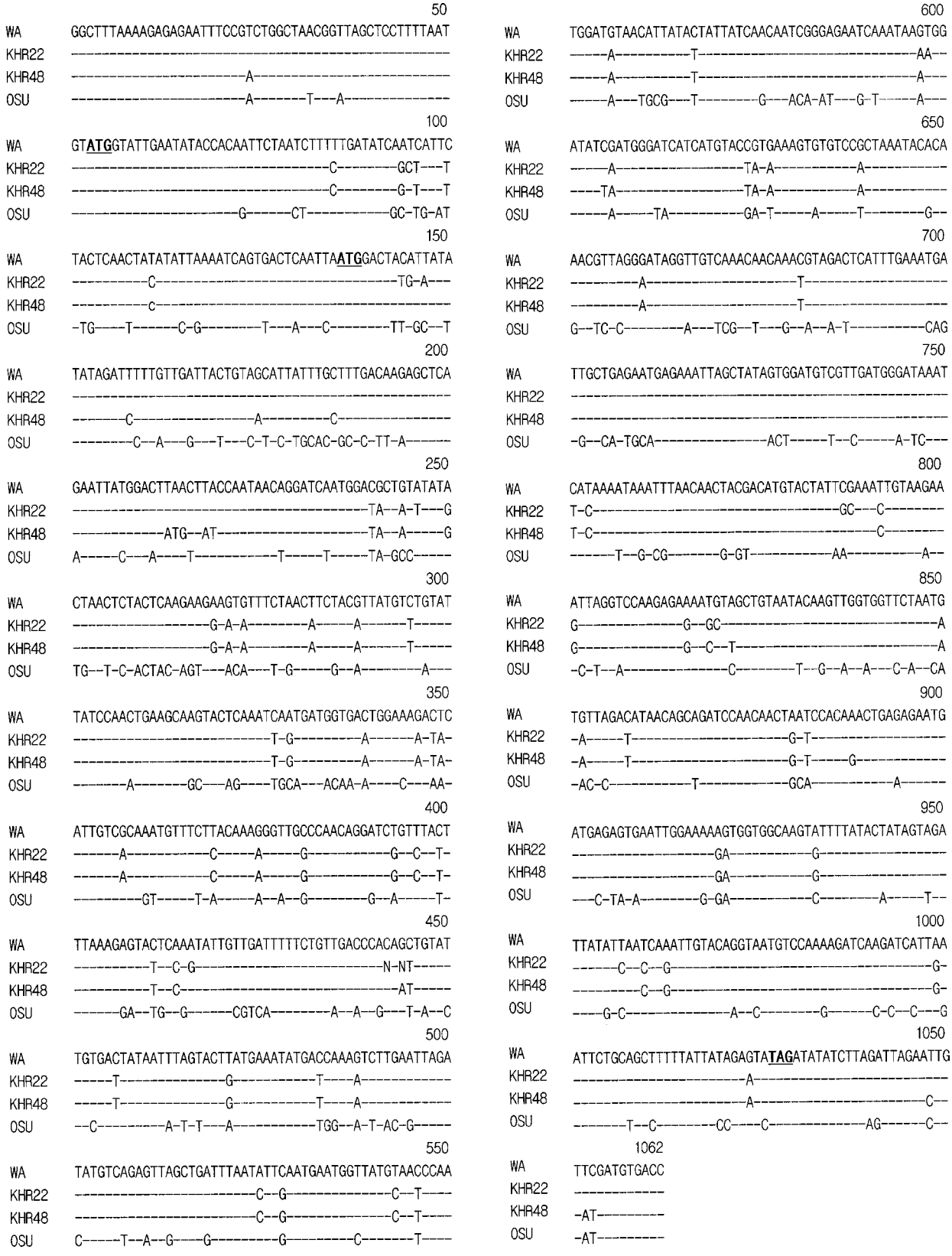


**Fig. 1.** Complete nucleotide sequence multi-alignment of VP7 genes from human rotaviruses WA, KHR22, and KHR48, and porcine OSU strain.
The initiation and termination codons are underlined.

antibody (VP7 specific mAb or polyclonal BRV antiserum) overnight. Next, the membrane was treated with the biotinylated secondary antibody for 1 h and washed. An avidin-biotin reaction was performed for 1 h at room temperature. The antigen-antibody reaction was detected by color development after the addition of a DAB solution.

## RESULTS

### Nucleotide Sequence of the VP7 Gene

Rotavirus RNAs were used for the cDNA preparation by RT-PCR. The ampified VP7 gene was cloned into pTZ19R, resulting in KHR22 and KHR48. The nucleotide and its deduced amino acid sequences are shown in Figs. 1 and 2, respectively. The cDNA of VP7 was 1,062 base pairs in length and contained an open reading frame of 326 amino acid residues. A comparison of the nucleotide and amino acid sequences of the VP7 genes from WA (representative serotype 1), KHR22, and KHR48 revealed

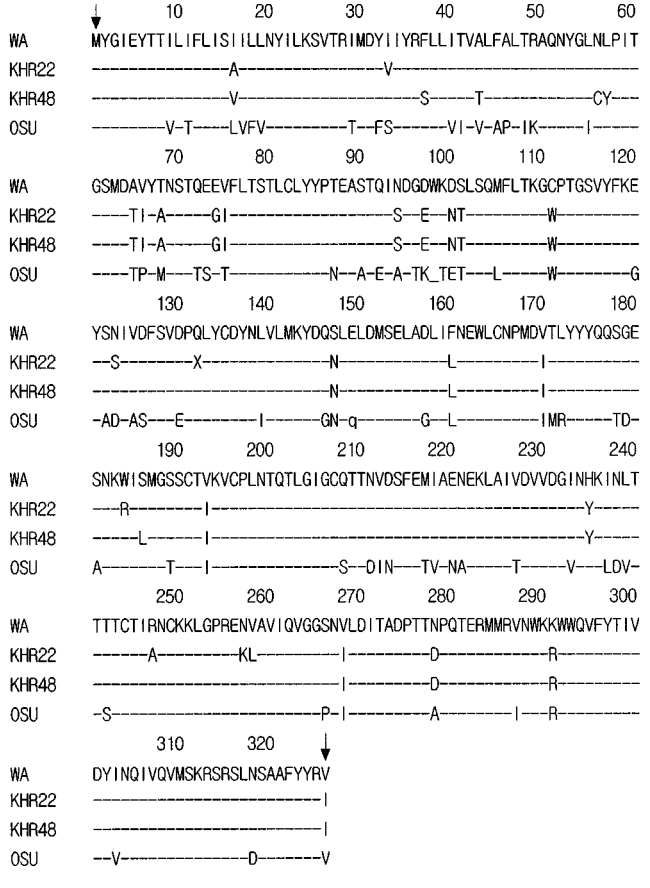


**Fig. 2.** Multi-aligned amino acid sequences of the VP7 gene from WA, KHR22, KHR48, and OSU strains.
The amino acid substitutions found in the KHR22, KHR48, and OSU sequences corresponding to the WA standard strain are indicated for comparative purposes. The inverted arrows indicate the start and stop codons.

92.3% to 92.6% homology in the nucleotide sequences and 92.0% to 92.2% homology in the amino acid sequences among the human strains. As for the Korean isolates, higher degrees of homology were observed; 97.3% homology in the nucleotide sequences and 95.9% homology in the amino acid sequences. However, only 72% homology in the nucleotide sequences and 78% homology in the amino acid sequences were found between the human and porcine rotavirus OSU strains (Figs. 1 and 2).

### Expression of Rotavirus Proteins VP6 and VP7 in the Cytoplasm of BHK-21 Cells

A question of whether the rotavirus proteins VP6 and VP7 could be produced in the cytoplasm of BHK-21 cells using an SFV-based expression system was investigated. Thus, the rotavirus VP6 and VP7 genes were amplified by PCR and cloned into an SFV vector, pSFV-1. These cloning steps resulted in the recombinant plasmids pSFV-VP6 and pSFV-VP7. Capped RNAs transcribed *in vitro* from the SP6 promoter in pSFV-VP6 and pSFV-VP7 were transfected into BHK-21 cells by electroporation using helper RNAs. The BHK cells were then fixed on a slide glass with methanol 48 h post-transfection and observed immunocytochemically, as described in Materials and Methods. The results showed that the rotavirus proteins VP6 and VP7 were expressed in the cytoplasm of the BHK cells (dark brown-stained patterns in Fig. 3), but not in the cytoplasm of the mock transfected cells.

### Electron Microscopy

Post-transfection, the cell culture medium was ultracentrifuged to harvest the recombinant virus, rSFV-VP6 or rSFV-VP7, containing the rotavirus RNAs. The results revealed that the morphology of the recombinant virus, rSFV-VP7, was similar to that of a Semliki Forest virus in nature (Fig. 4).

### Conformational Properties of Expressed Protein VP6

We also investigated whether the expressed VP6 proteins would assemble into oligomeric structures, as previously reported in a natural infection [19] and baculovirus expression using insect cells [28]. Thus, the recombinant virus, rSFV-VP6, was infected into BHK cells, the infected cells were harvested, and lysed 48 h post-infection. The cell lysates were divided into two aliquots. One was left untreated, while the other was boiled for denaturation. Under non-reducing conditions, the expressed protein VP6 was detected in a trimeric form with a molecular mass of 120–140 kDa (Fig. 5A), whereas under reducing conditions it was detected in a monomeric form with a molecular mass of 43 kDa, using polyclonal guinea pig serum against BRV (Fig. 5B). As such, when expressed in a monomeric form, the VP6 then assembled into a trimer form to produce its native conformation.

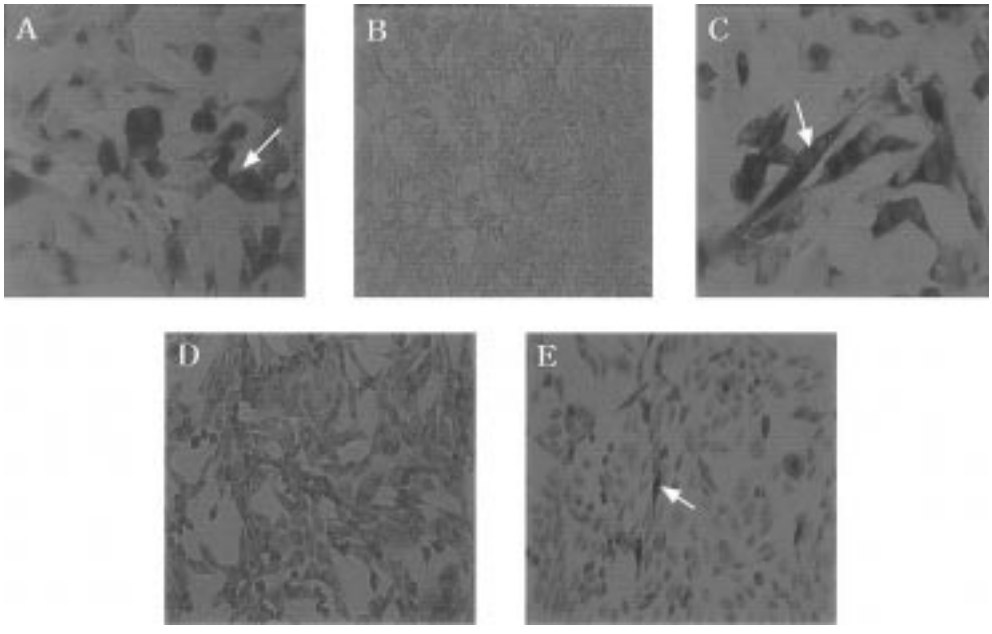


**Fig. 3.** VP6 and VP7 expression in cytoplasm of BHK cells.
A, pSFV-VP6 transfected; B, Mock transfected; C, pSFV-VP7 transfected; D, Mock transfected; and E, pSFV-VP7 transfected. Anti-Wa (A, B, and C) and VP7-specific mAb (D and E) were used for the immunocytochemistry.

### Identification of Expressed Protein VP7

To determine the expressed protein VP7, the recombinant virus rSFV-VP7 was infected into BHK cells, as described under Materials and Methods. The infected cells were harvested and lysed 24 to 48 h post-infection. The cell lysate was separated by SDS-PAGE and detected by Western immunoblotting using an anti-VP7 monoclonal antibody. Two bands were detected in the infected cell lysates with a molecular mass of 34–37 kDa, yet not in the mock-infected cell lysates (Fig. 5C). Previous biochemical studies of VP7 in infected cells and the cell-free translation of VP7 in wheat-germ lysates [4] suggest that the two bands related to VP7 may be due to distinctive processing of VP7 initiated from each of the initiation codons.

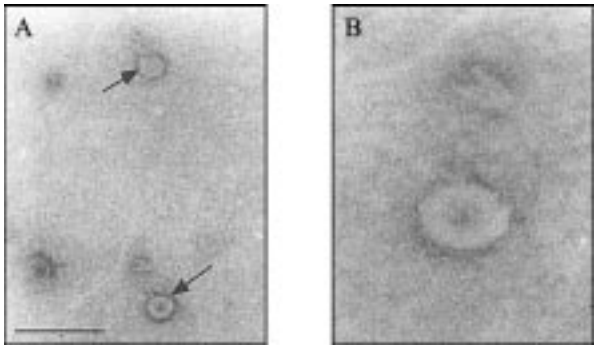


**Fig. 4.** Electron microscopy of recombinant virus (rSFV-VP7) exhibiting similar size and morphology to native SFV.
Bar indicates 200 nm in size (A: 85,000×; B: 125,000×).

## DISCUSSION

In the current study, we investigated the outer capsid glycoprotein VP7 of HRV in Korean isolates and the inner capsid glycoprotein VP6 of BRV produced in mammalian cells using an SFV-based expression system. The PCR-amplified VP6 and VP7 genes were cloned into the SFV vector, pSFV-1. The cloned VP6 gene revealed a coding frame of 1,194 nucleotides for a polypeptide of 397 amino acid residues, similar to the Indiana strain of the group C bovine rotavirus. The VP7 gene of the human rotavirus, isolated from Korean patients, was cloned and

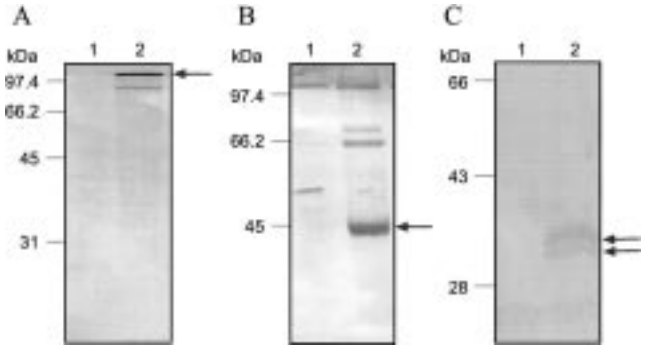


**Fig. 5.** Western immunoanalysis of VP6 expression (A and B) and VP7 expression (C) in BHK cells after infection by rSFVs.
The cell lysates infected with rSFV-VP6 were analyzed under non-reducing (A) and reducing (B) conditions. Lane 1 indicates the mock-infected cell lysates, while lane 2 of each panel indicates the rSFV-infected cell lysates.

sequenced. It exhibited a high homology of its nucleotide and amino acid sequences with the serotype 1 strain WA of group A, and was also found to contain two on-phase initiations and an ORF of 326 amino acids carrying two potential glycosylation sites at Asn-69 and Asn-238. After cleavage of the signal peptide, the mature protein contained 276 amino acids with a molecular mass of approximately 37 kDa. Capped RNAs transcribed *in vitro* from the SP6 promoter in pSFV-VP6 or pSFV-VP7 were transfected into BHK-21 cells by electroporation using helper RNA. The recombinant genome was replicated and transcribed the heterologous protein gene VP6 or VP7 instead of the viral structural proteins in the cytoplasm of the BHK cells.

Immunocytochemistry demonstrated that the rotavirus capsid protein VP6 or VP7 was efficiently expressed in the cytoplasm of BHK-21 cells. The recombinant RNAs were packaged by the cotransfected helper RNA and assembled into defective, yet infectious, SFV particles. Electron microscopy revealed that this recombinant SFV containing the RNA of rotavirus VP6 or VP7 had a similar morphology to SFV in nature. These recombinant virus particles, rSFV-VP6/rSFV-VP7, underwent one round of multiplication when infected into various host cells. This property could advantageously be used in the construction of prototype vaccines [29, 30].

The recombinant defective viruses were infected into BHK-21 cells, and the expressed capsid proteins were then detected using specific antibodies against rotaviruses. The results showed that the molecular mass of the recombinant VP7 expressed in mammalian cells was similar to that of the natural VP7. Also, the recombinant VP7 was expressed in the mammalian cells in two different forms. The VP7 included two in-phase initiations, due to distinctive processing of VP7 initiated from each of the initiation codons, as in the native infection.

The expressed VP6 protein was detected in a monomer form with a molecular mass of 43 kDa under reducing conditions and in a trimer form with a molecular mass of 120–140 kDa under nonreducing conditions. As such, these results demonstrated that the capsid proteins VP6 and VP7 expressed by the SFV expression system retained the intrinsic properties of the native conformation, together with the ability to react with an antiserum against rotaviruses.

SFV was recently developed as a vaccine vector and has been used for protein expression and the production of viral capsids. Due to many advantages of an SFV replicon, this system seems to be a good candidate for the expression of the primary immunogen of a rotavirus in its native conformation.

In conclusion, the current study demonstrated that an SFV-based expression system for the VP6 and VP7 of rotaviruses is an efficient tool to produce useful subunit components for candidate vaccines.

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