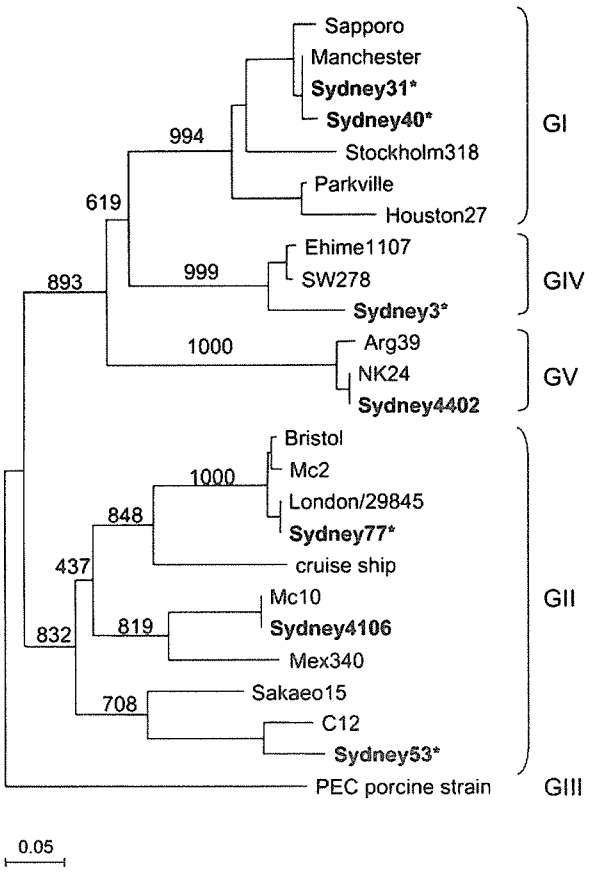

Figure. Phylogenic tree of Sapovirus (SaV) sequences isolated in this study (represented in **boldface**). SaV nucleotide sequences were constructed with the partial N-terminal capsid region, using SaV PEC strain (a porcine SaV) as an outgroup. The numbers on the branches indicate the bootstrap values for the clusters. Bootstrap values >950 were considered statistically significant for the grouping (8). Asterisks indicate specimens collected from outpatients. The distance scale in nucleotide substitutions per position is shown. Sydney isolates have been deposited in GenBank (accession nos. DQ104357–DQ104363). GenBank accession numbers for the reference strains are as follows: Arg39, AY289803; Bristol/98, AJ249939; C12, AY603425; cruise ship/2000, AY289804; Ehime1107, DQ058829; Houston/27/90, U95644; London/29845/90, U95645; Manchester, X86560; Mc2, AY237419; Mc10, AY237420; Mex340/1990, AF435812; NK24, AY646856; Parkville, U73124; PEC, AF182760; Sapporo/82, U65427; Stockholm/318/97, AF194182; Sakaeo15, AY646855; and SW278, DQ125333.

identity with C12. These findings suggest that Sydney4106 and Sydney53 were also recombinant strains and indicate the widespread distribution and genetic stability of recombinant SaV strains. One sequence (strain Sydney3) belonged to genogroup GIV and had ≈99% nt identity with the SW278 sequence, which recently caused an outbreak of gastroenteritis in adults in Sweden in March 2004 (*1*). Another sequence (strain Sydney4402) belonged to genogroup GV and had 100% nt identity with the NK24 sequence, which was isolated from an infant with gastroenteritis in Thailand in December 2002 (*10*). White blood cells were detected in the stool specimens of 3 children infected with SaV genogroups GII, GIV, and GV (strains Sydney4106, Sydney3, and Sydney4402, respectively). In our previous study (*10*), an infant infected with NK24 (SaV genogroup GV) had a fever for 11 days and vomiting for 3 days, which was notably longer than the duration of symptoms in other infants infected with SaV GI and GII strains (unpub. data). These results suggest that some SaV genogroups could be more virulent than others, although additional studies are needed.

Conclusions

Little is known about SaV infections in Australia (*11–14*). Data from these reports indicate that SaV is an uncommon cause of acute gastroenteritis in Australia. When the proportion of SaV present in the total calicivirus isolations was used, SaV was estimated to be the etiologic agent of gastroenteritis in 0.56% (*11*), 0.32% (*12*), and 0.46% (*14*) of cases. Our results have shown that SaV is an important cause of acute gastroenteritis in children in Sydney, with a minimum prevalence of 4.1%, which is higher than previously reported. This is the first report of SaV GIV genogroup-associated infection in Australia and widespread distribution of SaV. However, a more comprehensive study is needed to determine whether predominant SaV strains are circulating, as observed with noroviruses (*7,11,15*).

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Enhancement of sapovirus recombinant capsid protein expression in insect cells

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Abstract Human sapovirus (SaV) is uncultivable, but expression of the recombinant capsid protein (rVP1) in insect cells results in the formation of virus-like particles (VLPs) that are morphologically similar to the native viruses. However, the SaV rVP1 expression levels are considerably low. We have found that inclusions of short foreign nucleotide sequences inserted directly upstream from the predicted rVP1 AUG start codon lead to increased yield of VLPs. This method allowed us to express a SaV rVP1, which could not have been expressed to measurable or practical levels otherwise.

Keywords: Baculovirus; Virus-like particles; Capsid protein; Western blotting

1. Introduction

The family *Caliciviridae* is made up of four genera, *Sapovirus*, *Norovirus*, *Lagovirus*, and *Vesivirus*, which contain *Sapporo virus* (SaV), *Norwalk virus* (NoV), *Rabbit hemorrhagic disease virus*, and *Feline calicivirus*, respectively. Human SaV and NoV strains are agents of gastroenteritis. SaV strains were recently divided into five genogroups (GI–GV), of which GI, GII, GIV, and GV strains infect humans, while GIII strains infect porcine species [1]. The SaV GI, GIV, and GV genomes are each predicted to contain three main open reading frames (ORFs), whereas SaV GII and GIII have two ORFs. SaV ORF1 encodes for non-structural proteins and the major capsid protein (VP1). SaV ORF2 (VP2) and ORF3 (VP3) encoded proteins of yet unknown functions.

Human SaV and NoV strains are non-cultivable, but expression of the recombinant VP1 (rVP1) in a baculovirus expression system with insect cells results in the self-assembly of virus-like particles (VLPs) that are morphologically and antigenically similar to the native SaV [2,3]. To date, we have expressed SaV GI, GII, and GV VLPs in insect cells [3], however the expression levels of SaV in insect cells was much lower than that of NoV and some strains cannot be expressed to detectable levels [4,5]. In an earlier SaV expression study, an upstream sequence was found to be a crucial element for SaV VLP formation [6]. In a recent baculovirus expression study, expression levels of exogenous genes was shown to be enhanced by the addition of 21-nucleotides upstream [6]. The 21-nucleotide sequence, derived from a lobster tropomyosin, is A-rich and part of it is found in the polyhedrin leader sequence. In addition, a Kozak sequence is known to greatly facilitate the initial binding of mRNA to the small subunit of the ribosome, which can improve expression levels [7].

In an earlier study, we found that nucleotide point mutations in the SaV rVP1 gene increased the yield of VLPs in insect cells [4]. However, when these point mutations were introduced into other SaV rVP1 sequences we did not observe an increase in expression levels or yield of VLPs (Hansman et al. unpublished data). In the current study, we analyzed the expression levels of SaV genogroups GI and GIV with short foreign nucleotide sequences inserted directly upstream from the predicted VP1 AUG start codon.

2. Materials and methods

SaV GI Mc114 strain (GenBank Accession Number, AY237422) was isolated from a male infant seven months of age from the McCormic Hospital, Chiang Mai, Thailand on the 7th May 2001 [8]. SaV GIV SW278 strain (Accession Number DQ125333) was isolated from an outbreak of gastroenteritis in Sweden, in 2004 [9]. SaV Mc114 rVP1 was designed with the 21-nucleotide-lobster sequence directly upstream from the VP1 AUG start codon (termed Mc114 lob21 construct) as shown in Table 1. RNA extraction and RT-PCR were performed as previously described [3] and the PCR-amplified fragment was cloned into the Gateway Expression System (Invitrogen, Carlsbad, Calif.) as previously described [3], except for a slightly different donor vector (pDONR221). The sequence was verified as previously described [3]. A recombinant bacmid was transfected into Sf9 cells (Riken Cell Bank, Japan) and the recombinant baculovirus was collected as previously described [3]. EM, Western blotting, and antigen ELISA were performed as described previously [3,10,11].

The time-course expression of the Mc114 lob21 construct was compared to a construct without the additional upstream sequence (Mc114 Wt construct; [4]). The expression of the rVP1 constructs were analyzed by infecting recombinant baculoviruses at a MOI of 10 in 2.7×10^6 confluent Tn5 cells in 1.0 ml of Ex-Cell 405 medium followed by incubation at 26 °C. The total culture medium was harvested 1, 2, 3, 4, 5, and 6 dpi. The culture medium was centrifuged for 5 min at $3000 \times g$, and further centrifuged for 40 min at $10000 \times g$. The VLPs in the culture medium were further concentrated by ultracentrifugation for 2 h at 50000 rpm at 4 °C (Beckman TLA-55 rotor), and then resuspended in 20 μl of Grace's medium and stored at 4 °C. For Mc114 Western blotting, we used rabbit and guinea pig antisera that was raised against Mc114 VLPs [3,10,11]. Mc114 VP1 had a predicted molecular weight of approximately 60 kDa.

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Table 1
Primer names and sequences

Primer	Sequence 5′–3′
Mc114 lob21	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC**AACTCCTAAAAAACCGCCACC** ATGGAGGGCAATGGCTCCAACTCA
Mc114 Wt	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGAGGGCAATGGCTCCAACTCA
SW278 lob3	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC**ACC**ATGGAGGGCAATGGCTCCCGACTTG
SW278 Wt	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGAGGGTAATGGCCTACCCCAGGCTGGA
SW278 lob21	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACC**AACTCCTAAAAAACCGCCACC** ATGGAGGGTAATGGCCTACCCCAGGCTGGA

The bold represents the lobster sequences. For Mc114 we used the entire 21-nucleotides of the lobster sequence (Mc114 lob21 construct). For SW278 we used three nucleotides from the 3′ end of the 21-nucleotide lobster sequence, i.e., nucleotides ACC (SW278 lob3 construct) and the entire 21-nucleotides of the lobster sequence (SW278 lob21 construct). The underlined sequence represents the putative VP1 sequences.

3. Results

EM confirmed that both Mc114 constructs, Mc114 lob21 and Mc114 Wt, formed VLPs morphologically similar to the native SaV with 38 nm in diameter (Fig. 1A). The antigen ELISA indicated that the Mc114 lob21 yield of VLPs was greater (between 1.5 and 3.0×) than that of the Mc114 Wt at 4, 5, and 6 dpi (Fig. 1B). The differences in expression levels between these two constructs was also evident when we performed CsCl ultracentrifugation as described previously [12]. A viral band was visible with the Mc114 lob21 construct, but no viral band was visible with the Mc114 Wt construct (data not shown). These results indicated that the 21-nucleotides upstream from the SaV GI Mc114 VP1 AUG start codon increased the yield of VLPs.

Following the Mc114 lob21 results, we investigated whether a construct with an even shorter sequence directly upstream from the VP1 AUG start codon could form VLPs and whether this increased the expression levels and yield of VLPs. We decided to use the three nucleotides from the 3′ end of the 21-nucleotide lobster sequence, i.e., nucleotides ACC. We also used a SaV strain that could not be expressed to practical levels, i.e., SaV GIV SW278 strain. We compared the expression levels of the construct with nucleotides ACC directly upstream from the VP1 AUG start codon (termed SW278 lob3 construct) with that of a construct without the upstream sequence (termed SW278 Wt construct) (Table 1). These two constructs were developed and expressed as described for the Mc114 constructs, including the same MOI of 10. The total culture medium was harvested 1, 2, 3, 4, 5, and 6 dpi. Initially, EM was used to determine the expression levels. We found that both constructs formed VLPs morphologically similar to the native SaV (Fig. 2A), however the yield of VLPs appeared to be greater with the SW278 lob3 construct than that of the SW278 Wt construct. The different expression levels were obvious when we performed CsCl ultracentrifugation. We visualized a viral band with the SW278 lob3 construct, but this viral band was not visible with the SW278 Wt construct. The purified SW278 lob3 viral band contained approximately 10 μg of VLPs when 20 flasks (75 cm^2) were used, whereas less than 1 μg of SW278

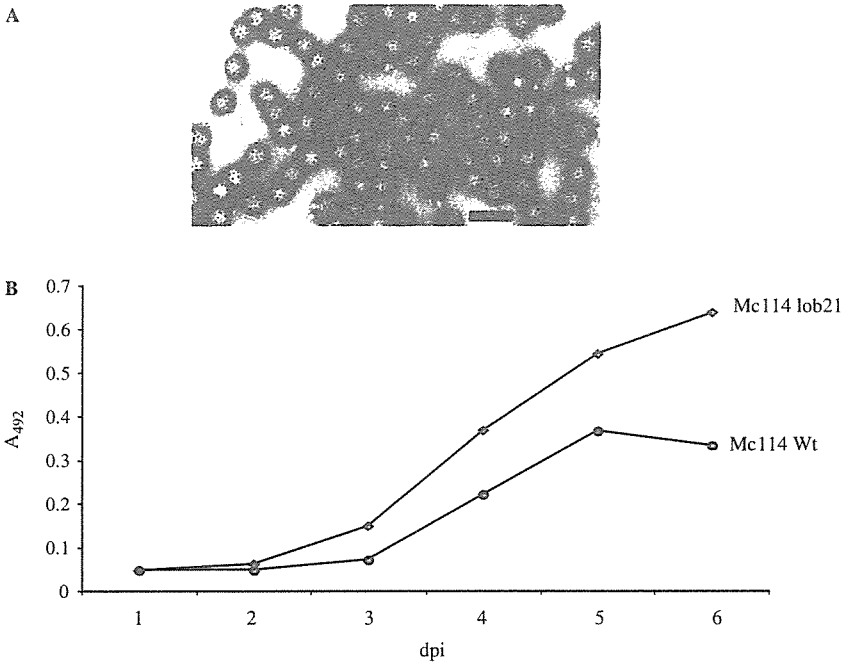

Fig. 1. Analysis of Mc114 expression in insect cells. (A) EM analysis of Mc114 lob21 VLPs. The bars represent 100 nm. (B) Antigen ELISA of the time-course expression of Mc114 lob21 and Mc114 Wt using hyperimmune rabbit and guinea pig antisera raised against Mc114 VLPs [4].

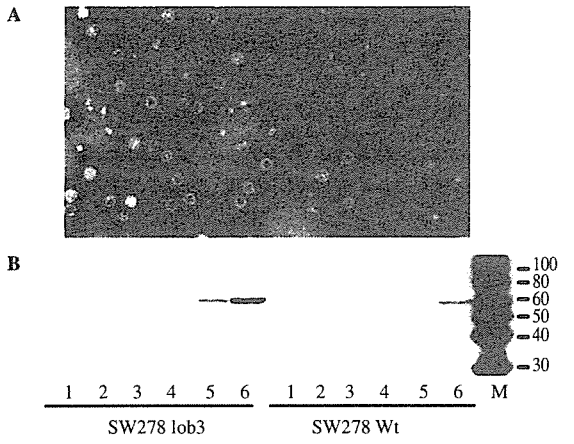

Fig. 2. Analysis of SW278 expression in insect cells. (A) EM analysis of SW278 lob3 VLPs. The bars represent 100 nm. (B) Western blotting analysis of the time-course expression of SW278 lob3 and SW278 Wt using hyperimmune rabbit antiserum raised against SW278 lob3 VLPs.

Wt VLPs were purified when 20 flasks (75 cm^2) were used (data not shown). The purified SW278 lob3 VLPs was used to develop hyperimmune antiserum as previously described [12], and then the rVP1 time-course expression of the SW278 lob3 and SW278 Wt constructs were compared by Western blotting. SW278 VP1 had a predicted molecular weight of approximately 60 kDa. The 60-kDa SW278 lob3 rVP1 was clearly detected at 5 dpi, whereas a very faint 60-kDa SW278 Wt rVP1 was detected at 5 dpi (Fig. 2B). The expression level of rVP1 increased at 6 dpi with both constructs, however the rVP1 band intensity was greater with the SW278 lob3 construct. We estimated the SW278 lob3 had approximately 1.6× and 2.3× rVP1 increase at 5 dpi and 6 dpi, respectively [13]. These results indicated that the three nucleotides upstream from the SaV GIV SW278 VP1 AUG start codon increased the expression levels and yield of VLPs. Interestingly, we found that a construct that included the entire lobster 21-nucleotides (SW278 lob21 construct) formed VLPs, but the expression level was very low, i.e., less than 1 μg of VLPs when 20 flasks (75 cm^2) were used (data not shown).

4. Discussion

Only three other expression studies of human SaV VLP formation have been reported using insect cells [14–16]. In one of those studies, the native upstream sequence of 73 nt from the predicted VP1 AUG codon was a crucial element for VLP formation [14], whereas one of the other two reports included the native 39 nucleotides upstream [15]. Porcine enteric calicivirus of SaV GIII also expressed VLPs in insect cells using a construct that contained nine foreign nucleotides upstream of the VP1 AUG codon, i.e., GTGTTCGTGATGGA (underlined) [17]. Jiang suggested that an upstream sequence could be responsible for initiating transcription or translation; alternatively, this element could serve as a spacer between the polyhedrin promoter and the VP1 gene, or as a stabilizer of the RNA after transcription [6,14]. Interestingly, Jiang also tried to express two other constructs that included the native 9 and 29 nucleotides upstream, respectively, but those also failed to express rVP1 or form VLPs [14]. We also expressed Mc114 rVP1 constructs that included the native 84 and 39 upstream nucleotide sequences, though they formed VLPs, they did not have increased the expression levels when compared to the Mc114 Wt construct (unpublished data). As mentioned earlier, the Kozak sequence, i.e., nucleotides ACCAUGG, is known to improve expression levels [7]. However, the Mc114 Wt construct included the Kozak sequence in the sense primer for the Gateway expression system (ACCAUGG, where the underlined AUG represented Mc114 VP1 start codon) (Table 1). Likewise, SW278 Wt also had the same Kozak sequence in the sense primer (Table 1). When we included the lobster 21-nucleotides in the Mc114 construct, we observed increased expression levels and yield of VLPs (Fig. 1), likewise when we included the ACC sequence upstream for the SW278 construct, we also observed increased expression levels and yield of VLPs (Fig. 2).

In conclusion, these results indicated that upstream sequences have increased the expression levels for SaV GI and GIV rVP1 by approximately 3× and 2.3×, respectively, in insect cells. Without the ACC foreign upstream sequence, we were unable to produce sufficient quantities of SW278 VLPs for development of hyperimmune antiserum. Since the SaV expression levels in insect cells were quite low and some strains cannot be expressed to detectable levels, we recommend that either the lob21 and/or the lob3 upstream sequences be incorporated into difficult to express constructs.

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Reverse genetics system for introduction of site-specific mutations into the double-stranded RNA genome of infectious rotavirus

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We describe here the successful establishment of a reverse genetics system for rotavirus (RV), a member of the *Reoviridae* family whose genome consists of 10–12 segmented dsRNA. The system is based on the recombinant vaccinia virus T7 RNA polymerase-driven procedure for supplying artificial viral mRNA in the cytoplasm. With the aid of helper virus (human RV strain KU) infection, intracellularly transcribed full-length VP4 mRNA of simian RV strain SA11 resulted in the rescue of the KU-based transfectant virus carrying the SA11 VP4 RNA segment derived from cDNA. In addition to the rescued transfectant virus with the authentic SA11 VP4 gene, three more infectious RV transfectants, into which silent mutation(s) were introduced to destroy both or one of the two restriction enzyme sites as gene markers in the SA11 VP4 genome, were also rescued with this method. The ability to artificially manipulate the RV genome will greatly increase the understanding of the replication and the pathogenicity of RV and will provide a tool for the design of attenuated vaccine vectors.

rescue of transfectant virus | viral selection system | *Reoviridae*

Rotavirus (RV) is the leading etiological agent of severe gastroenteritis in infants and young children worldwide and is estimated to cause 440,000 deaths and 140 million episodes of diarrhea each year (1, 2). As a member of the *Reoviridae* family, RV is a dsRNA virus that possesses an 11-segment genome (3). Most positive- and negative-stranded RNA viruses (reviewed in refs. 4–8) can be altered through site-specific mutagenesis by using cloned cDNA. Such reverse genetics systems allow artificial manipulation of viral genomes at the cDNA level by site-directed mutagenesis, deletion/insertion, and rearrangement and have led to the accumulation of significant new knowledge relating to the replication, biological characteristics, and pathogeneses of these viral genera and families (5, 9). For dsRNA viruses, which comprise three families, the *Reoviridae*, *Birnaviridae*, and *Cystoviridae*, such achievements have so far been restricted to the low-numbered segmented dsRNA viruses: two segmented birnaviruses (10, 11) and three segmented $\phi 6$ bacteriophage of the *Cystoviridae* (12). The *Reoviridae* viruses that possess 10–12 segmented genomes have been proven to be very refractory to this approach, except for the reoviruses. Roner and Joklik (13–15) developed a unique but complicated reovirus reverse genetics system involving temperature-sensitive mutants and transformed cells that stably express a particular viral protein encoded by the gene segment to be manipulated. However, there have been no reports on the performance of this method in other laboratories or its application to other *Reoviridae* members so far.

Since the first development of an RV template-dependent *in vitro* replication system in 1994 (16) in which the RV open core can direct the synthesis of genomic dsRNA from viral mRNA in a cell-free system, no infectious RV transfectants have been rescued at all as far as we know, despite intensive attempts at the establishment of RV reverse genetics systems around the world in the last decade. This long lack of progress has led to pessimism regarding the possibility of the development of a reverse genetics system for RV and has made it difficult to determine which methodology is feasible for RV reverse genetics. In the RV replication cycle, mRNAs transcribed from the respective genome segments by viral RNA-dependent RNA polymerase (RdRp) are also being used as templates for the synthesis of minus-strand RNAs, resulting in the formation of genome dsRNAs (16, 17). Thus, theoretically, a cDNA-derived artificial plus-strand RNA corresponding to the natural viral mRNA introduced into cells should be recognized and replicated to yield dsRNA by means of RdRp supplied by the helper virus, and packaged into the virus particles to give rise to an infectious virus containing a gene segment derived from the cDNA.

Here we show that an infectious RV containing a previously undescribed gene segment derived from cDNA was generated with a reverse genetics system. We also describe the rescue of previously undescribed RVs that have site-specific mutations in their VP4 gene genomes derived from an artificially manipulated cDNA genome. Manipulation of the RV genome at the DNA level will allow detailed analysis of its replication and pathogenicity and will provide a tool for the design of attenuated vaccine vectors. This system will also be applicable to other *Reoviridae* viruses.

Results

Structure of SA11 VP4 Gene cDNA. To generate an infectious RV transfectant that contains the previously undescribed cDNA-derived gene segment, we used the helper virus-driven reverse genetics procedure, which was developed originally for influenza viruses by Palese and colleagues (18–20), with some modifications. This procedure requires a strong selection system for the transfectant virus containing the cDNA-derived gene because the majority of virus in the culture fluid is helper virus. Because we obtained some neutralizing monoclonal antibodies (N-MAbs) against VP4 spike protein that specifically neutralize human RV strains exhibiting P[8]-type specificity, such as strain KU, without affecting the replication of viruses with VP4 of the non-P[8] type, such as strain SA11 (P[2]), we attempted to rescue a transfectant virus that contains the SA11 cDNA-derived VP4 gene segment in the background of helper KU viruses by passages in the presence of such N-MAbs. This strategy is also conceivably advantageous, because the growth of RV with the SA11 VP4 gene was found to be much better than that of the original RV in reassortment experiments (21).

First, we cloned the full-length VP4 gene of SA11 flanked by the T7 RNA polymerase promoter and the hepatitis delta virus (HDV) ribozyme followed by the T7 RNA polymerase terminator (Fig. 1 *A* and *B*). Thus, transcription of the resulting vector, pT7/VP4(SA11), allows production of a 2,362-nt-long positive-strand

Conflict of interest statement: No conflicts declared.

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Abbreviations: HDV, hepatitis delta virus; N-MAbs, neutralizing monoclonal antibodies; RV, rotavirus; TLP, triple-layered particle.

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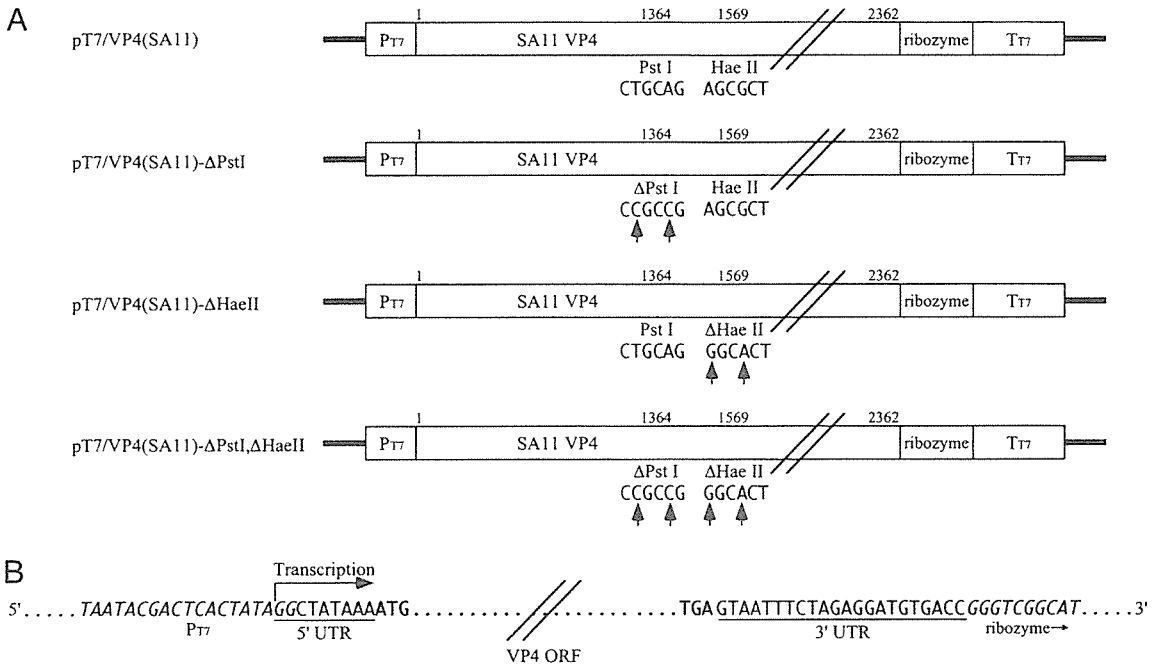

Fig. 1. Schematic representation of transcription plasmids encoding the full-length VP4 gene derived from RV SA11. (*A*) Plasmid pT7/VP4(SA11) contains the authentic full-length VP4 gene cDNA of SA11, flanked by the T7 RNA polymerase promoter, and HDV ribozyme, followed by the T7 RNA polymerase terminator. Manipulation of the VP4 gene by means of silent mutations (positions are indicated by arrows below the sequence) was carried out in pT7/VP4(SA11): the mutant plasmids, pT7/VP4(SA11)-ΔPstI, pT7/VP4(SA11)-ΔHaeII, and pT7/VP4(SA11)-ΔPstI,ΔHaeII, contain the destruction mutation(s) at unique PstI and HaeII sites, and both, respectively. Numbers indicate the nucleotide positions in the SA11 VP4 gene sequence. (*B*) The sequences at the 5′ and 3′ terminus of the SA11 VP4 gene in the transcription vectors. PT7, ribozyme, TT7, and UTR denote the T7 RNA polymerase promoter, HDV ribozyme, T7 RNA polymerase terminator, and untranslated region, respectively.

RNA with the correct 5′ and 3′ ends corresponding to the ends of authentic SA11 VP4 mRNA.

Transfection of cDNA Encoding the Full-Length SA11 VP4 Gene and Rescue of Viruses Containing the cDNA-Derived VP4 Genome. The transfection experiment on the constructed transcription plasmid, pT7/VP4(SA11), was carried out with COS-7 cells that had been infected 1 h earlier with the recombinant vaccinia virus to supply the T7 RNA polymerase. The recombinant vaccinia virus strain, rDIs-T7pol, was selected for its low pathogenicity toward COS-7 and other mammalian cell lines (22). One day after transfection, the transfected cells were infected with KU as the helper virus and then cultured for a further 24 h until harvest. The culture fluid collected was subjected to passages in MA104 cells in the presence of two N-MAbs, ST-1F2 and YO-2C2, that specifically neutralize the KU helper virus. Although significant cytopathic effect could not be observed in the first passage, MA104 cells in the second passage showed cytopathic effect, suggesting the generation of transfectant viruses with the rescued VP4 gene derived from cDNA. The rescued virus was biologically cloned by three successive plaque-to-plaque purifications in CV-1 cells to isolate clones and to exclude the possibility of sequestrated irrelevant dsRNAs by core particles as described for avian reovirus (23) and bluetongue virus (24). After amplification in MA104 cells, the virus particles of transfectant clones were purified by isopycnic CsCl gradient centrifugation and subsequent fractionation, followed by a single major band formation (data not shown). The virus particles in the band were identified as triple-layered particles (TLPs) by their density and viral protein composition (data not shown) and then used for subsequent experiments.

RNA Analysis of Rescued Viruses. Virion dsRNAs were extracted and then analyzed by PAGE. Fig. 2*A* shows the profiles of the dsRNAs from the KU used for the helper virus (lanes 1 and 7) and SA11 virus used for the VP4 gene cloning (lane 6). The VP4 gene dsRNA in lane 2 migrated to the same position as the corresponding dsRNA of SA11 (lane 6), the mobility being slower than that of the VP4 gene segment of the KU helper virus (lanes 1 and 7). Furthermore, sequence analysis of the RT-PCR fragment of the VP4 gene of the rescued virus showed it was derived from SA11 (data not shown). The VP4 gene of KU and SA11 is 2,359-nt and 2,362-nt long, respectively, and the identity at the nucleotide level between them is 71.0% (data not shown). Thus, an infectious RV transfectant containing the cDNA-derived VP4 gene segment, named KU//rVP4(SA11) virus, could be recovered as a result of cDNA transfection, followed by infection with helper virus.

Serological Characterization of Rescued Viruses. The VP4 protein produced by rescued viruses was analyzed by antigen capture ELISA with anti-VP4 N-MAbs, ST-1F2 and KU-6B11. As shown in Fig. 2*B*, KU//rVP4(SA11) showed no reactivity with ST-1F2 that selectively reacts with P[8] type, such as KU helper virus (G1P[8]), but reacted with KU-6B11 that is found to commonly react with both P[8] and P[2] types, including SA11 virus (G3P[2]), whereas the rescued virus reacted with anti-VP7 N-MAb KU-4 specific for G1 type but not with YO-1E2 for G3 type. These serological results were consistent with the results of the RNA analysis, confirming that rescued KU//rVP4(SA11) is the KU-based transfectant virus carrying the SA11 VP4 proteins expressed from the cDNA-derived VP4 genome and that the rescued viruses are quite stable in repeated cell cultures.

Introduction of Site-Specific Mutations into the Genome of Infectious RV. We then introduced four silent mutations as gene markers into the coding region of the VP4 gene in plasmid pT7/VP4(SA11) by PCR-based site-directed mutagenesis. The four mutations in the cDNA comprised T-to-A at position 1,365 and A-to-C at position 1,368 as well as A-to-G at position 1,569 and G-to-A at position

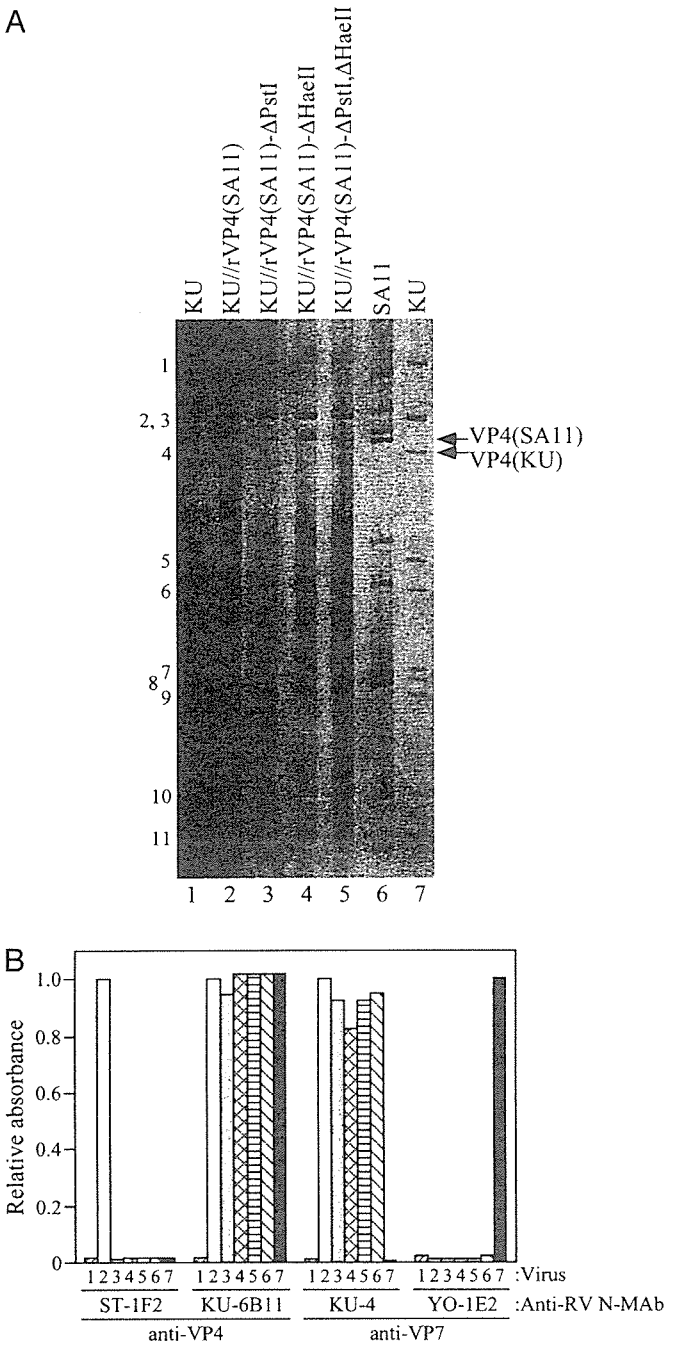

Fig. 2. Rescue of viruses containing the cDNA-derived VP4 genome. (*A*) PAGE of dsRNAs extracted from the rescued VP4 gene transfectants. Lanes 1 and 7, dsRNAs from the KU helper virus; lanes 2–5, dsRNAs from the mutant viruses with the rescued VP4 gene named KU//rVP4(SA11) (lane 2), KU//rVP4(SA11)-ΔPstI (lane 3), KU//rVP4(SA11)-ΔHaeII (lane 4), and KU//rVP4(SA11)-ΔPstI,ΔHaeII (lane 5); lane 6, dsRNAs of SA11 virus used for VP4 gene cloning. The numbers on the left indicate the order of the genomic dsRNA segment of the KU helper virus. (*B*) Reactivity patterns of the rescued viruses with anti-VP4 and VP7 N-MAbs. The rescued viruses and KU and SA11 were subjected to the reaction with anti-VP4 (ST-1F2 and KU-6B11) and anti-VP7 (KU-4 and YO-1E2) N-MAbs in antigen capture ELISA: PBS alone (no virus) (virus 1; right hatched bars), KU (virus 2; open bars), KU//rVP4(SA11) (virus 3; dotted bars), KU//rVP4(SA11)-ΔPstI (virus 4; crosshatched bars), KU//rVP4(SA11)-ΔHaeII (virus 5; horizontal hatched bars), KU//rVP4(SA11)-ΔPstI,ΔHaeII (virus 6; left hatched bars), and SA11 (virus 7; filled bars). Values are expressed as the relative absorbance corresponding to KU (in cases of ST-1F2, KU-6B11, and KU-4) or SA11 (in case of YO-1E2) normalized to 1.0. The experiment was repeated three times with similar results, and representative results are shown.

1,572, destroying the unique PstI site at position 1,364 and the HaeII site at position 1,569, respectively. The previously undescribed plasmids, pT7/VP4(SA11)-ΔPstI, pT7/VP4(SA11)-ΔHaeII, and pT7/VP4(SA11)-ΔPstI,ΔHaeII, contained the destruction mutation(s) at the PstI site, the HaeII site, and at both, respectively. Transfection of each mutagenized plasmid into COS-7 cells, which had previously been infected with the recombinant vaccinia virus, followed by superinfection with the helper virus, resulted in the rescue and isolation of transfectant virus clones named KU//rVP4(SA11)-ΔPstI, KU//rVP4(SA11)-ΔHaeII, and KU//rVP4(SA11)-ΔPstI,ΔHaeII, respectively, that grew in the presence of the helper virus-specific N-MAbs. After the third plaque-to-plaque purification of each virus clones, individual TLPs of the previously undescribed viruses were purified by CsCl gradient density centrifugation and then used for subsequent characterization experiments. The virion dsRNAs were extracted and subjected to PAGE and RT-PCR of the entire VP4 sequences, and the VP4 proteins of the virions were characterized by ELISA analysis. On PAGE analysis, the mobility of the fourth segment of the recovered viruses was found to be the same as that of strain SA11 (Fig. 2*A*, lanes 3–5). On RT-PCR-restriction enzyme digestion analysis, the 2,386-bp product of the authentic VP4 gene from KU//rVP4(SA11) virus, which includes extra 24 bp encoded by 5′ terminus of primer 2, was digested with PstI or HaeII into 1,392 and 994 bp, or 1,597 and 789 bp, ones, respectively (Fig. 3*A*). In contrast, the corresponding DNA fragments from previously undescribed transfectant viruses could not be cut with PstI and/or HaeII, as planned in the plasmid constructions (Fig. 3*A*). Furthermore, direct sequence analysis of the PCR products also revealed the correct presence of these silent mutations within the genomes of the rescued viruses (Fig. 3*B*). In antigen capture ELISA analysis, the rescued viruses showed no reactivity with anti-VP4 N-MAb ST-1F2 (P[8]-specific) but reacted with KU-6B11 (cross-reactive) (Fig. 2*B*). Thus, successful rescue of infectious RVs containing site-specific mutations in their genomes was achieved via transfection of cloned cDNA, followed by helper virus infection in a recombinant vaccinia virus T7 RNA polymerase-driven system.

Discussion

The approach described here represents a successful reverse genetics system for RVs and involves infection with a recombinant vaccinia virus expressing T7 RNA polymerase and superinfection with a helper virus. Concerning the viruses in the family *Reoviridae*, Roner and Joklik (13–15) have reported reverse genetics to generate reovirus transfectants, but the system requires very complicated techniques and very tedious preparation of temperature-sensitive mutants and transformed cells that stably express a particular viral protein encoded by the gene segment to be manipulated. There have been no reports on its application in other laboratories or to other *Reoviridae* viruses. In contrast, we demonstrated that the reverse genetics system involving the traditional T7 RNA polymerase expression system (25) developed by Palese and colleagues (18–20) could be applied to RVs with some modifications. The recombinant vaccinia virus T7 RNA polymerase-driven system allows extremely high expression of artificial viral mRNAs mimicking the natural RV mRNAs in the cytoplasm, which is favorable for the development of reverse genetics for RVs whose entire replication cycle is completed in the cytoplasm.

RV mRNAs are unique in that they possess a 5′ cap structure but lack 3′ poly(A) tails (26, 27), and several studies have revealed the importance of these structures for efficient genome replication and translation (28–30). In this study, each of the authentic 5′ and 3′ end structures were planned to be obtained with vaccinia virus-encoded capping enzyme and the HDV ribozyme sequences inserted in the transcription plasmids (Fig. 1), respectively. Intracellularly transcribed viral mRNA derived from cDNA in the recombinant vaccinia virus T7 RNA polymerase-driven system was used, with

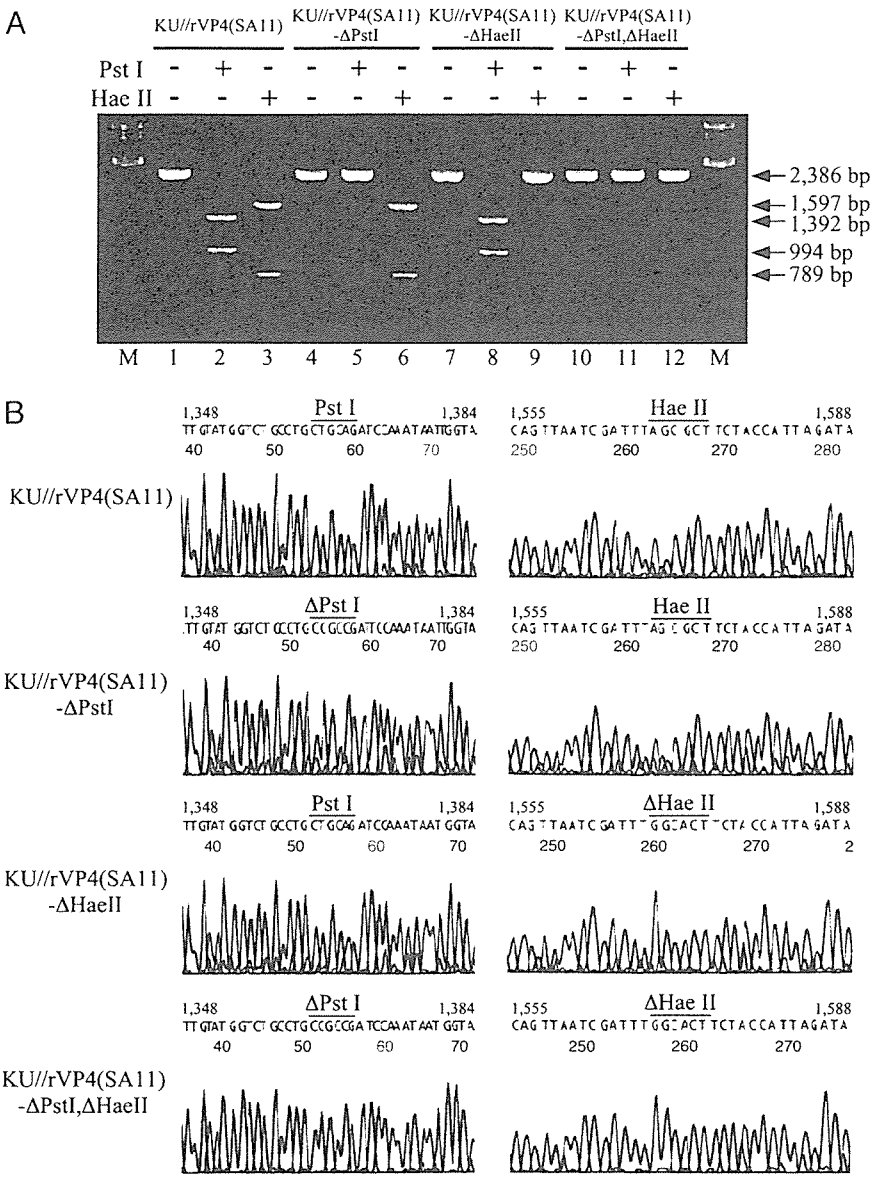

Fig. 3. Site-specific mutations introduced into the genomes of the rescued VP4 gene transfectants. The full-length VP4 gene of each virus was amplified by RT-PCR to yield a 2,386-bp product. (*A*) The amplified fragments were digested with PstI or HaeII, followed by separation in a 1.25% agarose gel. KU//rVP4(SA11) (lanes 1–3), KU//rVP4(SA11)-ΔPstI (lanes 4–6), KU//rVP4(SA11)-ΔHaeII (lanes 7–9), and KU//rVP4(SA11)-ΔPstI,ΔHaeII (lanes 10–12). The 2,386-bp fragments (lanes 1, 4, 7, and 10) were digested with PstI (lanes 2, 5, 8, and 11) or HaeII (lanes 3, 6, 9, and 12). M, 1-kb ladder weight markers. (*B*) The 2,386-bp fragments were directly sequenced, which demonstrated the site-specific mutations introduced within the genomes of the infectious VP4 gene transfectants.

the aid of helper virus infection, to rescue an infectious RV that contains the previously undescribed cDNA-derived gene segment. For the selection of such transfectant viruses, we chose a system that requires the presence of a simian RV strain SA11-like VP4 gene in the rescued viruses. The growth of viruses containing this gene was not restricted in the presence of N-MAbs that selectively neutralize the helper virus (human RV strain KU)-derived VP4 gene, but rather the virus with the SA11-VP4 gene could multiply much better than the helper virus. In the experiments presented, we first recovered KU//rVP4(SA11) virus, which contains the authentic SA11-origin VP4 gene derived from plasmid pT7/VP4(SA11) (Fig. 1*A*). KU//rVP4(SA11) virus exhibits the expected RNA pattern (Fig. 2*A*), VP4 gene sequence (data not shown), and reactivity with anti-VP4 and VP7 N-MAbs (Fig. 2B) and grew to reach almost the same titers in MA104 cells to those of the SA11 virus used for the VP4 gene cloning (data not shown). The different growth characteristics of KU//rVP4(SA11) virus from those of the parental KU were expected from the previous finding that VP4 protein often largely confers RV infectivity (17, 31, 32).

We also rescued three transfectant viruses that have silent mutations to destroy the original restriction enzyme site(s) as gene markers in their VP4 genomes, derived from plasmids pT7/VP4(SA11)-ΔPstI, pT7/VP4(SA11)-ΔHaeII, and pT7/VP4(SA11)-ΔPstI,ΔHaeII (Fig. 1). We verified the presence of these mutations in the rescued mutant viruses (KU//rVP4(SA11)-ΔPstI, KU//rVP4(SA11)-ΔHaeII, and KU//rVP4(SA11)-ΔPstI,ΔHaeII) by RT-PCR-restriction enzyme digestion analysis and sequence analysis of the virion dsRNAs (Fig. 3). These mutations did not result in any amino acid change in VP4 protein and, thus, were not expected to change the biological properties of KU//rVP4(SA11) virus with the authentic SA11-derived VP4 gene. In fact, all of the rescued mutant viruses showed no reactivity to the P[8]-specific anti-VP4 N-MAb ST-1F2 (Fig. 2*B*) and growth characteristics (data not shown)

Table 1. PCR primers used for plasmid construction

Primer	Nucleotide positions	Nucleotide sequence (restriction enzyme site)*
1	2338–2362	5′-GGTCACATCCTCTAGAAATTACTCA-3′
2	1–23	5′-cc<u>AAGCTT</u>*TAATACGACTCACTATAGG*CTATAAAATGGCTTCGCTCAT-3′ (HindIII)
3	1359–1382	5′-GCCTGCC**C**GC**C**GATCCAAATAATGG-3′
4	1350–1377	5′-ATTTGGATC**G**GC**G**GCAGGCAGACCATAC-3′
5	1560–1585	5′-AATCGATTT**G**GC**A**CTTCTACCATTAG-3′
6	1553–1580	5′-GGTAGAAG**T**GC**C**AAATCGATTAACTGCG-3′

*The restriction enzyme site is underlined. The italic letters denote the T7 RNA polymerase promoter sequence, and the bold letters represent the nucleotides substituted in the VP4 gene of SA11 virus. Lowercase letters for primer 2 indicate the nonviral nucleotides added to the 5′ terminus of the primer.

indistinguishable from those of KU//rVP4(SA11) virus. Thus, we confirmed the utility of the reverse genetics system developed here for RVs. However, there is a problem to be considered. In a typical experiment, at least one series of three transfections on 60-mm dishes were required to isolate each transfectant virus under the present conditions (data not shown). The cause for the low efficiency of the transfectant virus recovery might be the very strict control of packaging of the exact number of genome segments per virion, and/or the hard accessibility of artificial RV mRNAs to viroplasm as reported by Silvestri *et al.* (33); however, the efficiency of the system must be improved.

The introduction of mutations that change the biological characteristics of RVs would help to define the precise functions of all of the viral proteins, including those of nonstructural proteins. Concerning the VP4 protein, for example, researchers could soon be able to perform direct assessment of the relationship between trypsin cleavability at the conserved arginines and the virulence of RVs by using the reverse genetics system. The untranslated regions of the genome could also be studied by means of mutagenesis, leading to a better understanding of the regulatory signals present in viral RNAs. Furthermore, this reverse genetics system provides the opportunity to identify attenuation markers within the RV genome segments and, thus, will facilitate the development of attenuated recombinant viruses that could be used as vaccine or vaccine vector candidates. Clearly, there are limitations to our helper virus-dependent reverse genetics because of the requirement of a strong selection system, such as the N-MAbs selection system used in this study. For gene segments that do not encode viral surface proteins, other gene-specific selection procedures, such as host-range restriction and temperature sensitivity, will be required. Nevertheless, the current approach has immediate promise in several areas, particularly with respect to the study of the functions of viral proteins and the signals involved in the transcription, replication, and packaging of RV genome dsRNAs, which will also lead to a further goal: establishment of a helper virus-free system to generate infectious RVs entirely from cloned cDNAs.

Materials and Methods

Viruses. Human RV strain KU (G1P[8]) (34) and simian RV strain SA11 (SA11-L2) (G3P[2]) (35) were pretreated with trypsin (type IX, from porcine pancreas and crystallized) (10 μg/ml; Sigma) and then propagated in MA104 cells in the presence of trypsin (1 μg/ml). Recombinant vaccinia virus rDIs-T7pol (22), kindly provided by Y. Matsuura (Research Institute for Microbial Diseases, Osaka University, Osaka), which was designed to express the T7 RNA polymerase, was grown in chicken embryo fibroblasts.

Cell Culture and Infection. Epithelial monkey kidney cell lines COS-7, MA104, and CV-1 were cultured in Eagle's minimum essential medium supplemented with 5% FCS (complete medium).

Monolayers of the cells were inoculated with RV pretreated with trypsin (10 μg/ml) for 30 min at 37°C. After adsorption for 1 h at 37°C, the infected cells were washed with minimum essential medium without FCS (incomplete medium) and then cultured in incomplete medium containing trypsin (1 μg/ml) until harvest. The cultures were subjected to three cycles of freezing and thawing and then centrifuged at low speed to remove cell debris. The supernatants were used for RV infection experiments.

Construction of Authentic VP4 Gene cDNA of SA11. dsRNA of SA11 was extracted from the culture fluid with a disruption solution (1% SDS/0.1% 2-mercaptoethanol/60 mM EDTA) and then with phenol-chloroform. cDNA of the SA11 VP4 gene was synthesized from virion dsRNA with avian myeloblastosis virus reverse transcriptase (Seikagaku Kogyo, Tokyo) and primer 1 (Table 1) as described in ref. 36. cDNA encoding the full-length VP4 gene (2.4 kb) was amplified by PCR with KOD-Plus (TOYOBO, Osaka) and primers 1 and 2. Primer 2 contains the T7 promoter sequence and a 23-base sequence corresponding to the 5′ terminus of the SA11 VP4 gene. After digestion with HindIII, amplified cDNA was ligated into the HindIII and SmaI sites of T7 expression vector pX8dT (37) (kindly provided by Y. Matsuura, which was originally developed by K. K. Conzelmann (Ludwig-Maximilians-University, Munich). The generated plasmid, pT7/VP4(SA11), contains the authentic full-length VP4 gene cDNA of SA11, flanked by the T7 RNA polymerase promoter and the HDV ribozyme, followed by the T7 RNA polymerase terminator.

Introduction of Genetic Markers into the RV Genome. Manipulation of the VP4 gene was carried out in plasmid pT7/VP4(SA11). Destruction of the unique PstI and HaeII sites (positions in the VP4 gene of SA11: 1,364 and 1,569, respectively) by artificial introduction of silent mutations was achieved with a QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Briefly, pT7/VP4(SA11) was amplified with mutated nucleotide primers 3 and 4 and primers 5 and 6 to change the SA11 PstI and HaeII site nucleotide sequences, respectively. After digestion with DpnI to eliminate the pT7/VP4(SA11) used as a template, the PCR products were used to transform *Escherichia coli*-competent cells. The generated plasmids, pT7/VP4(SA11)-ΔPstI and pT7/VP4(SA11)-ΔHaeII, have silent mutations at the PstI and HaeII site, respectively. Furthermore, plasmid pT7/VP4(SA11)-ΔPstI,ΔHaeII, which has mutations at both the PstI and HaeII sites, was also prepared by using template pT7/VP4(SA11)-ΔHaeII and primers 3 and 4 by a similar method to that described above. Newly generated plasmids were all identified to be appropriate clones by confirming their sequences.

Reverse Genetics System. COS-7 cells were seeded at a concentration of 2.5×10^5 cells/ml in 60-mm dishes. After overnight culture, 5 μg of pT7/VP4(SA11), pT7/VP4(SA11)-ΔPstI, pT7/VP4(SA11)-ΔHaeII, or pT7/VP4(SA11)-ΔPstI,ΔHaeII plasmid DNA was transfected into COS-7 cells, which had been infected 1 h before

with rDIs-T7pol at a multiplicity of infection (MOI) of 3, with the use of TransIT LT-1 (Mirus, Madison, WI) according to the manufacturer's instructions. Twenty hours after transfection, the transfected cells were washed twice with incomplete medium and then infected with KU as the helper virus at a MOI of 3. Twenty-four hours after the helper virus infection, the cultures were harvested, and transfectant viruses with the VP4 gene segment derived from SA11 cDNA were selected as described below.

For selection of transfectant viruses containing the cDNA-derived VP4 gene segment, the harvested viruses were activated with trypsin, followed by inoculation onto MA104 cell monolayers cultured in 60-mm dishes. After 1 h adsorption, the infected cells were washed with incomplete medium twice and then subjected to 7 days' culture in incomplete medium with trypsin (1 μg/ml) and anti-VP4 N-MAbs ST-1F2 (38) and YO-2C2 (39) that selectively neutralize KU helper virus of the P[8] type. After single mock passage, the RV-induced cytopathic effect was observed, and then the cultures were harvested and subjected to a further passage to amplify the transfectant viruses. The rescued transfectant viruses were then triply plaque-to-plaque purified in CV-1 cells by picking up every five plaques as described in ref. 38.

Purification of RV Particles of the Rescued Transfectants Containing the cDNA-Derived VP4 Genome Segment. The plaque-purified transfectant viruses were propagated in MA104 cells. After the virus particles were pelleted from the supernatants by centrifugation at 100,000 $\times$ *g* for 3 h and resuspended in PBS containing 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS), it was followed by isopycnic CsCl (0.475 g/ml) gradient density centrifugation at 100,000 $\times$ *g* for 18 h and subsequent fractionation as described in ref. 39. The virus band corresponding to the TLPs was collected and pelleted by centrifugation at 100,000 $\times$ *g* for 3 h and then dissolved in PBS.

Identification of the cDNA-Derived VP4 Gene in the Rescued Viruses. The genomic dsRNAs of rescued viruses were extracted from the purified TLPs. The precipitated virion dsRNAs were used for (*i*) PAGE analysis, (*ii*) RT-PCR restriction enzyme digestion analysis, and (*iii*) sequence analysis. For PAGE analysis, the dsRNAs were electrophoresed in a 10% acrylamide gel (2-mm thick) for 16 h at 20 mA at room temperature and then visualized by silver staining (35). For RT-PCR restriction enzyme digestion analysis, dsRNAs were converted to cDNAs by incubation with SuperScript II RT (Invitrogen) with primer 1, and full-length VP4 genes were then amplified with primers 1 and 2. PCR products were digested with PstI or HaeII, and the resulting fragments were separated by 1.25% agarose gel electrophoresis in Tris-acetate-EDTA buffer. Gels were stained with ethidium bromide. For sequence analysis, the PCR products were sequenced directly with an ABI PRISM 310 automated sequencer (PerkinElmer) by using a DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia Biosciences).

Identification of the VP4 Protein Produced by the Rescued Viruses. To identify the VP4 protein produced by the rescued viruses, antigen capture ELISA was carried out as described in ref. 40 with anti-VP4 [ST-1F2 (38) and KU-6B11 (38)] and anti-VP7 [KU-4 (41) and YO-1E2 (41)] N-MAbs. Purified TLPs of rescued viruses were incubated with N-MAbs coated on ELISA plates. For the anti-VP4 N-MAbs, ST-1F2 selectively reacts with P[8] type, such as KU helper virus (G1P[8]), whereas KU-6B11 commonly reacts with both P[8] and P[2] types, including SA11 virus (G3P[2]). The anti-VP7 N-MAbs, KU-4 and YO-1E2, selectively react with G1 and G3 type, respectively.

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Reverse genetics systems of segmented double-stranded RNA viruses including rotavirus

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The rotavirus genome is composed of 11 segments of double-stranded (ds)RNA. Recent studies have elucidated the precise mechanisms in transcription and replication of rotavirus RNA mainly by *in vitro* experiments. However, the ideal methodology for the molecular study of rotavirus replication is reverse genetics, which enables the viral genome to be artificially manipulated. Since the development of the first reverse genetics system for RNA virus in bacteriophage QB in 1978, the methodology has been developed for a variety of RNA viruses with plus-strand, minus-strand or dsRNA as a genome. However, there have been no reports on the reverse genetics of the viruses in the family *Reoviridae* with a genome of 10–12 segmented dsRNA, except for reovirus. This review describes the replication cycle of rotavirus with the aim of providing a general background to the development of rotavirus reverse genetics, and summarizes the reverse genetics system for dsRNA viruses, including rotavirus.

Rotaviruses, which are members of the family *Reoviridae*, are the leading pathogen for causing acute gastroenteritis in the young of a variety of mammals and birds worldwide. In humans, dehydration due to diarrhea and vomiting induced by rotavirus infection results in high mortality rates, and 500,000 deaths per year have been estimated in developing countries. In developed countries, rotavirus infection is a significant cause of morbidity [1,2].

Rotavirus particles exhibit three concentric capsid layers that enclose a genome of 11 segments of double-stranded (ds)RNA [3]. There are six structural proteins (the core proteins VP1, VP2 and VP3, the inner capsid protein VP6 and the outer capsid proteins VP4 and VP7) and six nonstructural proteins (nonstructural protein [NSP]1–6). The outer capsid proteins VP4 and VP7 carry independent neutralization and protective antigens. G serotype specificity is associated with VP7, and 15 different G serotypes (G1–G15) have been reported. VP4 defines P serotype and at least 14 P serotypes have been assigned; however, since it is difficult to differentiate P serotypes serologically, P genotypes based on the VP4 sequence have been proposed and 26 P genotypes (P[1]–P[26]) have been reported. There are seven rotavirus groups (A–G) with distinct RNA profiles and a lack of serological cross-reactivity. Only group A rotaviruses, which are the most characterized, are considered in this review.

The segmented nature of the rotavirus genome allows reassortment of genome segments during mixed infections. Reassortment experiments, a classical approach, are a very powerful tool and have provided a number of answers with regard to rotavirus phenotypes and replication mechanisms. However, the limitation of this technique is that genetic analysis has been restricted to genetic markers with phenotypes that can be identified easily in the progeny of mixed infections. Most positive- and negative-stranded RNA viruses [4–9] can be altered through site-specific mutagenesis by using cloned cDNA. Such reverse genetics systems allow artificial manipulation of viral genomes at the cDNA level by site-directed mutagenesis, deletion/insertion and rearrangement, and have led to the accumulation of a significant amount of new knowledge relating to the replication, biological characteristics and pathogenesis of these viral genera and families. For the viruses with segmented dsRNA as a genome, which comprise three families; *Reoviridae*, *Birnaviridae* and *Cystoviridae*, such achievement has currently been restricted to the low-numbered segmented dsRNA viruses: two segmented birnaviruses [10–12] and three segmented bacteriophages of Φ6 [13,14] and Φ8 [15] of the *Cystoviridae* family. The *Reoviridae* viruses that possess 10–12 segmented genomes have been demonstrated to be very refractory to this, except for retrovirus. Roner and colleagues developed a unique, but complicated, reovirus reverse genetics system involving transformed cells that stably express a particular viral protein encoded by the gene segment to be manipulated [16–19].

Detailed genetic analyses of rotaviruses have been delayed considerably by the inability to perform reverse genetics, and rotavirologists

Keywords: double-stranded RNA virus, *Reoviridae*, rescue of transfectant virus, reverse genetics, RNA segment, rotavirus, viral selection system

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have been struggling without the advent of a working reverse genetics system. Recent work in our laboratory generated an infectious rotavirus containing a gene segment derived from cloned cDNA by reverse genetics involving the traditional T7 RNA polymerase-driven procedure for supplying artificial viral single-stranded (ss)RNA intracellularly [20].

This review describes the nature of the rotavirus genome and the rotavirus replication cycle in order to highlight the background of rotavirus reverse genetics. Furthermore, we summarize the method and application of reverse genetics for segmented dsRNA viruses including rotavirus.

Rotavirus genome

Each of the 11 segments contains an open reading frame (ORF) with the exception of segment 11, which has two out-of-frame initiation codons resulting in two ORFs. Viral dsRNA species are present within virus particles in equimolar proportions, representing one copy of each segment per virion. The entire rotavirus genome has been sequenced for several rotavirus strains. The total rotavirus genome contains 18,555 base pairs (bp; a simian strain SA11) with the length of individual segments ranging from 3302 (segment 1) to 663 bp (segment 11) [21]. The 5′ terminus of the positive-sense RNA strand of each segment contains a cap structure. By contrast, the 3′ terminus of mRNAs lack polyA tails. Rotavirus group A-specific and segment-specific sequence motifs appear to be necessary for successful replication, translation and encapsidation. The RNA segments have conserved terminal sequences at both ends; 5′-GGC(A/U)(A/U)U(A/U)A(A/U)(A/U)...(A/U)(G/U)(G/U)(G/U)(A/G)CC-3′. These terminal sequences may act as recognition signals for the viral transcriptase and replicase functions. Sequences near the 5′ and 3′ termini of the positive-sense viral RNAs share extensive complementarity to form stem-loops and secondary structures.

It is well known that rotavirus shows extensive genome diversity [22], which can be detected readily by polyacrylamide gel electrophoresis (PAGE). The variation in the rotavirus genome involves three distinct mechanisms: accumulation of point mutations, reassortment and rearrangement. In the reassortment mechanism, when a cell is infected with two different, but compatible, rotaviruses, a high percentage (nearly 50% *in vitro* and 70–80% *in vivo*) of progeny contain novel assortments of gene segments [23,24]. During rearrangement, alteration of considerable tracts of sequence occurs within single genome segments, occasionally in the form of deletions and often as partial duplications [25].

Rotavirus replication cycle

The replication cycle is completed in 10–12 h for the SA11 strain which has a highly efficient growth ability, and 18–22 h for human strains at 37°C. All the replication processes occur entirely in the cytoplasm. Numerous studies highlight the importance of electrodense viroplasms or virus inclusion bodies, occurring in localized areas of the cytoplasm during replication of rotavirus. The viroplasms appear to function as the sites of viral mRNA synthesis, genome packaging, assortment of genomic segments and replication. Early in infection, viroplasms scatter as numerous small punctuate structures, and at a later stage, they mature to fewer, large and highly organized structures. NSP2 and NSP5 are two essential elements for the assembly of functional viroplasms and are therefore relevant for virus replication. Since precise reviews on transcription and replication have already been published [26–31], an outline of the rotavirus life cycle will be described in this section

Adsorption & penetration

It has been demonstrated that sialic acid residues are required for binding of rotaviruses to cells, since the treatment of the cells with neuraminidase (NA) resulted in a drastic decrease in the infectivity of some animal rotavirus strains. However, the infectivities of some animal and most human rotavirus strains were not affected even after the NA treatment of cells [32–34]. It appears that NA-sensitive strains bind to external sialic acid residues in gangliosides, while NA-resistant strains recognize gangliosides with internal sialic acid. The rotavirus receptor(s) is suggested to be a complex of several cell molecules, most likely immersed in cholesterol and glycosphingolipid-enriched plasma membrane microdomains [33]. Integrins are found to be more specific receptors that facilitate rotavirus entry into the cell [35]. It has been proposed that VP8 molecules of rotavirus strains initially contact with receptors containing sialic acid, VP5 molecules then interact with the integrin $\alpha 2\beta 1$ and, finally, interactions between VP7 molecules, $\alpha v\beta 3$ and $\alpha x\beta 2$, and between VP5 molecules and heat shock cognate protein 70 enable the virus to penetrate into the cell [34]. The virus entry process removes the outer capsid proteins, VP4 and VP7, and thereby

converts triple-layered particles (TLPs) to the transcriptionally active double-layered particles (DLPs) in the cytoplasm of the infected cell. It has been suggested that some of the DLPs then serve as focal points for the accumulation of newly synthesized proteins into viroplasms and are thereby incorporated into the inclusions.

Transcription

Rotaviruses contain all the necessary enzymatic machinery to synthesize complete mRNA transcripts within their core without the need for disassembly. The RNA-dependent RNA polymerase (RdRp) of the DLP functions as a transcriptase to synthesize the 11 viral plus-strand RNAs. The presence of all the enzymatic activities is required to generate the 5′ cap required by the eukaryotic translation machinery. The innermost capsid protein, VP2, acts as a scaffold on which the core components of the transcription apparatus are assembled [36].

DLP-associated enzymes, RdRp VP1 and guanylyltransferase VP3, produce 5′ capped, non-polyadenylated mRNAs, which are full-length transcripts from the minus-strand of each of 11 dsRNA segments of a genome. The plus-strand RNAs are extruded from DLPs through channels at the icosahedral apices that extend through both the VP2 and VP6 protein layers [36]. Plus-strand RNAs not only direct protein synthesis but serve as templates for the synthesis of the segmented dsRNA genome. Recently, it was suggested that trafficking pathways to transport transfected plus-strand RNAs to viroplasms do not exist within the cytosol [37]. Therefore, it has been hypothesized that there are two pools of plus-strand RNAs in the cells infected with rotavirus; one located within the viroplasm that serves as a template for dsRNA synthesis and the other present outside of the viroplasm that serves as a template for translation [37]. DLPs initiate transcription within the cytoplasm, leading to the synthesis of plus-strand RNAs that are incorporated into polysomes and translated to produce viral proteins. Plus-strand RNAs synthesized within viroplasms are the primary source of templates for genome replication. The necessary cyclized conformation appears to be required for efficient multiple rounds of transcription [38].

Translation

Translation stages proceed in the cytoplasm. Since rotavirus mRNA does not have polyA at the 3′ end, it has been proposed that NSP3 could catalyze the circularization of rotavirus mRNA within polysomes in place of polyA binding protein (PABP), thereby increasing translation efficiency. The N-terminal domain (residues 4–164) of NSP3 specifically binds the 3′ end consensus sequence (5′-GUGACC-3′) of viral mRNA, and the C-terminal domain interacts with a cap-associated eukaryotic translation initiation factor termed eIF4G [39–41]. These interactions are functionally equivalent to the interactions between PABP and both eIF4G and the polyA tail at the 3′ end of eukaryotic mRNA. Through these interactions, the rotavirus achieves circularization of its mRNA and selectively boosts the efficiency of translation. NSP3 evicts PABP from eIF4G [42]. eIF4E, a cap-binding protein, binds the 5′ cap and the initiation factor eIF4G, a multipurpose adaptor protein that is responsible for delivering mRNAs to the ribosome. Since the binding sites for NSP3 and PABP on eIF4G overlap and the affinity of eIF4G for NSP3 is stronger than for PABP, translational activity of the viral mRNA increases while the translation of host mRNAs is reduced [43–46]. Thus, the virus hijacks the host translation machinery to enhance the synthesis of viral proteins and simultaneously prevents the production of host proteins [44,45]. By contrast, in knockdown experiments using RNA interferences, Montero and colleagues [47] have recently demonstrated that NSP3 is not necessary for the translation of viral mRNAs in cell culture. This remarkable difference in the role of NSP3 in translation might reflect the distinctness of the experimental conditions; a cell-free system or cell culture system.

Gene expression is regulated by the level of transcription from individual genomic segments, with differences evident in both the kinetics and level of production of different mRNAs. There is more than a 250-fold difference in the level of expression between the most (NSP4) and least (VP1) abundant protein. Rotavirus X protein associated with NSP3 (RoXaN), a 110 kDa novel cellular protein forming a ternary complex with NSP3 and eIF4GI, has been found to be related to translation regulation [48].

Replication

Based on extensive studies, it has been suggested that RNA replication is performed in the viroplasms, where NSP2 and NSP5 are accumulated [49,50]. The mRNAs are used as templates for a single round of minus-strand synthesis, thereby reforming the dsRNA genome segments of a progeny virus particle. *Cis*-acting replication

elements, such as the seven 3′ terminal nucleotides GUGUACC on the mRNAs, which assist minus-strand synthesis, have been identified using an *in vitro* replication system [51,52]. The synthesis of dsRNA occurs simultaneously with the packaging of mRNA templates into core-like replication intermediates (core RIs), composed of structural proteins VP1, VP2, VP3, NSP2 and NSP5 [37]. All the core RI proteins have affinity for ssRNA. The mRNA templates may pass into cores through channels that exist at the five-fold axes of the VP2 shell, which serve as platforms on which mRNA binds VP1. Multiple, specifically recognized elements exist at the 3′ end that promote dsRNA synthesis, including RdRp-recruitment signals and a minus-strand initiation sequence. The 5′ end contains a base-specific recognition signal that has an important role in the assembly of RdRp and cofactors into a stable initiation complex for minus-strand RNA synthesis [38]. The dsRNA genome segments are usually packaged in precise equimolar ratios (one copy of each genome segment per particle). The selection of viral mRNAs for packaging is therefore thought to be highly specific, involving recognition signals on each mRNA species.

The highly frequent occurence of reassortment in the mixed infection raises a question on the role of viroplasms in replication of rotavirus. How do NSP2 and NSP5 from distinct strains participate in the formation of viroplasms? Further research examining the role of viroplasms in rotavirus replication is necessary if this question is to be solved.

Assembly & release

Through the acquisition of VP6, core RIs maturate to DLPs. NSP4 is an intracellular receptor of DLPs and, thus, is a function necessary for the recruitment of DLPs to the endoplasmic reticulum, where they are converted to TLPs (this assembly process has been reviewed precisely [53]). The matured virions are then released from the cell by lysis.

Reverse genetics for segmented dsRNA viruses

Temperature-sensitive (ts) mutants [54–58], genome reassortment [59,60] and *in vitro* reconstitution studies [61–67] have been the main approaches used to understand the function of rotavirus proteins. The RNA replication process has been elucidated by *in vitro* replication systems [51,68,69]. Recently, knockout strategies using RNA interference [37,70–73] and intracellular antibody-capture technologies [74–76] have been employed for that purpose.

However, reverse genetic systems are the most powerful experimental approaches. A reverse genetics system has been established for segmented dsRNA viruses, including Φ6 [13,14] and Φ8 phages [15], infectious bursal disease virus (IBDV) [10,12], infectious pancreatic necrosis virus (IPNV) [11] and reovirus [16]. Finally, a reverse genetics system has recently been developed for rotavirus in our laboratory [20], which will provide investigators with new methods to investigate rotavirus biology and pathogenesis.

Bacteriophage Φ6

Bacteriophage Φ6 is an organism of the family *Cystoviridae*. For a long time, Φ6 represented the only member of this family and was the only known dsRNA virus infecting bacteria [77]. Eight similar viruses (Φ7 to Φ14) have been further isolated [78]. Virions contain three segments of dsRNA as a genome inside of a procapsid that is covered with a lipid-containing envelope. Each segment, designated large (L), medium (M) and small (S), contains four or five ORFs found closely together, with untranslated regions (UTRs) of several hundred bases at both 5′ and 3′ ends [79].

To study Φ6 viral proteins required for transcription and replication, Gottlieb and colleagues cloned the L segment into an expression vector, which encodes the P1, P2, P4 and P7 proteins that form procapsids [80,81]. The procapsids expressed and formed in *Escherichia coli* or *Pseudomonas phaseolicola* exhibited *in vitro* dsRNA replicase and transcriptase activity. The cDNA-derived procapsids were capable of packaging and replicating viral positive-stranded mRNA to dsRNA genomic segments, in addition to producing transcripts using the dsRNA formed as a template *in vitro* (*in vitro* packaging-replication system). Conversely, Ojala and colleagues developed a technique for infecting *P. phaseolicola* spheroplasts with the purified procapsids [82].

In 1990, Olkkonen and colleagues developed a novel reverse genetics system to generate infectous Φ6 viruses that have a cDNA-derived segment by combining the techniques of Gottlieb and Ojala [13]. A plasmid encoding the M segment under the control of T7 RNA polymerase promoter was constructed and the ssRNA of the M segment with correct 5′ and 3′ ends mimicking natural mRNAs was then transcribed *in vitro*

with T7 RNA polymerase. The cDNA-derived M ssRNA was mixed with natural L and S ssRNAs purified in sucrose gradients, and was subjected to an *in vitro* packaging-replication reaction. After coating on outer shell protein P8 [82], the products (nucleocapsids) were infected onto *P. phaseolicola* spheroplasts [13] and many infectious Φ6 viruses were yielded. This system was confirmed by introducing a 4 bp deletion as a gene marker into the M segment genome of the infectious Φ6 virus. The pioneers, Mindich and colleagues, also constructed Φ6 virions with reporter gene insertions, such as *kan* and *lacα*, in the noncoding regions of the genome segments and these are very stable [83,84]. This *in vitro* packaging-replication system made it possible to study the packaging and replication of the genome in great detail and prepare engineered derivatives of the genome. Using this system, they also elucidated the basic elements of the packaging and replication of three-segmented dsRNA genomes [85–96].

Onodera and colleagues devised the first system to create the replication and packaging of the Φ6 viral genome *in vivo* [14]. They prepared a culture of *P. phaseolicola* carrying a plasmid that transcribes the intact Φ6 L segment ssRNA with SP6 RNA polymerase in the cells. This plasmid also codes for SP6 RNA polymerase and expresses it in the cells. The cells were then infected with replication-incompetent Φ6 derivatives that contain a deleterious deletion in the L segment. The Φ6 derivatives were prepared by the *in vitro* packaging-replication system. This mutant Φ6 could acquire the cDNA-derived transcript encoding the intact L segment genome while infecting the host cell, resulting in the production of reverted infectious Φ6 viruses that possess the functional cDNA-derived L segment as a genome. Furthermore, they created novel engineered live viruses that possess genomic segments joined together so that the number of each segment can be only one or two [97]. In this system, although the transcripts contain an extra sequence at the 5′ and 3′ ends, the resulting segments of isolated infectious viruses are tailored perfectly. This tailoring is achieved by host cellular activities before being packaged into the virion. This new *in vivo* transcript acquisition system has made it possible to isolate a desired mutant virus with greater ease than the previous *in vitro* packaging-replication system. The genome segment(s) of Φ8 has also been manipulated using this system [15,98]. Finally, it is now possible to electroporate three plasmids encoding L, M and S segments, respectively, under T7 or SP6 RNA polymerase promoter into the spheroplasts expressing T7 or SP6 RNA polymerase. This results in the production of many infectious virions [98,99]. The development of the excellent reverse genetics systems for Φ6 and Φ8 phage viruses has already advanced research on dsRNA bacteriophage represented by Φ6, and promises to continue to do so in the future [100]. However, since their host is bacteria, applying the system to the viruses affecting humans is unlikely to be feasible. Conversely, Bamford's group developed an *in vitro* assembly system, in which infectious Φ6 nucleocapsids can be assembled from purified proteins and ssRNA segments in a test tube [101,102]. This system has revealed the sequence of molecular interactions operating during virion assembly [100,101,103]. Although they did not introduce any mutations into the viral genome, this cell-free system apparently has the potential to be another reverse genetics system for *Cystoviridae* members. The application of this cell-free system to reconstitute infectious rotaviruses in a test tube has been hampered, presumably due to the complexities of viral replication processes taking place within the viroplasms in infected cells.

Birnaviruses

IBDV, a member of the family *Birnaviridae*, is the causative agent of a highly immunosuppressive disease in young chickens [104]. This virus contains the bi-segmented dsRNA as a genome surrounded by a single-shelled capsid [105]. The larger segment, A, contains two partly overlapping ORFs and encodes the minor (VP3) and major (VP2) capsid proteins [106]. The smaller segment, B, encodes only one protein, VP1, with RdRp activity [107,108]. VP1 has multiple functions since it is also linked covalently to the 5′ ends of the genomic dsRNA segments (viral protein genome-linked [VPg]) [109]. Both segments contain UTRs of approximately 100 bases at both 5′ and 3′ ends [110]. Some controversies exist regarding the nature of the 5′ ends of birnavirus dsRNA and ssRNA. Whether the viral ssRNAs also contain VPg at the 5′ ends, as dsRNA does, is still unknown. Spies and colleagues have reported that viral ssRNAs contain a cap structure at their ends [108], while Dobos, and Magyar and colleagues reported that the ssRNAs are not capped but most likely contain a VPg at their 5′ ends [111,112]. Conversely, the ssRNAs of these segments lack 3′ polyA tails.

In 1996, Mundt and colleagues developed a reverse genetics system to generate infectious IBDV with the use of synthetic transcripts

derived from cloned cDNAs [10]. Two independent full-length cDNAs containing the entire coding regions and UTRs of each segment A and B under the T7 RNA polymerase promoter were constructed and then used for an *in vitro* transcription reaction with T7 RNA polymerase and cap analog. The synthesized 5′-capped positive-stranded RNAs of each segment were combined and subsequently transfected into Vero cells. The cells generated infectious IBDV as early as 36 h post-transfection. This system was confirmed by introducing new restriction enzyme sites and silent mutations as gene markers into either segment A or B. This advent of a reverse genetics system was soon followed by the generation of IPNV, a prototype virus of the *Birnaviridae* family [11]. These *in vitro* transcription systems demonstrated that only the ssRNAs of both segments are sufficient to initiate the replication cycles of birnaviruses. Furthermore, it was also shown that 5′ capping of the transcripts is required for transfectant virus generation and that the extra sequence at the 3′ end is tailored precisely during replication by an unknown mechanism. Subsequently, Boot and colleagues devised the first system to generate viral ssRNAs intracellularly [12]. They prepared plasmids carrying the full-length IBDV sequences of each segment A or B, flanked by a T7 RNA polymerase promoter and hepatitis delta virus (HDV) ribozyme and followed by a T7 RNA polymerase terminator. The HDV ribozyme has the advantage that only sequences downstream of the cleavage site are required for autocatalytic activity, and they are generally indiscriminate with regard to upstream sequences. Thus, transcription of the resulting vectors in QM5 cells, which had been infected with the recombinant fowlpox virus (FPV-T7) to supply T7 RNA polymerase, allows intracellular production of ssRNAs with virus-like 5′ and 3′ ends, corresponding to both segments A and B. This approach also allowed the generation of transfectant IBDV possessing cDNA-derived genome segments. Recently, Peters and colleagues reported that coexpression of viral structural proteins (VP2 and VP3) *in trans* with viral ssRNAs further improves the efficiency of IBDV production [113]. Both of these reverse genetics systems on birnaviruses have opened the door to the study of all aspects of viral replication, infectivity and pathogenicity [114–132]. Thus, the reverse genetics for the birnaviruses are unexpectedly simple. This might be attributed to the small number (only two) of genome segments in these viruses. The applications of the systems to the *Reoviridae* viruses have all been unsuccessful, presumably due to the high complexity of the viruses.

Reovirus

Mammalian orthoreovirus (reovirus) is a member of the family *Reoviridae*. The name reovirus involves an acronym for a respiratory and enteric orphan, reflecting that the initial isolates were derived from human respiratory and enteric tracts but were not associated with any serious human disease. There are three major reovirus serotypes; serotype (ST)1, -2 and -3. The genome of reovirus consists of ten segmented dsRNAs inside a double-shelled capsid, and the segments are classified into L, M and S size classes that encode the corresponding λ, μ and σ-class viral proteins [133]. Each segment encodes one or two protein(s) and contains short UTRs at both 5′ and 3′ ends. As with a rotavirus, positive-stranded RNAs of this virus are unique in that they possess a 5′ cap structure and lack 3′ polyA tails. The dsRNA genome consists of three L (L1, -2 and -3), three M (M1, -2 and -3) and four S (S1, -2, -3 and -4) segments, and 12 viral proteins are synthesized from the segments in infected cells. Eight of the 12 proteins (λ1, λ2, λ3, μ1, μ2, σ1, σ2 and σ3) are structural, whereas the other four (μNS, μNSC, σNS and σ1S) are nonstructural.

In 1990, Roner and colleagues developed a unique but complicated reverse genetics system [16], in which transfected reovirus RNAs are infectious; this was the only reverse genetics system for the *Reoviridae* family over the last decade. The protocol of this system is as follows; first, the intact ssRNAs and dsRNAs of reovirus ST3 to be transfected were prepared. The ssRNAs were synthesized *in vitro* by a transcription reaction via viral cores and the dsRNAs were purified from virions. L929 cells were transfected with the ten species of prepared reovirus ST3 ssRNA or dsRNA, and with a rabbit reticulocyte lysate (RRL) in which ssRNAs or melted dsRNAs have been translated for 60 min. After 8 h, the transfected cells were infected with reovirus ST2 as the helper virus. The transfected and infected cells were cultured for an additional 23 h and, subsequently, harvested and assayed for the presence of infectious ST3 viruses by plaque assay (ST3 virus forms plaques after 5 days, whereas ST2 helper virus only forms plaques after 12 days), demonstrating the isolation of infectious ST3 viruses. Although the data demonstrating that the isolated viruses were created from the transfected RNAs and the

genetic stability of the viruses were not provided in this study, these were confirmed in their repeated studies [17–19]. The features of this infectious reovirus RNA system are summarized as follows: the mixture of ST3 RNAs (ssRNAs or dsRNAs) and RRL primed with the ST3 RNAs is infectious when introduced into L929 cells that are infected with a ST2 helper virus. It was demonstrated that the material necessary for the generation of progeny genomes is ssRNAs, while dsRNAs activate ssRNAs infectivity in this system. However, it is unclear how dsRNA enhances the ssRNA infectivity, and what the precise role of each ssRNA-primed RRL and ST2 helper virus is, respectively. Irrespective of these uncertainties, they have further generated a double ts mutant using virally produced RNAs obtained from two different single ts mutants [134].

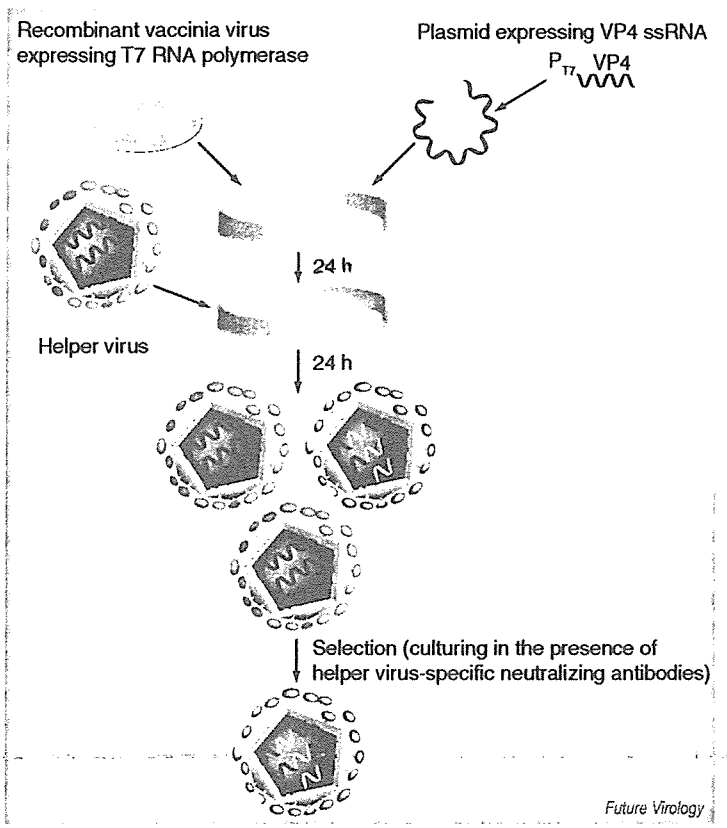
Subsequently, Roner and Joklik optimized their system to rescue a transfectant reovirus possessing the unique cDNA-derived S2 RNA segment [17]. A plasmid carrying the truncated S2 gene and full-length chloramphenicol acetyl transferase (CAT) gene sequences was prepared, flanked by a T7 RNA polymerase promoter and HDV ribozyme, and followed by a T7 RNA polymerase terminator. Thus, transcription and translation of the resulting vector allows the expression of the CAT protein fused with the portion of σ2 protein. Since the σ2-CAT protein no longer exercises σ2 function(s), a cell line (L-ST3.S2) was newly established, which stably expresses intact σ2 proteins. All ten virally produced ssRNAs were incubated with oligonucleotides of the target S2 gene segment to form an RNA–DNA hybrid, which was digested with RNaseH. To rescue a CAT-expressing reovirus, the S2 ssRNA-depleted set of nine ST3 virus ssRNAs was then mixed with *in vitro*-synthesized S2-CAT ssRNA mimicking virus-like 5′ and 3′ ends. Approximately half of the ssRNA mixture was translated in RRL and both this mixture and the untranslated mixture were transfected into L-ST3.S2 cells that had been infected with a ST2 helper virus, yielding stable transfectant ST3 viruses that express CAT proteins. The encapsidation signal sequences on reovirus S2 ssRNA was also calculated using this system [18,19]. Thus, the system has recently worked well, although in the initial process the studies made slow progress. However, there have been no reports on its application in other laboratories or to other *Reoviridae* viruses. One of the reasons why this system has not been adapted by other laboratories may be that it is technically and materially demanding and is relatively complicated.

Rotavirus

As far as is known, since the development of the first rotavirus template-dependent *in vitro* replication system in 1994 [51], whereby the rotavirus open core can direct the synthesis of genomic dsRNA from the cDNA-derived viral mRNA in a cell-free system, no infectious rotavirus transfectants had been rescued at all until our recent breakthrough. This was despite intensive attempts worldwide at establishing a rotavirus reverse genetics system. This long lack of progress had led to pessimism regarding the possibility of the development of a reverse genetics system for rotavirus and made it difficult to determine which methodology was feasible for rotavirus reverse genetics. In the rotavirus replication cycle, mRNAs transcribed from the respective genome segments by viral RdRp are also being used as templates for the synthesis of minus-stranded RNAs, resulting in the formation of genome dsRNAs. Thus, theoretically, a cDNA-derived artificial positive-stranded RNA corresponding to the natural viral mRNA introduced into cells should be recognized and replicated to yield dsRNA by means of RdRp supplied by the helper virus [135], and packaged into the virus particles to produce an infectious virus containing a gene segment derived from the cDNA.

An infectious rotavirus possessing a previously undescribed gene segment derived from cDNA was generated recently by a reverse genetics system with a modified helper virus-driven procedure [20], which was developed originally for the influenza virus by Palese and colleagues (Figure 1) [136–139]. We cloned the full-length VP4 gene of the simian rotavirus strain SA11 (G3P5B), flanked by a T7 RNA polymerase promoter and HDV ribozyme and followed by a T7 RNA polymerase terminator. The resulting plasmid was transfected into COS-7 cells that had been infected previously with the recombinant vaccinia virus to supply T7 RNA polymerase. Each of the authentic 5′ cap and 3′ polyA tail-lacking structure at mRNA ends was to be obtained with vaccinia virus-encoded capping enzyme and the HDV ribozyme sequences, respectively. The transfected cells were infected with the human rotavirus strain KU (G1P1A) as the helper virus 24 h after transfection and then cultured for a further 24 h until harvest. The harvested cultures were passaged in MA104 cells in the presence of P1A-specific antibodies that specifically neutralize the KU helper virus with P1A specificity. Rescued viruses were biologically cloned by three

Figure 1. Reverse genetics system for rotavirus.



A plasmid carrying full-length VP4 sequences under the T7 RNA polymerase promoter is transfected into cells which have been infected with recombinant vaccinia virus to supply the T7 RNA polymerase. The transfected cells are infected with the helper virus 24 h after transfection and then cultured for a further 24 h until harvest. The generated transfectant rotavirus containing the VP4 RNA segment derived from cDNA is rescued from the background of (wild-type) helper viruses by a strong selection system.
ss: Single-stranded.

successive plaque-to-plaque purifications in CV-1 cells. The rescued and cloned virion dsRNAs were subjected to PAGE and reverse transcriptase-PCR analyses and the expressed VP4 proteins on the virions were characterized by enzyme-linked immunosorbent assay analysis with strain-specific antibodies. All of these results revealed the successful rescue of the transfectant virus containing the SA11 VP4 RNA segment derived from cDNA. In addition to the rescued transfectant virus with authentic SA11 VP4 genes, three more infectious rotavirus transfectants, into which silent mutation(s) were introduced to destroy one or both of the two restriction enzyme sites as gene markers in the SA11 VP4 genome, were also rescued with this method. Thus, the utility of this reverse genetics system for rotavirus was confirmed. Furthermore, the rescued viruses are quite stable during serial virus passage in cell culture. However, there is a problem to be considered; the low efficiency of transfectant virus recovery under the presented conditions, indicating a problem with the tight packaging control of the viral genome in rotavirus and other members of the *Reoviridae*. The cause of the problem might be the very strict control of the packaging of the exact number of genome segments per virion, and/or the restricted accessibility of artificial rotavirus mRNAs to viroplasm as suggested by Silvestri and colleagues [37]. However, our development of the first reverse genetics system for rotavirus will enable the production of future prosperous research products with the aid of reverse genetics.

Conclusion

The dsRNA viruses represent a large, diverse group of pathogens (affecting a very wide range of host species) several of which are of medical, veterinary or agricultural importance. For example, rotavirus is recognized as a major global cause of infant mortality. Reverse genetics, a term used in molecular virology, describes the generation of viruses possessing a genome derived from cloned cDNA(s). Now, reverse genetics systems for these dsRNA viruses have been established for members of the *Cystoviridae*, *Birnaviridae* and *Reoviridae* families, although the extent of the accomplishment varies depending on the viruses (Table 1). In cases of the low-numbered segmented dsRNA viruses (*Cystoviridae* and *Birnaviridae*), only mRNAs are sufficient to initiate virus replication cycles. By contrast, in the case of the *Reoviridae* family, attempts to reconstitute infectious viruses by introducing the viral mRNAs into susceptible cells alone have all been unsuccessful in many laboratories. This suggests that generating infectious synthetic *Reoviridae* viruses entirely from cDNAs may require *trans*-complementation by additional viral and host factors involved in virus replications. Recently, a reverse genetics system for rotavirus with a helper virus-driven approach has been established. This approach was modified from the method developed originally for the influenza virus (segmented negative-stranded RNA virus). The ability to alter the genomes of segmented rotaviruses will undoubtedly have a dramatic impact on the way we perceive these viruses. For example, the availability of a new reverse genetics system allows us to elucidate previously ill-defined steps in viral replication that escaped detection with conventional methods of genome manipulation.