Generation of Genetically Stable Recombinant Rotaviruses Containing Novel Genome Rearrangements and Heterologous Sequences by Reverse Genetics

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The rotavirus (RV) genome consists of 11 segments of double-stranded RNA (dsRNA). Typically, each segment contains 5' and 3' untranslated regions (UTRs) that flank an open reading frame (ORF) encoding a single protein. RV variants with segments of atypical size owing to sequence rearrangements have been described. In many cases, the rearrangement originates from a partial head-to-tail sequence duplication that initiates after the stop codon of the ORF, leaving the protein product of the segment unaffected. To probe the limits of the RV genome to accommodate additional genetic sequence, we used reverse genetics to insert duplications (analogous to synthetic rearrangements) and heterologous sequences into the 3' UTR of the segment encoding NSP2 (gene 8). The approach allowed the recovery of recombinant RVs that contained sequence duplications (up to 200 bp) and heterologous sequences, including those for FLAG, the hepatitis C virus E2 epitope, and the internal ribosome entry site of cricket paralysis virus. The recombinant RVs grew to high titer ($>10^7$ PFU/ml) and remained genetically stable during serial passage. Despite their longer 3' UTRs, rearranged RNAs of recombinant RVs expressed wild-type levels of protein *in vivo*. Competitive growth experiments indicated that, unlike RV segments with naturally occurring sequence duplications, genetically engineered segments were less efficiently packaged into progeny viruses. Thus, features of naturally occurring rearranged segments, other than their increased length, contribute to their enhanced packaging phenotype. Our results define strategies for developing recombinant RVs as expression vectors, potentially leading to next-generation RV vaccines that induce protection against other infectious agents.

Rotaviruses (RVs), members of the *Reoviridae* family, are the primary cause of life-threatening gastroenteritis in infants and young children (1, 2). The RV genome consists of 11 segments of double-stranded RNA (dsRNA) and is encapsidated within an icosahedral virion formed by three concentric protein layers (3, 4). During RV entry into the cell, the outer protein layer shell is lost, yielding a double-layered particle (DLP) that is transcriptionally active and produces viral positive-sense RNAs (+RNAs) (5–7). The +RNAs have 5'-methylated caps but are not polyadenylated (8). Most RV +RNAs contain a single open reading frame (ORF) that is flanked by 5' and 3' untranslated regions (UTRs) (9, 10). Segment 11 is the exception for group A RVs, as it includes two overlapping, out-of-frame ORFs (11). The length and sequences of the UTRs differ among the 11 genome segments, except at the extreme termini of the +RNAs, where highly conserved sequences (consensus sequences [CSs]) are present (1, 9, 10). The 3' CS contains *cis*-acting elements that promote the use of +RNAs as the templates for dsRNA synthesis and translation (12–16). Sequence complementarity between the 5' and 3' UTRs is predicted to cause RV +RNAs to fold such that they have long 5'-3' panhandle structures (9, 17). Projecting from the panhandles are stem-loop structures that are unique for each of the 11 types of +RNAs (9). The stem-loop structures are proposed to function as segment-specific packaging signals (15).

The dsRNA genome of prototypic group A RVs is approximately 18,600 bp in size (10). Analyses of RV variants that contain one or more genome segments of unusually large size indicate that the RV capsid can hold an additional 1,800 bp of dsRNA (18). These atypical segments contain viral sequence rearrangements, typically originating from partial head-to-tail duplication of seg-

ment sequences (19, 20). In many cases, the duplication initiates after the stop codon of the ORF and generates a genome segment with an extended 3' UTR (21–25). RVs with sequence duplications involving segments encoding NSP1 (gene 5 RNA) (25, 26), VP6 (gene 6) (27), NSP2 (gene 8) (28), NSP3 (gene 7) (21, 26, 29), NSP4 (gene 10) (22), and NSP5/6 (gene 11) (23, 24) have been described. Rearrangements that involve sequence deletions have also been reported, but these are relatively rare (30, 31). RVs with rearranged genomes have been isolated from chronically infected immunodeficient children (32–34) and can be generated *in vitro* by serial passage of virus at a high multiplicity of infection (MOI) (21, 28, 35). The mechanism by which rearrangements are formed is not understood but may involve dissociation and reassociation of the viral RNA polymerase with the template during transcription (19, 23, 24, 36).

High-MOI serial passage of wild-type RVs with RVs that have rearranged segments yields virus progeny that predominantly contain the rearranged segment (35, 37). The molecular basis for this phenomenon is not entirely clear but may result from duplication of packaging signals in the rearranged segment, thereby

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TABLE 1 Primers used in constructing recombinant rotaviruses

Primer	Sequence
g8 ^R -1	AACGGATAGAAAAATGGACGAAGTTTCTCAAGTTGGCGTTTAAGACTACAAGGACGACGATGACAAGTTAAT
g8 ^R -2	TAACCTTGTCATCGTCGTCCTTGTAGTCTTAAACGCCAACTTGAGAACTTCGTCCATTTTCTATCCGTT
g8 ^R -3	AACGGATAGAAAAATGGACGAAGTTTCTCAAGTTGGCGTTTAAGTATTAAACCAACGGCAGCCAGTTAAT
g8 ^R -4	TAACCTGGCTGCCGTGGTGTAAATCAGTTAAACGCCAACTTGAGAACTTCGTCCATTTTCTATCCGTT
g8 ^R -5	GCGCTTAATTAAGCAAAAATGTGATCTTGCTTG
g8 ^R -6	GCGCTTAATTAAGGTAAATTTCTTAGGTTTTTC

increasing its probability of encapsidation. Indeed, Troupin et al. (38) were able to develop an RV reverse genetics system based on a previously described, spontaneously occurring rearrangement in the gene 7 RNA of the bovine RV strain RF (29). By extensive serial passage of lysates from cells infected with wild-type RV RF and transfected with a T7 expression plasmid producing the rearranged gene 7 RNA, the authors recovered a recombinant RV containing the rearranged RNA (38).

In the present study, we assessed the capacity of the RV genome to accommodate additional genetic information using a recently developed reverse genetics system (39, 40). The results show that both synthetic rearrangements and heterologous (nonrotaviral) sequences can be introduced into the 3' UTR of RV genome segments and that recombinant RVs containing these sequences grow to high titers and are genetically stable. The ability to engineer an internal ribosome entry site (IRES) into the viral genome provides a foundation for developing recombinant RVs as expression vectors, potentially leading to next-generation RV vaccines that induce protection against other infectious agents.

MATERIALS AND METHODS

Cells and viruses. Fetal rhesus monkey kidney (MA104 and MA104-g8D) cells were grown in medium 199 containing 5% fetal bovine serum (FBS), while African green monkey kidney (COS-7) fibroblasts were grown in Dulbecco's minimum essential medium (D-MEM; Quality Biological) containing 10% FBS (41). Production of a MA104 cell line that expresses a short hairpin RNA (shRNA) specific to the SA11 genome segment (gene) 8 RNA (MA104-g8D cells) was described previously (39). Stocks of the RV SA11 temperature-sensitive (*ts*) mutant *tsE* (genotype G3P[2]N5) and a replication-defective vaccinia virus expressing T7 RNA polymerase, rDIs-T7pol, were amplified, and titers were determined as previously described (39, 41). RV was treated with 5 µg per ml of porcine pancreas trypsin (type IX-S; Sigma-Aldrich) for 1 h at 37°C prior to use (41).

Plasmid construction. The plasmid pBS-SA11g8^R contains a full-length gene 8 cDNA that is flanked upstream by the T7 RNA polymerase promoter sequence and downstream by a hepatitis delta virus (HDV) ribozyme and T7 polymerase terminator (31). The plasmid pBS-SA11g8^R_{PacI} was made by digesting pBS-SA11g8^R with the restriction enzymes HincII and RsrII, allowing deletion of the last 43 bp of the NSP2 ORF, the 3' UTR, and the first 30 bp of the HDV ribozyme. The deleted region was replaced with an identical chemically synthesized sequence (GenScript Corp.), except that it also included a PacI site at the beginning of the 3' UTR. To introduce sequence duplications into the SA11g8^R_{PacI} cDNA, sequences that contained the last 43 bp of the NSP2 ORF (including its stop codon), followed by a duplication of the 3'-terminal 25, 50, 100, 200, 400, or 800 bp of the NSP2 ORF, and that were flanked by restriction sites HincII and PacI were chemically synthesized. Ligation of the sequences into pBS-SA11g8^R_{PacI} (that had been digested with HincII and PacI) yielded plasmids pBS-SA11g8^R-3'25D (duplication), -50D, -100D, -200D, -400D, and -800D. To generate pBS-SA11g8^R-3'FLAG, the primer pair g8^R-1 and g8^R-2 (Table 1) containing FLAG epitope sequences was annealed and ligated into the pBS-SA11g8^R_{PacI} plasmid (di-

gested with HincII and PacI). Similarly, to generate the plasmid pBS-SA11g8^R-3'HCV2, the primer pair g8^R-3 and g8^R-4 (Table 1) containing hepatitis C virus type 2 (HCV2) E2 epitope sequences was annealed and ligated into the pBS-SA11g8^R_{PacI} plasmid. To generate pBS-SA11g8^R-3'CrPV, the primer pair g8^R-5 and g8^R-6 (Table 1) was used to amplify the cricket paralysis virus (CrPV) IRES sequence in the plasmid pMK-g8^Rre-CrPV-EGFP (GeneArt, Inc.) and to add flanking PacI sites. The sequence of the 190-nucleotide (nt) CrPV IRES in the plasmid is given under GenBank accession number AF218039. The amplified CrPV IRES was digested with PacI and ligated into pBS-SA11g8^R_{PacI}.

Generation and recovery of recombinant viruses. Recombinant viruses were generated using the reverse genetic system developed by Trask et al. (39, 40). In brief, COS-7 cells were infected with rDIs-T7pol and transfected with pBS-SA11g8^R or a derivative plasmid, providing for the transcription of gene 8 + RNAs. Cells were then infected with *tsE*, a mutant virus encoding a *ts* NSP2 protein, and maintained at the permissive temperature (30°C). Recombinant viruses were recovered by sequential passage of infected cell lysates on MA104-g8D cells at a nonpermissive temperature (39°C), followed by three cycles of plaque-to-plaque purification. Recombinant gene 8 RNAs are insensitive to the effects of the gene 8-specific shRNA produced in MA104-g8D cells due to the introduction of mutations in the siRNA target site (39).

RT-PCR and sequencing. Total RNA was isolated from infected cell lysates using a QIAamp viral RNA minikit (Qiagen). A SuperScript One-Step RT-PCR kit (Invitrogen) was used to carry out reverse transcription-PCR (RT-PCR). RT-PCR products were purified using a QIAquick PCR purification kit (Qiagen). Sequencing was performed using an ABI Prism BigDye v3.1 Terminator sequencing kit and an Applied Biosystems 3730 DNA analyzer. The sequencing data were analyzed with Sequencher v4.9 software (Gene Codes Corp.).

Electrophoretic analysis of dsRNA. Viral RNA recovered from infected cell lysates was analyzed by polyacrylamide gel electrophoresis (PAGE) on 12% polyacrylamide gels containing sodium dodecyl sulfate (SDS) (42). dsRNA was detected by staining gels with SYBR Gold (Invitrogen).

Electrophoretic analysis of protein. MA104 cells were infected with RV at an MOI of 2. Infected cell lysates were mixed with Novex Tris-glycine SDS sample buffer (Invitrogen) containing 2-mercaptoethanol, denatured by heating to 95°C for 10 min, and subjected to electrophoresis on 4 to 20% polyacrylamide gels (Invitrogen). After transferring the resolved proteins onto nitrocellulose membranes, the membranes were treated with Odyssey blocking solution (LI-COR) and incubated overnight at 4°C with mouse monoclonal anti-NSP2 (5E2) antibody (kindly provided by C. D. Kirkwood) diluted 1:3,000 and guinea pig polyclonal anti-VP6 antibody (43) diluted 1:2,500 in blocking solution with 0.2% Tween 20. The membranes were washed three times with phosphate-buffered saline (PBS) containing 0.2% Tween 20 and then incubated with IRDye680- or IRDye800-conjugated secondary antibodies (diluted 1:15,000; LI-COR) in PBS containing 0.2% Tween 20 and 1% dry milk (Carnation). Following incubation with secondary antibody, membranes were washed three times with PBS containing 0.2% Tween 20. Membranes were analyzed using an Odyssey infrared imaging system (LI-COR).

FIG 1 Conservation of predicted panhandle (PH) and stem-loop (SL) structures of recombinant gene 8 + RNAs. Portions of the predicted secondary structures of SA11 gene 8 + RNAs showing panhandle and stem-loop structures formed by interactions of 5' and 3' UTRs (boxed) are shown. Panhandle and stem-loop structures were conserved among all recombinant gene 8 + RNAs introduced into a stem-loop; however, variations in the predicted structures of sequences outside the panhandle and stem-loop regions were noted (e.g., $g8^R$ versus $g8^R$ -3'CrPV). Also labeled are the start and stop codons (black and red arrowheads, respectively) of the NSP2 ORF. The location of the 8-mer UUAUUUCG sequence that was changed to UUAUUUAA to create a PacI site is shown. The amino acid triplet that forms the stop codon is underlined. Secondary structures associated with minimum free energy were calculated using *RNAfold* (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) and color coded to indicate base-pairing probability (44, 45).

Single-cycle growth kinetics. MA104 cells were infected with RV at an MOI of 5 PFU per cell. At the indicated times after infection, cells were freeze-thawed twice in their medium, and the cell lysate was centrifuged at $850 \times g$ for 5 min to remove large cellular debris. The titers of virus in clarified cell lysates were determined by plaque assay on MA104 cells (41).

Serial passage of recombinant viruses. MA104 cells were initially infected with RV at an MOI of 2, and when cytopathic effects (CPEs) were complete, the cells were freeze-thawed twice in their medium and the lysates were clarified by centrifugation at $850 \times g$ for 5 min. Virus in clarified lysates was passaged serially 10 times, using 1 volume of the lysate and 2 volumes of fresh medium to prepare the inoculum (high MOI). Double-stranded RNA in clarified lysates was examined by SDS-PAGE, and the gene 8 RNA of passaged virus was analyzed by sequencing.

Competition assays. MA104 cells were coinfecting with two different RVs, each at an MOI of 0.5. Viruses in infected cell lysates were serially passaged 10 times (as described above), and the dsRNA recovered from lysates was analyzed by SDS-PAGE.

Modeling of RNA secondary structures. Minimum free energy secondary structure and base pair probabilities were calculated for the RV SA11 gene 8 + RNA using the *RNAfold* web server (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) (44, 45).

Nucleotide sequence accession numbers. The gene 8 RNA sequences of recombinant viruses are given under the following GenBank accession numbers: $g8^R$, [KC881021](#); $g8^R$ -PacI, [KC881022](#); $g8^R$ -3'25D, [KC881023](#); $g8^R$ -3'50D, [KC881024](#); $g8^R$ -3'100D, [KC881025](#); $g8^R$ -3'200D, [KC881026](#); $g8^R$ -3'FLAG, [KC881027](#); $g8^R$ -3'HCV2, [KC881028](#); and $g8^R$ -3'CrPV, [KC881029](#).

RESULTS

Strategy to introduce a unique cloning site into the 3' UTR of gene 8 cDNA. The predicted secondary structure of the gene 8 + RNA (SA11 strain) was analyzed to identify an appropriate position at which to introduce a cloning site that could be used for insertion of additional genetic material. As explained above, it is presumed that the panhandle and stem-loop structures formed by the 5' and 3' UTRs of the gene 8 + RNA represent *cis*-acting signals important for RNA packaging (Fig. 1). Guided by this, we prepared pBS-SA11 $g8^R$ -PacI, a T7 transcription vector that contains a full-length gene 8 cDNA with a PacI site positioned in the gene 8 3' UTR, immediately downstream of the NSP2 ORF stop codon (Fig. 2). Introduction of the PacI site at this position did not alter

FIG 2 Organization of recombinant gene 8 RNAs engineered into RVs. Names given to RVs containing the indicated recombinant gene 8 RNA are shown on the left. Also labeled are locations of the NSP2 ORF and 5' and 3' CSs and UTRs. The engineered PacI site shown in the SA11g8^R_{PacI} virus was used to produce viruses that contained viral sequence duplications (SA11g8^R-3'-25D, -50D, -100D, and -200D) and heterologous sequences (SA11g8^R-3'-FLAG, -HCV2, and -CrPV). The green-and-white-striped box identifies the NSP2 ORF region that was selected for duplication in the gene 8 RNA, and the green-and-gray-striped box identifies the position of the duplicated sequence. The blue and red boxes identify heterologous sequences. Note that the duplications and insertions were placed into 3' UTR immediately downstream of the NSP2 stop codon, thus increasing the length of the 3' UTR.

the predicted structure of the panhandle or stem-loop of the gene 8 + RNA (Fig. 1, g8^R versus g8^R_{PacI}).

To evaluate whether insertion of the PacI site influenced RV replication, plasmid pBS-SA11g8^R_{PacI} was used to generate the recombinant SA11g8^R_{PacI} virus by reverse genetics (20). Sequence analysis of the gene 8 RNA of plaque-purified SA11g8^R_{PacI} demonstrated the presence of the PacI site (Fig. 3B), verifying its recombinant origin. Electrophoretic analysis of the genome of SA11g8^R_{PacI} showed that the gene 8 (PacI) RNA had the same electrophoretic mobility as the gene 8 RNA of SA11g8^R (Fig. 3A). We determined that the SA11g8^R_{PacI} virus reached a titer equivalent to that of SA11g8^R in single-cycle growth experiments (Fig. 3C), suggesting that SA11g8^R_{PacI} was wild type-like in its characteristics and that the gene 8 (PacI) site was a suitable target for further mutagenesis.

Introduction of sequence duplications into the RV genome.

Once we had determined that placement of the PacI site into the 3' UTR did not affect viral replication, we used the site for introducing gene 8 sequence duplications into the viral genome. To accomplish this, duplications of the 3'-most 25, 50, 100, 200, 400, and

800 bp of the NSP2 ORF were inserted into the gene 8 3' UTR of pBS-SA11g8^R_{PacI} (Fig. 2) immediately upstream of the PacI site, resulting in plasmids pBS-SA11g8^R-3'25D, -50D, -100D, -200D, -400D, and -800D, respectively. These insertions did not alter the NSP2-coding potential since the sequence duplications initiated downstream from the stop codon of the gene 8 ORF. Moreover, the predicted secondary structures of gene 8 +RNAs with sequence duplications retained the same panhandle and stem-loop as those of wild-type gene 8 +RNA (Fig. 1). Using reverse genetics, we successfully recovered recombinant RVs that contained 25-, 50-, 100-, and 200-bp duplications. We were unable to recover recombinant viruses from plasmids pBS-SA11g8^R-3'400D and -800D, despite multiple attempts. Sequence analysis was used to confirm the presence of duplicated viral sequences in the recombinant viruses (data not shown). As expected, electrophoretic analysis of the dsRNA genomes of recombinant RVs showed a decrease in mobility of the gene 8 RNA that was generally proportional to the increase in the length of the inserted duplication (Fig. 4A). Single-cycle growth curves of recombinant RVs with gene 8 sequence duplications did not significantly differ from the curve of the recombinant virus SA11g8^R (Fig. 4B), indicating that the gene 8 sequence duplications had little or no effect on virus replication.

To determine whether viruses carrying engineered gene 8 sequence duplications were genetically stable, recombinant RVs containing the 25-, 50-, 100-, and 200-bp duplications were serially passaged 10 times at a high MOI in MA104 cells. The genomes of the passaged viruses were examined by gel electrophoresis, and their gene 8 RNAs were sequenced. As illustrated in Fig. 5 for the SA11g8^R-3'200D virus, no changes in the electropherotype of the viruses were noted during sequential passage (data of the same nature were obtained for SA11g8^R-3'25, -50D, and -100D; not shown). Likewise, sequencing revealed no changes in the gene 8 RNA of the recombinant viruses during serial passage (data not shown).

Introduction of duplications in SA11g8^R-3'25, -50D, -100D, and -200D viruses resulted in the synthesis of gene 8 +RNAs in infected cells with 3' UTRs (84, 109, 159, and 259 nt, respectively) that were much longer than the 59-nt 3' UTR of wild-type RV. Because features of the 3' UTR may impact the translational activity of mRNAs, we probed whether the extended 3' UTRs of the recombinant viruses affected NSP2 expression. To address this question, MA104 cells were infected with the recombinant viruses, and at 8 h postinfection (p.i.), the levels of NSP2 relative to those of VP6 were determined by immunoblot assay. The results showed no evidence that the extended 3' UTRs of the recombinant viruses affected NSP2 expression (Fig. 6), indicating that sequence duplications in the 3' UTR (up to 200 bp) did not alter the translational efficiency of RV +RNAs. These results are consistent with the observation that the SA11g8^R-3'25, -50D, -100D, and -200D viruses grew to titers comparable to the titer of SA11g8^R, a virus with a wild-type gene 8 RNA (Fig. 4B).

Introduction of genetically stable nonviral sequences into the RV genome. The capacity to produce genetically stable recombinant RVs containing sequences of non-RV origin would represent an important advance in developing RVs as expression vectors and for creating next-generation vaccines. Toward this end, we inserted sequences of either the 24-nt FLAG tag or HCV2 E2 epitope (46, 47) into the 3' UTR PacI site of pBS-SA11g8^R_{PacI} (Fig. 2). Because these sequences were placed downstream of the

FIG 3 Characteristics of the SA11g8^R_{PacI} virus. (A) Genomes of recombinant SA11g8^R and SA11g8^R_{PacI} viruses resolved by SDS-PAGE and stained with SYBR Gold. The positions of the 11 viral genome segments are labeled. The position of the gene 8 RNA containing the engineered PacI site is denoted with an arrow. (B) Sequencing chromatograph of the region of the gene 8 RNA modified to contain a PacI site. (C) Single-cycle growth curves of SA11g8^R and SA11g8^R_{PacI} viruses in MA104 cells infected at an MOI of 5.

NSP2 stop codon, polypeptides for FLAG and the HCV2 E2 epitope were not expressed by the gene 8 + RNAs (g8^R-3'FLAG, g8^R-3'HCV2) made from these plasmids. The predicted secondary structures of g8^R-3'FLAG and g8^R-3'HCV2 RNAs contained the same panhandle and stem-loop as wild-type gene 8 + RNA (Fig. 1). The mutagenized plasmids were used in reverse genetics

experiments to generate the recombinant RVs SA11g8^R-3'FLAG and SA11g8^R-3'HCV2. Sequencing (data not shown) and electrophoretic analysis (Fig. 7A) of the viruses confirmed the presence of the heterologous FLAG and HCV sequences in the gene 8 RNAs. Attempts to create a recombinant RV in which a FLAG epitope was fused to the end of NSP2 were unsuccessful. This result is

FIG 4 Characteristics of SA11 viruses containing gene 8 sequence duplications. (A) Genomes of SA11g8^R and SA11g8^R-3'-25D, -50D, -100D, and -200D viruses analyzed by SDS-PAGE. The positions of the 11 viral genome segments are labeled. (B) Single-cycle growth curves of viruses were performed as described in the legend to Fig. 3.

FIG 5 Genetic stability of the SA11g8^R-3'200D virus. The genomes of SA11g8^R (first lane) and SA11g8^R-3'200D viruses serially passed through 10 cycles (lanes P2 to P10) in MA104 cells were analyzed electrophoretically. The positions of the 11 viral genome segments are labeled. The position of the gene 8 RNA containing the 200-nt duplication is denoted with an arrow.

consistent with that of an earlier analysis performed with an NSP2 complementation system, which indicated that addition of extra residues to the C terminus of NSP2 rendered the protein nonfunctional in supporting replication (48).

Single-cycle growth curves revealed that, despite the presence of heterologous sequences, SA11g8^R-3'FLAG and SA11g8^R-3'HCV2 grew well ($>10^7$ PFU/ml), reaching peak titers that were only slightly reduced (approximately 4-fold) from the titer of the wild-type SA11g8^R (Fig. 7B). To examine the genetic stability of the modified gene 8 RNAs, the recombinant viruses were passaged at a high MOI for 10 cycles in MA104 cells. Electrophoresis and sequence analysis of SA11g8^R-3'FLAG (Fig. 8) and SA11g8^R-3'HCV2 (data not shown) indicated that the modified gene 8 RNAs were stable. As was observed for recombinant RVs containing duplicated gene 8 sequences, SA11g8^R-3'FLAG and SA11g8^R-3'HCV2 expressed levels of NSP2 in infected cells comparable to the level of SA11g8^R (Fig. 6).

Introduction of the CrPV IRES into the RV genome. As a next step in developing RVs as vector systems for expressing foreign proteins, we explored the possibility of introducing an IRES into the *PacI* site of the SA11g8^R_{*PacI*} virus. For this purpose, we selected the 190-nt CrPV IRES because of its small size (relative to other IRESs) and the limited number of translation factors associated with its function (49, 50). The CrPV IRES sequence was inserted into the *PacI* site of the pBS-SA11g8^R_{*PacI*} plasmid, creating the plasmid pBS-g8^R-3'CrPV (Fig. 2). Despite the 190-nt size of the IRES insertion, the calculated secondary structure of the gene 8 + RNA transcribed from the plasmid retained the panhandle and stem-loop of wild-type gene 8 + RNA (Fig. 1). The plasmid was successfully used in reverse genetics experiments to generate the recombinant virus SA11g8^R-3'CrPV. Electrophoresis (Fig. 7A) and sequencing (data not shown) of SA11g8^R-3'CrPV confirmed incorporation of the CrPV IRES sequence into the gene 8 RNA of the virus. Because the IRES was inserted downstream of the NSP2 ORF, SA11g8^R-3'CrPV produced wild-type NSP2, and its level of NSP2 expression in infected cells was indistinguishable

FIG 6 Expression of NSP2 and VP6 by recombinant viruses. MA104 cells were mock infected or infected at an MOI of 2 with the indicated virus and harvested at 8 h p.i. Cellular lysates were resolved by SDS-PAGE and analyzed by immunoblot assay using mouse anti-NSP2 monoclonal antibody and guinea pig anti-VP6 polyclonal antiserum. Purified SA11 RV virions were analyzed in parallel as a control (lane RV). Lane MW, molecular weight marker (numbers on the left are molecular weights in thousands).

from that of SA11g8^R (Fig. 6). The single-cycle growth curves of SA11g8^R versus SA11g8^R-3'CrPV over a 24-h period were analyzed by plaque assay. As shown in Fig. 7C, the replication kinetics of the two viruses were essentially identical, indicating that insertion of the IRES had no effect on virus growth. Likewise, SA11g8^R-3'CrPV produced NSP2 at levels similar to those at which it was produced by SA11g8^R. On the basis of serial passage (10 cycles) of SA11g8^R-3'CrPV in MA104 cells, the recombinant gene 8 RNA was genetically stable (data not shown).

Engineered gene 8 RNAs with duplicated or heterologous sequences are at a competitive disadvantage over wild-type gene 8 RNAs during virus replication. Previous reports have shown that high-MOI serial passage of wild-type RVs with RVs that have naturally occurring rearranged genome segments produces progeny that predominantly contain the rearranged segment (35, 37). To determine whether the synthetic rearranged gene 8 segments of the recombinant RVs produced in this study similarly outcompeted the wild-type gene 8 segment, we coinfect MA104 cells with RVs containing a sequence duplication (SA11g8^R-3'25D, -50D, -100D, or -200D) or heterologous sequence (SA11g8^R-3'FLAG or -CrPV) and with the SA11g8^R wild type. After passage of infected cell lysates for 10 cycles at a high MOI, the genomes of virus progeny were evaluated by SDS-PAGE and by sequencing of gene 8 RNAs. The results showed that relative to wild-type gene 8 RNA, RNAs with duplicated or heterologous gene 8 sequences were barely detectable in virus progeny after even a single round of virus passage in MA104 cells (Fig. 9). After 10 rounds of passage, progeny with duplicated or heterologous gene 8 sequences were undetectable (Fig. 9). These results indicate that introduction of artificially duplicated and heterologous sequences into its 3' UTR did not provide a replication advantage to the gene 8 RNA. In fact, our results indicate that the synthetic rearranged gene 8 RNAs were replicated less efficiently than the wild-type gene 8 RNA.

DISCUSSION

RV genome rearrangements result from the partial head-to-tail duplication of RNA sequence (19, 20). In the most extreme cases, such duplication increases the length of an RNA segment by 80 to 90% relative to that of the wild type (21, 23). Indeed, the only head-to-tail duplication described to date for the gene 8 RNA increased its length by 88% (28). This rearranged gene 8 RNA was detected in Wag7/8re, an RV variant generated by serial passage of

FIG 7 Characteristics of viruses containing heterologous sequences. (A) Genomes of SA11g8^R, SA11g8^R-3'FLAG, SA11g8^R-3'HCV2, and SA11g8^R-3'CrPV viruses. The positions of the 11 viral genome segments are labeled. (B, C) Single-cycle growth curves of SA11g8^R, SA11g8^R-3'FLAG, and SA11g8^R-3'HCV2 (B) and SA11g8^R and SA11g8^R-3'CrPV (C).

human Wa virus for 11 cycles in gnotobiotic pigs and 33 cycles in MA104 cells (28) (annotation for GenBank accession number FJ423142). In general, though, duplications detected in RV RNA segments increase the length of the RNA by much less than 80 to

90% and commonly less than 50% (19, 21, 25, 36). Besides RVs, other members of the *Reoviridae* with genome segments containing sequence rearrangements have been described; these include bluetongue virus (51), cypovirus (52), epizootic hemorrhagic disease virus (53), mycoreovirus (54), and rice dwarf virus (55). There are no previous reports of heterologous (nonrotaviral) sequences being successfully introduced into the RV genome by reverse genetics.

Here, we have used reverse genetics to produce recombinant RVs that contain duplicated ORF sequences inserted into the 3' UTR of the gene 8 RNA. These viruses grew well and were genetically stable. Unlike the duplicated sequences engineered by Troupin et al. (38) into the 3' UTR of gene 7 (NSP3), the duplicated gene 8 sequences engineered in our study were not based on previous descriptions of naturally occurring rearrangements. For the naturally occurring variant Wag7/8re, the gene 8 sequence duplication initiates 12 nt downstream of the NSP2 stop codon, while in the engineered gene 8 sequences of recombinant viruses, the duplication initiates immediately downstream of the stop codon (28). Thus, the gene 8 duplications do not affect the NSP2-coding capacity of either Wag7/8re or the recombinant viruses. A significant feature of the gene 8 segment of Wag7/8re is that it contains a 927-nt duplication, yielding a dsRNA with a total length of 1,986 bp (28). Since the Wag7/8re virus is viable, the gene 8 RNA must retain functional signals for packaging, replication, and translation, despite its long sequence duplication. Notably, the predicted secondary structure of the Wag7/8re gene 8 + RNA includes the same panhandle and stem-loop structures present in the gene 8 + RNAs of wild-type and recombinant viruses (data not shown).

FIG 8 Genetic stability of the SA11g8^R-3'FLAG virus. Electrophoretic analysis of the genomes of SA11g8^R and SA11g8^R-3'FLAG serially passed through 10 cycles (results for passages 2 to 10 are shown in lanes P2 to P10) in MA104 cells. Due to partial loss of sample, less dsRNA was loaded into the P2 lane. The positions of the 11 viral genome segments are labeled. The position of the gene 8 RNA containing the FLAG sequence site is denoted with an arrow.

FIG 9 Viral progeny formed in cells infected with mixtures of recombinant viruses. MA104 cells were initially infected with SA11g8^R and SA11g8^R-3'25D, -50D, -100D, -200D, -FLAG, or -CrPV, each at an MOI of 0.5. During subsequent passages, cells were infected with inoculum prepared by combining 1 part infected cell lysate with 2 parts fresh medium. Genomes of viruses in passage 1 (P1) and 10 (P10) lysates were analyzed by SDS-PAGE. Genomes of viruses in the initial inoculum were resolved in lanes labeled I. The positions of the 11 viral genome segments are labeled. Arrowheads indicate the position of the rearranged segment in virus samples in lanes I and P1. Rearranged segments were not detectable in samples in lane P10.

Despite repeated attempts, we were unable to recover recombinant viruses with 400- and 800-nt duplications in the gene 8 RNA (39, 40). This failure may represent a technological limitation of the reverse genetics system, one that could be due to the decreased transcriptional efficiency of longer versus shorter elongated gene 8 +RNAs by T7 RNA polymerase in rDis-T7pol/*tsE*-infected cells. As a result, the formation of recombinant viruses with 400- and 800-nt duplications may have occurred at levels too low to overcome the threshold of background viruses generated by back mutation of the *tsE* helper virus to a wild-type phenotype. Alternatively, it is possible that the 400- and 800-nt duplications in the gene 8 +RNAs generated transcripts incapable of supporting one or more aspects of viral RNA function. On the basis of comparison with the gene 8 RNA of Wag7/8re, it is unlikely that insertion of 400- and 800-nt sequences into the 1,059-nt gene 8 RNA would produce rearranged RNAs too long to be functional. Analysis of the predicted secondary structures of gene 8 +RNAs with the 400- and 800-nt duplications showed that they retained the panhandle and stem-loop of wild-type gene 8 +RNAs, suggesting the presence of key *cis*-acting signals necessary for packaging and replication (data not shown). We cannot rule out the possibility, though, that the 400- and 800-nt insertions may have altered the tertiary structures of the RNAs, such that interactions of viral proteins or other RNAs with the *cis*-acting signals were sterically hindered.

Despite the apparent limitation of inserting longer sequence duplications into the gene 8 RNA using the reverse genetics system, we reasoned that it should be possible to introduce foreign genetic material into the RV genome. Indeed, we were able to create recombinant RVs containing sequences for the FLAG tag, the HCV2 E2 epitope, and the CrPV IRES in the gene 8 3' UTR. These sequences were genetically stable and had little or no effect on the growth characteristics of the virus; the insertions also did not affect the level of NSP2 expression. Similarly, we have been able to generate recombinant RVs containing the 59-nt HIV Tar

sequence (56) or 1 or 2 copies of the 25-nt PP7 and 19-nt MS2 operators that are recognized by the PP7 and MS2 bacteriophage coat proteins, respectively (57; S. D. Trask, A. Navarro, and J. T. Patton, unpublished data), confirming the capacity of gene 8 RNA to tolerate insertions of highly structured RNA elements into its 3' UTR at the *PacI* site. Application of reverse genetics technologies has shown that heterologous sequences can be inserted into the genomes of at least some other members of the *Reoviridae* as well (58–60).

RV RNAs with naturally occurring sequence duplications have been identifiable in mixed virus pools because of their capacity to outcompete homologous wild-type RNAs at some step during virus replication (35, 37). It is possible that this preferred replication phenomenon results from the duplication of packaging signals in the rearranged RNA. However, this explanation is not entirely satisfactory, since RNAs with naturally occurring rearrangements are typified by the presence of partially duplicated ORF sequences and not UTR sequences and thus lack duplication of the 3' UTR panhandle and stem-loop structures presumed to function in RNA packaging. Our analyses of recombinant RVs containing synthetic gene 8 duplications indicate that their rearranged RNAs were not preferentially packaged. Indeed, the opposite was true; the introduction of wild-type gene 8 RNAs into progeny viruses was vastly favored over that of the synthetic rearranged gene 8 RNAs (Fig. 9). This observation also held true for gene 8 RNAs containing heterologous sequences. Extrapolating from these observations, it may be the case that rearrangements of the RV genome occur more often than previously thought in infected cells but that many (and perhaps most) of the rearranged RNAs are less fit than wild-type RNAs and thus are lost from the population. Taken together, our results indicate that the preferred packaging phenotype of naturally occurring rearranged RNA is not explained simply by its increased length and that instead other factors, perhaps including the tertiary structure of the RNA, may be primary determinants of packaging efficiency.

The fact that RV grows efficiently and remains genetically stable even upon introduction of heterologous sequences supports the concept that RVs can be developed as vectors for expression of foreign proteins. On the basis of our results, it seems plausible that foreign protein expression could be driven through the introduction of an IRES followed by an ORF inserted into the 3' UTR of RV RNAs. Alternatively, foreign protein expression could be directed through insertion of 2A StopGo elements (61) or stop codon read-through elements (62, 63) between an upstream RV protein and a downstream foreign protein in a single ORF. Maximizing the potential of RV as an expression vector will require the development of an improved reverse genetics system, particularly one that can support the efficient manipulation of viral genes other than gene 8 and the insertion of long heterologous sequences. Our results suggest that reverse genetics technologies will ultimately enable the insertion of markers and tags (e.g., green fluorescent protein) into the RV genome, creating recombinant viruses that can be used to better understand RV biology. Perhaps the most profound impact that reverse genetics systems can have is to allow the development of next-generation vaccines capable of encoding not only RV proteins but those of other infectious agents as well. Such vaccines may be engineered such that they provide protection not only against RV disease but also against diseases caused by other enteric pathogens, including norovirus, hepatitis A viruses, and HCV.

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