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Generation of an Avian-Mammalian Rotavirus Reassortant by Using a Helper Virus-Dependent Reverse Genetics System

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ABSTRACT

The genetic diversity of rotavirus A (RVA) strains is facilitated in part by genetic reassortment. Although this process of genome segment exchange has been reported frequently among mammalian RVAs, it remained unknown if mammalian RVAs also could package genome segments from avian RVA strains. We generated a simian RVA strain SA11 reassortant containing the VP4 gene of chicken RVA strain 02V0002G3. To achieve this, we transfected BSR5/T7 cells with a T7 polymerase-driven VP4-encoding plasmid, infected the cells with a temperature-sensitive SA11 VP4 mutant, and selected the recombinant virus by increasing the temperature. The reassortant virus could be stably passaged and exhibited cytopathic effects in MA-104 cells, but it replicated less efficiently than both parental viruses. Our results show that avian and mammalian rotaviruses can exchange genome segments, resulting in replication-competent reassortants with new genomic and antigenic features.

IMPORTANCE

This study shows that rotaviruses of mammals can package genome segments from rotaviruses of birds. The genetic diversity of rotaviruses could be broadened by this process, which might be important for their antigenic variability. The reverse genetics system applied in the study could be useful for targeted generation and subsequent characterization of distinct rotavirus reassortant strains.

Rotavirus A (RVA) infections are a leading cause of diarrhea in young children worldwide, causing an estimated 450,000 deaths every year (1). RVAs also are widely distributed in several animal species, and zoonotic transmission has been demonstrated between humans and other mammals (2). They are also widely distributed among avian species, including chicken and turkey (3). However, genetic analyses of avian RVA strains indicated that they are only distantly related to their mammalian counterparts (4, 5, 6), and the zoonotic potential of avian RVA remains elusive.

The RVA particle is triple-layered and nonenveloped. The antigenic determinants are VP4 and VP7, which are located at the outer surface of the virus particle. VP4 is the spike protein that binds to cellular receptors and is proteolytically activated by trypsin-like proteases (7, 8). A large genetic variability of VP4 has been described, allowing a classification of 37 protease-sensitive genotypes (9). Although the recently introduced human rotavirus vaccines Rotarix and Rotateq have been shown to significantly decrease the number of severe rotavirus cases (10), their efficiency against the broad variety of rotavirus strains so far is not known.

The genome of RVA is composed of 11 double-stranded RNA segments, which contain conserved 5′ and 3′ noncoding regions and encode one or two proteins each. The exchange of genome segments between two different RVA strains can result in replication-competent reassortants. Whereas reassortants among mammalian RVAs have been identified frequently (2), only one study reported the generation of a turkey RVA containing the VP4 gene of a simian RVA using coinfection of cell cultures (11). Several efforts have been made to develop genetic systems that allow a targeted generation of RVA reassortants using genome segments cloned into plasmid vectors. Recently, reverse genetics systems have been successfully established for some genome segments using RVA helper viruses. Recombinant vaccinia virus-mediated T7 RNA polymerase expression facilitated the transcription of the target segment, and antibody-, short interfering RNA (siRNA)- or temperature-sensitive mutation-based selection systems allowed the selection of the generated reassortants (12, 13).

In order to test if mammalian RVA can package genome segments of avian RVA strains, a reverse genetics system was adopted and modified here. A temperature-sensitive simian RVA VP4 mutant and a plasmid containing the VP4 segment of a chicken RVA were used to generate this reassortant virus. The resulting reassortant initially was characterized to obtain information on its host cell specificity and growth characteristics in cell culture. Our study provides insights into the ability of avian and mammalian rotaviruses to exchange genome segments and the properties of the resulting reassortants.

MATERIALS AND METHODS

Generation of VP4-encoding plasmid. The whole VP4-encoding genome segment of chicken RVA strain 02V0002G3 (5, 6) was amplified by reverse transcription-PCR (RT-PCR) using the Qiagen LongRange 2Step RT-PCR kit (Qiagen, Hilden, Germany) from virus-containing MA-104 cell culture supernatant. The product was cloned into the vector pIDTSmart Amp, which was generated by gene synthesis and contained a T7 RNA polymerase promoter, a hepatitis delta virus ribozyme, and a T7

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RNA polymerase terminator sequence (Integrated DNA Technologies, Leuven, Belgium). Constructs were confirmed by Sanger DNA sequencing using an ABI 3730 DNA analyzer (Applied Biosystems). The plasmid was purified using the Qiagen plasmid minikit (Qiagen) prior to transfection.

Generation of reassortant virus. BSR5/T7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; supplemented with nonessential amino acids, L-glutamine, and gentamicin; all cell culture solutions were by Pan-Biotech GmbH, Aidenbach, Germany) containing 1 mg/ml G418 (Biochrom, Berlin, Germany) and 10% fetal calf serum at 37°C and 5% CO_2. For use in transfection experiments, the cells were grown in T25 flasks under the conditions described above, but without G418, until a confluence of 80% was reached. For transfection, 10 μg of DNA from plasmid pT7-VP4(02V0002G3) was mixed with 400 μl DMEM and 20 μl Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and incubated for 15 min at room temperature. BSR5/T7 cells were washed two times with phosphate-buffered saline (PBS) and one time with DMEM. A total of 1,600 μl DMEM was added to the transfection mixture and mixed by pipetting, and the whole mixture was added to the cell culture flask. The cells were incubated for 5 h at 37°C and 5% CO_2. Thereafter, the solution was removed and 5 ml DMEM (supplemented with nonessential amino acids, L-glutamine, gentamicin, and 10% fetal calf serum) was added. The cells were incubated overnight at 37°C and 5% CO_2.

Simian rotavirus SA11-tsA (14) was grown in MA-104 cells at 31°C and 5% CO_2 until a marked cytopathic effect (CPE) was visible. Cellular debris was removed by low-speed centrifugation, and the supernatant was stored at −80°C. For infection, 1 ml of the supernatant was thawed at 31°C, and 40 μl trypsin solution (stock concentration of 0.25% trypsin and 0.1% EDTA in water) was added. The mixture was incubated for 30 min at 31°C and 5% CO_2. The transfected BSR5/T7 cells were washed two times with PBS and the trypsin-activated SA11-tsA was added. The cells were incubated for 1 h at 31°C and 5% CO_2. Thereafter, the solution was removed, the cells were washed with PBS, and 5 ml DMEM (supplemented with nonessential amino acids, L-glutamine, gentamicin, and 2.5 μl trypsin solution) was added. The cells were incubated for 5 days at 31°C and 5% CO_2. No CPE was evident during this time of incubation. Thereafter, the supernatant was stored at −20°C.

MA-104 cells were grown in T25 flasks in MEM (supplemented with nonessential amino acids, L-glutamine, gentamicin, and 10% fetal calf serum) at 37°C and 5% CO_2 until confluence was reached (minimum age of 8 days after seeding). The supernatant from the transfection/infection experiment described above was thawed at 31°C, and 10 μl supernatant was mixed with 990 μl MEM and 40 μl trypsin solution. The mixture was incubated for 30 min at 31°C and 5% CO_2. MA-104 cells were washed two times with PBS, and the mixture was added to one flask and incubated for 1 h at 31°C and 5% CO_2. Thereafter, the supernatant was removed and 5 ml MEM (supplemented with nonessential amino acids, L-glutamine, gentamicin, and 20 μl trypsin solution) was added. The cells were placed into the CO_2 incubator, which was switched to a temperature of 39°C. After the occurrence of a CPE or after 7 days, the supernatant was stored at −20°C.

Isolation of reassortant virus. For endpoint dilution, confluent MA-104 cells (minimum age of 8 days after seeding) grown in a 96-well plate were infected with a 10-fold dilution series (8 lines with 10^0 to 10^{-11} dilutions each) of the supernatant from the experiment described above. Trypsin activation and infection was performed as described above but at 37°C, and the infected cells were incubated at 37°C and 5% CO_2 for 7 days. The supernatants of the wells inoculated with the highest dilutions, which showed CPE, were tested for their genome composition by strain- and segment-specific RT-PCRs using the primers listed in Table S1 in the supplemental material. Supernatants containing the VP4 gene of the avian rotavirus 02V0002G3 were used in plaque purification assays as described previously (15). Isolated plaques were picked, inoculated, and propagated in MA-104 cells at 37°C and 5% CO_2 as described above. Electron microscopy of the culture supernatants was performed after negative staining using uranyl acetate.

RVA replication kinetics. Virus growth curves were established by infection of T25 flasks of confluent MA-104 cells or primary chicken embryo fibroblasts (CEF) with the different rotaviruses at 37°C. For MA-104 cells, the conditions described above were applied. For infection of CEF, the activation of rotavirus was performed by adding 100 μl of a 1:50-diluted trypsin solution (final concentration, 0.005%) to 1 ml diluted virus-containing supernatant. Only 2.5 μl of the 1:50-diluted trypsin solution was added to 5 ml culture medium after infection. Two hundred-microliter aliquots of the culture supernatants were removed at days 0, 1, 2, 3, 4, and 7 after infection. The aliquots were tested in triplicates for the presence of the viral genome by quantitative real-time RT-PCR as described previously (3, 16). Endpoint dilution assays of the aliquots were performed with 8 replicates for each sample on 96-well plates of MA-104 cells as described above. The Spearman and Kärber algorithm (17) was used for the calculation of the 50% tissue culture infectious doses ($TCID_{50}$).

Nucleotide sequence accession number. The complete sequence of the plasmid designated pT7-VP4(02V0002G3) is available under the GenBank accession number KT239165.

RESULTS AND DISCUSSION

To determine if mammalian RVAs can package genome segments from avian RVA strains, we generated a helper virus-dependent reverse genetics system that is based on published protocols (12, 13) but does not require a vaccinia helper virus, antibodies, or siRNA (Fig. 1). A plasmid was constructed (GenBank accession no. KT239165) containing the complete sequence of the VP4-encoding genome segment of the chicken rotavirus strain 02V0002G3 under the control of the T7 RNA polymerase promoter, terminator, and hepatitis delta virus ribozyme sequence, which facilitates the correct processing of the 5′ and 3′ ends of the genome segment. Sequencing of the plasmid revealed 9 point mutations leading to 7 amino acid exchanges compared to the sequence available at GenBank (GenBank accession no. FJ169856). Almost all amino acid changes also are present in other avian and mammalian RVA strains (see Table S2 in the supplemental material). Therefore, it was suspected that either these mutations represent a quasispecies that arose during cell culture passaging or that the originally determined sequence (5, 6) contained some errors induced by RT-PCR. As the mutations are common to replication-competent viruses, the newly generated plasmid was considered suitable for use in the following experiments.

The plasmid was transfected into BSR5/T7 cells, which constitutively express the T7 RNA polymerase (18). The transfected cells were infected at low temperature with the simian rotavirus SA11-tsA, which contains a temperature-sensitive mutation in its VP4 gene (14). Thereafter, the generated reassortant viruses were selected on MA-104 cells at high temperature. After an endpoint dilution assay, the supernatants of the wells with the highest dilutions showing cytopathic effects (CPE) were tested by RT-PCR for the presence of the chicken and simian RVA VP4 genes (not shown). In 2 of 8 wells, clonal reassortant virus containing the chicken VP4 gene was detected. Despite negative selection, 4 of 8 supernatants were positive only for the simian VP4 gene, and 2 of 8 supernatants were positive for both genes. Reassortant viruses containing the chicken VP4 gene were obtained in two independent experiments at similar recovery rates. Trask et al. (13) identified sequences of 21 reassortant rotaviruses out of 24 rotavirus-specific RT-PCR products cloned from a supernatant produced by a dual-selection reverse genetics approach. However, these recov-

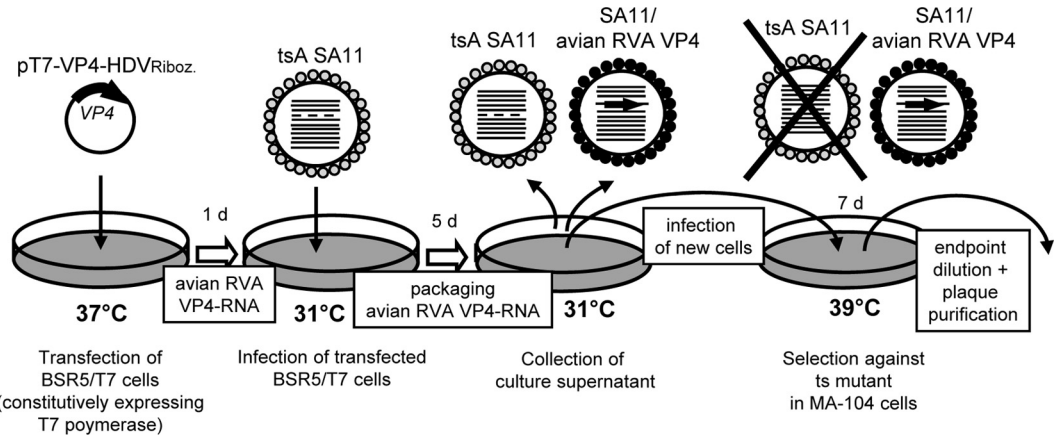

FIG 1 Schematic of the method for generation of the avian-mammalian reassortant RVA using helper virus-dependent reverse genetics. ts, temperature sensitive.

ery rates cannot be directly compared, as different genome segments and virus strains were used and different analytic approaches were applied in the experiments.

Plaque purification was used to ensure the clonality of the stocks of the reassortant viruses. One of the plaque-purified viruses was propagated in MA-104 cells and further analyzed. Segment- and strain-specific RT-PCRs were performed, which resulted in fragments of approximately 400 bp for the simian RVA genes and approximately 200 bp for the chicken RVA genes. The analysis revealed that the reassortant virus indeed contained 10 genes from the SA11 parent virus and the VP4 gene from the chicken strain 02V0002G3 (Fig. 2A). Sequencing of the complete VP4-encoding genome segment of the reassortant virus showed no mutations compared to the sequence of the cloned VP4 gene. By electron microscopy, typical RVA-like particles were found for the reassortant virus that were indistinguishable from those of the parental viruses (Fig. 2B).

Growth kinetics were performed with the reassortant virus, the chicken strain 02V0002G3, and a simian SA11 strain in the simian kidney cell line MA-104 and in primary chicken embryo fibro-

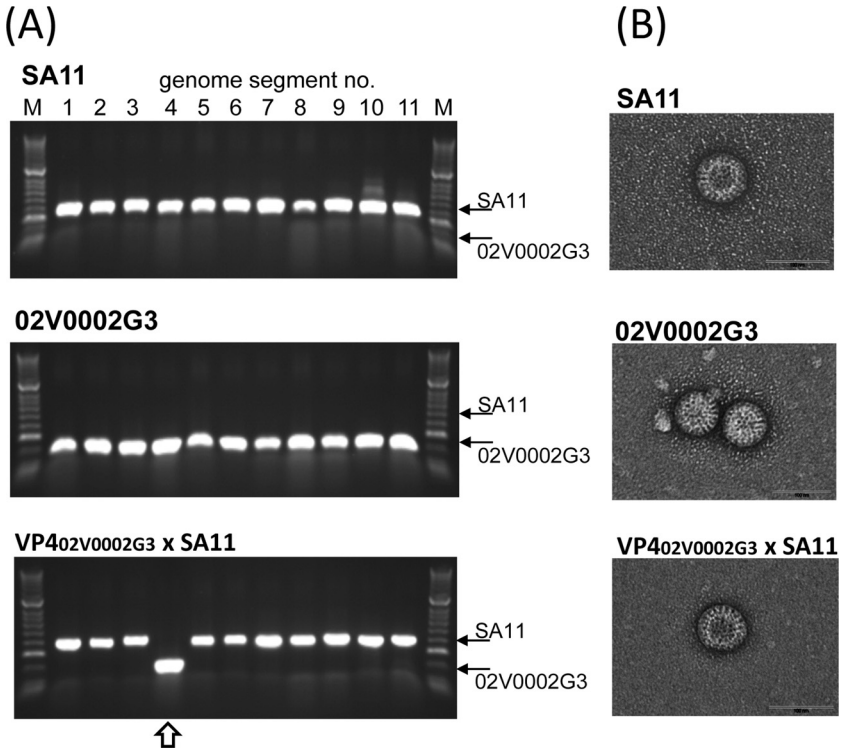

FIG 2 Characterization of the generated avian-mammalian reassortant RVA and its parent viruses, SA11 and 02V0002G3. Cell culture supernatants were analyzed 4 days after infection of MA-104 cells. (A) RT-PCR analysis using segment- and strain-specific primers (listed in Table S1 in the supplemental material), which generate products of approximately 400 bp in the case of strain SA11 and those of approximately 200 bp in the case of strain 02V0002G3. (B) Negative-stain electron microscopy, uranyl acetate staining.

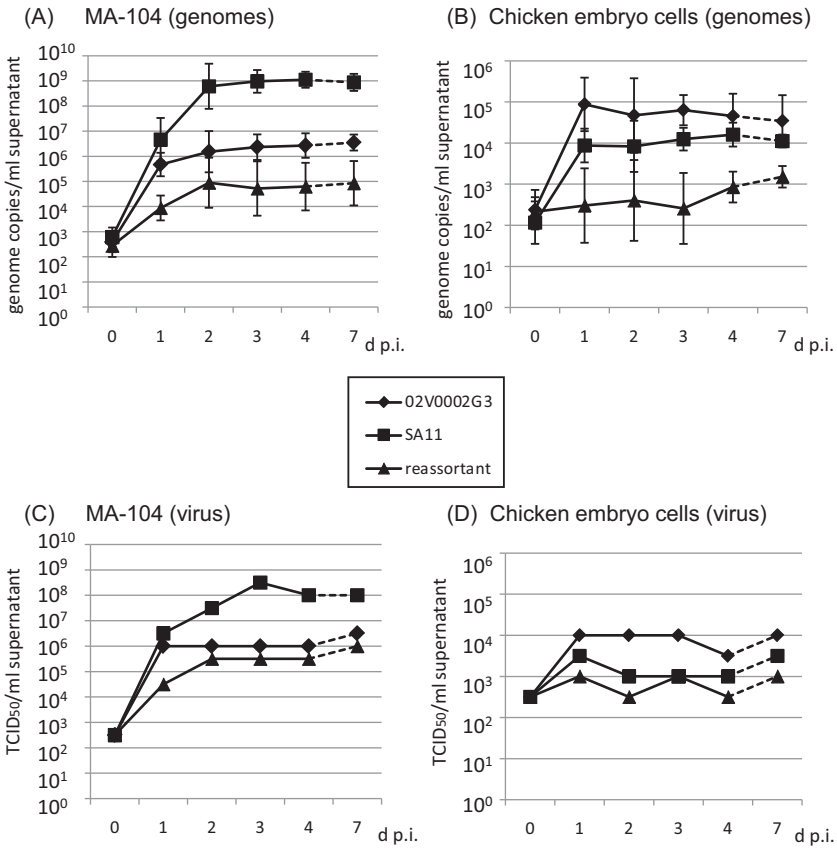

FIG 3 Growth curves of the avian-mammalian reassortant RVA and its parent viruses, SA11 and 02V0002G3, after infection of MA-104 cells (A and C) or chicken embryo cells (B and D). Cell culture supernatants were analyzed by RT-qPCR (A and B) or endpoint dilution assay (C and D) to determine the $TCID_{50}$.

blasts (CEF) at 37°C. As the parent SA11 strain originally used for the generation of the tsA mutant was not available, an original non-temperature-sensitive SA11 strain (ATCC VR-1565) was used for these experiments. Conditions of infection were identical between the cell types, except that trypsin concentration was 50-fold reduced for CEF due to their high trypsin sensitivity. In MA-104 cells, all viruses induced a CPE at 1, 3, or 4 days after infection. Virus replication was detected for all viruses by RT-quantitative PCR (qPCR) analysis and by determination of the $TCID_{50}$ of culture supernatants; however, the reassortant virus replicated slower than both of the other viruses (Fig. 3A and C). The growth kinetics of the SA11 strain used (ATCC VR-1565) were comparable to those described for the $SA11g8^{R}$ strain, which is based on the same parent SA11 strain as the tsA mutant (13, 14). In CEF, only minimal CPEs were observed, and replication of all viruses was slower (Fig. 3B and D). In this cell type, the reassortant showed only marginal growth. The generally observed low replication efficiency of all three RVA strains in CEF indicates that these cells only poorly support RVA replication. Also, consecutive weekly passages of the reassortant virus in CEF were not successful and led to the absence of the virus beginning with the 3rd passage as assayed by RT-PCR. In contrast, in six consecutive weekly passages of the reassortant virus on MA-104 cells, the typical onset of the CPE at 3 or 4 days after infection was not changed. In addition, the genome constellation of the virus did not change after six serial passages as determined by segment- and species-specific RT-PCR (data not shown). These results indicate that the reassortant replicates stably in MA-104 cells.

Our data show that rotavirus reassortants can be generated using a modified reverse genetics system. The established protocol is simpler than those previously described (12, 13), as neither vaccinia virus, antibodies, nor siRNA is required. Further studies will be needed to compare the efficiency of our system to those of other rotavirus reverse genetic systems.

Our study demonstrates that mammalian RVA can package genome segments of avian RVA strains, which can result in replication-competent reassortant strains. One prerequisite for successful reassortment is the presence of conserved sequences at the genome segment termini, which enable recognition by the replication and packaging complex (19, 20). Genome sequence analyses recently showed that avian and mammalian RVA contain nearly identical sequences at their genome segment termini (6, 9). In contrast, a nucleotide sequence analysis of the coding regions of the VP4 genes of the simian and chicken rotavirus strains used in this study shows only a 65.8% nucleotide and 62.2% amino acid identity. The results of the study indicate that the divergent avian gene is sufficient to provide the essential functions of VP4 in the genetic backbone of a mammalian RVA strain. However, the lower replication efficiency of the reassortant virus compared to that of its parental strains indicate that the interplay between the virus genes or their products has a negative effect on virus replication. It would be intriguing to analyze if compensatory muta-

tions will occur after a higher number of virus passages, which would allow a more efficient replication of the reassortant.

There is increasing evidence that avian and mammalian RVAs can be zoonotically transmitted between their hosts (21, 22, 23). A reassortant turkey RVA containing a simian VP4 gene has been generated by coinfection trials (11), and a mammalian-like VP4 gene has been detected recently in an avian RVA field isolate from pheasants (9). Although the frequency of successful avian RVA transmission to mammals and the spontaneous generation of replication-competent reassortants seems to be rather low, our study further supports the concept of a common gene pool of mammalian and avian RVAs. Most avian-mammalian RVA reassortants may be of low fitness, explaining why these variants are not often observed in the field. Further studies should analyze the pathogenic and antigenic properties of the generated reassortant virus to assess the risk of spread between animals and the human population.

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