Preferential Assortment of Rearranged NSP3 Gene in the Genetic Background of Simian Rotavirus SA11

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Selective assortment of rotavirus NSP3 gene in the genetic background of simian rotavirus strain SA11 was analyzed by using single-NSP3 gene-substitution reassortants having NSP3 gene from human rotavirus strain KU (KU-R) or porcine rotavirus strain OSU (OSU-R) in the SA11 background, and an SA11-derived variant Ga613 having a rearranged SA11-NSP3 gene which is 937 nucleotides longer than the original SA11-NSP3 gene. Through mixed infection and multiple passages in MA104 cells among these strains and SA11, the rearranged NSP3 gene of SA11 was selected most preferentially among the four NSP3 gene segments analyzed. The original NSP3 gene of SA11 was selected less efficiently than OSU-NSP3 gene, while more preferentially than KU-NSP3 gene. Growth rate of SA11 was similar to those of KU-R and Ga613, and slightly higher than that of OSU-R. These findings suggested that the rearranged SA11-NSP3 gene may have more functional advantage for being assorted with other SA11 gene segments, compared with intact NSP3 genes of SA11 as well as heterologous strains.

Keywords Rotavirus; RNA segment; NSP3 gene; Rearrangement; Assortment

1. Introduction

Rotavirus is the leading cause of severe acute diarrhea among infants and young children worldwide, and is widely distributed to various mammalians and avian species as an intestinal pathogen. The rotavirus comprises the genus Rotavirus within the family *Reoviridae*, and has 11 segments of double-stranded (ds) RNA as a genome which encodes six structural proteins (VP1-VP4, VP6, and VP7) and six nonstructural proteins (NSP1-NSP6). The 11 RNA segments of rotavirus are separated by polyacrylamide gel electrophoresis (PAGE) and observed as an RNA pattern. Rotavirus has a nonenveloped particle composed of three concentric layers, i.e., outer capsid, inner capsid, and core [1]. The outer capsid of rotavirus particle consists of two structural proteins VP7 and VP4 which have neutralization antigens and define G and P serotype of group A rotavirus, respectively. Based on the sequence diversity of VP7 and VP4 genes, group A rotaviruses are discriminated into G genotype (G type) and P genotype (P type), respectively [1].

Molecular evolution of rotavirus occurs via three major mechanisms, i.e., point mutation, reassortment, and rearrangement [2]. Reassortment is a recombination of RNA segments peculiar to viruses with segmented genome [3]. Reassortment of rotavirus is observed in coinfection of two different strains *in vitro* as well as *in vivo*, resulting in progeny viruses, i.e., reassortants, having RNA segments derived from both rotavirus strains coinfected (parental strains). Reassortment between human and animal rotaviruses has also been identified in human rotavirus isolates with unusual genetic and antigenic characteristics, suggesting occasional occurrence of reassortment in nature [4-7]. In contrast, rearrangement is a rare genetic event in which radical change in the size of the RNA segment occurs by concatemerization or deletion within a single RNA segment [8,9]. The most frequent type of rearrangement is a head-to-tail sequence duplication, which has been reported for RNA segments encoding NSP1-NSP5 and VP6 [9].

In the process of reassortment, selection of RNA segments from parental strains is not considered to be random, since reassortants with specific genome constellation are preferentially generated, or certain RNA segments are preferentially selected from one of the parent viruses and assorted into reassortants [10-12]. Although the precise mechanism has not yet been elucidated for the preferential selection of reassortants or RNA segments from either of parental strains, two explanations have been described to date. First, an increased infectivity of reassortants possessing certain foreign RNA segment(s) in a genetic background of a parental virus allows predominance of such reassortant viruses. It has been documented that introduction of a single foreign VP4, VP7, or NSP1 gene segment could confer a replication advantage on reassortants [13-16]. Secondly, specific RNA segments from a parental strain could be preferentially selected into progeny irrespective of alteration of infectivity. This case was considered to be related to genetic and functional adaptability of the RNA segment in a genetic background of a given strain, which was observed for VP7 gene and NSP1 gene in our previous studies [17-21]. In the genetic background of simian G3 rotavirus strain SA11, the homologous (SA11-) VP7 gene and homotypic (G3-) VP7 gene were preferentially selected over heterologous or heterotypic VP7 genes, although growth rates were same among rotaviruses with or without the preferentially selected RNA segments [17-19]. In contrast, four among the five heterologous NSP1 genes were more preferentially selected than homologous (SA11-) NSP1 gene in the genetic background of SA11 [21]. Therefore, functional adaptability of

homologous or heterologous RNA segments to the SA11 background is considered to be different depending on viral protein encoded by the RNA segment, although only VP7 gene and NSP1 gene have been analyzed.

In the present study, assortment of NSP3 gene in the genetic background of SA11 strain (SA11-L2 clone) was analyzed by using single NSP3 gene-substitution reassortants and a variant clone having a rearranged NSP3 gene. The rotavirus NSP3, like NSP1, has an ability to bind viral mRNA during the replication of rotavirus in cells, although the binding site to mRNA is different from that of NSP1. The aim of this study is to clarify whether selection mode of NSP3 gene is similar to NSP1 gene or VP7 gene, and to determine selection mode of rearranged gene segment.

2. Materials and Methods

The NSP3 gene of the strain SA11 is the 7th segment of the RNA pattern in PAGE, although the migration speed of the 7th segment is very close to the 8th segment which encodes NSP2. In the present study, to discriminate the SA11-NSP3 gene segment from the SA11-NSP2 gene segment for preparation of single NSP3 gene-substitution reassortants, a rotavirus variant clone Ga613 derived from SA11-L2 clone was used. The clone Ga613 has a rearranged NSP3 gene segment which possesses an additional non-coding 937 nucleotide sequence-duplication in a head-to-tail orientation at the site following the termination codon, and all other gene segments are derived from SA11, as reported previously [22] (Fig.1).
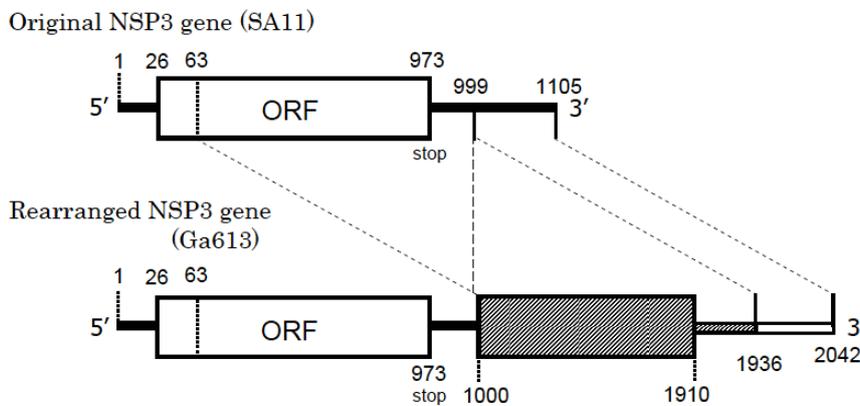

Fig. 1 Schematic diagram of the rearranged NSP3 gene detected in the clone Ga613 compared to the original NSP3 gene of SA11 [22]. In the Ga613 NSP3 gene segment, nucleotide (nt.) 1-999 containing an intact ORF is followed by an additional sequence corresponding to nt. 63-1105 of the original NSP3 gene. Actually duplicated portion (nt.63-999) is shaded.
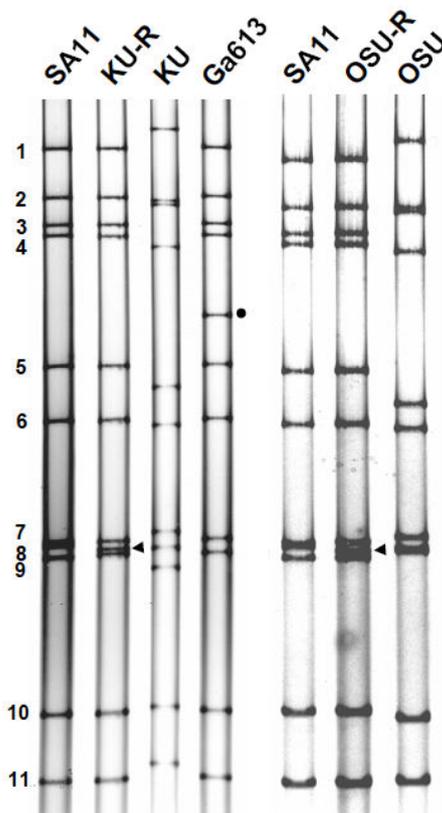

Fig. 2 RNA patterns in PAGE of reassortants KU-R and OSU-R, and rotavirus strains SA11, KU, OSU, and a clone Ga613. Arrowheads indicate NSP3 genes from KU or OSU. Closed circle indicates a rearranged NSP3 gene of Ga613. RNA segments 1 to 11 are indicated on the left.

Thus, despite possessing an original ORF of NSP3, NSP3 gene segment of Ga613 is located between gene segment 4 and 5 in PAGE, allowing differentiation from the NSP2 gene segment (8th RNA segment). In the present study, SA11-L2 clone [15] was used for the analysis of its NSP3 gene selection as well as preparation of single gene reassortants, as in the previous studies on VP7 gene and NSP1 gene selection in the SA11 background [19,21]. Single NSP3-gene reassortants KU-R and OSU-R were isolated from coinfection culture of Ga613 and human rotavirus strain KU or porcine rotavirus strain OSU, respectively. As shown in Fig.2, KU-R and OSU-R have only the 8th RNA segment (NSP3 gene) from strains KU and OSU, respectively, in the genetic background of SA11. These clones were purified three times by plaque isolation in CV-1 cells and propagated in MA104 cells for the present experiment. NSP3 genes of the strains KU and OSU (genotype T1) show 88.5% nucleotide identity (92.3% amino acid identity) each other. The SA11-NSP3 gene (genotype T5) has 75.9% and 74.3% nucleotide identities (78.2% and 77.6% amino acid identities) to KU and OSU, respectively.

SA11-L2 and one of the reassortants (KU-R and OSU-R) or Ga613, and Ga613 and one of the two reassortants were simultaneously inoculated onto MA-104 cell monolayer in a six well plate at a multiplicity of infection (m.o.i.) of five plaque forming unit (p.f.u.)/cell for each virus. Activation of virus with acetylated trypsin, inocuation of viruses and serial passage of the mixed infection culture were performed as described previously [21]. Presence of NSP3 genes from individual parental viruses used for coinfection after serial passage was examined by PAGE of RNA segments extracted from the culture fluid of mixed infection. Subsequently, RNA patterns of propagated virus clones isolated from the mixed infection culture fluid were observed to identify derivation of their NSP3 genes. Furthermore, single-step growth curves of SA11, Ga613, KU-R and OSU-R were prepared as described previously [18].

3. Results

Selection of NSP3 gene segment from either of parental virus strains in the 5th and 10th passage of mixed infection culture was analyzed. As shown in Fig.3, in the passage no. 10 of the coinfection culture with SA11 and KU-R, only NSP3 gene from SA11 could be observed.
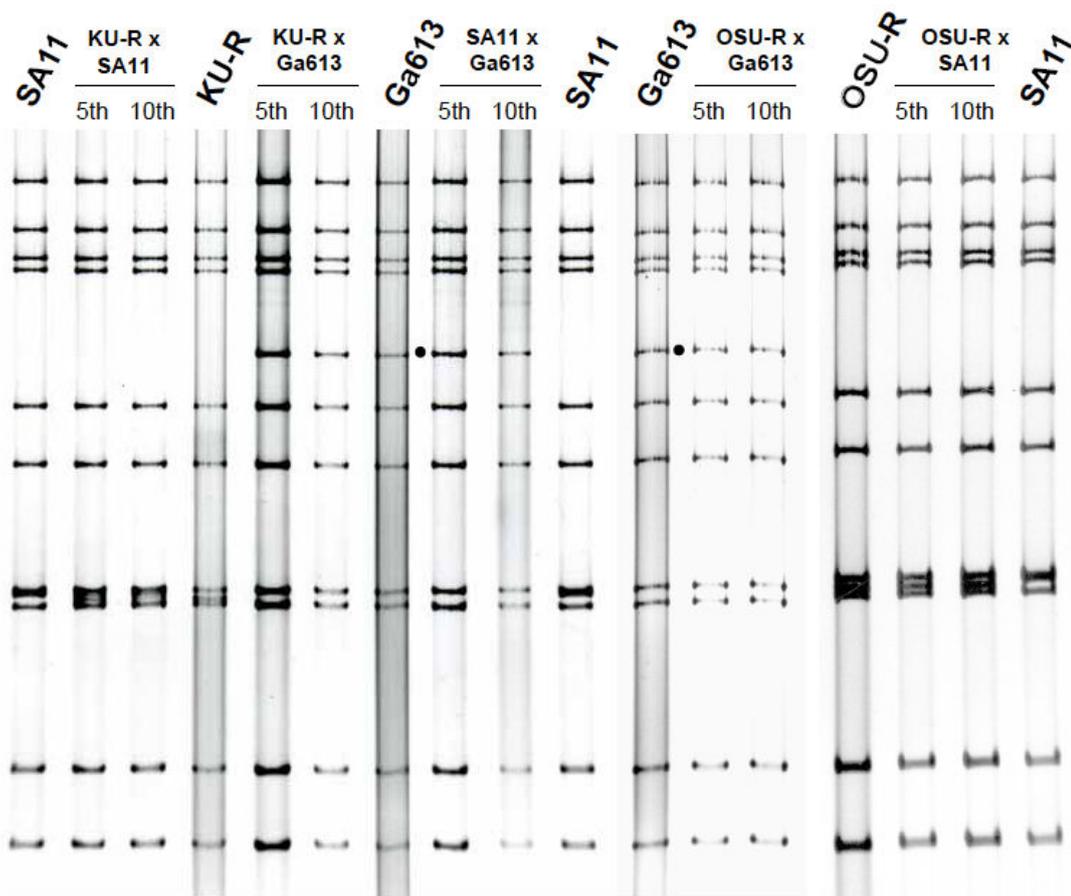

Fig. 3 RNA profiles from coinfected cultures (SA11 and KU-R, KU-R and Ga613, Ga613 and SA11, Ga613 and OSU-R, and OSU-R and SA11, from the left) after multiple passages (passage no. 5 (5th) and 10 (10th)). Closed circle indicates the rearranged NSP3 gene from Ga613.

Consistently with this finding, most of the clones (75%) isolated from the 10th passage had NSP3 gene from SA11 (Table 1). In contrast, in the coinfection of SA11 and OSU-R, NSP3 gene from OSU-R was evidently detectable in the 10th passage (Fig.3), and all the clones isolated from the 10th passage had OSU-NSP3 gene segment (Table 1).

In the mixed infection of SA11 and Ga613, the rearranged NSP3 gene segment from Ga613 surpassed the original NSP3 gene even at the 5th passage (Fig.3), and all the clones at the 10th passage had the rearranged NSP3 gene (Table 1). Similarly, in the mixed infections of Ga613 and KU-R, and Ga613 and OSU-R, predominance of the rearranged NSP3 gene was evidently observed in the RNA patterns in PAGE of mixed infection cultures (Fig.3) as well as in the isolated clones at the 10th passage (Table 1).

As shown in Fig.4, Ga613 and KU-R exhibited almost the same growth rate as that of SA11. The reassortant virus OSU-R showed slightly lower rate than other virus clones until 12 hours after inoculation to cells, although virus titer reached same level 16 hours after infection.

Table 1 Frequency of NSP3 genes selected from the 10th passage of mixed infections of rotavirus strains having different NSP3 genes in the genetic background of SA11

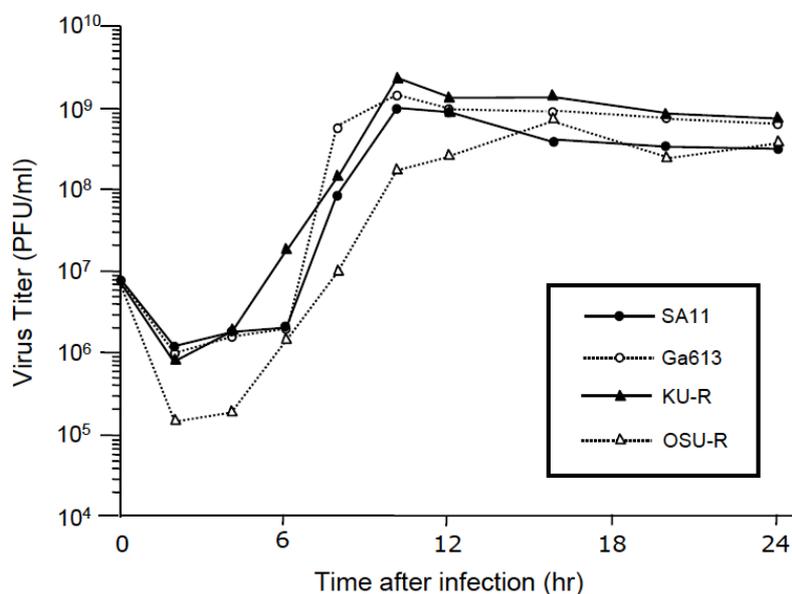| Mixed infection	Numbers of clones examined	Number (%) of NSP3 gene from rotavirus strains coinfected
SA11 x KU-R	52	SA11 : 39 (75) KU-R : 13 (25)
SA11 x OSU-R	36	SA11 : 0 (0) OSU-R : 36 (100)
SA11 x Ga613	36	SA11 : 0 (0) Ga613 : 36 (100)
KU-R x Ga613	36	KU-R : 0 (0) Ga613 : 36 (100)
OSU-R x Ga613	36	OSU-R : 0 (0) Ga613 : 36 (100)

Fig. 4 Single-step growth curves of SA11, Ga613, and reassortants KU-R and OSU-R. Virus titers at individual time points are average values obtained from three independent experiments of viral growth in MA-104 cells.

4. Discussion

In the present study, it was observed that a heterologous NSP3 gene from OSU was more preferentially selected than the normal SA11 NSP3 gene, while another heterologous NSP3 gene from KU was selected less efficiently than SA11-NSP3 gene. In our previous study, some NSP1 genes from heterologous viruses including both OSU and KU were found to be selected more preferentially than SA11 NSP1 gene [21]. Thus, it is suggested that heterologous NSP genes could more fit in the SA11 background genetically or functionally, dependent on the heterologous strains, unlike the VP7 gene selection [17-19].

Rotavirus NSP3 interacts specifically with the 3'-end of the non-polyadenylated rotavirus mRNAs, and binds also to eukaryotic translation initiation factor eIF4G, resulting in the stabilization of the mRNA-eIF complex and stimulating translation of rotavirus mRNAs [23,24]. The NSP3 is composed of three functionally distinct domains; an N-terminal RNA-binding domain, a central domain for dimerization of this protein, and a C-terminal domain responsible for the association with eIF4G [25]. Among the NSP3 amino acid sequences of SA11, KU, and OSU, C-terminal domain is more divergent than other two domains, although sequence divergence is found throughout the sequence (data not shown). More divergent nature of C-terminal region of NSP3 was also reported by comparative analysis for various rotavirus strains [26]. Therefore, it may be speculated that the preference of NSP3 gene selection may be related to the sequence divergence at the C-terminal region of NSP3.

The most intriguing finding was that the rearranged gene segment having unnecessary long additional nucleotides was selected by far the most preferentially among the four NSP3 gene segments including homologous original SA11 NSP3 gene. Since both the original SA11-NSP3 gene and the rearranged NSP3 gene of Ga613 have an identical ORF which encodes an identical protein product, only difference of these gene segments may be the length of these gene segments. This fact suggests that efficacy of genome segment packaging in virus particle may be different between original gene and the rearranged gene. Pesavent and coworkers [27] hypothesized a model of RNA packaging in rotavirus particle, through observation of isometric and concentric nature of rotavirus RNA. In this model, each RNA segment is folded and surrounds a transcription enzyme complex in a shape of cone with its base facing at the 5-fold vertex of icosahedron. Although the actual status of RNA folding and arrangement of individual RNA segments in the virus particle are unknown, it is probable that interactions may occur among neighboring RNA-enzyme complexes in the particle and may affect efficacy for generating virion. It may be speculated that a rearranged SA11 NSP3 gene having partially duplicated sequence with an appropriate length may form an RNA-enzyme complex having the best fit with those of other SA11 genes.

So far, rotaviruses with rearranged gene has been isolated from serial passage of rotavirus strains at high m.o.i. or from chronically infected children with immunodeficiency [8], suggesting that the occurrence of rearrangement may be related to extremely higher rate of viral replication in cells, although its reason is not clear. In the present study, rotavirus strains were inoculated to cells at high m.o.i. (5 pfu/cell) throughout the serial passages, as our previous studies of VP7 gene or NSP1 gene selection [17-21]. Thus, it is conceivable that such a condition, i.e., high m.o.i. for viral infection may be ascribable to the efficient persistence of the rearranged gene segment during the multiple passage. As further study, selection of the rearranged gene segment at a condition with low m.o.i. for viral infection should be performed to know the contribution of m.o.i. to the selectivity of rearranged gene segment.

Some plausible explanations for possible mechanism which causes rearrangement of rotavirus gene have been described, e.g., dissociation of RNA-dependent RNA polymerase from an RNA template and reassociation to a distant site on the same template targeting identical or similar sequence to that at the dissociation site [8], or to a remote site which is actually a closely located by the formation of secondary panhandle structure of the single stranded RNA [28]. However, occurrence of all the rearrangements cannot necessarily be explained by above mechanisms, and it has been observed that some specific forms of rearrangement occurs preferentially only in a limited gene segment [22]. Thus, it may be also suggested that only a few rearranged genes which highly fit with other gene segments within a virus particle may be sustained in the progeny viruses, although actually many undetectable rearranged genes are possible to be generated.

In the present study, it was revealed that the mode of NSP3 gene selection in the genetic background of SA11 is similar to NSP1, however, the rearranged RNA segment is highly adapted to SA11 background than original NSP3 gene. To understand the mechanism of the preferential selection of the rearranged gene, more study on assortment of rearranged RNA segments in other viral protein genes and other genetic background will be necessary.

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