

Reverse Genetic Analysis of the Recombination in Theilovirus based on the Infectious cDNA Clones

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Abstract

Saffold virus (SAFV) which belongs to the species *Theilovirus* of the genus *Cardiovirus* of the family *Picornaviridae* is a novel human cardiocivirus identified in 2007. However, the pathogenicity of SAFV to humans still remains unclear. Recent studies by the phylogenetic and recombination analyses of *Theilovirus* suggest that there are no recombination events between viruses of the different type (e.g. SAFV and Theiler's murine encephalomyelitis virus (TMEV)). Information on the recombination events of these viruses will be helpful to better understand the host specificity and pathogenicity of SAFV. In the present study, we performed the reverse genetic analysis to investigate the possibility of the recombination between SAFV and TMEV. The recombination of the capsid protein (VP1 and/or VP2) by reverse genetics between SAFV and TMEV did not happen, although the recombination of the non-capsid protein, L, occurred. These results strongly suggest that the shift of host range from rodents to humans or from humans to rodents by natural recombination of capsid protein(s) within Theiloviruses does not happen. The present results will provide the valuable information for the studies on the pathogenicity of SAFV.

Keywords: Theilovirus; TMEV; SAFV; Recombination

The genus *Cardiovirus* which belongs to the family *Picornaviridae* is divided into two species: *Theilovirus* and *Encephalomyocarditis virus* (EMCV). The natural hosts for *Cardioviruses* have been thought to be the rodents. However, in 2007, a human cardiocivirus, designated Saffold virus (SAFV), was identified from an infant with a fever of unknown origin [1]. Its nucleotide sequences showed a strong similarity to Theiler-like rat virus (TRV), which was isolated from rats in Japan [2]. In the aid of phylogenetic analysis, SAFV was classified with TRV, Theiler's murine encephalomyelitis virus (TMEV) and Vilyuisk human encephalomyelitis virus (VHEV) into the species *Theilovirus* and eight genotypes of SAFV have been reported [1, 3-6].

The recombination is a common mechanism of evolution and antigenic variability for picornaviruses [7]. Recently, seven potential recombination events were reported by the phylogenetic and recombination analyses of *Theilovirus* species over the complete genomes [8]. According to this report, no recombination events were identified between viruses of the different type (e.g. SAFV and TMEV), although the potential recombination events were identified between the different SAFV strains. In addition, a previous study reported that there are apparently no recombination events between SAFV and TMEV [9]. To understand the host-specificity and pathogenicity of each virus, further information of the recombination within these viruses is valuable. Since previous studies [8,9] were only performed by using the computer software, in the present study, we performed the reverse genetic analysis by using the infectious cDNA clones to investigate whether the recombination between SAFV and TMEV potentially occurs or not.

The homology of capsid proteins (VP1, VP2, VP3 and VP4) between SAFV-3 (JPN08-404) and TMEV (DA and GDVII) was summarized in Table 1. The homology of amino acid sequence of VP1 is strikingly low between SAFV-3 and TMEV (56-57%). Furthermore, amino acid sequences of CD loop in VP1 and EF loop in VP2 of SAFV are markedly different from those of TMEV-DA (Figure 1). CD and EF loops have important roles to interact with the receptor of host cells [10]. If the recombination of these regions happened between SAFV and TMEV, the host range of this virus could be changed. Therefore, we generated the constructs for VP1 and/or VP2 recombinant viruses based on the infectious cDNA clone of SAFV-3, pSAF404 [11], the

infectious cDNA clone of DA strain of TMEV (TMEV-DA), pDAFL3 [12], and the infectious cDNA clone of GDVII strain of TMEV (TMEV-GD), pGDVIIIFL2 [13] to perform the reverse genetic analysis (Figure 2). pDAFL3 and pGDVIIIFL2 were kindly provided from Dr. Raymond P. Roos (University of Chicago, IL). L protein (L) of TMEV is known to be important for cell-to-cell propagation [14], virus growth in macrophage cells and persistent infection [15]. However, it is not essential for virus production [14]. Therefore, the constructs for the L recombinant viruses were generated as a control for reverse genetics of recombinant viruses (Figure 2). Infectious RNAs transcribed from the constructs based on pSAF404 and the constructs based on pDAFL3 were transfected to HeLa and BHK-21 cells, respectively, as described previously [11]. On the cells transfected with synthesized RNA of SAFV-3, cytopathic effects (CPEs) were observed within 48 hours.

Capsid protein	homology (%)	
	vs TMEV-DA	vs TMEV-GDVII
VP4	68.1	68.1
VP2	70.1	68.6
VP3	79.3	81.0
VP1	56.7	56.0

This table shows the % of homologies of capsid proteins between SAFV-3 and TMEV (DA and GDVII). The homology of amino acid sequences was analyzed by genetyx ver. 10. Capsid proteins were arranged from the top according to the order from N-terminus of polyprotein

Table 1: The homologies of capsid proteins between SAFV-3 and TMEV (DA and GDVII).

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CD loop (VP1)

```
SAFV-3  LTPLPS---DIVNNSVLPEQE-RWISFASPTTQKPPYKTKQC
      ***** * * * * *
TMEV-DA LTPLPSFCPDSTSGPVKTKAPVQWRWVRSGGTTNFPLMTKQC
```

EF loop (VP2)

```
SAFV-3  PEFDTSNHSTEVEPRADTAFKVDANWQKHTQILTGHAYVNTTTKVNVPALALNHQNF
      *** * * * * * ** * * **** *
TMEV-DA PEFYTGKGTKTGDMEPTDPFTMDTTWRAPQGAFTGYR---DSRTGF-FAMNHQNQW
```

Figure 1: Amino acid similarity of CD and EF loops (the structures of the part of VP1 and VP2 proteins, respectively) between SAFV-3 (JPN08-404) and TMEV-DA. Asterisks indicate identical amino acid residue.

Original Viruses



Potential recombinant viruses

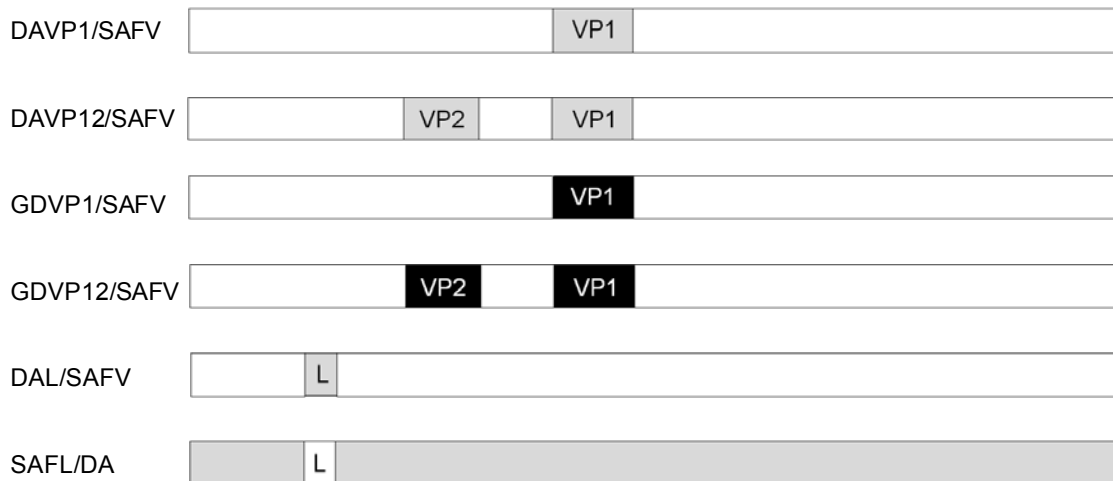


Figure 2: Diagrams of the constructs for recombinant viruses. The top three diagrams represent the genome structures of original viruses. White, gray and black boxes are SAFV-3, TMEV-DA and TMEV-GDVII, respectively. In DAVP1/SAFV, the VP1 coding region of SAFV-3 was replaced with that of TMEV-DA. In DAVP12/SAFV, the VP1 and VP2 coding regions of SAFV-3 were replaced with those of TMEV-DA. In GDVP1/SAFV and GDVP12/SAFV, the same recombination was designed. In DAL/SAFV, the L coding region from SAFV-3 was replaced with that of TMEV-DA. Vice versa recombination was designed in SAFL/DA. DAL/SAFV and SAFL/DA were generated as a control for reverse genetics of recombinant viruses.

The CPEs were also observed on the cells transfected with synthesized RNAs of DAL/SAFV, which is SAFV containing L of TMEV-DA. In the case of SAFL/DA, which is TMEV-DA containing L of SAFV, CPEs were also observed within 48 hours. These viruses were prepared by three freezing/thawing cycles, and then the plaques were isolated. The isolated viruses were propagated by several passages and the titers of recombinant viruses based on SAFV and TMEV-DA were determined by a standard plaque assay on HeLa and BHK-21 cells, respectively. The titers of SAFV-3 and SAFL/DA reached to 7.3×10^7 pfu/ml and 2.6×10^8 pfu/ml, respectively, after three passages. The titer of DAL/

SAFV reached to 1.9×10^8 pfu/ml after six passages. This information suggests that the recombination of L between SAFV and TMEV could occur, although the propagation periods were not similar. The CPEs were not observed on the cells transfected with RNAs of SAFV which is recombined with VP1 and/or VP2 of TMEV (DAVP1/SAFV, DAVP12/SAFV, GDVP1/SAFV and GDVP12/SAFV) within 72 hours. Infectious viral particles of these recombinant viruses were not obtained even after three blind passages on HeLa and BHK-21 cells. Additionally, we analyzed the viral protein synthesis after transfection by Western blotting with mouse anti-VP1 antibody (DAmAb2) specifically reacted

to VP1 protein of TMEV-DA [16]. VP1 of TMEV-DA was detected in the lysate of the cells transfected with RNA of DAVP12/SAFV (data not shown). The result suggests that the translation from the transfected RNA and the maturation of VP1 by viral protease occurred. The reason for the lack of recombination could be 1) viral particles were not produced because of the failure of assembly by the changes of VP1 and/or VP2, 2) the recombinant viruses could not be attached to the receptor on HeLa and BHK-21 cells because of the collapse of the higher order structure of receptor binding regions by the changes of VP1 and/or VP2. Although further studies are required to confirm these propositions, it is strongly suggested that the recombination of VP1 and/or VP2 between SAFV and TMEV does not naturally occur.

In conclusion, the recombination of the capsid protein (VP1 and/or VP2) between viruses of the different type within *Theilovirus* species did not happen although the recombination of the non-capsid protein, L, occurred. These results agree with the previous reports by the phylogenetic and recombination analyses [8, 9]. From these observations, it is suggested that the changes of host range by natural recombination of capsid protein(s) between SAFV and TMEV do not occur. The present study will provide the valuable information for further research of the recombination among the species *Theilovirus*.

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