INTRODUCTION

Severe fever with thrombocytopenia syndrome virus (SFTSV) belongs to the family Bunyaviridae, genus Phlebovirus (1). SFTSV possesses a negative-strand RNA genome that consists of large (L), medium (M), and small (S) segments. The L segment encodes an RNA-dependent RNA polymerase, the M segment encodes an envelope glycoprotein (GP, Gn and Gc), and the S segment encodes a nucleocapsid protein (N) and a nonstructural protein (NSs) (1,2). As is the case for other viruses in the family Bunyaviridae, SFTSV attaches to host cell receptors with the GP and is endocytosed in a receptor-mediated manner (3–5). After endocytosis, a conformational change in the GP occurs following acidification of the endocytic vesicles. This facilitates fusion of the viral membrane and vesicle membrane, releasing the viral genome into the cytoplasm.

Severe fever with thrombocytopenia syndrome (SFTS) is a lethal febrile illness characterized by leukopenia, thrombocytopenia, and fever. SFTS was first reported in China in 2009, and the causative virus was identified from a patient in 2011 (6). In Japan, SFTSV was first isolated in 2012 from a patient who died from a febrile illness of unknown origin. This virus was named strain YG1 (7). Recently, SFTSV and other genetically related viruses that are pathogenic for humans have been reported in East Asia and North America (8–13). However, their molecular biological characteristics and pathogenic mechanisms are not fully understood (5,14,15).

In our initial experiment, we analyzed the low pH-dependent cell fusion activity of the YG1 strain in infected cells, as previously described (16,17). Although infected cells typically form syncytia under acidic conditions, only a subset of the YG1 cells formed syncytia despite an infection rate of 100%. Therefore, we hypothesized that the YG1 strain harbored the original virus and various subclones. In this study, we isolated 3 subclones and the relationship between viral proteins and their functions.

SUMMARY: The first clinical case of the YG1 strain of the severe fever with thrombocytopenia syndrome virus (SFTSV) has been isolated in Japan. We found that only some of the cells underwent low pH-dependent cell fusion, although all of the cells were confirmed to have been infected with the virus. This suggested that the YG1 strain consists of a heterogeneous mixture of related viruses. Here, we established 3 subclones (term E3, A4, and B7) from the YG1 strain, using the limiting dilution method with the pH-dependent cell fusion activity. Subclone E3 showed weak fusion activity and cytopathic effects (CPE) in Vero E6 cells. The amino acid sequence of E3 was identical to the published sequence for the YG1 strain, and it likely comprises a subpopulation of the YG1 strain. Subclone A4 displayed strong fusion activity under acidic conditions. In contrast, subclone B7 showed strong fusion activity and CPE under neutral and acidic conditions. Two amino acid differences shared between B7 and A4 were found in the envelope glycoproteins. In addition, an amino acid variant of the RNA-dependent RNA polymerase was found only in B7. These subclones will be valuable tools to elucidate cell fusion mechanisms of SFTSV and the relationship between viral proteins and their functions.

MATERIALS AND METHODS

Cells: Vero E6 cells were maintained in minimum essential medium (MEM; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) as a growth medium, which was supplemented with 5% fetal bovine serum (FBS; Biowest, Nuaille, France), insulin-transferrin-selenium (Gibco), non-essential amino acids (Gibco), a penicillin-streptomycin mixture (P/S; Sigma, St. Louis, MO, USA), and gentamicin (Sigma). Huh7 human hepatoma cells, HeLa cells, and baby hamster kidney (BHK)-21 cells were grown in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% FBS and P/S.

Viruses: SFTSV strain YG1 was isolated in 2013 from the first SFTS patient in Japan. Culture supernatants from Vero E6 cells inoculated with a specimen of the patient’s serum were kindly provided by Dr. Ken Maeda, Yamaguchi University, Japan. The virus was propagated twice in Vero E6 cells, and the culture supernatant was collected following 3 passages in the presence of the virus. This virus stock was designated as the parent YG1 virus. The virus was dispensed into vials and stored at −80°C until use. The infectivity titers for the viruses were determined in Vero E6 cells by using the indirect immunofluorescence antibody (IFA)-focus assay, as described below.

Immune mouse serum: Six-week-old female BALB/
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c mice (SLC, Hamamatsu, Japan) were inoculated subcutaneously with the parent YG1 strain (5 × 10^3 FFU/mL). Three months after inoculation, the mice were euthanized and sera were collected. The pooled serum was used as a polyclonal antibody. The animal experiment was approved by the Institutional Animal Care and Use Committee of the National University Corporation at Hokkaido University. All mice were treated according to the Hokkaido University Manual for Implementing Animal Experimentation. Experiments involving viral infections were performed in a BSL-3 facility.

**IFA-focus assay:** Vero E6 cell monolayers were prepared on a 24-well glass slide. The cells were inoculated with the viral dilutions. After incubation at 37°C for 1 h, the inoculum was removed and the cells were overlaid with MEM supplemented with 1.5% carboxymethylcellulose (Wako, Osaka, Japan). After incubation for 48 h, the cells were washed with PBS and fixed with acetone. Viral antigens in the cells were stained by IFA using both the mouse immune serum (preparation described above) and an Alexa Fluor 488-labeled anti-mouse antibody (Thermo Fisher Scientific). Focus-forming units (FFU) were calculated by counting the number of infected cell foci.

**Low pH-dependent cell fusion:** Cells were inoculated with the viruses and incubated for 6–7 d. The medium was replaced with 50 mM acetate-buffered saline (pH 5.6). After incubation for 2 min at room temperature, the acetate buffer was replaced with the growth medium. After incubation at 37°C for 16 h, the cells were fixed with 10% neutral-buffered formalin and stained with Giemsa solution (Merck, Darmstadt, Germany) to visualize cell fusion.

**Establishment of subclones by the limiting dilution method:** Vero E6 cells on 96-well microplates were inoculated with tenfold dilutions of the virus. After incubation for 6–7 d, cultured supernatants were collected and the appearance of low pH-dependent cell fusion occurring in the infected cells was examined, as described above. To examine viral growth in each well, cultured supernatants were used to inoculate Vero E6 cells. After culturing for 48 h, the cells were fixed and viral antigens were detected using the IFA-focus assay. Viruses were selected based upon the pH-dependent cell fusion activity assay using syncytium formation as a criterion (Fig. 1A). The limiting dilution experiments for each clone were repeated once or twice. Selected clones obtained from the final limiting dilution plate were propagated in Vero E6 cells and stored at −80°C.

**Plaque-forming assay:** Vero E6 cells in a 6-well plate were inoculated with each subclone and cultured in 2 ml of overlay medium (MEM containing 0.8% SeaKem ME agarose [Takara, Kusatsu, Japan] and 4% FBS). After incubation for 7 d, 2 ml of neutral red solution (0.1 mg/mL) was added to the overlay medium. After incubation for 24 h, the neutral red solution was removed and the cells were incubated for an additional 3 d.

**Growth kinetics:** Vero E6 cells in a 6-well plate were inoculated with the viruses at a multiplicity of infection (MOI) of 0.005. After incubation for 1 h at 37°C, the cells were washed and the growth medium was added. The culture supernatant was collected at 1–7 days post-infection (dpi) and stored at −80°C for further titration. Viral titers were measured using the IFA-focus assay.

**Sequencing assay:** Viral RNA was extracted from the supernatants of infected cells using either a QIAamp Viral RNA Mini Kit (QIAGEN, Venlo, Netherlands) or ISOGEN-LS (Nippongene, Tokyo, Japan), according to the manufacturer’s protocol. The cDNA was synthesized from total RNA by using SuperScript II Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific). DNA fragments were amplified from the cDNA by using iP-roof High-Fidelity DNA Polymerase (BioRad, Hercules, CA, USA), according to the manufacturer’s protocol, with oligonucleotide primer pairs designed using the YG1 database sequences (AB817979, AB817987, and AB817995). Purified PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Thermo Fisher Scientific) and Ge-
RESULTS

Establishment of subclones: The process for performing the limiting dilutions is shown in Fig. 1B. Various degrees of syncytia formation were observed in the wells inoculated with a 10⁰ dilution of the parent YG1 strain (Fig. 1A). The culture supernatants from well D10 (where the most abundant syncytia formation [+++]) occurred) and well B3 (where the second-most abundant syncytia formation [++] occurred) were selected as viruses displaying strong cell fusion activity. In the microplate in which cells were inoculated with the parent YG1 strain diluted 10⁷-fold, 21 of the 32 wells showed viral growth, but none of the wells showed obvious syncytia formation. Among the wells that showed limited syncytia formation (+/-), the culture supernatant from well A9 was selected because it contained a virus that promotes weak cell fusion activity. The limiting dilution assay was repeated for each supernatant. The virus derived from well E3 from the second limiting dilution of well A9, which showed minimal syncytia formation (+/-), was selected and named subclone E3. The limiting dilution assay was repeated twice for wells D10 and B3. Strong syncytia formation (+++) was observed in 13 out of 32 wells at a 10⁵ dilution of D10B3. Since no virus growth was detected in the other 19 wells, these viruses were considered single cloned viruses obtained via 3 limiting dilutions. The virus derived from well D10B3B7 in the third limiting dilution was named subclone B7. Similarly, syncytia formation was observed in 10 out of 32 wells at a 10⁷ dilution of B3D4, and no viral growth was detected in the other 22 wells. The virus derived from well B3D4A4 was named subclone A4.

Comparisons of CPE and low pH-dependent cell fusion activity: Subclones B7 (2.5 × 10⁶ FFU/ml), A4 (2.0 × 10⁶ FFU/ml), and E3 (2.0 × 10⁶ FFU/ml) were diluted 10⁻² to 10⁻⁹-fold. Vero E6 cells were inoculated with these viral dilutions and treated for 2 min with pH 5.6 or neutral pH 7.0 buffer at 7 dpi (Fig. 2). After fixation and staining, patterns for the CPE and syncytia formation were compared. Strong syncytia formation appeared in B7-infected cells after treatment with low pH buffer. In addition, B7-infected cells showed a strong CPE and were sloughed off without low pH treatment following incubation with 10⁻², 10⁻⁴, and 10⁻⁹-fold dilutions of the viruses. A4-infected cells also showed obvious syncytia formation after low pH treatment, but no syncytia or CPE appeared under neutral conditions. On the other hand, E3-infected cells showed no obvious syncytia formation or CPE under either neutral or low pH conditions. In addition to Vero E6 cells, A4- and B7-infected Huh7 and HeLa cells displayed syncytia formation after low pH treatment (Fig. 2B). Only B7-infected Huh7 cells showed CPE under neutral conditions at 7 dpi (data not shown). In contrast, BHK-21 cells did not undergo syncytia formation or CPE. Although Huh7, HeLa, and BHK-21 cells were successfully infected by E3, they showed no obvious syncytia formation after low pH treatment. These results suggested that syncytia formation depended on the nature of the cell lines. Thus, the low pH-dependent cell fusion behavior observed in Vero E6 cells was also observed in other cell lines.

Plaque-forming activity of subclones: When we performed a plaque-forming assay with the YG1 parent strain, plaques of various sizes appeared from 8 to 11 dpi (data not shown). To determine the plaque-forming activity of these subclones, Vero E6 cells were inoculated with each subclone and the neutral red solution was added to the overlay medium at 7 dpi. Plaques of B7 began to appear 6 h after staining with neutral red solution, and then uniformly large and clear plaques were observed 24 h after staining (8 dpi; Fig. 3A). Plaques of A4 appeared 2 d after staining (9 dpi). Although the sizes of plaques for A4 and B7 were similar, microscopic observation showed that the cells in the plaques displayed different characteristics (Fig. 3B). Cells in B7 plaques did not incorporate neutral red and started to slough off, whereas cells in A4 plaques formed syncytia that incorporated neutral red. These findings indicated that cells in B7 plaques died, while those in A4 plaques survived. In contrast, plaques of E3-infected cells appeared 4 d after staining (11 dpi). These plaques were not as clear compared to those of B7 or A4, and they were similar to most of the parent YG1 plaques (data not shown).

Growth kinetics of subclones: The growth kinetics of each subclone were evaluated in Vero E6 cells (Fig.
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4). The growth of the parent YG1 strain and subclones was similar, and it reached nearly $10^6$ FFU/ml by 7 dpi. The parent YG1 strain showed the highest infectivity titer during the observation period. The growth rate of A4 was slightly lower than the parent YG1 strain, but it was similar to that of E3. Some of the infected cells of A4 displayed cell fusion at 9 dpi as the pH of the culture medium decreased (data not shown). B7 showed the lowest infectivity titer at 1 dpi, but it also displayed a sharp growth curve and reached its highest titer at 5 dpi. A CPE appeared at 5 dpi in cells inoculated with B7. Accordingly, the maximum infectivity titer was reached at 6 dpi. In contrast, cells inoculated with E3 did not show a CPE during the observation period, and the infectivity titer had not plateaued by 8 dpi.

Sequence analysis of subclones: The virological characteristics and genomic sequences of the subclones are summarized in Table 1. Nucleotide sequences of the S, M, and L segments of subclones B7, A4, and E3 were determined and compared to those of the parent YG1 strain. Nucleotide sequences for all 3 segments of E3 were identical to the published sequences. Two common nucleotide variants in the M segments of B7 and A4 were found: C at nucleotide 1,000 and T at nucleotide 1,888. Because of these differences, tyrosine at position 328 in Gn was replaced by histidine (Y328H), and arginine at position 624 in Gc was replaced by tryptophan (R624W). In addition, a nucleotide variant of A at nucleotide 5,689 in the L segment was found in B7. Owing to these differences, asparagine at position 1,891 in the RNA-dependent RNA polymerase was replaced by lysine (N1891K). A synonymous variant was also found at nucleotide 1,992 in the M segment of A4 and at nucleotide 1,487 in the S segment of B7.

DISCUSSION

In our initial experiment, we examined the low pH-dependent cell fusion activity of the parent YG1 viral strain-infected Vero E6 cells. We found that only some of the cells formed syncytia, even though all of the cells were confirmed to have been infected with the virus. From these results, we hypothesized that the parent YG1 strain consists of subclones with various low pH-dependent cell fusion activities. In this study, we used

<table>
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<th>Virus</th>
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<th>Neutral pH</th>
<th>Acidic pH</th>
<th>Plaque</th>
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<tbody>
<tr>
<td>YG1</td>
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<td>–</td>
<td>+</td>
<td>delay/small&lt;sup&gt;1)&lt;/sup&gt;</td>
</tr>
<tr>
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<td>+++</td>
<td>+++</td>
<td>early/large</td>
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<tr>
<td>A4</td>
<td>+++</td>
<td>–</td>
<td>+++</td>
<td>delay/large</td>
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<tr>
<td>E3</td>
<td>+</td>
<td>–</td>
<td>+</td>
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Table 1. Characteristics and mutations of subclones established from YG1

Nucleotide number / amino acid number

<table>
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<th>S segment</th>
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<th>L segment</th>
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<tr>
<td>1,487/231</td>
<td>1,000/328</td>
<td>1,888/624</td>
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<tr>
<td>G / L</td>
<td>T / Y</td>
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<td>A / L</td>
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<sup>1</sup): Large plaques were rarely observed.
the SFTSV YG1 strain, which had only been passaged 3 times, as a parental virus. We established subclones using the limiting dilution method. The subclones had different virological characteristics, such as low pH-dependent cell fusion activities, plaque formation, CPE, and related genome mutations. These results suggested that the parent YG1 strain contains quasispecies (18).

However, it is unclear whether the subclones existed in the original patient material or appeared through passages in Vero E6 cells. Yoshikawa et al. reported the nucleotide sequences of the YG1 virus isolated from Vero cells and registered them in the DNA database of Japan (DDBJ) (19). These submitted sequences are identical to those of E3. Since E3 was detected at the highest dilution in the limiting dilution experiments, the majority of the parent YG1 strain was likely to be E3. Yoshikawa et al. also demonstrated the existence of small degrees of synonymous and nonsynonymous variations in the genome of the YG1 virus by using next-generation sequencing, indicating that the YG1 viral strain contained quasispecies. At the same time, viral sequences from the original patient serum were also evaluated. Interestingly, the viral population from a parent virus stock might have caused by differences in polymerase activity.

As shown for other viruses (23,24), a quasispecies viral population from a parent virus stock might have a strong CPE under neutral conditions. Since one amino acid difference in the L segment is the only difference between A4 and B7, these variable activities might be caused by differences in polymerase activity.

In this study, we found that the CPE induced by B7 was clearly observed without a microscope, by using giemsa staining. Therefore, we performed a neutralization assay using B7 to identify the neutralizing titer of mouse immune sera, and we easily distinguished the positive wells with or without a CPE (data not shown). Thus, this property might be used to develop a simple assay for detecting neutralizing antibodies and screening for antiviral drugs and therapeutics.

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Conflict of interest None to declare.

REFERENCES
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