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VIRUS-CELL INTERACTIONS

The Combination of gQ1 and gQ2 in Human Herpesvirus 6A and 6B Regulates the Viral Tetramer Function for Their Receptor Recognition

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ABSTRACT Human herpesvirus 6A (HHV-6A) and HHV-6B use different cellular receptors, human CD46 and CD134, respectively, and have different cell tropisms, although they have 90% similarity at the nucleotide level. An important feature that characterizes HHV-6A/6B is the glycoprotein H (gH)/gL/gQ1/gQ2 complex (a tetramer) that each virus has specifically on its envelope. Here, to determine which molecules in the tetramer contribute to the specificity for each receptor, we developed a cell-cell fusion assay system for HHV-6A and HHV-6B that uses cells expressing CD46 or CD134. With this system, when we replaced the gQ1 or gQ2 of HHV-6A with that of HHV-6B in the tetramer, the cell fusion activity mediated by glycoproteins via CD46 was lower than that seen with the original-type tetramer. When we replaced the gQ1 or the gQ2 of HHV-6A with that of HHV-6B in the tetramer, the cell fusion mediated by glycoproteins via CD134 was not seen. In addition, we generated two types of C-terminal truncation mutants of HHV-6A gQ2 (AgQ2) to examine the interaction domains of HHV-6A gQ1 (AgQ1) and AgQ2. We found that amino acid residues 163 to 185 in AgQ2 are important for interaction of AgQ1 and AgQ2. Finally, to investigate whether HHV-6B gQ2 (BgQ2) can complement AgQ2, an HHV-6A genome harboring BgQ2 was constructed. The mutant could not produce an infectious virus, indicating that BgQ2 cannot work for the propagation of HHV-6A. These results suggest that gQ2 supports the tetramer's function, and the combination of gQ1 and gQ2 is critical for virus propagation.

IMPORTANCE Glycoprotein Q2 (gQ2), an essential gene for virus propagation, forms a heterodimer with gQ1. The gQ1/gQ2 complex has a critical role in receptor recognition in the gH/gL/gQ1/gQ2 complex (a tetramer). We investigated whether gQ2 regulates the specific interaction between the HHV-6A or -6B tetramer and CD46 or CD134. We established a cell-cell fusion assay system for HHV-6A/6B and switched the gQ1 or gQ2 of HHV-6A with that of HHV-6B in the tetramer. Although cell fusion was induced via CD46 when gQ1 or gQ2 was switched between HHV-6A and -6B, the activity was lower than that of the original combination. When gQ1 or gQ2 was switched in HHV-6A and -6B, no cell fusion was observed via CD134. HHV-6B gQ2 could not complement the function of HHV-6A's gQ2 in HHV-6A propagation, suggesting that the combination of gQ1 and gQ2 is critical for regulating the specificity of the tetramer's function for virus entry.

KEYWORDS gQ1, gQ2, tetramer, HHV-6A, HHV-6B, CD134, CD46

Human herpesvirus 6 (HHV-6) is a double-stranded DNA virus that belongs to the betaherpesvirus subfamily and has affinity for T lymphocytes (1–3). Although the homology of the HHV-6A and HHV-6B genome sequences is approximately 90% (4), these sequences are classified as different viruses based on differences in nucleotide sequence, antigenicity, and cell tropism (5, 6). HHV-6B is a causative virus of exanthema

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subitum in infancy (7) and, when reactivated in transplant patients, it causes serious diseases such as encephalitis (8, 9). Although the disease(s) caused by primary infection of HHV-6A are not yet established, HHV-6A was shown to be a risk factor for multiple sclerosis (MS) (10, 11) and, more recently, for the progression of Alzheimer's disease (12, 13). The reported entry receptors of each virus are human CD46 (hCD46) (14) and human CD134 (hCD134) (15, 16). Human CD46, a member of the regulator of complement activation (RCA) family (17, 18), is ubiquitously expressed on nuclear cells (with the exception of blood cells). Human CD134 (OX40), which is present on activated T cells (19), is a member of the tumor necrosis factor (TNF) receptor superfamily (20).

We demonstrated that HHV-6A and -6B have a specific envelope glycoprotein complex, i.e., a glycoprotein H (gH)/gL/gQ1/gQ2 complex (a tetramer), which is a viral ligand for each receptor (21–26). In order for the tetramer to be incorporated into the virus particle, it must go through the dimer formation of gH/gL and gQ1/gQ2 in the endoplasmic reticulum (ER), proceeded by the tetrameric complex in the post-ER compartment (21).

We also observed that the combination of tetramer components is important for the interaction between each tetramer and its typical receptor. The HHV-6A tetramer with HHV-6B gQ2 was able to bind to hCD46, although its affinity was lower than that of the wild-type tetramer of HHV-6A (27). In addition, the interaction of a chimeric tetramer containing gH, gL, and gQ2 of HHV-6A and gQ1 of HHV-6B with hCD46 was greatly decreased (27). Interestingly, when either gQ1 or gQ2 was switched between HHV-6A and -6B in the HHV-6B tetramer, a loss of binding to hCD134 was observed (16, 28). In contrast, the replacement of gH or gL between HHV-6A and -6B did not affect the tetramer-receptor interaction (16, 27, 28). We thus speculated that the combination of gQ1 and gQ2 may contribute to the receptor recognition of the HHV-6A or HHV-6B tetramer.

In the present study, to confirm which glycoprotein(s) are required for the tetramer-receptor interaction, we established a cell-cell fusion assay system for HHV-6A and HHV-6B that uses Chinese hamster ovary (CHO) cells expressing hCD46 or hCD134. With this assay, we observed that the combination of gQ1 and gQ2 in the tetramer of HHV-6A or -6B is important for the cell fusion mediated by gB, gH, gL, gQ1, and gQ2 via hCD46 or hCD134. We also identified the interaction domains of the gQ1 and gQ2 of HHV-6A. In addition, to determine whether the replacement of the gQ2 of HHV-6A with that of HHV-6B affects viral infection, we generated HHV-6A harboring the gQ2 of HHV-6B by using our bacterial artificial chromosome (BAC) system. The results demonstrated that the gQ2 of HHV-6B is unable to complement the function of the gQ2 of HHV-6A for the propagation of HHV-6A; that is, the gQ2 of HHV-6A plays a specific role in the life cycle of HHV-6A.

RESULTS

Confirmation of cell fusion mediated by HHV-6B gH/gL/gQ1/gQ2 and gB via CD134. We reported that the gQ1 of HHV-6A or HHV-6B is responsible for gH/gL/gQ1/gQ2 complex (tetramer)-receptor recognition (22, 28). Here, when we replaced the gQ1 of HHV-6A with the gQ1 of HHV-6B in the HHV-6A tetramer, the chimeric tetramer could bind only barely to human CD46 (hCD46). In addition, the HHV-6B tetramer with HHV-6A gQ1 could not bind to hCD134 at all. In case of the gQ2, when the gQ2 of HHV-6A was replaced with gQ2 of HHV-6B in the HHV-6A tetramer, the binding of chimeric tetramer to the hCD46 became weak. The binding of HHV-6B tetramer containing HHV-6A gQ2 to hCD134 became lost (16, 27, 28). In contrast, the homologous replacement of gH and gL did not affect the tetramer-receptor interaction (16, 27, 29).

In this study, to examine whether the replacement of gQ1 or gQ2 between HHV-6A and -6B affects cell fusion activity via hCD46 or hCD134, we used a recently established HHV-6 cell-cell fusion assay system based on the coexpression of gH, gL, gQ1, gQ2, and gB (Y822A) in 293T cells (30). The gB (Y822A) construct contains an alanine mutation at the tyrosine of the YXXφ motif in the cytoplasmic tail to express gB on the cell surface (30). First, to confirm the expression level of each tetramer on the cell surface,

we cotransfected 293T cells with plasmids of each glycoprotein using polyethylenimine (PEI) Max and then harvested the cells at 24 h after transfection. As described (16, 26), the gQ1 and gQ2 of HHV-6A or HHV-6B were not transported to the cell surface unless they were included in the tetramer. We thus used three types of anti-gQ1 monoclonal antibody (Mab) to detect tetramer expression on the cell surface (Fig. 1A).

The tetramers with HHV-6A gQ1 were reacted with Mab AgQ 1-1, which is a neutralizing antibody against HHV-6A gQ1, and the tetramers with HHV-6B gQ1 were detected with Mab KH-1, which is a neutralizing antibody against HHV-6B gQ1. As shown in the left panel of Fig. 1A, all of the tetramers could be recognized by Mab gQ1-1, which could react to both HHV-6A and HHV-6B gQ1. These results indicated that all of the tetramers expressed on the cell surface at similar levels. This experiment was conducted three times independently, and the results showed the same trend. We then performed the cell-cell fusion assay, as illustrated Fig. 1B. The effector cells were transfected with gH, gL, gQ1, gQ2, and gB with T7 polymerase (pCAGT7) expression vectors, and the target cells were transfected with firefly luciferase under the control of T7 promoter (pT7EMCV-Luc) and hCD134 (pCAGGS-hCD134), because 293T cells express endogenous hCD46 but not hCD134.

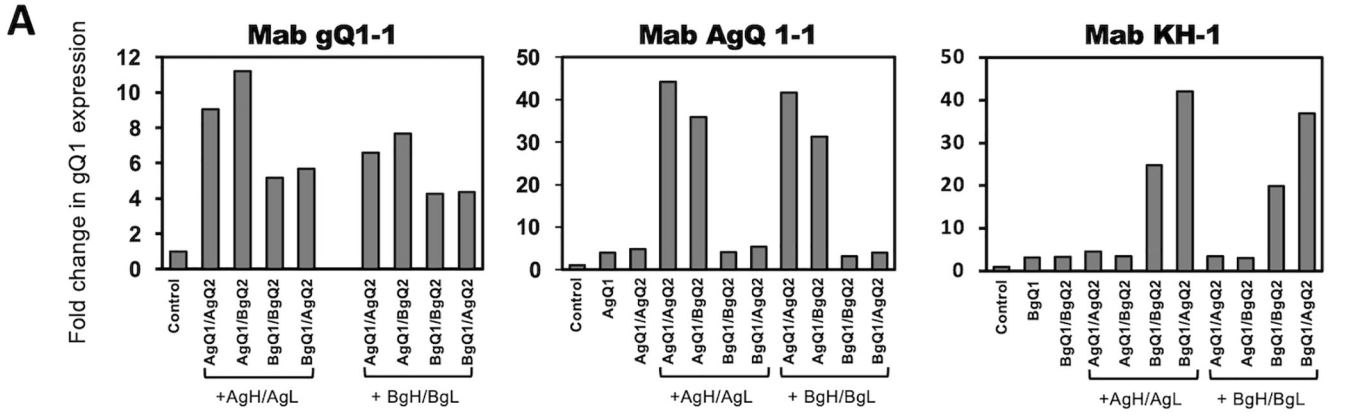
As shown in Fig. 1C, although we could detect CD46 on both the 293T and 293T-hCD134 cells, hCD134 could be detected only on 293T-hCD134 cells by the flow cytometry analysis. At 6 to 12 h after transfection, we combined the effector cells with the target cells and then incubated them for 6 to 12 h. The cells were harvested and lysed to quantify fusion activities using a Dual-Luciferase reporter assay system (Promega). As shown in Fig. 1D, when AgQ1 was in the tetramer, luciferase activity was detected on both 293T and 293T-hCD134 cells. Regarding HHV-6B tetramer, the luciferase activity was detected in only the 293T-hCD134 cells, not the 293T cells. On the other hand, the luciferase activity was observed in both of these cells when BgQ2 was replaced with AgQ2 in the HHV-6B tetramer.

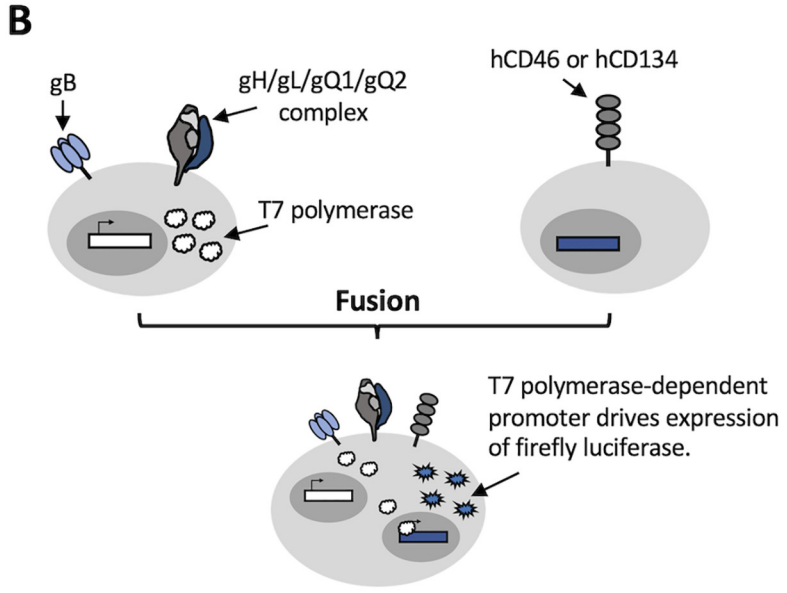
However, little luciferase activity was detected from the cells expressing the original HHV-6A tetramer because almost all of the cells were already dead when they were harvested due to rapid cell fusion, indicating that the kinetics of cell fusion mediated by HHV-6A tetramer and gB with hCD46 was very fast. We therefore separately compared the luciferase activity with the combination of AgQ1/AgQ2 and AgQ1/BgQ2 (Fig. 1E). The luciferase activity of the original HHV-6A tetramer was higher than that of the HHV-6A tetramer with HHV-6B gQ2 on both cell types. In addition, the results shown in Fig. 1D indicated that HHV-6B tetramer could not induce cell-cell fusion without the expression of hCD134, and the combination of gQ1 and gQ2 is a key factor for the induction of cell-cell fusion (Fig. 1D and E).

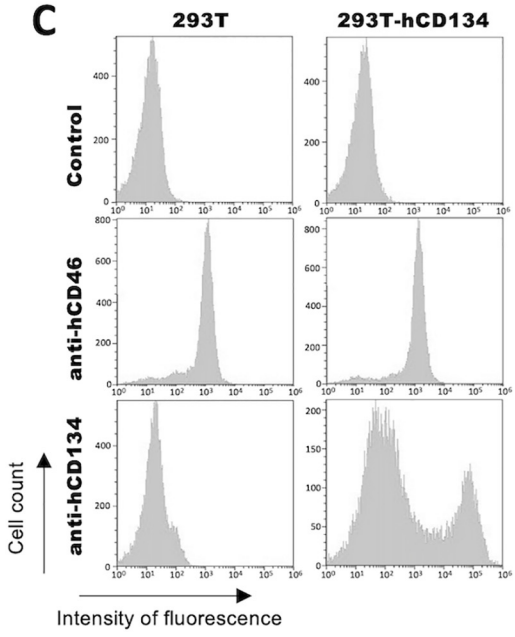
HHV-6A or -6B gH/gL/gQ1/gQ2 and gB induce cell fusion specifically to each receptor. In the assay using 293T cells, we could not analyze the critical interaction between the tetramer of HHV-6A or HHV-6B and each receptor because the 293T cells expressed hCD46. We therefore used a CHO cell line to which was introduced hCD46 (CHO-hCD46) (31) or hCD134 (CHO-hCD134). The expression of hCD46 or hCD134 on the surface of the CHO cells was confirmed by flow cytometry analysis (Fig. 2A).

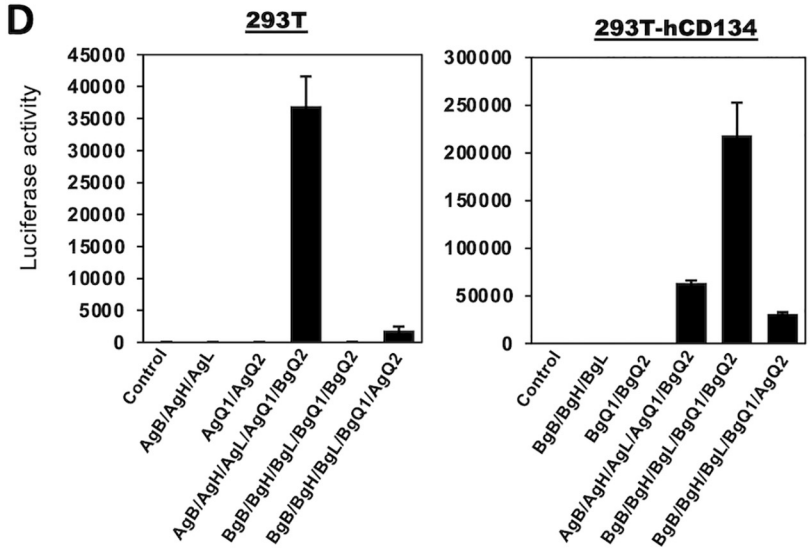
Next, to determine whether gH/gL/gQ1/gQ2 and gB of HHV-6A or -6B could induce cell fusion via hCD46 or hCD134, we cocultured the cells expressing gH, gL, gQ1, gQ2, and gB with CHO-hCD46 cells and CHO-hCD134 cells, respectively. The cells were harvested and stained with hematoxylin and eosin at 24 to 72 h after cocultivation (Fig. 2B). As expected, the cell fusion mediated by HHV-6A gH/gL/gQ1/gQ2 and gB was observed only when the cells were cocultured with CHO-hCD46 cells, and cell fusion mediated by HHV-6B gH/gL/gQ1/gQ2 and gB was observed only when the cells were cocultured with CHO-hCD134 cells.

To quantify the results shown in Fig. 2B, we used the cell-cell fusion assay system of HHV-6A and HHV-6B (Fig. 1B). We cocultured effector CHO cells expressing T7 polymerase with the glycoproteins and target cells (i.e., CHO-hCD46 or -hCD134 cells expressing firefly luciferase) and, a few hours or days later, the cells were harvested and the luciferase activities were measured with the Dual-Luciferase reporter assay system









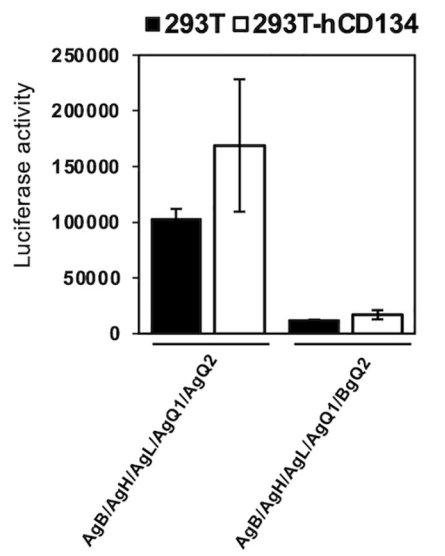


FIG 1 Cell-cell fusion mediated by HHV-6B gB-gH-gLgQ1-gQ2 occurs in 293T cells expressing hCD134. (A) 293T cells were transfected with individual molecule expression vectors and harvested at 24 h later. The cells were reacted with three types of anti-gQ1 Mab (gQ1-1, for both HHV-6A and -6B; AgQ 1-1, for HHV-6A; KH-1, for HHV-6B) at 4°C for 1 h and stained with secondary antibody (FITC-conjugated rabbit anti-mouse IgG) at 4°C for 30 min. The

(Continued on next page)

(Promega). The luciferase activity was detected only when CHO-hCD46 cells were cocultured with CHO cells expressing HHV-6A glycoproteins or when CHO-hCD134 cells were cocultured with CHO cells expressing HHV-6B glycoproteins. These results indicated that the induction of cell fusion mediated by HHV-6A or HHV-6B gH/gL/gQ1/gQ2 and gB was dependent on the expression of hCD46 or hCD134 (Fig. 2C).

Combination of gQ1 and gQ2 in a tetramer affects cell fusion activity. We investigated whether cell fusion via hCD46 occurs when AgQ2 is replaced with BgQ2 in the HHV-6A tetramer by using the method described in Fig. 1B. As shown in Fig. 3A, luciferase activity was detected via hCD46 when the tetramer contained HHV-6A gQ1. However, when AgQ2 was replaced with BgQ2 in the HHV-6A tetramer, the luciferase activity was lower than that of the original tetramer. Although no luciferase activity was detected when both the gQ1 and gQ2 of HHV-6A were replaced with those of HHV-6B, low luciferase activity was observed when AgQ1 was replaced with BgQ1 in the HHV-6A tetramer.

Regarding the CHO-hCD134 cells, no luciferase activity was detected when HHV-6A gQ1 or gQ2 was used in the tetramer (Fig. 3B). These results corresponded with the result presented in Fig. 1D and indicated that the combination of gQ1 and gQ2 is important for the cell fusion mediated by the tetramer with gB via hCD46 or hCD134. The exchanges of gB, gH, and gL between HHV-6A and HHV-6B scarcely affected the receptor-specific fusion.

AgQ2 amino acid residues 163 to 185 are important for the interaction with AgQ1. We previously demonstrated that the complex formation of gQ1 and gQ2 is required for the gQ1-folding and the interaction with gH/gL complex (21, 22).

To determine which domain in HHV-6A gQ2 is required for the interaction with gQ1, and to identify the essential domain for gQ1 and gQ2 heterodimer formation, we generated two mutants with C-terminal truncations in HHV-6A gQ2 (AgQ2). The sequence between amino acid (aa) residues 186 and 214 and the sequence between aa residues 163 and 214 of AgQ2 were deleted and named AgQ2(Δ186–214) and AgQ2 (Δ163–214), respectively. We then performed transfection with AgQ2 (wild-type), AgQ2 (Δ186–214), or AgQ2(Δ163–214) on 293T cells to confirm their expression by an immunofluorescence assay (IFA). The expression of both truncation mutants was detected using anti-gQ2 Mab, and their expressions were similar to that of the wild-type AgQ2 (data not shown).

To determine whether the AgQ2 C-terminal truncation mutants could form a tetramer with AgH, AgL, and AgQ1, we transfected 293T cells with glycoproteins and performed immunoprecipitation (IP) using an antibody against the gH/gL complex. As shown in Fig. 4A, AgH and AgL were detected using anti-gH Mab or anti-gL Mab. In contrast, AgQ1 and AgQ2 or AgQ2(Δ186–214) (but not AgQ1 and AgQ2[Δ163–214]) were precipitated with anti-gH/gL complex Mab and detected using an antibody against gQ1 (gQ1–119) or gQ2. We suspected that AgQ2(Δ163–214) did not form a tetramer with the other glycoproteins because the truncation mutant was not able to interact with AgQ1. We also confirmed the interaction between the tetramer with hCD46, which is the receptor for HHV-6A. Using anti-hCD46 Mab (M177), we detected hCD46 with AgH, AgL, AgQ1, and AgQ2 or AgQ2(Δ186–214), but not AgQ2(Δ163–214) (Fig. 4A).

To address this concern, we precipitated gH/gL/gQ1/gQ2 complex by using Mab gQ1–119. As a result, AgQ2 and AgQ2(Δ186–214) (but not AgQ2[Δ163–214]) were

FIG 1 Legend (Continued)
expression levels of gQ1 were confirmed by flow cytometry analysis, and all data are shown as the fold change relative to the control gQ1 expression. (B) Schematic representation of the cell-cell fusion assay using 293T or CHO cells. Effector cells expressing gB-gH-gL-gQ1-gQ2 (pCAGGS) with T7 polymerase (pCAGT7) were cocultivated with the target 293T or CHO cells expressing hCD46 or hCD134 (293T-hCD134, CHO-hCD46, or CHO-hCD134) and luciferase proteins (pT7EMCV-Luc) at 4 to 48 h posttransfection. At a few hours or days after the coculture, luciferase activities were measured by a Dual-Luciferase reporter assay system. (C) 293T cells were transfected with hCD134 expression vector, harvested 24 h later, and stained with anti-CD134 antibody followed by reacting secondary antibody (FITC-conjugated rabbit anti-mouse IgG). The hCD134 was detected by a flow cytometry analysis. (D and E) 293T cells expressing gB-gH-gL-gQ1-gQ2 of HHV-6A or HHV-6B were cocultured with 293T or 293T-hCD134 cells. Luciferase activity in the lysates was measured using the Dual-Luciferase reporter assay system. The values are the mean ± standard deviation (SD) of the results tested with triplicate samples.

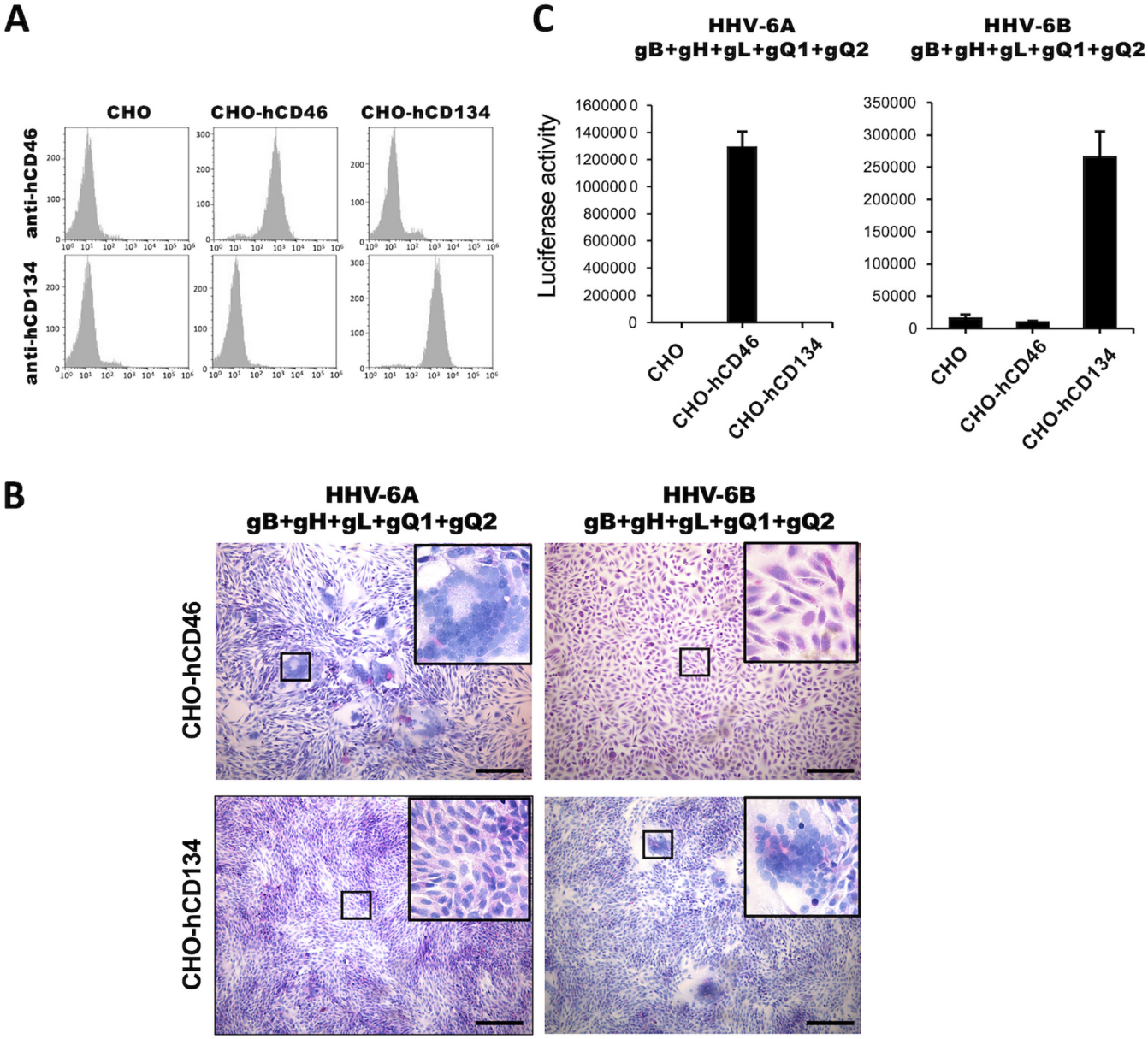


FIG 2 Cell-cell fusion mediated by HHV-6A or HHV-6B tetramer with gB occurs in a receptor-dependent manner. (A) The expression of hCD46 or hCD134 on CHO cells was confirmed by a flow cytometry analysis using anti-hCD46 Mab (J4.48) and anti–hCD134 Mab. (B) CHO cells were transfected with HHV-6A or HHV-6B gB, gH, gL, gQ1, and gQ2, and 8 to 12 h later, CHO cells expressing glycoproteins were cocultivated with CHO, CHO-hCD46 (30), or CHO-hCD134 cells. The cells were harvested and fixed in methanol for 30 min and then subjected to hematoxylin and eosin (HE) staining. Black boxes are enlarged images; scale bar, 20 μm. (C) CHO cells expressing gB-gH-gL-gQ1-gQ2 of HHV-6A or HHV-6B were cocultured with CHO, CHO-hCD46, or CHO-hCD134 cells.

detected using anti-gQ2 Mab (Fig. 4B). These results suggested that amino acid residues 163 to 185 in AgQ2 are involved in the interaction with AgQ1 that was required for tetramer formation.

AgQ2(Δ186–214) enhanced the cell fusion mediated by glycoproteins via hCD46. As shown in Fig. 4, there was no discernible difference in the interaction between AgQ1 and AgQ2(Δ186–214) and the binding to hCD46 compared with that of AgQ2. To analyze the function of AgQ2(Δ186–214) in more detail, we performed a cell-cell fusion assay. We first confirmed the expression of the tetramer with AgQ2, AgQ2 (Δ186–214), or AgQ2(Δ163–214) by flow cytometry, and 293T cells were transfected with plasmids expressing gH, gL, gQ1, and gQ2 (AgQ1, AgQ2[Δ186–214] or AgQ2 [Δ163-214]). Using anti-gQ1 Mab (AgQ 1-1), a tetramer with AgQ2(Δ186–214) could be

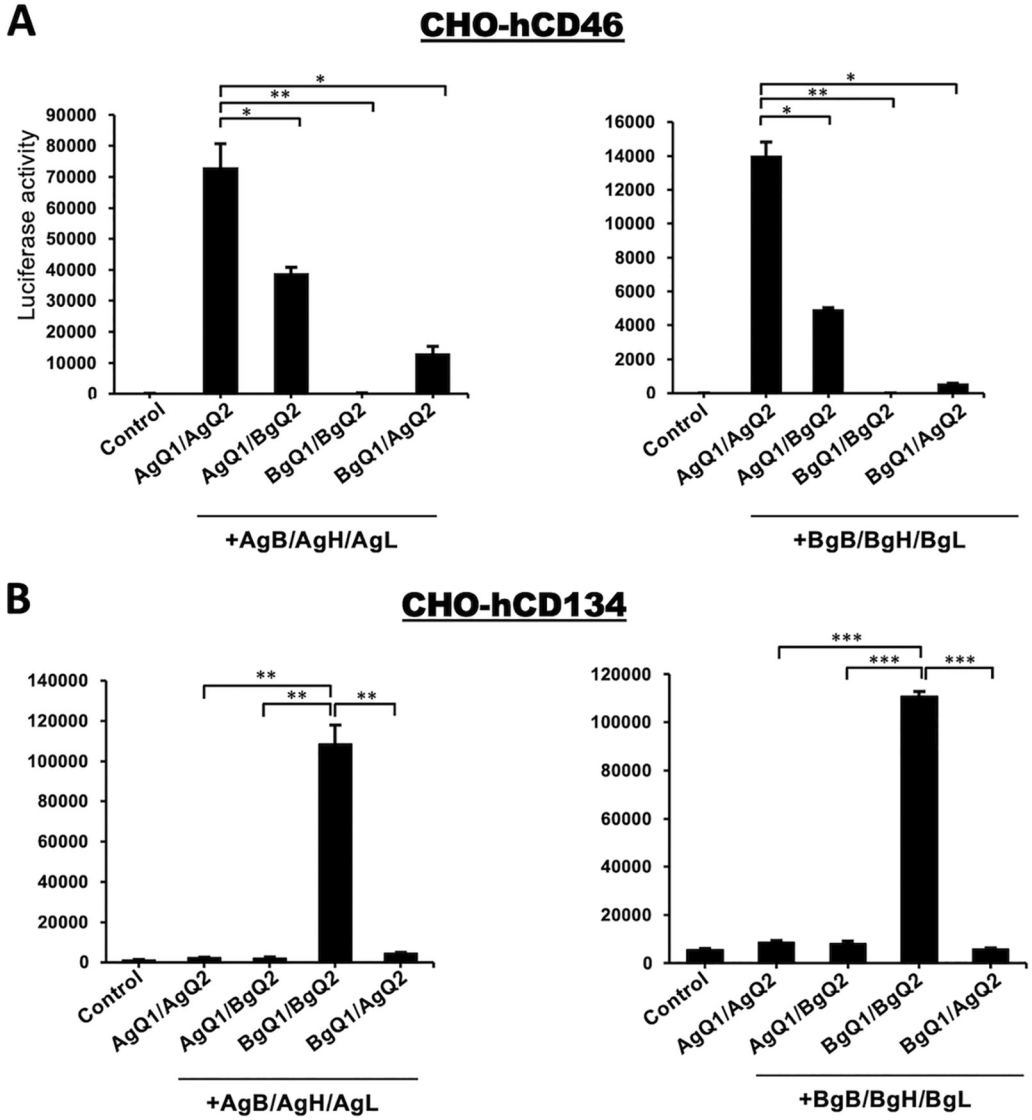


FIG 3 The combination of gQ1 and gQ2 between HHV-6A and HHV-6B in the gH/gL/gQ1/gQ2 complex is important for cell fusion efficiency. (A and B) CHO cells were transfected with individual genes and cocultivated with CHO-hCD46 or CHO-hCD134 cells harboring firefly luciferase. The cells were lysed, and then the luciferase activity in the lysates was measured using the Dual-Luciferase reporter assay system. The values are the mean ± SD (error bars) of the results tested with triplicate samples. Student's *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are representative of at least three independent experiments.

detected at the same level as that of AgQ2. As we expected, a tetramer with AgQ2 (Δ163–214) was not expressed on the cell surface, as seen in the control transfected with AgQ1 and AgQ2 (Fig. 5A).

The effector CHO cells were transfected with T7 RNA polymerase (pCAGT7), AgB, AgH, AgL, AgQ1, and AgQ2, AgQ2(Δ186–214), or AgQ2(Δ163–214). The target CHO-hCD46 or CHO–hCD134 cells were transfected with firefly luciferase (pT7EMCVLuc). At 24 h posttransfection, we cocultivated the effector and target cells. Luciferase activity was measured at 4 or 12 h after the coculture using the Dual-Luciferase reporter assay system, and the luciferase activity of AgQ2(Δ186–214) was higher than that of AgQ2 at both 4 and 12 h. We did not observe any luciferase activity in the cells transfected with AgQ2(Δ163–214) (Fig. 5B). These results thus indicate that the region between amino acids 163 to 185 of gQ2 is necessary for the interaction with gQ1, inducing the transport of the gQ1/gQ2 complex.

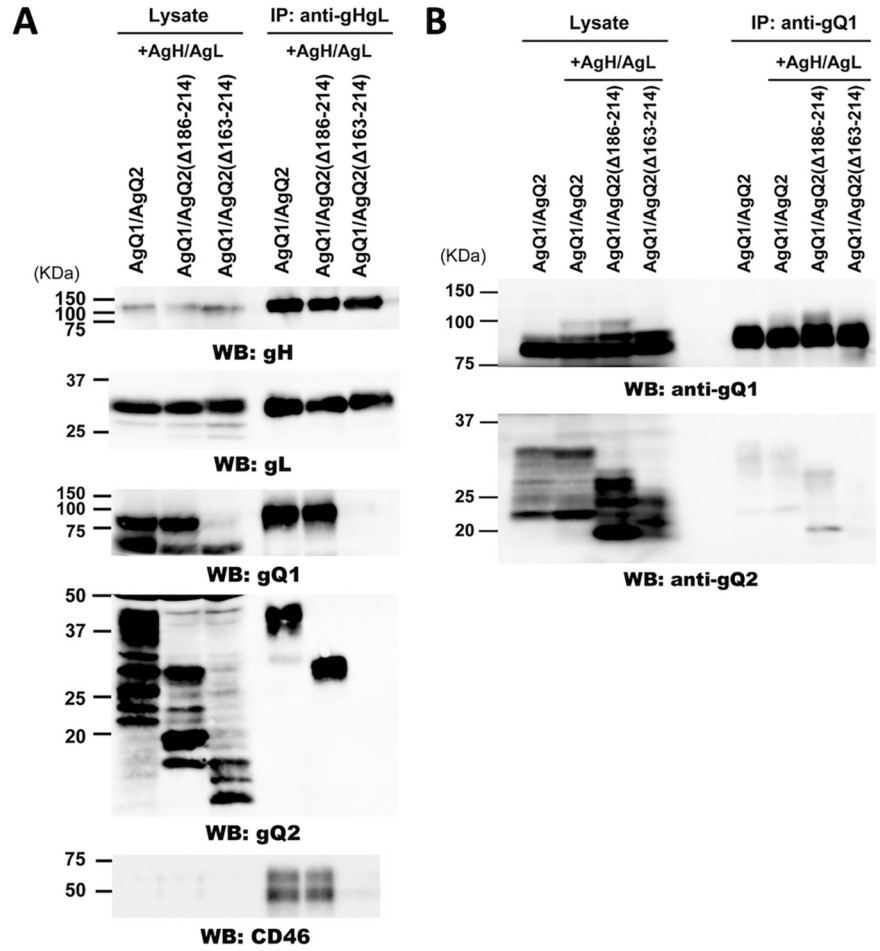


FIG 4 The interaction between AgQ2(Δ186–214) or AgQ2(Δ163–214) and AgQ1. (A) 293T cells were transfected with AgH, AgL, AgQ1 and AgQ2, AgQ2(Δ186–214) or AgQ2(Δ163–214) vectors. At 3 days posttransfection, the cells were harvested and lysed with TNE buffer for immunoprecipitation (IP) using an antibody against gH/gL complex. In lysates or precipitates, the gH, gL, gQ1, gQ2, or hCD46 was confirmed by Western blotting. (B) IP was performed using anti-gQ1 Mab (gQ1-119). The expression levels of gQ1 and gQ2 were detected using anti-gQ1 Mab (gQ1-119) or anti-gQ2 Mab.

Replacement of HHV-6A gQ2 with HHV-6B gQ2 in the HHV-6A genome. In an earlier investigation, we observed that gQ2 is essential for virus propagation (26). However, it is unknown whether gQ2 could be shared between HHV-6A and HHV-6B in virus infection, although it was reported that gH could be shared (29). To address this question, we generated recombinant viruses using our HHV-6ABAC system (29, 32–34). The amino-acid sequence alignment of gQ2 is shown in Fig. 6A. The gQ2 encoded in the U100 gene is 182 aa for HHV-6A strain U1102 and 214 aa for HHV-6B strain HST in size. They share approximately 68.7% amino acid sequence identity (35). We introduced the HHV-6B gQ2 (strain HST) sequence into the U100 gene of HHV-6A and established two clones that we named HHV-6A-BgQ2 clones 1 and 2. The revertant genome of HHV-6A-BgQ2 clone 1 was also generated and named HHV-6A-BgQ2rev (Fig. 6B).

HHV-6B gQ2 could not complement the function of HHV-6A gQ2 in HHV-6A propagation. We transfected BAC DNA of HHV-6A (wild type), HHV-6A-BgQ2 (clones 1 and 2), or HHV-6A-BgQ2rev extracted from *Escherichia coli* into JJhan cells (which are a permissive cell line for HHV-6A) by electroporation. The JJhan cells were then cocultured with cord blood mononuclear cells (CBMCs). Although we observed a high level of GFP expression with the cytopathic effect (CPE) in the cells transfected with HHV-6A or HHV-6A-BgQ2rev, no CPE was observed in HHV-6A-BgQ2 clones 1- or 2-transfected

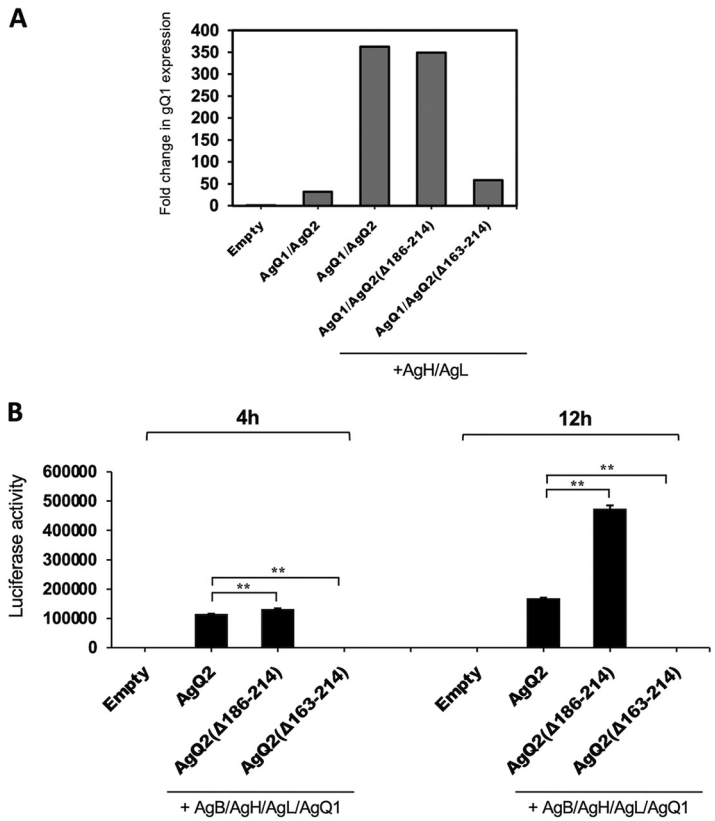


FIG 5 AgQ2(Δ186–214) enhanced the cell fusion mediated by glycoproteins via hCD46. (A) The expression levels of the tetramers with AgQ2, AgQ2(Δ186–214), or AgQ2(Δ163–214) were analyzed by flow cytometry using anti-gQ1 Mab (AgQ 1-1). The gQ1 expression was quantified as a fold change relative to the control. (B) Effector CHO cells expressing T7 RNA polymerase (pCAGT7), AgB, AgH, AgL, AgQ1 and AgQ2, AgQ2(Δ186–214) or AgQ2(Δ163–214) were cocultivated with the target CHO-hCD46 or CHO-hCD134 cells expressing firefly luciferase (pT7EMCVLuc) at 24 h posttransfection. At 4 or 12 h later, the luciferase activity was measured using a Dual-Luciferase reporter assay system. Bars are mean of the result determined with triplicate samples. **, $P < 0.01$ by Student's *t* test; error bars, SD. The data are representative of three independent experiments.

cells after coculture with CBMCs (Fig. 7). To confirm the expression of viral proteins in the cells infected with each virus, we performed Western blotting. The tegument protein (U11) (33) and two glycoproteins (gQ1 and gQ2) were detected in HHV-6A-infected cells and HHV-6A-BgQ2rev-infected cells (Fig. 8A). Although Fig. 7 implied that HHV-6A-BgQ2 does not propagate, we analyzed the expression of viral proteins from the cells in which BAC DNA of HHV-6A-BgQ2 was transduced. As shown in Fig. 8B, we could not detect gQ1 of HHV-6A-BgQ2 using an anti-gQ1 antibody, even though the expression of gQ1 from HHV-6A or HHV-6A-BgQ2rev infected cells was observed. These results indicated that HHV-6A-BgQ2 did not produce an infectious virus.

DISCUSSION

We used our cell-cell fusion assay system to determine which of the molecules of HHV-6A or HHV-6B gH/gL/gQ1/gQ2 complex are important for receptor recognition. We previously reported on a HHV-6A cell-cell fusion assay using 293T cells (30). Here, we created a novel system of HHV-6A or HHV-6B using CHO cells which stably express hCD46 (CHO-hCD46) or hCD134 (CHO-hCD134) because it seemed difficult to determine the essential role of each tetramer-receptor interaction when 293T cells were used, as 293T cells express endogenous CD46 but not CD134 (Fig. 1C). As shown in Fig.

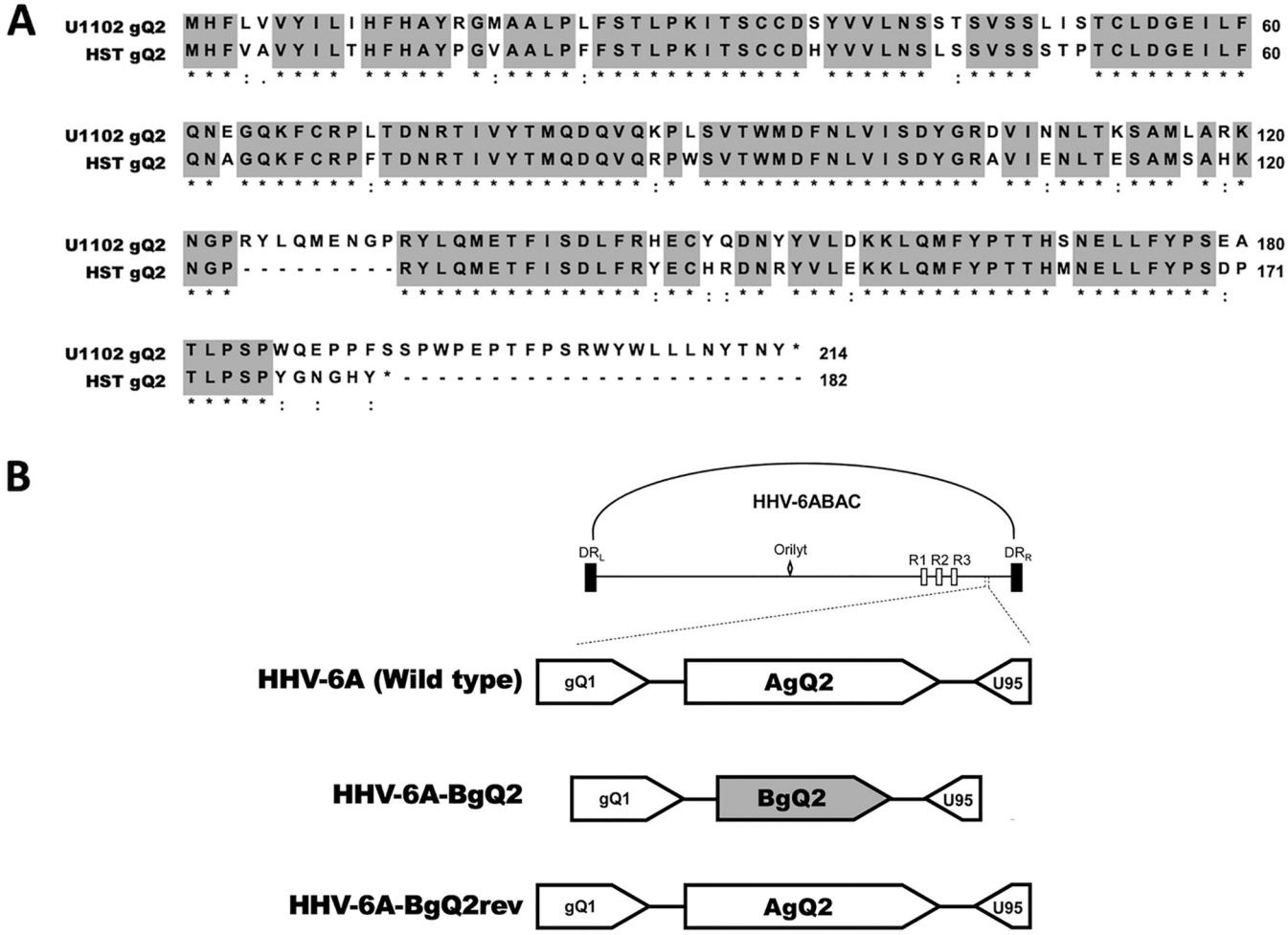


FIG 6 Construction of HHV-6A harboring HHV-6B gQ2. (A) Amino acid sequence alignment of HHV-6A and HHV-6B gQ2. The two viruses have 68.7% amino acid identity. (B) The HHV-6ABAC genome contains an origin of replication (Orilyt), a direct repeats region on the left and right sides (DR_L and DR_R), and three major internal repeat elements (R1, R2, and R3). HHV-6A gQ2 (AgQ2) exists within the gQ1 and U95. HHV-6A gQ2 (AgQ2) is replaced with HHV-6B gQ2 (BgQ2) (which is named "HHV-6A-BgQ2") and its revertant, HHV-6A-BgQ2rev.

2B and C, the cell-cell fusion mediated by HHV-6A or HHV-6B tetramer was dependent on hCD46 or hCD134.

To identify the molecules that contribute to this receptor-specific cell fusion, we performed the cell-cell fusion assay with the gB, gH, gL, gQ1, or gQ2 switched between HHV6A and HHV-6B. In Fig. 1A and Fig. 5A, we used 293T cells to analyze cell surface expression of the tetramers because the expression levels of tetramer were not enough to be detected by flow cytometry in CHO cells. As shown in Fig. 1A, analysis of Mab KH-1 against both HHV-6B gQ1 showed the expression level of the tetramer with BgQ1/AgQ2 was higher than that of BgQ1/BgQ2. Although we confirmed cell surface expression several times, there were variations in the results of expression levels. Thus, we also used gQ1-1 antibody for checking the expression of the tetramer. As shown with the gQ1-1 antibody, we speculate there was no big differences between expression levels of BgQ1/BgQ2 and BgQ1/AgQ2 (with gH/gL).

As shown in Fig. 1D, 1E, 3A, and 3B, when gQ1 or gQ2 was replaced with its original in each tetramer, the cell fusion significantly decreased or did not occur. In particular, when gQ1 was replaced, cell fusion was rarely seen. These results indicated that gQ1 is critical for the receptor binding, and that its combination with gQ2 is essential for its correct folding.

Interestingly, when HHV-6B gQ1 formed a complex with HHV-6A gQ2 in the tetramer, a slight amount of cell fusion could be induced via hCD46 (Fig. 1D and 3A). Our

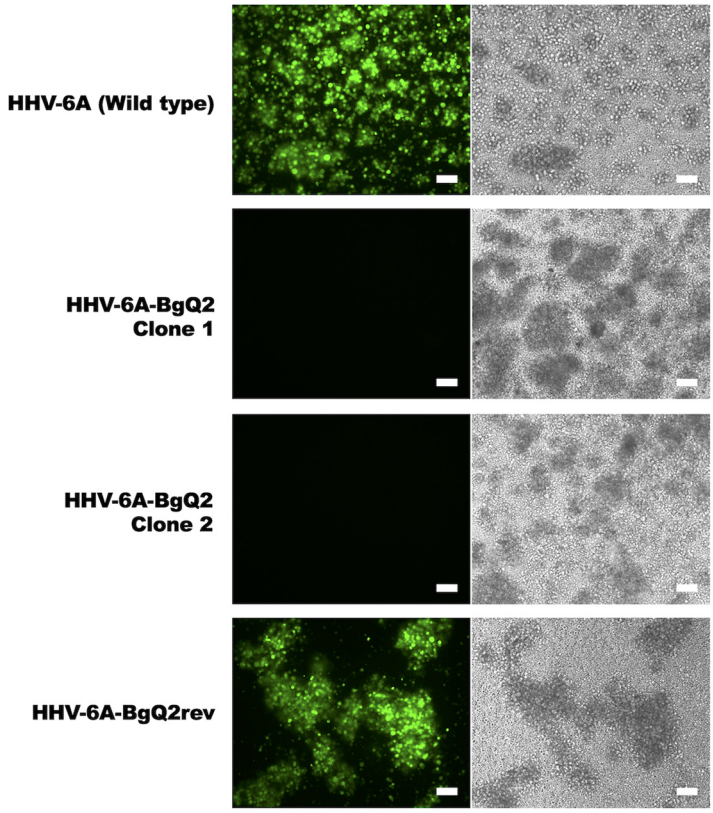


FIG 7 The reconstitution of HHV-6A-BgQ2 and its revertant. JJhan cells were transfected with BAC DNA of HHV-6A, HHV-6A-BgQ2 clone 1, clone 2, or HHV-6A-BgQ2rev. The transfected cells were cocultivated with CBMCs at 4 to 6 days posttransfection, and viruses were propagated. GFP fluorescence images (left panels) and light microscopy images (right panels) of each type of virus-infected cells are shown. Scale bar indicates 100 μm.

prior study's flow cytometry analysis revealed that the HHV-6B tetramer, including HHV-6B gQ1 and HHV-6A gQ2, bound slightly to hCD46 (27). These results may indicate that HHV-6B gQ1 has a recognition site for hCD46 in addition to the hCD134 recognition site, and HHV-6A gQ2 may work to expose its hCD46 binding site inside HHV-6B gQ1, forming a BgQ1/AgQ2 complex. However, its function in HHV-6B virus itself seems to be disrupted, because its fusion activity was very low.

We have shown that gH, gL, gQ1, and gQ2 form a tetramer. To accomplish this, gH and gL (gH/gL) and gQ1 and gQ2 (gQ1/gQ2) should each dimerize in the endoplasmic reticulum. The gH/gL and gQ1/gQ2 complexes subsequently form tetramers through a process of translocation to the *trans*-Golgi network, and then are ultimately located on the virus particle. Since gQ1 and gQ2 cannot migrate out of the endoplasmic reticulum unless they form a complex, their interaction in the endoplasmic reticulum is a very important event (21, 26). The results of the present cell-cell fusion assay revealed that the combination of gQ1 and gQ2 affected the cell fusion mediated by HHV-6 glycoproteins. We therefore focused on the dimer formation of gQ1 and gQ2 of HHV-6A in the tetramer, and we determined the site(s) of gQ2 that are required for the binding to gQ1.

As shown in Fig. 4A, when the C-terminal domain of gQ2 was deleted up to 186 amino acid (aa) (AgQ2[Δ186–214]), AgQ2(Δ186–214) could form a tetrameric complex with gH, gL, and gQ1. When gQ2 was deleted up to 163 aa (AgQ2[Δ163–214]), AgQ2 (Δ163–214) could not form the complex. This is because AgQ2(Δ163–214) was unable to interact with gQ1 (Fig. 4B). The tetramer was not expressed on 293T cells when gH, gL, gQ1, and AgQ2(Δ163–214) were introduced (Fig. 5A). The aa region 163 to 185 of

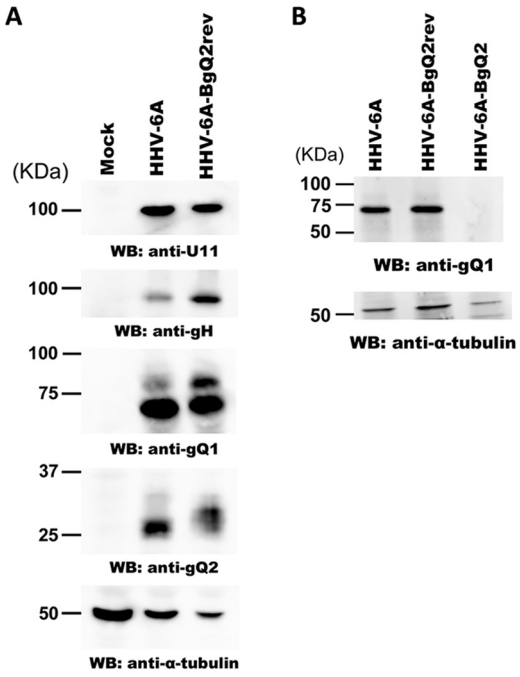


FIG 8 The expression of viral proteins from CBMCs infected with HHV-6A, HHV-6A-BgQ2rev, or HHV-6A-BgQ2. (A) Cells infected with HHV-6A or HHV-6A-BgQ2rev were harvested, lysed, and resolved by SDS-PAGE. Western blotting was performed using antibody against U11, gH, gQ1(gQ1-119), gQ2, and tubulin (loading control). (B) HHV-6A-BgQ2 transfected cells were used for Western blotting by using gQ1(gQ1-119).

AgQ2 may be involved in the dimer formation with AgQ1, and if dimer formation does not occur, it cannot form a tetramer with the gH/gL complex and thus cannot bind to hCD46 (Fig. 4A). On the other hand, there is the possibility that the large deletion of the C-terminal region of gQ2 may have an adverse effect on the correct folding of gQ2, resulting in the loss of function for binding to gQ1. Further analyses are needed to identify the critical domain for interaction between gQ1 and gQ2.

The results of the cell-cell fusion assay with AgQ2(Δ186–214) are also interesting (Fig. 5B). The cell fusion by gB-gH-gL-gQ1 with AgQ2(Δ186–214) was induced faster and stronger than that with wild-type AgQ2, and the cell fusion was clearly visible by microscopy. Although the gQ2 of the U1102 strain (HHV-6A) consists of 214 aa, in our laboratory, U1102-infected cells frequently showed a short gQ2-encoding virus in which tryptophan was substituted into the stop codon aa residue at 186 aa due to a point mutation, i.e., TGG to TGA. In an earlier investigation, we analyzed the ability of AgQ2(Δ186–214) to form tetramers and observed that it showed no difference from wild-type AgQ2 (26). As shown in Fig. 4B, AgQ2(Δ186–214) did not affect the interaction with gH, gL, and gQ1 in the tetramer and subsequent binding with hCD46. It is not yet known how this single nucleotide polymorphism (SNP) introducing a stop codon could contribute to the promotion of cell fusion. This SNP has also been reported by another laboratory (36), suggesting that the mutation may be naturally introduced by long-term passage.

The gQ2 has 68.7% amino acid identity between HHV-6A and HHV-6B, which is the lowest among the molecules constituting the tetramer. We suspected that gQ2 may function specifically for HHV-6A or HHV-6B infection. In a previous study, we demonstrated that HHV-6A gH could be exchanged with HHV-6B gH in the HHV-6A genome (29). This may be due to the 94.3% amino acid homology between the two viruses (29) and because, above all, gH and gL are conserved in the *Herpesviridae* family (37) and thus the basic function of gH related to membrane fusion may be common.

Here, to clarify whether HHV-6A gQ2 functions for only HHV-6A infection, we replaced the HHV-6A gQ2 with the gQ2 of HHV-6B by using a Red recombination system in *E. coli*. As shown in Fig. 7 and 8, despite our attempts to analyze two clones (HHV-6A-BgQ2 clones 1 and 2), they could not be obtained as infectious viruses after their introduction to JJhan cells. In contrast, the revertant (HHV-6A-BgQ2rev) of clone 1 could be reconstituted and expressed viral proteins at the same level as the wild type (HHV-6A). Fig. 8A illustrates our observation that the gQ2 band of HHV-6A and that of HHV-6A-BgQ2rev were at different positions. This seems to be due to the difference in the infection efficiency and cycle between HHV-6A and HHV-6A-BgQ2rev, and this may have led to differences in the stage of the glycosylation of gQ2. In another study, we demonstrated that gQ1 and gQ2 vary in molecular weight in infected cells depending on where they undergo glycosylation (21, 26). Although there were gQ1 bands at 74 and 80 kDa, it was reported that gQ1 (80 kDa) is incorporated into virus particles (21).

These results indicated that HHV-6B gQ2 does not function in HHV-6A infection, unlike HHV-6A gQ2. Although gQ2 is thought to be a molecule that supports the function of gQ1, our present findings suggest that the structure of gQ1 changes depending on the combination with gQ2 in order to exert a fatal effect on viral infection. Interestingly, previous cell-cell fusion assays using hCD46 or the direct binding of tetramers to hCD46 (27) did not show any lethal effects such as loss of cell fusion or hCD46 binding by the combination of HHV-6A gQ1 and HHV-6B gQ2. However, in actual virus infection, as shown in Fig. 7 and 8, merely replacing HHV-6A gQ2 with HHV-6B gQ2 in the HHV-6A genome did not produce an infectious virus.

Our present findings suggest that the combination of gQ1 and gQ2 is important for viral propagation, probably for determining the receptor specificity of HHV-6A or HHV-6B for virus entry.

MATERIALS AND METHODS

Cells. Cells of the human embryonic kidney cell line 293T were cultured in Dulbecco's modified Eagle medium (DMEM) containing 8% fetal bovine serum (FBS). Chinese hamster ovary (CHO) cells (38) and CHO cells stably expressing human CD46 (hCD46) with the vectors containing the cDNA of the ST^{C}/CYT2 isoform (31) were cultured in Ham's F-12 medium with 8% FBS. CHO cells stably expressing human CD134 (hCD134) were prepared as described (31).

Briefly, the CHO cells were transfected with pCAGGS vector containing human CD134 (hCD134) cDNA and the puromycin resistance gene by using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. The cells were then maintained in Ham's F-12 medium (8% FBS) containing 0.1 mg/ml of puromycin. Correct colonies resistant to puromycin were selected, and the hCD134 expression level was confirmed by flow cytometry analysis.

The human T-cell line JJhan was cultured in RPMI 1640 medium containing 8% FBS. Umbilical cord blood mononuclear cells (CBMCs) were propagated in RPMI 1640 medium with 10% FBS, phytohemagglutinin (5 μg/ml), and interleukin-2 (IL-2) (2 ng/ml) (29, 39). CBMCs were purchased from RIKEN (Institute of Physical and Chemical Research School of Medicine; Ibaraki, Japan). The use of CBMCs was approved by the Ethics Committee of Kobe University Graduate School of Medicine.

Antibodies. The following mouse monoclonal antibodies (Mabs) have been described: Mabs for HHV-6A gH (gH1-1) (26), gL (AgL3), gH/gL complex (gHgL A2) (26), gQ1 (i.e., gQ1-119 [40], AgQ 1-1 [35], and gQ1-1 [27]), gQ2 (gQ24-2) (26), and hCD46 (M177 [41] and J4.48 [16, 23]) and hCD134 (28). Rabbit polyclonal antibody specific for HHV-6A U11 was reported (33). Anti-α-tubulin (Sigma-Aldrich, St. Louis, MO), FITC-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark), and Alexa Fluor488 goat anti-human IgG (heavy plus light chain [H+L]) (Invitrogen, Carlsbad, CA) antibodies were purchased.

Plasmid construction. The gB tyrosine mutant of HHV-6A in which Y822 is replaced by A, i.e., AgB (Y822A), has been described (30). We also prepared a gB tyrosine mutant of HHV-6B as described (30); its name is BgB(Y822A). Plasmid for the expression of human CD134 was described previously (16). In the present study, we generated dual expression vectors. The template plasmids expressing gH, gL, gQ1 and gQ2 of HHV-6A or HHV-6B that are inserted into a pCAGGS vector (42) have been described (16, 21, 26, 35). For the plasmid expressing both the gH and the gL of HHV-6A or HHV-6B, the gL fragment was amplified from pCAGGS-AgL or -BgL by the appropriate primer pair, digested with HindIII, and ligated into a digested pCAGGS-AgH or -BgH vector. Plasmids expressing both gQ1 and gQ2, AgQ1AgQ2, AgQ1BgQ2, BgQ1BgQ2, or BgQ1AgQ2 were prepared as described above. Their gQ2 fragments were ligated into a digested pCAGGS-AgQ1 or -BgQ1 vector.

For the construction of plasmids expressing the HHV-6A gQ2 carboxyl-terminal deletion mutants AgQ2(Δ163–214) and AgQ2(Δ186–214), each gQ2 fragment was amplified from pCAGGS-AgQ2 vector by the appropriate primers, AgQ2m185stop_547F (5'-CCTAGCCCCTAAggtaccctcgaGAGATC-3') and AgQ2m185stop_539R (5'-gatctctcgagggtaccTTAGGGGCTAGGCAGGGTGGCC-3') or AgQ2m162stop_486F (5'-GAAACTCCAGTAAggtaccctcgaGAGATC-3') and AgQ2m162stop_486R (5'-cgagggtaccTTACTGGAGTT

TCTTGTCCAG-3'), and then digested with HindIII and ligated into a digested pCAGGS-AgQ1 vector. All plasmids were verified by sequence analysis.

Hematoxylin and eosin staining. Each type of cells was stained with Carrazzi's hematoxylin solution (Wako, Tokyo) for 5 min and then rinsed with tap water at 60°C. We used 0.5% EosinY ethanol solution (Wako, Tokyo) for staining the cytoplasm for 1 to 2 min. The cells were then dehydrated using 90% to 100% ethanol (Nacalai Tesque, Kyoto, Japan) and penetrated with xylene after the stain was washed away.

Flow cytometry analysis. Human 293T cells were transfected with glycoproteins or hCD134 expression vectors using polyethylenimine (PEI) Max (molecular weight [MW] 40,000) (Polysciences, Warrington, PA) according to the manufacturer's instructions. The cells were harvested at 24 h posttransfection and then prepared for analysis with anti-hCD46(J4.48), anti-hCD134, or anti-gQ1 Mab (gQ1-1, AgQ 1-1, or KH-1). The analysis was performed with a spectral cell analyzer (SA3800, Sony Biotechnology, Tokyo). CHO-hCD46 or CHO-hCD134 cells were established as stable cell lines. The cells were stained with anti-hCD46 (J4.48) or anti-hCD134 followed by secondary antibody.

HHV-6A or HHV-6B cell-cell fusion assay. 293T cells were co-transfected with HHV-6A or HHV-6B gB, gH, gL, gQ1, and gQ2 expression vectors, creating 293T effector cells. Separately, 293T cells were transfected with hCD134 expression plasmid as target 293T cells (293T-hCD134). At 6 to 12 h posttransfection, the effector cells were cocultivated with 293T or 293T-hCD134 cells, and then cell-cell fusion was observed 6 h (for 293T) or 12 h (for 293T-hCD134) later. Regarding CHO cells, the effector CHO cells that express HHV-6A or HHV-6B tetramer and gB were cocultivated with target cells that stably express hCD46 or hCD134 at 8 to 12 h after transfection. Cell-cell fusion was observed 4 to 12 h later in 293T-hCD134 or CHO-hCD46 cells, or at 24 to 48 later in CHO-hCD134 cells. For the quantification of fusion efficiency, T7 RNA polymerase (pCAGT7) was also cotransfected with gB, gH, gL, gQ1, and gQ2 into effector cells. Firefly luciferase under the control of T7 promoter (pT7EMCVLuc) was transfected into target cells (43). At 4 to 48 h after the coculture, we measured the firefly luciferase activities by using the Dual-Luciferase reporter assay system (Promega, Madison, WI) and a TriStar LB941 multimode reader (Berthold Technologies, Wildbad, Germany) as described (30).

Western blotting. Western blotting was performed as previously described (21). Briefly, 293T cells were transfected with glycoproteins by the calcium phosphate method and harvested at 48 h posttransfection. The cells were resolved with sample buffer (32 mM Tris-HCl [pH 6.8], 7.5% SDS, 5% glycerol, 2.5% 2-mercaptoethanol), boiled for 5 min at 100°C for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes for Western blotting. After blocking with blocking buffer (5% skim milk, 0.001% Tween 20, phosphate-buffered saline [PBS]), the membranes were incubated for 1 h with the first antibody in blocking buffer. For the visualization of the target bands, the membranes were incubated for 30 min with horseradish peroxidase (HRP)-linked sheep anti-mouse or donkey anti-rabbit IgG secondary antibody (GE Healthcare Biosciences, Uppsala, Sweden). The target bands were detected by using chemiluminescence (Nacalai Tesque) and an ImageQuant LAS 4000 mini-imager (GE Healthcare Biosciences).

Immunoprecipitation assay. An immunoprecipitation (IP) assay was done as described (21, 22, 26, 33). Antibodies were bound to protein G-Sepharose (GE Healthcare) by incubation at 4°C for 16 h. Whole-cell extracts were then immunoprecipitated with the appropriate protein G-Sepharose-bound antibody by incubation at 4°C for 16 h. The bound proteins were eluted with 0.1 M glycine (pH 2.8) at 4°C and neutralized with 1 M Tris-HCl (pH 8.8) to pH 7.0 to 8.0. The samples were then subjected to SDS-PAGE. For Western blotting analyses, the antibodies were cross-linked with protein G-Sepharose using dimethyl pimelimidate (DMP; Thermo Fisher Scientific) according to the manufacturer's instructions.

Construction of HHV-6A-BgQ2 and HHV-6A-BgQ2rev. HHV-6A-BgQ2 and its revertant (HHV-6A-BgQ2rev) were constructed by using two-step Red-mediated mutagenesis (29, 32–34, 44). First, to construct HHV-6A-BgQ2, we amplified the kanamycin resistance gene from pEP-KanS using the following two pairs of primers, (BgQ2)kan_936F (5'-CCCTACGTTGCCCTCACCTTATGGCAACGGACATTATTAAaggat gacgacgataag-3') and (BgQ2)kan_1898R (5'-catttaataaagaacatgtgacacgatttctgactctcTTAATAATGTCCGTT GCCATAAGGTGAGGGCAACGTAGGGtaca-3').

The BgQ2 sequence was amplified from the HST genome using the primers BgQ2_17F (5'-tcattgtttgaataacaaactatattgctgctagataattATGCATTTCGTGGCTGT-3') and BgQ2NheR (5'-accGCTAGCttaa taatgtccgttgccataag-3'). Using U1102_146559F (5'-ctgtgtattgtaaaagggaagatttgtaaggacaaagttcagtaacatt-taataaagaac-3') and U1102_147627R (5'-gctgtaaatattcatattatatttaaatgaagataaaagaatcattgtttgaataac-3'), we amplified the target fragment to elongate the homologous region. Next, the PCR products were transformed into *Escherichia coli* GS1783 electroporation-competent cells harboring HHV-6ABAC (wild type). The resultant clones were confirmed by sequence analysis using the appropriate primer, U1102_146451F (5'-ttctatgtaaacttctgc-3'), U1102_147712R (5'-aatataacccccgataac-3'), or Kan 388R (5'-tccgtcagccagtttagtctg-3').

A second recombination was then performed to excise the kanamycin resistance gene after inducing the expression of I-SceI restriction enzyme. A chloramphenicol-resistant but kanamycin-sensitive clone was selected, and we named the resultant BAC "HHV-6A-BgQ2 clones 1 and 2." We next constructed the HHV-6A-BgQ2 revertant (rev) of HHV-6A-BgQ2 clone 1. We amplified the kanamycin resistance gene from pEP-KanS by using the primers U1102BgQ2rev_(AgQ2kan)_F (5'-TTACACAAATTACTGAaggatgac gacgataag-3') and U1102BgQ2rev_(AgQ2kan)_R (5'-catttaataaagaacatgtgacacgatttctgactctcTCAGTA ATTTGTGTAATTTAATAAGAGCCAATACCAACGTcaaccaattaaccaat-3'). To amplify the AgQ2 gene from U1102, we used the primers, AgQ2_16F (5'-cattgtttgaataacaaactatattgctgctagataattATGCATTTCTTGG TTG-3') and AgQ2Rpstxho (5'-ctcgagctgcagTCAGTAATTTGTGTAA-3').

We then elongated the target fragment by using U1102_146451F (5'-ttctatgtaaacttctgc-3') and

U1102_147712R (5'-aatataaccccgataac-3') for the first homologous recombination. The PCR product was transformed into *E. coli* GS1783 harboring HHV-6ABAC (wild type), and we then excised the kanamycin resistance gene by using I-SceI restriction enzyme followed by secondary Red-mediated recombination. We named the resultant BAC "HHV-6A-BgQ2Rev."

Reconstitution of BAC DNA. BAC DNA of HHV-6A (wild type), HHV-6A-BgQ2 (clones 1 and 2), or HHV-6A-BgQ2rev was extracted from *E. coli* using NucleoBond BAC100 (Macherey-Nagel, Düren, Germany) and then resuspended in pH 8.0 Tris-EDTA (10 mM Tris-HCl, 1 mM EDTA). To introduce 3 μg of BAC DNA into 1 to 2 $\times$ 10^6 JJhan cells, we performed electroporation using the Cell Line Nucleofector kit V and Nucleofector 2b device (Lonza, Walkersville, MD) and Program T-008 (Lonza) following the manufacturer's instructions. The cells were incubated for a few days after electroporation and then co cultivated with 2 to 3 $\times$ 10^6 CBMCs. At 4 to 5 days later, the infected cells were cocultured with fresh CBMCs again. An increase in the number of cells expressing green fluorescent protein (GFP) with the cytopathic effect (CPE) was observed after 2 to 3 cocultures with CBMCs.

ACKNOWLEDGMENTS

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A.W. and Y.M. designed the research; A.W., M.N., A.K., C.Y., A.L.P., L.H.T., and J.A. performed the research; A.W. and Y.M. wrote the paper.

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