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SHORT PAPER

Acute Megakaryocytic Leukaemia (AMKL)-like Disease in a Cynomolgus Monkey (*Macaca fascicularis*)

S. Okabayash*,†, C. Ohno* and Y. Yasutomi†

* *The Corporation for Production and Research of Laboratory Primates and* † *Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Hachimandai 1-1, Tsukuba-shi, Ibaraki 305-0843, Japan*

Summary

A 5-year-old male cynomolgus monkey (*Macaca fascicularis*) with a clinical history of bleeding tendency, severe anaemia, thrombocytopenia and elevated serum concentration of liver-related enzymes was examined *post mortem*. Ecchymotic haemorrhages were present on the left eyelid and forehead. The liver, kidney and spleen were markedly enlarged and the kidneys had capsular petechiae. Microscopically, numerous atypical cells resembling myeloid cells were observed in the bone marrow, and myelofibrosis was present. Atypical cells were also present in the blood vessels of the liver, kidney, spleen, lymph nodes, lung, heart, bladder, adrenal gland and brain. Some neoplastic cells had oval or pleomorphic macronuclei and others were multinucleated. Immunohistochemically, the majority of the neoplastic cells had granular cytoplasmic expression of the megakaryocyte-associated antigens Von Willebrand Factor and CD61-IIIa, but were negative for myeloperoxidase. A diagnosis of acute megakaryocytic leukaemia (AMKL)-like disease was made. This would appear to be the first report of AMKL-like disease in non-human primates. This monkey was infected with simian retrovirus type D and it is possible that this viral infection was associated with the development of neoplasia.

Keywords: acute megakaryocytic leukaemia; cynomolgus monkey; immunohistochemistry; simian retrovirus type D

Haematological malignancy has been infrequently documented in monkeys infected by the simian immunodeficiency virus (SIV) (Fortgang *et al.*, 2000). Simian T-cell leukaemia virus (STLV) is also linked to the development of simian T-cell malignancies that closely resemble human T-lymphotropic virus (HTLV) associated leukaemia and lymphoma (Hubbard *et al.*, 1993). Furthermore, simian retrovirus type D (SRV/D) is a common cause of simian acquired immunodeficiency syndrome (SAIDS), a fatal immunosuppressive disease of macaques. SRV/D-infected monkeys may develop lymphadenopathy, splenomegaly, anaemia, bone marrow hyperplasia, lymphoid depletion, neutropenia, weight loss, diarrhoea or malignant neoplasia (Guzman *et al.*, 1999). Although a number of clinical and pathological studies have described lymphoma in non-human primates (Hubbard *et al.*, 1993; Paramastri *et al.*, 2002), there are no reports of myeloid leukaemia in these animals. The present report describes the first case of acute megakaryocytic leukaemia (AMKL)-like disease in a non-human primate.

A 5-year-old male cynomolgus monkey (*Macaca fascicularis*) was housed in the Tsukuba Primate Research Center (TPRC) in an individual cage and maintained according to the National Institute of Biomedical Innovation rules and guidelines for experimental animal welfare. On routine haematological examination, the animal was found to have mild anaemia (red blood cells [RBC] 4.28×10^{12}/l; reference range $5.55–6.68 \times 10^{12}$/l; haemoglobin [Hb] 99 g/l; reference range 105–125 g/l; haematocrit [HCT] 32.7%; reference range 35.4–41.4%) and severe thrombocytopenia (platelets [PLT] 27×10^9/l; reference range $195–339 \times 10^9$/l). The number of white blood cells (WBC) was normal (6.9×10^9/l; reference

Correspondence to: S. Okabayash (e-mail: okarin@primate.or.jp).

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range 4.2–9.2 × 10^9/l). Although the animal care staff regularly monitored the health of the animal, at this time no clinical signs were observed. Repeat haematological examinations were performed one and four weeks later, but there was no progression of the anaemia and thrombocytopenia was not present on these occasions. The monkey continued to have normal appetite, faeces and activity.

Three months after the initial haematological examination a spot of blood was detected under the monkey's cage. At this time the animal displayed clinical signs including emaciation, pallor of mucous membranes and haemorrhage on the cutaneous side of one eyelid. Haematological examination revealed severe anaemia (RBC 1.66 × 10^{12}/l, Hb 39 g/l, HCT 13.3%) and thrombocytopenia (PLT 28 × 10^9/l). The WBC count was normal (4.1 × 10^9/l). Serum biochemical examination revealed elevation in the concentration of aspartate aminotransferase (AST, 176 U/l; reference range 31–47 U/l); alanine aminotransferase (ALT, 303 U/l; reference range 21–65 U/l); lactate dehydrogenase (LDH, 7660 U/l; reference range 292–975 U/l), and C reactive protein (CRP, 12.8 mg/l; reference range 0.3–1.7 mg/l).

Cynomolgus monkeys in the TPRC breeding colony are SIV and STLV negative, but most are infected by SRV/D (Hara *et al.*, 2005). The animal described here was seronegative for SRV/D antibody by western blotting, but tested positive by polymerase chain reaction (PCR) for the detection of virus genetic material, consistent with current viraemia. On the basis of the clinical and laboratory data, haematological malignancy was suspected.

The monkey was deeply anaesthetized with a lethal dose of pentobarbital and necropsy examination was performed. Ecchymoses were noted on the left eyelid and forehead. The liver was markedly enlarged and the gallbladder was distended. The kidneys were enlarged, pale red-brown in colour and had capsular petechiation. The spleen was also enlarged, but there were no distinct lymphoid follicles on the cut surface. A dark red nodule (1 cm diameter) was present within each of the inferior lobes of the lung. The femoral bone marrow had a brownish-red appearance.

Tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (3 μm) were stained with haematoxylin and eosin (HE), periodic acid-Schiff (PAS) and Masson's trichrome stains. Microscopically, many atypical cells resembling myeloid cells were observed in the bone marrow and the blood vessels of the liver, kidney, spleen, lymph nodes, lung, heart, bladder, adrenal gland and brain. Some hepatic and renal vessels contained neoplastic emboli (Fig. 1). There was extensive infiltration of the liver, kidneys and adrenal glands by the same neoplastic population, with associated parenchymal degeneration or necrosis. Neoplastic cells were present in the spleen and lymph nodes, and in both tissues there was atrophy of lymphoid follicles. Sternal and femoral bone marrow contained many abnormal blast cells, with a marked reduction in normal haemopoietic tissue.

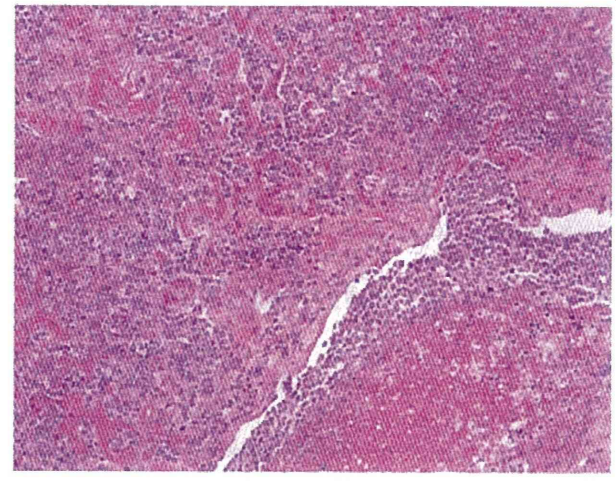

Fig. 1. Extensive infiltration of neoplastic cells into the hepatic parenchyma with associated degeneration and necrosis. A tumour embolus has formed in the central vein. HE. ×100.

The neoplastic cells were generally poorly differentiated, with a medium-sized round nucleus, dense nuclear chromatin and either scant or abundant cytoplasm. Some larger cells had oval or pleomorphic macronuclei, whilst others were multinucleated, with a lower nuclear to cytoplasmic ratio (Fig. 2). There

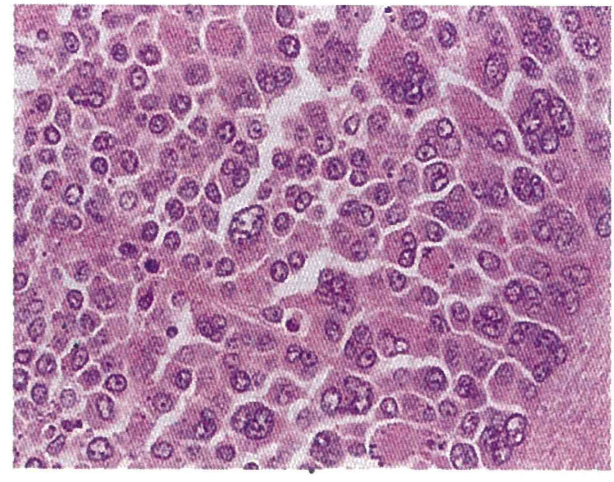

Fig. 2. Neoplastic cells within the liver. These vary greatly in size and are generally 2–3 times larger than normal lymphocytes. The majority of cells have large, round to oval nuclei with stippled chromatin and abundant cytoplasm. Some cells have macronuclei and others are multinucleated, with a lower nucleus to cytoplasmic ratio. HE. ×400.

were numerous abnormal mitoses. The majority of blast cells did not stain with PAS, but occasional individual cells were weakly stained. Masson's trichrome staining demonstrated severe fibrosis of the bone marrow (Fig. 3).

To further identify the neoplastic cells, immunohistochemical studies of femoral bone marrow, liver and kidney were performed. Sections were dewaxed, pre-treated with 0.5% H_2O_2 in methanol and then subjected to antigen retrieval with citric acid buffer (pH 6.0) and heating in an autoclave for 10 min at 121°C. Sections were then incubated with primary antibody overnight at 4°C. The primary antibodies employed were rabbit polyclonal antibodies specific for myeloperoxidase (Novocastra Laboratories, Newcastle, UK; 1 in 150 dilution); Von Willebrand Factor (Dako Cytomation, Denmark; 1 in 400 dilution); CD3 (Dako, 1 in 100 dilution); lysozyme (clone EC 3.2.1.17, Dako; 1 in 400 dilution) and monoclonal mouse antibodies specific for CD235a (clone JC159, Dako; 1 in 200 dilution); CD61-IIIa (cloneY2/51, Dako; 1 in 100 dilution); CD20 (clone L26, Dako; 1 in 100 dilution); HLA-DR alpha-chain (clone TAL.1B5, Dako; 1 in 40 dilution) and CD68 (clone KP1, Dako; 1 in 100 dilution). Following brief washes with buffer, the sections were incubated with the EnVision™ + Dual Link-HRP system (Dako) as secondary stage for 30 min. Labelling was "visualized" by treating the sections with the chromogen 3-3′-diaminobenzidine tetroxide (Dojin Kagaku, Japan) and H_2O_2. The sections were then counterstained with haematoxylin.

The majority of the neoplastic cells had granular cytoplasmic expression of the megakaryocyte-associated antigens Von Willebrand Factor (Fig. 4) and CD61-IIIa, but were negative for all other markers. On the basis of these findings, a diagnosis of AMKL (M7)-like disease with myelofibrosis was made. The neoplastic cells in AMKL often have granular cytoplasmic PAS staining when examined in a blood or a bone marrow smear (Wu *et al.*, 1996; Shukla *et al.*, 2004). The absence of significant PAS staining in the cells of the present case may relate to the formalin fixation process.

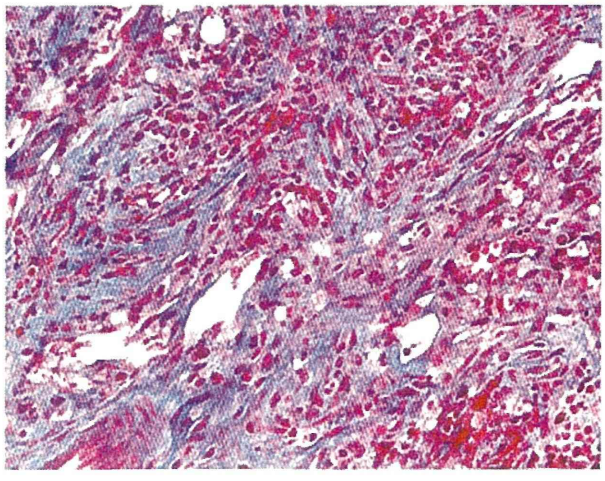

Fig. 3. Marked fibrosis (blue staining) is present within the bone marrow and admixed with neoplastic cells. Masson's trichrome. ×200.

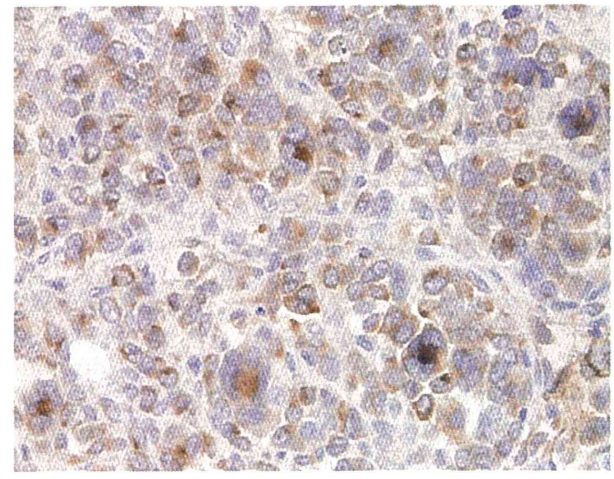

Fig. 4. Expression of Von Willebrand Factor by neoplastic cells within the bone marrow. IHC. ×400.

AMKL was first described as a subtype of acute myeloid leukaemia (AML) (von Boros and Korenyi, 1931) and was incorporated into the French–American–British (FAB) classification of AML as M7 (Bennett *et al.*, 1985). AMKL is rare, accounting for 3–5% of all human AML (Brunning *et al.*, 2001), but there is a higher incidence in children, partly due to an association with Down's syndrome (Athale *et al.*, 2001; Paredes-Aguilera *et al.*, 2003). Although AMKL is well characterized in man (Koike, 1984; Akahoshi *et al.*, 1987), in animals it has been reported only in the dog and cat (Colbatzky and Hermanns, 1993). Disrupted haematopoiesis leads to cytopenia, particularly thrombocytopenia, which becomes manifest as cutaneous petechiae, epistaxis and bleeding gums. In leukaemic patients there is often elevation of serum LDH concentration (Ferrara and Mirto, 1996). Since megakaryocytes, which store various growth factors in their alpha granules, are known to be involved in the pathogenesis of myelofibrosis, AMKL is frequently accompanied by myelofibrosis (Terui *et al.*, 1990). AMKL typically has a more guarded prognosis than other types of leukaemia (Athale *et al.*, 2001).

Differential diagnoses for AMKL include minimally differentiated AML (M0), pure erythroid

leukaemia (M6b) and acute lymphocytic leukaemia (ALL). M0, M6b and ALL are all generally negative for expression of myeloperoxidase in immunohistochemistry (IHC), as is AMKL. The neoplastic cells in AMKL occasionally have a lymphoblast-like appearance similar to M0 and ALL (Brunning *et al.*, 2001). Furthermore, neoplastic multinucleate cells are observed in both M6b and AMKL, and are often positively stained by PAS (Brunning *et al.*, 2001). Megakaryoblasts do not express myeloperoxidase, but are labelled by one or more of the megakaryocyte-associated antigens CD41, CD61 and Von Willebrand Factor (Brunning *et al.*, 2001; Daniel and Arber, 2001). The cytological and immunohistochemical features of the neoplastic population in the present case were not consistent with M0, M6b or ALL.

Further differential diagnoses for AMKL with myelofibrosis, as described in the present case, include acute panmyelosis with myelofibrosis (APMF), blastic transformation of chronic myeloid leukaemia (CML) or idiopathic myelofibrosis (IMF). APMF is characterized by multi-lineage myeloid proliferation, with a less numerous population of blast cells than in acute megakaryoblastic leukaemia (Orazi *et al.*, 2005). The cells in APMF do not express megakaryocyte-related antigens, which is inconsistent with the findings in the present case. CML is a clonal bone marrow stem cell disorder with proliferation of mature granulocytes (Travis *et al.*, 1987; Bourantas *et al.*, 1998) whereas IMF is a clonal myeloproliferative disorder that is characterized by abnormal deposition of collagen within the bone marrow (Hirose *et al.*, 2001). Human patients with CML or IMF also develop terminal blastic transformation, and these blast cells have frequently been identified as megakaryoblasts (Travis *et al.*, 1987; Bourantas *et al.*, 1998; Hirose *et al.*, 2001). Although the present case most likely represents AMKL with myelofibrosis, it is difficult to entirely exclude the alternative interpretation of blastic transformation of CML or IMF. For this reason, the present case has been described as an AMKL (M7)-like disease.

To our knowledge, this is the first case of spontaneously arising AMKL-like disease in non-human primates. The affected monkey had SRV/D infection, which may have contributed to the development of the neoplastic disease (Guzman *et al.*, 1999). Alternatively, a genetic mechanism may be proposed as humans with Down's syndrome have predisposition to the development of AMKL associated with a somatic mutation in the gene encoding the GATA1 transcription factor protein (Shimizu *et al.*, 2008). Further cases of such leukaemia in non-human primates should be subject to genetic investigation.

Acknowledgments

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Human herpesvirus 6 major immediate early promoter has strong activity in T cells and is useful for heterologous gene expression

Masaaki Matsuura[1,2], Masaya Takemoto[1], Koichi Yamanishi[1], Yasuko Mori[1,3*]

Abstract

Background: Human herpesvirus-6 (HHV-6) is a beta-herpesvirus. HHV-6 infects and replicates in T cells. The HHV-6-encoded major immediate early gene (MIE) is expressed at the immediate-early infection phase. Human cytomegalovirus major immediate early promoter (CMV MIEp) is commercially available for the expression of various heterologous genes. Here we identified the HHV-6 MIE promoter (MIEp) and compared its activity with that of CMV MIEp in various cell lines.

Methods: The HHV-6 MIEp and some HHV-6 MIEp variants were amplified by PCR from HHV-6B strain HST. These fragments and CMV MIEp were subcloned into the pGL-3 luciferase reporter plasmid and subjected to luciferase reporter assay. In addition, to investigate whether the HHV-6 MIEp could be used as the promoter for expression of foreign genes in a recombinant varicella-zoster virus, we inserted HHV-6 MIEp-DsRed expression casette into the varicella-zoster virus genome.

Results: HHV-6 MIEp showed strong activity in T cells compared with CMV MIEp, and the presence of intron 1 of the MIE gene increased its activity. The NF-κB-binding site, which lies within the R3 repeat, was critical for this activity. Moreover, the HHV-6 MIEp drove heterologous gene expression in recombinant varicella-zoster virus-infected cells.

Conclusions: These data suggest that HHV-6 MIEp functions more strongly than CMV MIEp in various T-cell lines.

Background

Human herpesvirus 6 (HHV-6) was first isolated in 1986 from the peripheral blood of patients with lymphoproliferative disorders and AIDS [1,2]. The virus was subsequently shown to be ubiquitous in healthy adults [3]. HHV-6 has been isolated from infants with exanthema subitum, a common childhood disease [4]. Later, HHV-6 isolates were classified into two variants, A and B (HHV-6A and HHV-6B), based on molecular and biological criteria [5-8]. HHV-6B causes exanthema subitum [4], while the pathogenesis of HHV-6A is still unknown. HHV-6 has the unique feature of being able to replicate and produce progeny in T cells [9,10]. The HHV-6 genome is a double-stranded DNA of approximately 160 kbp, consisting of a unique long region of 140 kbp flanked by 10-kbp direct repeats, and there is 90% identity between the two variants [11].

HHV-6 belongs to the beta-herpesvirus subfamily, which includes human cytomegalovirus (HCMV) and human herpesvirus 7 (HHV-7) [12]. The betaherpesviruses have extensive domains of similar genomic organization, with conserved herpesvirus gene blocks in the unique region of their genome [13]. HCMV's major immediate early (MIE) enhancer-containing promoter has been developed [14,15]; it is currently commercially available and is used to drive the expression of various genes. The MIE promoter controls the expression of two IE transcripts, designated IE1 (UL123) and IE2 (UL122) [16]. HHV-6 has positional homologs of UL123 and UL122; they are U89 and U86, which are designated IE1 and IE2, respectively [11,13,17,18]. The HHV-6 IE1 and IE2 transcripts are formed by alternative splicing [19,20]. Recently Takemoto et al. reported that the R3 region in the right end of HHV-6 is a strong enhancer

* Correspondence: ymori@nibio.go.jp
[1]Laboratoy of Virology, Division of Biomedical Research, National Institute of Biomedical Innovation, 7-6-8, Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
Full list of author information is available at the end of the article

of another HHV-6 immediate early gene, U95 [21]. R3 is positioned between U95 and U89; therefore, the region containing R3 is predicted to also contain promoter activity for the IE1 and IE2 genes. In other words, this location is predicted to be a positional homolog of the HCMV MIE promoter.

In this study, we identified the promoter region that regulates the HHV-6 MIE gene, and analyzed its activity. As expected, part of the R3 region was critical for the promoter activity. We also found that the first intron encoded by the IE1 gene enhanced HHV-6 MIE promoter (HHV-6 MIEp) activity, and that HHV-6 MIEp with the first intron had significantly stronger activity than the HCMV MIE promoter, especially in T-cell lines. The HHV-6 MIEp was able to express heterologous genes in a recombinant varicella-zoster virus, indicating that it could be useful for expressing various genes in a similar manner as the CMV MIE promoter.

Results

The HHV-6 major immediate-early promoter had stronger activity than the CMV promoter in T-cell lines

The 5' end of the mRNA encoded by the HHV-6 immediate early 1 (IE1) gene is located at base 139442 of the HHV-6 strain HST genome [11,22]. The 971-bp region upstream of the IE1 gene, including the R3 repeat, was suspected to include the HHV-6 major immediate-early promoter (HHV-6MIEp). The promoter region used in this study is illustrated in Figure 1A. First, to investigate the relative strength of the HHV-6 MIE promoter in various cell types, reporter gene assays were performed using the luciferase gene expression system. A plasmid containing the luciferase gene under the HHV-6MIEp was transfected into MRC-5, MeWo, U373, Molt-3, SupT1, and Jurkat cells. The pRL-TK plasmid, encoding Renilla luciferase under the transcriptional control of the herpes simplex virus thymidine kinase (HSV-TK) promoter, was co-transfected to normalize the transfection efficiency. The data show the fold-increase relative to the value of cells transfected with a blank plasmid, pGL3-basic (Promega). As shown in Figure 1B, the activity of the HHV-6 MIE promoter was weaker than that of the CMV promoter (CMV MIEp) in MRC-5, U373 and Mewo cells, while the activity was stronger than that of the CMV promoter in Molt-3, SupT1, and Jurkat cells.

The mRNAs encoded by the HHV-6 IE1 gene are produced by alternative splicing (Figure 1A). It is known that introns within some genes can elevate the protein expression level by either enhancing the promoter activity or stabilizing the mRNA [23]. In HCMV, the addition of intron A from the IE1 gene to the IE promoter/enhancer increases the promoter activity [24]. Therefore, we examined the role of the introns encoded by the

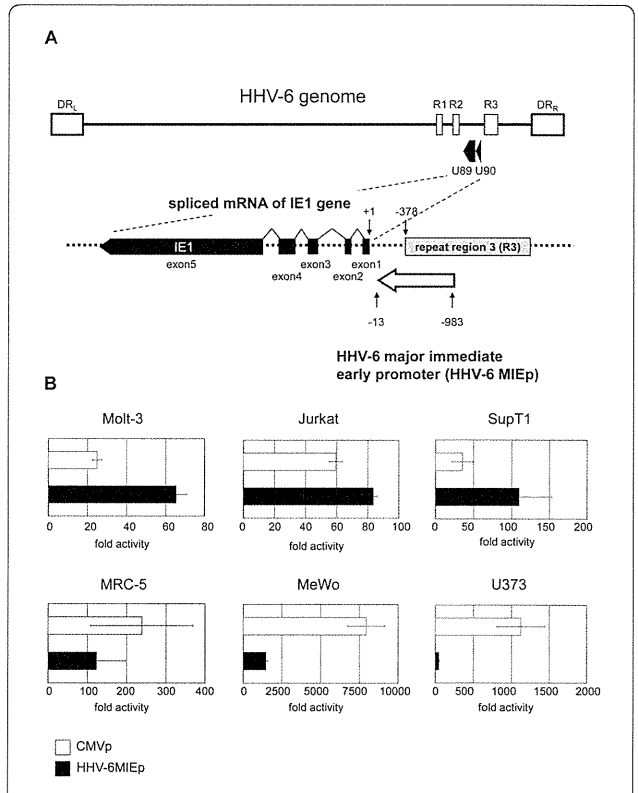

Figure 1 Comparison of the activities of the HHV-6 MIE promoter and CMV promoter. (A) The HHV-6 genome is a double-stranded DNA molecule of approximately 160 kbp, and is composed of a single long unique sequence (U) flanked by identical direct repeats (DR_L and DR_R). IE1 maps to open reading frames U90 and U89. The HHV-6 MIE promoter of 971 bp, located upstream of exon 1 of IE1, was amplified by PCR from the HHV-6B strain HST genome. Bases are numbered starting with the transcriptional start site for the IE1 gene. (B) The HHV-6 MIE promoter and CMV promoter were subcloned into the pGL3-basic plasmid, and the resultant plasmids were transfected into various cell lines (Jurkat, Molt-3, SupT1, MRC-5, MeWo, and U373 cells). At 24-hr post-transfection, the cells were harvested and subjected to the luciferase activity assay. The mean fold-activity relative to that of blank pGL3-basic plasmid-transfected cells and standard deviation for three independent experiments were plotted.

HHV-6 MIE genes in the HHV-6 MIE promoter activity. To examine this, several HHV-6 MIE promoter variants containing introns 1-4 were constructed (Figure 2A), and the activities were compared by performing the reporter assay in various cells. As shown in Figure 2B, in the presence of intron 1, the promoter activity was significantly upregulated in all the cells compared to the HHV-6 MIE promoter without intron 1. In contrast, the further addition of introns 1-2, 1-3, or 1-4 downregulated the promoter activity (Figure 2B). Therefore the HHV-6 MIE promoter containing intron 1 (HHV-6MIEp-in1), whose length is 1245-bp, was included in the remaining experiments.

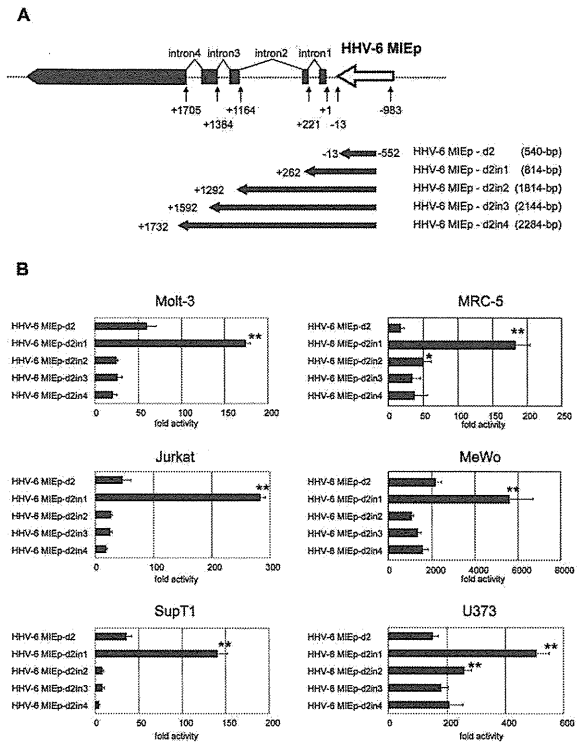

Figure 2 The activity of the HHV-6 MIE promoter including intron(s) of the IE gene. Schematic representation of the HHV-6 MIE promoter variants, including introns 1-4 of the IE1 gene, used for analysis of the introns. The numbers indicate the nucleotide position from the 5' end of mRNA encoded by the IE1 gene. The +1 indicates the 5' end. The name and size of each promoter used here are shown at the right. (B) Luciferase assays were performed after transfection in various cell lines (Molt-3, Jurkat, SupT-1, MRC-5, Mewo, and U373). The mean fold-activity of each HHV-6 MIE promoter variant relative to that of the blank pGL3-basic plasmid is shown by the horizontal bar. Standard deviation for three independent experiments is indicated. One asterisk indicates that the P value is < 0.05 and two asterisks indicate that the P value is < 0.01 in comparison with HHV-6MIEp-d2 (without intron 1), as determined by Student's unpaired two-tailed t-test.

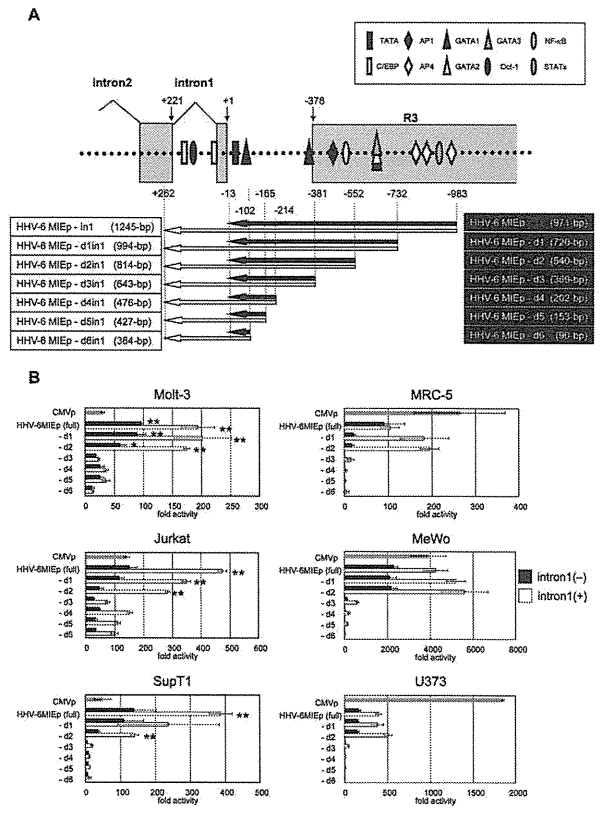

Figure 3 Comparison of promoter activity in deletion mutants of HHV-6MIEp and HHV-6MIEp-in1. (A) Schematic representation of 5'-deletion mutants of the HHV-6MIEp (black arrows) and of the HHV-6MIEp-in1, which is the HHV-6MIEp including intron 1 (white arrows). The name and size of each promoter are shown at the right (without intron 1) and left (with intron 1). Putative transcription factor-binding sites, predicted by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html), are shown. (B) Luciferase assays were performed in various cell lines. The mean fold-activity relative to that of the blank pGL3-basic plasmid is indicated by the horizontal bars. The standard deviation for three independent experiments is indicated. The activities of CMV MIEp, deletion mutants of the HHV-6MIEp, and deletion mutants of the HHV-6MIEp-in1, are indicated by gray, black, and white bars, respectively. One asterisk indicates that the P value is < 0.05 and two asterisks indicate that the P value is < 0.01 in comparison with the CMV MIEp, as determined by Student's unpaired two-tailed t-test.

Next, to determine the region that contributes to the promoter activity, various deletion mutants of both HHV6MIEp and HHV6MIEp-in1 were constructed (Figure 3A), and their activities were examined and compared by reporter assays in various cell lines. As shown in Figure 3B, the HHV-6MIEp-d3 promoter activity decreased compared to that of HHV-6MIEp-d2 (both with and without intron 1), showing that the region at nt positions from -381 to -552, which lies within R3, is important for the activity. In addition, the activities of HHV-6MIEp and HHV-6MIEp-in1 were significantly stronger than CMV MIEp activity in Jurkat, Motl-3, and SupT1 cells, suggesting that the HHV-6 MIE promoter has higher activity than the CMV promoter in certain cells, especially in T cells. This property of the HHV-6 MIE promoter might render it as a promising candidate for efficient protein expression in T cells.

The region at nt positions -381 to -552, which lies within R3, is predicted to have an NF-κB-binding site and AP-1-binding site (Figure 3A). Takemoto et al. reported that the NF-κB-binding site in the R3 region plays an important role in U95 promoter activity [21]. We hypothesized that the NF-κB-binding site plays a major role in the HHV-6MIEp promoter activity as well. To investigate this, we constructed a promoter in which the NF-κB-binding site was deleted (HHV-6MIEpΔNF-

κBin1) (Figure 4), and examined its activity in various cell lines. As shown in Figure 4, the NF-κB-binding site-deleted promoter HHV-6MIEpΔNF-κBin1 exhibited significantly decreased promoter activity in all cell lines, indicating that the NF-κB-binding site in the HHV-6MIEp plays an important role in its promoter activity.

The HHV-6 MIE promoter could drive the expression of foreign gene in a recombinant varicella virus

We recently constructed a recombinant varicella vaccine Oka strain (vOka) expressing the MuV (mumps virus) HN (hemaglutinin-neuraminidase) gene, as a possible candidate for a polyvalent vaccine for both varicella zoster virus (VZV) and MuV infections [25]. In that study, the CMV promoter was used to control the HN gene. Since the HHV-6 MIE promoter and CMV promoter showed similar activity in MRC-5 cells and MeWo cells, which are susceptible to VZV infection, we next examined whether the HHV-6 MIE promoter could control the expression of foreign genes in VZV.

To investigate this, we incorporated the HHV-6 MIE promoter, with the DsRed2 gene and BGH poly (A) signal sequence, into the VZV vOka BAC genome by Tn7-mediated site-specific transposition (Figure 5). Since the full-length HHV-6 MIE promoter including intron 1 (HHV-6MIEpin1) had the strongest activity of all the promoter variants, we used it for this construct. The DsRed2 gene, which encodes a red fluorescent protein, was used as a reporter gene. The insertion of foreign gene cassette was confirmed by RFLP analysis using *Bam*HI and soutern blot analysis. As shown in

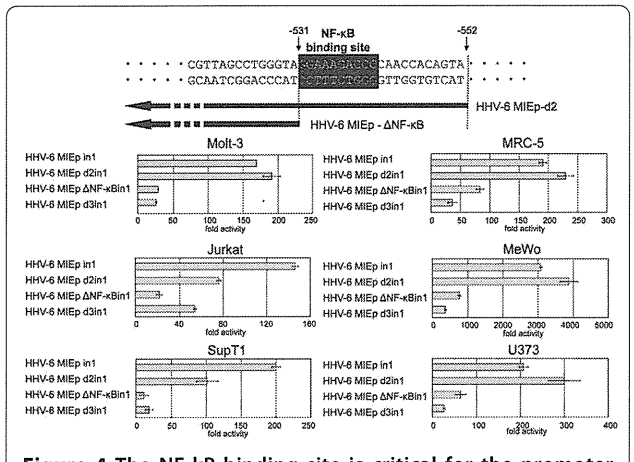

Figure 4 The NF-kB-binding site is critical for the promoter activity of 6MIEp. (A) To investigate the importance of the NF-κB-binding site for the promoter activity of 6MIEp, a 5'-deletion mutant of the 6MIEp lacking the NF-κB-binding site (white letters in black box) was constructed. (B) The Luciferase assay was performed in various cell lines. The mean fold-activity relative to that of the blank pGL3-basic plasmid is indicated by the horizontal bars. The standard deviation for three independent experiments is indicated.

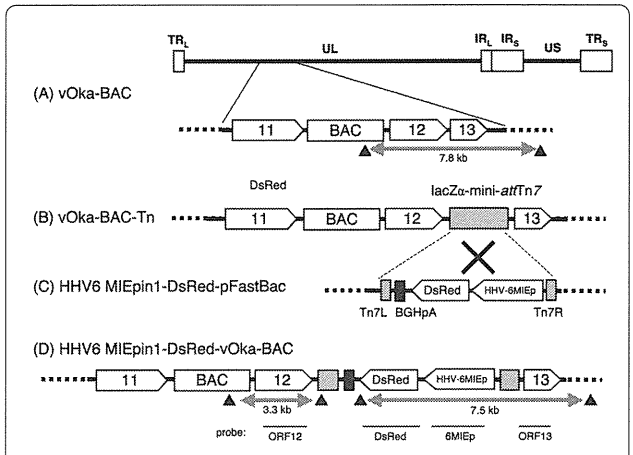

Figure 5 Construction of the 6MIEpin1-DsRed-vOka genome. The varicella vaccine Oka strain (vOka)-BAC genome (A) is about 125-kbp long and includes terminal repeats (TRL and TRS), a unique long (UL) domain, internal repeats (IRL and IRS), and a unique short domain (US). The LacZα-mini-attTn7 sequence was inserted between ORF12 and ORF13 of the vOka-BAC genome by RecA-mediated recombination, generating vOka-BAC-Tn (B). The LacZα-mini-attTn7 sequence in the vOka-BAC-Tn genome permitted site-specific insertion of the HHV-6MIEpin1-DsRed-BGH poly(A) signal sequence casette (C) by Tn7-mediated transposition, resulting in the HHV-6MIEp-DsRed-vOka-BAC genome (D). Black arrowheads indicate the *Bam*HI sites. Horizontal bars indicate the region of the probe used for Southern blot analysis.

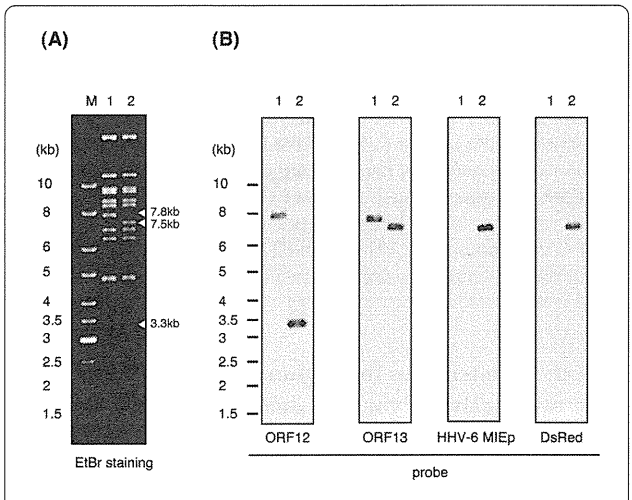

Figure 6 Confirmation of the insertion of HHV-6MIEp-DsRed into the vOka-BAC genome by Southern blot. (A) The HHV-6MIEpin1-DsRed-vOka-BAC DNA and the vOka-BAC DNA were digested with *BamHI*, loaded onto a 0.5% agarose gel, and separated by electrophoresis. The DNA fragments were visualized with a UV transilluminator. Arrowheads indicate the band shift following transposition. Each DNA size is shown on the right side of the panel. (B) The blots were hybridized with ORF12, ORF13, DsRed, or HHV-6MIEp probes. Bands were detected by the Enhanced Chemiluminescence (ECL) Direct Nucleic Acid Labeling and Detection System. Lane M: size markers, lane 1: vOka-BAC DNA, lane 2: HHV-6MIEp-DsRed-vOka-BAC DNA.

Figure 6A, there was a shift in size from 7.8-kbp in the vOka-BAC DNA to 7.5-kbp in the HHV-6MIEpin1-DsRed-vOka-BAC DNA. Furthermore, in the Southern blot analysis, the probes for HHV-6MIEp and DsRed detected bands only in the HHV-6MIEpin1-DsRed-vOka-BAC genome (Figure 6B), indicating that the HHV-6MIEpin1-DsRed cassette had been inserted into the vOka genome.

To reconstitute infectious virus from the HHV-6MIEpin1-DsRed-vOka-BAC DNA, MRC-5 cells were transfected with the BAC DNA. Five days after the transfection, typical cytopathic effects (CPEs) were shown. Along with the CPEs, green fluorescence from green fluorescent protein (GFP), which gene was included in BAC sequence, and red fluorescence from DsRed2 were observed by fluorescence microscopy (Figure 7A); this indicated that the HHV-6MIEpin1-DsRed-vOka-BAC had been reconstituted as an infectious recombinant virus expressing DsRed under control of the HHV-6 MIE promoter.

The expression of the DsRed was confirmed by Western blotting analysis (Figure 7B). Recombinant vOka-infected MRC-5 cell lysates were separated by SDS-PAGE and analyzed by Western blotting with an anti-DsRed mAb or anti-VZV gB Ab. The expression of gB, which is a late gene [26], was examined as a positive control of VZV infection. As shown in Figure 7B, the expression of gB was found in lysates from cells infected with either the control rvOka-BAC or HHV-6MIEpin1-DsRed-rvOka-BAC, while the anti-DsRed mAb specifically reacted with a 29-kDa band only in the HHV-6MIEpin1-DsRed-rVoka-BAC-infected cell lysates. These data indicated that the HHV-6 MIE promoter can be used to drive the expression of foreign genes in VZV-infected cells.

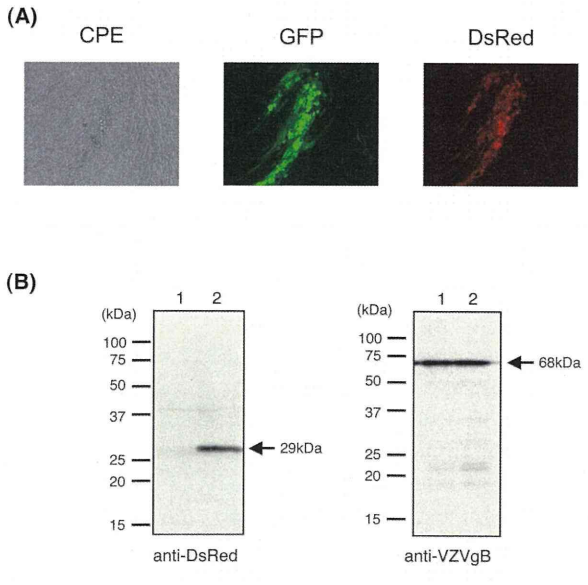

Figure 7 The expression of heterogous gene under the HHV-6 promoter in recombinant VZV-infected cells. (A) The HHV-6MIEpin1-DsRed-vOka-BAC DNA was transfected into MRC-5 cells. The infectious virus, reconstituted from the HHV-6MIEp-DsRed-vOka-BAC DNA, caused typical cytopathic effects along with green fluorescence and red fluorescence at 5 days post-transfection. (B) The HHV-6MIEpin1-DsRed-vOka-BAC-infected MRC-5 cells and vOka-BAC-infected MRC-5 cells were lysed in sample buffer, and subjected to Western blot analysis. Blots were reacted with an anti-DsRed mAb or anti-VZV gB Abs. The position and molecular mass in kDa of marker proteins are indicated at the left. Lane 1: vOka-BAC-infected MRC-5 cells, lane 2: HHV-6MIEpin1-DsRed-vOka-BAC-infected MRC-5 cells.

Discussion

The HCMV major immediate early promoter (HCMV MIEp) has been established and used as a tool to drive gene expression by researchers worldwide. HHV-6 also belongs to the beta-herpesviruses, and has a positional homolog of the HCMV MIE gene. As described in the Introduction, HHV-6 replicates and produces progeny in T cells very well; we therefore speculated that the MIE promoter would have stronger promoter activity in T cells than in other cells. Here we identified the region of the HHV-6 major immediate early promoter (HHV-6 MIEp), described in Figure 1. The promoter activity of HHV-6 MIEp was stronger than that of HCMV MIEp in T- cell lines, but not in other adherent cell lines. This feature of HHV-6 MIEp activity is consistent with the fact that HHV-6 is T-cell tropic.

HHV-6 MIEp is predicted to have an NF-κB-binding site. The activity of a mutant HHV-6 MIEp, with the NF-κB-binding site deleted, was dramatically decreased, indicating that the NF-κB-binding site is critical for the promoter activity of HHV-6 MIEp. However, the HCMV MIEp activity was weak compared to that of HHV-6 MIEp in T-cell lines in our study, even though HCMV MIEp also has an NF-κB-binding site that plays a major role in its promoter activity [27,28]. Therefore, another binding site in addition to the NF-κB-binding site might contribute to the T-cell-specific promoter activity of HHV-6 MIEp, or another binding site in HCMV MIEp might have a repressive effect in T cells.

Although the AP-2 and PEA3 binding sites were not found in HHV-6 MIE promoter region by TFSEARCH, R3 region has these binding sites [17,29]. However, in the study of U95 promoter, it has been reported that PEA3 binding sites in R3 region did not bind any proteins[21]. Therefore, PEA3 binding site might have no or low effect on the MIEp activity. The deletion promoter, HHV-6 MIEp-d1, lost two complete AP-2 binding sites and one AP-2 binding site with one nucleotide mutation, compared to full length promoter. Nevertheless, the activity of HHV-6 MIEp-d1 was similar to that

of HHV-6 MIEp. Therefore, the AP-2 binding sites might have low effect on the MIEp activitiy.

Adding the first intron (intron 1) of IE1 to HHV-6 MIEp enhanced the promoter activity significantly. When intron 1 was added, the activity of HHV-6 MIEp became markedly greater than that of HCMV in T cells. In adherent cell lines such as MRC-5 and MeWo cells, the activity of HHV-6 MIEp with intron 1 became similar to that of HCMV MIEp. Intron1 of the IE1 region is predicted to have two CCAAT enhancer binding protein (C/EBP) binding sites and an OCT-1-binding site (Figure 3). The transcriptional regulators that bind to these sites might enhance the promoter activity of HHV-6 MIEp. Interestingly, the promoter construct that contained introns 1 and 2 was less active than the promoter containing only intron 1. Further investigation is needed to elucidate the mechanisms involving the intron regions.

We recently developed a recombinant VZV vaccine strain containing the mumps virus HN gene. In this study, we examined whether the HHV-6 MIEp containing intron 1 functioned as a heterologous expression promoter in the VZV vaccine strain. Indeed, in the recombinant VZV, HHV-6 MIEp functioned to drive the expression of the DsRed gene, which is a heterologous gene. These findings indicate that, like the commercially available HCMVp, HHV-6 MIEp is useful for expressing heterologous genes in a VZV vaccine strain.

Conclusions

Our results show that HHV-6 MIE promoter functions more strongly than CMV MIEp in various T-cell lines. Moreover, the first intron of HHV-6 IE1 gene enhances the promoter activity of HHV-6 MIEp. In addition, the HHV-6 MIEp could drive heterologous gene expression in recombinant varicella-zoster virus-infected cells. These results suggest that HHV-6 MIEp can be used for driving gene expression.

Methods

Cells

MRC-5 cells, human lung fibroblasts, were cultured in modified minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). MeWo cells, a human melanoma cell line, and U373 cells, a human astrocytoma cell line, were cultured in Dulbecco's modified Eagle's medium supplemented with 8% FBS. Molt-3 cells, SupT1 cells, and Jurkat cells, which are lymphoblastic T-cell lines, were cultured in RPMI1640 medium supplemented with 8% FBS.

Plasmids for the luciferase reporter assay

The HHV-6 major immediate-early promoter (HHV-6MIEp) sequence and its deletion mutants were amplified by PCR from the HHV-6B strain HST [30]. The primer sequences are shown in Table 1. The 971-bp fragment located from -983 to -13 bp upstream of exon 1 of IE1, which was amplified using the primer pair 6MIEpF and 6MIEpR, was defined as 6MIEp. The 5' primers named 6MIEpF-732, 6MIEpF-552, 6MIEpF-531, 6MIEpF-381, 6MIEpF-214, 6MIEpF-165, and 6MIEpF-102 were used to generate a series of 5'-deletion mutants. The 3' primers named 6MIEpex2R, 6MIEpex3R, 6MIEpex4R, and 6MIEpex5R were used to amplify HHV-6MIEp including introns 1 to 4, respectively. These amplified fragments were digested and inserted into the pGL3-basic vector (Promega) at the *Hin*dIII and *Xho*I or *Kpn*I site.

The CMV MIE promoter sequence was excised with *Nru*I and *Bam*HI from pcDNA3.1(+) (Invitrogen), and inserted into pGL3-basic (Promega) at the *Sma*I and *Bgl*II sites.

The pRL-TK plasmid (Promega), which contains the Renilla luciferase reporter gene under the HSV TK promoter, was used to normalize the transfection efficiency.

Luciferase reporter assay

Adherent cells (MRC-5, MeWo, and U373) were plated on 24-well plates at a density of 1×10^5 cells per well on the day before transfection, and were transfected with 1 μg of reporter plasmid and 0.25 μg of pRL-TK plasmid (Promega), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Samples containing 4×10^5 suspended cells (Molt-3, Jurkat, or SupT1) were transfected with 1 μg of reporter plasmid and 0.25 μg of pRL-TK using Lipofectamine2000.

Firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol, using a luminometer (Berthold, TriStar LB941). Cells were lysed in 1 × lysis buffer (50 μL/well) for 15 min at room temperature, and each cell lysate was added to a luminometer tube containing 100 μL of assay reagent. The mixture was blended quickly by flicking, and placed in the luminometer for a 1-sec measurement. The transfection efficiency was normalized to the Renilla luciferase activity. The data (mean + SD) were collected from three independent transfections.

Generation of a recombinant vOka-BAC genome containing HHV-6 MIE promoter

To generate the HHV-6MIEpin1-pFastBac plasmid, the gentamicin-resistance gene and the polyhedrin (PH)-promoter region of the pFastBac1 plasmid (Invitrogen) were replaced with 6MIEp including the intron 1 (HHV-6MIEpin1) sequence.

The DsRed fragment was amplified by PCR using the primer pair DsRed2-HindF and DsRed2-HindR, and

Table 1 Primers

Primer	Sequence*
6MIEpF	5'-tct ctc gag agt taa aga tca gcg ggt ac-3'
6MIEpF-732	5'-agt cgg tac cgg cga atg aga act cta aaa gct c-3'
6MIEpF-552	5'-agt cgg tac cta ctg tgg ttg ggg tct ttc cta c-3'
6MIEpF-531	5'-acc ggt acc tac cca ggc taa cga gaa cc-3'
6MIEpF-381	5'-agt cgg tac cac att cct gtt tca tga tgt gta gc-3'
6MIEpF-214	5'-agt cgg tac ctc ctg ttt ttg agt aag ata tga c-3'
6MIEpF-165	5'-agt cgg tac cag cta att tcc att cca tat ttg tc-3'
6MIEpF-102	5'-agt cgg tac cta cag cga ttg gct cct tca tcc tc-3'
6MIEpR	5'-agt cct cga gca ctg aac tgg ctg taa ctt ctg c-3'
6MIEpex2R	5'-tct aag ctt cag caa tcc aat aat tga tg-3'
6MIEpex3R	5'-cat aag ctt gca tac gtt cct cat tgg at-3'
6MIEpex4R	5'-cat aag ctt cca aag ttt tga att ctt ca-3'
6MIEpex5R	5'-cat aag ctt ttt gga tgc aag tgc caa cg-3'
DsRed2-HindF	5'-acc aag ctt tac cgg tcg cca cca tgg cct-3'
DsRed2-HindR	5'-acc aag ctt tta tct aga tcc ggt gga tcc-3'
ORF12TnFw	5'-tat ctc gag agg tac cgg tga ctt cag ag-3'
ORF12TnRv	5'-cga gga tcc aat caa cca atc aga cct-3'
ORF13TnFw	5'-gag gat ccg tac cca caa tat caa gtg gt-3'
ORF13TnRv	5'-gac tcg agc cta ttc gtg tca tct aga tgg-3'

*:underlines indicate restriction enzyme sites.

*Hin*dIII sites were introduced at both the 5' and 3' ends. The pDsRed2-C1 plasmid (Clontech), in which the *Hin*dIII site had been eliminated, was used as the PCR template. Following amplification, the PCR products were inserted into the HHV-6MIEpin1-pFastBac plasmid at the *Hin*dIII site, generating the HHV-6MIEpin1-DsRed-pFastBac plasmid (Figure 5C). The BGH poly (A) signal sequence was derived from pFastBac plasmid.

The vOka-BAC was obtained using pHA-2 cloning vector (a kind gift from Dr. Ulrich Koszinowski[31]), as described previously[32]. The LacZα-mini-*att*Tn*7* cassette was inserted into vOka-BAC (Figure 5A) to produce vOka-BAC-Tn (Figure 5B) using RecA-mediated recombination, essentially as described previously [32]. In brief, *E. coli* DH10B electrocompetent cells harboring circular vOka-BAC DNA were co-transformed with 1 μg of the targeting vector, pKO5M-Tn (pKO5M is a kind gift from Dr. Kawaguchi[33]), which contain the LacZα-mini-attTn7 region[33,34], and 3 μg of pDF25 (Tet)– (a kind gift from Dr. J. Heath [35]) by electroporation, using a Gene Pulser II (Bio-Rad, Hercules, CA). The surviving co-integrant colonies, selected by their resistance to chloramphenicol and zeocin, and by a Lac + phenotype on an LB plate containing X-Gal and IPTG, were made electrocompetent and transformed with 1 μg of pDF25(Tet). The *E. coli* DH10B colonies containing the correct survival recombination were then selected by the following criteria: resistance to chloramphenicol, sensitivity to zeocin, and a Lac + phenotype on LB containing X-Gal and IPTG. The insertion of the LacZα-mini-attTn7 sequence into the BAC genome was confirmed by PCR and Southern blotting (Data not shown).

The HHV-6MIEpin1-DsRed cassette was inserted into the vOka-BAC-Tn genome using Tn7-mediated site-specific transposition, essentially as described previously [34]. In brief, *E. coli* DH10B harboring the vOka-BAC-Tn genome was transformed with HHV-6MIEpin1-DsRed-pFastBac and pMON7124 (Invitrogen), a helper plasmid for transposition. The pMON7124 plasmid DNA was isolated from DH10Bac cells (Invitrogen). The transformed *E. coli* was cultured on LB containing X-gal and IPTG for blue/white selection. The white colonies were analyzed by PCR to verify the insertion of the DsRed expression cassette (data not shown). This completed the construction of the HHV-6MIEpin1-DsRed-vOka-BAC genome (Figure 5D).

Southern blot analysis

The HHV-6MIEpin1-DsRed-vOka-BAC DNA was extracted using a NucleoBond BAC 100 kit (Macherey-Nagel) following the manufacturer's instructions.

The BAC DNA was then digested with *Bam*HI, loaded onto a 0.5% agarose gel, and separated by electrophoresis at 20 V for 72 hrs. The DNA fragments were visualized with a UV transilluminator and then transferred onto a nylon membrane (Hybond-N+) (GE Healthcare Bio-sciences). The blots were hybridized with ORF12, ORF13, DsRed, or HHV-6MIEp probes labeled with horseradish peroxidase. These probes were amplified by PCR using the following primer pairs: ORF12TnFw/ORF12TnRv, ORF13TnFw/ORF13TnRv, DsRed-HindF/DsRed-HindR, and 6MIEpF-552/6MIEpex2R, respectively (the primer sequences are shown in Table 1). Bands were detected by the Enhanced Chemiluminescence (ECL) Direct Nucleic Acid Labeling and Detection System (GE Healthcare Bio-sciences) following the manufacturer's instructions.

Reconstitution of infectious virus from vOka-BAC DNA

Reconstitution of the recombinant virus, named HHV-6MIEpin1-DsRed-rvOka, was performed as described previously [32,36]. Briefly, MRC-5 cells were transfected with 1 μg of HHV-6MIEpin1-DsRed-vOka-BAC DNA by electroporation, using a Nucleofection unit (Amaxa Biosystems). The transfected cells were then cultured in MEM supplemented with 3% FBS for 3-5 days, and were observed under a microscope until a typical cytopathic effect with green and red fluorescence appeared.

Western blot analysis

The HHV-6MIEp-DsRed-vOka-BAC-infected MRC-5 cells were lysed in sample buffer [32 mM Tris-HCl (pH 6.8), 1.5% SDS, 5% glycerol, 2.5% 2-mercaptoethanol], separated by SDS-polyacrylamide gel electrophoresis (PAGE), and electrotransferred onto PVDF membranes (Bio-Rad Laboratories). A monoclonal antibody (mAb) against DsRed (Clontech) was purchased, and an anti-VZV gB monospecific antibody (Ab) was produced in our laboratory [26]. Blots were blocked with blocking buffer (PBS, 5% skim milk, 0.1% Tween-20) and reacted with the anti-DsRed mAb or anti-gB Ab in blocking buffer. The protein bands were developed with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and ECL detection reagents (GE Healthcare Bio-Sciences), following the manufacturer's instructions.

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Author details

[1]Laboratoy of Virology, Division of Biomedical Research, National Institute of Biomedical Innovation, 7-6-8, Saito-Asagi, Ibaraki, Osaka 567-0085, Japan. [2]Kanonji Institute, the Research Foundation for Microbial Diseases of Osaka University, 2-9-41, ahata-cho, Kanonji, Kagawa, 768-0061, Japan. [3]Division of Clinical Virology, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, 650-0017, Japan.

Authors' contributions

MM performed and analyzed the experiments, and drafted the manuscript. TM participated in the design of the study partly and performed the experiments partly. KY analyzed the study. YM participated in its design and coordination, analyzed the study, and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Human herpesvirus 6 encoded glycoprotein Q1 gene is essential for virus growth

Huamin Tang [a], Akiko Kawabata [a,b], Mayumi Yoshida [b], Hiroko Oyaizu [a,b], Takahiro Maeki [a,b], Koichi Yamanishi [c], Yasuko Mori [a,b,*]

[a] Laboratory of Virology and Vaccinology, Division of Biomedical Research, National Institute of Biomedical Innovation, 7-6-8, Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[b] Division of Clinical Virology, Kobe University Graduate School of Medicine, Kobe, Japan
[c] National Institute of Biomedical Innovation, 7-6-8, Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

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ABSTRACT

Human herpesvirus 6 (HHV-6) glycoprotein Q1 (gQ1), a unique gene in HHV-6, forms a complex with glycoproteinH (gH) and gL, which is the viral ligand for its cellular receptor, CD46. However, whether gQ1 is essential for virus growth is unknown, because a system is lacking for making gene knockouts for HHV-6. Recently, bacterial artificial chromosome (BAC) and *E. coli* mutagenesis techniques have been applied to herpesvirus investigation. Here we successfully inserted the HHV-6A genome into a BAC, and obtained reconstituted infectious virus from the HHV-6A-containing BAC DNA. Using this system, we generated a gQ1 mutant virus genome, which failed to yield reconstituted infectious virus, whereas its revertant virus could be produced, indicating that the HHV-6 gQ1 gene is essential for virus growth. Therefore, we successfully applied BAC and *E. coli* mutagenesis techniques to the study of HHV-6, and discovered that HHV-6 gQ1 is an essential gene for virus growth.

Introduction

Human herpesvirus 6 (HHV-6) is a T-cell tropic virus, belonging to the betaherpesvirus subfamily and related to human herpesvirus 7 (HHV-7) and human cytomegalovirus (HCMV). It was first isolated from the peripheral blood lymphocytes of patients with lymphoproliferative disorders and AIDS (Salahuddin et al., 1986). The clinical isolates of HHV-6 can be categorized into two variants, HHV-6A and HHV-6B (Ablashi et al., 1991; Aubin et al., 1991; Campadelli-Fiume et al., 1993; Wyatt et al., 1990). Recently, HHV-6 was proposed to be an environmental factor contributing to diseases like multiple sclerosis, epilepsy, and others. However, the mechanisms underlying HHV-6's involvement in these diseases remain to be elucidated (Pietilainen et al., 2009; Theodore et al., 2008), in part because of a lack of biological tools for HHV-6.

Glycoproteins or their complexes on the surface of enveloped viruses play crucial roles in the viruses' infectivity. In HIV, a trimeric complex of gp120 forms a unique conformational structure that binds to the cellular HIV-1 receptor, CD4 (Amess et al., 1998). In several herpesviruses, unique glycoproteins have been reported that function by themselves or with other glycoproteins to promote virus entry. The binding of glycoprotein D (gD) of herpes simplex virus 1 (HSV-1), to its cellular receptor is a critical step for virus entry (Spear et al., 2000). In Epstein–Barr virus (EBV), gp42 forms a complex with gH and gL. This complex binds to human leukocyte antigen (HLA) class II, and is required for the virus's entry into B cells (Li et al., 1997). The glycoprotein complex gH/gL/UL128-132 in HCMV is required for the virus' entry into epithelial and endothelial cells (Ryckman et al., 2008a), although its cellular partner remains to be identified.

The gQ proteins are unique to HHV-6 and HHV-7. Mori et al. (2003) first identified the gQ1 protein in HHV-6 and found that it forms a complex with gH and gL. They found that this complex binds to CD46, the cellular receptor for HHV-6 (Mori et al., 2004). Later, another gQ protein, gQ2 was identified, and found to be part of the gH/gL/gQ1 complex (Akkapaiboon et al., 2004; Mori, 2009). We speculated that gQ1 is essential for HHV-6 propagation, based on the functions of gD in HSV-1, gp42 in EBV, and UL128-132 in HCMV, but there were no direct experimental data to support this hypothesis. To test this hypothesis, new biologic research tools for studying HHV-6 were needed.

In recent years, a number of human herpesvirus genomes have been successfully cloned into F plasmids as BACs, including HSV-1 and HSV-2 (Meseda et al., 2004; Stavropoulos and Strathdee, 1998), varicella-zoster virus (VZV) (Nagaike et al., 2004), EBV (Kanda et al., 2004), HCMV (Borst et al., 1999), and Kaposi's sarcoma associated herpesvirus (KSHV) (Zhou et al., 2002). This technique allows the stable maintenance of a viral genome as a BAC in *E. coli*, and the mutagenesis of viral genome in *E. coli* using the bacterial recombination machinery (Wagner et al., 2002). Reconstituted infectious viruses are generated by the transfection of the BAC plasmids into mammalian cells. The phenotypes of mutant viruses can help

* Corresponding author. Division of Clinical Virology, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, 650-0017, Japan. Fax: +81 78 382 6879.
E-mail address: ymori@med.kobe-u.ac.jp (Y. Mori).

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characterize the function(s) of their disrupted genes, and this information might lead to new treatments for controlling virus infections. In HHV-6, Borenstein and Frenkel successfully ligated a BAC construct with the HHV-6A genome and maintained it in *E. coli*; however, they could not reconstitute infectious virus using the BAC containing the HHV-6A genome in a T-cell line (Borenstein and Frenkel, 2009). Arbuckle et al. (2010) constructed an infectious HHV-6A virus harboring GFP and BAC sequences in the U53/U54 intergenic region, however, they did not take it through *E. coli* and back to human cells.

Here we cloned the HHV-6A strain U1102 genome as a BAC plasmid, and found that this HHV-6A BAC could be stably maintained in *E. coli*. Infectious virus was successfully reconstituted by transfecting the HHV-6A BAC into a T-cell line, followed by co-culturing with umbilical cord blood mononuclear cells (CBMCs). The expression of viral proteins was detected by Western blotting analysis, and the reconstituted virions were confirmed by electron microscopy (EM). We then generated in the BAC genome a deletion mutant of the gQ1 gene, and found that this mutation resulted in a failure to reconstitute the virus. The revertant could be reconstituted, suggesting that gQ1 was essential for HHV-6A propagation *in vitro*.

Results

Strategy of HHV-6A BAC construction and reconstitution of infectious virus

The plasmid pHA-2, containing the guanine phosphoribosyl transferase gene (gpt), gfp, loxP, and BAC sequences, was used in this study (Adler et al., 2000). The BAC construct was inserted into the HHV-6A genome between the U53 and U54 poly A sequences. The DNA fragments of U53 and U54 were amplified from the HHV-6A genome by PCR using the primers shown in Table 1. The PCR product was digested with NotI and PacI and ligated with pBluescript SK (−) (Stratagene) plasmid that had been digested with NotI. The resultant plasmid was then digested with PacI and ligated with the fragment containing the BAC sequence released from the pHA-2 digested with PacI. The construct was named pHA-U5354. After digestion with NotI, the plasmid was transfected into CBMCs by electroporation using a Nucleofection unit (Amaxa).

On the next day, the CBMCs were co-cultured with HHV-6A (U1102 strain)-infected CBMCs. On the third day after the transfection, the recombination selection drugs mycophenolic acid and xanthine were added to the culture medium to a final concentration of 12.5 μg/ml and 110 μg/ml, respectively. The recombinant viruses were enriched by performing the cell-to-cell infection three times in the selection-drug medium, and then the circular viral DNAs were isolated from the infected CBMCs and transformed into *E. coli* DH10B (Invitrogen) by electroporation using a Bio-Rad *E. coli* Pulser. The transformants were transferred to agar plates containing 17 μg/ml chloramphenicol. The recombination strategy is shown in Fig. 1.

The BACs from *E. coli* DH10B were isolated using NucleoBond AX purchased from Macherey-Nagel. HHV-6ABAC DNA was transfected into JJhan cells by electroporation using a Nucleofection unit (Amaxa). After electroporation, the cells were cultured in RPMI medium containing 8% FBS. On the second day after electroporation, the cells were co-cultured with CBMCs in RPMI medium containing 8% FBS. Green fluorescence from the infected CBMCs was easily confirmed after 4–5 days in co-culture. After three cell-to-cell infections (using CBMCs as the uninfected cells), the cells were harvested and prepared for Western blot analysis and EM.

Successful reconstitution of infectious virus using HHV-6ABAC DNA

After the recombination between PHAU5354 and HHV-6A genome in CBMCs, the resultant circular DNAs were isolated and then transformed into DH10B competent cells. We cultured the positive colonies from the chloramphenicol plates and isolated the BACs as described above. These BAC DNAs were digested with BamH1 and separated on agarose gels. As shown Fig. 2A, different digestion patterns of the BAC DNAs were observed, which might have reflected genetic variances within the U1102 strain before the insertion of the BAC into the HHV-6A genome. We then transfected the HHV-6ABAC DNAs into JJhan cells, and enriched for the reconstituted virus by co-culturing the transfected JJhan cells with CBMCs. Infectious virus could only be successfully reconstituted using BAC DNA from the G-1 strain (lane 1 in Fig. 2A). The proportion of GFP-positive cells increased dramatically when we performed the cell-to-cell infection three times (Fig. 2B).

Confirmation of viral gene expression and infectious virions in HHV-6ABAC-transfected cells

Borenstein et al. successfully inserted a BAC sequence into the HHV-6A genome, but neither the viral protein nor HHV-6A virions could be identified in the HHV-6A BAC-transfected Sup T 1 cells. Viral protein expression could only be confirmed in the case of complementation by UV light-inactivated virus (Borenstein and Frenkel, 2009). We transfected JJhan cells with HHV-6ABAC and then co-cultured them with CBMCs, which are commonly used for propagating HHV-6. After three rounds of cell-to-cell infection, the GFP expression from the BAC gfp gene was greatly increased (Fig. 2B).

We harvested these cells and prepared samples for Western blot analysis. The expression of U14, an early gene product of HHV-6 and gQ1, gH, and glycoprotein B (gB), which are late gene products of HHV-6, could be detected as in wild-type HHV-6-infected CBMCs

Table 1
Primer sequence.

Primer name	Sequence
AgQ1Xho1F	5-accctcgagccaccatggcaaccgcaagactg-3
AgQ1693 Ecor1R	5-accgaattcacatagagtctctttcactttg-3
Agp105F922	5-tcttctaacctcatggactcag-3
AgQ1spstnotR	5-accctgcaggcggccgcgacggagaatggcaaccgcaag-3
AgQ1de2F	5-acgaaggcttccagccccatagtttctactctgaaaggatgtagtcttcgtctcagtcttgtgaaggatgacgacgataagtaggg-3
AgQ1de2R	5-gtttgtctcgcgcatacgaaattttcacaagactgagacgaagactacatcctttcagagtagaaaccaaccaattaaccaattctgattag-3
ReF1	5-atgattacgaaggcttccag-3
ReR1	5-acaggatccatggcaaccgcaaggctgag-3
ReF2	5-acaggatccaggatgacgacgataagtag-3
ReR2	5-tgtttgtctcgcgcatacgaaattttcacaagactgagacgaagaatggcaaccgcaaggctgagcgctatgaaaccgccgagatccaaccaattaaccaattctgattag-3
ReR3	5-tgtttgtctcgcgcatacgaaattttc-3
U53Fnot1	5-accagcggccgcgtttgggtaggtggattcctttg-3
U531650rpac1	5-accttaattaacactctgaatcttccttataac-3
U541651Fpac1	5-accttaattaaaattcctggacatggtgaaac-3
U54Rnot13650	5-accagcggccgcagacgcatgtcttcaagctgtatc-3

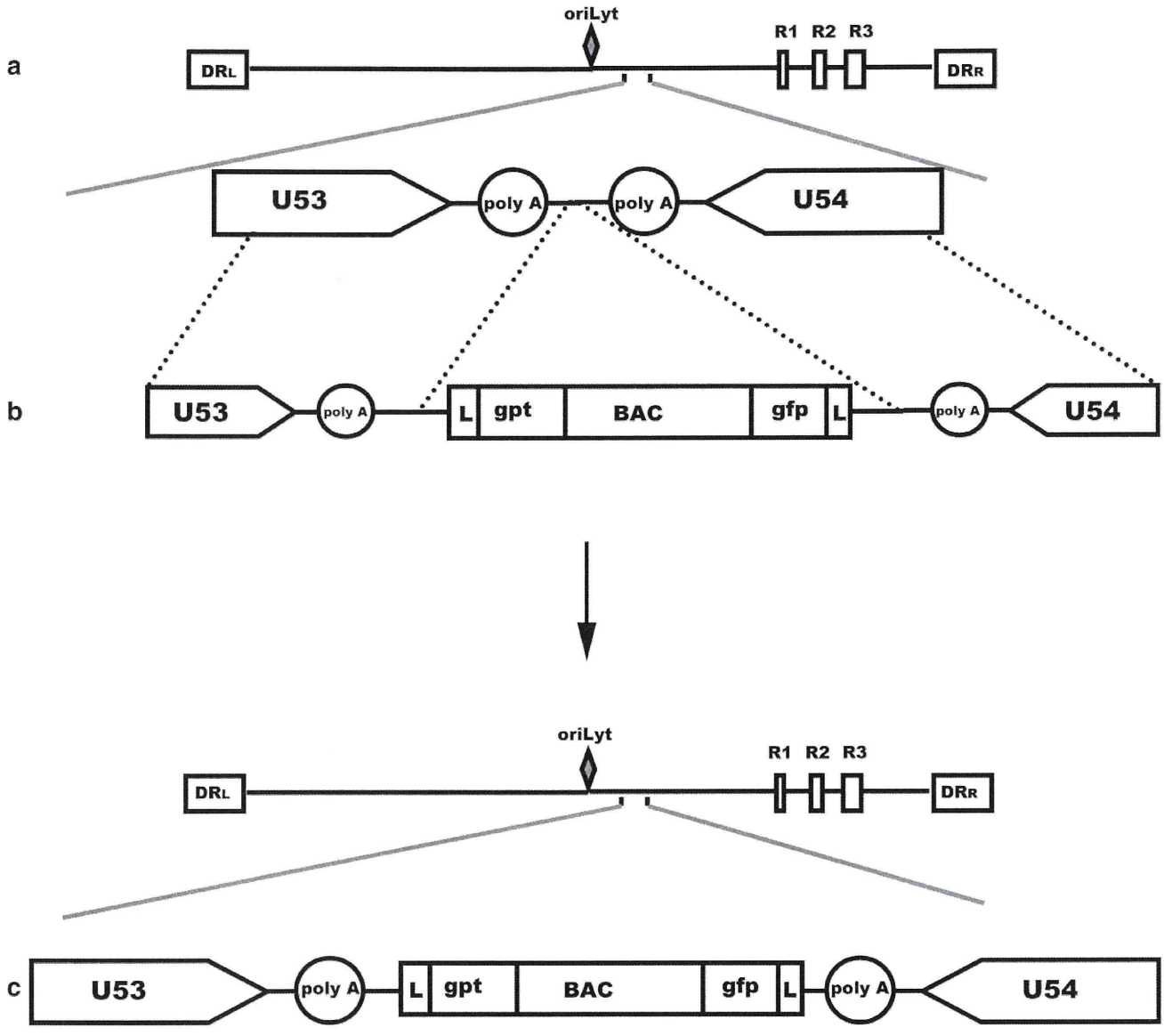

Fig. 1. Strategy for cloning the HHV-6A BAC. (a) The HHV-6A genome consists of three major internal repeat elements (R1–R3), the origin of replication (oriLyt) and the direct repeat termini (DR_L and DR_R). (b) The recombinant plasmid, PHAU5354 contained an approximately 1.5-kbp sequence from U53, and 2.0-kbp sequence from U54 amplified from the HHV-6A genome by PCR, and the BAC vector sequence including the gpt gene, gfp gene, and *loxP* sites at both termini. The recombinant plasmid was digested with NotI and electroporated into CBMCs. These cells were then infected with HHV6A. Homologous recombination occurred between PHAU5354 and the HHV-6A genome. (c) The resultant HHV-6ABAC is depicted at the bottom of the figure. The circular recombinant viral genomes were then harvested and electroporated into DH10B. Single BAC clones were isolated.

(Fig. 3A). Interestingly, the 80-kDa gQ1 gene product (Akkapaiboon et al., 2004), which is incorporated into HHV-6A virions, was also detected. Next, we asked whether mature HHV-6 virions were also produced in these cells. We therefore observed these samples under EM. As shown in Fig. 3B, mature virions could be identified in both the cytoplasmic and extracellular spaces. This observation confirmed that we had successfully reconstituted the infectious virus of HHV-6A by transfecting HHV-6ABAC DNA into JJHAN cells and co-culturing them with CBMCs.

Generation of a gQ1 deletion mutant virus and its revertant virus

The main purposes of cloning intact viral genomes as BACs are to manipulate *E. coli* mutagenesis systems to disrupt the viral genes and to analyze the biocharacteristics of the mutant viruses. The gQ gene is unique to HHV-6. The gQ1 protein is reported to form a tetrameric complex with gQ2, gH, and gL that acts as viral ligand for CD46, the cellular receptor for HHV-6A (Akkapaiboon et al., 2004). However, the specific function of gQ1 in HHV-6-infected cells, or even whether it is essential for HHV-6 propagation, has been unknown. In addition, even if gQ1 is non-essential, it is not known what, if any, phenotype results when the gQ1 gene is knocked out.

We therefore constructed a gQ1 mutant virus, and analyzed its function in HHV-6 infection. The N-terminal segment of gQ1 was deleted from the HHV-6A genome, and its revertant was also generated. (The detailed procedure can be found in Materials and methods, and is depicted in Fig. 4). The resultant BAC DNAs (HHV-6ABACΔgQ1 and HHV-6ABACΔgQ1re) and HHV-6ABAC DNA were examined by restriction digestion with BamH1.

As shown in Fig. 5, the digestion of HHV-6ABACΔgQ1re (lane 3) with BamH1 yielded the same pattern as HHV-6BAC DNA (lane 1); however, a different digestion pattern was seen with HHV-

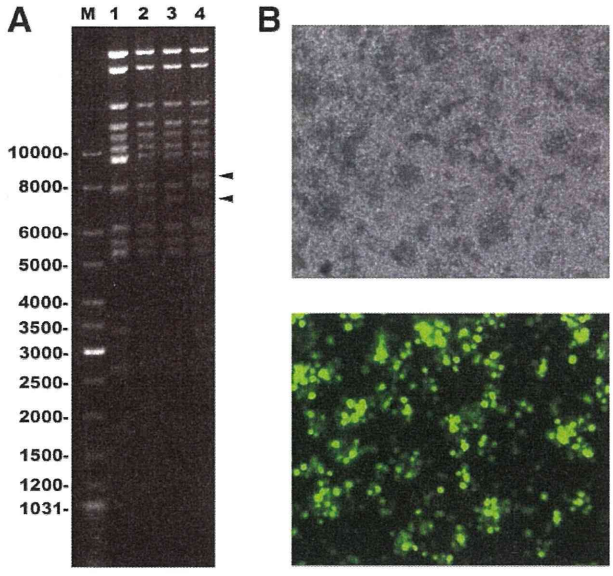

Fig. 2. Restriction enzyme digestion of HHV-6ABAC clones and GFP expression in reconstituted-virus-infected cells. (A) The BAC DNAs isolated from single clones (lanes 1–4) of HHV-6ABACs were digested with BamH1 and analyzed with molecular weight markers (M) by electrophoresis on 0.5% agarose gels in 0.5× Tris–borate–EDTA buffer for 48 h at 2.5 V/cm. DNA fragments were visualized by ethidium bromide staining. The different-sized bands among the clones are indicated by arrowheads. (B) HHV-6ABAC DNA isolated from the G-1 clone (lane 1 in A) was transfected into JJhan cells. The transfected cells were co-cultured with CBMCs on the third day post-transfection. The results of light-microscopic examination are shown at the top. The green fluorescence of the cells in the same sample is shown at the bottom.

6ABACΔgQ1 (lane 2). Southern blot analysis was performed using probe 1 (part of the deleted gQ1 sequence) and probe 2 (part of the residual gQ1 sequence). As expected, we detected positive bands in all three digested samples using probe 2, but the only positive bands in the HHV-6A BAC and HHV-6 BACΔgQ1re samples were detected using probe 1. These data indicated that we had successfully generated gQ1-

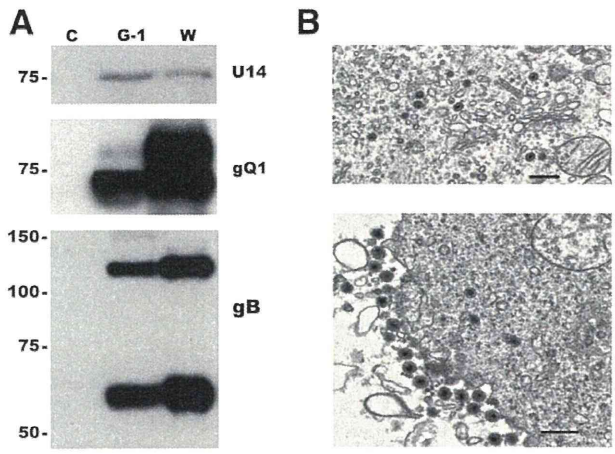

Fig. 3. Confirmation of viral protein expression and virion formation in the reconstituted-virus-infected cells. (A) JJhan cells were transfected with HHV-6ABAC (G-1) and then co-cultured with CBMCs. These cells were subjected to three rounds of cell-to-cell infection and lysed with RIPA buffer on the fourth day after the last cell-to-cell infection. The samples were resolved by SDS-PAGE under reducing conditions, electrotransferred to PVDF membranes, and reacted with anti-U14 Abs (top), the MAb AgQ-119 (anti-gQ1) (middle), and anti-gB Abs (bottom). We used HHV-6A-infected CBMCs (W) as a positive control. Uninfected CBMCs were used as a negative control (c). (B) The reconstituted-virus-infected CBMCs described in (A) were prepared for EM analysis. HHV-6A virions could be found in the cytoplasm (top) and extracellular space (bottom). Scale bars: 0.5 μM.

deletion and revertant BAC DNAs in *E. coli* and that we could use this *E. coli* mutagenesis system to construct HHV-6A BAC mutants.

gQ1 is essential for the propagation of HHV-6A in vitro

We next transfected JJhan cells with HHV-6ABAC, HHV-6ABACΔgQ1re, and HHV-6ABACΔgQ1 DNAs, and then co-cultured them with the CBMCs from the same donor. The GFP expression in the CBMCs increased in the HHV-6ABAC- and HHV-6ABACΔgQ1re-co-cultured samples, but not in the HHV-6ABACΔgQ1 sample (data not shown). We then performed the cell-to-cell infection three times, and harvested the cells for Western blot analysis. gQ1 expression could be detected in both the HHV-6ABACΔgQ1re- and HHV-6ABACΔgQ1-expressing cells (Fig. 6), suggesting that the gQ1 deletion revertant was successfully constructed. gQ1 is a component of gH/gL/gQ1/gQ2 complex, which acts as a viral ligand for the cellular receptor, CD46 (Akkapaiboon et al., 2004). The partial deletion of the gQ1 gene from HHV-6A genome resulted in a failure to reconstitute infectious virus. These data supported the idea that gQ1 plays a crucial role in HHV-6 infection.

Discussion

In this study, we successfully cloned and performed mutagenesis of the HHV-6A genome as an infectious BAC in *E. coli*. The BAC sequence was inserted into a site between the U53 and U54 polyA signal sequences in the HHV-6A genome, using homologous recombination in CBMCs. The circular intermediates of the recombinant viral genome were isolated and transformed into *E. coli*. Infectious virus was reconstituted by transfecting JJhan cells with the HHV-6ABAC DNA and co-culturing them with CBMCs. A selected viral gene, the gQ1 gene, was disrupted using the two-step Red recombination system. BACs containing the mutant viral genome failed to yield reconstituted virus, which demonstrated that gQ1 was essential for the propagation of HHV-6A *in vitro*.

In HSV-1, the insertion of foreign genes between selected intergenic polyA signal sequences does not disrupt viral genes or transcriptional units, nor does it affect viral growth in cell culture or virulence in mice (Morimoto et al., 2009). In addition, the insertion of the GFP and BAC sequences into the region between the U53 and U54 polyA signal sequences of the HHV-6A genome does not affect the propagation of infectious virus in SupT1 cells (Arbuckle et al., 2010). Therefore, we inserted the BAC cassette into the U53 and U54 polyA signal sequences using homologous recombination in CBMCs. The proportion of recombinant virus increased dramatically in the presence of mycophenolic acid and xanthine, which was indicated by the expression of GFP from the BAC cassette (as shown in Fig. 2B). Moreover, infectious virus could be reconstituted using the HHV-6ABAC DNA isolated from *E. coli*. Therefore, the intergenic region of the U53 and U54 of HHV-6A is a suitable site for the insertion of foreign genes, even large sequences like the BAC.

Digestion of the HHV-6ABAC DNA with the restriction enzyme BamH1 showed different digestion patterns. This result might indicate that the U1102 strain consisted of a mixture of populations before it was inserted into the BAC. In the U1102 strain, the region from DR1 to DR6 is reported to be deleted when it is propagated in T-cell lines, and this deletion has no effect on the virus propagation in T-cell lines (Borenstein et al., 2010). However, we still do not know the precise differences between these variant clones, or why only the G-1 clone could be efficiently reconstituted in our experiments. Some mutation in the other clones might retard or be lethal to HHV-6A propagation.

Like the gH/gL/UL128-131 complex in the β-herpesvirus HCMV, the gH/gL/gQ1/gQ2 complex is unique in HHV-6. UL128, UL130, and UL131 are all required for the gH/gL/UL128-131 complex formation, which functions in the entry of HCMV into epithelial and endothelial cells (Ryckman et al., 2008b). In HHV-6, we did not know whether gQ

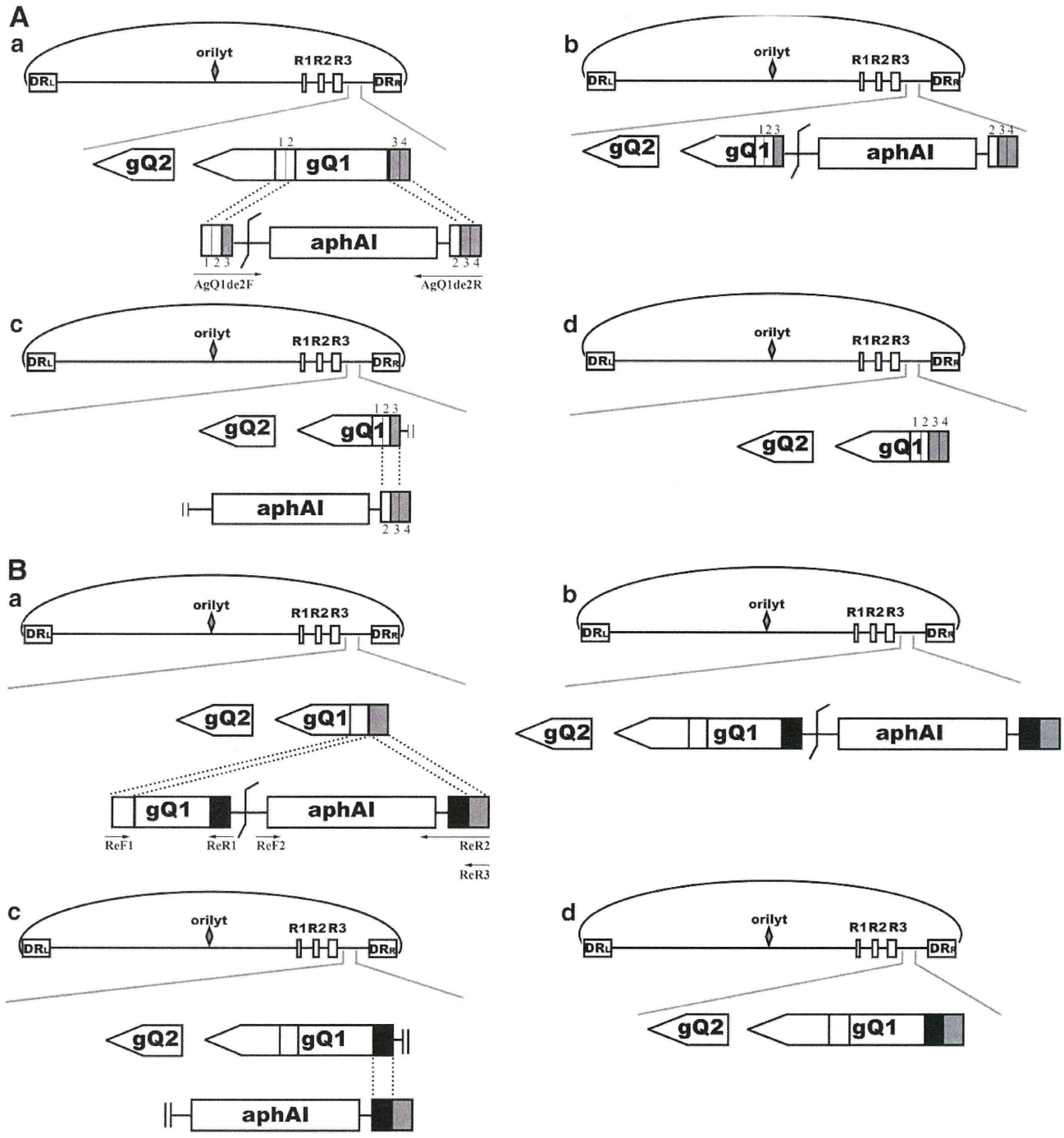

Fig. 4. Strategy for constructing HHV-6ABACΔgQ1 (A) and HHV-6ABACΔgQ1re (B). In all the figures, the regions encoding the gQ1 and gQ2 cDNAs are expanded in the HHV-6A BAC DNAs. (A) About 20-bp sequential sequences in downstream of the deleted sequences of gQ1 gene are labeled as 1 and 2 in the figure, similarly, the upstream sequences are labeled as 3 and 4. The kanamycin-resistance gene (aphAI) amplified with long primers (AgQ1de2F and AgQ1de2R) flanked with gQ1 upstream labeled as 3 and 4 (gray) and interim sequences labeled as 1 and 2 (white) recombines with the same gQ1 upstream and interim sequences (a), which results in the deletion of N-terminal segment of gQ1 and the insertion of the kanamycin-resistance gene (b). The I-sec1 site between the gQ1 sequence and the aphAI gene is cleaved (c), and the second recombination occurs (c), which results in deletion of the kanamycin-resistance gene (d). (B) The deleted part of gQ1 is amplified from HHV-6A genomic DNA using ReF1 and ReR1 primers, and ligated with the kanamycin-resistance (aphAI) gene which is amplified with ReF2 primer and ReR2 primer flanked with part of gQ1 deleted sequences (dark) and the upstream sequences of gQ1 (gray). The resulting DNA fragment flanked with gQ1 upstream (gray) and interim (white) sequences recombines with the same sequences in the HHV-6ABACΔgQ1 DNA (a), which results in the insertion of the DNA fragment (b). The I-sec1 site between the gQ1 sequence and aphAI is cleaved (c), and the second recombination occurs (c), which results in the deletion of the kanamycin-resistance gene (d).

was also needed for infection. We therefore constructed a gQ1 deletion mutant and its revertant using the Red recombination system in *E. coli*. Our data showed that without the gQ1 gene, no infectious virus could be reconstituted using HHV-6ABAC (HHV-6ABACΔgQ1), which indicated that gQ1 was essential for HHV-6A propagation *in vitro*.

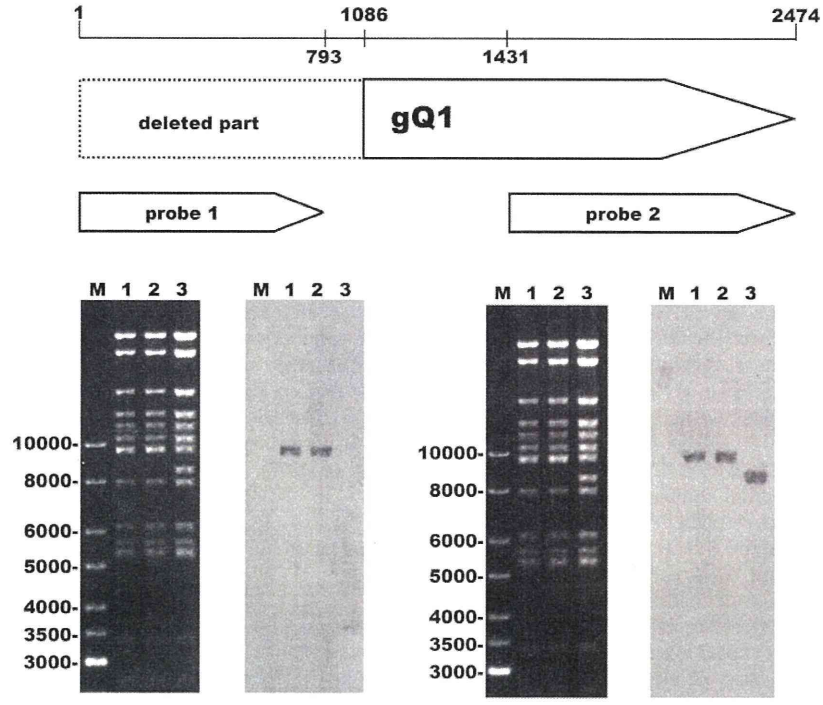

Fig. 5. Confirmation of the construction of HHV-6ABACΔgQ1 and HHV-6ABACΔgQ1re. The scale at the top indicates the length of the gQ1 region, the deleted part of the gQ1 region (dotted line), and the probes used for Southern blot analysis. The DNA of HHV-6ABAC (lane 1), HHV-6ABACΔgQ1 (lane 3), and HHV-6ABACΔgQ1re (lane 2) were digested with BamH1 and separated on a 0.5% agarose gel with markers (M). Bands were visualized with ethidium bromide, transferred to a Hybond-N + nylon membrane, and hybridized using the probes indicated above the figures.

In summary, we successfully constructed an HHV-6ABAC and reconstituted infectious virus by transfecting it into mammalian cells. This constructed BAC could also be manipulated in *E. coli* using an *E. coli* mutagenesis system, which should be applicable for generating any selected gene of HHV-6A. Using these systems, we demonstrated that the gQ1 of HHV-6 is essential for the virus propagation. This novel approach in HHV-6A will contribute to the genetic analysis of HHV-6A, and should lead to new breakthroughs in HHV-6 biology and treatments for HHV-6 infections.

Materials and methods

Cells, viruses, and antibodies

The JJhan T-cell line was cultured in RPMI-1640 medium supplemented with 8% fetal bovine serum. CBMCs were separated on a Ficoll–Conray gradient and cultured in RPMI medium containing 10% fetal bovine serum, and stimulated with phytohemagglutinin (5 μg/ml) for 3 days before use. The HHV-6A strain U1102 was propagated in stimulated CBMCs. The monoclonal antibodies (MAbs) for HHV-6A gQ1 (AgQ1-119) and for U14 were described previously (Akkapaiboon et al., 2004; Takemoto et al., 2005). The polyclonal antibody for gB was also described previously (Mori et al., 2008).

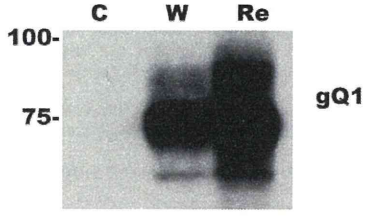

Fig. 6. JJhan cells were transfected with HHV-6ABAC and HHV-6ABACΔgQ1re DNA, and then co-cultured with CBMCs. The cells were subjected to three rounds of cell-to-cell infection, lysed with RIPA buffer, separated by gel electrophoresis, electrotransferred to a PVDF membrane, and reacted with the MAb AgQ-119 (anti-gQ1). We used uninfected CBMCs as a control (c). W: wild type, Re: revertant.

Isolation of viral DNA and BAC plasmids

The total cellular DNA was isolated from U1102-infected CBMCs. Briefly, HHV-6A-infected cells were harvested on the third day post-infection and washed with phosphate-buffered saline (PBS). The cell pellet was resuspended in Tris–EDTA buffer (100 mM Tris–HCl, 20 mM EDTA, pH 8.0), and an equal volume of 1% sodium dodecyl sulfate and proteinase K (500 μg/ml) was applied. The mixture was incubated at 55 °C for 3 h. The DNA was then extracted twice with phenol–chloroform and once with chloroform–isoamylalcohol (24:1), then precipitated with isopropanol and washed with 70% ethanol. The isolated DNA was eluted in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5). Circular viral DNA was isolated by the method of Hirt as described previously (Hirt, 1967).

Western blot

Western blotting was performed as described previously (Akkapaiboon et al., 2004).

DNA analysis

BAC DNA isolated from *E. coli* was cleaved with BamH1 and separated on a 0.5% agarose gel. The DNA fragments were transferred to a Hybond-N+ nylon membrane (GE Healthcare). Probe 1 was amplified with the AgQ1Xho1F and AgQ1693 Ecor1R primers, and probe 2 was amplified with the Agp105F922 and AgQ1spstnotR primers (shown in Table 1) by PCR. Southern blot hybridization was then performed according to the company's instructions (GE Healthcare).