Detection of a Gene Cluster That Is Dispensable for Human Herpesvirus 6 Replication and Latency

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The U3-U7 gene cluster of human herpesvirus 6 (HHV-6) was replaced with an enhanced green fluorescent protein-puromycin gene cassette containing the cytomegalovirus major immediate-early promoter. Neither viral replication in T cells nor latency and reactivation in macrophages was impaired. During HHV-6 latency, the cytomegalovirus promoter used the transcription start sites employed in cytomegalovirus latency.

Human herpesvirus 6 (HHV-6) normally produces relatively mild diseases (2, 3, 5, 7, 10), and thus it has been suggested as a viral vector candidate for gene therapy (36). However, the establishment of a recombinant HHV-6 is difficult and has not been reported. The difficulty is partly due to a technical issue that we describe below. It is also due to the nature of the HHV-6 genome. Many HHV-6 genes are homologous to those of human cytomegalovirus (HCMV); however, HHV-6 has a smaller number of genes than HCMV, and it does not encode homologues of the HCMV genes that are dispensable for viral replication and are deleted in making recombinant HCMV (9, 12, 14, 26). To make a recombinant virus by homologous recombination, a dispensable gene(s) that can be replaced with a drug-resistant gene needs to be found.

To identify genes that are dispensable for viral replication, we replaced a gene cluster of the HHV-6 genome with EGFP-puro, a gene cassette containing the gene for enhanced green fluorescent protein (EGFP) under control of the HCMV major immediate-early promoter.

FIG. 1. Structure of H6R28. At the top is a map of the HHV-6B HST genome, with the region U1 to U9 expanded below. In the middle, shaded arrows show the U3-U7 open reading frames (coordinates 10315 to 16302) that were replaced by the EGFP-puro cassette. The locations of the PCR primers used for the cloning of the U2 and U8 DNA fragments (U2 XbaI, U2 AflII, U8 BamHI, and U8 EcoRI) and the primers for the verification of the recombinant virus are depicted. Primer sequences are shown in Table 1. The bottom diagram represents the EGFP-puro cassette pU2-U8 EGFP-puro. The open box represents the EGFP gene and HCMV MIEP that was derived from pEGFP-C1 (nucleotide numbers 8 to 1640) (Clontech). Multiple cloning sites of pEGFP-C1 including PstI were deleted. The puromycin-N-acetyl-transferase gene (pac) and SV40 early promoter were derived from pPUR (nucleotide numbers 408 to 1392) (Clontech). The annealing sites of the primers used are depicted by small solid arrows. The recognition sites of the restriction enzymes used (PstI, AflII, and BamHI) are also shown. The sizes of the amplified or digested products are indicated by dotted arrows.

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immediate-early enhancer-promoter (MIEP) and the puromycin resistance gene under control of the simian virus 40 (SV40) early promoter (Fig. 1). To insert the EGFP-puro cassette into the HHV-6 genome by homologous recombination, 1-kb segments of viral genome were inserted into each end of the cassette (Fig. 1). The following gene clusters were examined: the DR2-DR7 genes, which are duplicated in the viral genome (9, 12, 14); U95, the positional homologue of the murine cytomegalovirus (MCMV) immediate-early (IE) 2 gene, which is known to be dispensable for viral replication (4); and the U3-U7 genes. Of these, we found that replacement of the U3-U7 genes with EGFP-puro resulted in a successfully replicating virus. For the construct, the U2 gene was amplified by PCR with primers U2XbaI and U2AfluII, and the U8 gene was amplified with U8BamHI and U8EcoRI (primers used in this study are listed in Table 1). The amplified products were then inserted into each end of pEGFP-puro (pU2-U8 EGFP-puro in Fig. 1). The cloned plasmid was transfected into phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells by us-

FIG. 2. PCR amplification of viral DNA from wt virus and H6R28. (A) Viral DNA from wt virus (lanes 1 and 3) and H6R28 (lanes 2, 4, 5, and 6) was amplified with double-nested PCR using primer pairs U2R2-U8F2 and U2R1-U8F1. The amplified products were digested with PstI (lanes 3 and 4), AflIII (lane 5), or BamHI (lane 6). The undigested products were separated on a 0.6% agarose gel (lanes 1 and 2), and the digested ones were separated on a 1.0% gel (lanes 3 to 6). The expected sizes of the digested products from the wt virus are shown by open arrows, and the expected products from H6R28 are depicted with solid arrows. The positions of the products are depicted in Fig. 1. (B) DNA from H6R28 was amplified by PCR with primers U2R1-EGFPprim (lane 1) or U8F1-PACprim (lane 2). The amplified products were separated on a 1.0% agarose gel. The expected sizes of the products (1,582 bp for U2R1-EGFPprim and 1,760 bp for PACprim-U8R1) are depicted by arrows. (C) PCR amplification of the deleted open reading frames. Viral DNA from wt virus (lanes 1 to 5) and H6R28 (lanes 6 to 10) was amplified by PCR using primer pairs U3F1-U3R1 (lanes 1 and 6), U4F1-U4R1 (lanes 2 and 7), U5F1-U5R1 (lanes 3 and 8), U6F1-U6R1 (lanes 4 and 9), or U7F1-U7R1 (lanes 5 and 10). The amplified products were separated on a 1.0% agarose gel. The expected sizes of the products, indicated by arrows, are as follows: U3F-U3R, 1,161 bp; U4F-U4R, 1,338 bp; U5F-U5R, 1,275 bp; U6F-U6R, 171 bp; U7F-U7R, 1,094 bp. Lanes M, 1-kbp ladder Plus (Invitrogen).
ing a Nucleofector electroporator (Amara Biosystems, Germany) according to the manufacturer's recommended protocol. Briefly, 5 × 10^6 cells were mixed with 5 μg of the plasmid and 100 μL of Nucleofector solution for T cells, and electroporation was performed with the Nucleofector using the program U-14. Alternatively, a conventional electroporation method was used. In this case, 1 × 10^7 cells were mixed with 50 μg of the plasmid suspended in 500 μL of K–phosphate-buffered saline (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na2HPO4, 1.46 mM KH2PO4, and 25 mM MgCl2), and the mixture was placed in an electroporation cuvette (Gene Pulser cuvette, 0.4-cm diameter; Bio-Rad). Electroporation was performed with the Gene Pulser II using the protocols shown in Fig. 1, giving rise to the expected bands in both CBMCs and H6R28. CBMCs were infected as described above, and infected cells were harvested at the indicated times and frozen at −80°C. Progeny viruses were titrated on CBMCs using IFA staining (1). Virus titer was indicated as 50% tissue culture infective doses per milliliter. Titers from cells infected with wt virus (C), H6R28 clone 1 (A), clone 2 (B), and clone 3 (C) are shown. Values shown are mean values of results for three replicate cultures. Values on day 0 represent the titers of the input viruses. Data shown are mean values of results for three replicate cultures.

To confirm the insertion of the EGFP-puro cassette into the expected region, viral DNA was amplified by double-nested PCR with KOD Plus DNA polymerase (TOYOBO, Otsu, Japan) using primers against regions outside the homologous hinge regions (U2R2-U8F2 and U2R1-U8F1) (Fig. 1). An amplified product of approximately 8.5 kb was observed in the wild-type (wt) virus, and 5.0-kb bands were observed in three clones of H6R28 (representative cases are shown in Fig. 2A). The amplified products were digested with the restriction enzymes shown in Fig. 1, giving rise to the expected bands in both wt virus and H6R28 (Fig. 2A). The amplified products were confirmed by partial sequencing (data not shown). The 8.5-kb product was not observed in the recombinants, which indicated that they were not contaminated with the wt virus. The inserted position of EGFP-puro was also examined using primers ho-

![FIG. 3. Productive infection of H6R28. (A) Kinetics of the increase in cells infected with wt virus and H6R28. CBMCs were infected with wt virus and three independent clones of H6R28 at an MOI of 0.05 (0.05) to 0.15 tissue culture infectious doses/cell, and the percentages of cells reacting with a mixture of monoclonal antibodies to gB and gH were determined by IFA staining using monoclonal antibodies (29). The percentages of cells infected with wt virus (C), H6R28 clone 1 (A), clone 2 (B), and clone 3 (C) are shown. Data shown are mean values of results for three replicate cultures. (B) Growth curves for wt virus and H6R28. CBMCs were infected as described above, and infected cells were harvested at the indicated times and frozen at −80°C. Progeny viruses were titrated on CBMCs using IFA staining (1). Virus titer was indicated as 50% tissue culture infective doses per milliliter. Titers from cells infected with wt virus (C), H6R28 clone 1 (A), clone 2 (B), and clone 3 (C) are shown. Values shown are mean values of results for three replicate cultures.](image)

![FIG. 4. Latent infection and reactivation of H6R28. (A) Percentages of HHV-6 DNA-positive cells. The percentages of HHV-6 DNA-positive cells were examined 4 and 6 weeks postinfection. The data shown are mean values and standard deviations of results for three replicate cultures of wt virus and three clones of H6R28. Open column, wt virus; shaded column, H6R28. (B) Percentages of reactivation-positive cells. Viral reactivation was induced, and the percentages of reactivation-positive cells were calculated. The data shown are mean values and standard deviations of results for three replicate cultures of wt virus and three clones of H6R28. Open column, wt virus; shaded column, H6R28.](image)
mologous to EGFP-puro, and the expected PCR products were observed (Fig. 2B). To address the possibility of the ectopic expression of U3-U7 genes, we attempted to amplify each gene from H6R28 by PCR, and no amplified products were obtained (Fig. 2C).

We produced three independent isolates of H6R28 by three individual electroporations and examined the replication kinetics in CBMCs. Virus titration was performed using CBMCs as described previously (1). CBMCs were infected at a MOI of 0.05, and the three H6R28 clones and the wt virus showed similar levels of viral spreading (Fig. 3A) and virus production (Fig. 3B) over time.

We next investigated H6R28 for its ability to establish latency and its efficiency of reactivation. To evaluate the establishment of latency, peripheral blood macrophages were infected with wt virus and H6R28 and the percentage of HHV-6 DNA-positive cells was monitored as described previously (17, 19, 20). Briefly, peripheral blood macrophages were cultured in RPMI 1640 supplemented with 25% horse serum on plastic plates coated with collagen (Sumitomo Bakelite Co., Ltd, Japan). The macrophages were infected with HHV-6 on day 7 and cultured for 4 to 6 weeks. The infected macrophages were detached from the plates, and the absence of viral replication was confirmed by immunofluorescent antibody (IFA) staining using monoclonal antibodies against glycoproteins B and H (29). The cells were serially diluted (104 to 1 cell per tube) into sample tubes using four tubes for each dilution, and the DNA was isolated from each sample tube. Viral DNA was detected by double-nested PCR (18), and the numbers of HHV-6 DNA-positive cells were calculated by the Reed-Muench method (30). This experiment showed similar percentages of HHV-6 DNA-positive cells in the wt virus- and H6R28-infected cultures (Fig. 4A).

To study the reactivation efficiency, viral reactivation was induced by tetradecanoyl phorbol acetate (TPA) treatment as described previously (17, 20). Briefly, latently infected cells were detached from the culture dish, serially diluted, and cocultivated with an uninfected macrophage feeder layer. Subsequently, the cells were treated with TPA (20 ng/ml) for 7 days and cocultivated with CBMCs for 7 days. The efficiency of the viral reactivation was calculated by the Reed-Muench method (30) and found to be similar for the wt virus and H6R28 (Fig. 4B). From these data, we concluded that the establishment of latency and the reactivation process was not impaired by the deletion of the U3-U7 genes.

Interestingly, during HHV-6 latency, we failed to detect the expression of EGFP that was driven by the HCMV MIEP (25, 35) (Fig. 5A). On the other hand, EGFP expression was observed in the latently infected macrophage transfected with the plasmid pU2-U8 EGFP-puro illustrated in Fig. 1 (Fig. 5B), reactivation-induced macrophages (Fig. 5C), productively infected CBMCs and Molt-3 cells (Fig. 5D and E), and abortively infected HeLa cells (Fig. 5F).
FIG. 6. Function of the HCMV promoter in the latently infected HHV-6. (A) HCMV IE1/IE2 promoter and PCR primers. The EGFP transcript initiating at cDNA ends (RACE) was performed as described previously (19). The 5′ end of the transcript was dA tailed and annealed with anchor primer RL-1. Primer sequences are shown in Table 1. (B) 5′-RACE amplification of the EGFP transcripts. RNA from 1 × 10^6 latently infected macrophages (M6) (lane 1), 1 × 10^5 latently infected Molt-3 cells (lane 4), and 1 × 10^5 abortively infected HeLa cells (lane 5) was analyzed by the 5′-RACE method. The RACE method used was the same as that used in previous studies (19-21). The 5′ end of the transcript was dA tailed and annealed with the anchor primer RL-1 (Fig. 6A) and amplified first with primers N2-EGFP R2 and then with primers N1-EGFP R1. The 5′ end of the transcript initiating at PSS (~360 bp), LSS1 (~720 bp), and/or LSS2 (~650 bp) were detected. HaeIII-digested φX174 DNA fragments were used as size markers (lane φX).

infected HeLa cells (Fig. 5F). To investigate the gene expression from the IE1/IE2 promoter, 5′-rapid amplification of cDNA ends (RACE) was performed as described previously (19-21). Briefly, the 5′ end of the cDNA was dA tailed and annealed with an anchor primer, RL-1. The initial 10 cycles of PCR were performed with Taq polymerase (Roche Diagnostics) using the following conditions: denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. PCR amplification was performed with PCR with KOD Plus DNA polymerase (TOYOBO) using primers N2 and EGFP-R2 followed by primers N1 and EGFP-R1 (Fig. 6A) under the following conditions: denaturation for 1 min at 94°C, annealing for 30 s at 65°C, and extension for 1 min at 68°C (15 cycles per amplification). The amplified products were sequenced. In the latent cells, transcription of the mRNA from the usual transcription start position (productive infection transcription start site [PSS]) was not detected (Fig. 6B); however, small amounts of mRNA were transcribed from the latent infection transcription start sites (LSSs) 1 and 2 of HCMV, which are used to express the latency-associated transcripts of HCMV (21). In contrast, the PSS was used in the latently infected macrophage transfected with the plasmid pU2-U8 EGFP-puro, reactivation-induced macrophages, and the productively infected Molt-3 cells and the abortively infected HeLa cells (Fig. 6B). Since HCMV MIEP showed the latency-associated performance in the context of HHV-6 latency, it is suggested that the transcriptional control of HHV-6 latency may share some common mechanism with HCMV latency. These findings may be related to the fact that HCMV shows some similarity with HHV-6, such as the site of latency (8, 15–17, 24, 33, 34).

Overall, the recombinant virus H6R28 revealed that the fairly large gene cluster U3-U7 was dispensable for viral replication, latency, and reactivation. Of the deleted genes, the characteristics of U4 and U6 have not been reported. Genes U3, U5, and U7 belong to the US22 family gene (9, 12, 28), whose members are related to the HCMV US22 gene (6). Every betaherpesvirus encodes several US22 family genes that encode at least one of four conserved motifs (28). Although the functions of most of the US22 family genes are unknown, some of them, such as the MCMV IE2 gene and the HCMV UL36-38 genes, encode proteins with transactivating functions. However, MCMV IE2 is known to be dispensable for viral replication and latency and reactivation (4). Deletion of the US22 family genes of H6R28 showed them to have similar properties; HHV-6 U3 encodes a protein with a weak transactivating function (27), and we failed to find any difference in the viral replication or latency and reactivation between the wt and recombinant virus. The US22 family genes UL36 and UL37 of HCMV have an antiapoptotic function (11, 22, 31). However, we did not observe increased apoptosis in H6R28-infected cells in the present study. Other US22 family genes, such as the MCMV M140 and M141 genes, confer altered cell and tissue tropism (13). Since the in vivo host tissue range of HHV-6 is broad and since the virus infects various types of cells (2, 3), it is possible that the HHV-6 US22 family genes contribute to the broad organ tropism of this virus.

H6R28 appears to be a useful tool for the study of HHV-6 latency and reactivation. Moreover, this large dispensable locus can be a useful site for inserting a large gene, such as a bacterial artificial chromosome vector (23, 32).

We believe this is the first report of a successful recombinant HHV-6, and we can provide HHV-6 investigators with a detailed protocol for making it.

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