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Identification and Analysis of a Lytic-Phase Origin of DNA Replication in Human Herpesvirus 7

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Human herpesvirus 7 (HHV-7) DNA sequences colinear with the HHV-6 lytic-phase origin of DNA replication (*ori*Lyt) were amplified by PCR. Plasmid constructs containing these sequences were replicated in HHV-7-infected cord blood mononuclear cells but not in HHV-6-infected cells. In contrast, plasmids bearing HHV-6 *ori*Lyt were replicated in both HHV-6- and HHV-7-infected cells. Finally, the minimal HHV-7 DNA element necessary for replicator activity was mapped to a 600-bp region which contains two sites with high homology to the consensus binding site for the HHV-6 origin binding protein. At least one of these binding sites was shown to be essential for replicator function of HHV-7 *ori*Lyt.

Human herpesviruses 6 and 7 (HHV-6 and -7) are closely related, ubiquitous, T-lymphotropic betaherpesviruses which were first isolated and cultured in 1986 and 1990, respectively (12, 35). Since then, HHV-6 has been identified as an agent of roseola (exanthem subitum) (34, 38) and the virus has also been associated with some neurologic syndromes (seizures, encephalitis, and possibly multiple sclerosis) (4, 11, 15, 20, 23, 29) as well as with disease in immunosuppressed individuals (5, 10, 25). The pathogenesis of HHV-7 infection is more uncertain, although it too appears to cause some cases of roseola (2, 17, 33), possibly as a result of its ability to reactivate HHV-6 during primary infection or in vitro culture (2, 13, 21). The potential interplay between HHV-6 and HHV-7 is also suggested by in vitro data, which have shown (i) that HHV-6 infection leads to de novo expression of the CD4 receptor on cells of hematopoietic lineage, including $CD8^+$ T cells, γδ T cells, and NK cells (24, 26, 27), and (ii) that HHV-7 binds to the CD4 receptor (6, 28, 39). This interaction between HHV-7 and HHV-6 is likely to be relevant in vivo, since (i) both viruses infect over 90% of children by 3 years of age, (ii) both viruses are tropic for T lymphocytes, (iii) HHV-7 infection generally occurs after HHV-6 infection (i.e., it occurs in children who already harbor HHV-6), and (iv) HHV-6 reactivation occurs in transplant recipients (most of whom presumably harbor HHV-7), with results that can sometimes be quite serious (3, 5, 10, 11).

In this study, we set out to compare the replicative strategies used by HHV-6 and HHV-7 through analysis of the *cis*-acting elements involved in viral DNA replication. In order to do this, PCR amplification was used to clone a DNA fragment from HHV-7 located at a genomic position equivalent to those of previously mapped lytic-phase origins of DNA replication (*ori*Lyt) in the human betaherpesviruses, HHV-6, and human cytomegalovirus (1, 8, 16). This DNA fragment corresponds to a region immediately 3′ to the open reading frame for the major DNA-binding protein in HHV-6 (U41) and human cytomegalovirus (UL57).

Oligonucleotide primers were designed, based on available sequence information, from the flanking regions of the HHV-7 genome (H7O3, TTGTTACTGTAAAGTATCGAATTC, and H7O4, TCTCTTGCGATTATTGAGGAAGCTT); these primers contain an *Eco*RI and a *Hin*dIII restriction site, respectively (underlined), which are known to flank the viral DNA fragment of interest. PCR with these primers was then carried out with 1 to 10 ng of template DNA from purified HHV-7 virions (isolate R-2) (36). Initial amplification reaction mixtures included *Pfu* DNA polymerase (Stratagene), but unexpectedly, multiple internal *Eco*RI and *Hin*dIII sites were encountered in the PCR amplimer, making it difficult to clone into an appropriately digested plasmid vector. For this reason, *Taq* DNA polymerase (Promega) was used in subsequent PCRs and cloning of PCR products was achieved with a T-overhang vector (pGEM-T; Promega). In all, 35 cycles of amplification were performed with standard reaction buffer (Promega) and sequential incubations at 94°C (30 s), 55°C (45 s), and 72°C (3 min).

Two plasmid clones (pH7O3 and pH7O8) were selected for sequence analysis. These clones were 2.45 and 2.25 kb in length, respectively, and exhibited 99.7% nucleotide identity (the difference in sequence length was due to a spontaneous deletion of a short DNA segment of roughly 200 bp at the 3′ end of clone H7O8, which occurred during cloning). Analysis of the DNA contents of these plasmid clones revealed a high degree of similarity to the corresponding locus in HHV-6, with greater than 60% nucleotide identity. However, both of the HHV-7 DNA clones were found to have an internal deletion of approximately 1.1 kb, relative to the corresponding region in HHV-6, raising the possibility that the HHV-7 DNA clones were deleted during cloning. For this reason, Southern blot analysis was performed with purified virion DNA from HHV-7 (R-2) and the DNA was then hybridized to radiolabeled DNA from plasmid clone pH7O3.

The results of digestion of viral DNA with *Eco*RI, *Hin*dIII or both enzymes, are shown in Fig. 1. Major hybridizing species of 2.0 kb (*Eco*RI), 1.45 kb (*Hin*dIII), and 1.3 kb (*Hin*dIII plus

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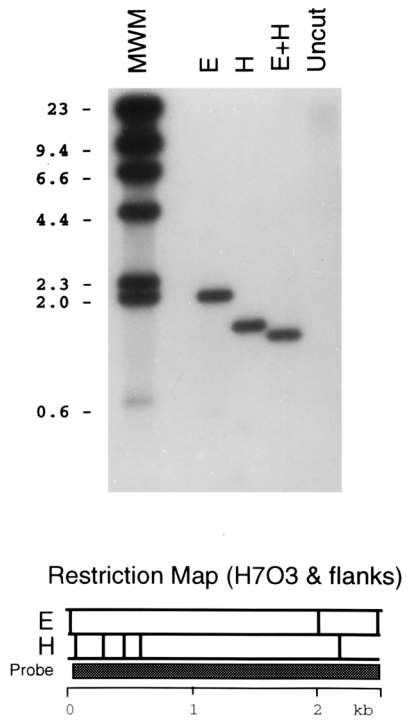

FIG. 1. Restriction mapping of HHV-7 *ori*Lyt region. Purified HHV-7 (R-2) was digested with the indicated restriction enzymes (E, *Eco*RI, and H, *Hin*dIII), and DNA fragments were resolved on an agarose gel. Shown is a Southern blot of this DNA with a radiolabeled probe corresponding to pH7O3. Numbers on the left denote molecular weight markers (MWM; sizes are in kilobases; *Hin*dIII digest of λ DNA), and the diagram at the bottom of the figure presents a restriction map for plasmid pH7O3.

*Eco*RI), which correspond precisely to the predicted sizes of DNA fragments, based on a restriction map of plasmid pH7O3, were detected in viral DNA (Fig. 1). Thus, we concluded that the plasmid clones pH7O3 and pH7O8 did indeed represent intact, full-length, HHV-7 DNA clones. This interpretation was also supported by inspection of the recently released DNA sequence of HHV-7 strain JI (32), to which plasmids pH7O3 and pH7O8 exhibit 99.6 and 99.5% DNA sequence identity, respectively.

A comparison of the HHV-6 and HHV-7 DNA sequences in the region corresponding to HHV-6 *ori*Lyt is presented in Fig. 2A. A large DNA segment (approximately 1.1 kb) present in HHV-6 *ori*Lyt was found to be absent in the putative HHV-7 *ori*Lyt. This HHV-6 DNA fragment contains a presumed DNA-unwinding element (DUE) and 3′ auxiliary replication domain and also spans the terminal one-third of the minimal essential origin domain, which includes one of two binding sites for the HHV-6-encoded origin-binding protein (OBP_{H6}) (8, 18, 19). As noted in Fig. 2B (analysis of predicted DNA helical stability with the Thermodyn algorithm), there is no obvious extended region of DNA instability (i.e., no putative DUE) anywhere within the immediate vicinity of the HHV-7 *ori*Lyt (in contrast to the HHV-6 *ori*Lyt).

Although lacking the DUE and 3′ auxiliary elements present in the HHV-6 genome, consensus alphaherpesvirus *ori*Lyt motifs, including an AT-rich spacer element as well as two DNA sequences with high homology (9 of 10 bases) to a previously defined consensus OBP_{H6} binding site, YGWYCWCCYN, were well conserved in HHV-7 (Fig. 2B) (19). The HHV-7 OBP-II motif is particularly interesting in that it is very similar (9 of 10 bases) to one of the two wild-type OBP_{H6} binding sites present in HHV-6 *ori*Lyt (HHV-6 OBP-2, 5′-CgTCCACCTC; the lowercase lettering denotes a mismatched residue in HHV-7 OBP-II).

Both of the putative OBP binding sites within HHV-7 *ori*Lyt map to a local (~100-bp) region of DNA instability (Fig. 2B), which supports the contention that DNA replication initiates at this site. In addition, a potential binding site for the cellular transcription factor Oct-1 was also mapped to this region of HHV-7 *ori*Lyt. This may have implications for HHV-7 DNA replication, since Oct-1 and/or other cellular transcription factors are known to interact with simian virus 40 and herpes simplex virus type 1 replication origins (7, 22).

In light of the sequence similarity between the putative HHV-7 *ori*Lyt region and its counterpart in HHV-6, we set out to determine whether this HHV-7 DNA element has replicator function, as assessed by transient replication complementation experiments, in HHV-7-infected cells. For these experiments, we elected to use primary cord blood mononuclear cells (CBMC), since HHV-7 has (to date) been shown to replicate only in CBMC or in the $CD4^+$ T-cell line SupT1. Of these cells, we obtained the best efficiency of DNA transfer (<1%) with CBMC, using electroporation conditions previously optimized for J-Jahn cells (1 pmol of DNA per 5×10^6 cells, with electroporation at 300 V and 960 μF in RPMI 1640 medium with 10 mM dextrose–0.1 mM dithiothreitol) (9). Results of one representative experiment using plasmid pH7O3 are shown in Fig. 3A and demonstrate that this plasmid was efficiently replicated in HHV-7-infected CBMC but not in uninfected control cells. Furthermore, only a single DNA fragment of the expected size was generated upon linearization of replicated (*Dpn*I-resistant) DNA with *Not*I, indicating that the plasmid had replicated autonomously and not by integration into the viral genome (integration was expected to generate a distinct pattern of restriction fragments).

Functional analysis of a number of deletion constructs allowed us to map the minimal essential domain for replicator activity to a region of HHV-7 DNA that was approximately 600 bp in size (Fig. 3B and C). This DNA element contains all of the putative OBP binding sites previously identified in our sequence analysis (Fig. 2B). Flanking DNA elements almost certainly enhance replication from this minimal domain, as they do in HHV-6, since smaller plasmid clones replicated with noticeably lower efficiency than larger clones (data not shown). However, the limited availability of CBMC precluded our being able to perform simultaneous analyses of multiple plasmids in a single experiment (5×10^6 cells are needed per plasmid transfection, and only about 20×10^6 virus-infected cells were available at any one time). Thus, we did not attempt a quantitative analysis of the replication experiments due to the difficulty of adequately controlling for interexperimental variation in viral infection parameters and replication efficiencies.

The presence of a sequence motif within HHV-7 *ori*Lyt (OBP-II) with almost complete identity to the OBP-2 motif in HHV-6 (18) prompted us to test whether this element, like its counterpart in HHV-6 *ori*Lyt (9), is required for origin function. To answer this question, a mutated derivative of plasmid pH708Δ11 was prepared (this plasmid contains the minimal essential HHV-7 *ori*Lyt element of approximately 600 bp). Briefly, pH708Δ11 was subjected to site-directed mutagenesis with the QuikChange system (Stratagene) with oligonucleotides directed against OBP-II (M702NC, 5′-GTTTATGCAA ATTAGCATC**GGG**CTCACTCGTAATAGTATTTATTT TTC, and M7O2C, 5′-GAG**CCC**GATGCTAATTTGCATAA ACTAT [opposite strand]; the OBP site is underlined and mutated bases are in boldface type [the wild-type OBP-II se-

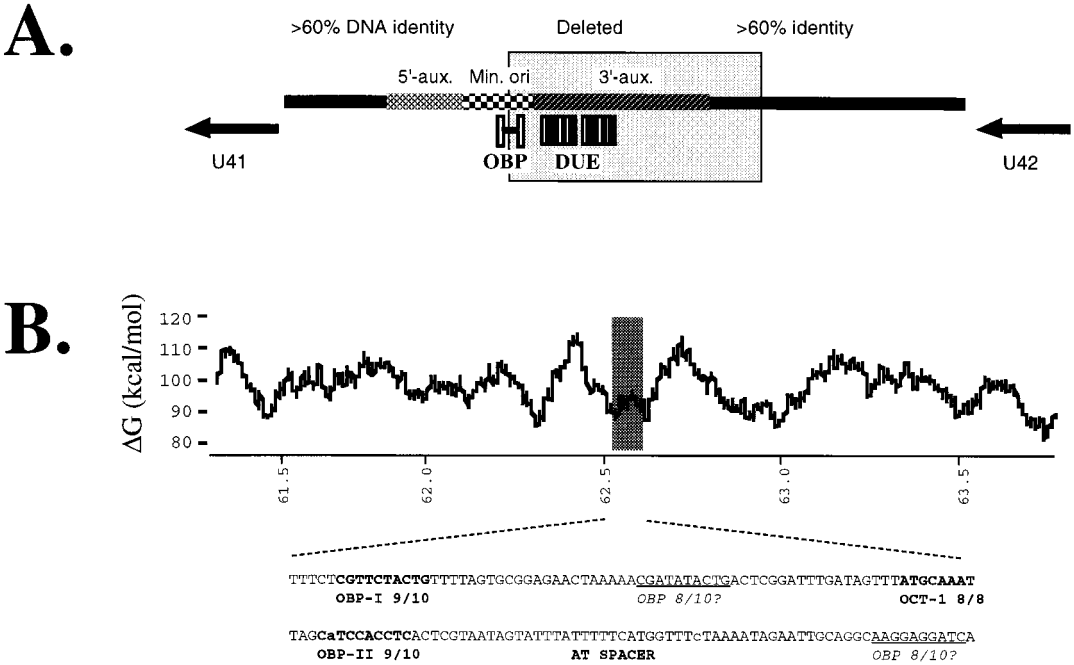

FIG. 2. Sequence analysis of the HHV-7 *ori*Lyt region. (A) A map of the HHV-6 *ori*Lyt region is presented. Sequence blocks denoted as 5′-aux. (5′ auxiliary), Min. ori (minimal essential origin), and 3′-aux. (3′ auxiliary) refer to functionally identified domains of the HHV-6 *ori*Lyt (8), while the motifs DUE and OBP (OBP recognition sequence) refer to specific sequence elements (8, 18, 19). The relative conservation of these structures within the HHV-7 *ori*Lyt region is shown along the top line and by the large shaded box (which shows the region in HHV-6 that is missing in HHV-7). (B) Predicted helical stability of the HHV-7 *ori*Lyt region. DNA helical stability (ΔG) was calculated with the Thermodyn algorithm (30) and plotted by nucleotide position. Numbers correspond to nucleotide positions in the intact HHV-7 genome (32). The shaded box denotes a region of HHV-7 DNA which contains potential OBP_{H7} binding sites, as judged by their homology to a previously defined consensus OBP_{H6} binding site (19). The sequence content of this region is presented at the bottom of the panel (the sequence shown corresponds to that of plasmid pH7O8). Two regions with high homology (9 of 10 bases match) to the consensus OBP_{H6} binding site, YGWYCWCCYN (Y is T or C and W is T or A), are in boldface type (OBP-I and OBP-II, respectively), and two sites with weaker homology (8 of 10 bases match) to this consensus are underlined. In addition, a potential AT-rich spacer element is indicated, as is a consensus binding site for the cellular transcription factor Oct-1. Finally, lowercase lettering denotes nucleotide differences between pH7O8 and plasmid pH7O3. It should be noted that the nucleotide change within OBP-II (pH7O8 has A and pH7O3 has G) does not appear to interfere with replicator activity in HHV-7-infected cells, since pH7O3 replicates efficiently (Fig. 3A), as do derivatives of pH7O8 (Fig. 3C).

quence is CATC**CA**CCTC]). After the resulting plasmid (pH708Δ11-M2) was sequenced to verify that the desired mutation had been introduced, its replicator function was evaluated. The results are presented in Fig. 3B and show that mutation of OBP-II completely abrogates the replicator function of pH708Δ11. This finding (i) indicates that replication of pH708Δ11 is specific and not recombination dependent, since an otherwise identical plasmid with a 3-nucleotide substitution fails to replicate, and (ii) shows that the OBP-II site in HHV-7 *ori*Lyt is both positionally and functionally equivalent to its counterpart in HHV-6 *ori*Lyt, since it is essential for replicator function (9).

The presence of putative OBP binding motifs within the minimal essential replicator element from HHV-7 also prompted us to test (i) whether HHV-6 *ori*Lyt might possess replicator function in HHV-7-infected cells and (ii) whether the HHV-7 *ori*Lyt element might have replicator activity in HHV-6-infected cells. These experiments revealed that an HHV-6 *ori*Lyt-bearing plasmid (clone pH6Δ7) was replicated in HHV-7-infected cells (Fig. 4A). This observation is consistent with the interpretation that a fully functional complex of HHV-7 replication proteins can assemble at the HHV-6 *ori*Lyt element and can initiate DNA synthesis. In contrast, plasmids containing HHV-7 *ori*Lyt were not replicated in HHV-6-infected cells (Fig. 4B, upper gels), although they were able to interfere with the replication of plasmids bearing an HHV-6 *ori*Lyt element (Fig. 4B, lower gels). The simplest explanation for these findings is that HHV-6 OBP can bind to at least one of the consensus OBP binding sites in HHV-7 *ori*Lyt but that a fully functional complex of HHV-6 replication proteins does not assemble at this site. This could be the result of subtle differences in the recognition sequences of OBP_{H6} and OBP_{H7}, i.e., differences in their structural properties or differences in the abilities of these proteins to interact with cellular factors that may be required for viral DNA replication (e.g., Oct-1). Studies are under way to delineate the DNA sequence motif that is recognized by OBP_{H7} and to compare this with the element recognized by OBP_{H6}. Additional experiments are also ongoing to test whether OBP_{H6} does indeed bind to the HHV-7 *ori*Lyt, as suggested by the competition experiment shown in the lower gels of Fig. 4B.

In summary, our data show that the region of the HHV-7 genome which contains the viral *ori*Lyt is closely related to, but considerably shorter than, its counterpart in HHV-6. This compaction of the HHV-7 replication origin can be traced in large measure to the absence of a DUE that is found in HHV-6 *ori*Lyt. The functional significance of this remains uncertain. Finally, the observation that HHV-7-encoded proteins can stimulate DNA replication from a plasmid bearing an HHV-6 *ori*Lyt element may offer at least a partial explanation as to how HHV-7 triggers the hypothesized reactivation of HHV-6 infection (2, 13). This may have important implications with respect to understanding the pathogenesis of HHV-7 infection. In addition, the functional mapping of a lytic-phase origin in HHV-7 makes possible a number of future studies, including

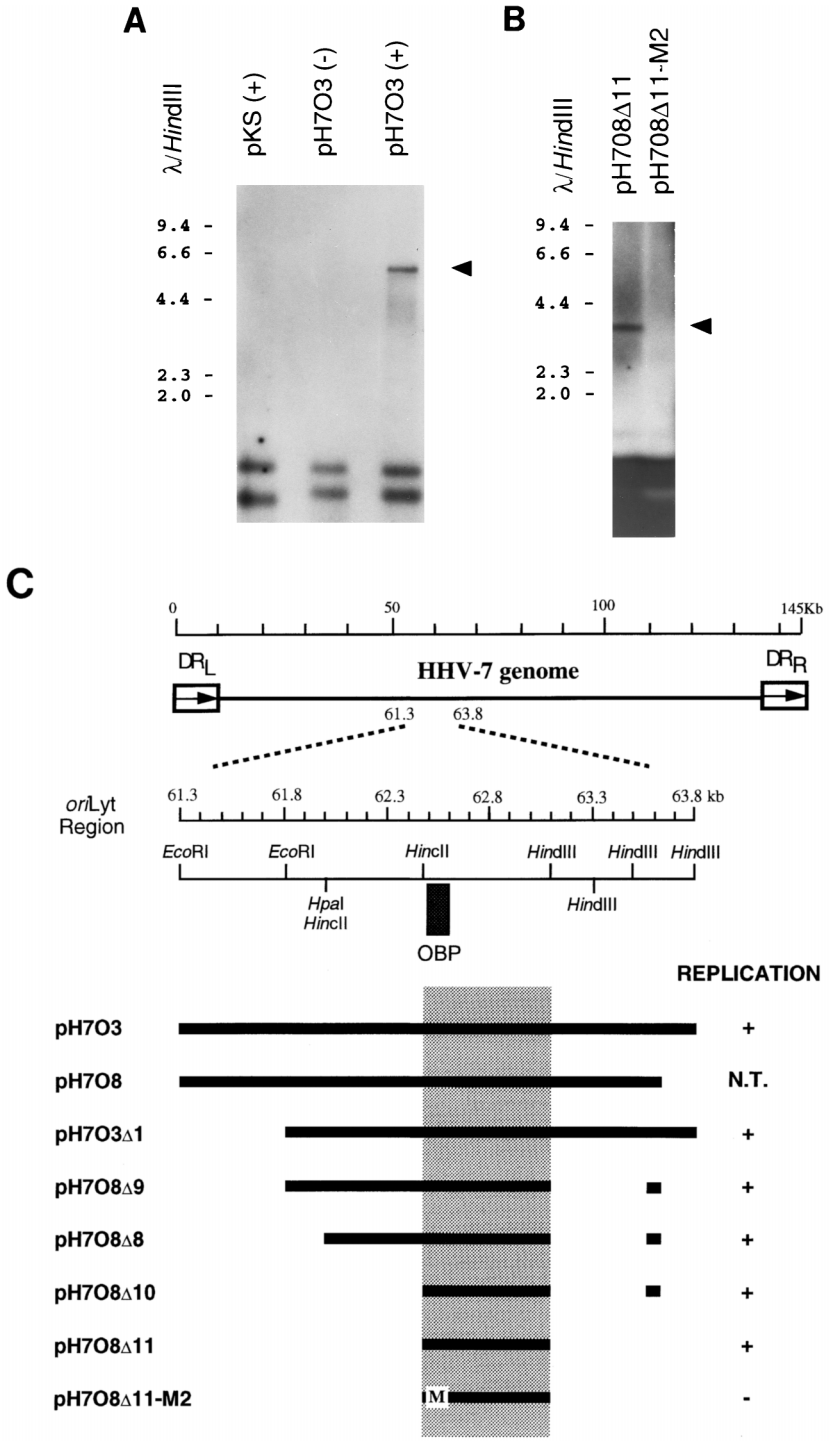

FIG. 3. Mapping of the HHV-7 *ori*Lyt. (A) Transient replication assay of pH7O3 in CBMC infected with HHV-7 (R-2). At least 10% of cells in these cultures stained positive for HHV-7 antigen, as detected by immunofluorescence assay, at the time at which replication assays were performed. CBMC (5×10^6) were transfected with 1 pmol of CsCl-gradient purified plasmid DNA by electroporation, and replication assays were performed as described previously (8). Hirt-extracted DNAs were digested with *Dpn*I and *Not*I, and DNA fragments were resolved on an agarose gel. Shown is a Southern blot of this DNA with a radiolabeled pKS probe. (+) denotes DNA harvested from cells infected with HHV-7, while (−) denotes DNA from virus-negative cells. pKS is a negative control plasmid which lacks HHV-7 DNA. The arrowhead denotes the position of replicated, linearized pH7O3 (5.5 kb), while the numbers on the left correspond to molecular weight markers (sizes in kilobases; *Hin*dIII digest of λ DNA [λ/*Hin*dIII]). (B) OBP-II is essential for replication from the HHV-7 *ori*Lyt. The result of a transient replication assay of pH7O8Δ11 (minimal essential origin construct) and its mutated derivative pH708Δ11-M2 is shown (performed with CBMC infected with HHV-7). The arrowhead denotes the position of replicated, linearized pH7O8Δ11 (3.6 kb), while numbers correspond to molecular weight markers (note that the autoradiogram shown in this panel is overexposed, relative to that shown in panel A). (C) Summary of the results of transient replication assays with derivatives of the parental plasmids pH7O3 and pH7O8. Map units are relative to those of the intact HHV-7 genome (31). DR_L, left direct repeat; DR_R, right direct repeat; N.T., not tested; M, mutated.

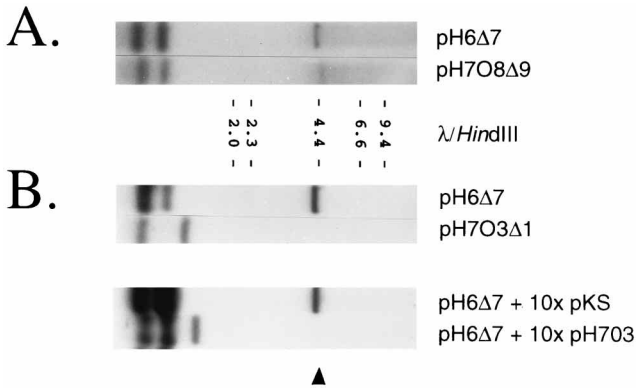

FIG. 4. Functional cross-complementation of HHV-6 and HHV-7 replication machinery. (A) Transient replication assays in HHV-7-infected cells. Replication of plasmids containing the HHV-6 *ori*Lyt (pH6Δ7) (8) or the HHV-7 *ori*Lyt (pH7O3Δ9) was analyzed in CBMC infected with HHV-7 (R-2). Shown is a Southern blot of replicated (*Dpn*I-resistant) and linearized plasmid DNA, after hybridization to a radiolabeled pKS probe. (B) Transient replication assays in HHV-6-infected cells. (Upper gels) Replication of plasmids containing the HHV-6 *ori*Lyt (pH6Δ7) or the HHV-7 *ori*Lyt (pH7O3Δ1) was analyzed in J-Jahn cells infected with HHV-6 (R-1). (Lower gels) Replication of pH6Δ7 was analyzed in HHV-6-infected cells, in the presence of a 10-fold molar excess of either a control plasmid (pKS) or a plasmid bearing the intact HHV-7 *ori*Lyt element (pH7O3). In both gels, a Southern blot of replicated (*Dpn*I-resistant) plasmid DNA is shown, after linearization and hybridization to a radiolabeled pKS probe. The arrowhead denotes the approximate positions of linearized, replicated, plasmid DNAs (in pH6Δ7, 4.3 kb; in pH7O8Δ9, 4.4 kb; and in pH7O3Δ1, 5.0 kb); pH6Δ7 was linearized with *Xho*I, while all other plasmids were linearized with *Not*I. Numbers correspond to molecular weight markers (sizes are in kilobases; *Hin*dIII digest of λ DNA [λ/*Hin*dIII]).

the construction of gene transfer vectors potentially capable of targeting $CD4^+$ cells (14, 36, 37).

Nucleotide sequence accession number. Sequences described in this paper have been submitted to GenBank under the accession number L40417.

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