The terminal repeats and latency-associated nuclear antigen of herpesvirus saimiri are essential for episomal persistence of the viral genome

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The simian herpesvirus saimiri (HVS) induces malignant T cell lymphomas and is closely related to Kaposi’s sarcoma-associated herpesvirus (KSHV or HHV-8). Both belong to the gamma-2 herpesvirus subgroup. The viral genome of HVS consists of a unique region (L-DNA) that contains all of the viral genes flanked by non-coding terminal repeats (H-DNA). Here we describe the cloning of a 113 kb restriction fragment containing the L-DNA of an oncogenic HVS strain in an F́ replicon-based E. coli vector. Cloned DNA was infectious and the ends of the progeny viral genome consisted of amplified tandem alternating repeats of vector and a single H-DNA unit. T cells infected with these viruses contained the linear DNA typically found a few weeks after infection, but were unable to form episomal circular viral DNA, which is the latent form of the viral genome. Recombinant viruses with reconstructed H-DNA were generated and T cells infected with these rescued viruses contained high copy numbers of episomal DNA. Plasmids expressing the latency-associated nuclear antigen (LANA) and containing various numbers of H-DNA repeats stably replicated as episomes, but constructs containing three repeat units produced the highest copy numbers. These data show that intact and multiple terminal repeats are essential components for episomal replication in latently infected T cells. Moreover, LANA and terminal repeats are sufficient for stable plasmid persistence. Cloned HVS can also be utilized for mutagenesis of HVS and for the expression of foreign genes through efficient manipulation of plasmids in E. coli.

Introduction

Lymphotropic or gamma-herpesviruses are implicated as causative agents of malignant diseases. They have been further classified into gamma-1 and gamma-2 subgroups. Epstein–Barr virus (EBV) represents the gamma-1 subfamily. The recently discovered Kaposi’s sarcoma-associated herpesvirus (KSHV), also referred to as human herpesvirus-8 (HHV-8), and the simian herpesvirus saimiri (HVS), as well as other herpesviruses of numerous other species, belong to the gamma-2 group.

KSHV/HHV-8 is closely related to HVS because of a high degree of homology of open reading frame sequences and the architecture of the viral genomes (Albrecht et al., 1992; Russo et al., 1996).

HVS can be isolated from healthy squirrel monkeys (Melendez et al., 1968). In contrast, HVS induces CD8 lymphomas in several other species of New World monkeys and rabbits (Fleckenstein, 1980; Medveczky et al., 1989; Medveczky, 1995; Melendez et al., 1968). HVS also efficiently immortalizes New World monkey and human T cells (Biesinger et al., 1992; Desrosiers et al., 1986; Medveczky et al., 1993; Szomolanyi et al., 1987). The genome of HVS consists of 113 kb of unique sequences designated as L-DNA (light or low G+C), which contains at least 75 open reading frames (Albrecht et al., 1992; Fleckenstein, 1980). These L-DNA sequences are flanked by tandem repeats of non-coding H-DNA (heavy or high G+C) (Fleckenstein, 1980).
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Using the lack of sequence homology in the 2 kb at the left end of the genome as a criterion, the various isolates have been classified into three subgroups: A, B and C (Medveczky et al., 1984). Group C strains are the most potent in both immortalization and oncogenesis assays, and are the only strains able to immortalize human T cells (Biesinger et al., 1992; Medveczky et al., 1993). HVS group C strain 484-77 is also highly oncogenic in New Zealand White rabbits (Medveczky et al., 1989).

During latent/persistent infection, the viral genome of herpesviruses exists as a circular episome, and HVS is no exception. Tumour tissues and cell lines established from tumours or by in vitro immortalization carry multiple copies of the viral DNA in covalently closed circular form, and circularization occurs by joining the ends of the viral genome (Fleckenstein, 1980; Gardella et al., 1984; Medveczky, 1995; Schirm et al., 1984). No evidence has been published indicating that HVS integrates into the host genome.

Most of the viral genes encoded by HVS are inactive in immortalized T cells, and only a limited number of gene products required for oncogenesis, such as the STP and TIP proteins, and small RNAs encoded by the left end of the L-DNA have been described (for reviews, see Fleckenstein, 1995; Schirm et al., 1984; Medveczky et al., 1993). Extensive methylation of the episomal HVS DNA at CG residues in immortalized T cells has been described and is thought to correlate with the lack of gene expression in mammalian cells (Desrosiers et al., 1982; Desrosiers et al., 1979; Youssoufian & Mulder, 1981). Consistent with this concept, the left end of the L-DNA is hypomethylated. The high G + C H-DNA sequences are also heavily methylated, and all attempts to find transcription in the repeats have failed (Desrosiers, 1982; Desrosiers et al., 1979; Youssoufian & Mulder, 1981). No known functions of H-DNA have so far been described.

Most KSHV genes are also inactive in immortalized cells. One of the proteins that is expressed in cells latently infected with KSHV is the latency-associated nuclear antigen (LANA), which is encoded by orf 73. LANA and epimorphs containing H-DNA have been shown to co-localize with mitotic chromosomes (Ballestas et al., 1999), and persistence of these epimorphs in cell culture is mediated by LANA binding to a specific sequence within the H-DNA (Ballestas & Kaye, 2001). LANA has also been shown to bind to histone H1 (Cotter & Robertson, 1999). These data suggest a mechanism by which LANA tethers viral DNA to mitotic chromosomes through its interaction with H-DNA, allowing efficient segregation of viral episomes to progeny cells.

HVS encodes a homologue of KSHV LANA, and although there is limited similarity between KSHV and HVS LANA, both proteins contain a central glutamate-rich domain as well as several potential phosphorylation sites (Albrecht et al., 1992; Russo et al., 1996). HVS LANA also localizes in the nucleus in a distinctive speckled distribution, similar to that of KSHV LANA (Hall et al., 2000). These findings suggest that HVS LANA may play a role in episomal maintenance similar to that of KSHV LANA.

Here we describe the cloning of an infectious L-DNA restriction fragment of the highly oncogenic HVS strain C484-77 in E. coli. Analysis of T cells infected with progeny viruses recovered from cloned DNA showed a complete absence of episomal viral genomes while recombinants with restored terminal repetitive H-DNA were able to replicate as epimorphs, indicating that intact H-DNA is essential for latent episomal replication. Moreover, LANA expression vectors containing terminal repeats stably replicated in 293 cells as epimorphs, indicating that LANA is essential and sufficient for episomal replication.

Methods

Cloning of L-DNA and remnants of H-DNA in E. coli. The main steps used to clone HVS L-DNA are depicted in Fig. 1. To isolate full-length L-DNA, owl monkey kidney (OMK) cells (10⁷ cells/ml) infected with HVS strain C484-77 for 3 days at an m.o.i. of 2 were embedded in 0.7% low melting point agarose blocks (NuSieve GTG; FMC Bioproducts). DNA was digested by dialysis of agarose blocks in a 10-fold volume of buffer containing 100 μg/ml proteinase K, 1% SDS, 10 mM Tris–HCl, pH 8.0, 100 mM NaCl and 10 mM EDTA for 24 h at 37 °C. Agarose blocks were extensively dialysed against NotI buffer (150 mM NaCl, 6 mM Tris–HCl, pH 7.9, 6 mM MgCl₂, 1 mM dithiothreitol) for 48 h at 4 °C. NotI (Promega; 500 U/ml) was added every 2 h and blocks were incubated with occasional gentle shaking at 37 °C for 8 h. L-DNA and H-DNA fragments were separated by pulse-field electrophoresis in low melting point agarose gels. Agarose blocks containing the approximately 110 kb L-DNA fragment (not shown) were digested with β-agarase (Sigma) followed by dialysis against 10 mM Tris–HCl, pH 8.0, and 0.1 mM EDTA.

The cloning vector pBeloBac was selected to clone the L-DNA. This vector contains two NotI sites in its multiple cloning site, encodes an F' replicon and is capable of accepting inserts of at least 200 kb (Shizuya et al., 1992). The vector was digested with NotI, dephosphorylated with alkaline phosphatase and ligated to the NotI L-DNA fragment. E. coli DH10B was transfected by electroporation and HVS-containing recombinant clones were selected by colony hybridization with purified viral DNA labelled by nick translation. Several dozen clones hybridized with radiolabelled viral DNA (not shown) and plasmid DNA from 40 clones were prepared using an alkaline lysis mini-prep kit (5 Prime → 3 Prime, Inc.).

Infection and immortalization of T cells. Human peripheral blood mononuclear cells were purified by ficoll gradients and infected as described earlier (Schirm et al., 1984). Briefly, OMK cells were infected with approximately 10⁶ p.f.u. of virus. Two to three days after infection, when early signs of cytopathic effects of the virus were observed, the medium was removed and 2 × 10⁵ mononuclear cells in 10 ml AIM V medium (Gibco BRL) containing 10% fetal calf serum were added to the cultures. Infected lymphocytes were cultured in the same medium. Control uninfected cells were grown in the same medium, or, in some experiments, supplemented with 100 U/ml human recombinant IL-2.

Transfection of DNA and generation of rescued virus. The main steps for generating virus and recombinants from pBeloBac-HVS are depicted in Fig. 1. Owl monkey kidney cells were transfected with pBeloBac-HVS clones or co-transfected with pBeloBac-HVS and pl-H, a construct containing 5 kb of L-DNA and attached H-DNA units, by the
These viruses formed episomes in T cells. Repeats and the L-DNA junction generated progeny with normal H-DNA. Clones co-transfected with the pL-H plasmid containing several H-DNA fragments of L-DNA was cloned in pBeloBac. Transfection of Bac clones into OMK cells yielded infectious progeny virus with rearranged H-DNA. From this cell suspension was gently liberated by an agarose gel method of Gardella et al. (1984). Viral DNA was visualized by restriction enzymes, which cut in both H-DNA and L-DNA (Albrecht et al., 1992; Bankier et al., 1985), and compared to viral DNA isolated from virions. The results indicated that five clones contained full-length 484-77 HVS L-DNA, since all unique enzymes, which cut in both H-DNA and L-DNA (Albrecht et al., 1992; Bankier et al., 1985), and compared to viral DNA isolated from virions. The results indicated that five clones contained full-length 484-77 HVS L-DNA, since all unique bands co-migrated with virion DNA. However, no supermolar DNA that resulted from cloning the viral DNA in E. coli DNA was visualized by Southern hybridization using random primer-labelled pBCKMV as a probe. To determine the copy number of episomes per cell, the hybridized blots were analysed with a PhosphorImager (Molecular Dynamics) using the ImageQuant program of the manufacturer.

Results

Analysis of L-DNA clones

To clone the L-DNA of HVS strain C484-77, viral DNA was digested with several restriction endonuclease sequences and were predicted to cut in H-DNA but not in L-DNA. NolI and SmalI were found to cleave the repetitive H-DNA but failed to cut the L-DNA (data not shown). The 113 kb NolI fragment was cloned in pBeloBac, as described in Methods.

Clones were digested with PstI and ScaI restriction enzymes, which cut in both H-DNA and L-DNA (Albrecht et al., 1992; Bankier et al., 1985), and compared to viral DNA isolated from virions. The results indicated that five clones contained full-length 484-77 HVS L-DNA, since all unique bands co-migrated with virion DNA. However, no supermolar H-DNA bands were observed. Restriction fragment patterns of a representative clone #3 are shown in Fig. 2(A). Supermolar H-DNA bands were absent in the clone, and extra 3 and 3-9 kb vector bands appeared in the PstI digest of the clone while a single extra 8 kb band appeared in the ScaI digest. An identical banding pattern was obtained with clone #17 (not shown). These data show that clones #3 and #17 contain the entire L-DNA region and remnants of H-DNA adjacent to L-DNA that were not cleaved off by NolI. The additional bands present in the digests of cloned viral DNA are contaminating E. coli DNA that resulted from cloning the viral DNA in E. coli. Viral DNA does not contain these bands because this DNA was isolated from purified virus.

HVS DNA cloned in E. coli is infectious

DNA of the five clones containing full-length L-DNA was transfected into OMK cells by the calcium co-precipitation method and three clones, #3, #17 and #29, were found to be infectious. Viral DNA was isolated from clone-derived progeny viruses #3, #17 and #29, cut with restriction enzymes, which cut in both H-DNA and L-DNA (Albrecht et al., 1992; Bankier et al., 1985), and compared to viral DNA isolated from virions. The results indicated that five clones contained full-length 484-77 HVS L-DNA, since all unique bands co-migrated with virion DNA. However, no supermolar H-DNA bands were observed. Restriction fragment patterns of a representative clone #3 are shown in Fig. 2(A). Supermolar H-DNA bands were absent in the clone, and extra 3 and 3-9 kb vector bands appeared in the PstI digest of the clone while a single extra 8 kb band appeared in the ScaI digest. An identical banding pattern was obtained with clone #17 (not shown). These data show that clones #3 and #17 contain the entire L-DNA region and remnants of H-DNA adjacent to L-DNA that were not cleaved off by NolI. The additional bands present in the digests of cloned viral DNA are contaminating E. coli DNA that resulted from cloning the viral DNA in E. coli. Viral DNA does not contain these bands because this DNA was isolated from purified virus.

■ Episomal replication assay. Plasmids were transfected into 293 cells using GenePORTER transfection reagent (Gene Therapy Systems) according to the manufacturer's protocol. G418 was added 72 h post-transfection to a final concentration of 850 µg/ml. Cells were trypsinized when confluency was reached, and 20% were passed to new plates with fresh media and G418. Low molecular mass DNA was isolated from the remaining cells by the method of Hirt (1967). To distinguish replicative DNA from DNA used for transfection, isolated DNA was digested with the restriction enzymes DpnI and MboI. Plasmid DNA amplified in dam methylase-positive E. coli is efficiently digested by DpnI while DNA methylated by mammalian cells is resistant to DpnI digestion. Conversely, the DpnI isoschizomer MboI cleaves DNA that is replicated and methylated in mammalian cells but does not efficiently digest DNA produced by dam methylase-positive E. coli. After restriction digestion, DNA was electrophoresed on a 0.8% agarose gel and the DNA was transferred to nitrocellulose. Plasmid DNA was detected by Southern hybridization using random primer-labelled pBCKMV as a probe. To determine the copy number of episomes per cell, the hybridized blots were analysed with a PhosphorImager (Molecular Dynamics) using the ImageQuant program of the manufacturer.

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Fig. 2. Restriction patterns and Southern blotting of virion, cloned HVS and HVS rescued from cloned DNA. (A) DNA samples were purified from virions and clone g3, digested with PstI and SacI, and separated by agarose gel electrophoresis. DNA bands were visualized by SybrGreen staining and scanning with a Molecular Dynamics scanner and ImageQuant program for blue fluorescent gels. (B) Image of DNA fragments from virions, clones g3 and g17 and virion DNA recovered after transfection of clone g3, digested with SacI. (C) Southern blot of HindIII-digested DNA from virion, cloned HVS and HVS rescued from cloned DNA, showing amplification of vector DNA in rescued virus. Marker, lambda DNA cleaved with HindIII. H, H-DNA; S, supermolar band containing vector and H-DNA sequences; V, vector band; L, left junction of L-DNA and H-DNA; wt, wild-type.

enzymes, and compared with wild-type virus and cloned HVS plasmids.

Fig. 2(B) shows that the progeny viral genome of clone g3 contained an 8 kb supermolar band when cleaved with SacI, suggesting amplification of the vector DNA. Identical banding patterns of virion DNA recovered from clones g17 and g29 were obtained (not shown).

Fig. 2(C) shows viral DNA that consists of the H-DNA at the left end of the genome and the L-DNA up to the first HindIII site. Multiple bands were present due to varying numbers of terminal repeats among the individual viruses. However, when viral DNA cloned in E. coli and DNA from clone-derived progeny was digested with HindIII, which also cleaves once at the right end of the cloning vector, only two bands were present. The smaller band is the fragment between the HindIII restriction site at the left end of the L-DNA and the HindIII site at the right end of the vector. The large band is the vector, which consists of the fragment between the HindIII site in the vector and the HindIII site at the right end of the L-DNA. In the clone-derived progeny, the vector band was present at a much higher level of intensity, indicating that it had been amplified (Fig. 2C).

The E. coli plasmids of clones g3, g17 and the virion progeny DNA cleaved with NolI showed the expected 7-3 kb vector band (Fig. 3A). No H-DNA repeats were detected in the E. coli clones. Surprisingly, the clone-derived viral genome contained a supermolar band that hybridized with repetitive H-DNA (Fig. 3B).

Analysis of H-DNA in viral DNA cloned in E. coli and progeny virus

Because digestion of the viral genome with NolI does not remove all of the H-DNA from the ends of the L-DNA, the remnants were cloned and sequenced. The viral clone was digested with HindIII, which cuts frequently in the L-DNA but has no recognition sites within the H-DNA, and NolI. The HindIII–NolI fragment from each end was isolated and cloned. For comparison, the 1-4 kb NolI fragment of HVS strain C484-77, which consists of one terminal repeat, was also cloned and sequenced (GenBank accession no. AF276733). Sequence analysis revealed that the H-DNA consisted of 1443 nucleotides and shared 94% identity with H-DNA from HVS strain A11. Moreover, the packaging cleavage site was conserved between the two strains (Bankier et al., 1985).

Sequence analysis the HVS DNA cloned in E. coli revealed that the remnant at the left end consisted of the sequence between the NolI site and the cleavage site, whereas the
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**Fig. 3.** (A) DNA samples purified from virions, clones and progeny of clone #3 were digested with NotI and separated by agarose gel electrophoresis. Bands were visualized as described for Fig. 2(A). (B) DNA was transferred to nitrocellulose and hybridized with repetitive H-DNA. (C) DNA was transferred to nitrocellulose and hybridized with vector DNA. Marker, lambda DNA cleaved with HindIII. V, vector band; H, H-DNA.

**Fig. 4.** Schematic illustration of sequencing results of the ends of the 113 kb NotI fragment and the H-DNA fragment of the progeny viral genome obtained by transfection. (A) Left and right ends of HVS pBeloBac L-DNA clone #3 were subcloned into pSK Bluescript as NotI–HindIII fragments (HindIII cuts near the terminal repeats). Several additional subclones were generated and the inserts of both clones were sequenced. (B) The 144 bp NotI H-DNA fragment of progeny viral DNA from clone #3 was subcloned into pSK Bluescript as NotI–HindIII fragments. Several additional subclones were generated and the entire insert was sequenced.

remnant at the right end consisted of the sequence between the cleavage site and the NotI site (Fig. 4A). In progeny virus produced from this cloned DNA, the sequence flanking the L-DNA consisted of alternating repeats of H-DNA and vector DNA (Fig. 4B).

**Viruses recovered from cloned DNA are unable to undergo episomal replication, but replication can be rescued by restoration of terminal repeats**

Human T cells infected with strain C484-77 maintain the viral genome as multiple copies of episomes (Medveczky et al., 1989, 1993). Cultures infected with #3, #17 and #29 viruses were grown for weeks to several months and were analysed for episomes in several independent experiments by the method of Gardella et al. (1984). A representative experiment, shown in Fig. 5, indicates the lack of viral episomes but the presence of some linear DNA in T cells infected with viruses lacking intact terminal repeats. This linear DNA is typically found in cells latently infected with HVS (Medveczky et al., 1993; Szomolanyi et al., 1987). No episomes were detected in nine other experiments (not shown).

Since the loss of episomal replication could be due to a mutation in a region located within the unique L-DNA, H-DNA was restored by marker rescue. Cloned DNA #29 and a plasmid containing 6 kb of left-end unique sequences and four repeat units were co-transfected in OMK cells. Several virus clones were analysed by restriction fragment analysis to determine whether any of these viruses contained restored terminal repeats. Four clones were found to contain contiguous
terminal repeats, as indicated by the reappearance of a SacI fragment (which cleaves once in H-DNA) that was absent in the cloned constructs (not shown).

T cells were infected with these rescued viruses and analysed for viral episomes. The amount of episomal DNA that was detected in all four cell cultures was similar to that found in the established HVS-immortalized cell line 484Th (Fig. 5). The second, faster-migrating band in the control cell line 484Th is a result of development of a deletion in a subpopulation of cells often seen in cell lines serially passaged over time. Viral DNA was also detected in the linear range of the gel. This linear species is always present in cultures infected with HVS for up to 3 months after infection.

HVS LANA is sufficient for replication and maintenance of plasmids containing H-DNA

Because KSHV LANA is involved in the maintenance of KSHV episomes through its interaction with the H-DNA (Ballestas et al. 1999; Ballestas & Kaye, 2001), the ability of HVS LANA to support maintenance of plasmids containing HVS H-DNA was tested. A LANA expression vector was constructed by inserting the LANA open reading frame into pBKCMV, designated as pLANA. One, two and three repeats of H-DNA were inserted into pLANA, creating pLANAH1, pLANAH2 and pLANAH3, respectively. KSHV LANA and four units of H-DNA, which were previously shown to be sufficient for replication (M. M. Medveczky, G. Fejer, E. Horvath, B. Lane, Y. Chang, P. S. Moore, B. Chandran & P. G. Medveczky, unpublished results), were used as a positive control. These plasmids contain a G418 resistance marker, and will confer long-term G418 resistance to cells harbouring them if they are maintained in transfected cells. 293 cells were transfected with these constructs as well as pLANA, which expresses LANA but does not contain H-DNA, and pBKH3, which contains three repeats of H-DNA but does not express LANA. Transfected cells were passaged under selection of G418. Low molecular mass DNA was isolated from G418-resistant cells by Hirt extraction (Hirt, 1967), digested with DpnI and MboI, and the DNA separated by gel electrophoresis.

Fig. 6(A) shows two representative ethidium bromide-stained gels of Hirt-extracted DNA after eight (left blot) and nine (right blot) passages from two independent experiments. Each lane represents DNA extracted from 3·75 × 10⁶ cells, and equal loading of DNA from each culture was determined by comparing the amount of mitochondrial DNA present. Mitochondrial DNA is replicated by the mammalian replication machinery and is therefore resistant to digestion by DpnI but sensitive to digestion by MboI. The gel indicated no significant difference in the amount of loaded DNA quantities among lanes and showed that MboI completely digested the mitochondrial DNA.

The gels were blotted on to nitrocellulose and Southern hybridization with radiolabelled vector DNA was performed. Only plasmids that expressed LANA were replicated, as DpnI-resistant and MboI-sensitive bands were detected by autoradiography. Replication of these plasmids was not stringently dependent on the presence of H-DNA. However, plasmids that contained H-DNA were detected at a much higher level. To determine the copy number of episomes per cell after nine passages, the amount of episomal DNA present was compared to 0·1 ng of vector DNA (equivalent to approximately 53 copies per cell). Constructs that expressed LANA but contained no H-DNA were detected at less than one copy per cell. Constructs that expressed LANA and contained one or two repeats were present at a copy number of at least 1·6 and 1·3 copies per cell, respectively. Constructs that expressed LANA and contained three repeats were present at a copy number of at least 4·9 copies per cell. These calculations are based on the assumption that all episomal DNA was completely extracted by the Hirt method. Extraction of 100% of the episomal DNA is unlikely; therefore, these figures are probably an underestimate of the actual copy number.

Plasmids that expressed LANA and contained three H-DNA units were maintained in cell culture for much longer. pLANA was undetectable after 10 passages. Plasmids con-
Fig. 6. HVS LANA and H-DNA are sufficient for replication. (A) LANA expression vectors containing 0, 1, 2 or 3 repeats (pLANA, pLANAH1, pLANAH2 and pLANAH3, respectively) or vector containing H-DNA alone (pBK3) were transfected into 293 cells. A vector expressing KSHV LANA and containing four KSHV terminal repeats was used as a positive control. Episomal DNA was extracted by the method of Hirt (1967) from cell cultures grown under G418 selection after eight (right) and nine (left) passages from two independent experiments. DNA was digested with DpnI or MboI and electrophoresed on a 0.8% agarose gel. (B) Gels were blotted on to nitrocellulose membranes and DNA was detected by Southern hybridization using radiolabelled pBKCMV as a probe. M, closed circular mitochondrial DNA.

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taining one or two repeats could still be detected after 16 passages, whereas plasmids containing three repeats could still be detected after 25 passages. It should be noted that, although the HVS LANA expression vectors were different sizes due to varying amounts of H-DNA, all replicated plasmid DNA migrated the same distance during electrophoresis. This would
seem to indicate that rearrangement or amplification of episomal DNA had occurred, although we cannot say for certain what happened. Attempts to clone this replicated DNA in *E. coli* for further analysis were unsuccessful, possibly due to its large size.

**Discussion**

Cloning of several other herpesvirus genomes has recently been described. Messerle *et al.* (1997) cloned the entire mouse cytomegalovirus (MCMV) genome by insertion of an *E. coli* vector into a non-essential region of the viral DNA. This was accomplished through recombination of the targeting vector via homologous flanking sequences into the viral genome followed by isolation of circular replication intermediates containing the vector and by transformation of *E. coli* with circular DNA. Delecluse *et al.* (1998) transfected EBV-transformed B cells with an *E. coli* targeting vector with flanking EBV sequences, and homologous circular EBV recombinants were cloned in *E. coli*. We elected a different approach to clone HVS, taking advantage of the unique and uneven distribution of restriction sites in the viral L-DNA and H-DNA. This method is simple and is probably applicable to most gammaherpesviruses with high G+C terminal repeats by using partial digestion of the genome with enzymes that recognize G+C nucleotides.

It is somewhat surprising that the HVS L-DNA fragment was found to be infectious. Purified HVS DNA digested with *SmaI* (which cleaves three times in H-DNA but not in L-DNA) was found to be non-infectious (G. Keil & B. Fleckenstein, personal communication). It is plausible that *SmaI* cleavage leads to loss of essential elements required for packaging and cleavage of replicative intermediates. *NotI*, which cuts only once in each repeat unit, presumably does not eliminate H-DNA sequences essential for lytic replication.

Another interesting question is the mechanism of amplification of the vector and generation of rearranged H-like DNA repeated between vector sequences during transfection of cloned DNA. Since the length of cloned HVS DNA is shorter than wild-type virion DNA by as much as 40 kb it is likely that amplification of the vector occurred to ‘fill’ the mature virion capsid with DNA of required length. Another issue is how the vector–H-DNA repeats were generated. One possible mechanism involves recombination between the H-like DNA in the *E. coli* clone, which would result in formation of a circular vector–H-DNA molecule. Amplification of this sequence could then occur by generation of concatamers by a rolling circle mechanism followed by joining of these concatamers and the termini of L-DNA.

To evaluate whether H-DNA is required for episomal replication, recombinant viruses with rescued H-DNA were constructed. T cells infected with rescued viruses contained high copy numbers of episomal DNA. This is the first report showing that intact terminal repeats of a herpesvirus are essential for the maintenance of episomes in latently infected cells.

To determine if LANA is involved in replication and maintenance of the HVS genome, LANA expression vectors were constructed. LANA was able to support maintenance of plasmids that contained H-DNA, and constructs that had three repeats were detected at much higher levels and persisted significantly longer than plasmids containing one or two repeats. Maintenance of plasmids that contained H-DNA but did not express LANA could not be detected. Surprisingly, plasmids that expressed LANA but contained no terminal repeats were also maintained, but were quickly lost through passage in culture. One possible explanation for this is that LANA may non-specifically bind the vector and tether it to the host cell chromosome, but much less efficiently than H-DNA. Because of this inefficient tethering, plasmids lacking H-DNA are not maintained in cell culture over a long period of time.

Data from studies on the closely related virus KSHV suggest that the terminal repeats play a role in anchoring the viral genome in the nucleus. Cloned KSHV terminal repeats can stably persist in KSHV-immortalized B cells over a 5 month period (unpublished). KSHV LANA tethers the viral episome to metaphase chromosomes (Ballestas *et al.*, 1999; Cotter & Robertson, 1999) by binding to a cis-element within the H-DNA (Ballestas & Kaye, 2001). These data, taken together, are consistent with the working hypothesis that terminal repeats of gamma-2 herpesviruses contain cis-acting sites essential for LANA binding and episomal maintenance.

Maintenance and replication of the latent EBV genome have been studied extensively. Two components required for persistence of EBV episomal DNA have been identified. The EBNA-1 protein interacts specifically with DNA and trans-activates a cis-acting element termed oriP (origin of plasmid DNA replication) (Chittenden *et al.*, 1989; Reisman *et al.*, 1985; Yates & Guan, 1991; Yates *et al.*, 1985). The cis-acting element consists of a dyad symmetry element (DS) and a family of 20 tandem copies of a 30 bp repeat (FR), which are located near the left end of the viral genome. Oligomers of EBNA-1 bind to both elements resulting in DNA bending and initiation of DNA replication in the dyad symmetry component. At least seven copies of the 30 bp repeat of FR are required for efficient maintenance of the EBV genome (Wysokenski & Yates, 1989). Because the LANA expression vector containing three terminal repeats was maintained much longer and at higher levels than plasmids containing only one or two repeats, it is tempting to speculate that the terminal repeats of HVS and other gamma-2 herpesviruses serve a function analogous to FR of EBV.

Replication origins contain not only a dyad symmetry element but are also rich in A+T residues. Therefore, it is unlikely that the terminal repeats of HVS and related gamma-2 herpesviruses contain the origin of replication because of their unusually high G+C content. A 2 kb fragment of HVS C484-77 encodes a dyad symmetry element near the left end,
and E. coli plasmid clones containing this fragment replicated autonomously in C484-77-transformed T cells (Kung & Medveczky, 1996). Therefore, it is possible that the dyad symmetry element contains an origin of replication. On the other hand, deletion mutants lacking the dyad symmetry element still formed episomes in T cells, showing that the dyad symmetry element is not essential (Kung & Medveczky, 1996). HVS encodes other dyad symmetry elements that may serve as alternative origins (Albrecht et al., 1992). Replication has also been shown to initiate at multiple sites in the EBV genome, primarily in a large region extending leftward from oriP (Little & Schildkraut, 1995), and EBV mutants lacking the dyad symmetry element are still capable of establishing latent infection (Norio et al., 2000). Therefore, although dyad symmetry elements serve as recruitment sites for the host DNA replication machinery, they are different from those found in small DNA viruses. Papovavirus genomes contain precise origins of replication whereas herpesviruses appear to replicate like mammalian cellular DNA, where replication initiates at broad initiation zones (Hamlin et al., 1994).

Identification of transformation-related viral genes and cis-acting elements of HVS episomal maintenance is an essential step for the comprehensive understanding of the molecular mechanisms involved in latency and HVS-induced oncogenesis. The Bac clones of HVS described here can now be efficiently and rapidly mutagenized in E. coli using a variety of extremely efficient methods (Zhang et al., 1998). The HVS clones can also provide a new tool to address general questions about the latent replication of gamma-2 herpesviruses. HVS, unlike KSHV, can be readily mutagenized and propagated in tissue culture. One possible advantage of the HVS system is that it provides all the technical tools to determine whether LANA is required for lytic or latent DNA replication of the virus. Another future use of the HVS system could be the generation of KSHV–HVS hybrids to understand the role of LANA and the terminal repeats in the biology of gamma-2 herpesviruses.

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References


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