Identification of a Herpesvirus Saimiri *cis*-Acting DNA Fragment That Permits Stable Replication of Episomes in Transformed T Cells

SZU-HAO KUNG AND PETER G. MEDVECZKY*

Department of Medical Microbiology and Immunology, University of South Florida,
Tampa, Florida 33612-4799

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Herpesvirus saimiri is a lymphotropic herpesvirus capable of immortalizing and transforming T cells both in vitro and in vivo. Immortalized and transformed T cells harbor several copies of the viral genome as a persisting episome. The mapping of the *cis*-acting genetic segment (oriP) required for viral episomal maintenance is reported here. Viral DNA fragments that potentially contain oriP were cloned into a plasmid that contains the hygromycin resistance gene. After several rounds of subcloning followed by transfection, oriP was mapped to a 1.955-kb viral segment. This viral fragment permits stable plasmid replication without deletion or rearrangement as well as episomal maintenance without integration or recombination. The function of oriP depends on a *trans*-acting factor(s) encoded by the viral genome. The 1.955-kb viral segment includes a dyad symmetry region located between two small nuclear RNA genes and is located upstream of the dihydrofolate reductase gene homolog. Therefore, this oriP contains novel elements distinct from those of other DNA viruses.

Animal viruses have provided useful systems for the study of DNA replication in eukaryotic cells. Characteristic features of the replication of latent genomes of DNA viruses are the presence of specific viral sequences that serve as origins of DNA replication and at least one specific viral protein whose direct interaction with these sequences is thought to be involved in the initiation of the replication process (11, 14). These viral origins of replication may be models for the regulated replication of cellular chromosomes or, alternatively, adaptations that latent viruses evolved to use the normal cell cycle controls for maintenance of viral genomes.

Herpesvirus saimiri (HVS) is the prototype of the lymphotropic γ -2 subfamily of herpesviruses (46). The natural host of the virus is the New World primate squirrel monkey, which is normally persistently infected without causing any diseases (39). However, HVS infection of numerous other New World primates as well as New Zealand White rabbits results in rapidly proliferating tumors of T lymphoid cells (13, 38, 40). Cell lines established from infected animals show no signs of producing viruses after long-term culture (27, 53). Moreover, human primary T cells can be transformed by group C strains of HVS to continuously proliferating T-cell lines with either the CD4⁺ or CD8⁺ phenotype (7, 36). Immortalized T cells harbor the HVS viral genome as a persisting circular form in high copy number (7, 27, 36, 53).

HVS is closely related to Epstein-Barr virus (EBV), whose episomal replication has been studied in great detail. EBV episomal DNA replication starts from a fixed region of the viral genome, termed the origin of plasmid replication (oriP) (48, 56). The EBV oriP consists of a family of direct repeats (FR) and a dyad symmetry element (45). This *cis*-acting genetic element requires a virus-encoded protein, EBNA1 (for EBV nuclear antigen 1), in *trans* for episomal replication and persistence in latently infected cells (34, 57).

The complete nucleotide sequence of the genome of strain 11 HVS has recently been reported (2). The majority of HVS genes have open reading frame sequence similarities with the EBV genome, and most of these genes are arranged in a collinear order (2, 21). There are differences between HVS and EBV regarding genes relevant to transformation and latent replication. In HVS, there are no EBNA1 and oriP-like sequences found in the regions of the genome that correspond to these sites in EBV.

The persistent HVS episome is partially deleted and rearranged in different HVS-infected lymphoid cell lines. However, while large portions of the “unique light” portion of the DNA (L-DNA) can be deleted, regions of the HVS genome that contain about 15 kb from both the left and right ends of L-DNA have always been preserved (1, 15, 17, 27, 47). These observations suggest that the HVS oriP is located within these two 15-kb regions of the HVS genome.

In this study, the two 15-kb regions were cloned. After several rounds of transfection into HVS-transformed T cells, hygromycin selection, and DNA analysis, a 1.955-kb *cis*-acting genetic element from the right end of the HVS genome was the smallest segment found to permit stable replication and episomal maintenance. There was no measurable integration or recombination of the transfected plasmid. This HVS fragment contains a dyad symmetry region flanked by two small nuclear RNAs (snRNAs) and is located upstream of the viral dihydrofolate reductase gene homolog, representing a novel replication origin.

MATERIALS AND METHODS

Chemicals and enzymes. Chemicals and reagents were purchased from Sigma (St. Louis, Mo.). All restriction and DNA-modifying enzymes were from Promega (Madison, Wis.) and were used according to the manufacturer's instructions.

Cell lines. Two T-lymphocyte lines designated 484-spleen and 484-blood (37) were established from New Zealand White rabbits infected by HVS strain 484. Jurkat cells (from the American Type Culture Collection, Rockville, Md.) are human leukemic lymphoid T cells susceptible to HVS 484 infection and supportive of HVS 484-77 latent replication (36).

Molecular cloning. phyg-L9 contains the *Kpn*I E subfragment, which was obtained by cleavage of PWD7-*Kpn*IE with *Sma*I and *Kpn*I (30). The *Kpn*I E

* Corresponding author. Mailing address: Dept. of Medical Microbiology and Immunology, University of South Florida, MDC 10, 12901 Bruce B. Downs Blvd., Tampa, FL 33612. Phone: (813) 974-5541. Fax: (813) 974-4151.

FIG. 1. Locations of various cloned viral fragments relative to the HVS genome and hygromycin resistance of the transfectants. Vertical bars, repetitive H-DNA; long open boxes, unique L-DNA region; +, hygromycin resistant; -, hygromycin sensitive; SM, *Sma*I; KP, *Kpn*I; SL, *Sal*I; SU, *Sau*3A; SP, *Spe*I; AV, *Ava*III; SA, *Sac*I; HD, *Hind*III; BM, *Bam*HI.

fragment was given blunt ends by using the Klenow fragment and then cloned into *Eco*RV- and calf intestine phosphatase-treated phyg.

To construct the phyg-L16 clone, HVS DNA was prepared as described previously (36). The viral DNA was digested with *Sma*I and *Sal*I, fragments were separated by pulsed-field gel electrophoresis, and the 16-kb *Sma*I-*Sal*I fragment was purified by electroelution. phyg was digested with *Eco*RV and *Sal*I and was used to clone the 16-kb *Sma*I-*Sal*I fragment.

The phyg-R15H clone was previously described (36).

phyg-R6.3H was subcloned from phyg-R15H by *Ava*III and *Cla*I digestion of phyg-R15H followed by Klenow treatment and self-ligation.

phyg-R6 was subcloned from phyg-R6.3H by *Apa*I and *Sal*I cleavage of phyg-R15H followed by Klenow treatment and self-ligation.

phyg-H was derived from phyg-R15H by *Bam*HI digestion of phyg-R15H followed by self-ligation.

phyg-R3H was subcloned from phyg-R15H by *Hind*III and *Cla*I digestion of phyg-R15H followed by Klenow treatment and self-ligation.

phyg-R2 was subcloned from phyg-R6.3H by *Sal*I and *Sac*I digestion of phyg-R6.3H followed by Klenow treatment and self-ligation.

phyg-R2.4D was subcloned from phyg-R15H by *Spe*I digestion of phyg-R15H followed by self-ligation. The clone was subjected to further digestion with *Apa*I and *Sal*I to delete repetitive H-DNA followed by self-ligation.

Plasmid transfection and selection. Transfection of 484-spleen and 484-blood cells was conducted by using the lipofectin method described by Whitt et al. (55). Because of significant differences in the molecular weights of the plasmids, the amount of DNA was adjusted to 1 µg/kb of DNA for each transfection. Transfection of Jurkat cells was conducted by electroporation (PG 200 ProGenetor II; Hoefer Scientific Instruments, San Francisco, Calif.) under the conditions described by Anderson et al. (3). About 48 h later, hygromycin was added to a final concentration of 200 µg/ml and was kept in medium up to the time of harvest. After the transfectants emerged in medium containing hygromycin, they were harvested for DNA analysis (typically 30 to 40 days after transfection).

Analysis of DNA from hygromycin-resistant cells. Low-molecular-weight DNA was extracted by the method of Hirt (25). To distinguish replicated DNA from input DNA, DNA was purified from the Hirt extracts and digested with *Dpn*I or *Mbo*I restriction endonuclease. Total DNA was extracted by the method of Gross-Bellard et al. (22). The method used for lysis of intact cells and DNA electrophoresis was that described by Gardella et al. (18), modified so that the voltage was 30 V and the duration was 15 h, in a 20-cm vertical electrophoresis apparatus (BRL, Gaithersburg, Md.). Southern blotting was conducted by the protocol described by Medveczky et al. (36).

RESULTS

Molecular cloning of viral DNA fragments and establishment of hygromycin-resistant cells. As discussed above, about 15 kb at each end of the L-DNA was hypothesized to contain the HVS oriP. The phyg plasmid (48, 57) was used to clone viral DNA fragments from HVS. This plasmid contains the hygromycin resistance gene; however, it does not have an origin for replication and cannot replicate as an episome in mammalian cells. Thus, viral DNA fragments harboring oriP will confer the ability to replicate to the recombinant plasmid in *cis*, allowing long-term expression of the hygromycin resistance gene.

Cloned DNA was transfected into the two HVS strain 484-77-transformed T-cell lines (484-spleen and 484-blood) derived from tumor-bearing rabbits. These cell lines have been cultured for several years and harbor circular viral genomes (37). No signs of a linear form of the viral genome were found in repeated experiments, indicating latent replication (18). These cell lines were used for transfection because they could provide a *trans*-acting factor(s) required for replication that is possibly encoded by the resident HVS genome.

In the first series of experiments, phyg-R15H (Fig. 1) transfectants showed hygromycin resistance and were detected 30 to 40 days after transfection. From phyg-R15H, several subclones were constructed; this was followed by transfection and selection. The smallest clone that conferred hygromycin resistance contained a 1.955-kb viral DNA segment (Fig. 1). Conversely, transfectants containing phyg-R2.4D, which had a deletion in this region, could not confer hygromycin resistance, suggesting the importance of this viral fragment. The clones tested in this

FIG. 2. Southern blot analysis of Hirt extracts from phyg-R2-transfected HVS-transformed T cells. At 45 days posttransfection, low-molecular-weight DNAs from phyg-R2 transfectants showing hygromycin resistance were prepared by the Hirt protocol and digested with either *DpnI* or *MboI*. The 1 and 10 copy controls are phyg-R2 from *E. coli* mixed with Hirt extracts from untransfected rabbit spleen cells. The blot was hybridized with a ^{32}P -labeled phyg probe. Sp, 484-spleen cells; Bl, 484-blood cells; n, nicked; s, supercoiled. *HindIII*-digested lambda DNA was used as size marker (positions [in kilobases] are shown at the right).

study and their abilities to confer hygromycin resistance are summarized in Fig. 1.

Analysis of the status of the transfected DNA. The phyg-R2-transfected cells showing growth in hygromycin medium were harvested, and low-molecular-weight DNA was extracted by Hirt's procedure (25). Southern blotting of Hirt supernatant DNA revealed several bands that comigrated with uncut plasmid DNA (Fig. 2), suggesting that the transfected DNA replicated as an episome. To distinguish input *Escherichia coli*-replicated DNA from that replicated in the mammalian cells, restriction endonucleases with different methylation sensitivities were employed. *DpnI* cleaves only DNA methylated at adenine residues by *E. coli*, and *MboI* is inhibited by this type of methylation. Opposite methylation sensitivities are observed when DNA is replicated in mammalian cells. Southern blotting showed that DNAs from Hirt extracts obtained from two experiments were resistant to *DpnI* but susceptible to *MboI* digestion (Fig. 2). Therefore, the transfected DNA was replicated in rabbit cells. Bands migrating between supercoiled and nicked DNAs could be linear DNA, as they were within a size range similar to the size of phyg-R2, or they could represent supercoiled dimers.

To test the possibility that transfected constructs integrated into the cellular genome, total DNA was prepared from transfected cells and digested with *HindIII*, which has only one cutting site in phyg-R2 (Fig. 3). Total DNA cut with *HindIII*

FIG. 3. Southern blot analysis of total DNA from phyg-R2-transfected HVS-transformed T cells. Total DNAs from approximately 10^6 phyg-R2 transfectants showing hygromycin resistance were digested with *HindIII*. The 1 and 10 copy controls represent phyg-R2 digested with *HindIII*. The blot was hybridized with a ^{32}P -labeled phyg probe. Sp, 484-spleen cells; Bl, 484-blood cells. Numbers on the left are molecular sizes in kilobases.

showed only a 6.3-kb band, which is the size of phyg-R2. This experiment indicated that random unique and single integration into the cellular genomic DNA probably did not occur, or at least that such an event was not detectable. These data did not exclude the possibility of integration of concatemeric DNA into the host genome.

To confirm the episomal status of transfected constructs by another method, intact cells were loaded in vertical agarose gels and analyzed as described by Gardella et al. (18). In this assay, about 10^6 Ficoll-Hypaque-purified cells were loaded in a vertical agarose gel and overlaid with a solution containing sodium dodecyl sulfate and pronase, which resulted in gentle in situ lysis of cells. Southern blotting of the gel hybridized with ^{32}P -labeled phyg showed similar bands in control DNA lanes and in lanes loaded with transfected cells (Fig. 4A). Bands between the supercoiled and nicked DNAs could represent linear or dimer forms. The same blot was stripped and re-probed with ^{32}P -labeled cloned repetitive H-DNA (pBR-HDNA) (5), which was expected to hybridize with both the transfected DNA and the resident viral genome. In addition to the bands representing the transfected DNA, high-molecular-weight bands representing nonintegrated viral genomic DNA were detected (Fig. 4B). EBV-based vector p220.2 (obtained from B. Sugden, University of Wisconsin) (23) Jurkat transfectants were used as a positive control for the Gardella gel assay. It is noteworthy that no viral DNA bands were detected by the phyg probe, indicating that homologous recombination did not take place between the transfected DNA and the resident viral genome, or at least not within the detectable range.

As a fourth method to study the episomal status of transfected DNA, Hirt extracts were used to transform *E. coli*, and several hundred bacterial colonies were obtained. DNA was prepared from these colonies, and this was followed by restriction endonuclease digestion with either *EcoRI* or *PstI* (data not shown). Five representative DNA preparations were ana-

FIG. 4. Detection of phyg-R2 episomes by the Gardella gel method. After *in situ* lysis electrophoresis (18) of cell suspensions, the gel was hybridized with ^{32}P -labeled phyg (A) and then stripped and rehybridized with ^{32}P -labeled pBR-HDNA (B). The 1 and 10 copy controls represent phyg-R2 mixed with Jurkat cells. phyg-R2-Sp and phyg-R2-BI, 484-spleen cells and 484-blood cells stably transfected with phyg-R2, respectively; p220-Jur, p220.2 Jurkat cells transfected with p220.2, showing hygromycin resistance as well. About 10^6 cells were loaded in each well. o, loading well origin; v, viral genome; n, nicked; s, supercoiled.

lyzed and showed band patterns identical to those of the controls. These data indicate that no deletion or rearrangement was detected in the transfected DNA.

***trans*-acting factor(s) encoded by the viral genome needed for episomal replication.** The phyg-R2 clone was transfected into Jurkat cells, a human leukemic T-cell line that does not contain HVS episomes, by electroporation followed by hygromycin selection. Total DNA isolated from hygromycin-resistant cells and control DNA mixed with genomic DNA from Jurkat cells were analyzed by Southern blotting. Compared with the location of the chromosomal DNA on an ethidium bromide-stained gel (Fig. 5, left panel), the labeled phyg probe hybridized at the position of chromosomal DNA (Fig. 5, right panel), indicating that the transfected DNA was integrated into the cellular genome. Several experiments were also performed with the Gardella method, and no episomal DNA was detected (data not shown). Therefore, no episomes were detected in transfected Jurkat cells in spite of the fact that HVS 484-77 can readily infect and establish latent genomes in these cells (37). These experiments support the hypothesis that episomal replication of the viral genome requires a viral *trans*-acting factor(s) and that the viral *trans*-acting factor(s) may prevent integration.

DISCUSSION

This report describes the identification of a *cis*-acting element of the HVS genome that can support stable extrachromosomal replication in rabbit cells harboring latent HVS genomes. A virus-encoded *trans*-acting factor(s) is required for the establishment and/or maintenance of the HVS-derived plasmid. We have analyzed HVS-transformed cells stably expressing the hygromycin resistance gene, and in all cases extrachromosomal plasmid DNA was detected. Since HVS-negative Jurkat cells did not support episomal replication of this plasmid, the transformed rabbit T cells containing latent HVS

FIG. 5. Southern blot analysis of total DNA indicates integration of phyg-R2 into uninfected Jurkat cells. Total DNA from phyg-R2 Jurkat transfectants (phyg-R2-Jur) was loaded uncut along with five copies of phyg-R2 mixed with uncut genomic DNA from Jurkat cells. The left panel shows the ethidium bromide-stained gel. The gel was then blotted and hybridized with a ^{32}P -labeled phyg probe (right panel).

probably provide an essential *trans*-acting factor(s). Restriction enzyme cleavage analysis of DNA from cells transfected with phyg-R2 showed no unexpected bands, which would have arisen if the DNA had randomly integrated into chromosomal DNA. In addition, Gardella gel analysis showed only bands consistent with episomal, not integrated, phyg-R2. Therefore, the transfected plasmid did not integrate into resident HVS episomes. Restriction enzyme analysis of the DNA rescued by *E. coli* also showed no signs of rearrangement or deletion. Taken together, these results imply that the 1.955-kb HVS fragment must have elements that specifically allow stable replication in the presence of a *trans*-acting factor(s) probably provided by the resident HVS genome.

The 1.955-kb viral DNA insert of phyg-R2 is known to contain 352 bp of the potential open reading frame 3, genes encoding two snRNAs (20), and 259 bp of the dihydrofolate reductase gene homolog (2, 19, 20, 50, 54) (Fig. 6A).

Several special features of this DNA element are worthy of discussion. There is a striking dyad symmetry region with 23 perfectly matched pairs (Fig. 6). This DNA structure is predicted to have a moderately low free energy and thus probably easily unwinds (44) (Fig. 7). Dyad symmetry sequences are generally seen in different sources of replication origins (11, 28), including animal viruses such as polyomaviruses and EBV (29). The dyad symmetry sequences are the location where the large T antigen of polyomaviruses or EBNA1 of EBV begins unwinding DNA and bidirectional replication initiates (14). There are also dyad symmetry regions at similar locations in HVS group C strain 488 and group A strain 11 (2, 8) (Fig. 6B). However, while there is 84% homology between the dyad symmetry regions of strains 484 and 488, there is a low level of sequence homology between those of strains 484 and 11 (Fig. 6B). Interestingly, there is another dyad symmetry region, with 27 perfectly matched pairs and located between snRNAs 1 and

FIG. 6. Maps and features of the HVS oriP. (A) Location of the 1.955-kb viral insert relative to the strain 484 viral genome. ORF1, -2, and -3, open reading frames; DHFR, dihydrofolate reductase; 1, 2, 3, and 4, four snRNAs; DS, dyad symmetry. (B) Sequence variation and conservation within the similar locations of dyad symmetry regions among HVS strains 484, 488, and 11 (GenBank accession numbers, HSSRNAG [nucleotides 1635 to 1681], HSV488A [nucleotides 5332 to 5379], and HSGEND [nucleotides 5848 to 5896], respectively). Dots represent nucleotide sequences conserved among dyad symmetry regions of strains 484, 488, and 11. Underlined nucleotides are noncomplementary in the dyad symmetry regions.

2 of strain 11 (2), which is not found within the corresponding regions of strains 484 and 488. Strain 11 deletion mutant viruses with deletions at this region were not affected in either latent or lytic replication (16, 43). Further mutational analysis of this dyad symmetry region will be needed to dissect its function.

A replication origin (*ori*) has been mapped to the upstream region of the amplified dihydrofolate reductase locus in the methotrexate-resistant CHO cell line CHO C 400 (35). Because the definitive sequences of *ori* have not been determined, no comparison of HVS *oriP* sequences with those of *ori* can be accomplished. It has been found that herpesviruses contain genes with identifiable cellular counterparts (6, 12, 42, 52). It is possible that the HVS *oriP* region was transduced from host cells. However, more data are needed to evaluate this possibility.

The viral insert of phyg-R2 contains two snRNAs, snRNA3 and snRNA4, which are also expressed in the HVS-transformed cells used in this study. In addition, two more snRNAs are located upstream of the viral DHFR homolog, similar to the location of *oriP* of EBV. EBV contains two genes encoding small RNA genes (EBER 1 and 2) which are abundantly transcribed during latent infection (26, 41). It was reported that a set of replication fork pause sites are located in between the two EBER genes and *oriP* of EBV, possibly because the directions of replication and transcription are opposite to each

FIG. 7. Predicted helical stability (ΔG) of the rightmost 6.5-kb L-DNA of HVS strain 484. (1 kcal = 4.184 kJ). The DNA helical stability was calculated with the Thermodyn computer program (44) by using default parameters and was plotted with the Slidewrite program. The dyad symmetry region is indicated by an arrow and has a free energy moderately lower than that of the surrounding area. Nucleotide positions are depicted according to the sequence information deposited in GenBank (accession numbers HSSRNAG and HSVCLS).

other (33). It has also been demonstrated that site-specific barriers to replication exist in the rRNA genes of several organisms. In these genes, replication forks that are proceeding in the direction opposite the direction of transcription are arrested at the 3' end of the transcription unit (9, 24, 31, 32). Consistent with this idea, mapping the HVS oriP to a region with transcriptionally active RNA implies that similar replication-transcription regulation may take place in HVS oriP.

It was recently reported that a 5.3-kb minor transcript spanning from snRNA3 to the 3' end of open reading frame 2 was detected in HVS strain 484-transformed rabbit cell lines (54). This transcript overlaps with the HVS oriP region. Considering that chromosomal replication origins are generally located in a nontranscribed region (4, 10, 51), the HVS oriP may represent a unique replication origin, although many viral replication origins, such as the EBV oriP, overlap with promoters and are surrounded by transcriptionally active regions. In line with this finding, origins of bidirectional DNA replication were recently identified within transcriptionally active regions, i.e., the Chinese hamster PRS14 locus and the Syrian hamster CAD locus (carbamoyl-phosphate synthetase, aspartate carbamoyltransferase, dihydroorotase and genes) (28, 49). The data suggest that in a subset of replicons, transcription may not interfere with initiation of replication, possibly because these events are tightly regulated in these regions.

It was previously reported that a spontaneously generated mutant of HVS strain 484 contains a 9-kb deletion at the right end of the genome which includes the 1.955-kb region (36). This deletion mutant was able to establish circular genomes (although at a very low copy number) in human peripheral blood T cells for about 4 weeks, a result contradictory to those of the present work. Little and Schildkraut (33) recently showed that initiation of latent DNA replication in the EBV genome can occur at sites other than that currently defined as oriP. The frequencies with which these other initiation sites are utilized relative to oriP differ among cell lines examined by using two-dimensional agarose gel electrophoresis. These data suggest that HVS may have an origin(s) other than this genetically defined oriP. This may explain why the 9-kb deletion mutant of HVS can latently replicate in spite of the deletion at this region.

From the data described above, we conclude that the HVS oriP shares some features generally found in other replication origins, such as the dyad symmetry element. However, this region contains small U-type RNAs and the DHFR homolog, which represent novel characteristics among DNA viruses. Continuation of this work should provide new insight into DNA replication in eukaryotic cells.

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