Herpesvirus Papio Contains a Plasmid Origin of Replication That Acts in *cis* Interspecies with an Epstein-Barr Virus *trans*-Acting Function

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Herpesvirus papio (HVP) and Epstein-Barr virus (EBV) are closely related biologically and biochemically; lymphoblastoid cells infected with either virus contain episomal viral DNA. The putative origin of replication for EBV plasmids (*oriP*) has been assigned to a 1,790-base-pair fragment (*cis*) in the short unique region of the genome which requires a viral function supplied in *trans* from elsewhere in the genome (J. Yates, N. Warren, D. Reisman, and B. Sugden, Proc. Natl. Acad. Sci. USA 81:3806-3810, 1984). We report here the identification of the putative origin of replication (*cis*) in HVP; we assigned it to the HVP *EcoRI* K fragment. The results indicate that the HVP replication process requires both a *cis* and a *trans*-acting function, analogous to that found in EBV.

In Epstein-Barr virus (EBV)-infected cells, the EBV genome persists in an episomal form (9). The putative origin of plasmid replication in the EBV genome (*oriP*) has been assigned to a 1,790-base-pair (bp) subfragment of the short unique region of the genome (17). The functional domain is composed of a 30-bp A+T-rich tandem repeat, approximately 600 bp in length, and a 114-bp region displaying a 65-bp dyad symmetry, both of which are required for episomal maintenance of chimeric plasmids (15). For episomal maintenance, a *trans*-acting function is encoded within a fragment known to encode EBV nuclear antigen I (EBNA I). Deletion of a 700-bp repetitive triplet which encodes part of EBNA I did not affect the *trans* function (18).

Based on other studies, there is an indication that EBNA I directly interacts with the essential regions of *oriP* (14). Filter binding assays and DNase I footprinting have revealed that a fusion protein representing the carboxy-terminal domain of EBNA I specifically protects binding sites within both the 30-bp tandem and the 65-bp dyad symmetry of *oriP*. In addition, a large in-frame deletion and a linker-insertion frameshift mutation within the coding region of EBNA I, which altered its carboxy terminus, destroyed its *trans*-acting ability (10).

Herpesvirus papio (HVP) and EBV are closely related both biologically and biochemically. HVP is a lymphotropic virus of baboons and is able to immortalize both human and baboon B lymphocytes in vitro (13). The growth-transformed cells contain early antigen, membrane antigen, and an intranuclear antigen designated HVP nuclear antigen, all of which show cross-reactivity with human anti-EBV serum (12, 13).

DNA-DNA hybridization studies indicated that HVP and EBV B95 DNA share a 40% homology that is not restricted to certain regions of the viral DNA but is dispersed (8). In addition, the restriction endonuclease digest patterns of EBV B95 DNA and HVP DNA are distinctly different. HVP-permissive lymphoblastoid cells contain both unit-length linear and episomal viral DNA. The circular mole-

cules are approximately the same size as episomes found in EBV growth-transformed Raji cells, 170×10^3 nucleotide bp (2). Physical maps of HVP show colinearity with EBV, and the genome contains both terminal and internal repeat sequences (5, 7) similar to those in the EBV genome. The internal direct repeats separate both genomes into short unique and long unique regions (1, 5).

HVP provides an ideal opportunity to generalize these mechanisms of episomal maintenance to another virus. We decided to test whether, despite the loss of 60% sequence homology with EBV, these functions are retained in HVP. We identified a region in HVP with sequence and functional homology to the putative EBV origin of replication. Also, HVP provides a unique opportunity better to define the essential region containing the EBV origin of replication, as well as the recently identified *trans*-acting function that seems to be necessary for episomal maintenance.

Identification of HVP DNA fragment homologous to the fragment of EBV containing *oriP*. Since HVP and EBV share approximately 40% base-pair homology, in our first attempts to find a plasmid origin of replication functionally analogous to the EBV *oriP* in the HVP genome, we used the EBV *oriP* fragment as a probe to search for a homologous HVP fragment. Extracellular viral DNA from 594S/F9, a baboon cell line productive for HVP (gift of H. Rabin), was digested with *Bam*HI, *Hind*III, and *Eco*RI, electrophoresed on agarose gels, and analyzed by the method of Southern (16). There was hybridization at reduced stringency between the EBV *oriP* fragment and two HVP fragments, HVP *Eco*RI-K and the terminal HVP *Eco*RI-J (Fig. 1). HVP *Eco*RI-K was subsequently cloned into G-418-selectable plasmid vector pKan2 (Fig. 1). pKan2 (gift of B. Sugden) contains the gene encoding aminoglycoside phosphotransferase II from the bacterial transposon Tn5 for selection in mammalian cells. Transcriptional control is afforded by the herpes simplex virus type 1 TK transcription initiation and termination signals. Southern blots of *Eco*RI-digested HVP were probed with pKan2:*Eco*RI-K. The resulting signals showed that *Eco*RI-K hybridized with *Eco*RI-K and *Eco*RI-J, indicating that homologous sequences present in *Eco*RI-K are also represented in *Eco*RI-J.

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FIG. 1. (A) Southern blot hybridization of *EcoRI* fragments of HVP with EBV DNA and HVP DNA as probe. Lanes: 1, HVP extracellular viral DNA digested with *EcoRI* with ^{32}P -HVP total extracellular viral DNA as probe; 2, HVP extracellular viral DNA digested with *EcoRI* probed with the ^{32}P -labeled *EcoRI*-*SstII* subfragment of EBV *BamHI*-C (EBV *oriP*-containing fragment); 3, HVP extracellular viral DNA digested with *EcoRI* probed with ^{32}P -HVP *EcoRI*-K. HVP fragments *EcoRI* J and K are indicated by arrows in lanes 2 and 3. (B) Plasmid vector pKan2 (18) containing HVP *EcoRI*-K. The gene encoding aminoglycoside phosphotransferase II of the bacterial transposon Tn5 (shaded area) provides G-418 resistance and is under the transcriptional regulation of the herpes simplex virus type 1 TK gene (open boxes). Restriction endonuclease sites are shown for *BamHI* (B), *ClaI* (C), *EcoRI* (E), *HindIII* (H), *SalI* (S), *SmaI* (Sma), *SstII*, *BclI*, and *BglII*. Kbp, Kilobase pairs. (C) *EcoRI* restriction digest map of HVP DNA (8). Kbp, Kilobase pairs; US, short unique region; UL, long unique region; TR, terminal repeat; IR, internal repeat.

High frequency of stable transfection of HVP *EcoRI*-K. To determine whether HVP *oriP* is functionally similar to EBV *oriP*, we transfected the pKan2:*EcoRI*-K construct by electroporation (11) into D98/HR1, a somatic hybrid cell line between human epithelial cell line D98 and non-virus-producing lymphoblastoid cell line P3JHR1 (3), which contains multiple copies of the EBV genome. The cell population was subsequently placed under G-418 (600 $\mu\text{g}/\text{ml}$) selection, and resistant colonies were isolated. Table 1 shows that plasmid construct pKan2:*EcoRI*-K gave rise to greater numbers of G-418-resistant colonies when compared with a control plasmid after electroporation into EBV-genome-positive cell line D98/HR1. As shown in Table 1, approximately 5,000 of 10^7 D98/HR1 cells electroporated with pKan2:*EcoRI*-K (0.05%) gave rise to G-418-resistant

colonies. No G-418-resistant cells were established in HeLa cells, which lack an endogenous EBV genome.

HVP *oriP* can replicate autonomously as plasmid in EBV-positive cells. The above-described assay, that of selection of G-418-resistant colonies, depends on the fact that the pKan2 construct remains stably in the cell line. This phenotype can be elicited by either stable integration of the construct or autonomous replication of the plasmid. We then asked whether the latter explanation was correct. DNA isolated from G-418-resistant D98/HR1 cells electroporated with pKan2:*EcoRI*-K was analyzed for autonomously replicating plasmid molecules. Low-molecular-weight DNA was isolated from pools of G-418-resistant colonies after approximately 20 passages posttransfection by the method of Hirt (6). The resulting low-molecular-weight DNA was digested

with *EcoRI*, electrophoresed, and analyzed by the method of Southern using pKan2:*EcoRI*-K as probe. The major hybridization pattern observed was identical to the mobility of an *EcoRI* digest of original construct pKan2:*EcoRI*-K, the HVP *EcoRI* K fragment, and the two *EcoRI* vector fragments, indicating that the plasmids did not undergo rearrangement (Fig. 2). Undigested DNA was also analyzed as described above, and the resulting hybridization pattern was identical to the mobility of supercoiled plasmid DNA (data not shown). In two independent transfections, one by the calcium phosphate coprecipitation method (4) and the other by electroporation, plasmid molecules were easily detected in Hirt supernatant fluids.

To determine the number of plasmids containing HVP *oriP*, total cellular DNA was isolated from G-418-resistant D98/HR1 colonies previously transfected with pKan2:*EcoRI*-K. The DNA was electrophoresed on agarose gels and analyzed by the method of Southern (16), by using pKan2 as probe. Plasmid reconstructions were made at 1, 10, and 100 copies per cell; the resulting hybridization signals indicated a plasmid copy number of approximately 5 per cell (Table 1). Low plasmid copy numbers could represent inefficient plasmid maintenance resulting from a divergence in sequence homology from EBV *oriP*.

To determine whether the putative HVP origin of replication might utilize the EBNA-I function of EBV as a *trans*-activating factor for replication, HeLa cells were co-transfected with pKan:*EcoRI*-K and a second plasmid, pKan2 *Sall*-F; the *Sall*-F component from EBV DNA encodes EBNA-I. G-418-resistant cells were selected, and DNA was extracted from mass cultures and digested with *HindIII* to linearize the pKan2:*EcoRI*-K plasmid. After electrophoresis and Southern transfer, the HVP *EcoRI*-K fragment was used as a probe. The hybridization signal showed a single band migrating at a molecular weight equal to that of linearized pKan2:*EcoRI*-K (data not shown). The single hybridization band is indicative of either a tandem integration event or an autonomously replicating molecule in the cells, suggesting that the EBV EBNA-I-encoding region is the *trans* factor that allows maintenance of the plasmid.

We report here the identification of a HVP *cis*-acting element homologous to EBV *oriP* which can function as a plasmid origin of replication in cell lines containing an endogenous EBV genome. The HVP element was localized to a 6.2-kilobase fragment within the short unique region of the HVP genome (Fig. 1). Analogous to *oriP* in EBV, this element requires a function supplied in *trans* from a latent viral genome for maintenance of the episome. Recent evidence suggests that EBNA I is required in *trans* for the maintenance of EBV *oriP*-containing plasmid constructs (18). Furthermore, DNA binding studies indicate that EBNA I may have sequence-specific DNA binding properties,

TABLE 1. Selection of G-418-resistant colonies in different cell lines after electroporation with various plasmids constructs

Cell line	Plasmid	No. of G-418-resistant colonies	No. of plasmid copies per cell
D98/HR1	pKan2	0	
D98/HR1	pKan2:HVP <i>EcoRI</i> -K	5,000	5
HeLa	pKan2	0	
HeLa	pKan2:HVP <i>EcoRI</i> -K	0	
HeLa	pKan2:HVP <i>EcoRI</i> -K + pKan2:EBV <i>Sall</i> -F	5	ND ^a

^a ND, Not determined.

FIG. 2. pKan2 construct containing HVP *EcoRI*-K in D98/HR1 cells. D98/HR1 cells were electroporated with pKan2:*EcoRI*-K and selected with G-418. Colonies (5×10^3) were pooled and passaged approximately 20 times in medium containing 600 μ g of G-418 per ml and extracted by the method of Hirt (6). The DNA in the supernatant was digested with *EcoRI*, and Southern blots were probed with ³²P-pKan2:*EcoRI*-K. The arrows indicate the 6.2-kilobase HVP *EcoRI* K fragment and the two *EcoRI* fragments of pKan2, 2.5 and 2.1 kilobases, respectively.

showing preferential binding to the essential regions of EBV *oriP*, those being the 30-bp tandem repeats and the 65-bp dyad symmetry (14). It remains to be determined whether EBNA I from EBV is the essential *trans* element allowing episomal maintenance of the HVP plasmid construct. We can, however, predict that if EBV *oriP* represents the origin of replication used to maintain the episomal form, a direct analogy can be made in the HVP system. There exists in the HVP-transformed cell line a protein designated HVP nuclear antigen which is antigenically cross-reactive with serum from EBV-infected individuals. If HVP *EcoRI*-K represents the fragment analogous to EBV *oriP*, the HVP nuclear antigen and EBNA I could be structurally similar enough for EBNA I to function as the *trans* element in a cooperative fashion with the HVP plasmid origin of replication. Cotransfections of the EBNA I-encoding fragment (EBV *Sall*-F) and the HVP *EcoRI* K fragment resulted in the establishment of a G-418-resistant cell line which may contain the HVP construct replicating autonomously. If this is the case, EBNA I may be the responsible *trans*-acting element. We were unable to detect EBNA I in the cell line by standard anticomplement immunofluorescence techniques, although this may have been the result of a lack of sensitivity

in the assay, since the EBV *SalI* F fragment may represent a single-copy integration event.

HVP provided us with a unique opportunity to define better the essential region containing the EBV origin of replication. Despite a loss of approximately 60% sequence homology between EBV and HVP, the *cis* function appears to be maintained in HVP, and the fragment is located colinearly with EBV. The HVP *trans*-containing segment remains to be discovered; our data indicate that it probably exists. Eventually, its identification will help to define the general essential genome structure of EBV. By comparing sequences from functionally identical fragments in two related viruses, we should be able to pinpoint the common sequence required for these functions in the two genomes. It remains to be determined whether other distantly related herpesviruses, especially those, such as herpesvirus saimiri and herpesvirus ateles, that are known to form episomes, retain such functions, and what sequence divergence is tolerated.

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