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The Lytic Origin of Herpesvirus Papio Is Highly Homologous to Epstein-Barr Virus ori-Lyt: Evolutionary Conservation of Transcriptional Activation and Replication Signals

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Herpesvirus papio (HVP) is a B-lymphotropic baboon virus with an estimated 40% homology to Epstein-Barr virus (EBV). We have cloned and sequenced ori-Lyt of herpesvirus papio and found a striking degree of nucleotide homology (89%) with ori-Lyt of EBV. Transcriptional elements form an integral part of EBV ori-Lyt. The promoter and enhancer domains of EBV ori-Lyt are conserved in herpesvirus papio. The EBV ori-Lyt promoter contains four binding sites for the EBV lytic cycle transactivator Zta, and the enhancer includes one Zta and two Rta response elements. All five of the Zta response elements and one of the Rta motifs are conserved in HVP ori-Lyt, and the HVP DS-L leftward promoter and the enhancer were activated in transient transfection assays by the EBV Zta and Rta transactivators. The EBV ori-Lyt enhancer contains a palindromic sequence, GGTCAGCTGACC, centered on a *Pvu*II restriction site. This sequence, with a single base change, is also present in the HVP ori-Lyt enhancer. DNase I footprinting demonstrated that the *Pvu*II sequence was bound by a protein present in a Raji nuclear extract. Mobility shift and competition assays using oligonucleotide probes identified this sequence as a binding site for the cellular transcription factor MLTF. Mutagenesis of the binding site indicated that MLTF contributes significantly to the constitutive activity of the ori-Lyt enhancer. The high degree of conservation of *cis*-acting signal sequences in HVP ori-Lyt was further emphasized by the finding that an HVP ori-Lyt-containing plasmid was replicated in Vero cells by a set of cotransfected EBV replication genes. The central domain of EBV ori-Lyt contains two related AT-rich palindromes, one of which is partially duplicated in the HVP sequence. The AT-rich palindromes are functionally important *cis*-acting motifs. Deletion of these palindromes severely diminished replication of an ori-Lyt target plasmid.

Epstein-Barr virus (EBV) establishes a latent infection in B cells and undergoes its lytic replicative cycle in epithelial cells of the oropharynx. The parotid salivary gland has been documented as a significant source of virus, and lytic replicative antigens have also been demonstrated in mucosal epithelial scrapings (48). In oral hairy leukoplakia, lytic cycle antigens and virus particles have been demonstrated in the spinous epithelial layer of the tongue (19). A reactivated lytic replicative cycle can also occur in B cells.

Some progress has been made toward identifying EBV-encoded replication genes. Episomal DNA replication during latency requires only a single viral protein, EBNA-1 (46, 55). In contrast, lytic DNA replication is dependent on multiple viral products. The differential drug sensitivity of latent and lytic viral DNA replication pointed to the existence of an EBV-encoded DNA polymerase (12). The DNA polymerase gene (BALF5) and that of the polymerase processivity factor (BMRF1) have now been cloned and shown to be active in in vitro assays (30, 33, 39). We recently described a cotransfection-replication assay that allowed the identification of four additional lytic cycle replication genes: BALF2, the single-stranded-DNA-binding protein homolog; BSLF1 and BBLF4, the primase and helicase homologs; and BBLF2/3, a potential homolog of the UL8 gene of herpes simplex virus (HSV) and possible component of the helicase-primase complex (16). In addition, ori-Lyt replication in the cotransfection assay is dependent on the three EBV lytic cycle transactivators Zta, Rta, and Mta (16a).

The *cis*-acting signals for lytic EBV DNA replication lie within the lytic origin of replication, ori-Lyt (22). The EBV genome contains two copies of ori-Lyt, one in DS-L and one in DS-R. The minimal DS-L origin covers 690 bp and lies in the *Bam*HI H fragment between the divergent BHLF1 and BHRF1 open reading frames. The origin can be divided into three operational segments, two of which contain signals for transcriptional regulation of the adjacent genes. The leftmost segment consists of the promoter and leader of the BHLF1 gene, whose transcript is the most abundant early mRNA (27, 28). The BHLF1 promoter contains four binding sites for the Zta transactivator, and its activity is stimulated by Zta in cotransfection assays (34, 36, 37). The rightmost segment is a transcriptional enhancer element that is activated synergistically by the Zta and Rta transactivators and contains one binding site for Zta and two for Rta (6, 11, 21, 24, 37). Between these segments lies a *Kpn*I fragment that is not essential for ori-Lyt function and a 225-bp region that is likely to be the site of initiation of DNA synthesis. This latter region contains related 18- and 20-bp AT-rich palindromes and a homopyrimidine tract. In other characterized origins of replication, sequences of this type have been implicated

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as sites of localized melting and loss of double-stranded DNA structure (52).

The presence of a Zta-responsive promoter and an Rta-responsive enhancer within the defined boundaries of ori-Lyt along with the apparent requirement for Zta and Rta for replication of ori-Lyt in the cotransfection-replication assay focuses attention on the potential role of transcriptional signals in activation of the EBV lytic origin. To gain further insight into the significance of these transcription elements and to identify other important *cis*-acting motifs, we have examined the degree of conservation and the arrangement of sequences in the lytic origin of a closely related baboon B-lymphotropic virus, herpesvirus papio (HVP). This virus shows 40% cross-hybridization with EBV and has a colinear genomic organization (25, 26, 32). A cosmid clone containing the HVP ori-Lyt was identified by hybridization with an EBV DS-L ori-Lyt probe. DNA sequencing revealed an extremely high degree of homology between the EBV and HVP origins and an almost total conservation of the transcriptional signals located within the boundaries of ori-Lyt. EBV ori-Lyt is the only gammaherpesvirus lytic origin previously identified, and it has no obvious homolog in herpesvirus saimiri, a gamma two herpesvirus, by sequence comparison (1, 43). The present identification and sequencing of the HVP ori-Lyt provides evidence for a highly conserved ori-Lyt in a second gamma one herpesvirus.

MATERIALS AND METHODS

Cells. Ba65 cells latently infected with HVP were obtained from Elliott Kieff (Harvard University) and grown in Optimen medium supplemented with 2.5% fetal calf serum (FCS). Vero cells were grown in Dulbecco's modified Eagle's medium containing 10% FCS; Raji and DG75 cells were maintained in RPMI 1640 medium supplemented with 10% FCS.

Cosmid cloning. Cell pellets from 2 liters of Ba65 cells were washed in phosphate-buffered saline, frozen and thawed, and then lysed in 2 ml of buffer containing 10 mM Tris HCl (pH 8.0), 10 mM EDTA, 2% sodium dodecyl sulfate (SDS), and 100 μg of proteinase K per ml. After overnight incubation at 37°C, the mixture was diluted to 4 ml with water and sodium acetate (pH 5.2) was added to a final concentration of 0.3 M. Samples were extracted with phenol-chloroform and chloroform and then precipitated with ethanol. DNA (1 mg/ml) was dissolved in water, treated with RNase A, reprecipitated with ethanol, and then banded in CsCl density gradients. Aliquots (250 μl) were layered over 56% (wt/vol) CsCl solution and centrifuged in a TV850 vertical rotor at 36,000 rpm for 20 h at 20°C. Fractions (0.7 ml) were assayed for total DNA content by optical density measurements at 260 nm and for HVP DNA content by dot blot hybridization using an EBV *Bam*HI-W DNA probe that had been labeled with ^{32}P by random priming (15). Fractions which contained HVP DNA (density of 1.71 to 1.73 g/ml) and did not overlap significantly with the peak of cellular DNA were pooled and desalted by using Centricon filtration concentrators with a cutoff of 30 kDa. One hundred micrograms of DNA was partially digested with *Sau*3A for 6 min, reextracted, precipitated, assessed for size on a 3% agarose gel, and then loaded on 10 to 40% preformed sucrose gradients. Samples were spun at 27,000 rpm in an SW41 rotor for 16.5 h at 4°C. High-molecular-weight fractions were pooled and desalted in filtration concentrators. DNA was ligated to a pWE15 cosmid vector which had been cleaved with *Bam*HI and treated with calf intestinal phosphatase. Ligated DNA was packaged into lambda phage, using commercially available reagents (Stratagene, La Jolla, Calif.). The library was screened by hybridization of replica colonies on Nytran filters (Schleicher & Schuell, Keene, N.H.) with the EBV ^{32}P-labeled *Bam*HI-W probe, and a colony that was positive on secondary screening was mapped by restriction enzyme analysis of the cosmid DNA (cJR4), using *Eco*RI, *Hin*dIII, *Sal*I, and *Xba*I. The presence of insert sequences homologous to EBV ori-Lyt was confirmed by Southern blot hybridization to cleaved cJR4 DNA with an EBV *Bam*HI-H probe.

DNA sequencing. The 7-kb HVP *Xba*I I fragment within cJR4, which hybridized to the EBV *Bam*HI-H probe, was subcloned into Bluescript SK+ as pJR7. A 900-bp *Sma*I fragment and an overlapping 2,500-bp *Apa*I fragment of pJR7 also hybridized with EBV *Bam*HI-H and were further subcloned into pBSSK for sequencing (pJR9 and pJR10, respectively). Polymerase chain reaction (PCR) fragments of each of these plasmid inserts were generated by using T3 and T7 primers and a 3:1 mix of deazaguanosine to dGTP, with dATP, dCTP, and dTTP for preliminary sequence analysis. The complete overlapping sequence from each strand was then obtained by using a series of unique oligonucleotide primers and standard dideoxy sequencing procedures.

Plasmid constructions. Plasmids pDH123, which contains the 1,000-bp duplicated region (DS-L) of the EBV B95-8 genome linked in the leftward orientation to the reporter gene chloramphenicol acetyltransferase (CAT), and pPL52, containing the BHLF1 promoter (positions −155 to +40) driving CAT, have been described previously (36). Plasmid pJR16 contains the corresponding HVP BHLF1 promoter cloned into pCATb′ as a *Hin*dIII-*Bam*HI fragment generated by PCR amplification using HVP template plasmid pJR9, the forward primer 5′-CTAGAAGCTTACCAACAACCTTGT TGAC, and reverse primer 5′-CTAGGATCCGTGTTGA CGACGCTGG. The EBV ori-Lyt enhancer-CAT constructs (pYNC146A and pYNC146B; wild-type and reverse orientations, respectively) were made by adding *Bgl*II linkers onto the 260-bp *Sma*I enhancer fragment of EBV ori-Lyt and ligating this fragment into the *Bgl*II site of an E1b-CAT plasmid, pGH262. Similar wild-type- and reverse-orientation HVP enhancer E1b-CAT constructs (pJR18 and pJR19, respectively) were made in pGH262 by inserting a *Bgl*II-cleaved 270-bp PCR fragment generated by using HVP template plasmid pJR10, the forward primer 5′-CTAGAGAT CTACGGTTGCCTGAGCCAT, and reverse primer 5′-CTA GAGATCTACGGTTGCCCTGAGCCCAT. The ΔZRE5-ΔZRE6 (Zta response element) enhancer plasmid pYNC157 was generated as described for pYNC146 except that the 260-bp mutated *Sma*I fragment was derived from pYNC127. Plasmid pYNC123, containing the ori-Lyt enhancer linked to the EBV BHRF1 promoter, was generated by using PCR primers (forward, 5′-TGGAAGATCTTACGGTTGCCTGA GCCCAT; reverse, 5′-TCGCGGATCCAACCTCTTCAGG CCCGG) to amplify the EBV (B95-8) sequences from positions 53390 to 53820. The PCR product was cleaved with *Bam*HI and *Bgl*II and cloned into the *Bam*HI site of pGH262.

Single mutations within the wild-type BHRF1 promoter/enhancer background in plasmid pYNC123 were made by using a combination of uracil selection (31) and gapped DNA techniques. Plasmid pYNC123 DNA grown in *dut ung Escherichia coli* was denatured and annealed with a mixture of DNA grown in *dut*$^+$ *ung*$^+$ bacteria that had been cleaved with restriction enzymes on either side of the element targeted for mutation and with a single-stranded oligonucle-

otide carrying the mutation. The gap was filled in with Klenow polymerase, and the DNA was ligated and transferred into a *dut*$^+$ *ung*$^+$ strain of *E. coli*. The colonies were screened for the generation of plasmids carrying the restriction sites incorporated into the mutant oligonucleotides. The oligonucleotides used were the following:

ΔZRE5	5′-GACTTGTCACCTTTAAACATTTGGTCAGCT	(*Dra*I)
ΔZRE6	5′-TAGCAGGGGCTTAGTGATATCATGGTGAGGCAGG	(*Eco*RV)
ΔMLTF	5′-GGCGAGCATCGGGAAGCTTCCCAAATGTGCAA	(*Hin*dIII)
Δ"TRE"	5′-GGCACAGGCCAGCTCTAGAGCCAGGAAGTGGC	(*Xba*I)

Double and triple mutations were made by subjecting these initial clones to PCR-mediated mutagenesis. Overlapping pairs of PCR products containing the desired mutations were amplified by using plasmids containing single mutations as templates in PCR reactions primed with the external forward and reverse primers used to generate pYNC123 together with the following sets of internal primer pairs:

ΔZRE5 5′-CCTTACTGACTTGTCACCTTTAAACATTTG and 5′CAAATGTTTA AACCTGACAAGTCAGTAAGG

ΔZRE6 5′-TAGCAGGGGCTTAGTGATATCATGGTGAGGCAGG and 5′ CCTG CCTCACCATGATATCACTAAGCCCCTGCTA

Δ"TRE" 5′-GGCACAGGCCAGCTCTAGAGCCAGGAAGTGGC and 5′ GCCACT TCCTGGCTCTAGAGCTGGCCTGTGCC

The amplified overlapping PCR products were subjected to a second round of PCR amplification with the external forward and reverse primers and then cloned into pGH262 as described for pYNC123. Plasmids were screened for the acquisition of new restriction sites, and mutations (indicated in parentheses) were confirmed by DNA sequencing; constructions generated in this way were pYNC125 (ΔMLTF [major late transcription factor]), pYNC127 (ΔZRE5 and ΔZRE6), pYNC131 (ΔZRE5 and Δ"TRE" [thyroid response element]), pYNC143 (ΔZRE5, ΔZRE6, and ΔMLTF), and pYNC144 (Δ"TRE"). Plasmid pSV2CAT contains the complete simian virus 40 early promoter/enhancer (18).

The target of the replication assay, plasmid pEF51A, was generated by a multistep protocol that deleted the two AT-rich palindromes and their intervening sequences (nucleotides 53262 to 53334). The protocol also resulted in the deletion of a 592-bp *Bgl*II-*Bcl*I fragment of *Bam*HI-H that lies outside ori-Lyt. The control plasmid pEF52 contained only the *Bgl*II-*Bcl*I deletion. Plasmid pPDL7, used as a probe in the replication assays, contains the EBV (strain MABA) EBNA-2 open reading frame.

Transfections and CAT assays. Vero cells were transfected by calcium phosphate precipitation as previously described (38). Lymphoblastoid cells were transfected by the DEAE-dextran method (36). Cells were harvested 48 h after transfection, and CAT assays were performed as previously described (18).

Preparation of nuclear extracts. Nuclear extracts were prepared from HeLa and Raji cells according to the method of Dignam et al. (14). The Raji extract was then fractionated on a heparin-agarose affinity column (29). Protein was eluted with increasing KCl (100 mM steps). The 300 mM KCl column fraction was used in the gel retardation and footprinting assays.

Mobility shift assays for DNA binding. The following oligonucleotide pairs were synthesized by the Johns Hopkins School of Hygiene Core Facility:

EBV MLTF 5′-GATCCATTTGGTCAGCTGACCGATCTAGAA and 5′-GATCTTCTAGATCGGTCAGCTGACCAAATG

Mutant EBV MLTF 5′-GATCCATTTGGGAAGCTTCCGATCTAGAA and 5′-GATCTTCTAGATCGGGAAGCTTCCCAAATG

EBV "E" 5′-GATCCATTTTGTCACGTGACCGATCTAGAA and 5′-GATCTTCTAGATCGGTCACGTGACAAAATG

AdML 5′-GATCCAGTAGGCCACGTGACCGGCAGCTGA and 5′-GATCTCAGCTGCCGGTCACGTGGGCTACTG

μE3 5′-GATCTTCTAGATCGGCCACCTGCCTAAATG and 5′-GATCTTCTAGATTTGCCACATGACCAAATG

κE2 5′-GATCTTCTAGATCGGCCACCTGCCTAAATG and 5′-GATCCATTTAGGCAGGTGGCCGATCTAGAA

Complementary 30-mer oligonucleotides (with 4-bp 5′ overhangs) were annealed at 50 μg/ml in buffered 150 mM NaCl at 65°C for 5 min and then slowly cooled. Annealed oligonucleotides (0.1 μg) were incubated with the Klenow fragment of *E. coli* DNA polymerase in 10 mM Tris–5 mM $MgCl_2$–7.5 mM dithiothreitol (DTT) (pH 7.5) buffer plus [^{32}P]dCTP (3,000 Ci/mmol) and unlabeled dTTP, dGTP, and dATP. Protein-DNA complexes were formed by mixing equal portions of each protein sample with 20 μg of poly(dI-dC) · poly(dI-dC) per ml at 20°C in 20 μl of binding buffer (10 mM [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 50 mM KCl, 1 mM EDTA, 0.1 mM DTT, 0.1 M phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 5% glycerol [pH 7.5]). After 15 min, ^{32}P-labeled probe DNA (approximately 25 fmol and 5,000 to 20,000 cpm) or probe DNA plus unlabeled competitor oligonucleotides was added, and the mixture was incubated for 30 min at 20°C. Samples were then electrophoresed through a 4.5% nondenaturing (30:0.8 polyacrylamide/bisacrylamide) gel in HEPES buffer (100 mM HEPES, 1 mM EDTA, 0.5 mM EGTA [pH 7.5]) at 20°C.

DNase I footprinting assay. The 260-bp EBV ori-Lyt enhancer fragment was isolated by using *Bam*HI and *Bgl*II sites in the polylinker of plasmid pMH103 (24) and end labeled with ^{32}P at the *Bgl*II polylinker site at the left-hand (ZRE5) boundary. The fractionated Raji nuclear extract and labeled DNA were mixed in 25 mM HEPES (pH 7.9)–70 mM KCl–0.5 mM EDTA–1 mM DTT–0.5% Nonidet P-40–5% glycerol–20 μg of poly(dI-dC) per ml in a 100-μl volume and incubated at 23°C for 20 min before treatment with DNase I (5 ng/ml; 10 mM $MgCl_2$, 1 mM $CaCl_2$; Worthington Diagnostics). Reactions were quenched after 30 s with 100 mM NaCl–50 mM EDTA, extracted with phenol, and ethanol precipitated. DNA was resuspended in formamide-dye and electrophoresed a 7% polyacrylamide–8 M urea sequencing gel (37).

DNA replication assay. The cotransfection-replication assay was performed as previously described (16). Briefly, an EBV or HVP ori-Lyt-containing plasmid and a complete set of plasmids encoding EBV replication genes were cotransfected into Vero cells. DNA from cells harvested 92 h after transfection was digested with *Bam*HI and *Dpn*I, separated by electrophoresis through a 1% agarose gel, and transferred to a Nytran membrane (Schleicher & Schuell). After prehybridization, the membrane was incubated overnight at 60°C with, at 2×10^6 to 5×10^6 cpm/ml each, HVP *Xba*I-I and EBV pPDL7 (EBNA-2 open reading frame) DNA probes labeled with ^{32}P by random priming to a specific activity of 5×10^8 cpm/μg. The membrane was washed twice in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 65°C for 45 min and subjected to autoradiography.

Nucleotide sequence accession number. The GenBank accession number for the HVP ori-Lyt sequence is L13013.

RESULTS

Cloning and sequencing of HVP ori-Lyt. Cross-hybridization between the genomes of HVP and EBV and their colinear organization enabled us to use EBV probes and

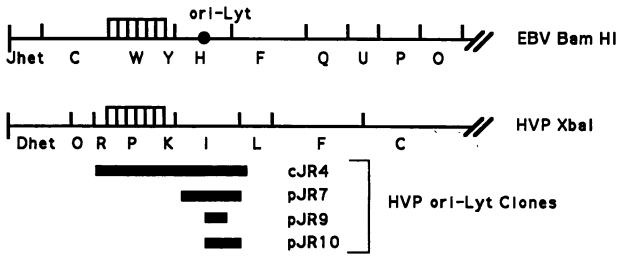

FIG. 1. Map of the left half of the EBV and HVP genomes indicating the location of ori-Lyt and of the clones used for sequencing.

gene-walking techniques to clone HVP sequences. Cosmid cloning of high-G+C DNA from the HVP-infected Ba65 cell line led to the identification of a 41-kb cosmid, cJR4, which hybridized to both EBV ori-Lyt and *Bam*HI-W probes. The *Xba*I I fragment and two smaller *Sma*I and *Apa*I subfragments identified by hybridization to the EBV ori-Lyt probe were subcloned into Bluescript plasmids for sequencing of both DNA strands (Fig. 1).

Alignment of EBV and HVP ori-Lyt sequences revealed an extraordinary level of conservation (Fig. 2) and an identical overall organization (Fig. 3A). All three regions, the BHLF1 promoter and leader, the enhancer, and the adjacent sequences containing two AT-rich palindromes and a stretch of pyrimidine-biased DNA, were present in HVP. In addition, the sequence between the *Kpn*I sites, which is not essential for lytic replication, was also highly conserved. Consensus binding motifs for both viral and cellular transcription factors were well preserved. The level of nucleotide homology within the 950-bp *Sst*II-*Nsi*I ori-Lyt region (22) averages 89% but fell to nearly 50% outside those boundaries (Fig. 3B). The near identity of the origins contrasts with the estimate of 40% overall similarity for the two genomes based on cross-hybridization studies (25, 26) and with our own observation that DNA sequence homology at the neighboring EBNA-2 locus is less than 50% (40).

Structure and function of the BHLF1 promoter. Transcription elements within the promoter and leader for the BHLF1 gene were highly conserved in HVP ori-Lyt (Fig. 4A). All four of the Zta binding sites within the minimal promoter are positionally conserved. There is a C-for-T substitution in the first of these response elements, which alters its sequence to that of the higher-affinity ZRE5 found in the enhancer, TGTGCAA (5, 37). The CAAT box just upstream of the promoter is preserved, but there is a mutation of the TATA-binding protein (TBP) binding site GATAAA in EBV to an atypical TATA sequence AATAAA. To test the ability of this region to function as a promoter, we cloned the 160-bp EBV promoter fragment and the homologous region of HVP into reporter plasmids. Transfection of the unmodified CAT reporter plasmid or the plasmids containing the entire EBV ori-Lyt sequence, the EBV BHLF1 promoter, or the putative HVP promoter into Vero cells gave negligible levels of CAT activity (Fig. 4B). However, cotransfection with a plasmid expressing the EBV transcriptional activator Zta resulted in 20-fold stimulation of CAT activity from the EBV promoter plasmids. Cotransfection of the Zta expression plasmid and the HVP promoter-CAT reporter plasmid consistently resulted in a greater activation, 110-fold in Fig. 4C. Thus, the putative HVP promoter, like the homologous region of EBV, is both functional and Zta responsive.

Structure and function of the enhancer element. Transcriptional elements were also conserved within the ori-Lyt enhancer. The high-affinity Zta binding site TGTGCAA (ZRE5) is fully conserved. One of two binding sites for the lytic cycle transactivator Rta is conserved. The other site has three base pair changes which make it an unlikely

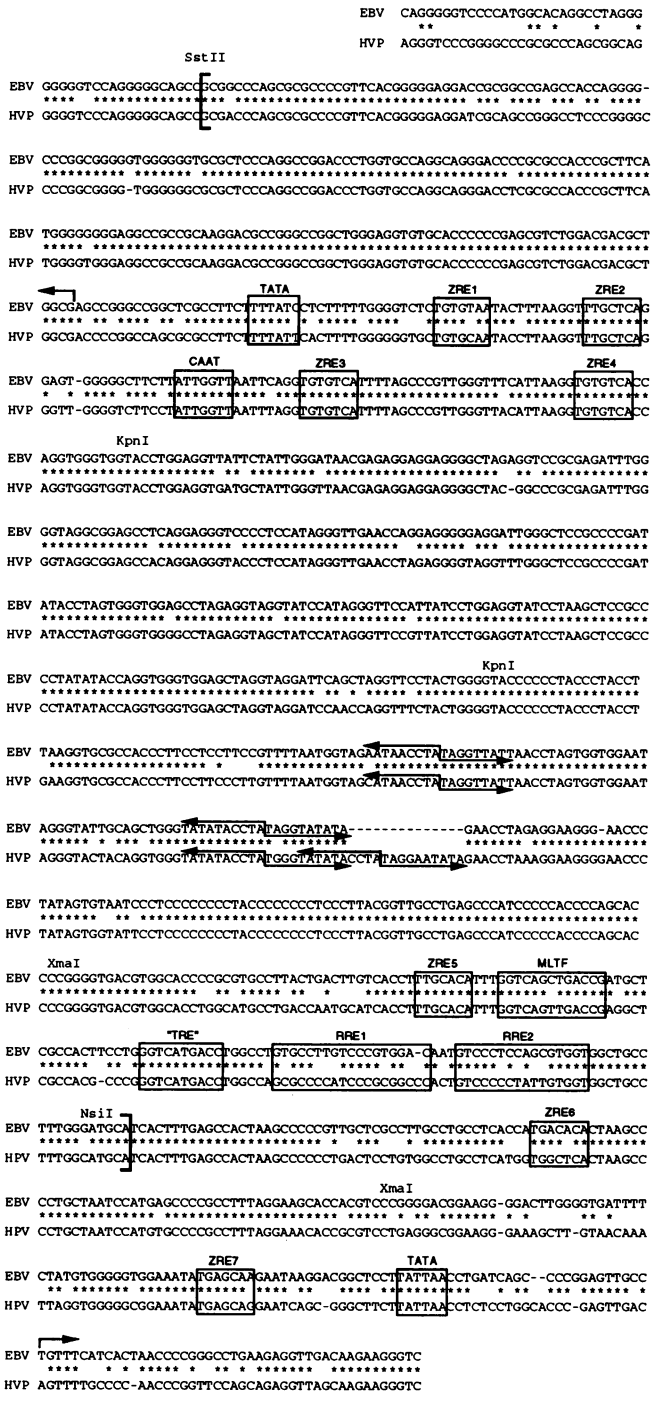

FIG. 2. Alignment of EBV B95-8 (4) and HVP sequences. Asterisks indicate identity. The boundaries of ori-Lyt are shown as bold brackets. Transcription start sites are indicated by bent arrows. Palindromes are shown as diverging arrows. Binding sites for transcription factors in the promoter and enhancer are indicated by boxed sequences. Abbreviations are defined in the text.

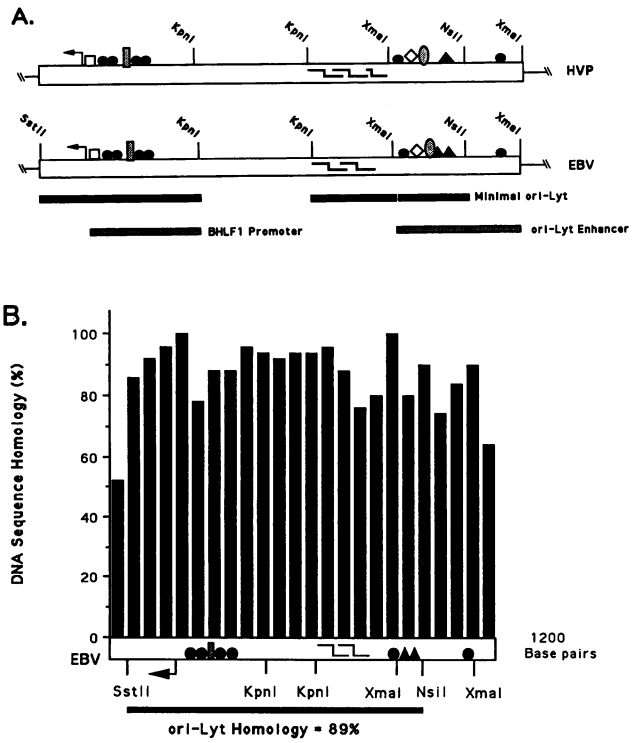

FIG. 3. (A) Comparison of the structures of ori-Lyt of EBV and HVP. The three regions required for origin function are indicated by the black bars. Locations of the BHLF1 promoter and enhancer are shown as grey bars. ●, ZRE; ▲, RRE; □, TATAA box; ▨, CAAT box; ◇, potential MLTF binding site; ⬯, putative TRE. (B) Percent homology between EBV and HVP sequences calculated for successive 50-bp blocks.

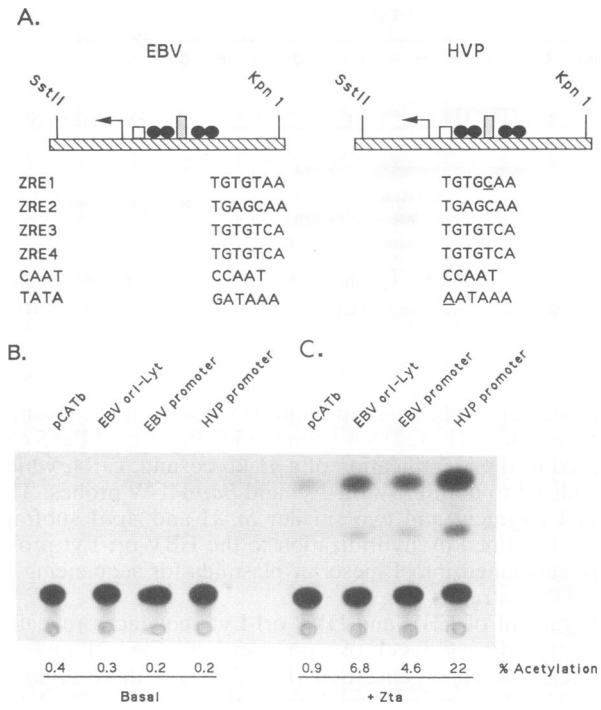

FIG. 4. (A) Comparison of transcriptional elements in the EBV and HVP ori-Lyt promoters. Nucleotide changes are underlined. ●, ZRE; □, TATAA box; ▨, CAAT box. (B and C) Activities of CAT reporter plasmids containing EBV and putative HVP promoter sequences in Vero cells. (B) Basal activities of EBV ori-Lyt-CAT (pDH123), EBV promoter-CAT (pPL52), and HVP promoter-CAT (pJR16). (C) Transactivation after cotransfection with a Zta expression plasmid.

binding site for Rta (Fig. 5A). We wondered whether these changes would alter the synergistic effect of Zta and Rta on transcriptional transactivation of the enhancer. To answer this question, we cloned the complete 260-bp enhancer from each of the viruses into a heterologous expression plasmid, E1b-CAT. These constructions extend beyond the *NsiI* site that defines the boundary of the minimal ori-Lyt to encompass the sequences (including the ZRE6 binding site) which constitute the 260-bp enhancer originally defined in transient expression assays (6, 11). When the EBV enhancer was cloned in front of the E1b promoter, cotransfection with either Zta or Rta stimulated low levels of CAT activity in the EBV-negative B-cell line DG75. A marked synergistic effect was seen when the transactivators were added together (Fig. 5B). Synergy between Zta and Rta was abrogated when the Zta binding sites (ZRE5 and ZRE6) in the EBV enhancer were mutated to prevent ligand binding (Fig. 5B, ΔZRE enhancer), indicating that cooperation between Zta and Rta requires that both proteins bind to the DNA. The HVP enhancer also showed orientation-independent synergy between Zta and Rta in B cells (Fig. 5C) and Vero cells (Fig. 5D). Thus, the changes within the second HVP Rta response element (RRE) did not affect synergy between the two transactivators.

Identification of an MLTF binding site within the EBV and HVP enhancers. A previously recognized palindromic sequence, GGTCAGCTGACC, located at the *PvuII* site in the EBV enhancer (37) was altered by one base pair to GGT CAGTTGACC in HVP. We looked for evidence of protein binding to this site by DNase I footprinting using Raji cell nuclear extracts fractionated on a heparin-agarose column. A well-defined footprint covered an area centered on this site (Fig. 6).

The EBV *PvuII* (P2) palindrome contains the core CANNTG motif recognized by helix-loop-helix group proteins and in particular resembles the binding site for MLTF (USF) found in the adenovirus major late promoter (AdML) and in the immunoglobulin M heavy-chain enhancer (μE3) (Table 1) (20). To determine whether the EBV sequence binds MLTF, we used mobility shift assays to compare binding of a Raji nuclear extract to ^{32}P-labeled 30-bp oligonucleotides containing the EBV sequence or known MLTF binding sites. Protein binding to the P2 EBV probe and to a related sequence (EBV "E") found at position 92952 in the *BamHI* E fragment of EBV upstream of the promoter for the membrane glycoprotein gp350/220 was compared with binding to the MLTF site of AdML and to the μE3 enhancer. In addition, a related site that binds another helix-loop-helix protein but does not bind MLTF, the E2 site in the immunoglobulin κ light-chain enhancer (κE2) (20), and a P2 probe (EBVΔP2) mutated to abrogate MLTF binding were tested (Fig. 7A; Table 1). In the presence of Raji nuclear extract, both EBV probes formed complexes that migrated through agarose gels with mobilities indistinguishable from those of complexes formed with the AdMLTF and μE3 probes. In contrast, the probe carrying the mutated P2 sequence, ΔP2, failed to form a protein-DNA complex, and the κE2 oligonucleotide bound poorly. To determine specificity, binding

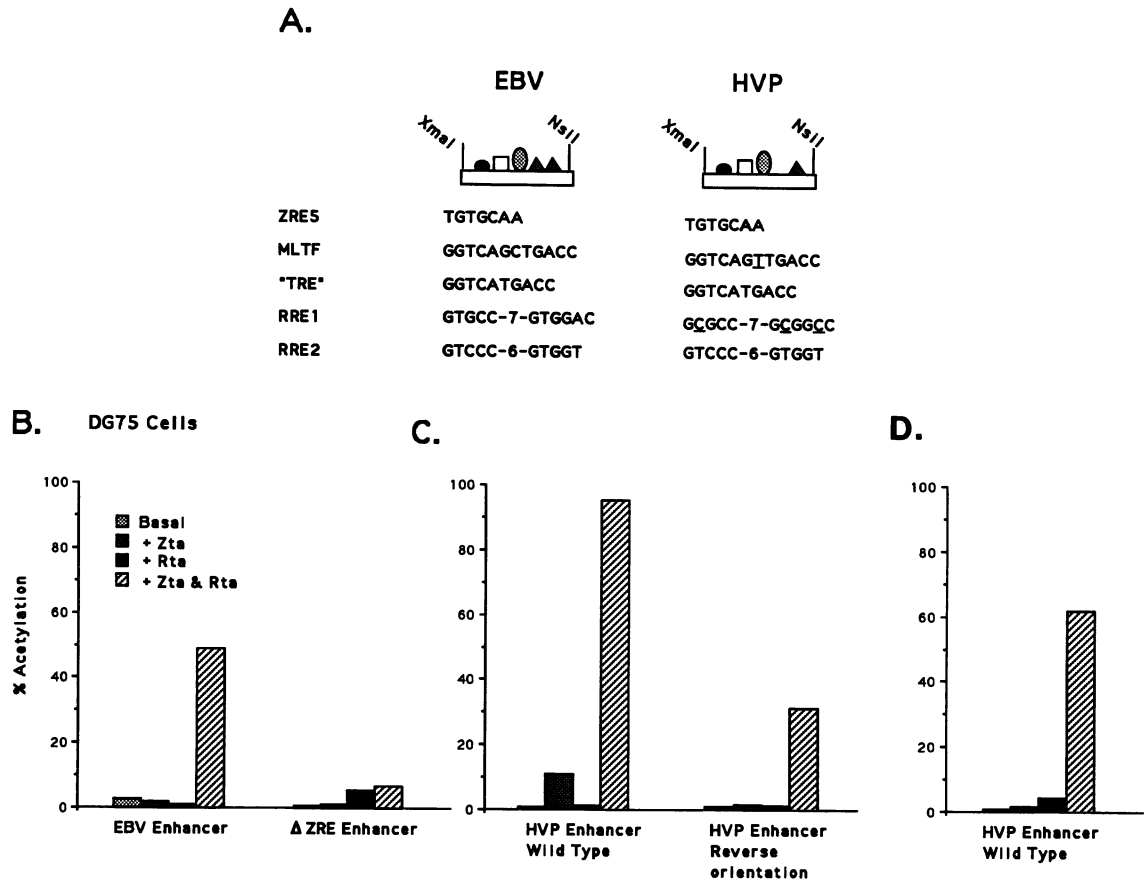

FIG. 5. (A) Comparison of transcriptional elements in the EBV and HVP ori-Lyt enhancers. Nucleotide changes are underlined. ●, ZRE; ▲, RRE; ◇, potential MLTF binding site; 0 , putative TRE; (B and C) Transactivation of the EBV and HVP enhancer-E1bCAT plasmids in EBV-negative DG75 cells. The EBV enhancer-E1bCAT plasmid (pYNC146A), an EBV enhancer-E1bCAT plasmid with mutations in the ZRE5 and ZRE6 sites (pYNC157), and an HVP enhancer-E1bCAT plasmid in wild-type (pJR18) or reverse (pJR19) orientation were cotransfected with expression plasmids for Zta, Rta, or Zta plus Rta. (D) Transactivation of the HVP enhancer in Vero cells. The plasmid constructions are as described for panels B and C. Percent acetylation of a 1:10 dilution of cell extracts is shown.

of the Raji nuclear extract was competed for with unlabeled oligonucleotides (Fig. 7B). Binding to the EBV P2 probe was completely blocked by high concentrations of unlabeled P2, AdML, EBV "E", and μE3 oligonucleotides. In contrast, the κE2 oligonucleotide and the mutated P2 oligonucleotide were unable to compete with the wild-type EBV P2 probe for binding of the protein from Raji nuclear extracts. Thus, the EBV sites bound to the same protein as did the adenovirus and μE3 sites.

To confirm the identity of the protein from Raji cells that bound to the EBV sequence, we incubated the protein-DNA complex with antibody to MLTF prior to gel electrophoresis. Addition of polyclonal MLTF antibody to the complex formed with the AdML probe resulted in the generation of two supershifted bands and a decrease in the amount of the original complex. A similar supershift pattern was seen when the MLTF antibody was incubated with the complex formed with the EBV P2 probe. In each case, some of the supershifted complex was also retained in the loading wells (Fig. 8A). The generation of two supershifted species with antibody against MLTF (USF) has been reported previously (44). The two supershifted complexes may contain different ratios of the 43- and 44-kDa polypeptides that comprise purified MLTF. We then tested the ability of the related HVP sequence to bind MLTF. Both Raji and HeLa cell extracts bound to the labeled HVP probe to produce a shifted complex with the same mobility as that formed with the EBV probe, although binding to the HVP probe was less than that to the EBV sequence, especially with the Raji cell extract (Fig. 8B). Thus, ori-Lyt of both EBV and HVP contains an MLTF binding site. The data obtained from the mobility shift assays are summarized in Table 1.

The preservation of the MLTF site within the HVP enhancer suggested that this site might be functionally important. The wild-type enhancer confers constitutive transcriptional activity in Vero cells in the absence of the EBV Zta and Rta transactivators (Fig. 9) (6). We therefore examined the role of the MLTF site in this enhancer activity. Wild-type or mutated EBV enhancer sequences were cloned into the heterologous promoter E1b-CAT construction. Transfection of the reporter plasmids and subsequent assay of CAT activity showed that the MLTF site was a major factor contributing to constitutive enhancer activity. Enhancer activation was decreased nearly 20-fold when the MLTF site was mutated singly or in combination with the Zta binding sites ZRE5 and ZRE6. In contrast, mutation of the Zta binding sites alone or of the putative TRE, whose sequence, GGTCATGACC, is similar to a consensus thyroid

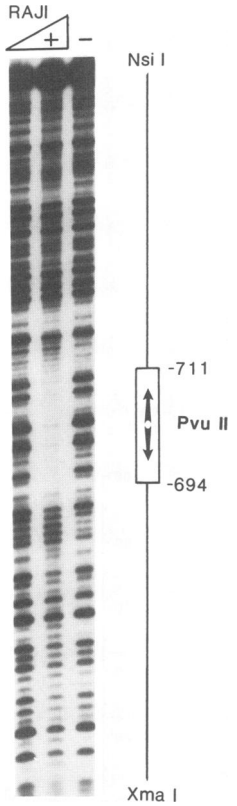

FIG. 6. DNase I footprint of the ori-Lyt enhancer. The 260-bp EBV enhancer end labeled in the polylinker sequence adjacent to the *Xma*I site was incubated with an increasing amount of fractionated Raji cell nuclear extract (+) or without extract (−) and then subjected to treatment with DNase I and electrophoresis. Location relative to the *Pvu*II restriction site and the BHLF1 RNA start site is indicated.

hormone receptor binding site sequence, decreased CAT activity only twofold (Fig. 9). The enhancer with the MLTF site mutation remained capable of a synergistic response to Zta and Rta (data not shown).

Conservation and function of the AT-rich palindromes. HVP contains conserved copies of both 18- and 20-bp AT-rich palindromes and an additional partially duplicated copy of the 20-bp palindrome. AT-rich sequences are common features of DNA origins of replication. We therefore tested the effect of deletion of these palindromes on the ability of EBV replication proteins to replicate an ori-Lyt target in transfection-replication assays. In these assays, plasmids encoding EBV replication genes and the lytic cycle transactivators Zta, Rta, and Mta were cotransfected into Vero cells along with plasmids containing either EBV or HVP ori-Lyt. DNA from transfected cells was purified and cleaved with *Bam*HI (EBV) or *Xba*I (HVP) plus *Dpn*I before electrophoresis. Replicated DNA appeared as a *Dpn*I-resistant band on Southern blots after hybridization with a probe specific for the target ori-Lyt DNA. An EBV ori-Lyt plasmid with a deletion of the two palindromes together with their intervening sequence was replicated sevenfold less efficiently than the control EBV ori-Lyt plasmid (Fig. 10A). (Quantitation of the level of replication was obtained from a densitometer tracing of the original autoradiograph.) Furthermore, EBV proteins were capable of efficiently replicating a plasmid containing the putative HVP ori-Lyt. Replication was dependent on the EBV DNA polymerase, as illustrated by the complete lack of unmethylated target DNA when the plasmid encoding the viral polymerase was omitted from the transfection mix (Fig. 10B). Thus, the structural homology between the EBV and HVP lytic origins reflects a high level of functional homology.

TABLE 1. Presence of an MLTF binding site in the ori-Lyt enhancer

Probe[a]	Sequence	Binding
MLTF (AdML)	GGCCACGTGACC	+++
EBV ori-Lyt (EBV P2)	GGTCAGCTGACC	++
HVP ori-Lyt (HVP P2)	GGTCAGTTGACC	+
EBV *Bam*HI-E (EBV "E")	TGTCACGTGACC	++
Ig enh μ E3 (μE3)	GGTCATGTGGCA	+
Ig enh κ E2 (κE2)	AGGCAGGTGGCC	−
ΔEBV ori-Lyt (EBVΔP2)	GGGAAGCTTCCC	−

[a] Ig enh, immunoglobulin enhancer.

DISCUSSION

Prior to this study, EBV ori-Lyt was the only gammaherpesvirus lytic origin that had been described. EBV ori-Lyt has no obvious sequence homology with other known alphaherpesvirus or betaherpesvirus origins. The origins of replication of the betaherpesviruses human and simian cytomegalovirus (CMV) are very large, complex, and as yet relatively uncharacterized (2, 3, 7, 23). Ori-Lyt of human CMV requires more than 2,400 bp for activity in a transient replication assay, and the origin of simian CMV spans 1,300 bp. These origins contain numerous transcription factor binding sites, clusters of repeated elements, and two large elements with dyad symmetry. Of the alphaherpesviruses, lytic replication of HSV is the best characterized (41, 49, 51). HSV contains three origins of replication, two copies of ori-S and one copy of ori-L. Ori-S and ori-L share sequence homology but differ in size: the minimal ori-L consists of a perfect 144-bp palindrome, while ori-S contains an imperfect 45-bp palindrome within a 75- to 90-bp minimal origin. Each origin contains binding sites for the virus-encoded UL9 origin-binding protein surrounding a central 18-bp AT-rich region. Transcriptional regulatory elements flank each of these minimal replication origins. Ori-L is located between the divergent promoters for the DNA polymerase and single-stranded-DNA-binding protein genes, while ori-S lies between the divergent promoters for immediate-early (IE) genes IE175 and IE68. The level of replication of a plasmid bearing ori-S is dramatically increased in transient replication assays when the transcriptional regulatory elements for the flanking IE genes are present (53).

EBV has two copies of ori-Lyt, one in DS-L and the other in DS-R. The DS-L origin lies between the divergent promoters for the BHLF1 and BHRF1 early genes. The minimal EBV ori-Lyt is also relatively large (690 bp) and complex. It comprises two domains with transcriptional regulatory function, the BHLF1 promoter and the upstream enhancer, located between which is a central region that contains related 18- and 20-bp AT-rich palindromes and a homopyrimidine cluster. Transcription from the promoter and the enhancer element is highly responsive to the EBV-encoded transactivators Zta and Rta. Thus, the organization of EBV ori-Lyt and the incorporation of response elements for viral transactivators within the minimal origin sequences distin-

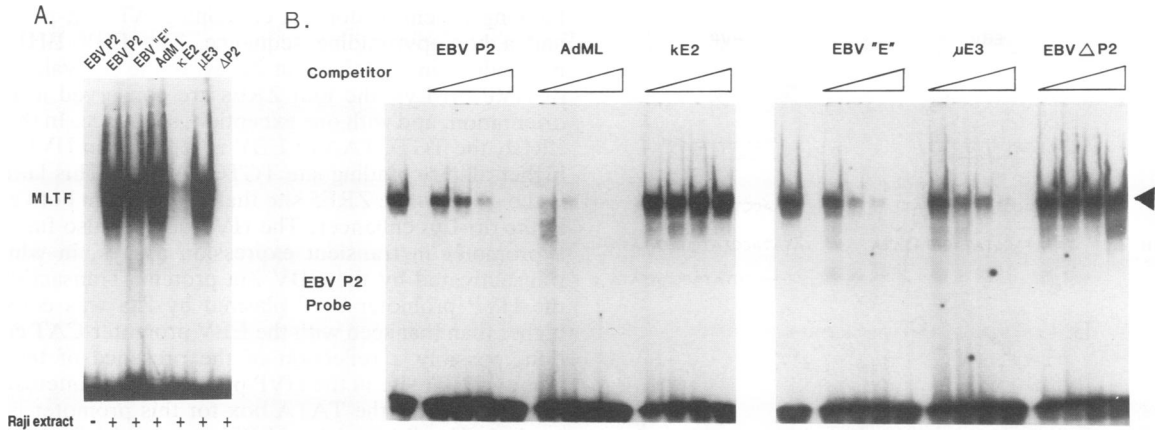

FIG. 7. (A) Mobility shift assays comparing binding of the Raji nuclear extract to the EBV ori-Lyt oligonucleotide, EBV P2, and oligonucleotides containing known or potential MLTF sites. The sequences contained within the different probes are given in Table 1. End-labeled oligonucleotides were incubated with heparin-agarose-fractionated Raji nuclear extracts, and the protein-DNA complexes were separated on a nondenaturing polyacrylamide gel. The DNA probe is given above the lanes, and the presence or absence of Raji nuclear extract is indicated below the lanes. (B) Effects of competitor oligonucleotides on binding to the EBV P2 oligonucleotide. Increasing amounts of the oligonucleotides listed above the lanes were used to compete for binding of the Raji extract to a ^{32}P-labeled EBV P2 probe. The position of the bound complex is marked with an arrowhead.

guish it from the origins of the alphaherpesviruses and betaherpesviruses. To ascertain whether the structure of EBV ori-Lyt is typical of a gamma one herpesvirus lytic origin and to obtain information on the degree of conservation and contribution of individual motifs, particularly those representing transcriptional signals, we cloned and sequenced the lytic origin from the related baboon gammaherpesvirus HVP.

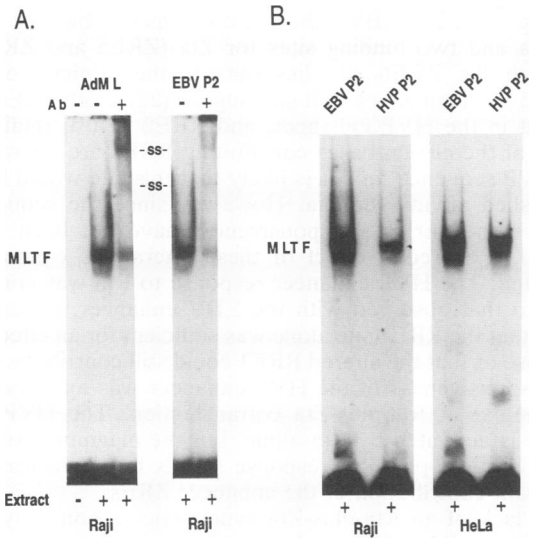

FIG. 8. Confirmation that the protein binding to the EBV P2 site is the cellular factor MLTF and demonstration of the formation of a similar complex with an HVP enhancer probe. (A) Mobility shift assay demonstrating that the shifted complex formed with Raji nuclear extract and the AdML or EBV P2 probes was supershifted (-ss-) by the addition of antibody (Ab) raised against purified MLTF. (B) Mobility shift assay using the EBV P2 and HVP P2 probes. The shifted species formed with Raji or HeLa cell extracts and the HVP probe was identical in mobility to that formed with the EBV P2 probe.

The HVP ori-Lyt proved to be highly homologous to the EBV ori-Lyt, having 89% DNA sequence identity within the boundaries of the origin, compared with 50 to 65% sequence identity in the immediate flanking sequences and 47% sequence identity in the open reading frame for the downstream EBNA-2 protein (40). The high degree of homology over the entire 950-bp (*Sst*II-*Nsi*I) ori-Lyt also contrasts with the situation in the latency replication origin, ori-P. Although the HVP and EBV latency origins are similar in general arrangement, there are significant differences in the details of their structures and a much lower level of sequence identity (42). EBV ori-P consists of 20 copies of a 30-bp repeat unit (the family of repeats) separated by 987 bp of intervening nonessential sequences from two pairs of 30-bp repeats (the dyad symmetry region) (54). The HVP ori-P consists of 10 copies of a 26-bp repeat unit separated by 764 bp of intervening sequence from a domain containing four complete copies and one partial copy of the 26-bp repeat. The intervening sequence also contains two diverged copies

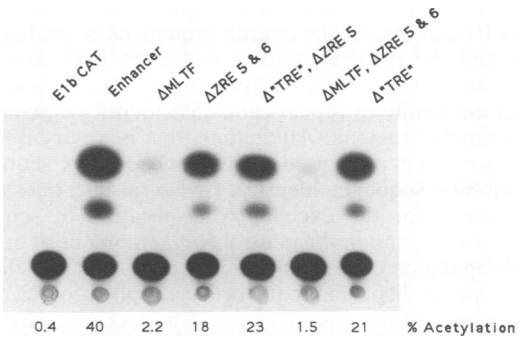

FIG. 9. Abrogation of constitutive enhancer activity upon mutation of the MLTF binding site. Vero cells were transfected with an E1b-CAT reporter plasmid or E1b-CAT carrying either the wild-type EBV ori-Lyt enhancer or enhancers with mutations in the Zta binding sites (ΔZRE), the TRE motif (Δ"TRE"), or the MLTF binding site (ΔMLTF). The percent acetylation is indicated.

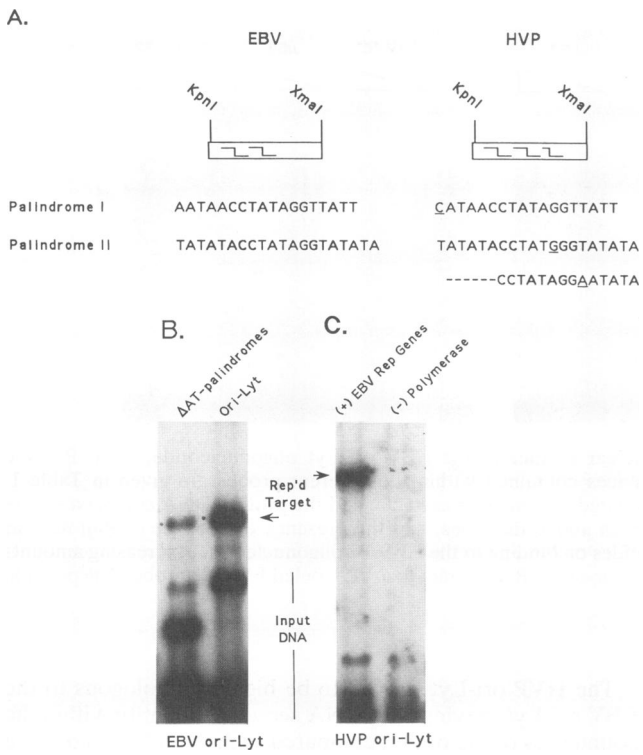

FIG. 10. Structure and function of the AT-rich palindromes. (A) Sequence comparison of the EBV and HVP AT-rich palindromes. Nucleotide changes are underlined. Palindromes are indicated by bent lines. (B and C) Cotransfection-replication assays. (B) Comparison of the replication of a control plasmid containing EBV ori-Lyt (Ori-Lyt) and an EBV ori-Lyt with a deletion of the AT-rich palindromes (ΔAT-palindromes) cotransfected with the complete set of EBV replication proteins (16). (C) Replication of a plasmid containing the HVP ori-Lyt cotransfected with the complete set of EBV replication proteins [(+) EBV Rep Genes]. No replication was observed when the EBV DNA polymerase was omitted from transfection mix [(−) Polymerase].

of the 26-bp repeat unit. The dyad symmetry element of EBV ori-P is formed from two of the repeat units together with their flanking sequences, whereas that of HVP ori-P is generated by all four repeat units and does not involve flanking DNA (42). Although there is a high level of homology between the core EBNA-1 binding sites of EBV and their HVP equivalents, the overall sequences of the family of repeats and the dyad symmetry region of EBV and HVP have a much lower degree of matching, i.e., 54% identity between the family of repeats and 55% identity between the dyad symmetry regions. Altogether, this is a very different picture from that presented by the two ori-Lyt sequences with their 89% sequence identity. It also raises a question as to the reason for the excellent conservation of sequence (93% identity) found in the central *Kpn*I fragment of ori-Lyt. This 260-bp region contains a series of repeats of the element GGAGG and is dispensable for ori-Lyt replication (22). The *Kpn*I region may negatively regulate transcription from the BHLF1 promoter (36), and the sequence identity may thus reflect preservation of transcriptional elements as opposed to replication signals.

Not only is there a high degree of sequence homology in HVP ori-Lyt, but the overall organization of the origin is conserved, with functional promoter and enhancer domains flanking a central domain containing AT-rich palindromes and a homopyrimidine sequence. The EBV BHLF1 promoter domain contains four ZREs. In the equivalent domain of HVP ori-Lyt, the four ZREs are conserved in location, orientation, and with one exception, sequence. In the case of ZRE1, the TGTGTAA of EBV is replaced in HVP with the higher-affinity binding site TGTGCAA (5). This latter motif is the same as the ZRE5 site that is present in both genomes in the ori-Lyt enhancer. The HVP domain also functions as a promoter in transient expression assays, in which it is transactivated by the EBV Zta protein. Transactivation of the HVP promoter-CAT plasmid by Zta was consistently higher than that seen with the EBV promoter-CAT construction, possibly a reflection of the presence of the higher-affinity ZRE1 site in the HVP promoter. It is interesting that in both viruses, the TATA box for this promoter is noncanonical. The GATAAA of EBV and the AATAAA of HVP would be anticipated to have a relatively low affinity for TBP, whose binding provides the initial step in the formation of the transcription initiation complex. Certainly both promoters have extremely low basal transcriptional activities. Zta has been shown to contact TBP and stabilize its binding to the GATAAA sequence (35). The end result is a promoter that is tightly regulated by and highly responsive to Zta. Replication origins are commonly associated with transcriptional regulatory elements (13). One of the functions that transcription factors serve in replication may be to render the origin sequences available to the replication complex by interfering with nucleosome assembly (8, 9). The promoter domain of EBV and HVP ori-Lyt is thus ideally designed to exist in a fully repressed state during latency and to be highly activated during the lytic cycle by the very viral Zta protein that is responsible for triggering lytic cycle gene expression (10).

HVP ori-Lyt also retains a functional enhancer domain with a highly conserved arrangement of transcription factor binding sites. The EBV enhancer contains two binding sites for Rta and two binding sites for Zta (ZRE5 and ZRE6), although the ZRE6 site lies outside the minimal origin defined by Hammerschmidt and Sugden (22). Both ZREs are present in the HVP enhancer, and RRE2 is also retained. Three of the eleven bases constituting RRE1 are altered in the HVP sequence, and it is likely that this site would have diminished affinity for Rta. However, since the sequence requirements for Rta responsiveness have not been fully defined, the precise effect of these alterations cannot be predicted. The HVP enhancer response to Rta was comparable to that observed with the EBV enhancer, indicating either that the RRE2 site alone was sufficient for an effective response or that the altered RRE1 could still contribute. The Rta responsiveness of the HVP enhancer was examined in the context of Rta-plus-Zta cotransfection. The HVP enhancer is activated by Rta alone, but the magnitude of the synergistic Zta-plus-Rta response serves to emphasize the important contribution of the enhancer ZREs.

The lack of an Rta-plus-Zta synergistic response by the control ΔZRE5-ΔZRE6 enhancer is interesting since the enhancer contains an additional potential Zta binding site, TGAGCCA, immediately adjacent to the *Nsi* restriction site at the right-hand boundary of ori-Lyt. This same sequence occurs in the Zta promoter (ZRE IIIb), and DNase I footprinting of this promoter indicated that Zta binds to the IIIb sequence (17, 34). In the present experiments, when the ZRE IIIb site was the only ZRE remaining in the enhancer, no synergistic response between Zta and Rta was observed. This result is consistent with weak binding of Zta to the ZRE

IIIb site and further emphasizes the gradient of Zta responsiveness that is conferred by the presence of multiple ZREs of differing affinities in the regulatory regions of the EBV lytic genes.

Both the EBV and HVP enhancers contain palindromic sequences, GGTCAGCTGACC (EBV) and GGTCAGT TGACC (HVP), whose function was previously unknown. A Raji nuclear extract fractionated on a heparin-agarose column gave a DNase I footprint centered over this sequence in the EBV enhancer. Mobility shift assays using the same nuclear extract indicated that the bound protein was the ubiquitous cell transcription factor MLTF (USF). MLTF is a member of the c-Myc/MyoD/E12 family of helix-loop-helix plus leucine zipper proteins and, like c-Myc, binds to the core sequence -CACGTG- (20). However, MLTF tolerates the change to the -CAGCTG- sequence found in the EBV enhancer site, whereas the functional E12:E47 and c-Myc: c-Max heterodimers, which are both likely to be present in Raji cells, are not expected bind to this sequence (45). MLTF binds the HVP enhancer site with its central -GT- pairing with an intermediate affinity similar to that seen with the μE3 sequence, which carries a central -TG- pair. A cooperative interaction has been demonstrated between MLTF and the TATA-binding protein TFIID (47). Since MLTF has a role in the expression of several tissue specific and developmentally regulated genes, it is believed that MLTF can interact with cell-type-specific as well as general basal transcription factors (20). It had previously been noted that the ori-Lyt enhancer, in addition to its Zta- and Rta-responsive enhancer activity, had constitutive enhancer activity in epithelial cells in the absence of the viral transactivators (6). Using an EBV enhancer that had been modified by site-directed mutagenesis, we demonstrated that the MLTF binding site was largely responsible for this constitutive enhancer activity. A similar consensus palindromic MLTF binding site is also present in the CMV lytic origin (23), but its contribution in CMV has not yet been examined.

Comparison of the EBV and HVP ori-Lyt DNAs revealed a single example of a sequence duplication event. In the central domain of HVP ori-Lyt, the second AT-rich palindrome is partially duplicated to create a third overlapping palindrome. AT-rich sequences are present in many origins of replication, where they are believed to facilitate strand separation. Mutation of individual bases in the AT-rich region of the SV40 origin to G or C resulted in a reduced efficiency of DNA unwinding by T antigen and a corresponding reduction in DNA replication to 0 to 20% of wild-type levels (50). The HSV origin contains an 18-bp AT-rich sequence. Conversion to a GC sequence or reduction to a single AT pair abolishes replication (41). In the case of EBV ori-Lyt, deletion of both copies of the AT-rich palindromes resulted in an origin that replicated with less than 20% of the wild-type efficiency.

The structure of HVP ori-Lyt with its conserved promoter and enhancer domains that respond to the viral Zta and Rta transactivators reemphasizes the potential of the Zta and Rta proteins to regulate not only viral lytic gene transcription but also lytic DNA replication. The contribution of Zta and Rta to ori-Lyt replication is being further explored in cotransfection-replication assays (16a).

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