Transcriptional Activation Signals Found in the Epstein-Barr Virus (EBV) Latency C Promoter Are Conserved in the Latency C Promoter Sequences from Baboon and Rhesus Monkey EBV-Like Lymphocryptoviruses (Cercopithicine Herpesviruses 12 and 15)

EZEQUIEL M. FUENTES-PANANA´, 1 SANKAR SWAMINATHAN, 2 AND PAUL D. LING 1 *  
Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030, 1 and Sealy Center for Oncology and Hematology and Division of Infectious Diseases, Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas 77555-1048 2  
Received 10 August 1998/Accepted 7 October 1998

The Epstein-Barr virus (EBV) EBNA2 protein is a transcriptional activator that controls viral latent gene expression and is essential for EBV-driven B-cell immortalization. EBNA2 is expressed from the viral C promoter (Cp) and regulates its own expression by activating Cp through interaction with the cellular DNA binding protein CBF1. Through regulation of Cp and EBNA2 expression, EBV controls the pattern of latent protein expression and the type of latency established. To gain further insight into the important regulatory elements that modulate Cp usage, we isolated and sequenced the Cp regions corresponding to nucleotides 10251 to 11479 of the EBV genome (−1079 to +144 relative to the transcription initiation site) from the EBV-like lymphocryptoviruses found in baboons (herpesvirus papio; HVP) and Rhesus macaques (RhEBV). Sequence comparison of the approximately 1,230-bp Cp regions from these primate viruses revealed that EBV and HVP Cp sequences are 64% conserved, EBV and RhEBV Cp sequences are 66% conserved, and HVP and RhEBV Cp sequences are 65% conserved relative to each other. Approximately 50% of the residues are conserved among all three sequences, yet all three viruses have retained response elements for glucocorticoids, twopositionally conserved CCAAT boxes, and positionally conserved TATA boxes. The putative EBNA2 100-bp enhancers within these promoters contain 54 conserved residues, and the binding sites for CBF1 and CBF2 are well conserved. Cp usage in the HVP- and RhEBV-transformed cell lines was detected by S1 nuclease protection analysis. Transient-transfection analysis showed that promoters of both HVP and RhEBV are responsive to EBNA2 and that they bind CBF1 and CBF2 in gel mobility shift assays. These results suggest that similar mechanisms for regulation of latent gene expression are conserved among the EBV-related lymphocryptoviruses found in nonhuman primates.

Epstein-Barr virus (EBV) is a human lymphocryptovirus associated with various malignancies, including Burkitt’s lymphoma (BL), Hodgkin’s disease, nasopharyngeal carcinoma, and lymphomas in immunosuppressed individuals (34). EBV establishes a lifelong infection in the human host, where B lymphocytes are the primary latent reservoir from which occasional virus reactivation occurs (34).

EBV also establishes latent infection in B lymphocytes in vitro and transforms them into continuously proliferating lymphoblastoid cell lines (LCLs). Molecular genetic techniques have demonstrated that several EBV nuclear antigens (EBNAs) and an integral membrane protein (LMP1) are required for lymphocyte transformation in vitro (19, 36). EBNA2 plays a central role in the process of transformation, as it activates the expression of the other EBNAs, LMP1 and LMP2, proteins, and various cellular proteins associated with the transformed phenotype (1, 5, 7, 20, 25, 35, 41, 52–56, 60, 61).

The regulation of EBNA expression in EBV-infected cells is likely to be important in viral persistence and the development of EBV-associated malignancies. With the exception of EBNA1, all EBNAs possess epitopes which induce a strong cytotoxic T-cell response (22, 34). Downregulation of EBNA expression may allow a latently infected B cell to escape immune surveillance and also permit proliferation of malignant EBV-infected cells. In healthy individuals, latent EBV infection appears to be primarily confined to resting B cells. The only EBV gene expressed in these cells is LMP2a, a pattern of gene expression termed latency 0 (29, 30, 51). In BL cells, only EBNA1 is expressed (latency I) (34, 51). In Hodgkin’s disease, nasopharyngeal carcinoma, and T-cell lymphomas, EBNA1 and one or both LMPs are expressed (latency II) (34, 51). All EBNAs and LMPs are expressed during acute infectious mononucleosis, in lymphoproliferative syndromes in immunocompromised individuals, and in LCLs (latency III) (34, 51). The association of these specific patterns of EBNA expression with various physiologic and pathologic states underscores the importance of EBNA gene regulation in EBV persistence and EBV-associated oncogenesis.

Upon in vitro infection of B lymphocytes, the first EBV genes expressed are EBNA-LP and EBNA2, transcribed from the W promoter (Wp) as bicistronic mRNAs (2, 3, 40, 56). Thirty-six hours postinfection, transcription of EBNA2 and the other EBNAs switches to the upstream C promoter (Cp) (2, 3, 40, 57). EBNA2 protein is first detectable at 12 h postinfection and reaches maximal levels between 32 and 46 h (2). These findings are consistent with a role for EBNA2 in activating Cp.
and mediating the switch from Wp transcription. Elucidating the mechanisms that regulate transcription of EBNA2 and the other EBNA\textsubscript{s} is therefore important for understanding the process of EBV-mediated B-lymphocyte transformation.

Transcription from Cp leads to concomitant expression of EBNA2 and the other EBNA\textsubscript{s}. Thus, Cp activity is the major difference between latency III and the other latency programs. Several mechanisms that may regulate Cp activity have been described. First, EBNA1 binds to an enhancer 3 kb upstream of Cp and activates Cp in transfection assays (32, 33, 47). Second, glucocorticoid response elements (GRE\textsubscript{s}) about 900 bp upstream of Cp respond to glucocorticoids and activate Cp in vitro but are not required for transformation in vitro (21, 45, 46, 49). Transient transfections have also demonstrated that the Cp GRE\textsubscript{s} are capable of responding to glucocorticoids (21, 45, 49). Third, the EBNA2-responsive enhancer (E2RE) in Cp is critical for Cp function in vitro and in vivo (17, 23, 48, 59). Deletion of E2RE from the viral genome results in loss of Cp activity in infected cells (59). Finally, methylation of CpG sequences in the promoter region downregulates Cp (28, 38, 44). Whereas LCLs (latency III) show no evidence of Cp methylation, B cells in latencies I and II are hypermethylated in different regions of Cp (28, 38, 44). Consistent with a role for methylation in the silencing of Cp, in vitro treatment of type I BL cells with 5-azaacytidine, an inhibitor of DNA methylation, led to activation of Cp (27, 38).

In order to gain further insight into the significance of Cp and the transcriptional elements that regulate its expression, a segment of the genome from the BCRF1 poly(A) site to the end of the first Cp exon was sequenced from the EBV-like lymphocryptoviruses herpesvirus papio (HVP) and rhesus macaque EBV (RhEBV). These sequences were compared to EBV sequences, and the abilities of these promoters to respond to EBNA2 and to bind CBF1 and CBF2 cellular proteins were evaluated.

DNA sequences of the Cps from HVP and RhEBV and their alignment with the EBV Cp. Several cell lines were used for our studies. DG75 and CA46 are EBV-negative BL cell lines. B95-8 is a type 1-EBV-transformed marmoset cell line, and P3HR1 is a type 2-EBV-transformed cell line containing an immortalization-defective EBV strain. 26CB-1 (obtained from the American Type Culture Collection as CRL-1495) and Baf65 are baboon cell lines transformed with HVP (12), H254 (gift from Micheal Murphy-Corb) and LCL 8664 (CRL-1805) are rhesus macaque cell lines transformed with RhEBV. Previous sequence analysis of a cosmid clone derived from the Baf65 cell line revealed sequences identical to B95-8 EBV DNA in the C1 end of the HVP Cp (unpublished data and reference 25). Based on this and preliminary analysis by rapid amplification of cDNA 5\textsuperscript{\textprime} ends of the BCRF2 open reading frame of HVP (unpublished observations), PCR primers that would amplify the putative BCRF2 and Cp sequences from HVP were designed. Briefly, 26CB-1 or Baf65 cells were lysed in PBS–1% Triton X-100, boiled, and treated with proteinase K for 1 h. Samples were boiled again for 10 min, and the cleared lysates were used for PCR analysis. From each of the cell lines a 2.0-kb DNA molecule was amplified and cloned directly into pT7Blue-2 (Novagen). The resulting plasmids, pCRL-3 and pBaf65 Cp, were derived from 26CB-1 and Baf65 cells, respectively. Plasmid inserts were sequenced on both strands with an automated sequencing system (Applied Biosystems). With the sequence information from the HVP clones, two primers were designed for common sequences also present in EBV that amplify RhEBV Cp sequences from the end of the BCRF2 open reading frame to the end of the putative C1 exon. LCL 8664 and H254 were lysed, and DNAs were prepared for PCR amplification as described above. Two clones, pPDL 380A and pPDL 380B, from PCR products of independent cell preparations from H254 were obtained. Two clones, pPDL 394A and pPDL 394B, were also obtained from LCL 8664 in a similar manner. During all PCR amplifications, control samples containing all essential PCR components except cell lysates were consistently negative. Both strands of the RhEBV plasmid inserts were also sequenced with an Applied Biosystems automated sequencing system.

For HVP, clones pCRL-3 and pBaf65 Cp were sequenced. The sequence for CRL-3 is shown in Fig. 1A. Six nucleotide differences were observed between these particular isolates and are listed in Fig. 1B. Since only one clone was analyzed from each cell line, it is unclear whether these changes are strain specific or the result of PCR error. For RhEBV, both clones derived from the H254 cell line in independent PCRs were identical. Surprisingly, clones isolated from RhEBV LCL 8664 were also identical in sequence to those derived from H254. Both cell lines were established at the Delta Regional Primate Center and may contain identical or similar viral strains. While the passage history of H254 is somewhat unclear, it may have been established from a virus obtained from LCL 8664 or an animal in the same colony. A sequence alignment of the Cp sequences from the three related viruses is shown in Fig. 1A.

Sequence comparison of the Cps derived from these primate viruses reveals that EBV and HVP Cp sequences are 64% conserved, EBV and RhEBV Cp sequences are 66% conserved, and HVP and RhEBV Cp sequences are 65% conserved relative to each other. The overall GC content of each sequence is 52, 52, and 54% for the EBV, HVP, and RhEBV Cps, respectively. Several sequence elements in the EBV Cp that have been proposed to be important for promoter function have been described. Among them are three GRE\textsubscript{s} (GRE I, II, and III), E2RE, distal and proximal CCAAT boxes, and the TATA box. The sequence alignment in Fig. 1 shows that there are four regions that contain clusters of either functional or putative transcription factor binding sites (regions 1, 3, and 4 [R1, R3, and R4, respectively] and a clustered region containing a high concentration of CpG dinucleotides (R2). R1 contains putative binding sites for PEA-3 as well as a TPA response element (TRE) (8, 13, 26). Three GRE\textsubscript{s} have been described in the EBV Cp, which is localized about 900 bp upstream of the transcription initiation site (21, 45). The in vivo importance of these elements remains unclear. Only one of these GRE\textsubscript{s} is conserved in both HVP and RhEBV (GRE III). However, HVP Cp has one other potential GRE localized just upstream of GRE I while RhEBV appears to have positionally retained GRE I. Neither prime sequence appears to have conserved GRE II.

R2 contains an island of CpGs in all three sequences even though the levels of sequence homology are significantly diverged between EBV and the primate viruses. The overall GC content of R2 is slightly higher than for the Cp overall and is 65, 63, and 58% for EBV, HVP, and RhEBV, respectively. However, while the average CpG frequencies for the Cp sequences in Fig. 1 are 4.3, 3.3, and 4.0% for EBV, HVP, and RhEBV, respectively, the CpG frequencies nearly double or triple in R2 (11.0, 9.0, and 8.0% for EBV, HVP, and RhEBV, respectively). In Cp-negative cell lines such as Rael, nearly all CpG within the first upstream 400 bp proximal to the transcription initiation site are methylated (37, 38, 44). Extensive analysis of CpG methylation within R2 has not been performed with these cells. Addition of 5-azaacytidine, which inhibits methyltransferases, results in demethylation and activation of Cp (27, 38). In addition, previous studies have shown that meth-
ylation primarily targets two regions, called methylation-hyper-sensitive region I (MHRI; which encompasses the EBNA2 enhancer) and MHRII (from 220 to 25 or positions 826 to 1023 in Fig. 1) (37). MHRI seems to control Cp transcriptional activation by EBNA2, while MHRII controls the basal activity of the promoter. The reported important CpG elements in MHRI are localized downstream of the CBF2 binding site, and these sites do not appear to be conserved. However, some of the potential methylation sites present in MHRII are conserved in both HVP and RhEBV.

R3 is part of the EBNA2 enhancer and contains the binding sites for the cellular DNA binding proteins CBF1 and CBF2 (11, 24). The CBF1 recognition sequence is fully conserved in all three promoters, supporting the important role of this protein in Cp activation by EBNA2. We have recently published that the most critical region contributing to CBF2 binding is the GGTTCA sequence found downstream of the CBF1 site (11). In addition to this sequence, the surrounding nucleotides also contribute to the affinity of binding. With the exception of a G→C change found in the primate viral sequences, these sites do not appear to be conserved. However, some of the potential methylation sites present in MHRII are conserved in both HVP and RhEBV.

FIG. 1. (A) Nucleotide sequences of aligned EBV, HVP, and RhEBV Cp sequences from the poly(A)′ site of BCR2 to the end of the C1 exon. Asterisks indicate conserved nucleotides. Binding sites for transcription factors are indicated by boxed sequences. The boundaries of the EBNA2 enhancer are indicated with brackets inside the sequence diagram, and the different functional regions of the Cp are denoted with brackets outside the sequence diagram and are labeled as R1 to R4. The horizontal arrow above the bases shown in bold indicates the transcriptional initiation site. The CpG dinucleotides are underlined. (B) Nucleotide differences between HVP Cp sequences obtained from the 26CB-1 and Ba65 cell lines. Shown are the nucleotide positions from panel A where base differences were detected and the nucleotides present in each strain.
our mutagenesis results are consistent with the fact that critical sequences required for CBF2 binding are conserved. A distal CCAAT box identified as functionally significant is also conserved (32). Also within this region is a putative Sp1 binding site.

R4 contains the proximal CCAAT box, TATA box, and the transcription initiation site. Just downstream of the TATA box a conserved NFI site is present (15). The conservation of sequences surrounding these important elements suggests that they are also important for Cp expression.

**Analysis of Cp expression from HVP- and RhEBV-transformed B lymphocytes.** Analysis of Cp usage and mapping of the site for initiation of transcription in HVP and RhEBV B cell lines were performed by S1 nuclease protection analysis. Based on the sequence analysis in Fig. 1, oligonucleotide probes that could detect Cp usage in the primate and EBV-based species were designed. The probes were tested in transient transfections using HVP and RhEBV B cell lines. Oligonucleotides were prepared by chemical synthesis (version 2.0; Invitrogen). Fifty to 100 fmol of kinased oligonucleotide was hybridized to 2 μg of poly(A) + RNA in 50 μl of hybridization buffer {40 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.4], 1 mM EDTA, 0.4 M NaCl, 0.1% sodium dodecyl sulfate, 50% (vol/vol) formamide} at 37°C for 12 h. The hybridization reaction mixture was diluted to 400 μl with buffer with final concentrations of 0.28 M NaCl, 50 mM NaOAc (pH 4.6), and 4.5 mM ZnSO4 and digested with 20 U of S1 nuclease (Gibco BRL) for 30 min at 37°C. The reaction mixtures were phenol-chloroform extracted and ethanol precipitated. The resulting precipitate was resuspended in loading buffer and fractionated on a 10% denaturing polyacrylamide gel.

As shown in Fig. 2A, an EBV-specific probe detected transcripts from B95-8 (type 1 EBV) of the predicted sizes that were not detected in the EBV-negative B-cell line DG75 or the other baboon or macaque cell lines. This result is consistent with the results of other studies, and the start site is indicated in Fig. 1 (4, 57). In contrast to previously published work, we detected a minor RNA species in both B95-8 and P3HR1 that was approximately 3 bp larger than the major 44- and 45-bp protected fragments. More stringent hybridization conditions failed to abolish these protected species. It is unclear at this time whether these RNAs were derived from the Cp or were some other cross-reactive RNA derived from another part of the viral genome. Wp-specific probes detected W-specific transcripts from B95-8 and RhEBV-transformed cells with the Cp probe specific for those viruses. Again the minor RNA species detected in both B95-8 and P3HR1 were also detected with the HVP Cp probe but not the RhEBV Cp probe. The HVP probe also had some nonspecific hybridization with apparent cellular RNA that appears as a ladder of bands in both virus-positive and -negative cells. As a control for RNA abundance and quality, a cellular α-actin probe was also used to detect α-actin RNA in the cell lines (Fig. 2D). The transcription start sites for HVP and RhEBV are indicated in Fig. 1. Even though there is some sequence divergence around the EBV transcription initiation site and among the TATA boxes, it appears that HVP- and RhEBV-transformed cells also initiate Cp transcripts at an analogous location.

**Analysis of CBF1 and CBF2 binding to the HVP and RhEBV Cp sequences.** The sequence alignment of Fig. 1A shows that the EBNA2 enhancer lies in R3. The CBF1 binding site is fully conserved in HVP and RhEBV. The CBF2 binding site has a single change in a critical region for binding. Electrophoretic mobility shift assays (EMSA) were performed as described previously to test the ability of these enhancers to bind CBF1 and CBF2.

By using the full-length HVP and RhEBV EBNA2 enhancers as probes, we found that CBF1 binds with high affinity and that EBNA2 interacts with CBF1 as detected in gel mobility shift assays (data not shown). The CBF2 activity associated with the EBV Cp enhancer was stronger than that associated with the HVP and RhEBV enhancers, indicating that these promoters possess a reduced affinity for this protein.
To quantify the affinity of CBF2 for the response elements present in HVP and RhEBV, competition assays were performed. The affinities of the HVP and RhEBV CBF2 sites were measured by quantifying their ability to compete for CBF2 binding on the EBV sequence. An example of the competitions is shown in Fig. 3A, and a summary of the data is presented in Fig. 3B. This analysis showed that while only 3.2 nM EBV CBF2 oligonucleotide is required to compete 50% of the binding, 15.7 and 12.5 nM HVP and RhEBV oligonucleotides, respectively, are required for the same level of competition. This represents 4.7-fold (HVP) and 3.9-fold (RhEBV) reduced affinities for binding compared with that for the EBV binding site.

We have previously published that a double transversion mutation in the EBV CBF2 binding site from TT to GG (GGTTCA to GGGGCA) abolishes CBF2 binding (11). When this mutation is introduced into the Cp, the ability to respond to EBNA2 transactivation in transient-transfection assays is strongly reduced. Oligonucleotides representing the CBF2 binding site sequences from HVP and RhEBV were synthesized, and a TT→GG change was introduced into both the HVP and RhEBV CBF2 sequences (Fig. 3B). These mutants were called HVP mut.1 CBF2 and RhEBV mut.1 CBF2 (Fig. 3B). Interestingly, the HVP sequence has two putative CBF2 binding sites. One site lies in the same position as in EBV, and the other site is 3 nucleotides downstream of the first site (site 1, GCTTCA, and site 2, GGTTGA) (Fig. 3B). An oligonucleotide carrying the TT→GG change in the putative second site was also synthesized, and the resulting mutant was called HVP mut.2 CBF2. This mutant was also analyzed in the competition experiments, as shown in Fig. 3B. Competition experiments with HVP mut.1 and mut.2 showed that, as in EBV, changes in the TTGGCA sequence result in decreased affinity for CBF2. RhEBV mut.1 had a 25-fold-reduced binding affinity. In HVP, both sites were found to contribute to CBF2 binding, with the upstream site being the most important. Mutation of the first site completely abolished the binding, while mutation of the downstream site did not abolish binding although it led to a 2.7-fold-reduced activity compared with that of the HVP wild-type sequence.

Figure 3C shows the direct binding of HVP CBF2 wild-type
and mutant oligonucleotides in the gel retardation assay. As expected, HVP mut.1 CBF2 was not able to bind CBF2 (lane 7) while HVP mut.2 was still able to bind but with reduced affinity (lane 10). These results confirm that, although the upstream site is the most important for binding, the second site contributes to the binding of the wild-type sequences.

**Activation of HVP and RhEBV Cp by EBNA2.** The 1-kb regions from the HVP and RhEBV sequences that correspond to the EBV Cp construction (Cp −1021 to +3) were PCR amplified, and these fragments were cloned upstream of the luciferase reporter gene in the pGL3-Basic vector (Promega). DNA transfections were carried out by using the DEAE dextran method. DG75 cells (5 × 10^6) were transfected with 2 μg of target plasmid and the amounts of effector plasmid indicated in the legend to Fig. 3. The final DEAE dextran concentration in the transfection reaction mixture was 1 mg/ml. Transfected cells were harvested after 2 days of incubation and lysed, and the luciferase activity was measured according to the instructions of the manufacturer. A constitutively expressing chloramphenicol acetyltransferase reporter vector (pCAT control; Promega) was used as the internal control for transfections, and the values from luciferase assays were normalized to the chloramphenicol acetyltransferase activity.

DG75 cells were transfected with the Cp constructs, and their ability to respond to an EBNA2-expressing plasmid was evaluated. EBNA2 activated the EBV Cp to a maximum of 11-fold, while it activated the HVP and RhEBV promoters 6-fold and 9-fold, respectively (Fig. 4). At the highest doses of EBNA2 these differences between the promoters are not statistically significant. At lower doses of EBNA2 (0.8 and 1.6 μg of EBNA2), there was a statistically significant difference in the ability of EBNA2 to transactivate the EBV Cp about 5-fold and it was able to transactivate the HVP and RhEBV Cp promoters only 1.5- to 3-fold.

In summary, to gain further insight into the regulatory elements that modulate the expression of the viral latency Cp, the Cp regions corresponding to nucleotides 10251 to 11479 of the EBV genome (+144 to −1079 relative to the transcription initiation site) from the EBV-like lymphocryptoviruses HVP and RhEBV were cloned and sequenced. The Cp regions from these viruses are about 65% identical. Earlier studies have shown that HVP and EBV have approximately 40% overall DNA homology and indicate that Cp is more highly conserved than the rest of the genomes (9, 16). No experiments to determine general hybridization kinetics between EBV and RhEBV have been carried out. Among some of the HVP gene sequences, the DNA identities between LMP1 (43%), Fp (also called Op) (86%), EBNA1 (63%), and ori-Lyt (89%) range from 43 to 89% compared to the identities between similar regions in EBV (10, 42, 43, 58). The RhEBV sequences for LMP1 (44%), Fp/Op (80%), EBNA2 (41%), and GP350 (57%) range from 44 to 80% compared to the identities between similar regions of EBV (10, 14, 31, 43). While these genes represent only a small portion of the genome, it appears that Cps are somewhat less homologous than other regulatory elements but more homologous than latent protein coding regions. The significance of this is unclear, since the evolutionary pressures that are present for regulatory regions may be different than those for coding regions or genes involved in the lytic cycle. The fact that Cp is generally more conserved than the rest of the genome suggests that there are universal regulatory elements within Cp which are required for the virus life cycle.

An alignment of these sequences suggests that the function and regulation of these promoters have been conserved. Initial surveys of the individual Cp sequences by computer analysis reveals that there are many potential transcription factor binding sites in each promoter. Given the relative degrees of homology between EBV and the other lymphocryptoviruses, we reasoned that crucial cis-acting elements required for Cp function are conserved between these viral promoters. Using these criteria, we have identified three regions within the Cp that contain clusters of conserved transcription factor binding sites and a fourth region that contains a high frequency of CpG residues. For ease of discussion we have called these regions R1 to R4.

Previous studies identified three GREs in the EBV Cp located between nucleotides 10240 and 10440 (−1077 to −894 relative to the transcription start site) of the EBV genome and demonstrated that this region could confer responsiveness to glucocorticoids both in the context of Cp and when fused to a heterologous promoter (21, 45). In contrast, using a large plasmid construction containing both Cp and OriP, Woisetschlaeger et al. found that deletions of sequences containing the GRE had little overall effect on basal or activated promoter activity in transient-transfection experiments (56). Finally, recombinant EBV containing a deletion encompassing the three GREs display on average fivefold-higher expression from Cp than wild-type recombinant viruses (6). In light of these data, the biological role of the GRE remains unclear. R1 contains two conserved glucocorticoid elements. One of these, GRE III, appears to be positionally conserved, while GRE I in the HVP Cp is not. No obvious counterpart to EBV GRE II was identified in our sequence comparisons. While the role of glucocorticoids in modulating Cp activity during the immortalization process remains unclear, the conservation of two GRE sites suggests an important role for these elements in vivo. Two other conserved sequence elements that resemble ETS/PEA3 and TRE were also identified in R1 (8, 13, 26). The roles these factors have for Cp function remain unknown, but previous results obtained with mutants with R1 deleted may also be due to deletion of ETS or TRE. Introduction of more subtle point mutations in the GRE and putative ETS and TRE sites will clarify the role of these control elements in Cp function.

Several studies have described a role for CpG methylation in silencing of Cp activity (28, 37–39, 44, 50). CpG methylation of the EBNA2 enhancer region as well as downstream sequences from the enhancer to the TATA box approaches 100% of available CpG in Rael cells, a type I-phenotype cell line (38,
Using a methylated cassette assay, Robertson and Ambinder have demonstrated that CpG methylation of Cp sequences in the EBNA2 enhancer silence EBNA2-mediated Cp activity (37). In addition, CpG methylation from the EBNA2 enhancer downstream to the TATA box abolished Cp activity completely in transient-transfection assays (37). Site-specific methylation has been proposed to inhibit binding of cellular factors required for Cp activity (37, 38). R2 contains a high proportion of CpG sites. However, these previous studies have not shown a role for this region in regulation of Cp activity. A recent analysis of the methylation sites in two Mutu clones (BL cell lines) that differ from each other in the EBV latent gene expression pattern, have shown that all of the CpG sites present in the region homologous to R2 are methylated in the latency type I clone (50). No differential methylation was found in any other region of the promoter, including the CBF1 and CBF2 binding sites. Site-specific methylation has been proposed to inhibit binding of cellular factors required for CBF2 activity (37, 38). However, an alternative mechanism for how methylation may work to silence promoter activity is through recruitment of DNA binding proteins with affinities for methylated CpG sites. These proteins may function as transcriptional repressors by (i) altering the structure of neighboring chromatin, (ii) excluding positive transcription factors from binding their cognate recognition sites, or (iii) directly exposing a repressor domain (18). The nature of chromatin assembly on plasmid DNA during transient-transfection analysis may not reflect the status of EBV chromosomes in infected cells, and methylation effects on chromatin structure may be manifest only in the context of the viral genome in immortalized cells. The contribution of R2 to Cp silencing may be revealed only under these circumstances.

R3 retains elements for the binding of the cellular factors CBF1 and CBF2, which along with EBNA2 are the primary mediators of Cp EBNA2 enhancer function. We have recently reported that the requirement for a functional CBF2 binding site is more apparent at low concentrations of EBNA2 (11). Quantification of CBF2 binding to HPV and RhEBV promoters revealed 4.7- and 3.9-fold-reduced affinities for CBF2, respectively. However, this analysis was done with extracts derived from a human B-cell line and it is possible that the affinities of the HVP and RhEBV Cps for human CBF2 do not accurately reflect their affinities for the CBF2 homologue present in their natural host cells. When testing these promoters in transient-transfection assays with increasing amounts of EBNA2, we observed a reduction of the ability of the HVP and RhEBV promoters to respond to low concentrations (0.8 µg of effector) (Fig. 4) of EBNA2 compared with the responsiveness of the EBV Cp. At higher concentrations of EBNA2, the three promoters had similar levels of induction. A similar phenomenon was also observed when the wild-type EBV Cp was compared to the EBV Cp containing mutations in the CBF2 binding site (11). The physiological significance of the reduced ability of the EBV-related viruses to respond to low concentrations of EBNA2 is uncertain. The conservation of the CBF2 binding site in the Cp EBNA2 enhancer of HVP- and RhEBV-related viruses argues in favor of CBF2 serving an important role in promoter function.

R4 contains a second conserved CCAAT box, also important for Cp function, as well as the TATA box (32). S1 nucleosome protection assays revealed that all of the nonhuman primate cell lines immortalized by their species-specific lymphocryptovirus use Cp and that the transcription start sites are located in analogous positions relative to the TATA box, even though there is some heterogeneity in the nucleotides surrounding the +1 initiation site.

The presence of transcriptional control elements conserved in both HPV and RhEBV Cp sequences that were previously identified as important mediators of Cp activity, combined with data that Cp is used in cells immortalized by these viruses and that HVP and RhEBV Cp reporter constructions are transactivated by EBNA2, suggests that these nonhuman lymphocryptoviruses have retained similar mechanisms for regulation of latency gene expression. The presence of conserved transcriptional control elements that have not been examined in the context of Cp will provide opportunities to identify additional cellular factors that regulate Cp activity. The Cp sequence comparison also reveals several additional conserved stretches of nucleotides that will aid in identifying new or novel transcription factors that regulate Cp activity.

This work was supported by NIH grants R29 CA69437 (P.D.L.) and R29 CA60067 (S.S.) and an award from the William Stamps Farish Foundation (P.D.L.).

We thank Elliott Kieff for the Ba65 cell line and Fred Wang for RhEBV LCL 8664.

REFERENCES

20. Knudson, J. C. 1990. The level of c-fgr RNA is increased by EBNA-2, an