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cis-Acting Regulatory Elements near the Epstein-Barr Virus Latent-Infection Membrane Protein Transcriptional Start Site

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Epstein-Barr virus (EBV) latent-infection membrane protein (LMP) gene *cis*-acting regulatory sequences were assayed in human B lymphocytes by using chloramphenicol acetyltransferase (*cat*) gene expression as a reporter. The activities of progressively longer upstream elements from bases −55 to −2350 were compared. At least two positive *cis*-activating regulatory components (−155 to −147 and −234 to −205) upstream of the LMP promoter were defined. LMP promoter *cat* gene constructs were more active in a Burkitt's lymphoma cell line latently infected with the B95 EBV strain than in the same cells latently infected with the P3HR1 EBV strain. Since the P3HR1- and B95-infected cells differ in EBNA-2 and EBNA-LP expression, EBNA-2 or EBNA-LP is a likely transactivator of the LMP promoter. Probable cognate sequences for known transcription factors in the LMP promoter are discussed.

Epstein-Barr Virus (EBV) infects and efficiently growth transforms human B lymphocytes. The entire EBV genome persists in such cells, usually as an episome, and a restricted set of viral genes is expressed (for reviews, see references 3 and 9). An initiation site for L-strand transcription has been identified in the unique DNA 0.5 kilobases from the terminal repeats at the right end of the linear EBV genome (4). The leftward transcript gives rise to an mRNA which encodes the latent-infection membrane protein, LMP (4, 6). The *lmp* gene is of particular interest, since LMP expression in rodent fibroblasts causes oncogenelike effects and expression in B lymphocytes induces B-lymphocyte activation and adhesion molecule expression (25–27). In latently infected lymphocytes, *lmp* mRNA is the most abundant EBV mRNA (4). Surprisingly, transcriptional run-on experiments indicate that *lmp* mRNA is less heavily transcribed than the much less abundant EBV nuclear protein mRNAs (20a). Further, *lmp* expression is down regulated in some EBV-infected Burkitt's lymphomas and in Burkitt's lymphoma cells infected with the nontransforming EBV strain P3HR-1 (15, 20). Thus, *lmp* gene expression is tightly regulated at the transcriptional level and may also be posttranscriptionally regulated. Further, *lmp* regulation may be different in EBV-transformed lymphoblastoid cell lines and Burkitt's lymphoma cells.

The objective of this series of experiments was to dissect the *cis*-acting regulatory elements 5′ to the *lmp* start site by assaying the activity of progressively longer upstream sequences in stimulating transient *cat* reporter gene expression in human B-lymphocyte cell lines. Expression was assayed in EBV-transformed lymphoblastoid cell lines and in infected or noninfected Burkitt's lymphoma cell lines so that cell- or virus-specific effects could be distinguished.

cat constructs were generated by ligation of cloned EBV, B95-8 strain, *lmp* DNA fragments into a site in pCAT3M (12). Plasmid DNAs were purified through two cycles of cesium chloride gradient centrifugation. Transfections were by electroporation with either an ISCO 494 power supply or a Progenitor I apparatus (Hoefer) supplemented with a 50-μF capacitor. In the latter transfection setup, cells were subjected to a 70-ms pulse at a field strength of 1,125 V/cm. Pulses were applied to a suspension of 10^7 cells in 0.5 ml of ice-cold 1× phosphate-buffered saline with 20 μg of DNA (17). Chloramphenicol acetyltransferase (CAT) assays (5) used extracts made by three cycles of freeze-thawing of cells harvested 72 h after transfection. Percent acetylation was determined by thin-layer chromatography and scintillation counting. All CAT assay results were considered as relative activity and normalized to either pCAT3M (activity, 1) or pSV2CAT (activity, 100), both of which were transfected in parallel in every experiment. Statistical analyses were performed by the Mann-Whitney U statistic test or the Wilcoxon signed-rank test by using individual CAT activity values as raw sample data. Multiple constructs were assayed in parallel so that relative effects were readily evident.

lmp promoter-*cat* constructs which extend from −2350 to +40, −1280 to +40, or −512 to +40 relative to the *lmp* cap site were assayed in noninfected human B-lymphoma cell line BL41 (2) or Louckes (24); in an EBV-infected human B-lymphoma cell line LY65 (13), which was naturally infected, in BL41/B95 or BL41/P3HR1, which were infected in vitro with the B95 or P3HR1 EBV strain (2); or in a latently EBV-infected, growth-transformed lymphoblastoid cell line IB4 (10). The (fold) increased CAT activities of the *lmp* promoter *cat* constructs relative to the parental pCAT3M vector in these lines are shown in Table 1. The most impressive difference observed was between the −512 to +40 *lmp-cat* construct in EBV-infected (IB4, BL41/B95, and LY65) versus EBV-negative Burkitt's lymphoma cell lines (BL41 and Louckes) ($P < 0.005$; Mann-Whitney U test). *lmp* promoter activity was also assayed in EBV-negative Burkitt's lymphoma BL41 cells infected with the B95-8 or P3HR1 EBV strain to establish more directly whether these differences were due to EBV infection. The EBV P3HR-1 strain differs from the EBV B95-8 strain in that the P3HR-1 genome is deleted for the last two exons of EBNA-LP and the entire EBNA-2 gene (9, 18, 28). Hence, P3HR-1-infected BL41 cells differ from B95-8-infected BL41 cells in their lack of EBNA-2 or a complete EBNA-LP (15), and comparison of *lmp-cat* activities in P3HR-1- versus B95-8-infected cells is an indication of the dependence of an EBV effect on expression of these two latent-infection cycle gene products. The

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TABLE 1. Relative activities caused by *lmp-cat* distal deletions

Cell line	Mean ± SEM CAT activity (no. of expts) caused by *lmp-cat* construct:		
	−2350 to +40[a]	−1280 to +40	−512 to +40
IB4	13 ± 0.3 (2)	18 ± 7.5 (2)	210 ± 90 (5)
LY65	2.2 ± 1.0 (2)	9.2 ± 1.7 (2)	31 ± 5.4 (2)
BL41/B95	2.6 ± 1.8 (2)	31 ± 13 (2)	27 ± 14 (2)
BL41/P3HR1	2.4 ± 1.0 (7)	8.1 ± 3.2 (7)	3.6 ± 1.5 (7)
BL41	1.2 ± 0.4 (3)	3.0 ± 1.1 (3)	1.3 ± 0.3 (3)
Louckes	0.9 ± 0.4 (2)	8.1 ± 2.8 (2)	5.3 ± 2.7 (2)

[a] Segments of *lmp* upstream DNA (numbers indicate bases relative to the *lmp* cap site) inserted 5′ to *cat*. Values for CAT activity represent fold activation above the background (pCAT3M = 1). A 27-fold activation in BL41/B95 cells electroporated with −512 to +40 *lmp-cat* corresponds to 15% chloramphenicol conversion versus 38% with pSV2CAT. Independent experiments were done in parallel with pCAT3M and pSV2CAT controls.

−512 to +40 *lmp-cat* construct was 21-fold more active in EBV-infected BL41 (BL41/B95) cells than in uninfected BL41 cells. Notably, BL41 cells infected with the P3HR1 EBV strain were similar to BL41 and differed from B95-8 EBV-infected BL41 cells ($P < 0.05$; Mann-Whitney U test), indicating that the EBV-specific effect is one that does not occur or occurs at much lower levels in the BL41/P3HR1 line.

The −1280 to +40 *lmp-cat* construct tended to give lower CAT activity than the −512 to +40 *lmp-cat* construct in some cell lines; the −2350 to +40 construct consistently gave lower activity than the −1280 to +40 construct in all cell lines, irrespective of EBV infection ($P < 0.05$ by the Wilcoxon test; $P < 0.005$ by the Mann-Whitney U test). These results could indicate an upstream EBV-independent negative *cis*-acting regulatory element or silencer, which largely overrides the EBV-specific positive effect on these *cat* constructs.

RNA analysis was performed on cells transiently expressing the −512 to +40 *lmp-cat* construct. Single-stranded antisense ^{32}P-labeled RNA probes were generated by T7 RNA polymerase by using a construct containing an *lmp-cat* fragment cloned into pBluescriptKS+ (Stratagene). A 594-base labeled probe which contained bases +256 to 1 of *cat* and +40 to −234 of *lmp* was purified on a 6% denaturing polyacrylamide gel. Total cellular RNA was isolated by the guanidinium isothiocyanate-cesium chloride method and treated with RNase-free DNase (Promega Biotec) to remove residual transfected plasmid DNA. Whole-cell RNA (5 μg) was hybridized for 20 h at 40°C with 10^6 cpm of the probe in a 50% formamide hybridization buffer. The initiation site and the relative quantities of the resultant RNA species were determined by RNase protection. A 320-base protected RNA fragment was barely detected with RNA from cells transfected with −512 to +40 *lmp-cat* (Fig. 1) but was absent with RNA from cells transfected with vector pCAT3M lacking any eucaryotic promoter or with RNA from cells transfected with a less active lmp-cat construct (−147 to

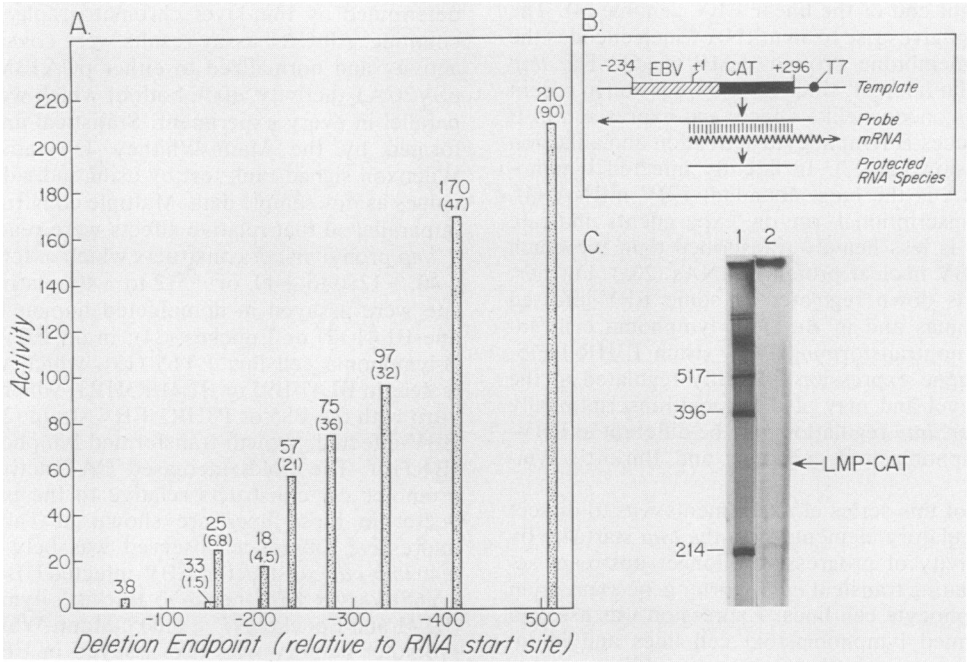

FIG. 1. (A) Functional dissection of the positive upstream *lmp* promoter-regulatory element. CAT activities caused by *lmp-cat*-proximal deletions in IB4 cells are plotted as a histogram. Values represent means ± the standard errors and include the results of transfection with the −55 to +40, −147 to +40, −155 to +40, −205 to +40, −234 to +40, −276 to +40, −334 to +40, −411 to +40, or −512 to +40 *lmp-cat* construct. Activities are expressed as fold increases relative to the parental pCAT3M vector and are the mean results of 1, 11, 7, 7, 7, 7, 9, 4, and 5 experiments, respectively, for each construct. A 210-fold stimulation with the −512 to +40 *lmp-cat* construct corresponds to 42% chloramphenicol conversion versus 82% conversion with pSV2CAT in the same experiments. (B) Schematic of *lmp-cat* RNase protection assay. The template was linearized pKSLCΔ1 containing EBV *lmp* DNA −234 to +40 upstream of the *cat* DNA (bases 1 to 256) in pBluescript-KSt. An internally labeled antisense *lmp-cat* probe was synthesized with T7 polymerase, purified, and hybridized to whole-cell RNA, treated with RNase, and run on a denaturing polyacrylamide gel. (C) Autoradiogram of the RNase-protected RNA analyzed on a denaturing polyacrylamide gel. Lanes: 1, ^{35}S-labeled *Hin*fI-cut pUC18 DNA markers; 2, RNase protection following hybridization of 106 cpm of the probe with 5 μg of cell RNA after transfection with the −512 to +40 *lmp-cat* construct. The arrow indicates a band corresponding to approximately 320 bases, near the 296-base size of the expected fragment.

+40; see below). The 320-base protected RNA fragment is close in size to that expected (296 bases) from the lmp-cat constructs. Other, smaller protected RNA fragments, evident at around 214 bases, were seen with RNA from cells transfected with lmp-cat or with control plasmids lacking *lmp* promoter sequences (Fig. 1; data not shown).

The positive EBV *lmp cis*-regulatory element was dissected by examining the CAT activity caused by lmp-cat constructs with progressively shorter upstream elements from −512 to +40 to −55 to +40 in EBV-infected, growth-transformed B lymphocytes (IB4). The results (Fig. 1) indicate the existence of at least two *cis*-activating components and suggest that there are other *cis*-activating components in the −512 to +40 sequence. Statistically significant increases occurred between −147 and −155 ($P < 0.05$ and 0.005 by the Wilcoxon and Mann-Whitney tests, respectively) and between −205 and −234 ($P < 0.05$ and $P = 0.05$ by the Wilcoxon and Mann-Whitney tests). The magnitudes of these increases in activity were 7.6-fold at −147/−155 and 3.2-fold at −205 to −234, as determined by the ratio of the means of the signals from these constructs. No statistically significant differences could be found between any other adjacent pair of deletions. However, analysis of the entire set of datum points derived from constructs between −147 and −512 indicated a positive upward trend (P between 0.01 and 0.001; Friedman 2-way analysis of variance). Other positively activating components upstream of −234, therefore, may further contribute to the −512 to +40 positive effect.

These data indicate that there are positive and negative EBV DNA regulatory sequences upstream of the *lmp* transcriptional start site. Complex regulation is probably significant, since LMP has such profound effects on B-cell activation and adhesion molecule expression (26). The effect of LMP on B lymphocytes or rodent fibroblasts requires the expression level ordinarily conferred by EBV in B lymphocytes, since expression at lower levels has little effect on cell growth (25–27). *lmp* is not only likely to be essential for B-lymphocyte transformation, it is also important in infected B-lymphocyte conjugation and adhesion to T lymphocytes and in recognition by cytotoxic T cells (14, 19, 27). The existence of positive and negative *cis*-regulatory elements upstream of this gene could provide the virus with a mechanism at the transcriptional level through which an optimum amount of LMP is expressed to facilitate transformation, as well as an acceptable level of T-cell recognition, thereby favoring latent-virus persistence without disease in a normal host.

The *lmp cis*-acting regulatory sequences are affected by EBV infection. Specifically, strain B95 virus infection conferred activity to the *lmp* upstream region, while P3HR1 virus infection did not. The principal difference between B95 and P3HR1 is the expression of EBNA-2 and a complete EBNA-LP by the B95 virus (15, 28). Thus, the data suggest that *lmp* promoter activity is regulated by EBNA-2 or EBNA-LP transactivation.

Our data define two specific components, 8-base (−147 to −155) and 29-base (−205 to −234) DNA segments, which contribute to up regulation of the *lmp* promoter. Analysis of the EBV *lmp* upstream sequences for similarity to known nuclear factor cognate sequences by using the algorithm of Korn et al. (11) at the zero- or single-mismatch level revealed similarity to the Oct-1 (1, 21) recognition sequence at −140 to −152 and to the NF-I (8) recognition sequence at −230 to −242 (Fig. 2). The sequence ACGTCA at −45 to −40 is also identified as the cognate sequence for the CREB or ATF

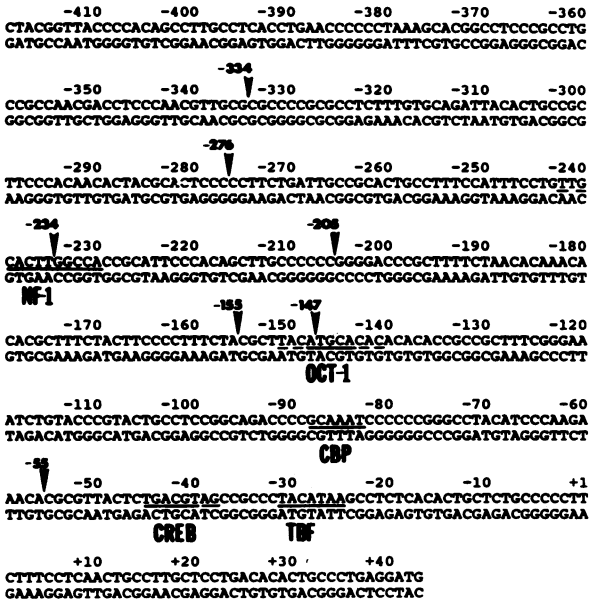

FIG. 2. Putative *lmp* promoter-regulatory elements between positions −479 and +43 relative to the site of endogenous lmp RNA initiation. The arrowheads refer to endpoints of the EBV sequences used in *lmp* promoter deletion mutants. Similarities to known transcription factor recognition sequences are indicated, and they include NF-I (−242 to −230), Oct-1 (−152 to −140), the CAAT box-binding factor (−88 to −83), CREB (−45 to −38), and the TATA box-binding factor (−31 to −25).

transcription factor (1, 7; M. Horikoshi, T. Hai, Y. Lin, and R. Roeder, J. Cell. Biochem., Suppl. 12d, abstr. O161, 1988). The apparent *lmp* promoter requirement for EBV EBNA-2 or EBNA-LP and the presence of these putative recognition sequences is intriguing, since ACGTCA is involved in cyclic AMP-mediated (21), E1A-mediated (7, 22), and possibly c-*myc*-mediated (16) regulation.

It has been recently shown that infection with the B95 versus the P3HR1 strain of EBV is a determinant in the expression of the *lmp* gene (15), suggesting that the activity of the endogenous EBV genome *lmp* promoter is affected by EBNA-2 and/or EBNA-LP expression. The results of this study suggest that the activity of the B95 *lmp* promoter, independently of its gene, parallels endogenous *lmp* expression. This report defines *cis*-acting elements which affect *lmp* upstream regulatory activity in latently infected cells and enables direct testing of the biochemical mechanisms by which the sequences effect *lmp* expression. While our data are most consistent with the hypothesis that this regulation is at the transcriptional level, we have not rigorously excluded the unlikely possibility that the *lmp* +1 to +40 sequences which are part of the *lmp-cat* mRNA (or other *lmp* upstream sequences which could be part of undetected minor transcripts through this region) are posttranscriptionally acting *cis*-regulatory elements.

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