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Mutually exclusive use of viral promoters in Epstein–Barr virus latently infected lymphocytes

(S1 nuclease protection/chloramphenicol acetyltransferase assays/viral promoters/transfection of lymphocytes)

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ABSTRACT **Of the eight viral antigens known to be expressed during Epstein–Barr virus latency, six are transcribed from a major rightward transcriptional unit, which gives rise to mRNAs containing common 5′ exons. Analysis of cDNA clones has identified the use of two different promoters (W_p and C_p), located near the left-hand end of the viral genome, in generating these viral messages. Characterization of the activities of these two viral promoters in a number of Burkitt lymphoma and lymphoblastoid cell lines has revealed exclusive usage of only one of these promoters in all cell lines examined. Transfection of reporter constructs containing W_p and/or C_p linked to the bacterial chloramphenicol acetyltransferase gene into several different Epstein–Barr virus-infected cell lines generally supports a model in which the mutually exclusive use of C_p or W_p is determined by cellular factors and not by viral strain variation.**

Epstein-Barr virus (EBV) infection of umbilical cord blood lymphocytes predominantly results in a latent infection in which little or no virus is produced, accompanied by a concomitant growth transformation of the infected lymphocytes. During viral latency, only a limited number of viral genes are expressed. To date six viral nuclear antigens (EBNAs) and two viral membrane proteins have been identified (for review, see refs. 1–3). The promoters giving rise to all these gene products are clustered around the origin of plasmid replication termed oriP (Fig. 1).

All the genes encoding the EBNAs are transcribed from the same strand in a major transcriptional unit, which spans the left-hand ≈110 kilobases of the viral genome, and the resulting mRNAs all share 5′ exons in common. Two small exons (W_1 and W_2) encoded within the major internal repeat (IR1 *Bam*HI W repeats) are present in all the EBNA mRNAs that have been analyzed. Cloning these messages has identified two viral promoters, referred to as C_p and W_p, which give rise to the EBNA mRNAs (4–7). Those transcripts initiating at C_p contain at their 5′ ends two small exons (C_1 and C_2) encoded near the right-hand end of the unique region (U1) of the viral *Bam*HI C fragment, whereas transcripts initiating from W_p contain at their 5′ ends a short exon (W_0) encoded in IR1, located about 150 base pairs (bp) upstream of the region encoding the W_1 exon (6, 7). Both the C_2 and W_0 exons are spliced to the first W_1 exon, followed by a variable number of W_1/W_2 repeat exons (4, 8, 9).

In this paper we show that C_p and W_p activity are mutually exclusive and that the activities exhibited by exogenous promoter constructs introduced into EBV-transformed human lymphocytes appear to be generally consistent with the promoter activity of the endogenous genome.

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MATERIALS AND METHODS

RNA Preparation and S1 Nuclease Analysis. Total cellular RNA was prepared by the method of Auffray and Rougeon (10). Poly(A)$^+$ RNA was isolated by fractionation on oligo(dT)-cellulose (11). S1 analyses were carried out with synthetic oligonucleotides that were complementary to the region spanning the 5′ end of either the C_1 or W_0 exon (C_1 58-mer: 5′-CTGGGGGTCTTCGGTGTCCTTGTCTCTATGCCATCTGATCTAAAATTTGCAGCAGAAC-3′; W_0 48-mer; 5′-ATTTGTGTGGACTCCTGGCGCTCTGATGCGACCAGAAATAGCTGCAGG-3′). Fifty to 100 fmol of kinased oligonucleotide (12) was hybridized to 2 μg of poly(A)$^+$ RNA in 50 μl of hybridization buffer [40 mM Pipes, pH 6.4/1 mM EDTA/0.4 M NaCl/0.1% SDS/50% (vol/vol) formamide] at 37°C for 12 hr. The hybridization reaction was then treated with S1 nuclease (Bethesda Research Laboratories) at a concentration of 100 units/ml (in 0.28 M NaCl/50 mM NaOAc, pH 4.6/4.5 mM $ZnSO_4$) for 30 min at 37°C. The protected fragments were fractionated on a 10% denaturing polyacrylamide gel.

Cell Culture, Transfections, and Chloramphenicol Acetyltransferase (CAT) Assays. Cells were grown at 37°C in RPMI 1640 supplemented with 10% (vol/vol) newborn calf serum as described (9). Transfection was done using liposome-mediated DNA transfer according to the manufacturers protocol (Bethesda Research Laboratories), with minor modifications. Basically, 2 μg of CsCl-purified plasmid DNA was mixed with 5 μl of lipofectin reagent in 1 ml of Opti-MEM I reduced serum medium (GIBCO). Cells (5×10^6–1×10^7) were resuspended in 100 μl of Opti-MEM I, added to the DNA liposomes, and incubated at 37°C for 6–18 hr, depending on the cell line, followed by addition of medium containing 10% fetal calf serum to a cell density of 5×10^5 cells per ml. Transfectants were harvested 72 hr posttransfection, and CAT assays were performed as described (13).

Plasmid Constructions. Fusions between EBV exons and the CAT gene were generated by converting 5′ overhangs of *Hin*fI (W_0 exon), *Avr* II (W_1 exon), and *Acc* I (Y_2 exon) into *Hin*dIII sites by using the Klenow fragment to make the ends blunt and by adding *Hin*dIII linkers (New England Biolabs). The C_1 exon–CAT gene fusion was achieved by using a *Bgl* I/*Hin*dIII adapter with the sequence 5′-AGAAGCTTC-TACA-3′.

RESULTS

Endogenous Activity of Latent EBV Promoters. To determine the relative activity of C_p and W_p in different cell lines, S1 nuclease protection experiments were performed employing specific oligonucleotides. Total cellular poly(A)$^+$ RNA

Abbreviations: EBV, Epstein–Barr virus; CAT, chloramphenicol acetyltransferase; EBNA, EBV nuclear antigen.

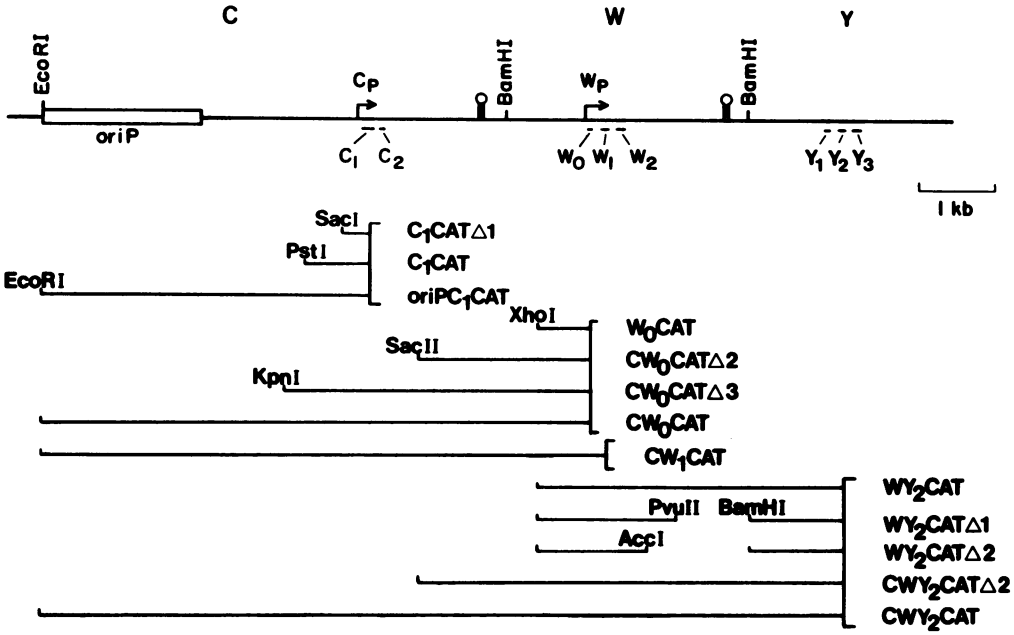

FIG. 1. Organization of the viral promoters involved in transcription of the EBNA genes. The structure of four different sets of CAT constructs is shown with respect to a viral *Bam*HI restriction map. Vertical brackets indicate fusion with the reporter gene. Small horizontal lines denote exons, arrows mark the positions of the promoters under study, and potential hairpin loop structures are drawn schematically.

from a variety of EBV-positive lymphoblastoid cell lines, as well as cell lines established from Burkitt lymphoma tumor explants, were hybridized to oligonucleotides that were homologous to either the 5′ end of the C_1 exon or the W_0 exon and extended upstream of the cap site (see *Materials and Methods*) and were therefore diagnostic of transcripts initiating either from C_p or W_p (Fig. 2). Employing the W_0 oligonucleotide, a major protected fragment of 30 nucleotides was detected with RNA from the cell lines Daudi, clone-13, IB4, and X50-7, which is indicative of W_p being transcriptionally active in these cells. No specific protection was detected with RNAs from BJAB/B95.8, Raji, Jijoye, JY, JC5, and B95.8 cell lines (or control RNA from the EBV-negative BJAB cell line), indicating that W_p is inactive in these cell lines. The length of the protected fragments indicates that transcription initiation from W_p occurs at 14,378–14,380 bp on the standard B95.8 map (14), which corresponds very closely to the 5′ end of the previously described IB4-WY1 cDNA clone (7). A significant portion of the W_0 oligonucleotide was completely protected from exonuclease digestion by RNA from some cell lines. However, when this analysis was repeated employing cytoplasmic RNA, protection of the entire probe was not apparent (data not shown).

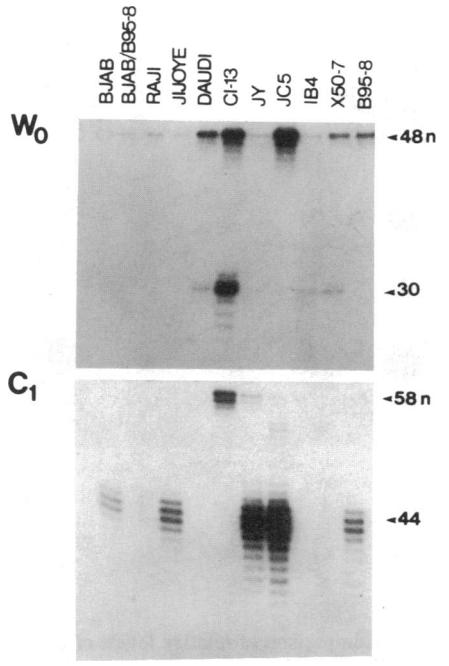

FIG. 2. S1 nuclease protection experiments with specific oligonucleotides. Identical amounts of RNA were subjected to the analysis with the W_0 oligonucleotide (*Upper*) or the C_1 oligonucleotide (*Lower*) as a hybridization probe. n, Nucleotides.

When the same RNAs were assayed with the C_1 oligonucleotide to determine C_p usage, a distinctly different pattern was observed (Fig. 2). RNAs from those cell lines that did *not* exhibit W_p activity did protect the appropriate size fragment of the C_1 oligonucleotide (44 nucleotides), demonstrating transcriptional activity of C_p in these cell lines. Conversely, those cell lines exhibiting W_p activity did not show protection of any appropriate size fragments of the C_1 oligonucleotide and therefore do not appear to utilize C_p. The 5′ end of the C_1 exon was determined to be near position 11,337 bp on the standard B95.8 genome, which is in good agreement with previous results (15). RNA from the Burkitt lymphoma cell line Raji did not appear to employ either promoter. This may be due to either multiple mutations in the C_1 or W_0 exons that prevented proper binding and subsequent protection of the oligonucleotide probes or that transcription of the EBNA genes initiates from a distinct, and as yet unidentified, promoter. The absence of any appropriate protection of the C_1 oligonucleotide in W_p-active cell lines clearly demonstrates that in a given cell line only one of these two latent EBV promoters is used for generating EBNA transcripts.

Activities of Transfected C_p and W_p Constructs. The mutually exclusive utilization of either C_p or W_p can most likely be explained by (*i*) viral strain differences in cis-acting elements that favor one promoter over the other and/or (*ii*) variation in host cell transcription factors. To begin to address this phenomenon, various cell lines were transfected with a number of promoter constructs employing the bacterial CAT gene as a reporter (see Fig. 1). The constructs in which the CAT gene is fused to the C_1 exon do *not* contain W_p, and therefore the resulting activities can presumably be unam-

biguously ascribed to C_p. The same consideration also applies to the two shorter W_0CAT and WY_2CAT constructs, in which the CAT gene is fused to the W_0 and the Y_2 exon, respectively, since they contain only W_p and *not* C_p. However, the longer constructs of these series as well as the CW_1CAT construct contain both C_p and W_p, which makes an interpretation of the results difficult. Given the splicing pattern that has been identified so far from cDNA clones, a functional CAT mRNA from transfection with CW_0CAT would be expected to arise from W_p, although expression from C_p, which normally utilizes the C_1 and C_2 exons together with W_1, cannot be ruled out. Both C_p and W_p should be capable of generating functional CAT mRNAs with the CW_1CAT and CWY_2CAT plasmids.

Utilization of exogenous W_p. Two Burkitt lymphoma cell lines (Jijoye, which utilizes C_p with its endogenous viral genome, and clone-13, which is transcriptionally active with W_p) and two latently infected lymphoblastoid cell lines (JY, which uses C_p, and X50-7, which employs W_p) were transfected with the various CAT constructs. Each individual cell line gave a characteristic profile of CAT activity, although the overall picture was quite similar (Fig. 3). The activities of the W_0CAT constructs revealed several important regulatory regions acting on W_p. (*i*) A positive regulatory element, which maps to a 1439-bp *Sac* II/*Xho* I fragment spanning the *Bam*HI junction between *Bam*HI C and W, was apparent when the activities of W_0CAT and CW_0CATΔ2 were determined in the clone-13, X50-7, and Jijoye cell lines but not in the JY cell line (Fig. 3). This up-regulation was also observed in the EBV-negative Burkitt lymphoma line Ramos (data not shown), indicating that cellular factor(s) presumably govern this effect. Clone-13 cells were particularly active with these short W_0CAT constructs, compared to the other W_p cell line (X50-7), which correlates with the high level of W_p activity that was observed in the S1 nuclease protection experiments (see Fig. 2). (*ii*) The addition of another 2 kilobases of upstream sequences (CW_0CATΔ3) caused a consistent decrease of CAT expression in all cell lines examined. (*iii*) A strong positive effect, however, was seen when additional upstream sequences, including the latent origin of replication (oriP), were included in the test plasmid (CW_0CAT), thereby supporting previous studies that identified an EBNA-1-dependent enhancer linked to oriP (16, 17). This also underscores the pronounced effect of distal sequences on W_p (and possibly C_p) activity.

Utilization of exogenous C_p. Transfection of CAT plasmids in which the reporter gene was fused to the C_1 exon to examine the activity of C_p revealed a weak positive regulatory element, located on a 575-bp *Sac* I/*Pst* I fragment

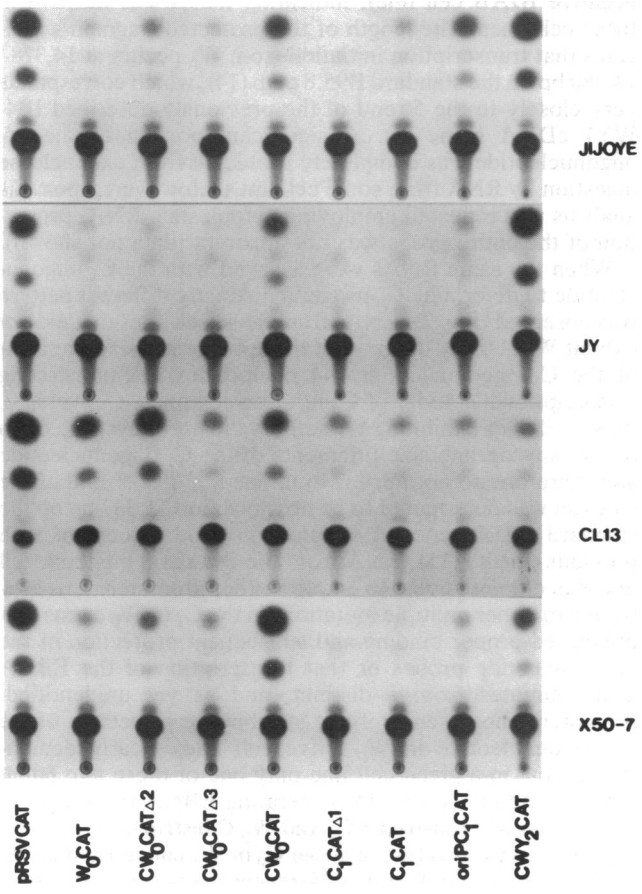

FIG. 3. CAT activity profile of different promoter test plasmids in two W_p-using cell lines (clone-13 and X50-7) and two lines that employ C_p (JY and Jijoye). CAT assays were performed as described in ref. 13. Quantitation of acetylated reaction products was done by cutting out relevant radioactive spots from the TLC plate and assaying them in 5 ml of Econoflour (DuPont) in a scintillation counter.

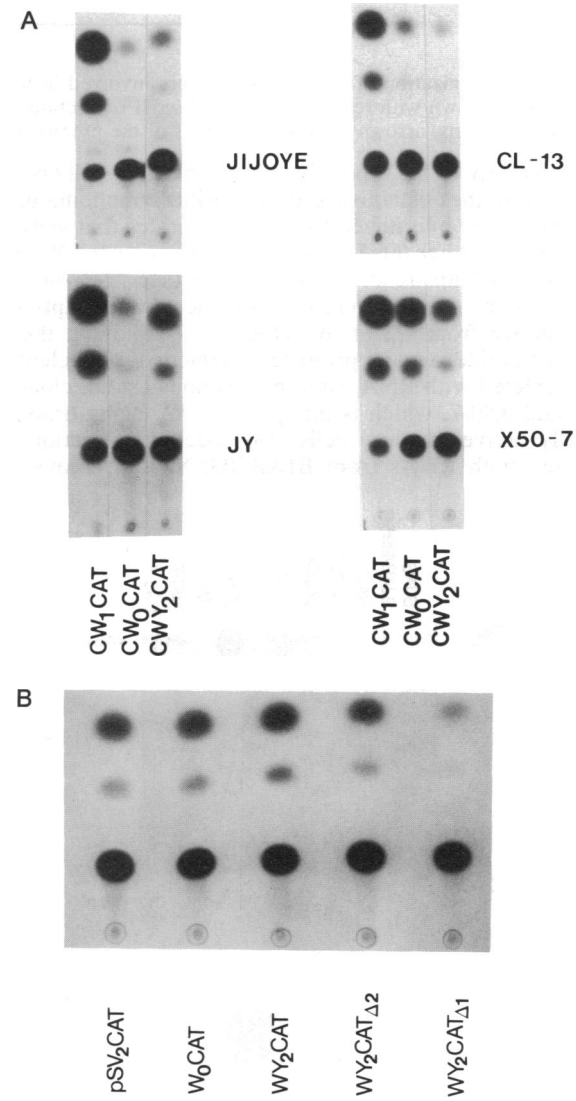

FIG. 4. (*A*) Comparison of relative levels of CAT gene expression of three different CAT constructs in two W_p-using and two C_p-using cell lines. The structures of the plasmids are given in Fig. 1. Quantitation is shown in Table 1. (*B*) Effect of deletions in the W_2/Y_1 intron on W_p activity in clone-13 cells. See Fig. 1 for plasmid structures.

Table 1. Comparison of the activities of several CAT constructs in C_p- and W_p-using cell lines

Cell line	% acetylation of [^{14}C]chloramphenicol			Relative activities		
	CW_1CAT	CW_0CAT	CWY_2CAT	CW_0CAT/ CWY_2CAT	CW_1CAT/ CW_0CAT	CW_1CAT/ $oriPC_1CAT$
Clone-13 (W_p/BL)	31.8	3.4	1.3	2.6	9.3	28.1
X50-7 (W_p/LCL)	81.3	38.5	11.7	3.3	2.1	262.2
Jijoye (C_p/BL)	77.9	1.8	3.0	0.6	42.8	48.1
JY (C_p/LCL)	59.8	2.0	10.7	0.2	29.5	249.2

The quantitation of the CAT assays shown in Fig. 4*A* is given. BL, Burkitt lymphoma; LCL, lymphoblastoid cell line.

(compare $C_1CAT\Delta1$ and C_1CAT), which was apparent in the X50-7, Jijoye, and JY cell lines but was not apparent in the clone-13 cell line (Fig. 3). As was observed with the CW_0CAT plasmid, the presence of the oriP enhancer in $oriPC_1CAT$ significantly increased CAT expression in all lines tested except clone-13, again underscoring the potential importance of this element in viral promoter regulation.

The overall picture revealed from the activities of the W_0CAT and C_1CAT plasmids does *not* clearly indicate a mutually exclusive usage of either C_p or W_p with transfected exogenous constructs; for example, Jijoye cells, which utilized C_p exclusively, and X50-7, which appeared to be a W_p-using cell line (Fig. 2), could both drive the CAT gene from C_p in $oriPC_1CAT$ and from W_p in $CW_0CAT\Delta2$. A better indication of relative C_p and W_p activities was obtained when the activity of CW_0CAT (Wp) was compared to that of CW_1CAT (Cp and Wp) (Fig. 4*A*). Transfection of CW_1CAT resulted in a marked increase of CAT activity relative to CW_0CAT in all cases, but the relative increase in C_p-using cell lines (Jijoye and JY) was at least 3 times higher than that in the W_p cell lines (clone-13 and X50-7) (Table 1), consistent with Jijoye and JY cell lines utilizing C_p with the exogenous CW_1CAT plasmid. Furthermore, the general increase of CAT expression with CW_1CAT suggests the presence of a positive regulatory element located in the intron between the W_0 and the W_1 exon.

Effect of sequences downstream of W_p. In an attempt to mimic more closely the physiological splicing pattern and also to study the influence of sequences downstream of W_p in the context of upstream sequences, the reporter gene was fused to the Y_2 exon (CWY_2CAT, see Fig. 1). This construct displayed much less CAT activity than CW_1CAT in all cell lines (Fig. 4*A*), indicating that the additional sequences had a negative effect on promoter activity, either at the level of transcription or possibly of splicing efficiency. This negative influence was much more pronounced in the two Burkitt lymphoma cell lines than in the lymphoblastoid cell lines (Table 1). The level of expression of CWY_2CAT relative to CW_0CAT (i.e., addition of downstream sequences) was consistently decreased in the W_p-using cells, whereas the opposite effect was observed with the C_p cell lines (Table 1).

The presence of regulatory elements downstream of W_p was confirmed by examining the effect of deletion of portions of the W_2/Y_1 intron in the WY_2CAT construct (see Figs. 1 and 4*B*). Deletion of a fragment (*Pvu* II/*Bam*HI, $WY_2CAT\Delta1$) containing a potentially large hairpin loop structure (18) resulted in a dramatic loss in activity in the clone-13 cells. Interestingly, as discussed above, these same sequences occur upstream of W_p, and a positive regulatory element has been mapped to that region. A further deletion of the W_2/Y_1 intron (*Acc* I/*Bam*HI, $WY_2CAT\Delta2$) resulted in loss of a strong negative element (Fig. 4*B*).

Although it appears that neither the CW_0CAT nor the CW_1CAT construct exhibit a strict mutually exclusive promoter usage, the combined analyses with these two plasmids in conjunction with the CWY_2CAT construct indicate that the exogenous DNAs are able to distinguish C_p and W_p cell lines. Furthermore, the observed differences in the activities of the exogenous constructs argues in favor of a model for the mutually exclusive promoter usage involving differences in host cell factors. It should be emphasized that the mutually exclusive usage of C_p or W_p may depend on interactions between distal elements located upstream and downstream of these promoters. Thus, this phenomenon may only be exhibited with longer constructs such as CWY_2CAT and awaits further analysis.

DISCUSSION

In this paper, utilization of C_p or W_p in EBV-infected B lymphocytes has been shown to be mutually exclusive. Furthermore, reporter plasmids, containing either W_p, C_p, or both promoters from the B95.8 strain of EBV exhibited distinct activities in C_p versus W_p cell lines. The mutually exclusive behavior of these promoters may involve differences in cellular transcription factors in EBV-positive B cells. However, viral strain variation, such as differences in cis-acting elements or in viral antigens that ultimately influence (either directly or indirectly) transcription factors, may also play a role in this phenomenon. The existence of a major variation in EBV strains has been documented (type A and B EBV strains) (19–22). The requirement for two distinct promoters may relate to a need for the virus to exist in lymphocytes at different stages of differentiation, and this may be (at least in part) influenced by the strain of EBV. The other obvious factor that may affect the differentiation state of infected lymphocytes is the initial phenotype of the host cell.

Comparison of W_p- and C_p-utilizing cell lines does *not* reveal any clear correlation between promoter usage and cell type (Burkitt lymphoma or lymphoblastoid cell lines). In addition, B95.8 virus can give rise to both C_p and W_p cell lines [B95.8 and BJAB/B95.8 are both C_p users, while the IB4 cell line established with B95.8 virus (23) is a W_p user], indicating that the viral strain alone does *not* determine promoter usage. However, it is possible that the B95.8 cell line produces a heterogeneous mixture of viruses, which results in establishment of both C_p and W_p cell lines. It is also of some interest to note that both the Daudi and clone-13 Burkitt lymphoma cell lines, which contain nearly identical deletions of the carboxyl terminus of the EBNA-4 gene and the entire EBNA-2 gene, are both W_p using cell lines. Some caution should be taken, however, in interpreting the results obtained with Burkitt lymphoma cell lines since these may *not* accurately reflect the normal viral program. Thus, a detailed analysis of a large panel of newly established lymphoblastoid cell lines is clearly warranted and should provide a statistically better picture of the relative usage of C_p and W_p.

An unexpected result was the observed activity of the CW_0CAT construct in the C_p-using cell lines (JY and Jijoye). Given the known splicing pattern, W_p in this plasmid should be functional while, due to the absence of the W_1 exon, its C_p should be unable to give rise to a functional CAT mRNA. This activity may then reflect W_p usage with the exogenous

DNA, which would suggest that other cis-acting elements (e.g., downstream sequences) may be required for mutually exclusive promoter usage or that transfection of exogenous DNA into these cells disrupts the exclusive usage of C_p (i.e., titration of a repressor of W_p). Alternatively, CAT expression in those cells may reflect C_p activity, which could arise (*i*) from the failure to splice at the C_2 splice donor junction (resulting in an mRNA containing a fused C_2–W_0 exon along with the intervening sequences) or (*ii*) by splicing from the C_2 splice donor junction to a splice acceptor sequence upstream of the W_0 exon.

The activities of the C_1CAT constructs, which must utilize C_p, in the clone-13 cell line were also inconsistent with the lack of C_p activity of the endogenous genome. Again, it is not clear whether (*i*) downstream sequences are necessary for mutually exclusive promoter usage or (*ii*) transfection of exogenous DNA into clone-13 disrupts the exclusive usage of W_p, which may explain the observed C_p activity. It is also possible that the endogenous clone-13 virus contains a mutated C_p and that the cell line contains the necessary transcription factors for C_p activity. With regard to this point, it should be noted that the clone-13 cell line is a subclone of the P3HR-1 cell line, which spontaneously arose from the Jijoye cell line. Thus, the clone-13 cell line is a descendant of a C_p-using cell line.

Whether the exogenous promoter constructs can mimic the mutually exclusive utilization of C_p or W_p requires further investigation. However, the data presented here do provide an additional important insight—namely, the pronounced influence of distal elements on C_p and W_p activity. Since its identification, it has been speculated that oriP might be a central regulatory region for viral gene expression during latency (16, 17). Consistent with this hypothesis, inclusion of the upstream sequences containing oriP did indeed enhance CAT activity. Furthermore, other elements located several kilobases upstream or downstream significantly altered activity. These results underscore the need to characterize C_p and W_p activity in the presence of these regions of the EBV genome and *not* as short discrete promoter elements.

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