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Host Cell and EBNA-2 Regulation of Epstein-Barr Virus Latent-Cycle Promoter Activity in B Lymphocytes

CLIONA M. ROONEY,[1,2]* MATTHEW BRIMMELL,[2] MICHAEL BUSCHLE,[1,2] GORDON ALLAN,[2] PAUL J. FARRELL,[2] AND JOHN L. KOLMAN[3]

St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, Tennessee 38103[1]; Ludwig Institute for Cancer Research, St. Mary's Branch, Norfolk Place, London W2 1PG, United Kingdom[2]; and Yale University School of Medicine, New Haven, Connecticut 06510[3]*

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The six latent-cycle nuclear antigens (EBNAs) of Epstein-Barr virus (EBV), whose genes share 5′ leader exons and two promoters (Cp and Wp), are differentially expressed by cells of the B lineage. To examine the possibility that EBNA gene expression is regulated through selective use of Cp and Wp, we monitored the activity of promoter-chloramphenicol acetyltransferase (CAT) gene constructs transfected into EBV-positive and EBV-negative B lymphocytes and Burkitt's lymphoma cells. Wp was a much stronger promoter than Cp in EBV genome-negative B-cell lines and was used exclusively in primary B cells. When B cells were infected with transforming EBV, Cp became the stronger promoter. This switch was not observed when B cells were infected with an immortalization-deficient virus, P3HR-1, which lacks the EBNA-2 open reading frame and expresses a mutant leader protein (EBNA-LP). Cp function was transactivated when EBV-negative or P3HR-1-infected B cells were cotransfected with Cp and a 12-kb fragment of DNA (*Bam*HI-WWYH) that spanned the P3HR-1 deletion. This activity was mapped to the EBNA-2 gene within WWYH; constructs expressing EBNA-LP did not induce Cp function, and the deletion of 405 bp from the EBNA-2 open reading frame abolished transactivation. This research demonstrates host cell and EBNA-2 regulation of latent-cycle promoter activity in B lymphocytes, a mechanism with implications for persistence of EBV-infected lymphoid cells in vivo.

Resting B cells infected with Epstein-Barr virus (EBV) become immortalized as permanently growing lymphoblastoid cell lines (LCLs). Although in most of these cells the virus is latent or nonproductive, LCLs express 11 or more viral genes; 6 encode nuclear proteins (EBNA-1, -2, -3A, -3B, and -3C and leader protein EBNA-LP), 3 encode membrane proteins (LMP and the TP1 and TP2 terminal proteins), and 2 encode small nuclear RNAs (EBERs) (10, 15). Expression of latent-cycle genes results in the transition of resting B cells from G_0 into the cell cycle, where they remain indefinitely. The protein products of at least four latent-cycle genes are highly immunogenic (6, 19, 20); thus, LCL-like cells are not found in immunocompetent seropositive individuals. However, EBV-infected B cells do exist in vivo, but it is not known what viral genes they express. One would predict that the survival of such cells in a normal host would require suppression of the immunogenic proteins and expression of only those viral genes required for maintenance of the virus episome. Indeed, many EBV-carrying tumor cells do not express the full complement of latent-cycle genes seen in LCLs in vitro (28, 29), unless they are proliferating in an immunocompromised host (42). For example, endemic Burkitt's lymphoma (BL) cells express only EBNA-1 (28), the minimal requirement for viral episome maintenance in a dividing cell (41). Since EBNA-1 does not induce the cell adhesion molecules required for cytotoxic T-cell-mediated killing (28), BL cells are not recognized by HLA-restricted cytotoxic T cells (27, 28). This downregulation of the other latent-cycle genes demonstrates the importance of the tissue-specific regulation of latent-cycle genes in vivo.

It is not known how latent-cycle genes are suppressed in malignant or nonmalignant EBV-positive B cells, but the promoters for these genes represent a potential target for regulation. The six EBNA genes expressed in LCLs are controlled by two separate promoters (4, 30, 31, 33): Cp, which is located in the first unique segment of the virus, and Wp, which is reiterated within the large internal repeat (32). Each promoter can potentially initiate the expression of all six EBNAs by a mechanism involving differential splicing and 3′ cleavage site selection (4, 32). The leader sequences of all EBNA mRNAs share the W1 and W2 exons derived from the large internal repeat. Whether W1 and W2 are translated into EBNA-LP depends on selection of the first W1 splice acceptor site. Selection of the W1′ acceptor creates an initiator ATG codon, whereas selection of the W1 acceptor, only five nucleotides upstream, creates an untranslatable leader. Why the virus has two promoters to express one set of genes and how the promoters are selected or individually regulated are not clear. Woisetschlager et al. (39) suggested that use of one promoter excludes the use of the other within any cell line, but they were unable to determine whether this restriction is determined by viral or cellular factors. In group 1 BLs, in which most latent-cycle genes are not expressed, EBNA-1 is expressed from an alternative promoter, Fp, located downstream of the EBNA-2 gene (30). Presumably, in these cells Cp and Wp are suppressed, while Fp is not expressed in LCLs (30).

Our previous work suggested that both viral and cellular factors can influence latent-cycle gene expression (26). Comparison of viral gene expression after infection of two types

* Corresponding author.

TABLE 1. Characteristics of BL cell lines

Cell line	Infecting virus	EBNA-2 expression[a]	Source
BL2	None	None	G. Lenoir, International Agency for Research on Cancer, Lyons, France
BL30	None	None	G. Lenoir
BL41	None	None	G. Lenoir
BL2/CL16	P3HR-1	−	C. Rooney
BL30+P3	P3HR-1	−	A. Rickinson, Birmingham, United Kingdom
BL41/CL16	P3HR-1	−	C. Rooney
BL2/B95-8	B95-8	+	C. Rooney
BL30+B95-8	B95-8	+	A. Rickinson
BL41/B95-8	B95-8	+	C. Rooney
P3HR-1 (clone 16)	P3HR-1	−	G. Miller, Yale University, New Haven, Conn.
B95-8	B95-8	+	G. Miller

[a] −, weak expression; +, strong expression.

of B cell with two different strains of EBV demonstrated that while all the latent-cycle genes were expressed sequentially after infection of primary B cells with standard EBV, the same cells infected with an immortalization-deficient strain, P3HR-1 (21), expressed only a mutant LP and not EBNA-1, -3A, -3B, or -3C. The EBNA-2 gene and 3′ unique exons of EBNA-LP are deleted in P3HR-1 (5, 16, 17, 21), suggesting that virus-encoded factors are important in latent gene expression. Conversely, P3HR-1 expressed all the EBNA genes not affected by the deletion in BL cells; this indicated that cellular factors were also important in viral gene expression (26). Thus, both virus-encoded factors, deleted in P3HR-1, and cellular factors, present in BL but not in primary B cells, may control the expression of other EBNA genes during primary infection. EBNA-2 has been shown to induce viral gene expression from the latent-cycle promoters of LMP and TP1 (1, 11, 38), and therefore this protein may be important in determining the pattern of latent-cycle genes expressed in EBV-infected cells.

In this work we investigated the possibility that Cp and Wp are prime targets for the regulation of EBNA gene expression. We found that Wp is used constitutively in EBV-negative primary B lymphocytes, while Cp requires EBV-encoded factors for its expression in B cells. Control of this flexible use of promoters maps to expression of the EBNA-2 gene.

MATERIALS AND METHODS

Culture conditions. All cells were grown in RPMI 1640 medium supplemented with 1 mM glutamine, 8% fetal calf serum, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml.

Cell lines. The cell lines and their relevant characteristics are summarized in Table 1. The IARC/BL2, IARC/BL30, and IARC/BL41 BL lines lack EBV genomes (8). The BL41/B95-8, BL2/B95-8, and BL30+B95-8 lines were 100% EBNA positive after conversion with the B95-8 strain of EBV. BL30+P3, BL2/CL16, and BL41/CL16 were converted with P3HR-1 or a subclone of P3HR-1 (clone 16) which lacks rearranged (defective) virus populations.

Preparation of tonsillar B cells. Lymphocytes were extracted from fresh tonsils by scraping with a sterile scalpel blade. Cells (5×10^7) in 1 ml of medium were incubated for 15 min at 37°C with a mixture containing 1 ml of fetal calf serum and 2 ml of 2% sheep erythrocytes. The cells were centrifuged at 200 × *g* for 10 min and incubated on ice for 30 min. The pellet was gently resuspended, layered over lymphocyte separation medium, and then centrifuged at 200 × *g* for 30 min. The interface was enriched for tonsillar B cells (fewer than 1% formed rosettes with sheep erythrocytes), which were transfected with promoter-chloramphenicol acetyltransferase (CAT) constructs. Tonsillar B cells were stimulated with 1 μg of pokeweed mitogen per ml for 3 days before transfection. The BL cell lines were treated with 2 μg of phorbol ester (TPA [12-*O*-tetradecanoylphorbol-13-acetate]) per ml immediately after transfection where indicated.

Immunofluorescence. EBNA-2 expression was measured by immunofluorescence 24 h after transfection by using both well-characterized human sera and a rabbit monospecific serum raised against an EBNA-2–TrpE fusion protein in a standard complement-mediated assay (22).

Plasmids. To test EBV promoter function, we linked Cp and Wp to a promoterless CAT gene and monitored the activity of the enzyme by thin-layer chromatography as described by Gorman et al. (13). The constructs are illustrated in Fig. 1. The negative control CAT plasmid pUC-CAT is a promoterless CAT gene derived from pA10-CAT; the *Hin*dIII-to-*Aat*II fragment of pA10-CAT was cloned into the large *Hin*dIII-to-*Aat*II fragment of pUC18. Wp was cloned into the *Pst*I site of the polylinker of pUC-CAT; −921W-CAT and −3092W-CAT contained EBV genomic nucleotides 44188 to 45089 and 42017 to 45089, respectively (3). −3888C-CAT and −1425C-CAT contained EBV nucleotides 7448 to 11337 and 9911 to 11337, respectively, linked to the *Bgl*II-to-*Bam*HI fragment of pSVOCAT cloned in both orientations into the *Bam*HI site of pUC18. CMVLTR-CAT, a gift from I. Chen which contains the cytomegalovirus immediate-early (CMV-IE) enhancer linked to a human T-cell lymphotrophic virus type I promoter (9), served as the positive control. $pKan_2$-WWYH, which was derived from p1040.30 (a gift from B. Sugden, Madison, Wis.), had the EBV fragment consisting of nucleotides 40863 to 52940 inserted into the large *Bam*HI-to-*Sal*I fragment of $pKan_2$. A 405-bp *Sph*I fragment was removed from this plasmid to generate the EBNA-2 deletion mutant E2ΔA6. WWYΔ, also derived from p1040.30, comprised EBV positions 40863 to 48040 and was used as a control for promoter competition; it did not contain EBNA-2 or BHLF-1. 3R, 5R, and 7R were EBNA-LP cDNAs cloned into a plasmid containing the CMV-IE promoter and enhancer (a gift from P. O'Hare, Marie Curie Institute, Oxted, Surrey, United Kingdom).

Transfections. Cells fed 1 day earlier were transfected in duplicate with 20 μg of each construct. This procedure was

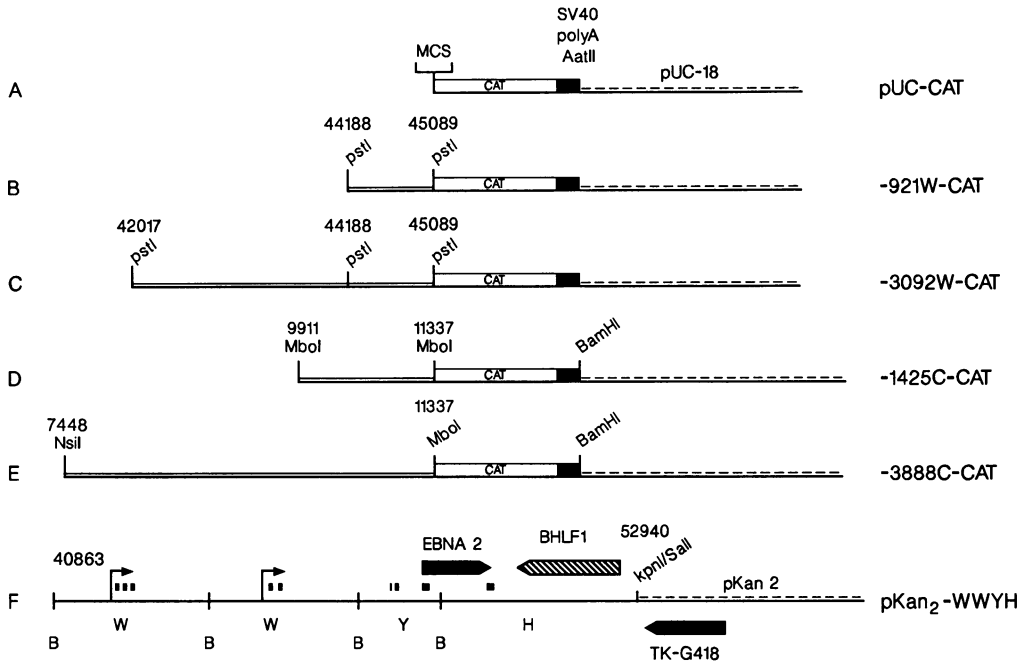

FIG. 1. Constructs used in transfections. (A) pUC-CAT, the negative control plasmid, contains the *Hin*dIII-to-*Aat*II fragment of pA10-CAT cloned into the *Hin*dIII-to-*Aat*II fragment of pUC18. (B and C) −921W-CAT and −3092W-CAT contain EBV genomic nucleotides 44188 to 45089, and 42017 to 45989, respectively, cloned into the *Pst*I site of pUC-CAT; each plasmid contains the Wp TATA box at position 45072 (3). (D and E) −1425C-CAT and −3888C-CAT contain EBV genomic nucleotides 9911 to 11337 and 7448 to 11337, respectively, plus the *Bgl*II-to-*Bam*HI fragment of pSVOCAT, cloned into pUC18; each contains the Cp TATA box at nucleotide 9631 (3). (F) $pKan_2$-WWYH contains EBV genomic nucleotides 40863 to 52940 cloned into the *Bam*HI-to-*Sal*I site of $pKan_2$. The thin arrows and small black rectangles represent the Wp TATA box. MCS, multiple-cloning site; SV40 polyA, simian virus 40 polyadenylation site; TK-G418, neomycin resistance gene driven by the herpes simplex virus thymidine kinase promoter; B, *Bam*HI. W, Y, and H are *Bam*HI fragments of EBV.

repeated on at least three separate occasions under conditions described by Buschle et al. (7). Briefly, 8×10^6 cells in 250 μl of ice-cold medium containing 20 μg of DNA were electroporated in a Bio-Rad Gene Pulser at 250 V and 960 μF. The shocked cells were cultured for 24 h before extraction in 0.25 M Tris, pH 7.5. For the CAT assay, 20 μl of cell extract, representing 2×10^6 cells, was incubated with 5 μl of 4 mM acetyl coenzyme A and 1 μl of [^{14}C]chloramphenicol (50 nCi) for 1 h at 37°C. The samples were extracted with ethyl acetate and assayed by thin-layer chromatography. The results were quantified by counting the amount of radioactivity associated with acetylated and nonacetylated chloramphenicol and then calculating the percentage of acetylated product.

S1 nuclease protection assays. Fifty micrograms of total RNA was incubated with 5×10^4 cpm of end-labeled oligonucleotide probe (2 pmol of oligonucleotide labeled with [γ-^{32}P]dATP [3,000 μCi/mmol]), for 10 min at 75°C and overnight at 56°C in aqueous buffer containing 1 M NaCl, 0.16 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5), and 0.33 mM EDTA. Each reaction mixture was then treated with 300 U of S1 nuclease (Bethesda Research Laboratories) for 45 min at 37°C. The reaction was terminated with EDTA and ice-cold ethanol, and the mixture was precipitated and resuspended in 7 μl of Tris-EDTA (TE) plus formamide loading buffer before being loaded on a sequencing gel (8% polyacrylamide, 7 M urea). The W0W1 oligonucleotide was 5′-CACTTCGGTCTCCCC TAGGATTTGTGTGGACTCCTGGCGCTCTGATGCGAC CAGAAA-3′; the W0W1′ oligonucleotide was 5′-GG GCCTTCACTTCGGTCTCCCATTTGTGTGGACT CCTGGCGCTCTGATGCGACCA-3′; the C_1 oligonucleotide was 5′-ATGAGGGCTCTGGGGGTCTTCGGTGTCCT TGTCTCTATGCCATCTGATCTAAAATTTGCA-3′; the protected fragments are predicted to be 46, 48, and 50 nucleotides long, respectively.

RESULTS

EBV-negative B cells constitutively utilize Wp. To determine how Cp and Wp function in the absence of viral influences, we transfected Wp-CAT and Cp-CAT gene constructs into a variety of EBV genome-negative cell lines, including three BL cell lines, a multiple myeloma line, and tonsillar B cells that were either untreated or activated with pokeweed mitogen for 3 days (Fig. 2A). We used two constructs for each promoter. The shorter of each, −921W-CAT and −1425C-CAT, has previously been described (14), and we included −3092W-CAT, which contains an entire IR-1 repeat, to determine whether the additional sequences within IR-1 had positive or negative regulatory effects. The longer Cp construct was included to assess the effects of ori-P on promoter function. ori-P has been shown to contain a transcriptional enhancer which is transactivated by EBNA-1 (23). In general, we found that the ori-P-containing construct was more active than the shorter construct, even in EBV genome-negative cell lines which use Cp poorly and do not contain EBNA-1 (Fig. 2B).

Figure 2A compares the function of the short C promoter (−1425C-CAT) with that of the short W promoter (−921W-CAT) in different EBV-negative B cell lines. In every comparison, Wp was more active than Cp. In most instances

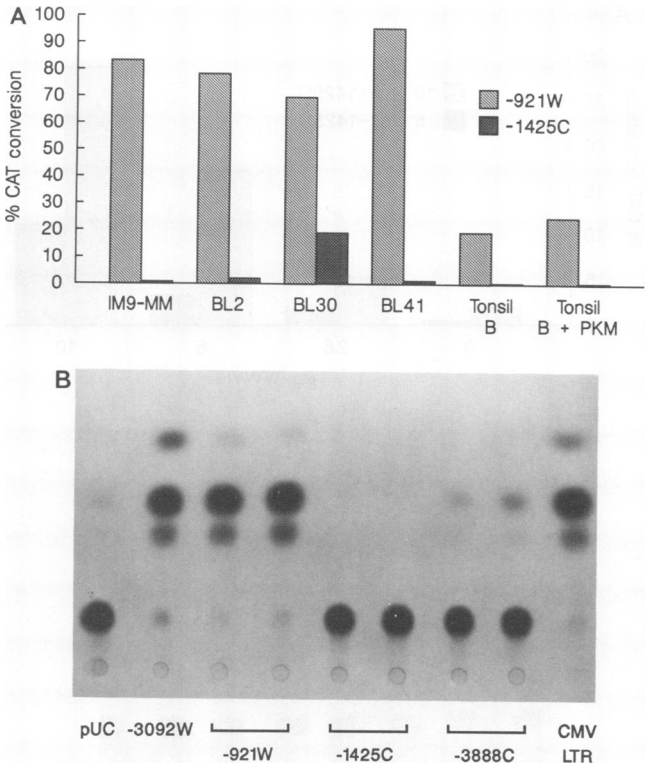

FIG. 2. Cp and Wp function in EBV-negative cell lines. (A) Composite of experiments with four EBV-negative cell lines and primary B lymphocytes. IM9 is a multiple myeloma (MM) cell line; primary tonsillar B cells were untreated or activated for 3 days with pokeweed mitogen (PKM). Only results for −921W-CAT and −1425C-CAT are shown. (B) Cells were assayed for CAT activity after 24 h; the lowest spot is the nonacetylated form, while the higher spots are acetylated forms indicating enzyme activity. Results of a typical experiment in which BL2 cells were transfected with 20 μg of DNA by electroporation are shown. pUC-CAT and CMVLTR-CAT were negative and positive controls, respectively.

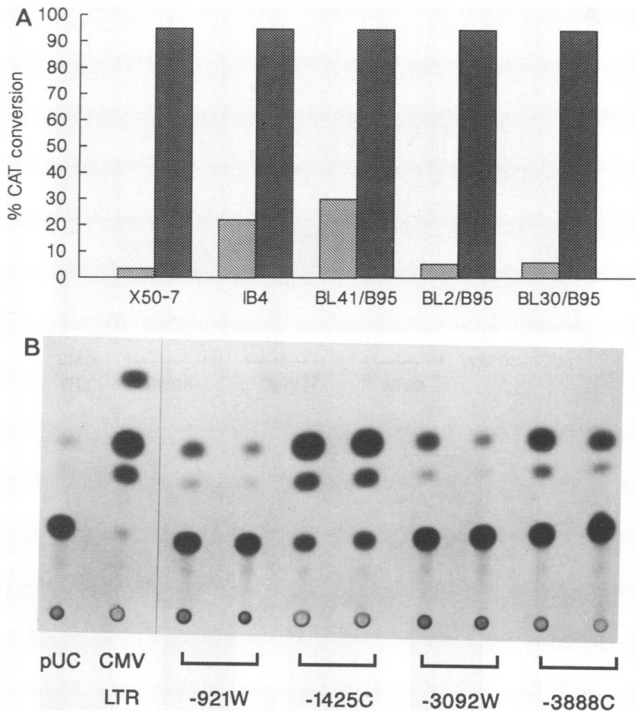

FIG. 3. Effect of EBV infection on latent-cycle promoter use. (A) Composite of different experiments with EBV-transformed cell lines (B95-8-converted BL cells or EBV-infected normal B cells [X50-7 and IB4]). Only results with −921W-CAT (▨) and −1425C-CAT (■) are shown. (B) BL2 cells that had been converted with B95-8 virus (BL2/B95-8) were transfected with Cp-CAT and Wp-CAT constructs; CAT activity was measured after 24 h.

Cp was not used at all; in others (e.g., BL30), its activity was appreciable but still much lower than that of Wp. A typical result is shown in Fig. 2B. Both −921W-CAT and −3092W-CAT were strong promoters in BL2 cells, whereas both Cp constructs were weak. pUC-CAT, with no detectable activity, and CMVLTR-CAT, with very strong activity, were negative and positive controls, respectively. The shorter Wp construct usually showed greater activity than the longer construct, but every cell line tested used both promoters or neither. These experiments show that Wp is constitutively active in B cells.

Infection of B cells with EBV increases Cp activity. To examine the effect of EBV on Cp and Wp function in B cells, we transfected promoter-CAT gene constructs into normal B cells transformed with EBV in vitro (IB4 and X50-7) and EBV-negative BL cell lines that had been converted to EBV positivity with B95-8 in vitro. All of these cell lines expressed the full complement of latent-cycle genes, except that TP expression was not verified and the EBERs were not expressed in BL41/B95-8 cells (26). All of the EBV-positive lines, used Cp much more strongly than Wp (Fig. 3A), and even though Wp activity was uniformly detected it, appeared weaker than in EBV-negative B cells (Fig. 2A). Figure 3B shows the results of a typical experiment in which both Cp and Wp constructs were active in BL2/B95-8 cells, with Cp showing more activity than Wp. Thus, EBV induced Cp function and effectively switched promoter preference from Wp to Cp, suggesting that Cp requires virus-encoded factors for its optimal function.

The immortalization-deficient mutant P3HR-1 does not induce the Wp-to-Cp switch. The differential use of Cp and Wp by EBV-negative and EBV-positive cells suggested that some factor induced or encoded by EBV transactivates Cp. To determine whether the same factor is induced by the immortalization-deficient EBV P3HR-1, which lacks the EBNA-2 gene and has a mutant EBNA-LP gene, we studied Cp and Wp function in three BL cell lines transformed by P3HR-1: BL2/CL16, BL41/CL16, and BL30+P3 (26). All three retained their original specificity for Wp after conversion. Figure 4A shows that in BL41/CL16 cells, both −921W-CAT and −3092W-CAT were both active promoters, while −1425C-CAT and −3888C-CAT activities were barely detectable. To determine whether a shift in cell differentiation could induce a shift to Cp function, BL41/CL16 cells were treated with the differentiation-inducing phorbol ester TPA. TPA increased Wp activity slightly but had no effect on Cp activity. The same pattern was seen with the BL2/CL16 line (not shown). TPA downregulates Cp and Wp function in cells in which the lytic cycle is inducible (unpublished results). However, the lytic cycle is not TPA inducible in BL41/CL16 or BL2/CL16, suggesting that the downregulation of Cp and Wp is dependent on induction of differentiation of the cell. P3HR-1 infection did induce a slight increase in Cp function in BL30 cells (Fig. 4B), but this

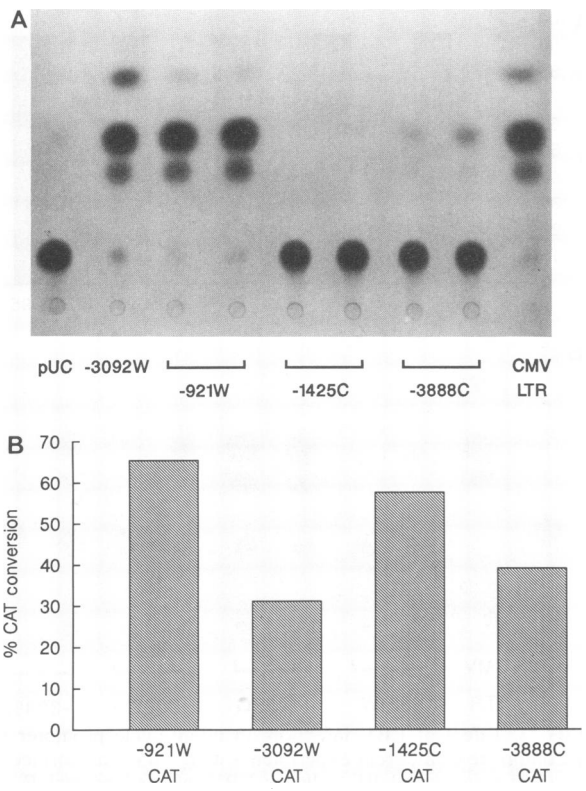

FIG. 4. Effect of P3HR-1 infection on Cp and Wp activity in BL cell lines. (A) BL41/CL16 cells were transfected with plasmids as shown. In this experiment, the cells were untreated or were treated with TPA immediately after transfection and harvested after 48 h. (B) Histogram showing Cp and Wp activity in BL30+P3 cells (converted with P3HR-1) transfected in duplicate.

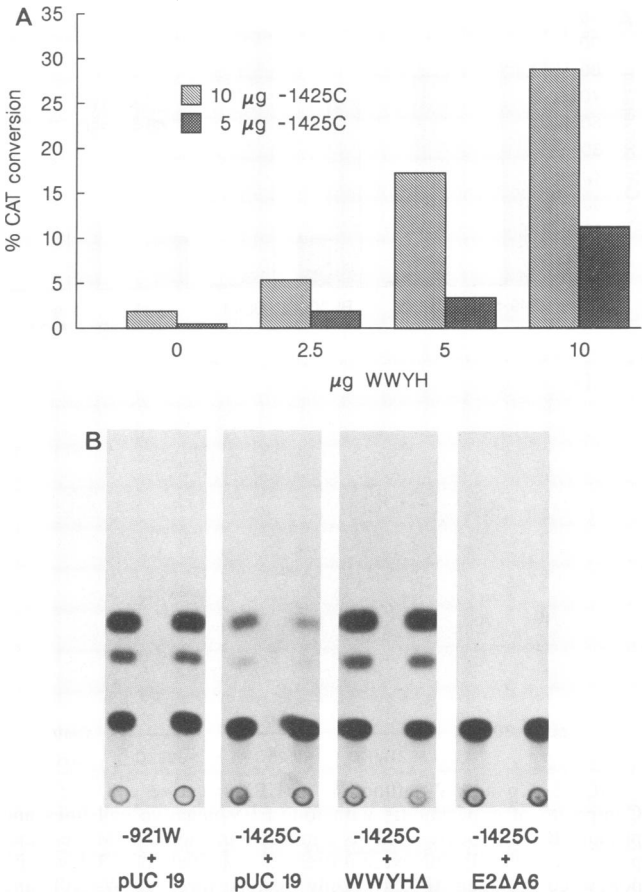

FIG. 5. Effect of EBNA-2 on Cp and Wp promoters. (A) BL41/CL16 cells cotransfected with −1425C-CAT (10 or 5 μg) and increasing amounts of pKan$_2$-WWYH. CAT activity was measured after 24 h; percent acetylation is represented on the histogram. (B) BL41 cells cotransfected in duplicate with 20 μg of −921W-CAT or −1425C-CAT. E2ΔA6 is pKan$_2$-WWYH with a deletion from EBV genomic nucleotides 49246 to 49650 inclusive, which removes 405 bp from the EBNA-2 open reading frame.

Cp activity was consistently weaker than that of Wp (uninfected BL30 cells also showed some Cp activity [Fig. 2A]). Thus, infection of BL cells with the EBNA-2-negative P3HR-1 virus does not induce Cp function as strongly as does infection with the EBNA-2-positive B95-8 virus.

EBNA-2 induces Cp function. The deletion in the P3HR-1 virus affects not only the EBNA-2 and EBNA-LP genes but also an early lytic-cycle gene, BHLF-1 (Fig. 1). Since Cp is a latent-cycle gene promoter, the two EBNA genes seemed good candidates for the role of Cp transactivation. To determine whether complementing its EBNA-2 gene deletion would restore Cp-inducing activity to P3HR-1, we cotransfected Cp-CAT with a plasmid derived from genomic B95-8 EBV, pKan$_2$-WWYH, that spanned the P3HR-1 deletion. In BL41 cells, pKan$_2$-WWYH induced Cp activity in a dose-dependent manner (Fig. 5A). This effect could not be attributed to competition for negative regulatory elements because a plasmid, pKan$_2$-WWYΔ, which contains the EBV promoter region but lacks functional open reading frames, had no such effect on Cp (not shown).

Plasmid pKan$_2$-WWYH expresses EBNA-2 in BL cells and potentially expresses a short EBNA-LP from two W promoters within the *Bam*HI W fragments and the BHLF-1 gene from its early lytic-cycle promoter. Although we could detect the expression of EBNA-2 in BL41 cells by immunofluorescence after transfection with pKan$_2$-WWYH (not shown), we were not able to detect EBNA-LP or BHLF-1 gene expression. EBNA-2 was expressed from an EBV promoter, probably Wp since it was still expressed when WWYH was excised from the pKan$_2$ vector. To identify which of the three EBV genes within WWYH was responsible for Cp transactivation, we cotransfected Cp-CAT with plasmids expressing EBNA-LP or an EBNA-2-deletion mutant of pKan$_2$-WWYH, E2ΔA6. EBNA-LP was expressed from cDNAs with three, five, or seven W1W2 repeats linked to the CMV-IE promoter (3R, 5R, and 7R), but none of these constructs had any effect on Cp function, although their expression of EBNA-LP could be detected by immunofluorescence and by Western immunoblotting (not shown). The EBNA-2 deletion mutant contains a 405-bp in-frame deletion in the EBNA-2 open reading frame and expresses a mutant EBNA-2. The deletion does not affect EBNA-LP or BHLF-1. This deletion of 135 amino acids from the EBNA-2 protein abrogated transactivation and even appeared to reduce the small amount of expression detected from −1425C-CAT alone. This experiment suggests that EBNA-2 either directly or indirectly transactivates Cp function.

S1 analysis confirms transient transfection assay results. We were concerned that the results of transient transfection assays using a heterologous reporter gene might not reflect

the in vivo situation. This concern was emphasized by the suggestion of Woisetschlager et al. (39) that within a given cell line, Cp and Wp function is mutually exclusive, while our results showed that both promoters were used in all B-cell lines infected with B95-8 virus. We therefore performed S1 nuclease protection experiments on the EBV-negative BL cell lines and on the B95-8 and P3HR-1 converts. We used end-labeled oligonucleotides that were complementary to and overlapped the 5′ ends of mRNAs originating downstream of Cp (C1 oligonucleotide) and Wp (W0W1 oligonucleotide, which crossed the W0W1 splice junction, and W0W1′ oligonucleotide, which crossed the W0W1′ splice junction). We detected activity from both genomic promoters in BL41/B95-8, BL30+B95-8, and BL30+P3, just as we had found in our CAT assays (Fig. 6). Only Cp activity was detected in BL2/B95-8, and only Wp activity was detected in BL41/CL16. The signal seen in BL41/CL16 with the C1 oligonucleotide is undigested oligonucleotide, and no Cp activity was seen in repeated experiments (Fig. 6B). Wherever Wp activity was detected, both splice sites were used, with one creating a message that could translate EBNA-LP (W0W1′) and the other creating an untranslatable leader sequence. No message could be detected in BL2/CL16 in repeated experiments. Parental P3HR-1 (clone 16) cells used both promoters, as we had found in the transient transfection assays, and B95-8 cells used only Cp, as was found by Woisetschlager and coworkers (39). Figure 6B depicts an experiment in which Wp activity in BL41/CL16 and BL41/B95-8 cells is clearly in evidence. Although the S1 data in Fig. 6A suggest that in BL41/B95-8, Wp activity is greater than Cp activity, this may be a result of the relative specific activities of the probes. These results show that our transient transfection studies do reflect the situation in cells in that both promoters are functional in cell lines infected with B95-8 virus. They also showed that mutually exclusive promoter function is not a rule for all cell lines, although only in situ hybridization studies would determine whether both promoters are used in one cell.

DISCUSSION

We have found that one of the two promoters of EBNA gene transcription, Wp, is used constitutively in EBV genome-negative primary B cells and in EBV-negative B-cell lines. This suggests that Wp is an immediate early promoter of EBV that does not require any other EBV gene product for its expression. The other promoter, Cp, is used weakly in EBV-negative BL cell lines and not at all in primary tonsillar B cells or in a human multiple myeloma cell line. Infection of primary B cells or EBV-negative B-cell lines with transforming EBV causes a switch in promoter preference from Wp to Cp, implying that some virally encoded factor alters promoter specificity in B cells and is required for optimal Cp function. The immortalization-deficient mutant P3HR-1 does not induce the Wp-to-Cp switch, suggesting that one of the three genes affected by the P3HR-1 deletion—EBNA-2, EBNA-LP, or BHLF-1—must be important in inducing Cp function. When the region deleted in P3HR-1 is complemented, only EBNA-2 induces a Wp-to-Cp preference switch in EBV-infected B cells. This confirms the recent finding of Sung et al. (34) that EBNA-2 transactivates a lymphoid-specific enhancer in the *Bam*HI C promoter. Our observations also show that there is differential specificity in the function of these two EBNA promoters, and they extend the findings of Ricksten et al. (24, 25), who mapped an

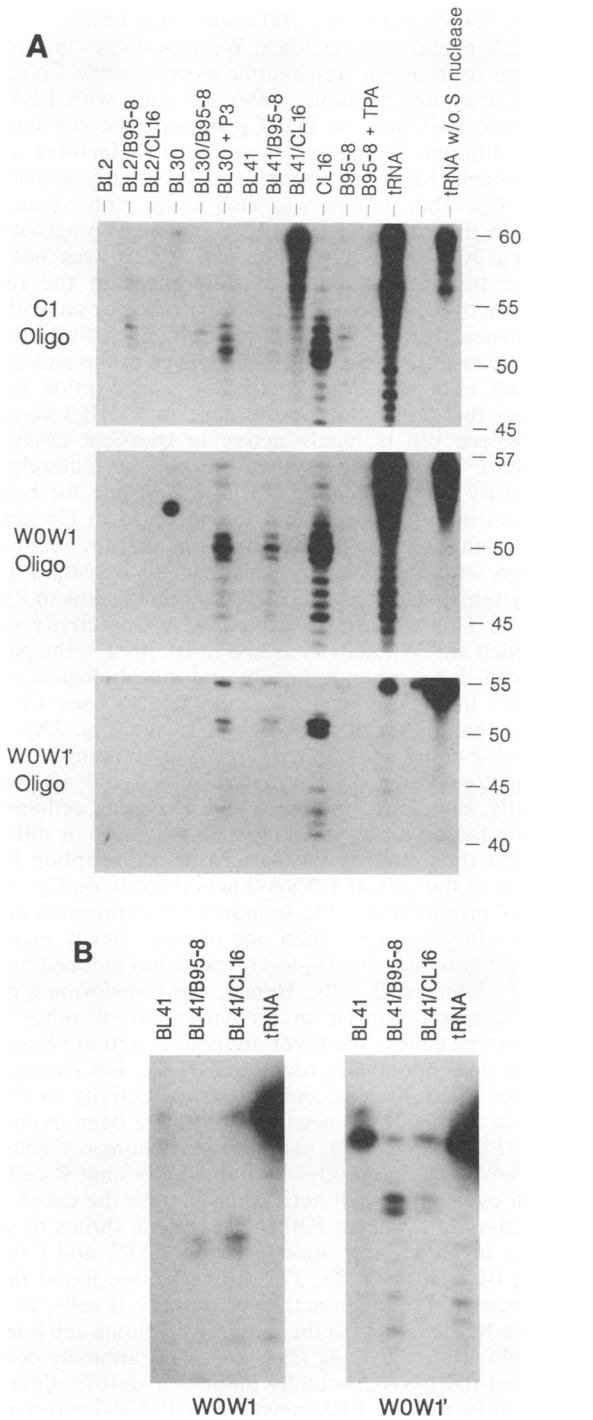

FIG. 6. S1 analysis of Wp and Cp function in EBV-negative BL cells and their B95-8 or P3HR-1 converts. Cytoplasmic RNA was incubated with oligonucleotides spanning the W0/W1 and W0/W1′ exons (W0W1 Oligo and W0W1′ Oligo) to monitor Wp function and the C1 exon (C1 Oligo) to monitor Cp function. (A) Controls were clone 16 (CL16) of P3HR-1 RNA, B95-8 RNA, tRNA, and tRNA without S1 nuclease. Numbers at the right indicate nucleotides. (B) BL41 and its converted subclones were probed with W0W1 and W0W1′.

enhancer region upstream of Wp and showed that Wp functions as an EBNA-2 promoter in an EBV-negative BL line (DG75) but not in the HeLa epithelial line.

In EBV-negative B-cell lines, Wp was always the stronger promoter in transient transfection assays, while Cp activity was weak or undetectable. After infection with EBV, this preference switched, so that Cp became the stronger promoter, although Wp activity was always detectable (except in B95-8 cells [data not shown]) but was much weaker than that of Cp. This pattern was also found with S1 analysis, except in the case of BL41/B95-8, where Wp activity was more easily detected than Cp activity. It was not clear whether this was a result of differences in the relative specific activities of the Cp and Wp probes or an artifact of the transient transfection assay. Wp-CAT activity may be weak because the repeated W sequences in the endogenous virus act as a sink for Wp-specific transcription factors, although this could not be the case in P3HR-1-converted BLs, where Wp is highly active in transient assays. An alternative explanation is that the cellular differentiation induced by B95-8 infection (8) is responsible for both the reduction in Wp activity and the increase in Cp activity. These changes in cell differentiation are not induced by infection with P3HR-1 (8), which would explain why Wp activity remains high and Cp activity remains low in P3HR-1 converts. This would also explain why Cp activity is relatively high and Wp activity is low in BL30+P3; the parental BL30 cell line is phenotypically and morphologically more activated than BL2 or BL41, and it also uses Cp more strongly than either of these two cell lines (Fig. 2A).

EBNA-2 could activate Cp directly by binding to specific sites upstream of Cp. Alternatively, it could activate Cp indirectly, either by binding to and activating cellular transcription factors or by inducing cell activation or differentiation and thus altering the pattern of transcription factors available in the cell. If EBNA-2 acts directly on Cp (and on the LMP promoter [11, 43]) to induce the expression of other latent-cycle genes (26), then one of these newly expressed genes might induce the G_0-to-G_1 transition induced by EBV in newly infected B cells. Hence, the transforming role of EBNA-2 might consist in its activation of other EBV-transforming genes. We favor an indirect action because Cp function does not always require EBNA-2. For example, Cp functions in BL30 cells and has strong activity in P3HR-1 cells that are EBNA-2 negative, but have been exposed to EBNA-2 in the past (21), and in primary human T cells (6a). If EBNA-2 acts indirectly, by inducing resting B cells into the cell cycle, then cell activation may be the cause of Cp transactivation. Indeed, EBNA-2 has been shown to induce markers of B-cell activation, such as CD23 and CD21, in cycling BL cell lines (36, 37). However, we found that Cp was inactive in mitogen-activated primary B cells, in EBV-negative BL cells, and in the multiple myeloma cell line IM9, all of which were cycling (Fig. 2A). This anomaly could be explained if EBNA-2 actually induces a shift in differentiation; that is, cycling EBV-negative or P3HR-1-positive BLs would be slightly less differentiated than B95-8-positive or EBNA-2-positive BL lines and consequently would contain a different complement of transcription factors. The idea that EBNA-2 might bind to and alter the activity of a cellular transcription factor is attractive because of our finding that the EBNA-2 deletion mutant reproducibly reduces the slight Cp activity seen in BL41 cells (Fig. 5B), i.e., the deletion mutant may retain transcription factor-binding activity but might inactivate rather than activate the factor.

P3HR-1 neither activates B cells (12) nor induces the expression of EBNA-1, EBNA-3A, EBNA-3B, or EBNA-3C in primary B cells (26), even though Wp is active in these cells and even though EBNA-1, EBNA-3A, EBNA-3B, and EBNA-3C can be expressed by P3HR-1 virus in BL cells. This failure could be interpreted as the inability of Wp to promote transcription of EBNA-1, EBNA-3A, EBNA-3B, and EBNA-3C. However, in two cell lines in which Cp is deleted, X50-7 and IB4 (40), the full complement of EBNAs is expressed from Wp (39). Thus, cell-cycle-specific post-transcriptional regulation at the level of pre-mRNA processing may determine whether EBNA-1, EBNA-3A, EBNA-3B, and EBNA-3C are expressed from Wp. The difference in EBNA expression from P3HR-1 in primary B cells and established cell lines could then be explained by the activation or differentiation states of these cells. We attempted to see whether we could induce Cp function in BL41/CL16 cells by treatment with TPA (Fig. 4A); however, TPA had no effect. Therefore, we suggest that after infection of primary B cells with EBV, the first proteins to be expressed are EBNA-2 and EBNA-LP. EBNA-2 expression results in both a G_0-to-G_1 transition and the induction of the other latent-cycle proteins. We cannot predict the order of these two events, but both require EBNA-2.

Transactivation of Cp by EBNA-2 may explain the promoter switch from Wp to Cp seen between 40 and 140 h after infection of primary B cells with transforming EBV (40). EBNA-2 and EBNA-LP are the first proteins to appear, at about 12 h postinfection (2, 18, 26). EBNA-1 and EBNA-3 appear later, at a time that coincides with the promoter switch; hence, the expression of these proteins might require Cp induction by EBNA-2. Our results extend the finding that Wp is used before Cp after infection of primary B lymphocytes with EBV; we show in this article that Wp function is constitutive in B cells and does not require the expression or function of any other EBV protein.

Enhancer sequences downstream of the Cp transcriptional start site must be considered in a discussion of the selective use of Cp or Wp, since they have been reported by Walls and Perricaudet to be important for transfected Cp function in EBV-positive cells (35). In that study, transfected Cp functioned in all three cell lines tested, but the endogenous viral Cp functioned in only two of them. Hence it was not clear whether the constructs containing the downstream elements reflected the true biological behavior of the virus. One of the downstream Cp enhancer regions is situated in IR-1 upstream of Wp and has been shown to contain enhancers for Wp (24); the other region resides partially within IR-1. If these enhancers serve both promoters, how are they individually regulated? Specificity could be conferred through repressor signals, which are position dependent. For example, in primary B cells, Cp would be specifically suppressed, and the IR-1 enhancers would act on Wp. Infection with EBV would remove this suppression, and the IR-1 enhancers would act on Cp. Unsuppressed Cp function may repress Wp function because of its upstream position, since Wp activity usually decreases as Cp function increases (compare Fig. 2A and 3A).

A major question in the study of EBV biology is how EBV-infected B cells survive in vivo in an immunocompetent host. One obvious manner would be by suppression of all immunogenic latent-cycle genes, and their promoters would be one target for their suppression. For example, both Cp and Wp may be suppressed in group 1 BL cells, which express only EBNA-1 from a newly described downstream promoter, Fp (30). Suppression of Wp and Cp and activation of Fp may be dependent on the differentiation state of a B

cell, or it may require virus-mediated gene regulation. Latent-cycle genes could be suppressed in vivo via Wp. This would prevent EBNA-2 expression and the consequent expression of the remaining EBNAs from Cp or the latent-cycle membrane proteins from their promoters (1, 11, 34, 43). As a result, the G_0-to-G_1 transition would not occur, and the virus would not require plasmid replication to be maintained. The cell could then carry EBV anonymously until activated by a specific antigen at sites appropriate for infection of epithelial cells or other B cells. Suppression of Wp activity would be one way in which normal B cells in vivo could avoid expressing immunogenic latent-cycle genes and thus survive in an immune host. Our work demonstrates the importance of the interplay of virus and host functions in determining promoter usage and latent-cycle gene expression in infected cells. This interaction carries implications for the survival of EBV-infected B cells in different stages of activation or differentiation.

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