Methylation Status of the Epstein-Barr Virus (EBV) BamHI W Latent Cycle Promoter and Promoter Activity: Analysis with Novel EBV-Positive Burkitt and Lymphoblastoid Cell Lines

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The Epstein-Barr virus (EBV) latent cycle promoter Wp, present in each tandemly arrayed copy of the BamHI W region in the EBV genome, drives expression of the EB viral nuclear antigens (EBNAs) at the initiation of virus-induced B-cell transformation. Thereafter, an alternative EBNA promoter, Cp, becomes dominant, Wp activity declines dramatically, and bisulfite sequencing of EBV-transformed lymphoblastoid cell lines (LCLs) shows extensive Wp methylation. Despite this, Wp is never completely silenced in LCLs. Here, using a combination of bisulfite sequencing and methylation-specific PCR, we show that in standard LCLs transformed with wild-type EBV isolates, some Wp copies always remain unmethylated, and in LCLs transformed with a recombinant EBV carrying just two BamHI W copies, Wp is completely unmethylated. Furthermore, we have analyzed rare LCLs, recently established using wild-type EBV isolates, and rare Burkitt lymphoma (BL) cell clones, recently established from tumors carrying EBNA2-deleted EBV genomes, which express EBNAs exclusively from Wp-initiated transcripts. Here, in sharp contrast to standard LCL and BL lines, all resident copies of Wp appear to be predominantly hypomethylated. Thus, studies of B cells with atypical patterns of Wp usage emphasize the strong correlation between the presence of unmethylated Wp sequences and promoter activity.

Epstein-Barr virus (EBV), a B-lymphotropic herpesvirus implicated in the pathogenesis of several human malignancies, efficiently transforms resting B cells in vitro into permanently proliferating lymphoblastoid cell lines (LCLs). Such LCLs all display a latency III form of infection characterized by the constitutive expression of six EBV-encoded nuclear antigens, EBNA1, -2, -3A, -3B, -3C, and -LP, and three latent membrane proteins, LMP1, -2A, and -2B (38). While the LMP genes are transcribed from their own individual promoters, all six EBNA mRNAs are generated by the splicing of long primary transcripts which initiate from one of two alternative promoters, Wp or Cp (9, 10, 41, 47). Wp, present in each BamHI W repeat of the EBV genome, is selectively activated immediately postinfection (3, 56). While these Wp-initiated transcripts can potentially encode all six EBNAs, at these early time points there appears to be a preferential expression of EBNA2 and EBNA-LP; subsequently, these two antigens activate the alternative EBNA promoter, Cp (39, 48, 54), leading to the broadening of virus antigen expression to all six EBNAs, and up-regulate the expression of the LMP promoters (1, 17, 53, 62). The early stages of B-cell transformation are, therefore, characterized by a marked switch in EBNA promoter usage, with Cp becoming dominant over Wp, leading to the outgrowth of Cp-using LCLs (43, 55, 56).

While there has been some progress in identifying the cellular factors important for the initial activation of Wp in resting B cells (8, 28, 50), how Wp is subsequently repressed remains poorly understood. This is an important question, however, since the flexibility of latent promoter usage is central to the virus’ strategy for persistence in vivo (49), and the Wp-to-Cp switch provides a rare opportunity in which changes in promoter usage can be followed in vitro in real time. The first clue that DNA methylation may play a role in the initiation or in the subsequent maintenance of Wp silencing came from earlier studies of EBV-positive Burkitt lymphoma (BL) cell lines displaying a restricted latency I form of infection (5, 16, 24, 30–32). In such cells, the Wp, Cp, and LMP promoters are all silent and hypermethylated, and only a single latent protein, the genome maintenance protein EBNA1, is expressed from an alternative promoter, Qp (33, 42). However, to what extent Wp methylation status and Wp activity are linked remains a subject of debate.

One of the constraints in this regard is the lack of cell culture models available for analysis and, in particular, the absence of well-characterized lines in which Wp is the exclusive EBNA promoter. Here we attempt to overcome this limitation by (i) identifying rare Wp-using LCLs in which Cp, though present in the resident EBV episomes, is silent; and (ii) studying recently isolated Wp-restricted BL cell lines in which Wp, rather than Qp, is active and leads to the expression of EBNA1, -3A, -3B, -3C, and -LP in the continued absence of EBNA2, LMP1, and LMP2 (25). A second constraint is the difficulty of analyzing Wp sequence methylation exhaustively by the usual methods of PCR analysis and bisulfite sequencing (20). Very large numbers of sequences need to be analyzed in this way in order to gain a representative picture of promoter methylation since,
within most latently infected cells, there are multiple copies of the viral genome and multiple copies of Wp within each genome (4, 7). Here we attempt to overcome this limitation by (i) establishing LCLs using a recombinant virus with only 2 copies of Wp per genome, (ii) monitoring EBV genome load in all the lines analyzed, and (iii) developing a sensitive methylation-specific PCR assay (22) that more accurately reflects the full range of methylated and unmethylated Wp sequences present in any cell line.

MATERIALS AND METHODS

Cell lines. The panel of LCLs carrying natural EBV isolates included lines established by spontaneous transformation from EBV-infected donors (IM53.1 to IM107.1 and EBH41.2) or by virus infection of EBV-naïve donor B cells in established LCLs using a recombinant virus with only 2 copies of Wp within the viral genome and multiple copies of Wp within each genome (8a). Quantitative reverse transcription (RT)-PCR assays to detect Wp- and Cp-initiated EBNA transcripts, as described previously (44).

Preparation and use of recombinant EBVs. The recombinant EBV B95.8 strain genome from which the immediate-early BZLF1 gene was deleted has been described previously (18). Recombinants carrying different numbers of BamHI W repeats were made using the same technique (R. Tierney, unpublished data). Briefly, a vector was designed that contained a BamHI C-derived 5'-flanking region and a BamHI Y-derived 3'-flanking region into which premigrated BamHI W fragments were inserted and then introduced into the EBV bacterial artificial chromosome 2089 (13) by homologous recombination. Clones were screened to determine the numbers of BamHI W repeats present, and recombinant genomes with 2, 4, 6, 8, and 11 Wp copies were selected. Genomes were transfected into 293 producer cells, virus preparations were generated, and the EBV genome content was assayed as described previously (44).

Peripheral blood mononuclear cells were prepared from buffy coat samples (Blood Transfusion Service, Birmingham, United Kingdom), and B cells were isolated by positive selection using CD19 Dynabeads (Dynal). Resting B cells were exposed to virus overnight at 37°C at a multiplicity of infection of 50. Following infection, B cells were cultured in standard medium.

Quantitative PCR assays for EBV gene expression and genome load. Total RNA was extracted from 2 × 106 to 5 × 106 cells using a Nucleospin RNA extraction kit (Macherey-Nagel) according to the manufacturer's instructions. Four hundred nanograms of RNA was reverse transcribed into cDNA by using a mix of primers specific for numerous EBV transcripts, as described previously (8a). Quantitative reverse transcription (RT)-PCR assays to detect Wp- and Cp-initiated transcripts, EBNA2 transcripts, and BamHI Q-U-K-spliced EBNA1 and BamHI Y-U-K-spliced EBNA1 transcripts were performed. EBV transcripts were normalized to cellular GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcripts and expressed relative to an appropriate reference cell line, assigned an arbitrary value of 1. Reference cell lines included X50-7 (Wp-initiated transcripts), CD+Oku (CP-initiated, EBNA2, and BamHI Y-U-K-spliced EBNA1 transcripts), and Rael-EBL (BamHI Q-U-K-spliced EBNA1 transcripts). To determine EBV genome load, genomic DNA was extracted using standard methods and assayed by quantitative PCR amplification of the EBV DNA polymerase (BALF5) gene in parallel with the cellular beta 2-microglobulin gene, as described previously (26).

Western blot analysis of EBV latent proteins. Immunoblotting was performed using monoclonal antibodies H4 (anti-EBNA1) (21), JF186 (anti-EBNA-LP) (19), PE2 (anti-EBNA2) (61), and CS1 to CS4 (anti-LMP1) (40).

Bisulfite genomic sequencing. Genomic DNA was treated with sodium bisulfite, and for each sample, 2- to 5-μl aliquots of bisulfite-modified and unmethylated DNA were amplified in strand-specific PCRs using primers specific for the regulatory regions of Cp as described previously (51) or for the regulatory region of Wp, as follows. Unmodified Wp DNA was amplified in nested PCRs using the following primers and conditions: Wp outer1 (5'-CTTATCCACCGGGGTGCAG-3'), B95.8 coordinates 13796 to 13816) and Wp outer2 (5'-TGGAGGTGTG-3'). Wp inner1 (5'-CGGCGTCTCAGGGCGTCCT-3') (B95.8 coordinates 14485 to 14469) amplified for 30 cycles of 95°C for 30 s, 42°C for 30 s, and 72°C for 30 s.

RESULTS

Analysis of Wp and Cp during B-cell transformation. The Wp and Cp promoters are shown in their relative genomic positions in Fig. 1A, along with the downstream exon structures of the individual EBNA mRNAs expressed from both promoters. Note that Wp (and the first two exons of the EBNA-LP mRNA) lie within a BamHI W fragment that is tandemly repeated in the viral genome; thus, all natural EBV isolates contain multiple copies of Wp and express EBNA-LP species with multiple copies of a repeat domain.

The first set of experiments sought to reexamine the kinetics of Wp and Cp activation over time in freshly infected B-lymphocyte cultures, taking advantage of newly developed quantitative RT-PCR assays for Wp- and Cp-initiated EBNA transcripts. Here we used a recombinant B95.8 virus (with 11 Wp copies), rendered incapable of lytic virus replication by deletion of the BZLF1 immediate-early gene (18), in order to ensure that all viral RNA analyzed in emerging LCLs was episomal and not contaminated with newly replicated progeny virions. In repeated experiments, we found that Wp was activated to high levels within 24 to 48 h of infection and then gradually declined over the following 14 days, after which time it remained stable at a low level. However, as illustrated by the data from one such experiment (Fig. 1B), residual Wp activity was still detectable up to day 75, by which time the resultant LCL had undergone more than 20 population doublings in vitro. In the same experiment, Wp was not detectable until 60 h postinfection but then rose quickly as Wp activity declined.

Aliquots of the infected cells were harvested at regular intervals throughout this same experiment, and the methylation status of both Wp and Cp promoters was analyzed by bisulfite sequencing.
This analysis focuses on a 570-bp region of Wp encompassing two regulatory regions, the promoter-proximal “B-cell-specific” upstream activation sequence 1 (UAS1 -87 to -264 relative to the transcription start site) and the promoter-distal “lineage-independent” UAS2 (-264 to -352) (8). As illustrated in Fig. 1C, UAS1 contains binding sites for YY1, BSAP, RFX, and CREB, together with a recently identified second YY1 site between -270 and -276 relative to the transcription start site. Also marked are 20 CpG dinucleotides (black lollipop-shaped symbols, a to t) which represent potential methylated cytosines (B95.8 coordinates 13956, 13976, 14077, 14085, 14101, 14103, 14105, 14115, 14143, 14161, 14259, 14261, 14288, 14290, 14296, 14381, 14391, 14445, and 14462). CpG sites j, k, and n to p (boxed) have been shown previously to abrogate factor binding when methylated. (D) Results of bisulfite sequencing analysis of Wp in B cells 8 to 28 days postinfection. The Wp regulatory region was PCR amplified, cloned, and sequenced. Bisulfite-treated DNA was amplified with primers specific for Wp, and several PCR clones were sequenced for each sample. Individual CpG dinucleotides are identified as either methylated (+, shaded) or unmethylated (−).
Analysis of Wp and Cp in established LCLs. We then examined Wp and Cp usage in a panel of 25 LCLs carrying natural EBV isolates which had been established either by spontaneous or experimentally induced transformation in vitro. The quantitative RT-PCR data from a representative set of lines (Fig. 2) show that all but two LCLs expressed typical levels of Cp-initiated transcripts accompanied by some Wp transcription, while the two exceptions (EBH41.2 and IM53.1) showed relatively high levels of Wp but no detectable Cp activity. These two “Wp-only” LCLs were not obviously different from the Cp/Wp-using LCLs in terms of viral genome load (determined by quantitative PCR assay of acyclovir-treated cell lines) (Fig. 2) and showed similar levels of viral latent antigen expression and similar cell growth phenotypes (data not shown). Sequencing showed that Cp was, nevertheless, intact in these lines, at least up to 1 kb upstream of the transcription start site.

The two “Wp-only” LCLs were then compared with several standard Cp/Wp-using LCLs in bisulfite sequencing assays. The standard Cp/Wp-using LCLs all showed extensive Wp methylation by this criterion, typical for results illustrated by the IM100.1 and CD+Oku lines (Fig. 3). Interestingly, as seen in the earlier transformation experiments, we consistently noted the sparing of CpGs q and r immediately adjacent to the Wp transcription start site and of CpGs b and c upstream of UAS2. In addition, some, but not all, standard LCLs showed the sparing of CpGs j and k within one of the two BSAP sites in UAS1. However, the bulk of the Wp regulatory region, including CpGs n, o, and p in the BSAP/CREB site in UAS1, was clearly hypermethylated in Cp/Wp-using LCLs. By contrast, the two “Wp-only” LCLs gave a markedly different pattern. In both cases, there was extensive (but not total) methylation in the promoter-distal UAS2 “lineage-independent” region. However, the promoter-proximal UAS1 “B-cell-spe-
methylated Wp sequences are present in the Cp/Wp-using LCLs.

Analysis of LCLs with reduced Wp copies. Although there is no way to reduce the EBV episomal copy number in in vitro-transformed LCLs, it is nevertheless possible to reduce the numbers of Wp copies per episome by manipulating the cloned EBV episome as a bacterial artificial chromosome. By cloning from the original 2089 B95.8 recombinant (containing 11 Wp copies), we selected recombinant EBV genomes with 2, 4, 6, 8, and 11 Wp copies, transferred them into 293 producer cells, and rescued the corresponding infectious viruses. Panels of LCLs with different numbers of Wp copies per episome were then generated from the same initial B-cell population, and the LCLs were analyzed for Wp and Cp usage. Figure 5B shows data from cell lines transformed with 2-Wp-copy (2W LCL) virus or with 11-Wp-copy (11W LCL) virus; these data are representative of six lines of each type that were studied. Both sets of LCLs showed roughly equivalent levels of Wp activity, whereas Cp activity was consistently stronger in the 2W LCLs. The overall levels of EBNA mRNA transcription, assayed here using the BamHI Y1-U-K-spliced EBNA1 mRNA that is expressed from Wp and Cp, were not markedly different between the sets of cells. Furthermore, there were no major differences in the steady-state levels of EBNA1, EBNA2, or LMP1 between the LCLs detected by immunoblotting with specific monoclonal antibodies (Fig. 5C). Note that immunoblotting for EBNA-LP, a protein whose size is determined by the number of BamHI W-encoded repeat domains, confirmed that the 2W LCLs did indeed express a low-molecular-weight EBNA-LP consistent with the presence of only two repeats, whereas the 11W LCLs expressed a ladder of species with one dominant isof orm.

These same LCLs were then analyzed for Wp methylation by bisulfite sequencing (Fig. 6A). The 11W LCLs showed the pattern typical of extensive Wp methylation but with particular CpGs spared, as seen earlier for standard LCLs (Fig. 3). By contrast, the 2W LCLs were almost entirely unmethylated at Wp, a pattern that we have never observed before with established LCLs. Subsequently, these same LCLs and additional LCLs carrying 4-, 6-, and 8-Wp-copy virus strains were analyzed using the Wp-specific MSP assay (Fig. 6B). This confirmed that the 2W LCLs were almost entirely devoid of methylated Wp sequences, whereas LCLs with 4 to 11 Wp copies per episome carried both methylated and unmethylated Wp sequences.

Analysis of BL cell lines with different patterns of EBNA promoter usage. We then turned our attention to examining the possible relationship between Wp usage and Wp methylation status in a unique series of BL cell lines and derived cell lines, recently established in this laboratory (25, 26), and to displaying three different programs of restricted EBV latent gene expression (Fig. 7A). The transcriptional profiles of these BL lines, as determined by quantitative RT-PCR assays, are presented in Fig. 7B, which also includes for comparison two standard Cp/Wp-using LCLs to show how each of the BL programs described above differs from the standard latency III (LCL-like) form of infection.

First, we compared the Akata-BL and Rael-BL lines, displaying the classical latency I form of infection in which Wp and Cp are silent and EBNA1 is expressed selectively from the
Qp promoter, with the recently described Ava-BL, Oku-BL, and Sal-BL lines displaying a “Wp-restricted” form of latency (25). These latter lines carry EBNA2-deleted genomes and show exclusive use of Wp, in the absence of Cp or Qp activity, and express EBNA1, -3A, -3B, -3C, and -LP in the absence of EBNA2 or the LMPs. Because the parental Ava-BL, Oku-BL, and Sal-BL lines also carry a silent wild-type genome in many cells, here we used clones of these lines which retain the Wp-restricted pattern of virus antigen expression but carry only EBNA2-deleted genomes (26). All of these latency I and Wp-restricted BL cell populations carried multiple episomes (Fig. 7B).

Results from bisulfite sequencing analysis of Wp in these same lines (Fig. 8A) showed that Akata-BL (and Rael-BL [data not shown]) adheres to the previously published pattern for Qp-using latency I cells, where Wp is almost entirely methylated, except for the upstream CpG c and the CpGs q and r near the transcription start site. Interestingly, in the Wp-restricted BL cells, bisulfite sequencing showed that the dominant Wp species was again heavily methylated. However, we note that both the Ava-BL and Oku-BL clones nevertheless contained minor Wp copies that were almost entirely nonmethylated. Subsequent MSP analysis (Fig. 8B) showed that there were indeed unmethylated Wp copies in all three Wp-restricted BL clones, whereas no such unmethylated sequences could be detected in the latency I Akata-BL clone.

We next analyzed a series of clones recently established from early passages of the Awia-BL tumor line. This unique line is heterogeneous at the single-cell level and yielded clones which, though all derived from the same malignant BL population and all showed the same single-cell pattern of BL growth in vitro, nevertheless display three different forms of restricted virus latency (26a). These three different latency programs are illustrated in Fig. 7A, and the corresponding transcriptional data are shown in Fig. 7B. Some clones (9 and 20) carry multiple wild-type EBV episomes and display the classic latency I form of infection like Akata-BL and Rael-BL. Other clones (3 and 4) resemble Ava-BL, Oku-BL, and Sal-BL and show Wp-re-

![Diagram of EBV genome](image-url)
FIG. 6. (A) Bisulfite sequencing analysis of Wp in 2W and 11W LCLs. Data are presented as described in the Fig. 3 legend. (B) Results of MSP analysis of Wp methylation status in 2W, 4W, 6W, 8W, and 11W LCLs. Data are presented as described in the Fig. 4 legend.

FIG. 7. (A) Diagrammatic representation of three programs of EBV latent gene expression found in different Awia-BL clones. Conventional latency I clones express EBNA1 alone from the BamHI Q promoter Qp. Atypical Wp-restricted clones carrying only the EBNA2-deleted form of the genome express EBNA1, -3A, -3B, -3C, and -LP from the BamHI W promoter Wp. Novel EBNA2+/LMP1- clones express all six EBNAs from an unidentified promoter in the absence of the LMPs. (B) Analysis of EBV latent gene expression in BL lines and Awia-BL clones. The histograms show the results of quantitative RT-PCR assays used to measure BamHI Q-U-K-spliced EBNA1, Wp-initiated, Cp-initiated, and EBNA2 transcripts. Error bars indicate standard deviations of results of duplicate assays. Also shown are mean EBV genome loads for the corresponding acyclovir-treated cell lines determined by quantitative DNA PCR using a primer-probe combination specific for the EBV BALF5 gene. Included as controls were the standard Cp/Wp-using LCLs IM100.1 and CD + Oku.
restricted latency but in this case carry just a single EBNA2-deleted genome. A third set of Awia-BL clones (1 and 2) are unique in that they express all six EBNA proteins (in the absence of LMP1) but show no detectable Wp, Cp, or Qp usage in the quantitative RT-PCR assays of latent gene transcription (Fig. 7B); these EBNA2⁺ LMP1⁻ clones were also unusual in that they carry just a single copy of a wild-type EBV genome (26a).

Bisulfite sequencing analysis of these different sets of Awia-BL clones revealed that the latency I Awia-BL clone 9 (and clone 20 [data not shown]) resembled Akata-BL in showing extensive Wp methylation at all sites except for CpGs c, q, and r (Fig. 9A). Interestingly, the Wp-restricted Awia-BL clones 3 and 4, where the EBNA2-deleted genome load per cell was much lower than in the Wp-restricted Ava-BL, Oku-BL, and Sal-BL clones studied earlier, gave a distinct pattern (Fig. 9A); although there was extensive methylation in the UAS2 region of Wp, CpGs in the B-cell-specific UAS1 region were only partially methylated, a pattern similar to that seen with the Wp-only LCLs (Fig. 3). By contrast, the EBNA2-positive, LMP1-negative Awia-BL clones 1 and 2, also carrying low genome loads but where Wp was silent, showed the same extensive levels of methylation throughout Wp UAS1 and UAS2, as typically seen in latency I BL clones. These patterns were subsequently confirmed by MSP analysis (Fig. 9B). Thus, latency I BL clones and also the EBNA2-positive, LMP1-negative clones showed almost no unmethylated Wp sequences, whereas in the Wp-restricted clones, it was clear that Wp usage was associated with the presence of some unmethylated Wp copies.

**Summary of Wp methylation status in different LCL and BL lines.** Table 1 presents a summary of the overall results. For each cell line studied, the pattern of EBNA promoter usage (Wp, Cp, or Qp) is shown alongside the methylation status of Wp as determined by MSP assay and by bisulfite sequencing. In the case of the bisulfite sequencing data, the results are expressed as the percentage of methylation of all 20 CpGs analyzed or, specifically, of those methylation-sensitive CpGs lying within the upstream BSAP sites (j and k) or the adjacent BSAP/CREB sites (n, o, and p) in UAS1. By focusing on this bisulfite sequencing data, it can be seen that (with the exception of Ava-BL, Oku-BL, and Sal-BL clones with high genome loads) all cell lines showing Wp transcription tend to have low levels of methylation of CpGs j and k, and cell lines using Wp selectively also have relatively low levels of methylation of CpGs n, o, and p and of overall CpGs in the Wp region. Most importantly, Table 1 emphasizes the absolute correlation between detectable Wp activity in a cell line and the presence of unmethylated Wp sequences, as revealed by MSP analysis.

**FIG. 8.** (A) Bisulfite sequencing analysis of Wp in latency I Akata-BL and Wp-restricted clones of Ava-BL, Oku-BL, and Sal-BL. Data are presented as described in the Fig. 3 legend. (B) Results of MSP analysis of Wp methylation status in latency I Akata-BL and Wp-restricted clones of Ava-BL, Oku-BL, and Sal-BL. Data are presented as described in the Fig. 4 legend.
Wp is the first viral promoter to be activated during the in vitro transformation of primary resting B cells, but thereafter, levels of Wp-initiated transcripts decline and Cp becomes the dominant EBNA promoter in most established LCLs. While this Wp-to-Cp switch is well documented (3, 43, 56, 58), the mechanism of promoter switching remains unknown. The high levels of Wp-initiated transcripts seen in the early stages of infection might reflect the fact that the incoming viral genomes are unmethylated and nucleosome-free and, thus, are readily accessible to the transcriptional machinery. By contrast, the viral genome in established LCLs is known to adopt a structure similar to that of host chromatin (14, 45). If this structural change occurs early postinfection, perhaps linked to genome circularization which is detectable within the first 24 h (23), then it may contribute to the rapid decline in Wp activity. Indeed, a similar mechanism may be involved in silencing other regions of the EBV genome such as the BHRF1 and BALF1 lytic cycle genes which are transiently expressed following EBV infection (6). An alternative hypothesis is that the activation of the distal EBNA promoter Cp blocks the activity of the downstream copies of Wp through a transcriptional interference mechanism (35, 36, 59).

In previous work, we suggested that DNA methylation might be implicated in the downregulation of Wp, since we observed that Wp sequences were progressively methylated between 7 and 21 days postinfection, in experiments where peak Wp activity was not reached until day 7 (51). By contrast, in the present study, using newly developed quantitative RT-PCR assays to monitor virus promoter usage, we noted kinetics of Wp methylation that were similar to those described above but in circumstances where Wp activity clearly peaked much earlier. These discrepancies may be due in part to differences in RT-PCR methods and/or the dose of transforming virus used in the two studies. Importantly, the present findings make it clear that for Wp, the kinetics of promoter methylation lags significantly behind down-regulation, implying that Wp methylation does not initiate promoter silencing but may serve to maintain promoter sequences in an inactive state (34).

In line with recent findings from other groups (15, 59), the present analysis of a panel of LCLs carrying different EBV strains revealed that Cp was the dominant EBNA promoter in most lines but that Wp was never completely silenced (Fig. 2). This is in contrast to early studies, often based on long-established LCLs, that propose that Wp and Cp are mutually exclusive in their usage (55). The persistence of Wp activity in LCLs also calls into question the relevance of promoter methylation as a regulatory factor, since our bisulfite sequencing data, like those already in the literature (34, 51), showed extensive Wp methylation in all amplified sequences. However, bisulfite sequencing itself gives limited information, since, un-

FIG. 9. (A) Bisulfite sequencing analysis of Wp in latency I, Wp-restricted, and EBNA2+ LMP1– Akata-BL clones. Data are presented as described in the Fig. 3 legend. (B) Results of MSP analysis of Wp methylation status in latency I Akata-BL and Wp-restricted clones of Ava-BL, Oku-BL, and Sal-BL. Data are presented as described in the Fig. 4 legend.
less very large numbers of amplified products are analyzed, the pattern obtained reflects only the most abundant Wp species. Therefore, we used our understanding of the organization of Wp transcription factor binding sites to design an MSP assay that would detect Wp sequences that had not been methylated in the critical UAS1 regulatory region that is sensitive to methylation. The MSP assay revealed that there was heterogeneity in the critical UAS1 regulatory region that is sensitive to methylation. The MSP assay revealed that there was heterogeneity in the critical UAS1 regulatory region that is sensitive to methylation.

Studies of Wp methylation are further complicated by the presence of multiple Wp copies in each EBV episome. We attempted to overcome this problem experimentally by reducing the Wp copy number in a recombinant EBV genome context. We therefore specifically generated a recombinant with only two BamHI W repeats, thought to be the minimum required for transformation (27), and compared this construct with recombinants generated on the same B95.8 background but containing 4, 6, 8, and 11 BamHI W repeats. This work showed that the overall Wp methylation status was critically affected by the Wp repeat number. Thus, Wp sequences were almost entirely unmethylated in 2W LCLs, based on both bisulfite sequencing and the more-sensitive MSP analysis, whereas 4W, 6W, 8W, and 11W LCLs contained both unmethylated and methylated sequences. Interestingly, Elliott et al. (15) recently reported the hypomethylation of Wp in LCLs transformed by a recombinant virus that was fortuitously low in BamHI W repeats, but the present work clearly shows the significance of this finding in an experiment with internal high-Wp-copy-number control viruses. The presence of only unmethylated Wp sequences in 2W LCLs strongly suggests that Wp methylation in standard LCLs preferentially targets the downstream copies of Wp and supports the hypothesis that this difference was apparent even in bisulfite sequencing (Fig. 3 and 4). These unmethylated Wp species could therefore account for the low level of Wp transcription observed in standard LCLs.

Importantly, we found two LCLs (EBH41.2 and IM53.1) where Wp was the only active EBNA promoter yet where Cp was apparently intact. These lines are therefore quite distinct from the long-established Wp-only LCLs X50-7 and IB4 which have deletions in BamHI C encompassing Cp (56, 57). Interestingly, our recently established Wp-only LCLs, in contrast to conventional Wp/Cp users, were substantially hypomethylated in the promoter-proximal UAS1 region (Fig. 3), and the fact that this difference was apparent even in bisulfite sequencing assays suggests that it affects the majority of Wp copies in the resident EBV episomes. Further studies of these unusual LCLs, both of which arose by spontaneous transformation in peripheral blood mononuclear cells from EBV-infected donors, could help identify the controls governing the interrelationship between Cp and Wp activities.

Studies of Wp methylation are further complicated by the presence of multiple Wp copies in each EBV episome. We attempted to overcome this problem experimentally by reducing the Wp copy number in a recombinant EBV genome context. We therefore specifically generated a recombinant with only two BamHI W repeats, thought to be the minimum required for transformation (27), and compared this construct with recombinants generated on the same B95.8 background but containing 4, 6, 8, and 11 BamHI W repeats. This work showed that the overall Wp methylation status was critically affected by the Wp repeat number. Thus, Wp sequences were almost entirely unmethylated in 2W LCLs, based on both bisulfite sequencing and the more-sensitive MSP analysis, whereas 4W, 6W, 8W, and 11W LCLs contained both unmethylated and methylated sequences. Interestingly, Elliott et al. (15) recently reported the hypomethylation of Wp in LCLs transformed by a recombinant virus that was fortuitously low in BamHI W repeats, but the present work clearly shows the significance of this finding in an experiment with internal high-Wp-copy-number control viruses. The presence of only unmethylated Wp sequences in 2W LCLs strongly suggests that Wp methylation in standard LCLs preferentially targets the downstream copies of Wp and supports the hypothesis that only the most-S’ copies remain active and unmethylated (59). Analysis of the same 2W LCLs showed the Cp activity to be consistently higher than that in the corresponding 11W LCLs. This may reflect a compensatory mechanism whereby Cp transcription is increased to ensure that overall EBNA expression is maintained at the optimal levels required for B-cell trans-
formation. However, another interesting possibility is that in standard LCLs, the methylation of downstream Wp sequences has a negative effect on Cp transcription. Such a scenario, first proposed by Paulson and Speck (34), is further supported by earlier findings that sequences in the Wp regulatory region are also important for Cp activity (35, 52, 60).

A complementary approach to studying the relationship between Wp activity and methylation status was provided by the recent identification of BL cell lines with an unusual Wp-restricted form of latency (25). These lines are phenotypically very similar to conventional latency I BL lines (expressing EBNA1 from Qp) yet have a quite different pattern of viral transcription in which Qp is silent and EBNA1, -3A, -3B, -3C, and -LP mRNAs are expressed exclusively from Wp. Thus, such lines provide the opportunity to look at an active Wp in the unusual context of a cell with a germinal-center-like (BL) phenotype, rather than a lymphoblastoid (LCL) phenotype. This work revealed interesting parallels with the data from the Oku-BL, Sal-BL, and Ava-BL lines carrying multiple viral episomes, where we found by bisulfite sequencing that the dominant Wp species were hypermethylated, just as they are in latency I BLs. However, MSP analysis revealed the presence of unmethylated Wp sequences in these Wp-restricted BLs, a situation not seen in conventional latency I lines. This suggests that in these Wp-restricted BLs, as in standard LCLs, Wp is active in only a minority of the resident Wp copies. A more interesting picture emerged from the analysis of the Wp-restricted clones of the Awia-BL line which carried only a single EBV genome. Here the dominant Wp species were hypomethylated in the critical UAS1 region, extending up to the CpG site nearest the UAS2-UAS1 boundary, a pattern similar to that seen in the Wp-only LCLs. Thus, a pattern which appears to be imposed on every resident copy of the virus genome in Wp-only LCLs is only seen in Wp-restricted BLs when the genome copy number is low; this implies that in Wp-restricted BLs with multiple copies of the genome, there may be heterogeneity in methylation patterns between individual genomes.

It is important to note, however, that the Wp-restricted clones of Awia-BL carry a single integrated EBV genome rather than an episomal copy of the EBV genome (26a), and therefore, care must be taken in interpreting the general relevance of these particular findings. It is nonetheless interesting to note the contrast between these Wp-restricted Awia-BL clones and clones derived from the same tumor, again with a single integrated virus genome, in which Wp (and Cp) is silent and the cells display an EBNA2+ LMP1− form of infection. Unlike the Wp-restricted clones, where Wp is hypomethylated, in the EBNA2+ LMP1− clones, Wp is hypermethylated. Therefore, even in these unusual circumstances, a correlation is maintained between Wp activity and hypomethylation of promoter sequences.

The broader significance of these findings stems from the relationship between DNA methylation and other epigenetic regulatory controls (29). Thus, methylated DNA, through its interaction with methyl CpG binding factors, can recruit histone deacetylases and chromatin remodeling complexes that can alter chromatin structure and interfere with access to the transcription machinery. It is very likely that the EBV episome can be remodeled in the same way as cellular chromatin (11, 12, 46). Indeed, this would be consistent with the finding that several EBV-encoded transcription factors, notably EBNA2 and EBNA3C (2, 37), exploit interactions with chromatin remodeling complexes to regulate viral latent gene expression. The novel cellular models described here may be useful in the dissection of these epigenetic processes.

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