Variable Methylation of the Epstein-Barr Virus Wp EBNA Gene Promoter in B-Lymphoblastoid Cell Lines

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During the initial stages of Epstein-Barr virus (EBV) infection of peripheral resting B cells, transcription of the six genes encoding the EBV latency-associated nuclear antigens (EBNAs) is driven from Wp, a promoter that is present in multiple copies within the EBV major internal repeat. As infection progresses, transcription from Wp is downregulated following upregulation of EBNA gene transcription driven from a promoter, Cp, located ca. 3 kb upstream of the first copy of Wp. Recently published data have provided evidence that, concomitant with the switch in EBNA gene promoter usage, Wp becomes heavily methylated (R. J. Tierney et al., J. Virol. 74:10468-10479, 2000). Based on this observation, it has been argued that methylation of Wp plays a pivotal role in suppressing Wp activity in EBV-immortalized B-lymphoblastoid cell lines (LCLs). Here we present data compiled from analyses of Wp methylation in eight randomly selected low-passage-number B-LCLs. These data demonstrate that there is considerable variability in Wp methylation, both between different cell lines and within clonal LCLs. Overall, less methylation of Wp was noted in established, low-passage-number LCLs than was previously observed in bulk cultures of infected B cells at days 18 and 21 postinfection. Importantly, the majority of LCLs examined harbored both unmethylated and methylated copies of Wp. In addition, all low-passage-number LCLs examined contained both Cp- and Wp-initiated EBNA transcripts, arguing for the presence of some transcriptionally active copies of Wp. Taken together, these data argue that other factors, perhaps in conjunction with Wp methylation, play a role in suppressing Wp activity in LCLs.

Background. Epstein-Barr virus (EBV), a DNA tumor virus and member of the herpesvirus family, efficiently transforms primary B cells and is an etiologic agent of infectious mononucleosis. In addition, numerous studies have implicated EBV in the development of various human lymphoid and epithelial malignancies, including Burkitt’s lymphoma, nasopharyngeal carcinoma, Hodgkin’s lymphoma, immunoblastic lymphoma, peripheral T-cell lymphoma, and gastric carcinoma (4, 13, 19).

EBV primarily establishes a latent infection in which the viral genome persists as an episome (2). Several different programs of latency-associated EBV gene expression have been described. During infectious mononucleosis, during ex vivo infection of B cells, and in EBV-associated posttransplant lymphoproliferative disease, EBV enters a program of gene expression known as latency III, which results in B-cell growth transformation (13). The latency III program is characterized by the expression of a wide array of viral proteins, including six different Epstein-Barr nuclear antigens (EBNAs 1, 2, 3a, 3b, 3c, and 4) and three latency-associated membrane proteins (LMPS 1, 2a, and 2b). Transcription of the EBNAs initiates at one of two promoters, Cp or Wp (Fig. 1) (18). Notably, Wp is encoded within the major internal repeat (IR-1) of EBV and as such is present in multiple copies within the EBV major internal repeat. As infection progresses, transcription from Wp is downregulated following upregulation of EBNA gene transcription driven from a promoter, Cp, located ca. 3 kb upstream of the first copy of Wp. Recently published data have provided evidence that, concomitant with the switch in EBNA gene promoter usage, Wp becomes heavily methylated (R. J. Tierney et al., J. Virol. 74:10468-10479, 2000). Based on this observation, it has been argued that methylation of Wp plays a pivotal role in suppressing Wp activity in EBV-immortalized B-lymphoblastoid cell lines (LCLs). Here we present data compiled from analyses of Wp methylation in eight randomly selected low-passage-number B-LCLs. These data demonstrate that there is considerable variability in Wp methylation, both between different cell lines and within clonal LCLs. Overall, less methylation of Wp was noted in established, low-passage-number LCLs than was previously observed in bulk cultures of infected B cells at days 18 and 21 postinfection. Importantly, the majority of LCLs examined harbored both unmethylated and methylated copies of Wp. In addition, all low-passage-number LCLs examined contained both Cp- and Wp-initiated EBNA transcripts, arguing for the presence of some transcriptionally active copies of Wp. Taken together, these data argue that other factors, perhaps in conjunction with Wp methylation, play a role in suppressing Wp activity in LCLs.

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The methylation status of Wp was assessed by bisulfite PCR...
TABLE 1. Both Cp and Wp are active in low-passage-number LCLs

<table>
<thead>
<tr>
<th>LCL</th>
<th>% of EBNA transcripts initiated from Cp</th>
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<tbody>
<tr>
<td>X50-7</td>
<td>&lt;1</td>
</tr>
<tr>
<td>JY</td>
<td>99</td>
</tr>
<tr>
<td>B95-A</td>
<td>2</td>
</tr>
<tr>
<td>B95-C</td>
<td>37</td>
</tr>
<tr>
<td>Ak-C1</td>
<td>ND</td>
</tr>
<tr>
<td>Ak-C2</td>
<td>86</td>
</tr>
<tr>
<td>25-3</td>
<td>72</td>
</tr>
<tr>
<td>22-19</td>
<td>67</td>
</tr>
<tr>
<td>17-20</td>
<td>57</td>
</tr>
<tr>
<td>28-8</td>
<td>47</td>
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* Relative abundances of Cp- and Wp-initiated EBNA gene transcripts were determined by using a quantitative real-time PCR assay and primers specific for splicing either from the C2 exon to the W1 exon or from the W0 exon to the W2 exon. To adjust for slight differences in the efficiencies of RT-PCR amplification of Cp- and Wp-initiated transcripts, the levels of Cp- and Wp-initiated transcripts were standardized to those of the positive controls JY (for Cp-initiated transcripts) and X50-7 (for Wp-initiated transcripts). Total RNA was prepared as previously described (12), and RT-PCR analyses were carried out with the QuantiTect SYBR green kit (QIAGEN) in accordance with the manufacturer’s protocol. The primers used for PCR amplification were as follows for Cp-initiated transcripts: for the C2 exon, 5′-TTG TCC AGG GCC TTC ACT TC-3′ and for the W1 exon, 5′-TGG TCC AGG GCC TTC ACT TC-3′. For detection of Wp-initiated transcripts, the following primers were used: for the W0/W1 splice site, 5′-GGA GTC CAC ACA AAT CCT AGG GGA G-3′, and for the W2 exon, 5′-TTC TTA CCA TGC GGC CAT GTA-3′. For de

analysis, as previously described (9, 10). As a positive control for the presence of hypermethylation around Wp, we utilized DNA recovered from the Rael Burkitt’s lymphoma cell line, which exhibits a restricted EBV latency phenotype (latency I) (8, 16). As expected, all bisulfite PCR clones recovered from the Rael cell line demonstrated that Wp is hypermethylated in this Burkitt’s lymphoma cell line (Fig. 2) (17). Furthermore, in all the LCLs analyzed, there was evidence of methylation of some of the copies of Wp (Fig. 2). However, the extent of methylation was quite varied between cell lines and overall was significantly lower than that previously reported for bulk culture peripheral blood mononuclear cell infection at day 21 (21). Except with the B95-C LCL, the presence of at least one unmethylated Wp clone was detected in each cell line, indicating that unmethylated copies of Wp exist within these LCLs. Notably, the majority of bisulfite PCR products recovered from the Ak-C1 (six of eight clones analyzed) and 17-20 (six of seven clones analyzed) LCLs were devoid of any detectable methylation, suggesting that most of the IR-1 repeat region was hypomethylated. Importantly, all clones analyzed were obtained from independent bisulfite PCRs and thus do not represent the analysis of a single preferentially amplified PCR product.

Conclusions. Analysis of eight low-passage-number LCLs demonstrated that the IR-1 region of the EBV genome was indeed methylated, as previously reported (21). However, the extent of methylation within this region was quite varied. Furthermore, we detected in all but one of the LCLs examined the presence of unmethylated copies of Wp. Thus, the importance of DNA methylation in extinguishing Wp activity during the establishment of LCLs is unclear. As discussed above, previous data have provided strong evidence that transcriptional interference is the primary mechanism of quenching downstream Wp-initiated transcription (11, 12, 24–26).

Analysis of the methylation status of the EBV genome dur-

FIG. 2. Analysis of Wp methylation in low-passage-number LCLs. Genomic DNA was isolated from the indicated LCLs and from the EBV-positive Burkitt’s lymphoma cell line Rael. As a negative control, DNA was also isolated from the EBV-negative Burkitt’s lymphoma cell line DG75. DNA was bisulfite treated, amplified with primers designed to amplify bisulfite-modified DNA, cloned, and sequenced as previously described (9, 10). The genome coordinates of the CpGs in the amplified region are shown on the left side of the panel. Also shown are the positions of known regulatory regions. O, unmethylated CpGs; ●, methylated CpGs. Each numbered column shows the data obtained from an independent bisulfite PCR amplification. The vertical arrows denote those bisulfite PCR clones for which no CpG methylation was observed. The B95-A and B95-C LCLs were established by infection of peripheral blood B cells with B95.8 virus. The Ak-C1 and Ak-C2 LCLs were established by infection of peripheral blood B cells with Akata virus. The 25-3, 22-19, 17-20, and 28-8 LCLs were established by infection of peripheral blood B cells with immortalizing virus recovered from P2HR-1 clone 16 cells transfected with a plasmid containing the EBV BamHI W, Y, and H fragments, the 3′ EBNA-leader protein-encoding exons, the entire EBNA 2 coding exon, and flanking sequences, as previously described (20, 27).
ing restricted viral latency in the memory B-cell compartment has provided a strong correlation between methylation of Wp and Cp and the absence of detectable Cp- or Wp-initiated transcripts (10, 15). Based on those data, we have argued that targeted methylation of the IR-1 region of the viral genome is important for long-term maintenance of virus infection in vivo (10). Perhaps the hypermethylation of Wp observed at day 21 postinfection (21) reflects (i) viral genome methylation in B cells that then fail to progress to LCLs and (ii) the establishment of a restricted form of EBV latency in tissue culture. Thus, only those EBV-infected B cells in which there was low-to-modest methylation of the IR-1 repeat region would become immortalized and grow out in culture. Because the previously reported RT-PCR analyses of Cp- and Wp-initiated EBNA gene transcription (21) were not carried out with a limiting dilution analysis, this group could not assess whether the EBNA gene transcripts detected arise from B cells harboring viral genomes in which Wp is hypermethylated. The analyses of EBV genome methylation and viral gene transcription in peripheral B cells during the acute phase of infectious mononucleosis (21) also suffer from the same experimental limitation. Comparison of the levels of Wp methylation observed at day 21 postinfection (21) to the levels found in our analysis of eight low-passage-number LCLs argues that there is selection against EBV genomes in which Wp is hypermethylated. Further careful analysis of the relationship between IR-1 methylation and the transition to Cp utilization will be required before it can be resolved whether methylation plays a role in EBNA gene promoter utilization.

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REFERENCES


