Expression of Cyclin D2 in Epstein-Barr Virus-Positive Burkitt’s Lymphoma Cell Lines Is Related to Methylation Status of the Gene

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The cyclin D2 gene is not expressed in resting primary B lymphocytes or in group I Burkitt’s lymphoma (BL) cell lines that retain the characteristics of authentic BL cells. Expression of cyclin D2 is induced in primary B lymphocytes following infection with Epstein-Barr virus (EBV) or transfection of the EBV genes EBNA-LP and EBNA-2. However, attempts to induce cyclin D2 expression in BL cell lines by the enforced expression of EBV genes were unsuccessful. Since the demethylation agent 5-azacytidine has been shown to modulate viral gene expression in BL cells, we explored the possibility that methylation plays a significant role in the control of cyclin D2 expression. We show that 5-azacytidine treatment of the Mutu Cl 179 BL cell line led to expression of cyclin D2 RNA and that expression correlated with differences in the methylation status of a CCGG restriction enzyme site near the transcription initiation region of the cyclin D2 gene. Thus, methylation appears to play a direct role in the regulation of the cyclin D2 locus in BL.

The cyclin D family is involved in the control of both proliferation and differentiation (12, 16, 18–20). We reported previously that the expression of cyclin D2 correlated with the expression of the full set of Epstein-Barr virus (EBV) immortalizing genes in cell lines derived from Burkitt’s lymphoma (BL) (17). Group I BL cell lines, which retain the phenotype characteristic of BL in vivo and express a subset of the EBV immortalizing genes, do not express cyclin D2, whereas group III BL cells, which arise spontaneously from group I BL cell lines and express the full complement of EBV immortalizing genes, express cyclin D2 RNA. This finding suggested that the expression of EBV genes may regulate the expression of cyclin D2, and we have since demonstrated this to be true for primary B lymphocytes; infection with EBV or transfection with two EBV genes, EBNA-LP and EBNA-2, results in the induction of cyclin D2 expression (21). We investigated whether the cyclin D2 gene could be regulated by EBV in the same manner in BL cell lines.

Coexpression of EBNA-2 and EBNA-LP in group I BL cell lines. The Mutu Cl 179 cell line displays the group I BL phenotype, with EBV expression restricted to EBNA-1 and the EBER RNAs (10), and no detectable cyclin D2 RNA is found in this cell line (17). We introduced vectors for EBNA-LP and EBNA-2 (21) into Mutu Cl 179 cells by electroporation (4) and investigated whether cyclin D2 expression could be induced. Western blotting (immunoblotting) of extracts from the transfected cells confirmed that both viral proteins were expressed (Fig. 1A). However, no cyclin D2 RNA was detectable in the transfected cells as assayed by reverse transcription (RT)-PCR (21). A PCR product was readily amplified from the Jijoye cell line, which is known to express cyclin D2 (Fig. 1B). Reconstruction experiments showed that the cyclin D2 signal could be detected in a 10^3-fold dilution of the Jijoye RNA, demonstrating the sensitivity of this assay (data not shown). The integrity of the RNA was verified by amplifying a segment of the cdc2 coding sequence.

We also attempted to induce the expression of cyclin D2 by transfection of individual viral genes or following infection with EBV. EBV-negative BL cell lines that had previously been either infected (5) with EBV (BL41, BL41/P3HR1, and BL41/B95-8) or transfected (9) with EBV genes (BJAB, BJAB-EBNA-2, BJAB-EBNA-LP, BJAB-LMP1, BJAB-EBNA-1, BJAB-EBNA-3a, and BJAB-EBNA-3C) were found to be negative for cyclin D2 expression. Thus, the introduction and stable expression of EBV genes was not sufficient to induce the expression of cyclin D2 RNA in these established cell lines (data not shown).

Methylation of the cyclin D2 locus in BL cells. Since these results are in sharp contrast to our findings with primary B lymphocytes, we decided to investigate the differences. One possible explanation for the difference in the ability of EBV to induce cyclin D2 in primary B lymphocytes and BL cell lines was that alterations to the cyclin D2 locus in BL cell lines might make this locus refractory to transactivation by EBV genes. Southern blotting analysis using EcoRI and PstI revealed no gross alterations in group I BL cell lines (Fig. 2). Since methylation has been shown to play a role in the silencing of many viral genes in group I BL cells (1, 2, 6, 7, 11, 13–15, 22), we next asked whether the cyclin D2 locus was differentially methylated in cell lines that differ in their cyclin D2 expression. Two subclones from the Mutu BL cell line, Mutu Cl 179 (group I) and Mutu Cl 62 (group III), were chosen for this analysis since these cell lines are isogenic yet display different phenotypes; Cl 179 has a quasi-resting phenotype, expresses a restricted set of EBV genes (10), and has no detectable cyclin D2 RNA, whereas Cl 62 has an activated phenotype, expresses the full complement of EBV immortalizing genes, and also expresses cyclin D2 RNA (17).

For comparison, we analyzed DNA from an EBV-immortalized cell line (LCL), from primary B lymphocytes isolated from human peripheral blood (21), and from the two Mutu...
clones. The genomic DNAs were digested with the isoschizomers \textit{HpaII} and \textit{MspI}, which differ in sensitivity to methylation at the second cytosine in the CCGG recognition site. Figure 3A shows the expected cleavage pattern for these enzymes based on the DNA sequences surrounding the transcription start sites of the cyclin D2 gene (16a). Complete digestion would generate a series of fragments ranging from 38 to 541 bp that would be detectable with a probe specific for this region. However, the 156-, 144-, and 189-bp fragments are unlikely to be resolved by electrophoresis in agarose gels, and the smaller fragments would be undetectable by Southern blotting. The primary B-lymphocyte and LCL DNAs yielded two major products which we interpret to be the 541-bp fragment (band 2 in Fig. 3B) and the mixed 156-, 144-, and 189-bp fragments (band 4, 5, and 6 in Fig. 3B). In these cells, there appears to be no distinction between \textit{HpaII} and \textit{MspI}. In both Mutu cell lines, an additional fragment of about 700 bp was observed with \textit{HpaII} but not \textit{MspI} (band 1). This is likely to reflect a methylation difference specific for BL. Significantly, the group I BL cell line had another novel fragment of about 300 bp which was not present in the group III counterpart or in the primary B lymphocytes and the LCL.
expression by RT-PCR. Total cell RNA was prepared and analyzed for cyclin D2 and cdc2 expression by RT-PCR.

Effect of 5-azacytidine on cyclin D2 expression in group I BL. To further investigate whether methylation is involved in the regulation of cyclin D2 in these BL cell lines, we tested the effect of the demethylating agent 5-azacytidine on the expression of cyclin D2 in the group I BL cell line Mutu Cl 179. This agent has been previously shown to activate the expression of methylated viral genes in a small fraction of treated cells (10, 14). Mutu Cl 179 cells were cultured with 15 μM 5-azacytidine for 3 days, and the expression of cyclin D2 was determined by using the RT-PCR assay (21). Cyclin D2 was detected in Mutu Cl 179 only after treatment with 5-azacytidine, whereas the control cdc-2 transcript was identified in both samples (Fig. 4).

These data show that at least one site in the 5’ flanking region of cyclin D2 is hypermethylated in a group I BL compared with a group III BL and that the difference in expression of cyclin D2 is reflected by the differential methylation. We propose that methylation of the 5’ flanking region of cyclin D2 plays a role in silencing its expression in BL. The control of gene expression by selective methylation has been observed for a variety of differentiation of cell-type-specific cell genes (3) and for EBV genes in BL (1, 2, 6, 7, 11, 13–15, 22). There is evidence to suggest that methylation may play a direct role in down regulating gene expression; for example, the methylation of specific restriction enzyme sites in the LMP-1 promoter and the Wp and Cp promoters reduces the level of their expression (13, 15). However, it is also possible that methylation may play a secondary role (3) in silencing gene expression. Since 5-azacytidine treatment of Mutu Cl 179 also induces the expression of viral genes, it is not possible to distinguish whether the induction of cyclin D2 is a direct result of demethylation of the flanking regions or if it also requires the expression of EBV genes.

B lymphocytes in vivo are subject to different fates, depending on their antigen specificity, and can be selected to apoptosis, to amplify and mutate the immunoglobulin genes, to differentiate into antibody-secreting plasma cells, or to stop dividing and become memory cells (23). A lack of monospecific reagents has so far precluded the analysis of the normal pattern of cyclin D2 expression during B-lymphocyte differentiation in vivo. However, since members of the cyclin D2 family influence both cell proliferation and differentiation (20), it is likely that cyclin D2 plays a role in normal B-lymphocyte differentiation. The close correlation between cyclin D2 expression and the activated B-cell phenotype suggests that cyclin D2 expression is an integral component of B-lymphocyte activation. Although they proliferate, BL cells in vivo display a quasi-resting phenotype, which implies that down regulation of the activation phenotype is important for the development of BL. The control of cyclin D2 expression by differential methylation may therefore provide a selective advantage to precursor BL cells during the development of the lymphoma.

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REFERENCES


10. Hunt, T. 1989. Methylation of discrete sites in the LMP-I promoter and the Wp and Cp promoters reduces the level of their expression (13, 15). However, it is also possible that methylation may play a secondary role (3) in silencing gene expression. Since 5-azacytidine treatment of Mutu Cl 179 also induces the expression of viral genes, it is not possible to distinguish whether the induction of cyclin D2 is a direct result of demethylation of the flanking regions or if it also requires the expression of EBV genes.

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