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Patterned CpG Methylation of Silenced B Cell Gene Promoters in Classical Hodgkin Lymphoma-derived and Primary Effusion Lymphoma Cell Lines

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Hodgkin and Reed–Sternberg (HRS) cells of classical Hodgkin lymphoma (cHL) and primary effusion lymphoma (PEL) are derived from germinal center (GC) and post-GC B cells, respectively. Neither express many of the B cell genes or surface markers typically expressed by other GC-derived B cell lymphomas or normal B cells. This loss of B cell gene expression is not due to a lack of essential transcription factors, as studies have shown that the ectopic expression of missing transcription factors failed to reactivate endogenous target genes. These results implicate epigenetic mechanisms extinguishing B cell gene expression. Silenced endogenous B cell genes representing a surface receptor, *B29* (Igβ, CD79b), a signaling molecule, *TCL1*, and a transcription factor, *Bob1* (OCA-B, OBF-1), were reactivated by 5-aza-2′-deoxycytidine, indicating that gene silencing in HRS and PEL cells is due to DNA methylation. Genomic bisulfite sequencing corroborated this prediction and revealed three distinct patterns of methylation for the silenced *B29* and *TCL1* promoters. These distinct patterns consisted of 5′ promoter CpG methylation alone, 5′ and 3′ promoter CpG methylation sparing sites in the central cores, and complete CpG methylation throughout the promoter regions. The silenced *Bob1* promoter showed one pattern of dense CpG methylation at essentially all sites. These consistent patterns predict that, although gene silencing in many HRS and PEL cells mimics appropriate gene silencing, in some cases of complete CpG methylation throughout entire promoters both the activation and targeting of methylation is abnormal.

Keywords: classical Hodgkin lymphoma; CpG DNA methylation; gene reactivation; gene silencing; primary effusion lymphoma

Abbreviations used: GC, germinal center; HRS, Hodgkin and Reed–Sternberg; PEL, primary effusion lymphoma; cHL, classical Hodgkin lymphoma; Ig, immunoglobulin; TSA, trichostatin A; 5-aza, 5-aza-2′-deoxycytidine; RT, reverse transcribed.

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Introduction

Most B cell lymphomas derive from transformed germinal center (GC)-experienced B cells that retain patterns of gene expression like their non-malignant counterparts. These B cell lymphomas remain dependent on the B cell-specific gene expression program for continued survival and growth.[1] Classical Hodgkin lymphoma (cHL) and primary effusion lymphoma (PEL) are two striking exceptions whose lack of expression of immunoglobulins (Ig) and other B cell-specific genes previously raised questions regarding their cellular origins.[2,3]

cHL is a relatively common lymphoma in Europe and North America and accounts for 95% of all Hodgkin disease cases.[4] The malignant cells of cHL are mononucleated Hodgkin cells and multinucleated Reed–Sternberg (HRS) cells which make up less than 1% of the total tumor mass and are embedded in a background of non-malignant reactive T cells, B cells, eosinophils, histiocytes and neutrophils. The B cell derivation of HRS cells was only resolved when single-cell PCR showed that they contain clonally rearranged, somatically hypermutated Ig genes. HRS cells are now considered to correspond to pre-apoptotic GC B cells.[3]

PEL is an unusual, aggressive B cell lymphoma that is characterized by liquid growth in body cavities.[2] PEL cells are almost always infected with Kaposi sarcoma-associated herpesvirus/human herpes virus 8 (KSHV/HHV8) and occur mainly in AIDS patients and other immunocompromised individuals. Like cHL, the origin of PEL was uncertain until rearranged *Ig* genes with somatic hypermutation patterns that reflect antigen selection were identified, classifying them initially as mature B cells of GC or post-GC derivation.[2] Recent gene expression profiling has shown that PEL have features intermediate between post-GC immunoblasts and plasma cells, suggesting a peculiar origin from "plasmablast"-type cells.[5] The survival and growth of both HRS and PEL cells has been correlated with constitutive activation of NF-κB along with immortalization by Epstein-Barr (EBV) in HRS cells and KSHV/HHV8 in PEL.[2,3]

HRS and PEL cells no longer transcribe multiple B cell-specific genes including *Ig*, *B29* (*Igβ*, *CD79b*), *mb-1* (*Igα*, *CD79a*), *CD19*, and *CD20*. The loss of B cell-specific gene expression in HRS and PEL cells has been attributed to the lack of select essential transcription factors Oct-2, PU.1, and Bob1 (OBF-1, OCA-B) in cHL and Oct-2, PU.1, Bob1, and Pax-5 in PEL.[3,6] Ectopic expression of Oct-2, Bob1, EBF, and PU.1 was found to support transcription of co-transfected reporter constructs containing B cell gene promoters. However, transcription of silenced endogenous B cell genes was not reactivated in these transfection studies.[6–10] Thus, these findings do not resolve the mechanism(s) underlying the loss of transcription factor expression or the silencing of target B cell genes.

The failure of ectopic transcription factors to reactivate endogenous B cell gene expression suggests that the B cell gene program in HRS and PEL cells may be silenced by epigenetic mechanisms. We found that the loss of *B29* gene transcription in a non-*B29*-expressing plasma cell line was related to promoter DNA methylation.[11] Accordingly, we examined the nature and role of epigenetic mechanisms in B cell gene silencing in cHL and PEL. Of the numerous silenced genes in cHL and PEL, we chose three genes *B29*, *TCL1*, and *Bob1* that were consistently silenced in all of the cells evaluated. These silenced endogenous genes were reactivated with 5-aza-2′-deoxycytidine (5-aza), an inhibitor of DNA methyltansferases in all HRS and PEL cells. In contrast trichostatin A (TSA), an inhibitor of histone deacetylases only reactivated these genes in certain HRS and PEL cells. Sodium bisulfite sequencing of *B29*, *TCL1*, and *Bob1* revealed three distinct patterns of CpG island promoter methylation in HRS and PEL cells. These patterns provide insight into possible mechanisms of aberrant gene silencing.

Results

Silenced B cell genes in HRS and PEL lines

We chose the widely used L428, L591, and L1236 HRS cell lines, BC-1, BCBL-1, and KS-1 PEL cell lines, and the GC-derived Ramos B cell line for these studies. The HRS and PEL cell lines have been characterized extensively and shown to correspond to the primary lymphoma cells of the parent tumors (data not shown).[12,13] The three most consistently silenced genes among the HRS and PEL cell lines, *B29*, *TCL1*, and *Bob1*, were selected as examples of a cell surface receptor gene, a signaling molecule gene, and a transcription factor gene, for gene reactivation and DNA methylation studies (Figure 1, and data not shown). The characterized promoters of these three silenced genes all contain multiple potential CpG methylation sites in CpG islands and are therefore compelling targets for silencing by DNA methylation. Other commonly silenced genes in cHL and PEL include *PU.1*, *EBF*, *Pax5*, *mb-1*, and *CD19*, but were not included in our study as they either do not contain CpG island promoters or they are inconsistently silenced among the cell lines tested (data not shown).

The *B29* gene encodes a B cell receptor (BCR) molecule that relays surface Ig signals required for continued, non-malignant B cell survival.[14] *Bob1* is a B cell-specific co-activator required for GC formation that functions with octamer-binding transcription factors to enhance the expression of *B29* and *Ig* genes.[15,16] *TCL1* encodes a key co-activator of the Akt/PKB cell survival and growth kinase and is expressed throughout B cell development with the exception of post-GC B cells.[17,18] By RT-PCR, all six HRS and PEL lines lacked detectable *B29* gene expression (Figure 1). HRS and PEL lines generally lacked *Bob1* expression, although L1236 and BCBL-1 expressed low, but detectable *Bob1* (Figure 1).

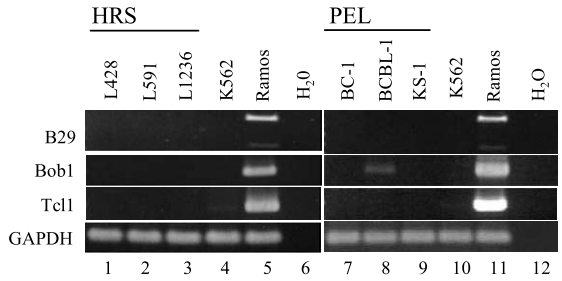

Figure 1. Expression of *B29*, *TCL1* and *Bob1* by RT-PCR in HRS and PEL cell lines. Ethidium bromide-stained RT-PCR gels using 100 ng of total RNA. Lane 1, L428 (HRS); lane 2, L591 (HRS); lane 3, L1236 (HRS); lanes 4 and 10, K562 (TCL1+); lanes 5 and 11, Ramos (B29+, Bob1+, TCL1+); lanes 6 and 12, water; lane 7, BC-1 (PEL); lane 8, BCBL-1 (PEL); lane 9, KS-1 (PEL).

None of the HRS and PEL lines tested showed *TCL1* expression despite a derivation, at least for the HRS lines, from GC B cells (Figure 1).

Reactivation of silenced endogenous B cell genes

We first used two reactivation strategies to assess the nature of epigenetic silencing mechanisms operating on silenced B cell genes in HRS and PEL cells. HRS and PEL lines were treated with TSA or 5-aza.[19,20] RNA from TSA or 5-aza-treated L428 and L1236 (HRS) and BC-1 and KS-1 (PEL) cell lines was analyzed using a highly sensitive RT-PCR and Southern blot protocol to facilitate detection of endogenous gene reactivation. Representative results from these reactivation experiments are shown in Figure 2 and a compilation of the four lymphoma cell lines tested is summarized in Table 1.

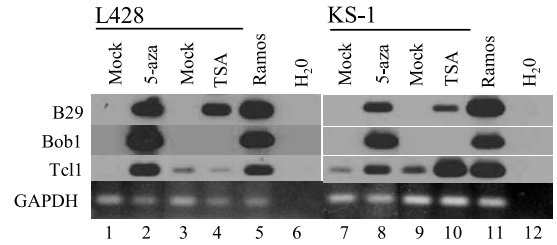

Figure 2. Reactivation of endogenous *B29*, *Bob1*, and *TCL1* gene expression by 5-aza and TSA in HRS and PEL cell lines. Southern blots of RT-PCR gels were used to detect gene reactivation. L428 and KS-1 cell lines were treated with 5-azacytidine (2.5 μM) and TSA (50 nM). GAPDH is an EtBr-stained gel. (a) HRS cell line L428. Lane 1, mock 5-aza; lane 2, 5-aza; lane 3, mock TSA; lane 4, TSA; lane 5, untreated Ramos 1:100 dilution; lane 6, water. (b) PEL cell line KS-1. Lane 1, mock 5-aza; lane 2, 5-aza; lane 3, mock TSA; lane 4, TSA; lane 5, untreated Ramos 1:100 dilution; lane 6, water.

TSA treatment only reactivated the endogenous *B29* gene in L428 and KS-1 cells and the *TCL1* gene only in L1236 and KS-1 cells. TSA failed to induce *Bob1* expression in any of the cell lines tested (Table 1). Treatment with 5-aza reactivated expression of silenced endogenous *B29*, *Bob1*, and *TCL1* genes in all four cell lines tested, indicating that silencing of these genes is due to epigenetics (Table 1). These results predict differing extents of DNA methylation for specific genes in HRS and PEL lines.

Unique DNA methylation patterns in silenced B cell gene promoters

We next used genomic DNA bisulfite sequencing to directly identify patterns of DNA methylation in silenced *B29*, *TCL1*, and *Bob1* promoters in the three HRS and the three PEL cell lines. Genomic DNA samples from these HRS and PEL cell lines were treated with sodium bisulfite, PCR amplified, and sub-cloned. Ten clones were isolated for each promoter in each cell line and were sequenced to obtain DNA methylation patterns. Representative electrophoretograms of the genomic bisulfite sequencing analyses are shown in Figure 3.

The individual methylation patterns for ten independent *B29* promoter clones from the HRS and PEL cell lines are shown in Figure 4. The majority of the CpG sites across the *B29* promoter were methylated in all three HRS cell lines and in two PEL cell lines, BC-1 and BCBL-1. In contrast, CpG sites in the *B29* promoter from the KS-1 PEL cell line were less frequently methylated overall. Methylation in KS-1 cells primarily occurred 5′ (sites 1–10) and minimally in the 3′ region (sites 15–19) from the start of transcription. Interestingly, the central portion of the *B29* promoter (sites 11–14) was not methylated in any KS-1 clone. Even this

Table 1. Reactivation of endogenous *B29*, *Bob1*, and *TCL1* by 5-aza and TSA

	B29		Bob1		TCL1	
Lines	5-Aza	TSA	5-Aza	TSA	5-Aza	TSA
L428	I	I	I	U	I	U
L1236	I	U	I	U	I	I
BC-1	I	U	I	U	I	U
KS-1	I	I	I	U	I	I

HRS and PEL cell lines were treated with a range of 5-aza and TSA concentrations up to a maxium tolerated dose for three days with addition of new drug and medium every 24 hours. RT-PCR followed by Southern blot was used to detect reactivation of endogenous genes. I, induced; U, uninduced.

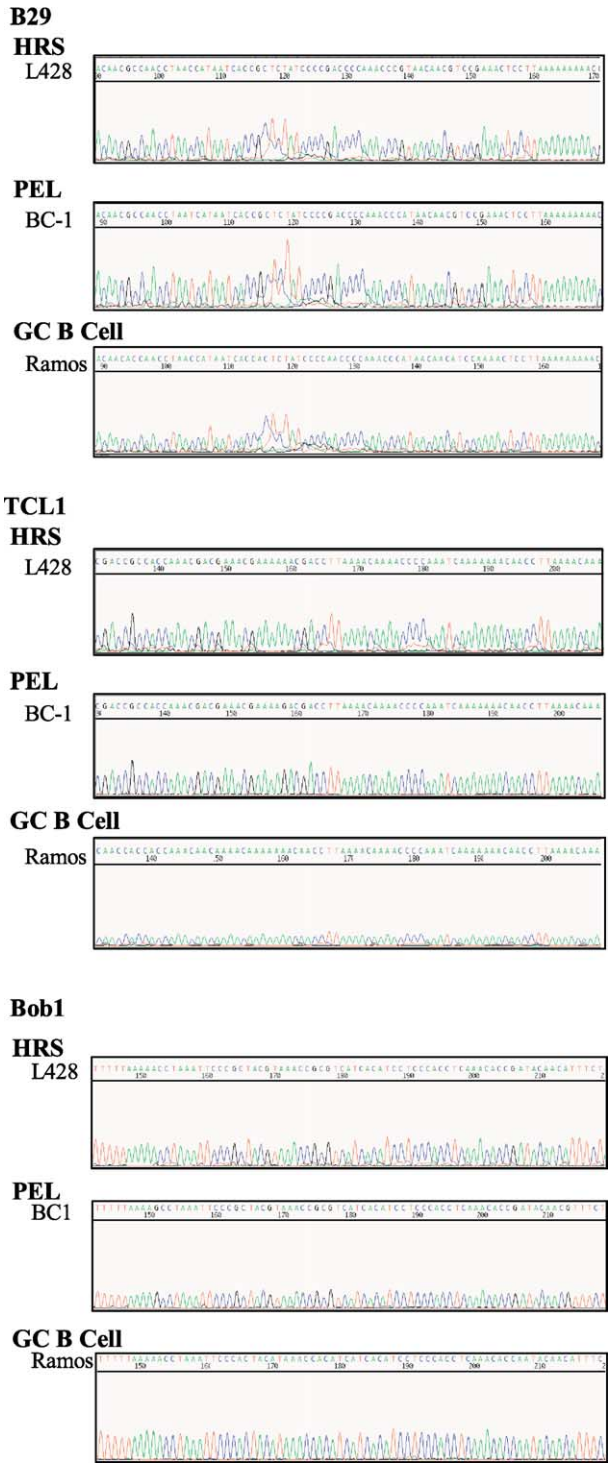

Figure 3. Representative electrophoretograms of bisulfite-sequenced clones of the *B29*, *TCL1*, and *Bob1* promoters in HRS and PEL cell lines. Genomic DNA from HRS and PEL cell lines was subjected to sodium bisulfite conversion, PCR amplification, sub-cloning, and cycle sequencing. Methylated and therefore bisulfite unconverted cytosine residues appear as guanine after sub-cloning and cycle sequencing. Representative *B29* promoter clone sequences span CpG sites 14 to 20 in reverse orientation. Representative *TCL1* promoter clone sequences span CpG sites 20 to 30 in reverse orientation. Representative *Bob1* promoter clone sequences span CpG sites 23 to 28 in reverse orientation.

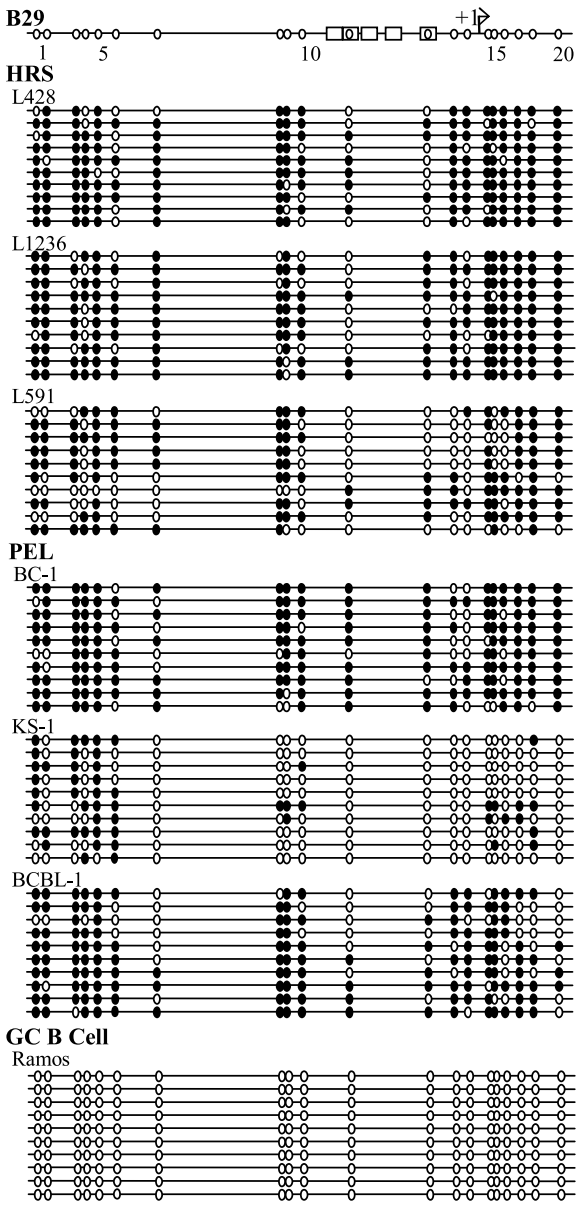

Figure 4. DNA methylation patterns of individual bisulfite-sequenced clones of the *B29* promoter in HRS and PEL cell lines. Genomic DNA from HRS and PEL cell lines was subjected to sodium bisulfite conversion, PCR amplification, cloning, and cycle sequencing. Unmethylated CpG sites are indicated by open circles and methylated CpG sites are indicated by filled circles. Ten clones from each cell line were bisulfite-sequenced to obtain a representative sampling of methylation patterns. The cartoon is drawn to scale. Transcription factor binding sites are indicated by boxes: EBF, Sp1. +1 indicates the major transcription start site.

minimal methylation in conjunction with histone code changes was sufficient to facilitate silencing of *B29* expression in KS-1 cells. The complete lack of methylated CpG sites over an extended region of the promoter containing functional *B29* promoter motifs in KS-1 cells also likely accounts for the reactivation of *B29* expression by TSA treatment (Table 1). The CpG methylation patterns in the

individual *B29* promoter clones with extensive methylation from the other cell lines showed a biphasic distribution. Complete modification of most CpG sites was observed in the 5′ and 3′ flanking regions of the *B29* promoter (sites 1–10 and 13–20) with infrequent or no modification of the two central core promoter CpGs (sites 11 and 12) located in known essential EBF and Sp1 transcription factor motifs.[21–23] The only exception to this noted biphasic pattern was the PEL line, BC-1, where essentially all *B29* promoter CpG sites, including the central CpGs (sites 11 and 12), were modified in all ten clones analyzed (Figure 4). No methylated CpG sites were detected in any of the ten clones from the GC-derived *B29* expressing B cell line Ramos (Figure 4), showing that the "normal" counterpart of HRS have an unmethylated *B29* gene promoter and verifying that the bisulfite conversion of gDNA in these studies was complete.

The *TCL1* promoters in L428, L1236, BC-1, and BCBL-1 cells also exhibited reproducible biphasic methylation patterns (Figure 5). Extensive CpG methylation was detected in 5′ CpG sites 1–17 and 3′ CpG sites 25–33. These flank the central core of the promoter which was methylated minimally, particularly in the three known functional Sp1 motifs (sites 18, 20, and 24).[24] The *TCL1* promoter in the PEL line, KS-1, had a similar pattern of CpG methylation as the *B29* promoter in these cells. Methylated CpGs were largely restricted to the 5′ region, (sites 2–11), with only rare methylated CpG sites over the 3′ region, (sites 25–33), of the promoter and with none in the essential core Sp1 promoter motifs (Figure 5). In view of this limited pattern of methylation, it is not surprising that TSA reactivated relatively robust *TCL1* expression in KS-1 cells (Figure 2). All CpG sites were methylated in the *TCL1* promoter in the HRS cell line, L591, with the exception of four sites (18, 20, 22 and 31). Two of these sites (18 and 20) are located in functional Sp1 motifs (Figure 5). No CpG methylation was detected in the *TCL1* promoter in *TCL1*-expressing GC-derived Ramos B cells (Figure 5).

In contrast to the biphasic methylation patterns seen in the majority of cell lines in the *B29* and *TCL1* promoters, essentially all CpG sites were methylated in the *Bob1* promoter cloned from all HRS and PEL cell lines except the BCBL-1 PEL cell line (Figure 6). The only exceptions were CpG sites 5–7 and 22–23, which were partially methylated in several lines. Half of the individual *Bob1* promoter clones from BCBL-1 (i.e. five out of ten) were not methylated at any CpG site, suggestive of monoallelic *Bob1* silencing. The other half of the clones from BCBL-1 were completely methylated at most CpG sites in a pattern similar to that seen with the other HRS and PEL cell lines (Figure 6). This lack of methylation in half of the *Bob1* promoter clones may account for the small RT-PCR amount of *Bob1* expression detected by RT-PCR in BCBL-1 cells. Only three CpG sites were methylated in GC-derived Ramos B cells, which express *Bob1* (Figure 6). The dense hypermethylation of the *Bob1* promoter in all HRS and PEL cell lines, except BCBL-1, is consistent with our finding that *Bob1* reactivation was only achieved with 5-aza (Table 1).

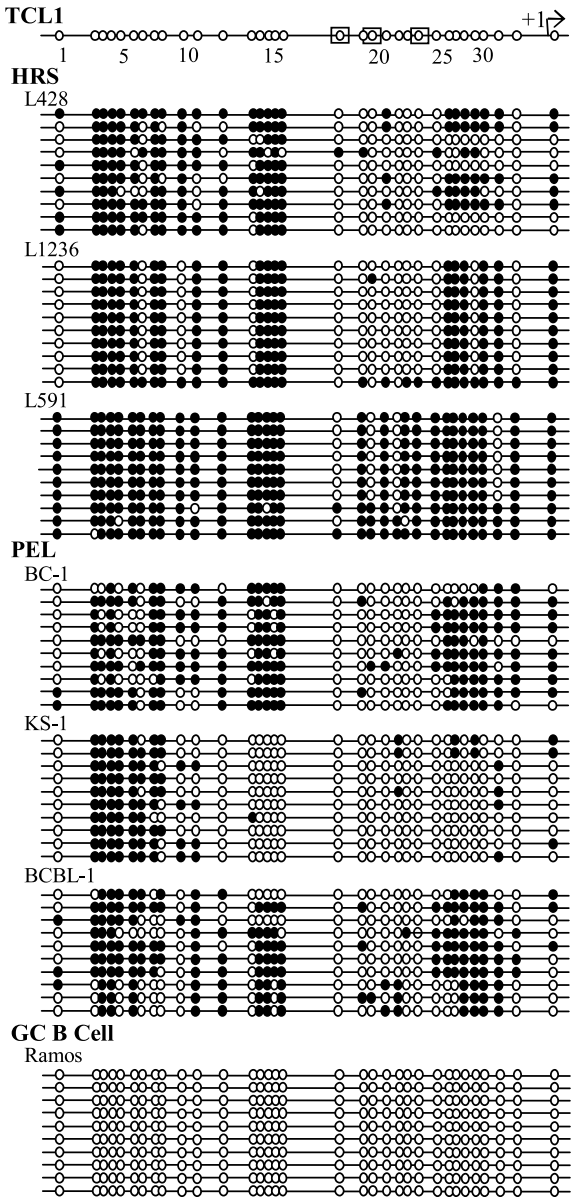

Figure 5. DNA methylation patterns of individual bisulfite-sequenced clones of the *TCL1* promoter in HRS and PEL cell lines. Genomic DNA from HRS and PEL cell lines was subjected to sodium bisulfite conversion, PCR amplification, cloning, and cycle sequencing. Unmethylated CpG sites are indicated by open circles and methylated CpG sites are indicated by filled circles. Ten clones from each cell line were bisulfite-sequenced to obtain a representative sampling of methylation patterns. The cartoon is drawn to scale. Transcription factor binding sites are indicated by boxes: Sp1. +1 indicates the major transcription start site.

Discussion

Results from the three representative CpG island

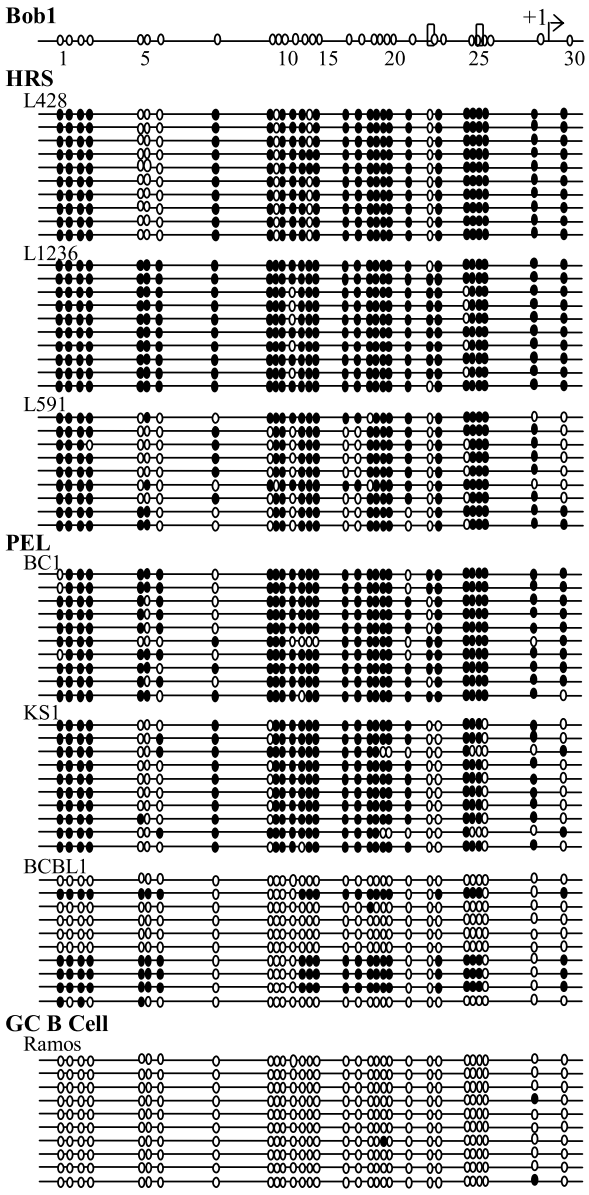

Figure 6. DNA methylation patterns of individual bisulfite-sequenced clones of the *Bob1* promoter in HRS and PEL cell lines. Genomic DNA from HRS and PEL cell lines was subjected to sodium bisulfite conversion, PCR amplification, cloning, and cycle sequencing. Unmethylated CpG sites are indicated by open circles and methylated CpG sites are indicated by filled circles. Ten clones from each cell line were bisulfite-sequenced to obtain a representative sampling of methylation patterns. The cartoon is drawn to scale. Transcription factor binding sites are indicated by boxes: NFAT, CREB/ATF. +1 indicates the major transcription start site.

genes used here indicate that advanced epigenetic mechanisms involving CpG methylation account for the silencing of B cell transcription factors and target genes in cHL and PEL. In general, the differential reactivation of silenced endogenous genes seen with TSA and 5-aza treatments in tested HRS and PEL cell lines correlates with varying degrees of CpG DNA methylation and associated chromatin changes. The reactivation of silenced *B29* and *TCL1* genes by 5-aza in all and by TSA treatment in most HRS and PEL cell lines evaluated predicted accurately that these promoters would be less densely CpG methylated than *Bob1* promoters. *Bob1* promoters were found to be completely CpG methylated and were only reactivated by 5-aza treatment, but not TSA treatment, in all tested HRS and PEL cell lines. A major finding was the existence of three patterns of CpG DNA methylation in the *B29* and *TCL1* promoters. As discussed below, these patterns suggest a novel staged progression in CpG methylation events. Ushmorov *et al*. recently reported reactivation using 5-aza and chromatin precipitation studies in L1236 and L428 HRS cell lines, indicating that the loss of Ig gene expression is due to epigenetic silencing.[25] Although genomic bisulfite sequencing was not performed in this study, the reactivation by 5-aza but not TSA predicts that the Ig gene silencing in these HRS cells is due to an advanced state of CpG methylation.

We previously reported a novel type of DNA methylation at C^mCWGG sites (W = A or T) that was inversely correlated to CpG methylation in the core promoter of silenced *B29* genes in a non-expressing plasma cell line (RPMI 8226) and a PEL cell line (BC-3) not studied here.[11] In view of the dense overall CpG methylation in the *B29*, *TCL1*, and *Bob1* promoters in the HRS and PEL lines, we did not expect to find many C^mCWGG sites. Only five individual C^mCWGG methylated sites were detected out of 180 clones sequenced, with four in *B29* promoter clones and one in a *Bob1* promoter clone (data not shown).

Our bisulfite sequencing studies revealed distinct and consistent patterns of DNA methylation, which have not been noted before in silenced promoters with CpG islands. We intentionally extended the length of bisulfite-sequenced DNA segments to >500 bp stretches to include most or all of the CpGs within the CpG islands of the silenced gene promoters. The *B29* promoter contains 20 CpG sites including two centrally located CpGs in known functional EBF and Sp1 transcription factor binding motifs.[21–23] The *TCL1* promoter also conforms to a classic CpG island and contains 33 CpG sites including three in centrally located and functional Sp1 binding motifs.[24] Sp1 binding is not sensitive to DNA methylation, but Sp1 is thought to be important in maintaining CpG islands free of methylation.[26,27] The *Bob1* promoter has 30 CpG sites and fulfils the definition of a classical CpG island. Two CpG sites are located in a central CREB/ATF site that is reported to be critical for promoter activity.[28] Interestingly, CREB/ATF DNA binding is blocked by CpG methylation.[29]

Three methylation patterns were reproducibly detected by bilsufite sequencing of the silenced *B29* and *TCL1* promoters while only one methylation pattern was found in the silenced *Bob1* promoter in the HRS and PEL cells analyzed. In one methylation pattern found in the silenced *B29* promoters (HRS

cell lines L428, L591 and PEL cell line BCBL-1) and in the silenced *TCL1* promoters (HRS cell lines L428, L1236 and PEL cell lines BC-1, BCBL-1), CpGs were densely methylated in flanking 5′ and 3′ DNA sequences while CpGs within the central core promoter were either unmethylated or only minimally methylated. In particular, CpGs located in known functional transcription factor binding motifs in the *B29* and *TCL1* promoters, such as Sp1 and EBF, were largely unmethylated. Our previous studies have shown dense CpG promoter methylation of *B29* and *TCL1* in the non-B lineage silencing of these genes in Jurkat T cells.[11,24] Dense CpG methylation was also found in the *B29* promoter in non-lymphoid lineage HeLa cells (our unpublished results). Interestingly, these dense CpG promoter methylations are also biphasic and spare the central promoter core and are therefore similar to the silencing seen in most HRS and PEL cells evaluated. Our previous studies using the plasma cell line RPMI 8226 where *B29* is aberrantly silenced, showed that the *B29* promoter is CpG methylated similar to the pattern seen in Jurkat T cells, in HeLa cells, and here where only the core promoter is spared from dense CpG methylation (our unpublished results).[11] This suggests that the aberrantly methylated and silenced promoters in HRS and PEL cells are similar to the normal methylation pattern in appropriate gene silencing, as would be expected if the targeting mechanism remains intact but its activation is abnormal. The second methylation pattern was found in the *B29* and *TCL1* promoters in the PEL cell line KS-1. The unmethylated core promoters were flanked by methylated CpGs selectively clustered in the 5′ region with mostly unmethylated CpGs located in the 3′ and core promoter regions. Significantly, *B29* and *TCL1* promoters with this flanking sequence methylation pattern could be reactivated by treatment with TSA. The third pattern of methylation was found in the *B29* promoter in the PEL cell line BC-1 and in the *TCL1* promoter in the HRS cell line L591. This pattern consisted of dense CpG methylation throughout the entire promoter regions, including methylation of the core promoter sites that were essentially unmethylated in the first two noted patterns. The *Bob1* promoter is a classic example of this third DNA methylation pattern. *Bob1* reactivation could only be achieved with 5-aza treatment, and the *Bob1* promoter was found to be densely methylated at essentially all CpG sites in all cell lines tested with one interesting exception. The PEL cell line, BCBL-1, showed hypermethylation of presumably only one allele, and this cell line does express low levels of *Bob1* mRNA (Figure 1). These data suggest that in some cases, the cHL and PEL silencing machinery has not only inappropriately silenced the gene, but has also "over-targeted" the methylation pattern necessary for appropriate silencing.

The discernible specific methylation patterns noted in our studies suggest that the progression of DNA methylation of CpG island promoters may occur in successive stages. The advancement to complete CpG island promoter methylation may occur *via* a targeted process beginning with methylation of CpGs in 5′ flanking regions, followed by methylation of CpGs in 3′ flanking regions, and then culminating with total methylation of promoter CpG sites. The presence of Sp1 sites within the *B29* and *TCL1* core promoters may be related to the consistent absence of CpG methylation in these core regions, since Sp1 has been shown to exclude CpG methylation in other studies.[26,27] In fact, what appears to be appropriate *B29* and *TCL1* gene silencing in non-B-lineage Jurkat T cells supports this notion, in that the core promoter regions harboring Sp1 sites exclude CpG methylation as well.[11,24]

Our bisulfite sequencing studies in the *B29*, *TCL1*, and *Bob1*-expressing germinal center-derived B cell line Ramos (Figures 1 and 2) confirmed the lack of promoter methylation our previous studies of *B29* and *TCL1* in other expressing B cell lines have shown.[11,24] Additionally, isolated primary tonsilar B cells show completely unmethylated *B29* promoters as well (our unpublished data). These GC-derived B cell lines and primary B cells correspond to the normal counterpart of HRS cells,[3] are *B29*, *TCL1*, and *Bob1*-expressing, and have completely unmethylated promoters. At present, there are no available cell lines that correspond to the presumed "plasma-blast" stage of development to represent the normal counterpart of PEL cells.[5] Although we cannot comment on the *B29*, *TCL1*, and *Bob1* promoter methylation at this developmental stage, we have previously shown that the *B29* promoter is completely unmethylated in all plasma cell lines analyzed that express both *B29* and *Bob1*.[11] Therefore, at least for the *B29* gene, the "plasma-blast" stage between that of the GC-derived B cell and plasma cell is likely to have unmethylated promoters as well.

Finally, what is the origin and role of epigenetic B cell gene silencing in cHL and PEL? Normal B cells are well known to require the expression of BCR genes and other B cell-specific genes for survival, activation and resistance to apoptosis.[30] This requirement is superceded by alternative transforming pathways in HRS and PEL cells. Both HRS and PEL cells express constitutively activated NF-kB *via* multiple mechanisms, such as from mutations in IκBα in cHL.[3,31] A considerable fraction of cases are also infected with distinct human herpesviruses that express viral genes that may further relieve these transformed B cells of any dependence on BCR signaling for continued survival and growth.[2,3] Expression of EBV-encoded LMP1 and LMP2a in cHL, and of HHV8-encoded FLIP in PEL leads to NF-κB activation and tumor cell survival.[3,31] Additionally, HRS cells express constitutively active c-FLIP, which inhibits CD95/FAS-induced apoptosis and promotes HRS cell survival.[3] Thus, although they were derived from B cells, HRS and PEL cells no longer function as B cells.

We postulate two, not mutually exclusive mechanisms, producing B cell gene silencing in cHL and PEL. In one mechanism, epigenetic silencing is preceded by deleterious somatic mutations of transcription factors during GC maturation that effect the expression and/or activity of essential transcription factors required for B cell gene expression. The lack of critical transcription factors could result in the loss of expression of multiple B cell genes, unleashing an epigenetic silencing cascade that would culminate in dense CpG hypermethylation. It is well known that the loss of transcription targets genes with CpG-rich promoters for epigenetic silencing and CpG hypermethylation.[32] However, all transcription factors involved in B cell gene expression need not be affected. Two important B cell transcription factors, EBF and Pax-5, continue to be expressed in cHL (data not shown).[3] In the second mechanism, the acquisition of transformation-specific growth and survival programs, such as constitutive NF-κB expression and herpesvirus infection, would obviate the requirement for the normal B cell gene program and initiate a selection process against maintaining such a program. Evolving tumor cells that rapidly discard B cell effector gene expression in a trial-and-error process could out-compete those that inefficiently retain non-essential B cell gene expression. Loss of B cell-specific gene expression in this context resembles tumor cell de-differentiation, although we suggest the alternative, that it is advantageous for an evolving tumor to discard the normal growth-regulating program in favor of the transformed program.

In either scenario, the majority of our results indicate that B cell genes in HRS and PEL cells are aberrantly inactivated by methylation of their CpG island-containing promoters causing terminal gene extinction much like that seen in appropriate B cell gene silencing in non-B lineage cells. However, the existence of total methylation at essentially all sites of the B cell gene promoters suggests that in some cases both the activation and the targeting of methylation is abnormal in cHL and PEL.

Materials and Methods

DNA and RNA extraction

Genomic DNA was isolated from 1×10^7 cells using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Total RNA was isolated from 1×10^7 cells using the RNeasy Mini Kit in combination with the Qiashredder (Qiagen, Valencia, CA).

RT-PCR

RNA (100 ng/sample) was reverse transcribed with gene-specific primers using the SuperScript One-Step RT-PCR System and PLATINUM Taq polymerase (Invitrogen, Carlsbad, CA). Reverse transcription proceeded for 30 minutes and PCR was performed for 30 s at 94 °C, 30 s at the gene-specific annealing temperature, and 1.5 minutes at 68 °C. The RT and annealing temperatures for each gene were as follows: *B29* and *mb-1*, RT at 55 °C and PCR at 59 °C; *Bob1*, RT at 59 °C and PCR at 59 °C; *EBF*, RT at 51 °C and PCR at 56 °C; *GAPDH*, RT at 54 °C and PCR at 57 °C; *Pax-5*, RT at 44 °C and PCR at 49 °C; *PU.1*, RT at 42 °C and PCR at 48 °C; and *TCL1*, RT at 50 °C and PCR at 55 °C. RT-PCR products were gel purified with the Geneclean kit (Q-BIOgene, Carlsbad, CA), cloned into the TOPO TA Cloning vector (Invitrogen), and cycle sequenced (Laragen, Los Angeles, CA). Gene-specific primer sequences were 5′ to 3′:

B29, GGAGCCTCGGACGTTGTCA and CGACCTGG CTCT-CACTCCT;
mb-1, AACTCAAACTAACCAACCCACT and ACTA ACGAGGCTGCTACAAT;
Bob1, CGGCTTCAAAGAGAAAAGGCAAC and TGGAGGTGGGTAGTGTGGAAAG;
EBF, TCGGGCATCTTCTCCTTCTCAC and TGGCAT TCCTATTCTGTCCCATAC;
Pax-5, CCAAAGTGGTGGAAAAAATCGC and TGA ATACCTTCGTCTCTCTTGCG;
PU.1, ATACCAACGCCAAACGCACGAG and GCTT CTTCTTCACCTTCTTGACCTC;
TCL1, CTTCAGCCTCTTTCTGTCCCTTC and TGCC TATTCTCACTTCTCTCCACC;
GAPDH, GTCATACCAGGAAATGAGCTTG and GATGACATCAAGAAGGTGGTG.

DNA demethylation and histone deacetylase inhibition

Cell lines were treated with either 5-aza-2′-deoxycytidine (5-aza) (Sigma-Aldrich, Milwaukee, WI) at 5 μM, 2.5 μM, or 1 μM or trichostatin A (TSA) (Sigma-Aldrich) at 200 nm, 100 nM, 50 nM, or 25 nM for three days with addition of fresh medium and drug every 24 hours. RNA isolation and RT-PCR (as described above) was followed by standard Southern blot analysis and the SpotLight Random Primer Labeling Kit and Chemiluminescent Hybridization and Detection Kit (BD Biosciences, Palo Alto, CA). cDNA products generated with the gene-specific RT-PCR primers shown above were used as Southern blot probes.

Genomic DNA bisulfite sequencing

Genomic DNA (2 μg/sample) was digested with ApaI for *B29*, NcoI and HindIII for *TCL1* and MslI and XcmI for *Bob1* and subjected to sodium bisulfite treatment as described[11,33] with slight modifications including use of 40 μg of glycogen to precipitate DNA, resuspension of DNA in 20 μl of water, and the addition of 1 μl of 6.3 M NaOH to denature the DNA at 39 °C for 30 minutes before and after denaturation at 97 °C followed by the addition of 200 μl of sodium bisulfite solution. Sodium bisulfite-treated DNA (2 μl) was PCR amplified for 50 cycles using sense-strand primer pair combinations followed by 35 cycles of nested PCR amplification. PCR products were cycle sequenced as described above. Sense-strand primer sequences were 5′ to 3′:

B29, TTTAGATGTTTGATTTGGGTTTGTGGTTGT and CCACCATCCAATAACTAAACAC
Bob1, TTTGAAGAGATGATAGGTGTTTTT and AAC AAAATATTACCTTTTCTCTTTA
TCL, GGGGGGGTTTTTTAGAAGAAGAAAGGGT and CAAAAACCAAAACCTCTCAAAA

Nested primer sequences were 5′ to 3′:

B29, GGGTGAGGAATAGTTTAGGATAGAGGAGT and CTATACCTAAAAACAACAACAACAAC
Bob1, TTTTTTTAGTTGAGAATTAGTGATT and CA ATTTAACTTCTTTAATATAAA
TCL1, GAGTTTTTAGTAGAGGTTTAGAGTT and CCAAAACCTCTCAAAACCACTC

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