

C^mC(A/T)GG DNA methylation in mature B cell lymphoma gene silencing

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DNA methylation has been linked to gene silencing in cancer. Primary effusion lymphoma (PEL) and myeloma are lymphoid malignancies that arise from terminally differentiated B cells. Interestingly, PEL do not express immunoglobulins or most B lineage-specific genes. The B cell-specific *B29* (*Igβ/CD79b*) gene is silenced in PEL and some myelomas but is expressed in other normal and malignant B cells. *B29* expression was reactivated in PEL by demethylating and histone deacetylase inhibiting treatments. Bisulfite sequencing revealed two types of DNA methylation in silenced *B29* promoters: at conventional CpG and at CC(A/T)GG *B29* promoter sites. The pattern of methylated CpG (mCpG) and C^mC(A/T)GG *B29* promoter methylation observed was similar to that recently reported for epigenetic silencing of an integrated retrovirus. Methylation of C^mC(A/T)GG sites in the *B29* promoter significantly repressed *in vivo* transcriptional activity. Also, methylation of a central conserved C^mCTGG *B29* promoter site blocked the binding of early B cell factor. This methylated motif formed DNA–protein complexes with nuclear extracts from all cell types examined. Therefore, C^mC(A/T)GG methylation may represent an important type of epigenetic marker on mammalian DNA that impacts transcription by altering DNA–protein complex formation.

PPrimary effusion lymphoma (PEL) is an infrequent neoplasia of severe immunodeficiency and is usually observed in those with longstanding AIDS (1, 2). PEL exhibit features both in common with and distinct from other B cell neoplasms. Like myeloma, PEL arise from postgerminal center, developmentally mature B lymphocytes (1–3). Unlike myeloma, PEL grow in body cavities as liquid effusions, are almost always infected with human herpesvirus-8 (HHV-8) and lack expression of B-lineage-specific genes (reviewed in ref. 2). The loss of B lineage gene expression in PEL could be caused by DNA methylation and epigenetic silencing. In accord with this idea, γ -herpesvirus genomes such as HHV-8, herpesvirus saimiri, and Epstein–Barr virus, are commonly methylated (4, 5). The same mechanism(s) responsible for viral DNA methylation may also be involved in methylation and silencing of B lineage-specific genes in PEL.

DNA methylation in mammalian cells largely occurs on cytosines in symmetric CpG dinucleotides and is associated with repressed gene transcription (6–10). Methyl-binding domain proteins engage methylated CpG (mCpG) and recruit histone deacetylase (HDAC) and transcriptional repressors to form stable repression complexes that induce local chromatin remodeling and gene silencing (11–16). Early mammalian embryos and germ cells, like plants and fungi, also methylate non-CpG cytosines (17–21). A recent report indicates that peripheral blood leukocytes may methylate the internal cytosine of symmetric CCTGG but not CCAGG motifs (22). However, little is known about the gene specificity, frequency, and functional significance of this latter type of symmetric non-CpG methylation.

The *B29* (*Igβ/CD79b*) component of the B cell surface receptor is encoded by the *B29* gene and is absent in all PEL lines

and some myelomas (1, 23). The B cell-specific *B29* promoter is well characterized and provides an ideal model to analyze DNA methylation in B lineage gene silencing in PEL and myeloma. Early B cell factor (EBF), in concert with Octamer, Ets, Sp1, and Ikaros transcription factors, regulates *B29* promoter activity in early B cell development, whereas non-EBF factors control *B29* gene expression at later stages of B cell differentiation (24). The human *B29* promoter contains 20 CpG dinucleotides and 6 CCAGG or CCTGG motifs within a 450-bp span (25). CpG dinucleotides are present in single Sp1 and EBF consensus-binding sites, whereas the CCAGG and CCTGG motifs are concentrated in a central promoter control region containing essential EBF sites.

Here we report that the *B29* promoter in PEL and nonexpressing myeloma cells is methylated at CpG and CC(A/T)GG sites. Because the methylation pattern observed at these *B29* promoter sites is similar to that reported in epigenetic retroviral silencing, we propose that B cell gene extinction occurs through a similar mechanism. We also find that CC(A/T)GG methylation repressed *B29* promoter activity and replaced transcription factors with new protein complexes.

Materials and Methods

DNA Demethylation and HDAC Inhibition. BCBL-1 cultures were treated with 5-aza-2'-deoxycytidine (400 nM, 64 h, Sigma) and trichostatin A (TsA, 50 nM, 24 h, Sigma), alone or in combination, or with sodium butyrate (0.3 mM, 64 h, Aldrich). BCBL-1 PEL cells were stably infected with *MSCV-GFP-IRES-PURO*, a green fluorescent protein-expressing retrovirus, to monitor the effects of demethylating and HDAC-inhibiting treatments. Growth, viability, and general gene expression were assessed by cell counts, trypan blue exclusion, and green fluorescent protein intensity. Total RNA isolation and reverse transcription with random primers was followed by 40 cycles of PCR amplification with *B29*-specific primers (5'-GGAGCCTCGGACGTTGTCA-3' and 5'-CGACCTGGCTCTCACTCCT-3'). Southern blot analysis was performed by using a random-primed α^{32} -dCTP-labeled *B29* cDNA fragment (PrimeIt II, Stratagene).

Genomic Bisulfite Sequencing. Two micrograms of isolated genomic DNA was restriction digested with *ApaI* and subjected to sodium bisulfite treatment as described (26, 27). Two micro-

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Abbreviations: PEL, primary effusion lymphoma; HDAC, histone deacetylase; EBF, early B cell factor; TsA, trichostatin A; IVT, *in vitro* translated; dsDNA, double-stranded DNA; mCpG, methylated CpG; EMSA, electrophoretic mobility-shift assay.

See commentary on page 10034.

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liters of sodium bisulfite-treated DNA was PCR amplified for 47 cycles by using multiple sense-strand primer pair combinations followed by 30 cycles of nested PCR amplification. Primer pairs that amplified the largest fragment possible from sodium bisulfite treated genomic DNA were used (published as supplemental data on the PNAS web site, www.pnas.org). PCR products were subcloned into the pCR2.1-TOPO vector (Invitrogen) and cycle sequenced.

Transient Transfection Assay. Cell line sources are listed in the supplemental data. Transient cotransfection of BL-41 B cells (1×10^7 cells/sample) with 10 μ g of *Renilla* reporter constructs pRL-null, pRLB29, or pRLSV40 was performed with 5 μ g of firefly reporter construct pGL3 control for transfection efficiency normalization by the DEAE-Dextran method (28) and analyzed with the Dual Luciferase Assay System (Promega). pRLB29 contains the genomic *B29* promoter fragment from -193 to +47. Reporter constructs were passed through *dcm* + DH5 α or *dcm* - *scs110* bacteria and bisulfite sequenced to confirm the methylation status of C^mC(A/T)GG sites (data not shown).

Protein Preparation and Electrophoretic Mobility-Shift Assay (EMSA). Human EBF protein was *in vitro* transcribed and translated (IVT) from pCDNA3hEBF, kindly provided by Mikael Sigvardsson (Lund University, Lund, Sweden) by using the T7 TNT Quick Coupled Transcription and Translation kit (Promega). Preparation of crude nuclear extracts was as described (29). EMSA was performed by using either 20 μ g of nuclear extract or 2 μ l of IVT hEBF, as described (28). EMSA probes and cold competitors were complementary double-stranded (ds)DNA oligonucleotides: 5'-CTGGTGCCTCCCCTGGGTCCCAA-TT-3' (C⁻⁹¹); 5'-CTGGTGCCTCCC^mCTGGGTCCCAAATT-3' (C^mC⁻⁹¹); 5'-CATGAATGGGGGTGGCAGAGA-3' (Ikaros); 5'-GATCGATCGGGGCGGGGCGATC-3' (Sp1); 5'-GATCTCGAGCAGGAAGTTCGA-3' (Ets-1); and 5'-GAGAGAGACTCAAGGAATTGTGGCCAGCC-3' (EBF). BL-41 and Ramos genomic cell line DNA competitors were prepared by using the Wizard genomic DNA Prep kit (Promega). *Saccharomyces cerevisiae*, adult *Drosophila melanogaster*, and *dcm* + *Escherichia coli* competitor genomic DNAs were kindly provided by Michael Grunstein, Utpal Banerjee, and Jeffrey Miller (University of California, Los Angeles), respectively.

Results

B29 Gene Expression in PEL Is Activated by Demethylation and HDAC Inhibition. Twenty B cell lines from distinct developmental stages and one T cell line were examined for *B29* gene expression. All four PEL lines, the myeloma line RPMI-8226, and Jurkat T cells lacked *B29* mRNA by Northern blot and reverse transcription-PCR analyses (see supplemental data). Transient transfections of *B29* promoter-luciferase reporter constructs were performed to determine the transcription competency of the *B29* negative lines. *B29*-expressing cell transfectants resulted in a 4- to 20-fold luciferase activity, whereas PEL and RPMI-8226 cells exhibited lower but significant ($P < 0.05$) 3- to 5-fold activations over control background transfections (data not shown). These results are within experimental variability observed for *B29* reporter constructs in other B and plasma cell lines (24). The data argue that factors needed for *B29* expression are present in PEL and RPMI-8226 cells and that silencing of the endogenous locus may be because of DNA methylation. Therefore, *B29*-negative BCBL-1 PEL cells were treated with demethylating and HDAC-inhibiting agents to test whether these treatments could activate *B29* gene expression. Treatments with 5-aza-2'-deoxycytidine, TsA, and sodium butyrate, alone or in combination, activated *B29* gene expression (Fig. 1; refs. 5, 30). The extent of activation was similar to the levels of activation reported for other silenced

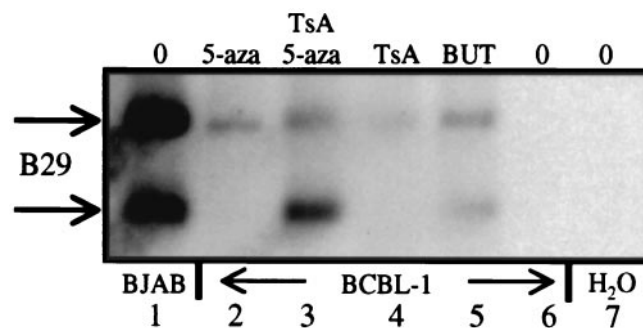


Fig. 1. DNA demethylation and HDAC inhibition activates endogenous *B29* gene expression detected by reverse transcription-PCR. Products (750 and 438 bp) correspond to the normal and alternately spliced forms of *B29* mRNA (48). Lanes: (1) BJAB untreated; (2) BCBL-1 + 5-aza-2'-deoxycytidine (5-aza); (3) BCBL-1 + 5-aza/TsA; (4) BCBL-1 + TsA; (5) BCBL-1 + sodium butyrate; (6) BCBL-1 untreated; and (7) water blank.

genes with these drug treatments (31). These results show that in BCBL-1 PEL cells, the direct or indirect effects of DNA methylation and HDAC activity repress *B29* gene transcription.

CpG and CC(A/T)GG Methylation of the B29 Minimal Promoter. Multiple *B29*-expressing and nonexpressing lines were surveyed by genomic bisulfite sequencing to determine whether silencing is because of DNA methylation of the *B29* promoter (Fig. 2A and B and supplemental data; ref. 32). Results from 17 independent DNA clones derived from seven *B29*-expressing cell lines indicate that *B29* expression is correlated with promoter region hypomethylation (Fig. 2A). In contrast, 13 clones from three different *B29* silenced PEL lines exhibited dense CpG methylation that ranged from 40 to 92% of sites (Fig. 2B). Interestingly, several clones from *B29* nonexpressing BC-3 PEL and RPMI-8226 myeloma lines had minimal or no CpG methylation. Instead, these clones showed specific methylation of three or four non-CpG cytosines at positions -119, -91, -60, and +58 relative to the major transcriptional start site, all localizing to the internal cytosine within the consensus sequence CC(A/T)GG (Fig. 2B and supplemental data). One clone from RPMI-8226 myeloma cells was not methylated; however, the only PCR primer set that worked for this bisulfite-converted DNA amplified a short span of the *B29* promoter in these cells. We cannot exclude the influence of DNA methylation outside of the examined region in this clone. Strikingly, CC(A/T)GG forms the target sequence for *dcm* methyltransferase activity in bacteria (33). These bisulfite sequencing results indicate that C^mC(A/T)GG also occurs in mammalian cells, as recently reported in studies of genomic Southern blotting of the myogenic *Myf-3* gene (22). We did not detect *B29* promoter methylation at additional symmetric and nonsymmetric non-CpG sites, including 22 C(A/T)G and 5 CpCpG sites. ^mCpG and C^mC(A/T)GG are strongly correlated with extinguished gene activity, suggesting that both forms of methylation may be involved in *B29* gene silencing.

C^mC(A/T)GG Represses B29 Promoter Activity in B Cells. To determine whether C^mC(A/T)GG sites repressed *B29* promoter activity *in vivo*, we tested CC(A/T)GG site-specific methylated and unmethylated reporter constructs by transient transfections of BL-41 B cells. *B29* reporter constructs were propagated in *dcm* + DH5 α or *dcm* - *scs110* *E. coli* and were bisulfite sequence confirmed. The *B29* promoter in DH5 α -propagated constructs was methylated on three distinct C^mC(A/T)GG sites, whereas *scs110*-propagated constructs were unmethylated (data not shown). The *dcm*-methylated *B29* promoter constructs reproducibly showed statistically significant reductions of 50% in

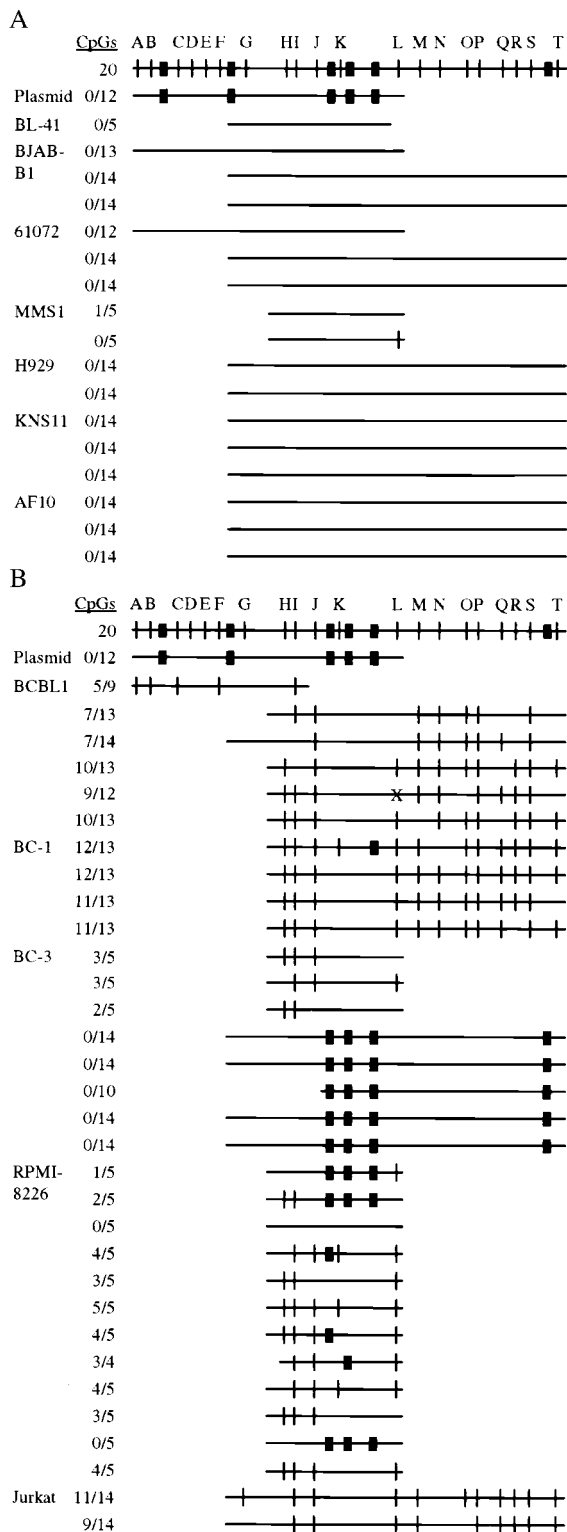


Fig. 2. Genomic bisulfite sequencing of the endogenous human *B29* promoter. Compiled results of bisulfite sequencing from *B29*-expressing (A) and nonexpressing (B) cell lines. The CpG positions analyzed are labeled A-T in alphabetic order (see supplemental data). The cell lines (Left) list the *B29* promoter clones examined. Data for BC-3 cells are from two independent bisulfite conversions on distinct days. The span sequenced for each DNA clone is indicated by the extent of the horizontal line. A vertical slash indicates a ^mCpG at that position; a darkened box indicates a methylated C^mC(A/T)GG at that position. X = C to G polymorphism. The plasmid is the *B29* minimal promoter that was bisulfite sequenced after passage through *dcm*⁺ DH5 α bacteria and demonstrates positions of C^mC(A/T)GG sites.

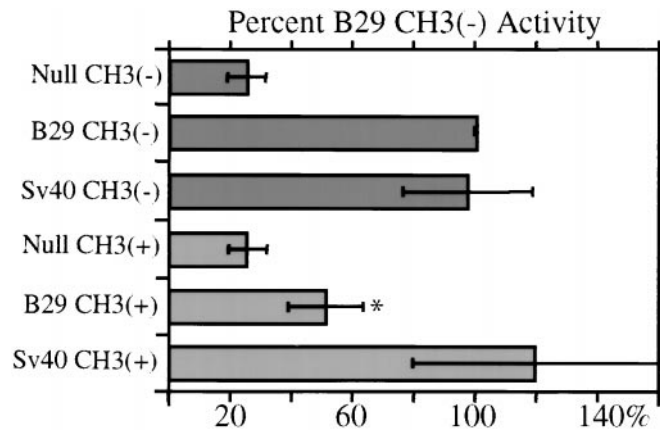


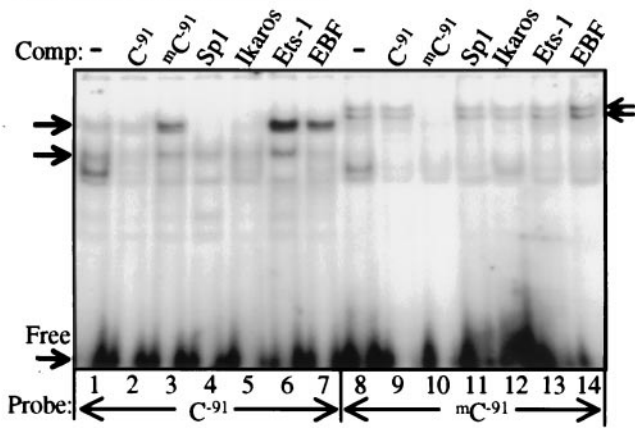
Fig. 3. Effect of C^mC(A/T)GG methylation on *B29* promoter activity in *B29* expressing BL-41 B cells. *Renilla* luciferase *B29* promoter, *SV40* promoter and promoter-less (null) reporter constructs were propagated in *dcm*⁽⁺⁾ DH5 α [CH3(+)] or *dcm*⁽⁻⁾ scs110 [CH3(-)] *E. coli*. *Renilla* luciferase activities are *SV40* firefly luciferase normalized (pGL3 control) with the \pm SD of at least three transfections by using three preparations of DNA. The data are presented as percent activity relative to the scs110-propagated *B29 Renilla* luciferase construct, [B29 CH3(-)]. The activity of *SV40* constructs, which contain no CC(A/T)GG sites, is statistically equivalent whether passed through *dcm*⁺ or *dcm*⁻ bacteria (Student's two-sided *t* test, *P* > 0.50). *, statistical significance, *P* < 0.05.

reporter gene activity compared with *dcm*-unmethylated constructs in *B29*-expressing BL-41 B-cells (*P* < 0.05; Fig. 3). Importantly, control simian virus 40 promoter driven constructs that do not contain CC(A/T)GG sites showed no statistically significant difference in activity between DH5 α - and scs110-propagated constructs (*P* > 0.50).

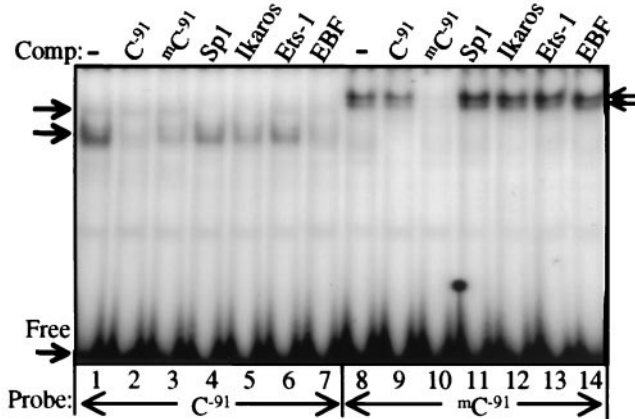
C^mCTGG Methylation Yields a Unique DNA-Protein Complex in Multiple Cell Types. Because *B29* promoter activity was repressed by C^mC(A/T)GG methylation, we analyzed the effect of this methylation on transcription factor binding in EMSA. The human and mouse *B29* core promoters contain three and two CC(A/T)GG motifs, respectively. One CCTGG site is highly conserved between species in a central critical region of the promoters, with the methylated internal cytosine located at position -91 (C⁻⁹¹) relative to the major transcriptional start site in the human sequence (25, 28). Prior DNase I footprint and EMSA have shown that purified Sp1, Pu.1, Ets-1, Ikaros, and EBF transcription factors bind the mouse *B29* promoter in this region (24, 28). The human *B29* promoter contains these same consensus sequences, although binding of specific transcription factors has only been inferred by use of these consensus motifs as cold competitors in EMSA (34).

Two dsDNA oligonucleotides were synthesized with and without symmetrical methylation of the target C⁻⁹¹ residue. Nuclear extracts were prepared from multiple cell lines that either express (Ramos, Raji, 729, and BL-41) or do not express (BC-1, BC-3, KS-1, BCBL-1, Jurkat, and HeLa) the human *B29* gene. Several specific protein complexes were observed in EMSA by using the unmethylated human *B29* dsDNA oligonucleotide as a probe with Ramos nuclear extracts (Fig. 4A). Similar complexes were also seen with nuclear extracts from all of the *B29*-expressing cell lines and from the nonexpressing PEL cell lines (BC-1, BC-3, BCBL-1, and KS-1; Fig. 4 and data not shown). This observation confirms that PEL contain transcription factor complexes similar to those seen in *B29*-expressing cell lines. Sp1, Ikaros, and EBF cold competitor oligonucleotides inhibited several DNA-protein complexes with the unmethylated target probe, consistent with results previously obtained with the

A. Ramos Extracts



B. BC-3 Extracts



C. HeLa Extracts

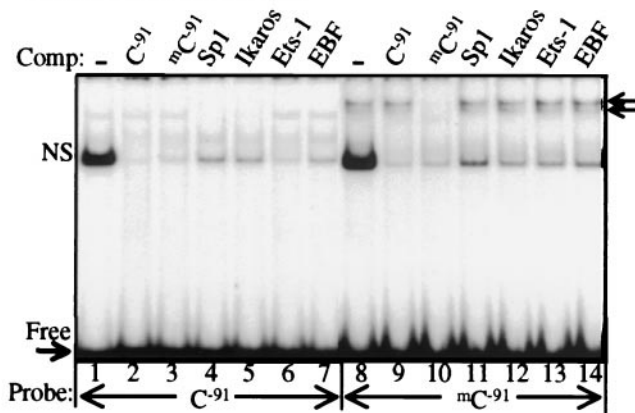


Fig. 4. Methylation of C^{-91} in C^m CTGG dramatically alters transcription factor binding and results in a unique DNA–protein complex. Symmetrically mC^{-91} -methylated or unmethylated dsDNA oligonucleotide probes were analyzed by EMSA with 20 μ g of nuclear extracts from (A) Ramos, a $B29$ -expressing B cell line, (B) BC-3, a $B29$ nonexpressing PEL cell line, and (C) HeLa, a $B29$ nonexpressing carcinoma cell line. Oligonucleotide cold competitor (500-fold) was added as indicated. EMSA banding patterns were similar in all the B cell extracts tested (data not shown). Unlabeled arrows denote specific complexes. NS, nonspecific complex.

corresponding mouse $B29$ promoter region (24, 28). Importantly, cold competitor dsDNA oligonucleotides with symmetric methylation of C^{-91} (mC^{-91}) residues failed to inhibit formation

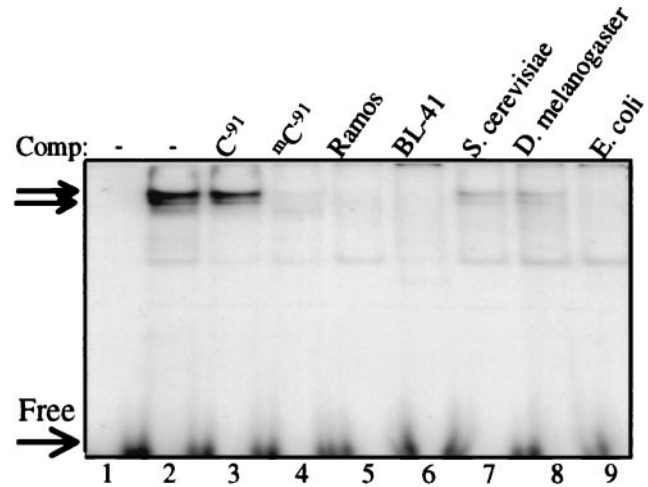


Fig. 5. Methylation negative genomic DNA does not compete for the mC^{-91} DNA–protein complex. Symmetrically mC^{-91} -methylated dsDNA oligonucleotide probe was analyzed by EMSA with 20 μ g of Ramos B cell nuclear extracts. One microgram of genomic DNA cold competitor was added, as indicated. Unlabeled arrows denote specific complexes.

of these protein complexes. Moreover, when the mC^{-91} probe was used as the target, the specific DNA–protein complexes seen with both Ramos and BC-3 nuclear extracts were not observed (Fig. 4 A and B). Instead, mC^{-91} showed new specific bands of reduced mobility, indicating alterations in DNA–protein complex formation. Neither unmethylated cold competitor probe (C^{-91}) nor transcription factor-specific cold competitors inhibited this banding pattern. Fig. 4C shows that this reduced mobility banding pattern was not unique to B cell lines. These complexes were also seen with non-B lineage HeLa and Jurkat nuclear extracts incubated with the mC^{-91} probe (Fig. 4C and data not shown). In fact, all 10 cell line extracts tested displayed an identical banding pattern with the mC^{-91} probe (data not shown). This banding pattern was not inhibited by a symmetrically CpG-methylated probe, indicating that the mC^{-91} complexes were distinct from CpG methylation-specific complexes (see supplemental data). Also, 1 μ g of genomic DNA from organisms that do not or perhaps minimally methylate their DNA, such as *S. cerevisiae* and adult *D. melanogaster*, could not compete for this DNA–protein complex, whereas organisms that methylate their DNA, such as humans and *E. coli*, completely inhibited complex formation (Fig. 5; ref. 35). $B29$ -expressing Ramos and BL-41 genomic DNAs also abrogated complex formation, predicting that genes other than $B29$ contain $C^mC(A/T)GG$ methylated sites in these lines. These observations indicate that C^m CTGG site-specific methylation in the $B29$ promoter replaced normal factor binding with distinct methylation-dependent DNA–protein complexes of ubiquitous protein factors.

C^m CTGG Methylation Blocks EBF Transcription Factor Binding. The conserved mC^{-91} region contains a consensus EBF-binding site that binds purified EBF in EMSA studies of the mouse $B29$ promoter (24). This site has not been investigated directly for EBF binding in the human promoter sequence, except as a cold competitor in EMSA (34). Therefore, we performed EMSA to determine whether an IVT EBF binds the C^{-91} site in the human sequence and whether mC^{-91} alters this binding. Fig. 6 demonstrates that IVT EBF bound the unmethylated C^{-91} probe and binding was completely inhibited by competition with itself but not by competition with cold mC^{-91} probe or a cold Sp1 consensus oligonucleotide. In contrast, when the mC^{-91} se-

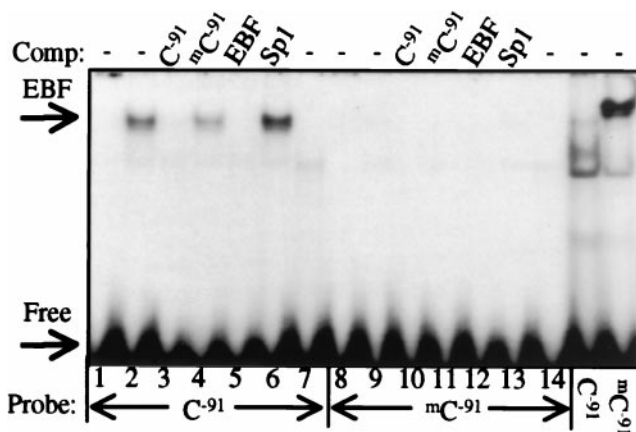


Fig. 6. Methylation of C⁻⁹¹ in C^mCTGG blocks EBF binding. Symmetrically ^mC⁻⁹¹-methylated or unmethylated dsDNA oligonucleotide probes were analyzed by EMSA with human IVT EBF (lanes 2–6 and 9–13), control IVT luciferase (lanes 7 and 14), or 20 μ g Ramos nuclear extracts (lanes 15 and 16). Oligonucleotide cold competitor (250-fold) was added, as indicated.

quence was used as a probe, IVT EBF binding was completely abolished (Fig. 6). These results indicate that C^mCTGG methylation is sufficient to block known transcription factor binding.

Discussion

PEL and RPMI-8226 myeloma cells must have expressed *B29* during earlier times in normal development. Multiple results strongly argue that *B29* silencing in PEL and RPMI-8226 cells is directly attributable to promoter region methylation. First, *B29* expression is reactivated by DNA demethylation and HDAC inhibition. Second, the *B29* promoter is active in transfections of both *B29* producing and silenced B cells. Third, symmetric methylation at both ^mCpG and C^mC(A/T)GG is associated with *B29* gene silencing in PEL and RPMI-8226 lines. The pattern of methylation at these two motifs in the *B29* promoter is similar to that reported for epigenetic silencing of an integrated retrovirus, where the density of C^mC(A/T)GG methylation is negatively correlated with the density of ^mCpG methylation (36).

This is, to our knowledge, the first clear example of C^mC(A/T)GG methylation in endogenous gene silencing detected by direct DNA sequencing in mammalian cells. Previously, symmetric C^mC(A/T)GG methylation was detected and implicated in retroviral gene silencing after viral integration into mammalian DNA (36). Also, methylation was detected in the *Myf-3* gene by genomic Southern analysis at C^mCTGG sequences and not at C^mCAGG sites (22). Although our study used tumor cell lines, C^mCTGG methylation of the *Myf-3* gene was found in peripheral blood lymphocytes, indicating that this type of symmetric methylation occurs in normal and malignant lymphoid cells. However, this latter study did not address the functional significance of C^mCTGG methylation, and the exclusion of C^mCAGG methylation is distinct from results reported here and by Lorincz *et al.* (36). Our study provides evidence of methylation on C^mC(A/T)GG in endogenous genes in mammalian cells, indicating that this pattern is not merely a cellular response to invading nucleic acids (18).

C^mC(A/T)GG methylation does not depend on the presence of γ -herpesviruses, because *B29*-silenced human herpesvirus-8-negative RPMI-8226 cells exhibit this type of methylation. Also, multiple *B29*-expressing Epstein–Barr virus-positive Burkitt lymphomas do not have C^mC(A/T)GG. This finding predicts that an enzyme with bacteria-like *dcm* methyltransferase activity is present in mammalian cells. A potential candidate would be Dnmt2, a mammalian structural homologue of the *Schizosac-*

charomyces pombe *pmt1*, which can specifically bind C^mC(A/T)GG sequences *in vitro* (37). However, methyltransferase activity for Dnmt2 has not been demonstrated (38). We can exclude contamination with *dcm*⁺ bacteria as the source of these results by noting the co-occurrence of both ^mCpG and C^mC(A/T)GG methylation in the same DNA clones from RPMI-8226 cells (39). The detection of abundant ^mCpG methylation in BCBL-1 DNA clones with no unconverted CC(A/T)GG sites confirms complete bisulfite treatment conditions despite nearby methylated cytosines (40, 41). Also, the 5' cytosine in CC(A/T)GG sites was always converted with bisulfite treatment, indicating that structural changes caused by the methylation of an adjacent internal cytosine did not impede the reaction (40). Furthermore, we show that different types of symmetric methylation may be present singly or concurrently in distinct DNA sequences. Overall, these observations suggest that the enzymatic activities responsible for ^mCpG and C^mC(A/T)GG are separable, perhaps independently regulated and active within a single cell.

Among the *B29*-silenced cell lines, only BC-3 and RPMI-8226 had abundant C^mC(A/T)GG methylation in distinct sequences. Interestingly, for both cell types, there is an apparent negative correlation between ^mCpG and C^mC(A/T)GG methylation in that sequences with abundant methylation of one type tend to lack methylation of the other type and vice versa (Fig. 2B). This pattern of methylation has been reported previously for silencing of a retroviral gene in mouse erythroleukemia cells (36). To determine whether the patterns are likely to be equivalent, we applied two statistical tools to the data derived for methylation of 12 RPMI-8226 sequences in Fig. 2B and 24 sequences from clone 18 in figure 4 of Lorincz *et al.* The standard Pearson correlation coefficient (*r*) between ^mCpG and C^mC(A/T)GG methylation density was *r* = -0.82 for the RPMI-8226 sequences (excluding the one sequence that was not methylated at all) and *r* = -0.63 for data derived from clone 18. Each correlation coefficient is significantly different from zero (1-sided *P* < 0.005), suggesting that each data set represents a negative correlation between ^mCpG and C^mC(A/T)GG methylation density. Furthermore, Kendall's rank correlation coefficient (τ) corrected for ties allows a direct comparison of the two data sets (42). The estimated τ (and asymptotic standard error) for RPMI-8226 and clone 18 sequences are -0.532 (0.200) and -0.374 (0.176), respectively. Each τ is significantly different from zero (1-sided *P* = 0.02 and 0.01, respectively, by exact permutation test), indicating it is likely they represent the same degree of negative correlation because this hypothesis cannot be rejected. If the unmethylated sequence is included in the RPMI-8226 data, the estimated coefficients are somewhat reduced in magnitude (*r* = -0.55 and τ = -0.352) but are even closer to the corresponding estimates for clone 18 sequences. These results suggest that the differential methylation mechanism operating on an integrated retrovirus may be similar to that operating on the endogenous *B29* gene in RPMI-8226 cells.

Most strikingly, our results show that the EBF transcription factor is displaced and that a unique methylation-dependent DNA–protein complex forms instead on a symmetrically methylated C^mCTGG site. ^mCpG competitors did not inhibit complex formation, indicating that the protein(s) bound to this C^mCTGG site are distinct from those that bind ^mCpG. Our data further demonstrate that EBF, along with other transcription factors (on the basis of EMSA competition assays with nuclear extracts) was displaced by a symmetrically methylated C^mCTGG site, similar to descriptions of transcription factor displacement by ^mCpG sites (reviewed in ref. 9). Although some transcription factors, such as Sp1, MTF-1, and Krox-20, are still able to bind ^mCpG methylated consensus sequences, other transcription factors, such as ATF-like and RBF1, are blocked by ^mCpG methylation in their consensus sequences (43, 44). Apparently, some factors

are insensitive to ^mCpG, although data indicate that Sp1 is blocked from DNA binding if methylation occurs on two consecutive cytosine residues in the consensus-binding region (45). Interestingly, inhibition of ATF-like and RBF1 binding by ^mCpG methylation strongly correlates with repression of *Rb1* promoter activity *in vivo* (44). Similarly, we demonstrate that *B29* promoter activity *in vivo* is ≈50% inhibited by methylation of three C^mC(A/T)GG sites. In both cases, this repression is likely because of recruitment of HDAC and chromatin remodeling complexes to the methylated, *in vivo* chromatinized plasmids, as has been shown directly for Ikaros-mediated repression of reporter gene transcription (46). Our *B29* expression results with methylated C^mC(A/T)GG sites are also comparable to the reductions produced by site-directed mutations that block EBF binding to the mouse *B29* promoter. These site-directed mutations caused a 30–40% reduction in *B29* promoter activity in mature B cells (24). Additionally, site-directed mutations within EBF consensus sites in both the *CD19* and *λ5* promoters resulted in a similar 40–65% reduction in promoter activity (34, 47). Furthermore, previous data have shown that multiple transcription factors are capable of binding the critical EBF-consensus

region of the mouse *B29* promoter, indicating that mutations or DNA methylation in this region may effect more than just EBF factor binding (24, 28). Taken together, promoter inhibition and blockage of EBF binding by C^mC(A/T)GG methylation in our studies are consistent with data generated from EBF-binding site mutation studies. Our study is, to our knowledge, the first to demonstrate a link between site-specific non-CpG methylation, methylation-specific DNA–protein complex formation, and cellular gene repression. These findings indicate that C^mC(A/T)GG methylation contributes to promoter activity control in mammalian cells and may have a companion role with CpG methylation in epigenetic gene regulation.

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