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# Identification of a Functionally Impaired Positive Regulatory Domain I Binding Factor 1 Transcription Repressor in Myeloma Cell Lines[1]

**Ildikó Győry,\* György Fejér,[2]\* Nilanjan Ghosh,\* Ed Seto,\* and Kenneth L. Wright[3]\*†**

B cell differentiation into a plasma cell requires expression of the positive regulatory domain zinc finger protein *1* gene (PRDM1) that encodes the positive regulatory domain I binding factor 1 (PRDI-BF1 or Blimp-1) protein. It represses the transcription of specific target genes, including c-*myc*, the MHC class II *trans*-activator, Pax-5, and CD23b. In this study we demonstrate the presence of an alternative protein product of the PRDM1 gene. The new protein, PRDI-BF1$\beta$, has a disrupted PR domain and lacks the amino-terminal 101 aa of the originally described protein. PRDI-BF1$\beta$ has a dramatic loss of repressive function on multiple target genes, but maintains normal DNA-binding activity, nuclear localization, and association with histone deacetylases and deacetylase activity. Myeloma cell lines express the highest levels of PRDM1$\beta$ mRNA relative to the full-length form, while primary cells and several other cell lines have very low, but detectable, levels of PRDM1$\beta$. RNA analysis and analysis of the PRDM1 promoters demonstrate that PRDI-BF1$\beta$ is generated from the same gene by alternative transcription initiation using an internal promoter. These newly described features of the PRDM1 gene are highly analogous to the PRDM2 (RIZ) and PRDM3 (MDS1-EVI1) genes, in which each express a truncated protein missing the PR domain. The expression of each of the truncated proteins is elevated in cancerous cells and may play an important role in the disease. *The Journal of Immunology,* 2003, 170: 3125–3133.

T he positive regulatory (PR)[4] domain zinc finger protein *1* (PRDM1) gene plays an essential role in the differentiation of B lymphocytes into plasma cells, and its gene product has been identified as Blimp-1 in the mouse and PRDI-BF1 in the human. In vivo expression of Blimp-1 is restricted to plasma cells derived from T-dependent or independent responses, bone marrow-resident long-lived plasma cells, and a subset of germinal center B cells (1). Memory B cells lack Blimp-1 expression. Ectopic expression of Blimp-1 in the BCL1 mature B cell line induces many of the phenotypic changes associated with plasma cell differentiation, including IgM secretion, J chain up-regulation, and Syndecan-1 expression (2, 3). Expression of Blimp-1 in earlier stage B cell lines leads to increased apoptosis (3), consistent with a central role for Blimp-1 or PRDI-BF1 in the cell fate choice of B cells.

There are four identified target genes that are directly repressed by PRDI-BF1 in the B lineage: c-*myc*, MHC class II *trans*-activator (CIITA), CD23b, and Pax-5 (4–8). The c-*myc* gene plays roles in growth control, apoptosis, and differentiation, and repression of c-*myc* is important, but not sufficient, for plasma cell differentiation (9). The down-regulation of CIITA by PRDI-BF1 results in silencing of MHC class II expression, another phenotypic feature of plasma cells (7, 8). CD23b is a type II integral membrane protein that is down-regulated in activated B cells and remains silenced during later stages of B cell development. Blimp-1 plays a role in maintaining low levels of CD23b by interfering with activation of the CD23b promoter by IFN regulatory factor-4 (5). The B cell lineage-specific activator protein (BSAP/Pax-5) is indispensable for B cell lineage commitment and B cell development and is silenced at the transcriptional level in terminally differentiated B cells by Blimp-1 (6). PRDI-BF1 also represses the IFN-$\beta$ promoter after viral induction in nonlymphoid cell lines (10).

The PRDM1 gene belongs to the PRDM gene family of transcriptional repressors, which is characterized by the simultaneous presence of Kruppel-type zinc fingers and the PR domain. The zinc fingers are required for direct DNA binding and function (10, 11). The PR domain is a subclass of the SET domain named for an evolutionarily conserved domain initially characterized in the *Drosophila* proteins SU(VAR)3–9, E(Z)30, and TRX31. It has been implicated as a protein interaction domain and in chromatin-mediated gene regulation (12–14). However, the function of the PR domain is not known. All known members of the PRDM family, including PRDM1, map to chromosomal regions that commonly undergo deletions in human cancer (15–17). Several family members share the feature of expressing two protein products that differ in the presence or the absence of the PR domain. Studies of the PRDM2 gene product RIZ have revealed that multiple tumor types often overexpress the PR domain-deleted protein relative to the full-length protein (18, 19). An important advance in understanding the activity of the PRDM family was made by Steele-Perkins

et al. (20) when they showed that targeted disruption of only the full-length form of PRDM2 results in a significant increase in tumor formation in multiple tissues. A similar observation has been made for PRDM3 (MDS1-EVI1) (21). Thus, the imbalance between the two forms may be critical for oncogenesis.

PRDI-BF1 elicits its repressive function by several different mechanisms. It interferes with the DNA binding of different IFN response factors to their recognition sites. This mechanism plays a role in silencing the IFN-β and CD23b promoters (5, 10). PRDI-BF1 also recruits corepressor proteins from the Groucho/TLE family, and histone deacetylases (HDAC) (22, 23). Interestingly, Groucho/TLE has been shown to interact with HDACs (24, 25), and proteins of the Groucho/TLE family as well as HDAC-2 have been shown to associate with the same proline-rich region of PRDI-BF1 (22, 23). This raises the possibility that all three proteins cooperate to repress some PRDI-BF1 target genes. While histone deacetylation is essential for repression of the c-*myc* promoter, it is not required for repression of CIITA (8). CIITA repression is diminished by removal of the PR domain. These findings indicate that PRDI-BF1 has multiple functional domains, which may be active on different subsets of PRDI-BF1 target genes.

This report now demonstrates the presence of an alternative protein product of the PRDM1 gene, referred to herein as PRDI-BF1β. For clarity, we suggest that the full-length form of the protein be referred to as PRDI-BF1α. The new protein, PRDI-BF1β, is abundantly expressed in myeloma cell lines through alternative transcription initiation. It has a disrupted PR domain and lacks the N-terminal acidic region, similar to the oncogenic form of PRDM2 (RIZ). Importantly, PRDI-BF1β has a significantly impaired transcription repressor function on multiple target genes.

## Materials and Methods
### Cell culture

The B cell line, CA46, and the myeloma cell lines, U266 and NCI-H929, were grown according to American Type Culture Collection (Manassas, VA) specifications. HeLa and HEK-293 cells were cultured in DMEM (Invitrogen, San Diego, CA) supplemented with 10% FBS (HyClone, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin. Human peripheral blood monocytes and primary macrophages and dendritic cells were cultured as previously described (26) and were gifts from Dr. S. Wei (Moffitt Cancer Center). Peripheral blood-derived B lymphocytes were isolated by negative selection as previously described (27) and were provided by Dr. E. Sotomayor (Moffitt Cancer Center).

### DNA constructs, recombinant adenovirus, and transient transfections

The CIITA and c-*myc* reporter constructs have been described previously (8). The pCDNA-FLAG-PRDI-BF1α and pCDNA-FLAG-PRDI-BF1β plasmids were constructed by PCR amplification of cDNA using appropriate primers and cloned into the pCDNA3.1 vector (Invitrogen) that had been modified by inserting a Flag epitope sequence between the *Kpn*I and *Eco*RI sites. The modified vector was a gift from Dr. T. Maniatis (Harvard University, Boston, MA) (22). The Gal4 fusion vectors were made by inserting the PRDI-BF1α, PRDI-BF1β, and Δ-PR sequences (8) into the *Eco*RI-*Xba*I sites of the Gal4 fusion vector PM1 (28). The Gal4 reporter construct pG2G5tk has been described previously (29). PRDI-BF1α and PRDI-BF1β expressing recombinant adenoviruses were constructed with the Ad-Easy system, provided by Dr. B. Vogelstein (The Johns Hopkins University, Baltimore, MD) and produced in the HEK-293 cell line (ATCC CRL1573; American Type Culture Collection). In all experiments the amount and integrity of the expressed proteins were determined by Western blot analysis. The PRDM1α promoter construct was created by subcloning the 2.7-kb *Sac*I-*Sma*I DNA fragment from the genomic clone RP1–101 M23 into the pGL3 basic vector (Promega, Madison, WI). The PRDM1β promoter construct was created by PCR amplification of the region from −684 to 32 bp relative to the PRDM1β transcription start site and inserted into the pGL3 vector. Transient transfection of CA46 cells was conducted by electroporation as described previously (8). HeLa cells were transfected using the FuGene6 reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions using 0.4 μg of DNA and 1.3 μl of Fugene6. All transfections were normalized to the signal obtained from a constant amount of cotransfected pRL-thymidine kinase (Promega).

### RNA isolation and Northern blot analysis

Total cellular RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions. Poly(A)+ RNA was purified from 500 μg of total RNA with the Poly(A)Ttract mRNA Isolation System (Promega). The RNA samples were separated by electrophoresis and blotted onto a Hybond-N+ membrane (Amersham Pharmacia Biotech, Arlington Heights, IL), Hybridization was conducted in Denhardt's hybridization solution. The PRDM1 probe was prepared by random priming labeling of the 2800-bp *Kpn*I fragment of the pCDNA-FLAG-PRDI-BF1α plasmid. The probe that selectively detects PRDM1β was prepared by random priming of a 900-bp DNA fragment containing the 150-bp PRDM1β-specific exon 1β flanked by nonspecific vector sequences.

### 5′ RACE and RT-PCR

The 5′ ends of PRDM1α and -β were determined by First Choice RLM-RACE (Ambion, Austin, TX), using 500 ng of poly(A)-selected RNA as the starting material. The specific primer sequences for the RACE-PCR reaction were the following; exon 2 outer reaction, 5′-CAGGGGTGG TCGTTCACAATGTATG-3′; exon 2 inner reaction, 5′-GAGTCATATC CGCATCCTCCATGTC-3′; exon 5 outer reaction, 5′-CTCTTTGGGACA TTCTTTGGGCAG-3′; and exon 5 inner reaction, 5′-GTTCATTTTTC TCAGTGCTCGGTTGC-3′. The products were cloned and sequenced (GenBank AY198414, AY198415). RT for the subsequent PCR reactions were conducted with Superscript II Reverse Transcriptase (Invitrogen) using 5 μg of total RNA and oligo(dT) as the primer. The PCR conditions for each primer pair were optimized using the PCR Optimizer kit (Invitrogen), a 1/200 dilution of the cDNA, and 34 cycles of amplification.

### RNase protection assay (RPA)

The RPA probes were generated by subcloning the appropriate PCR products to pCR2.1 (Invitrogen) and transcribing antisense RNA by T7 polymerase (30). RPAs were conducted with the RPA III kit (Ambion) following the manufacturer's instructions with minor modifications: 20 μg of total RNA was coprecipitated with $8 \times 10^5$ cpm of labeled probe. The hybridization was conducted in 8 μl of hybridization buffer overnight at 56°C. The samples were digested with RNaseA/RNaseT1 mix diluted 1/100.

### Nuclear extract preparation and EMSAs

Nuclear extracts were prepared as described by Dignam et al. (31). EMSA was performed as described previously (8). The PRDI-BF1 oligonucleotide spans from −190 to −158 bp of the CIITA-p3 promoter. The wild-type sequence is 5′-GTCCACAGTAAGGAAGTGAAATTAATTTCAGAG-3′, and the mutant sequence is 5′-CACAGTCCACAGTAAGGAgtcGAcgTTA ATTTCAGAGAGGTG-3′.

### Immunoprecipitation and Western blotting

The transiently transfected or adenovirus-infected cells were washed twice in PBS and lysed in PBS containing 0.1% IGEPAL (Sigma-Aldrich, St. Louis, MO), 10% glycerol, and Complete EDTA-free protease inhibitor (Roche). One hundred microliters of lysis buffer was used per $2 \times 10^6$ cells. Nuclei were collected by centrifugation for 5 min at 4°C, and the supernatant was used as the cytoplasmic extract. The nuclear extract was prepared by resuspending the pellet in the same volume of lysis buffer and sonicating. The immunoprecipitations were performed from the nuclear extract using Anti-FLAG-M2 affinity Gel (Sigma-Aldrich). Western blotting was conducted with Hybond-C Extra membrane (Amersham Pharmacia Biotech) and was visualized with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Abs to the amino and carboxyl termini of PRDI-BF1 have been described previously (8). The anti-FLAG Ab was purchased from Sigma-Aldrich.

### HDAC assay

The immunoprecipitated samples described above were incubated with tritium-labeled core histones (3000 cpm total input) prepared from cycloheximide-treated HeLa cells as previously described (32). Briefly, samples were incubated overnight at room temperature, then extracted with ethylacetate and centrifuged, and the amount of free acetate was measured from the water-soluble phase by scintillation counting. Immunoprecipitates prepared with nonspecific Ab and specific immunoprecipitates assayed in the presence of 600 nM TSA served as negative controls (33).

## Results

### The PRDM1 gene uses two major transcription start sites in myeloma cell lines

Northern blot analysis of PRDM1 mRNA from human myeloma cell lines suggested that the reported 5′ end of the message is under-represented relative to the 3′ two-thirds of the PRDM1 mRNA (data not shown). Since genes of the PRDM family characteristically encode alternatively initiated mRNA products, we hypothesized the existence of an alternative 5′ end. To test this hypothesis, poly(A)$^+$ mRNA from the human myeloma cell line U266 was subjected to 5′ RACE using specific nested oligonucleotide primers corresponding to sequences of either the second or fifth exon.

RACE from the second exon yielded a single product. The 5′ terminus of the RACE product is within 16 bp of the previously reported end (10) (GenBank, NM 001198) and confirms that transcription initiation occurs within this region. Sequencing of this product revealed that it contains an additional 123 bp between the reported exons 1 and 2 (Fig. 1A). Comparison with the genomic sequence (GenBank, NT 033944) shows that the additional sequences are directly adjacent to exon 1 and are created by an alternative splice donor site. This mRNA is referred to as PRDM1α throughout the report (GenBank, AY198414). The PRDM1α mRNA contains an in-frame translation initiation codon that would potentially add 36 aa to the PRDM1α-encoded protein (PRDI-BF1α), but it does not conform to the Kozak consensus sequence. Whether this initiation site is used in vivo remains to be tested.

RACE from the fifth exon of PRDM1 yielded a primary product that spanned from exon 5 through exon 4 and included 155 bp of unique sequence at the 5′ end. The unique sequence contains a translation initiation codon with an appropriate Kozak consensus sequence (34) and is in-frame with the remainder of the PRDI-BF1 protein. Comparison with the genomic sequence revealed that the unique sequence is found as a single novel exon 205 bp upstream of exon 4 (Fig. 1A). This novel short mRNA species is designated PRDM1β, and the protein encoded by it is PRDI-BF1β (GenBank,

AY198415). For clarity, the originally described full-length PRDI-BF1 protein (10) will be referred to as PRDI-BF1α.

Confirmation of the two different start sites was made by RPA. We designed the RPA probe for the PRDM1α 5′ end to contain 400 bp of genomic sequences, 200 bp of which overlaps the mRNA sequences of the first exon of PRDM1α. Several protected bands were detected, the longest of which corresponds to the sequenced 5′RACE product. The other two major protected products were 40 and 50 bp shorter, consistent with multiple transcription initiation sites typical of TATA-less promoters (Fig. 1B). RNase protection analysis of the predicted PRDM1β 5′ end in myeloma cell lines revealed three protected bands (Fig. 1C, lanes 1 and 2). The longest protected product corresponds to the expected PRDM1β form and confirms the abundant presence of PRDM1β mRNA in the myeloma cell lines. The shortest product represents PRDM1α that is protected over the common exon 4 sequences. A less intense middle band is consistent with an mRNA containing exon 4 and part of the unique exon 1 of PRDM1β. This may represent a secondary start site for PRDM1β or may be a degradation product. Interestingly, this band is the more prominent of the two in THP-1 and B cells, consistent with the hypothesis that there are two start sites for PRDM1β.

### Characterization of the PRDM1 mRNA

The expression pattern of the newly discovered PRDM1β mRNA was investigated on an array of different primary cells and cell lines. We found the myeloma cell lines U266 end NCI-H929 expressed an especially high level of PRDM1β message relative to PRDM1α (Fig. 1C). Body cavity-based lymphoma (BCBL) is a tumor type derived from postgerminal center B cells, with the BCL-6-/syndecan-1$^+$ phenotype (35, 36). In good correlation with their differentiation stage, a BCBL-derived cell line (BCBL-1) expresses high levels of PRDM1α as well as PRDM1β (lanes 15–18). Importantly, in an enriched primary B cell preparation, PRDM1β was detectable, albeit at a low ratio, with PRDM1α, indicating that the β form is not unique to cell lines (lane 10). The reason why the shorter PRDM1β protected band is predominant in
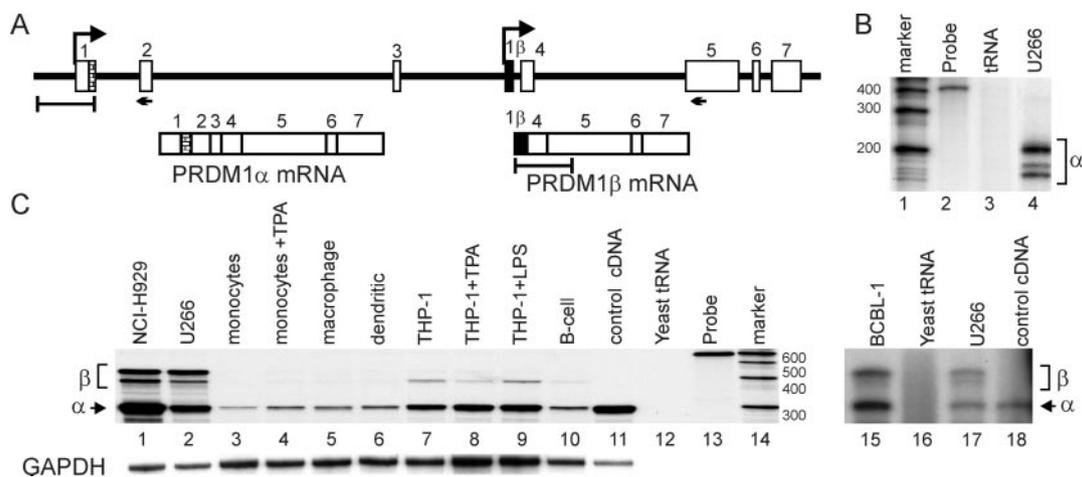


**FIGURE 1.** The PRDM1 gene expresses two major mRNA types. A, Schematic representation the genomic structure and the expressed mRNAs of PRDM1. Numbered open boxes represent the exons. The hatched exon represents the extended region of exon 1. The arrows pointing to the *left* represent the gene-specific primers for 5′RACE, while the arrows pointing to the *right* indicate the transcriptional start sites. RPA analysis of the 5′ end of PRDM1α (B) and PRDM1β (C). RPA probes are indicated (⊢—) on the genomic map for PRDM1α and on the mRNA map for PRDM1β. Each lane represents 20 μg of total RNA of the indicated cell line or control yeast tRNA used in the assay. NCI-H929 and U266 are myeloma cell lines, THP-1 is a monocytoid cell line, and BCBL-1 is a BCBL cell line. The other cell type names represent primary cells of human origin. The α and β symbols mark the protected bands specific for PRDM1α and PRDM1β, respectively. Figures are representative of at least four independent experiments, except for the primary cell RNA, which was analyzed twice.
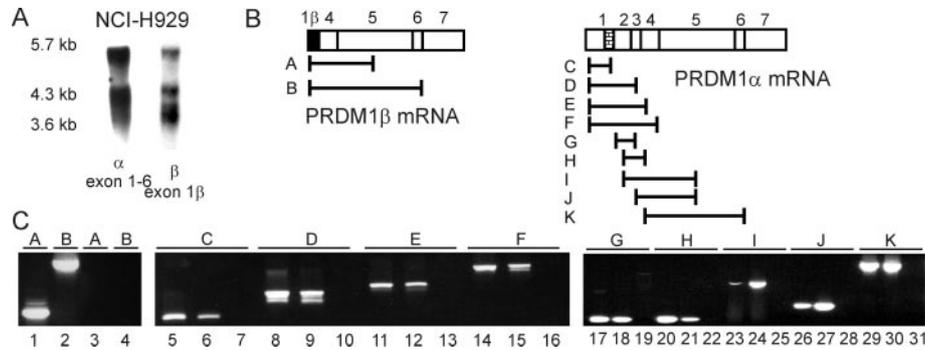
**FIGURE 2.** Differential transcription initiation is the only variation in the mRNA structure of the PRDM1 gene. *A*, Northern blot analysis of RNA from NCI-H929 myeloma cells. The probes are complementary to either the full-length PRDM1α cDNA (*lane 1*) or the unique exon 1β of PRDM1β (*lane 2*). *B*, Schematic representation of the PCR primer sets used in *C* to amplify specific regions of PRDM1α and -β. *C*, RT-PCR of PRDM1α or -β from mRNA isolated from the U266 myeloma cell line. The primer set is indicated above the lanes. *Lanes 1–4*, PRDM1β-specific PCR products from U266 cDNA (*lanes 1* and *2*) or the negative control lacking cDNA (*lanes 3* and *4*). Amplification by primer sets C–K (*lanes 5–31*) is presented in sets of three, including U266 cDNA (5, 8, 11, 14, 17, 20, 23, 26, 29), cDNA from HeLa cells overexpressing recombinant PRDM1α (*lanes 6, 9, 12, 15, 18, 21, 24, 27,* and *30*), and a negative control (*lanes 7, 10, 13, 16, 19, 22, 25, 28,* and *31*).

the B cells and THP-1 (*lane 7*) is unknown, but could be due to a change in promoter usage. PRDM1β is also present in the myeloid lineage. The monocytoid cell line THP-1 expresses substantial amounts of the α form, while expressing relatively small amounts of PRDM1β. Both forms appear to be inducible by bacterial LPS (*lanes 7–9*), thus keeping the ratio of PRDM1α and PRDM1β constant. PRDM1α is weakly detectable in primary macrophages and dendritic cells, while PRDM1β is not detectable (*lanes 3–6*).

Murine PRDM1 mRNA is found in three major isoforms of 5.7, 4.3, and 3.6 kb, determined by Northern blot analysis, which are known to differ in their 3′ noncoding region (2, 37). A similar analysis using a cDNA probe corresponding to the 155-bp unique first exon of PRDM1β was performed to determine whether usage of the downstream start site was coupled to alternative splicing or polyadenylation (Fig. 2*A*). Both the PRDM1β-specific probe and the PRDM1α probe detect three similar major mRNA forms. Fine mapping of further possible splice variants in the coding region of PRDM1 was conducted by a series of RT-PCR reactions. Primers were chosen to span one or more exon junctions (Fig. 2*B*). These results confirmed the presence of PRDM1β mRNA and demonstrate that it is identical with PRDM1α from exon 4 through the end of the messages (*lanes 1–4*). No other variations were detected in either PRDM1α or β (*lanes 5–31*). Taken together the RACE, RPA, and RT-PCR data indicate that PRDM1β is a specific and unique isoform of PRDM1.

### PRDM1β mRNA encodes a novel protein

PRDM1β mRNA encodes a predicted protein 700 aa long in which the amino-terminal three amino acids are unique, and the remainder of the protein is identical with aa 102–798 of PRDI-BF1α (Fig. 3, *A* and *B*). This generates a PRDI-BF1β protein missing the amino-terminal acidic domain and disrupting the PR motif. The entire coding region of PRDM1β was subcloned into a mammalian expression vector and transiently transfected into HeLa cells. A protein of ~80 kDa was easily detectable on a Western blot using an Ab specific for the shared C terminus of the two PRDI-BF1 isoforms (Fig. 3*C*). In comparison, a similar expression construct for PRDI-BF1α produced the expected 97-kDa protein. We next tested for endogenous expression of the two PRDI-BF1 isoforms. Western blot analysis of the human myeloma cell line NCI-H929 with the C-terminal Ab clearly detected both the 80-kDa PRDI-BF1β and the 97-kDa PRDI-BF1α proteins (Fig. 3*C*, *lane 4*). The Burkitt lymphoma line CA46 does not contain either PRDI-BF1
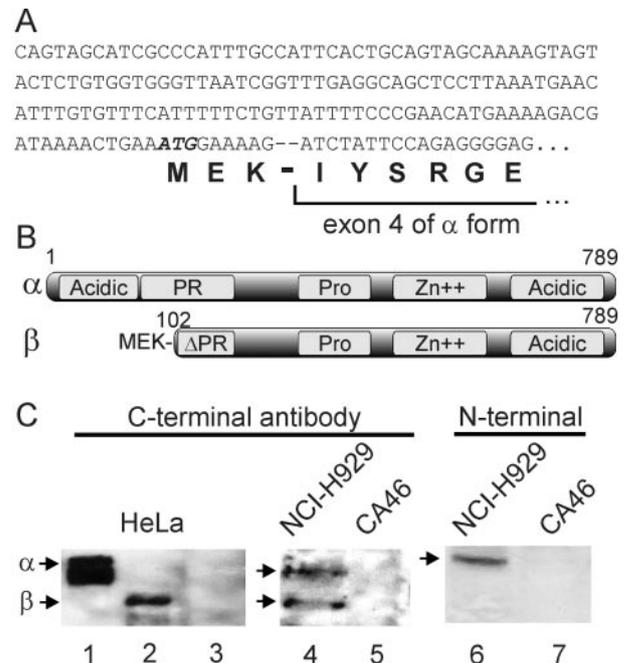


**FIGURE 3.** Myeloma cell lines express two PRDI-BF1 proteins of ~97 and 80 kDa. *A*, 5′ sequences of the PRDM1β mRNA and unique amino terminus of the PRDI-BF1β protein. The unique first exon of the mRNA is 155 bp long and is joined in-frame to exon 4 of the PRDM1α mRNA. The splice junction is indicated by dashes. The translation start codon is in bold, and the encoded amino acids are shown below. The remainder of the mRNA and protein is identical with PRDM1α. *B*, Schematic of the predicted proteins. *C*, The Ab raised against a C-terminal peptide of PRDI-BF1 recognizes both PRDI-BF1α and PRDI-BF1β on a Western blot (*lanes 1–5*). *Lanes 1–3* represent cell lysates from $2 \times 10^6$ HeLa cells infected with either PRDI-BF1α-expressing adenovirus (*lane 1*), PRDI-BF1β-expressing virus (*lane 2*) or GFP-expressing adenovirus (*lane 3*). *Lane 4* shows the endogenous expression of both PRDI-BF1α and PRDI-BF1β in the myeloma cell line NCI-H929, while the Burkitt lymphoma CA46 cell line (*lane 5*) does not express either. The Ab against an N-terminal peptide of PRDI-BF1α recognizes only the α form (*lanes 6* and *7*). Whole-cell lysates from either $1 \times 10^8$ cells (*lanes 4* and *5*) or $2 \times 10^7$ cells (*lanes 6* and *7*) were analyzed. In some experiments, overexpressing PRDI-BF1α or -β results in an additional smaller band that may arise from partial degradation of the protein (*lane 1*; see also Fig. 4, *lane 3*).

protein (*lane 5*). A similar analysis using the amino-terminal PRDI-BF1 Ab only detected PRDI-BF1α in the myeloma cell lines (*lanes 6* and *7*). These protein expression findings are consistent with the mRNA analysis and confirm that PRDM1β is lacking the amino-terminal portion of PRDM1α. Importantly, it demonstrates that myeloma cell lines endogenously express similar levels of PRDI-BF1β relative to PRDI-BF1α.

### PRDI-BF1β protein localizes to the nucleus and binds DNA

PRDI-BF1α is a direct DNA binding transcriptional repressor requiring nuclear localization. To determine whether PRDI-BF1β maintains a similar nuclear localization, cytoplasmic and nuclear extracts were prepared from cells transfected with either FLAG-tagged PRDI-BF1α, PRDI-BF1β, or the empty expression vector. Immunoblotting for the expressed PRDI-BF1 proteins revealed that both isoforms localize to the nucleus (Fig. 4A). Consecutive immunoblotting with Abs against 14-3-3 and HDAC-2, uniquely cytoplasmic or nuclear, respectively, demonstrates that the cyto-
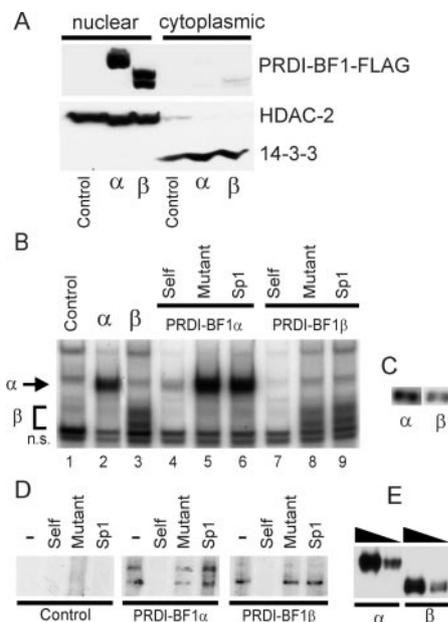


**FIGURE 4.** Both PRDI-BF1α and PRDI-BF1β are localized to the nucleus and bind DNA. *A*, Nuclear and cytoplasmic extracts were prepared from HEK-293 cells transfected with empty vector, FLAG-PRDI-BF1α, and FLAG-PRDI-BF1β. Western blot was performed with anti-FLAG Ab to show the localization of PRDI-BF1 and with HDAC-2 and 14-3-3 to demonstrate the correct separation of the nuclear and cytoplasmic extracts. *B*, EMSA was performed with nuclear extracts using the known PRDI-BF1 binding site in the CIITA promoter. Nuclear extracts were obtained from HeLa cells infected with GFP- (*lane 1*), PRDI-BF1α- (*lane 2*), or PRDI-BF1β (*lane 3*)-expressing adenovirus. This binding was specific for both proteins, as shown by oligonucleotide competition. Unlabeled PRDI-BF1 binding site oligonucleotide abolished the indicated complexes (*lanes 4* and *7*), while a mutant PRDI-BF1 binding site or an unrelated oligonucleotide had no effect (*lanes 5*, *6*, *8*, and *9*). α and β, protein complexes specific for PRDI-BF1α and PRDI-BF1β; n.s., proteins bound nonspecifically. *C*, Western blot of the nuclear extracts used in *B* demonstrates the relative amounts of PRDI-BF1α and PRDI-BF1β used in the EMSA. The protein was detected with an anti-FLAG Ab. *D*, PRDI-BF1α and PRDI-BF1β proteins were purified from adenovirus-infected HeLa cells by immunoprecipitation with M2 beads and subsequent washes. Control represents immunoprecipitates from HeLa cells infected with a GFP expressing adenovirus. The proteins were used in EMSAs as described in *B*. *E*, Western blot of 3-fold dilutions of the purified PRDI-BF1α and -β samples demonstrates equal loading of proteins in the EMSA. The proteins were detected with anti-FLAG Ab.

plasmic and nuclear extracts do not significantly contaminate each other.

We next examined whether PRDI-BF1β maintained similar DNA-binding properties as PRDI-BF1α. EMSAs with nuclear extracts obtained from cells transfected with either PRDI-BF1α or PRDI-BF1β revealed that both proteins can bind to the known PRDI-BF1 binding site in the CIITA promoter (Fig. 4B, *lanes 1–3*). This binding was specific for both proteins, as shown by oligonucleotide competition. Unlabeled PRDI-BF1 binding site oligonucleotide abolished the indicated complexes (*lanes 4* and *7*), while a mutant PRDI-BF1 binding site or an unrelated oligonucleotide had no effect (*lanes 5*, *6*, *8*, and *9*). Although this type of experiment does not reveal binding affinities, Western blot analysis of the nuclear extracts indicates that the relative expression level of the two isoforms is similar to the relative intensities in the EMSA analysis (Fig. 4C). To further confirm PRDI-BF1β DNA binding activity FLAG-tagged PRDI-BF1α and -β proteins were affinity purified and used in an EMSA analysis (Fig. 4D). The purified proteins both specifically bound the known PRDI-BF1 binding site. PRDI-BF1β bound as a single complex, while PRDI-BF1α bound as an expected slower migrating complex similar to that seen in Fig. 4B and a faster migrating complex that may be a proteolytic breakdown product during the purification. These findings suggest that PRDI-BF1β has the potential to bind target DNA sequences in the cell and modulate gene expression.

### PRDI-BF1β protein has diminished repressive activity compared with PRDI-BF1α

The transcriptional repressor function of PRDI-BF1β was investigated in three different, well-established in vitro systems. First, we created fusion proteins between the DNA binding domain of Gal4 and PRDI-BF1α, PRDI-BF1β, or PRDI-BF1α lacking the PR domain (Fig. 5A). We tested the repressor capacity of these constructs in HeLa cells on a luciferase reporter gene driven by the herpes simplex thymidine kinase minimal promoter and five consecutive Gal4 consensus binding sites immediately upstream of the promoter. Consistent with previous studies (22, 23), PRDI-BF1α potently represses transcription from the heterologous promoter in a dose-dependent manner, reaching as much as 40-fold repression (Fig. 5B). In sharp contrast, PRDI-BF1β shows a markedly reduced capacity at any given dose. At the maximum dose, PRDI-BF1β has only 15% of the repression activity compared with PRDI-BF1α. To test the importance of the PR domain in the repressive activity, an internal deletion of only the PR domain was used. Removal of the entire PR domain partially attenuated the repression activity to ∼50% of PRDI-BF1α. Western blotting demonstrated that the differences between the repressive activities of the different PRDI-BF1 proteins is not due to the differences in protein expression levels (Fig. 5B, inset). This indicates that the amino-terminal acidic domain as well as the PR domain play critical roles in transcriptional repression.

PRDI-BF1β activity was next examined on natural PRDI-BF1 target promoters. CIITA promoter III is well established as a highly repressed target of PRDI-BF1 (7, 8). CIITA promoter III luciferase reporter constructs were cotransfected with expression constructs for PRDI-BF1α or PRDI-BF1β into the B cell line CA46. Consistent with previous reports, PRDI-BF1α strongly repressed activity 11-fold relative to the empty vector control (Table I). PRDI-BF1β also repressed CIITA in this assay; however, its activity was significantly attenuated to 58% of that of PRDI-BF1α. The c-*myc* promoter is also a well-described target for PRDI-BF1 repression (4, 9). Cotransfection of a c-*myc* promoter reporter construct with the PRDI-BF1 expression vectors also demonstrated
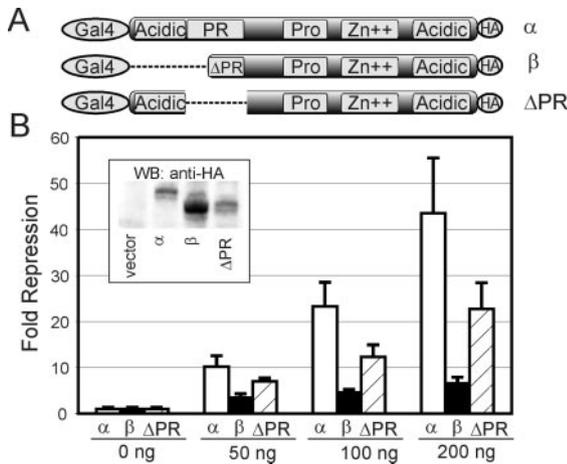
**FIGURE 5.** PRDI-BF1β has impaired repressive activity in a Gal4-luciferase assay compared with PRDI-BF1α. *A*, Schematic representation of Gal4-PRDI-BF1α, Gal4-PRDI-BF1β, and Gal4-PRDI-BF1α engineered to lack the PR domain. *B*, Fold repression of transcription by PRDI-BF1α, PRDI-BF1β, and ΔPR-PRDI-BF1α. The PRDI-BF1 expression vectors were cotransfected with a luciferase reporter vector (200 ng) containing five Gal4 binding sites upstream of the thymidine kinase minimal promoter. The amount of expression vector used is indicated below the graph. Fold repression was calculated relative to the activity measured in the control sample that was cotransfected with 200 ng of empty Gal4 expression vector (average of 7,476,391 light units). The data represent the average of three independent experiments, each performed in duplicate or triplicate, with the SDs shown and $p < 0.01$ in all cases. The inset shows a Western blot of the expressed proteins to demonstrate that the PRDI-BF1β protein was present at high levels.

that while PRDI-BF1α can repress transcription, PRDI-BF1β has ~50% attenuated activity (Table I).

### PRDI-BF1α and PRDI-BF1β associate with HDACs

A key component of PRDI-BF1 repressor activity is the recruitment of HDACs. It has been previously demonstrated that HDAC-2 can directly bind PRDI-BF1α (23). To determine whether the attenuation of PRDI-BF1β-mediated repression is a function of its ability to recruit HDACs, the PRDI-BF1 isoforms were immunoprecipitated and examined for associated HDAC-2. Recombinant adenoviruses expressing FLAG-tagged PRDI-BF1α, PRDI-BF1β, or only green fluorescent protein (GFP) were used to express the proteins in HeLa cells before immunoprecipitation with an anti-FLAG Ab. HDAC-2 clearly coimmunoprecipitated

**Table I.** *PRDI-BF1β has diminished repressive function[a]*

| | Luciferase Activity | | Fold Repression | |
|---|---|---|---|---|
| | CIITA | c-*myc* | CIITA | c-*myc* |
| pCDNA | 8.8 ± 0.8 | 5.7 ± 0.5 | 1.0 | 1.0 |
| PRDI-BF1α | 0.8 ± 0.1 | 3.2 ± 0.2 | 11.0 | 1.8 |
| PRDI-BF1β | 1.5 ± 0.3 | 4.3 ± 0.5 | 5.9 | 1.3 |

[a] The CA46 B cell line was cotransfected with 15 μg of either the 545-CIITA promoter-3 luciferase reporter or the 1100 bp human c-*myc* promoter luciferase reporter and 5 μg of the indicated expression vector. Each transfection is normalized to the expression of an internal control vector (RL-TK; Promega). Fold repression is calculated based on the activity of the promoters when cotransfected with the empty pCDNA expression vector. The average luciferase activity for CIITA and c-*myc* was 54,971 and 719,723 respectively when cotransfected with pCDNA. Data is an average of three independent experiments each done in duplicate or triplicate. All of the differences between the group of samples were found significant by the *t* test, with the following *p* values for both CIITA and c-*myc* promoters: PRDI-BF1α to pCDNA: <0.001; PRDI-BF1β to pCDNA: <0.005; PRDI-BF1β to PRDI-BF1α: 0.01.
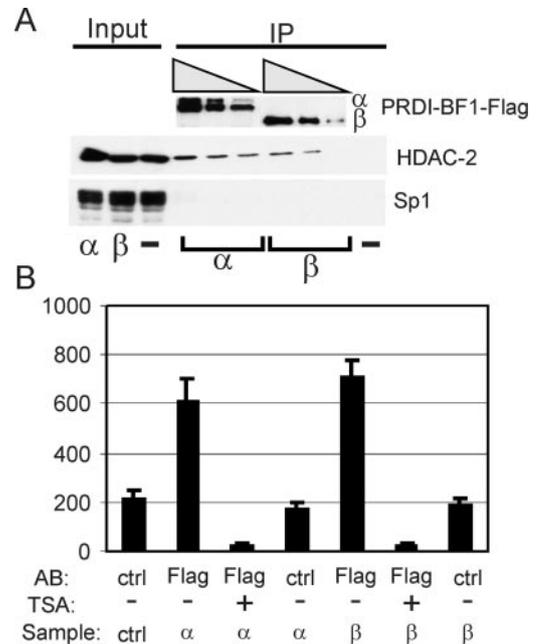


**FIGURE 6.** Both PRDI-BF1α and -β associate with HDAC-2 and co-purify with HDAC activity. *A*, FLAG-PRDI-BF1α and FLAG-PRDI-BF1β were expressed in HeLa cells and immunoprecipitated, and associated proteins were analyzed by Western blot. The precipitated proteins were loaded as a 3-fold dilution series to allow semiquantitative comparison of the amounts of different immunoprecipitated proteins (IP). The most diluted sample represents immunoprecipitated proteins from $0.2 \times 10^6$ cells. Lanes marked input are used as a positive control and represent $0.4 \times 10^6$ cells present in the original lysate. The same membrane was serially probed with Abs to HDAC-2, Sp1, and FLAG to show the amount of PRDI-BF1α or -β expressed. *B*, HDAC assays were performed using immunoprecipitates as described in *A*. Lysates of FLAG-PRDI-BF1α- and FLAG-PRDI-BF1β-expressing HeLa cells were immunoprecipitated with anti-Flag M2 beads or with nonimmune Abs coupled to agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Tritium-labeled core histones were used as substrates, and the specific (anti-Flag) immunoprecipitates were assayed in the absence or the presence of 600 nM Trichostatin-A. The values represent the average of three independent experiments.

with both PRDI-BF1α and PRDI-BF1β (Fig. 6*A*). Binding of endogenous HDAC-2 to PRDI-BF1 was specific, since the control sample expressing only GFP did not immunoprecipitate HDAC2 protein, and the PRDI-BF1 did not coimmunoprecipitate an unrelated nuclear protein, Sp1. Furthermore, the affinity to coimmunoprecipitate HDAC-2 is similar between PRDI-BF1α and PRDI-BF1β.

Since there are >10 different histone deacetylases known, we considered the possibility that PRDI-BF1α and PRDI-BF1β could differentially recruit HDAC complexes, which might cause a difference in the deacetylase activity. We performed an in vitro HDAC assay to detect the enzymatic activity bound to the PRDI-BF1α and -β proteins isolated by coimmunoprecipitation. Tritium-labeled core histones were used as substrates. We observed that the functionally recruited HDAC activity of PRDI-BF1β was not significantly different from that of PRDI-BF1α (Fig. 6*B*). These findings suggest that the difference in repression by PRDI-BF1α and PRDI-BF1β is not due to altered HDAC interaction.

### PRDM1β is generated by alternative promoter usage

Combined, these studies demonstrate that a functional PRDI-BF1β protein and mRNA are present at elevated levels in myeloma cell lines. The mRNA is initiated from an independent transcription
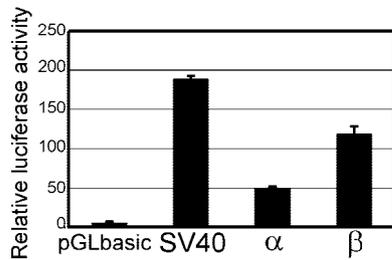
**FIGURE 7.** PRDM1α and PRDM1β mRNA are initiated from independent promoters. The NCI-H929 myeloma cell line was transfected with 15 μg of the indicated luciferase reporter plasmid. Each transfection was normalized to a cotransfected internal control *Renilla* luciferase plasmid, and the data represent the average of three independent experiments. The activity of the PRDM1α promoter (α) was tested using 2700 bp of promoter sequences upstream of PRDM1α transcription start site. The activity of the PRDM1β promoter (β) was tested using the 700 bp encompassing the region upstream of the PRDM1β transcription start site.

site, which is not part of the PRDM1α message. The presence of an alternative initiation site predicts the existence of an independent promoter from which PRDM1β mRNA is initiated. To demonstrate that functional promoters exist upstream of both initiation sites, the genomic sequences encompassing 700 bp upstream of the PRDM1β mRNA initiation site and 2700 bp upstream of the PRDM1α initiation site were cloned into a luciferase reporter vector. Upon transfection into the myeloma cell line NCI-H929, both promoters displayed significant activity compared with an SV40 control promoter (Fig. 7). Indeed, the PRDM1β promoter consistently displayed higher activity than the PRDM1α promoter in myeloma cell lines. Similar experiments in a B cell line that does not express endogenous PRDM1 showed very little PRDM1α or -β promoter activity (data not shown). These findings are consistent with transcription of the PRDM1α and PRDM1β genes being driven by two independent promoters. Remarkably, the PRDM2-encoded RIZ1 and RIZ2 mRNAs have similar independent promoters (38), and the PRDM3-encoded EVI1 mRNA is also expressed from an internal promoter (39).

## Discussion

The PRDM family of proteins has diverse biological functions; however, all are transcriptional repressors defined by a zinc finger DNA binding motif and a SET-like PR domain. A critical discovery was the observation that both the protein products of the PRDM2 (RIZ) and PRDM3 (MDS1-EVI1) genes are expressed as two distinct protein isoforms (38, 40). The isoforms differ in the presence or absence of the PR domain and amino terminus. Importantly, high relative expression of the isoform missing the PR domain is linked to oncogenesis (41–45).

In this paper we show that the PRDM1 gene encodes both a full-length protein of 798 aa and an alternative smaller product of 697 aa, designated PRDI-BF1α and PRDI-BF1β, respectively. Both products are recognized by an affinity-purified rabbit polyclonal Ab that recognizes a carboxyl-terminal peptide of PRDI-BF1. An Ab that recognizes an amino-terminal peptide sequence detects only PRDI-BF1α, demonstrating that the two products differ in their amino termini. Detailed analysis of the PRDM1 mRNAs revealed the presence of a novel mRNA lacking the first three exons of PRDM1. This mRNA encodes the PRDI-BF1β protein and begins with a novel 155-bp exon located upstream of exon 4. This novel exon contains a translation initiation codon and encodes three additional amino acids fused in-frame with the remainder of the PRDI-BF1α protein. Thus the PRDI-BF1β protein lacks the N-terminal acidic domain and disrupts the PR domain.

RIZ (PRDM2) is very similar in structure to PRDM1 and is found as a full-length protein and a short protein, RIZ1 and RIZ2, respectively (38). RIZ2 differs from RIZ1 in the absence of the amino terminus, including the entire PR domain. The RIZ2 mRNA is generated by activation of an internal promoter (38). The findings presented in this report indicate that PRDM1β is also expressed through use of an internal promoter. Initial analysis of the DNA region upstream of the PRDM1β transcription start site demonstrates that it is sufficient to promote strong transcriptional activation in myeloma cell lines. Although detailed analysis of RIZ1 and RIZ2 transcriptional control has not been reported, the significance of altering the relative expression levels of RIZ1 and RIZ2 is evident. The RIZ gene is located at the site of frequent deletions and frame-shift mutations in multiple tumor types, including breast, liver, and colon cancers as well as lymphomas and leukemia (17, 19, 46, 47). Predominately, the RIZ1 isoform is lost or significantly decreased in tumors, while the RIZ2 isoform is not affected (18, 19, 48). This suggests that the balance between RIZ1 and RIZ2 is an important factor. Overexpression of RIZ1 has also been shown to induce $G_2/M$ arrest and/or apoptosis in tumor cell lines (18, 19, 48). The recent development of mice with normal expression of RIZ2, but a selective disruption of the RIZ1 isoform, confirms a role in tumor development (20). The RIZ1$^{-/-}$ mice displayed significantly increased tumor formation of multiple types and accelerated tumor formation in a p53 heterozygous background. The most striking increase was in the development of diffuse large B cell lymphomas.

The PRDM3 (MDS1-EVI1) gene is also expressed in two forms (40). The full-length protein product, MDS1-EVI1, has an additional 188 aa at the amino terminus of EVI1. This extended region of MDS1/EVI1 encodes the PR domain; thus, EVI1 lacks the PR domain. EVI1 is a transforming gene originally identified as a common integration site of murine leukemia retrovirus (49). It is mapped to human chromosome 3q26, where several reoccurring chromosomal rearrangements are observed in myeloid leukemia and myelodysplastic diseases (39, 41, 43, 44). These rearrangements inappropriately activate EVI1 expression, leading to elevated levels of EVI1 compared with the PR domain containing MDS1/EVI1 protein. Importantly, a mouse model expressing the AML1/EVI1 translocation fusion gene in bone marrow cells develops acute myelogenous leukemia (45).

In this report we demonstrate that the shorter PRDI-BF1β protein is present in both primary cells and cell lines that correspond to the tissue specificity of PRDI-BF1. Importantly, elevated expression of PRDI-BF1β is detected in the transformed cell lines, while normal primary cells contain only a low level of PRDI-BF1β relative to PRDI-BF1α. PRDI-BF1 is not expressed ubiquitously, but only at the later stages of B lymphoid and myeloid differentiation (1, 2, 50). PRDI-BF1β appears to be the most abundant in myeloma and body cavity-based lymphoma cell lines. These two types of tumor cell lines are different in their origin and exact differentiation stage, but share the BCL-6$^-$/syndecan$^+$ phenotype (35, 51). It will now be important to determine whether increased expression of PRDI-BF1β is linked to the development of myeloma or if it is characteristic of mature plasma cells. In addition, since deletion of 6q21 is very frequent in non-Hodgkin lymphomas, melanomas, and breast cancer (16), it is possible that aberrant expression of the PRDI-BF1 isoforms also occur in these tumors.

PRDI-BF1 has been clearly defined as a potent repressor of multiple genes, including c-*myc* and CIITA (4, 7, 8). Our results show that PRDI-BF1β has a severely impaired ability to repress transcription. When multiple copies of PRDI-BF1 are tethered to an artificial promoter, PRDI-BF1β has only 20% the repressive activity of PRDI-BF1α. More importantly, in the context of the

natural promoters of CIITA and c-*myc*, PRDI-BF1β has only one-half the repressive activity. The RIZ1/RIZ2 proteins have a similar repression profile on the SV40 promoter, where RIZ2 is much less effective (52). However, this appears to be promoter dependent, because RIZ1 and RIZ2 repress the HSV-thymidine kinase promoter equally well. The activities of MDS1-EVI1 and EVI1 are not as well defined, but one study using an artificial promoter indicated that both repress transcription to a similar level (53). These observations are consistent with the multiple mechanisms proposed to be important for PRDI-BF1 function (8, 23) and further emphasize that the promoter context in which PRDI-BF1 is located will alter its function. Potentially, only a subset of PRDI-BF1-responsive genes will be altered by overexpression of the PRDI-BF1β isoform. Interestingly, deletion of only the PR domain of PRDI-BF1α results in a partially diminished repressive activity that is still significantly stronger than that of PRDI-BF1β. This suggests an important functional role for the amino terminus of the protein in addition to the PR domain and is consistent with previous studies of the murine PRDM1 protein, Blimp-1 (23).

Recruitment of HDACs is a common characteristic of transcription repressors (54). Immunoprecipitation of PRDI-BF1 shows that it interacts with endogenous HDAC-2. A recent report by Calame and colleagues (6, 23) directly demonstrates by chromatin immunoprecipitation that overexpression of Blimp-1 leads to loss of acetylated histones at the target promoters. Our studies of the PRDI-BF1β isoform indicate that it maintains the ability to interact with HDAC2 and that the HDAC activities associated with PRDI-BF1α and PRDI-BF1β are similar. This suggests that its loss of function is not due to a failure to recruit deacetylases. Furthermore, both PRDI-BF1α and -β similarly localize to the nucleus and are capable of self-interaction (I. Györy and K. L. Wright, unpublished observation). Thus, the altered function of PRDI-BF1β is not due to loss of any of the known activities of PRDI-BF1.

The function of the PR domain has not yet been defined. It has high homology to the SET domain, which is found in >100 mammalian proteins (55). Originally proposed to be a protein-protein interaction domain, it has recently been demonstrated to be part of the catalytic region of histone methyltransferases involved in chromatin remodeling (13, 56). Multiple SET-containing proteins have also been implicated in cancer (57). The regions flanking the SET domain have been described to play a role in determining substrate specificity and in modulating the activity (58). Transcriptional repression through methylation of histone H3 would be consistent with the role of PRDM1. However, the regions flanking the PR domain do not conform to the known histone methyltransferases. This may indicate that either PRDI-BF1 has a unique substrate target, or that PRDI-BF1 is not itself an intrinsic protein methyltransferase. Defining the potential methyltransferase activity of PRDI-BF1 will be an important area of investigation.

In summary, our results define a new truncated form of PRDI-BF1 expressed in myeloma cell lines that has an impaired ability to repress gene transcription. This establishes a defining feature of all PRDM family members studied, which is the expression of two isoforms, either with or without the PR domain. Furthermore, an imbalance in the amounts of the two protein isoforms appears to be an important factor in the development of cancer (17–19). A clear understanding of the functional differences between the isoforms will probably be critical in deciphering their role in oncogenesis.

## Acknowledgments

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