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BLyF, a Novel Cell-Type- and Stage-Specific Regulator of the B-Lymphocyte Gene *mb-1*

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The *mb-1* gene encodes an integral membrane protein that appears to be required for the surface expression and signalling function(s) of the immunoglobulin receptor on B lymphocytes. The gene is expressed in a lineage-restricted manner. It is activated early in B-cell ontogeny, continues to be expressed in mature B cells, but is turned off in terminally differentiated plasma cells. We have identified the *mb-1* promoter and functionally tested its activity by transient transfections. A 737-bp promoter fragment preferentially stimulates accurately initiated transcription in *mb-1*-expressing B cells. Deletion analysis of the promoter suggests the presence of two functional domains, proximal and distal. Both domains independently activate transcription from a heterologous promoter. The distal domain functions in a cell-type- and stage-specific manner, activating transcription in B cells but not in T cells or plasma cells. A 25-bp element within this domain is necessary and sufficient for activity. This element is recognized by a novel cell-type- and stage-specific transcription factor termed BLyF. The binding of BLyF completely correlates with the ability of the regulatory element to stimulate transcription. Thus, BLyF appears to positively regulate transcription of the *mb-1* gene. Our results also suggest that the inactivity of the *mb-1* locus in plasma cells is not simply due to the loss of BLyF activity.

The ordered rearrangement and expression of immunoglobulin (Ig) genes are central events in the B-cell developmental pathway. Recently, four new B-lineage-restricted genes that encode Ig-associated proteins have been isolated. These include VpreB, λ5, *mb-1*, and B29 (8, 15, 22, 23). VpreB and λ5 encode surrogate light-chain proteins that associate with the μ protein on the surface of differentiating pre-B cells (3, 12, 28). The *mb-1* and B29 gene products are integral membrane proteins that are associated with the Ig receptor on B cells (2, 10, 17, 21). The MB-1 and B29 proteins form a disulfide-linked heterodimer which is required for surface expression of IgM, IgA, and IgE (2, 11, 29). The cytoplasmic tails of both proteins contain a conserved sequence motif found in the γ, δ, and ζ chains of the T-cell antigen receptor-CD3 complex (21). This complex functions as a signal transducer. Therefore, the MB-1 and B29 proteins are likely to be similarly involved in regulating the signalling function(s) of the Ig receptor. All four genes appear to be activated early in B-cell ontogeny in precursor cells undergoing heavy-chain gene rearrangement (8, 15, 22, 23). VpreB and λ5 expression is turned off in mature B cells, whereas *mb-1* expression is turned off in terminally differentiated plasma cells. Analysis of function and regulation of activity of these genes is likely to provide insight into mechanisms that control differentiation events in the B lineage.

In this report, we present a structural and functional analysis of the promoter of the murine *mb-1* gene. This region contains developmental regulatory information which preferentially promotes transcription in *mb-1*-expressing B cells. We identify a domain in the promoter which functions to activate transcription in a cell-type- and stage-specific manner. The activity of this domain appears to be regulated by a novel cell-type- and stage-specific activator termed BLyF.

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MATERIALS AND METHODS

Cell lines and culture. 38B9, 70Z/3, WEHI-231, and EL-4 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol. J558L cells were grown in Dulbecco modified Eagle medium with L-glutamine supplemented with 10% fetal calf serum. Resting B lymphocytes were isolated from a suspension of murine spleen cells by anti-Thy-1 depletion of T lymphocytes followed by enrichment on Percoll gradients (18).

Isolation of *mb-1* cDNA and genomic clones. Two oligonucleotide primers, DM-1 (5′-CTTCGAATTCATGCCAGG GGGTCTAGAAGCC-3′) and DM-4 (5′-CTTCGAATTCT GGCTTTTCCAGCTGGGCATG-3′), were synthesized on the basis of the murine *mb-1* cDNA sequence (22). By using these two primers, the *mb-1* coding region was amplified by polymerase chain reaction of a double-stranded cDNA pool synthesized from WEHI-231 poly(A)$^+$ RNA. The 660-bp amplified product was subcloned into pBS+ and sequenced (Sequenase 2.0) to confirm its identity. This fragment was used to screen a cDNA library prepared from WEHI-231 poly(A)$^+$ RNA. Several positive clones were isolated, and the largest (1.1 kb) was subcloned into pBS+ and sequenced.

mb-1 genomic clones were isolated by screening a murine C57BL/6J-pWE15 cosmid library (Stratagene), using the 1.1-kb *mb-1* cDNA as a probe. Four overlapping clones were isolated, and one clone that contained the entire *mb-1* gene, as determined by Southern analysis with the *mb-1* cDNA probes, was used for subsequent analyses.

Plasmid constructs. Target-1, Target-3, OVEC-Ref, and pSP6βTS (19, 30) were kind gifts from W. Schaffner. ΔTATA was generated by digestion of Target-1 with *Sac*I

(−43) and *Pst*I (−10); the 3′ overhangs were blunt ended with T4 DNA polymerase and religated. P737 was made by cloning a *Hin*dIII-*Xba*I *mb-1* genomic fragment (−695 to +42) into pBluescript SK and then cloning a *Sal*I-*Xba*I fragment into the *Sal*I (−37)-*Pst*I (−10) sites of Target-1. P327 was generated by cleaving at the unique internal *Pst*I site (position −285) in P737 and blunt ligating to the *Sac*I site. P157 was constructed by cloning an *Nde*I-*Xba*I *mb-1* genomic fragment (−115 to +42) into the *Eco*RI-*Xba*I sites of pBluescript SK and then cloning a *Sal*I-*Xba*I fragment into the *Sal*I (−37)-*Pst*I (−10) sites of Target-1. P64 was constructed by cloning a *Kpn*I-*Xba*I genomic fragment (−22 to +42) into the *Eco*RI-*Xba*I sites of pBluescript SK and then cloning a *Sal*I-*Xba*I fragment into the *Sal*I (−37)-*Pst*I (−10) sites of Target-1. The riboprobe template for mapping the *mb-1* transcriptional start sites was a *Bam*HI-*Xba*I *mb-1* genomic fragment (−253 to +42) subcloned into pBluescript (pBSmb295). The riboprobe template for mapping accurately initiated *mb-1* transcripts from transfected promoter constructs was a *Bam*HI fragment from P737 (−253 *mb-1* to +340 globin) subcloned into pBluescript SK (pSKmb-globin).

Nine oligonucleotide primers which contain 5′ *Sac*I sites were used to amplify by polymerase chain reaction the distal and proximal domains of the *mb-1* promoter. The nine primers are as follows: mbp-1, −253 to −236 (GGCGAG CTCGGATCCTTTCTCAGGGAT); mbp-2, −168 to −185 (GGCGAGCTCCCTTGAGTCTCTCTCTAG); mbp-2b, −157 to −160, (GGCGAGCTCTGGCCACAATTCCCTTGA); mbp-3, −178 to −160 (GGCGAGCTCAGACTCAAGG GATTGTGG); mbp-3b, −178 to 161 (GGCGAGCTCAGAC TCAAGGGAATTGTG); mbp-3d, −219 to −201 (GGC GAGCTCCCTTGAACCACCCTCTCC); mbp-4, −107 to −124 (GGCGAGCTCTTGCCATATGTGTGGGCT); mbp-5, −116 to −99 (GGCGAGCTCCATATGGCAAATAAA GGG); and mbp-6, −27 to −46 (GGCGAGCTCCCACTC CAAACTCCGCCT). The proximal domain, positions −116 to −27, was amplified by using primers mbp-5 and mbp-6. The distal domain (D), positions −253 to −107, was amplified by using primers mbp-1 and mbp-4. The distal domain was divided into five overlapping regions, D1 through D5. Each region was amplified by using the indicated primers: D1, positions −253 to −157, mbp-1 and mbp-2b; D2, positions −218 to −157, mbp-2b and mbp-3d; D3, positions −178 to −107, mbp-3b and mbp-4; D4, positions −178 to −107, mbp-3 and mbp-4; and D5, positions −253 to −169, mbp-1 and mbp-2. Each amplified region was cloned into the *Sac*I site (−43) of Target-1 and sequenced.

The minimal BLyF binding site (MBS) and the mutant binding site (mbs) span positions −283 to −259 and include *Sal*I cloning sites.

MBS: TCGACAGAGAGAGACTCAAGGGAATTGTGG
GTCTCTCTCTGAGTTCCCTTAACACGAGCT

mbs: TCGACAGAGAGAGACTCActtGAATTGTGG
GTCTCTCTCTGAGTgaaCTTAACACCAGCT

Three nucleotide changes are present in mbs at positions −170 to −168, converting AGG to CTT. Both MBS and mbs were cloned into the *Sal*I site (−37) of Target-1 and sequenced.

Primer extension and RNase protection assays. Total RNA was isolated according to Davis et al. (4). For riboprobe synthesis, the pBSmb295 construct was linearized with *Bam*HI, and a 335-nucleotide probe was synthesized by using the T3 promoter. The RNase protection assay was performed as previously described (5). Primer extension reactions were performed as previously described (24), using two oligonucleotide primers. PE1 (AGACCCCCTGGC ATCGTCTCCCAGTGAGTC, +39 to +10) was used to map the upstream start sites, whereas PE2 (GTTCAGCGT CAGGGATGGATGGTGGACC, +148 to +125) was used to map the downstream start sites. Extension products were resolved on a 6% polyacrylamide–7 M urea denaturing gel along with a sequencing ladder generated with the same primers.

DNA transfections and RNA analysis. Test constructs were cotransfected with the reference plasmid (OVEC-Ref) into WEHI-231, J558L, and EL-4 cells by the DEAE-dextran method as previously described (6) except that the DEAE-dextran concentration was 0.25 mg/ml. In these transfections, 15 μg of the test construct and 5 μg of the reference plasmid were used. Cytoplasmic RNA was isolated 40 to 42 h after transfection and analyzed by the RNase protection assay. pSKmb-globin was linearized with *Eco*RI, and a 720-nucleotide riboprobe was synthesized by using the T3 polymerase. The riboprobe detected accurately initiated transcripts from the *mb-1* promoter constructs. These protection products were quantitated by scanning densitometry (LKB Ultrascan XL enhanced laser densitometer), and their values were normalized to those of the reference protection products. Accurately initiated globin transcripts were detected by using a globin riboprobe as previously described (19).

Gel electrophoresis DNA binding assays. Nuclear extracts were prepared and assayed as described by Singh et al. (26). Binding reaction products were resolved in 5% polyacrylamide gels (30:1), using either Tris-glycine-EDTA or 0.25× Tris-borate-EDTA as the running buffer. The BSAP and PU.1 oligomers were derived from the H2B-2.1 (1a) and MHCII Aβ (14) genes, respectively.

Methylation interference assay. The methylation interference assay was performed essentially as previously described (25). After electrophoresis, autoradiography was performed on the wet gel. Free DNA and DNA in the BLyF complex were electroeluted. After cleavage with piperidine, the DNA fractions were analyzed by urea–10% polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Identification of the *mb-1* promoter. The *mb-1* mRNA is approximately 1.0 kb in size. Previously, both murine cDNA and genomic clones have been characterized (13, 22). The longest cDNA clones contain nine nucleotides of 5′ noncoding sequence which has been used to tentatively define the transcriptional start site. To unambiguously define the site of transcription initiation, we mapped the 5′ end of the *mb-1* mRNA by RNase protection and primer extension assays. Figure 1A shows the results of the RNase protection assay using an antisense genomic probe that spans the 5′ ends of the cDNA clones (see Materials and Methods). Multiple protection products were detected with use of RNA from various *mb-1*-expressing pre-B- and B-cell lines (70Z/3, 38B9, and WEHI-231). The lower bracket in Fig. 1A denotes major protection products observed in both pre-B and B-cell lines. The upper bracket denotes a major product preferentially observed in the B-cell line WEHI-231. None of these products were detected in control reactions using either tRNA or RNA from the B-cell line J558L, a plasma cell in which the *mb-1* gene is not expressed. Primer extension assays using WEHI-231 RNA generated products whose 5′

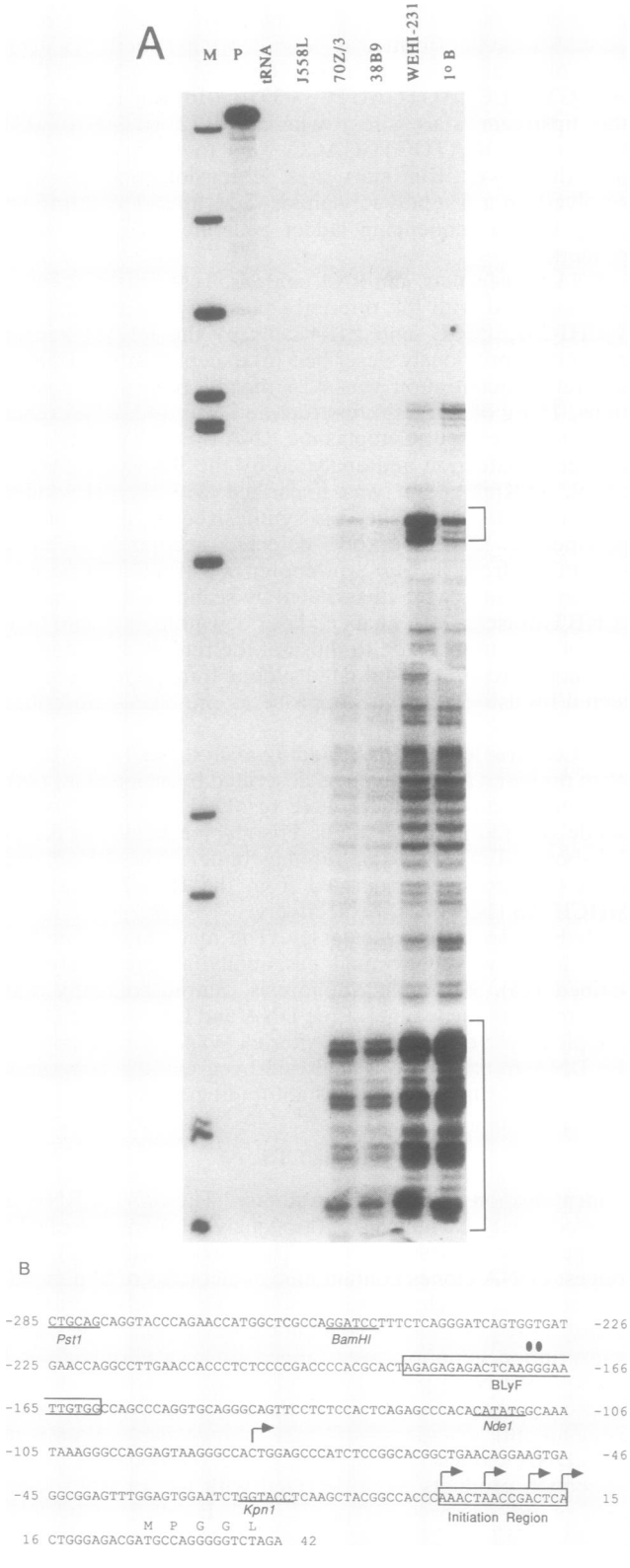

FIG. 1. Determination of the *mb-1* transcriptional start sites. (A) RNase protection assay. An antisense *mb-1* riboprobe (−253 to +42) was hybridized with 20 μg of tRNA or 20 μg of total RNA from the indicated cells. After RNase digestion, the protected fragments were resolved on a 6% denaturing gel along with DNA molecular weight markers. The markers (lane M) are 404, 238, 242, 190, 157, 147, 110, 67, 57, 34, and 26 bp. Lane P contains the riboprobe. The predominant protection products are indicated with brackets. (B) Sequence of the *mb-1* promoter. The arrows denote major transcriptional start sites determined by RNase protection and primer extension assays (see Materials and Methods). The 5′-most initiation site in the major cluster is designated +1. Key restriction sites are indicated on the sequence. The minimal BLyF binding site is boxed. The guanine residues contacted by BLyF are indicated by filled ovals. MPGGL represent the first five amino acids of the MB-1 protein.

ends corresponded with those indicated by brackets in the RNase protection assay (Fig. 1A and data not shown; see Materials and Methods). These sites of transcription initiation are indicated by arrows in Fig. 1B. The same pattern of transcription initiation was observed with RNA isolated from primary splenic B cells (Fig. 1A). Inspection of the sequence immediately upstream of the clustered start sites (Fig. 1B) failed to reveal TATA motifs.

Functional analysis of the *mb-1* promoter. The *mb-1* gene is expressed in a lineage-restricted and stage-specific manner in pre-B and B cells but not in terminally differentiated plasma cells. Transient transfection assays were used to determine whether the promoter contains regulatory information that controls lineage- and stage-specific activity of the *mb-1* locus. Derivatives of the OVEC vector (19, 30) containing the simian virus 40 enhancer downstream of the β-globin gene were used to functionally test the *mb-1* promoter. The control construct, ΔTATA, has a β-globin test gene deleted of its TATA box and upstream promoter sequence to position −10 (see Materials and Methods). The P737 construct contains a 737-bp *mb-1* promoter fragment (spanning positions −695 to +42) linked to the β-globin reporter gene at position −10. The three constructs P327, P157, and P64 represent 5′ deletions to the *Pst*I (−280), *Nde*I (−114), and *Kpn*I (−18) sites, respectively (Fig. 1B; see Materials and Methods). Each test construct was cotransfected with the β-globin reference construct (OVEC-Ref) to normalize for transfection efficiency. OVEC-Ref, like the test constructs, contains the simian virus 40 enhancer. RNA from transiently transfected cells was analyzed for accurately initiated transcripts from the *mb-1* promoter region by RNase protection using an antisense *mb-1*/globin probe (see Materials and Methods). Such transcripts are expected to generate two major sets of protection products centered about 405 and 490 nucleotides. These products were observed in transfections of WEHI-231 cells with the P737 construct but not with the ΔTATA construct (Fig. 2). The slowest-migrating band for each of the two constructs represents read-through transcription. The 325-nucleotide protected fragment represents transcription from the reference plasmid. Deletion to position −280 (P327) did not affect the level of accurately initiated transcription. Further deletion to position −114 (P157) reduced accurately initiated transcription approximately 14-fold, as determined by scanning densitometry (see Materials and Methods). Deletion to position −18 (P64) completely abolished accurately initiated transcription. As was the case for P737, the slowest-migrating band seen with each deletion construct represents read-through transcription. Thus, the *mb-1* promoter fragment (−695 to +42) shows the same pattern of transcription initiation as does the endogenous gene. Furthermore, the deletion analysis indicates the presence of at least two functional domains between positions −280 and −18.

To test for the cell-type-specific activity of the *mb-1*

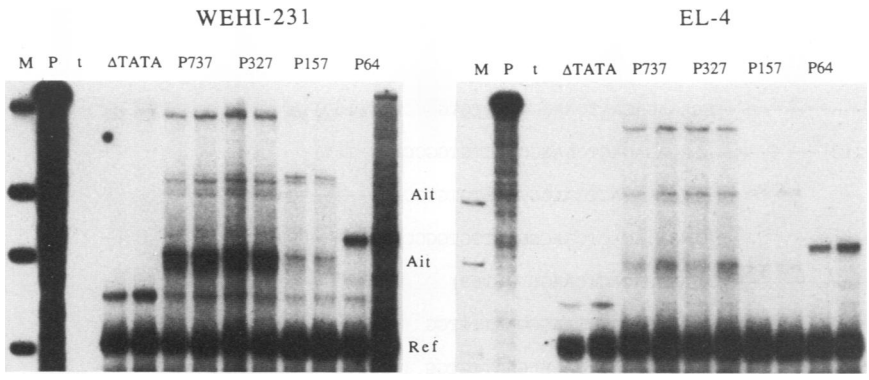

FIG. 2. Cell type and deletion analysis of the *mb-1* promoter. Each of the indicated constructs was transiently cotransfected with OVEC-Ref into WEHI-231 and EL-4 cells. Cytoplasmic RNA (20 μg) was assayed for accurately initiated transcripts from the *mb-1* promoter, using an antisense *mb-1*/globin riboprobe (−253 *mb-1* to +340 globin). Protected bands representing accurately initiated transcripts from *mb-1* test constructs and OVEC-Ref transcripts are indicated by Ait and Ref, respectively. The molecular weight markers (lanes M) are 713, 489, 404, and 328 bp. Lanes P contains the riboprobe, and lanes t represent control hybridizations with tRNA.

promoter, the same panel of constructs was transfected into the T-cell line EL-4. Accurately initiated transcripts were detected in transfection assays with P737 (Fig. 2), albeit at a level 10-fold lower than that observed in WEHI-231 cells. Deletion to position −114 reduced transcription approximately fourfold, while deletion to position −18 failed to show any accurately initiated transcription. In contrast, none of the four test constructs promoted accurately initiated transcription in the plasma cell line J558L (data not shown). Thus, the *mb-1* promoter is preferentially active in B cells.

Two independently functioning domains of the *mb-1* promoter. Deletion analysis of the *mb-1* promoter in WEHI-231 cells suggested the presence of two functional domains between positions −280 and −18. Deletion to the *Bam*HI site (−253) had no effect on promoter activity in WEHI-231 cells (data not shown). The proximal region (−116 to −27) and the distal region (−253 to −107) were cloned upstream of the minimal β-globin promoter in Target-1 (see Materials and Methods) to test whether each could independently stimulate transcription in a cell-type-specific manner. These constructs as well as Target-1 and Target-3 were cotransfected with OVEC-Ref into WEHI-231, J558L, and EL-4 cells. Target-3 contains the Ig gene octamer element, ATT TGCAT, upstream of the β-globin TATA box. This construct is activated by the lymphoid-specific transcription factor Oct-2 (19). RNA from transiently transfected cells was analyzed by RNase protection using an antisense β-globin probe. Accurately initiated globin transcripts from the Target constructs should yield a 179-nucleotide protected fragment. Reference transcripts protect a 160-nucleotide fragment. In WEHI-231 cells, both the distal and proximal regions strongly stimulate transcription (Fig. 3). However, in EL-4 cells, the proximal but not the distal region activates transcription. In contrast, neither of the two regions significantly stimulates transcription in J558L cells. Thus, the distal domain activates transcription in a cell-type- and stage-specific manner, thereby mimicking the activity of the *mb-1* locus.

Deletion analysis of the distal domain. To identify the functional element(s) within the distal domain, a series of deletion derivatives, D1 through D5, was constructed in Target-1 (see Materials and Methods and Fig. 4). This series of plasmids was cotransfected with OVEC-Ref into WEHI-231 cells. Figure 5 shows the results of the RNase protection assay. Three deletion derivatives, D1 (−253 to −157), D2 (−218 to −157), and D3 (−178 to −107) activated transcription to levels equivalent to that observed with the entire distal domain (D, −253 to −107). These derivatives overlap between positions −178 to −157. In contrast, two derivative constructs, D4 and D5, which lack an intact −178 to −157 region fail to stimulate transcription (Fig. 5). D4 differs from D3 by a single-nucleotide deletion at position −166. D5 differs from D1 by 12 nucleotides at the 3′ end (Fig. 4). The simplest interpretation of these results is that the distal domain consists of a single activating element between positions −178 and −157. Consistent with this interpretation, a 25-bp segment, −184 to −160 (MBS), activated transcription from the β-globin promoter, whereas a mutant

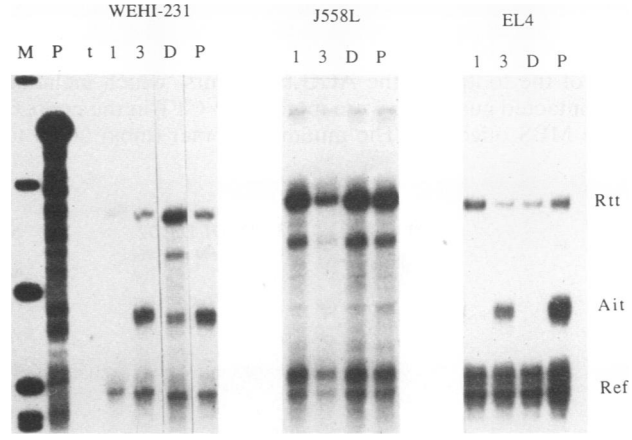

FIG. 3. Cell-type-specific functioning of the distal and proximal domains of the *mb-1* promoter. Each of the indicated constructs (1, Target-1; 3, Target-3; D, distal domain; P, proximal domain [the latter three are derivatives of Target-1; see Materials and Methods]) were transiently cotransfected with OVEC-Ref into WEHI-231, J558L, and EL-4 cells. Cytoplasmic RNA (20 μg) was assayed for accurately initiated transcripts from the β-globin promoter, using an antisense β-globin riboprobe. Protected bands from read-through transcripts, accurately initiated transcripts, and OVEC-Ref transcripts are indicated by Rtt, Ait, and Ref, respectively. The molecular weight markers (lane M) are 328, 242, 190, 157, and 147 bp. Lanes P and t are as indicated in the legend to Fig. 2.

		Transcription Activation	BLyF Binding
D	(-253)----------AGAGAGAGACTCAAGGGAATTGTGGCCA---------(-107)	+	+
D1	(-253)----------AGAGAGAGACTCAAGGGAATTGTGGCCA (-157)	+	+
D2	(-218)---------AGAGAGAGACTCAAGGGAATTGTGGCCA (-157)	+	+
D3	(-178) AGACTCAAGGGAATTGTGGCCA---------(-107)	+	+
D4	(-178) AGACTCAAGGG-ATTGTGGCCA---------(-107)	-	-
D5	(-253)----------AGAGAGAGACTCAAGG (-169)	-	-
MBS	(-184) AGAGAGAGACTCAAGGGAATTGTGG (-160)	+	+
mbs	(-184) AGAGAGAGACTCActtGAATTGTGG (-160)	-	-

FIG. 4. Summary of the ability of various deletion derivatives of the *mb-1* promoter distal domain to activate transcription from a heterologous promoter and to bind BLyF.

segment (mbs) containing three nucleotide substitutions failed to do so (Fig. 4 and 5).

BLyF, a cell-type- and stage-specific regulator of the distal domain. The gel electrophoresis DNA binding assay was used to identify nuclear factors capable of interacting with the distal domain. The probe was an end-labelled *Bam*HI-*Nde*I restriction fragment (−253 to −113). A single predominant complex was detected with this probe, using a nuclear extract from WEHI-231 cells (Fig. 6). The distal domain (D) as well as various deletion derivatives (D2 through D5) were used in competition binding reactions. Segments D, D2, and D3 strongly competed for binding, D5 weakly competed, and D4 failed to compete. Thus, this complex represents a sequence-specific interaction requiring nucleotides between positions −178 to −157 for its formation. This conclusion was corroborated by using the MBS oligomer (−184 to −160), which strongly inhibited binding (Fig. 6). Contact residues on the purine-rich coding strand were identified by dimethyl sulfate interference. A pair of guanine residues within the motif AAGGGAA interfered with binding (Fig. 1B and 6). Shorter exposures of the footprint did not reveal noticeable interference at the third guanine residue. On the basis of the footprint, the AGG base pairs, which included the contacted guanines, were mutated to CTT in the context of the MBS oligomer. The mutant oligomer (mbs) failed to compete for binding of the factor to the distal domain (Fig. 6). Figure 4 summarizes the properties of distal domain segments to activate transcription as well as to bind the B-cell factor. A complete correlation exists between DNA binding in vitro and transcription activation in vivo. Even though the D5 segment weakly competed for binding, it failed to show detectable binding when used as a labelled fragment. Thus, the B-cell factor likely regulates the function of the distal domain of the *mb-1* promoter. Binding of the B-cell factor to the distal domain was not competed for by an oligomer containing a BSAP binding site (1a) (Fig. 6). BSAP appears to represent a B-lineage specific transcription factor whose distribution parallels the activity of the *mb-1* locus (see Discussion). Binding of the B-cell factor was also not competed for by the purine-rich motif GAGGAA that PU.1, a B-cell- and macrophage-specific transcription factor, recognizes (14). Thus, on the basis of these criteria as well as the cell type distribution shown below, we propose the term BLyF (B lymphoid factor) for this novel transcription activator.

The cell type distribution of BLyF was determined by using the gel electrophoresis DNA binding assay. The distal domain was used as a probe, and the two oligomers (MBS and mbs) were used as competitor DNAs. This procedure permitted both relative mobility and DNA binding specificity

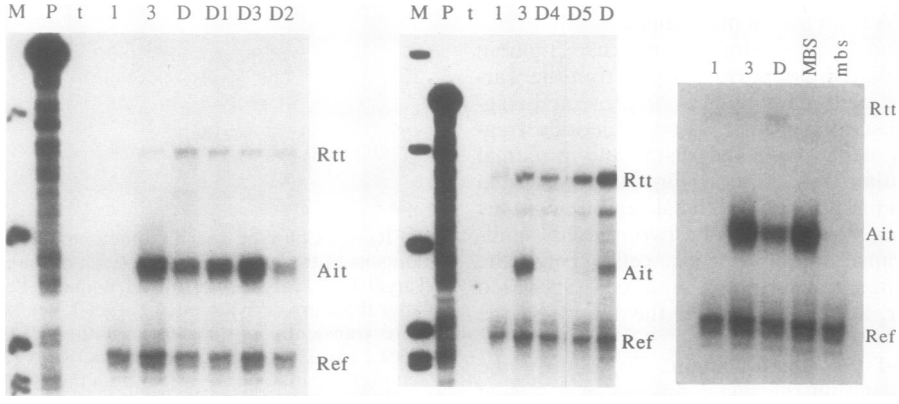

FIG. 5. Mutational analysis of the distal domain of the *mb-1* promoter. Each of the indicated constructs (see Fig. 4 and Materials and Methods) was transiently cotransfected with OVEC-Ref into WEHI-231 cells. See legend to Fig. 3 for additional details. Lanes M, P, t, 1, 3, and D of the middle panel represents the same data shown in the WEHI-231 panel of Fig. 3. They are reshown alongside D4 and D5 since all of these constructs were analyzed in the same experiment.

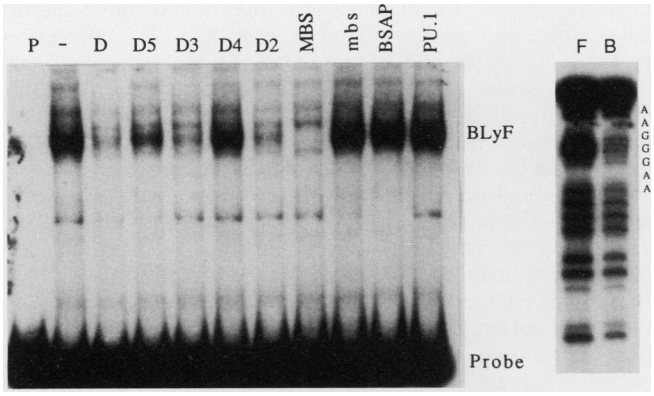

FIG. 6. Characterization of the BLyF binding site in the distal domain of the *mb-1* promoter. A labelled *Bam*HI-*Nde*I (−253 to −113) fragment (see Fig. 1B) was used in a gel electrophoresis DNA binding assay along with nuclear extract protein from WEHI-231 cells. In addition to poly(dI-dC) · poly(dI-dC), the binding reaction mixtures contained 50 ng of the indicated competitor DNAs (see Fig. 4 and Materials and Methods). Lane P, probe in the absence of nuclear extract protein; lane −, binding reaction with WEHI-231 nuclear extract in the absence of any specific competitor DNA. The positions of the BLyF complex and free probe are indicated. The right panel shows a methylation interference analysis of the coding strand in the BLyF complex. A labelled *Bam*HI-*Msc*I (−253 to −160) fragment was used for footprinting. F and B denote the free and bound probe fractions, respectively. In the indicated purine-rich motif, the lower pair of guanine residues, upon methylation, interfere with BLyF binding.

to be used as criteria for documenting the presence of BLyF in nuclear extracts of other cell types. The BLyF complex was detectable in 38B9 pre-B cells but not in J558L plasma cells or in EL-4 T cells (Fig. 7). The absence of BLyF in J558L and EL-4 cells appears not to be due to proteolysis, since intact Oct-1 and Oct-2 complexes were detected in these extracts (data not shown). The absence of BLyF in J558L and EL-4 cells correlated with the inability of the

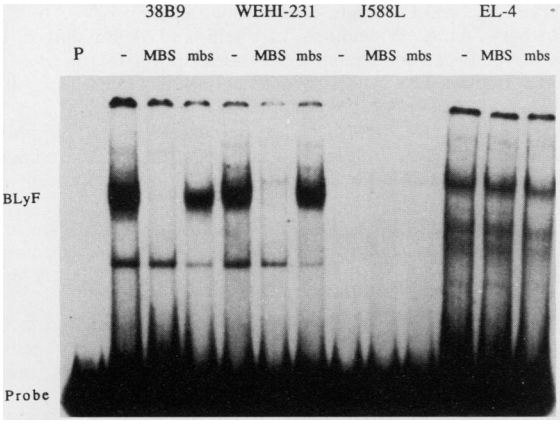

FIG. 7. Cell type distribution of BLyF. Nuclear extracts of the indicated cells were assayed for BLyF, using the *Bam*HI-*Nde*I probe and native gel electrophoresis (see legend to Fig. 6 for details). Binding reactions with each extract were carried out in the absence of specific competitor DNA (lane −) or in presence of the wild-type (MBS) or mutant (mbs) BLyF binding site oligomers. Lane P is as in Fig. 6. The positions of the BLyF complex and free probe are indicated.

distal segment to stimulate transcription in these cells. Thus, BLyF appears to be a cell-type- and stage-specific regulator of the distal domain. BLyF is also present in 70Z/3 pre-B cells as well as mature B lymphocytes isolated from murine spleen (data not shown). The BLyF complex is not detected in MOPC315 plasma cells or in 3T3 fibroblasts (data not shown). Thus, BLyF appears to represent a cell-type- and stage-specific transcriptional activator whose distribution completely correlates with the activity of the *mb-1* locus.

DISCUSSION

The *mb-1* gene encodes an integral membrane protein that appears to be required for the surface expression and signalling function(s) of the Ig receptor on B lymphocytes (21). It may also be involved in signalling pathways regulating pre-B-cell differentiation (20). The *mb-1* gene is expressed early in B-cell development in precursor cell lines undergoing heavy-chain gene rearrangement (22). However, unlike expression of Ig genes, expression of *mb-1* is turned off in terminally differentiated plasma cell lines. Thus, this gene provides an attractive model for analyzing the control of gene activity in both early- and late-stage B-lineage cells. By using murine cDNA and genomic clones as well as mapping the 5′ ends of the mRNA, we have identified the *mb-1* promoter. Functional analyses of a 737-bp promoter fragment have revealed that it encodes lineage- as well as stage-restricting regulatory information. A novel cell-type- and stage-specific transcription activator, BLyF, appears to play an important role in developmentally regulating the activity of the *mb-1* promoter.

Transcription of the *mb-1* gene is initiated from multiple start sites. The predominant sites are clustered in a 15-bp region 10 nucleotides upstream of the AUG. Transcription is also initiated upstream of this region with a preferred site at position −82 (Fig. 1B). The *mb-1* promoter does not contain a consensus TATA box. In this regard, the *mb-1* promoter is similar to promoters of the other B-lineage genes that encode Ig-associated proteins. The λ5, VpreB, and B29 gene promoters also show heterogeneous initiation and lack TATA boxes (8, 15, 23). A 737-bp *mb-1* promoter fragment linked to a β-globin test gene shows accurately initiated transcription when transiently transfected into WEHI-231 B cells (Fig. 2). The same pattern of transcription initiation is observed in EL-4 T cells, albeit with significantly reduced efficiency. In contrast, no accurately initiated transcription is detectable in J558L plasma cells. In a T-lineage cell, the *mb-1* gene is never activated, whereas in a plasma cell, a previously active *mb-1* gene is shut off. Our observations raise the intriguing possibility that the inactivity of the *mb-1* locus in T cells and plasma cells may be accomplished in part by different mechanisms (see below).

Deletion analysis of the *mb-1* promoter in WEHI-231 cells reveals two functionally important regions (Fig. 2). Each domain promotes transcription from a heterologous promoter. The proximal domain (−116 to −27) also stimulates transcription in EL-4 T cells but not in J558L plasma cells. In contrast, the distal domain (−253 to −107) is inactive in both of these cell lines. Thus, the activity of the distal domain parallels the activity of the *mb-1* locus. Deletion analysis of this domain identified a 25-bp segment (−184 to −160) which is a necessary and sufficient activation element. Gel electrophoresis DNA binding assays revealed a cell-type- and stage-specific factor, BLyF, which binds the distal element. The complete correlation between BLyF binding and transcription activation by binding site fragments argues that

BLyF positively regulates transcription of the *mb-1* gene. In the absence of a fine mutational analysis of the distal element, we cannot rule out the possibility that BLyF, while being necessary, is not sufficient to activate transcription from the distal element. Deletion of a region (−280 to −114) containing the distal element in the *mb-1* promoter reduces transcription 14-fold in WEHI-231 cells. Mutational analysis of this region in the context of a heterologous promoter fails to reveal additional activation elements.

BLyF appears to represent a novel B-lineage-specific transcription factor. Its distribution in B-lineage cells is the same as that of a previously described activator protein termed BSAP (1a). However, BLyF is distinct from BSAP, since a BSAP binding site fails to compete for BLyF binding to the *mb-1* promoter. Furthermore, BSAP generates a faster-migrating DNA complex than does BLyF in native acrylamide gels (17a). Even though BLyF recognizes a purine-rich motif, it is not PU.1 (14) or Ets-1 (9), since neither of the cloned proteins recognize the BLyF binding site (1). On the basis of its cell type distribution, BLyF is also distinct from the recently described lymphoid factor LyF-1 (16). The structure of BLyF awaits its purification and cloning.

The presence of BLyF in pre-B cells (38B9 and 70Z/3) suggests that it may play a key role in activating the *mb-1* locus in B-cell ontogeny. It may therefore be involved in B-cell commitment or determination. It will be interesting to determine when active BLyF appears during B-cell development as well as to identify the developmental signal and the mechanism of activation. Furthermore, future work will attempt to determine whether BLyF participates in the activation of other early B-lineage genes.

The absence of BLyF in plasma cells suggests that part of the mechanism responsible for shutting off of the *mb-1* gene involves loss of a positive activator. It will be interesting to determine whether signalling through the antigen receptor during B-cell activation and terminal differentiation results in loss of BLyF activity and to determine the mechanism by which this loss is effected.

Our results suggest that the inactivity of the *mb-1* locus in plasma cells involves a mechanism(s) in addition to the loss of BLyF activity. Deletion of the BLyF binding site in the *mb-1* promoter reduces but does not abolish accurately initiated transcription in WEHI-231 B cells (Fig. 2). In contrast, no accurately initiated transcription is detectable in J558L plasma cells (data not shown). Consistent with these results, the proximal domain which lacks a BLyF binding site activates transcription from a heterologous promoter in WEHI-231 cells but not in J558L cells (Fig. 3). Interestingly, the proximal domain contains binding sites for an uncharacterized nuclear factor as well as the Ets-1 (9) and PU.1 (14) transcription factors (17a). The nuclear factor is present in both WEHI-231 and J558L cells. Ets-1 and PU.1 appear to be expressed in both B and plasma cells. Thus, if one or more of these factors control the activity of the proximal domain, they may be rendered inactive in plasma cells via mechanisms other than loss of expression or DNA binding. The proximal domain of the *mb-1* promoter is very active in EL-4 T cells (Fig. 3). It therefore may account for a significant portion of the *mb-1* promoter activity in T cells (Fig. 2). In this regard, deletion of the distal region containing the BLyF binding site reduces transcription fourfold in EL-4 cells. However, this reduction is due to a BLyF-independent mechanism, since no BLyF is detectable in EL-4 cells (Fig. 7) and the distal domain is inactive in these cells (Fig. 3). The activity of the proximal domain in EL-4 cells may involve the nuclear factor mentioned above and/or Ets-1, since both are expressed in these cells. The difference in the activity of the native *mb-1* promoter as well as promoter segments between EL-4 cells and J558L cells strengthens the possibility that different BLyF-independent mechanisms may be additionally operative in keeping the *mb-1* locus inactive in T versus plasma cells.

After submission of this report, the Grosschedl laboratory reported a similar analysis of the *mb-1* gene promoter (7, 27). They have also identified and characterized a B-lineage-restricted transcriptional regulator and termed it EBF (7). BLyF and EBF appear to represent the same transcription factor, as determined from DNA binding specificity and cell type distribution. UV cross-linking of the EBF protein-DNA complex reveals two polypeptides of 80 to 85 kDa and 70 to 75 kDa (7). Purification and structural characterization of EBF/BLyF will be necessary to determine whether it is a heterodimer. Hagman et al. (7) suggest that the EBF/BLyF binding site requires an additional element(s) within the distal domain of the *mb-1* promoter to enhance transcription from a heterologous promoter. However, we demonstrate that the minimal binding site can stimulate transcription from a heterologous β-globin promoter in conjunction with the simian virus 40 enhancer. Therefore, although BLyF/EBF may function to activate transcription in a context-dependent manner, it is not strictly dependent on an additional *mb-1* proximal domain element(s) for its functioning.

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