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# Multiple motifs regulate the B-cell-specific promoter of the B29 gene

(tissue-specific transcription/transcriptional regulation/lymphocyte gene)

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**ABSTRACT** **The B-cell-specific B29 and mb1 genes code for covalently linked proteins (B29 or Ig$\beta$ and mb1 or Ig$\alpha$, respectively) associated with membrane immunoglobulins in the antigen receptor complex on B cells. We have functionally analyzed the upstream region of the B29 gene and have identified a 164-bp region which comprises the minimal promoter responsible for B-cell-specific transcription. Linker scanning mutagenesis of this minimal promoter has established that both the previously identified octamer motif and a DNA motif that binds an unknown protein factor are critical for B29 gene expression in a pre-B-cell and B-cell line. Further mutations showed that binding motifs for Ets, $\mu$B/LyF1, and Sp1 also significantly contributed to the overall activity of the minimal B29 promoter. However, the relative contribution of certain motifs to promoter activity was different in a pre-B versus a B-cell line. The $\mu$B/LyF1 motif was necessary for full promoter activity in the pre-B cells but was not required in the B cells.**

The B-cell-specific B29 gene is expressed at all stages of B-cell differentiation and encodes a cell-surface glycoprotein with a single immunoglobulin-like domain (1). The B29 protein (Ig$\beta$) is present on the surface of B cells in a disulfide-linked heterodimeric complex with the mb1 gene product (Ig$\alpha$) (2–4). The B29–mb1 heterodimer is associated with all membrane immunoglobulin isotypes (2, 5–10) and comprises the B-cell antigen receptor complex, analogous to the CD3 complex associated with the T-cell receptor. Translocation of cytoplasmic immunoglobulins to the cell surface requires association with B29–mb1 heterodimers (4, 7, 11), and crosslinking of surface IgM causes the phosphorylation of the B29 and mb1 cytoplasmic tails, ultimately resulting in signal transduction (reviewed in ref. 12).

B29 is a member of a growing class of genes whose promoters lack a TATA box (13). A number of these genes are expressed in lymphocytes, including the surrogate light chains $\lambda$5 (14) and VpreB (15), as well as terminal deoxynucleotidyltransferase (TdT) (16), CD11a (17), CD18 (18), CD19 (19), CD20 (20), CD22 (21), and mb1 (22, 23). Like these TATA-less genes, the B29 and mb1 genes initiate transcription at multiple sites (13, 23). This is in contrast to the TdT gene, where transcription is initiated at a unique site determined by an element called an initiator (24). We have carried out a functional characterization of the B29 promoter and show that sequences up to −164 are sufficient for B-cell-specific gene transcription. Linker scanning mutations of the minimal promoter indicate that multiple cis-acting sequences contribute to B29 promoter activity. The transcription factors binding to these functional motifs were confirmed by DNA-binding studies. Interestingly, this spectrum of transcription factor motifs is also present in the promoter of the coexpressed mb1 gene.§

## MATERIALS AND METHODS

**Plasmid Construction and Mutagenesis.** The chloramphenicol acetyltransferase (CAT) reporter constructs pbCAT.B29(−1230) and pbCAT.B29(−164) were made by subcloning the 1.2-kb and 200-bp fragments, respectively, into the promoterless pCAT-Basic vector (Promega) (henceforth referred to as pbCAT). The endogenous ATG for B29 was destroyed so that the first methionine codon was that of the CAT gene. Linker scanning mutations of the 200-bp fragment were generated by incorporating the mutations into the primers used in subsequent polymerase chain reactions (PCRs).

**DNA Transfections and CAT Assays.** Lymphoid cell lines were transfected by the DEAE-dextran method (25) and fibroblasts by the calcium phosphate method (26). Cells were cotransfected with 10 $\mu$g of CAT reporter plasmid and 5 $\mu$g of pRSV-luciferase (27). Extracts were prepared 44–48 hr later in 200 mM $K_3PO_4$, pH 7.8/2 mM dithiothreitol/30 mM $MgSO_4$/10 mM ATP and assayed for luciferase and CAT activity (28). CAT assays were incubated for 6 hr at 37°C using *n*-butyryl-CoA instead of acetyl-CoA, and products were separated by TLC and quantitated by liquid scintillation. Results are normalized to luciferase activity and are the averages of at least three independent transfections using two preparations of DNA unless otherwise stated.

**Nuclear Extracts, Purified Proteins, and DNA-Binding Assays.** Preparation of crude nuclear extracts from cell lines and DNase I footprinting were done as described (29). For the gel shift assays, 10 $\mu$g of crude nuclear extract was preincubated with a 1000-fold molar excess of nonradioactive competitor in HGED.05 [20 mM Hepes, pH 7.9/0.2 mM EDTA, 20% (vol/vol) glycerol/50 mM KCl/1 mM dithiothreitol plus 4 $\mu$g of poly(dI·dC) and 10 $\mu$g of bovine serum albumin in 20 $\mu$l for 10 min on ice. Probe (10,000–20,000 cpm) was then added, and the mixtures were incubated for another 30 min on ice before they were loaded onto a 5% polyacrylamide gel made with 0.25× TBE (1× is 89 mM Tris/89 mM boric acid/2 mM EDTA). Gels were run at 150 V for 2 hr at room temperature.

Purified Ets-1 and PU.1 proteins were generated as fusion proteins from the bacterial expression vectors pGEX-2T (glutathione *S*-transferase fusion partner, Pharmacia) and pET-15b ($His_6$ tag, Novagen), respectively. Proteins from isopropyl $\beta$-D-thiogalactopyranoside-induced cultures were purified on glutathione-Sepharose 4B (Pharmacia) or His-

Abbreviations: IgH, immunoglobulin heavy-chain; CAT, chloramphenicol acetyltransferase; TdT, terminal deoxynucleotidyltransferase.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M26184).

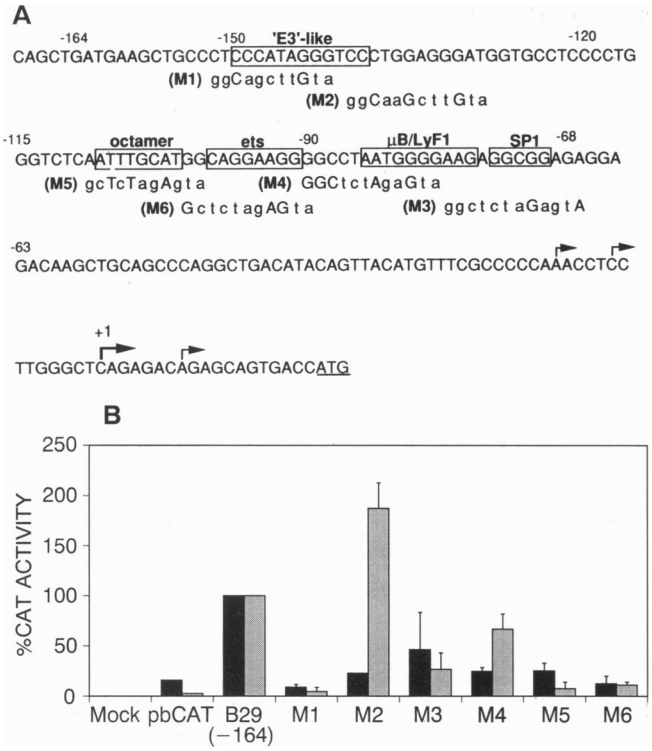


FIG. 1. Linker scanning mutagenesis of the B29 minimal promoter region. (*A*) Sequence of the minimal promoter region of the B29 gene from −169 to +22. The linker scanning mutations are shown below the wild-type sequence preceded by the identification of the mutation in parentheses. Mismatches to the wild-type sequence are in lowercase letters. The major (+1) and minor start sites defined by nuclease S1 analysis are indicated by the arrows, and the translation initiation site is underlined. Potential transcription factor binding sites are boxed. (*B*) CAT activity from pre-B cells (3-1; black bars) and B cells (M12; gray bars) transfected with the wild-type promoter construct [pbCAT.B29(−164)] or mutagenized promoter constructs (M1–M6). The activity of the wild-type construct is set at 100%.

Bind resin (Sepharose 6B fast-flow conjugate, Pharmacia) according to the manufacturer's instructions. The purified proteins were >95% pure by SDS/PAGE.

The sense-strand probe for DNase I footprinting was generated by 5′ end labeling with [$\gamma$-$^{32}$P]ATP at the *Hin*dIII site of pbCAT.B29(−164) followed by digestion with *Eco*RI. The antisense-strand probe was generated by 5′ end labeling with [$\gamma$-$^{32}$P]ATP at the *Eco*RI site of pbCAT.B29(−164) followed by digestion with *Hin*dIII. Gel shift probes were double-stranded oligonucleotides labeled at both 5′ ends with [$\gamma$-$^{32}$P]ATP. All probes were purified by electrophoresis in polyacrylamide gels.

## RESULTS

**Defining the Minimal Promoter.** A previous paper (13) reported that B29 is a member of a growing class of genes which lack a TATA box. To more precisely define individual transcription initiation sites of the B29 gene, a 200-bp fragment containing upstream B29 sequence (see *Materials and Methods*) was cloned into the replicating vector T$\beta$Py (23) to amplify mRNA products for nuclease S1 analysis (25). S1 analysis performed on total cytoplasmic RNA isolated from transfected pre-B cells (PD36) and B cells (M12) revealed four distinct transcriptional start sites over a 25-bp region: one major start site, designated +1, and three minor start sites (Fig. 1*A*). To define the minimal promoter responsible for B29 gene expression, 5′ deletion analysis was done on the DNA sequence 1.2 kb upstream of the B29 start sites. The deleted fragments were then cloned upstream of the CAT reporter gene and tested for activity in transient transfections of pre-B cells (3-1), B cells (M12), T cells (RLm11, BW5147), and fibroblasts (3T3). As shown in Table 1, maximal activity was contained in a 164-bp fragment located immediately upstream of the transcription start sites [pbCAT.B29(−164)]. Additionally, this region was sufficient to direct B-cell-specific expression, as significantly less CAT activity is observed in the T-cell and fibroblast lines transfected with the same 164-bp fragment. We have designated this region as the minimal promoter for B29 expression. Interestingly, the construct containing the entire 1.2-kb fragment [pbCAT.B29(−1230)] exhibited 3-fold less activity than the 164-bp fragment in both the pre-B-cell and B-cell lines tested. This presumably indicates the presence of negative regulatory elements within the region 5′ of the minimal promoter.

**Linker Scanning Mutagenesis of the B29 Promoter.** Linker scanning mutants were generated to delineate critical motifs within the 164-bp minimal B29 gene promoter. These mutated promoters (Fig. 1*A*) were cloned upstream of the CAT gene and tested for their activity relative to the wild-type pbCAT.B29(−164) construct in transient transfections of 3-1 pre-B cells and M12 B cells (Fig. 1*B*). The results indicate that multiple motifs contribute to the activity of the B29 promoter. The DNA sequences identified by mutations M1, M3, M5, and M6 are critical for full promoter activity in 3-1 and M12 cells. Further, a fifth and a sixth region, identified by the M2 and M4 mutations, are also required for full expression in the 3-1 pre-B cells. Mutation of either the E3-like binding site (M1) or the octamer binding site (M5), which has been shown to bind the Oct-1 and Oct-2 proteins (13), completely abrogated promoter activity in both the pre-B- and B-cell lines. Thus, both sites are required for full promoter activity. However, the M1 and octamer sites are still not sufficient for full activity because mutation of either the M3 or M6 region alone also reduced promoter activity by 6-fold in both the pre-B and B-cell lines even though the M1 and octamer sites were intact. Finally, the additional requirement for the region identified by the M2 and M4 mutations in 3-1 pre-B cells suggests that changes in the transcriptional regulation of B29 gene expression occur with differentiation. This type of change in transcription factors during differentiation is also seen in the regulation of the immunoglobulin heavy-chain (IgH) genes (30, 31) and the mb1 gene (23).

**Identification of Transcription Factors Binding in the B29 Promoter.** The critical B29 promoter sequences were searched

Table 1. Transcriptional activity of B29 gene segments in transient transfections

| | Normalized CAT activity | | | | |
|---|---|---|---|---|---|
| Transfected plasmid | 3-1 | M12 | BW5147 | RLm11 | 3T3 |
| pbCAT | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| pbCAT.B29(−1230) | 2.3 ± 1 | 13.8 ± 6.4 | 1.6 ± 0.5 | 0.8 ± 1.4 | 0.9 ± 0.4 |
| pbCAT.B29(−164) | 6.2 ± 2.5 | 38.6 ± 15.1 | 3.7 ± 2.3 | 1.3 ± 2.3 | 2.3 ± 0.6 |

Values for fold increases in pbCAT activity with either 1230 or 164 bp of 5′ B29 sequence are normalized to luciferase activity and are presented as the mean ± SEM of measurements from triplicate transfection experiments. 3-1 is a pre-B-cell line, M12 is a B-cell line, BW5147 and RLm11 are T-cell lines, and 3T3 is a fibroblast line.

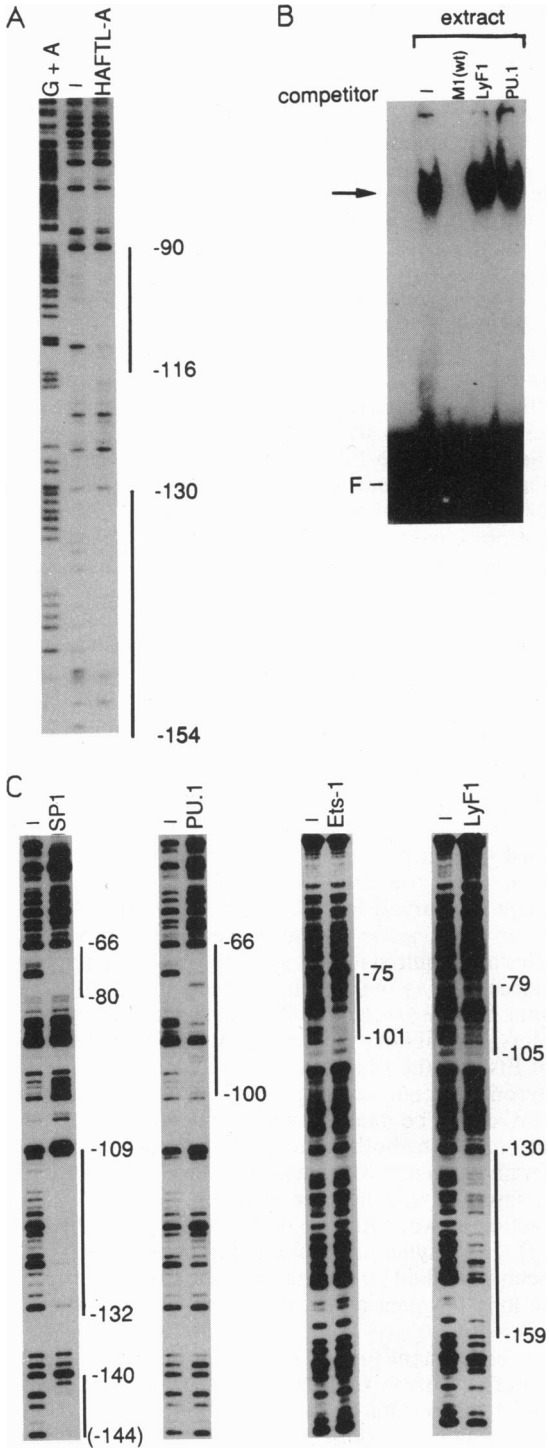


FIG. 2. Transcription factor binding to the B29 minimal promoter region. (*A*) Representative DNase I footprint of the minimal promoter region (sense strand) using 10 μg of crude nuclear extract from the pre-B-cell line HAFTL-A. Lane without extract is indicated (−), and footprints are identified by a bold line and sequence position with respect to the major transcription initiation site (+1) in accord with the numbering in Fig. 1*A*. (*B*) Gel shift assay using a double-stranded oligonucleotide [M1(wt)] encompassing the region −154 to −122. The probe was incubated in the absence or presence (bracketed lanes) of 10 μg of HAFTL-A crude nuclear extract. Samples containing extract were incubated in the absence (−) or presence of specific [M1(wt)] or nonspecific [LyF1, PU.1] nonradioactive competitor. The specifically shifted band is indicated (arrow). Sequences for the nonspecific competitor oligonucleotides with the relevant sequences underlined: LyF1, 5′-AGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCCTTTGGGAGAGGATCCCCGGGTACCGAGCTCG-3′; PU.1, 5′-GCTACAAGGAGGAAGTGAAACCTGGGGCGGGA-3′. F, free probe. (*C*) DNase I footprints of the minimal promoter region (sense strand) using purified proteins. Lanes without protein are indicated (−), and footprints are identified as in *A*. Approximately 50 ng of purified protein was used for each reaction. The number in parentheses for the Sp1 footprint indicates that the full 5′ extent of the footprint was not determined.

for homologies to transcription factor binding sites. Matches were found to the motifs for the Ets family of transcription factors (M6), an Sp1 site (M3), a μB (30, 31) or LyF1 site (M4) (29), in addition to the previously identified octamer (M5) and E3-like (M1) sites (13). DNase I footprinting of both the coding and noncoding strands using nuclear extracts or purified proteins was done to identify factors binding to these regions of the B29 promoter. A representative footprint of the sense strand for crude nuclear extracts from pre-B-, B-, plasmacytoma, and T-cell lines is shown in Fig. 2*A*. Crude extracts protected a region of DNA containing the octamer and Ets binding sites (−90 to −116) and a region over the E3-like site which also extends 10–15 bp further 3′ (−130 to −150 or −154). Although there is a footprint from nuclear extracts over the E3-like site, other data suggest that this is not an E3 binding site. Binding to oligonucleotides containing sequences from −151 to −142 or from −151 to −136, which encompass the entire E3-like site, was not observed in a gel shift assay using *in vitro* transcribed and translated mTFE3 (data not shown). mTFE3 was shown to bind the known E3 site located in the IgH intronic enhancer (32). In contrast, an oligonucleotide containing sequence from −154 to −122, which contains the entire 5′ extent of the nuclear extract footprint and sequence further 3′, did give rise to a shifted band with pre-B-cell (HAFTL-A, Fig. 2*B*) and B-cell (WEHI 231, data not shown) nuclear extracts in gel shift assays. This band was specifically competed by nonradioactive probe [M1(wt)] (Fig. 2*B*) but not by a wild-type E3 oligonucleotide (data not shown) or by other nonspecific competitors, including an LyF1 site from the TdT promoter or a consensus Ets binding site (PU.1) (Fig. 2*B*). Finally, protection was not observed over the μB/LyF1 or Sp1 sites.

Lack of Sp1 binding from nuclear extracts is consistent with earlier reports (33). However, purified Sp1 bound strongly to the putative Sp1 site in the B29 promoter (−66 to −100) (Fig. 2*C*). Interestingly, two other sites showed high-affinity binding of Sp1 (−109 to −132 and −140 to −144). It has been reported that Sp1 will bind with reduced affinity to GT boxes (33), suggesting that these two other footprints may be due to two small GT boxes.

Purified PU.1 was found to bind −66 to −100, suggesting that both the Ets site and the μB/LyF1 site were being bound simultaneously (Fig. 2*C*). PU.1, a B-cell- and macrophage-specific protein (34), binds the μB site in the IgH intronic enhancer (35). As with PU.1, purified Ets-1 appeared to bind the same two sites (−75 to −101) (Fig. 2*C*), the only qualitative difference being the 3′ extent of the footprint and the location of the hypersensitive site characteristic of the binding of all Ets family members (data not shown) (36).

Partially purified LyF1 protein from RLm11 nuclear extracts (10,000- to 20,000-fold purification) (29) bound three regions of DNA; one with high affinity and two with very low affinity (Fig. 2*C*). The high-affinity binding site corresponds to the binding site for LyF1 shared with the μB site (−79 to −87). The two low-affinity sites correspond to the region identified as an Ets binding site (−90 to −105) and the region originally identified as an E3-like binding site (−130 to −159). This footprint over the E3-like site coincides well with the footprint observed with nuclear extracts. However, the gel shift analysis described earlier identifies a binding protein in this region whose expression pattern does not correlate with that of LyF1 (29) and whose binding is not blocked when an

LyF1 binding site is used as competitor (see Fig. 2*B*). Therefore, this suggests that, like the PU.1 footprint of this region, the footprint observed with purified LyF1, represents a fortuitous but not relevant interaction.

**Comparison of the B29 and mb1 Promoter Regions.** Since the B29 and mb1 genes are coordinately activated and coexpressed through the B-cell stage, it is logical to expect that these two genes might be similarly regulated. Comparative sequence analyses of the −164 to +1 region of the B29 and mb1 promoters (23) revealed multiple elements in common between the two (Fig. 3). Further, the spatial relationships among these elements appear to be reasonably conserved. Both promoters contain an Ets binding site, an Sp1 site (whose sequence is identical in both promoters), a μB/LyF1 site, and an octamer site (the last site is a tentative identification in the mb1 promoter, as it has not been tested for binding). The relevance of these similarities is substantiated by functional criteria which show that three of the shared sites, Ets, Sp1, and μB/LyF1, are important for both mb1 and B29 promoter activity in pre-B cells (23), and two out of those three sites (Ets and Sp1) are also important for B29 promoter activity in the B-cell line tested. However, these shared promoter elements are by no means the only level of control for these two genes. For example, lack of plasma-cell-stage expression of mb1 has been correlated with the absence of EBF binding at a site 5′ from the promoter sequence shown here (23, 37, 38). B29, which is expressed at the plasma cell stage, has no binding site for EBF (data not shown) and hence is not regulated at any level by EBF expression.

## DISCUSSION

We have shown that the minimal promoter controlling tissue-specific B29 gene expression lies in a 164-bp DNA fragment immediately upstream of the multiple transcriptional start sites. Within this region, binding sites for lymphocyte-specific and ubiquitous transcription factors have been identified. The binding data, together with the functional mutagenesis studies, show that the effects of the mutations on B29 promoter activity can be correlated with the disruption of the binding of these transcription factors. However, each of the binding sites identified can bind different members of the same family. Hence, definitive assignment of a binding protein to the functional motifs found in the B29 promoter will require further study.

The octamer motif is one major determinant of B29 promoter activity. Recently, Corcoran *et al.* (39) have shown that the B29 gene is still expressed in *Oct-2*-knockout mice. Our results show that the octamer is essential for B29 promoter activity. Three possibilities might account for this result. (*i*) B29 gene expression may be regulated early in development by the ubiquitously expressed Oct-1 protein, rather than the lymphocyte-specific Oct-2 protein. In support of this, a number of groups have shown that the IgH promoter can be activated in HeLa cells which only contain Oct-1, not Oct-2, *in vitro* (40, 41) and *in vivo* (42) and that the activity in these cells cannot be boosted by overexpression of Oct-1 or Oct-2 because the amount of Oct-1 present is already saturating (42). Further, Luo *et al.* (43) have purified a B-cell specific co-activator, OCA-B, that binds to both Oct-1 and Oct-2, but preferentially to Oct-1. (*ii*) A possibility not mutually exclusive from the first is that Oct-1 can functionally compensate for the lack of Oct-2 in B-cells from these *Oct-2*-knockout mice, thus maintaining B29 gene expression. This hypothesis is also supported by the aforementioned experiments. (*iii*) Other B29 regulatory sequences (e.g., enhancers) may compensate for the loss of the octamer site in the complete gene.

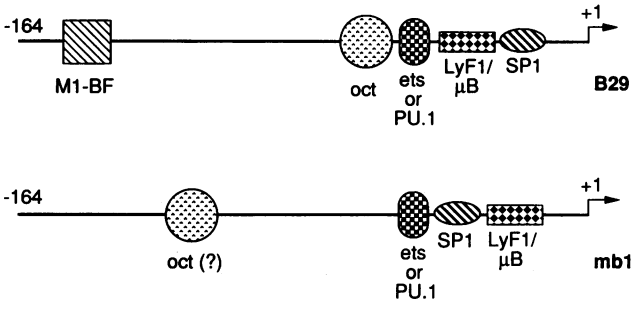


FIG. 3. Comparison of the B29 and mb1 promoter regions from −164 to +1. Binding sites for transcription factors for both promoters are shown. Question mark indicates potential binding site that has not been verified. Drawings are not to scale.

Tentative assignments of Ets-1 and PU.1 as the binding proteins for the Ets site and the μB/LyF1 site, respectively, can be made for the B29 promoter from experiments on the regulation of IgH intronic enhancer activity. The B29 promoter has an Ets site located adjacent to a μB/LyF1 site. This arrangement of binding sites is also seen in the IgH intronic enhancer, where the μB site has been shown to bind the Ets family member PU.1 (34, 35). Furthermore, binding of PU.1 to the μB site in the IgH intronic enhancer appears to be part of a cooperative interaction with binding of the Ets-1 protein to the Ets site. The spacing between the Ets site and the μB site in the B29 promoter suggests that this type of interaction may be taking place in the B29 promoter as well.

Two pieces of evidence support the hypothesis that both proteins are required for B29 promoter function. (*i*) Nelsen *et al.* (35) have shown that COS cells transfected with expression vectors for both Ets-1 and PU.1 can transactivate a minimal IgH intronic enhancer fragment containing just the Ets site (μA) and the μB site. On the other hand, this fragment is relatively inactive when the expression vector for Ets-1 or PU.1 alone is transfected, suggesting that both proteins are required for full activity. Additionally, both sites are necessary, as the μB site alone is inactive (30, 31). (*ii*) Hagman and Grosschedl (44) suggest that the Ets-1 protein contains an inhibitory C-terminal domain that prevents binding of Ets-1 to the Ets site in the mb1 promoter. By forming a heteromeric complex with another protein, the inhibitory domain could be masked and thus increase the binding affinity of one or both proteins. Heteromeric complexes of Ets family members with themselves and other proteins have been observed in a number of cases (45–48). This type of interaction between Ets-1 and PU.1 is suggested by Nelsen *et al.* (35), and a synergistic interaction between Sp1 and Ets-1 has been described for activation of the Ets-responsive region in the long terminal repeat of human T-lymphotropic virus type I (49).

The requirement for PU.1 binding at the μB site for B29 promoter activity is further supported by an indirect application of the results on the regulation of the IgH intronic enhancer. Whereas both the octamer and μB sites are required for full enhancer activity in pre-B cells, only one of the two sites is necessary at later B-cell stages (30, 31). The B29 gene similarly requires both the octamer site and the μB site for full promoter activity in the pre-B-cell line examined, as mutation of either of these sites (M5 and M4, respectively) decreased activity of the promoter substantially. Only one of the sites, the octamer, is required for full activity in the B-cell line tested. This slight difference in the necessity for octamer in B29 as opposed to the IgH intronic enhancer, which can use either the octamer or μB site for full activity in B cells, may simply reflect the fact that one is a promoter and the other is an enhancer. However, the possibility that the M4 mutation affects an LyF1 site rather than a μB site cannot be ruled out. The LyF1 site was originally identified in the Tdt promoter (29) and can bind the μB site in the IgH intronic

enhancer (29). The finding that PU.1 binds to the μB site in the IgH enhancer shows that the μB-binding protein (PU.1) is clearly distinct from the LyF1-binding protein even though the two binding sites are the same in these instances. This distinction is also supported by the fact that the LyF1 protein, although not yet cloned, is probably not a member of the Ets family (36).

One factor that could be largely responsible for the B-cell-specific expression of the B29 gene is the PU.1 protein. However, the PU.1 gene is expressed in macrophages as well as B cells (34). This contradiction can be resolved by proposing that it is the PU.1 protein in combination with the other lymphocyte-specific factors implicated in B29 gene expression that confers B-cell specificity on the B29 gene. T cells, which contain the Ets-1 and octamer proteins, do not contain PU.1 and therefore do not express the B29 gene. Similarly, macrophages and monocytes which contain PU.1 either lack or have very low amounts of Ets-1 (50) and therefore do not express B29. As an extension of this combinatorial hypothesis, one can speculate that the regulatory regions of co-regulated genes will have the same complement of factors. Such similarities can be seen in the promoters of the B29 and mb1 genes (see Fig. 3). Each of these regulatory regions contains an octamer (or octamer-like site in mb1), an Ets site, and a μB site, although in different spatial orientations, and each of these sites binds factors that are found in other hematopoietic cells in addition to B cells. These similarities suggest that tissue-specific gene expression may be due to the combinatorial activities of multiple lymphocyte-specific transcription factors rather than the activity of a single tissue-specific factor.

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