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*J Immunol* 1998; 160:2287-2296;
http://www.jimmunol.org/content/160/5/2287

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PU.1/Spi-1 Is Essential for the B Cell-Specific Activity of the Mouse CD72 Promoter

Han Ying, Ju-Fay Chang, and Jane R. Parnes

CD72 is a 45-kDa glycoprotein that is predominantly expressed on cells of the B lineage, except for plasma cells. Its expression pattern is representative of many B cell-specific proteins, which are essential for B cell development and activation but are down-regulated after B cells become terminally differentiated plasma cells. We have examined the promoter region of the mouse CD72 gene to identify sequences responsible for this regulatory pattern. The CD72 gene does not have an obvious TATAA box. Primer extension assays identified multiple transcription initiation sites. Deletion analyses have identified the 255-bp minimal promoter required for tissue-specific and developmental stage-specific expression. DNase I footprinting analysis of the CD72 minimal promoter revealed three protected elements: FP I, FP II, and FP III. Sequences corresponding to FP I or III gave increased reporter gene activity specifically in B cells, but not in T cells or NIH-3T3 cells. Sequences corresponding to FP II gave increased reporter gene activity in mature B cells, but not in plasma cells or non-B cells. Electrophoretic mobility shift assays and DNase I protection analyses revealed that FP I was bound by the transcription factor PU.1/Spi-1. Transient reporter analyses with plasmid bearing the mutated PU.1 binding site showed that binding of PU.1 is necessary for the increase in CD72 promoter activity in B cells. These results suggest that the 255-bp CD72 promoter confers both tissue specificity and developmental stage specificity, and that the B cell and macrophage-specific transcription factor PU.1 is essential for regulating the tissue specificity of the mouse CD72 promoter.


The differentiative stages of B lymphocytes from committed precursors to Ab-producing plasma cells are defined by the ordered rearrangement of Ig genes as well as by sequential induction and extinction of developmental stage-specific gene products. According to their expression pattern, B-lineage-specific gene products can be divided into three groups. The first group represents proteins that are expressed at very early stages of B cell development. Such proteins include the RAG-1 and RAG-2 gene products (1–3), terminal deoxynucleotidyl transferase (TdT) (4, 5), and λ5 and VpreB (6, 7). These proteins play important roles in early B cell development, as they are required for Ig rearrangement and pre-B cell receptor complex formation. The second group represents proteins that are expressed at all stages of B cell development, except for terminally differentiated Ab-producing plasma cells. Such proteins include transmembrane proteins Igα (5), Igβ (5, 6), CD40 (7), CD72 (8, 9), and CD19 (10). Moreover, it includes cytoplasmic proteins such as Src family members Btk (11) and Blk (12), and nuclear proteins such as the transcription factors B cell-specific activator protein (BSAP) (13) and early B-cell factor (14). The last group represents proteins that are not present at the mature B cell stage, but are specifically expressed after B cell activation. Such proteins include J chain (15), syndeacan (16) and Blimp-1 (17). Although many advances have been made toward characterizing their functions in B cell development and activation, mechanisms involved in regulating their tissue specificity and developmental stage specificity remain largely unknown.

CD72 is a 45-kDa type II transmembrane glycoprotein (8, 9, 18). Functional studies using anti-CD72 mAbs have demonstrated that CD72 plays important roles in B cell activation, proliferation, and plasma cell differentiation (19–25). CD72 is predominantly expressed on early B cells. Its expression is lost at the Ab-producing plasma cell stage (8, 9, 23, 26, 27). Thus the CD72 gene, whose expression represents that of the second group of genes, provides a good model system for studying tissue-specific and developmental stage-specific gene regulation during B lymphopoiesis. Previously, we have cloned and sequenced the mouse CD72 gene isolated from the C57L mouse (28). In this article we report the identification of the 255-bp minimal CD72 promoter, which is capable of tissue-specific and developmental stage-specific expression, reflecting in vivo CD72 expression. The specificity of the minimal promoter is regulated by several cis-acting elements in the proximal region of the CD72 minimal promoter. We demonstrate that the transcription factor PU.1, an Els family member (29) that is highly expressed in B lymphocytes, macrophages, monocytes, and, to a lesser extent, immature erythroid cells (30, 31), specifically binds to one of the cis elements encompassing nucleotides −162 to −132 of the CD72 promoter. The interaction of PU.1 with the CD72 promoter is essential for the tissue-specific activity of this promoter.

Materials and Methods

Genomic cloning and sequencing

The isolation and sequencing of the mouse CD72 gene was described previously (28). The mouse CD72a and CD72c promoter fragments (528
bp were cloned by PCR using the oligonucleotide Oligo 584, encompassing nucleotides −528 to −512 of the mouse CD72 promoter (5′-ATG GTTGAGGACGGAGC-3′), and the oligonucleotide Oligostart, which lies within exon 1 of the mouse CD72 gene (5′-CTAGATGTTTGGTAT GCGC-3′), as primers. Total genomic DNA isolated from the tail of BALB/c (CD72a) or AKR (CD72b) mice was used as the template. PCR was performed under conditions described previously (28). CD72 genomic fragments were then subcloned into pBluescript vectors (Stratagene, La Jolla, CA), and sequence analysis was performed according to standard protocols.

The isolation of the human CD72 genomic clone cos-hu-Lyb-2/CD72 was described previously (32). The CD72 genomic fragment was cleaved by HindIII and subcloned into pBluescript vectors (Stratagene). One subclone containing a 2.1-kb HindIII fragment of the CD72 gene was partially sequenced, and the 840-bp human CD72 promoter sequence was compared with mouse CD72 promoter sequences to identify homologous sequence elements.

**Plasmid constructions and in vitro mutagenesis**

The initial clone of the CD72a gene was a 15.2-kb fragment isolated from a pre-B cell library in the vector Lambda Fix (Stratagene, La Jolla, CA); the vector was then subcloned into a pBluescript vector (Stratagene). Two fragments of this 15.2-kb fragment were obtained by restriction enzyme digestion. Appropriate DNA fragments were then cloned into the enhancerless, promoterless luciferase reporter vector pSV0AL (6) at the HindIII site, which is immediately upstream of the luciferase gene (33). All inserted CD72 genomic fragments representing the 5′ flanking sequence of the CD72 gene have identical 3′ ends, which were generated by cleavage of the insert from the XhoI site of the cosmid insert just upstream of the ATG site, and whose 5′ ends extend varying distances upstream of the ATG site (Fig. 2A). All constructs were analyzed by both restriction enzyme digestion analysis and sequencing of the pertinent DNA junctions to verify copy number and orientation of inserts. Control vectors included pGL2 positive, in which the luciferase gene is regulated by the SV40 promoter/enhancer (Promega, Madison, WI), and pON 405, in which the β-galactosidase gene is controlled by the immediate early CMV promoter (provided by D. E. Mocarski, Stanford University, Stanford, CA). The pGL2-positive plasmid was used to measure maximum reporter activity, and the latter was used to normalize transcription efficiency.

Mutations in the P.1 site were generated using oligonucleotide carrying point mutations. The nucleotides 5′-TTCCC-3′, which are critical for binding of P.1, were replaced by the nucleotides 5′-GCTG-3′, as indicated by underlined sequence in the oligonucleotides shown below. Mutant luciferase constructs were generated by PCR according to standard protocols and were confirmed by sequence analysis. Two oligonucleotides used for site-specific mutagenesis were: PU.1 mut1, 5′-GACCCTTCGGT CTTTTGAGTTCCG-3′; and PU.1 mut2, 5′-CATAAAAGAACA GGAAGCTTGGGCAAGA-3′.

**Cell culture and transfection assays**

The mouse pre-B cell line L1.2 (provided by D. I. Weissman, Stanford University, Stanford, CA), the pre-B cell line HAFITL1, the pre-B cell line HAFITL1.clon6 (provided by D. Davidson, National Institutes of Health, Bethesda, MD), the B lymphoma cell line M12.4.1 (provided by M. Lieber, Washington University, St. Louis, MO), the plasmacytoma cell line MOPC315p (provided by Dr. M. Davis, Stanford University), and thymoma cell line BW5147 (American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 5% FCS (Sigma Chemical Co., St. Louis, MO), 50 μM 2-ME (Sigma Chemical Co.), and 25 μg/ml each of penicillin and streptomycin (Life Technologies).

The cells were transfected by electroporation (Bio-Rad Gene Pulser, Bio-Rad, Hercules, CA). Cells (1 × 10⁶/ml) were harvested and resuspended in 0.4 ml of Cytomix buffer (120 mM KCl; 0.15 mM CaCl₂; 10 mM K₂HPO₄/KH₂PO₄, pH 7.6; 25 mM HEPES, pH 7.6; 2 mM EGTa, pH 7.6; and 5 mM MgCl₂; pH adjusted by KOH) (34) containing 10 μg of the luciferase reporter plasmid and 5 μg of the plasmid pON405 containing lacZ driven by the immediate early CMV promoter (provided by E. Mocarski, Stanford University). Electroporation was performed in a 0.4-cm cuvette (Invitrogen, La Jolla, CA) using the following parameters: M12.4.1 at 280 V and 960 mM capacitance, MOPC315p at 260 V and 960 mM capacitance, BW5147 at 320 V and 960 mM capacitance, and NIH-3T3 cells at 260 V and 960 mM capacitance.

After 24 h, transfected cells were harvested for luciferase and β-galactosidase assays. Luciferase activity was measured from 50 μl of the cell extract with the luciferase reagents as described by the supplier (Analytical Luminesence Laboratory, San Diego, CA). The light emission was measured with a Monolight 2010 instrument (Analytical Luminesence Laboratory, San Diego, CA), reading relative light for 10 s. Luciferase activities were normalized for transfection efficiency as determined by β-galactosidase activity. The β-galactosidase assay was performed as previously described (35).

**Electrophoretic mobility shift assay (EMSA)**

Nuclear proteins were prepared from cultured cells as described previously (36). The ds oligonucleotides were end labeled with [γ-³²P]ATP (Amer sham, Arlington Heights, IL). One to three femtomoles of the probe was incubated with 15 μg of nuclear protein extract and 1 μg poly(dI-dC) in a final volume of 30 μl of a buffer consisting of 8 mM HEPES (pH 7.9), 2.5 mM Tris-HCl (pH 7.9), 60 mM NaCl, 1 mM DTT, 10% glycerol, 1 mM EDTA, and 2.5 mM MgCl₂ for 30 min at 20°C. Samples were analyzed on a 4% native polyacrylamide gel.

The ds oligonucleotide 31–32 encompassing nucleotides −154 to −124 of the mouse CD72 promoter (note, numbering is from the translation start site) was used as a probe and a specific competitor; ds oligonucleotide 33–34 (derived from the CD72 gene), which does not contain a P.1 binding site, was used as a nonspecific competitor. Their sequences are as follows: 31–32, 5′-GATCCITTCCTTCTTTATGACTTGGC-3′ and 33–34, 5′-GATCCAGGAGCTTGGTATTTATGACTTGGC-3′.

**Footprinting analysis**

The pBluescript plasmid containing the 255-bp minimal CD72 promoter was digested with HindIII, dephosphorylated with calf intestinal phosphatase, 5′ end labeled with [γ-³²P]ATP (Amer sham), and then cleaved with either BamHI or SalI to label one end of the noncoding and the coding strand, respectively. Probes were purified on a native polyacrylamide gel. Binding reactions were conducted at 20°C for 30 min with 1 to 3 fmol of the probe in the presence or the absence of 40 to 100 μg of nuclear protein extract and 1 μg of poly(dI-dC) (Pharmacia, Piscataway, NJ) as a nonspecific competitor. Digestions were performed at 20°C with 0.2 U (without nuclear extract) or 1 to 2 U (with nuclear extract) of DNase I (Promega) for 90 s. Reactions were stopped, and phenol/chloroform was extracted. Samples were analyzed on a sequencing gel together with a G+A sequencing ladder.

**Results**

Sequence analysis of the 5′ flanking region of the CD72 gene

As the expression patterns of the mouse and human CD72 genes are identical, it is highly likely that tissue-specific cis-acting elements may be conserved between these species. The 5′ flanking sequence of mouse CD72a was thus compared with the 5′ flanking sequence of the human CD72 gene to identify homologous sequence elements. As shown in Figure 1A, all conserved sequence elements between mouse and human lie within 250 bp upstream of the ATG site, suggesting that this region might be important for CD72 promoter activity. Two of the conserved sequence elements are highlighted and labeled BSAP and P.1 (Fig. 1A). The BSAP fragment, encompassing nucleotides −187 to −169, is homologous to the consensus sequence recognized by the B cell-specific transcription factor BSAP (13). The P.1 element, extending from nucleotide −149 to −143 (5′-TTCTTCC-3′), is the reverse complement of the consensus sequence (5′-GAGGA-3′) recognized by the B cell- and macrophage-specific transcription factor P.1.

Neither the human nor the mouse CD72 gene contains an obvious TATAA box. Primer extension analysis identified several transcription initiation sites that could only be detected in normal B cells or B cell lines (Fig. 1), not in cells (BW5147, MOPC315) that do not express CD72 (data not shown). As shown in Figure 1A, the major initiation site is at nucleotide −145, which is in the middle of the putative P.1 site. One minor site is at −27, and another at −229. We have also identified another possible minor start site that is several hundred base pairs upstream of the major initiation site. The nature of this minor initiation site needs to be further characterized.
Recent studies from our laboratory showed that the CD72b allele, but not the CD72a or CD72c alleles, is also expressed on a fraction of peripheral T cells and activated thymocytes (37). It is unclear whether this phenomenon is only due to a genetic leakage or implies a function for CD72b in T cells. To understand whether the allelic differences in the CD72 expression pattern in mice are due to polymorphism in the promoter region, the 5′ flanking regions of the CD72b and CD72c genes were cloned and sequenced (see Materials and Methods) and compared with that of CD72α (Fig. 1). In the 528-bp region examined there are only 11 base pair differences between CD72b and the consensus of CD72a and CD72c. These allelic polymorphisms lie in the region upstream of −255, where there is very little homology between the mouse and human sequences. Whether this polymorphism is responsible for the allelic differences in the expression of CD72 is not yet known. There is >99% identity among the three mouse CD72 alleles within the 528-bp regions compared. In addition, there are only two base pair mismatches within the region −255 to +1, suggesting that this region might be potentially important for CD72 gene regulation.

Deletional analysis of the CD72 promoter

To functionally characterize the CD72 promoter, a series of deletions of the mouse CD72a 5′ flanking sequence was inserted in front of the luciferase gene in the promoterless and enhancerless pSVOAL5′ (see Materials and Methods). Reporter constructs were named according to the size of the inserted CD72 gene fragments. Transient transfections were performed by transfecting reporter constructs into cells by electroporation. Luciferase activity was determined 24 h after transfection. In the B lymphoma cell line M12.4.1 (CD72α + ), maximum luciferase activity was observed when cells were transfected with the −8300 construct (Fig. 2B), which contains the largest 5′ flanking fragment of the CD72 gene. Further deletional analyses of this 8.3-kb fragment identified several sequence elements that yielded increased luciferase activity relative to shorter fragments in M12.4.1 cells. The luciferase activity of construct −131 was 5.6-fold higher than that of reporter construct −63. Other DNA fragments that gave increased luciferase activities in M12.4.1 cells were −162 to −132 (3-fold), −196 to −163 (4-fold), and −1113 to −531 (2.5-fold).

The cell type-specific activity of the CD72 promoter was examined by comparison of the luciferase activities of the reporter constructs in different cell lines (Fig. 3). M12.4.1, which represents the mature B stage, displayed the greatest reporter activity. Results of reporter gene assays with another mature B cell line, L10A6, were similar to those obtained with M12.4.1 (data not shown). In contrast, luciferase activity in MOPC315p, which represents the plasma cell stage (CD72−), and BWS5147 cells, which represents thymic T cells (CD72−), were significantly lower. The fibroblast line NIH-3T3 (CD72−), on the other hand, displayed negligible activity. Therefore, the luciferase activity in these cell types was indeed reflective of the expression pattern of CD72 in those cell types.

### FIGURE 1

CD72 5′ flanking sequence and transcription initiation sites. 

**A.** Sequence comparison of the 5′ flanking region of the mouse and human CD72 gene. CD72 5′ flanking regions from mouse CD72 alleles a, b and c, as well as from human were cloned and sequenced as described in Materials and Methods. The sequence shown is the 5′ flanking sequence of the mouse CD72 gene. The translation start site ATG is designated +1. Transcription start sites determined by primer extension analysis are indicated by arrows. Uppercase letters represent nucleotides that are identical among the three mouse CD72 alleles. Lowercase letters represent nucleotides that are different among the three mouse CD72 alleles. The sequence elements homologous between the mouse and human CD72 promoters are indicated by asterisks. The underlined sequence element −187 to −169 is homologous to the consensus sequences recognized by BSAP, and the underlined sequence element −149 to −143 is reverse complementary to the consensus sequences recognized by PU.1. 

**B.** Analysis of the transcription initiation sites by primer extension. A 32P-labeled 33-mer oligonucleotide (5′-GGGGCACTTTCACAAAGCGCAGGTCTCGATACG-3′) was annealed to mRNA extracted from a B lymphoma cell line (L10A6; lane 1), purified splenic B cells from DBA2 mice (lane 2), α B lymphoma cell line HAFT1 (lane 3), and a B lymphoma cell line BAL17 (lane 4) and extended using SuperScript reverse transcriptase (Life Technologies). The reactions were analyzed on a sequencing gel together with a sequencing reaction as a size marker. The three detected transcription initiation sites at −229 (minor), −145 (major), and −27 (minor) are indicated in A. Similar results were obtained using mRNA from other B-lineage cell lines, including L10A6, M12.4.1, L1.2, A20, and HAFT1clone6 (data not shown).
Several DNA fragments contributed to the tissue-specific activity of the mouse CD72 promoter. The DNA fragment from nucleotide −162 to −132 produced a 3-fold increase in the luciferase activity in M12.4.1, a 7-fold increase in MOPC315p, and a 2-fold increase in BW5147 cells relative to the reporter construct −131. In addition, the relative luciferase activity was higher in M12.4.1 (16.1 ± 0.8) and MOPC315p (27.8 ± 2.9) than in BW5147 (5.1 ± 0.8) cells. Therefore, this DNA fragment may contain a cis-acting element contributing to the B cell-specific activity of the CD72 promoter. By contrast, the DNA fragment from nucleotide −196 to −163 produced a 4-fold increase in luciferase activity relative to the reporter construct −162 in M12.4.1, but little increase in MOPC315p or BW5147 cells. In addition, the relative luciferase activity of reporter construct −196 was higher in M12.4.1 (63.2 ± 1.8) than in MOPC315p (30.2 ± 3.3) and BW5147 (8.1 ± 0.8) cells. Therefore, this fragment may contain a regulatory element contributing to the B cell-specific and developmental stage-specific activity of the mouse CD72 promoter. The DNA fragment from nucleotide −1113 to −531 conferred a 2.5-fold increase in luciferase activity in M12.4.1 cells, but not in other cell types. Unlike the fragment from −196 to −163 and the fragment from −162 to −132, which contain homologous sequence elements present in both human and mouse, there is very little homology between mouse and human CD72 gene in the upstream region.

The 255-bp CD72 5′ flanking sequence contains three major binding sites for nuclear factors

The interaction of nuclear proteins with the CD72 promoter in the region up to −255 was examined by DNase I protection analysis. The 5′ end-labeled DNA fragment −255 to −6 was incubated with nuclear extract prepared from the HAFTL1 clone6 cell line (pre-B), then digested with DNase I and analyzed by denaturing PAGE (Fig. 4). Similar footprint patterns were detected using nuclear protein extracts from B cell lines L10A6, M12.4.1, and L1.2 (data not shown). Three regions, referred to as FP I, II, and III, were protected in both the coding (Fig. 4A) and noncoding (Fig. 4B) strands. All three protected fragments contain sequence elements that are conserved between mouse and human (Fig. 4C). FP I, encompassing nucleotides −161 to −141, lies within the B cell-specific cis-element −162 to −132. In addition, it contains the sequence element 5′'-TTCTCT-3′, which is reverse complementary to a PU.1 binding site, 5′'-GAGGAA-3′. FP II, encompassing nucleotide −190 to −168, lies within the developmental stage-specific cis-element −196 to −163. FP II contains the sequence homologous to the consensus sequence recognized by BSAP. Examinations of FP III did not reveal any sequences homologous to any known lymphocyte-specific transcription factor binding sites. This fragment is highly conserved between mouse and human, suggesting the functional importance of this element.

**PU.1 binds to the DNA fragment −161 to −141**

The DNA fragment from nucleotide −154 to −124 was end labeled and incubated with purified recombinant PU.1 in the presence or the absence of unlabeled ds oligonucleotides as competitors, and samples were electrophoresed on a polyacrylamide gel. As shown in Figure 5, incubation of the probe with recombinant PU.1 protein resulted in the formation of a DNA-protein complex (lanes 2–4). The formation of the complex was completely inhibited by the unlabeled probe (lanes 5 and 6) and by a ds oligonucleotide competitor containing a known PU.1 binding site (lanes 7 and 8). By contrast, a ds oligonucleotide that does not contain a PU.1 binding site had no effect on the formation of the complex (lanes 3 and 4).

The binding of PU.1 to the CD72 promoter was further examined by DNase I footprinting analysis using the radiolabeled fragment −255 to −6 of the CD72 promoter as the probe (Fig. 6). The probe was incubated with different amounts of purified recombinant PU.1 protein. Samples were electrophoresed on a sequencing gel in parallel with a G+A sequencing ladder. As shown in Figure 6, the sequence element protected by recombinant PU.1 (lane 2) is identical with the FP I fragment (from nucleotide −161 to −141). In addition, there was only one sequence element protected in this...
analysis, suggesting that there is only one PU.1 binding site in the 255-bp CD72 promoter.

To further determine whether native PU.1 binds to the fragment −154 to −124, the radiolabeled fragment −154 to −124 was incubated with nuclear protein extract from L1.2 (pre-B) or A20 (mature B) in the presence or the absence of anti-PU.1 antiserum, which recognizes the DNA binding domain of PU.1 (31). As shown in Figure 7, the shifted band representing the DNA-PU.1 complex was specifically inhibited by the anti-PU.1 antiserum (Fig. 7, lanes 4 and 7), but not by rabbit IgG (Fig. 7, lanes 3 and 6), suggesting that native PU.1 binds to the fragment −154 to −124. Identical competition results were reproduced using nuclear extract from L10A6, M12.4.1, HAFTL1, and HAFTL1.clone6 (data not shown). The above three in vitro assays strongly suggest that PU.1 specifically binds to the sequence element from nucleotide −161 to −141 in the mouse CD72 promoter.

**FIGURE 3.** Mouse CD72 promoter activity in different cells. CD72-based reporter gene constructs were electroporated into M12.4.1, MOPC315p, BW5147, and NIH-3T3 cells, and luciferase activity was determined 24 h after transfection. Luciferase activity is expressed as described in Figure 2. CD72 promoter activities in M12.4.1 (M12; stippled), MOPC315p (white), BW5147 (striped), and NIH-3T3 (black) cells are compared in A. The CD72 promoter activities in MOPC315p, BW5147, and NIH-3T3 cells are shown separately in B, C, and D, respectively, with scales chosen for better resolution. The promoter activity in M12.4.1 is shown in Figure 2.

**Mutational analysis of the PU.1 binding site in the CD72 promoter**

To characterize whether the binding of PU.1 to the fragment −161 to −141 is responsible for the increase in luciferase activity in M12.4.1 and MOPC315p cells (with reporter construct −162 compared with construct −131; Fig. 3), the PU.1 site in the reporter constructs −255 and −162 were mutated by site-specific mutagenesis (see Materials and Methods). The 5′-TTCC-3′, which is critical for PU.1 binding, was replaced by 5′-GCTG-3′ in the mutant luciferase constructs (Fig. 8A). The mutated site was not bound by PU.1 in EMSAs using the ds oligonucleotide containing the mutated PU.1 site as a probe (data not shown). Luciferase analysis comparing the luciferase activity of wild-type reporter constructs with the mutant constructs showed that knocking out the PU.1 site in the reporter construct −162 completely eliminated the increase in luciferase activity in both these cell lines (Fig. 8, B and C). In addition, mutations in the −255 construct significantly decreased the luciferase activity in M12.4.1 cells (Fig. 8B) and MOPC315p cells (Fig. 8C). These assays demonstrate that PU.1 plays a very important role in determining the CD72 promoter activity in B cells and plasma cells. In contrast, the decrease in luciferase activity in T cells (Fig. 8D) was much less than that in B cells and plasma cells (note the difference in the scale of the x-axis in Fig. 8, B, C, and D). Since PU.1 is expressed in M12.4.1 (mature B) cells and MOPC315p (plasma) cells, but not in BW5147 (thymic T) cells, the decrease seen in BW5147 could be an effect of an interaction of this region with nuclear factors present in BW5147 cells. Most likely other Ets family members, such as Ets-1, Ets-2, T-cell factor-1, Elf, or GA-binding protein-α, may interact with this region as the 5′-TCC-3′ is reverse complementary to 5′-GGA-3′, which is the consensus sequence recognized by all Ets family
This is further supported by the footprinting using an end-labeled 255 to 26 probe incubated with nuclear protein extract from BW5147 cells; the FP I fragment was also protected (data not shown). However, this interaction played a less significant role than the PU.1-DNA interaction in B cells, because the relative luciferase activity of reporter construct 2162 in BW5147 cells was much lower than that in M12.4.1 or MOPC315p cells.

Discussion

In this study we have cloned and sequenced the 5' flanking region of the mouse CD72a, CD72b, and CD72c genes as well as from the human CD72 gene. Sequence comparisons showed that the sequence within 255 bp upstream of the ATG site exhibits the greatest homology. Within this region, there are only two base pair mismatches among the three mouse CD72 alleles. In addition, there are several sequence elements that are homologous between the mouse and human genes in this region. In contrast, there is little homology between mouse and human CD72 genes upstream of −255. Analysis of the CD72 promoter sequences suggests that there may be important regulatory sequence elements within 255 bp upstream of the ATG site.

Like many lymphoid-specific genes, both the mouse and human CD72 gene lack obvious TATAA boxes (28). It is generally believed that the initiator (Inr) element, which is present in promoters
In adribosomal protein Inr element 9
9
CD72 promoter bears 10 of 11 bp identity with the mammalian
9
encompassing nucleotides
the Inr element of TdT. However, the sequence element 5
5
-CTCA(N)0– 5 GAGNC-3
represented by the lymphocyte-specific TdT promoter, uses the
according to sequence homology (41). One type of Inr element,
however, understanding of the mechanism of initiation on TATA-
transcription initiation complex in TATA-less promoters (40).

lanes 2– 4
lanes 4
lanes 5–7

Rabbit antimouse PU.1 antiserum (gift from Dr. Richard Maki, La Jolla,
CA) was added in lanes 4 and 7. Rabbit IgG was added in lanes 3 and 6
as an isotype-matched control.

Comparison of luciferase activities of construct −162, which
contains the PU.1-binding site, among different cell lines demon-
strated that luciferase activity was higher in cells expressing PU.1
(i.e., M12.4.1 and MOPC315) than in cells that do not (i.e.,
BW5147 and NIH-3T3). In addition, mutations in the construct
−162 that eliminated PU.1 binding also eliminated the increase in
luciferase activity compared with that in the wild-type construct.
These results suggest that PU.1 is essential for the B cell-specific
activity of the mouse CD72 promoter. Our results also suggest
that the expression of PU.1 at its physiologic level is sufficient for
activation of the minimal promoter to a certain extent, as deter-
mined in reporter gene assays. For example, the luciferase activity
of the construct −162 (containing the PU.1 binding site) was
higher than that of the construct −131 (no PU.1 binding site) in
both M12.4.1 and MOPC315 (both cells express PU.1). On the
other hand, the luciferase activity of −255 in MOPC315 (plasma
cell stage, CD72” and PU.1”) was much lower than that in
M12.4.1 (mature B stage, CD72” and PU.1”). Mutation of the
PU.1 site in −255 decreased, but did not eliminate, luciferase ac-

tivity (Fig. 8). The above results suggest that PU.1 expression is
sufficient for activation of the minimal promoter to some degree,
but that full activation requires other factors that interact with the
minimal CD72 promoter.

Our studies together with those of others indicate that PU.1 ex-
pression is not sufficient for the expression of CD72 protein or
mRNA. First, CD72 mRNA is not expressed in plasmacytoma
(8, 9), although PU.1 is expressed (31). We have recently
shown that CD72 surface expression decreases 10-fold when rest-
ing B cells are driven to become plasma cells (syndecan7+/−, B220
low, J chain+, Blimp-1+/−) in the presence of LPS.4 In a gel-shift
experiment performed with a probe containing a known PU.1 bind-
ing site and nuclear protein from resting B cells or LPS-induced
plasma cells, the intensity of the band representing the PU.1-DNA
complex remained the same between resting B cells and plasma
cells (our unpublished observation). These studies suggest that
the expression of PU.1 at the plasma cell stage is not sufficient for
maintaining CD72 protein expression or transcription. Finally, we
have not been able to detect CD72 expression in macrophages
from DBA2 mice, even though these cells express PU.1. There-
fore, additional positive and/or negative factors must be involved
in the physiologic regulation of CD72 expression.

The essential role of PU.1 in regulating tissue-specific promoter
activities has been demonstrated in several different cell lineages,
including B cells, macrophages, and monocytes (29). Targeted dis-
ruption of the PU.1/Spi-1 gene is lethal to the resultant mutant
mice. The mutant embryos present multilineage defects character-
ized by defective development of progenitors of monocytes, gran-
ulocytes, and T and B cells and variable impairment of erythroid
maturation (46). Interestingly, the PU.1 protein level and message
level remains relatively constant throughout B cell development

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gene expression during B lymphocyte development. Submitted for publication.
Additionally, the PU.1 mRNA level is similar among different lineages (29). In fact, many target genes for PU.1 have been identified in B cells (κ 3' enhancer (47, 48), λ 2–4 enhancer (49), heavy chain Eμ enhancer (50), J chain promoter (51), and Btk promoter (52)), myeloid cells (CD11b promoter (43, 53), CD18 promoter (44, 54), c-fes tyrosine kinase promoter (55), M-CSFR (macrophage CSF) (42), G-CSFR (granulocyte CSF) (56), scavenger receptor (57), FCgRIIIA (58), FCgRI (59), and IL-1β (60)), mast cells (IL-4 gene enhancer (61)), and erythroid cells (β-globin intervening sequence 2 (62)). These studies suggest that PU.1 is a key regulator that is essential for multilineage development during hemopoiesis, possibly through regulating the expression of lineage-specific genes.

Although our studies have established an important role for PU.1 in the regulation of mouse CD72 promoter activity, they do not explain how CD72 expression is lost at the plasma cell stage where PU.1 is still expressed. In plasma cells, the luciferase activity of the construct −162 was higher than that in cells that do not express PU.1, suggesting that PU.1 is not responsible for the change in CD72 expression at the stage of plasma cell terminal differentiation, which may involve concerted action of several factors that interact with other cis-regulatory elements of the mouse CD72 promoter (data not shown). In contrast to FP I, FP II conferred both B cell-specific and developmental stage-specific activity that correlates with the expression pattern of the endogenous CD72 gene (Figs. 2 and 3). In a separate report (manuscript submitted) we show that FP II is specifically recognized by BSAP, a zinc finger protein that belongs to the paired domain family Pax (63). The full CD72 promoter activity requires interactions with both BSAP and PU.1. On the other hand, BSAP expression is lost at the plasma cell stage. It is likely that the absence of positive regulators such as BSAP accounts for at least part of the down-regulation of CD72 expression in the terminally differentiated plasma cells.

**FIGURE 8.** Comparison of luciferase activity between PU.1 mutant reporter constructs and wild-type constructs. Site-specific mutagenesis was performed as described in Materials and Methods. The 5'-TTCC-3', which is critical for PU.1 binding, was replaced by 5'-GCTG-3' in the mutant luciferase constructs, as shown in A. Relative luciferase activity in M12.4.1 (M12; B), MOPC315p (C), and BW5147 (D) cells, corrected for transfection efficiency, is expressed as fold activity above the background activity conferred by the promoterless control plasmid in those cells.
Finally, sequence fragments upstream of −255 may also contribute to the tissue-specific activity of the CD72 promoter. First, the luciferase construct −3100 gave the second highest luciferase activity in M12.4.1 (comparable to the activity of the luciferase construct −8300) and the second lowest luciferase activity in the rest of the cells examined (Fig. 3), suggesting that B cell-specific control elements exist between nucleotides −3100 and −255. Secondly, there was a significant decrease in luciferase activity in MOPC315 cells between the construct −255 and −530, and luciferase activity remained at a similar level for the constructs −1113, −3100, and −8300, suggesting that there may be a negative regulatory element in the region between −255 and −530 that down-regulates the CD72 promoter activity in plasma cells, although sequence comparison between the mouse and human CD72 genes did not reveal any significant homologous elements within this region (data not shown). In addition, between the luciferase constructs −530 and −1113, luciferase activity was significantly increased in M12.4.1, but was decreased in MOPC315, suggesting that more positive control elements may exist in the region between −530 and −1113. These studies indicate that sequence 5′ of −255 may also play an important role in determining the B cell-specific expression of the CD72 gene. Further analysis of the functional role of sequence 5′ of −255 in CD72 gene regulation is underway.

References


