Identification and Characterization of a Transcriptional Regulator for the lck Proximal Promoter*

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The lck gene encodes a protein-tyrosine kinase that plays a key role in signaling mediated through T cell receptor (TCR) and pre-TCR complexes. Transcription of the lck gene is regulated by two independent promoter elements: the proximal and distal promoters. Previous studies employing transgenic mice demonstrated that the sequence between −584 and −240 from the transcription start site in the mouse lck proximal promoter is required for its tissue-specific expression in the thymus. In this study, we demonstrate that a Krüppel-like zinc finger protein, mtβ (BFCOL1, BERF-1, ZBP-89, ZNF148), previously cloned as a protein that binds to the CD3ζ gene enhancer, binds to the −365 to −328 region of the lck proximal promoter. mtβ is ubiquitously expressed in various cell lines and mouse tissues. Overexpressed mtβ is more active in T-lineage cells than B-lineage cells for transactivating an artificial promoter impaired by mutating the mtβ binding site or by reducing mtβ protein expression level by using antisense mRNA. Our results indicate that mtβ activity is regulated in a tissue-specific manner and that mtβ is a critical transactivator for the lck proximal promoter.

The lck gene encodes a lymphocyte-specific protein-tyrosine kinase, p56$lck$, a member of the src kinase family (1). It has been demonstrated by co-immunoprecipitation that p56$lck$ associates with the cytoplasmic domains of CD4 and CD8 coreceptors (2) and with the acidic region of the IL-2$R$β-chain in T cells (3). By a series of biochemical analysis, it has been shown that p56$lck$ plays a key role in signal transduction mediated through the T cell receptor (TCR) complex in mature T cells (4, 5). It also contributes to signaling through the pre-TCR complex, thereby playing an essential role in thymocyte development. lck-deficient mice and transgenic mice overexpressing a dominant negative form of p56$lck$ exhibit severe impairment in the expansion of CD4/CD8-double negative immature thymocytes (6, 7). A simple doubling of wild type p56$lck$ expression levels in immature thymocytes in transgenic mice was sufficient to block maturation of thymocytes (8). These findings suggest that the transcriptional control of the lck gene must be tightly regulated to express adequate amounts of p56$lck$ at the right developmental stage during thymopoiesis.

The lck gene is transcribed from two structurally unrelated promoters (9–13). The lck proximal promoter is positioned immediately adjacent to the first coding exon, and is active in the thymus, but is essentially silent in peripheral T cells. The distal promoter is located far 5′-upstream from the proximal promoter and is active during all developmental stages of T-lineage cells. Since the proximal promoter becomes active only at an early developmental stage of T-lymphopoiesis (14, 15), and since the level of p56$lck$ greatly influences thymocyte maturation (6–8), the transcriptional regulators of this promoter play a critical role in the developmental program for T-lineage cells.

The 5′-flanking sequence of the lck proximal promoter that is critical for the thymocyte-specific and developmental stage-specific expression has been defined by transgenic mouse models (16). Transgenic animals bearing truncations in the mouse lck proximal promoter revealed that as little as 584 bases of the 5′-flanking sequence can confer appropriate developmentally regulated expression of heterologous reporter genes. The 5′ sequence critical for the promoter activity contains several binding sites for nuclear proteins. Among those nuclear proteins, “B-factor,” which binds to the G-rich stretch within the −365 to −328 region was reported as a candidate for the critical transcriptional regulator. B-factor is only found in cells expressing the lck transcript derived from the proximal promoter, namely thymocytes and thymoma cell lines such as LSTR and EL4 (16).

In this study, we characterized the B-factor and identified an 86-kDa Krüppel-type zinc finger protein, which had been cloned previously as a binding protein to the CD3ζ gene enhancer, as a component of the B-factor. The NH2-terminal half of the protein is 90% identical to htβ, a 49-kDa protein that binds to the human TCR Vβ8.1 gene promoter and the TCR α gene silencer (17), indicating that mtβ is the murine homologue of htβ, and the reported amino acid sequence of htβ is a part of its full-length protein. mtβ is ubiquitously expressed in various
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cell lines and tissues. We re-evaluated distribution of the B-factor and found it is also expressed in various cell lines and tissues. However, the transcriptional activity of mtβ measured by reporter constructs carrying the B-factor binding site is greatly impaired by introducing mutations in the B-factor binding site or by expression of mtβ antisense mRNAs. Our results demonstrate that mtβ is one of the critical transactivators driving the lck proximal promoter and that its activity is regulated in a tissue-specific manner.

**EXPERIMENTAL PROCEDURES**

**Cells**—EL4, LSTRA, WEHI231, and BALB/c were grown in RPMI 1640 medium supplemented with 8% FCS and 50 μg 2-mercaptoethanol. FDC-P1 and Ba/F3 cells were grown in RPMI 1640 medium supplemented with 8% FCS, 50 μg 2-mercaptoethanol, and 5 units/ml IL-3. Murine IL-3 was prepared as a culture supernatant of XbAg8.6.63 cells transfected with ml-3 cDNA (18). COS7 cells were grown in RPMI 1640 medium supplemented with 8% FCS. MTH cells were grown in RPMI 1640 medium supplemented with 8% FCS, 50 μg 2-mercaptoethanol, and 1.25 ng/ml IL-2 (Roche Molecular Biochemicals).

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSAs)**—Cells were washed twice with ice-cold phosphate-buffered saline and once with hypotonic buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 0.1 mM EGTA, 2 mM MgCl2, 1 mM Na2VO4, 20 mM NaF, 1 mM dithiothreitol, 0.1 mM Fabeloc SC (Roche Molecular Biochemicals), 10 μg/ml leupeptin). Cells were suspended in buffer I (hypotonic buffer containing 0.2% Nonidet P-40) and incubated for 5 min on ice. Nuclei were pelleted by centrifugation at 15,000 × g for 20 min and resuspended in buffer K (hypotonic buffer supplemented with 420 mM NaCl and 20% glycerol). After vigorous shaking for 30 min at 4 °C, the supernatants were collected and used as nuclear extract (19). Oligonucleotides corresponding to the −365 to −328 region of the lck proximal promoter (−365/−328, see section below) were subcloned into the KpnI site of pBluescript II (Stratagene). The fragment was cut out by Asp718I, labeled with [32P]dATP by Klenow large fragment (TaKaRa) and activated and boiled in SDS-PAGE sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 1% SDS) and separated on 12% acrylamide gel. Autoradiography was carried out after 2 h exposure to X-ray film.

**Plasmid Construction**—The full-length mtβ cDNA was subcloned into the EcoRI site of pcDNA3 (Stratagene), a eukaryotic expression vector driven by the human cytomegalovirus enhancer and promoter, resulting in pcDNA3-mtβ. For the mtβ antisense plasmid (pcDNA3-Asmtβ), the full-length mtβ cDNA was subcloned in the opposite direction into the EcoRI site of pcDNA3. Various truncated fragments from the mouse lck proximal promoter were subcloned into the pGL2-Basic plasmid (Promega), which has a firefly luciferase gene without promoter or enhancer. For −3200/pGL2, the Notl-BamHI (positions −3206 to +37) fragment of the p1017 plasmid (20) containing the entire lck proximal promoter region was blunt-ended and ligated to the XhoI, HindIII-digested, blunt-ended pGL2-Basic plasmid. For −3433GL2, two Small fragments (position −3200 to −1675 and −1675 to −3433) were removed from −3200/pGL2 and self-ligated. For −240/pGL2, two KpnI fragments (position −3200 to −358 and −358 to −240) were removed from −3200/pGL2. For −584/pGL2, the KpnI fragment (position −584 to −240) from −3200/pGL2 was inserted into the KpnI site of −240/pGL2. To construct reporter plasmids carrying the B-factor binding site and TATA box, −365/−328 and −365/−328mut oligonucleotides (see section above) were inserted upstream of the TATA box of pGL2 using the SmaI and BglII-digested pGL2-Basic plasmid. Point mutations were introduced into the mtβ binding sites of −3200/pGL2 and −433/pGL2 by PCRamdirected mutagenesis using −365/−328mut oligonucleotides to generate −3200-mut/pGL2 and −433-mut/pGL2, respectively.

**RNA Isolation and Northern Blot Analysis**—Total RNA was extracted from various cell lines using the acid guanidinium thiocyanate-phenol-chloroform method. About 5 μg microsomal RNA were fractionated through electrophoresis on 1% agarose gel in the presence of 0.66% formaldehyde, transferred to nylon membranes (Genescreen, DuPont). Mouse multiple tissue Northern blot was purchased from OriGene Technology (Rockville, MA). Membranes carrying RNA were hybridized with a 2.0-kilobase EcoRI fragment of mtβ cDNA labeled with [α-32P]dCTP by the random priming method. After hybridization, membranes were washed with 0.6× SSC and 0.1% SDS, then exposed to X-ray film.

**Preparation of Cell Lysates and Western Blotting**—Cells were harvested and boiled in SDS-PAGE sample buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol) for 5 min. After centrifugation at 15,000 × g for 15 min, resulting clear cell lysates were analyzed on 7×100 Bio-Image Analyser (Fuji Film, Tokyo, Japan). After removing the mtβ probe, the membranes were re-hybridized with a human β-actin probe to normalize the amount of RNA loaded per lane.

**Generation of Polyclonal Antibodies**—A 1135-base pair EcoRV-ApaI fragment encoding Ser32 to Glu33 and a 1510-base pair XhoI-NolI fragment encoding Thr448 to Gly572 of mtβ protein were subcloned into pGEX-4T-1 expression vector (Amersham Pharmacia Biotech), respectively. The resulting plasmids were used to transform BL21(DE3)/pLysS (Novagen, Madison, WI). Recombinant glutathione S-transferase-mtβ fusion proteins were induced with 1 mM isopropyl-β-D-thiogalactopyranoside and affinity-purified by binding to glutathione-linked Sepharose beads (Amersham Pharmacia Biotech). The fusion proteins were further purified by gel filtration and used to immunize rabbits. Rabbit polyclonal anti-mtβ antibodies were immunopurified on Sepharose-4B beads covalently coupled with the respective glutathione S-transferase-mtβ fusion proteins used as immunogens.

**RT-PCR**—Total RNA or mRNA purified using Micro-FastTrack kit (Invitrogen) was subjected to cDNA synthesis using random hexamers (Takara Bio, Tokyo, Japan) and Superscript II reverse transcriptase (Life Technologies, Inc.). Serial dilution (3- or 2-fold) of the cDNA synthesis mixtures was subjected to PCR amplification using the following primers: distal-sense, 5'-ATGTTGAGCTTCACATACTGGAG-3'; proximal-sense, 5'-TTCAAGGTTGACATCCATTGGCTTCG-3'; lck antisense, 5'-GATTCTTGAAG-3'; β-actin-sense, 5'-ACATGGTGGCACCATTCCACAAC3'; β-actin antisense, 5'-CTGAGGACATCTTGGTGCA-3'; G3PDH (glycerolaldehyde-3-phosphate dehydrogenase)sense, 5'-ACACGAGTGCCATTGAGCG-3'; G3PDH antisense, 5'-CTGACCACGATTCTTCCCA-3'. Primer sequences were used in a total of 320 cycles of 90 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min for the lck or HPRT transcripts and 27 cycles of the above for β-actin or G3PDH transcripts using a PCR Thermal Cycler MP (Takara). The resulting PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

Transfections and Luciferase Assays—Cells (3 × 104) were suspended

2 K. Georgopoulos, unpublished data.
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Identification of a Nuclear Protein Binding to the lck Proximal Promoter—A nuclear factor termed “B-factor” binds to a G-rich stretch located at −365 to −328 from the transcriptional initiation site of the lck proximal promoter, and its expression correlates well with the activity of the promoter (16). Because lck expression driven by the proximal promoter occurs early during lymphopoiesis (14, 15), and the level of p56\(^{cK} \) greatly influences thymocyte maturation (6–8), we hypothesized that the B-factor would be one of the transcription factors playing critical roles in early lymphopoiesis. Ikaros regulates the early lymphopoiesis or the commitment for lymphocytes as demonstrated in the Ikaros-deficient mice (22). Ikaros has been shown to bind to a G-rich sequence of the CD3\(^{\gamma}\) gene enhancer (23), although the high affinity binding sites for Ikaros are not G-rich (24). We examined whether the B-factor contains Ikaros or Ikaros-related proteins by EMSAs. Nuclear extract of a thymoma cell line, LSTRA, contains the B-factor binding to the −365 to −328 G-rich sequence (−365/−328) of the lck proximal promoter (Fig. 1A) as shown previously (16). The binding was specific, since it was competed by unlabeled −365/−328 oligonucleotides, but not by the −365/−328 oligonucleotides carrying mutations in the G-rich sequence (−365/−328mut). As shown in Fig. 1B, the binding of B-factor to the −365/−328 probe was competed by \(\alpha\)A sequences, a functional element of the CD3\(^{\gamma}\) gene enhancer. The \(\alpha\)A consists of a CRE (cyclic AMP response element)-like region and a G-rich site similar to the B-factor binding site (25). The binding of B-factor to \(\alpha\)A was mediated by the G-rich site, because mutation at the G-rich site (\(\alpha\)A-G) but not at the CRE-like site (\(\alpha\)A-CRE) abrogated the competition with the −365/−328 probe. This binding characteristic of B-factor to \(\alpha\)A sequence was similar to that of Ikaros (23). However, the high affinity Ikaros binding oligonucleotides (IkarosBS) (24) did not show any competition with −365/−328. During the cloning of Ikaros, a cDNA clone encoding a zinc finger protein (mt\(\beta\)) that binds to \(\alpha\)A sequence is a component of the B-factor. The B-factor complex was supershifted by anti-mt\(\beta\) antibodies. E, the B-factor present in nuclear extracts from EL4.

RESULTS

Identification of a Nuclear Protein Binding to the lck Proximal Promoter—A nuclear factor termed “B-factor” binds to a G-rich region located at −365 to −328 from the transcriptional initiation site of the lck proximal promoter as a probe. The −365 to −328 (−365/−328) oligonucleotides or the oligonucleotides carrying mutations in the G-rich region (−365/−328mut) were used as competitors (10- and 50-fold molar excess over the labeled probe) to determine the binding specificity of the B-factor. B, the B-factor binds to \(\alpha\)A, the core enhancer sequences of the CD3\(^{\gamma}\) gene enhancer. Unlabeled oligonucleotides with various G-rich sequences were used as competitors. \(\alpha\)A-CRE, \(\alpha\)A carrying the mutation in the CRE binding site; \(\alpha\)A-G, \(\alpha\)A carrying the mutation in the G-rich sequence; and Ikaros BS, a high affinity Ikaros binding sequence. C, Ikaros is not a component of the B-factor. Anti-Ikaros antiserum did not react with the B-factor. D, zinc finger protein; mt\(\beta\) that binds to \(\alpha\)A sequence is a component of the B-factor. The B-factor complex was supershifted by anti-mt\(\beta\) antibodies. E, the B-factor present in nuclear extracts from EL4.
tein that binds to the Vβ8.1 promoter and the Vα silencer of the T cell receptor genes (17). The cDNA sequence encoding the NH₂-terminal half of the mtβ is 90% identical to hTβ, and their deduced amino acid sequences are 95% identical. The 3'-half of the mtβ coding region has 91% identity with the 3'-untranslated region of the reported hTβ cDNA. These indicate that mtβ is the murine homologue of hTβ and that the reported hTβ cDNA sequence has a one-base deletion that causes a frameshift and a premature stop codon. During this study, several cDNAs that have identical sequences with mtβ have been reported: BFCOL1 that binds to the proximal promoters of the type I collagen genes (27) and BERF-1, a 89-kDa protein that binds to a muscle-specific enhancer of the type I collagen genes (27) and ZBP-89, has been shown to bind to promoter regions of the gastrin gene (29) and the ornithine decarboxylase promoter (30). It has subsequently been reported that the same zinc finger protein also binds to the p21WAF1 gene (31), the matrix metalloproteinase-3 gene (32), the pTα gene (33), and the vimentin gene (34).

Recombinant mtβ Binds to the lck Proximal Promoter—We then asked whether recombinant mtβ forms the B-factor complex. Recombinant mtβ expressed in COS7 cells was detected as a band around 105 kDa in immunoblots (Fig. 2A). An endogenous simian homologue of mtβ in COS7 cells was detected at the same position as the recombinant mtβ when the blot was overexposed (data not shown). mtβ protein appeared to migrate more slowly in SDS-PAGE than its estimated molecular size, as is consistent with the observation for BFCOL1 by Hasegawa et al. (27). In EMSA, a residual amount of the B-factor complex was increased by expressing the recombinant mtβ protein. Anti-mtβ antibodies supershifted the B-factor complex.

Expression of mtβ in Various Cell Lines and Tissues—In a previous study, the strong correlation between the lck proximal promoter activity and amounts of B-factor has been reported (16). We therefore examined the expression of mtβ mRNA in various cell lines and tissues. As shown in Fig. 3A, two mRNA species, with estimated sizes of 9.0 and 4.2 kilobase, were detected. Mtβ mRNA expression was observed in all cell lines tested and was independent of the lck proximal promoter activity. In mice, the mtβ transcripts were ubiquitously expressed in various tissues. The mRNA was abundant in thymus where the proximal promoter is active; however, significant amounts of mRNA were also detected in all tissues, especially in the heart, kidney, and liver (Fig. 3B). We conclude that there is no correlation between lck proximal promoter activity and the expression levels of mtβ mRNA.

The expression level of mtβ protein might be controlled by post-transcriptional mechanisms. To test this possibility, we measured mtβ protein levels by immunoblots of whole cell extracts isolated from various cell lines and primary mouse lymphoid cells. The mtβ protein was detected in all tested cell lines, LSTRA (thymoma), MTH (mature T), Ba/F3 (pro-B), WEHI231 (immature B), BAL17 (mature B), and FDC-P1 (myeloid). Total RNA (15 μg) was separated and transferred onto a nylon membrane. A prepared multi-tissue membrane was purchased from OriGene Technologies. The blots were hybridized with mtβ cDNA. The blots were stripped and subsequently hybridized with a β-actin probe to normalize for the amount of loaded RNA.

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**Fig. 2. The B-factor complex formation by recombinant mtβ.** A. Western blot analysis. COS7 cells were transfected with 10 μg of mtβ expression vector or control plasmid. Cells were harvested at 48 h after transfection, and nuclear extracts were prepared. The resulting extracts (1 × 10⁶ cells/lane) were separated, transferred to membrane, and were probed with anti-mtβ antibodies. Molecular size markers are indicated on the left. B, EMSA. The amount of B-factor complex (arrow) was increased by expressing the recombinant mtβ protein. Anti-mtβ antibodies supershifted the B-factor complex.

**Fig. 3. Northern blot analysis.** Expression of mtβ transcripts in various cell lines (A) and mouse tissues (B). RNA was prepared from LSTRA (thymoma), MTH (mature T), Ba/F3 (pro-B), and WEHI231 (immature B), BAL17 (mature B), and FDC-P1 (myeloid). Total RNA (15 μg) was separated and transferred onto a nylon membrane. A prepared multi-tissue membrane was purchased from OriGene Technologies. The blots were hybridized with mtβ cDNA. The blots were stripped and subsequently hybridized with a β-actin probe to normalize for the amount of loaded RNA.
nuclear extract prepared from thymocytes as reported previously (16). We initially failed to detect either mtβ protein or B-factor complex in extract from total splenocytes. However, the B-factor was present in the nuclear extract prepared from purified splenic B cells as well as mature T cells (Fig. 4B). High proteinase activity in total splenocytes may have caused deg-

FIG. 4. Expression of mtβ protein and the B-factor is not restricted to T lineage cells. A, Western blot analysis of mtβ in LSTRA (thymoma), BAL17 (mature B), Ba/F3 (pro-B), MTH (mature T), EL4 (lymphoma), thymocytes, purified splenic T cells (95% was CD3+), and purified splenic B cells (95% was B220+). Cells (2 × 10^5) were boiled in 1 × SDS-PAGE sample buffer, and the insoluble materials were removed by centrifugation. Resulting cell lysates were separated, transferred to membrane, and were probed with anti-mtβ antibodies. Molecular size markers are indicated on the left. B, EMSA. The B-factor (arrow) was detected in nuclear extracts prepared from thymocytes, purified splenic B cells (95% was B220+), BAL17, Ba/F3, MTH, and purified splenic T cells (95% was CD3+). Anti-mtβ antibodies supershifted the B-factor complexes in all of tested extracts.

FIG. 5. Transcriptional activity of mtβ on the lck proximal promoter. A, schematic representation of the lck gene promoter regions, and position of primers used for RT-PCR analysis are shown (left). Type I and II lck mRNAs are transcribed from the proximal and distal promoters, respectively. The relative ratio of type I and type II mRNAs in EL4 and BAL17 cells were measured by semiquantitative RT-PCR analysis. Serial dilutions (3-fold) of cDNA prepared from each cell line were subjected to PCR using sets of primers for type I (primers A and C) and type II (primers B and C) lck transcripts. β-Actin cDNA was amplified (right lower panel) to calibrate the amounts of cDNA templates in each sample. The proximal lck promoter is mainly active in EL4, while the distal promoter is active in BAL17. B, mtβ activates transcription from an artificial promoter consisting of the mtβ binding site of the lck proximal promoter and a TATA-box (pLuc-wild) in EL4 but not in BAL17. Cells were transfected with 10 μg of reporter plasmid and 10 μg of mtβ expression (+) or vector plasmid (−). Twelve hours after transfection, cells were harvested, lysed, and subjected to luciferase activity measurement. The luciferase activities are represented as percent activity of that produced by pGL2 control vector driven by the SV40 promoter. In pLuc-mut, mutations were introduced into the mtβ binding site. The activity produced by a promoterless plasmid (0) is also shown. The results represent mean ± S.D. of multiple independent transfections. C, the binding of mtβ is critical for the lck proximal promoter activity. Cells were transfected with 10 μg of luciferase reporter constructs carrying the various lengths of the lck proximal promoter region (−3200, −584, −433, −240, and 0) or the mutated promoter sequences (−3200-mut and −433-mut). The mtβ binding site was destroyed by point mutations in the −3200-mut and −433-mut reporter constructs. The results are represented as percent luciferase activity observed with pGL2 control vector driven by SV40 promoter. The results represent mean ± S.D. of multiple independent transfections. In each experiment, the luciferase activities were normalized for transfection efficiency by measuring renilla luciferase activities encoded by a co-transfected pRL-TK plasmid. ND, not determined.
transfected with vector control plasmid.

Expression of mtβ antisense reduces the lck proximal promoter activity. A. Reduction of mtβ protein level by expression of antisense mtβ. EL4 cells were transfected with 10 μg of pcDNA3-ASmtβ or pcDNA3, together with 1 μg of pEGFP-N1. Cells expressing GFP were sorted at 36 h after transfection. GFP-positive cells (5 × 10⁵ cells) were lysed, and mtβ protein levels were analyzed by immunoblot. Molecular size markers are indicated on the left. The blots were stripped and subsequently probed with anti-tubulin antibodies to normalize the amount of loaded proteins. The relative expression levels of mtβ are indicated by mtβ/tubulin ratio, which is set to 1 in cells transfected with vector control plasmid. B, Relative luciferase activities of cells expressing mtβ antisense plasmid. EL4 cells were transfected with 15 μg of pcDNA3-ASmtβ or control pcDNA3, together with 3 μg of reporter plasmids (~3200/pGL2 or pGL2) and 1 μg of pRL-TK. Cells were harvested at 30 h after transfection, and luciferase activity in cell lysates was measured. The results represent mean ± S.D. of three independent transfections. C, reduced expression of the endogenous lck transcripts by expression of antisense mtβ. EL4 cells were co-transfected with pcDNA3-ASmtβ or control pcDNA3, together with pEGFP-N1 and GFP-positive cells were sorted at 30 h after transfection. cDNAs were synthesized, and serial dilutions (2-fold) of cDNA templates were subjected to PCR amplifications for mtβ, G3PDH, and HGPRT.

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FIG. 6. Expression of mtβ antisense reduces the lck proximal promoter activity. A. Reduction of mtβ protein level by expression of antisense mtβ. EL4 cells were transfected with 10 μg of pcDNA3-ASmtβ or pcDNA3, together with 1 μg of pEGFP-N1. Cells expressing GFP were sorted at 36 h after transfection. GFP-positive cells (5 × 10⁵ cells) were lysed, and mtβ protein levels were analyzed by immunoblot. Molecular size markers are indicated on the left. The blots were stripped and subsequently probed with anti-tubulin antibodies to normalize the amount of loaded proteins. The relative expression levels of mtβ are indicated by mtβ/tubulin ratio, which is set to 1 in cells transfected with vector control plasmid. B. Relative luciferase activities of cells expressing mtβ antisense plasmid. EL4 cells were transfected with 15 μg of pcDNA3-ASmtβ or control pcDNA3, together with 3 μg of reporter plasmids (~3200/pGL2 or pGL2) and 1 μg of pRL-TK. Cells were harvested at 30 h after transfection, and luciferase activity in cell lysates was measured. The results represent mean ± S.D. of three independent transfections. C. reduced expression of the endogenous lck transcripts by expression of antisense mtβ. EL4 cells were co-transfected with pcDNA3-ASmtβ or control pcDNA3, together with pEGFP-N1 and GFP-positive cells were sorted at 30 h after transfection. cDNAs were synthesized, and serial dilutions (2-fold) of cDNA templates were subjected to PCR amplifications for mtβ, G3PDH, and HGPRT.

DISCUSSION

It has been reported that a close correlation exists between transcriptional activities of the lck proximal promoter and the presence of the B-factor complex binding to the G-rich stretch of the promoter (16). In this work, we characterized and identified the B-factor, a potential transcriptional regulator of the lck proximal promoter. Our results indicated mtβ, which has been previously cloned by its binding to the CD3 enhancer in vitro, is a component of the B-factor. Anti-mtβ antibodies supershifted the B-factor complex, and the recombinant mtβ expressed in cell lines formed the B-factor complex.

mtβ is an 89-kDa zinc finger protein belonging to the Krüppel-type subfamily, whose members recognize the GC-rich or GT-rich sequences with their conserved DNA-binding zinc finger domains (26, 35). Mtβ (identical to BFCOL1, BERF-1) and its human and rat homologues (htmtβ, ZBP-89, ZFP148) are reported to bind regulatory regions of various genes, such as the Vβ8.1 promoter and the Vα silencer of the T-cell receptor genes (17), the gastrin promoter (29), the type I collagen promoter (27), the e-lens enhancer (28), the ornithine decarboxylase promoter (30), the p21WAF1 promoter (31), the matrix metalloproteinase-3 promoter (32), the pTα enhancer (33), and the silencer element of the vimentin gene (34). Our data showing that mtβ is ubiquitously expressed at both mRNA and protein levels are consistent with previously published reports and the fact that mtβ functions in the various promoters and enhancers in various tissues. However, it should be noted that mtβ regulates genes critical for maturation of T cells, such as lck, pTα, the TCRδ gene, as well as TCR α and β genes. Thus, mtβ is one of the key transcriptional regulators controlling the T cell development.

In addition to its binding to a broad range of target genes, mtβ appears to act as both transcriptional activator and repressor. As shown in this study, mtβ is required for transactivating the proximal lck promoter. It also activates transcription...
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...tion from the V\( \beta \)8.1 promoter of the TCR gene and counteracts the silencing effect of the TCR \( \alpha \) gene silencer (17), and increased promoter activity of the p21WAF1 gene (31) and the matrix metalloproteinase-3 gene (32). Moreover, the binding site of mt\( \beta \) appears to be important for the pTo enhancer element (33). In contrast, mt\( \beta \) represses transcription from the gastrin gene (29), the \( \beta \)-enolase gene (28), the ornithine decarboxylase gene (30), and the vimentin gene (34). It is currently unknown how mt\( \beta \)/BFCOL1/BERF-1 (ht/ZBP-89/ZFP148 in humans) manifests opposite activities on transcription of different genes. It has been shown that ZBP-89 competes with Sp1 for binding to the same element in the gastrin promoter (29) and inhibits the activation of the ornithine decarboxylase promoter by Sp1 (30). This might be one of the potential mechanisms by which mt\( \beta \)/BFCOL1/BERF-1 suppresses transcription from several promoter elements. A fascinating possibility is that mt\( \beta \) has different isoforms derived from alternative splicing, and each isoform has distinct transcriptional activities. To test this hypothesis, we performed RT-PCR to amplify various fragments of mt\( \beta \) cDNA using several combinations of primers. However, we could not detect any splicing variants of mt\( \beta \) cDNA either in thymocytes or splenocytes (data not shown). Another possibility is that interacting proteins exist that determine the DNA binding specificity and the transactivating activities of the mt\( \beta \) complex and whose expression is regulated in a tissue-specific manner. It is also possible that mt\( \beta \) receives post-transcriptional modifications in a tissue-specific manner. Endogenous as well as overexpressed mt\( \beta \) transactivated transcription from an artificial promoter consisting of B-factor binding sites and TATA-box in EL4 but not in BAL17. Moreover, mt\( \beta \) generally transactivates genes expressed in T cells such as \( \text{lck} \) and TCR \( \alpha \) and \( \beta \) genes, but represses gastrin, collagen, and \( \beta \)-enolase genes expressed in non-T cells. These observations support the idea that there are tissue-specific mechanisms regulating activity of mt\( \beta \). Basic Krüppel-like factor (BKLF), which is widely expressed in various tissues and binds to the CACC motifs, is also a member of the Krüppel-like zinc finger protein subfamily (36). Although BKLF positively regulates the transcription from a promoter containing a single BKLF binding site, it represses activity of glucocorticoid receptor-mediated activation of a promoter containing three copies of CACC motifs and glucocorticoid-responsive elements (36, 37). The NH\(_2\)-terminal domain of BKLF is responsible for its repressive activity and interacts with a co-repressor protein, murine COOH-terminal-binding protein 2 (mCtBP2) (37). mCtBP2 interacts with BKLF and a number of mammalian transcription factors, such as Evi-1, AREB6, ZEB, and FOG, via the Pro-X-Asp-Leu-Ser (PXDLS) motif on the transcription factors (37). The mt\( \beta \) also carries several PXDLS-like motifs (PVDLQ (amino acids 112–116), PKDINS (amino acids 282–286)). mt\( \beta \) may associate with mCtBP2 or related molecules and exhibits suppressing activity in cells that fail to support proximal \( \text{lck} \) promoter activity. Our initial attempts, however, to detect associating molecules using glutathione S-transferase-mt\( \beta \) fusion proteins or modification of mt\( \beta \) such as phosphorylation have not been successful.

mt\( \beta \) is critical for the full activation of the \( \text{lck} \) proximal promoter activity, since the mutation of the mt\( \beta \) binding site of the promoter or the reduction of the mt\( \beta \) protein level signifi-
cantly impaired the promoter activity. However, the overex-
pression of mt\( \beta \) in EL4 did not augment the \( \text{lck} \) proximal activity (data not shown). This may indicate that the coordinated interaction of mt\( \beta \) with T cell-specific transcription factors (whose expression level is limiting) is involved in the full activation of the \( \text{lck} \) proximal promoter in thymocytes. Binding sites for the T cell-specific factors TCF-1, LEF, and TCF-1\( \alpha \) are present in a region highly homologous between the murine and human proximal promoters (16). TCF-1 expressed ectopically in BAL17 cells, however, failed to drive the \( \text{lck} \) proximal promoter activity with endogenous mt\( \beta \) (data not shown), suggesting a complex cooperation of multiple transcription factors in transactivating the \( \text{lck} \) proximal promoter. A \(-240 \text{lck} \) promoter fragment lacking a mt\( \beta \) binding site is still active in EL4. It should be noted, however, that the \(-240 \) fragment (but not the \(-584 \) promoter fragment carrying a mt\( \beta \) binding site) failed to support thymocyte-specific transcription of the lacZ-hGH transgene construct in mice (16). The EL4 cell line might lack a negative regulator expressed in primary cells that suppresses the \(-240 \) promoter activity in the absence of mt\( \beta \). Alternatively, EL4 might abundantly express positive transactivators binding to the \(-240 \) fragment whose activity is repressed by proteins bound to the \(-584 \) to \(-240 \) region. The mechanism that accounts for the discrepancy between the \(-240 \) promoter activity in EL4 and that in thymocytes remains unknown.

In addition to the positive regulators directing the \( \text{lck} \) proximal promoter activity in thymocytes, the silencers suppressing the promoter are likely to play roles in peripheral T cells. It has been reported that A2 complex binding to the sequence from \(-477 \) to \(-460 \) in the murine proximal promoter was detected in extracts from cells negative for the \( \text{lck} \) proximal promoter activity (16). It has also been reported that the \(-474 \) to \(-466 \) region in the human \( \text{lck} \) proximal promoter acts as a strong repressor in human tumor cell lines that do not express \( \text{lck} \) and binds proteins with molecular masses of 35 and 75 kDa (38). Deletion of the \(-584 \) to \(-433 \) region from our luciferase reporter constructs resulted in the enhancement of the promoter activity. These suppressive elements and binding factors are also critical in achieving tissue-specific expression of the \( \text{lck} \) proximal promoter in concert with the positive regulators, including mt\( \beta \).

In summary, we identified a Krüppel-type zinc finger protein, mt\( \beta \), as a transactivator of the \( \text{lck} \) proximal promoter. mt\( \beta \) is ubiquitously expressed and manifests a broad range of activities on various genes. However, mt\( \beta \) is presumably a critical transcription factor for the T cell development, since it positively regulates \( \text{lck} \) and pTo genes as well as TCR \( \alpha \) and \( \beta \) genes. Overexpressed mt\( \beta \) is active only in T-lineage cells, suggesting that there exists tissue-specific regulatory mechanisms to control mt\( \beta \) activity. Understanding the function of mt\( \beta \) should provide important insight into how T cell development and the thymocyte-specific expression of the \( \text{lck} \) proximal promoter are controlled and how one DNA-binding protein regulates different promoters positively and negatively.

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