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Differential lineage-specific regulation of murine CD45 transcription by Oct-1 and PU.1

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Abstract

Although it has been established that CD45 expression is regulated at the transcriptional level, neither the regulatory elements that are responsible for its unique expression pattern nor the relevance of its three distinct transcriptional start sites (P1a, P1b, and P2) has been fully characterized. We studied the contribution of the three start sites to CD45 mRNA production in haematopoietic cell lines and primary haematopoietic cells. In myeloid and lymphoid cells and cell lines most CD45 transcripts originate from P1b with the exception of the thymoma-derived T cell line EL4, in which ~90% of CD45 transcripts originate from P1a. The degree of contribution of P1a is highest in lymphoid cells and increases in T cells following mitogen stimulation. In vitro evaluation of sequence upstream of the start sites shows that the P2 start site is sufficient for CD45 expression in lymphoid but not in myeloid cells, confirms the presence of a PU.1-binding site essential for myeloid expression of CD45, and reveals an Octamer-binding site that interacts with both Oct-1 and Oct-2 and activates CD45 transcription in lymphoid and myeloid cells. These findings are the first evidence that Octamer-binding factors are involved in the control of CD45 expression.

Keywords: CD45; PTPRC; Transcription factor; Transcriptional regulation; Alternative start site; PU.1; Octamer; Oct-1; Oct-2

CD45 (formerly known as the leukocyte common antigen) is an abundant cell surface glycoprotein whose expression is restricted to cells of the haematopoietic system [1,2, p. 56]. Numerous isoforms, ranging in size from 180 to 240 kDa, are generated by alternative exon splicing and are expressed in a cell type-specific pattern on functional subpopulations of lymphocytes [3–5]. CD45 is a transmembrane protein tyrosine phosphatase (PTPase), and is a critical component of the antigen-dependent signal transduction machinery of lymphocytes [6] and also regulates integrin-mediated signalling in neutrophils and monocytes [7]. In addition to these functions, CD45 has recently been shown to suppress Janus kinase (JAK) signalling, thereby acting as a negative regulator of cytokine receptor signalling in haematopoietic cells [8]. CD45 expression is typically deficient in leukaemic blasts [9], fueling speculation that the loss of this regulator of proliferation contributes to the leukaemic phenotype.

All nucleated haematopoietic cells express CD45 [1], and the protein is detectable from the earliest identifiable stage of haematopoietic development, [10] permitting its use as a marker of mesodermal commitment to a haematopoietic fate in embryogenesis. Studies of mRNA abundance [4] and nuclear run-on experiments [11] have demonstrated that the expression of CD45 is regulated at the level of transcription, rather than by mRNA stability or by post-translational mechanisms. However, little is known concerning the mechanisms by which the unique expression pattern of CD45 is initiated or maintained.

The mouse *CD45* locus spans 120 kb, including a 50 kb intron following exon 2, and comprises 34 exons [12].

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Alternative transcriptional start sites have been mapped [12] for exons 1a, 1b, and 2, and are referred to as P1a, P1b, and P2. The P1b start site is the major initiation site for *CD45* transcription, and substantial nucleotide sequence conservation between the mouse and human loci has been observed upstream of this site [1]. A potential PU.1-binding site has been identified in this region [13], and the absence of CD45 expression in PU.1 null myeloid cells suggests that this transcription factor is an important regulator of myeloid CD45 expression. DiMartino et al. [14] reported functional studies of the minor P2 start site. They identified a cluster of nucleotides, which they named the TC box, that can act as a minimal promoter for the P2 site. This sequence showed activity only in constructs from which the major P1b start site had been deleted, and the physiological relevance of the TC box remains unclear.

The regulation of CD45 expression in the lymphocyte lineage has not been well characterized. Virts and Raschke [15] studied the expression of *CD45* cDNA constructs in lymphocyte-derived cell lines. They found that *CD45* cDNA, driven either by a retroviral LTR promoter or by the T cell-specific *lck* promoter, failed to result in detectable CD45 protein expression in the T cell lines 27J or BW5147. Transient transfections of the *CD45* 3′ untranslated region fused downstream of a β-galactosidase reporter gene revealed no influence of the 3′UTR on reporter expression. A *CD45* minigene, comprising a genomic DNA fragment containing exons 4–8 flanked by cDNA containing exons 1–3 and exons 9–33, expressed strongly in lymphoid cell lines when driven by the LFA-1 promoter. However, when the LFA-1 promoter was replaced by 19 kb of native *CD45* upstream sequence, relatively weak *CD45* expression was detected. These findings suggest that these *CD45* upstream and intronic sequences may not be sufficient to produce high-level *CD45* expression in T cells. Regulatory elements in the upstream region may however be necessary for high-level, tissue-specific *CD45* expression in other cell types.

In summary, three transcriptional start sites of *CD45* have been mapped, although the importance of the P1a and P2 start sites in lineage- or stage-specific expression of CD45 has not been determined. Sequence analysis of the *CD45* locus suggests the presence of important 5′ regulatory elements immediately upstream these sites. No systematic functional analysis of this region has been published. Here we report an analysis of lineage- and stage specificity of CD45 start site choice and the characterization of regulatory elements upstream of the major promoter of *CD45*.

Experimental procedures

Plasmids. The wild-type CD45 promoter and immediate upstream region from position −438 to +251 relative to the P1b transcriptional start site were amplified by PCR, using an upstream primer containing a *Sac*I site and cloned into pPCRScript (Clontech; Mountain View, CA). A *Sac*I–*Nco*I fragment of this promoter clone was subcloned into pGL3B (Promega; Madison WI) such that the ATG codon of the luciferase gene was substituted for that of *CD45*. Deletion mutants were generated using upstream PCR primers at positions −420, −379, −215, −170, −155, −125, −110, and −100. A construct lacking both the P1a and P1b start sites was generated using the *Nhe*I restriction site at position +25. The ΔOct and ΔPU.1 substitution mutants were generated by a two-step PCR method. The DNA sequences of all clones generated by PCR were verified prior to use in luciferase assays.

Cells, cell culture, and flow cytometry. Murine neutrophils and promyelocytes were obtained by culture of bone marrow from C57Bl/6 and hCG-NuMA-RAR transgenic mice [16], respectively. Thymocytes and T cells from thymus and spleen were isolated from 6-week-old C57Bl/6 mice by fluorescence-activated cell sorting (FACS) after staining with CD4 and CD8 antibodies. Similarly, B220-expressing B cells were isolated from spleens of C57Bl/6 mice by FACS.

The RAW264.7 murine macrophage cell line [17] and NIH3T3 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS: GibcoBRL), L-glutamine and antibiotics. HCD57 erythroleukaemia cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 0.05 mM 2-mercaptoethanol (2-ME), 0.5 U/mL erythropoietin, and 20% FBS. WEHI-3 and EL-4 cells were grown in DMEM with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.05 mM 2-ME and 10% FBS. MEL3 and M12 cells were grown in RPMI (Gibco) with 4 mM L-glutamine and 10% FBS. Rα30G cells were maintained in Opti-MEM (Gibco) supplemented with 4% WEHI-3 conditioned supernatant and 20% FBS.

Cell surface expression of CD45 was determined in primary cells and cell lines by flow cytometry following incubation with an antibody recognizing all isoforms of CD45 (BD Biosciences; San Jose, CA).

RNA extraction and real-time reverse transcription PCR. RNA was extracted from cell lines or primary cells using Trizol reagent and was purified by column chromatography (RNeasy; Qiagen, Netherlands). Total RNA was reverse transcribed from oligo(dT) primer with MMLV reverse transcriptase (Invitrogen; Burlington, ON, Canada). Quantitative real-time PCR was performed with an Applied Biosystems 7700 Analyzer, according to manufacturer's instructions. Data were analysed by the ΔC_T method, with normalization of all transcript levels to levels of the housekeeping gene *β-actin*. The levels of endogenous CD45 transcripts were quantified by amplification of the 5′ region of either the P1a, P1b, or P2 transcript, using the following primers: sense P1a/P2 (5′-CTT GTC ATA TCT TGG GGA GAC-3′); sense P1b/P2 (5′-AAG ACA GAG TGC AAA GGA GAC-3′); sense P2 (5′-TGC AAA GTA TGC GTT CTT TTC TTT TAG-3′); antisense CD45 exon 2/3 (5′-TGT AGG TGT TTG CCC TGT GAC AAA GAC-3′), sense β-actin (5′-TTC CAG CAG CTG TGG CTA CGA-3′), antisense β-actin (5′-AGT CCG CCT AGA AGC ACT TGC-3′).

Transfections. RAW264.7 cells were transfected transiently by electroporation [18]. Cells were suspended in DMEM with 10% FBS at a density of 3.75×10^7/mL, and 200 μL of cells plus 10 μg of luciferase plasmid and 10 μg of β-galactosidase reporter were electroporated in a 0.4 mm cuvette (Bio-Rad, Hercules CA) in a Bio-Rad Gene Pulser at 250 V and 950 μF. Following a 10 min recovery period at room temperature cells were washed with 5 mL PBS and resuspended in 5 mL DMEM plus 10% FBS. Forty-eight to sixty hours after electroporation cell extracts were prepared in 500 μL lysis buffer (Promega, Madison WI). Luciferase activity was determined from a 20 μL aliquot of cell extract, and β-galactosidase activity from 10 μL of cell extract by the manufacturer's protocol (Promega, Madison WI). Transfections were performed in triplicate.

Nuclear extracts and DNA-binding assays. DNA-binding assays were performed as described by Vyas [19]. Cells (5×10^7) were pelleted, washed twice with PBS, and resuspended in 500 μL of buffer 1, comprising 10 mM Hepes, pH 8, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and protease inhibitor (Complete Protease Inhibitor Cocktail, Roche, Mannheim). After 15 min incubation on ice, 35 μL of 10% NP40 was added and the suspension was mixed well by vortex. Cell debris and nuclei were then pelleted by centrifugation and resuspended in 50 μL of buffer (20 mM Hepes, pH 8, 400 mM NaCl, 1 M EDTA, 1 mM EGTA, 1 mM DTT, and protease inhibitor). After 15 min incubation on ice, the nuclear fraction was isolated by collecting the supernatant after centrifugation.

Table 1
Oligonucleotide sequences for EMSA experiments

−130	ATA ATT ACT TGA ATT AAG GAA GTA
ΔPU.1	ATA ATT ACT TGA ATT AA**T** **TCG** **AG**A
−438	CTC CTT ATG GTA AAT AAC AG
Δoct	CTC C**CG** ATG **C**T**G** **G**AT AAC AG
Oct-c	CCC ATG CAA ATT CCC CCC ATG CAA ATC CCC
TFIID	GCA GAG CAT ATA AGG TGA GGT AGG A

Electrophoretic mobility shift assay (EMSA) probes were prepared by annealing dephosphorylated single-stranded oligonucleotides that had been cassette-purified (ACGT Corporation, Toronto, ON). Probe (800 ng) was end-labelled with [γ-^{32}P]ATP using T4 kinase. Labelled probes were purified by chromatography through a Sephadex G50 column. Nucleotide sequences of oligonucleotide probes are displayed below (Table 1). For EMSA, probe (10^5 cpm) was added to 2 μg of nuclear extract plus 0.25 μM poly(dI–dC) in binding buffer (40 mM Tris–Cl [pH 7.5], 250 mM NaCl, 2.5 mM dithiothreitol, 2.5 mM EDTA, and 20% glycerol) and binding was performed at room temperature for 30 min. EMSA products were separated on a 5% polyacrylamide 0.4× Tris-borate-EDTA gel run at room temperature for 1 h at 25 mA. Unlabelled competitor oligonucleotides were added to binding reactions at 50× excess 10 min prior to addition of probe. For supershift experiments, 1 μL of antibody was added to binding reactions at room temperature 20 min after the first incubation with the probe. Gels were dried and exposed to Kodak Biomax film at −80 °C in the presence of an intensifying screen.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) was utilized to detect in vivo protein–DNA interactions. Cells were grown to a concentration sufficient for the experiment (1×10^7 cells per antibody per time point) and 1% formaldehyde was added to the tissue culture medium to crosslink DNA–protein interactions. After stopping the reaction by the addition of glycine, the cells were washed with PBS and resuspended in cell lysis buffer. The nuclei were recovered by centrifugation and resuspended in nuclei lysis buffer. The nuclear lysate was sonicated to shear the chromatin to an average length of about 600 bp. After centrifugation, the supernatant was precleared by the addition of blocked Staph A cells. Following overnight incubation with 1–3 μg of antibody, an aliquot of G plus beads was added to the samples and the chromatin–protein–antibody-G bead complex was collected by centrifugation. The supernatant from the "no antibody" sample was isolated as the "total input chromatin." The chromatin–protein–antibody complex was eluted from the G bead and incubated with RNase A and NaCl at 67 °C for 5 h to reverse the formaldehyde crosslinks. The chromatin was isolated by ethanol precipitation and resuspended in water. The relative quantity of chromatin between the various samples was analysed by PCR.

Results

P1b is the predominant start site for CD45 transcription in myeloid and lymphoid lineages

Three distinct transcriptional initiation sites—P1a, P1b, and P2—have been defined for murine CD45. It has previously been suggested that P1a transcripts are more abundant in lymphoid than in myeloid cells, but no quantitative data comparing output from the three initiation sites within a lineage or across lineages have been published. We used quantitative real-time reverse transcription polymerase chain reaction (QRT-PCR) to evaluate CD45 start site choice in the haematopoietic cell lines RAW264.7 (late myeloid), WEHI-3 (early myeloid), M12 (B-lymphoid), and EL4 (thymoma-derived T-lymphoid) (Fig. 1A). In RAW264.7 and WEHI-3

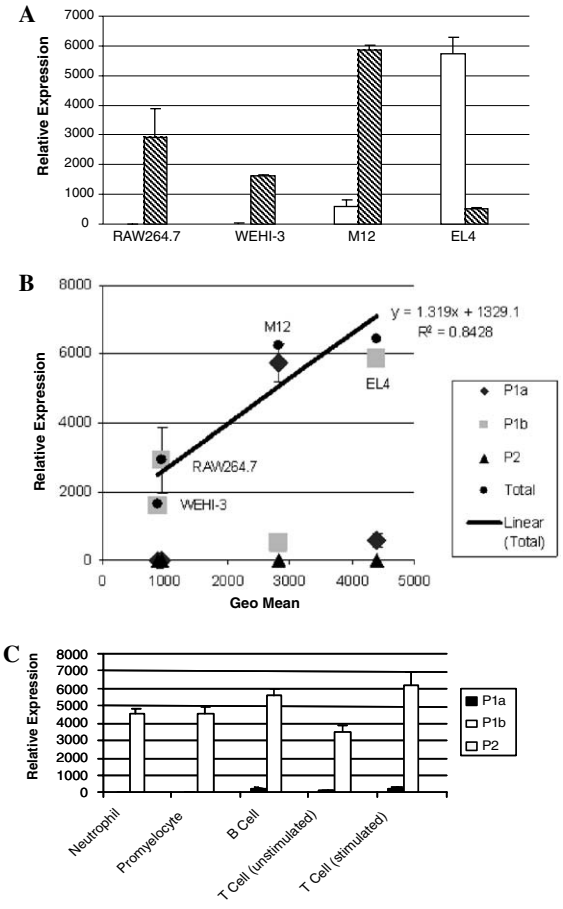

Fig. 1. Quantitative analysis of CD45 mRNA expression. Real-time RT-PCR was used to quantify endogenous CD45 transcripts originating from three mutually exclusive transcriptional start sites (P1a, P1b, and P2) in murine haematopoietic cell lines (A) and primary murine haematopoietic cells (C). Transcript levels were normalized against levels of β-actin mRNA in each specimen and are expressed relative to the P1a transcript level in RAW264.7 cells (for A) or to P1a transcript level in neutrophils (for B). Total transcript levels (P1a + P1b + P2) correlate significantly with cell surface CD45 protein expression as measured by flow cytometry (C).

the output of P1b is much greater than that of P1a (P1b:P1a 2916:1 and 217:1, respectively). In M12 cells the contribution of P1a is greater (ratio = 10:1), while in EL4 cells the ratio is reversed, with P1a transcripts being more abundant than P1b transcripts (ratio = 1:10). However, cell surface expression of CD45 reflects total CD45 transcript level, irrespective of initiation site (Fig. 1B).

CD45 transcript initiation site choice in primary haematopoietic cells reflects that seen for immortalized cell lines. All detectable transcripts in murine myeloid cells (neutrophils and promyelocytes) were P1b-derived. While B- and T-lymphocytes had detectable P1a transcripts, with the highest P1a transcript levels seen in GP33-stimulated T cells, P1b transcripts remained overwhelmingly predominant in lymphocytes as well (Fig. 1C). Notably, P2 transcripts accounted for less than 0.1% of CD45 transcripts in all cell lines and cells tested.

Functional analysis of 5′ deletion mutants of the CD45 upstream region

Sequence in the vicinity of the CD45 transcriptional start sites exhibits substantial sequence similarity between the murine and human loci (Fig. 2). These blocks incorporate the start sites and corresponding exons, as well as a portion of intron 1a. Since P1b is functionally predominant we elected to examine the sequence immediately upstream of it for potential regulatory elements. An upstream segment containing P1b and flanking sequence from position −379 to the ATG codon was cloned in the luciferase reporter vector pGL3B. This construct was transfected transiently into the CD45-expressing murine cell lines RAW264.7, M12, and EL4, representing myeloid, B-lymphoid, and T-lymphoid lineages, respectively. Assessment of luciferase activity confirmed that this upstream region is sufficient to support the accurate initiation of transcription (Fig. 3). To identify potential regulatory elements in the *CD45* upstream region, we undertook an unbiased mutational analysis employing 5′ deletions. Six deletion mutants were cloned in pGL3B and were tested for ability

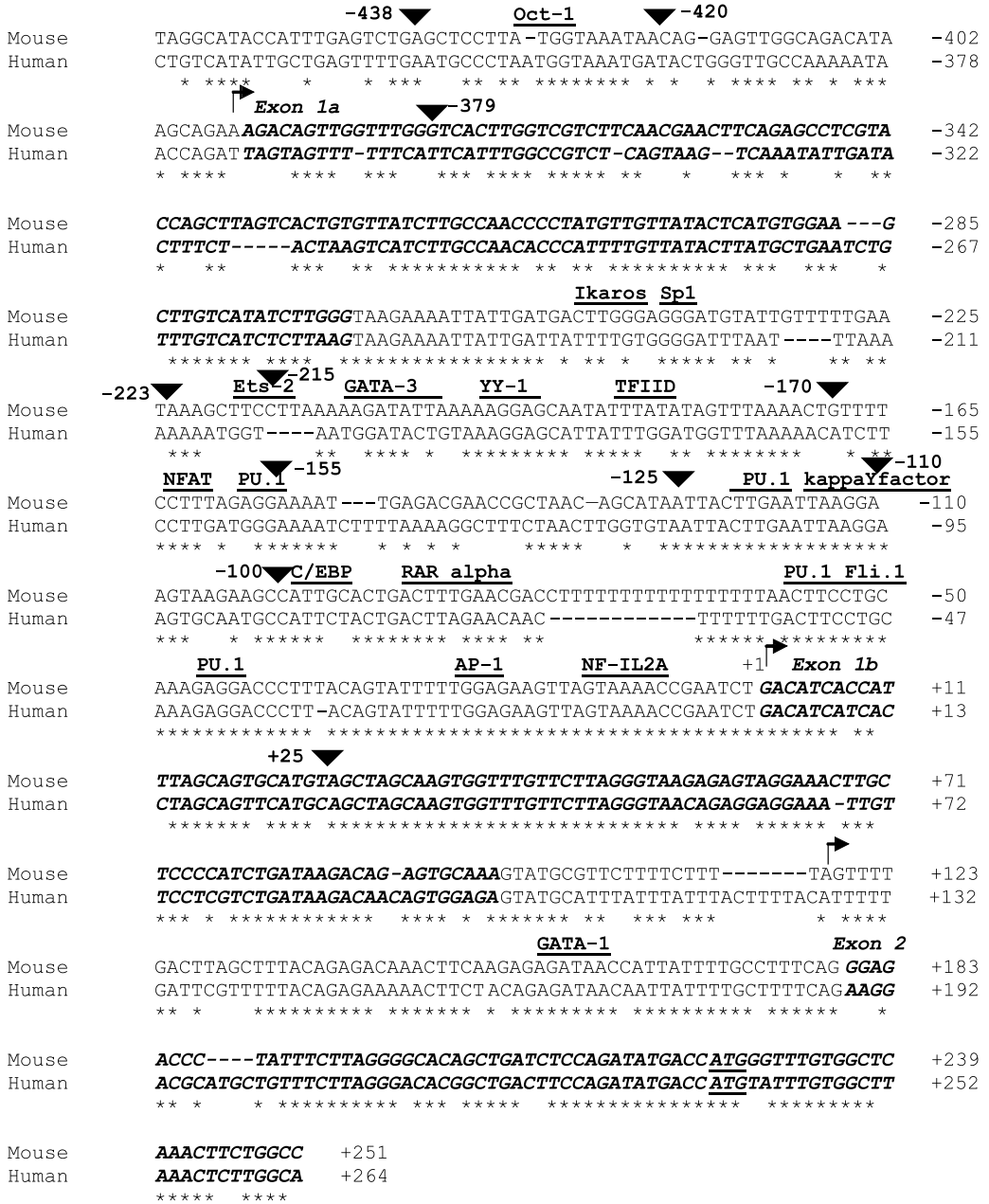

Fig. 2. Murine and human genomic sequence in the region of the transcriptional start sites. Comparison of genomic sequences of the mouse and human CD45 proximal homology region (PHR). The PHR contains the alternative 5′ exons 1a, 1b, and 2. Exonic sequence is italicized. The major transcriptional start site at P1a is designated +1. Asterisks indicate nucleotide identity between mouse and human sequences. The translational start site is underlined. Putative transcription factor binding sites are labelled. The endpoints of 5′ deletion mutants are indicated with triangles.

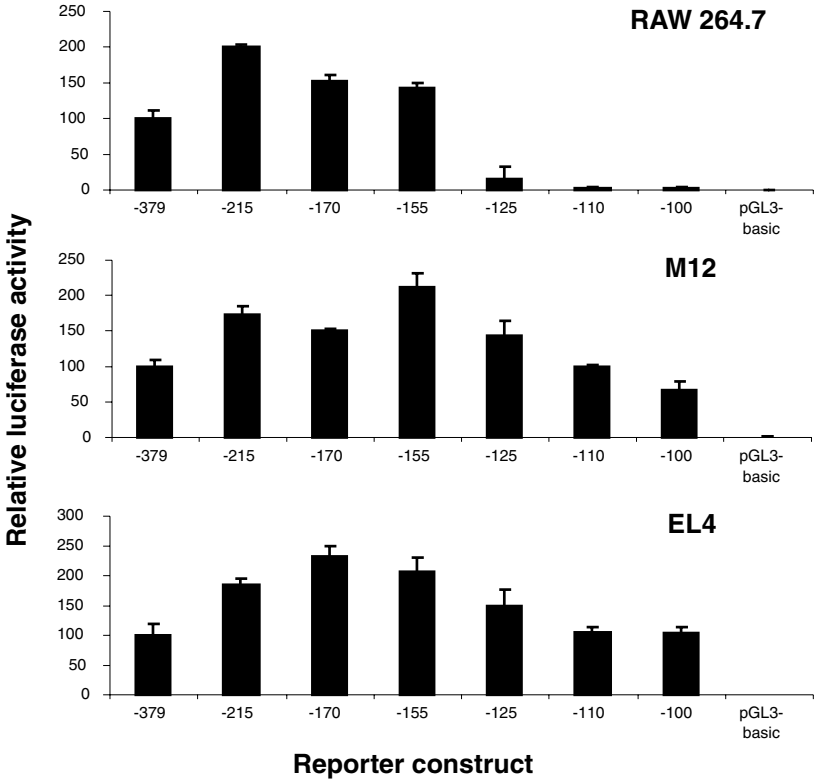

Fig. 3. Transcriptional activation in myeloid and lymphoid cells by sequences upstream of P1b. Luciferase assays were performed after transient transfection of reporter constructs into RAW264.7, M12, and EL4 cells as indicated. Each experiment was performed in triplicate and repeated on at least two separate occasions. Co-transfection of a β-galactosidase plasmid was used to normalize for transfection efficiency. CD45 upstream fragments with 5′ ends at positions −379, −215, −170, −155, −125, −110, and −100 were cloned upstream of the luciferase reporter gene in pGL3basic. Results are expressed as luciferase activity relative to the −379 construct. Error bars show standard deviation.

to activate luciferase transcription in transiently transfected RAW264.7, M12, and EL4 cells.

Distinct patterns of reporter activity were seen in myeloid vs lymphoid cells. In RAW264.7 cells, reporter activity declined sharply after deletion of sequence between −155 and −125, and constructs with 5′ endpoints at −110 and −100 showed no activity over empty vector controls. In M12 and EL4 cells, however, reporter activity was maintained even in the −100 construct.

These data are consistent with the presence of an indispensable positive regulatory element for transcription of CD45 in myeloid cells in the interval between −125 and −110. This interval lies within the block of strongly conserved sequence between exon 1a and exon 1b (Fig. 2) and contains a previously described ets-binding site (Fig. 3) [13].

P2 is sufficient to support transcription in lymphoid but not myeloid cells

To test whether an intact P1b start site is required to support initiation of transcription in myeloid and lymphoid cells, further 5′ deletions were made through P1b to position +25, removing P1a but preserving the P2 start site. While no reporter activity was seen in RAW264.7 (myeloid) cells in the +25 construct, activity similar to that seen for the −379 construct was seen for the +25 construct in both M12 (B-lymphoid) and EL4 (T-lymphoid) cells (Fig. 4). Thus, the presence of the P2 start site is sufficient to support CD45 reporter transcription in lymphoid cells.

PU.1 activates CD45 transcription in myeloid cells

An oligonucleotide probe that spans the critical interval (−125 to −110) was designed for EMSA experiments. EMSA with this probe as well as with a probe in which the PU.1-binding sequence is mutated (ΔPU.1) revealed PU.1-specific binding activity in RAW264.7 nuclear extracts (Fig. 5A). It has previously been reported that CD45 expression is absent from myeloid cells in PU.1 null mutant mice, suggesting that PU.1 is required specifically for expression of CD45 in the myeloid lineage [13]. Incubation with PU.1-specific antibody resulted in supershift of the most prominent DNA–protein complex, identifying it as PU.1.

To confirm the importance of the ets site in regulation of CD45 transcription, the ets-binding sequence was mutated in the context of the −379 and −223 CD45 upstream luciferase reporter constructs. Both mutant constructs exhibited markedly reduced ability to support transcription in RAW264.7 (Fig. 5B). In contrast, in cells representing erythroid (HDC57), B-lymphoid (MEL3), and T-lymphoid

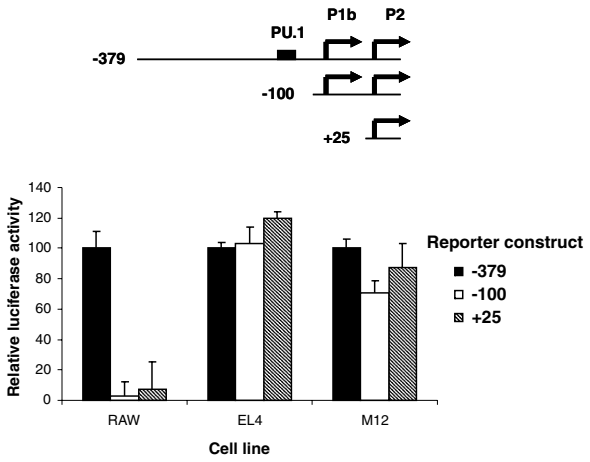

Fig. 4. P2 supports initiation of CD45 transcription in B- and T- lymphoid cells but not in myeloid cells. 5′ deletion constructs ending at −379, −100, and +25 were assayed for their ability to support luciferase reporter gene expression after transient transfection into RAW264.7, M12, and EL4 cells. Each experiment was performed in triplicate, and repeated on at least two separate occasions. Co-transfection of a β-galactosidase plasmid was used to normalize for transfection efficiency. Results are expressed as luciferase activity relative to the −379 construct. Error bars show standard deviation.

(EL4) lineages, no reduction in reporter activity was seen following mutation of the ets site (data not shown). These data confirm the myeloid lineage-specificity of PU.1 in regulation of CD45 expression.

We used chromatin immunoprecipitation (ChIP) of nuclear extracts from haematopoietic cell lines representing early and late myeloid cells to confirm the in vivo binding of PU.1 to the CD45 ets site (Fig. 5C). PU.1 binding was seen in both early (WEHI-3) and late (RAW264.7) myeloid cell nuclear extracts.

An Oct site upstream of P1a activates transcription in myeloid and lymphoid lineages

A consensus Octamer-binding site (Oct site) at position −430, upstream of P1a, is conserved in the mouse and human CD45 loci. We used luciferase reporter assays to assess the importance of this sequence in regulation of CD45 transcription. Deletion or mutation of the Oct site resulted in ∼30% reduction in reporter activity in RAW264.7, M12, and EL4 cells (Fig. 6). EMSA confirms that Oct-1 protein binds the Oct-1 site in RAW264.7 and M12 extracts, while both Oct-1 and Oct-2 binding is seen in EL4 extracts.

EMSA was performed to detect protein interactions with this sequence, using a labelled oligonucleotide that spans the −438/−420 interval (Fig. 7A). A single DNA–protein complex (Complex I) that comigrates with that formed with an Octamer consensus probe was seen in nuclear extracts of RAW264.7 and M12 cells. In EL4 cells two additional complexes (Complexes II and III) are seen. All three complexes are competed with a 50-fold excess of an unlabelled Octamer consensus oligonucleotide but not with an unrelated cold oligonucleotide, establishing that the interactions seen occur specifically with the Octamer sequence. Antibody supershift experiments were undertaken to identify the proteins binding to the Oct site in these cells

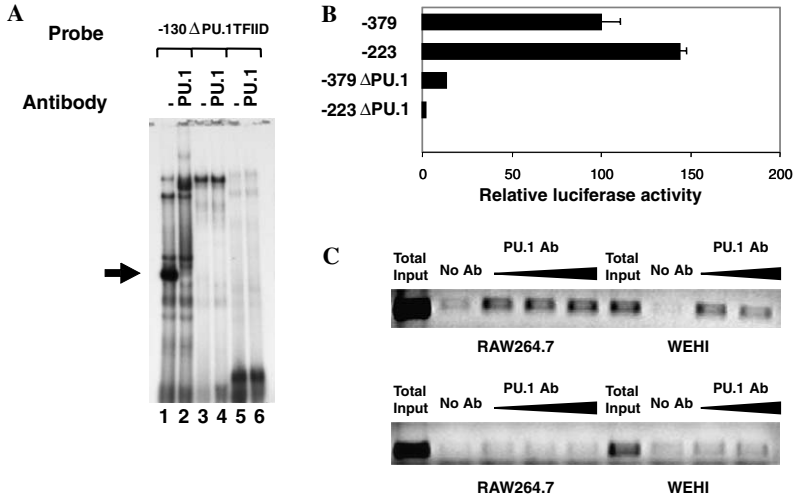

Fig. 5. A PU.1-binding site at −125/−110 is essential for reporter activity in myeloid cells. (A) Electrophoretic mobility shift assay and antibody supershifts. RAW264.7 nuclear extract was incubated with a radiolabelled double-stranded oligonucleotide probe containing the PU.1-binding site (−130), an oligonucleotide with a mutation of the PU.1 site (ΔPU.1) or with an irrelevant oligonucleotide (TFIID), and then electrophoresed through a non-denaturing polyacrylamide gel. Autoradiography reveals a prominent gelshift (arrow) with the −130 probe that is not seen with the TFIID or ΔPU.1 probes and which is supershifted by co-incubation with a specific PU.1 antibody. (B) Luciferase reporter assays show near total loss of transcriptional activity in CD45 constructs with endpoints at position −379 and −223 when the PU.1 site is mutated. (C) Chromatin immunoprecipitation. Cells from the myeloid lines RAW264.7 and WEHI-3 were treated with 1% formaldehyde to crosslink NDA–protein complexes. After fragmentation by sonication, chromatin was immunoprecipitated with 1, 2, or 3 μg of PU.1 antibody. DNA extracted from immunoprecipitates was used as template for polymerase chain reaction with primers flanking the PU.1-binding sequence at −125/−110 (between positions −215 and +254; upper panel) or with primers further upstream (between positions −689 and −391; lower panel). PCR products were analysed by agarose gel electrophoresis.

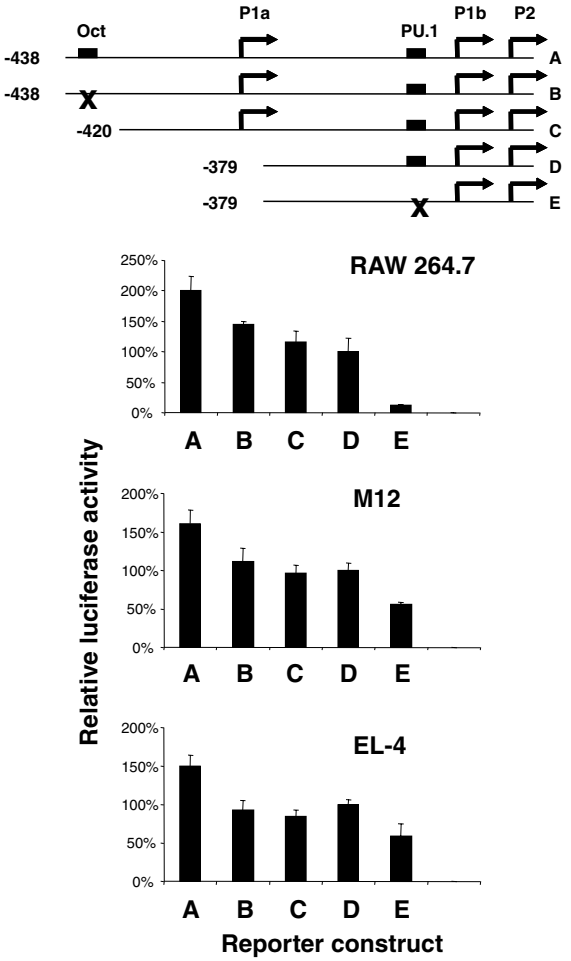

Fig. 6. An Octamer-binding site upstream of P1a activates CD45 transcription in myeloid and lymphoid cells. CD45 reporter constructs with 5′ endpoints at positions −438 (construct A), −420 (construct C), and −379 (construct D) were assayed for their ability to support luciferase reporter gene expression after transient transfection into RAW264.7, M12, and EL4 cells. Deletion of sequence between −438 and −420 resulted in decreased reporter activity in all three cell lines. Specific mutation of a potential Octamer-binding site in this interval (construct B) caused a similar reduction in reporter activity, while the activity of a construct in which the PU.1 site at −125/−110 was mutated (construct E), which showed no activity in RAW264.7 cells, was unaffected in M12 or EL4 cells. Each experiment was performed in triplicate, and repeated on at least two separate occasions. Co-transfection of a β-galactosidase plasmid was used to normalize for transfection efficiency. Results are expressed as luciferase activity relative to the −379 construct. Error bars show standard deviation.

(Fig. 7B). In all three cell lines a partial supershift of Complex I was detected with Oct-1 antibody, identifying this binding activity as Oct-1. In EL4 cells Complex II was abrogated completely on pre-incubation with Oct-1 antibody, consistent with this complex containing a degradation product of Oct-1. Meanwhile, Oct-2 antibody resulted in abrogation and supershift of Complex III, identifying Oct-2 as the binding activity ion of this complex. Thus, the −438/−420 Octamer-binding site binds both Oct-1 and Oct-2 proteins, depending upon cell context.

Discussion

CD45 is a transmembrane protein tyrosine phosphatase that is expressed in all nucleated haematopoietic cells, and plays a key role in regulation of cellular proliferation by dephosphorylating Src- and Jak-family kinases [8]. The regulatory mechanisms permitting the initiation and maintenance of CD45 expression in multiple haematopoietic lineages are not understood.

The functional relevance of the three distinct transcriptional start sites of CD45, (P1a, P1b, and P2) is unknown. We used quantitative real-time RT-PCR to measure the relative contributions of the three start sites to CD45 transcription in cell lines and cells representing myeloid and lymphoid lineages. It has previously been suggested that the major start sites are lineage-restricted, with P1a utilized in lymphoid cells, and P1b in myeloid cells. Our data indicate that P1b transcripts predominate in both myeloid and lymphoid cells, although the relative contribution of P1a is greater in the lymphoid lineage. Interestingly, P1a transcripts are more abundant in GP33-stimulated T cells than in resting T cells; we conjecture that P1a may be utilized to allow rapid upregulation of CD45 transcription when needed. A striking exception to the general rule of P1b predominance is the EL4 cell line, in which P1a transcripts are ~10 times as abundant as P1b transcripts.

To gain insight into transcriptional regulation of CD45, we undertook a structure–function analysis of the region 438 bp immediately upstream of the coding region, including the P1a, P1b, and P2 promoters. Functional analysis of a series of 5′ deletion mutants through this region suggested the presence of an important positive regulatory element between −125 and −110. This interval contains a PU.1-binding sequence that has previously been reported to interact with PU.1 in the macrophage cell line RAW 264.7.

Anderson et al. [13] have postulated a role for PU.1 in control of CD45 transcription based upon observations in the PU.1 null mouse. CD45 is not expressed at detectable levels in $CD18^{+}$ myeloid cells cultured from PU.1 null foetal liver, whereas CD45 expression is normal in PU.1 null B- and T-lymphocytes. Furthermore, CD45 expression is restored to a PU.1 null myeloid cell line following forced expression of PU.1. They reported that in vitro translated PU.1 could bind to the same upstream PU.1 site that we have identified by unbiased mutational analysis in this report. Transcription of a reporter gene under the control of the CD45 upstream region was greatly enhanced by co-transfection of a PU.1 expression plasmid, and CD45 expression could be induced in PU.1 null myeloid cell lines by forced expression of PU.1. These authors concluded that PU.1 is necessary for CD45 expression in myeloid cells. The data we present here support this view. We find PU.1 interaction with the PU.1 site in mature macrophages and in immature myeloid cells (WEHI-3) but not in B- or T-lymphoid cells. Evidence that this binding is relevant to transcriptional regulation of this gene in myeloid cells comes from reporter assays of the ΔPU.1 substitution mutant, which show significant loss of

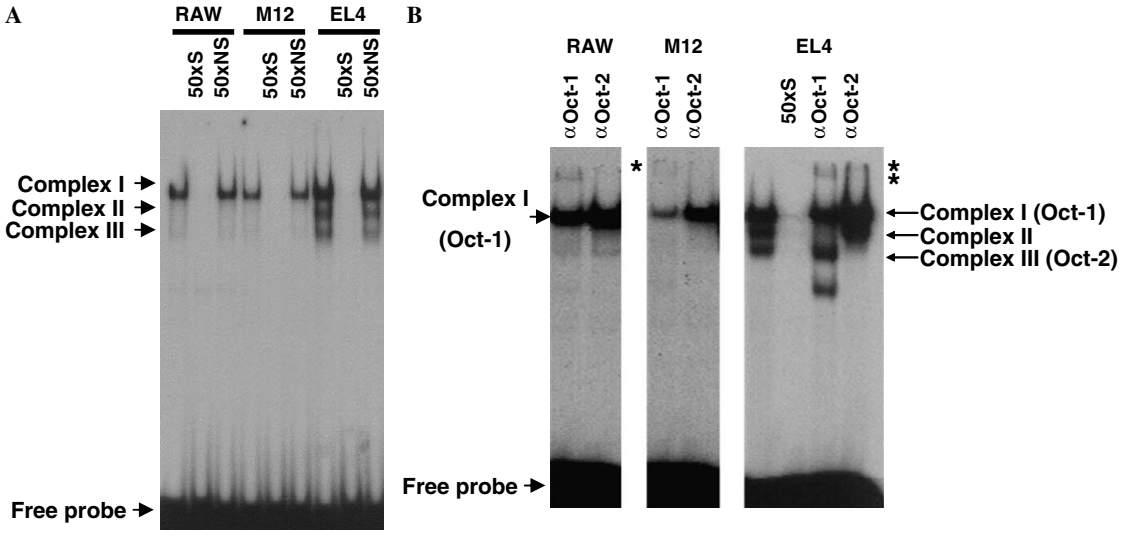

Fig. 7. The Octamer site at −438/−420 interacts with both Oct-1 and Oct-2 in haematopoietic cells. Electrophoretic mobility shift assay (A) and antibody supershifts (B). Nuclear extracts from RAW264.7, M12, and EL4 were incubated with a radiolabelled double-stranded oligonucleotide probe containing the Octamer sequence (P1aR) in the presence or absence of specific (Oct consensus) or non-specific (TFIID) cold competitor oligonucleotide, and then electrophoresed through a non-denaturing polyacrylamide gel. Supershifts with Oct-1 and Oct-2 antibodies are indicated by asterisks.

activity in WEHI-3 cells as well as in RAW264.7 cells. Using ChIP, we have demonstrated in vivo PU.1 binding to the CD45 PU.1 site in a mature macrophage cell line (RAW264.7) and in a primitive myeloid line (WEHI-3). Our observations, taken together with the data from the PU.1 null mouse, are consistent with a role for PU.1 in the maintenance of CD45 expression in myeloid cells, where PU.1 is very abundant. However, neither the initiation nor the maintenance of CD45 expression in the lymphoid lineages appears to be regulated by PU.1, while the control of its expression in very early haematopoietic progenitors remains an open question.

Octamer factors have not previously been implicated in the regulation of CD45 expression. The POU (Pit-Oct-Unc) family of transcription factors plays critical roles in the regulation of gene expression in multiple cell types. Two POU domain proteins, the ubiquitously expressed Oct-1 and the lymphoid-specific Oct-2, have been shown to be involved in haematopoietic differentiation. Oct-1 regulates expression of IL-2, IL-3, IL-5, IL-8, GM-CSF, and the light and heavy chains of immunoglobulins [20–26], while Oct-2 regulates CD36 [27] and CRISP-3 [28] expression. In several instances, as we have found for CD45, Octamer factors and PU.1 co-regulate lymphoid expression of genes [29–32].

The thymoma-derived T-lymphoid cell line EL4 has previously been shown to express Oct-2 constitutively at a high level [33]. Consistent with this we see prominent Oct-2 binding of the CD45 Octamer site in EMSA. It is tempting to conjecture that Oct-2 binding upstream of the P1a transcriptional start site contributes to the extraordinary activity of P1a observed in EL4 cells.

The initiation and maintenance of CD45 transcription in haematopoietic cells is complex and evidently relies on the interaction of multiple transcription factors with cis acting DNA elements. The evidence we present here indicates that overlapping regulatory systems operate for CD45 expression in myeloid cells, B-lymphocytes, and T-lymphocytes. We demonstrate that PU.1 directly activates myeloid CD45 transcription via interaction with the PU.1 site at −125/−110, while an Octamer consensus sequence at −438/−420, which can interact with Oct-1 and Oct-2, activates CD45 transcription in myeloid and B- and T-lymphoid cells. It is likely that other cis regulatory elements are necessary to establish the unique expression pattern of CD45; in particular, a block of sequence 2.5 kb upstream of the P1b start site that shows high interspecies conservation, and the long second intron of CD45 are strong candidates for these elements. Further work that elucidates the complexities of CD45 regulation will improve our understanding of early events in haematopoiesis, and may lead to development of very useful tools for directing transgene expression to the haematopoietic compartment of the mouse.

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References

[1] N.A. Johnson, C.M. Meyer, J.T. Pingel, M.L. Thomas, Sequence conservation in potential regulatory regions of the mouse and human leukocyte common antigen gene, J. Biol. Chem. 264 (1989) 6220–6229.

[2] I.S. Trowbridge, M.L. Thomas, CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development, Annu. Rev. Immunol. 12 (1994) 85–116.
[3] Y. Saga, J.S. Tung, F.W. Shen, E.A. Boyse, Sequences of Ly-5 cDNA: isoform-related diversity of Ly-5 mRNA [published erratum appears in Proc. Natl. Acad. Sci. USA 84 (7) (1987) 1991], Proc. Natl. Acad. Sci. USA 83 (1986) 6940–6944.
[4] W.C. Raschke, Cloned murine T200 (Ly-5) cDNA reveals multiple transcripts within B- and T-lymphocyte lineages, Proc. Natl. Acad. Sci. USA 84 (1987) 161–165.
[5] M. Streuli, L.R. Hall, Y. Saga, S.F. Schlossman, H. Saito, Differential usage of three exons generates at least five different mRNAs encoding human leukocyte common antigens, J. Exp. Med. 166 (1987) 1548–1566.
[6] K. Kishihara, J. Penninger, V.A. Wallace, T.M. Kundig, K. Kawai, A. Wakeham, E. Timms, K. Pfeffer, P.S. Ohashi, M.L. Thomas, et al., Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice, Cell 74 (1993) 143–156.
[7] T. Roach, S. Slater, M. Koval, L. White, E.C. McFarland, M. Okumura, M. Thomas, E. Brown, CD45 regulates Src family member kinase activity associated with macrophage integrin-mediated adhesion, Curr. Biol. 7 (1997) 408–417.
[8] J. Irie-Sasaki, T. Sasaki, W. Matsumoto, A. Opavsky, M. Cheng, G. Welstead, E. Griffiths, C. Krawczyk, C.D. Richardson, K. Aitken, N. Iscove, G. Koretzky, P. Johnson, P. Liu, D.M. Rothstein, J.M. Penninger, CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling, Nature 409 (2001) 349–354.
[9] R. Ratei, C. Sperling, L. Karawajew, G. Schott, M. Schrappe, J. Harbott, H. Riehm, W.D. Ludwig, Immunophenotype and clinical characteristics of CD45-negative and CD45-positive childhood acute lymphoblastic leukemia, Ann. Hematol. 77 (1998) 107–114.
[10] T. Jaffredo, R. Gautier, A. Eichmann, F. Dieterlen-Lievre, Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny, Development 125 (1998) 4575–4583.
[11] R. Taetle, H. Ostergaard, M. Smedsrud, I. Trowbridge, Regulation of CD45 expression in human leukemia cells, Leukemia 5 (1991) 309–314.
[12] Y. Saga, J.S. Tung, F.W. Shen, T.C. Pancoast, E.A. Boyse, Organization of the Ly-5 gene, Mol. Cell. Biol. 8 (1988) 4889–4895.
[13] K.L. Anderson, S.L. Nelson, H.B. Perkin, K.A. Smith, M.J. Klemsz, B.E. Torbett, PU.1 is a lineage-specific regulator of tyrosine phosphatase CD45, J. Biol. Chem. 276 (2001) 7637–7642.
[14] J.F. DiMartino, P. Hayes, Y. Saga, J.S. Lee, A novel initiator/promoter element within the CD45 upstream region, Int. Immunol. 6 (1994) 1279–1283.
[15] E.L. Virts, W.C. Raschke, The role of intron sequences in high level expression from CD45 cDNA constructs, J. Biol. Chem. 276 (2001) 19913–19920.
[16] M.A. Sukhai, X. Wu, Y. Xuan, T. Zhang, P.P. Reis, K. Dube, E.M. Rego, M. Bhaumik, D.J. Bailey, R.A. Wells, S. Kamel-Reid, P.P. Pandolfi, Myeloid leukemia with promyelocytic features in transgenic mice expressing hCG-NuMA-RARalpha, Oncogene 23 (2004) 665–678.
[17] P. Ralph, I. Nakoinz, Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: enhancement by PPD and LPS, J. Immunol. 119 (1977) 950–954.
[18] X. Ma, J.M. Chow, G. Gri, G. Carra, F. Gerosa, S.F. Wolf, R. Dzialo, G. Trinchieri, The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells, J. Exp. Med. 183 (1996) 147–157.
[19] P. Vyas, M.A. McDevitt, A.B. Cantor, S.G. Katz, Y. Fujiwara, S.H. Orkin, Different sequence requirements for expression in erythroid and megakaryocytic cells within a regulatory element upstream of the GATA-1 gene, Development 126 (1999) 2799–2811.
[20] K.S. Ullman, W.M. Flanagan, C.A. Edwards, G.R. Crabtree, Activation of early gene expression in T lymphocytes by Oct-1 and an inducible protein, OAP40, Science 254 (1991) 558–562.
[21] G.D. Wu, E.J. Lai, N. Huang, X. Wen, Oct-1 and CCAAT/enhancer-binding protein (C/EBP) bind to overlapping elements within the interleukin-8 promoter. The role of Oct-1 as a transcriptional repressor, J. Biol. Chem. 272 (1997) 2396–2403.
[22] K. Kaushansky, S.G. Shoemaker, C.A. O'Rork, J.M. McCarty, Coordinate regulation of multiple human lymphokine genes by Oct-1 and potentially novel 45 and 43 kDa polypeptides, J. Immunol. 152 (1994) 1812–1820.
[23] C.S. Malone, L. Patrone, R. Wall, An essential octamer motif in the mb-1 (Igalpha) promoter, Mol. Immunol. 37 (2000) 321–328.
[24] C.S. Malone, L. Patrone, K.L. Buchanan, C.F. Webb, R. Wall, An upstream Oct-1- and Oct-2-binding silencer governs B29 (Ig beta) gene expression, J. Immunol. 164 (2000) 2550–2556.
[25] V. Tovar, M.A. de la Fuente, P. Pizcueta, J. Bosch, P. Engel, Gene structure of the mouse leukocyte cell surface molecule Ly9, Immunogenetics 51 (2000) 788–793.
[26] P.C. Shah, E. Bertolino, H. Singh, Using altered specificity Oct-1 and Oct-2 mutants to analyze the regulation of immunoglobulin gene transcription, EMBO J. 16 (1997) 7105–7117.
[27] H. Konig, P. Pfisterer, L.M. Corcoran, T. Wirth, Identification of CD36 as the first gene dependent on the B-cell differentiation factor Oct-2, Genes Dev. 9 (1995) 1598–1607.
[28] P. Pfisterer, H. Konig, J. Hess, G. Lipowsky, B. Haendler, W.D. Schleuning, T. Wirth, CRISP-3, a protein with homology to plant defense proteins, is expressed in mouse B cells under the control of Oct2, Mol. Cell. Biol. 16 (1996) 6160–6168.
[29] H. Chen, P. Zhang, H.S. Radomska, C.J. Hetherington, D.E. Zhang, D.G. Tenen, Octamer binding factors and their coactivator can activate the murine PU.1 (spi-1) promoter, J. Biol. Chem. 271 (1996) 15743–15752.
[30] A. Sjoblom, A. Jansson, W. Yang, S. Lain, T. Nilsson, L. Rymo, PU box-binding transcription factors and a POU domain protein cooperate in the Epstein-Barr virus (EBV) nuclear antigen 2-induced transactivation of the EBV latent membrane protein 1 promoter, J. Gen. Virol. 76 (Pt. 11) (1995) 2679–2692.
[31] B. Kistler, P. Pfisterer, T. Wirth, Lymphoid- and myeloid-specific activity of the PU.1 promoter is determined by the combinatorial action of octamer and ets transcription factors, Oncogene 11 (1995) 1095–1106.
[32] H. Schwarzenbach, J.W. Newell, P. Matthias, Involvement of the Ets family factor PU.1 in the activation of immunoglobulin promoters, J. Biol. Chem. 270 (1995) 898–907.
[33] M.P. Kamps, L. Corcoran, J.H. LeBowitz, D. Baltimore, The promoter of the human interleukin-2 gene contains two octamer-binding sites and is partially activated by the expression of Oct-2, Mol. Cell. Biol. 10 (1990) 5464–5472.