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Transcriptional Regulation of Human CD5: Important Role of Ets Transcription Factors in CD5 Expression in T Cells

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CD5 is a surface receptor constitutively expressed on thymocytes and mature T and B-1a cells. CD5 expression is tightly regulated during T and B cell development and activation processes. In this study we show that the constitutive expression of CD5 on human T cells correlates with the presence of a DNase I-hypersensitive (DH) site at the 5′-flanking region of CD5. Human CD5 is a TATA-less gene for which 5′-RACE analysis shows multiple transcriptional start sites, the most frequent of which locates within an initiator sequence. Luciferase reporter assays indicate that a 282-bp region upstream of the initiation ATG displays full promoter activity in human T cells. Two conserved Ets-binding sites (at positions −239 and −185) were identified as functionally relevant to CD5 expression by site-directed mutagenesis, EMSAs, and cotransfection experiments. A possible contribution of Sp1 promoter activity in human T cells. Two conserved Ets-binding sites (at positions

D5 is a 67-kDa membrane glycoprotein belonging to the ancient and conserved family of receptors containing extracellular domains of the scavenger receptor cysteine-rich type (1). CD5 is a lymphoid-specific molecule expressed on thymocytes, mature T cells, and a subset of normal B cells, known as B-1a cells (2). CD5 also exists as a circulating protein in serum, resulting from proteolytic cleavage following lymphocyte activation (3).

The precise functional role of CD5 is not yet fully understood. However, it is currently accepted that CD5 behaves as a dual accessory receptor, able to modulate, either positively or negatively, activation and differentiation signals delivered by the Ag-specific receptor on different cell types and at different developmental stages (4). CD5 is found associated with the Ag-specific receptor complex expressed on T cells (5–7) and B-1a cells (8) and is recruited to the immunological synapse in an Ag-specific manner (9, 10). The CD5 cytoplasmic tail is devoid of intrinsic enzymatic activity, but it is well adapted for signal transduction. It has been shown that the CD5 signaling pathway involves several signal-transducing molecules, such as casein kinase II (11, 12), Src family tyrosine kinases (13, 14), phosphatidylinositol 3-kinase (15, 16), c-Cbl (15, 17), Ras GTPase-activating protein (17), phosphatidylinositol-specific phospholipase C (18), acidic sphingomyelinase (19), protein kinase Cζ (19), Ca2+/calmodulin-dependent kinases (20, 21), and members of the mitogen-activated protein kinases (19).

Surface expression of CD5 is tightly regulated during T cell development in the thymus. Low levels of CD5 are detected on immature CD4+CD8− (double-negative) thymocytes, independently of TCR rearrangement (22). A further increase in CD5 expression on double-negative thymocytes is seen after engagement of the pre-TCR (22). Higher surface CD5 levels are achieved later at both double-positive (CD4+CD8+) and single-positive (CD4+CD8− or CD4-CD8+) stages as a consequence of engagement of the TCRαβ by (positively or negatively) selecting ligands (22). Interestingly, the expression level of CD5 in mature thymocytes and circulating T cells has been correlated with the avidity of the TCR-ligand complex (22, 23).

In peripheral lymphocytes, CD5 surface levels are susceptible to either up- or down-regulation in response to different experimental and/or pathological situations. Protein kinase C activators have been shown to up-regulate CD5 expression on human T (24–26) and B cells (26–28). CD5 up-regulation is also observed on peripheral T cells after TCR/CD3 cross-linking (24, 25) and on murine B cells after IgM cross-linking (26, 29, 30). The v-H-Ras oncogene has been demonstrated as an inducer of CD5 expression on pre-B cell lines established from mouse bone marrow (31), indicating that the Ras signaling pathway could be involved in controlling CD5 expression. Interestingly, as described for CD28 and CD6 costimulatory molecules, reduced CD5 levels are found on T cells from patients suffering from acute herpes virus infections (32).

Human CD5 maps to chromosome 11q13 adjacent to CD6, another member of the scavenger receptor cysteine-rich family (33). A similar organization has been reported for their respective
mouse homologues at the syntenic region of mouse chromosome 19 (34). In both humans and mice, the 5′-flanking region of the CD5 gene is devoid of a TATA sequence and is flanked by a highly polymorphic microsatellite (CA)x repeat, which is positioned at −608 and −748 bp from the translation initiation codon, respectively (35). A high degree of overall sequence homology (70%) is detected when comparing the 5′-flanking regions of the human and mouse genes. Interestingly, the highest homology (80%) is found in a short DNA stretch (from −249 to −110 in the human sequence), including Ets (at positions −239 and −185) and E box (−198) binding sites. This suggests that these conserved regulatory elements may play an important role in controlling the tissue-specific expression of human and mouse CD5 (36). Accordingly, reduced levels of CD5 have been reported on T cells from mice deficient in some members of the helix-loop-helix family of transcription factors, which bind E boxes (38, 39). Although some information is available on the transcriptional regulation of CD5 in the mouse (40, 41), nothing is known about this in the human. As a first approach to decipher the regulatory elements controlling the expression of CD5 on human cells, the putative human CD5 promoter region was functionally characterized. In this study it is shown that the constitutive expression of CD5 on T cells correlates with the presence of a DNase I-hypersensitive (DH) site just at the 5′-flanking region of CD5. CD5 lacks a TATA box, but presents an initiator-like sequence and multiple transcription initiation sites. Transfection assays with gene reporter constructs showed that a 282-bp region upstream of the initiation ATG accounted for the T cell-specific expression of CD5. Within that minimal promoter region, two conserved Ets binding sites at positions −185 and −239 were shown to be critical for promoter activity in T cells. This indicates that Ets proteins are important transcriptional activators of human CD5, as reported for other T cell-specific and developmentally regulated genes, such as TCRα (42, 43), TCRβ (44, 45), IL-2R (46), IL-5 (47), and CD4 (48, 49). Furthermore, an inducible DH site has been located 10 kb upstream of the human CD5 in both T and B CD5+ cells. Interestingly, a sequence showing high homology with a murine IgM-inducible enhancer is found there, which suggests that the DH site may be a functional DNA element controlling CD5 expression in both T and B cells.

Materials and Methods
DH site analyses and Southern blot analysis

Nuclei isolations and DNase I digestions were performed essentially as previously described (50). Briefly, nuclei were isolated by cell disruption in 0.1% Nonidet P-40 and resuspended to −0.4 mg/ml total nuclear acid. Aliquots of nuclei were digested for 3 min at 22°C with DNase I (Worthington, Lakewood, NJ) at 2–60 U/ml in nuclei isolation buffer containing 1 mM CaCl2. After DNase I titration for each cell, optimally digested samples were digested either with MfeI, SacI, or XbaI; electrophoresed on a 0.8% agarose gel; and blotted onto a Hybond N nylon membrane (Amersham Pharmacia Biotech). Clones were sequenced by guest on May 24, 2022 http://www.jimmunol.org/ Downloaded from

Indirect immunofluorescence assays and FACS analysis

Indirect immunofluorescence was performed using FITC-conjugated goat anti-mouse polyclonal Ig antiserum (Sigma-Aldrich, St. Louis, MO) as secondary Ab. Primary Abs were Cris-1 (CD5), OKT-3 (CD3), and 9.1B3 (CD20) mouse mAb. Cris-1 and 9.1B3 mAb were produced in our laboratory by Dr. R. Villella (Hospital Clinic, Barcelona, Spain), and OKT-3 was obtained from Ortho Diagnostics (Raritan, NJ). Cells were analyzed by flow cytometry using the FACSscan analyzer and CellQuest software (BD Biosciences, Mountain View, CA).

Sequence analysis

The consensus binding sites for transcription factors were identified in the human CD5 promoter sequence from the PAC clone pDJ632c8 (accession no. AC003678) with the Transfac MatInspector program (51). Repetitive elements were identified by using the RepeatMasker program (http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker), and homologies between mouse and human sequences were identified using the Blast program in the National Center for Biotechnology Information web page (http://ncbi.nlm.nih.gov/).

5′-RACE assays

The identification of transcriptional start sites of the human CD5 gene was performed by 5′-RACE assays using the Marathon cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA) and total RNA from CEM cells. The 5′ end of human CD5 was amplified by PCR using the forward AP1 (5′-CCACCTCTATACGACTCACTATAGCCG-3′) primer and the nested gene-specific primers, CD5RACE3 (5′-TGGGTGTTCTAGGAGGGCGG-3′) and CD5RACE2 (5′-CTCGATTGGAAGAGGACCCCG-3′), located at the exon encoding for the first extracellular domain of the CD5 protein. This PCR product was gel-purified and subjected to a further nested PCR amplification with the forward AP2 (5′-ACTCAGTATAGGGCTGACGCCG-3′) primer and the gene-specific reverse primer CD5RACE1 (5′-TTTGGATCATTTTGGAGCTGTGAAGG-3′) primer. This final nested PCR product was cloned as Nol/BamHI into the pGEX 4T2 vector (Amersham Pharmacia Biotech). Clones were sequenced by automated dsDNA sequencing using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Warrington, U.K.).

Reporter constructs and site-directed mutagenesis

A 695-bp fragment of the human CD5 promoter region as well as serial 5′-deletions fragments were amplified by PCR from the PAC clone pDJ632c8 (accession no. AC003678). A common reverse primer (CD5-9HindIII Rv: 5′-maAGGTCTccctgtctgcagtgaggg-3′), which ends 9 bp upstream of the initiation methionine, was used in all cases (underlining, restriction enzyme sequences). The different forward primers used were CD5mutI (5′-CTACAGATTGTTGAGGAACGCAGG-3′), CD5mI (5′-GAACGACGCGAGGCCCACTAGG-3′), CD5mII (5′-CCCGAGGCCCAGCCTCTTG-3′) primers were used. This probe binds to a further nested PCR amplification with the forward AP2 (5′-ACTCAGTATAGGGCTGACGCCG-3′) primer and the gene-specific reverse primers CD5RACE1 (5′-TTTGGATCATTTTGGAGCTGTGAAGG-3′) primer. This final nested PCR product was cloned as Nol/BamHI into the pGEX 4T2 vector (Amersham Pharmacia Biotech). Clones were sequenced by automated dsDNA sequencing using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Warrington, U.K.).

Abbreviations used in this paper: DH; DNase I hypersensitive; Fw; forward; Rv; reverse; PAC; P1 artificial chromosome; GAPB, GA-binding protein; HEB, HeLa E-box binding protein.
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Cells

The human T cell lines Jurkat and CEM, the Burkitt’s lymphoma-derived Raji and Daudi cell lines, and the myeloid cell line K-562 were obtained from American Type Culture Collection (Manassas, VA). The human CD5+ B lymphoblastoid cell line L929 was derived by EBV immortalization of chronic lymphocytic leukemia cells and was provided by Prof. A. Rosen (University of Linköping, Linköping, Sweden) (52). T lymphoblasts were elicited from T cells by activating human PBMC with PHA for 3 days, then culturing the cells for 7 days in the presence of IL-2, by which time they were 99% CD3+ T cells. Cells were grown in RPMI 1640 medium with l-glutamine (Life Technologies, Gaithersburg, MD), supplemented with 10% FCS, and gentamicin. HeLa cells were grown in DMEM containing the same additives (Life Technologies). All cell cultures were maintained at 37°C in a 5% CO2 humidified atmosphere.

Transfection and cotransfection analyses

For transfection analyses, the promoter-less pGL3-Basic vector was used as a negative control. The SV40 promoter-luciferase pGL3-Control vector (Promega) was used as a positive control. The pRL-CMV vector (Promega), which encodes the luciferase gene under the control of CMV enhancer/promoter, was used as an internal control of transfection efficiency. Transfections were performed with DMRIE-C Reagent (Invitrogen, Life Technologies, Carlsbad, CA) following the manufacturer’s instructions. Briefly, for Jurkat and K-562 cells, 2 × 105 cells were mixed with 3 μl of DMRIE-C reagent, 2 μg of the indicated luciferase reporter plasmids, and 0.1 μg of pRL-CMV in a final volume of 1.2 ml of Opti-MEM I Reduced Serum Medium (Life Technologies). After 5 h of incubation at 37°C, 5 ml of RPMI 1640 plus 10% FCS were added. After 36 h, cells were lysed with 100 μl of Passive Lysis Buffer (Promega), and luciferase activity was measured in 20-μl samples by using a Luminoskan (Labsystems, Helsinki, Finland) and the manufacturer’s instructions. For CEM cell transfections, 4 μg of the luciferase reporter plasmids, 0.4 μg of pRL-CMV, and 3 μl of DMRIE-C were used. For transfection of I83-E95, Raji and Daudi cells, 4 μg of luciferase reporter plasmids, 0.2 μg of pRL-CMV, and 8 μl of DMRIE-C were used. All studies were performed in three different experiments with two independent transfections each time. Three different plasmid preparations were used.

For cotransfection experiments in HeLa cells, the following expression vectors for human Ets transcription factors were used: pSG5, Ets-1-pSG5, Ets-2-pSG5, Fli-1-pSG5, GA-binding protein (GABP)α-pSG5, GABPβ-pSG5, GABPγ-pSG5, Elf-1-pCDNA3, and Tel-pCDNA3. Dr. D. Watson (Hollings Cancer Center Medical University of South Carolina, Charleston, SC) provided all of them. The day before transfection, 106 HeLa cells were harvested and cultured in DMEM (Life Technologies, Carlsbad, CA) following the manufacturer’s instructions. Transient cotransfections were performed in triplicate in six-well plates using 5 μl of FuGene 6 reagent (Roche, Indianapolis, IN) according to the manufacturer’s instructions. One microgram of the corresponding luciferase Reporter vector, 0.05 or 0.2 μg of expression vector, and 0.02 μg of pRL-CMV were used. In all cotransfections, total DNA was kept constant with corresponding empty expression vectors. Cotransfections were repeated at least four times using two different plasmid preparations to ensure reproducibility. Luciferase activity was determined 36 h post-transfection in a Luminoskan. Three different plasmid preparations were used. For Western blot analysis, total protein/lane; Fli-1, 100 μg; CD5, 100 μg; and CD5– Fli-1, 100 μg/lane. The anti-human Ets-1 mouse mAb (Transduction Laboratories, Lexington, KY) was used at a 1:750 dilution, and the anti-human Fli-1 rabbit polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted at a 1:1000 dilution. After washing out the primary Ab excess with TBST, membranes were incubated with a 1:3000 dilution of HRP-conjugated secondary Abs. Detection of β-actin was performed by 2-h incubation with a 1:5000 dilution of an anti-human β-actin mouse mAb (Sigma-Aldrich). Immune complexes were detected by ECL (Amersham Pharmacia Biotech).

Results

A T cell-specific DH site is located at the 5′-flanking region of human CD5

The position of transcriptional regulatory elements in the genome strongly correlates with the location of DH sites, which are known to occur in and around regions where chromatin adopt altered structures (54). To test whether the 5′-flanking region of human CD5 showed cell-specific DH sites, nuclei from T lymphoblasts and the leukemic cell lines Jurkat (T cell), I83-E95 (CD5+ B cell), Raji (CD5– B cell), and K-562 (myeloid cell) were incubated in the presence or the absence of DNase I and then digested with MfeI. Before nuclei isolation, cells were treated with or without PMA (20 ng/ml) and A23187 (2 μM) for 6 h. In the absence of DNase I, Southern blot analysis showed the presence of a 15.4-kb MfeI genomic fragment extending from 6.68 kb upstream of the CD5 start ATG codon to 8.68 kb downstream (Fig. 1A). In DNase I-treated samples a T cell-specific DH site was detected, which mapped just 5′ to the start ATG codon of human CD5. This DH site resulted in the appearance of a 8.7-kb band in T lymphoblasts and Jurkat cells, but not in I83-E95, Raji, and K-562 cells (Fig. 1B). Fainter and smaller bands of 7.9 and 7.1 kb were detected in T cells, especially T lymphoblasts (Fig. 1B). These bands could correspond to additional DH sites located on the first intron of human CD5 (~800 and 1600 bp downstream of the ATG). Unexpectedly, the CD5+ B cell line I83-E95 (52) did not show any DH site within the MfeI genomic fragment analyzed. Nevertheless, the same cells revealed the presence of other specific DH sites within the CD5 locus, as will be considered below (see Fig. 7). Although there is a hint of a DH site at approximately −3.8 kb in I83-E95 cell samples (12.5-kb band in Fig. 1B), the existence of a B cell-specific DH site could not be confirmed when probing this region in other restricted genomic DNA fragments (data not shown). Furthermore, the potential DH site seen in the MfeI gel is confined within a repetitive region, and it is unlikely to be of significance. Fig. 1C shows the phenotypic analysis of the cell lines used in this study for T (CD3, CD5) and B (CD20) cell-specific surface markers.

Human CD5 displays multiple transcriptional start sites

The sequence analysis of the 5′-flanking region of human CD5 (Fig. 2A) revealed that this gene is devoid of a TATA box (36). The lack of this element is normally associated with the existence of multiple transcriptional start sites. To test whether this was the
case for human CD5, 5’-RACE analyses were performed from total RNA of the human T cell line CEM. The results obtained from 50 clones analyzed are summarized in Fig. 2B, and the exact location of the multiple start sites found in the 5’-flanking region of CD5 is shown in Fig. 2A. As expected, CD5 presented multiple transcriptional start sites going from −87 to +22 bp relative to the first initiation methionine. The finding of some start sites downstream of the ATG may correspond to the presence of a second ATG codon located at position −49 bp from the first one (Fig. 2A) or could represent incomplete amplification of the 5’ end. Interestingly, the major transcriptional start site located at position −58 lies within a sequence (CCAGAC) that strongly resembles the loose initiator consensus PyPyA+1NT/PyPy and is located 30–50 bp downstream of two potential Sp1 binding sites (Fig. 2A).

Identification of the minimal CD5 promoter

To assess the promoter activity of the 5’-flanking region of human CD5, serial 5’ deletions of a 665-bp fragment upstream of the initiation ATG were cloned into the firefly luciferase reporter vector pGL3. The existence of a highly repetitive region (including simple repeats and long terminal repeat/endogenous retrovirus-type repeats) extending to 5 kb upstream of that point prevented us from analyzing a longer region. The resulting reporter constructs were transiently transfected into CD5− (Jurkat, I83-E95) and CD5+ (K-562, Raji, and Daudi) cells. The transfection efficiency was monitored using the reporter pRL-CMV vector. The results obtained for three different experiments performed in triplicate are shown in Fig. 3. Among the constructs tested, pGL3−282 was shown to include the minimal region presenting T cell-specific full promoter activity. Almost complete loss of promoter activity was found for pGL3−121 (Fig. 3). Interestingly, the pGL3−282 construct showed no activity in the CD5− B cell line.
I83-E95, which agrees with the above-mentioned DH analysis results.

**Two conserved DNA motifs for Ets transcription factors are necessary for CD5 full promoter activity in T cells**

The pGL3−282 construct encompasses the most conserved region (from −249 to −110) found when comparing the 5′-flanking regions of the human and mouse CD5 genes (36). Sequence analysis reveals that the −282-bp fragment contains predicted binding sites for Ets (at −239 and −185), c-Myb (−177, −169), AP-1 (−151), and Sp1 (−115, −95) transcription factors (Fig. 2A). To further define specific elements in the 282-bp region that contribute to the proximal promoter activity in T cells, we generated a series of CD5 promoter luciferase-reporter constructs carrying single or multiple point mutations at potential binding sites (Fig. 2A). The site-specific mutations introduced in the pGL3−282 construct are indicated in Fig. 4A. Mutations at the Ets-239 or Ets-185 DNA binding elements resulted in 29 and 77% reductions in promoter activity, respectively (Fig. 4B, top panel). This agrees with the results obtained with the pGL3−282 (including both Ets motifs) and pGL3−213 (including only the Ets-185 motif) constructs (Fig. 3) and indicates that the two conserved Ets binding sites are important for human CD5 promoter activity. However, simultaneous mutation of both sites did not result in a much more marked reduction than that observed for the Ets−185 mutation alone, indicating a major con-

**FIGURE 3.** Delineation of the minimum promoter sequences required for human CD5 expression. The luciferase activities of pGL3 constructs containing various fragments of the 5′-flanking region of human CD5 are shown. The pGL3-Control and pGL3-Basic vectors are used as positive and negative controls, respectively. Luciferase activity was measured 36 h after transfection, and expressed in arbitrary units, assigning 100 to the activity of the pGL3 control vector. The pRL-CMV vector was cotransfected as an internal control of transfection efficiency. The results represent the mean of three experiments (each performed in duplicate).

**FIGURE 4.** A, Nucleotide sequence of the wild-type and mutated binding sites for Ets, c-Myb, Sp1, AP-1, and E proteins. The binding site core is underlined. The mutations introduced are indicated in bold. m, mutated binding site; wm, wider mutations of the binding sites. B, Site-directed mutagenesis of the minimal CD5 promoter. Single and double mutations were introduced at different binding sites present in the pGL3−282 reporter, then analyzed by transient transfection in Jurkat cells. At the top, a schematic representation of the region analyzed is shown. The pGL3-Basic vector was used as a negative control. Luciferase activity was measured 36 h after transfection and expressed in arbitrary units (x-axis), assigning a value of 100 to the activity of the pGL3−282 vector. The pRL-CMV vector was cotransfected as an internal control of transfection efficiency. The results represent the mean of three experiments (each performed in duplicate).
The potential interaction of Ets proteins with human CD5 promoter motifs was evaluated by EMSA using CEM nuclear extracts. Labeled oligonucleotides harboring wild-type Ets –239 and Ets –185 sites were able to form specific complexes, which were absent when similar oligonucleotides carrying mutated Ets cores were used (Fig. 5). Furthermore, the DNA-protein complexes for the Ets –185 site could be competed by adding excess of cold Ets wild-type oligonucleotides (Fig. 5). Moreover, an excess of an Ets-1 consensus oligonucleotide (5′-ATAACAGGAAGTGGT-3′) (55) showed inhibition as well (data not shown). Identical results were obtained when competition assays were performed with either the Ets –239 oligonucleotide or nuclear extracts from Jurkat cells (data not shown).

Ets family members are able to trans-activate the human CD5 promoter

Several Ets family members are preferentially expressed in lymphoid cells, and many lymphoid-restricted genes contain functionally important Ets binding sites (56). To further assess the contribution of Ets-related proteins to the activity of the CD5 promoter, cotransfection experiments with expression vectors for members of the Ets family of transcription factors were performed. These experiments were performed in the epithelial HeLa cell line, which is negative for CD5 surface expression and shows much lower endogenous Ets-1 and Fli-1 levels than the T cell lines Jurkat and CEM, as detected by Western blot analysis (Fig. 6A). Either the pGL3 –282 construct or its doubly Ets-mutated version (mEts–239 + wmEts–185) was cotransfected in HeLa cells with increasing amounts of expression vectors for several Ets proteins. As this family of transcription factors includes a large list of proteins, we focused on those members expressed in mature T lymphocytes: Ets-1, Ets-2, Fli-1, GABPα+β, Tel, and Elf-1 (57). The results of this experiment are shown in Fig. 6B. Significantly, Ets-1 and Ets-2 expression constructs induced 5- and 4-fold increases, respectively, in the transcriptional activity of the pGL3 –282 construct. The Elf-1 expression vector was also able to trans-activate human CD5 promoter only when used at high DNA concentrations. None of the other transcription factors tested (Fli-1, GABPα+β, and Tel) influenced the luciferase activity. The lack of trans-activation by Ets-1 on the doubly Ets-mutated reporter construct (mEts–239 + wmEts–185) indicated that the effect of this factor is driven specifically through the Ets motifs located in the CD5 promoter sequence.

Putative involvement of Sp1, c-Myb, and AP-1-like motifs in the control of human CD5 transcription

The ubiquitous transcription factor Sp1 has been involved in directing the formation of the preinitiation complex in TATA- and initiator-less promoters (58). Two Sp1 binding sites (−95 and −115 bp) are found in the human CD5 promoter. To assess the contributions of these two potential Sp1 motifs, we analyzed versions of the pGL3 –282 construct carrying single and double Sp1 mutations (Fig. 4, bottom panels). Mutations in Sp1 –95 and Sp1 –115 caused decreases in transcriptional activity of 22 and 24%, respectively. The introduction of broader mutations in both Sp1 motifs (wmSp1–95 and wmSp1–115; Fig. 4A) did not cause a further decrease in transcriptional activity (data not shown). The results indicate that the Sp1 –95 and –115 motifs could be relevant to human CD5 transcription.

c-Myb (−177, −169) and AP-1-like motifs (−151) can be identified within the highest conserved region of the human CD5 promoter (−249 to −110; Fig. 2A). Of the two motifs, only the former

FIGURE 5. Analysis by EMSA of the Ets –185 and Ets –239 motifs of the human CD5 promoter. EMSA analyses were performed using CEM nuclear extracts and 32P-labeled probes, including wild-type or mutated Ets –185 and Ets –239 binding sites. For competition analysis (right panel), the indicated molar excess of cold probes was added to the 32P-labeled Ets –185 probe. The sequences of the oligonucleotides probes used in the EMSA analysis are shown in Fig. 4A.

FIGURE 6. A, Expression of Ets-1 and Fli-1 proteins in Jurkat, CEM, and HeLa cells. Western blot analysis of human Ets-1 and Fli-1 protein content in total cell lysates is shown. Anti-Ets-1 mouse mAb and anti-Fli-1 rabbit polyclonal serum were used as primary Abs. The same blot was analyzed with an anti-β-actin mouse mAb. The blots were incubated with peroxidase-labeled secondary antisera and developed by chemiluminescence. B, Cotransfection experiments of HeLa cells with CD5 promoter constructs and expression vectors for Ets family members. The pGL3 –282 reporter construct, as well as a version of it carrying site-directed mutations for both the Ets–239 and Ets–185 binding motifs (mEts–239 + wmEts–185) were cotransfected in transient assays with different amounts (nanograms) of expression vectors for members of the Ets family (Ets-1, Ets-2, Fli-1, GABPα+β, Tel, and Elf-1). In all transfections total DNA was kept constant with the corresponding empty expression vector. pRL-CMV was used as an internal control of transfection efficiency. Data are expressed as relative luciferase activity, considering 1 as the activity of the pGL3 –282 construct in the absence of expression vectors. The results are the mean of at least four experiments (each performed in triplicate).
was conserved in the mouse. c-Myb transcription factors have been extensively related to the expression control of lymphocytic genes such as TCRβ (59), TCRγ (60), CD4 (61), and recombinase-activating gene 2 (62, 63). When the c-Myb –177 motif was mutated in the pGL3 –282 construct (mc-Myb-177), a 30% loss of transcriptional activity was observed in Jurkat cells (Fig. 4, bottom panels). Moreover, mutation of the AP-1-like sequence (GTGACGCAG), which differs in just one nucleotide from the AP-1 consensus sequence (TGACTCA), resulted in a 51% decrease in luciferase activity in unstimulated Jurkat cells (Fig. 4, bottom panels). The results suggest that c-Myb and AP-1-like elements could also contribute to human CD5 promoter activity.

To further investigate potential cooperative effects between different motifs of the human CD5 promoter, pGL3 –282 constructs carrying double mutations for the Ets, Sp1, AP-1-like, and c-Myb motifs were analyzed (Fig. 4, bottom panels). Compared with single mutated constructs, slight decreases in promoter activity were found for the double mutated constructs c-Myb –177 plus Ets –185 or Ets –239 (mEts-239+mc-Myb-177, mEts-185+mc-Myb-177) as well as Sp1 –95 plus Ets –185 or Ets –239 (mEts-239+msp1-95, mEts-185+msp1-95). However, none of the double-mutated constructs tested here showed severe losses in transcriptional activity, indicating the absence of significant additive or synergistic effects.

CD5+ human T and B cells presents an inducible DH site located at –10 kb from CD5 that shows high nucleotide sequence homology to a mouse anti-IgM inducible cd5 enhancer

The finding that CD5 promoter-pGL3 constructs did not respond to stimulation with either PMA alone or PMA plus A23187 in T cells (data not shown) led us to search for upstream regulatory regions. A region encompassing 20.5 kb upstream of CD5 was subjected to DH assays. Fig. 7A schematically shows the genomic fragments analyzed as well as the locations of the DH sites found. Three DH

FIGURE 7. A, Schematic map of the DH sites found in the human CD5 locus. A region from –20.5 to +8.68 relative to the CD5 translational start was analyzed for the presence of DH sites. All analyses included T lymphoblasts and the cell lines I83-E95, Jurkat, Raji, and K-562. Filled and open arrowheads represent DH sites found in T cells and I83-E95 B cells, respectively. An arrow points out a DH site mapping to a repeat DNA region. Boxes indicate inducible DH sites after 6-h treatment with 20 ng/ml PMA plus 2 μM A23187. The restriction enzymes used in the analysis, the relative positions of the genomic fragments, and the probes are indicated.

B, Analysis of the T- and B-inducible DH site located at –10 kb. Southern blot analysis of a SacI genomic fragment from T lymphoblasts and the I83-E95, Jurkat, Raji, and K-562 cell lines is shown. The arrows in the left panel indicate the 10.5-, 2.5-, and 1.8-kb bands corresponding to the DH 10, DH 18, and DH 18.7 sites represented in A. The right panel illustrates a SacI polymorphism that makes Jurkat and Raji cells not informative for the DH-10 site. Where indicated (+), cells were treated for 6 h with 20 ng/ml PMA and 2 μM A23187. C, Comparison of the human and murine CD5 loci. The relative position of the murine anti-IgM inducible enhancer and its homologous sequence in the human locus are indicated ( ], as are blocks of DNA repeat sequences ( ). D, Nucleotide sequence alignment of the murine anti-IgM-dependent enhancer and the homologous region found in the human. Predicted binding sites for several transcription factors, which are conserved in both species, are indicated. Bold letters in the mouse sequence indicate motifs that had already been analyzed in anti-IgM-induced murine splenic B cells.
sites were detected at −18.7, −18, and −10 kb by probing a SacI genomic fragment (Fig. 7, A and B). The DH −18.7 was detected in T lymphoblasts after PMA plus A23187 stimulation (Fig. 7B). The DH −18 was constitutively present in lymphoblasts and I83-E95 B cells (Fig. 7B). The DH −10 site was induced in T lymphoblasts and I83-E95 cells after treatment with PMA plus A23187 (Fig. 7B). Jurkat and Raji cells were hetero- and homozygous, respectively, for a polymorphism in the SacI genomic fragment (Fig. 7B) that rendered them not informative for the DH −10 site (Fig. 7B). The presence of the inducible DH −10 site was confirmed by analysis of the XbaI genomic fragment covering from −11.88 to +6.28 relative to the CD5 ATG (Fig. 7A and data not shown). Interestingly, the nucleotide sequence analysis of the DH −10 site showed an area with 89% identity to a previously reported 122-bp anti-IgM inducible murine cd5 enhancer (64) (Fig. 7D). This murine element is located approximately −2 kb relative to the cd5 translation start site, whereas the human homologue sequence locates at −9.6 kb in the PAC clone pDJ632c8 (accession no. AC003678; Fig. 7C) and −10.19 kb in the PAC clone CMB9-39H21 (accession no. AP000437). These different locations are due to the different lengths of the repetitive sequences present in the two human genomic clones. The repetitive sequences found in the human encompass −8 kb upstream of CD5 promoter, whereas only short areas of repeats are present in the mouse (Fig. 7C). Analysis of the enhancer sequence revealed the presence of two potential conserved composite sites (Pax-5/AP-1/NFAT and Ets/NFAT/NF-κB) apart from the E box, H4TF-1, Ets, and NFAT motifs previously reported to be functional in mouse splenic B cells (Fig. 7D). Our results suggest that the inducible DH −10 site found in the human could be a functional element not only in B cells (I83-E95), but also in T cells (lymphoblasts).

**Discussion**

Sequence analysis of the 5′-flanking region of the mouse and human CD5 genes reveals a high degree of nucleotide sequence conservation. This suggests that the two genes may share similar transcriptional regulatory elements. Although some information is available on the mouse cd5 promoter, nothing was previously known about the functionality of its human homologue. As a first attempt to understand the molecular basis for the tissue-specific expression of human CD5, chromatin configuration of its 5′-flanking region was assessed by DH assays. A T cell-specific DH site was found within that region, thus suggesting its functional relevance for CD5 expression. This DH site was absent from CD5− cells such as Raji (B cell), and K-562 (myeloid cell). Intriguingly, we did not find evidence for this DH promoter site in the CD5− human B cell line I83-E95. Strict correlation was found between the results of the DH assays and the transcriptional activity detected by luciferase reporter assays in the same cell types. The 5′-flanking region of mouse cd5 has been shown to direct tissue-specific expression of CD5 in both T and B cell lines in transient transfection assays with reporter vectors (40), but DH analyses of the cd5 locus remain to be performed. Our finding may indicate that constitutive CD5 expression is differentially regulated on human and mouse B cells. In fact, important anatomical differences can be found in the upstream sequences of the two genes. A highly repetitive region, including simple repeats and long terminal repeat/ERV-type repeats, extends from −665 bp to −8 kb upstream of human CD5 (Fig. 7C). No such region is found in mouse cd5. However, further analysis of human CD5+ B cells other than I83-E95 is needed to support the above-mentioned hypothesis. The scarcity of human CD5+ B cell lines as well as the limited availability of primary CD5+ human B cells makes this type of analysis difficult. An alternative explanation could be that the promoter activity in B cells is too weak to be detected by DNase I and transfection assays. In fact, surface CD5 expression in B cells is much lower than in T cells. Whatever the situation is in CD5+ B cells, an extended DH site analysis has allowed us to find putative distal control regions in the human CD5 locus that could account for the regulation of CD5 expression in B and T cells. As will be discussed below, one of these distal regions is highly homologous to a NFAT-dependent enhancer element that has been located at approximately −2 kb of the murine cd5 gene (64).

Within the 5′-flanking region of CD5, the minimal promoter region that accounts for full transcriptional activity in human T cells, but not B and myeloid cells consists of a 282-bp fragment upstream of the initiation ATG. This segment encompasses a region (from −249 to −110) with high nucleotide homology (80%) between the human and mouse genes (36). Putative regulatory elements located in this minimal promoter region include conserved Ets (−239 and −185), E box (−198), and c-Myb (−177 and −169) binding sites as well as nonconserved AP-1-like (−151) and Sp1 (−115 and −95) sites. All these sites, with the exception of the E box, seem to have a role in CD5 promoter activity in T cells as deduced from the results with luciferase reporter constructs. The major regulatory elements controlling the transcriptional activity of the human CD5 promoter are Ets binding sites. Preferential expression of some Ets family transcription factors (Ets-1, Elf-1, and Fli-1) in T lymphocytes has been associated with a role in controlling transcription of relevant T cell specific genes such as TCRα (42, 43), TCRβ (44, 45), IL-2R (46), IL-5 (47), and CD4 (48, 49). The two conserved Ets sites (−185 and −239) here identified are necessary elements for full transcriptional activity of human CD5 promoter, especially the Ets-185 motif. The mouse motif equivalent to human Ets-185 has also been shown to be relevant for mouse CD5 expression (41). However, the relevance of the mouse equivalent to the Ets-239 motif has not yet been addressed. Cotransfection experiments in HeLa cells with Ets members expressed in mature T cells (Ets-1, Elf-1, Fli-1, GABPα+β, Tel, and Elf-1) (57), indicated that Ets-1 and Ets-2 may have a role in the control of human CD5 expression. This is in agreement with the analysis of Ets-1/RAG-2-knockout mice, in which peripheral T cells expressed normal levels of TCRβ complex, but reduced levels of CD5 (37). Interestingly, the mRNA and protein levels of Ets-1 and Ets-2 parallel those observed for CD5 from early thymic development (22, 65, 66), which raises the question of whether Ets proteins may have a role in controlling CD5 expression from thymocyte to mature T cells. This possibility remains to be explored. Furthermore, some controversy exists about the roles of Ets-1 and Ets-2 in regulating gene expression on resting and activated T cells. It has been proposed that Ets-2 may be of particular importance in activated T cells, because its expression is up-regulated upon antigenic stimulation. Meanwhile, the high level of Ets-1 expression in resting T cells declines to an undetectable level after T cell activation (66). As CD5 is not only constitutively expressed in resting T lymphocytes, but is also up-regulated in activated T cells, its promoter may be an interesting model for studying the in vivo behavior of Ets-1 and Ets-2 transcription factors in resting vs stimulated T cells.

The conserved E box sequence (CANNTG) located at position −198 in the human CD5 promoter is a good candidate for regulating CD5 expression in T and B cells. The E box sequence is recognized by the helix-loop-helix family of transcription factors. This family includes members such as E2A, E2−2, and HeLa E-box binding protein (HEB). These proteins play important roles in T and B cell development (67). E boxes have been identified in the regulatory regions of numerous T and B lymphoid-specific genes such as CD4 (38), TCRα and -β (68, 69), Ig κ (70), and IgH
ruled out that E proteins control was partially performed in resting Jurkat cells (72). It cannot be that the study showing functional E boxes in the CD4 enhancer CD5 expression in thymocytes. It is worth mentioning, however, where mutation of the conserved E box resulted in only a minor in T cells. This is in agreement with results obtained in the mouse, where mutation of the conserved E box resulted in only a minor reduction in reporter activity (41). One possible explanation could be that the human (Jurkat) and mouse (EL4) T cells used in these studies are too mature to detect the observed role of E proteins in CD5 expression in thymocytes. It is worth mentioning, however, that the study showing functional E boxes in the CD4 enhancer was partially performed in resting Jurkat cells (72). It cannot be ruled out that E proteins control CD5 transcription by acting through more distal enhancer sequences or even that the E protein effect is not direct. Moreover, two additional nonconserved E boxes are found in the human CD5 promoter region (−50 and −37), which deserve further analysis.

The human CD5 promoter does not have a TATA sequence to determine the location of the transcriptional initiation site. As commonly reported for this kind of gene, 5′-RACE experiments showed that human CD5 has multiple transcriptional start sites, the most frequent one located at −58 bp from the ATG. Multiple start sites were reported in the mouse cd5 promoter, although the exact location and frequency of each one were not assessed (40). Interestingly, the two Sp1 (−115 and −95) motifs found in human CD5 seem to be functional in T cells. It is known that the Sp1 transcription factor has a role in directing the formation of preinitiation complexes to a window 40−100 bp downstream of its binding site in genes that lack both TATA and initiator elements (58). Furthermore, within that window, Sp1 may direct preinitiation complex formation at the DNA sequences that most closely resemble TATA or initiator elements (58). The Sp1 binding elements found in the human CD5 promoter are 57 and 37 bp upstream of the major transcription start site, which lies within the sequence CCAGAC, differing in only one nucleotide from the loose initiator consensus sequence PyPyA+1NT/PyPy (58). This indicates that Sp1 could be a component of the basal transcriptional machinery needed for CD5 expression.

The c-Myb (−177 bp) and AP-1-like (−151 bp) sites could also have a role in human CD5 promoter activity in T cells. Of the two sites, only the c-Myb motif has been analyzed in the mouse (41). There are two conserved c-Myb motifs (−169 and −177 bp; Fig. 1A). We only mutated the c-Myb −177 bp site, for which a slight reduction (30%) in transcriptional activity was observed. The c-Myb −169 bp site was not included in our analysis, but was shown to be active in the mouse. This c-Myb −169 bp site was included in the CD5X region reported in the mouse whose mutation affected transcriptional activity in T cells (41). Although a more detailed analysis is still needed, the available data indicate that c-Myb sites are of potential relevance to CD5 transcriptional control. The AP-1 and CREB/ATF transcription factors are b-ZIP proteins that are heavily involved in the regulation of lymphoid genes. They both bind DNA as dimers that interact via a structure referred to as the leucine zipper motif. AP-1 proteins are typically formed by dimerization of the Fos and Jun transcription factors and bind to the TGACTCA sequence (known as the 12-O-tetradecanoylphorbol-13-acetate-responsive element). The CREB/ATF dimers bind to the consensus sequence TGGGCA (known as the cAMP-responsive element) (73). AP-1 has been linked to the control of expression of numerous genes that are activated upon T cell activation, such as CD69 (74), IL-2 (75), GM-CSF (76), TNF-α (77), IFN-γ (78), IL-5 (79), and IL-9 (80). Moreover, several constitutively expressed, T cell-specific genes are known to have functional CREB/ATF sites in their transcriptional regulatory sequences, such as CD35 (81), TCRα (43), and TCRβ (44). Members of the Fos/Jun family form selective cross-family heterodimers with members of the ATF/CREB family. These cross-family heterodimers can bind to either the ATF/CRE or the AP-1 site depending on the dimer composition (82). As an example, the Enk-2 site from the proenkephalin regulatory region has been shown to bind ATF-2/Jun heterodimer better than does either homodimer (82). Interestingly, the AP-1 −151 site in the human CD5 promoter (5′-GTTGACCGACGGCC-3′) closely resembles the Enk-2 site (reverse sequence, GCTGACGCAGGCCC). The mutation of the AP-1-like motif (−151) in the CD5 promoter–luciferase construct caused a 50% reduction of transcriptional activity. The exact role of AP-1 or CREB/ATF transcription factors in the control of CD5 expression requires further exploration.

Analysis of the 20-kb region upstream of human CD5 has revealed the presence of an inducible DH site (DH −10) in both T lymphoblast and I83-E95 B cells stimulated with PMA plus A23187. This DH −10 site shows a high homology (89% nucleotide sequence identity) to the previously reported, 122-bp NFAT-dependent, anti-IgM-inducible murine enhancer, which is located 2 kb upstream of the murine cd5 gene (64). The functional analysis of the murine enhancer showed the relevance of conserved NFAT, Ets, and E protein motifs (64). Interestingly, two conserved composite (Pax-5/AP-1/NFAT and Ets/NFAT/NF-x-b) sites were detected in our sequence analysis for which no mouse data are available. The murine enhancer has only been studied in splenic B cells treated for 3 days with LPS that is induced to express CD5 after anti-IgM treatment (64). Nothing is known about the role of that enhancer in murine T cells or B-1a cells (constitutively CD5+). The fact that the human homologous sequence exists as an inducible DH site in both T lymphoblasts and I83-E95 (B CD5+) suggests that this DNA element could be important in the up-regulation of CD5 expression in both cell types. Further functional analyses are necessary to fully address this issue.

In summary, the data presented here constitute the first approach to understanding the regulatory elements controlling human CD5 expression. Although humans and mice share the minimal promoter region responsible for tissue-specific CD5 expression in T cells, some interspecies differences are found. The most intriguing difference relates to the control of CD5 expression in B cells. Much effort is needed to fully understand the transcriptional control of CD5 not only on B cells, but also during lymphocyte development and activation.

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