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The Role of Intron Sequences in High Level Expression from CD45 cDNA Constructs*

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Consistent expression from CD45 cDNA constructs has proven difficult to achieve. Through the use of new CD45 cDNA constructs and reporter genes, the role 5′, 3′, and intron sequences play in CD45 expression was determined. The CD45 polyadenylation signal sequence was fully functional in a β-galactosidase reporter construct. Furthermore, the CD45 3′-untranslated region and downstream sequences were shown to contain no negative regulatory elements. Several new CD45 cDNA constructs were designed that contain either the cytomegalovirus promoter, the leukocyte function-associated antigen (LFA-1; CD11a) promoter, or various CD45 5′ regions. Neither the cytomegalovirus nor the LFA-1 promoter was capable of generating detectable levels of expression in constructs with CD45 cDNA. However, when CD45 intron sequences between exons 3 and 9 were inserted in the cDNA construct to generate a CD45 minigene, the LFA-1 promoter was able to drive reproducible, significant expression of CD45. CD45 minigenes using the CD45 5′ sequences up to 19 kilobases upstream of the transcriptional start produced very little protein. The LFA-1 CD45 minigene construct produced correct cell type-specific isoforms when expressed in T and B lymphocyte lines. Therefore, we conclude that the regulation of CD45 expression and cell type-specific splicing requires elements within the intron sequences.

CD45 is a high molecular weight, transmembrane protein-tyrosine phosphatase expressed on all nucleated cells of hematopoietic origin. The protein is structurally heterogenous, consisting of isoforms ranging in size from 180 to 220 kDa. The heterogeneity of the protein results from differential RNA splicing of at least five and perhaps six exons encoding part of the extracellular domain (1–3). The CD45 exon usage pattern is highly regulated by the different leukocyte populations and is cell type-specific. The predominant isoform expressed by B cells contains all variable exons ($B220^+$) (4, 5). Upon antigen stimulation, lower molecular weight isoforms are detected (6, 7). Several subtypes of antigen-specific memory B cells have been identified, including an antibody-secreting subset producing the full-length splice variant and a nonsecreting subtype, which is $B220^-$ (8). In thymocytes three or four variable exons are removed to generate the lowest molecular weight isoforms (1, 3, 4, 9–11). Peripheral T cells express multiple splice variants and have a varied isoform expression pattern that is dependent upon the differentiation state, function, and prior antigenic exposure (2). Mast cells and monocytes also produce specific sets of CD45 isoforms, which are distinctive for each myeloid cell type (12).

In addition to the protein structural differences, polymorphic sequence variations in the CD45 gene led to the identification of three alleles in inbred murine strains, $Ly5^a$ (CD45.1), $Ly5^b$ (CD45.2), and $Ly5^c$ (13). The CD45.2 allele is expressed by most of the established strains, while the CD45.1 allele is found in only a few (13). The CD45.1 and CD45.2 alleles are distinguished antigenically by their reactivity to specific monoclonal antibodies, and the nucleotide changes that produce the antigenic differences have been identified (3).

CD45 is encoded by a single gene, and expression appears to be regulated at the level of transcription (14). The murine gene is characterized by 34 exons and a large 50-kilobase (kb)[1] intron between exons 2 and 3. CD45 transcription can initiate at three distinct sites in exon 1a, exon 1b, and downstream of exon 1b (15). However, the sequences responsible for the developmental and tissue-specific transcription of CD45 have yet to be identified.

Extensive analyses have demonstrated that CD45 is required to generate signaling through both the T and B cell receptors (16–21). In the case of antigen receptor signaling, CD45 is required for both the positive and negative regulation of the Src family kinases associated with the antigen receptors (22–25). Mice in which the expression of CD45 is impaired (26) or absent (27, 28) have greatly reduced numbers of peripheral T cells, suggesting that CD45 is required for T cell development (26, 27). In contrast to T cell maturation, there appears to be little effect on B cell development in the null mice (26, 27). The role of CD45 in signaling in other lymphoid lineages is less well defined, although reports suggest that the CD45 phosphatase activity is important in regulating signal transduction in mast cells through the high affinity receptor for IgE, FcεRI (29, 30).

Much less is known about the functional significance of the extracellular domain, although the isoform differences most likely convey ligand specificity. Studies have shown that the isoforms differentially affect activation through the T cell receptor (31, 32) and that the isoforms vary in their associations with the receptor (33, 34). The expression of specific isoforms has also been implicated in T cell apopotosis (35–37).

Many of the CD45 expression experiments were conducted with cDNA transgenes flanked either by retroviral sequences (38) or by the thymocyte-specific proximal *lck* promoter and the human growth hormone minigene (39, 40). Expression from these CD45 cDNA constructs is not obtained consistently (41),

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[1] The abbreviations used are: kb, kilobase(s); bp, base pair(s); PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; CMV, cytomegalovirus; FITC, fluorescein isothiocyanate; LFA-1, leukocyte function-associated antigen; UTR, untranslated region.

and in transgenic mice, the *lck*-controlled cDNAs only express 10–30% of endogenous CD45 protein levels (40). The 5′ and 3′ sequences used to regulate expression from these cDNA constructs differ considerably from those controlling the endogenous CD45 gene. The proximal *lck* promoter is expressed in thymocytes but not in peripheral T cells (42), a pattern of expression that is much restricted compared with endogenous CD45. In contrast, expression from the retroviral LTR promoter should be constitutive, but this construct lacks a discernible polyadenylation sequence in proximity to the CD45 cDNA. In addition, the 3′ sequences may contribute significantly to the expression of these cDNAs. Signals for controlling mRNA translation, stability, and localization have been found in the 3′-UTR of some genes, indicating that these sequences can contain key information for both positive and negative regulation of mRNA (43, 44). Therefore, the difficulty in consistently obtaining expression from these CD45 cDNA constructs may be due in part to the 5′ and 3′ regulatory elements used.

To address the roles of these gene elements, we designed new CD45 cDNA vectors. Sections of the 3′-end of the CD45 gene downstream of the translational termination codon were evaluated in reporter gene constructs and in CD45 cDNA vectors. Regions of the 5′-end of the CD45 gene as large as 19 kb upstream of the initiation codon were tested for promoter activity and compared with the promoter for the human LFA-1 gene, a gene with similar expression characteristics to CD45. We also tested the effect of the introns between exons 3 and 9 in CD45 expression constructs. These introns flank the principal alternatively expressed exons 4–8. Here we report that by using these novel constructs, we are able to identify components that generate reproducible expression of CD45 in a variety of hematopoietic cell types. These results suggest that the CD45 intron sequences contain information necessary for expression from the transgenes. Furthermore, the intron sequences flanking the alternatively spliced exons provide correct leukocyte lineage-specific splicing.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection—70Z/3 is a methylnitrosourea-induced murine pre-B lymphoblast cell line. Transfection of 70Z/3 was performed by electroporation in cuvettes with a 3.5-mm gap. After adding 25 μg of linearized DNA to 1×10^7 cells in phosphate-buffered saline (PBS), the DNA-cell complex was kept on ice for 10 min and then placed in a BTX Transfector 300 (BTX, La Jolla, CA) for electroporation (settings: capacitance, 1400 microfarads; voltage, 200 V). Following electroporation, the samples were incubated on ice for 10 min and then resuspended in growth medium for 48 h. Cells were subsequently grown in medium containing 1.5 mg/ml G418 sulfate (G418; Omega Scientific, Tarzana, CA).

MD.45–27J (27J) is a cytotoxic murine T cell hybridoma clone obtained from Joseph Lustgarten (Sidney Kimmel Cancer Center, La Jolla, CA) (45). Twenty micrograms of linearized plasmid DNA were mixed with 1×10^7 cells in PBS, and immediately electroporation (800 microfarads and 250 V) was performed. After electroporation, the cells were placed on ice for 10 min and then resuspended in growth medium for 36 h. Cells expressing the neomycin gene were selected using 2.0 mg/ml G418.

BW5147, a murine thymic lymphoma cell line, was transfected using 1×10^7 cells in PBS and 25 μg of linearized plasmid DNA. The mixture of cells and DNA was incubated on ice for 10 min prior to electroporation (250 V and 2200 microfarads). After electroporation, the cells were placed on ice for 10 min and then resuspended in growth medium. After 48 h, the cells were placed in medium containing 1.0 mg/ml G418.

Human embryonal kidney cells (293) were transfected using LipofectAMINE Reagent (Life Technologies, Inc.). One day before transfection, 1.2×10^6 cells were plated on 10-cm dishes treated with 0.1 mg/ml poly-D-lysine (Sigma). For transfection, 6 μg of supercoiled plasmid DNA, 40 μl of LipofectAMINE, and 300 μl of unsupplemented growth medium were mixed and kept for 15 min. at room temperature. After the 293 cells were washed two times with unsupplemented medium, the DNA/lipid mixture was added and incubated with the cells for 5 h at 37 °C. The cells were washed twice with supplemented medium and grown in 10 ml of medium for 48 h.

S49 is a murine T lymphoma cell line provided by Robert Hyman (The Salk Institute, La Jolla, CA). Transfection was performed by electroporation (250 V, 1400 microfarads). The human T cell leukemia line, Jurkat, was obtained from Javi Piedrafita (Sidney Kimmel Cancer Center, La Jolla, CA). Jurkat was transfected using DMRIE-C reagent (Life Technologies) according to the manufacturer's directions.

All cell lines were obtained from ATCC (Manassas, VA) unless otherwise indicated. The growth medium for the cell lines was Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 units/ml penicillin, and 10 μg/ml streptomycin except for 70Z/3, which was grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 1.0 mM sodium pyruvate, 10% fetal bovine serum, 0.05 mM 2-mercaptoetanol, 10 units/ml penicillin, and 10 μg/ml streptomycin. All media components were from Irvine Scientific (Irvine, CA).

Plasmids and Vector Constructions—To study CD45 cDNA expression in a variety of leukocyte populations, we obtained three constructs. The plasmid, pARV/CD45 (H), was kindly provided by K. Bottomly (Yale University School of Medicine, New Haven, CT). This plasmid is a modification of pARV/CD45 (38) in which the neomycin gene was replaced with the hygromycin gene (31). Since selection for hygromycin resistance in leukocytes is inefficient, we removed the hygromycin gene as a *Cla*I fragment and reinserted the neomycin gene (GKneo), creating pARV/CD45 (N). The neomycin gene from pGKneo^{c}bpA (Eva Lee, University of California, San Diego, La Jolla, CA) was used and contains the mammalian phosphoglycerate kinase promoter and the bovine growth hormone poly(A) sequence. The CD45 cDNA plasmids, pML84 and pML171, were obtained from J. Marth (Department of Medicine and Division of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, CA). The full-length cDNA is present in pML84 ($CD45R^{ABC}$), while pML171 carries the CD45 cDNA encoding the isoform missing exons 4–6 ($CD45R^{O}$). In these vectors, the transgenes are inserted 3′ of the proximal *lck* promoter and upstream of the human growth hormone gene (40). We added the GKneo *Not*I fragment into the *Not*I site of each plasmid to facilitate selection of stable transfectants. In all of the cDNA transgenes, the 3200-base pair (bp) *Xba*I fragment containing the CD45.2 allelic sequence was replaced with the corresponding *Xba*I fragment specifying the sequences for the CD45.1 allele.

To test the 3′ CD45 sequences, the plasmids pSPORT-(−A), pSPORT-BGA, pSPORT-XR, and pSPORT-EA, were generated (Fig. 1*A*). These plasmids are derivatives of pCMVSPORTβgal (Life Technologies). The SV40 small t/poly(A) site of pCMVSPORTβgal was removed creating pSPORT-(−A) (Fig. 1*A*). The SV40 small t/poly(A) was replaced by a 280-bp *Xba*I to *Xho*I bovine growth hormone poly(A) insert from pGKneo^{c}bpA to produce pSPORT-BGA (Fig. 1*A*). An 868-bp *Xba*I to *Eco*RI piece from CD45 exon 33 was introduced into pSPORT-(−A), generating pSPORT-X (not shown). The *Xba*I site lies 15 bp downstream from the CD45 stop signal, and the *Eco*RI site is 165 bp upstream from the 3′-end of the CD45 mRNA (3). An adjacent 800-bp *Eco*RI fragment containing the remaining 3′ portion of exon 33 and all CD45 polyadenylation signals were cloned into pSPORT-X, resulting in pSPORT-XR (Fig. 1*A*). This *Eco*RI fragment also was introduced into pCMVSPORTβgal upstream from the SV40 small t/poly(A) creating pSPORT-EA (Fig. 1*A*).

To study the effect of 5′ sequences on CD45 cDNA expression, the constructs pLFAT200 and pCMVT200 (Fig. 1*B*) were made by subcloning all components into pBluescript KS (Stratagene, La Jolla, CA). The 5′ cDNA from T200/3/pAX (38) containing all alternatively spliced exons (exons 4–8) was inserted as a *Cla*I/*Xba*I fragment. The 3′ CD45 DNA was subcloned from pSPORT-XR as a 1800-bp *Xba*I/*Eco*RI fragment (*striped box* in Fig. 1*A*). The remaining cDNA region obtained from a CD45.1 allele (3) extended from exon 9 through 33 and was inserted as a 3200-bp *Xba*I fragment. A 1800-bp *Sal*I/*Cla*I fragment containing the human leukocyte function-associated antigen (LFA-1, CD11a) promoter was inserted 5′ of the CD45 cDNA sequences. The LFA-1 promoter was obtained by PCR amplification from DNA isolated from the human T lymphocyte line, Jurkat. The sense primer extended from position −1694 to −1668 of the LFA-1 promoter (46) and contained unique *Sal*I and *Sfi*I sites at the 5′-end. The antisense primer extended from +77 to +96 of the LFA-1 promoter (46) and had unique *Cla*I and *Sgf*I sites engineered at the 3′-end of the LFA-1 sequence. The construct, pLFAT200 (Fig. 1*B*), was completed by the addition of the neomycin gene (GKneo). The LFA promoter was replaced with the cytomegalovirus (CMV) promoter to generate pCMVT200 (Fig. 1*B*) by digestion of pLFAT200 with *Sfi*I and *Cla*I and insertion of a *Nru*I/*Hin*dIII fragment from pcDNA3 (Invitrogen, Carlsbad, CA).

To investigate regulatory sequences within the introns, the CD45 minigene construct, pLFAiT200 (Fig. 1*C*), was made by inserting all

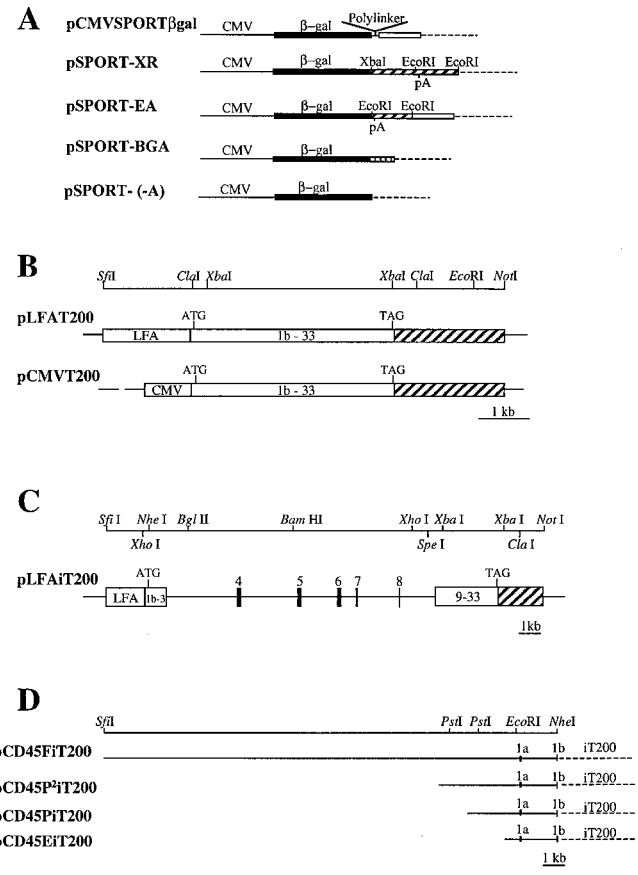

FIG. 1. **Structure of the plasmid constructs.** CD45 3′ sequences are represented by *diagonal striped boxes*. *CMV*, the cytomegalovirus promoter; *LFA*, the human LFA-1 (CD11a) promoter. *A*, all plasmids of the β-galactosidase expression constructs were derived from pCMVSPORTβgal shown at the *top*. The components of the plasmids are β-galactosidase (*filled box*), SV40 small t/poly(A) (*open box*), and bovine growth hormone poly(A) (*vertical striped box*). The CD45 element in pSPORT-EA lacks the *Xba*I to *Eco*RI 3′-UTR sequence present in pSPORT-XR. pSPORT-EA retains the 3′-terminal 165 bp of the 3′-UTR and the following 750 bp of downstream sequence (*diagonal striped box*). The polyadenylation site in the CD45 3′ sequence is indicated (*pA*). *B*, the structure of two CD45 cDNA plasmids is shown with restriction sites used for the construction in the map at the *top*. The positions of the translation start and stop codons are indicated. The CD45 cDNA region extending from exon 1b through the *Xba*I site of exon 33 is designated *1b-33*. *C*, the composition of the CD45 minigene and partial restriction map of the construct are shown. The CD45 5′ cDNA region that contains exons 1b through 3 is indicated as *1b-3*. The genomic DNA encoding the alternative spliced exons 4–8 is shown by the *solid line* with the exons indicated. The 3′ cDNA sequences are designated 9–33. Otherwise, the components are the same as in pLFAT200 (*B*). *D*, the structures of CD45 minigene constructs under the control of various extensions of CD45 upstream sequences and a partial restriction map are shown. The *Sfi*I site is not a naturally occurring site as discussed under "Experimental Procedures." The upstream region is indicated with the *solid line* with the relevant exons shown. The *Nhe*I site lies within exon 1b. The remainder of the iT200 minigene extends 3′ from this *Nhe*I site and is shown by the *dashed line*.

components into a modified mammalian expression vector pcDNA3 in which the CMV promoter had been replaced by the 1800-bp fragment containing the LFA-1 promoter. The 5′ CD45 cDNA/intron region in LFAiT200 starts in exon 1b at an *Nhe*I site, 27 bp from the beginning of the exon, continues through exons 2 and 3, and ends within the 3/4 intron at a *Bgl*II site, 500 bp downstream from exon 3. Two PCR products were used to generate this region. PCR amplification of cDNA extending from exon 1b to 3 produced the first fragment, and amplification of CD45 genomic DNA containing exon 3 and intron sequences 3′ to exon 3 resulted in the second. The fragment was completed by ligating the two PCR products. A λ genomic clone carrying CD45 exons 3–8 was used to obtain a *Bgl*II to *Bam*HI fragment containing exon 4. This fragment was inserted downstream from exon 3 at the *Bgl*II (intron) and *Bam*HI (vector) sites. The genomic sequences, extending from the *Bam*HI site 550 bp upstream of exon 5 to an *Xho*I site 600 bp downstream of exon 8, were taken from a λ genomic clone, placed into the plasmid vector pGEM11Z (Promega, Madison, WI) and subsequently transferred to the minigene construct as a *Bam*HI/*Not*I fragment generating plasmid pLFA1–8. The genomic region containing exons 8 and 9 was amplified by PCR from mouse genomic DNA and subcloned as a 2000-bp *Hin*dIII/*Eco*RI fragment into pGEM11Z, creating pG11(8–9). For this amplification, the 35-bp sense oligonucleotide primer was the complete exon 8 sequence, and the antisense primer extended from base 192 to 215 of exon 9. The PCR amplification fragment included the *Xho*I site downstream from exon 8 and the *Xba*I restriction site within exon 9. The *Xba*I to *Eco*RI 3′ CD45 DNA fragment in pSPORT-XR was inserted into pG11(8–9) as an *Xba*I/*Not*I fragment generating pG11(8–9)A. A CD45.1 cDNA region (3) extending from exon 9 to 33 was subcloned into pG11(8–9)A as an *Xba*I fragment producing pG11(8-A). The CD45 minigene construct, pLFAiT200, was completed by the introduction of the *Xho*I/*Not*I restriction fragment from pG11(8-A) into pLFA1–8. The final construct contains CD45 sequence consisting of a cDNA 5′ region (exons 1b–3), a genomic DNA region extending from exon 3 to exon 9, a cDNA segment from exon 9 to exon 33, and 3′ gene sequences from exon 33 to 750 bp downstream of the end of transcriptional termination.

The potential promoter region upstream of exon 1a was isolated from a λ genomic library constructed in the LambdaGEM-12 cloning vector (Promega). The longest λ clone extended ~19 kb upstream and 1800 bp downstream of the CD45 translational start site. The insert was flanked by *Not*I sites from the vector. A partial restriction map of a subsection of this DNA is shown in Fig. 1*D*; the *Nhe*I site maps within exon 1b. The entire upstream region was subcloned into plasmid pGEM5Z as three smaller fragments: a 15-kb *Not*I/*Pst*I fragment consisting of the most upstream 5′ sequences (pG5NP); a 1.3-kb *Pst*I fragment (pG5P); and a 4.5 kb, *Pst*I/*Not*I fragment including exons 1a, 1b, and 2 as well as 1.7 kb of intron sequence 3′ of exon 2 (pG5PN). The 5′ *Not*I site was changed to a *Sfi*I site using double-stranded oligonucleotides containing a *Not*I overhang and an internal *Sfi*I site. Using the natural *Eco*RI, *Pst*I, and *Nhe*I sites and the synthetic *Sfi*I site, the following plasmids were generated: pCD45FiT200, pCD45EiT200, pCD45PiT200, and pCD45P²iT200 (Fig. 1*D*).

RNA Isolation—Total RNA was purified from cells using TRIZOL (Life Technologies) following the manufacturer's directions. Fifty micrograms RNA isolated from cells transfected with plasmids pLFAT200 and pCMVT200 were treated with 1 unit of DNase I (Promega) for 1 h at 37 °C. The DNase I was removed by one extraction with phenol/chloroform/isoamyl alcohol followed by one extraction with chloroform/isoamyl alcohol as suggested by Promega. The RNA was precipitated with ethanol and resuspended in RNase-free water.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)—RT-PCR was performed using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) to distinguish transgene CD45.1 mRNA transcripts from the endogenous CD45.2 transcripts of the transfected cells. All RT and PCR reactions were assembled according to the manufacturer's recommendations. The antisense oligonucleotide used for cDNA synthesis, 5′-GAGACCAGAAACTCATAG-3′, contains the last 13 bp of exon 14 and the first 5 bp of exon 15. For the PCR component, the sense oligonucleotide primer was the complete exon 3 sequence, 5′-GGCAAACACCTACACCCAGTGATG-3′. The antisense oligonucleotides are specific for the CD45.1 (*Ly5*^{a}) and CD45.2 (*Ly5*^{b}) alleles (3, 47) and consist of bases 86–106 of exon 12. The sequences of these antisense primers are as follows with the allele-specific bases underlined: CD45.1, 5′-CCATGGGGTTTAGATGCAGGA-3′ and CD45.2, 5′-CCATGGGGTTTAGATGCAGAC-3′. The cDNA was synthesized for 1 h at 42 °C followed by 5 min at 95 °C. The cDNA was amplified for 34 cycles. The first cycle consisted of 2 min at 94 °C, 1 min at 63 °C, and 3 min at 72 °C. The remaining cycles were 1 min at 94 °C, 1 min at 63 °C, and 3 min at 72 °C. The amplified products were separated on a standard TBE-agarose gel (48), photographed using a DC120 zoom digital camera (Eastman Kodak Co.), and analyzed using Kodak 1D imaging analysis software.

β-Galactosidase Assays—293 cells were harvested 48 h after transfection. The plates were washed twice in PBS and then incubated with 2 ml of 0.5 g/liter trypsin, 0.2 g/liter EDTA solution (Irivine Scientific) for 5 min at 37 °C. The isolated cells were washed twice in PBS and resuspended in 1 ml of ice-cold PBS. Cellular extracts were prepared by three cycles of freeze-thaw followed by removal of the cellular debris and assayed for β-galactosidase activity (48). Total protein in the extracts was measured using the Bio-Rad Protein Assay (Bio-Rad).

Flow Cytometry and Antibodies—Cell suspensions were stained using directly conjugated antibodies from Pharmingen (La Jolla, CA): FITC-anti-CD45.1, FITC-anti-CD45.2, and FITC-anti-THP (IgG_{2a} isotype control). Approximately 1×10^6 cells were resuspended in 100 μl of Hanks' balanced salt solution (Mediatech, Herndon, VA) containing 0.1% bovine serum albumin and 0.02% sodium azide. Antibodies were added to a final concentration of 0.5 μg/ml and incubated in the dark for 20 min at 4 °C. Cells were washed one time in Hanks' balanced salt solution, resuspended in 500 μl of Hanks' balanced salt solution, and analyzed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA) using CellQuest software. Live cells were discriminated from dead cells using propidium iodide at a final concentration of 5 μg/ml.

Sequencing—Correct CD45 cDNA sequences in the constructs were confirmed by DNA sequencing. All DNA sequencing was performed on an ABI sequencer by the DNA core facility at the Burnham Institute (La Jolla, CA).

RESULTS

Examination of the Expression of CD45 cDNA Constructs—Following reports of expression of CD45 from cDNA plasmids, pARV/CD45 in thymic lymphoma cells (38), and pML84 and pML171 in transgenic mice (40), we tested expression of CD45 from these transgenes in the T cell hybridoma line, 27J. The full-length CD45 transgene of pARV/CD45 is under the control of the constitutive, retroviral 5′ long terminal repeat promoter, but the only discernible poly(A) site in the construct lies in the 3′ long terminal repeat ~2.4 kb downstream and beyond the neomycin gene. In contrast, the transgenes of pML84 and pML171, $CD45R^{ABC}$ and $CD45R^{O}$, respectively, are regulated by the thymus-specific, proximal *lck* promoter, and the human growth hormone gene provides the poly(A) signal. To facilitate our analysis of transgene-produced mRNA and protein, we replaced the CD45.2 allogenic determinants of all three cDNAs with sequences specifying the CD45.1 allele.

Stably transfected populations were generated using these constructs and were analyzed for CD45.1 expression by flow cytometry. No CD45.1 protein expression from the three cDNA constructs was detected in any of the transduced cell populations (Fig. 2). Since the CD45 cDNA in plasmids pML84 and pML171 is under the control of a thymus-specific promoter, the lack of expression from these plasmids may be due to the inability of the promoter to function in the cytotoxic T cell hybridoma line. Therefore, using pML84 and pML171, we produced stably transfected cell populations in the thymic lymphoma line, BW5147, and employed flow cytometry to analyze the populations for CD45.1 expression. No CD45.1 protein was detected in these transfected cells (Fig. 2).

The site of integration, levels of polyadenylation, transport to the cytoplasm, and stability of the mRNA are among the factors that can affect the expression from transgenes, and the structure of the transgene itself influences these elements (49–53). To better understand possible reasons behind the difficulty obtaining CD45 protein expression from cDNA constructs, we investigated the roles the 5′, 3′, and intron regions play in regulation.

Analysis of the 3′-Untranslated Region of CD45—To identify regulatory elements in the 3′-UTR of CD45, the 3′-UTR was fused downstream of the β-galactosidase coding region, and the effect on gene expression was analyzed following transient transfection into tissue culture cells. The plasmids constructed for this study are shown in Fig. 1*A*. The SV40 small t/poly(A) site normally regulating β-galactosidase mRNA processing in pCMVSPORTβgal (Fig. 1*A*) was replaced by the entire 3′-UTR of CD45 plus 750 bp of sequence downstream from the end of the mRNA (3) (pSPORT-XR; Fig. 1*A*). A smaller region beginning 887 bp downstream from the termination codon and extending 750 bp beyond the polyadenylation site was subcloned in front of the small t/poly(A) site (pSPORT-EA, Fig. 1*A*) to determine whether the 3′ region had any effect, positive or

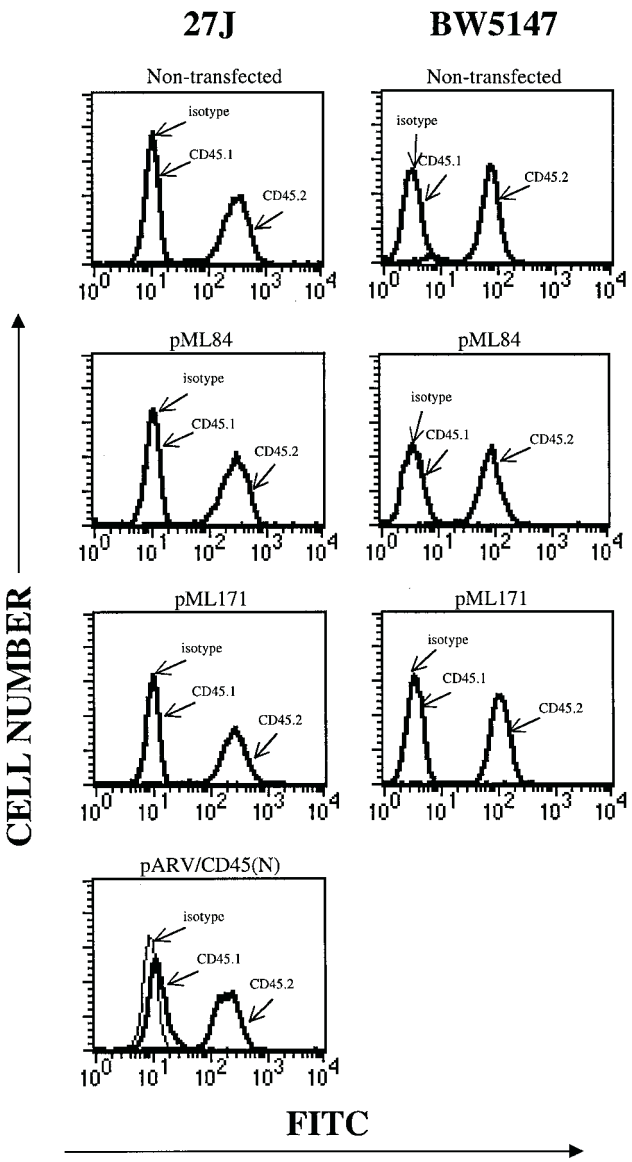

FIG. 2. **Flow cytometry analysis of cDNA transfectants.** The cell line is indicated *above* each column. The histograms are labeled according to the plasmid used to generate the stable cell populations. The cells were stained with either the FITC-labeled isotype control (isotype), FITC anti-CD45.1 (CD45.1), or FITC anti-CD45.2 (CD45.2) antibodies. The individual peaks are labeled with the antibody used for staining.

negative, on the function of an existing poly(A) sequence. pSPORT-BGA (Fig. 1*A*), in which the SV40 small t/poly(A) site was replaced with the bovine growth hormone poly(A), and pSPORT-(−A) (Fig. 1*A*), in which all poly(A) sites were removed, served as controls for these experiments.

Initially, the plasmids were transfected transiently into various lymphocyte cell lines (S49, 27J, and Jurkat). However, no β-galactosidase activity above background was detected (data not shown). This result is most likely due to the low transfection efficiency of these cells (S49 and Jurkat) or to high levels of endogenous β-galactosidase activity (27J). 293 cells, on the other hand, exhibit a high frequency of transfection and have low levels of endogenous β-galactosidase activity. Therefore, the analysis of the CD45 3′ region on transgene expression was completed using the 293 cell line.

β-Galactosidase activity was assayed from cellular extracts isolated 48 h after transfection. The results from four separate transfections were averaged and are summarized in Table I.

TABLE I
β-galactosidase activities from transfected 293 cells

	β-galactosidase activity	Percent activity relative to wild type pCMVSPORTβgal
	units/mg protein	%
pCMVSPORTβgal	38.1	100
pSPORT-XR	29.5	77
pSPORT-EA	47.0	123
pSPORT-BGA	40.5	100
pSPORT-(−A)	9.9	26

The β-galactosidase levels observed in cells transfected with pSPORT-XR, which contains the 3′-UTR and sequences downstream of the poly(A) site, were essentially equivalent to the activity seen in those harboring the parental vector, pCMVSPORTβgal. Introduction of the *Eco*RI fragment containing CD45 3′-UTR sequences upstream of the SV40 small t/poly(A) did not alter β-galactosidase expression substantially (pSPORT-EA; Table I). In fact, a slight increase in β-galactosidase levels was reproducibly observed in cells carrying this construct. Removal of all poly(A) sequences (pSPORT-(−A)) resulted in a significant reduction in β-galactosidase activity, and the bovine growth hormone poly(A) (pSPORT-BGA) restored activity.

Analysis of the Effect of Different 5′ Sequences on CD45 cDNA Expression—To investigate how 5′ sequences might influence expression, the cDNA encoding the full-length CD45 isoform (the 456789 isoform) was placed under the control of either the tissue-nonspecific CMV promoter (pCMVT200; Fig. 1*B*) or the leukocyte-specific human LFA-1 (CD11a) promoter (pLFAT200; Fig. 1*B*). Both transgenes utilize the CD45 poly(A) signal sequence plus 750 bp of sequence downstream from the mRNA termination site. In order to follow easily the expression of the CD45 from the transgene in transfected cells, the CD45 cDNA constructs were prepared containing the CD45.1 allele sequence. The CD45 cDNA in these constructs was sequenced to confirm an intact coding region. Transfections were performed using the T-cell hybridoma line, 27J, and the B lymphocyte cell line, 70Z/3. Both of these cell lines naturally express the CD45.2 but not the CD45.1 allele (Fig. 3). Stable transfectants were generated, and protein expression from the transgenes was monitored using flow cytometry and a CD45.1 allele-specific monoclonal antibody. CD45.1 protein production was not detected from either construct in the transfected cell populations (Fig. 3).

To determine whether there was a corresponding lack of transcription from the transgenes, total RNA was isolated from the transfected cells and analyzed with RT-PCR using allele-specific primers. The transfected 27J and 70Z/3 cell populations transcribed CD45 mRNA from both the pCMVT200 and pLFAT200 transgenes (data not shown). Therefore, the CD45 cDNA constructs, pCMVT200 and pLFAT200, produce some mRNA in both T and B cells, but detectable protein levels are not observed. Since the sequence of the 5′-end of the transgenes extending from the *Cla*I site across the *Xba*I junction (exon 1b to exon 9) was identical to published sequence, the lack of protein is not likely to be the result of a mutation introduced during construction. RT-PCR is a more sensitive assay for expression compared with flow cytometry. Therefore, the transgenes appear to be producing small amounts of mRNA that can be detected with RT-PCR, but the protein expressed, if any, is below the sensitivity of flow cytometry.

Analysis of CD45 Minigene Expression—Reports of transgene expression have indicated that the presence of intron sequences can increase gene expression (49–51). Since one of the interesting properties of the endogenous CD45 gene is the production of multiple isoforms through mRNA splicing, a

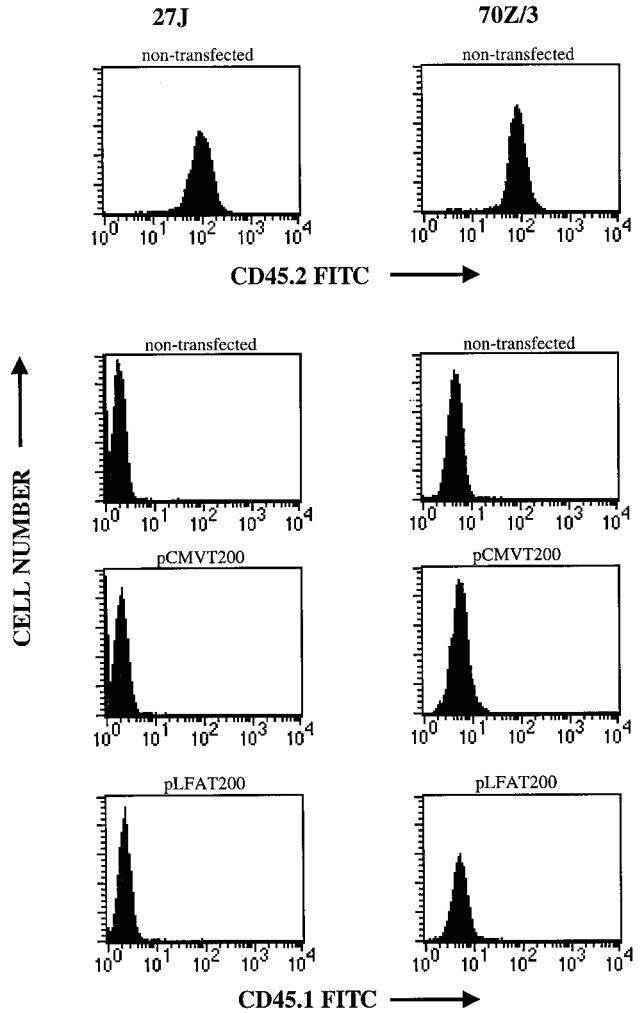

FIG. 3. **Flow cytometry analysis of cell lines transfected with pLFAT200 and pCMVT200.** The cell line tested is shown *above* each column. The transfection treatment is indicated *above* each histogram. The antibodies used for staining are given *below* the histograms (CD45.1 FITC, CD45.2 FITC).

CD45 expression construct that included the alternatively spliced exons and associated introns might not only exhibit increased levels of expression but also would provide the possibility of producing the various isoforms. Therefore, a CD45 minigene, encoding the CD45.1 allele, was generated and placed under the control of the LFA-1 promoter (pLFAiT200; Fig. 1*C*). In this plasmid, genomic DNA carrying the alternatively spliced exons 4–8 is flanked by two cDNA regions that span exons 1b–3 and 9–33.

The cell lines 27J and 70Z/3 were transfected with pLFAiT200, and stable populations expressing the neomycin resistance gene were selected. Flow cytometry with allele-specific antibodies was used to detect CD45 protein expression from the transgene. CD45.1 expression was dramatically increased in the transfected populations compared with the nontransfected control, suggesting that the pLFAiT200 minigene was producing substantial levels of CD45 protein (Fig. 4).

RT-PCR analysis of RNA isolated from the B and T cell transfectants was used to evaluate CD45 isoform expression from the minigene. Two CD45.1-specific mRNA species, corresponding to the 789 and 89 isoforms, were detected in the 27J transfected cells (Fig. 5*A*, *lane 4*). These are the same splice variants as produced by the CD45.2 endogenous allele (Fig. 5*A*, *lane 3*). The pattern of CD45 exon usage from the transgene

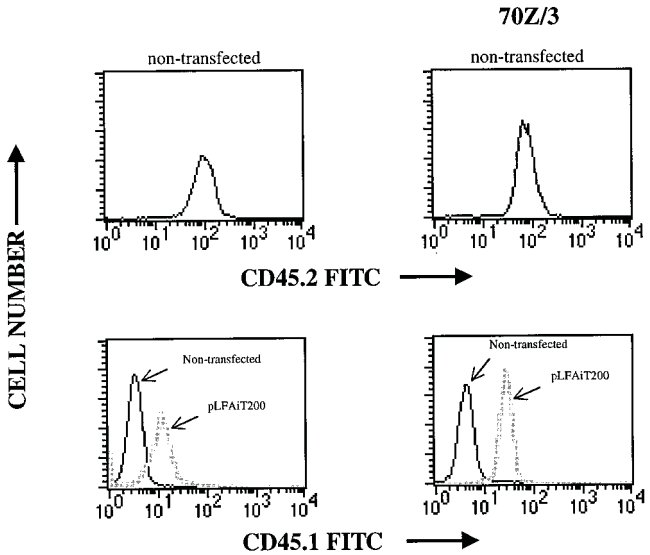

FIG. 4. **Flow cytometry analysis of pLFAiT200 transfectants.** The cell line is indicated *above* each column. The individual peaks of the *bottom* histogram are labeled with the transfection treatment. The staining antibodies are shown *below* the histograms (CD45.1 FITC, CD45.2 FITC).

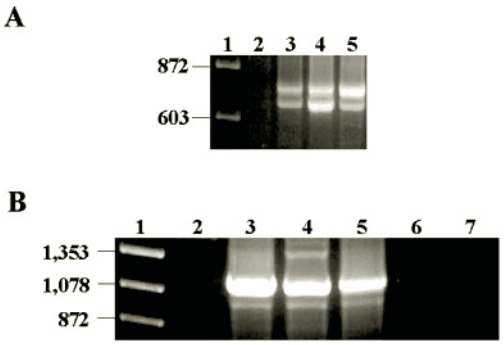

FIG. 5. **RT-PCR products from RNA isolated from T and B cell populations transfected with pLFAiT200.** In both *A* and *B*, *lane 1* contains molecular weight markers. Amplification products in all *even-numbered lanes* of both *panels* were generated using the CD45.1 allele-specific antisense primer, and the *odd-numbered lanes* contain fragments amplified with the CD45.2-specific primer. In *A*, the RNA was isolated from 27J cells either nontransfected (*lanes 2* and *3*) or transfected with pLFAiT200 (*lanes 4* and *5*). *B*, RNA was from 70Z/3 cells either nontransfected (*lanes 2* and *3*) or transfected with pLFAiT200 (*lanes 4* and *5*). *Lanes 6* and *7* are no RNA, negative controls.

and the endogenous gene also was identical in 70Z/3-transfected populations (Fig. 5*B*, *lanes 3–5*). The predominant isoform was 456789, with minor amounts of the 56789 form detected. No amplification products were seen using the CD45.1-specific primer with RNA isolated from nontransfected cells (Fig. 5, *A* and *B*, *lane 2*). Although the ratio of the two isoforms produced from the transgene and from the endogenous gene differs slightly in the 27J transfectants, both the 27J and 70Z/3 transfected populations express the same isoforms from the transgene as are expressed from the endogenous gene. Thus, the minigene carries the regulatory elements necessary for cell type-specific splicing.

Control of the Minigene with CD45 5′ Upstream Sequences—To identify upstream sequences responsible for regulating CD45 gene expression and to create a minigene that more closely resembles the endogenous gene, a λ genomic clone-containing sequence extending 19 kb upstream from the start of CD45 translation was utilized. The 19-kb region was inserted in place of the LFA-1 promoter in pLFAiT200, creating pCD45FiT200 (Fig. 1*D*). In the same fashion, minigene constructs containing 5′ truncations of the 19-kb CD45 upstream region were generated. pCD45P^{2}iT200, pCD45PiT200, and pCD45EiT200 extend 3.5 kb, 2.2 kb, and 839 bp, respectively, upstream from the translational start site (Fig. 1*D*).

Stable transfectants of the cell line 27J were generated with each of these transgenes. For a positive control, the cells were transfected with pLFAiT200. Expression from the transgenes was monitored by flow cytometry with the allele-specific antibodies (Fig. 6). As observed from previous experiments, CD45.1 protein was readily detected in the cells transfected with pLFAiT200. Compared with the isotype control, a minimal shift in the mean fluorescence intensity of CD45.1 was detected in cells harboring the pCD45EiT200 and pCD45FiT200 transgenes, while in pCD45PiT200 transfectants the CD45.1 peak exhibited a slightly more significant but still minor change. No CD45.1 protein expression was observed in cells containing pCD45P^{2}iT200.

Allele-specific RT-PCR experiments were carried out using these 27J transfected cell populations. The expected 789 and 89 isoform transcripts were expressed from all four constructs, and no fragments corresponding to CD45.1 transcripts were detected in nontransfected cells (data not shown)

DISCUSSION

For the analysis of the molecular basis of the developmental and functional properties of CD45, a reliable expression system is needed. However, obtaining stable high level expression from CD45 cDNA constructs has been difficult and inconsistent (41). Therefore, in an effort to optimize expression from CD45 cDNAs, we tested 5′, 3′, and intron regions for their effect on transgene expression.

A number of examples are known for translational control of mRNA through sequences in the 3′-UTR (43, 44, 54). In many cases, these regulatory elements interact with positive or negative trans-acting factors, thereby controlling mRNA stability or localization (43, 44, 54). To assess the possible role of the CD45 3′-UTR in expression, the 3′-UTR and downstream sequences were fused 3′ of the β-galactosidase coding region. Our results show that the CD45 polyadenylation signal was fully functional in conjuction with the β-galactosidase reporter gene. Furthermore, the presence of the CD45 3′ sequences had no effect on the levels of β-galactosidase expressed in the transient transfection assays, suggesting that no negative cis-acting regulatory factors are present. Since these experiments measured the effect on a heterologous gene (β-galactosidase) in a nonlymphocyte system, the possibility of leukocyte-specific controls was not eliminated by this analysis.

Stable lymphoid cell populations generated by transfection with constructs containing CD45.1 coding sequences show dramatic differences in levels of CD45.1 protein expressed. pLFAT200 and pLFAiT200 only differ in the inclusion of the six introns present in pLFAiT200. The addition of the intron sequences led to a substantial increase in protein expression as measured by flow cytometry. Both the LFA-1 promoter and the strong CMV promoter did not produce detectable protein levels in the CD45 cDNA constructs without the introns. Although the exact mechanism by which the introns affect expression is not known, it has been shown that introns can contain enhancers (55–59), transcriptional silencers (60, 61), and elements controlling cell-specific expression (61–64). Furthermore, splicing can influence the levels of cytoplasmic mRNA through posttranscriptional mRNA processing (49). Examples of this processing include increased nuclear mRNA stability, increased polyadenylation, and increased transport to the cytoplasm. Utilizing RT-PCR, we found that the cell populations transfected with the CD45 cDNA constructs all produced RNA from the transgene, supporting the possibility that a posttran-

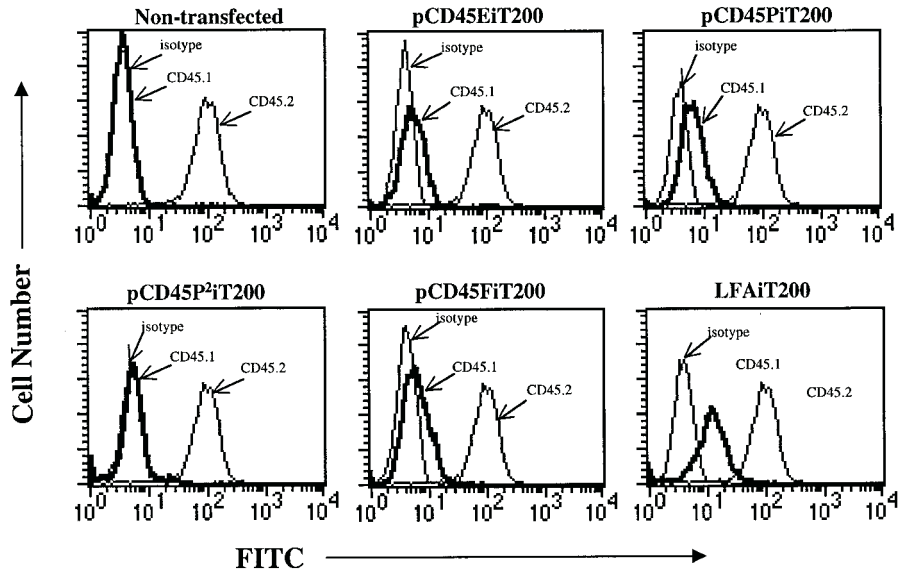

FIG. 6. **Flow cytometry of the T cell populations transfected with the CD45 upstream constructs.** The plasmids used to generate the stable cell populations are given at the *top* of each histogram. The cells were stained with either the isotype control FITC anti-THP (isotype), FITC anti-CD45.1 (CD45.1), or FITC anti-CD45.2 (CD45.2) antibodies. The individual peaks are labeled with the antibody used for staining.

scriptional mechanism is responsible for the intron-related increase in expression. RT-PCR also showed that T and B cells transfected with pLFAiT200 produced the distinctive isoforms characteristic of each cell type. Therefore, the controls for correct splicing of the alternative exons lie within the pLFAiT200 construct. Previous splicing studies also concluded that the sequences within the introns and exons in this region are sufficient to regulate alternative splicing (65–68). Finally, it should be noted that both pLFAT200 and pLFAiT200 utilize an identical CD45 3′ region extending from exon 33 to 750 bp downstream from the polyadenylation site, suggesting that this sequence contains no cis-acting elements responsible for controlling expression in lymphoid cells.

Although the CD45.1 protein levels were enhanced in pLFAiT200-transfected cells, they were below endogenous CD45 expression in both cell types. One simple explanation would be that the transgene is subject to integration-dependent positional effects. However, since these transfected populations are not clonal, it would be expected that a positional effect in a few cells would be averaged out over the entire population. Also, the lower level of expression from the transgene may be due to particular properties of the cell lines used in this study. Another potential explanation is related to the importance of the introns for expression. The included introns are located between exons 3 and 9 in pLFAiT200, and the remainder of the construct (exons 9–33) is strictly cDNA. Therefore, additional splicing events that could contribute to endogenous CD45 gene expression cannot occur.

Perhaps the most important feature of the construct affecting expression levels is the human LFA-1 promoter that controls the transgene expression in mouse cell lines. The expression level may reflect a diminished ability of the human promoter to interact with a murine regulatory factor. Furthermore, the protein levels of endogenous CD45 are very high, ~10% of lymphocyte surface proteins (69), and the human LFA-1 promoter may not be able to drive expression to the same levels. The LFA-1 promoter, however, contributes several features that make it an attractive choice for a heterologous promoter in these studies. First, the CD11a gene is expressed in a cell type-specific pattern similar to CD45 (46, 70, 71). Second, in mice the human LFA-1 promoter directed transgene expression in a pattern parallel to endogenous murine CD11a (71). However, T cell developmental signals do play a role in the regulation of CD11a. Low level expression of CD11a is thought to characterize naive T cells, while expression of high levels of CD11a ($CD11a^{bright}$) is a property of memory T cells (72). The 27J T cell hybridoma line exhibits the $CD11a^{bright}$ phenotype (data not shown).

In an effort to improve expression, the natural CD45 upstream region was investigated. Examination of the sequences upstream from the CD45 translation initiation sites reveals neither a consensus TATA box near positions −30 to −25 nor a pyrimidine-rich initiator usually located near the transcription start site. Typically, recognition sites for DNA-binding transcription factors lie upstream (−50 to −200 bp relative to the transcription start) from these core promoter elements (73). In addition, transcriptional enhancers and silencers located far upstream or downstream of a gene's promoter may regulate transcription (73, 74). Although the CD45 upstream region does contain canonical binding sites for many transcription factors, the possible role of these sites in CD45 regulation has not been reported. To test the promoter activity of CD45 upstream sequences, the CD45 minigene was placed under the control of different CD45 upstream regions, extending to 19,000 bp 5′ of the translational start. Stably transfected cell populations were generated with these transgenes and mRNA, but little or no protein, was detected. The CD45 protein levels did not vary significantly between these various CD45 upstream constructs, indicating that the low level of expression most likely is due to the absence of an enhancer. One previously identified control element, a TC box located between exons 1b and 2 that acts as a tissue-specific initiator for a minor transcriptional start position 3′ of exon 2 (15), is missing in our constructs. However, the two major transcriptional start sites at exons 1a and 1b with surrounding sequence are present in these constructs. An intriguing candidate for the location of additional transcriptional control elements is the 50-kb intron between exons 2 and 3, an intron whose unusual size and location is conserved between species.

Our experiments indicate that regulation of CD45 is a complex process requiring elements that lie within the introns of the gene and possibly well outside of the coding region. The 3′-UTR and associated downstream sequences contain no positive or negative regulatory elements. The CD45 region up to 19 kb upstream of the transcriptional start is not sufficient to produce readily detectable levels of expression. However, the intron sequences, located within the genomic DNA segment encoding the alternatively spliced exons, enhance expression of the transgene dramatically and contain the controls necessary for correct splicing of the alternative exons. Using the

pLFAiT200 minigene, we were able to reproducibly generate stable lymphoid cell populations producing correctly spliced CD45.1 mRNA and protein.

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