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CD69 Gene Is Differentially Regulated in T and B Cells by Evolutionarily Conserved Promoter-Distal Elements

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CD69 is a type II C-type lectin involved in lymphocyte migration and cytokine secretion. CD69 expression represents one of the earliest available indicators of leukocyte activation and its rapid induction occurs through transcriptional activation. In this study we examined the molecular mechanism underlying mouse CD69 gene transcription in vivo in T and B cells. Analysis of the 45-kb region upstream of the CD69 gene revealed evolutionary conservation at the promoter and at four noncoding sequences (CNS) that were called CNS1, CNS2, CNS3, and CNS4. These regions were found to be hypersensitive sites in DNase I digestion experiments, and chromatin immuno-precipitation assays showed specific epigenetic modifications. CNS2 and CNS4 displayed constitutive and inducible enhancer activity in transient transfection assays in T cells. Using a transgenic approach to test CNS function, we found that the CD69 promoter conferred developmentally regulated expression during positive selection of thymocytes but could not support regulated expression in mature lymphocytes. Inclusion of CNS1 and CNS2 caused suppression of CD69 expression, whereas further addition of CNS3 and CNS4 supported developmental-stage and lineage-specific regulation in T cells but not in B cells. We concluded CNS1–4 are important cis-regulatory elements that interact both positively and negatively with the CD69 promoter and that differentially contribute to CD69 expression in T and B cells.

of distal enhancers, silencers, and insulators for efficient gene expression, and may require DNA methylation and histone modifications to regulate access to transcription factors. Epigenetic regulation has been shown to be crucial for inducible expression of a variety of immune system genes, including IL-4, IFN-γ, IFN-β, and IL-12 (18–21). Therefore, the goal of this study was to define the epigenetic changes and cis-acting elements important for regulated CD69 gene expression in lymphocytes in vivo. We searched for distal regulatory elements using cross-species sequence comparison, DNase I hypersensitivity mapping, and transcriptional activity analysis in transient transfection assays and we then used a Tg approach to test the functional significance of candidate cis-regulatory elements in vivo. Our results indicate that unusual as well as common transcriptional regulatory mechanisms control expression of the CD69 gene in different lymphocyte populations.

Materials and Methods

Comparative genomic analysis

Sequence comparison of mouse, human, and dog CD69 was performed using VISTA Browser from the Lawrence Berkeley National Laboratory (available at http://pipeline.lbl.gov/cgi-bin/gateway2).

DNase I hypersensitivity assay

Rag2<sup>−/−</sup> X Tcrb Tg mice (Rxβ) and hemaggulitin (HA)-TCR Tg mice were described previously (22, 23). BRC were lysed in 0.1 M NH₄Cl, 1 mM KHC0₃, 1 mM Na₂EDTA (pH 7.4) for 3 min at 4°C and subsequently washed in 30 ml of cold PBS. Thymocytes (10⁷/ml) were permeabilized with 0.067 mg/ml lysolecithin in buffer C (0.15 M sucrose, 80 mM KCl, 30 mM HEPES (pH 7.4), 5 mM MgCl₂, 5 mM CaCl₂) for 5 min. DNase I was added at a final concentration of 0, 4, 8, 12, 16, 20, 24, 28, or 32 U/ml for 10 min on ice. Reactions were stopped by the addition of EDTA, SDS, and proteinase K to final concentrations of 10 mM, 0.4% (v/v), and 0.4 mg/ml, respectively, and were incubated overnight at 37°C. DNA was purified by phenol, phenol/chloroform, and chloroform extractions and ethanol precipitation, taking care not to shear the genomic DNA. Purified DNA (10 µg) was incubated overnight at 37°C with an excess of EcoRI restriction enzyme. Digests were separated by 0.7% (w/v) agarose gel electrophoresis and were analyzed by Southern blot with 3²P-labeled DNA probes (primers to generate probes; supplemental Table I).

Reporter constructs and Tg mice

hCD2 reporter constructs were generated by modification of the mCD8 reporter vector (24). To generate construct 1 (see Fig 4B), the promoter of mCD8 (−645/+1) was PCR amplified (Expand High Fidelity PCR System; Roche) and cloned into psiBlue vector (Vector Acceptor Kit; Novagen). Restriction sites introduced during PCR amplification were used to excise the mCD8 promoter from this plasmid with Apal and ClaI and ligate it to the Apal/ClaI-digested hCD2 construct. To ensure that we incorporated conserved sequences and DNase I hypersensitive sites, primers for genomic regions encompassing conserved noncoding sequence (CNS1) (233 bp), CNS2 (451 bp), CNS3 (439 bp), and CNS4 (181 bp) were designed (primer sequences, supplemental Table I). PCR products for CNS1–4 fragments were 807, 932, 600, and 707 bp in length, respectively. To generate construct 2, CNS1 and CNS2 were first combined into psiBlue cloning vector. CNS2/CNS1 fragment was then excised from this construct using Apal and XbaI and inserted into XbaI/Apal sites upstream of the mCD8 promoter in construct 1. Similarly, to make construct 3, CNS3 and CNS4 were combined into psiBlue, digested with XbaI, and inserted into the XbaI site upstream of CNS2 in construct 2. Importantly, NotI restriction sites were flanking all three hCD2 reporter constructs. Construct 1 had a size of 3.7 kb and was separated from vector backbone using Apal/NotI digestion. Construct 2 was 5.4 kb and was separated with XbaI/NotI digestion. Construct 3 was 6.7 kb, was separated with NotI digestion. Purified DNA was then microinjected into fertilized eggs. Founders were identified by PCR and copy number was determined by quantitative real-time PCR. Input DNA was determined using primers ExonIIIF and ExonIIIR (primers sequences, supplemental Table I). Handling of mice and experimental procedures were in accordance with institutional requirements for animal care and use.

Purification of cell populations and cell culture

To enrich for T cell populations, lymph node cell suspensions were passed through a nylon wool column (Polysciences) following the manufacturer’s instructions. T cell purity, determined by flow cytometry, was >85%. Splenocytes of TCRβ<sup>−/−</sup>T cells were used to obtain B cells >95% B220<sup>+</sup> by flow cytometry.

Before chromatin immunoprecipitation (ChIP) analysis of peripheral lymphocyte populations, cells were stimulated in vitro with 10 ng/ml PMA (Sigma-Aldrich) and 0.5 µM calcium ionophore (Sigma-Aldrich) for 6 h at 37°C. CD69 expression was then analyzed by flow cytometry.

For hCD2 analysis in peripheral lymphocytes, splenocytes of wild-type and Tg mice were activated by incubation with 10 ng/ml PMA and 1 µM to or 5 µg/ml plate-bound anti-CD3 (clone 145-2C11; eBioscience) and anti-CD28 (clone 37.51; eBioscience) mAbs overnight at 37°C. In experiments using PMA plus Io, data were collected from experiments in which activated lymphocytes were >90% CD69<sup>+</sup>.

Poly(I:C) treatment

Mice were injected i.p. with 500 µg of poly(I:C) (Sigma-Aldrich). After 18 h of treatment, spleens were obtained from animals and lymphocyte suspensions were prepared for FACS analysis.

Chromatin immunoprecipitation

For analysis of thymocytes, immunoprecipitations were performed on purified mononucleosomes as described previously (25). Briefly, thymocytes were lysed and nuclei were treated with micrococcal nuclease to produce a partial chromatin digest. After removal of linker histone H1, chromatin was fractionated on a sucrose gradient. For analysis of peripheral lymphocytes, immunoprecipitations were performed on parafomaldehyde-cross-linked chromatin prepared as described previously (26). Sonication was used to obtain DNA fragments ranging from 300 to 500 bp. With either approach, anti-diacylated H3, anti-dimethylated H3 K4, and control rabbit-IgG Abs (Upstate Biotechnology) were used for immunoprecipitation and bound and input fractions were quantified using SYBR Green real-time PCR (Roche). Analysis of the constitutively active carbamoyl-transferase dihydroorotase (CAD) gene was used to normalize values of different samples. Primer sequences are provided in supplemental Table I.

Flow cytometry

Cell suspensions from spleen, thymus, and bone marrow were stained with FITC-CD69 (BD Biosciences), PE-hCD2 (Caltag Laboratories), PerCP-CD4 (BD Biosciences), aliphosphorycin-CD8 (BD Biosciences), FITC-CD24 (HSA; eBioscience), PE-Cy5 IgM (eBioscience), or aliphosphorycin-B220 (eBioscience) Abs. Data were collected on a FACSCalibur or FACSCan (BD Biosciences) and were analyzed using FlowJo software.

Luciferase reporter constructs and cell transfections

For the firefly luciferase vector we used the pXPG vector (27). Primers used to amplify fragments were the same as for the hCD2 reporter constructs with modifications in the restriction sites (primer PromF contained a XhoI restriction site and other primers a BamHI restriction site). The CD69 promoter was digested with XhoI and BamHI and ligated to XhoI BamHI-digested pXPG-Luc vector. CNS1–4 fragments were digested with BamHI and ligated to BamHI-digested CD69 prom-pXPG-Luc vector. Construct sequences were confirmed by restriction enzyme digestion and sequence analysis.

For transient transfection assays, a total of 5 × 10<sup>5</sup> Jurkat cells were plated into a 24-well plate and transfected with 1 µg of specific firefly luciferase test plasmid, 20 ng of pRl-TK Renilla luciferase control plasmid, and 4 µl of SuperFect (Qiagen) according to the manufacturer’s instructions. Twenty-four hours after transfection, cells were stimulated with 10 ng/ml PMA and 1 µM Io or 5 µg/ml plate-bound anti-CD3 (clone OKT3; eBioscience) and anti-CD28 (clone CD28.2; eBioscience) mAbs or were mock incubated. A luciferase assay was performed 24 h later using the Dual-Luciferase Reporter Assay System (Promega). Transfections were performed in duplicate and values were normalized to Renilla luciferase activity.

Results

Conservation of the CD69 locus

CD69 is a type II transmembrane C-type lectin encoded in the NK complex on mouse chr6 and human chr12 (Fig. 1A). The gene spans ~7.5 kb and contains 5 exons. The first two exons encode the cytoplasmic and transmembrane domains, and exons III, IV,
and V encode the extracellular portions of the molecule. The defined murine CD69 promoter (−656 to +1 relative to the transcription start site) is the only \textit{cis}-acting element known to regulate CD69 expression (13). We wanted to identify other potential \textit{cis}-acting sequences involved in CD69 gene regulation. Because cross-species genome analysis has been useful for this purpose (28), we compared mouse, human, and dog genomic sequences by the means of VISTA Browser (29). Using the default parameters for defining a conserved CNS element (70% identity over 100-bp length), four elements upstream of the CD69 gene were identified (Fig. 1B). CNS1 was upstream of and contiguous to the promoter, and CNS2, CNS3, and CNS4 were 9, 28, and 40 kb away from the main site of transcription initiation, respectively. Marked conservation was also observed at the promoter. Because there are substantial differences in genomic organization of the human and mouse NK complexes upstream of CD69 (Fig. 1A), we hypothesized that CNS1–4 may regulate CD69 rather than neighboring gene expression.

**Accessibility of the CD69 locus**

Chromatin regulatory regions are typically characterized by DNase I hypersensitive sites (HSs) that reflect NF binding and disrupted nucleosome organization. We conducted DNase I HS assays and Southern blots to determine whether CNS1–4 represent conserved DNase I HSs. CD69 is up-regulated by thymocyte-positive selection, and different TCR Tg mouse strains express characteristic quantities of CD69 based on both the selecting background and the strength of TCR-MHC/peptide interactions (30). We used thymocytes from B10.D2 H-2Kd mice expressing a Tg TCR specific for a HA peptide presented by H-2Kd to isolate thymocytes with high CD69 expression and thymocytes from Rag2\(^{-/-}\) mice that express a Tcrb transgene (Rx\(^{b}\)) to isolate thymocytes with low CD69 expression (Fig. 2A). Total thymocytes from these strains were treated with varying concentrations of DNase I, and genomic DNA was extracted, digested with EcoRI, and analyzed by Southern blot. Size markers and relevant bands are denoted.

**FIGURE 1.** Genomic organization and conservation of the CD69 locus. A, Genomic organization of the human and mouse CD69 gene and neighboring genes in the NK complex. The diagram is drawn to scale according to the most recent gene annotations (July 2007 for mice and March 2006 for human) at the University of California Santa Cruz website (http://genome.ucsc.edu). Boxes are genes and arrows indicate transcriptional initiation sites and orientation. B, VISTA Browser diagram identifying conserved noncoding sequences upstream of the CD69 gene. The mouse sequence is shown on the $x$-axis and percentage of similarity to the human and dog genomes is shown on the $y$-axis. Noncoding sequences of at least 100 bp long with $>70\%$ sequence identity are indicated with arrows.

**FIGURE 2.** Conserved noncoding sequences are constitutive HSs. A, Flow cytometry analysis showing CD69 expression in Rx\(^{b}\) and HA Tg thymi. B, Map of the CD69 locus showing locations of CNSs and the genomic fragments expected from EcoRI digestion. Probes used for Southern blot are shown and HSs are indicated. C, Representative DNase I analyses using the indicated probes. Total thymocytes from Rx\(^{b}\) and HA thymocytes were treated with increasing amounts of DNase I. EcoRI digested DNA was examined by Southern blot. Size markers and relevant bands are denoted.
CD69 locus histone modifications

One mechanism by which chromatin structure participates in the regulation of gene expression is through the modification of histone tails (31). Histone H3 acetylation and lysine 4 dimethylation are associated with active and poised chromatin, respectively (32). We assessed their levels by ChIP at conserved genomic regions (CD69 proximal promoter, CNS1, CNS2, CNS3, and CNS4) as well as at several nonconserved regions (−3.4, −20, and −30 kb). The β2-microglobulin gene was used as positive control.

Mononucleosomes were prepared from Rxβ and HA Tg thymocytes and were then immunoprecipitated with anti-acetylated histone H3, anti-dimethylated histone H3 K4, or control IgG Abs. Coprecipitated DNA was purified and subjected to real-time PCR to quantify the recovery of regions of interest (Fig. 3A). Results are expressed as the ratio of immunoprecipitated DNA and input DNA normalized to the abundance of the constitutively active control CAD gene. The data are representative of two experiments and are expressed as the means ± SEM of triplicate PCRs.

FIGURE 3. Epigenetic profile of the CD69 locus in T and B cells. ChIP with antisera against acetylated H3 and dimethylated H3 K4. A. ChIP performed on purified mononucleosomes obtained from total thymocytes of Rxβ and HA Tg mice. B. ChIP performed on sonicated chromatin from purified T cells either unstimulated or stimulated with 10 ng/ml PMA plus 0.5 µM Iq for 6 h. Purity as determined by flow cytometry was >95%. C, ChIP performed on sonicated chromatin from B cells isolated from TCRβ−/− B γ−/− mice, either unstimulated or stimulated with 10 ng/ml PMA plus 0.5 µM Iq for 6 h. Flow cytometry analysis showed that >95% of spleen cells were B220+.

To investigate CD69 locus chromatin structure in peripheral lymphocytes, lymph node T cells and splenic B cells were treated in vitro with PMA and Iq for 6 h to up-regulated CD69 expression or were left untreated. Chromatin was then cross-linked with paraformaldehyde, fragmented by sonication, and immunoprecipitated as above. As in thymocytes, acetylation of H3 and dimethylation of H3 K4 were enriched at the promoter in resting and activated T and B cells (Fig. 3, B and C). Also as in thymocytes, H3 acetylation and H3 K4 dimethylation increased substantially at CNS1 upon activation of both peripheral T and B cells (Fig. 3, B and C). However, unlike in thymocytes, we detected only very low H3 acetylation at CNS2, CNS3, and CNS4 in both resting and activated peripheral T cells. Moreover, H3 K4 dimethylation at these sites, although mildly elevated in resting T cells, was not inducible. B cells displayed increased amounts of both modifications at CNS2, CNS3, and CNS4, but only CNS3 showed inducible modification. Moreover, in striking contrast to thymocytes, H3 acetylation and H3 K4 dimethylation were constitutively high at CNS4 in B cells.

The above experiments revealed three important aspects of CD69 gene chromatin structure. First, the CD69 promoter is constitutively associated with active chromatin modifications in all three cell types. Second, CNS1 becomes hyperacetylated and hypermethylated upon CD69 induction in all three cell types. Third, histone modifications at CNS2, CNS3, and CNS4 undergo dynamic changes during T cell development but are differentially modified in peripheral T and B cells. This suggests the possibility of distinct mechanisms of CD69 gene regulation in the various cell types.

Analysis of promoter and CNS1–4 function

The correlation between DNA conservation and the presence of HSs and positive histone marks at the promoter and CNS1–4 prompted us to test their regulatory properties. We prepared luciferase reporter constructs under the control of the CD69 promoter linked to CNS fragments (Fig. 4A). Plasmids were transiently transfected into Jurkat cells, cultured for 24 h, and then stimulated or not with PMA/Iq for 24 h more before cells extracts were harvested for luciferase activity. Stimulation conferred a 4-fold increase in the activity of the CD69 promoter. Inducible activity of the CD69 promoter, however, was greatly enhanced in the presence of CNS2. This fragment conferred a 2-fold increase in basal activity and 20-fold increase over basal activity under conditions of stimulation. CNS4 conferred an ~5-fold increase in basal transcription activity and a further 2-fold increase under conditions of stimulation. In contrast, reporter activity was not altered when CNS1 or CNS3 was linked to the CD69 promoter. Luciferase activity of these constructs was also determined upon stimulation with immobilized Abs against CD3 and CD28 (supplemental Fig. 1). CNS2 was found to confer inducibility to this stimulus, although the magnitude (3-fold) was lower than with PMA/Iq. These results provide initial evidence that CNS2 and CNS4 may be enhancer elements for CD69 gene transcription.

To assess their in vivo functional properties, Tg mice were generated using a hCD2 expression construct as a reporter. Transgenic constructs containing the mCD69 promoter alone upstream of hCD2 (construct 1) or together with all four CNSs (construct 3)
were generated (Fig. 4B). Due to the strong enhancer activity observed for CNS2 element in luciferase assays, Tg mice containing CNS1 and CNS2 upstream of the promoter (construct 3) were also generated. Two Tg lines were obtained for construct 1, three for construct 2, and five for construct 3 (Fig. 4C). Copy numbers were determined by quantitative real-time PCR (Fig. 4C).

We assessed the magnitude and fidelity of hCD2 reporter expression on gated CD69+ and CD69− thymocytes and on purified resting or activated mature lymphocyte populations by flow cytometry (Fig. 4D). Tg lines 1A-23 and 1B-8 (containing 23 and 8 copies of the transgene, respectively) that contained only the CD69 promoter expressed hCD2 in both thymocytes and peripheral lymphocyte populations (Fig. 5A and supplemental Figs. 2A and 3A). hCD2 expression in double-positive (DP) and single-positive (SP) thymocyte populations was strictly correlated with endogenous CD69 expression, suggesting that the promoter was sufficient to confer specificity to CD69 expression during positive selection (Fig. 5A). However, aberrant expression of hCD2 was observed in resting peripheral T (Fig. 5A) and B (Fig. 6A) lymphocytes, suggesting the need for additional elements.

We tested for inducibility of the mCD69 promoter by overnight activation of isolated peripheral T and B lymphocytes with PMA and Io. Because even resting cells expressed hCD2, we used the mean fluorescence intensity (MFI) of the whole population to measure changes in hCD2 expression. Inducibility was calculated as the ratio of the MFI for the hCD2 staining between activated and resting cells. We detected 1.5- to 2.5-fold hCD2 inducibility in activated T cells (Fig. 5, B and C) and 1.5- to 2-fold inducibility in activated B cells (Fig. 6B), suggesting that promoter elements support some inducibility in vivo.

Unexpectedly, inclusion of CNS1 and CNS2 upstream of the CD69 promoter (Tg lines 2A-40, 2B-15, and 2C-15) suppressed hCD2 expression in both thymocytes and peripheral T and B cell populations (Figs. 5A and 6A and supplemental Figs. 2B and 3B). hCD2 expression was substantially reduced in CD69+ DP and SP thymocytes, and it was essentially eliminated in resting and activated peripheral CD4+ and CD8+ T cells (Fig. 5A). Expression was also eliminated in resting and activated peripheral B cells in two of the three Tg lines (Fig. 6A and supplemental Fig. 3B). These data suggest that CD69 gene expression in lymphocytes is regulated by silencer elements mapping to CNS1 or CNS2.

In contrast to the results obtained with construct 2, Tg lines carrying construct 3, including the CD69 promoter and all four CNSs (Tg lines 3A-134, 3B-80, 3C-45, 3D-9, and 3E-6), revealed a recovery of hCD2 expression (Figs. 5A and 6A and supplemental Figs. 2C and 3C). Expression of the hCD2 reporter correlated well with CD69 expression in all thymocyte populations, ranging from 92 to 22% in DP thymocytes and 89 to 15% in CD4SP and CD8SP thymocytes (supplemental Fig. 2C). Within the T cell compartments, hCD2 expression also correlated with mCD69 expression, since both were detected 2 h after poly(I:C) treatment, indicating that CNSs can also induce hCD2 expression following in vivo administration of poly(I:C) (supplemental Fig. 4A).

Analysis of construct 3 expression in mature CD4+ and CD8+ T cells stimulated with PMA/Io revealed hCD2 expression to be restricted to activated cells in four of five Tg lines (3E-6, 3C-45, 3B-80, and 3A-134) with percentages ranging from 99 to 10% (supplemental Fig. 2C). However, hCD2 expression was inducible in all five lines (range, 23-fold to 3.5-fold), and inducibility was substantially higher than for Tg lines containing construct 1 with the promoter alone. Tg line 3E-6, with the lowest copy number, was the most tightly regulated, with negligible hCD2 expression in resting cells. We detected 1.5- to 2-fold inducibility in T cells (Fig. 5A) and 1.5- to 2 fold inducibility in mature T cells (Fig. 5B and C) and 1.5- to 2-fold inducibility in mature B cells (Fig. 6B), suggesting that promoter elements support some inducibility in vivo.
suggest that the combination of CNS1–4 contributes to regulated CD69 expression in both thymocytes and peripheral T cells under a variety of activation conditions.

We further examined the expression of hCD2 in B cells. The combination of CNS1–4 clearly enhanced hCD2 expression as compared with the promoter alone (Fig. 6A and supplemental Fig. 3C). However, hCD2 expression was not concordant with mCD69 expression, since substantial numbers of resting B cells were hCD2⁺/H11001 in all five Tg lines (range, 22–79%) (supplemental Fig. 2C). This may reflect the constitutive and abundant H3 acetylation and H3 K4 dimethylation detected at CNS4 in B cells (Fig. 3C).

Stimulation with PMA and Io resulted in 2- to 2.5-fold inducibility in the various lines (Fig. 6B). However, the failure to appropriately suppress expression in resting B cells suggests that there are different requirements for regulated CD69 gene expression in B and T cells.

Because dysregulated expression of hCD2 was observed in mature B cells, we evaluated whether CNSs and the promoter may have an effect on hCD2 expression at different stages of B cell development (supplemental Fig. 7). Results indicate that hCD2 expression was already present in B220⁻/IgM⁺ immature B cells in Tg lines containing the promoter alone or in combination with CNS1–4 elements and suggest that specific signals received at this stage of development induce the dysregulated expression of hCD2 that is also observed in mature B cells.

Collectively, these experiments indicate that the CD69 promoter has strong activity and by itself can faithfully direct CD69 transcription in developing thymocytes. CNS1 and CNS2 appear to repress promoter activity, whereas CNS3 and CNS4 appear to counteract this repression, resulting in more tightly regulated expression in peripheral T lymphocytes and enhanced expression in both thymocytes and peripheral T lymphocytes. The same elements are insufficient to faithfully direct CD69 expression in the B cell lineage.

Discussion

In this study we have provided insights into the mechanism that regulate CD69 gene expression in vivo by the identification of new cis-regulatory elements and analysis of their chromatin structure and function. We identified four conserved noncoding sequences upstream of the CD69 promoter. Chromatin and functional analyses indicated that the CD69 promoter adopts an open chromatin conformation and can direct reporter gene transcription in Tg mice. However, the reporter, unlike mCD69, was expressed in unstimulated peripheral lymphocytes, suggesting that other elements must participate to achieve appropriate specificity. We detected several DNase I hypersensitive sites that mapped to CNSs and that displayed epigenetic profiles that were distinct in T and B cells and dynamic during T cell development. Analysis of CNS function in transient transfection assays revealed constitutive and inducible enhancer activity for both CNS2 and CNS4, but no apparent activity for CNS1 and CNS3. However, we found that Tg mice bearing CNS1 and CNS2 reduced reporter expression in T and B cells and that this inhibition was only overcome by inclusion of CNS3.
The combination of all four CNSs allowed for high level reporter expression that was appropriately regulated in T cells but not in B cells.

DNA sequence comparison has become a useful tool to identify specific remote elements that participate in the regulation of gene expression (19, 33, 34). We observed discrete islands of conservation in a 45-kb region upstream of the CD69 gene and found them to correspond to sites displaying hypersensitivity to DNase I digestion. It is noteworthy that these HSs were equally sensitive to DNase I digestion in CD69- and CD69+ lymphocytes. CD69 is one of the earliest Ags expressed upon activation and, therefore, it is possible that these regions are occupied by proteins even before activation. In agreement with our results, the first genome-wide map of DNase I HSs in human CD4+ T cells identified HSs lying within the CD69 promoter, CNS2, CNS3, and CNS4 in the human CD69 locus (35).

Previous work showed that the CD69 promoter can support rapid induction of reporter gene expression in transient transfection assays. Here we found that the CD69 promoter is always associated with an “active” chromatin configuration, even in situations where CD69 is not expressed. The promoter displayed high-level histone H3 acetylation and H3 K4 dimethylation in all thymocyte and peripheral lymphocyte populations. These two modifications have been shown to mark transcriptional competence of the IL-4 and IL-2 loci, respectively, in T cells (36, 37) and may be important to maintain transcriptional competence of the CD69 gene as well. Interestingly, genome-wide analysis of chromatin modifications in unstimulated human CD4+ T cells demonstrated enrichment of both histone H3 K4 trimethylation and RNA polymerase II at the promoter, exon I, and intron I of mCD69 (34). These data suggest that, as for other genes that require rapid induction, the CD69 promoter may constitutively harbor a promoter-paused RNA polymerase II (38, 39). CNS1 is the distal region of the C69 promoter, and one notable result is that this region showed a peak of permissive histone marks that correlated with CD69 expression in all cell populations analyzed. However, the promoter alone showed inducible activity in both transient and Tg reporters, and at least in the case of transient assays, CNS1 had no influence on basal or inducible promoter activity. This suggests that inducible histone modifications at this site may depend on other elements.

Tissue- and developmental stage-specific gene expression depends on both activators and silencers. A well-studied case is that of CD4, in which a silencer represses the CD4 promoter and enhancer in double-negative and CD8 SP thymocytes (40, 41). Similarly, regulated IL-4 transcription requires the action of a 3’ silencer element that can suppress reporter gene expression in Tg mice (18) and whose germline deletion results in the aberrant expression of IL-4 in Th1 cells (34). Moreover, there are examples of bifunctional elements, which function as both enhancers and silencers in a developmental stage-specific way (42, 43). Our results from Tg mice indicate that CNS1, CNS2, or the combination of the two elements can play a repressive role in CD69 gene expression in thymocytes, as well as stimulated T and B cells. Nevertheless, CNS2 displayed strong, inducible enhancer activity when tested in isolation by transient transfection into Jurkat cells. Notably, the detected enhancer activity is consistent with the inducible histone H3 acetylation detected at this site in thymocytes. We suggest that CNS2 is an inducible thymocyte enhancer whose activity in the context of chromatin requires the activity of other CD69 regulatory elements. Remarkably, inclusion of CNS3 and CNS4 in Tg reporter constructs led to both a recovery of reporter gene expression and tightly regulated reporter gene expression in thymocytes and peripheral T cells. Based on its inducible enhancer activity in Jurkat cells and inducible histone acetylation in thymocytes, CNS4 appears to function as a thymocyte enhancer as well. Thus, we suggest that CNS4 may not only function as an enhancer in the context of the endogenous CD69 locus, but may, perhaps in conjunction with CNS3, function as an antisilencer that switches CNS2 from silencer to enhancer activity. We propose that all four CNSs may converge to interact physically and functionally in the form of an active chromatin hub, as initially described for the β-globin locus (44). Perhaps consistent with this, a previous study suggested that CD69 may be regulated by the architectural protein SATB1 (45).

We note that our conclusions about the activities of CNS1–4 in vivo assume that these elements function normally in the context of our relatively compact Tg reporter. Prior studies have validated this approach in other systems (18, 46). However, we cannot rule out that our Tg constructs lack relevant elements from DNA segments that normally separate the CNSs or cannot adopt important three-dimensional chromatin configurations that are important for physiological regulation. To further study mechanisms of CD69 regulation in vivo we will analyze mice transgenic for a bacterial artificial chromosome containing the mCD69 locus.

As compared with thymocytes, the mechanism of induction of CD69 gene expression in mature T cells appears distinct, as only CNS1 displayed inducible H3 histone acetylation and H3 K4 dimethylation in the latter cell population. This was true despite the fact that, as for thymocytes, CNS2–4 were needed for tightly regulated, inducible expression in peripheral T cells of Tg mice. We
suggest that CNS2–4 activity during T cell development may establish a specific chromatin context that is required for proper regulation of the CD69 locus in mature T cells. Although the combination of all four CNSs was capable of promoting high-level and tightly regulated reporter gene expression in thymocytes and peripheral T lymphocyte populations, it did not do so in all Tg lines. One explanation for this is that additional elements may be required to reconstitute a CD69 locus control region that can consistently overcome chromosomal position effects. However, we note that hCD2 expression was both low and variated in those Tg lines containing particularly high copy reporter arrays. Thus, it is possible that RNA interference may cause gene translocation in these lines (47).

The unexpected finding that Tg mice with all CNSs misexpressed the hCD2 reporter in unstimulated B cells indicates that CD69 gene transcription is differentially controlled in B and T cells. Thus, it seems likely that the construct lacked an element required to repress the expression in unstimulated B cells. Further evidence for differential regulation in T and B cells was obtained from the epigenetic profile of CD69, which revealed distinct patterns of histone modifications at CNS2, CNS3, and CNS4 between T and B cells. In this regard, IL-2 transcription is thought to be regulated by different mechanisms in CD4 and CD8 lymphocytes, and IL-4 and IL-13 transcription is reduced in Th2 cells but not in mast cells in mice deficient for the CNS1 region (48, 49). Interestingly, Tg mice bearing a 30-kb genomic fragment containing Ly49A, another gene in the NK complex that encodes a C-type lectin, also showed aberrant expression in B cells. Perhaps similar mechanisms are used to suppress CD69 and Ly49A expression in B cells (50).

Based on our findings, we propose that the CD69 promoter and upstream elements display an accessible chromatin structure before CD69 transcription to support rapid gene induction upon stimulation. Gene induction requires the combined activity of multiple upstream CNSs, two of which display classical enhancer activity in thymocytes. The interdependence of these CNSs may also allow for physiological repression of CD69 expression by disrupting CNS3 and CNS4 interactions with CNS1 and CNS2. Finally, an as yet uncharacterized B cell-specific element may be required to suppress CNS activity in unstimulated B cells. The above results indicate that CD69 gene regulation is complex and likely differs in different cell types. Future studies are required to elucidate the nature of CD69 regulatory elements and the mechanism through which they regulate CD69 expression.

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Disclosures

The authors have no financial conflicts of interest.

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