Positive selection induces CD4 promoter and enhancer function

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Abstract

Developmental expression of the CD4 gene in mature T cells is controlled by at least four transcriptional control elements: a promoter, two enhancers and a silencer. In this report we use a transgenic approach to study the mechanisms in which these elements interact to convey appropriate tissue- and cell-specific expression at all stages of T cell development. Our data indicate that the control of CD4 gene expression requires the interaction of multiple elements functioning in different combinations at different stages of T cell development. Expression of the CD4 gene in immature CD4⁺CD8⁺ thymocytes requires a third enhancer element located in the 3' flanking region of the CD4 gene. Interestingly, the CD4 promoter and proximal/distal enhancers first begin to function at the HSA⁺ CD69⁺ H-2K⁺ CD4 single-positive stage; cells of this phenotype are believed to have survived positive selection. These data indicate that the CD4 promoter and late enhancer elements are induced by positive selection; thus, the final maturation process is an active event that requires the initiation of a novel program of gene expression.

Introduction

The control of CD4 gene expression provides an excellent model system in which to study the molecular events driving T cell development and selection. The expression or lack of expression of CD4 and CD8, a similar accessory molecule, is linked directly to each stage of thymic development (for review, see 1,2). CD4 is first expressed at low levels in early pluripotent hematopoietic stem cells (3). T cell precursors that first arrive in the thymus from the bone marrow maintain this low level of expression (CD4⁺CD8⁻ TCR⁻) (4). CD4 expression is then down-regulated, resulting in the CD4⁺CD8⁻ thymocyte. These cells subsequently express low levels of CD8 alone, followed by increasing levels of both CD4 and CD8, and the TCR. These cells, referred to as double-positive (DP or CD4⁺CD8⁺) cells, represent the bulk of thymocytes and are the first population of thymocytes to undergo the receptor-mediated selection processes that are critical for generating the appropriate repertoire of antigen-specific, non-self-reactive T cells (5–13). The survivors will then down-regulate either CD4 or CD8, leading to the mature CD4⁺CD8⁻ or CD4⁻CD8⁺ populations (6,7,9,11,13). These latter cells, referred to as the single-positive (SP) T cells, represent the mature thymocyte; these cells eventually migrate to the periphery to become helper T cells (Th) or cytotoxic T cells (Tc) respectively. Thus, the temporal expression of CD4 during T cell development is very complicated; CD4 gene expression is first initiated early in development and is either maintained in Th cells or turned off in Tc cells. The factors that turn CD4 gene expression on and off are believed to be linked both to the thymic selection process and to the process that eventually determines the functional subclass of the mature SP T cell (14–20).

We and others have identified multiple transcriptional regulatory elements in the CD4 locus, including a promoter (21–23), two enhancers (24–26) and a transcriptional silencer (27–29). As described above, the expression of CD4 during T cell development and activation is very complex; we were therefore interested in studying how these transcriptional control elements interact to control CD4 gene expression during these processes. Our previous characterization of the promoter revealed that an enhancer element was necessary to obtain reporter gene expression in transient transfection experiments using DP thymoma cell lines (21). Here we demonstrate that the previously identified promoter and enhancer elements are insufficient to induce CD4 expression in immature DP cells in transgenic mice. There is an uncharacterized control element in the CD4 locus that is capable of inducing CD4 gene expression in immature CD4⁺CD8⁺ thymocytes and in different non-T cell hematopoietic cells.

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The CD4 silencer can function on this enhancer to limit expression to CD4⁺ cells; thus this element may represent the putative DP enhancer. In addition, we have conducted a detailed multiparameter flow cytometric analysis of mice transgenic with constructs lacking the putative DP enhancer element(s). Our analysis indicates that the onset of marker expression in these mice does not coincide with the down-regulation of endogenous CD8; first expression of the marker gene occurs late in development of the CD4 SP T cell. These data indicate that control of expression of CD4 in DP and CD4 SP cells requires different elements and that the function of the CD4 promoter and distal/proximal enhancers is linked to the successful completion of positive selection. Thus, we believe that the control of CD4 gene expression in vivo requires the interaction of multiple elements functioning in different combinations at different stages of T cell development.

Methods

Synthesis of transgenic constructs

For the cosC, λB, pASUA and pTG transgenic constructs, restriction enzyme fragments that contained each DNase hypersensitive (DH) site were subcloned into the reporter construct vector using standard techniques [see Fig. 1; details of the cosC and λB construct synthesis have been reported previously (30)]. The cosC construct contains all 17 DH sites in the CD4 locus, whereas λB contains DH sites 1–6 and 11–17 (27). The pASUA construct contains all six DH sites (DH 1–6) located in the CD4 5' flanking region. DH1 was isolated on a 1.3 kb BglII restriction enzyme fragment, and DH2 and DH3 were isolated on a 5 kb SacI restriction enzyme fragment from the 2J3 cosmid. The CD4 promoter region containing DH sites 4-6 was isolated as a 3.0 kb XhoI restriction enzyme fragment from the J4 cosmid. In addition to the restriction enzyme fragments containing the minimal promoter and distal and proximal enhancers, the pF and pG constructs contain the following CD4 genomic restriction enzyme fragments: a 600 bp PstI–BglII fragment containing DH site 2, a 700 bp KpnI–BstXI fragment containing DH site 4 and a 1.0 kb XhoI–KpnI fragment containing DH site 5, all from cosmid 2J3. In addition, these constructs contained a 1.7 kb BglII–PstI fragment containing the promoter and the entire CD4 first exon, and a 1.2 kb BamHI fragment within the first intron containing a potential faint DH site not previously identified; these latter fragments were obtained from the J4 cosmid and are contained either within the promoter or the 6 kb XhoI–HindIII first intron fragment used in the cosC and λE constructs (27). A splice acceptor site was cloned immediately upstream of the HLA-B7 marker gene in these constructs, thus permitting the direct splicing of the first CD4 exon to the marker gene.

Generation of transgenic mice

Transgenic mice were generated using each of the constructs described above using previously published protocols (31). Construct DNA was excised from the vectors and purified on a 10–40% continuous sucrose gradient. Fractions containing the appropriate insert DNA were then pooled and dialyzed against injection buffer (0.1 mM EDTA, 5 mM Tris, pH 7.5). The DNA was then sterile-filtered and microinjected into the pronuclei of fertilized mouse eggs obtained from (C57BL/6 × CBA)F₁ crosses. The injected eggs were then implanted into Swiss-Webster foster mothers as previously (30). The cosC construct contains all 17 DH sites described previously (31). Founders were identified using a combination of PCR techniques and flow cytometric analysis on peripheral blood and by genomic Southern blotting on tail DNAs as described previously (29). Multiple expressing founders were analyzed for each construct: we have analyzed the results from five cosC founders, three λB founders, three pASUA founders, three pTG founders, eight pF and four pG founders (Fig. 5 and data not shown). The results from one representative founder for each construct are shown; all...
other founders were consistent with those presented (data not shown).

RNA purification and Northern analyses
Spleen and thymus tissue were harvested from transgenic mice, and RNA purified as described (32). Briefly, tissue was homogenized in 2 ml of denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate; 0.1 M β-mercaptoethanol) using a Fisher Polytron for 1 min at maximum speed. The homogenate was then recovered and the following solutions were added sequentially, with thorough mixing immediately after each addition: 200 µl 2 M sodium acetate (pH 4.0), 1 ml water-saturated phenol and 1 ml 49:1 chloroform:isoamyl alcohol. The solution was then centrifuged, the aqueous phase recovered and the RNA precipitated with the addition of 1 volume of isopropyl alcohol. After the recovery of the RNA by centrifugation, the pellet was resuspended in 300 µl of denaturing solution and reprecipitated with 1 volume of isopropyl alcohol. The RNA pellet was then resuspended in 100 µl DEPC-treated H2O. Northern analyses were performed as described (30). Ten micrograms of total RNA were resolved on a 1% agarose formaldehyde gel and blotted to nitrocellulose and hybridized using either the HLA-B7 cDNA probe or with a murine β-actin cDNA probe.

Flow cytometric analyses
Cells were analyzed on a dual-laser FACStar Plus (Becton Dickinson, Mountain View, CA) located at the Columbia Presbyterian Cancer Center. Calibration was performed before each experiment using Fluoricon multi-fluorescent beads (Baxter Healthcare, McGaw Park, IL) and Shiva, a computer calibration program (Stanford University). A single-color compensation control for each of the four fluorescence channels was prepared for each experiment and compensation was done by eye. Data were collected and analyzed using the Electric Desk software package (W. Moore, Stanford University) running on a MicroVax 3400 (Digital Equipment, Maynard, MA). The 5% probability contour plots contain an equal number of cells at each contour level. Histograms represent the percentage density of cells at a particular fluorescence level, rather than the actual number of cells at each fluorescence level. All plots were gated by propidium iodide fluorescence to exclude dead cells.

All analyses were performed on 4- to 7-week-old littermates housed in the pathogen-free Cancer Center Animal Facility at Columbia University. The following mAb reagents were obtained from PharMingen (San Diego, CA) to identify splenic and thymic cells: allophycocyanin (APC)-conjugated RM4-5 (anti-CD4), FITC-conjugated 53-6.7 (anti-CD8α), biotin-conjugated PK136 (anti-NK-1.1), FITC-conjugated H57-597 (anti-TCRβ), biotin-conjugated GL3 (anti-TCRγδ), biotin-conjugated TER-119 (anti-erythroid cells), biotin-conjugated RB6-8C5 (anti-Gr-1), biotin-conjugated 145-2C11 (anti-CD3ε), biotin-conjugated AF6-88.5 (anti-H-2Kb), biotin-conjugated 36-7.5 (anti-H-2Kd), biotin-conjugated M1/69 (anti-HSA) and biotin-conjugated H1.2F3 (anti-CD69). FITC-conjugated 4A5 (anti-platelet cells) was kindly provided by Dr Samuel Burstein (33). The following monoclonal reagents were made available with the generosity of Dr Alan Stall: FITC-conjugated 331 (anti-IgM); FITC-, phycoerythrin (PE)- and APC-conjugated 6B2 (anti-B220); APC-conjugated M1/70 (anti-MAC-1); and FITC-conjugated GK1.5 (anti-CD4). Biotin reagents were revealed with a second-step stain of Texas Red-conjugated Avidin from Caltag (South San Francisco, CA). The transgenic marker was stained with ME-1 (anti-HLA-B7 (29)), which was revealed by a second-step stain of PE-conjugated polyclonal goat anti-mouse IgG1 from Caltag.

The different hematopoietic cell populations were identified using a variety of different combinations of antibodies and preparations. The different subclasses of splenic T cells were identified from a whole splenocyte preparation; γδ T cells were identified from spleen using anti-CD3 and anti-TCRγδ antibodies; B cells and macrophages were excluded by gating out B220+ and Mac-1+ cells. Splenic NK cells were identified using NK1.1 antibodies after gating out TCRβ+ B220+ and Mac-1+ cells. Mature splenic B cells were identified using B220 and gating out T cells using anti-TCRβ. Splenic pre/pro B cells were identified from bone marrow by gating on B220+ IgM+ cells. Splenic macrophage and granulocytes were identified using antibodies directed against Mac-1 and Gr-1; T and B cells were gated out using anti-B220 and anti-TCRβ. Erythroblasts and platelets were identified from bone marrow and peripheral blood respectively; erythroblasts were identified with TER-119 and side scatter, whereas platelets were identified with 4A5 and forward scatter. For the analysis of the pTG transgenic mice, thymocytes were stained with anti-CD4, anti-CD8, anti-ME-1 and either anti-CD3, anti-HSA, anti-CD69 or anti-H-2K. For the CD4 SP analysis, the CD8+ thymocytes were gated out, and the marker-positive and marker-negative populations were then gated and analyzed for CD3, HSA, CD69 or H-2K expression. Similarly, for the CD8 SP analysis the CD4+ cells were gated out.

Results
The transcriptional control elements in the CD4 locus function in a limited subpopulation of hematopoietic cells
Using DH and transgenic assays, we and others have identified many transcriptional control elements in the CD4 locus, including the promoter, two enhancers and a silencer (27,28). In addition to the five DH sites that included these four transcriptional control elements, our original transgenic constructs contained 12 other DH sites (Fig. 1). To characterize the role that these DH sites (Fig. 2). As we have described previously, the cosC construct contains all of the DH sites in the CD4 locus, including all four known transcriptional control elements. The λB construct contains the two enhancers, the promoter, and DH sites 2, 4 and 11–17. The pASUA construct is identical to λB with the exception of DH sites 11–17, which are not present on the pASUA construct. The pTG construct contains only the two enhancers and the promoter.

Transgenic mice were generated from each of these constructs and analyzed for marker gene expression in different tissues and cell types. We have generated and analyzed multiple founders from each transgenic series; typical results...
from each founder series are shown. Total RNAs were purified from different tissues from each founder transgenic line obtained and subjected to Northern analyses using cDNAs encoding the HLA-B7 marker cDNA or the endogenous murine β-actin gene as radioactive probes (Fig. 3 and data not shown). As can be seen in Fig. 3, for the cosC transgenic mice we can detect marker gene expression primarily in spleen and thymus; as these are lymphoid organs that contain large numbers of T cells, these data are consistent with previous data that indicate that constructs containing the silencer express the marker gene only in the appropriate T cell subclasses. We can detect marker gene expression primarily in spleen and thymus in the λB, pASUA and pTG transgenic mice (Fig. 3 and data not shown), indicating that expression of the marker gene in these mice is limited to lymphoid organs as well. We can detect low levels of expression in the lung in the λB transgenic mice. Although it is possible that this expression is the result of ectopic expression on lung epithelia, we can also detect Ig κ light chain gene mRNA in these samples and thus we believe this positive signal to be the result of contaminating blood lymphocytes (data not shown). As mentioned above, the λB, pASUA and pTG constructs do not contain the silencer; thus, these data indicate that even in the absence of the silencer the transcriptional control elements in the CD4 locus are sufficient to limit expression of CD4 to the hematopoietic compartment.

To determine the precise specificity of the CD4 locus transcriptional control elements, we have analyzed each cell type within the hematopoietic compartment in the cosC, λB, pASUA and pTG founder mice for marker gene expression (Fig. 4). The cosC and pTGSil transgenic mice did not express the marker gene in any CD4- cell type; these data are consistent with previous data showing that the CD4 silencer is capable of inhibiting CD4 expression in non-T cells (27). Interestingly, the λB transgenic mice express the marker gene in many different hematopoietic cell types. These include all classes of T cells, including all thymic T cells and γδ T cells. In addition, we can detect expression of the marker gene in both pre/pro B and mature B cells, NK cells, and Mac-1+ macrophages and Mac-1+Gr-1+ granulocytes. However, we cannot detect expression of the marker gene in the 4A5+ megakaryocyte or the TER-119+ erythroid lineages. As the λB transgenic construct does not contain the silencer, these data indicate that the combination of DH sites on this transgenic construct is capable of driving marker gene expression in a wide variety of different hematopoietic cell types.

In contrast, mice that are transgenic with constructs that contain only the CD4 promoter and enhancers have a much more limited pattern of expression. The pASUA construct contains the identical 5’ flanking region DNA as the cosC and λB constructs and differs from λB only in that the latter construct contains DH sites 11–17, which are located in the far 3’ flanking region (Figs 1 and 2). Transgenic mice containing this construct express the marker gene only in peripheral αβ and possibly γδ T cells but not in cells in the NK, B cell, myeloid, erythroid or megakaryocytic lineages. These data indicate that there is an additional enhancer region in DH sites 11–17 that is capable of driving expression of CD4.
Fig. 4. Marker expression in CD4 minilocus transgenic mice in different hematopoietic lineages. Splenocytes were isolated from the cosC, λB, pA5UA and pTG mice, and analyzed for marker expression using either the anti-HLA-B7 antibody ME-1 (solid lines) or the MOPC-31c isotype-matched control antibody (dashed lines). Specific hematopoietic populations were identified for analysis using lineage-specific antibodies (see Methods for details). (A) T and NK lineage cells. (B) B cells and monocyte lineage cells. (C) Erythroblasts and platelets.
in this population does not correlate completely to surface TCR αβ or γδ expression. Taken together, these data indicate that there are additional transcriptional control regions not present in the pTG or pTGSil constructs that are responsible for inducing CD4 gene expression in immature T cells.

We presumed that this putative DP enhancer element is present on the cosC and λB transgenic constructs, since we can detect marker expression in DP T cells in mice transgenic for these constructs (Fig. 5). To localize this enhancer further, we generated and analyzed additional transgenic mice using constructs that contain different combinations of the DH sites that are also present in the λB construct (Figs 1 and 2). As mentioned above, the pA5UA construct contains all of the 5′ flanking region DNA contained in the cosC and λB constructs. As can be seen in Fig. 5, similar to the pTG series transgenic mice we cannot detect expression of the marker in the DP population. To localize the putative DP enhancer further, we analyzed all of the previous murine CD4 transgenic constructs that have been generated to date by ourselves (27,29) and other groups (28,35), and we have generated additional transgenic constructs containing the flanking regions and DH sites that all of the previous constructs have in common (Fig. 6). In addition to the proximal enhancer and the promoter, the previous constructs that contain the smallest amounts of CD4 genomic sequences overlap only in the first intron (Fig. 6). To determine if this region contains enhancer activity, we generated additional transgenic constructs (Fig. 2). The pF and pG constructs contain all six DH sites identified in the 5′ flanking region of the CD4 gene, including the two enhancers and the promoter region. All of the DH sites were included to control for the possibility that the putative DP enhancer functions indirectly through one of the previously characterized elements. Both the pF and pG constructs contain the intact first CD4 exon, which consists entirely of 5′ untranslated region, and portions of intron sequence that were contained in all previous transgenic constructs. The splice acceptor site from the 5′ end of the second CD4 exon was cloned 5′ to the HLA-B7 marker gene; thus, these constructs contain an intron and have maintained the intron/exon structure of the CD4 gene. Transgenic mice were generated with these constructs and multiple founders were obtained and analyzed (Fig. 5 and data not shown). Both the pF and pG transgenic mice had the same phenotype; surprisingly, we were unable to detect marker expression in DP thymocytes in either of these founder lines (Fig. 5 and data not shown).

In contrast to the previously published results and analyses (27,28,35), these data indicate that the DP enhancer is not present in either the 5′ flanking region or the first intron. To try to localize the enhancer further we reanalyzed progeny from all of our founder transgenic mice for marker expression in the thymus. The only transgenic mice in which we detect consistent expression in DP thymocytes in all founders contain either the cosC or λB constructs; these two constructs contain in common DH sites 11–17 in the far 3′ flanking region of the CD4 gene. Deletion of this region results in the loss of expression of the marker gene in DP thymocytes (compare the λB construct and the pA5UA construct transgenic mice). As mentioned above, the CD4 silencer can limit the function of this enhancer to CD4+ cells (compare the cosC and λB transgenic mice). Because this enhancer fulfills all of the

The identity of this population is unknown; marker expression in a wide variety of hematopoietic lineages. Similarly, mice transgenic for the pTG construct also express the marker only in the peripheral T cell lineages; these data indicate that the CD4 enhancers and promoter are capable of functioning in cells of these lineages, and that the additional DH sites in the 5′ flanking region of the CD4 gene do not appear to play a role in the control of CD4 gene expression.

The CD4 enhancers alone cannot drive CD4 expression in DP thymocytes

To determine the function of the CD4 promoter and enhancers during early development, thymocytes from each of the transgenic mice were isolated and analyzed for marker expression in each different developmental subclass (Fig. 5). As we previously reported, the cosC transgenic mice express the marker in the CD4+CD8− and CD4+CD8+ thymocytes only, whereas the λB transgenic mice, due to the lack of the silencer in the construct, express the marker in all thymocyte subclasses [Fig. 5 and (27)]. The pTG and pTGSil transgenic mice express the reporter marker in both the CD4+CD8− and CD4+CD8+ SP populations or the CD4+CD8− SP populations only respectively; again, the difference reflects the absence of the silencer in the pTG construct (29). Interestingly, neither the pTGSil nor the pTG transgenic mice express the marker in the CD4+CD8+ DP thymocyte population. We have obtained this result with multiple founders and with multiple constructs (see below and data not shown); thus we believe that this result reflects the lack of a stage-specific enhancer element (or elements) and is not due to transgene expression variation as has been seen in other systems (34). In addition, these mice do not express the marker in the CD4+CD8− double-negative (DN) populations (Fig. 5). We can detect a small marker-positive population seen in the DN populations.

The identity of this population is unknown; marker expression in this population does not correlate completely to surface TCR αβ or γδ expression. Taken together, these data indicate that there are additional transcriptional control regions not present in the pTG or pTGSil constructs that are responsible for inducing CD4 gene expression in immature T cells.

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Stage-specific CD4 promoter/enhancer function

Fig. 6. Minilocus construct maps. Restriction enzyme fragments contained in all of the murine CD4 minilocus transgenic constructs published to date are shown (27–29,35). DH sites are indicated with arrows; black box represents the first CD4 exon; enhancers and promoter are shown as white boxes. Dashed arrow indicates position in intron of a faint T-cell-specific DH site not previously reported (data not shown). For the cosC and \( \lambda \) constructs, the first CD4 intron is separated by the HLA-B7 marker gene (see 27 and Fig. 2). Restriction enzymes used to generate each DH-containing fragment are indicated underneath each construct. Additional sequences contained in each transgenic construct (if any) and the status of expression of the marker gene in DP thymocytes for mice transgenic with each construct are listed on the right.

The CD4 enhancers initiate function in mature CD4 SP T cells

In our analyses, we noted that most if not all peripheral SP T cells in all founders expressed high levels of the marker gene. However, expression of the marker on CD4 SP thymocytes in mice that are transgenic for the pTG-based constructs was always bimodal, representing subpopulations that both expressed and did not express the marker gene (Fig. 5). In most cases, ~50% of the CD4 SP thymocytes expressed the marker, whereas 50% did not. For the transgenic constructs that did not contain the silencer, we also observed bimodal marker expression in the CD8 SP thymocytes; however, unlike the CD4 SP thymocytes, there are relatively fewer marker-negative CD8 SP thymocytes; indeed, in many cases all of the CD8 SP thymocytes are expressing the transgenic marker gene (Fig. 5 and data not shown). To characterize the SP populations that are and are not expressing the marker gene further, we have conducted multiple-parameter flow cytometric analysis on these cells. Thymocytes obtained from the pTG transgenic mice were analyzed with antibodies against HSA, CD69 and the MHC class I molecule H-2K. HSA is expressed on all immature thymocytes and is down-regulated late in development in the SP stage (36). CD69 is an activation marker for mature T cells that is expressed transiently in the CD4+CD8lo/early SP CD4 population, possibly as the result of activation of these cells during positive selection (37,38). The immature SP thymocyte that presumably survives positive selection then down-regulates CD69; the resting mature SP T cell expresses low levels of CD69 but can be induced to express high levels upon activation with antigen (37,39). The MHC class I molecule H-2K is expressed at low levels early in thymic development but is up-regulated at the final maturation stage of the SP thymocyte (40–43).

Thus, these three antigens are useful for defining late stages of T cell development.

Thymocytes from pTG transgenic mice were isolated and stained with antibodies against CD4 and CD8 and the anti-marker antibody ME-1 along with different combinations of the anti-H-2K, CD69 and HSA antibodies as described in the Methods. We then plotted marker expression in the remaining thymocytes against the expression of either CD4 or CD8, gated on the marker-negative and marker-positive populations, and determined if these populations are expressing any of these maturation markers (see Methods for details). As can be seen in Fig. 7, the entire CD4 SP population, including both the transgenic marker-positive and marker-negative populations, express high levels of CD3. However, the CD4 SPs are bimodal in expressing HSA, CD69 and H-2K, indicating that this population consists of both mature and immature SP cells. Interestingly, the CD4 SP transgenic marker-negative cells are primarily CD69+HSA+H-2Klo, which as discussed above is the phenotype of those SP cells that are undergoing positive selection. In contrast, the CD4 SP transgenic marker-positive populations are mostly CD69+HSA+H-2Khi cells, which represent the more mature CD4 SP thymocytes that have recently undergone and survived positive selection. As described above, the marker gene in the pTG construct is under the transcriptional control of the CD4 promoter and the proximal and distal enhancers only; our data thus imply that these elements first become functional late in CD4 SP thymocyte development immediately after the thymocyte successfully survives positive selection. Therefore, these data indicate that the CD4 promoter and enhancer function is directly linked to thymic positive selection events.

We conducted similar analyses on the CD8 SP T cells isolated from the pTG transgenic mice thymus (Fig. 7). Unlike the CD4 SP population, total CD8 SP T cells express both high and low levels of CD3, indicating that this population contains a mixture of both mature CD8 SP T cells and...
Control of CD4 expression in DP thymocytes CD4 promoter and enhancers first begin to function at a later levels of CD8, which is usually seen in the immature CD4 – that the CD8SP marker-negative population expresses lower positive selection the immature CD4 –CD8loCD3– population that serves as the cell types (compare cosC and analyzed for expression of each cell-surface marker (right). enhancer in this region that can mediate CD4 gene expression marker-negative individual populations were then gated on and of DH sites 11–17; we therefore conclude that there is an in Methods; for analysis, DP thymocytes were gated out and the expression in DP thymocytes in the pA5UA transgenic miceSP ... 5 and data not shown). The only difference betweenpopulations using two-color flow cytometry (two-color plots on left). Either the total SP populations or the transgenic marker-positive and marker-negative individual populations were then gated on and analyzed for expression of each cell-surface marker (right).

Discussion

Control of CD4 expression in DP thymocytes

We present evidence for the existence of DP stage-specific enhancer elements in the CD4 locus. The requirement for at least one DP enhancer element was initially determined using transient transfection analyses; our characterization of the promoter revealed that an enhancer element was necessary to obtain reporter gene expression in the AKR1G1 DP thymoma cell line (21). In addition, we determined that the CD4 promoter and distal/proximal enhancers were insufficient to drive marker expression in DP thymocytes of transgenic mice (29). Using a transgenic construct containing the human CD4 promoter and a synthetic enhancer containing a 3-fold multimerization of the human proximal enhancer, Salmon et al. also reported the lack of marker expression in DP thymocytes and HSA CD4 SP thymocytes (35); taken together, these results were surprising in light of the results of Sawada and coworkers (24,28), who reported that the murine proximal enhancer functions in DP cells in transient transfections and in transgenic mice. These contrasting results may be due to non-overlapping sequences in the different transgenic constructs (27–29), interspecies differences (35) or complications arising from the non-physiological state of the multimer-ized enhancer (35).

We have localized the putative DP enhancer further by generating additional constructs and transgenic mice. Mice transgenic with constructs that contain all of the restriction fragments in common between all of the published DP-expressing constructs do not express the marker gene in DP thymocytes (constructs pF and pG; Fig. 5 and data not shown). We therefore analyzed the expression of our marker gene in all of our different transgenic mice. We can reliably detect marker expression in DP thymocytes in all cosC and λB transgenic mice; in contrast, we cannot detect marker expression in DP thymocytes in the pA5UA transgenic mice (see Fig. 5 and data not shown). The only difference between the pA5UA construct and the λB construct is the presence of DH sites 11–17; we therefore conclude that there is an enhancer in this region that can mediate CD4 gene expression in DP thymocytes. In addition, this enhancer can controlled by the CD4 silencer in that the addition of the silencer to the transgenic construct limits marker expression only to CD4+ cell types (compare cosC and λB marker expression; Fig. 4).

Therefore, this novel enhancer fulfills the functional requirements of the DP enhancer and thus may play a role in the control of developmental CD4 gene expression. We had earlier reported expression in DP thymocytes in λE transgenic mice. This construct is identical to pA5UA with the addition of first-intron sequences (27). However, further analyses with additional λE founders have shown inconsistent expression in this population. We therefore conclude that the λE construct cannot mediate CD4 expression in DP thymocytes (data not shown).

CD4 promoter and enhancer function as a marker for positive selection

As discussed above, mice transgenic with constructs that contain only the proximal and distal enhancers do not express the marker gene until the SP stage of development. Interest-ingly, these mice do not express the marker immediately after commitment to the CD4 SP lineage; our data indicate that the CD4 promoter and enhancers first begin to function at a later stage of SP development. In addition, our data indicate that the CD4 enhancers are not functional at the initial stage after the DP thymocyte commits to the CD4 SP lineage; specifically, at the CD4+CD8+CD3+HSA+CD69+H-2Khi (‘immature CD4 SP’) stage. These elements become functional once the SP T cell matures to the CD4+CD8–CD3+HSA–CD69+H-2Khi (‘mature CD4 SP’) stage. Bendelac and colleagues (37) have proposed that the transient expression of CD69 in the immature CD4 SP population is the result of intrathymic activation that may be occurring due to the positive selection process; thus, this cell-surface marker can be used to distin-
Fig. 8. CD4 transcriptional control element function during T cell development. The ‘+’ and ‘–’ indicate the ability or lack of ability of the transcriptional control element to mediate CD4 expression. Subclasses of thymocytes that are believed to be undergoing selection events are indicated. See text for details.

From the comparisons of our data with those of Bendelac (37), we propose that the CD4 transcriptional control elements are intimately linked to the thymic selection process. Early in T cell development, the CD4 silencer functions to inhibit enhancer function in the DN thymocytes. Upon maturation to the DP stage, the silencer stops functioning and a DP enhancer element or elements initiate(s) function, thus permitting expression of CD4. The DP thymocyte then undergoes both negative and positive selection; T cells that survive positive selection develop first into the immature CD4 SP and subsequently to the mature CD4 SP stage. It is during this latter transition that the DP enhancer ceases function and is replaced by the CD4 distal and proximal enhancers; in conjunction with the promoter, these elements then maintain CD4 expression in mature CD4 SP T cells. Since our pTG constructs contain the distal and proximal enhancers but not the putative DP enhancer, the onset of marker expression in the CD4 SP thymocytes in these mice correlates to a CD4 SP developmental stage immediately following positive selection and before the final maturation of the T lymphocyte. These data have several interesting implications. These data imply that the maturation of the CD4 SP T cell is an active process that involves the initiation of expression of a battery of transcription factors that activate the CD4 promoter and proximal/distal enhancers and possibly other genes important in mature T cell function (37). The recovery from positive selection, therefore, is not a passive event but rather part of an overall developmental program (see Discussion below). These data also indicate that the function of the CD4 promoter and enhancers is linked directly to distinct steps in thymocyte development. This transgenic system may therefore be useful in generating populations of thymocytes at stages both before and after positive selection for biochemical analyses.

Control of CD4 gene expression requires multiple elements functioning at different stages during T cell development

Our data indicate that the control of CD4 gene expression is extremely complex. Based on our results we can devise a model to account for the control of CD4 expression at all stages of T cell development (Fig. 8). At early stem-cell stages, the low level of CD4 expression is mediated primarily by the DP enhancer and the silencer. In DN thymocytes, the DP enhancer is still capable of functioning, but the CD4 silencer inhibits its action and thus CD4 expression is turned off. Once the DN thymocyte matures into the DP thymocyte, the silencer ceases function and CD4 expression is then mediated once again by the DP enhancer. The down-regulation of CD4 during development of the CD8 SP T cell is accomplished primarily by the CD4 silencer. However, the maintenance of CD4 expression as the DP thymocyte becomes a CD4 SP T cell requires multiple steps. First, the DP enhancer remains functional as the CD4 SP T cell down-regulates CD8; thus, the early CD4 SP T cell undergoing selection (the CD69⁺HSA⁺H-2Klo CD4 SP cell) is transcribing the CD4 gene primarily because of the action of the DP enhancer. However, once the CD4 SP T cell completes the selection process (the CD69⁺HSA⁺H-2Khi CD4 SP cell), the DP enhancer ceases to function and the proximal and distal enhancers concomitantly begin function and subsequently mediate CD4 gene transcription in resting mature CD4 SP T cells. Finally, CD4 promoter function can be induced in activated mature CD4 SP T cells, possibly as a mechanism to transiently increase CD4 expression to compensate for the down-regulation of surface CD4 during the activation process (23). Our data indicate that although the surface expression of CD4 is maintained at a relatively invariant level from the DP cell to the CD4 SP T cell, the transcription of the CD4 gene
requires multiple elements that are coordinately regulated. Previously, most models of CD4 SP T cell development proposed that the down-regulation of CD8 is the defining step, whereas the expression of CD4 is the result of maintenance of expression from the DP stage. Our data indicate that the expression of CD4 in the SP T cell differs fundamentally from the expression in the DP thymocyte in that different transcriptional enhancers are required for function. Thus, during repertoire selection the DP CD4 enhancer will cease function regardless of the developmental fate of the DP thymocyte. Concomitant with this, the CD4 locus distal and proximal enhancers will begin function; should the DP thymocyte be fated to develop into a CD4 SP T cell, the distal and proximal enhancers (and the promoter) will remain functioning. Alternatively, should the DP thymocyte be fated to develop into a CD8 SP T cell, the CD4 silencer will inhibit CD4 distal/proximal enhancer function and CD4 transcription will then remain off. Thus, this model indicates that the actual defining step in SP T cell development is the onset of distal/proximal enhancer function and whether or not the CD4 silencer is inhibiting their function. Recently, Lucas and Germain determined that both CD4 and CD8 expression during thymic development occurs in a dynamic and asymmetric fashion (45). Our results indicate that many of the changes in CD4 expression that they observed in DP thymocytes may be the result of changes in transcriptional control element utilization. A study of the factors that bind to these elements and control CD4 gene expression during DP development will help delineate the molecular signaling events that drive T cell differentiation. We are currently characterizing the DP enhancer further to address these issues.

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Abbreviations

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<tr>
<td>APC</td>
<td>aliphycocyanin</td>
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<tr>
<td>DH</td>
<td>DNase hypersensitivity</td>
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<td>DP</td>
<td>double positive</td>
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<td>DN</td>
<td>double negative</td>
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<tr>
<td>PE</td>
<td>phycoerythrin</td>
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<tr>
<td>SP</td>
<td>single positive</td>
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References

25. Sawada, S. and Littman, D. R. 1993. A heterodimer of HEB and E12-related protein interacts with the CD4 enhancer and whether or not the CD4 silencer proximal enhancer function and CD4...thymocyte be fated to develop into a CD8 SP T cell, the CD4 silencer will inhibit CD4 distal/proximal enhancer function and CD4 transcription will then remain off. Thus, this model indicates that the actual defining step in SP T cell development is the onset of distal/proximal enhancer function and whether or not the CD4...thymic development occurs in a dynamic and asymmetric fashion (45). Our results indicate that many of the changes in CD4 expression that they observed in DP thymocytes may be the result of changes in transcriptional control element utilization. A study of the factors that bind to these elements and control CD4 gene expression during DP development will help delineate the molecular signaling events that drive T cell differentiation. We are currently characterizing the DP enhancer further to address these issues.


Note added in proof

While this paper was in press, Uematsu et al. (Int. Immunol. 9:179, 1997) reported further evidence for additional human CD4 transcriptional control elements. Although they propose the existence of additional enhancers in the first intron, our Aβ transgenic mice express the marker in all thymocytes (27, this study). The Aβ construct does not contain intronic sequences, indicating that for the murine system this enhancer is not likely to be located in the first intron.