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A Viral Long Terminal Repeat Expressed in CD4$^+$CD8$^+$ Precursors Is Downregulated in Mature Peripheral CD4$^-$CD8$^+$ or CD4$^+$CD8$^-$ T Cells

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The long terminal repeat from a thymotropic mouse mammary tumor virus variant, DMBA-LV, was used to drive the expression of two reporter genes, murine c-*myc* and human CD4, in transgenic mice. Expression was observed specifically in thymic immature cells. Expression of c-*myc* in these cells induced oligoclonal CD4$^+$CD8$^+$ T-cell thymomas. Expression of human CD4 was restricted to thymic progenitor CD4$^-$ CD8$^-$ and CD4$^+$CD8$^+$ T cells and was shut off in mature CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells, known to be derived from the progenitor double-positive T cells. These results suggest the existence of similar and common factors in CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ T cells and support a model of differentiation of CD4$^+$CD8$^+$ T cells through common signal(s) involved in turning off the expression of the CD4 or CD8 gene.

DMBA-LV leukemia virus is a type B replication-competent thymotropic retrovirus which is highly related to mouse mammary tumor virus (MMTV) (5, 19). However, in contrast to MMTV, known to induce mammary carcinomas (36), DMBA-LV MMTV (MMTV^D) induces a high incidence of T-cell leukemias (thymomas) after a short latent period (6). Sequence analysis of the MMTV^D long terminal repeat (LTR) revealed that in addition to several point mutations, the U3 region has undergone structural alterations compared with the C3H MMTV LTR (7). These modifications introduce two additional copies of the distal glucocorticoid regulatory element and truncate the 3′ end of the open reading frame known to encode a protein exhibiting the characteristics of a superantigen (1, 15). However, the biological significance of these sequence alterations is still unclear. In another class of retroviruses, murine leukemia virus, it has clearly been established that the LTR, and specifically the U3 LTR, harbors the sequences determining the tissue (11, 21, 43) and disease (12, 13, 20, 48) specificity of these viruses.

The majority of the DMBA-LV-induced thymomas were found to be of an immature T-cell phenotype, Thy-1.2$^+$, CD4$^+$, CD8$^+$ (Lyt2$^+$), and CD5$^+$ (Lyt1$^+$) (37, 38). The earliest T-cell precursors, which constitute the majority of the early embryonic T cells, are a heterogeneous group of CD4$^-$CD8$^-$ Thy-1$^+$ cells (reviewed in reference 23). These cells represent only 3 to 5% of adult murine thymocytes. During thymic development, these CD4$^-$CD8$^-$ T cells differentiate into CD4$^+$CD8$^+$CD3$^+$ T cells, which eventually constitute the majority of adult murine thymocytes. The CD4$^+$CD8$^+$ T cells further differentiate terminally into CD4$^+$CD8$^-$ (helper) or CD4$^-$CD8$^+$ (cytotoxic) peripheral T cells, notably by turning off expression of the CD8 or CD4 gene, respectively, and most likely by altering the expression of several other genes. This last stage of differentiation is regulated so that the cells expressing class II-restricted T-cell receptor (TcR) are CD4$^+$CD8$^-$, while those expressing class I-restricted TcR are CD4$^-$CD8$^+$.

To determine whether the tissue specificity of DMBA-LV virus was determined by its LTR, and to identify the T-cell subpopulation in which this LTR was most active, we constructed transgenic mouse lines by using the MMTV^D LTR as a promoter to drive expression of two independent reporter genes, the murine c-*myc* proto-oncogene and the human CD4 cDNA. Transgenic mice expressing the MMTV^D/c-*myc* transgene consistently developed clonal or oligoclonal CD4$^+$CD8$^+$ T-cell lymphomas (thymomas). In addition, in mice bearing the MMTV^D/CD4 transgene, the human CD4 protein was preferentially expressed in CD4$^+$CD8$^+$ thymic T cells and their progenitors, and its expression was turned off in both CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ mature T cells. These experiments support a model of differentiation of CD4$^+$CD8$^+$ thymic T cells through common signals.

MATERIALS AND METHODS

Construction of the transgenes. The 3′ LTR of an integrated MMTV genome was obtained by digestion with *Bgl*II, located in the *env* region, and with *Hin*dIII, located in the cellular DNA. This 1.6-kbp purified fragment was cloned in pUC12 vector. This MMTV LTR subfragment was cleaved with *Bst*EII, located in the 3′ end of the U5 region, treated with Klenow enzyme, and ligated with either an *Xba*I or *Eco*RI linker. To construct the MMTV^D/c-*myc* transgene, the 1.6-kbp *Eco*RI-*Xba*I LTR fragment was ligated with the 4.6-kbp *Xba*I-*Bam*HI fragment containing exon 2 and exon 3 of the mouse c-*myc* gene (from plasmid pSV2/c-myc [27]) and the 0.75-kbp *Bam*HI-EcoRI fragment of pSV2gpt containing simian virus 40 (SV40) sequences. These ligated fragments were cloned in pUC18 vector cleaved with *Eco*RI. The MMTV^D/CD4 transgene was constructed from three fragments: the *Eco*RI LTR fragment, the 1.8-kbp *Eco*RI-

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*Bam*HI fragment containing the human CD4 cDNA (32), and the 1.4-kbp *Bgl*II-*Pst*I fragment of pSV2gpt containing SV40 sequences. These fragments were ligated and cloned in pUC18 vector cleaved with *Eco*RI-*Pst*I.

The 6.95-kbp MMTV^D/c-*myc* and 4.6-kbp MMTV^D/CD4 fragments to be microinjected were obtained by cleavage of plasmid DNA with *Eco*RI and with *Hin*dIII-partial *Eco*RI, respectively. These fragments were isolated by preparative agarose gel electrophoresis and further purified on CsCl gradients essentially as described previously (8, 26).

Construction of transgenic mice. One-cell (C57BL/6 × C3H)F_2 embryos were collected, microinjected, and transferred into pseudopregnant CD1 females essentially as described previously (8, 26). From 150 eggs microinjected with MMTV^D/c-*myc* and reimplanted, 33 mice were born, 6 of which were later found to be transgenic by Southern hybridization analysis of tail DNA with an SV40-specific probe. Four of these mice died at the age of 30 to 40 days. Five MMTV^D/CD4 transgenic founders were generated. These mice were evaluated by Southern hybridization analysis of tail DNA with a human CD4-specific probe. Transgenic founders MMTV^D/c-*myc* and MMTV^D/CD4 were then bred to CD1 or CFW mice, respectively. All mice were obtained from Charles River Laboratories, Saint-Constant, Canada.

Probes. The SV40 probe was the 1.2-kbp *Bam*HI-*Eco*RI fragment from pSV2gpt. The previously characterized 1.8-kbp *Eco*RI-*Bam*HI fragment harboring the human CD4 cDNA (32) was used as a probe to detect human CD4 sequences. The TcR β gene was analyzed with a 700-bp RBL5 DNA fragment containing most of the murine C region and 3′ untranslated sequences of $C_{\beta 1}$ (10). The J_H region was analyzed with a 6.2-kbp *Eco*RI germ line J_H DNA fragment (4). The C_κ probe was a 500-bp *Hpa*I-*Bgl*II fragment from the coding region and 3′ untranslated sequences of the C_κ region (31). All probes were labeled with [^{32}P]dCTP and dATP by the random primer method as described previously (22).

Analysis of the transgene RNA transcripts. RNA was isolated by the method of Chomczynski and Sacchi (16). For samples with very few cells, volumes of all solutions were reduced proportionately so that the extraction could be performed in a 0.4-ml tube. For Northern (RNA) blot analysis, the RNA was separated on 1% formaldehyde–agarose gels, transferred to Hybond-N membranes (Amersham Co.), and hybridized as described previously (8). For analysis of RNA by RNase protection, 20 μg of total RNA was hybridized overnight at 45°C with 5 × 10^5 cpm of a ^{32}P-labeled antisense RNA probe and digested for 1 h at 30°C with RNase A and RNase T_1. The protected fragments were then analyzed on a 6 or 8% denaturing polyacrylamide gel as described previously (34). The ^{32}P-labeled antisense RNA probes were synthesized as previously described (35). Briefly, the synthesis was done with T7 or SP6 RNA polymerase and [α-^{32}P]UTP (1,000 Ci/mmol; Amersham) as follows. A 300-bp fragment from the MMTV^D/c-*myc* transgene, spanning from the *Cla*I site in the MMTV LTR to the first *Pst*I site in c-*myc* exon 2, was cloned in pGEM-3 in the *Pst*I and *Acc*I sites. The resulting plasmid was linearized with *Ava*II for synthesis of probe A (see Fig. 4D). The total length of the probe A is 211 bp; it hybridizes to 193 bases of the transgene MMTV^D/c-*myc* RNA and to 138 or 173 bases of the spliced or unspliced endogenous c-*myc* mRNA, respectively. The same plasmid was also linearized with *Sac*I to generate probe B, encompassing the transcription start site (see Fig. 4D). This probe is 392 base long and is predicted to hybridize to 280 bases in the transgene mRNA or 374 bases in the transgene DNA. Probe C for protection of MMTV^D CD4 was prepared by subcloning, in *Sma*I-cleaved pGEM-3 vector, the 800-bp *Rsa*I MMTV^D/CD4 fragment spanning the transcription start point in the LTR and 220 bp of human CD4 coding sequence. The resulting plasmid was linearized with *Xba*I for synthesis of the probe (see Fig. 5B).

Cell transplantation to nude mice. Thymoma cells were dispersed in Dulbecco's modified Eagle's medium by mechanical shearing into a large-mesh grid and injected subcutaneously into 40- to 50-day-old nude mice. Approximately 10^7 cells were injected at each site.

Cell surface staining. Monoclonal antibodies fluorescein isothiocyanate (FITC)-30H12 anti-Thy-1.2, FITC-145-2C11 anti-CD3, and FITC-GK1.5 anti-murine CD4 were obtained from M. Julius. FITC-J11D anti-heat-stable antigen, biotin-12-15A anti-CD2 (provided by B. Kyewski), and FITC-PC61 anti-interleukin-2Rα were provided by T. Owens. FITC-Lyt1 anti-CD5, FITC- or biotin-coupled Lyt2 anti-CD8, phycoerythrin (PE)-L3T4 anti-murine CD4, PE-Leu3a anti-human CD4, FITC-Leu7 anti-human natural killer cells, and PE-Leu11 anti-human CD16 were purchased from Becton Dickinson. Staining with biotin-coupled antibodies was revealed by using PE-avidin (Southern Biotechnology) or FITC- or Duochrome-avidin (Becton Dickinson).

For single or double immunofluorescence analysis, 10^6 thymic or lymph node cells were incubated with saturating amounts of antibodies for 30 min on ice. The cells were washed twice with ice-cold phosphate-buffered saline before analysis. For three-color stainings, the cells were first incubated with biotin-coupled Lyt2 (CD8) for 30 min and then washed once before addition of Duochrome-avidin, FITC-GK1.5 (murine CD4), and PE-Leu3a (human CD4). Negative controls consisted of cells incubated with antibodies with irrelevant specificities (Leu7 or Leu11) or with secondary reagent alone. Cells were analyzed on a FACScan (Becton Dickinson). Ten thousand live cells were gated for each analysis.

Staining of fetal thymus. Day 0 of gestation was determined as the day of detection of vaginal plug. Females were sacrificed on day 14 or 16 of gestation, and fetal thymuses were harvested and stained for human CD4 and/or Thy-1.2 as described above.

RESULTS

Construction of transgenic mice. The LTR of MMTV^D was used as the promoter in front of the mouse c-*myc* gene or the human CD4 cDNA to construct the MMTV^D/c-*myc* and MMTV^D/CD4 transgenes (Fig. 1). Six transgenic founders harboring the MMTV^D/c-*myc* transgene were produced. Four of them died rather suddenly between 30 to 40 days, without apparent signs of discomfort and for unknown reasons. Two founders transmitted the transgene to their progeny in a Mendelian fashion. In both of them, the MMTV^D/c-*myc* sequences were present at one copy, as estimated by comparison with the endogenous c-*myc* sequences (data not shown). These transgene sequences appeared to be intact and localized at a unique integration site (data not shown). Five transgenic founders harboring the MMTV^D/CD4 transgene were produced. Four of these founders transmitted the transgene to their progeny in a Mendelian fashion, and the transgene, present at one copy, also appeared to be intact and localized at a unique integration site (data not shown). MMTV^D/c-*myc* lines M^D/M-1 and M^D/M-2 and MMTV^D/CD4 lines M^D/CD4-1 to M^D/CD4-4 were established by mating transgenic founders mice to

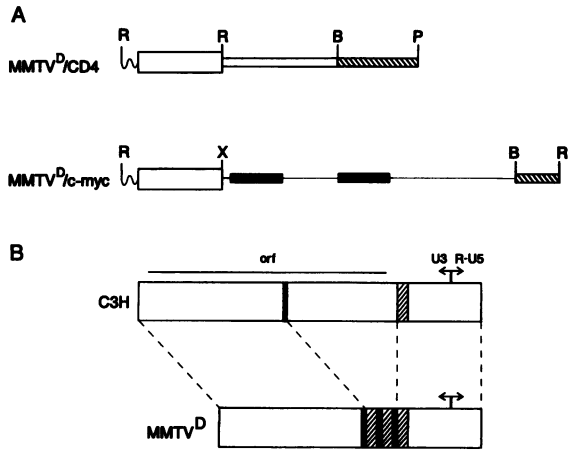

FIG. 1. (A) Structures of the DNA fragments used to produce transgenic mice. Symbols: large boxes, MMTV^D LTR; open bars, CD4 cDNA; close bars, c-*myc* exons 2 and 3; thin lines, introns and flanking sequences of c-*myc*; hatched bars, SV40 sequences; wavy line, MMTV^D *env* sequences and part of the pUC18 polylinker. Restriction sites: B, *Bam*HI; P, *Pst*I; R, *Eco*RI; X, *Xba*I. (B) Comparison of MMTV^D and C3H MMTV LTRs. Symbols: hatched boxes, glucocorticoid response element and surrounding nucleotides (43 bases); filled boxes, 15 nucleotides flanking the deletion in the U3 region. orf, open reading frame. The positions of 17 single-nucleotide differences outside of the deleted or duplicated regions are not shown. Data are from reference 7.

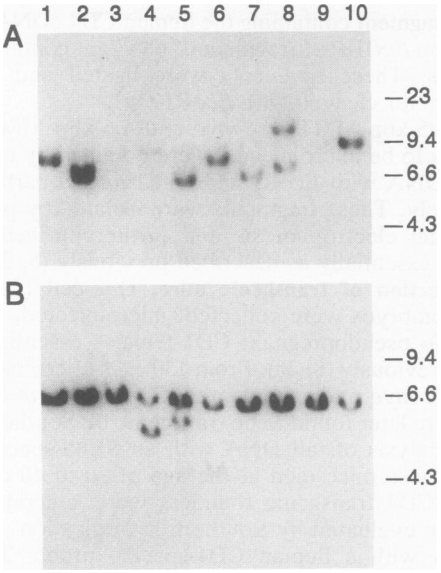

FIG. 3. Southern blot analysis of the immunoglobulin and TcR loci in thymomas of MMTV^D/c-*myc* transgenic mice. DNAs were digested with *Hin*dIII (A) or with *Eco*RI (B) and analyzed with the TcR RBL5 (A) or immunoglobulin J_H 6.2-kbp (B) ^{32}P-labeled probe. Numbers above each lane represent individual transgenic mice; lane 10, control DNA from a normal CD1 mouse. Numbers at the right represent the lengths of the *Hin*dIII-digested lambda phage DNA fragments used as molecular weight markers (in kilobase pairs).

outbred CD1 and CFW mice, respectively. All mice studied were heterozygous for the transgene.

Phenotype of transgenic mice. All mice of line M^D/M-1 bearing the MMTV^D/c-*myc* transgene developed a large thymoma which eventually filled the thoracic cage, leading to death of the animals (Fig. 2). No other signs of illness were observed at the time of death except that in a few mice, enlarged lymph nodes and spleens were also seen. Two of these thymomas were tested for their malignant phenotype by inoculation into nude mice. One of the two thymomas tested grew very well in nude mice, reaching a tumor diameter of 2 to 4 cm within 30 days postinoculation, thus indicating that these tumors are malignant. The phenotype observed in this transgenic line was very similar to that of the thymomas induced by the leukemogenic DMBA-LV MMTV. Mice of the other line (M^D/M-2) harboring the same transgene did not show any detectable phenotype since the transgene was not expressed, as measured by RNase protection analysis (data not shown).

Mice of the four lines harboring the MMTV^D/CD4 transgene did not develop any detectable macroscopic pathology.

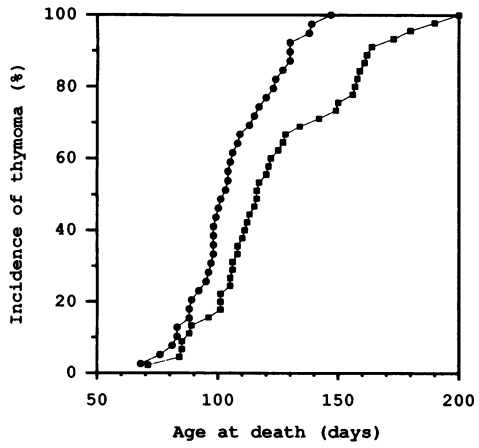

FIG. 2. Cumulative incidence of thymomas in transgenic MMTV^D/c-*myc* mice. Data are for 45 male (squares) and 39 female (circles) mice. Mice died naturally or were sacrificed 1 or 2 days before their anticipated death to collect organs.

Molecular characterization of the thymomas arising in mice carrying the MMTV^D/c-*myc* transgene. Several of the malignant tumors arising in transgenic mice or following inoculation with MMTV^D or with leukemogenic murine leukemia viruses have been found to be clonal or oligoclonal (19, 30, 47). To determine whether the thymomas arising in MMTV^D/c-*myc* transgenic mice were clonal and to identify the nature of the cells being transformed, we first used a molecular approach and studied the immunoglobulin and TcR gene rearrangements. In all of nine thymoma DNAs tested, the TcR β-chain gene was found to be rearranged or deleted in both alleles (Fig. 3A). One allele of the immunoglobulin heavy-chain gene was found rearranged in three of nine thymomas tested (Fig. 3B). These results indicated that these thymomas were clonal or oligoclonal in origin and that they belonged to the T-cell lineage.

RNA expression of the MMTV^D/c-*myc* and MMTV^D/CD4 transgenes. Expression of the MMTV^D/c-*myc* transgene was measured by an RNase protection assay. The ^{32}P-labeled RNA probes used encompass part of the c-*myc* gene exon 2 and either part of the U5 LTR (probe A) or the whole U5 and R regions plus part of U3 LTR (probe B) (Fig. 4D). With both probes, the endogenous c-*myc* spliced or unspliced RNA is predicted to protect a 138- or 173-base-long fragment, respectively, while RNA transcribed from the transgene should protect 193 bases in probe A and 280 bases in probe B.

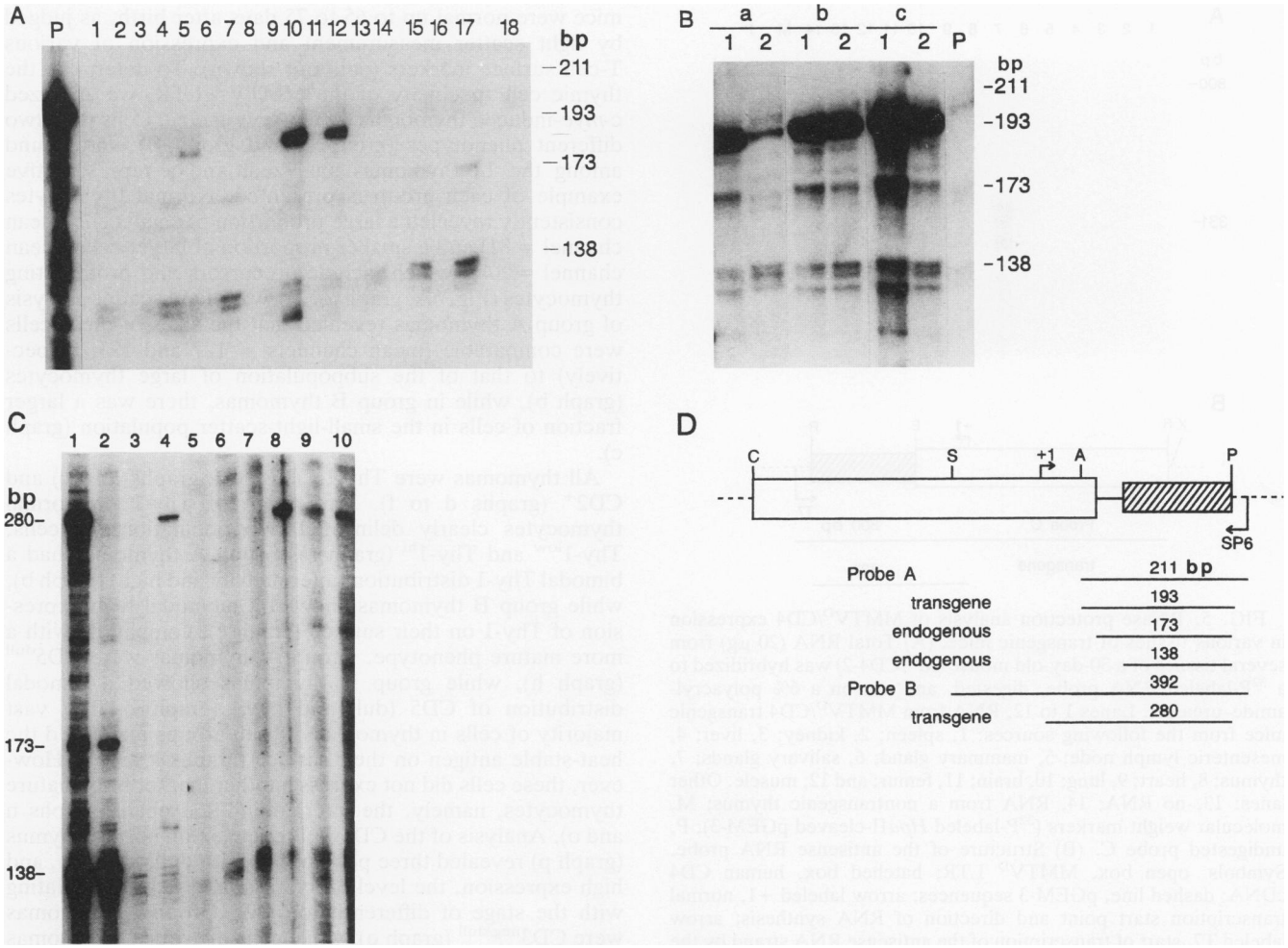

FIG. 4. RNase protection analysis of the MMTV^D/c-*myc* expression in various tissues of transgenic mice. (A) Total RNA (20 μg) from several tissues of a 34-day-old mouse was hybridized to ^{32}P-labeled RNA probe A, digested, and analyzed on an 8% polyacrylamide–urea gel. Lanes 1 to 14, M^D/M-1 mouse RNA from the following sources: 1, spleen; 2, kidney; 3, liver; 4, mesenteric lymph node; 5, small intestine; 6, ovary; 7, uterus; 8, mammary gland; 9, salivary gland; 10, thymus; 11, heart; 12, lung; 13, brain; and 14, bone marrow. Lanes 15 to 17, nontransgenic mouse RNA from the following sources: 15, spleen; 16, liver; and 17, thymus. Lane P, undigested probe; lane 18, digested probe. The positions and lengths of the transgene (193 bp) and endogenous spliced (138 bp) and unspliced (173 bp) c-*myc* protected fragments are indicated. (B) RNA (20 μg) from large thymoma, analyzed with probe A. Letters represent individual mice. Lanes: 1, thymoma; 2, spleen; P, digested probe. Mouse a had a normal size spleen (lane a-2); mice b and c had enlarged spleens (lanes b-2 and c-2), indicating the presence of infiltrated tumor cells. (C) RNA from embryonic day 15 thymus or from sorted T-cell subsets from the thymus or lymph node of a young adult transgenic mouse, probed with ^{32}P-labeled probe B. Lanes: 1, RNA from a pool of 4.5 thymus of transgenic embryos; 2, RNA from a pool of 4.5 thymus of nontransgenic embryos; 3, no RNA; 4, 100 ng of RNA from an adult thymus; 5, no RNA; 6, 100 ng of RNA from a nontransgenic thymus; 7, RNA from 2×10^5 sorted CD3$^+$ T cells from the mesenteric lymph node of an adult transgenic mouse; 8, RNA from 2×10^5 sorted CD4$^+$CD8$^+$ T cells from an adult thymus of transgenic mice; 9, RNA from 2×10^5 sorted CD4$^+$CD8$^+$ T cells from an adult thymus of transgenic mice; 10, RNA from 2×10^5 sorted CD4$^-$CD8$^-$ T cells from an adult thymus of transgenic mice. RNA in lanes 1 to 4, 5 to 8, and 9 and 10 were run in three independent assays. (D) Structures of the antisense RNA probes. Symbols: large open box, MMTV^D LTR; arrow labeled +1, normal transcription start point and direction of RNA synthesis; thin line, part of c-*myc* intron 1; hatched bar, part of c-*myc* exon 2; dashed line, pGEM-3 sequences; arrow labeled SP6, start of transcription of the antisense RNA strand by the SP6 RNA polymerase; horizontal lines with numbers, positions and lengths of the probes and of the expected protected fragments. Enzymes used in cloning or for linearizing the plasmid: A, *Ava*II; C, *Cla*I; S, *Sac*I; P, *Pst*I.

No RNA expression was detected in mice of line M^D/M-2. In normal 15-day-old transgenic mice of line M^D/M-1, strong expression was observed in the thymus, but lower expression could be detected in the spleen and in the salivary glands at a level comparable with that of the endogenous c-*myc* (data not shown). In the lungs, expression was found to be variable; in some animals it was low, while in others it was as high as in the thymus. In a slightly older animal (34 days old), whose thymus was the same size as that of a control littermate, transgene expression was highest in the thymus (Fig. 4A, lane 10). There was also expression in the lungs (lane 12) and much weaker or no expression in all other tissues tested (Fig. 4A). Expression of the c-*myc* transgene was also measured on specific T-cell subsets. Expression could be detected in sorted CD4$^+$CD8$^+$ and CD4$^-$CD8$^-$ thymocytes (2×10^5 cells) (Fig. 4C, lanes 8 to 10), but no signal was detected in the same number of sorted CD3$^+$ T cells from the peripheral lymph node (Fig. 4C, lane 7). In

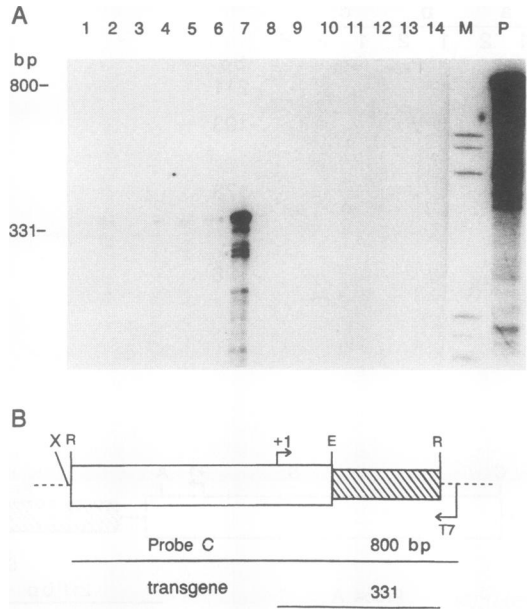

FIG. 5. RNase protection analysis of MMTV^D/CD4 expression in various tissues of transgenic mice. (A) Total RNA (20 μg) from several tissues of a 30-day-old mouse (M^D/CD4-2) was hybridized to a ^{32}P-labeled RNA probe, digested, and run on a 6% polyacrylamide–urea gel. Lanes 1 to 12, RNA from MMTV^D/CD4 transgenic mice from the following sources: 1, spleen; 2, kidney; 3, liver; 4, mesenteric lymph node; 5, mammary gland; 6, salivary glands; 7, thymus; 8, heart; 9, lung; 10, brain; 11, femur; and 12, muscle. Other lanes: 13, no RNA; 14, RNA from a nontransgenic thymus; M, molecular weight markers (^{32}P-labeled *Hpa*II-cleaved pGEM-3); P, undigested probe C. (B) Structure of the antisense RNA probe. Symbols: open box, MMTV^D LTR; hatched box, human CD4 cDNA; dashed line, pGEM-3 sequences; arrow labeled +1, normal transcription start point and direction of RNA synthesis; arrow labeled T7, start of transcription of the antisense RNA strand by the T7 RNA polymerase; horizontal lines with numbers, positions and lengths of the probe and of the expected fragments. Enzymes: E, *Eco*RI; R, *Rsa*I; X, *Xba*I.

addition, expression was found to be very high in the thymus of a 15-day embryo (Fig. 4C, lane 1).

In all of four thymomas tested, the levels of the c-*myc* transgene RNA were found to be as much as 10- to 20-fold higher than those of the endogenous c-*myc* (Fig. 4B). In these thymomas, the levels of endogenous c-*myc* were not significantly lower than those in a normal thymus.

In the MMTV^D/CD4 transgenic mice, RNA expression was analyzed by the Northern blot procedure with a human CD4-specific probe (data not shown) and by RNase protection analysis with a probe covering 580 bases at the 3′ end of the MMTV^D LTR and 220 bases in the human CD4 sequences (probe C). This probe protects a 331-base fragment in transgenic RNA (Fig. 5B). No expression was detected in mice of lines MMTV^D/CD4-1 and MMTV^D/CD4-3. In line MMTV^D/CD4-2, high expression was detected only in the thymus (Fig. 5A, lane 7). With longer exposure, much lower levels of transgene RNA could be detected in other tissues (Fig. 5A, lanes 1 to 12).

Together, these results indicate that the MMTV^D LTR has the ability to drive expression of surrogate genes preferentially within the thymus.

Characterization of the thymic cell population transformed by the c-*myc* oncogene. Thymocytes from MMTV^D/c-*myc* mice were normal up to 65 to 75 days after birth, as judged by light scatter measurement and expression of various T-cell surface markers (data not shown). To determine the thymic cell specificity of the MMTV^D LTR, we analyzed c-*myc*-induced thymomas by flow cytometry. Cells with two different phenotypes (group A and group B) were found among the 14 thymomas analyzed, and a representative example of each group is presented. Normal thymocytes consistently revealed a large proportion of small cells (mean channel = 81) and a smaller proportion of larger cells (mean channel = 144) which include precursors and proliferating thymocytes (Fig. 6A, graph a). Forward light scatter analysis of group A thymomas revealed that the sizes of these cells were comparable (mean channels = 127 and 135, respectively) to that of the subpopulation of large thymocytes (graph b), while in group B thymomas, there was a larger fraction of cells in the small-light-scatter population (graph c).

All thymomas were Thy-1$^+$ (Fig. 6B, graphs a to c) and CD2$^+$ (graphs d to f). Expression of Thy-1 on normal thymocytes clearly delineated two populations of cells, Thy-1^{low} and Thy-1^{hi} (graph a). Group A thymomas had a bimodal Thy-1 distribution (intermediate and high) (graph b), while group B thymomas showed a unimodal high expression of Thy-1 on their surface (graph c), compatible with a more mature phenotype. Group A thymomas were CD5^{dull} (graph h), while group B thymomas showed a bimodal distribution of CD5 (dull and high) (graph i). The vast majority of cells in thymomas of both groups expressed the heat-stable antigen on their surface (graphs k and l). However, these cells did not express another marker of immature thymocytes, namely, the interleukin-2R α chain (graphs n and o). Analysis of the CD3/TcR complex on normal thymus (graph p) revealed three populations with dull, medium, and high expression, the level of expression directly correlating with the stage of differentiation (42). Group A thymomas were CD3$^{neg/dull}$ (graph q), while cells of group B thymomas were CD3^{int} (graph r), again suggesting that group B thymomas have a more mature phenotype. In all thymomas tested, only the CD4$^+$CD8$^+$ subset of T cells was present. (Fig. 6C, graphs b and c). Therefore, two classes of CD4$^+$CD8$^+$ thymomas could be reproducibly identified: CD3/TcR$^{neg/dull}$ Thy-1^{int} CD5^{dull} (group A) and CD3/TcR^{int} Thy-1^{hi} CD5^{hi} (group B). These two classes of thymomas most probably represent two discrete stages of thymic development.

Characterization of the thymic cell population expressing the human CD4. To determine whether the MMTV^D/CD4 construct was also expressed exclusively in CD4$^+$CD8$^+$ thymocytes, expression of the human CD4 molecule in thymocytes was tested. In young adult mice of line M^D/CD4-2, the human CD4 was found to be expressed in the thymus (Fig. 7A) but not in peripheral lymphoid organs (Fig. 7B). Interestingly, expression of human CD4 in mouse lymphoid tissues did not significantly alter the levels of expression of murine CD4 and CD8 in the thymus, nor did it affect the frequency of mature CD4$^+$CD8$^-$ and CD8$^+$CD4$^-$ cells in peripheral lymphoid organ (Fig. 8C and D). In the thymus, cells expressing human CD4 molecules on their surface (Fig. 8A, inset) belong almost exclusively to the population of CD4$^+$CD8$^+$ T cells (Fig. 8A). We have not determined, however, whether minor subpopulations (<0.01%) of CD4$^+$CD8$^+$ thymic cells are negative for expression of the human CD4. By using three-color immunofluorescence, a minor subpopulation (0.1 to 0.5%) of CD3$^-$CD4$^-$CD8$^-$ cells expressing human CD4 was reproducibly detected in the adult thymus (data not shown).

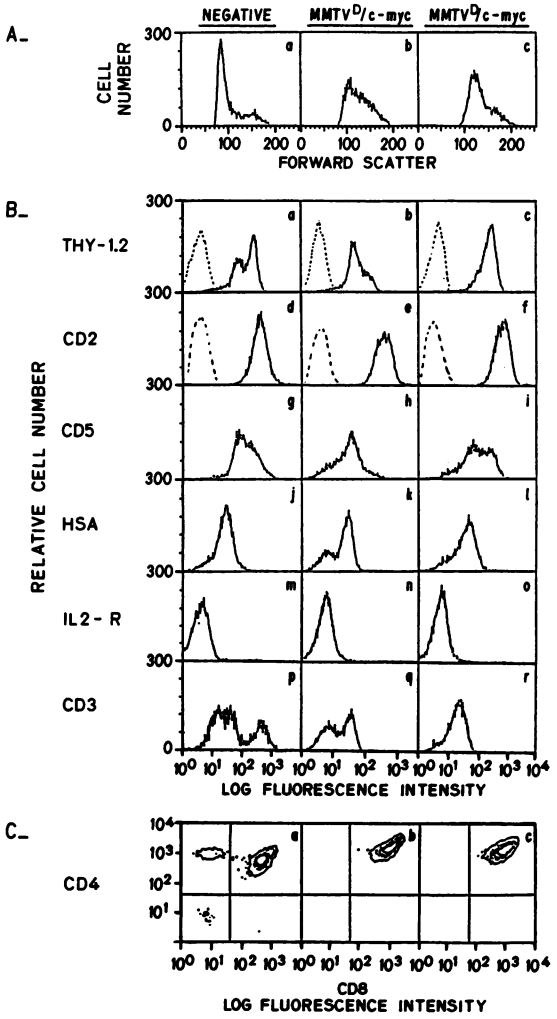

FIG. 6. Cell surface phenotype of thymomas induced by the MMTV^D/c-*myc* transgene. Thymocytes obtained from two 10-week-old MMTV^D/c-*myc* transgenic mice and from age-matched nontransgenic control littermates were stained with a panel of monoclonal antibodies specific for different T-cell surface markers. (A) Forward scatter analysis of thymic populations shows that thymoma cells are larger than most cells in normal thymus. (B) Cell surface stainings were carried out by using either a direct or an indirect immunofluorescence assay. Cell surface markers included Thy-1 (a to c), CD2 (d to f), CD5 (g to i), heat-stable antigen (j to l), interleukin-2R (m to o), and CD3 (p to r). (C) Thymomas were analyzed for expression of CD4 (PE-CD4) and CD8 (FITC-CD8), using a two-color immunofluorescence assay and directly labeled monoclonal antibodies. Stained cells were analyzed on a FACScan. Approximately 10,000 live cells are included in each fluorescence histogram. Negative controls consisted of cells stained with a directly labeled antibody with an irrelevant specificity (dashed lines panel B, graphs a to c). Negative controls stained with FITC-streptavidin (dashed lines in panel B, panels d to f).

Moreover, CD3$^-$CD4$^-$CD8$^+$ cells, which are thought to include the precursors of CD4$^+$CD8$^+$ cells, were found to express the transgene (data not shown).

To further determine the subset distribution of human CD4, its expression was correlated with levels of the CD3/TcR complex. In normal animals, CD4$^+$CD8$^+$ cells are known to express dull or intermediate levels of CD3 on their surface. Results shown in Fig. 8B confirmed that human

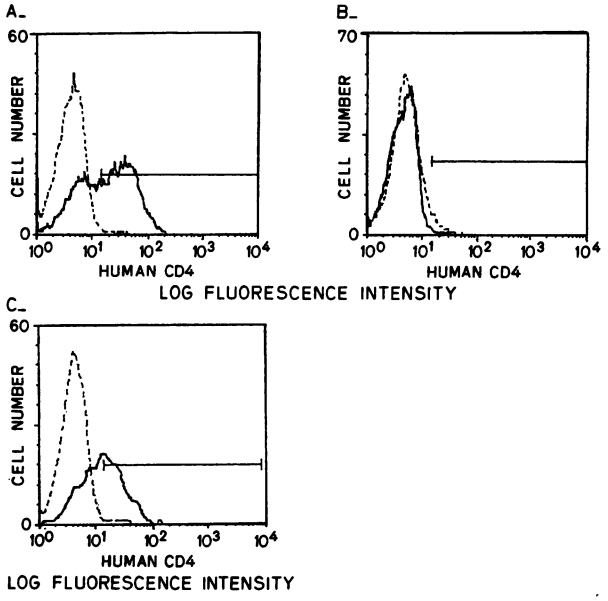

FIG. 7. Expression of human CD4 molecules in adult and fetal MMDT^D/CD4 transgenic mice. Thymocytes (A and C) or mesenteric lymph node cells (B) obtained from an 8-week-old young adult (A and B) or 14-day-old fetal (C) MMTV^D/CD4 transgenic mouse (solid lines) were stained for human CD4, using PE-Leu3a. Thymocytes and lymph nodes from nontransgenic littermates were used as negative controls (dashed line). Live cells were analyzed on FACScan equipped with a four-decade log scale. A minimum of 10,000 cells were analyzed for each fluorescence histogram.

CD4 is present on the surface of cells expressing dull or intermediate levels of CD3. CD3/TcR^{hi} cells, which represent mature CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$ thymocytes, were consistently negative for expression of human CD4. A similar flow cytometric analysis was performed on another MMTV^D/CD4 line (M^D/CD4-4). Although the percentage of cells expressing the transgene was lower in this line, expression of the human CD4 protein was also found to be restricted to immature thymocytes (CD4$^+$CD8$^+$, CD4$^-$CD8$^-$, and CD3$^-$CD4$^-$CD8$^+$ cells) and was undetectable in peripheral mature T cells (data not shown).

Altogether, this analysis confirmed the results obtained with the MMTV^D/c-*myc* transgenic mice and extended them by showing that the MMTV^D LTR is active almost exclusively in cells of the CD4$^+$CD8$^+$ phenotype and their precursors in young adult transgenic mice.

To determine whether the MMTV^D promoter was active during thymic ontogeny, day 14 fetal thymocytes were analyzed for expression of the human CD4 transgene. The vast majority of these cells are Thy-1$^+$ CD3$^-$CD4$^-$CD8$^-$ at that time. The human CD4 was found to be expressed in these cells (Fig. 7C), and levels of expression were comparable to that observed in CD4$^+$CD8$^+$ thymocytes of adult mice.

DISCUSSION

The LTR of DMBA-LV virus harbors the determinant of its thymotropism. Previous studies with the wild-type MMTV LTR in transgenic mice have revealed that its expression reflects the site of virus replication accurately (8, 45). Likewise, the pattern of expression of this variant MMTV^D LTR in transgenic mice appears to reflect the tropism of the

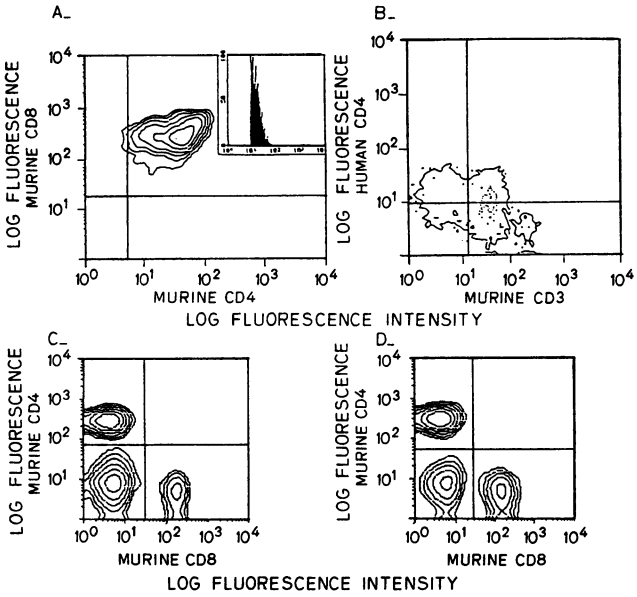

FIG. 8. Cell surface phenotype of human CD4+ T cells in MMTV^D/CD4 transgenic mice. (A) Thymocytes from young adult (8-week-old) transgenic mice labeled simultaneously with monoclonal antibodies specific for murine CD4, murine CD8, and human CD4 and analyzed on a FACScan, using a triple immunofluorescence assay. Expression of human CD4 was monitored by using PE-Leu3a. Live cells gated for their positive reactivity to a human CD4-specific monoclonal antibody (inset) were analyzed for expression of the murine CD4 (FITC-CD4) and CD8 (biotin-CD8 and Duochrome-avidin) molecules as described for Fig. 7. Note that most human CD4-positive cells are murine CD4$^+$CD8$^+$. (B) Two-color fluorescence analysis of thymocytes from MMTV^D/CD4 transgenic mice. Cells were incubated at 37°C for 4 h and stained with human CD4 PE-Leu3a and the murine CD3-specific monoclonal antibody FITC-145-2C11. Fluorescence contour plots were obtained and analyzed as described above. Note that most human CD4-bearing cells express low or intermediate levels of CD3 on their surface. (C and D) Two-color fluorescence analysis of murine CD4/CD8 expression in spleen cells of a control (C) or MMTV^D/CD4 transgenic (D) mouse. Cells were stained with conjugated monoclonal antibodies specific for murine CD4 (PE-CD4) and murine CD8 (FITC-CD8) as described above. Note that there is no significant difference in the levels of expression or cell distribution of these antigens between the two mice.

virus known to induce T-cell lymphoma predominantly of the Thy-1.2$^+$ CD5$^+$CD4$^+$CD8$^+$ phenotype (37, 38). With both reporter genes, expression was found to be highest in the thymus, strongly suggesting that the thymotropism of this virus is determined by its LTR. Our data suggest that the sequence differences, which distinguish the MMTV^D LTR from the wild-type MMTV LTR, are responsible for its novel tissue and cell tropism. These results extend the role of the LTR in determining tissue and disease specificity to a new class of leukemogenic retrovirus. Such a role has previously been recognized for MuLV (11–13, 20, 21, 43, 48).

The MMTV^D/c-*myc* transgene induces selective transformation of CD4$^+$CD8$^+$ T cells. The c-*myc* proto-oncogene expressed under the control of MMTV^D LTR was very efficient in transforming some (CD4$^+$CD8$^+$) but not all (CD4$^-$CD8$^-$) of the target cells in which it was expressed. These tumors were oligoclonal or clonal in origin, suggesting that the c-*myc* oncogene was not sufficient by itself to achieve a full transformation phenotype and that additional genetic events (second hit) contributed to the emergence of clonal populations having gained a growth advantage. Interestingly, we have observed that the endogenous c-*myc* was not downregulated by the high level of c-*myc* transgene expression either in thymomas or in thymic cells from young animals. This finding contrasts with those of a number of studies in which such downregulation was observed in *myc*-induced tumors (2, 3, 17, 24, 28, 29, 33, 39, 40).

Fluorescence-activated cell sorting analysis of these tumor cells showed that all of them belonged exclusively to the CD4$^+$CD8$^+$ cell lineage. As expected, most of these cells were blastic according to their light scatter properties. Surprisingly, no thymoma contained cells exhibiting the CD4$^-$CD8$^-$ phenotype despite the fact that the transgene was expressed in CD4$^-$CD8$^-$ embryonic thymic cells and in double-negative cells of adult mice. Interestingly, the constitutive expression of c-*myc* at this particular stage of embryonic differentiation did not seem to prevent further selection (positive or negative) of T cells, as substantiated by the normal distribution of T-cell subsets in the thymus and in peripheral cells before the appearance of thymomas (data not shown).

With additional cell surface markers, the CD4$^+$CD8$^+$ thymomas arising in our transgenic mice could be divided into two groups. One group (group B) of thymomas typed as Thy-1^{hi} CD3/TcR^{int} CD5^{hi}, with the majority of the cells exhibiting a smaller light scatter. This phenotype corresponds to a transition state between immature T-cell precursors and mature T cells and suggests that these cells may be going through positive or negative selection. The other group of thymomas (group A) was Thy-1^{int} CD3/TcR$^{neg/dull}$ CD5^{dull}, a phenotype which corresponds to the phenotype of immature T cells.

In contrast to several types of tumor cells, these double-positive tumor T cells have retained some of the characteristics of the normal CD4$^+$CD8$^+$ T cells, such as their rapid death by apoptosis when cultured in vitro (data not shown) and the absence of migration outside the thymus where they normally reside. These thymomas were indeed localized and surprisingly did not tend to metastasize in other peripheral lymphoid tissues. Only in a few older animals have we been able to detect metastases in the spleen or lymph nodes. Presumably these cells had reached a novel and more aggressive phenotype. However, the tumor T cells did not seem to have conserved all of the characteristics of normal CD4$^+$CD8$^+$ T cells, since no mature CD4$^+$CD8$^-$ or CD4$^-$CD8$^+$ tumor T cells were found among the populations of proliferating transformed cells. These results suggest that the *myc*-transformed cells may not respond to signals leading to their deletion (negative selection) or their positive selection.

The induction of T-cell lymphomas has previously been observed in mice expressing various transgenes, such as *pim-1* (9, 46), lymphotropic papovavirus early gene (14), BK virus T antigen (18), *bcr/ab1* (25), and c-*myc* (28, 44). The characterization of the subpopulations of transformed T cells was not complete enough in many of these studies to compare their phenotype with that of the T lymphomas induced by MMTV^D/c-*myc*. However, these thymomas did not seem to be restricted to a precise T-cell subpopulation, having the CD4$^+$CD8$^+$, CD4$^-$CD8$^+$, or CD4$^+$CD8$^-$ phenotype or consisting in a mixture of double-positive and single-positive cells in different proportions.

Therefore, although the CD3^{int}CD4$^+$CD8$^+$ T cells have been previously detected by using static multiparametric analysis of thymocyte subsets (23), this is the first report

demonstrating the isolation of clonal populations of cells with this phenotype maintained in a stable manner.

Common negative regulatory factors in mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells. The reporter human CD4 protein was also found to be expressed predominantly in the precursor $CD3/TcR^{dull/int}$ $CD4^+CD8^+$ T-lymphoid cell population of young adult mice. Interestingly, it was not expressed in mature $CD4^+CD8^-$ (helper) or in $CD4^-CD8^+$ (cytotoxic) T cells, both of which are known to be derived from the precursor $CD4^+CD8^+$ T cells (23). This pattern of expression indicates that the $MMTV^D$ LTR-driven transcription is turned off when the $CD4^+CD8^+$ cells differentiate into more mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells. To our knowledge, this LTR represents a new well defined regulatory element to be identified whose expression appears to be modulated by the commitment of the $CD4^+CD8^+$ T cells to differentiate. Our results suggest that an exogenous genetic element ($MMTV^D$ LTR) responds to the same cellular factors which are presumably responsible for turning off the transcription of CD4, CD8, and possibly other genes following differentiation of $CD4^+CD8^+$ into $CD4^+CD8^-$ or $CD4^-CD8^+$ mature T cells. This inference suggests that similar and common signals may be present in latter T-cell subpopulations. Whether the downregulation of these cellular genes and the $MMTV^D$ LTR occurs as a consequence of the positive selection (instruction model) or before and independently of this positive selection (selection model) (41) remains to be determined.

Very little is known about the biochemical and molecular events occurring during the critical stages of $CD4^+CD8^+$ T-cell maturation and differentiation. The capacity to obtain clonal populations of cells bearing this stable phenotype and to express various reporter genes in these cells should prove a valuable tool in the characterization of these events at the molecular level.

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