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Expression of T-Cell Receptor Alpha-Chain Genes in Transgenic Mice

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To examine the influences responsible for shaping the T-cell repertoire in vivo, we have introduced T-cell receptors of defined specificity into mice. In this report, we analyze transgenic mice carrying a T-cell receptor alpha-chain gene from a pigeon cytochrome *c*-reactive T-cell line. A variant of this construct, which has the immunoglobulin heavy-chain enhancer inserted into the JC intron, was also introduced into mice. Addition of the enhancer increased the steady-state level of transgene-encoded mRNA three- to fivefold in cultured T cells, leading to a two- to threefold increase in surface expression. In vivo, the difference between these two constructs was even more significant, increasing the number of transgene-positive cells from ~5 to 70% and the T-cell receptor surface density two- to threefold. Surprisingly, while surface expression of either type of transgene was limited to T cells, we found little tissue specificity with respect to transcription. In T cells expressing the alpha chain from the enhancer-containing construct, immunoprecipitation with a 2B4α-specific monoclonal antibody revealed the expected disulfide-linked dimer. Costaining of these T cells with the 2B4α-specific monoclonal antibody versus anti-CD3 indicated that expression of the transgene-encoded alpha chain precludes expression of endogenous alpha chains on the majority of cells; in contrast, 2B4α-chain expression from the construct lacking the enhancer is inefficient at suppressing endogenous alpha-chain expression. In mice of the enhancer lineage, Southern blot analysis indicated suppression of endogenous alpha-chain rearrangements in T-cell populations, consistent with the observed allelic exclusion at the cellular level. Interestingly, newborn, but not adult, mice of this lineage also showed an increase in retention of unrearranged delta-chain loci in thymocyte DNA, presumably resulting from the suppression of alpha-chain rearrangements. This observation indicates that at least a fraction of α:β-positive T cells have never attempted to produce functional delta rearrangements, thus suggesting that α:β and γ:δ T cells may be derived from different T-cell compartments (at least during the early phases of T-cell differentiation).

Both B and T lymphocytes are responsible for specificity in the immune systems of higher organisms. B cells use antibody heavy- and light-chain molecules to distinguish between the multitude of "self" and "non-self" antigens, while T cells use the α:β (and perhaps the γ:δ) heterodimer for this purpose. Each of the gene loci encoding these polypeptides randomly rearranges V, D (in some cases), and J gene segments adjacent to a C region to produce a large number of different molecules. In general, only one allele of each locus is functionally rearranged and expressed on the surface of a given cell. This phenomenon, referred to as allelic exclusion, was first established for immunoglobulin genes and has more recently been observed for T-cell receptor genes (10, 14, 24, 31, 32). In the case of immunoglobulin genes, much has been learned about the control of antibody gene rearrangements, particularly the phenomenon of allelic exclusion, through the analysis of mice carrying and expressing rearranged heavy- or light-chain genes (40, 46, 47, 54 [review]). In addition, since the delta-chain locus appears to be encoded between V_α and J_α coding regions, it cannot be expressed from any chromosome that has undergone a T-cell receptor alpha rearrangement by a deletion mechanism (8). Since the alpha chain is the last T-cell receptor chain to be rearranged and transcribed during T-cell ontogeny, the surface expression of α:β heterodimers is particularly dependent on alpha-chain expression. Because of this delay in T-cell receptor alpha expression, the γ:δ heterodimer appears well before α:β in thymic ontogeny (20, 43, 45, 51, 52). It is therefore of interest to determine what effect various T-cell receptor transgenes, and in particular alpha-chain transgenes, will have on this pattern of expression.

In this report, we describe the characterization of transgenic mice carrying a T-cell receptor alpha-chain gene from a T-cell hybridoma line, 2B4 (49), which recognizes a fragment of the pigeon cytochrome *c* molecule bound to the k allele of the I-E molecule (6). This alpha-chain gene was chosen because of the availability of a monoclonal antibody which recognizes the alpha-chain protein (49) but fails to stain detectable numbers of cells from normal mice (17, 48; A. Korman, personal communication) and can therefore be used to follow transgene expression in vivo. In an attempt to increase expression of the T-cell receptor alpha-chain gene, we have also inserted the immunoglobulin heavy-chain enhancer (2, 18) into the alpha-chain construct. We find that the immunoglobulin enhancer increases the steady-state amount of transgene-encoded mRNA in cultured T cells and has profound influences on the number of T cells which express the transgene in vivo. We also see evidence of allelic exclusion and suppression of both J_α and C_δ deletion,

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although, curiously, the latter is not evident in adult thymocytes. A preliminary report of some of these results has been published previously (25).

MATERIALS AND METHODS

Alpha-chain constructs. T-cell receptor alpha-chain constructs were derived from germ line gene segments and cDNA segments which were ligated to create pseudogenomic alpha-chain genes. The 2B4α gene was made as follows. A 2.3-kilobase (kb) fragment of germ line V_α gene sequence, containing 2 kb of 5′ flanking DNA and extending to the unique RI site within V_α, was fused to a 194-base-pair (bp) fragment of cDNA clone (3) covering the unique VJ junction. The remaining nucleotides of the J_α region, plus approximately 100 bp of JC intron sequence, was obtained from a germ line J_α clone. This reconstructed VJ gene segment was then inserted upstream of a 9.8-kb C_α clone which contained all the C_α exons and extended 0.5 kb downstream of the poly(A) addition signal. A 683-bp *Xba*RI fragment carrying the immunoglobulin heavy-chain enhancer (2, 18) was inserted at the junction between the VJ and C_α segments, yielding the 2B4αE_H construct. Both constructs were separated from the bacterial vector sequences prior to injection into fertilized eggs.

Transgenic mice. Fertilized eggs from superovulated (C57BL/6J × C3H/HeJ)F_1 females were injected with the purified 12.5-kb alpha-chain gene inserts. Injected eggs were implanted into foster mothers (5, 12, 23, 57, 58). The progeny were screened for new transgene-specific restriction fragments by Southern blot hybridization of tail DNA. All the mice used in these experiments were derived from the second through fourth backcross generation to C57BL/10SnJ or C3H/HeJ mice.

Transfections. 2B4α and 2B4αE_H constructs were inserted into a modified version of pSV2gpt (38) and transfected by electroporation (39, 44) into EL4 and J558L cells. EL4 cells were treated with 3 μg of mycophenolic acid per ml–15 μg of hypoxanthine per ml–200 μg of xanthine per ml, and J558L cells were treated with 5 μg of mycophenolic acid per ml–15 μg of hypoxanthine per ml–200 μg of xanthine per ml to select for expression of the *gpt* gene. Surviving cells in each well (not clonal populations) were expanded for RNA extraction.

RNA extractions and analysis. Cytoplasmic RNA from tissue culture cell lines was prepared as described by Brawerman et al. (4). RNA was prepared from mouse tissues by the method of Chirgwin et al. (9). Protection assays were performed with minor modification of the method of Zinn et al. (60). Cytoplasmic RNA (100 μg per sample) from tissue culture lines was used, and for mouse tissues, 2 to 5 μg was used per sample. RNA was hybridized with antisense single-stranded RNA probes for more than 12 h at 45 to 50°C in 80% formamide and then digested with RNase A and T1 at 14°C for 1 h. Samples were denatured and run on 6% urea-polyacrylamide gels. The probe used to detect T-cell receptor beta-chain transcription was the *Hpa*I-*Sac*I fragment of the fourth exon of the $C_{\beta 2}$ gene. An *Xho*I linker had been inserted into the *Eco*RV site within this fragment.

FACS analysis. For fluorescence-activated cell sorter (FACS) analysis, lymph node cells from transgenic and littermate control mice were stained with biotinylated A2B4-2 (49) or F23.1 (53) monoclonal antibody, followed by Texas Red-conjugated avidin (Organon Teknika). $Lyt2^+$ and $L3T4^+$ cells were detected by using fluorescein isothiocyanate (FITC)-conjugated 53-6.7 (30) and allophycocyanin-conjugated GK1.5 (15) monoclonal antibodies, respectively. CD3 was detected with a hamster monoclonal antibody (kindly provided by J. Allison) (1) followed by a FITC-conjugated anti-hamster reagent (Organon Teknika). Dead cells were excluded by propidium iodide staining (34). FACS analyses were performed essentially as described previously (21).

Immunoprecipitations. Spleen cells were depleted of erythrocytes by centrifugation onto a cushion of Lympholyte M (Cedarlane) before surface iodination by the lactoperoxidase method (28) followed by lysis in 0.5% Nonidet P-40. Extracts were precleared with goat anti-mouse immunoglobulin plus Staph A (Pansorbin; Calbiochem) and immune precipitated with either A2B4-2 ascites (kindly provided by L. Samelson) or normal mouse serum. Precipitated protein was eluted from the Staph A in nonreducing Laemmli buffer and loaded onto 7.5% polyacrylamide tube gels. After electrophoresis, tubes were freeze-thawed in reducing buffer, heated to 90°C for 3 min, and run on 10% sodium dodecyl sulfate slab gels.

DNA extractions and southern blot analysis. Thymus and liver DNA were prepared by standard methods. For each sample, 2 μg of DNA was digested with *Eco*RI and electrophoresed on a 0.8% agarose gel. DNA was blotted to nitrocellulose; blots were hybridized and washed under stringent conditions. Scanning laser densitometry was performed on an LKB Bromma Ultroscan XL laser densitometer. Multiple exposures of each Southern blot were scanned, and the resulting values were normalized by hybridizing the same filters to a single-copy, nonrearranging gene probe, mouse GM-CSF (kindly provided by J. F. Elliott).

RESULTS

T-cell receptor alpha-chain constructs and transcription in B- and T-cell lines. Numerous attempts to isolate the rearranged 2B4 VJ_α exon from genomic libraries were unsuccessful, apparently because of instability which was seen even when using $RecA^-$ or $RecBC^-$ host cells. For this reason, the 2B4α genomic clone depicted in Fig. 1 was constructed by combining the 5′ end of the germ line $V_{\alpha 2B4}$ ($V_{\alpha 11.1}$), a fragment of the 2B4α cDNA (3) covering the VJ junction, and a 3′ portion derived from the germ line $J_{\alpha 2B4}$ by using internal restriction sites (see Materials and Methods; Fig. 1A). This construct mimics the VJ region of the functionally rearranged 2B4 alpha gene. The reconstructed VJ exon and flanking sequences were then ligated to a restriction fragment containing the C_α exons plus 6 kb of 5′ and 0.5 kb of 3′ flanking sequence. A variant of this construct was made by inserting a 683-nucleotide (nt) fragment carrying the immunoglobulin heavy-chain enhancer (E_H) (2, 18) into the JC intron of the initial construct. The first construct was designated 2B4α, and the second construct was designated 2B4αE_H.

To assay for correct splicing and RNA stability, as well as to test the effect of the immunoglobulin heavy-chain enhancer on alpha-chain expression in T and B cells, the 2B4α and 2B4αE_H constructs were inserted into the pSV2gpt vector (38) and introduced into two murine tumor cell lines: EL4, a thymoma, and J558L, a plasmacytoma. Stable transfectants were selected in mycophenolic acid, and pools of positive colonies were analyzed for 2B4 alpha RNA expression by RNase protection assays. The probe used for this analysis was a 380-nt antisense RNA transcript with homology to 223 nt of the 3′ half of the VJ exon and 103 nt of the

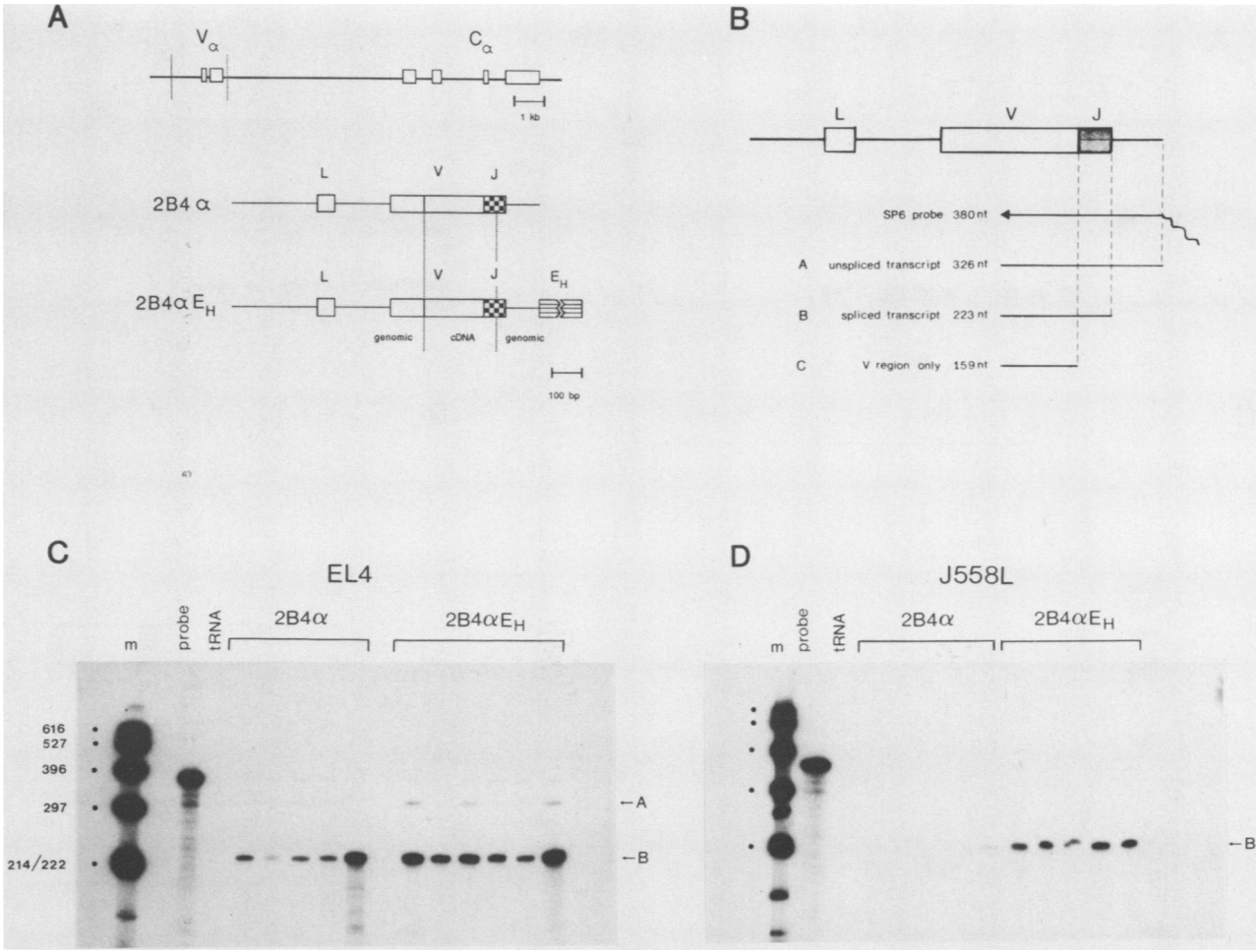


FIG. 1. Alpha-chain constructs and their expression in cultured cells. (A) Diagram of the two alpha-chain constructs showing the exon structure of the genes and an enlargement of the VJ region of each construct. The 683-bp immunoglobulin heavy-chain enhancer fragment (E_H) was inserted 80 bp downstream of the 2B4 J region in the construct designated 2B4αE_H. (B) Diagram of the Sp6 probe used for detecting transcription of the alpha-chain transgenes. The full-length antisense RNA probe was 380 nt in length, including 54 nt of vector polylinker sequences at the 5′ end (wavy line). Protection of unspliced transcripts yields a 326-nt fragment (line A); protection of the appropriately spliced alpha transcript yields a 223-nt fragment (line B) and protection of the homologous V region alone yields a 159-nt fragment (line C). (C and D) Expression of 2B4α and 2B4αE_H in EL4 cells (C) and J558L cells (D). The ^{32}P-labeled antisense probe was hybridized to equal quantities of cytoplasmic RNA, digested with ribonucleases and subjected to denaturing polyacrylamide gel electrophoresis. m, Size markers, indicated by dots; probe, undigested probe; tRNA, probe hybridized to tRNA alone. Arrows marked A and B are as designated in the diagram above (panel B).

JC intron of 2B4α. Correctly spliced 2B4α or 2B4αE_H mRNA yields a 223-nt fragment after hybridization and RNase digestion (Fig. 1B). In transfectants of the thymoma EL4, the level of stable 2B4 alpha mRNA was increased three- to fivefold by the immunoglobulin enhancer (Fig. 1C). FACS analysis of these transfectants and use of A2B4-2, the monoclonal antibody specific for the 2B4 alpha chain, indicated a concomitant two- to threefold increase in surface 2B4 alpha-chain expression (data not shown). In contrast, no 2B4 alpha transcription was detected in four of the five B-cell (J558L) transfectants carrying the 2B4α construct, and the fifth line had a barely detectable signal (Fig. 1D). Insertion of the heavy-chain enhancer, however, led to a significant level of 2B4 alpha transcription in all the J558L transfectants (Fig. 1D).

Expression of alpha-chain genes in transgenic mice. The two alpha-chain constructs were injected into (C57BL/6J × C3H/HeJ)F_2 fertilized mouse eggs (5, 12, 23, 57, 58). From these injections, four transgenic mice were obtained: three carrying the 2B4α construct and one carrying 2B4αE_H. One of the 2B4α founders failed to transmit the transgene to any of its progeny. The three lines of mice used for the analysis presented below, two 2B4α and one 2B4αE_H, have 4, 8, and 10 copies of the transgene, respectively (data not shown).

Thymus and spleen RNAs from transgenic and control offspring of one 2B4α and one 2B4αE_H lineage were analyzed by RNase protection by using the probe shown in Fig. 1B. In all cases, mice positive for the introduced DNA expressed an RNA transcript which protected the entire VJ junction, whereas normal littermates had no detectable transcripts of this length, suggesting that this rearrangement is rare in the endogenous population. We also detected a higher expression level in 2B4αE_H transgenic mice, an observation which was confirmed by subsequent FACS analysis (see below).

To assess the tissue specificity of transcription of the transgenic alpha-chain genes, RNA was prepared from a variety of transgenic mouse tissues. The analysis of two

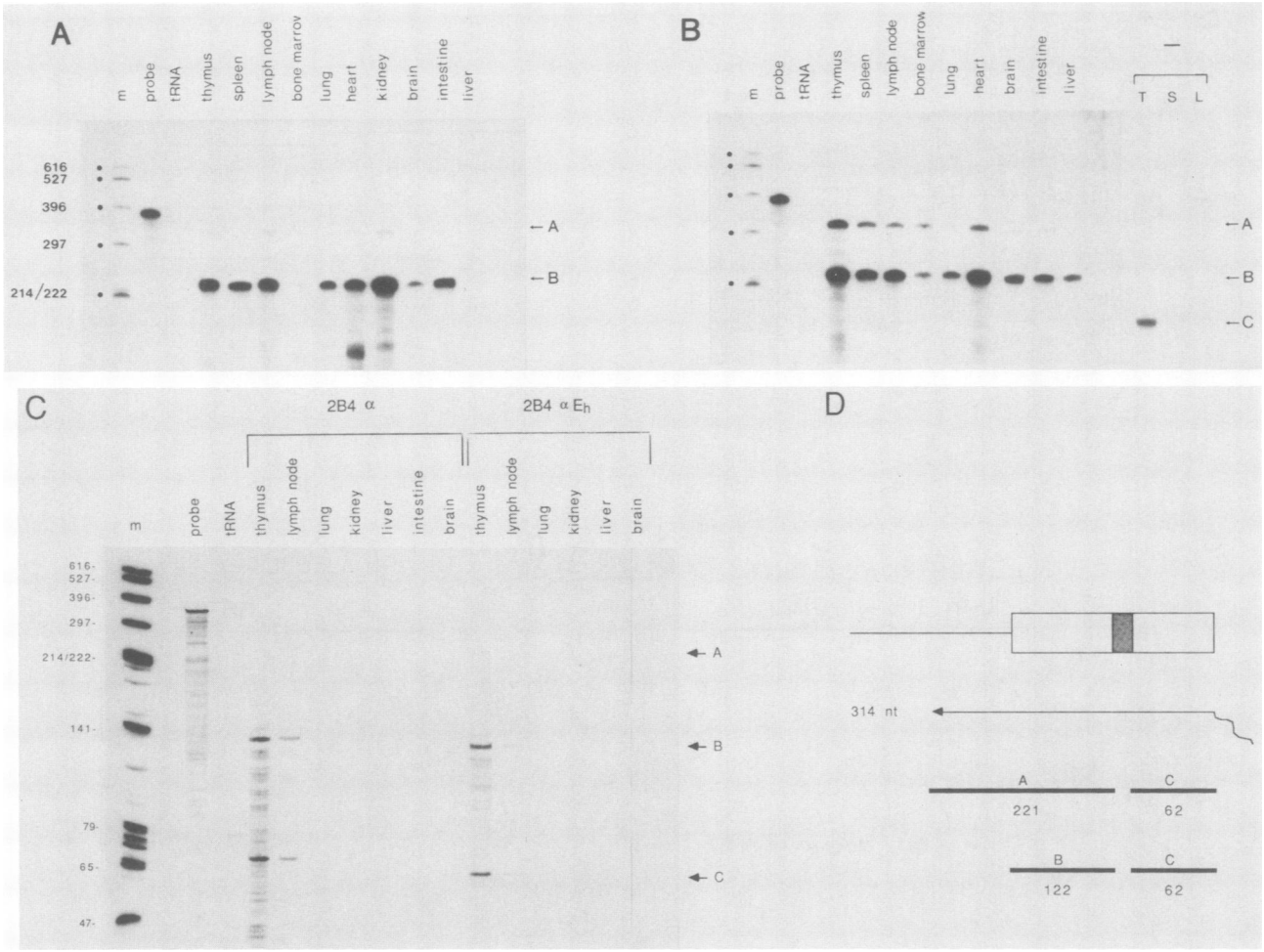


FIG. 2. Expression of alpha-chain genes in transgenic mice. RNA from various tissues of either a 2B4α (A) or a 2B4αE_H (B) transgenic mouse was analyzed by RNase protection for transcription of the transgene. Thymus (T), spleen (S), and liver (L) RNAs from a negative (−) littermate were also analyzed. For each sample, 5 μg of total RNA from the indicated tissue were hybridized to the ^{32}P-labeled probe. m, Size markers, indicated by dots; probe, undigested probe; tRNA, probe hybridized to tRNA alone. Arrows marked A, B, and C are as designated in the diagram in Fig. 1B. (C) The antisense ^{35}S-labeled beta-chain probe was hybridized to 2 μg of total RNA from the indicated tissues of a 2B4α or a 2B4αE_H transgenic mouse. Bands marked with arrows A, B, and C indicate protected fragments as shown in panel D. (D) Diagram of the antisense RNA probe (314 nt) used to detect T-cell receptor beta-chain transcription arising from the $C_\beta 2$ gene. The probe contained an *Xho*I linker inserted in the $C_\beta 4$ exon. Protection of unspliced beta-chain transcripts yields two fragments of 221 and 62 nt, while protection of spliced beta-chain mRNA yields fragments of 122 and 62 nt.

transgenic mice, one from each of the two lineages, is shown in Fig. 2. In both cases, transcription from the transgenic alpha-chain gene was not restricted to T cells but was apparent to various degrees in all tissues analyzed. Appropriately spliced 2B4 alpha mRNA was found at especially high levels in heart, kidney, and intestine of the 2B4α transgenic mouse (Fig. 2A, bands labeled B) and in heart, brain, and intestine of the 2B4αE_H transgenic mouse (Fig. 2B, bands labeled B). These results are especially surprising since, as described above, little or no transcription was observed after transfection of the 2B4α construct into J558L cells (Fig. 1D). The transfection results are consistent with the observation that bone marrow, which is composed of 20 to 30% pre-B and B cells (11) and less than 5% T cells (37), gave an extremely poor signal in the 2B4α compared to the 2B4αE_H transgenic mouse (Fig. 2A and 2B), suggesting that the 2B4α transgene might not be expressed in B cells. To test this possibility, RNA from T-cell-depleted spleen of a 2B4α transgenic mouse was prepared and analyzed for transcription of the transgene. In contrast to the expected outcome, we found significant levels of 2B4 alpha transcription, indicating that expression of the 2B4α construct in spleen is not limited to T cells, although the steady-state level of RNA is reduced compared with that seen in T-cell-depleted spleen RNA from a 2B4αE_H transgenic mouse (data not shown). These results strongly suggest that the 2B4α transgene is, in fact, expressed in B cells. From these data, both the transfection and the transgenic experiments, it is clear that the immunoglobin heavy-chain enhancer increases transcription of the T-cell receptor alpha-chain gene in B cells. A similar effect of the heavy-chain enhancer on T-cell-specific gene expression in transgenic mice has previously been reported (7).

We detected no protected fragments of the size expected for transgene-encoded transcripts in thymus, spleen, or liver of a nontransgenic littermate (Fig. 2B). The band labeled C, visible in thymus RNA of the nontransgenic littermate, represents transcription from the endogenous V_α gene corresponding to that present in the transgene (Fig. 1B). The bands labeled A in Fig. 2, most clearly visible in RNA from

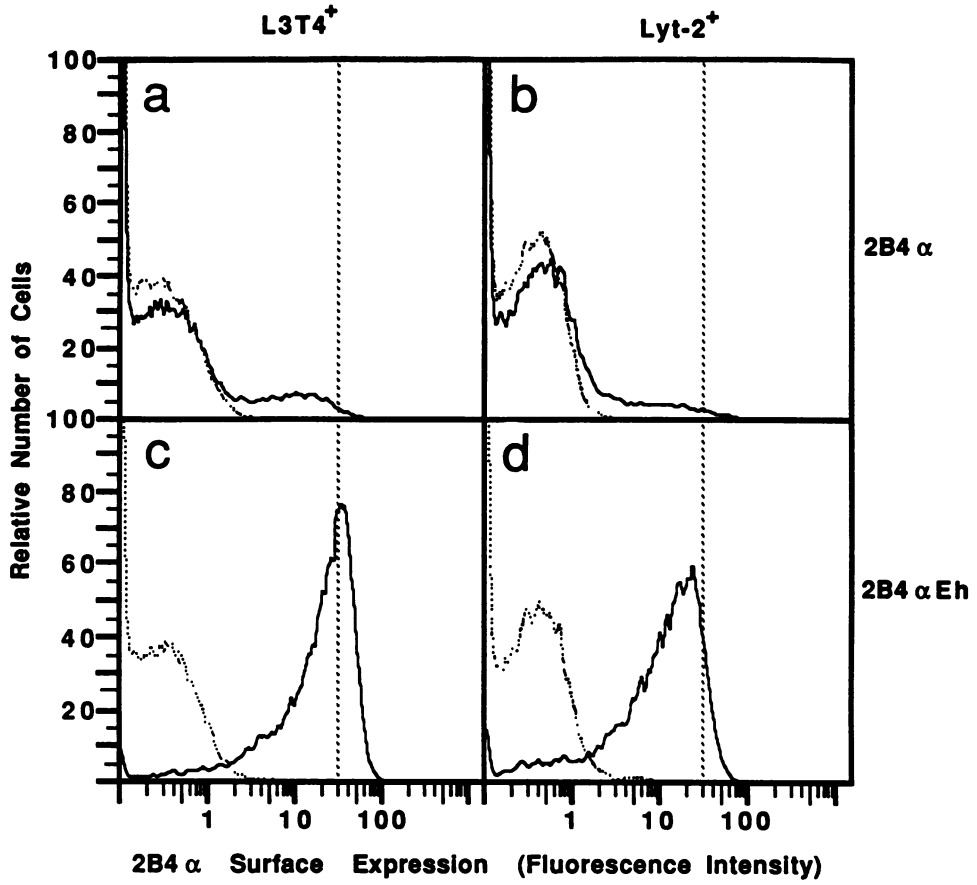


FIG. 3. Cell surface expression of 2B4 alpha chains in transgenic mice. Lymph node cells from transgenic (solid lines) and littermate control mice (dotted lines) were stained with the A2B4-2 monoclonal antibody and analyzed by FACS to detect surface 2B4 alpha-chain expression. Upper panels show A2B4-2 staining of $L3T4^+$ (a) and $Lyt\text{-}2^+$ (b) cells from mice of the 2B4α lineage; lower panels (c and d) show the same analysis for the $2B4\alpha E_H$ lineage. L3T4 and Lyt-2 surface expression was detected with GK1.5 and 53-6.7 monoclonal antibodies, respectively.

the $2B4\alpha E_H$ transgenic mouse, resulted from unspliced or aberrantly spliced transgene-encoded transcripts. These bands are clearly distinguishable from both the full-length probe and the correctly spliced transcript.

To confirm that the high level of 2B4α and $2B4\alpha E_H$ transcription in nonlymphoid tissues was not simply due to infiltration of these tissues with large numbers of T cells, RNA samples from various tissues were assayed for T-cell receptor beta-chain transcription. For this analysis, a single-stranded RNA probe homologous to the 3′ end of the $C_\beta 2$ gene was used (Fig. 2D). Hybridization of this probe to thymus RNA from a 2B4α or $2B4\alpha E_H$ mouse, followed by RNAse digestion, yielded two fragments of 122 and 62 nt; primary beta-chain transcripts present in these RNA preparations yielded an additional 221-nt protected fragment. Only trace amounts of beta-chain transcription were visible in lung, kidney, liver, intestine, and brain of a 2B4α transgenic mouse or in lung, kidney, liver, and brain of a $2B4\alpha E_H$ transgenic mouse (Fig. 2C). Thus, the observed transcription of the introduced alpha-chain genes in nonlymphoid tissues was not due to contamination by T lymphocytes. Recently, transgenic mice carrying rearranged T-cell receptor beta-chain genes have been described (29, 56). In these mice, the beta-chain transgene also appears to be transcribed in nonlymphoid tissues, although a marked preference for T- and B-cell expression is observed.

Surface-expression of alpha-chain transgenes. The experiments described thus far demonstrate transcription of the transgenic alpha-chain genes with little or no tissue specificity. However, previous studies have indicated that α:β T-cell receptor surface expression requires the presence of functional alpha and beta chains as well as the CD3 polypeptides (41). We therefore expected that surface expression of the transgenic alpha chain would be limited to T cells. To test this possibility and to quantitate the surface expression of the transgene-encoded 2B4 alpha chain at the single-cell level, FACS analysis of peripheral lymph node cells from 2B4α and $2B4\alpha E_H$ transgenic mice was performed with the A2B4-2 antibody. In the two independent lineages of 2B4α transgenic mice, approximately 3 to 5% of peripheral $L3T4^+$ ($CD4^+$) and $Lyt2/3^+$ ($CD8^+$) T cells expressed the 2B4 alpha chain on their surface (Fig. 3, panels a and b), whereas there was no detectable staining of cells from nontransgenic littermates. In the lineage with eight copies of the transgene, there were consistently higher numbers of T cells expressing the 2B4 alpha chain than in the lineage with only four copies. Mice from the $2B4\alpha E_H$ transgenic line expressed the 2B4 alpha chain on approximately 90% of their $L3T4^+$ and 80% of their $Lyt2^+$ peripheral T cells (Fig. 3, panels c and d). The level of 2B4 alpha surface expression per cell was two- to threefold higher in mice carrying the $2B4\alpha E_H$ construct, particularly in the $L3T4^+$ subset (Fig. 3, compare panels a and c). Thus, the immunoglobulin enhancer element appeared to increase both the percentage of total T cells

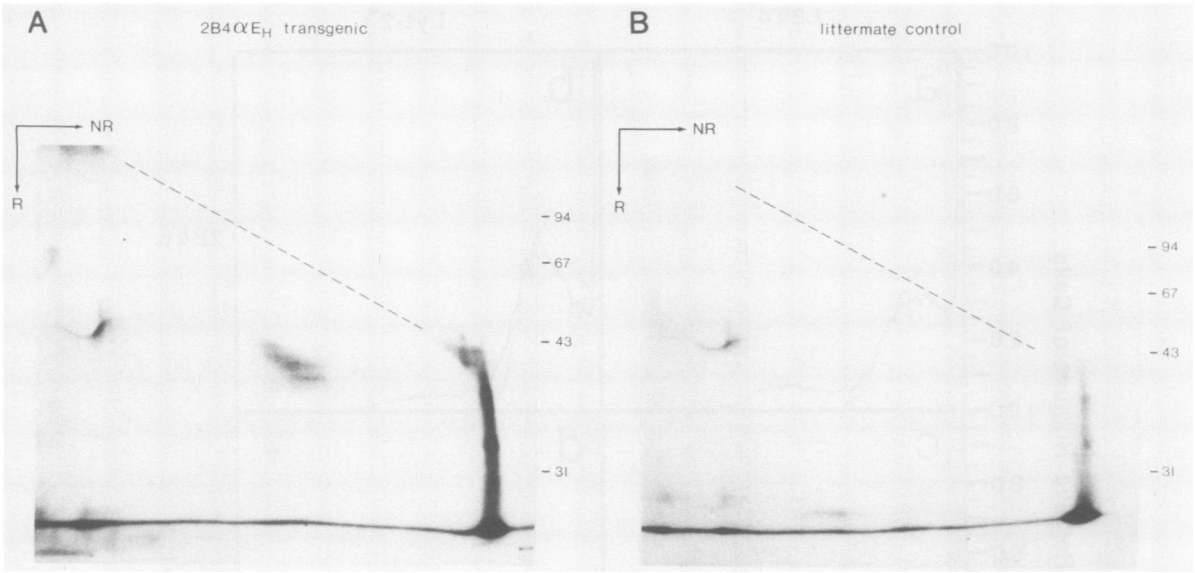


FIG. 4. Immune precipitation of ^{125}I-labeled transgenic and control spleen cells. Spleen cells from a 2B4αE$_H$ transgenic mouse (left panel) and normal littermate (right panel) were surface labeled with ^{125}I. Cell extracts were immune precipitated with the A2B4-2 monoclonal antibody and run on nonreducing (NR)-reducing (R) sodium dodecyl sulfate-polyacrylamide gels. The position of the diagonal is indicated by a dashed line. Molecular size markers for the reducing dimension are indicated in kilodaltons. The crescent-shaped band on the left side of each gel marks the position of the A2B4-2 immunoglobulin G2a heavy chain used for the precipitation.

expressing the transgene on their surface and the level of surface 2B4 alpha-chain expression per positive cell. None of the transgenic mice expressed the 2B4 alpha chain on the surface of cells carrying B-cell markers (B220 or BLA-1) or macrophage markers (Mac-1) (data not shown).

Immunoprecipitation of transgene-encoded T-cell receptor protein. To determine whether the 2B4 alpha chain expressed in these mice was part of a normal T-cell receptor heterodimer, we used the A2B4-2 antibody to precipitate ^{125}I surface-labeled protein from 2B4αE$_H$ transgenic and normal spleen cells. Immune precipitates were analyzed by two-dimensional nonreducing-reducing electrophoresis. As can be seen in Fig. 4, A2B4-2 precipitated T-cell receptor heterodimers from spleen cells of the transgenic mouse, while no disulfide-bonded dimer was precipitated from the spleen cells of the normal littermate. A control antibody did not precipitate any disulfide-bonded proteins from either mouse (data not shown).

Allelic exclusion of T-cell receptor alpha chains. The question of whether 2B4 alpha-chain expression suppresses endogenous alpha-chain rearrangement and expression was addressed first by costaining of peripheral lymph node cells with the A2B4-2 monoclonal antibody versus anti-CD3. As a control, lymph node cells from both a 2B4α and a 2B4αE$_H$ transgenic mouse were stained with the anti-Vβ8 monoclonal antibody, F23.1 (53), and an anti-CD3 monoclonal antibody (1) and analyzed by FACS. In both cases, the cells staining positively with both antibodies lay along a diagonal; individual cells which stained more brightly with anti-T cell receptor antibody also stained more brightly with CD3. An example of this is shown in Fig. 5a. In addition, staining of lymph node cells with an anti-V$_\alpha$ antiserum showed an identical diagonal pattern for endogenous alpha-chain versus CD3 expression (B. Fazekas de St. Groth and M. M. Davis, unpublished data). In contrast, costaining of lymph node cells from a 2B4α transgenic mouse and a 2B4αE$_H$ transgenic mouse with A2B4-2 and anti-CD3 showed distinctly different patterns. In the 2B4αE$_H$ transgenic lymph node, the majority of 2B4 alpha-positive cells lay along the diagonal while only 10 to 15% of the transgene-expressing cells fell below the diagonal (Fig. 5b). This pattern of staining indicates that 85 to 90% of the 2B4-positive cells express a single antigen receptor on their surface. In comparison, in the 2B4α transgenic mouse, only the cells brightest for 2B4 alpha expression appeared likely to be allelically excluded; the majority of the dull 2B4 alpha-positive cells were still bright for CD3 expression, indicating that additional T-cell receptor alpha chain(s) may be present on the surface of these cells (Fig. 5c).

Additional experiments support the conclusions drawn from the costaining data. Capping of the 2B4 alpha chain on the surface of purified 2B4-positive cells from a 2B4αE$_H$ transgenic mouse resulted in cocapping of all detectable cell surface CD3 (data not shown). In contrast, modulation of the 2B4 alpha chain off the surface of 2B4-positive cells from a 2B4α transgenic mouse resulted in only a twofold reduction in surface CD3 expression on the majority of the cells, consistent with the results shown in Fig. 5c that the 2B4α T cells are not allelically excluded (data not shown).

We next examined the effect of the transgene on endogenous alpha-chain rearrangements in the 2B4αE$_H$ transgenic mice. Because of the large size of the alpha-chain locus (22, 59), no single DNA probe is capable of detecting all possible alpha-chain rearrangements. However, a fragment from the most 5′ segment of the J$_\alpha$ cluster should detect sequences which are either rearranged or deleted as a consequence of alpha-chain rearrangements, apart from those occurring by inversion. In this analysis, loss of hybridization to the germ line fragment indicates rearrangement of the alpha chain locus. A map of the α:δ locus, indicating the position of this hybridization probe (J$_\alpha$), is shown in Fig. 6A (8); all J$_\alpha$ sequences identified to date mapped either within or 3′ to this fragment (22, 59). In all cases, the extent of rearrangements was estimated by hybridizing the filters to a nonrearranging single-copy gene probe (murine GM-CSF) and normalizing hybridization signals to this value.

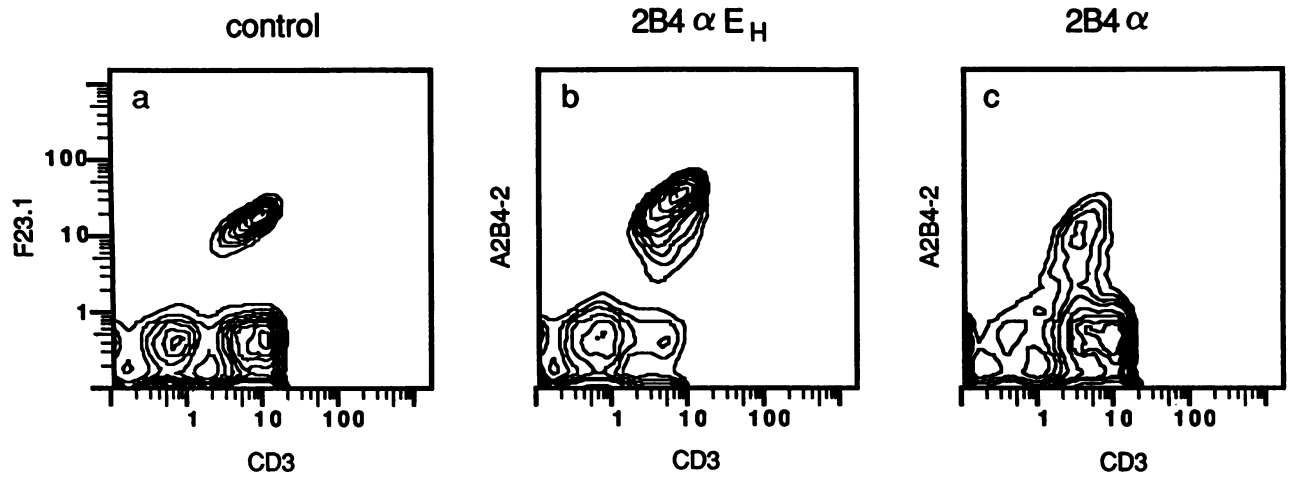


FIG. 5. Staining of transgenic lymph node cells with anti-T-cell receptor antibodies versus anti-CD3. Lymph node cells from transgenic mice were stained with either biotinylated F23.1 or A2B4-2 together with a hamster anti-CD3 monoclonal antibody (kindly provided by J. Allison), followed by avidin-Texas Red plus anti-hamster-FITC. Cells were then analyzed by FACS. (a) Staining of 2B4αE_H transgenic lymph node cells with F23.1 versus anti-CD3. (b and c) A2B4-2 versus anti-CD3 staining of lymph node cells from a 2B4αE_H transgenic mouse and a 2B4α transgenic mouse, respectively.

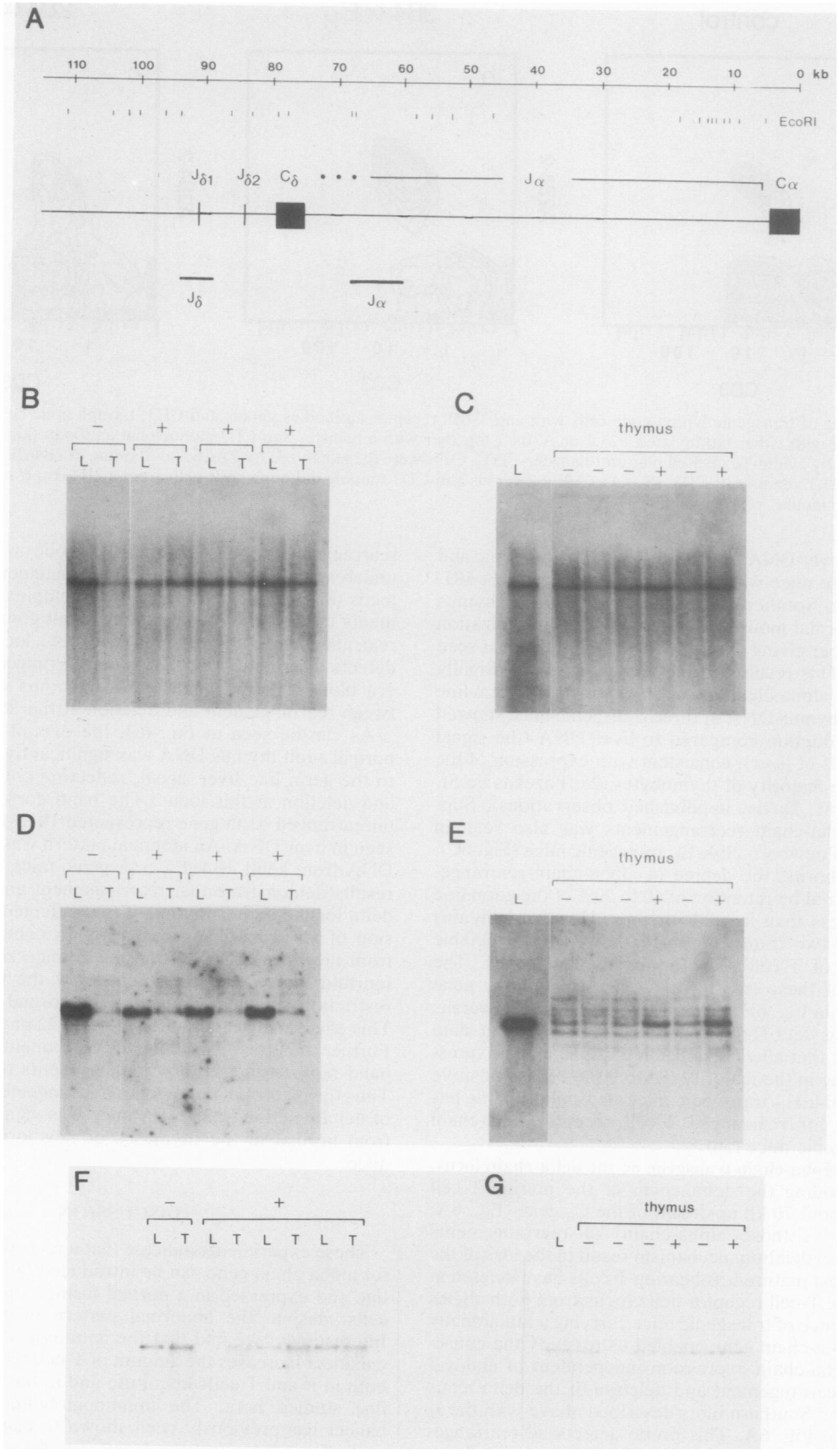
Thymus and liver DNA from adult 2B4αE_H transgenic and normal littermate mice was prepared, digested with *Eco*RI, and subjected to Southern blot analysis (Fig. 6B). Thymus DNA from a normal mouse exhibited reduced hybridization with the J_α probe, giving about 30 to 50% of the signal seen in liver DNA. This resulted from rearrangements normally occurring in the alpha-chain locus. In contrast, the germ line J_α fragment in thymus DNA of three transgenic mice showed only a slight reduction compared to liver DNA (the signal being 70 to 100% of liver), consistent with expression of the transgene in the majority of thymocytes (25; Fazekas de St. Groth and M. M. Davis, unpublished observations). Suppression of alpha-chain rearrangements was also seen in thymus DNA of newborn 2B4αE_H transgenic mice (Fig. 6C). In normal newborns, the degree of alpha-chain rearrangement, as measured by retention of 60 to 70% of the germ line J_α band, was less than in adult thymus. However, thymus DNA from the two transgenic mice showed no detectable rearrangement of T-cell receptor alpha-chain genes. The hybridization of these two filters with a single-copy gene probe is shown in Fig. 6F and G. Thus, both the CD3 versus A2B4-2 staining data (Fig. 5) and the rearrangement data (Fig. 6) suggest that allelic exclusion of alpha-chain expression is occurring in the majority (85 to 90%) of 2B4-positive T cells in the 2B4αE_H transgenic mice. A similar finding has been reported for rearranged T-cell receptor beta-chain genes in transgenic mice (56).

Effect of the alpha-chain transgene on the delta-chain locus. The locus encoding the delta chain of the murine T-cell receptor lies about 70 kb upstream of the C_α gene (Fig. 6A; 8); consequently, those alpha-chain VJ rearrangements which occur by a deletion mechanism result in the loss of the delta locus. Most mature α:β-bearing T cells have deleted at least part of the T-cell receptor delta locus from both alleles (33). The existence of transgenic mice carrying a functionally rearranged alpha-chain gene enabled us to study the consequences of alpha-chain expression independent of endogenous $V_\alpha \rightarrow J_\alpha$ rearrangement and deletion of the delta locus by reprobing the Southern blots described above with the J_δ probe shown in Fig. 6A. This probe detects all rearrangements involving the $J_{\delta 1}$ gene segment but not those involving $J_{\delta 2}$. However, as $J_{\delta 1}$ appears to be used in over 90% of delta rearrangements, use of such a probe will only slightly underestimate the extent of rearrangements at the delta locus (8, 16). Furthermore, as the complexity of rearrangements at the delta locus is quite small compared to that of rearrangements at the alpha and beta loci, the $J_{\delta 1}$ probe detects discrete delta-chain gene rearrangements on Southern blots of polyclonal T cells and thus distinguishes between rearrangement and deletion at that locus.

As can be seen in Fig. 6D, the overall hybridization to normal adult thymus DNA was significantly reduced relative to the germ line liver signal, reflecting both rearrangement and deletion at this locus. The band corresponding to the unrearranged delta gene represented 10 to 15% of the signal seen in liver DNA. An identical pattern was seen for thymus DNA from adult 2B4αE_H transgenic mice (Fig. 6D). These results demonstrate that rearrangement and deletion at the delta locus in adult thymus was unaffected by high expression of an alpha-chain transgene. In contrast, thymocytes from newborn 2B4αE_H transgenic mice showed a two- to fourfold increase in the intensity of the unrearranged $J_{\delta 1}$ restriction fragment compared to normal mice (Fig. 6E). This effect was seen in mice of up to 12 days of age (Fig. 7). Further analyses also indicated a frequent retention of the band representing $D_{\delta 1}$-$J_{\delta 1}$ joining events (data not shown). Thus the pattern of delta-chain rearrangement and the extent of delta deletion in $2B4_\alpha E_H$ mice was significantly different from normal in the thymuses of newborn, but not adult, mice.

DISCUSSION

These experiments indicate that a rearranged T-cell receptor alpha-chain gene can be introduced into the mouse germ line and expressed in a normal fashion on the surface of T cells, despite the abnormal pattern of RNA expression. Interestingly, we find that the immunoglobulin heavy-chain enhancer increases the amount of T-cell receptor alpha RNA both in B and T cells in culture and in the transgenic mouse line studied here. The immunoglobulin heavy-chain enhancer has previously been shown to cause B-cell expression of a predominantly T-cell-specific gene in transgenic mice (7). Although the increased expression seen in the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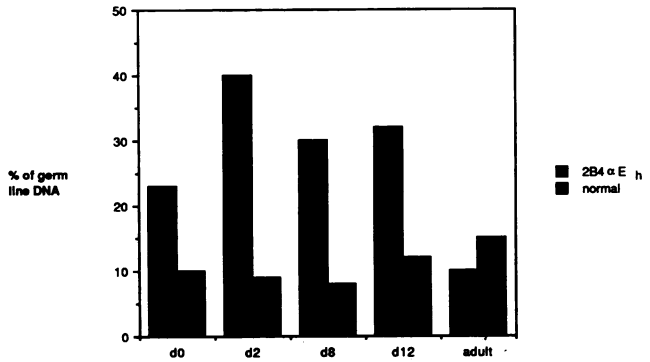

FIG. 7. Extent of delta-chain rearrangements in 2B4αE$_H$ transgenic versus normal mice. Southern blot analysis followed by scanning laser densitometry of liver and thymus DNA of 2B4αE$_H$ transgenic and normal littermate mice was used to determine the extent of rearrangements at the delta locus. The results are summarized for mice of the following ages: d0, newborn; d2, 2 days after birth; d8, 8 days after birth; d12, 12 days after birth; adult, over 8 weeks of age. In all cases, delta-chain rearrangements were detected by using the J$_{\delta 1}$ probe; hybridization with a single-copy nonrearranging gene probe was used to normalize for DNA content between lanes.

2B4αE$_H$ line may be an effect of the integration site of the transgene, we believe it to be the result of the immunoglobulin heavy-chain enhancer for two reasons. First, we have demonstrated a similar effect in vitro (Fig. 1); second, we have now seen similar increases in expression in transgenic mice with T-cell receptor beta genes carrying this enhancer element (L. Berg and M. M. Davis, unpublished observations). These results are in contrast to previously reported experiments demonstrating the absence of immunoglobulin heavy-chain enhancer activity in T cells (13, 36). The most reasonable explanation for this discrepancy is the difference in genes used for assaying enhancer function. Our ability to detect activity of the heavy-chain enhancer might be due to the high base-line transcription level of our 2B4α construct or, alternatively, to the appropriate combination of enhancer, T-cell receptor regulatory sequences, and factors present in T cells. Indeed, the observations that immunoglobulin heavy-chain transcripts can be found in T cells in vivo (27) and that immunoglobulin heavy-chain transgenes are commonly transcribed in T cells (19, 55), provide further evidence for the presence of heavy-chain enhancer-activating factors in T cells. In the 2B4αE$_H$ mice, this effect is manifested by an increase in both the number of T cells expressing the transgenic alpha chain and the level of expression per cell; the 15-fold increase in the number of positive cells is significantly greater than the three- to fivefold increase in steady-state RNA levels, arguing that a threshold level of RNA or protein is required to override endogenous alpha-chain rearrangement. It is also interesting that the increase in RNA levels attributable to the enhancer element (3- to 5-fold) is much less than that reported for immunoglobulin genes (20- to 400-fold; 18).

It is also interesting to contrast the ubiquitous expression of the alpha-chain transgenes in vivo with our observation that the construct lacking the heavy-chain enhancer is not expressed at all in 4 of 5 transfectants of a B-lineage tumor-cell line. One possible explanation is that the T-cell receptor alpha construct is expressed in a wide variety of tissues but, for some reason, not in B cells. Although the observation that the 2B4α transgene is expressed at very low levels in bone marrow is consistent with this possibility, preliminary experiments analyzing transgenic expression in T-cell-depleted spleen RNA suggest that this construct is expressed in B cells. A simple interpretation is that our alpha-chain construct does not contain all of the DNA sequences necessary for normal expression. These missing sequences apparently prevent T-cell receptor expression in inappropriate tissues rather than acting as an enhancer. In fact, the region homologous to the recently described human T-cell receptor alpha-gene enhancer, which lies within 1 kb 5′ of C$_\alpha$ (35), is clearly contained within our constructs.

The A2B4-2 versus anti-CD3 costaining experiment presented here indicates that the allelic exclusion of endogenous alpha-chain expression is not operating efficiently in 2B4-positive cells of the 2B4 alpha transgenic mice. This is most likely due to the low expression of the transgene, which results in both a small number of positive cells as well as a low level of surface expression per cell (Fig. 3a and 5b). There appears to be a threshold level of expression required to exclude rearrangement or expression of an additional alpha chain, as it is those cells with extremely low surface levels of the transgene which appear to have an additional receptor on their surfaces. In contrast, the costaining data indicate that the transgene-derived species is the only alpha chain on the surface of the majority of 2B4 alpha-positive T cells in the 2B4αE$_H$ transgenic mice. Furthermore, expression of the transgene in these mice appears to suppress rearrangements of the endogenous gene, as has been found for immunoglobulin transgenic mice (40, 46, 47, 54 [review]) and more recently for T-cell receptor beta-chain transgenics (56).

Our alpha-chain transgenic mice also provide an opportunity to test a model of T-cell differentiation recently proposed by Pardoll and colleagues (42), who suggested that all T cells pass through an obligatory γ:δ rearrangement stage; those cells that successfully rearrange functional gamma and delta genes become γ:δ-bearing cells, while those that fail go on to rearrange their alpha- and beta-chain genes. This model helps to rationalize the location of T-cell receptor delta within the alpha-chain locus (8). Such a linear pathway of T-cell development is schematized in Fig. 8A; the alternative pathway, in which the α:β and γ:δ cells occupy two different compartments deriving from a common progenitor (pro-T cell; 50), is shown in Fig. 8B. If we accept that endogenous alpha-gene rearrangement is suppressed in 2B4αE$_H$ transgene-expressing cells, then any observed differences at the delta locus when comparing bulk populations of T cells from normal and transgenic mice are likely to be the result of delta alleles retained in these cells. It therefore

FIG. 6. Southern blot analysis of adult and newborn thymus DNA from transgenic versus normal mice. (A) Diagram of the alpha/delta locus indicating the probes used in the Southern blot analysis. (B) Adult thymus (T) and liver (L) DNA hybridized with the J$_{\delta 1}$ probe. (C) Newborn thymus and liver (L) DNA hybridized with the J$_\alpha$ probe. (D) Adult thymus (T) and liver (L) DNA hybridized with the J$_{\delta 1}$ probe. (E) Newborn thymus and liver (L) DNA hybridized with the J$_\delta$ probe. (F) Adult thymus (T) and liver (L) DNA hybridized with the single-copy probe, GM-CSF. (F) Newborn thymus and liver (L) DNA hybridized with the GM-CSF probe. −, Normal littermate; +, 2B4αE$_H$ transgenic mouse.

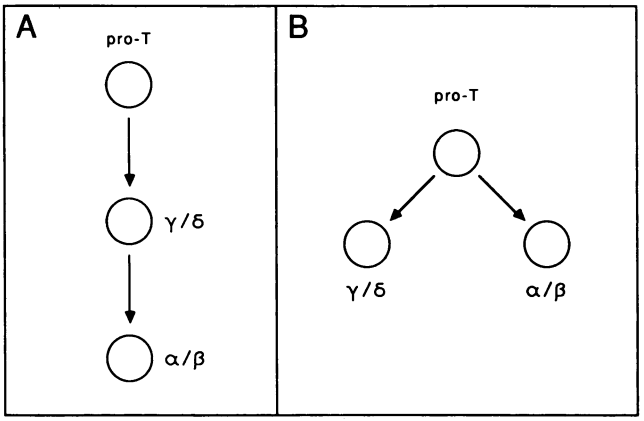

FIG. 8. Two alternative models for α:β and γ:δ T-cell lineages. (A) In this scheme, all T cells first attempt to rearrange and express T-cell-receptor gamma and delta genes. Successful T cells become the γ:δ cells, whereas unsuccessful cells then attempt to rearrange and express α:β T-cell receptor genes. (B) α:β-positive and γ:δ-positive lineages arise independently from a common precursor cell.

follows that an examination of the delta alleles present in the 2B4αE$_H$ transgenic T cells should reveal the state of the delta locus in normal cells at the time of endogenous alpha-gene rearrangement. In newborn thymuses from transgenic mice, we observe retention of both germ line delta loci and, to a lesser extent, a $D_{\delta 2}$-$J_{\delta 1}$ rearrangement, suggesting that these two configurations are overrepresented in cells destined to become α:β-bearing cells. This data provides support for the separate compartment model, although we cannot formally exclude the possibility that the observed increase in germ line and $D_{\delta 2}$-$J_{\delta 1}$ bands derives from another thymic subpopulation apart from α:β cells, as the balance between T-cell subpopulations is generally disturbed in 2B4αE$_H$ transgenic mice (Fazekas de St. Groth and Davis, unpublished observations). Surprisingly, in adult thymocytes the vast majority of delta loci are either rearranged or deleted in both transgenic and normal mice, suggesting that most transgene-expressing cells have deleted the delta locus despite suppression of alpha rearrangement as detected with a J_α probe (Fig. 6). This evidence, that newborn and adult thymocytes from transgenic mice differ in their pattern of delta-chain rearrangement and deletion, is consistent with several previous lines of evidence for distinct waves of T cells populating the mouse thymus (8, 16, 26).

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