

Domains of the TCR β -Chain Required for Early Thymocyte Development

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Summary

The T cell receptor β (TCR β) chain controls the developmental transition from CD4⁻CD8⁻ to CD4⁺8⁺ thymocytes. We show that the extracellular constant region and the transmembrane region, but not the variable domain or cytoplasmic tail of the TCR β chain are required for this differentiation step. TCR β mutant chains lacking the cytoplasmic tail can be found at the cell surface both in functional TCR/CD3 complexes and in a GPI-anchored monomeric form indicating that the cytoplasmic tail of the TCR β chain functions as an ER retention signal. The concordance between cell surface expression of the mutant chains as TCR/CD3 complexes and their capacity to mediate thymocyte differentiation supports the CD3 mediated feedback model in which preTCR/CD3 complexes control the developmental transition from CD4⁻CD8⁻ to CD4⁺CD8⁺ thymocytes.

The α and β chains of the TCR play a central role in the development of $\alpha\beta$ T cells (1–3). Both chains are members of the Ig superfamily and are encoded by gene segments that undergo rearrangements during T cell differentiation to yield functional genes (4). Rearrangement of both, TCR and Ig genes, requires the expression of the recombination activating genes RAG-1 and RAG-2 (5, 6).

During T cell development, rearrangement at the TCR β locus precedes rearrangement at the TCR α locus (7, 8). In transgenic mice, expression of a functional TCR β transgene was shown to block rearrangement and expression of endogenous TCR β genes, indicating that TCR β genes are subject to allelic exclusion (9). Expression of the TCR β protein was shown to be required for mediating allelic exclusion at the TCR β locus (10). However, a mutant TCR β chain lacking the variable domain (Δ V-TCR β), efficiently blocks rearrangements of endogenous TCR β alleles, while leaving recombination of the TCR α genes unimpaired (10, 11).

How does a TCR β chain block V-DJ rearrangements? Studies in TCR β transgenic mice are most consistent with the feedback model, first proposed for allelic exclusion at the Ig heavy chain locus (12). Before rearrangement at Ig light chain loci, Ig heavy chains assemble with surrogate light chains (VpreB and λ 5) as well as mb-1/B29 heterodimers (13–16). This pre-B cell receptor complex appears at very low levels at the cell surface and is thought to induce the development from pro-B cells to pre-B cells, concomitant with the shut off of the Ig heavy chain loci

through specific target elements (17–20). The identification of a surface-expressed pre-T cell receptor complex analogous to the pre-B cell receptor complex, has allowed extension of this model to the T cell lineage (21–24). Early in pre-T cell development, the pre-TCR complex is thought to signal a functional V-DJ rearrangement, impairing further V-DJ rearrangements. As is clear from studies of recombination-deficient mice reconstituted with a functional TCR β transgene (3, 22, 25) or with a mutant Δ V-TCR β transgene (26), the pre-TCR complex appears to be involved in further differentiation of pre-T cells including downregulation of the interleukin 2 receptor α (IL2-R α) chain, upregulation of CD4 and CD8 coreceptors, enhanced transcription at the TCR α locus and expansion of the thymocytes to normal numbers. More recently, a novel 33-kD glycoprotein (pT α) has been identified as the binding partner of the TCR β glycoprotein. This heterodimer is found at very low levels at the cell surface of pre-T cells (27). Subsequent cloning and gene-targeting of the pT α gene in mice demonstrated the crucial role of preT α chain in pre-T cell development (28, 29).

Evidence for an important role of CD3 components in signal transduction by the pre-TCR/CD3 complex has been provided (26, 30, 31). CD3 components are present at the surface of developmentally arrested thymocytes of RAG-deficient mice in the absence of TCR chains. Cross-linking of the CD3 modules on differentiation arrested CD4⁻CD8⁻ thymocytes of RAG-1^{-/-} mice in vitro (30) or in vivo (26, 31) with anti-CD3 ϵ mAb induces the tran-

sition from CD4⁻CD8⁻ thymocytes to CD4⁺CD8⁺ thymocytes, concomitant with all the changes described for recombination-deficient mice reconstituted with a functional TCR β transgene or the mutant Δ V-TCR β transgene (see above). Thus, cross-linking of the CD3 modules on CD4⁻CD8⁻ thymocytes apparently mimics the signaling mediated by the pre-TCR complex.

Earlier experiments have shown that the variable domain of the TCR β chain is not required to induce early thymocyte development (10, 11, 26). To determine the domains required to induce pre-T cell development, we have generated five different mutant TCR β transgenic mice with defined alteration in the TCR β constant region.

Materials and Methods

Construction of Mutant TCR Transgenes

Starting material for the construction of the $\beta\beta\beta\beta\Delta$, $\beta\beta\beta\alpha\alpha$, $\beta\alpha\alpha\beta\beta$, and $\beta\alpha\alpha\alpha\alpha$ mutants (see Fig. 1) was a 20-kb Asp 718 (KpnI) TCR β fragment from the cosmid clone cosHY β 9-1.14-5 (9). Mutations were restricted to the 6.1-kb BamHI fragment containing all the four exons of the C β 2 constant region; transcriptional control elements such as the TCR β promoter and the 3' TCR β enhancer region as well as the functional VDJ region were not altered.

Construction of a Mutant TCR β Transgene Lacking the Cytoplasmic Tail ($\beta\beta\beta\beta\Delta$). The $\beta\beta\beta\beta\Delta$ protein was generated by converting the 2nd codon of the 4th TCR β constant exon into a TAG stop codon that deletes three lysines, an asparagine and a serine. After three sequential subcloning steps, first a 6.1-kb C β 2 BamHI fragment, second a 1.940-kb PstI-HpaI fragment and third a 322-bp EcoRV fragment containing the TCR β constant exon 4 encoding the putative cytoplasmic tail of the TCR β chain was cloned into pSP72. By recombinant PCR the second codon AAG was mutated into a TAG stop codon, which introduces a XbaI restriction site (TCTAGA).

Two recombinant PCR primers, anti-sense (as) and sense (s), were used in combination with T7- and SP6-specific primers, respectively. Primer T7, 5'-AAATTAATACGACTCACTATAGGG3'. Primer SP6, 5'-ATACACATACGATTTAGGTGACAC-3'. Primer as, 5'-C TCAGGAATTTTTTCTAGACCTGAGAAAAGAGA-GA C-3'. Primer s, 5'-GTCTTCTCTTCTCAGGTCTA-GAAAAAAATTCCTGAG-3'.

PCR products were digested with XbaI and EcoRV restriction endonucleases yielding a 199-bp and a 123-bp XbaI/EcoRV fragment. These fragments were cloned into the polycloning sites of the pSP73 and pSP72 vectors respectively (Invitrogen, San Diego, CA). These plasmids were digested with XbaI and BglII and the appropriate fragments were ligated to generate a pSP72/73 hybrid vector containing the mutated 322-bp EcoRV fragment. Presence of the mutation was verified by sequencing. The reinsertion of the mutated 322-bp EcoRV fragment into the 20-kb Asp718 fragment was analogous to the subcloning procedure described above.

Construction of Mutant TCR α / β Chimeric Transgenes $\beta\beta\beta\alpha\alpha$, $\beta\alpha\alpha\beta\beta$, $\beta\alpha\alpha\alpha\alpha$. To generate the $\beta\alpha\alpha\alpha\alpha$ TCR chimeric gene, a 6.1-kb BamHI fragment containing the complete TCR β constant region (C β 2) was replaced by the 8.6-kb BamHI genomic fragment from the cosmid clone cos H-Y α 9.1-36 which contains the TCR α constant region (C α). Orientation was determined by ClaI and EcoRI digestions (see physical maps in refer-

ences 9, 32). For the $\beta\alpha\alpha\beta\beta$ and $\beta\beta\alpha\alpha\alpha$ TCR chimeric genes, a 5' and a 3' fragment of the C β 2 and C α fragments were cloned into pSP72 or pSP73 respectively (Invitrogen). The 5' fragments contain exon 1 and 2, and the 3' fragments contain exon 3 and 4 of the TCR constant regions. These plasmids were digested with PvuI and partially with BglII and the appropriate fragments were ligated to obtain the 8.2-kb 5'C β -3'C α and the 6.5-kb 5'C α -3'C β hybrid-fragments flanked by BamHI sites. This step introduces a BglII-EcoRV-ClaI-EcoI linker from the polycloning site of the pSP plasmids. Replacement of the 6.1-kb C β 2 BamHI fragment from TCR β with the 5'C β -3'C α and 5'C α -3'C β BamHI hybrid-fragments generates the $\beta\beta\beta\alpha\alpha$ and $\beta\alpha\alpha\beta\beta$ TCR transgenes, respectively. A 200-bp BamHI fragment 5' of the 6.1-kb C β 2 intron fragment was deleted at this step. Orientation was determined by ClaI digestion.

Construction of the $\beta\beta\beta$ -PI Transgene. Starting material for this mutant transgene was a 20-kb KpnI fragment harboring the functional TCR β gene of the encephalomyelitogenic T cell clone cloned into λ Charon 39 (33). In brief, the TCR β insert was digested to completion with BamHI. Three of the resulting restriction fragments were then subcloned into pKS(-) (Stratagene); a 8.3-kb BamHI fragment containing the functionally rearranged VDJB (pTCR β 8.3), a 7.3-kb BamHI fragment containing the genomic C β 2 region (pTCR β 7.3) and a 4.7-kb BamHI-KpnI fragment containing the 3' end of the phage clone (p4.7 kb). Site-directed mutagenesis was performed on the pTCR β 7.3 subclone to introduce an AccI restriction site 39 bp 3' of the start of the TM exon of C β 2 by using oligonucleotide-directed *in vitro* mutagenesis (Amersham Corp., Arlington Heights, IL); the resulting clone was called pC β 2Acc1. A 2.3-kb BamHI-SacI fragment from pTCR β 7.3 containing the first exon of C β 2 (Ig-like region), a 687-bp SacI-AccI fragment from pC β Acc1 containing the 2nd exon (encoding the connecting peptide) of C β 2 and the first 13 aa of exon 3 of C β 2 encoding part of the TM region, and a 1.4-kb AccI-BamHI fragment from Q72-6-220ApBG (a gift from Dr. Gerry Waneck, Massachusetts General Hospital, Boston, MA) containing the Q7^b PI anchor signal and SV40 polyadenylation signal was ligated into BamHI digested pKS(-) to generate pC β 2PI. To construct the mutated TCR β transgene used for injection a trimolecular ligation was performed with the 8.3-kb BamHI fragment from pTCR β 8.3, the 4.4-kb BamHI fragment pC β 2PI, and BamHI linearized dephosphorylated p4.7 BK plasmid. The resulting construct pTgC β 2PI was checked for orientation of the respective fragments relative to each other by restriction mapping. An ~17-kb NotI-KpnI restriction fragment was prepared from pTgC β 2PI and used for the production of transgenic mice.

Transgenic Mice

Fertilized mouse oocytes were recovered in cumulus from the oviducts of superovulated females that had mated with FVB males several hours earlier. The DNA-fragments (2-4 μ g/ml 10 mM Tris-HCl/0.1 mM EDTA [pH 7.5]) were injected into the most accessible pronucleus of each fertilized egg essentially as described (34). After overnight culturing, 2-cell stage embryos were implanted into the oviducts of day 1 pseudopregnant C57Bl/6 foster animals and carried to term. Several weeks after birth total genomic DNA was prepared from tail biopsies as described (35). Transgenic founders were crossed with FVB mice. To exclude insertion specific phenotypic effects, mice derived from three to six different founder lines were analyzed for each construct. The generation of TCR β ($\beta\beta\beta\beta\beta$) transgenic mice has been described (9).

DNA and RNA Blot Analysis

For Southern blot analysis 10 μ g of total genomic DNA of each mouse was digested with restriction enzymes as recommended by the supplier, separated on 0.6% agarose gels, and transferred to nitrocellulose. Filters were hybridized to 32 P-labeled probes and washed as described (36). Probes used for DNA analysis were V β 8.1 (37) and J β 2 (38). The final wash was at 0.1 \times SSC, 60°C and 0.1 \times SSC, 42°C, respectively.

For Northern blot analysis 20 μ g of total RNA, prepared by the LiCl-urea method, was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (39). Probes for V β 8.1 (37), C α (40), C β (41), and actin (42) were 32 P-labeled by random priming, hybridization conditions were as described (36) with the addition of 1% SDS to all solutions. The final wash was at 0.1 \times SSC, 60°C. Filters were exposed with Kodak X-Omat S or XAR-5 films at -70°C using intensifier screens.

Phospholipase C Treatment of Thymocytes

Thymocytes from $\beta\beta\beta\beta$, $\beta\beta\beta\Delta$, and $\beta\beta\beta$ -PI transgenic mice (10^6 /ml) were incubated for 40 min at 37°C in complete Iscove's medium either in the presence or absence of 1 U/ml phospholipase C (PLC)¹ (isolated from *Bacillus cereus*).

Antibodies and Immunofluorescence

Anti-CD3 ϵ 145-2C11 (43), anti-TCR β mAb H57-597 (44), anti-V β 6 mAb 44-22-1 (45), and anti-V β 8 mAbs KJ16-133 (46) and F23.1 (47) were purified from culture supernatants by HPLC. Purified Ig and FITC- or biotin-conjugates thereof were used for immunoprecipitation and immunofluorescence analysis as indicated. The mAbs directed against CD4 (clone RM-4-5), CD8 (clone 53-6.7) and V β 2 (clone B20.6) were purchased from PharMingen. Flow cytometry was performed on a FACScan[®] (Becton Dickinson, Mountain View, CA), using Lysis II software. For double labeling FITC- and PE or biotin-conjugated mAb were used. Cells incubated with biotin-conjugated mAb were subsequently stained by incubation with PE-conjugated streptavidin. Data are depicted as contour plots (log density 50%, threshold 1%, 1 \times smoothed) or dot plots.

Radiolabeling, Immunoprecipitation, and Gel Electrophoresis

For metabolic labeling splenocytes of transgenic and non-transgenic FVB mice were cultured in 15 ml Iscove's medium supple-

¹Abbreviations used in this paper: GPI, glycosyl-phosphatidylinositol; PLC, phospholipase C

mented with Con A and 10% FCS. After 3 d, activated lymphocytes were isolated on a density gradient. B cells were depleted with sheep anti-mouse Ig coupled to magnetic beads, according to the manufacturers procedure (Advanced Magnetics, Cambridge, MA). Enriched T lymphocytes were washed twice and starved for 1 h in methionine-free RPMI-medium (Selectamine kit; GIBCO BRL, Gaithersburg, MD) supplemented with 10% dialyzed serum and counted. Lymphocytes ($20\text{--}30 \times 10^6$) were labeled metabolically for 4 hours with 100 μ Ci [35 S]methionine in 1 ml methionine-free RPMI-medium in a humidified incubator at 37°C, 5% CO₂. Subsequently the cells were harvested and lysed for 30 min at 4°C in NP-40 containing lysis buffer (50 mM triethanolamine-Cl, pH 7.5, 5 mM MgCl₂, 1 mM PMSF, and 0.5% NP-40). Nuclear debris was removed by centrifugation for 15 min at 13,000 g.

Surface iodination, immunoprecipitation, SDS-PAGE, and autoradiography were carried out as described (11).

Results

The Experimental System. To dissect the domains within the TCR β constant region which are required to induce pre T cell development, five different mutant TCR β chain genes were constructed. The structure of each mutant TCR transgene product is illustrated in Fig. 1.

For transgenesis, the FVB mouse inbred strain was used. FVB mice lack V β 8 segments as demonstrated by Southern blot and flowcytometric analysis, allowing specific detection of the V β 8.2 domain common to all mutant TCR chains. To investigate whether the V β 8.2 domain had any detrimental effects on T cell development in the FVB background, e.g., by V β 8-specific superantigen(s), TCR β transgenic C57Bl/LiA mice were crossed with FVB mice. The T cell compartments in TCR β transgenic FVB \times C57Bl/LiA F1 and C57Bl/LiA mice did not show any changes in the size of V β 8.2⁺ T cell subsets in thymus, spleen and lymph nodes (data not shown).

Expression of the mutant transgenes was determined by Northern blot analysis using total RNA extracted from spleens of different founder lines and a V β 8 specific probe. The 3' untranslated region of the TCR α mRNA is \sim 200 bp longer than that of the 3' untranslated region of the TCR β message, whereas these two mRNAs are similar in length

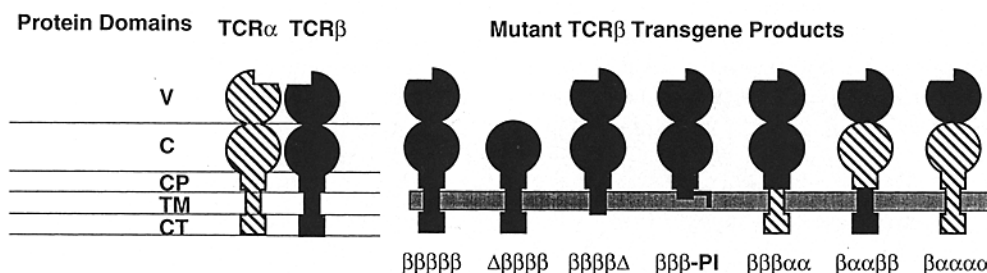


Figure 1. Schematic illustration of the proteins encoded by the mutant TCR transgenes. TCR α protein ($\alpha\alpha\alpha\alpha$, dashed) and TCR β protein ($\beta\beta\beta\beta$, black) share a common basic 5-domain structure: V, variable domain; Ig, Ig-like domain; CP, connecting peptide; TM, transmembrane region; CT, cytoplasmic tail. Beside the non-mutant TCR β transgene and the mutant

$\Delta\beta\beta\beta\beta$ chain, five additional alterations were introduced into the TCR β constant regions. The $\beta\beta\beta\Delta$ mutant resembles a TCR β protein lacking its cytoplasmic tail and the $\beta\beta\beta$ -PI mutant comprises the three extracellular constant regions of the TCR β chain and the first 13 aa of the TCR β transmembrane region connected to the PI-anchor sequence of Q7^b. The $\beta\beta\beta\alpha$, $\beta\alpha\alpha\beta$, and $\beta\alpha\alpha\alpha$ mutants were generated by domain swapping. The first β resembles the TCR β variable domain, the second, third, fourth, and fifth letter indicates the origin of the extracellular constant Ig-like domain, the connecting peptide, the transmembrane region and the cytoplasmic tail, respectively. Δ indicates deletion of that particular region.

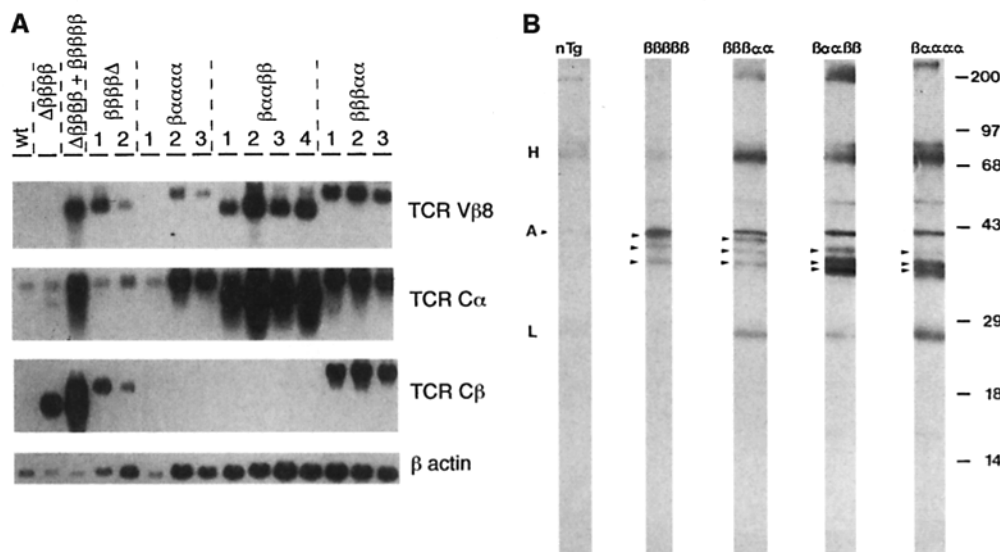


Figure 2. Expression of the mutant TCR transgenes. (A) Northern blot analysis to study expression of the mutant TCR transgenes. Total RNA extracted from spleens of different mutant TCR founder lines was analyzed for the presence of transgene specific transcripts. RNA samples from non-transgenic FVB mice (*wt*), $\Delta\beta\beta\beta\beta$ transgenic mice and $\beta\beta\beta\beta\beta/\Delta\beta\beta\beta\beta$ double-transgenic mice are included as controls. Northern blots were hybridized with V β 8-, constant TCR C α -, and TCR C β -specific probes. (B) SDS-PAGE analysis of anti-V β 8 immunoprecipitates from metabolically labeled Con A blasts of mutant TCR transgenic mice. V β 8-specific immunoprecipitates from NP40 lysates of metabolically

labeled Con A blasts derived from splenocytes of a non-transgenic FVB mouse (*nTg*) and mutant TCR transgenic mice were analyzed under reducing conditions by SDS-PAGE analysis on a 10–15% gradient gel. Arrow heads indicate the different glycosylation forms of the mutant TCR chains. Contaminating immunoglobulin heavy and light chain (*H* and *L*) are due to indirect precipitations with rabbit anti mouse Ig. *A* indicates the position of actin. The position of molecular weight markers are indicated (kD).

in the other regions. As expected, $\beta\beta\beta\alpha$ and $\beta\alpha\alpha\alpha$ transcripts co-migrated at the position of mature TCR α transcripts (1.5 kb) whereas the $\beta\beta\beta\beta\Delta$ and $\beta\alpha\alpha\beta\beta$ transcripts run at the position of mature TCR β transcripts (1.3 kb). The size of the $\beta\beta\beta$ -PI mRNA was also in accordance with the structure of the $\beta\beta\beta$ -PI transgene (not shown). Hybridization patterns with ^{32}P -labeled probes specific for the TCR α and TCR β constant regions were in accordance with the structure of the TCR α/β chimeric transgenes. The lack of detection of endogenous TCR β transcripts is not due to allelic exclusion but to the relatively short exposure of the autoradiograph in view of the relatively high expression of the mutant TCR β transgenes (Fig. 2 A).

Expression of the mutant TCR chains was further determined by SDS-PAGE analysis of V β 8-specific immunoprecipitates from NP40-lysates of metabolically labeled Con A activated splenocytes. T cell enriched splenocytes from non-transgenic FVB mice and transgenic $\beta\beta\beta\beta\beta$, $\beta\beta\beta\beta\alpha$, $\beta\alpha\alpha\beta\beta$, and $\beta\alpha\alpha\alpha$ mice were used. Immunoprecipitates were analyzed under reducing conditions on a 10–15% SDS-PAGE gradient gel. Whereas T cells from FVB mice lack V β 8-specific polypeptides, the T cells from the TCR β mutant mice were found to express anti-V β 8 reactive glycoproteins of the expected sizes (Fig. 2 B).

The $\beta\beta\beta\beta\alpha$ Transgene Perturbs the Transition from the CD4⁻CD8⁻ Stage to the CD4⁺CD8⁺ Stage. To study the effects of the different mutant TCR transgenes on thymocyte development, we first compared the number of thymocytes found in different mutant transgenic lines. With the exception of $\beta\beta\beta\beta\alpha$ transgenic mice, the absolute number of thymocytes in the mutant transgenic lines was comparable to that of non-transgenic mice (Fig. 3 A). In $\beta\beta\beta\beta\alpha$ transgenic mice the number of thymocytes was reduced to $36 \pm 8\%$ ($n = 6$) compared to non-transgenic

control littermates ($n = 7$). To assess which developmental stages were affected by the expression of the $\beta\beta\beta\beta\alpha$ chain, we determined the size of the different CD4⁻/CD8⁻ thymocyte compartments of control and mutant mice (Fig. 3, B and D). Although the absolute number of CD4⁻CD8⁻ thymocytes in $\beta\beta\beta\beta\alpha$ transgenic mice was normal, the number of the CD4⁺CD8⁺, CD4⁺CD8⁻ and CD4⁻CD8⁺ subsets were clearly reduced, suggesting that the $\beta\beta\beta\beta\alpha$ chain interferes either with the expansion of CD4⁺CD8⁺ thymocytes or with the transition of thymocytes from the CD4⁻CD8⁻ to the CD4⁺CD8⁺ stage. Mature $\alpha\beta$ T cells were significantly reduced in thymus, lymph nodes and spleen of $\beta\beta\beta\beta\alpha$ transgenic mice (Fig. 3, C and D). In all other mutant transgenic lines analyzed, the different thymocyte compartments were comparable to those of non-transgenic FVB mice (Fig. 3, A–D).

Usage of Endogenous V β Gene Segments in Mutant TCR Transgenic Mice. Two color immunofluorescence analysis using anti-V β 2 and anti-V β 6 mAbs was performed to assess functional rearrangements of endogenous alleles. In non-transgenic FVB mice the $\alpha\beta$ T cell subsets expressing V β 2- and V β 6-domains represent $13.8 \pm 0.7\%$ and $9.8 \pm 0.6\%$ ($n = 28$) of mature peripheral $\alpha\beta$ T cells, respectively (Fig. 4).

However, in $\beta\beta\beta\beta\Delta$ transgenic FVB mice the V β 2⁺ and V β 6⁺ subsets constituted only $0.2 \pm 0.1\%$ and $1.7 \pm 0.8\%$ of mature $\alpha\beta$ T cells, respectively ($n = 12$). These data strongly suggest that the $\beta\beta\beta\beta\Delta$ mutant protein product efficiently prevents rearrangements of endogenous TCR β alleles. Based on the usage of V β 2 and V β 6 gene segments in control FVB mice, the efficacy with which the $\beta\beta\beta\beta\Delta$ transgene prevented surface expression of rearranged V β 2- and V β 6-gene segments was 99% and 83%, respectively.

In $\beta\beta\beta\beta\alpha$ transgenic FVB mice, the V β 2-subset com-

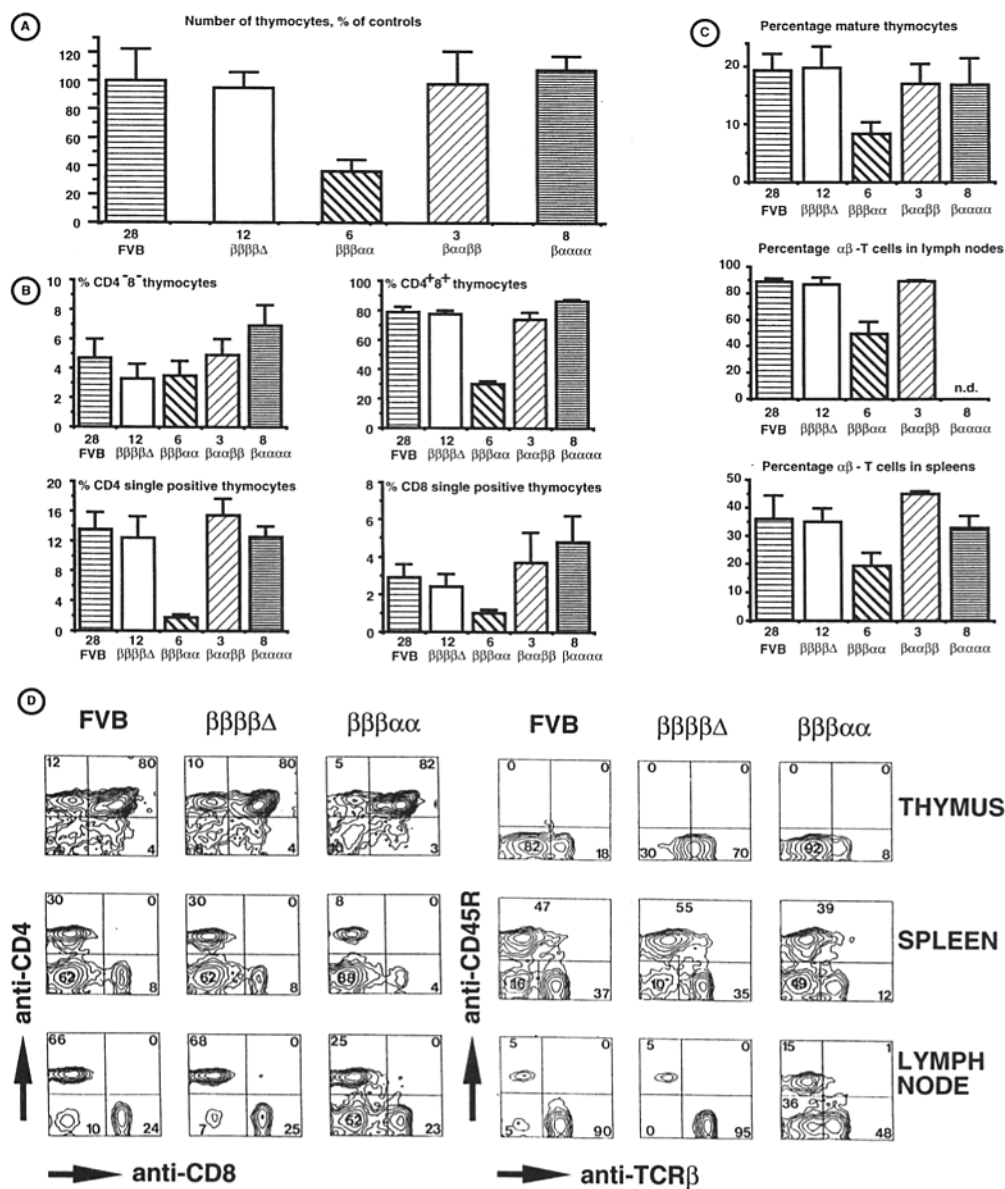


Figure 3. Quantitative comparison of the $\alpha\beta$ T cell compartment in mutant TCR transgenic mice. (A) Relative number of thymocytes in mutant TCR transgenic mice. The number of thymocytes was determined by flow cytometry on a Sysmex F800 and compared to nontransgenic littermates and/or age matched FVB mice. In $\beta\beta\beta\alpha\alpha$ transgenic mice the number of thymocytes was found to be reduced to $36 \pm 8\%$ of control mice. The thymocyte cellularity in all other mutant transgenic mice, including the $\beta\beta\beta$ -PI transgenics (not shown) does not differ from non-transgenic littermates. Number below each bar indicates the number of mice analyzed. (B) CD4/CD8 thymocyte subsets in mutant TCR transgenic mice. The percentage of CD4⁻CD8⁻ double-negative (DN), CD4⁺CD8⁺ double-positive (DP) and CD4⁺CD8⁻ or CD4⁻CD8⁺ single positive (SP) thymocyte subsets compared to the absolute number in FVB controls are depicted. The subsets were measured by two-color immunofluorescence and analyzed using Lysis II software (Becton Dickinson). Although the number of DN pro-thymocytes is normal in $\beta\beta\beta\alpha\alpha$ transgenic mice, the number of DP and SP are significantly reduced. Numbers below each bar indicate the numbers of mice analyzed. (C) $\beta\beta\beta\alpha\alpha$ transgenic mice lack mature T cells. The percentage of mature T cells in thymus, lymphnodes and spleens was determined by immunofluorescence analysis with anti-CD3 ϵ and anti-TCR β mAbs. A systemic reduction of mature $\alpha\beta$ T cells was found in $\beta\beta\beta\alpha\alpha$ transgenic mice. Numbers below each bar indicate the numbers of mice analyzed. (D) Two-color immunofluorescence analysis of thymus, spleen and lymph nodes with CD4/CD8 or CD45R/TCR $\alpha\beta$ specific mAbs. Typical examples of non-transgenic FVB control mice and transgenic $\beta\beta\beta\beta\Delta$ and $\beta\beta\beta\alpha\alpha$ mice are shown. Whereas the numbers and subsets of immature and mature $\alpha\beta$ T cells are comparable in non-transgenic FVB control mice and transgenic $\beta\beta\beta\beta\Delta$ mice, transgenic mice expressing the $\beta\beta\beta\alpha\alpha$ chain have a systemic reduction of mature T cells. Numbers in each quadrant indicate percentages.

prised $5.6 \pm 0.9\%$ ($n = 6$), a value significantly lower than the $13.8 \pm 0.7\%$ observed in nontransgenic FVB mice. However, the frequency of V β 6⁺ T cells was not significantly different in $\beta\beta\beta\alpha\alpha$ transgenic mice $10.6 \pm 0.7\%$ ($n = 6$) compared to the FVB control mice ($9.8 \pm 0.6\%$, $n = 28$).

Cell Surface Expression of the Mutant TCR Chains. Extremely high levels of $\beta\beta\beta\beta\Delta$ and $\beta\beta\beta$ -PI chains were found on the surface of thymocytes of $\beta\beta\beta\beta\Delta$ (Fig. 5) and $\beta\beta\beta$ -PI transgenic mice (not shown), as determined by flow cytometry. Low, but detectable levels of the $\beta\beta\beta\alpha\alpha$ chain were found on the surface of $\beta\beta\beta\alpha\alpha$ transgenic thymocytes and peripheral T cells (Fig. 5). The latter also ex-

press high levels of endogenous TCR/CD3 complexes (Fig. 3 D). The products encoded by the $\beta\alpha\alpha\beta\beta$ and $\beta\alpha\alpha\alpha\alpha$ transgenes could not be detected at the surface of thymocytes and mature T cells as determined by flow cytometry with a V β 8-specific mAb (data not shown). The failure of the $\beta\alpha\alpha\beta\beta$ and $\beta\alpha\alpha\alpha\alpha$ mutant TCR chains to appear on the cell surface could explain the absence of any observable effects on T cell development (Fig. 3) and usage of endogenous TCR β V-gene segments (Fig. 4).

Disproportional Surface Expression of TCR β and CD3 Components on $\beta\beta\beta\beta\Delta$ Thymocytes. Two-color immunofluorescence analysis of normal thymocytes using TCR β - and CD3 ϵ -specific antibodies demonstrates the characteristic

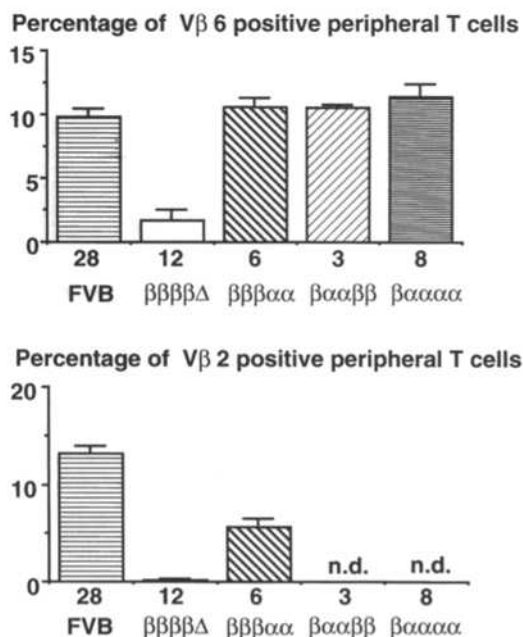


Figure 4. Frequencies of peripheral T cell subsets in mutant TCR β transgenic mice expressing V β 2- and V β 6- domains. The relative size of the V β 2- and V β 6- T cell subsets was determined by two-color flow cytometry and corrected for the percentage of mature T cells, as determined by anti-CD3 ϵ and anti-TCR β staining. The number of mice analyzed is indicated below each bar; nd, not determined. Mice from three to six different founder lines were included per mutant TCR construct.

staining pattern for the $\alpha\beta$ TCR/CD3 complex, with proportional levels of TCR β and CD3 ϵ components. Surprisingly, the surface levels of $\beta\beta\beta\beta\Delta$ and CD3 ϵ are disproportional on thymocytes from $\beta\beta\beta\beta\Delta$ mice (Fig. 6 A). Immature (i.e., CD3^{low} and CD3^{int}) and mature (CD3^{high}) thymocytes were found to express the $\beta\beta\beta\beta\Delta$ protein at unusually high levels.

The composition of the $\beta\beta\beta\beta\Delta$ /CD3 complex was further analyzed biochemically (Fig. 6 B). Analysis of CD3 ϵ -specific immunoprecipitates revealed a normal composition of disulfide-linked $\alpha\beta$ TCR heterodimers associated with CD3 δ , CD3 γ , CD3 ϵ , and disulfide-linked CD3 ζ components on thymocytes from non-transgenic FVB, transgenic TCR β and $\beta\beta\beta\beta\Delta$ mice. Beside normal levels of functional TCR/CD3 complexes, subsequent immunoprecipitations with anti-V β 8-specific mAbs indicated that $\beta\beta\beta\beta\Delta$ thymocytes express high levels of $\beta\beta\beta\beta\Delta$ monomers, apparently in the absence of CD3 components. This explains the disproportional $\beta\beta\beta\beta\Delta$ /CD3 ratio found on $\beta\beta\beta\beta\Delta$ transgenic thymocytes.

Anchoring of $\beta\beta\beta\beta\Delta$ Monomers through Glycosyl-phosphatidylinositol. The possibility that anchoring occurred through glycosyl-phosphatidylinositol (GPI) was tested by incubating thymocytes from $\beta\beta\beta\beta\Delta$ transgenic mice, and as a control from $\beta\beta\beta\beta\beta$ transgenic mice, with or without phosphatidylinositol-specific PLC, which can specifically release GPI-linked proteins from the cell surface. Subsequently, the cells were analyzed for surface TCR β expression by

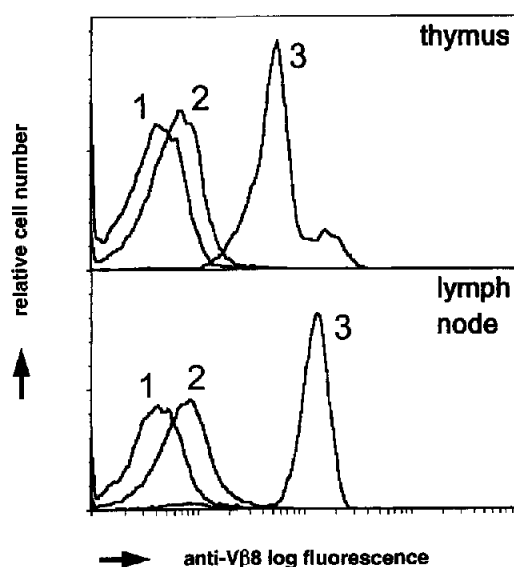


Figure 5. Surface expression of mutant TCR chains. Thymocytes and lymph node cells from non-transgenic FVB control mice (1) and transgenic $\beta\beta\beta\beta\alpha\alpha$ (2) and $\beta\beta\beta\beta\Delta$ (3) mice stained with transgene-specific FITC conjugated anti-V β 8 mAb.

flow cytometry. Upon PLC-treatment, thymocytes from $\beta\beta\beta\beta\Delta$ transgenic mice revealed a staining pattern, characteristic for thymocytes from control FVB mice and TCR β transgenic mice (Fig. 7), indicating that the $\beta\beta\beta\beta\Delta$ monomers appear in a GPI-linked form at the cell surface.

T Cell Development in RAG-1-deficient Mice Expressing the Mutant TCR β Chains. To study the effects of the mutant TCR chains on T cell development in the absence of endogenous TCR α or TCR β chains, we introduced the mutant transgenes into the V(D)J recombination-deficient RAG-1^{-/-} background (5). The $\beta\beta\beta\beta\Delta$ chain appears at the cell surface and was found to efficiently induce thymocyte development, as indicated by CD4/CD8 induction and expansion of the T cell pool to wild-type numbers (Table 1 and Fig. 8). Like the $\beta\beta\beta\beta\Delta$ chain, the $\beta\beta\beta$ -PI chain does appear at the cell surface but does not permit development of CD4⁻CD8⁻ thymocytes into CD4⁺CD8⁺ thymocytes in the RAG-1^{-/-} background, indicating that surface expression of the extracellular TCR β domains in the absence of the transmembrane region cannot promote this step in thymocyte development. Thymocytes of $\beta\beta\beta\alpha\alpha$ transgenic RAG-1^{-/-} mice expressed low but significant levels of the $\beta\beta\beta\alpha\alpha$ chain on the cell surface (Fig. 8 B). Interestingly, 7% of the thymocytes from $\beta\beta\beta\alpha\alpha$ transgenic RAG-1 mutant mice had a CD4⁺CD8⁺ phenotype. Thus, the $\beta\beta\beta\alpha\alpha$ chain is able to induce the transition from CD4⁻CD8⁻ to CD4⁺CD8⁺ thymocytes causing the formation of a small but significant CD4⁻CD8⁺ thymocyte subset (Fig. 8 A). However, the $\beta\beta\beta\alpha\alpha$ chain fails to increase the number of thymocytes (Table 1). The $\beta\alpha\alpha\beta\beta$ and the $\beta\alpha\alpha\alpha\alpha$ chains do not appear at the cell surface and do not promote thymocyte maturation (Fig. 8).

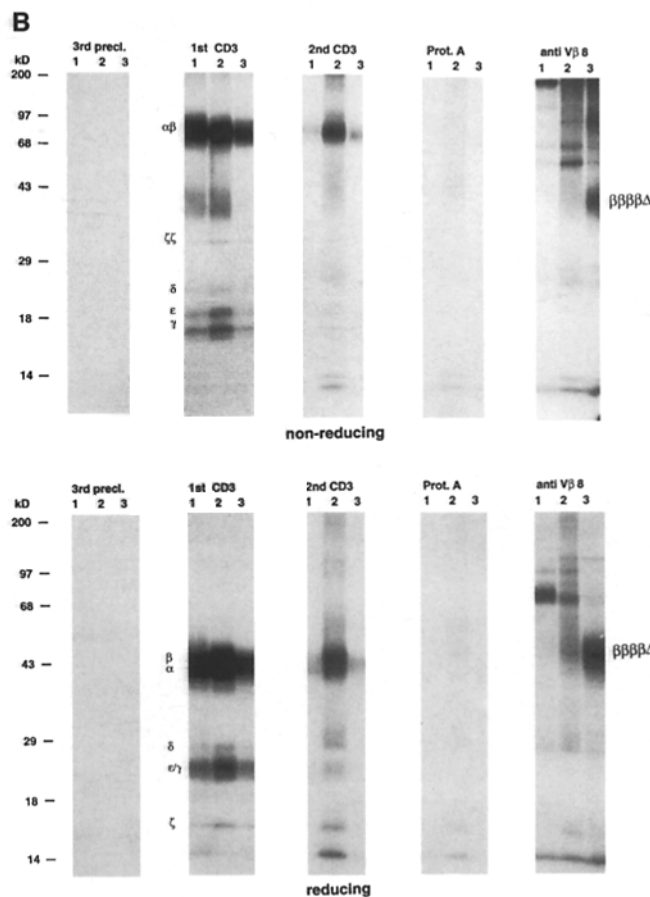
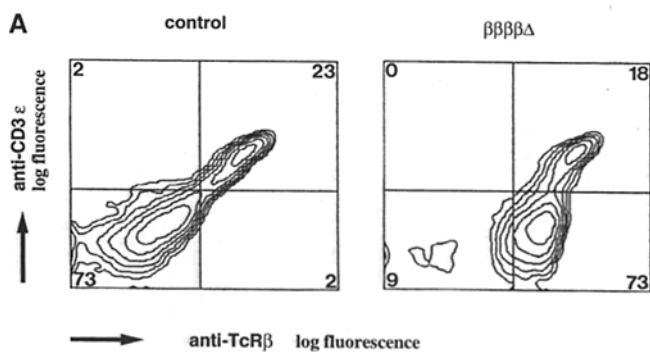


Figure 6. High levels of $\beta\beta\beta\beta\Delta$ monomers appear at the surface of immature thymocytes in the absence of CD3 components. (A) Disproportional surface expression of CD3 ϵ and TCR β on thymocytes from $\beta\beta\beta\beta\Delta$ transgenic mice. The unusually high levels of $\beta\beta\beta\beta\Delta$ chains on thymocytes from $\beta\beta\beta\beta\Delta$ transgenic mice are compared to CD3 ϵ levels by two-color immunofluorescence analysis. Thymocytes from nontransgenic (control) and $\beta\beta\beta\beta\Delta$ transgenic mice were stained with FITC-conjugated anti-CD3 ϵ (145-2C11) and biotinylated anti TCR β -(H57-597) mAbs. Biotinylated H57-597 was detected with PE-conjugated streptavidin. Numbers in each quadrant indicate percentages. (B) SDS-PAGE analysis of sequential anti-CD3 ϵ and anti-V β 8 immunoprecipitates from surface iodinated thymocytes from nontransgenic FVB mice, transgenic $\beta\beta\beta\beta$ and $\beta\beta\beta\beta\Delta$ mice. Thymocytes from nontransgenic FVB mice (lanes 1), transgenic TCR β (lanes 2) and $\beta\beta\beta\beta\Delta$ mice (lanes 3) were surface iodinated and lysed in digitonin containing lysis buffer (11). The composition of the CD3 ϵ and TCR β associated components of the $\alpha\beta$ TCR/CD3 complex were analyzed by sequential immunoprecipitations with CD3 ϵ and V β 8 specific mAbs. After three preclearing steps with normal mouse serum (3rd preclear), CD3 ϵ associated TCR/CD3 components were immunoprecipitated by two sequential immunoprecip-

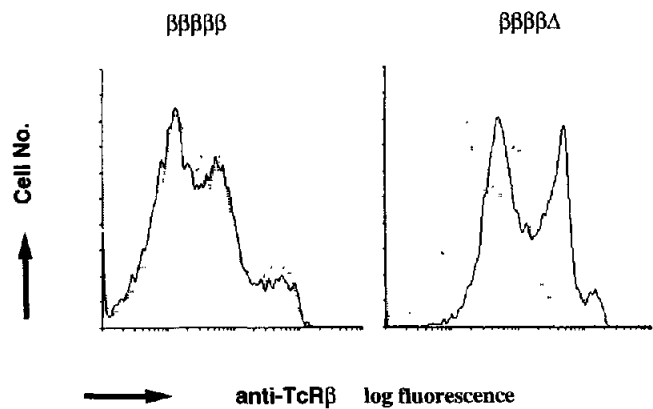


Figure 7. Release of $\beta\beta\beta\beta\Delta$ chains from the surface of $\beta\beta\beta\beta\Delta$ thymocytes by phosphatidylinositol-specific phospholipase C (PLC). PLC-treated (+PLC, dashed curve) and mock-treated (-PLC, solid curve) thymocytes from $\beta\beta\beta\beta\beta$ and $\beta\beta\beta\beta\Delta$ transgenic mice (10⁶/ml) were washed twice, stained with FITC-labeled anti-TCR β antibodies, and analyzed with FACScan[®] (Becton Dickinson).

Discussion

Previously, we have demonstrated that a mutant TCR β chain lacking the variable domain ($\Delta\beta\beta\beta\beta$) can induce thymocytes to differentiate from the CD4⁻CD8⁻ to the CD4⁺CD8⁺ stage (10, 26). To identify the regions within the TCR β constant region required for this step in thymocyte development, we have extended the mutational approach by generating five additional TCR β transgenic mice in which constant region domains were deleted or exchanged by corresponding regions of the TCR α chain. Although all mutant TCR transgenes are highly expressed at the mRNA and protein level, the $\beta\alpha\alpha\beta\beta$ and the $\beta\alpha\alpha\alpha\alpha$ chimeric chains could not be detected at the cell surface of thymocytes and peripheral T cells. Both proteins share a variable TCR β domain and the extracellular constant region of the TCR α chain, including the connecting peptide which contains the cysteine residue responsible for the covalent interchain bond with TCR β . The structure of the $\beta\alpha\alpha\beta\beta$ and $\beta\alpha\alpha\alpha\alpha$ chains might not allow assembly with endogenous TCR and CD3 components in the ER and subsequent transport to the cell surface (48). The failure of $\beta\alpha\alpha\beta\beta$ and $\beta\alpha\alpha\alpha\alpha$ chains to appear at the cell surface can explain the lack of any noticeable effects on thymocyte development in $\beta\alpha\alpha\beta\beta$ and $\beta\alpha\alpha\alpha\alpha$ transgenic mice, on both wild-type and RAG-1^{-/-} background.

The $\beta\beta\beta\beta\alpha$ chimeric chain resembles more closely a TCR β chain than the $\beta\alpha\alpha\beta\beta$ and $\beta\alpha\alpha\alpha\alpha$ chain. The

iterations with anti-CD3 ϵ (1st CD3 ϵ and 2nd CD3 ϵ). Any residual anti-CD3 ϵ mAbs were precipitated by an additional preclear step with protein A-Sepharose beads (Prot. A). Subsequently any residual $\beta\beta\beta\beta\Delta$ chains and associated molecules were precipitated with F23.1 mAbs indirectly coupled by rabbit anti-mouse IgG to protein A beads (anti-V β 8). Immunoprecipitates were analyzed under non-reducing and reducing conditions. Molecular mass markers are indicated. The 25–30-kD species and bands larger than 60 kD found in all V β 8-specific immunoprecipitations are Ig-light and Ig-heavy chains from contaminating B cells.

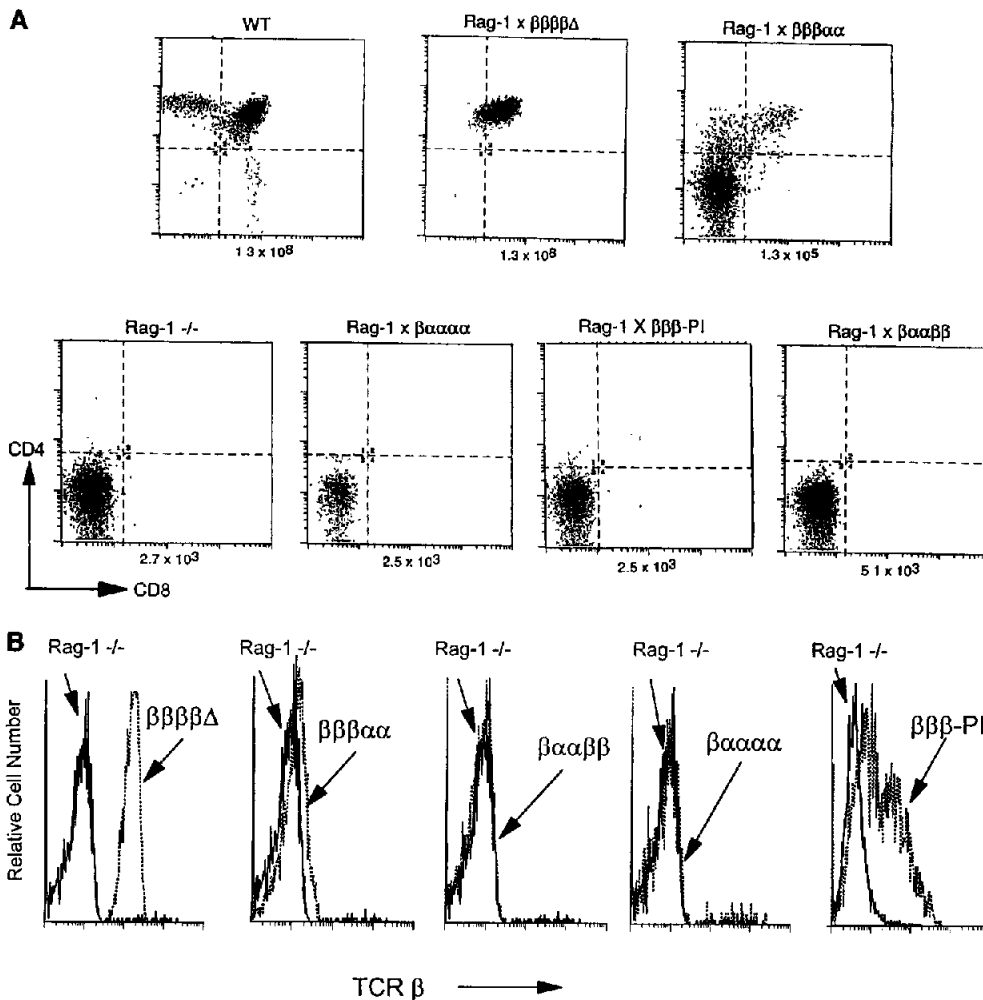


Figure 8. (A) Induction of CD4 and CD8 expression on thymocytes of mutant TCR transgenic RAG-1-deficient thymocytes. Thymocytes isolated from wild-type (WT), RAG-1-deficient (RAG-1 $^{-/-}$) and the five different transgenic RAG-1-deficient mice (RAG-1 $^{-/-}$ \times mutant TCR β transgene) were double stained using CD4- and CD8-specific mAb and analyzed by flow cytometry. Numbers below each dot plot indicate the absolute number of double-positive thymocytes. (B) Surface expression of mutant TCR β chains on RAG-1-deficient thymocytes. Thymocytes from control RAG-1-deficient (solid lines) and mutant TCR β transgenic RAG-1-deficient mice (dashed lines) were stained with H157-597 conjugated to FITC or PE and analyzed by flow cytometry. Shown are representative experiments. All experiments were repeated at least four times and gave consistent results. Similar data were obtained when cells were stained with F23.1 (anti-V β 8).

$\beta\beta\beta\alpha$ chain, which contains the extracellular cysteine required for covalent heterodimerization with TCR α and likely also with the pT α chain, appears at very low levels at the cell surface, as indicated by flow cytometric analysis. This suggests that the $\beta\beta\beta\alpha$ chain participates in the TCR/CD3 complex. However, the $\beta\beta\beta\alpha$ chain cannot fully substitute for the function of the TCR β chain in promoting differentiation to CD4 $^{+}$ CD8 $^{+}$ cells and in mediating allelic exclusion. The latter was indicated by the reduced usage of the V β 2 gene segment in mature T cells of $\beta\beta\beta\alpha$ transgenic mice. Mature thymocytes expressing high $\beta\beta\beta\alpha$ TCR/CD3 levels were not detected. Apparently, $\beta\beta\beta\alpha$ thymocytes depend on a non-mutant endogenous TCR β chain to mature into single positive thymocytes.

To specifically analyze the role of $\beta\beta\beta\alpha$ in the transition of thymocytes from the CD4 $^{-}$ CD8 $^{-}$ to the CD4 $^{+}$ CD8 $^{+}$ stage we determined the capacity of the $\beta\beta\beta\alpha$ chain to mediate thymocyte differentiation in $\beta\beta\beta\alpha$ transgenic, RAG-1 $^{-/-}$ mice. Expression of the $\beta\beta\beta\alpha$ chain induced the developmental transition from CD4 $^{-}$ CD8 $^{-}$ to CD4 $^{+}$ CD8 $^{+}$ cells as indicated by the presence of a low but significant number of CD4 $^{+}$ CD8 $^{-}$ thymocytes in the compound mutant mice, whereas such cells were not found in non-

transgenic RAG-1 $^{-/-}$ mice. Thus, the $\beta\beta\beta\alpha$ chain, although expressed at low levels at the surface of thymocytes, probably as a component of the preTCR/CD3 complex, can induce the expression of CD4 and CD8 receptors.

Table 1. Cellularity, Pre-T Cell Induction and Surface Expression in the Thymus of RAG-1 Mutant Mice Reconstituted with Mutant TCR Transgenes

Transgene	V	C	CP	TM	CT	Total No. of thymocytes	Absolute No. of DP cells	Surface expression
$\beta\beta\beta\Delta$	β	β	β	β	Δ	1.4×10^8	1.3×10^8	Yes
$\beta\beta\beta\alpha$	β	β	β	α	α	1.9×10^6	1.3×10^5	Yes
$\beta\alpha\alpha\beta$	β	α	α	β	β	3.7×10^6	5.1×10^5	No
$\beta\alpha\alpha\alpha$	β	α	α	α	α	7×10^5	2.5×10^3	No
$\beta\beta\beta$ -PI	β	β	β	PI		2×10^6	2.5×10^3	Yes
WT	β	β	β	β	β	1.6×10^8	1.3×10^8	Yes
RAG-1	NA					2×10^6	2.5×10^3	NA

Δ , Domain deleted, \dagger PI, Linked via Q7 b PI sequence.

The $\beta\beta\beta\beta\Delta$ transgene functions in most aspects as a normal $\beta\beta\beta\beta\beta$ transgene as judged by thymocyte numbers, size of the different CD4/CD8 subsets and induction of pre-T cell development in $\beta\beta\beta\beta\Delta$;RAG-1^{-/-} mice. Allelic exclusion in $\beta\beta\beta\beta\Delta$ transgenic mice appears to be efficient as indicated by the virtual absence of T cell subsets expressing rearranged endogenous V β 2 and V β 6 gene segments in these mice. Thus, the cytoplasmic tail of the TCR β chain is dispensable for its role in these aspects of T cell development. Remarkably, beside normal surface levels of disulfide linked TCR α / $\beta\beta\beta\beta\Delta$ heterodimers associated with CD3 components, $\beta\beta\beta\beta\Delta$ chains appear at high levels as GPI bound monomers in the absence of CD3 components on the surface of thymocytes and B cells (data not shown). Thymocytes are known to regulate their surface levels of TCR/CD3 complexes posttranslationally. Single subunits or partial complexes that are synthesized in excess are retained in the ER and degraded (49). Previous studies have shown that retention of individual subunits of oligomeric receptors is controlled by ER retention signals located in the cytoplasmic domain. A di-lysine motif at position -3 and -4 or -5 from the carboxyl terminus has been shown to control receptor assembly by retaining unassembled individual chains in the ER (50, 51). The cytoplasmic tails of TCR β proteins harbor such a di-lysine ER retention signals, VKRKNS in C β 1 and VKKKNS in C β 2. Indeed, $\beta\beta\beta\beta\Delta$ chains can escape ER retention, TCR/CD3 assembly and proteolytic degradation.

In normal TCR β chains GPI linkage might be prevented by the cytoplasmic tail. Therefore, the cytoplasmic tail of the TCR β chain which is encoded by a separate exon appears to be essential for determining the early processing events of unassembled TCR β chains rather than being required for TCR/CD3 assembly and signaling thymocyte differentiation.

When compared to nontransgenic littermates, the $\beta\beta\beta\alpha\alpha$ and the $\beta\beta\beta\beta\Delta$ transgenes were found to interfere with

the usage of the endogenous V β 2 segment in the $\alpha\beta$ T cell lineage. In all founder lines analyzed, the $\beta\beta\beta\alpha\alpha$ and the $\beta\beta\beta\beta\Delta$ transgenes consistently excluded endogenous V β 2-gene segments more efficiently than endogenous V β 6-gene segments. This phenomenon may relate to the position of the V segment in the TCR β locus (52). The V β 2 gene segment is the most 5' V β segments, distal of the D β /J β clusters whereas the V β 6 segment is located proximal to the D β /J β clusters. The suggested processivity of the recombination machinery from 3' to 5' would more profoundly affect V β genes lying distal from the DJ join (53-56). Alternatively, pre-T cell development induced by functional TCR β chains might signal the shut down of endogenous TCR β loci from 5' to 3', resulting in the preferential rearrangement of V β -segments lying proximal to the DJ region.

In conclusion, we have identified the extracellular constant domains and the transmembrane region of the TCR β chain to be required for initiating pre-T cell development as demonstrated by the efficient allelic exclusion of endogenous TCR β alleles in RAG-1^{+/+} mice and transition of CD4⁻CD8⁻ to CD4⁺CD8⁺ thymocytes in $\beta\beta\beta\beta\Delta$ -transgenic RAG-1^{-/-} mice. Allelic exclusion and pre-T cell development is inefficient in transgenic mice expressing the $\beta\beta\beta\alpha\alpha$ chain. Both the extracellular constant region and the transmembrane region of the TCR β chain, but not the variable domain and cytoplasmic tail, are required for its assembly with the other TCR/CD3 components. The cytoplasmic tail of the TCR β chain appears to be essential for retention of the TCR β chain in the ER but not for signal transduction. The finding that only those mutants that theoretically can form disulfide bonds to the pT α chain and assemble with CD3 components into a pre-TCR complex can catalyze the transition from CD4⁻CD8⁻ to CD4⁺CD8⁺ cells supports the notion that cell surface expression of the preTCR/CD3 complex is a prerequisite for pre-T cell differentiation (29).

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