

# Quantitative Analysis of the T Cell Repertoire Selected by a Single Peptide–Major Histocompatibility Complex

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## Summary

The positive selection of CD4<sup>+</sup> T cells requires the expression of major histocompatibility complex (MHC) class II molecules in the thymus, but the role of self-peptides complexed to class II molecules is still a matter of debate. Recently, it was observed that transgenic mice expressing a single peptide–MHC class II complex positively select significant numbers of diverse CD4<sup>+</sup> T cells in the thymus. However, the number of selected T cell specificities has not been evaluated so far. Here, we have sequenced 700 junctional complementarity determining regions 3 (CDR3) from T cell receptors (TCRs) carrying Vβ11–Jβ1.1 or Vβ12–Jβ1.1 rearrangements. We found that a single peptide–MHC class II complex positively selects at least 10<sup>5</sup> different Vβ rearrangements. Our data yield a first evaluation of the size of the T cell repertoire. In addition, they provide evidence that the single Eα52–68–I–A<sup>b</sup> complex skews the amino acid frequency in the TCR CDR3 loop of positively selected T cells. A detailed analysis of CDR3 sequences indicates that a fraction of the β chain repertoire bears the imprint of the selecting self-peptide.

Key words: thymus • major histocompatibility complex • T cell receptors • repertoire development • transgenic/knockout

During development, thymocytes undergo two steps of selection, each involving interaction with self-MHC molecules (1–4). Positive selection rescues thymocytes from programmed cell death and ensures that the mature T cell repertoire is directed against foreign peptides bound to self-MHC molecules (5–8). Subsequently, negative selection eliminates, through clonal deletion, T cells with potentially autoreactive receptors (1, 2, 9, 10).

Using in vitro fetal thymic organ culture system from β2 microglobulin- or transporters associated with antigen processing (TAP)-deficient mice, it was shown that peptides were important for positive selection of CD8<sup>+</sup> T cells (11, 12). Similar experiments with mice expressing rearranged TCR genes of known specificity revealed a stringent requirement for peptide recognition during the positive selection process (13–16). Affinity measurements of TCR-peptide–MHC interaction indicated that peptide–MHC complexes capable of positive selection were of low affinity for the TCR, whereas high affinity ones were deleting

ligands (17). More recently, natural self-peptides, extracted from MHC class I groove and capable of driving positive selection, were identified (18, 19). It was shown that different peptides could select thymocytes expressing different TCRs, suggesting that weak but specific interactions with self-peptide–MHC complexes promote positive selection of CD8<sup>+</sup> T cells. Hence, a particular TCR could be selected by different peptides.

Peptide involvement in positive selection of CD4<sup>+</sup> T cells was addressed using genetically engineered mice designed to express MHC class II molecules complexed to a single peptide. In mice lacking the MHC-encoded H-2M molecule and involved in the removal of the class II-associated invariant chain peptide (CLIP)<sup>1</sup> during the MHC class II maturation process (20–23), almost all MHC class II

<sup>1</sup>Abbreviations used in this paper: CLIP, class II-associated invariant chain peptide; Tg, transgenic mice.

molecules are occupied with the CLIP peptide (24). Transgenic mice (Tg) for the I-A $\beta$  chain connected to the E $\alpha$ 52-68 peptide (25, 26) backcrossed to MHC class II- and invariant chain-deficient animals express the single E $\alpha$ 52-68 peptide-I-A $\beta$  complex. Studies conducted with these two types of Tg suggested that a large and diverse repertoire of CD4 $^{+}$  T cells is selected (22–27). Staining with anti-V $\beta$  and -V $\alpha$  antibodies showed that a close to normal spectrum of V $\beta$  and V $\alpha$  was used by mature CD4 $^{+}$  T cells. Sequencing studies in the H-2M $^{-/-}$  model, from two V $\beta$ s and one V $\alpha$ , confirmed the polyclonality (24). Finally, positively selected cells were capable of responding to immunization with several peptide antigens (22–24, 27). In the E $\alpha$ 52-68-I-A $\beta$  model, Fukui et al. (26) have shown that the level of expression of this complex in the thymus affects the CD4 $^{+}$  T cell selection dramatically. Transgenic lines with low expression of E $\alpha$ 52-68-I-A $\beta$  complexes positively select CD4 $^{+}$  T cells, whereas such cells are eliminated in the thymus of another line with high expression (26). Furthermore, two thirds of the selected CD4 $^{+}$  T cells react with the syngeneic cells that express the same MHC class II molecules complexed to the natural set of self-peptides (25). In wild-type mice, such lymphocytes are eliminated by negative selection on bone marrow-derived cells expressing wild-type class II molecules in the thymus (23, 24). These results indicate that the Tg repertoire of CD4 $^{+}$  T cells is different from the wild type. Furthermore, it was shown using mice expressing various transgenic TCRs (which are positively selected in mice expressing wild-type class II molecules) that these TCRs are not selected in the CLIP mice or the E $\alpha$ 52-68-I-A $\beta$  Tg (22–24). These observations imply that the selecting peptide influences, to some extent, the emerging repertoire. However, the diversity of the repertoire selected by a single peptide–MHC class II complex has not been quantitated so far. In addition, the available evidence does not rule out that a few positively selected specific TCR rearrangements have not yet been detected over a polyclonal background.

These studies were designed to quantitate the number of positively selected T cells. We have analyzed the  $\alpha$  and  $\beta$  T cell repertoire of CD4 $^{+}$  T lymphocytes selected by the E $\alpha$ 52-68-I-A $\beta$  complex extensively. We show that CD4 $^{+}$  CD8 $^{-}$  NK1.1 $^{-}$  HSA $^{-}$  thymocytes selected on this complex bear TCRs that include all V $\beta$ s and 10 V $\alpha$ s tested. The J $\beta$  usage and CDR3 length distribution of V $\beta$  and V $\alpha$  chain rearrangements are indistinguishable from the repertoire of CD4 $^{+}$  CD8 $^{-}$  NK1.1 $^{-}$  HSA $^{-}$  thymocytes from normal C57Bl/6 mice. Extensive sequencing of particular V $\beta$ –J $\beta$  combinations with the same CDR3 length enabled us to calculate that a minimum of 10 $^5$  different V $\beta$  rearrangements are selected by the single peptide–MHC class II complex. Careful analysis of these sequences revealed some differences in the CDR3 amino acid composition of T cells selected by the single peptide–MHC complex or by wild-type MHC class II molecules. Altogether, our results provide a lower limit on the size of the selected CD4 $^{+}$  T cell repertoire in vivo and indicate that part of the repertoire bears the imprint of the selecting peptide.

## Materials and Methods

**Animals.** Mice used in this study have been described elsewhere (26) and were bred in the animal facility at the Medical Institute of Bioregulation. In brief, Tg were produced by injection of DNA encoding the  $\beta$  chain of I-A $\beta$  covalently linked to the peptide derived from MHC class II E $\alpha$  (E $\alpha$ 52-68) into fertilized eggs of I-A $\beta$ <sup>b</sup> gene knockout (I-A $^{-/-}$ ) mice carrying H-2<sup>b</sup> haplotype (28). To avoid peptide replacement, Tg were backcrossed with mice deficient for the invariant chain (Ii $^{-/-}$ ; reference 29). The B2L mice that express ~10% of the level of I-A $\beta$  found in C57Bl/6 animals were chosen for all studies.

**Purification of CD4 $^{+}$  T Cells.** Thymocytes were prepared from 6-wk-old mice and counted. CD4 $^{+}$  CD8 $^{-}$  NK1.1 $^{-}$  HSA $^{-}$  T cells were prepared using depletion and cell sorting as previously described (26). In brief, thymocytes were incubated with anti-CD8 and anti-HSA (J11D) monoclonal antibodies and killed by addition of rabbit complement. Remaining living cells were stained with anti-CD4-PE, anti-CD8-FITC, and anti-NK1.1-FITC (PharMingen, San Diego, CA). CD4 $^{+}$  CD8 $^{-}$  NK1.1 $^{-}$  HSA $^{-}$  T cells were analyzed and sorted on an Epics cell sorter (Coulter Corp., Miami, FL). For Immunoscope analysis of the T cell receptor repertoire, cells were washed with PBS and immediately frozen down into liquid nitrogen until further manipulation.

**mRNA Extraction and cDNA Synthesis.** Poly(A)<sup>+</sup> mRNA from CD4 $^{+}$  CD8 $^{-}$  NK1.1 $^{-}$  HSA $^{-}$  thymocytes was extracted (Quick mRNA Micro Prep Kit; Pharmacia, Piscataway, NJ). In brief, cells were lysed in guanidium thiocyanate and mRNA samples were purified by affinity chromatography on oligo-d(T) cellulose. After ethanol precipitation, mRNAs were reverse transcribed into cDNA using a cDNA synthesis kit (Boehringer Mannheim GmbH, Mannheim, Germany). RNA were denatured at 70°C for 10 min and then incubated with random primers (5  $\mu$ M), dNTP (1 mM), Rnasin (40 U; Promega, Madison, WI) and 2 U of reverse transcriptase from avian myeloblastosis virus (Boehringer Mannheim GmbH) at 43°C for 1 h, followed by an incubation at 53°C for 10 min.

**Immunoscope Analysis of V $\beta$  and V $\alpha$  Repertoires.** PCR was carried out in 50  $\mu$ l on 1/50 of the cDNA with 2 U of Taq polymerase (Goldstar, Eurogentec, Seraing, Belgium) in the buffer provided by the supplier. Each V $\beta$  mRNA was amplified using one of a set of 23 V $\beta$ -specific sense primers and an antisense primer designed to hybridize in the C $\beta$  gene (30). Similarly, V $\alpha$  mRNAs coding for V $\alpha$  1, 2, 3, 4, 5, 6, 8, 10, 11, 17, 18, and 19 were amplified with V $\alpha$ -specific sense primers and an antisense primer from the C $\alpha$  region (31).

Each amplified product was then used as a template for an elongation reaction with oligonucleotides labeled with a fluorescent tag (runoff reactions). The fluorescent runoff products, corresponding to the elongation of individual V $\beta$  or V $\alpha$  PCR products with various CDR3 sizes, were loaded on polyacrylamide gels and subjected to electrophoresis in an automated DNA sequencer. CDR3 size distribution and signal intensities were then analyzed with the Immunoscope software (30, 32, 33). The patterns observed from unprimed lymph node cells or splenocytes usually contain six to eight size peaks each spaced by three nucleotides, corresponding to the lengths of in-frame transcripts. The area of each size peak is proportional to the quantity of the TCR transcripts of the corresponding CDR3 length in the sample. It should be noted that each peak corresponding to a given CDR3 length is likely to contain multiple distinct sequences. Increase in the height and area of a size peak signals clonal expansion, occurring against polyclonal background. The fluorescent C $\beta$  (or C $\alpha$ ) primers used in the runoff reactions reveal all V $\beta$ –J $\beta$  (V $\alpha$ –J $\alpha$ )

rearrangements. Runoff reactions using specific fluorescent J $\beta$  primers reveal the CDR3 length distributions from particular V-J combinations. In brief, 2  $\mu$ l of each PCR product was subjected to three to five cycles of elongation with dye labeled specific primer (0.1  $\mu$ M) in a final volume of 10  $\mu$ l, containing 0.2 mM dNTP, 3 mM MgCl<sub>2</sub>, and 0.2 U of Taq polymerase (Promega) in buffer (Promega). The elongation starts with 1 min at 94°C, followed by three to five cycles each consisting of 45 s at 94°C, 45 s at 60°C, 45 s at 72°C, and ending with a step at 72°C for 3 min. Each runoff product was then diluted vol/vol in 30 mM EDTA-formamide solution. This mix was heat-denatured during 10 min at 80°C and 2  $\mu$ l aliquot was loaded on a 6% (or 4.25%) polyacrylamide 8 M urea gel. Gel electrophoreses were performed on a DNA sequencer (373A or 377; PE Applied Biosystems, Foster City, CA). Peak size and fluorescent intensity were determined with the Immunoscope software (32, 34–36). Because the technique is semiquantitative, we could evaluate the relative frequency of J $\beta$  usage for a given V $\beta$  population, as described in reference 37. The ratio of the area of the peak generated with a given J $\beta$  primer to the areas of all the J $\beta$  primers was calculated as a measure of the relative frequency of J $\beta$  usage in the V $\beta$ <sup>+</sup> cell population under study.

**Sequencing of Particular V $\beta$ -J $\beta$  Rearrangements with a Given Length.** Two PCR reactions were performed using specific V $\beta$ 11-J $\beta$ 1.1 or V $\beta$ 12-J $\beta$ 1.1 primers in 50  $\mu$ l on 1/50 of the cDNA with 2 U of Taq polymerase (Goldstar, Eurogentec) in the supplier's buffer. The elongation starts with 1 min at 94°C, followed by 40 cycles each consisting of 45 s at 94°C, 45 s at 60°C, 45 s at 72°C, and ending with a step at 72°C for 3 min. The PCR product was ethanol precipitated and resuspended in 10  $\mu$ l formamide containing 0.05% bromophenol blue and 0.05% xylene cyanol. This mix was heat denatured for 10 min at 80°C and then loaded on a 8% polyacrylamide/7 M urea gel. After migration, PCR products were visualized by a silver coloration (DNA Silver Staining System; Promega) following manufacturer's instructions. We usually obtained six to eight bands each spaced by three nucleotides, corresponding to in-frame transcripts of the V-J combinations. Bands of interest, corresponding to a given CDR3 length, were cut from the gel and disrupted in 40  $\mu$ l of water. A second PCR was carried out using the same primers on 2  $\mu$ l of the isolated PCR product with 2 U of Taq polymerase (Goldstar, Eurogentec) in the supplier buffer for 20 cycles. Further purification was realized on a 15% nondenaturing acrylamide gel in TBE. Staining of this gel was obtained with a 30 min bath in a 0.5 mg/ml ethidium bromide solution in TBE (Tris-borate-EDTA). PCR product was electroeluted in TBE and purity of the sample was estimated by a runoff reaction with the fluorescent J $\beta$  primer. We usually obtained 90–95% purity of the final product with the expected size. PCR products were then cloned in pCR<sup>®</sup>2.1 vector from the TA cloning kit (Invitrogen, Carlsbad, CA). 10–20 ng of DNA were ligated with an equimolar concentration of the vector. The ligation was carried out in 10  $\mu$ l with 4 U of T4 DNA ligase during 12 h at 14°C, following the manufacturer's instructions. Competent INV $\alpha$ F' bacteria (Invitrogen) were then transformed by heat shock at 42°C for 30 s and plated on Luria-Bertani medium containing 50  $\mu$ g/ml ampicillin and 60  $\mu$ g/ml X-Gal (ICN Biomedicals Inc., Aurora, OH).

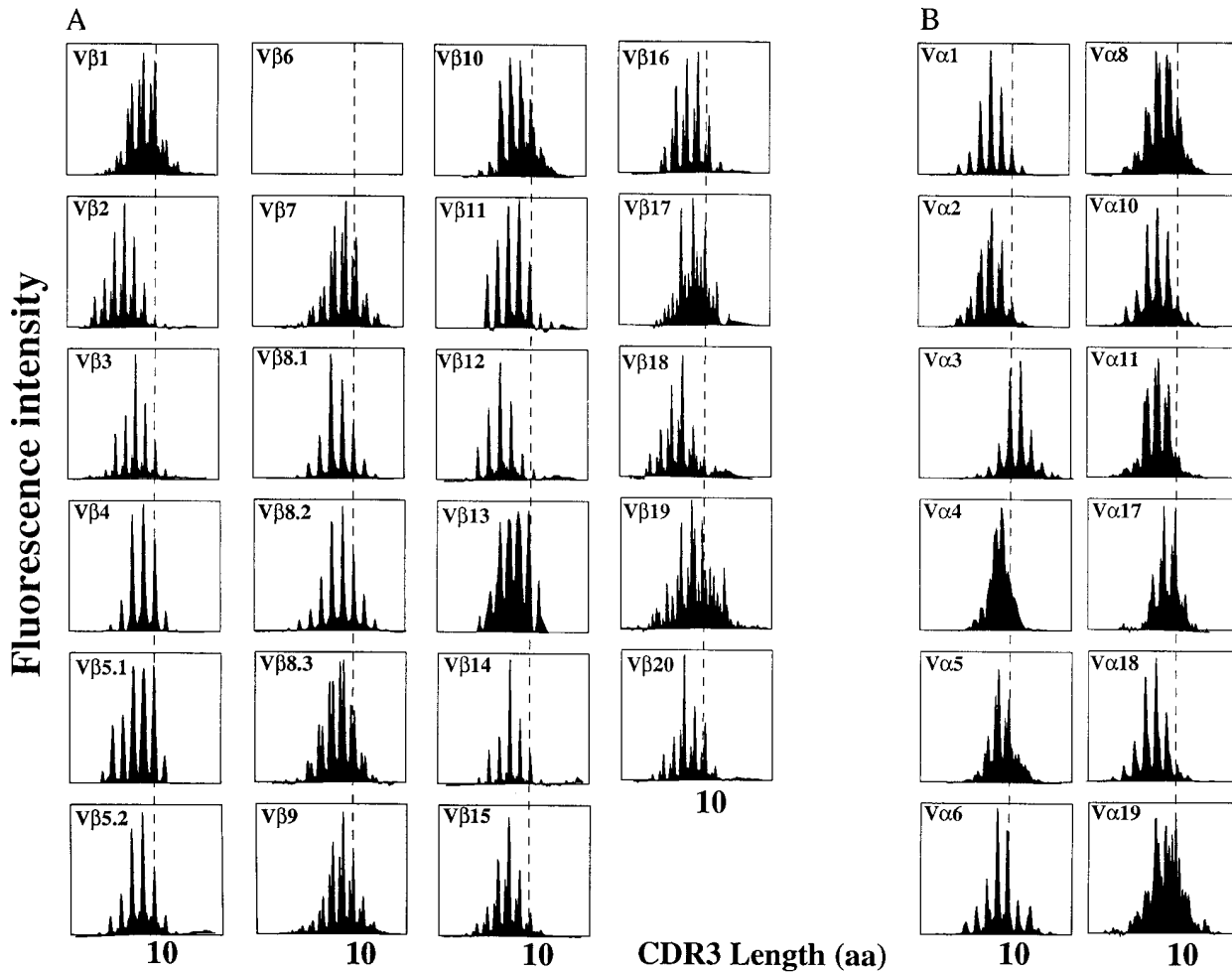
For the purpose of sequencing, PCR was carried out directly on LacZ<sup>-</sup> colonies in a final volume of 30  $\mu$ l using universal primers RP and M13(-40) designed to hybridize on each side of the polylinker where the V $\beta$ -J $\beta$  PCR product was cloned. 5  $\mu$ l of this PCR product was treated during 40 min at 37°C with 1 U of shrimp alkaline phosphatase (Nycomed Amersham, Bucking-

hamshire, UK) and 10 U of exonuclease (Nycomed Amersham) in a final volume of 10  $\mu$ l. Both enzymes were heat denatured at 80°C for 20 min. Sequencing reactions were carried out directly on these products using M13(-20) primer and the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit following the manufacturer's instructions (PE Applied Biosystems). Reaction products were loaded on a 4.25% polyacrylamide 8 M urea gel. Gel electrophoreses were performed on a DNA sequencer (377; PE Applied Biosystems), and CDR3 region corresponding sequences were extracted and analyzed using software designed for this purpose (Casrouge, A., E. Beaudoin, J.P. Levraud, L. Gapin, N. Garguier, D. Gautheret, J.M. Claverie, J. Kanellopoulos, P. Kourilisky, manuscript in preparation).

## Results

**CDR3 Size Distribution of TCR- $\alpha/\beta$  Repertoire from CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> Thymocytes Positively Selected by a Single Peptide-MHC Complex.** Single positive CD4 thymocytes were purified from the thymus of B2L, which are transgenic for a single peptide-MHC class II complex (26). The repertoire analysis did not include CD4<sup>+</sup>CD8<sup>-</sup> NK1.1<sup>+</sup> cells, which were eliminated by specific lysis with antibodies and complement, because it is known that such cells are selected by nonclassical MHC class I molecules such as CD1 (38).

mRNA from 3–4  $\times$  10<sup>5</sup> CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> thymocytes was extracted and reverse transcribed into cDNA. 23 V $\beta$ - and 12 V $\alpha$ -specific PCR reactions were then carried out. The product of each PCR was visualized by performing a runoff extension with internal C $\beta$ - (or C $\alpha$ -) specific fluorescent primer. Such primers enable elongation through the CDR3 region of each amplified product, and therefore reveal peaks of different sizes for all V-J combinations after separation on a sequencing gel. Previous studies from our laboratory, using unstimulated mouse splenocytes, have shown that, in all functional rearranged V $\beta$  or V $\alpha$  segments, the CDR3 size patterns display six to eight peaks spaced by three nucleotides. These peaks are distributed in Gaussian-like patterns that are characteristic of polyclonal T lymphocytes (34). The repertoire analysis performed on single positive CD4 cells selected on the E $\alpha$ 52-68 peptide-I-A<sup>b</sup> complexes is shown in Fig. 1. 22 out of the 23 V $\beta$  genes were amplified (Fig. 1 A). Amplification of V $\beta$ 6 gene failed for technical reasons. All V $\alpha$  genes tested were amplified (Fig. 1 B). The size distribution of CDR3 from each V $\alpha$  or V $\beta$  family gives a set of about seven peaks, each spaced by three nucleotides and corresponding to in-frame transcripts. For V $\beta$ 17 and V $\beta$ 19 genes that are pseudogenes in C57Bl/6 mice (39–41), in-frame transcripts are difficult to detect above the background of out-of-frame transcripts. To detect more subtle modifications of the V $\beta$  repertoire, we performed runoff reactions with 12 fluorescent primers that recognize individual J $\beta$  segments for the V $\beta$ 4, V $\beta$ 8.2, V $\beta$ 10, V $\beta$ 11, V $\beta$ 12, and V $\beta$ 14 families. The CDR3 distribution of specific V $\beta$ -J $\beta$  rearrangements was then obtained for the six different V $\beta$ s of two individual Tg. Our results clearly show that for each V $\beta$ , all J $\beta$ s are used. Furthermore, the



**Figure 1.** Profiles of the fluorescent V $\beta$ -C $\beta$  (A) and V $\alpha$ -C $\alpha$  (B) run-off products obtained with CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> thymocytes from single chain peptide-MHC class II complex Tg. Results are representative of two mice tested independently. The intensity of fluorescence is represented in arbitrary units as a function of the size of the single DNA fragments.

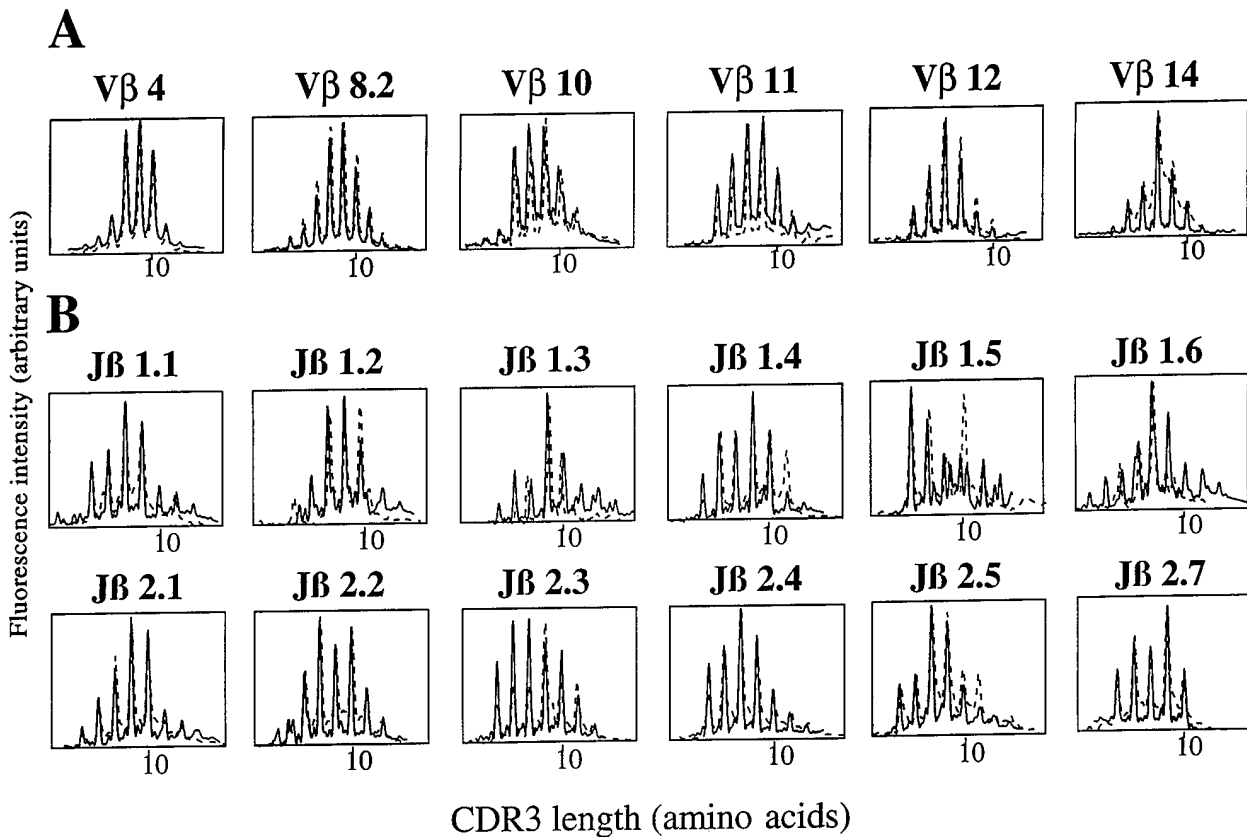
CDR3 distribution of these rearrangements has a Gaussian-like profile (V $\beta$ 11 in Fig. 2 B; otherwise, data not shown). We have observed previously that in every situation characterized by the expansion of specific clone(s) (35, 36, 42) or the presence of oligoclonal populations (43), the analysis with the Immunoscope technique always revealed perturbations of the CDR3 size profile. Therefore, the Gaussian-like profile obtained for CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> thymocytes selected by a single peptide-MHC class II complex indicates that the repertoire is polyclonal.

*Comparison of the  $\alpha/\beta$  Repertoire of CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> Thymocytes from Transgenic Mice for the E $\alpha$ 52-68 Peptide-I-A<sup>b</sup> Complex and C57Bl/6 Mice.* Repertoire analysis was performed on CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> thymocytes from C57Bl/6 mice as described in previous sections. CDR3 size distributions obtained with C $\beta$  or C $\alpha$  primers for each V $\beta$  or V $\alpha$  chain were compared with those from cells selected by the single peptide-MHC class II complex (Fig. 2 A and data not shown). Our results show that for all V $\beta$  and V $\alpha$ , the profiles are superimposable. On Fig. 2 A

are shown representative results for V $\beta$ 4, V $\beta$ 8.2, V $\beta$ 10, V $\beta$ 11, V $\beta$ 12, and V $\beta$ 14.

A more detailed analysis of V $\beta$ -J $\beta$  rearrangements was performed for V $\beta$ 4, V $\beta$ 8.2, V $\beta$ 10, V $\beta$ 11, V $\beta$ 12, and V $\beta$ 14. Representative results obtained with V $\beta$ 11 are shown on Fig. 2 B. The repertoires were superimposable and no significant modification was found with any J $\beta$  primer.

*Similar Usage of J $\beta$  Gene Segments by TCR from CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> Thymocytes Selected in Wild-type Mice or in Transgenic Animals Expressing a Single Peptide-MHC Class II Complex.* The relative J $\beta$  usage in the V $\beta$ 11, V $\beta$ 12, and V $\beta$ 14 T cell population measured from one individual mouse of each strain (B2L and C57Bl/6) is shown in Fig. 3. The ratio of the area of the peaks generated with a given J $\beta$  primer to the areas of all the J $\beta$  primers was calculated as a measure of the relative frequency of J $\beta$  usage. This analysis does not reveal any significant evidence of selection for a particular J $\beta$  and shows that the J $\beta$ 2 segments are used more frequently than the J $\beta$ 1 segments as previously de-



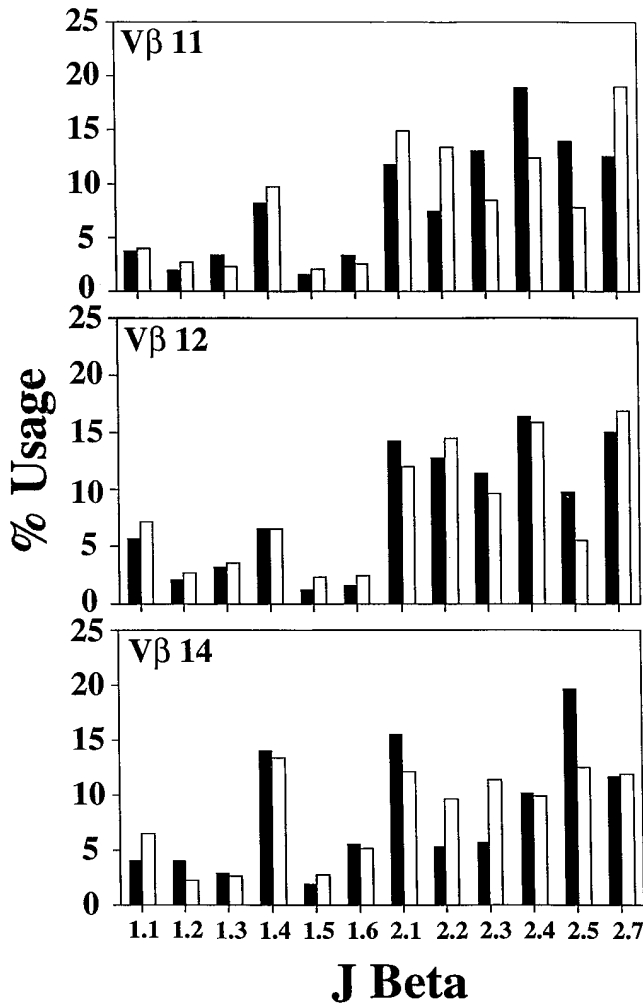
**Figure 2.** Comparison of the fluorescent runoff products profiles obtained from CD4<sup>+</sup> mature thymocytes from single chain peptide–MHC class II complex Tg (solid lines) and C57Bl/6 mice (dotted lines). Runoff extensions were performed for Vβ4, Vβ8.2, Vβ10, Vβ11, Vβ12, and Vβ14 with internal Cβ-specific fluorescent primer (A) and for Vβ11 with 12 Jβ-specific primers (B). The intensity of fluorescence is represented in arbitrary units as a function of CDR3 size in amino acids. Two mice were tested independently for each repertoire. Results were superimposable for the two mice tested in each combination.

scribed in the normal repertoire (44). Altogether, these results strongly suggest that the T cell repertoire selected by a single peptide–MHC complex is indistinguishable in terms of Vβ, Jβ, and Vα usage and CDR3 size distribution from the one selected by multiple peptide–MHC class II complexes.

*Sequence Complexity in a Given Vβ–Jβ Rearrangement with a Defined CDR3 Length.* Each peak corresponding to a given CDR3 length contains multiple distinct sequences. To evaluate the sequence complexity of the repertoire from CD4<sup>+</sup> T cells selected by the Eα52–68 peptide–I–A<sup>b</sup> complex, we have chosen to focus arbitrarily on two particular rearrangements with a given CDR3 length: Vβ11–Jβ1.1 with a CDR3 length of six amino acids and Vβ12–Jβ1.1 with a CDR3 length of eight amino acids. These two Vβs are well selected in B2L mice, but reasonably abundant in both B2L and C57Bl/6 animals so that the anticipated complexity would be more amenable to our analysis. The same consideration guided the choice of the CDR3 lengths.

Each peak corresponding to these rearrangements was isolated and purified. The sequence mixture contained in these peaks was cloned and every positive clone was se-

quenced. More than 700 sequences were collected. We found that, in the two rearrangements tested, the number of different sequences collected from each sample is in the same range of magnitude, with a mean of 30 different sequences in the Vβ11–Jβ1.1 peak with a CDR3 length of six amino acids and a mean of 70 different sequences in the Vβ12–Jβ1.1 peak with a CDR3 length of eight amino acids (Table 1). Hence, sequence diversity contained in a given Vβ rearrangement with a particular length is the same between wild-type mice and Tg. Since we could estimate the number of different sequences contained in a single peak, we calculated the minimal number of rearrangements positively selected by the Eα52–68–I–A<sup>b</sup> complex. The Vβ11–Jβ1.1 combination with a CDR3 length of six amino acids represents 13% of the rearrangements using the Vβ11 and Jβ1.1 gene segments. The Jβ1.1 is used in 5% of the rearrangements involving the Vβ11 gene segment. Staining with specific monoclonal antibody shows that 5% of the CD4<sup>+</sup>CD8<sup>–</sup>NK1.1<sup>–</sup>HSA<sup>–</sup> thymocytes from the Tg bear the Vβ11 chain (26). We then estimated that a minimum of 10<sup>5</sup> TCR–β rearrangements (30 × 7.7 [to correct for the CDR3 length] × 20 [to correct for all the Jβs] × 20 [to correct for all the Vβs]) are selected by the peptide–



**Figure 3.** J $\beta$  usage of V $\beta$ 11, V $\beta$ 12, and V $\beta$ 14 of CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> thymocytes from B2L Tg (black bars) and C57Bl/6 mice (white bars). In the abscissa are represented all members of the J $\beta$  family. In the ordinate is plotted the relative frequency of J $\beta$  usage, calculated as described in Materials and Methods. The results were generated from one mouse of each strain.

MHC class II complex. The same calculation was applied for the V $\beta$ 12-J $\beta$ 1.1 rearrangement and a total of  $7 \times 10^4$  TCR- $\beta$  rearrangements were found.

**Recurrent Sequences of the V $\beta$  Chain CDR3 Region from CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> Thymocytes of Wild-type Mice or Transgenic Animals Expressing E $\alpha$ 52-68-I-A<sup>b</sup> Complex.** Among the 700 sequences collected, we found some recurrent sequences between animals. The results obtained with the V $\beta$ 11-J $\beta$ 1.1 and V $\beta$ 12-J $\beta$ 1.1 rearrangements are shown in Fig. 4. For the V $\beta$ 11-J $\beta$ 1.1 rearrangement, only one recurrent amino acid sequence was found in all animals. This CDR3 is generated by the same nucleotide sequence and is encoded by the germline segments without trimming or N nucleotide addition (data not shown). For the V $\beta$ 12-J $\beta$ 1.1, four recurrent sequences were found between the C57Bl/6 mouse and one Tg, and seven between the same C57Bl/6 mouse and another Tg. Between the two Tg, nine amino

**Table 1.** Sequence Complexity in a Given V $\beta$ -J $\beta$  Rearrangement with a Defined CDR3 Length

Mice	Total No. of sequences	No. of different sequences	No. of unique sequences
V $\beta$ 11-J $\beta$ 1.1 CDR3 length: six amino acids			
C57Bl/6	159	34	12
C57Bl/6	180	27	0
Tg	33	25	18
Tg	58	34	21
V $\beta$ 12-J $\beta$ 1.1 CDR3 length: eight amino acids			
C57Bl/6	112	73	44
Tg	95	81	68
Tg	81	60	50

acid sequences of the CDR3 were recurrent. Among these, two CDR3 amino acid sequences were common to all animals tested: SLGANTEV and SLTANTEV (see Fig. 4). The SLGANTEV sequence was found one time in the C57Bl/6 animal, four times in one Tg, and two times in the other Tg tested (Table 2). Nucleotide sequence analysis of these rearrangements revealed that in each mouse, the sequence was generated differently by the recombination machinery (see Table 2). From the C57Bl/6 mice, the coding sequence was obtained with the trimming of three nucleotides from the V $\beta$ 12 germline segment, the usage of four nucleotides from the D $\beta$ 1 segment, 1 P nucleotide addition between the D and the J segments, and the germline sequence of the J $\beta$ 1.1 gene segment. This specific rearrangement was never found in the two Tg tested. In these two animals, recombination events (V $\beta$  trimming, D $\beta$  usage, N nucleotide addition) were different (see Table 2). However, one should notice that recurrent nucleotide sequences were also found between Tg. Comparable conclusions were reached when we analyzed the nucleotide sequences encoding the recurrent SLTANTEV (Table 2). Altogether, these results strongly suggest that these two CDR3 regions are not preferentially generated by the recombination machinery, but are positively selected at the amino acid level.

**Comparison of the Amino Acid Frequency at Different Positions in the CDR3 Region of V $\beta$ -J $\beta$  Rearrangements from CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> Thymocytes of Wild-type Mice or Transgenic Animals Expressing E $\alpha$ 52-68-I-A<sup>b</sup> Complex.** We compared the amino acid usage in the CDR3 region of the two sequenced rearrangements V $\beta$ 11-J $\beta$ 1.1 (CDR3 length of six amino acids) and V $\beta$ 12-J $\beta$ 1.1 (CDR3 length of eight amino acids) of the CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> thymocytes from Tg and wild-type mice. No difference was detected between mice when the individual percentage of amino acids in the CDR3 was plotted (data not shown). However, interesting differences became apparent when we plotted the frequency of individual amino acids at each position in the CDR3 loop (Figs. 5 and 6). Frequencies were calcu-

Vβ11-Jβ1.1 CDR3 6 aa

	Tg#1	Tg#2	B6#2
B6#1	SLDTEV SFNTEV SQNTEV	SLDTEV SPHTEV SQNTEV	SLDTEV SRHTEV SLRTEV
B6#2	SLDTEV	SSGATV SLDTEV	
Tg#2	SLVTEV SLKGV SLDTEV SQNTEV		

Vβ12-Jβ1.1 CDR3 8 aa

	Tg#1	Tg#2
B6	RGTANTEV SLGANTEV SLTGNTEV SLTANTEV	SYRTNTEV SLGNTEV SWNTEV SPGANTEV SLGANTEV SLTANTEV SWGANTEV
Tg#2	SLGANTEV SLQGNTEV SLTANTEV SLQANTEV SLARGTEV RROGNTEV SLGQGNTEV SLVGNTEV SLGNTEV	

**Figure 4.** Recurrent sequences of the Vβ chain CDR3 region from CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> thymocytes of wild-type mice or transgenic animals expressing Eα52-68-I-A<sup>b</sup> complex are displayed. Recurrent sequences between individual animals are shown and the sequences common to all animals are in bold.

lated with nonredundant sequences in order to eliminate bias due to overrepresented sequences. Positions 2 and 3 for the Vβ11-Jβ1.1 rearrangement and 2, 3, and 4 for the Vβ12-Jβ1.1 rearrangement are more variable due to N or P nucleotide additions and reading frame usage of the Dβ gene segment. One can notice that for each variable position, a limited number of amino acid residues are found and that they are similar in all mice. Furthermore, the percentage of amino acid residues found at each variable position (2, 3, and 4) is strikingly similar between C57Bl/6 mice and Tg. However, for the Vβ11-Jβ1.1 rearrange-

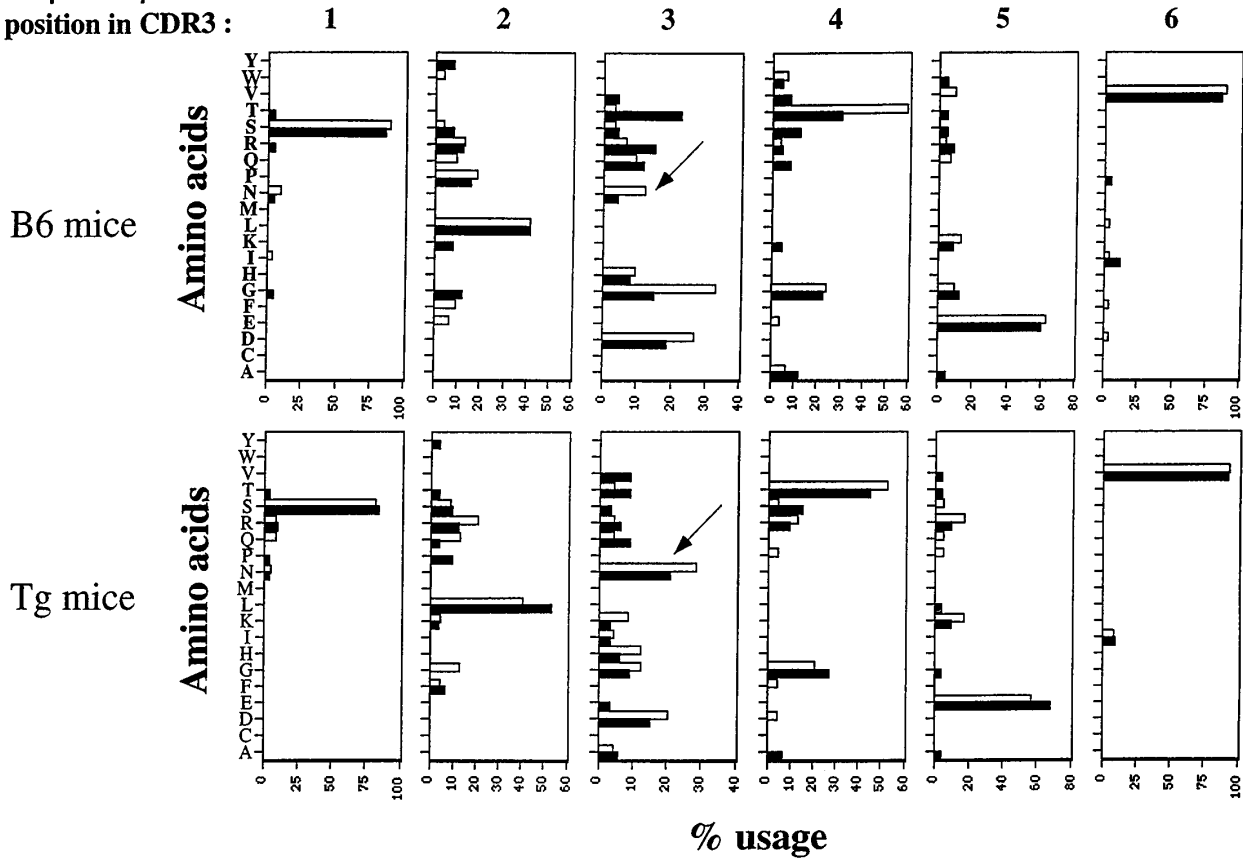
ment, it appears that at position three of the CDR3 from Tg, there is an increase in the frequency of the asparagine residue as compared with wild-type mice (24 versus 8%; see Fig 5). Similar skewing is also found with the Vβ12-Jβ1.1 rearrangement. When comparing amino acid frequency between wild-type mice and Tg, we found an increase in leucine at position 2, threonine and glutamine at position 3, and glycine at position 4 of the CDR3 loop of transgenic animals (Fig. 6). In contrast, in C57Bl/6 mice, glycine is preferentially found in position 3 of the CDR3. Analysis of nucleotide sequences encoding glycine or glutamine at this position shows that both amino acids are encoded by the Dβ1.1 gene segment in all CDR3s. Glycine can be encoded by all three Dβ1.1 reading frames. There are six possibilities to code for glycine. In Tg and wild-type mice, all encoding possibilities are used (Fig. 7), indicating that there is no bias due to the recombination machinery between these mice. Strikingly, in Tg animals, glutamine is found more frequently at this position (Fig. 6) even though this residue is only encoded by frame 2 of the Dβ1.1 gene segment. Altogether these results suggest that this residue has been selected for at the amino acid level. Furthermore, the preferential usage of the Dβ1.1 frame 2 in the transgenic animals should favor the appearance of a glycine residue at position 4. This increase in glycine frequency at position 4 is observed in transgenic animals and not in C57Bl/6 (Fig. 6). These differences in amino acid usage at different positions of the β chain CDR3 junctions reveal the imprint of the Eα52-68 peptide-I-A<sup>b</sup> complex on the selected T cell repertoire.

**Table 2.** Nucleotide and Amino Acid Translated Sequences of Vβ12-Jβ1.1 Recurrent Rearrangements with a CDR3 Length of Eight Amino Acids

Germline	Vβ12	N/P	Dβ1	N/P	Jβ1.1
C57Bl/6	TGT GCC AGC AGT TTA GC	GGG ACA GGG GGC	CA AAC ACA GAA GTC TTC		
1×*	TGT GCC AGC AGT TT	GGG A	G CA AAC ACA GAA GTC TTC	CASSLGANTEV	
Tg1	1× TGT GCC AGC AGT TTA G	GGG	CA AAC ACA GAA GTC TTC	CASSLGANTEV	
3×	TGT GCC AGC AGT TTA G	G GGC C	AAC ACA GAA GTC TTC	CASSLGANTEV	
Tg2	2× TGT GCC AGC AGT TTA G	GGG	CA AAC ACA GAA GTC TTC	CASSLGANTEV	
C57Bl/6	1× TGT GCC AGC AGT TTA	ACA G	CA AAC ACA GAA GTC TTC	CASSLTANTEV	
Tg1	2× TGT GCC AGC AGT TT	G ACA G	CA AAC ACA GAA GTC TTC	CASSLTANTEV	
Tg2	1× TGT GCC AGC AGT TTA	ACA G	CA AAC ACA GAA GTC TTC	CASSLTANTEV	
1×	TGT GCC AGC AGT TTA	AC	GG CA AAC ACA GAA GTC TTC	CASSLTANTEV	
3×	TGT GCC AGC AGT TT	G ACA G	CA AAC ACA GAA GTC TTC	CASSLTANTEV	

\*No. of times this sequence was found in the animal.

# V $\beta$ 11-J $\beta$ 1.1 position in CDR3 :



**Figure 5.** Amino acid frequency at the different positions in the CDR3 region of V $\beta$ 11-J $\beta$ 1.1 (length: six amino acids) rearrangements from CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> thymocytes. *Top*, the results obtained from two independent C57Bl/6 mice; *bottom*, the results obtained from two independent Tg. Amino acid frequencies were calculated with nonredundant sequences to avoid skewing due to overrepresented sequences (34 and 27 sequences and 25 and 34 sequences were used for C57Bl/6 mice and Tg, respectively).

## Discussion

*At Least 10<sup>5</sup> Distinct TCR- $\beta$  Rearrangements Are Selected by the Single MHC-Peptide Complex Displayed in B2L Mice.* The involvement of peptides presented by MHC molecules in the process of thymic positive selection of T cells has been investigated *in vivo* (25, 26, 45–49) and *in vitro* (11, 12, 14, 50). Recent studies (23, 24, 27) have shown that positive selection is a promiscuous process enabling the selection of many different TCR rearrangements by a single peptide–MHC class II complex, but their diversity has not been quantified so far. Here, we have estimated the number of TCR rearrangements in mature thymocytes positively selected in Tg B2L mice (26) designed to express a single peptide–MHC class II complex (the E $\alpha$ 52-68 peptide hooked to I-A<sup>b</sup>). Their Ii<sup>-/-</sup> genetic background guarantees that the physiological route of peptide loading is blocked (29). Similar mice, engineered by Ignatowicz et al. (25), have been shown (contrary to H-2M<sup>-/-</sup> mice) to present no other detectable self-peptides (22).

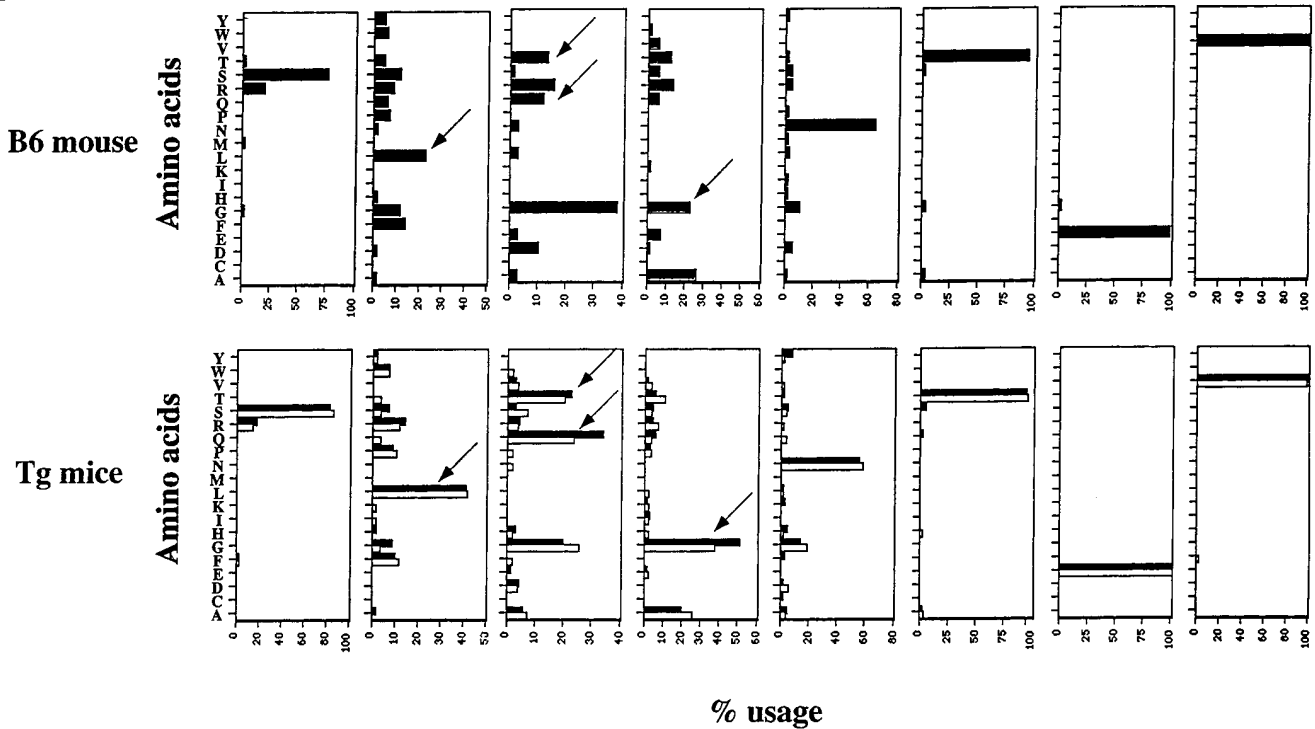
B2L mice express low levels of the E $\alpha$ 52-68–I-A<sup>b</sup> complex in their thymus and on ~5–10% of their splenocytes and positively select a number of CD4<sup>+</sup> T cells, which is

~20–50% of C57Bl/6 (26). We analyzed RNA from CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> T cells isolated from C57Bl/6 and from B2L mice by a PCR-based approach designed to determine the CDR3 lengths of the  $\beta$  and  $\alpha$  chains. All V $\beta$ -J $\beta$  combinations were used to visualize an overall picture of the  $\beta$  repertoire based upon some 2,000 measurements. Given the larger number of J $\alpha$  segments, the  $\alpha$  repertoire was not totally analyzed but sampled with 12 V $\alpha$  families. The normal mouse repertoire is characterized by bell-shaped, Gaussian-like, CDR3 length distributions (30). Distortions are observed upon antigenic stimulations that cause sufficient clonal expansion (34–36, 42). Oligoclonal distributions in pathological infiltrates are easily depicted (51–55). B2L CD4<sup>+</sup> T cell profiles were remarkably Gaussian-like and quasisuperimposable to wild type. The J $\beta$  usage for V $\beta$ 11, V $\beta$ 12, and V $\beta$ 14 closely matched that observed in C57Bl/6. Moreover, B2L and C57Bl/6 displayed overlapping CDR3 size distributions, even though the average CDR3 length is different for distinct V $\beta$  segments (30). No spikes suggesting clonal or oligoclonal expansions were detected. Therefore, the single MHC-pep-

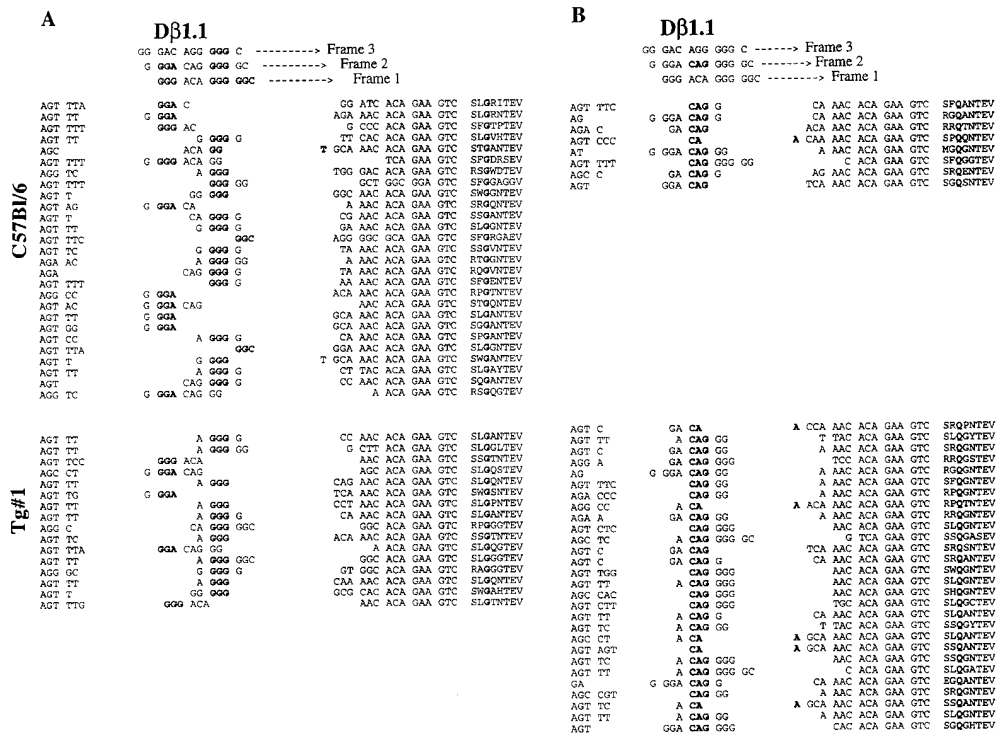


# Vβ12-Jβ1.1

position in CDR3 : 1 2 3 4 5 6 7 8



**Figure 6.** Amino acid frequency at the different positions in the CDR3 region of Vβ12-Jβ1.1 (length: eight amino acids) rearrangements from CD4<sup>+</sup>CD8<sup>-</sup>NK1.1<sup>-</sup>HSA<sup>-</sup> thymocytes. *Top*, the results obtained from one C57Bl/6 mouse; *bottom*, the percentage of usage obtained from two independent Tg. Amino acid frequencies were calculated with nonredundant sequences to avoid skewing due to overrepresented sequences (73 sequences and 81 and 60 sequences were used for C57Bl/6 mice and Tg, respectively).



**Figure 7.** Dβ reading frame usage coding for glycine or glutamine residues at position 3 of eight amino acids-long CDR3 regions of Vβ12-Jβ1.1 rearrangements. (A) The nucleotide sequences (*bold*) encoding a glycine residue at position 3 of the CDR3 region from C57Bl/6 (*top*) and Tg (*bottom*) thymocytes. (B) The nucleotide sequences (*bold*) encoding a glutamine residue at position 3 of the CDR3 region from C57Bl/6 (*top*) and Tg (*bottom*) thymocytes. All the sequences were aligned following the Dβ reading frame usage, regardless of the Vβ, Jβ gene segment trimming, and N addition.

ptide complex in B2L mice did not preferentially select a small number of clones over an otherwise polyclonal background.

We cloned and sequenced from B2L and C57Bl/6 mice the V $\beta$ 11-J $\beta$ 1.1 and V $\beta$ 12-J $\beta$ 1.1 size peaks with CDR3 lengths of six and eight amino acids, respectively. We could thus (Table 1) establish that the Tg and wild-type mice repertoires both include a minimum of  $10^5$   $\beta$  rearrangements, suggesting that the Tg repertoire is about as diverse as the wild-type one. In addition, the number of distinct  $\alpha$  chains capable of pairing with a given rearranged  $\beta$  chain has not been determined in physiological conditions. The work of Sant'Angelo et al. (56) showed that 20–30 different  $\alpha$  chains can associate with a unique transgenic rearranged  $\beta$  chain. If this figure can be extrapolated to physiological situations, the number of different TCRs would be much higher than  $10^5$ , leaving open the possibility that each positively selected CD4<sup>+</sup> thymocyte displays a unique TCR.

*The Transgenic Repertoire Is Skewed in at least Two Ways with Respect to the Wild-type Repertoire.* The above data show that the preferential selection of certain V $\beta$ s, observed in B2L mice by Fukui et al. (26), is not reflected in a few clonal expansions but in a larger number of rearrangements as revealed by the Gaussian distributions. This suggests that the E $\alpha$ 52-68 peptide prevents proper association with the subset of V $\beta$  segments that are not efficiently selected, that it is instrumental in binding those V $\beta$  segments that are selected (57), or both. The amino acid usage, compiled in Figs. 5 and 6, shows a second level of skewing now involving the CDR3 regions. We are confident that these differences in the amino acid composition at some CDR3 positions are significant. First, in conserved positions we find no variation of the amino acid usage between Tg and wild-type mice. Second, we evaluated the percentage of sequencing errors at conserved positions such as the C92<sup>-</sup>, A93<sup>-</sup>, S94<sup>-</sup>-encoded positions of the CDR3. The figure of 0.3% at each position cannot explain the results. Third, amino acid usage calculations were done with nonredundant sequences in order to avoid skewing due to overrepresentation. Fourth, such percentage variations are consistent from one mouse to another of the same lineage (Tg versus wild-type mice).

The elegant studies by Sant'Angelo et al. (56) have provided strong evidence for an imprint of the peptide in the process of positive selection. Here we had no direct way to evaluate the respective impact of positive and negative selection in shaping the B2L repertoire of CD4<sup>+</sup> thymocytes. However, the recurrent  $\beta$  chain rearrangements that we have observed (Fig. 4) are likely to reflect positive selection events, since they could hardly be the result of chance, or of a negative process that would delete all sequences but these ones. Remarkably, among these recurrent  $\beta$  chains, two were found in both B2L and C57Bl/6. They were encoded by nonidentical nucleotide sequences and generated

by distinct junctional events (Figs. 4 and 7), implying that they were selected at the amino acid level in a positive fashion. It is worth noting that C57Bl/6 mice do not express nor present the E $\alpha$ 52-68 peptide (58, 59). Therefore, in this case, the event that positively selected the  $\beta$  chain involved no peptide or self-peptides sharing amino acid residues with E $\alpha$ 52-68, or was mediated by the  $\alpha$  chain.

In the peripheral response to several defined antigens, one or a few specific CDR3 sequences, in given V $\beta$ -J $\beta$  and/or V $\alpha$ -J $\alpha$  combinations, have been found to be highly reproducible and shared by individual animals. They were named “public” (35) by analogy with recurrent idiotypes found in immunoglobulins (60). Whether these public rearrangements are selected in the periphery or in the thymus has not been determined so far. Since, as shown above, at least some recurrent rearrangements are positively selected in the thymus, it may be proposed that peripheral public rearrangements, in general, bear the imprint of thymic positive selection.

Altogether, our results also show that the impact of a peptide in positive selection may not be readily detected. First, our data indicate that the peptide may not be directly involved in all selection events regarding the  $\beta$  chain since the  $\alpha$  chain may also be involved in the selection process. This raises the possibility that not all TCR- $\beta$  rearrangements bear the imprint of the peptide. Second, in order to detect the latter, we had to focus on a specific V $\beta$ -J $\beta$  combination with a fixed CDR3 length, whereas Sant'Angelo et al. studied  $\alpha$  chain rearrangements associated with a  $\beta$  chain that had been fixed by transgenesis (56). This may explain some apparently conflicting results about peptide specificity in positive selection.

*How is the T Cell Repertoire Shaped in Wild-type Mice?* The physiological relevance of observations made in transgenic animals is questionable. Among the few reports on positive selection in nontransgenic animals (for review see reference 61), the comparison of positive selection of anti-ovalbumin TCR in K<sup>b</sup> and K<sup>bm8</sup> mutant mice has provided a clear indication for the involvement of presented peptides (45). The single peptide-MHC complex of B2L mice makes up  $\sim$ 10% of physiological complexes in the thymic cortex of B10-A(5R) mice. According to Grubin et al. (22), rare peptides (expressed, in their case, in the H-2M-deficient background) appear functional in positive selection whether the diversity of the T cell repertoire is built up only by the most abundant self-peptides like E $\alpha$ 52-68, or by rarer self-peptides as well. In the latter case, it remains to be determined to which extent the diversity of thymocytes is increased by rarer peptides in a cumulative fashion. We are currently performing an extensive repertoire analysis of B10-A(5R) mice in order to evaluate whether and to which degree it includes or overlaps with that of B2L animals.

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