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TCRβ and TCRα gene enhancers confer tissue- and stage-specificity on V(D)J recombination events

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We describe transgenic mice carrying germline variable gene segments associated with either the T cell receptor (TCR) β or α gene enhancers (Eβ or Eα). Transgenic constructs underwent high rates of site-specific rearrangements predominantly in T cells from independent mice. Rearrangements of the Eβ-containing transgenes began at different stages of T cell differentiation in embryonic and adult thymus than did the Eα-containing ones, with a pattern superimposable upon the patterns of TCRβ or TCRα gene expression, respectively. We demonstrate that sequences within the TCRβ and TCRα gene enhancers confer tissue- and stage-specificity upon the V(D)J recombination events affecting adjacent gene segments. The patterns of transgene expression also gave information on developmental events and lineage relationships (γδ versus αβ) during T cell development.
Key words: α gene enhancers/T cell differentiation/ TCRβ/VDJ recombination control

Introduction

Differentiation of T lymphocytes leads committed progenitors to two different subsets of mature T cells, bearing αβ or γδ T cell receptors (TCRs) on their cell surface. Differentiation along the αβ pathway occurs in the thymus through a series of complex steps involving negative and positive selection (Blackman *et al.*, 1990; von Boehmer, 1992); the γδ precursors are also present in the thymus, but the relationship between the two lineages is not clear. During T cell ontogeny, γδ cells appear first, representing the majority of TCR^+ thymocytes until day 16 of mouse gestation. After day 17, the proportion of γδ T cells diminishes with a concomitant increase of αβ T cells which predominate after birth and throughout adult life. The late appearance of αβ T cells is explained by the timing of the onset in expression of the TCRα gene, at day 17 of mouse gestation, which is later than the expression of the TCRγ, δ and β genes (Strominger, 1989; Allison and Havran, 1991).

During the early stages of T cell ontogeny and differentiation, genes that encode TCR variable regions are assembled somatically from germline gene segments. Like immunoglobulin (Ig) variable region genes in the B cell lineage, TCR variable region genes are assembled either from variable (V), diversity (D) and joining (J) segments (TCRβ and δ genes), or from V and J segments (TCRα and γ genes) (Lai *et al.*, 1989; Raulet, 1989). TCR and Ig variable gene segments are flanked by conserved signal sequences that target a site-specific recombination activity referred to as VDJ recombinase; this activity probably involves the interaction of lymphoid-specific and ubiquitous gene products (Alt *et al.*, 1992; Schatz *et al.*, 1992).

Recombination and transcription of TCR and Ig genes frequently correlate in developing lymphocytes. Accordingly, transcription of partially rearranged or unrearranged TCRβ and α gene loci have been described in cells undergoing TCRβ or α gene rearrangements (Snodgrass *et al.*, 1985; Fondell and Marcu, 1992). Recombinational and transcriptional activities are tissue-specifically and temporally regulated: for example, TCRβ and α variable gene segments are rearranged and transcribed in T-lineage cells only, with TCRβ variable regions being assembled before TCRα variable ones. These selective events may result from a modulation in the accessibility of the gene loci to the VDJ recombinase (Alt *et al.*, 1987). Although experimental results suggested that transcription increases V(D)J recombination of nearby variable segments (Blackwell *et al.*, 1986; Schlissel and Baltimore, 1989; Schlissel *et al.*, 1991), it is still controversial whether transcription plays an active role in inducing accessibility to the VDJ recombinase.

Transcription of Ig and TCR genes depend on *cis*-acting, lymphoid-specific, regulatory elements (Clevers and Owen, 1991; Libermann and Baltimore, 1991; Staudt and Lenardo, 1991; Leiden, 1992). They include the recently identified TCRβ and TCRα transcriptional gene enhancers (Krimpenfort *et al.*, 1988; McDougall *et al.*, 1988; Ho *et al.*, 1989; Winoto and Baltimore, 1989a; Gottschalk and Leiden, 1990). These enhancer elements are composed of multiple domains that bind T cell-specific and ubiquitous nuclear proteins (Ho *et al.*, 1989; Winoto and Baltimore, 1989a; Gottschalk and Leiden, 1990; Ho and Leiden, 1990; Takeda *et al.*, 1990). Interestingly, the purification of some of these factors has shown that a few of them bind to sequences shared by several lymphoid enhancers (Ho *et al.*, 1990, 1991; Waterman and Jones, 1990; Marine and Winoto, 1991; Ooesterwegel *et al.*, 1991; Travis *et al.*, 1991; van de Wetering *et al.*, 1991; Waterman *et al.*, 1991). Although these results support a model in which enhancers, through the binding of developmentally expressed or activated nuclear proteins, are involved in the coordinated expression of TCR genes, direct experimental data are still missing; notably, no evidence on the role of TCR gene enhancers on V(D)J recombination has been reported so far.

To identify *cis*-regulatory elements able to modulate the accessibility of nearby variable gene segments to the VDJ recombinase, a transgenic model has previously been developed, using a recombination substrate (Ferrier *et al.*, 1990). It was shown that the occurrence of lymphoid-specific

rearrangements within the reporter transgene was dependent on a *cis*-acting DNA fragment, behaving as a 'recombination enhancer'; significantly, this fragment contained the intronic transcriptional enhancer (Eμ) of the Ig heavy (H) chain gene. To address the hypothesis that TCR gene enhancers modulate the accessibility of their respective loci to the VDJ recombinase, TCRβ or TCRα gene enhancers (Eβ or Eα) were substituted for the Eμ-containing fragment within the recombination substrate. We report that both elements promote high levels of substrate rearrangements predominantly in T-lineage cells of independent lines of transgenic mice. Depending on Eβ or Eα, substrate rearrangements occurred at different stages of T cell ontogeny/differentiation with a pattern superimposable on normal TCRβ or TCRα gene expression. These experiments imply that the Eβ and Eα elements regulate the tissue-specificity and order of rearrangements of the TCRβ and α genes. The results also throw some light on developmental events and lineage relationships during T cell development.

Results

Generation of mice carrying TCR Eβ- or Eα-containing substrates

We constructed two recombination substrates from genomic fragments of the murine TCRα, TCRβ and IgH loci. The two substrates are similar to previously described constructs (Ferrier *et al.*, 1989a, 1990) and comprise a 7.7 kb variable minilocus composed of germline Vβ, Dβ and Jβ gene segments linked to a 10.5 kb IgH region containing the Cμ gene (Figure 1). In between the variable (VT) and constant (Cμ) regions, the substrates include either a 560 bp fragment (VTEβCμ construct) containing the TCRβ gene enhancer (Krimpenfort *et al.*, 1988), or a 515 bp fragment (VTEαCμ construct) containing the TCRα gene enhancer (Winoto and Baltimore, 1989a). The various types of rearrangement (Dβ to Jβ, Vβ to DβJβ or to Jβ, and Vβ to Dβ) that could theoretically occur by deletion within the variable minilocus are depicted in Figure 1.

DNA fragments (of ~20.7 kb) containing the VTEβCμ or VTEαCμ inserts were microinjected into pronuclei of fertilized (C57Bl/6J × CBA/J) F2 mouse eggs. Transgenic lines were derived from founders Eβ2, Eβ3, Eβ6, Eβ10 (VTEβCμ construct) and Eα1, Eα3, Eα6 (VTEαCμ construct) by crosses with (C57Bl/6J × CBA/J) F1 mice. Transgenic animals in the individual lines were shown to carry mostly intact copies of the substrates integrated in a head-to-tail configuration at a single site; number of intact copies varied from three to 85, depending on the line (data not shown and Figure 3B). Subsequent analyses were mainly conducted on lines Eβ2, Eβ6, Eα3 and Eα6.

Substrate rearrangements in tissues of the transgenic mice

To assay for rearrangements within the integrated substrates, genomic DNA from lymphoid (thymus, lymph nodes and spleen) and non-lymphoid (kidney) tissues of the transgenic mice was digested with appropriate restriction enzymes and tested for hybridization to probes specific for the Vβ and Jβ regions present in the construct. Site-specific

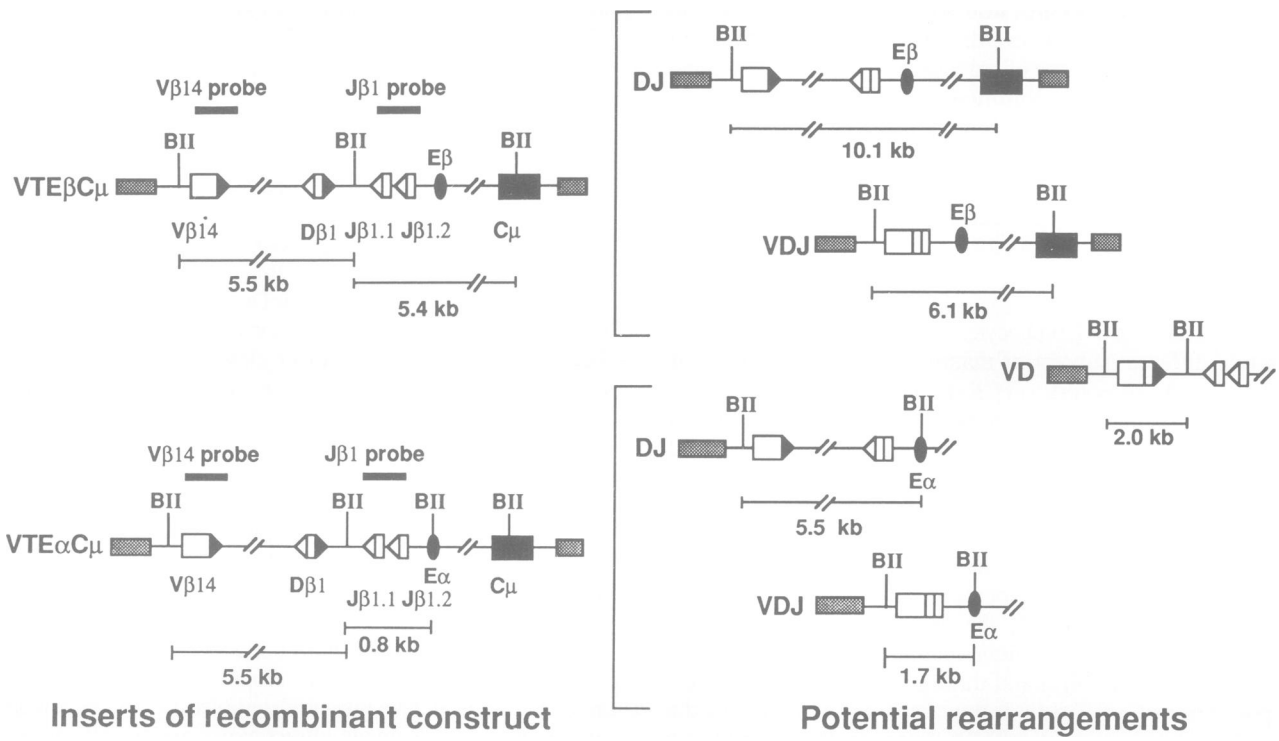

Fig. 1. Partial restriction endonuclease maps of the recombination substrates. Inserts of construct VTEβCμ (top) and VTEαCμ (bottom) are represented on the left; structures of the miniloci following potential VDJ recombinase-mediated rearrangements are on the right. DJ: DβJβ rearrangements; VDJ: Vβ(Dβ)Jβ rearrangements (only joins to Jβ1.2 gene segment are represented, joins to Jβ1.1 gene segment give larger fragments (by ~130 bp); VD: VβDβ rearrangements. The V, D and J variable gene segments are indicated by open boxes, their flanking recombination signal sequences by shaded (23 bp spacer) or open (12 bp spacer) triangles, the Eβ or Eα enhancers by shaded circles, the Cμ exons by a shaded box and the cosmid sequences by hatched boxes. Examples of the size of the *Bgl*II restriction fragments expected—when using the Jβ1 and/or Vβ14 probes—in Southern analyses of genomic DNA of the transgenic mice are shown below each map; BII: sites of restriction endonuclease *Bgl*II (not all *Bgl*II sites are shown).

rearrangements within both VTEβCμ and VTEαCμ transgenes were inferred from the labelling of fragments of predicted size in DNA from lymphoid versus non-lymphoid tissues.

Figure 2 shows representative data, obtained using a Jβ1 probe and *Bgl*II-digested DNAs from adult mice in transgenic lines Eβ2, Eβ6 (Figure 2A) and Eα1, Eα3 and Eα6 (Figure 2B). Thus, all transgenic animals contained significant proportions of DβJβ- and (to a lesser extent) Vβ(Dβ)Jβ-sized fragments in DNA from thymus and lymph nodes (Figure 2A, lanes 3, 6 and 9; Figure 2B, lanes 2, 6, 7 and 10), but not in DNA from kidney (Figure 2A, lanes 4 and 10; Figure 2B, lanes 3, 4 and 11). Those same fragments could also be detected at lower levels in spleen DNA (Figure 2A, lanes 5 and 11; Figure 2B, lanes 1 and 5; rearrangements were more evident on longer exposures). As expected, the Jβ1 probe also hybridized: (i) in all transgenic tissues, to fragments of either 5.4 kb (Figure 2A) or 0.8 kb (Figure 2B), corresponding to the unrearranged

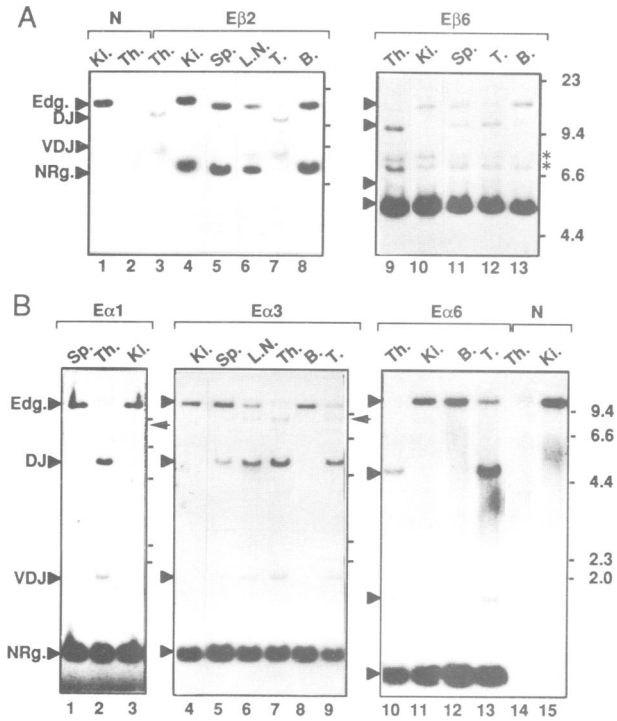

Fig. 2. Southern analysis of the Jβ-containing region in the transgenes. Genomic DNA (10 μg) from tissues or from peripheral lymphocytes of a 4 week old transgenic mouse in the indicated lines was digested with *Bgl*II and assayed by Southern blot for hybridization to the ^{32}P-labelled Jβ1 probe; (N): (C57Bl/6J × CBA/J) F1 mouse DNAs used as controls. Ki.: kidney; Th.: thymus; L.N.: lymph nodes; Sp.: spleen; T.: T-enriched cells; B.: B-enriched cells. FACS analyses were as follows: (Eβ2) T cells: (92% CD3$^+$/1% Ig$^+$), B cells: (83% Ig$^+$/4% CD3$^+$); (Eβ6) T cells: (82% CD3$^+$/5% Ig$^+$), B cells: (80% Ig$^+$/6% CD3$^+$); (Eα3) T cells: (84% CD3$^+$/3% Ig$^+$), B cells: (86% Ig$^+$/2% CD3$^+$); (Eα6) T cells: (80% CD3$^+$/4% Ig$^+$), B cells: (83% Ig$^+$/4% CD3$^+$). The positions of the *Bgl*II fragments containing the endogenous Jβ1 gene cluster (Edg.: endogenous), the unrearranged substrate (NRg.: non-rearranged) and the predicted normal rearrangements within the substrates [DJ and VDJ: DβJβ and Vβ(Dβ)Jβ rearrangements, respectively] are indicated on the left. Stars indicate truncated copies of the transgenes in line Eβ6; arrows indicate a 9.0 kb fragment seen in lines Eα1/Eα3. This latter fragment might result from joins involving Vβ and Dβ segments on separate constructs, as already described (Ferrier *et al.*, 1990). Fragment sizes of *Hin*dIII-digested λ phage DNA are indicated in kb on the right.

VTEβCμ or VTEαCμ transgenes, respectively; and (ii) in all tissues from normal or transgenic mice, to a 12 kb fragment containing the endogenous Jβ1 cluster in a germline configuration (hybridization is less intense in thymuses, due to TCRβ rearrangements in most adult thymocytes—Snodgrass *et al.*, 1985).

Hybridization of the same or other blots with the Vβ14 probe yielded concordant results (for example, see Figure 3A) and indicated lower levels of VβDβ rearrangements, suggesting that recombination events occur sequentially [DβJβ before Vβ(Dβ)Jβ] within the transgenes. However, VβDβ joins appeared consistently higher in Eα than in Eβ transgenic lines (Figure 3A and B). Eventually, additional Jβ1- and/or Vβ14-hybridizing fragments were detected in lymphoid tissues of the transgenic mice (for example, see Figure 2B, lanes 2, 5, 6 and 7; Figure 3A, lanes 4, 8 and 10). Their characteristics (size and hybridization pattern) suggested that they correspond to other types of VDJ recombinase-mediated events within the substrates, as previously reported (Ferrier *et al.*, 1990). Substrate rearrangements were observed at the highest rate (83%) in Eβ2 thymuses; in thymuses from other lines, rearrangements averaged almost one-third of total substrate (Figure 3B).

Substrate rearrangements in subpopulations of lymphoid cells

For both types of transgene, the intensities of the DβJβ- and Vβ(Dβ)Jβ-containing fragments varied in parallel among different lymphoid tissues in the same transgenic line. They were highest in thymus, lower in lymph nodes and even lower in spleen (Figure 2 and data not shown). The amounts of DβJβ and Vβ(Dβ)Jβ rearrangement thus decrease in parallel with the size of the T cell compartment in these organs (Hokama and Nakamura, 1982), suggesting that joining events within the constructs occur preferentially in the T cell lineage. This was surprising since previous studies had shown that Dβ to Jβ joins proceeded efficiently within substrates introduced into B cells, with only Vβ to (Dβ)Jβ joins being restricted to T cells (Ferrier *et al.*, 1989a, 1990). To clarify this issue, we purified peripheral T and B lymphocyte populations from the spleen and lymph nodes of transgenic lines Eβ2, Eβ6, Eα3 and Eα6. As expected, levels of DβJβ and of Vβ(Dβ)Jβ rearrangement found in the T cell enriched populations were roughly equivalent to those found in the corresponding thymus (Figure 2A: lanes 7 and 3 and 12 and 9; Figure 2B: lanes 9 and 7 and 13 and 10), whereas Vβ(Dβ)Jβ rearrangements were essentially absent in the B cell enriched populations (Figure 2A, lanes 8 and 13; Figure 2B, lanes 8 and 12). Moreover, in the latter populations, DβJβ rearrangements were also absent in the Eβ2, Eα3 and Eα6 lines or were present at a much lower level (as compared with thymus or T cells) in the Eβ6 line. Therefore, these data confirmed that T cells account for the vast majority of substrate rearrangements observed in the lymphoid tissues of the transgenic mice. However, the level of rearrangement found in B-enriched cells from the Eβ6 line (10% according to densitometric analysis) was higher than expected on the basis of T cell contaminants (see legend of Figure 2), suggesting that B cells might contribute to some DβJβ joins within the transgenes.

To analyse precisely the levels of substrate DβJβ rearrangement in T versus B cell lineages, we devised a

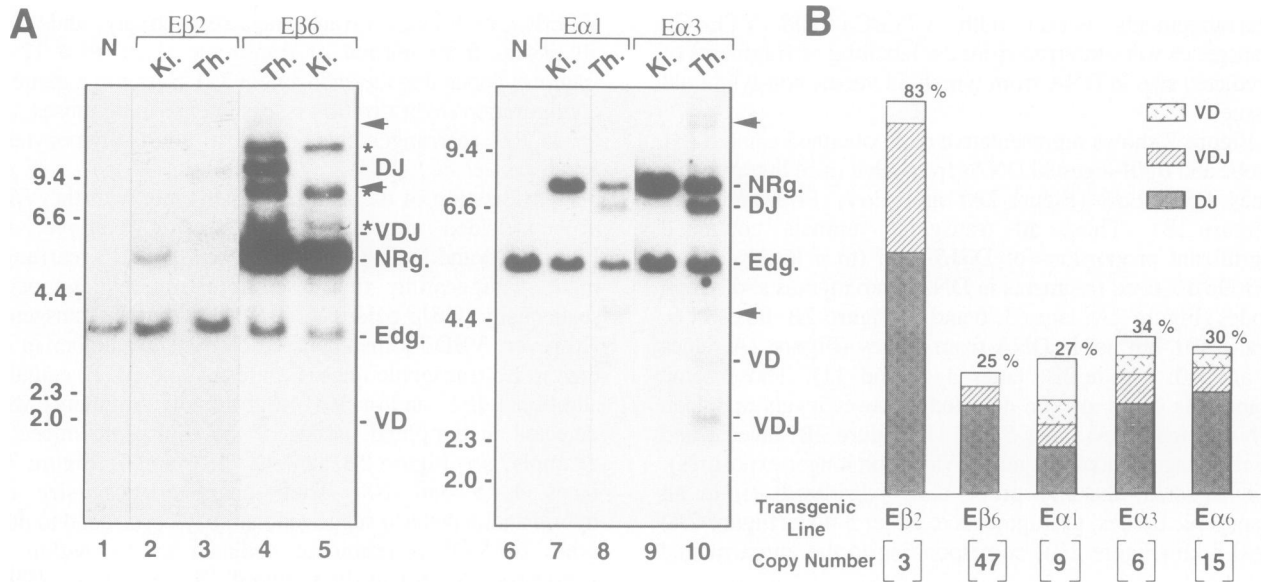

Fig. 3. Southern analysis of the Vβ14 gene region in the transgenes. (**A**) Examples of hybridization to the ^{32}P-labelled Vβ14 probe of *Bgl*II-digested (left panel) or *Pvu*II-digested (right panel) genomic DNA; N: kidney DNA from a (C57Bl/6J × CBA/J) F1 mouse; *Pvu*II digests are shown (instead of *Bgl*II) to individualize clearly the hybridizing fragments (in VTEαCμ, Vβ14-hybridizing unrearranged and DJ-rearranged *Bgl*II fragments co-migrate); *Pvu*II sites flank an ~7.0 kb fragment in the constructs, from 200 bp 5′ of the Vβ14 gene to 655 bp 3′ of the enhancer. Arrows indicate lymphoid-specific fragments which might represent: (i) pseudo-normal joins involving Vβ and Dβ segments on separate constructs (upper arrows) and (ii) aberrant deletional joins involving the Dβ segment (lower arrows), as already described (Ferrier *et al.*, 1990). (**B**) Graphic representation of substrate rearrangements in thymus; values were determined as mentioned in Materials and methods. The percentages of total substrate rearrangements within each line are indicated; the difference between these values and the sum of DJ + VDJ + VD substrate rearrangements (as indicated by the open rectangle on top of each column) most likely reflects the other types of VDJ recombinase-mediated joins mentioned in panel A.

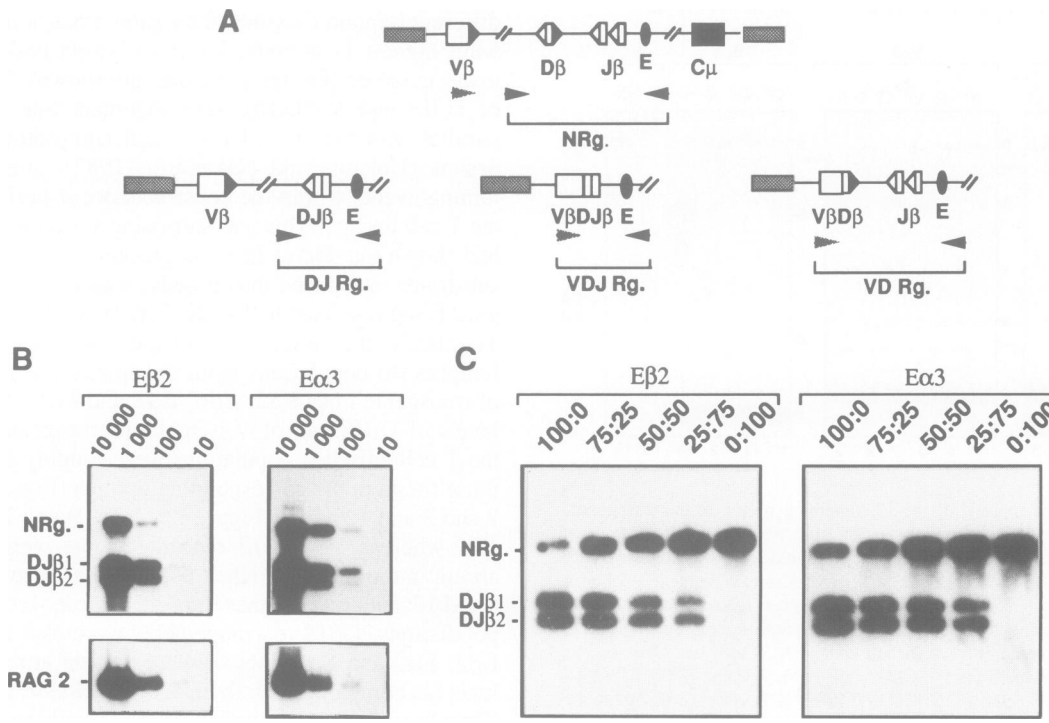

Fig. 4. DNA PCR assays. (**A**) Schematic representation of the PCR assays. Positions of oligonucleotide primers are indicated below diagrams of unrearranged and rearranged substrates; black arrows: Dβ and IgH primers; shaded arrow: Vβ primer. PCR products containing the unrearranged Dβ and Jβ segments (NRg.: non-rearranged; the unrearranged fragment corresponding to the Vβ/IgH primers is too large to be amplified) and DβJβ, Vβ(Dβ)Jβ and VβDβ rearrangements (Rg.: rearranged) expected—when using lymphoid cells from the transgenic mice—are schematized. (**B**) Analysis of decreasing number of transgenic adult thymocytes. (**C**) Titration of transgenic adult thymocytes: input DNA (equivalent to 2 × 10^3 cells) was kept constant using DNA from decreasing numbers of adult thymocytes and increasing amounts of kidney DNA in the proportions indicated above each lane. In panels B and C, PCR products were run on agarose gels, transferred to nylon membranes and hybridized with specific probes. B (top) and C: Jβ1-hybridization of PCR products following amplification with Dβ/IgH primers; DβJβ rearrangements appear as a doublet because the Dβ gene segment is joined to either Jβ1.1 or Jβ1.2 segments. B (bottom): PCR amplifications of a fragment in the RAG-2 gene (Oettinger *et al.*, 1990), used to estimate the amount of input DNA.

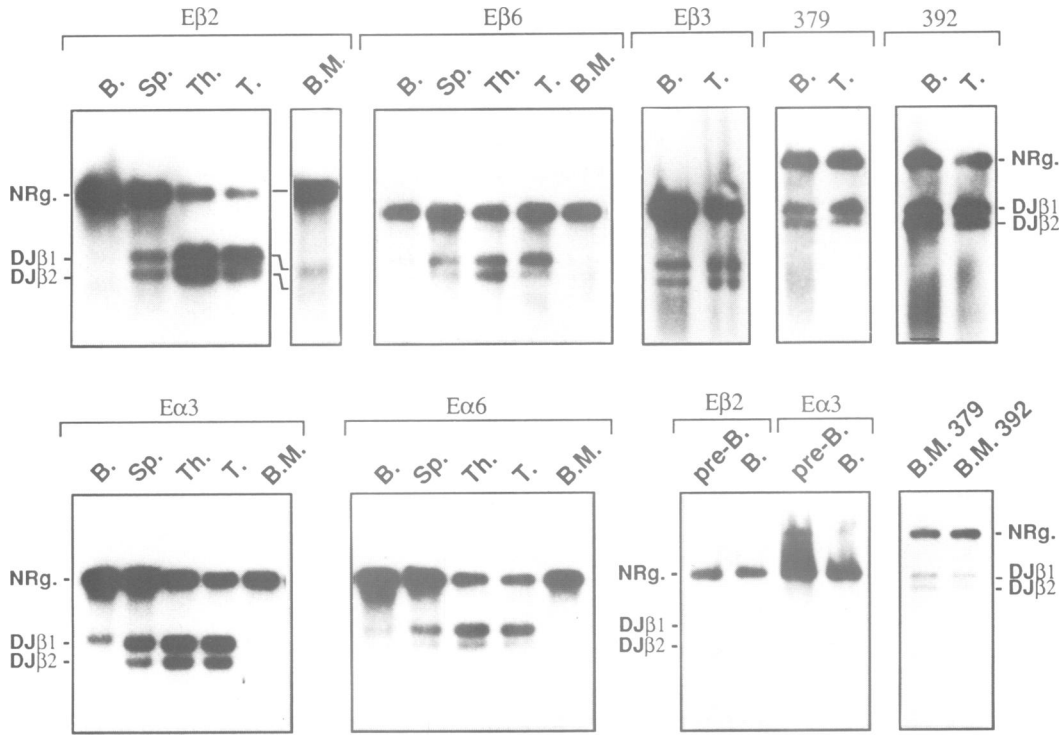

Fig. 5. Tissue-specificity of substrate DβJβ rearrangements. 2×10^3 cells from lymphoid tissues of 4 week old transgenic mice in the indicated lines were analysed as in Figure 4. Enriched-cell populations were as indicated in Figure 2, except for the Eβ2 B cells (top panels) which comprised 88% Ig^+ and 2% $CD3^+$ cells, or the Eβ6 B cells which were purified by cell sorting and were >98.5% pure. Other preparations were as follows: (379) T cells: (87% $CD3^+$/3% Ig^+), B cells: (89% Ig^+/4% $CD3^+$); (392) T cells: (83% $CD3^+$/4% Ig^+), B cells: (88% Ig^+/4% $CD3^+$). Eβ3 B and T cells (top panels) as well as Eβ2 and Eα3 B and pre-B cells (bottom panels) were purified by cell sorting and were >98.5% pure. Amplified fragments are larger in lines 379 and 392 due to the larger size of the enhancer-containing fragment in those transgenes.

quantitative PCR assay. This assay allows the simultaneous amplification of DNA fragments containing either unrearranged or rearranged Dβ and Jβ segments in the constructs (Figure 4A). Conditions were set up to get quantitative information, using thymocytes from adult transgenic mice in each line (see Materials and methods). Thus, the amount of amplified fragment decreased proportionally to the number of Eβ2 and Eα3 thymocytes used in the PCR reaction (Figure 4B). Moreover, the intensity of unrearranged versus rearranged fragments varied in opposite proportions when titrations were performed by diluting thymocyte DNA with genomic DNA from a non-lymphoid organ (Figure 4C), indicating that this assay can readily be used to compare levels of substrate DβJβ rearrangements. Similar results were obtained using thymocytes from Eβ6 and Eα6 transgenic mice (not shown).

Figure 5 shows results from analyses of cells isolated from lymphoid organs of transgenic (Eβ and Eα) mice and, as controls, of mice carrying an Eμ-containing substrate that undergoes significant levels of Dβ to Jβ rearrangements in T and B lineages (transgenic lines 379 and 392; Ferrier *et al.*, 1990). Thus, substrate DβJβ rearrangements in the Eβ2, Eβ6, Eα3 and Eα6 lines were strongly amplified from thymic or T-enriched cells and amplified to a lesser extent from spleen cells. However, rearrangements were markedly reduced in the corresponding B-enriched cells, although, as expected, they were detected at roughly equivalent levels in both B and T cells from control 379 or 392 mice. Consistently, analyses of bone marrow (the organ in which B cells differentiate in adults) reproduced this dual pattern (i.e. lower levels of substrate rearrangements in Eβ/Eα versus 379/392 lines); moreover, in lines Eβ2 and Eα3, we verified that rearrangements were barely detectable in ($B220^+$, $sIgM^-$) pre-B cells purified from adult bone marrow. Quantification by densitometric analyses indicated that DβJβ rearrangements in peripheral B cells represented (after normalization for input DNA) respectively, <2% (Eβ2), 16% (Eβ6), 6% (Eα3) and 3% (Eα6) of those found in T cells. Therefore, these results supported our previous Southern blotting analyses. Together, they strongly suggested that the enhancer fragment in VTEβCμ or VTEαCμ transgenes acts to restrict VDJ recombination to the T cell lineage. The discrepancy between the amount of rearrangement found in the Eβ6 lines and that in Eβ2 lines might be related to the significantly higher number of transgene copies in the former. First generation offspring from independent founders (Eβ3 and Eβ10), carrying even more copies of the VTEβCμ substrate (85 and 70 copies, respectively), also exhibited DβJβ rearrangements in peripheral B cells at ~25% of the level found in the corresponding T cells (Figure 5 and data not shown). Deregulation of tissue-specific rearrangements of a transgenic substrate when in high copy number has been reported (Bucchini *et al.*, 1987).

Substrate DβJβ rearrangements in embryonic thymus

During mouse embryogenesis, an ordered program of TCR gene expression takes place in the developing thymus; TCRγ, δ and β gene expression occurs between days 14 and 16 of gestation, whereas TCRα gene expression is delayed until day 17 (Strominger, 1989). To define the timing of substrate recombination in the course of thymus ontogenesis, we performed PCR assays on thymuses from transgenic fetuses at various ages of gestation. Analysis of several

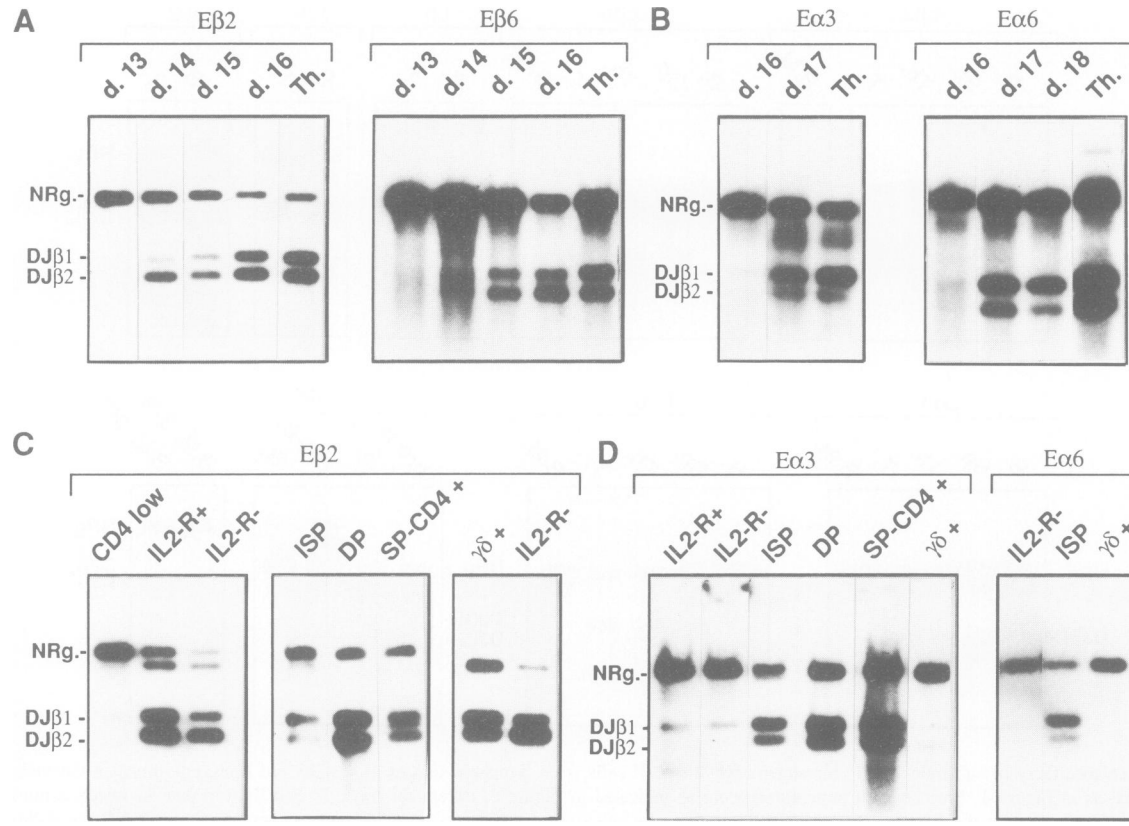

Fig. 6. Substrate DβJβ rearrangements in embryonic and adult thymocytes. Substrate rearrangements were analysed using 2×10^3 cells from embryonic thymuses (A and B) or from subpopulations in adult thymuses (C and D). (**A** and **B**) Thymuses were taken from individual transgenic embryos at different days of gestation, as indicated above each lane; Th.: thymus (2×10^3 cells) from a newborn transgenic mouse. (B, right panel) control (RAG-2) reactions indicated that substantially more DNA was available for amplification in the (Eα6/Th.) sample. (**C** and **D**) Subpopulations of thymocytes were purified from pooled thymuses of 4–6 week old transgenic mice. The phenotype of the cells (as mentioned in the text) is indicated above the lanes; $\gamma\delta^+$ samples correspond to a (DN, IL2-R$^-$, $\gamma\delta^+$) phenotype. (C, left panel) The additional signal detected in DN (IL2-R$^+$ and IL2-R$^-$) cells was encountered occasionally and probably corresponds to a PCR artefact; (D, left panel) Input DNA was substantially higher in the (SP-CD4$^+$) sample.

animals from each line gave consistent results. Characteristic results are shown in Figure 6. In transgenic lines Eβ2 and Eβ6, DβJβ rearrangements were detected at significant levels starting from day 14, with no or barely detectable rearrangements at day 13; the levels of DβJβ rearrangement at day 16 were comparable with those found in thymus from a newborn transgenic mouse in the corresponding line (Figure 6A). In contrast, high levels of rearrangement in the Eα3 and Eα6 lines were not detected until day 17 (Figure 6B). These results indicate that DβJβ rearrangements within the VTEβCμ construct start in embryonic thymuses on day 14 of mouse gestation and are completed by day 16, whereas most DβJβ rearrangements within the VTEαCμ construct occur from day 17 onwards. Using an independent assay to analyse substrate Vβ gene rearrangements, we found no evidence for ongoing Vβ joining events before day 14 (Eβ2 line) or day 17 (Eα3 line) (see below). Together, these results imply that Eβ and Eα confer distinct timing to the rearrangement events within the transgenes during T cell ontogeny. It is noteworthy that the onset of recombination within Eβ- or Eα-containing transgenes coincides with that of TCRβ or TCRα gene expression, respectively.

Substrate DβJβ rearrangements in adult thymus

The T cell differentiation pathway has been extensively characterized by following the behaviour of distinct markers on the cell surface of developing thymocytes in adult mice (Scollay *et al.*, 1988; Wilson *et al.*, 1988; Pearse *et al.*, 1989; Wu *et al.*, 1991). The earliest known intrathymic precursor cells express low levels of CD4 and Thy-1, but no CD8. As they develop towards mature T cells, they lose CD4 to become double negative (DN; CD4$^-$8$^-$) and acquire IL-2R (early DN; CD4$^-$8$^-$IL-2R$^+$), then lose IL-2R (late DN; CD4$^-$8$^-$IL-2R$^-$), before acquiring both CD4 and CD8 [double positive (DP); CD4$^+$8$^+$]. During the transition from DN to DP, some cells acquire either CD4 or CD8 first; these early single positive populations can be termed immature single positive (ISP; CD4$^+$8$^-$3$^-$ or CD4$^-$8$^+$3$^-$), distinct from mature single positive cells (SP; CD4$^+$8$^-$3$^+$ or CD4$^-$8$^+$3$^+$) by several criteria, including the level of TCR expression. To define precisely the timing of rearrangement within the VTEβCμ or VTEαCμ constructs, we purified these subpopulations from adult thymuses in line Eβ2, Eα3 and Eα6, and characterized by PCR the pattern of substrate rearrangement on individual cell samples.

Among the samples purified from Eβ2 transgenic mice, DβJβ rearrangements were strongly detected in early DN cells and remained at comparable levels in the subsequent cell stages of the pathway; conversely, rearrangements were scarcely detected in the CD4^{low} precursors (Figure 6C). Since the sorted cells were >98% pure on reanalysis (see Materials and methods), contaminants in the CD4^{low} preparation (i.e. cells not of the defined phenotype) would

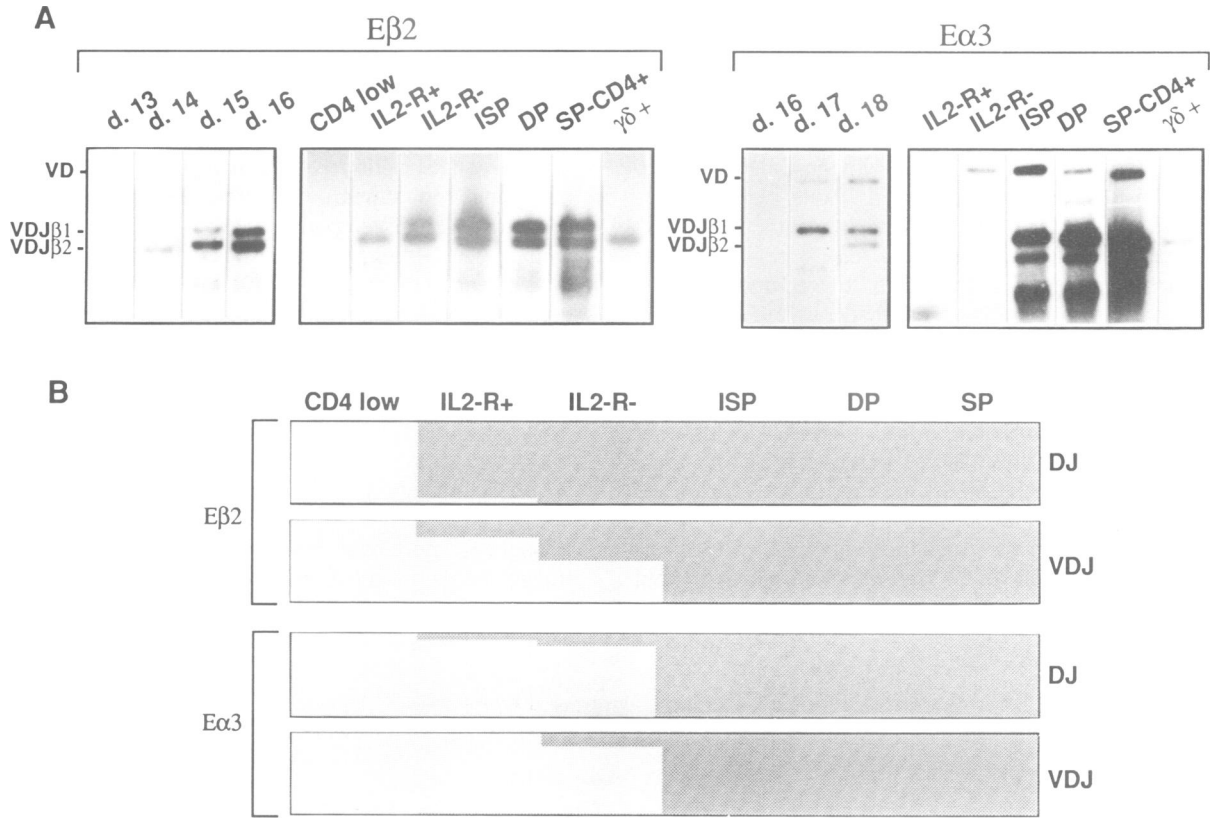

Fig. 7. Substrate Vβ rearrangements in embryonic and adult thymocytes. **(A)** Thymocytes from transgenic embryos at different age of gestation and thymic cell populations from adult transgenic animals were analysed as in Figure 6, except that the Vβ primer was used instead of the Dβ primer in the PCR reactions. **(B)** Relative levels of DβJβ and Vβ(Dβ)Jβ rearrangement in thymic populations were determined by densitometry. Substrate rearrangements (shaded areas) are expressed on a linear 0−100% scale, the values based on comparison with mature (SP, $CD4^+$) T cells; values were normalized according to RAG-2 PCRs.

be at about the limit of detection of the PCR assay (see Figure 4B). So the very faint bands visible in this population could be accounted for on the basis of contaminants, and their significance is therefore doubtful. We conclude that Dβ to Jβ joins within the VTEβCμ construct are initiated (and almost completed) at the early DN cell stage. Interestingly, high levels of rearrangements were also detected in $\gamma\delta^+$ thymocytes, indicating that substrate Dβ to Jβ joins proceed efficiently during γδ cell differentiation. The pattern of substrate rearrangements within the cell samples purified from the Eα3 and Eα6 mice differed from these results in two respects (Figure 6D). First, DN cell samples exhibited very low levels (at most) of DβJβ joins, whereas high levels were detected later on in the pathway starting from the ISP cell stage. Second, levels of rearrangement in $\gamma\delta^+$ thymocytes were also very low, and at a similar level to those observed in DN cell samples. We conclude that the vast majority of Dβ to Jβ joining events within the VTEαCμ transgenes occurred at a relatively late (ISP) stage of the αβ differentiation pathway on the one hand, and did not proceed efficiently during γδ differentiation on the other hand.

Substrate Vβ gene rearrangements in embryonic and adult thymus

To analyse substrate Vβ gene rearrangements, we performed PCR assays using a specific set of oligonucleotide primers; fragments containing substrate Vβ(Dβ)Jβ and VβDβ rearrangements can simultaneously be amplified in the assay (Figure 4A; PCR conditions allowed quantitative amplifications, as indicated by titration experiments—not shown). Analyses on embryonic thymuses and adult thymic cell populations in the Eβ2 and Eα3 lines are reported in Figure 7A.

In the Eβ2 line, substrate Vβ(Dβ)Jβ rearrangements were first detected at day 14 and in early ($IL2\text{-}R^+$) DN cells, at ~25% of the level found in mature (SP) cells. They increased on day 15 and in late ($IL2\text{-}R^-$) DN cells to reach their optimal level on day 16 and at the ISP cell stage; the level in $\gamma\delta^+$ cells was intermediate between those in the two populations of DN cells. In contrast, in the Eα3 line, high levels of Vβ(Dβ)Jβ joins were immediately detected in embryonic thymus at day 17 and in ISP cells, with only a small amount of rearrangements at day 16 and in DN or $\gamma\delta^+$ cells. Moreover, VβDβ rearrangements were essentially found among the Eα3 samples, a finding consistent with the Southern blotting results reported above.

In summary, these analyses indicated that rearrangements within the VTEβCμ transgenes took place between days 14 and 16 of thymic embryogenesis and mainly in DN cells in adult thymus, whereas rearrangements were delayed until day 17 in ISP cells within the VTEαCμ transgenes. Significantly, DN cells represent the vast majority of thymocytes until day 16 of mouse gestation, whereas the DN to DP cell transition occurs on day 17 (Scollay, 1987; Yagita *et al.*, 1989; Hugo *et al.*, 1990). These results imply that sequences in the Eβ or Eα fragments directed VDJ rearrangement within the transgenic miniloci to either of these discrete stages of the T cell differentiation pathway. These results also have implications on lineage relationships

($\gamma\delta$ versus $\alpha\beta$) during T cell development (see Discussion). Finally, these results imply a higher frequency of joining events involving simultaneously the Vβ and Dβ/Jβ segments in the Eα- versus Eβ-containing transgenes. This statement is evidenced by graphic representations of DβJβ and Vβ(Dβ)Jβ joins in adult Eβ2 or Eα3 thymuses (Figure 7B). The dual pattern is best interpreted if one assumes that, depending on the early or late activity of the associated recombinational enhancer, the D-J and V regions in the transgenes become fully accessible for recombination at distinct stages (Eβ activity) or simultaneously (Eα activity) during T cell developmental processes.

Expression of the transgenic substrates

To test the suggestion that transcriptional activation of germline segments/loci is a prerequisite for their rearrangement, we analysed the transcriptional status of the recombination substrates in tissues of the trangenic mice. Indeed, by Northern blot assays of poly(A)$^+$ RNA, we found no transcription of the transgenes in non-lymphoid organs from Eβ2, Eβ6, Eα3 and Eα6 mice, whereas in thymuses we readily detected, besides the expected TCR-Vβ(Dβ)Jβ/Ig-Cμ transcripts (i.e. hybrid transcripts generated following complete variable region gene assembly within the substrate—Ferrier *et al.*, 1989b), RNA messages that implied transcription of unrearranged regions in the transgenes (not shown). To investigate the latter activities further, we developed specific RNA PCR assays (Figure 8A). Primers were designed to amplify either a 370 nt fragment (Jβ and C_H primers) which corresponds to germline transcripts initiated upstream of the unrearranged Jβ1.1 segment in the constructs (hereafter referred to as Jβ transcripts), or a 431 nt fragment (Iμ and C_H primers) which has the properties of the so-called Iμ transcripts (i.e. early germline IgH transcripts initiated downstream of the intronic enhancer—Lennon and Perry, 1985). As indicated by Northern assays, Iμ-related transcripts are initiated downstream of the enhancer-containing fragment in the transgenes; moreover, in thymuses, Iμ transcription of the transgenes is more abundant than—and therefore easily

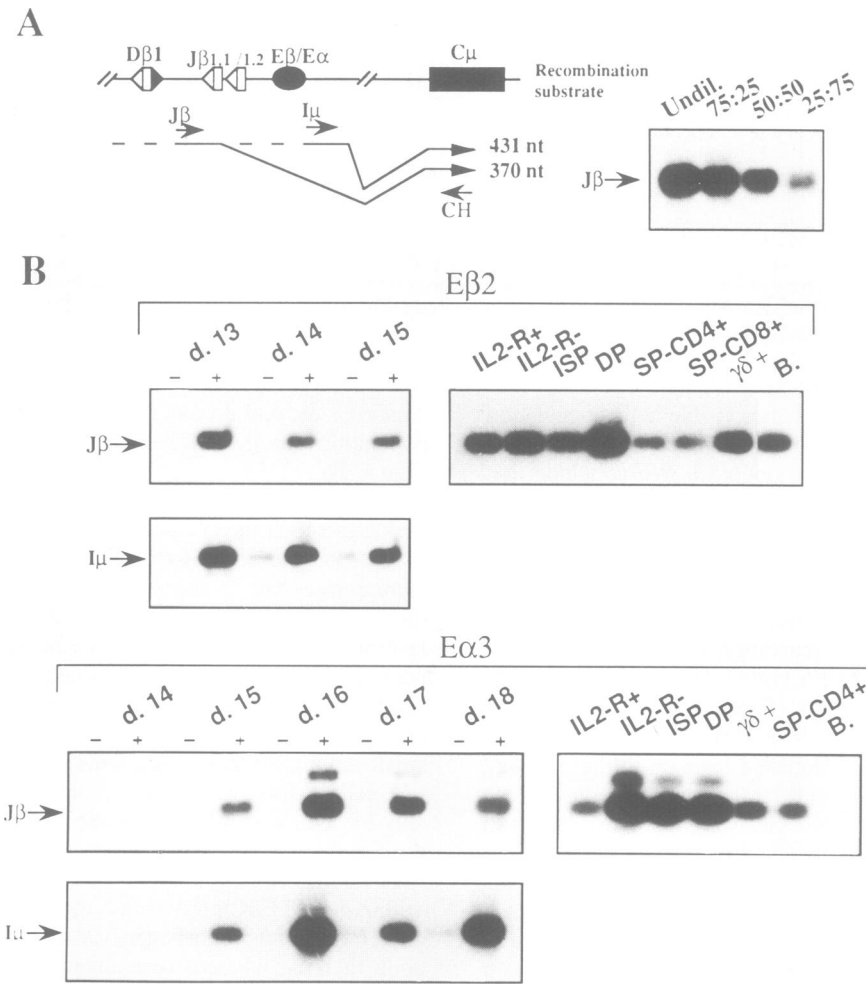

Fig. 8. Germline transcription of the transgenic substrates. (**A**) Diagram of the RNA PCR assays used to detect germline Jβ and Iμ transcripts. The locations of the Jβ and Iμ transcripts and their splices, along with the positions of the PCR primers, are indicated below a partial map of the transgenic miniloci; rearrangement events involving the Jβ segments result in loss of Jβ primer target sequences. Because the assays used primers which crossed intron–exon boundaries, DNA contamination could not account for the specific PCR signal. PCR (Jβ) analyses of serial dilutions of Eβ2 thymus RNA into non-transgenic thymus RNA are shown. (**B**) Analysis of transgene expression in the Eβ2 and Eα3 transgenic lines. Germline Iμ and/or Jβ transcripts were analysed by RNA PCR, using RNA from embryonic thymuses of transgenic (+) or wild type (−) littermates (left panels) and RNA from populations of thymic cells or from peripheral B cells of adult transgenic animals (right panels). Hybridizations used an IgH Cμ probe internal to the amplified fragments; the samples shown in the individual panels comprised roughly equivalent amounts of input RNA, as indicated by control β-actin PCRs.

distinguished from—endogenous Iμ transcription (C.Fernex, unpublished results). PCR conditions were established to obtain quantitative information; individual reactions were compared on the basis of parallel β-actin RNA amplifications (Figure 8A and data not shown).

Figure 8B shows characteristic results from analyses of embryonic thymuses and lymphoid cells from adult transgenic animals in the Eβ2 and Eα3 lines. In the Eβ2 line, transcripts with the properties of Jβ or Iμ were detected in transgenic thymuses at various ages of mouse gestation, the highest levels being consistently found as early as day 13 (Figure 8B and data not shown; note that, as expected from previous studies, endogenous IgH Iμ transcripts were detected at low levels in non-transgenic thymuses—Alt *et al.*, 1982). In adults, germline Jβ transcription was found at high levels in all the T cell precursors that we tested (including DN, ISP and DP cells), but was lower in mature (SP) thymocytes; however, significant amounts of Jβ transcripts were also detected in $\gamma\delta^+$ thymocytes and in peripheral B cells (a paradoxical result in view of the T cell specificity of substrate rearrangements in this line—but see below). Compared with these results, analyses of germline transcription in the Eα3 line pointed out some striking differences. Thus, Jβ and Iμ transcripts were detected later on (day 15) in thymic embryogenesis and only reached their maximum on day 16. Also, the level of Jβ transcripts was lower in early (IL2-R^+) DN cells than in the next cell stages of the differentiation pathway, or even than in terminally differentiated SP-$CD4^+$ and $\gamma\delta^+$ thymocytes (again despite substrate rearrangements being barely detected in Eα3 $\gamma\delta^+$ cells); conversely, almost no transcription was found in peripheral B cells. Using similar assays, substrate expression was only barely detected in thymocytes from independent transgenic mice carrying an enhancerless construct (M.Capone and P.Ferrier, unpublished results). Therefore, we conclude that germline transcription of the transgenes in the Eβ2 or Eα3 lines is differentially regulated with respect to lymphoid cell type and/or cell stage of differentiation. The implications of these results on the mechanism of accessibility to the VDJ recombinase and on the interpretation of the role of enhancer-associated sequences in targeting VDJ recombination are discussed in the next section.

Discussion

We have demonstrated that recombination substrates comprising the TCRβ or α gene enhancers (Eβ or Eα) undergo V(D)J assembly in lymphoid tissues from independent transgenic mice. A similar substrate was inert for rearrangement when integrated randomly into the murine genome, unless it included a DNA fragment containing the intronic IgH gene enhancer (Eμ) (Ferrier *et al.*, 1990). Therefore, Eβ and Eα, like the Eμ-containing fragment, promoted V(D)J recombination within the reporter transgenes. Further analysis of the tissue- and cell stage-specificity of the recombination events within the Eβ- and Eα-containing transgenes demonstrated patterns that were remarkably similar to those of the TCRβ and α gene, respectively. Altogether, the properties of activation and the tissue-restricted and temporally regulated patterns of rearrangement exhibited by Eβ and Eα within the reporter transgenes imply that both elements comprise *cis*-regulatory sequences which are able to modulate V(D)J recombination of associated variable gene segments and, most likely, play a dominant role in the control of TCRβ and α variable region gene assembly.

Eβ/Eα enhancers and control of V(D)J recombination

Previous analyses of mice carrying the Eμ-containing transgenes identified two separate regulatory elements within this type of recombination substrate (Ferrier *et al.*, 1990). One element, associated with the enhancer region, was necessary for the initiation of Dβ to Jβ rearrangements in lymphoid B and T cells, and a second element, most likely associated with the Vβ region and acting in conjunction with the enhancer-associated element, provided T cell specificity to the subsequent Vβ to (Dβ)Jβ joining events. Our current analyses of mice carrying the Eβ- and Eα-containing transgenes further emphasize the dominant role exerted by the enhancer-associated sequences in the control of V(D)J recombination. Thus, within the two substrates, all types of rearrangements, including DβJβ, occurred preferentially in the T cell lineage. Therefore, the regulatory sequences which, within Eβ and Eα, act to initiate rearrangements, also contribute to restrict the joining events to T cells. This is reminiscent of what has been described at the TCR gene loci, whose rearrangements can be found only rarely in B lymphoid cells (Traunecker *et al.*, 1986). Morever, consistent results in embryonic and adult transgenic mice demonstrated that the timing of rearrangement within the reporter minilocus during T cell differentiation is highly dependent on the associated (Eβ or Eα) enhancer element, with a pattern superimposable upon, respectively, TCRβ or TCRα gene expression (discussed below). Together, these results suggest that the recombinational activities of Eβ or Eα are sufficient to account for most of the characteristic patterns (tissue- and temporal-specificity) of V(D)J recombination at the TCRβ or TCRα genes, respectively.

However, considering the complex regulation of TCR gene expression, we stress the possibility that these elements might interact with other(s), located at unknown distances. At the level of our transgenic model, this would explain the dual pattern of Vβ gene rearrangement among the Eβ versus Eα lines, assuming that the Vβ-associated regulatory element mentioned above becomes active after Eβ but before Eα. Also, we generally found consistent patterns among independent lines of transgenic mice carrying a given type of substrate (including levels and tissue- and temporal-specificity of rearrangement—Ferrier *et al.*, 1990; this study). These findings suggest the presence, maybe within the enhancer fragments that we tested and in any case within the constructs, of element(s) that could be related to the locus control region (LCR) defined at the human β-globin gene locus (Grosveld *et al.*, 1987). By analogy to what was recently described at the LCR level (Fraser *et al.*, 1993), the putative elements might influence the developmental patterns that we describe.

Eβ/Eα enhancers and kinetics of TCR variable region gene assembly

The results of our transgenic experiments are in accordance with all the evidence that TCRβ gene expression precedes TCRα gene expression during αβ T cell differentiation. Moreover, we have demonstrated that the timing of rearrangement within the VTEβCμ and VTEαCμ transgenes in fetal thymus precisely reproduces the timing of,

respectively, TCRβ and TCRα gene expression during embryonic thymus development (Born *et al.*, 1985; Raulet *et al.*, 1985; Snodgrass *et al.*, 1985). In agreement with those results, we have demonstrated that the levels of rearrangements within the recombination substrates differed in the early stages of T cell differentiation. Thus, in transgenic line Eβ2, substrate rearrangements essentially took place in DN cells, probably being initiated at the junction between the CD4^{low} precursors and early (IL2-R$^+$) DN cells, whereas, in line Eα3, rearrangements were delayed until a later (ISP) cell stage. These results are in agreement with published analyses of TCRβ gene expression in thymocyte subpopulations, showing that early (IL2-R$^+$) DN cells are ~75% rearranged at the TCRβ locus and contain TCRβ RNA but not TCRα RNA, whereas CD4^{low} precursor cells still have TCRβ genes in a germline state (Pearse *et al.*, 1989; Held *et al.*, 1990; Wu *et al.*, 1991). Using *in situ* hybridization, Held and collaborators detected high levels of TCRα RNA at the DP cell stage (i.e. one cell stage later than the ISP stage), with ISP cells being mostly negative (Held *et al.*, 1990). However, other analyses using Northern blotting techniques showed that TCRα mRNAs could already be detected in the late (IL-2R$^-$) DN cells (Pearse *et al.*, 1989), a result that parallels our own transcriptional data. Most likely, the VDJ recombination profiles that we describe within the VTEβCμ and VTEαCμ transgenes in the course of T cell differentiation reflect faithfully those at the TCRβ and TCRα gene loci.

We found significant amounts of substrate rearrangement in $\gamma\delta^+$ thymocytes from transgenic mice of Eβ2 line but much less in those of Eα3/Eα6 lines. These results suggest that the recombinational activity of the mouse TCRβ gene enhancer is fully expressed in $\gamma\delta$ precursors while that of the TCRα gene enhancer is not. Other studies showing the presence of significant amounts of β RNA but not α RNA in $\gamma\delta$ T cells (Held *et al.*, 1990), and of TCRβ and δ gene rearrangements in thymic hybridoma (Thompson *et al.*, 1991) are in accord with this statement. Since TCRα gene rearrangements would result in the deletion of the δ gene due to the unique structure of the TCRδ/α gene locus, the lack of recombinational activity of the Eα element in $\gamma\delta$ precursors is certainly a prerequisite for the sophisticated regulation of δ/α gene expression during T cell ontogeny and differentiation. In the context of the relationship between TCRα gene transcription and rearrangement, the Eα element used in the VTEαCμ construct was reported to be transcriptionally active in $\alpha\beta^+$ and $\gamma\delta^+$ T cells, but this activity was strongly inhibited in $\gamma\delta^+$ T cells through sequences located upstream of Eα (the so-called Eα silencers—Winoto and Baltimore, 1989b). These silencer sequences were not included in the VTEαCμ construct. Accordingly, we found germline Jβ transcription of the transgenes in $\gamma\delta^+$ cells of the Eα3 line. Therefore, besides their implications on the mechanism of accessibility (discussed below), these results suggest that the delayed recombinational activity of the Eα element may not depend solely on the relief of an early silencing activity, as was proposed (Winoto and Baltimore, 1989b), but also on the appearance of a specific enhancing activity at a late stage of $\alpha\beta$ T cell maturation.

Because they contained relatively high amounts of VTEβCμ-rearranged substrates but few VTEαCμ-rearranged substrates, a significant proportion of the $\gamma\delta^+$ T cells must derive from precursor populations comprising the early (IL2-R$^+$) DN cells and the ISP cells (i.e. the earlier cells carrying high levels of substrate rearrangement in the Eβ2 and in the Eα3 transgenic mice, respectively). Accordingly, the pattern of rearrangement in $\gamma\delta^+$ thymocytes mimicked that of DN cell precursors in both Eβ2 and Eα3 transgenic lines—with a pattern intermediate between that found in early and late DN cells if one considers the profiles of substrate Vβ gene rearrangements. These results support recent data indicating that commitment to the TCR$\alpha\beta^+$ or $\gamma\delta^+$ T cell lineages can occur at a relatively late (DN; IL2-R$^-$) stage of intrathymic development (Petrie *et al.*, 1992). That the separation between the two lineages coincides with the onset of the recombinational activity of Eα, further emphasizes the role of this type of regulatory element in the T cell differentiation processes.

Eβ/Eα enhancers and VDJ recombinational accessibility

The characterization, in a transgenic model, of *cis*-regulatory sequences from different antigen receptor gene loci which are able to confer distinct patterns of recombination on to the same variable minilocus, strongly supports the proposed model ('accessibility model'; Alt *et al.*, 1987) of control of the VDJ recombinase activity. In addition, with respect to all the evidence that gene transcription is narrowly associated with ongoing VDJ rearrangements at the corresponding gene segments and/or loci (Blackwell and Alt, 1988), it is noteworthy that various DNA fragments, comprising distinct transcriptional enhancers, have been effectively shown to affect V(D)J recombination and germline expression within a transgenic reporter (Ferrier *et al.*, 1990; this study). Because the thresholds of the RNA and DNA PCR assays are difficult to compare, a definitive conclusion on the relative onset of germline transcription versus VDJ recombination within the reporter transgenes is not possible. Nevertheless, our data are compatible with the proposition that germline transcription precedes and thereby somehow activates VDJ recombination within the transgenic miniloci: in both Eβ2 and Eα3 lines, maximum levels of Iμ-related and Jβ transcripts were reached one day before maximum levels of substrate DβJβ rearrangements accumulated during thymic embryogenesis; similarly, in adult mice of the Eα3 line, one cell stage clearly separated the peak of the two processes (i.e. Jβ transcription and substrate DβJβ recombination) during thymocyte maturation.

Generally speaking, the transcriptional activities of the TCRβ and TCRα gene enhancers—i.e. the T cell predominance of both elements (Krimpenfort *et al.*, 1988; McDougall *et al.*, 1988; Ho *et al.*, 1989; Winoto and Baltimore, 1989a; Gottschalk and Leiden, 1990) and the lower transcriptional activity of the TCRα gene enhancer in immature versus mature T cells (Winoto and Baltimore, 1989a)—match the recombinational properties that we have defined within Eβ and Eα. Nevertheless, in B cells of Eβ2 transgenic mice and in $\gamma\delta^+$cells of Eα3 transgenic mice, we clearly detected germline transcription without a significant amount of rearrangement within the transgenes. These activities were not totally unexpected since the minimal β enhancer used in the VTEβCμ construct was reported to be transcriptionally active in some B-lineage cells (Takeda *et al.*, 1990) and, as already mentioned, the Eα element used in the VTEαCμ construct was reported to be transcriptionally

active in $\gamma\delta^+$ T cells (Winoto and Baltimore, 1989b). Assuming that Jβ transcription might also be active in the corresponding VDJ recombinase positive precursors (as suggested by our preliminary analyses of Eβ2 pre-B cells and Eα3 DN cells—M. Capone, unpublished data), these results would constitute a first piece of evidence that transcription through a germline segment is not sufficient *per se* to induce its recombination.

Because the Eβ and Eα elements correspond to relatively short DNA fragments comprising the minimal TCRβ and TCRα gene enhancers, it is likely that the recombinational activities that we describe are associated with one or several of the protein-binding domains defining these enhancers. Consequently, some of the nuclear factors that bind to lymphoid-specific enhancers would be directly involved in the control of accessibility to the VDJ recombinase. Recent experiments showing that, when it is overexpressed in a pre-T cell line, a nuclear factor that binds to the Eμ element activates IgH rearrangement (Schlissel *et al.*, 1991), and our unpublished analyses indicating that specific protein-binding domains account for the recombinational activity of the Eμ element, support this proposition. Our present results would suggest that this activation is more than a mere transcriptional activation of the substrate. One intriguing possibility would be that these domains and factors may be involved in inducing and/or in stabilizing some form of recombinational complex. The findings, in various systems, that enhancer-binding factors can mediate local bending of DNA (Giese *et al.*, 1992), fulfil a double role of activating chromatin and stabilizing transcriptional complexes (Burnol *et al.*, 1993), or may be required for joining of distant recombination sites at a recombinational enhancer (Heichman and Johnson, 1990) are all compatible with this statement.

Materials and methods

Construction of the recombination substrates

The 560 bp *Hpa*I−*Nco*I DNA fragment containing the TCRβ gene enhancer was obtained from plasmid pSP-Eβ2.1 and was blunt-ended; pSP-Eβ2.1 contains a 2.1 kb *Kpn*I−*Hpa*I insert from cosmid clone 40.1W7 (Malissen *et al.*, 1986). The 515 bp *Pvu*II DNA fragment containing the TCRα gene enhancer was obtained from plasmid pSN12.5; pSN12.5 contains 12.2 kb from the mouse TCRα locus (Wilson *et al.*, 1992). Both fragments were inserted into the *Sma*I site of cosmid cVβ14DβJβ (Ferrier *et al.*, 1990) in the same orientation as their endogenous counterparts relative to the Jβ or Jα clusters, yielding cosmids cVβ14DβJβEβ and cVβ14DβJβEα, respectively; the integrity and orientation of the Eβ and Eα fragments were verified by sequence analysis. The VTEβCμ and VTEαCμ substrates were derived by ligating the 10.5 kb pRI-Cμ *Eco*RI insert (Ferrier *et al.*, 1990) into the single *Eco*RI site of cVβ14DβJβEβ and cVβ14DβJβEα, respectively.

Production of transgenic mice

Preparation of the DNA inserts, microinjection into fertilized (C57Bl/6 × CBA/J) F2 eggs and identification of the transgenic mice were as previously described (Ferrier *et al.*, 1990). Embryos were aged based on a daily vaginal plug assessment, day 1 being the day on which a plug was detected.

Southern blot analyses

DNA preparation, restriction enzyme digests, agarose gel electrophoresis, DNA blotting, preparation and ^{32}P-labelling of DNA fragments used as probe and hybridization procedures were as previously described (Ferrier *et al.*, 1990). Quantifications were derived from densitometric analyses of either autoradiograms using a GS300 Scanning Densitometer (Hoefer Scientific Instruments) and/or hybridized filters using a Baf 1000 Imaging Plate Device (Fuji). Transgene copy numbers per diploid genome were determined in kidney DNA by comparing the intensities of the Vβ14-hybridizing fragments containing the transgenic Vβ14 gene and the germline endogenous Vβ14 gene, respectively. Percentages of total substrate, DJ, VDJ and VD substrate rearrangement were determined by comparing the unrearranged substrate-, the substrate DJ-, VDJ- and VD-containing fragments in thymus DNA versus the unrearranged substrate-containing fragment in kidney DNA; the amounts of DNA were normalized following re-hybridization of the blots with a RAG-2 probe (Oettinger *et al.*, 1990).

Cell separation

Peripheral B and T cells were purified either from a mixed cell suspension from spleen and lymph nodes using cell depletion techniques, or from spleen by cell sorting. T cell depletion was performed using an anti-Thy1 monoclonal antibody (mAb) + rabbit complement (Cedsarlane, Canada); B cell depletion was performed using anti-CD45 (B220) mAb + Dynabeads (Dynal A.S). Cell sorting was performed with a FACStar Plus (Becton-Dickinson) following direct staining of splenocytes with anti-CD3 and Fab'2 anti-sIg fluorescent mAbs. Cell purification was monitored by fluorescence-activated cell sorting (FACS).

Bone marrow pre-B cells and thymus cell populations were purified from pools of three to 15 transgenic mice. Pre-B cells corresponded to ($B220^+$, $sIgM^-$) cells; thymocytes were purified as described by Pearse *et al.* (1989) and Wu *et al.* (1991). Briefly, preliminary negative depletions were performed with antibodies and complement (followed by a density cut to remove dead cells) and then with magnetic beads. The resulting populations were stained with various fluorochrome-conjugated antibodies and sorted. Sorted populations were always tested for purity by reanalysis after sorting and were usually >98% pure. In most cases, the negative depletions included a cocktail of antibodies to remove myeloid and erythroid lineage cells. During all these procedures, no significant differences in the composition of the various thymic cell compartments were apparent between transgenic mice from Eβ2, Eα3 or Eα6 lines, or between transgenic and non transgenic mice.

DNA PCR assays

Lymphoid cells were incubated for 5 min at 95°C in 50 μl of lysis buffer (10 mM Tris, pH 8.4; 2.5 mM $MgCl_2$; 50 mM KCl; 200 μg/ml gelatin; 0.45% NP-40; 0.45% Tween-20) and 30 min at 56°C in the presence of proteinase K (60 μg/ml). 1−5 μl of this solution were amplified with a Perkin−Elmer Cetus DNA thermal cycler for 20 (Eβ6/Eβ3/Eβ10 lines) or 24 (Eβ2/Eα lines) cycles (1 min at 94°C, 0.5 min at 56°C and 2.5 min at 72°C) in a volume of 50 μl containing 0.25 μM of each oligonucleotide primer. One-fifth of each reaction mixture was separated on an agarose gel and analysed by Southern blotting.

RNA PCR assays

RNA and randomly primed first strand cDNA were prepared according to published protocols (Belyavsky *et al.*, 1989). cDNA was resuspended in 20 μl of water; comparative analyses were performed on cDNAs synthesized simultaneously. 2 μl of this solution were amplified for 22−25 cycles (1 min at 94°C, 0.5 min at 56°C and 2.5 min at 72°C) in a volume of 50 μl containing 0.25 μM of each oligonucleotide primer. Analysis was likewise done by blot hybridization.

Sequences of PCR primers

Oligonucleotides primers were as follows: Vβ: (5'-CCTTGGCTCT-TCTAGTATACT-3'); Dβ: (5'-TGGTTTCTTCCAGCCCTCAAG-3'); IgH: (5'-GGAGACCAATAATCAGAGGG-3'); RAG: (5'-AGATGG-AGACTCCTGACTGG-3'; 5'-GCAATATACCTGAGTCTGAGG-3') Iμ: (5'-CTGGGAATGTATGGTTGTGGC-3'); Jβ: (5'-CTCCTCATC-CTATGGCAC-3'); C_H: (5'-GCAGATCTCTGTTTTTGCCTCCG-3'); β-actin: (5'-GTGGGCCGCTCTAGGCACCAA-3'; 5'-CTCTTTGAT-GTCACGCACGATTTC-3').

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