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Separate elements control DJ and VDJ rearrangement in a transgenic recombination substrate

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We describe transgenic mice that carry an antigen receptor gene minilocus comprised of germline T cell receptor (TCR) β variable gene elements (V, D and J) linked to an immunoglobulin (Ig) Cμ constant region gene with or without a DNA segment containing the Ig heavy chain transcriptional enhancer (Eμ). Transgenic constructs lacking the Eμ-containing segment did not undergo detectable rearrangement in any tissue of six independent transgenic lines. In contrast, transgenic constructs containing this DNA segment underwent rearrangement at high frequency in lymphoid tissues, but not other tissues, of four independent lines. Analyses of purified B and T cells, as well as B and T cell lines, from transgenic animals demonstrated that the Eμ-containing segment within the construct allowed partial TCR gene assembly (D to J) in both B and T cells. However, complete TCR gene rearrangement within the construct (V to DJ) occurred only in T cells. Therefore, we have demonstrated elements that can control two separate aspects of TCRβ VDJ rearrangement within this construct. One lies within the Eμ-containing DNA segment and represents a dominant, *cis*-acting element that initiates lymphoid cell-specific Dβ to Jβ rearrangement; various considerations suggest this activity may be related to that of the Eμ element. The second element provides T cell-specific control of complete (Vβ to DJβ) variable region gene assembly; it correlates in activity with expression of the unrearranged Vβ segment.

Key words: IgH enhancer/VDJ recombinant control

Introduction

Genes that encode immunoglobulin (Ig) or T cell receptor (TCR) variable regions are assembled somatically from germline gene segments during B or T lymphocyte differentiation. Ig heavy (H) chain and TCRβ and δ variable region genes are assembled from variable (V), diversity (D) and joining (J) segments, whereas Ig light (L) and TCRα and γ variable region genes are assembled from just V and J segments. In general, germline J segments lie several kb 5′ to an associated constant (C) region gene with (D) and V segments lying 5′ to the J segments (reviewed by Tonegawa, 1983; Marrack and Kappler, 1987). Germline Ig and TCR variable region gene segments are flanked by conserved signal sequences that target a site-specific recombination activity referred to as VDJ recombinase (Aguilera *et al.*, 1987); current evidence suggests all Ig and TCR variable region gene segments are assembled by the same VDJ recombinase (Schuler *et al.*, 1986; Yancopoulos *et al.*, 1986).

Assembly of Ig and TCR variable region genes is tissue-specifically regulated; thus, neither Ig nor TCR genes are assembled in non-lymphoid cells, whereas Ig genes are assembled completely only in precursor (pre-) B lineage cells (in fetal liver or adult marrow) and TCR genes only in (pre-) T lineage cells (in thymus) (Kincade, 1987; von Boehmer, 1988). Within the T and B lineages, Ig or TCR variable region gene assembly also is stage-specifically regulated (reviewed by Alt *et al.*, 1986; Davis and Bjorkman, 1988); for example, IgH chain genes usually are assembled and expressed before IgL chain genes. Finally, these gene assembly events are regulated in the context of allelic exclusion, a process that involves functional assembly of a given antigen receptor locus on only a single allele (reviewed by Alt *et al.*, 1987). Regulation of variable region gene assembly appears to be controlled at the level of V gene segment rearrangement. For gene families that do not have D segments (like the Igκ L chain gene family), this simply involves regulation of V to J joining. However, for families that have V, D and J segments, it is the V to DJ joining step that appears to be regulated (Alt *et al.*, 1984; Uematsu *et al.*, 1988). In this context, assembly of a complete VDJ gene generally is an ordered process that involves initial D to J joining and subsequent appendage of a V segment to the DJ complex (Alt *et al.*, 1984, Okazaki *et al.*, 1987). Findings that VDJ recombinase activity is constitutive in precursor lymphocytes and generally absent in non-lymphoid cells (Blackwell *et al.*, 1986; Lieber *et al.*, 1987; Desiderio and Wolff, 1988; Schatz and Baltimore, 1988) account, at least in part, for lymphoid-specificity of variable gene assembly. However, given a single VDJ recombinase, stage-, tissue- and allele-specific activities must be regulated at another level. Previous studies suggested this regulation is effected by *cis*-acting DNA elements that target gene segments to recombinase activity; among candidate regulatory elements implicated are those involved in transcriptional control of antigen receptor gene expression (Yancopoulos and Alt, 1985; Blackwell *et al.*, 1986; Yancopoulos *et al.*, 1986; Schlissel and Baltimore, 1988; Diamond *et al.*, 1989; Ferrier *et al.*, 1989).

High-level transcription from promoters of rearranged V_H gene segments depends on the activity of the IgH enhancer element (Eμ). Transfection experiments into lymphoid and non-lymphoid cells indicated that Eμ activity

lies within a several hundred bp DNA segment in the intron between the J_H segments and the downstream C_μ gene (Banerji *et al.*, 1983; Gillies *et al.*, 1983; Mercola *et al.*, 1983; Neuberger, 1983; Grosscheldl and Baltimore, 1985; Gerster *et al.*, 1987). Although the mechanism by which Eμ regulates transcription remains unknown, protein binding and mutational analyses suggested several functional domains within this complex region (Ephrussi *et al.*, 1985; Augereau and Chambon, 1986; Petersen *et al.*, 1986; Sen and Baltimore, 1986a; Weinberger *et al.*, 1986; Lenardo *et al.*, 1987; Peterson and Calame, 1987; Kiledjian *et al.*, 1988; Perez-Mutul *et al.*, 1988; Tsao *et al.*, 1988). In addition, Eμ appears to be active throughout B cell differentiation, even in early stages before pre-B cells assemble complete Ig genes (Gillies *et al.*, 1983; Lenon and Perry, 1986; Nelsen *et al.*, 1988). Thus, both the location and early activity of Eμ are consistent with a role in activating the J_H locus for rearrangement. We have tested this hypothesis by creating transgenic mice that contain recombination constructs composed of unrearranged TCRβ V, D and J segments linked to a Ig Cμ gene with or without a segment of DNA containing the Eμ element. These experiments demonstrate that the Eμ-containing DNA segment acts dominantly in *cis* to initiate D to Jβ rearrangement and imply that enhancer elements associated with the J/C regions of endogenous antigen receptor loci may fulfil this function in normal physiology. Furthermore, our experiments demonstrate that additional sequences within the construct control tissue-specific Vβ to DJβ rearrangement.

Results

Experimental strategy

We constructed two recombination substrates from genomic fragments of the murine TCRβ and IgH loci (see Figure 1A). Each construct contained a TCRβ variable region gene minilocus comprised of the germline Vβ14 (Malissen *et al.*, 1986), Dβ1.1 (Siu *et al.*, 1984), and Jβ1.1 and Jβ1.2 (Gascoigne *et al.*, 1984) segments linked to the exons that encode the IgH Cμ constant region by a segment of DNA in which a 1126 bp DNA fragment containing the Eμ element was present (VβDJβECμ) or absent (VβDJβCμ). Thus, the Eμ element was placed in a similar position and in the same orientation relative to the Jβ segments as it normally occupies with respect to the Ig J_H cluster. Germline antigen receptor genes are flanked by signal sequences that consist of a palindromic heptamer and an AT-rich nonamer separated by a spacer of ~12 or ~23 bp; joining only occurs between segments flanked by signal sequences with different spacer lengths (the 12/23 rule; Early *et al.*, 1980; Sakano *et al.*, 1980). Based on the spacer lengths of the signal sequences flanking the Vβ (23 bp), Dβ (5′ 12 bp, 3′ 23 bp) and Jβ (13 bp) segments, several types of rearrangements could theoretically occur in accordance with the 12/23 rule; these include: Dβ to Jβ, Vβ to Jβ, Vβ to DJβ and Vβ to Dβ joins (Figure 1B). The V, D and J segments were oriented so that, within a given construct, joinings would occur by deletion. The design of the recombination substrates allowed us to detect easily the various types of site-specific rearrangements by assaying *Bgl*II-digested genomic DNA for hybridization to a set of diagnostic probes (see legend to Figure 1).

Transgenic mice carrying the recombination constructs

DNA fragments containing the VβDJβECμ (21.4 kb) or VβDJβCμ (20.2 kb) insert were micro-injected into fertilized (C57BL/6J × CBA/J)F2 mouse eggs. Transgenic lines were established by crossing founders 379, 382, 390, 392 (VβDJβECμ construct) and founders 1003, 1006, 1010, 1013, 1016, 1018 (VβDJβCμ construct) with normal (C57BL/6J × CBA/J)F1 mice. Analyses of several transgenic mice confirmed that each line contained mostly intact copies of the construct integrated in a head-to-tail configuration at a single site, but also suggested the presence

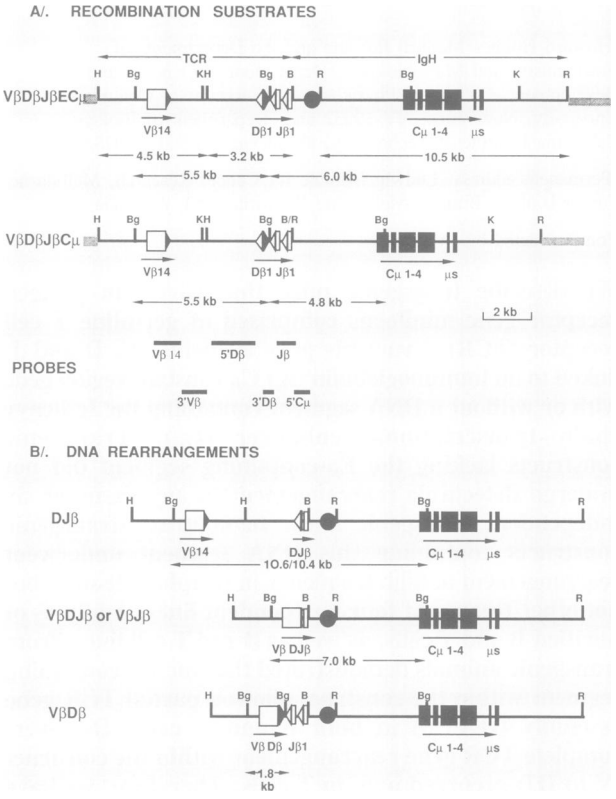

Fig. 1. Partial restriction endonuclease maps of the recombination substrates. (**A**) Inserts of recombinant cosmids VβDβJβECμ and VβDβJβCμ. The TCRβ V, D and J variable segments are indicated by open boxes, their flanking recognition heptamers and nonamers by shaded (23 bp spacer) or open (12 or 13 bp spacer) triangles, the IgH transcriptional enhancer elements by a shaded circle, the Cμ exons by shared boxes (these various elements are not drawn to scale) and the cosmid sequences by hatched boxes. The directions of transcription of the various coding elements are indicated by arrows. The location of the different probes (probe Vβ 14 used in the blotting analyses shown in Figures 2, 4 and 5; probes 3′Vβ, 5′Dβ, Jβ, 5′Cμ used to confirm the structure of the transgene rearrangements or to select the recombinant bacteriophages as mentioned in the text) is indicated below the VβDβJβCμ map. (**B**) Partial restriction maps of potential DJ, VDJ or VJ (only joins to the Jβ 1.2 gene segment are represented; VDJ and VJ rearrangements cannot be distinguished because the Dβ 1-1 segment is only 12 bp in length—Siu *et al.*, 1984) and VD rearrangements predicted from the structure of the VβDβJβECμ construct. Restriction endonuclease sites are indicated as follows: H, *Hin*dIII; Bg, *Bgl*II; K, *Kpn*I; B, *Bam*HI; E, *Eco*RI. The sizes of the *Bgl*II restriction fragments expected—when using the Vβ 14 probe—in Southern blotting analyses of genomic DNA purified from tissues or cells of the transgenic mice are indicated below each map.

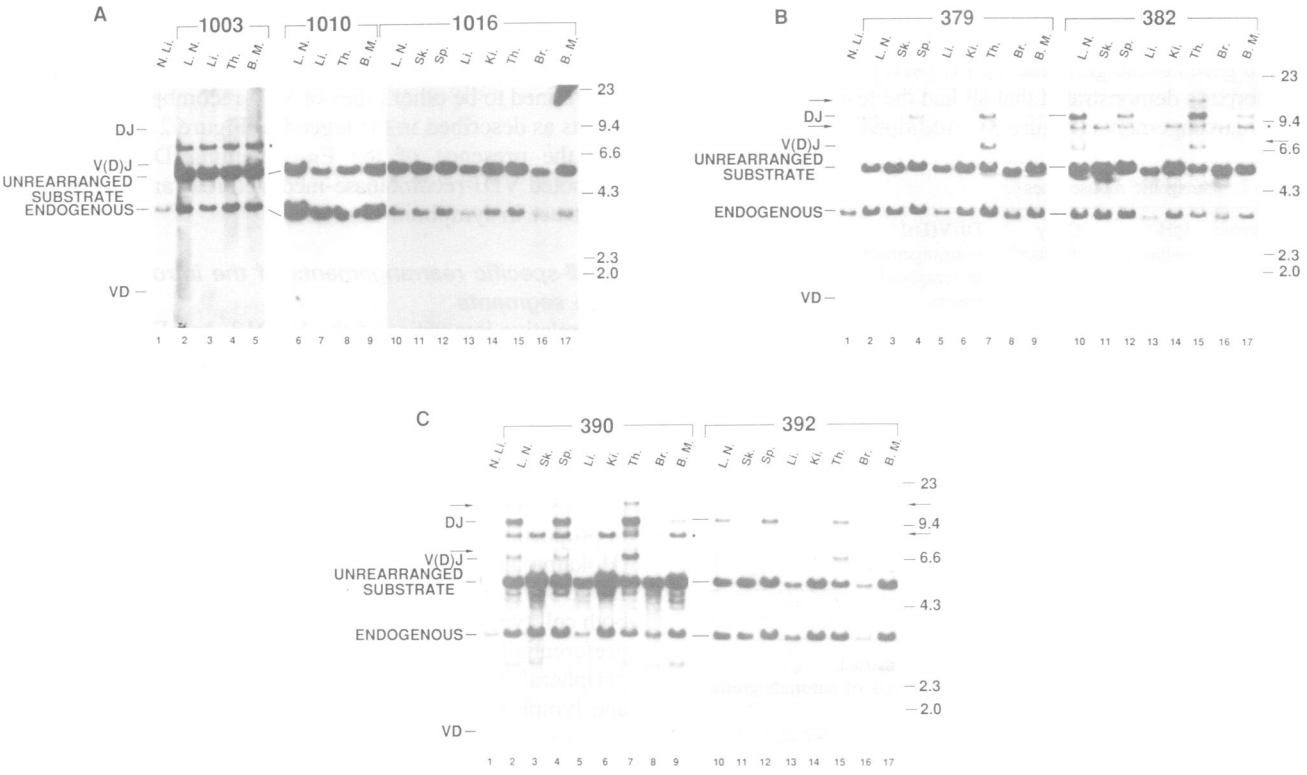

Fig. 2. Analysis of the micro-injected constructs in tissues of transgenic mice. Analysis of the Vβ 14-containing *Bgl*II fragments in enhancer-negative (**A**) and enhancer-positive transgenic lines (**B** and **C**). Genomic DNA (10 μg) from lymphoid [**lanes 2, 6, 10 (A)/ 2, 10 (B,C)**, lymph nodes; **lanes 12 (A) 4, 12 (B, C)**, spleen: **lanes 4, 8, 15 (A)/ 7, 15 (B,C)**, thymus; **lanes 5, 9, 17 (A)/ 9, 17 (B, C)**, bone marrow] and non-lymphoid [**lanes 11(A)/ 3, 11 (B, C)**, skin; **lanes 5, 7, 13 (A)/ 7, 13 (B, C)**, liver; **lanes 14 (A)/ 6, 14 (B, C)**, kidney; **lanes 16 (A)/8, 16 (B, C)**. Brain] tissues of individual 4 week old transgenic females were digested with *Bgl*II and analyzed by Southern blot analysis for hybridization to the ^{32}P-labeled Vβ 14 probe. DNA from normal (C57Bl/6J × CBA/J)F_2 liver served as the endogenous germ-line control (**lane 1**). The positions of the *Bgl*II fragments containing the endogenous Vβ 14 gene and the predicted substrate DJ, VDJ/VJ and VD normal rearrangements are indicated on the left. Stars and arrows indicate respectively the truncated copies of the transgene and the less abundant rearrangements mentioned in the Results. The structures of these rearrangements were determined by hybridization and direct molecular cloning analyses (data not shown); two result from a pseudo-normal join (Alt and Baltimore, 1982) between the signal sequences of a Dβ and Vβ segment located on separate constructs in head to tail orientation (generating Vβ-hybridizing *Bgl*II fragments of 9.2 or 14.5 kb depending whether it occurred before or after normal DJβ joins); the second type involves aberrant deletional joins between the Dβ segment and sequences in the Jβ-Cμ intron (consistently generating *Bgl*II fragments of 9.0 and 7.8 kb). Fragment sizes of *Hin*dIII-digested λ phage DNA are indicated in kb on the right.

of truncated copies in lines 382, 390, 1003 and 1006 (e.g. starred fragments in lines 1003, 382 and 390; see Figure 2). The number of intact copies varied from 2 to ~30 depending on the line (Table I). Initial DNA rearrangement and RNA expression analyses in lines 392 and 1016 showed no differences when comparing males versus females or newborn versus adult mice. Therefore, subsequent experiments were performed on tissues and cells from 4 week old females.

Tissue-specific rearrangements within the enhancer-positive construct

To assay for potential rearrangements of TCR variable segments in the constructs, genomic DNA from different lymphoid (lymph nodes, spleen, thymus and bone marrow) and non-lymphoid (skin, liver, kidney and brain) tissues of the transgenic mice was digested with *Bgl*II and tested for hybridization to a Vβ14-specific probe. Analyses of several animals from each line gave consistent results. Characteristic results are shown in Figure 2. As expected, in both normal (lane 1 contains normal liver DNA in all panels of Figure 2) and transgenic tissues, this probe hybridized to a 3.7 kb germline *Bgl*II fragment that contains the endogenous Vβ14 gene; in all transgenic tissues the probe also hybridized to the 5.5 kb *Bgl*II fragment containing the unrearranged V and D gene segments of the transgenes. No additional Vβ-hybridizing fragments were detected in any tissues of the six transgenic lines that contained the Eμ-negative construct (Figure 2A, Table I). Therefore, we conclude that the TCRβ variable gene segments in the Eμ-negative construct were not rearranged at readily detectable levels in any tissue of these animals.

In transgenic lines carrying the Eμ-positive transgene, we readily detected additional Vβ-hybridizing fragments of correct size to represent substrate DJβ (10.1 kb) and VβDJβ (7.0 kb) joins in DNA from lymphoid tissues but not that of non-lymphoid tissues (Figure 2B and C; faint hybridization to DJ-sized rearrangements in some non-lymphoid organs probably is due to resident lymphocytes in those organs). In contrast, Vβ-hybridizing fragments of the size predicted for VβDβ joins (1.8 kb) were not detectable or were barely detectable in lymphoid tissues and occurred at <5% the level of DJβ joins (Figure 2B and C, lanes 7 and 15). To confirm the structure of the observed rearrangements, we cloned six

VDJ and two DJ rearrangements from thymus DNA of line 392. Comparison of the nucleotide sequences of the rearranged genes to the germline Vβ14, Dβ1.1, Jβ1.1 and Jβ1.2 counterparts demonstrated that all had the features of normal VDJ rearrangements (Figure 3). Additional low abundance Vβ-hybridizing fragments (indicated by arrows on Figure 2B and C) detected in lymphoid tissues from lines containing the Eμ-positive construct were molecularly cloned and determined to be other types of VDJ recombinase-mediated events as described in the legend to Figure 2. We conclude that the presence of the Eμ-containing DNA fragment promoted VDJ-recombinase-mediated rearrangement of the construct in lymphoid tissues.

Table I. Transgenic mouse lines

Transgenic lines	IgH enhancer[a]	Copy number[b]	DJ/V(D)J rearrangement in lymphoid tissues	% rearranged substrate in thymus[c]		
				Total	DJ	VDJ
379	+	5	+	38	14	16
382	+	14	+	52	26	10
390	+	27	+	48	25	14
392	+	4	+	49	24	20
1003	−	6	−	−	−	−
1006	−	8	−	−	−	−
1010	−	2	−	−	−	−
1013	−	8	−	−	−	−
1016	−	20	−	−	−	−
1018	−	2	−	−	−	−

[a] +, VβDβJβECμ construct; −, VβDβJβCμ construct.
[b] and [c] were determined by densitometric analysis of autoradiograms similar to those shown in Figure 2.
[b]DNA copy number of micro-injected intact construct per diploid genome were determined by comparing in liver DNA the intensities of the Vβ-hybridizing *Bgl*II fragments containing respectively the unrearranged Vβ segment within the substrate and the germline endogenous Vβ segment.
[c]Percentage of total substrate, DJ and VDJ substrate rearrangements were determined by comparing the unrearranged substrate, the substrate DJ and VDJ containing fragments in thymus DNA versus the unrearranged substrate containing fragment in liver DNA; the amounts of DNA were normalized by scanning the same lanes hybridized to the pM.2 N-*myc*-specific probe (Zimmerman *et al.*, 1986).

T cell-specific rearrangements of the introduced Vβ gene segments

The relative intensities of the VβDJβ- and DJβ-containing fragments varied significantly among different lymphoid tissues. The ratio of VDJβ to DJβ rearrangements always was highest in thymus DNA (Figure 2B and C, lanes 7 and 15, and Table I), lower in lymph nodes and spleen (Figure 2B and C, lanes 2,10 and 4,12), and lowest in bone marrow (lanes 9 and 17). Thus, the VDJ/DJ intensity ratio varied in proportion to the T/B cell ratio in these lymphoid organs (Hokama and Nakamura, 1982), suggesting that substrate Dβ to Jβ rearrangements occurred at similar frequency in both cell types but that Vβ to DJβ rearrangements occurred preferentially in T cells. To confirm this point, we purified peripheral B and T lymphocyte populations from the spleen and lymph nodes of transgenic lines 379 and 392. Similar proportions of VβDJβ and DJβ rearrangements were found in DNA from the T cell-enriched populations (Figure 4A, lanes 6 and 11); in contrast. VβDJβ rearrangements were essentially absent in DNA from the B cell-enriched populations, whereas high levels of DJβ rearrangements were still observed (Figure 4A, lanes 5 and 10). Densitometric analyses of the autoradiograms from the four-copy line 392 confirmed that the levels of DJβ rearrangement were comparable in B and T cells; similarly, the estimated

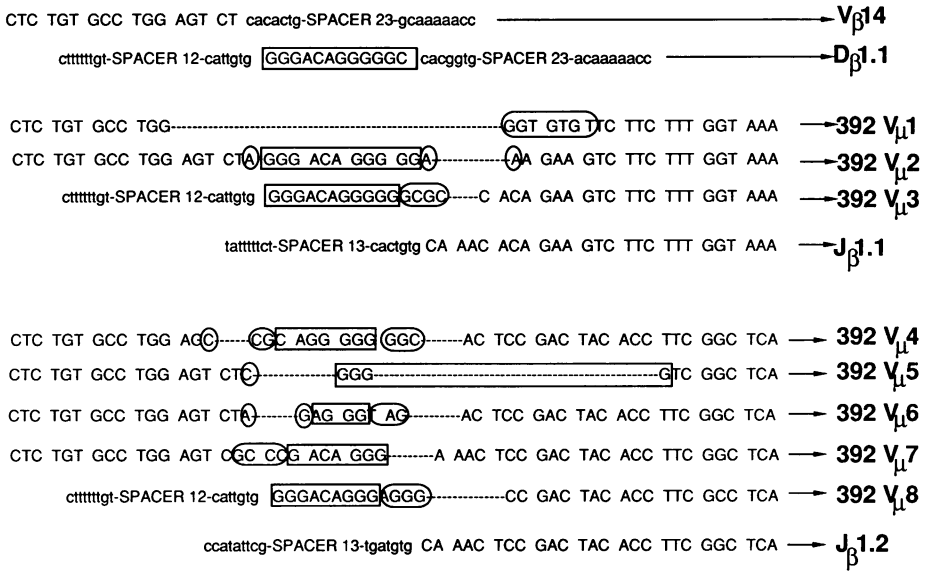

Fig. 3. Nucleotide sequence of normal joints involving the transgene variable gene segments. Vβ 14-Cμ-associated rearrangements were molecularly cloned from genomic DNA prepared from thymus of a transgenic mice in the four-copy line 392. The *Hin*dIII−*Bam*HI fragments containing the rearranged region were subcloned in M13 (mp19) bacteriophage or pUC19 plasmid and the nucleotide sequences of junctional regions determined by the dideoxy technique using synthesized oligonucleotides. The rearranged sequences, designated 392 Vμ 1−8, are compared to homologous germline sequences (Vβ 14, Malissen *et al.*, 1986; Dβ 1.1, Siu *et al.*, 1984; Jβ 1.1 and Jβ 1.2, Gascoigne *et al.*, 1984). The flanking recognition heptamers and nonamers are indicated with lower-case letters and the length of the spacers sequences are indicated. The regions of the Vβ 14 and Jβ 1.1 or Jβ 1.2 segments involved in each rearrangement have been aligned with the homologous germline sequences so that bases lost during joining are apparent. Potential Dβ-derived nucleotides are boxed and nucleotides added during the joining process (N-regions) are circled. The sequences are divided into codons according to the known Vβ 14, Jβ 1.1, and Jβ 1.2 reading frames to point out productive (392 Vμ1, 5) and unproductive (392 Vμ2, 4, 6, 7) VDJ rearrangements.

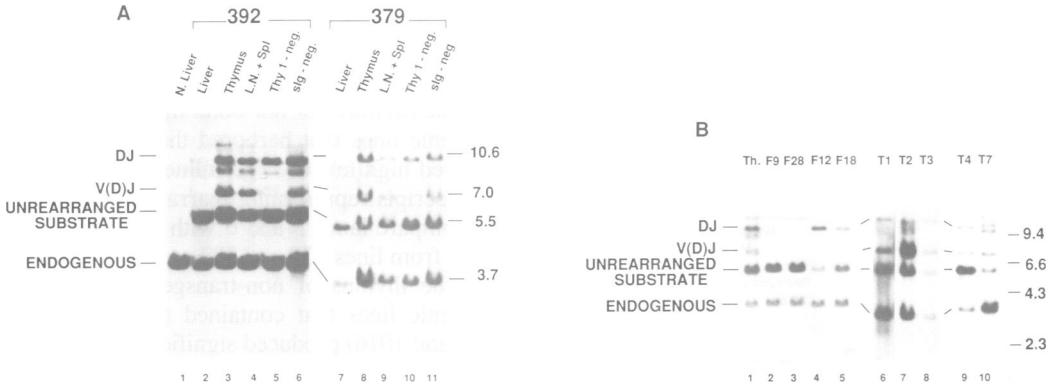

Fig. 4. Rearrangements of the VβDβJβECμ construct in lymphoid cells of the transgenic mice. Genomic DNA from various cell populations or cell lines was assayed for rearrangements of the construct as described in Figure 2. (**A**) Rearrangements in peripheral lymphoid cell populations from 4 week old transgenic females in lines 392 (**left panel, lanes 2–6**) and 379 (**right panel, lanes 7–11**). **Lane 1**: (C57Bl/6J × CBA/J)F_2 liver control; **lanes 2, 7** and **3,8**: respectively transgenic liver and thymus included as controls; **lanes 4** and **9**: peripheral lymphoid cells obtained by mixing cells from lymph nodes (LN) and spleen (Spl); **lanes 5/10**, B-enriched cells; **lanes 6/11**, T-enriched cells. In these experiments, the T cell-contaminations of the B cell-enriched populations [monitored by immunofluorescence using B cell-specific (Fab'2 anti-surface Ig) and T cell-specific (anti-Thy 1.2) antibodies—see Materials and methods] were <5%. Densitometric analyses performed as outlined in Table I gave the following percentages of total substrate, DJ and VDJ substrate rearrangements, respectively: 63%/59%, 21.3%/19% and <3% (B cells 379 and 392 respectively); 53%/40%, 9%/17% and 9%/13% (T cells 379 and 392 respectively). (**B**) Rearrangements in transgenic A-MuLV transformants. **Lane 1**, thymus of a transgenic mouse in line 382 included as control; **lanes 2–5**, fetal liver-transformed cells in line 392; **lanes 6–10**, thymic tumors in lines 379 (T7), 382 (T4), 390 (T2) and 392 (T1, T3). For T1, T2, T3, amounts of DNA and exposure times were varied to optimize visualization. Densitometric analyses performed as outlined in Table I gave the following percentages of total substrate, DJ and VDJ substrate rearrangements respectively: 67%/60%, 32%/20% and <1% (F12 and F18 B cells respectively); 73%/66%/69%, 7%/20%/13% and 13%/18%/10% (T1, T3 and T7 tumors respectively). F9 and F28 cells were characterized as non-lymphoid cells because they reacted with anti-mast cell/anti-macrophage B23.1 mAb (Katz *et al.*, 1983) and had unrearranged IgH and TCR loci (not shown). F12 and F18 were characterized as B cells because they reacted with specific anti-B cell B220 mAb (Coffman, 1982) and had IgH locus rearrangements (not shown); T1, T2, T3, T4 and T7 tumours arose from transformation of one or few different T cell(s) according to the following criteria (all data not shown): (i) when analyzed with an A-MuLV specific probe, one (T1, T3, T7), two (T4) and three (T2) copies of the viral genome were detected in tumor genomic DNA; (ii) all cells of the tumors were labeled with fluorescent anti-Thy 1.2 mAb; (iii) all tumor genomic DNAs contained endogenous rearrangements at the TCRβ locus; accordingly, normal TCRβ gene transcripts—but no normal Ig gene transcripts—were found in total RNA from the same tumors.

level of VβDJβ rearrangements in B cells (<5% of that in T cells) easily could have resulted from contaminating T cells (Figure 4A, legend).

Together, these results indicate that construct Dβ to Jβ rearrangements occurred at similar frequency in transgenic B and T cells; however, the vast majority of VβDJβ rearrangements observed in peripheral lymphoid tissues of these mice occurred in T lineage cells. Previous transgenic recombination constructs that employed rabbit Vκ and Jκ segments were rearranged in both B and T cells (Goodhardt *et al.*, 1987), whereas transgenic chicken Vλ and Jλ segments were rearranged specifically in murine B cells but identical constructs present at higher copy number (20 copies) were rearranged in T cells as well (Bucchini *et al.*, 1987). We see no obvious influence of copy number on the T/B cell specificity of the Vβ to DJβ joining event in our transgenic constructs; thus the ratio of VβDJβ to DJβ construct joins varies in proportion to T cell content in the lymphoid organs of transgenic lines containing from four to ~30 copies (Figure 2). The difference in specificity between the construct we now describe and previous ones may be related to the structure of individual constructs (which particular segments were used, the distance between the variable gene segments and the enhancer, etc.).

Construct rearrangements in transgenic T and B cell lines

To derive a system in which transgene rearrangements could be analyzed in clonal lymphoid cell populations and directly compared to the endogenous TCR and Ig rearrangements, we produced A-MuLV-transformed B and T cell lines from transgenic fetal livers and thymuses. A-MuLV transformation of transgenic fetal liver (from line 392) yielded multiple independent cell-lines; five were chosen at random for analysis of lineage-specificity and construct rearrangements. A-MuLV can infect and transform immature cells of the B lineage (reviewed by Alt *et al.*, 1986) and under certain circumstances, myeloid and mast cell lineages as well (Cook *et al.*, 1985; Pierce *et al.*, 1985). Accordingly, three fetal liver transformants were B lineage cells and two others were non-lymphoid cells—probably mast cells (see Figure 4B, legend). Independent A-MuLV-induced T lymphoid tumors were derived from thymuses of transgenic lines 379, 382, 390 and 392 (Figure 4B, legend); two tumors were adapted to growth in culture generating cell lines T3 and T7.

No rearrangements of the construct were detected in the A-MuLV-transformed non-lymphoid cells (Figure 4B, lanes 2 and 3). The A-MuLV-transformed pre-B cell lines had Ig V_HDJ_H rearrangements on at least one of their endogenous alleles (data not shown) and also showed substantial levels of construct DJβ rearrangements; yet these lines had barely detectable levels of construct VβDJβ rearrangements (e.g. Figure 4B, lanes 4 and 5). Thus, these cells had formed endogenous V_HDJ_H rearrangements and construct DJβ rearrangements before or shortly after transformation but generally had not formed construct VβDJβ rearrangements. Occasional subclones of the pre-B lines had Vβ to DJβ rearrangements (data not shown)—indicating that Vβ to DJβ rearrangements can occur at low frequency within the transgenic construct in B lineage cells. In contrast, genomic DNA from individual T cell tumors (Figure 4B, lanes 6–10) and from the T3 and T7 cell lines (not shown) contained the characteristic VβDJβ and DJβ construct rearrangements;

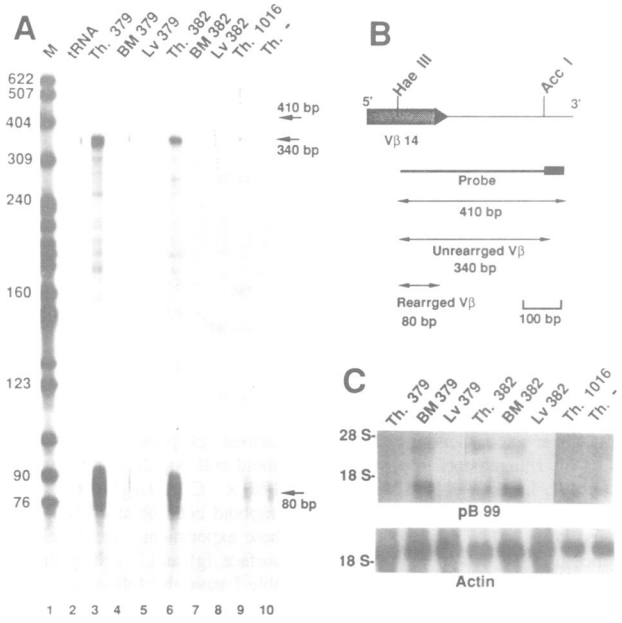

Fig. 5. Expression of the introduced Vβ14 gene. (**A**) Total RNA (15 μg) from thymus (**lanes 3, 6, 9** and **10**), bone marrow (**lanes 4** and **7**), and liver (**lanes 5** and **8**), of Eμ-positive (line 379 **lanes 3–5**; line 382, **lanes 6–8**), Eμ-negative (line 1016, **lane 9**) transgenic mice and of a non-transgenic normal mouse (**lane 10**) was assayed for hybridization to a probe comprising 340 bp of Vβ 14 3′ sequences and 70 bp of polylinker plasmid sequences by an S1 nuclease protection assay as outlined in (B). **Lane M**, ^{32}P-labeled *Msp*I-digested pBR322 marker DNA; the sizes of the marker fragments are indicated in bp on the left. **Lane tRNA**, 15 μg of yeast tRNA was used as a negative control in the S1 assay. The positions of the 410 bp probe, and of the protected fragments diagnostic for germline (340 bp) and rearranged (80 bp) Vβ14 gene transcripts are indicated on the right. (**B**) Strategy for the Vβ 14 gene expression assay. In the upper diagram the 3′ end of the Vβ 14 coding region is represented by a shaded box, the flanking recombination recognition sequences by a black triangle, and downstream flanking sequences by a line. The portion of the probe from this region is indicated by the line just below with the polylinker plasmid sequences in the probe indicated as a black box. The sizes of the expected protected fragments are diagrammed below and described in more detail in the text. (**C**) Total RNA (15 μg) was fractionated through formaldehyde–agarose gels, transferred to nitrocellulose filters and hybridized with the ^{32}P-labeled pB99 or β-actin probes; the RNA preparations employed were those used in the analyses of (A). The position of 28S and 18S ribosomal RNAs is indicated on the left.

whereas, again, none had readily detectable VβDβ rearrangements (not shown). These transgenic T cell lines also had formed rearrangements of their endogenous γ and β loci, but only two had rearranged TCRα genes (not shown) indicating that VβDJβ rearrangements within the construct occurred as early events in pre-T cell differentiation. Finally, individual T or B cells did not rearrange all copies of the construct (Figure 4B); in fact, the ratio of rearranged to unrearranged substrate in the clonal cell lines were comparable to that observed in total thymus and purified T (and B) cells (see Table I and Figure 4, legend). Together, these results indicate that the majority of developing normal T (and B) cells rearrange the transgenic construct.

Expression of the unrearranged Vβ14 gene segment

To assay for expression of Vβ14 gene segments, total RNA from various tissues of transgenic or control mice was assayed for ability to protect a Vβ14-specific probe from S1-nuclease digestion. The probe contained 80 bp of 3′ Vβ14 coding sequence plus 260 bp of downstream flanking sequences; thus, RNA transcripts from a rearranged Vβ14 gene segment should protect an 80 bp fragment, whereas transcripts from the unrearranged gene should protect a larger fragment of up to 340 bp (Figure 5B). Total RNA from the thymus but not bone marrow or liver of all four transgenic lines that harbored the Eμ-containing construct produced high levels of germline Vβ14 transcripts as well as transcripts representing rearranged Vβ14 genes (Figure 5A, compare lanes 3 and 6 with lanes 4, 5 and 7, 8; only results from lines 379 and 382 are shown). However, RNA from the thymus of non-transgenic lines and two tested transgenic lines that contained the Eμ-negative construct (1010 and 1016) produced significant but much lower levels of germline (and rearranged) Vβ14 transcripts (Figure 5a, representative data are shown in lanes 9 and 10). To estimate roughly the relative levels of precursor lymphocytes in the different tissues examined, we assayed RNA preparations used for S1 analyses for hybridization to a DNA probe representing a lymphoid-specific RNA sequence only expressed in pre-B and pre-T cells (PB99; G.D.Yancopoulos *et al.*, in preparation). This analysis indicated precursor lymphocytes were substantially represented in bone marrow RNA preparations (Figure 5C, top panel; the bottom panel is a β-actin control). Together, these data demonstrate that the unrearranged Vβ14 gene in the Eμ-containing, but not Eμ-negative, transgenic construct generates substantial levels of steady-state RNA in pre-T- but not pre-B-rich tissues.

Discussion

Initiation of the rearrangement process

We have defined elements that can mediate two distinct aspects of the control of TCRβ VDJ assembly within trangenic recombination substrates. The presence of a 1.1 kb DNA segment containing the Eμ element within recombination substrates integrated randomly into the murine genome promoted VDJ recombinase-mediated joining of associated Dβ and Jβ segments in T and B lymphoid cells. In the absence of this DNA segment, no Dβ to Jβ rearrangement within the transgene was observed in any cell type, whereas Eμ-associated Dβ and Jβ segments recombined in the majority of B and T lymphoid cells—emphasizing the dominant effect on recombination exerted by the Eμ-containing segment. An additional element within the construct, acting in conjunction with the Eμ-associated element (see below) provided T cell-specific control of the Vβ to DJβ joining step—allowing this process to proceed efficiently in T but not B lineage cells.

The accessibility model for the control of variable region gene assembly predicts *cis*-acting regulatory elements that modulate accessibility of different antigen-receptor gene segments (loci) to a common VDJ recombinase (Yancopoulos and Alt, 1985). The Eμ-containing DNA segment clearly can fulfil some requirements predicted for such recombinational control elements: in the context of the transgenic construct, this segment was prerequisite for efficient VDJ recombination in normal developing lymphoid cells. We refer to this dominant, *cis*-acting promotion of VDJ rearrangement as 'VDJ recombination enhancement' and the element(s) responsible as 'VDJ recombinational enhancers'. The Eμ-containing DNA segment is a complex region of DNA; Eμ itself contains multiple elements that play a role in regulating tissue-specific transcription of the IgH locus (Ephrussi *et al.*, 1985; Sen and Baltimore, 1986; Lenardo

et al., 1987; Peterson and Calame, 1987). We have not defined the exact sequences that generate the recombinational enhancement activity; however, it seems likely that they comprise, at least in part, the Eμ element itself. The Eμ element can enhance transcription of introduced genes in both B and T lymphoid cell lines (Grossschedl *et al.*, 1984; Storb *et al.*, 1986); in addition, Eμ appears to be active at the earliest stages of B cell differentiation (Gerster *et al.*, 1986; Lenon and Perry, 1986; Nelsen *et al.*, 1988). Thus, the transcriptional enhancement activity observed correlates well with the recombinational enhancement activity we see in developing transgenic cell populations. Finally, our preliminary evidence also indicates that the Eμ-containing fragment promotes transcription through the unrearranged Dβ/Jβ region of the construct in transgenic T cells (unpublished data), correlating the D to Jβ recombinational enhancement activity with transcriptional activation (see below).

Activation of Eμ in early B cell differentiation (before assembly of Ig variable region genes) may reflect a normal physiological function of this region—targeting the Ig J_H locus for VDJ recombinational activity. Likewise, other enhancers associated with IgL and TCR loci (Queen and Baltimore, 1983; Picad and Schaffner, 1984; Krimppenfort *et al.*, 1988; McDougall *et al.*, 1988; Meyer and Neuberger, 1989; Winoto and Baltimore, 1989) could fulfil enhancement requirements for normal rearrangement of those loci. For example, the κ IgL chain enhancer element becomes active at a time corresponding to the onset of IgL gene rearrangement during B cell differentiation (Parslow and Granner, 1983; Sen and Baltimore, 1986b; Atchison and Perry, 1987). The finding that the Eμ region contains a VDJ recombination enhancing activity also is consistent with apparently anomalous patterns of endogenous Ig gene transcription and rearrangement in lymphoid cells. For example, the germline Ig J_H-Cμ region often is transcribed in T lymphoid cells (Kemp *et al.*, 1980; Alt *et al.*, 1982b)—probably reflecting activity of the Eμ element in this lineage; correspondingly, DJ_H joins occur in T cells (Foster *et al.*, 1980); Kurosawa *et al.*, 1981). In contrast, germline transcripts of TCRβ genes generally are not found in B cell lines (perhaps reflecting inactivity of the TCRβ enhancer element in B cells; McDougall *et al.*, 1988); correspondingly, TCRβ genes undergo DJ rearrangements infrequently in B lineage cells (Kronenberg *et al.*, 1986). However, we clearly demonstrate that linkage of these segments to the Eμ-containing DNA segment dominantly overrides the relative T cell specificity of the TCRβ D to J joining process.

Tissue-specific control of VDJ recombinase activity

We have demonstrated that Vβ to (D)Jβ rearrangement within the Eμ-positive transgenic construct occurs efficiently in T cells but not in B cells. Likewise, we have observed much greater levels of Vβ to DJβ construct rearrangements in transgenic T cell lines as opposed to B cell lines. These findings demonstrate that elements other than Eμ, apparently associated with the TCR Vβ region of the construct, can effect tissue-specific control of Vβ to DJβ joining and thus provide lineage-specificity of VβDJβ assembly (and expression). These results, coupled with our additional finding that complete VβDJβ rearrangement within the transgenic construct usually proceeds via DJβ intermediate, are in accord with the suggestion that the V to DJ joining step is the regulated step in the context of endogenous allelically excluded and tissue-specific variable region gene assembly (Alt *et al.*, 1984, 1986, 1987; Uematsu *et al.*, 1988).

The known TCRβ transcriptional enhancer has been mapped to a region of the Cβ gene not included in our construct (Krimppenfort *et al.*, 1988; McDougall *et al.*, 1988; Hashimoto, 1989); therefore, this element could not have provided T cell specificity of Vβ to DJβ joining. Furthermore, no Vβ rearrangements were detected within the Eμ-negative construct. Therefore, activity of the Vβ-specific rearrangement elements must depend on the presence of the Eμ-containing segment in the construct (or on the occurrence of the DJβ joining event that the segment promoted). In this regard, we found high levels of transcripts from the unrearranged Vβ14 gene of the Eμ-containing construct in thymus but not bone marrow of transgenic mice harboring this construct. Conversely, we detected only a low level of unrearranged Vβ14 transcripts (equivalent to that of the endogenous gene in normal control mice) in thymuses of mice harboring the Eμ-negative construct. Therefore, expression, like rearrangement, of the unrearranged Vβ14 gene in the construct also depended on the presence of the Eμ element. Together, these data strongly support the notion that transcriptional activation of the Vβ14 segment is either directly or indirectly related to activation of this gene segment for recombination.

Expression of the germline Cκ locus and germline V_H gene segments occurs at or prior to the time of the rearrangements of these loci in a manner consistent with a cause–effect relationship (Van Ness *et al.*, 1981; Yancopoulos *et al.*, 1985; Schlissel and Baltimore, 1989). Furthermore, transcription of VDJ recombination substrates introduced into pre-B cell lines correlated with their rearrangement frequency (Blackwell *et al.*, 1986). Transcription and recombination activity also have been correlated in the context of a number of other types of recombination events, including heavy chain class-switch recombination (reviewed by Blackwell and Alt, 1988; Lutzker and Alt, 1989). We now show, for the first time, that direct linkage of substrate V, D and J sequences (and their associated VDJ recombinase signal sequences) to DNA segments containing lymphoid-specific transcriptional control elements can enhance assembly of the target sequences in a physiological setting. However, we emphasize that the mechanism by which transcriptional activation of these regions is linked to activation of recombination remains unknown and also that it remains possible that additional types of elements could be involved in mediating recombinational control. Finally, although we have described the Eμ(-associated) element as necessary to initiate the rearrangement process within the transgenic construct and the Vβ-associated element as responsible for lineage-specific control, combinations of such elements likely interact to effect the complex patterns of VDJ recombination observed at endogenous loci.

Materials and methods

Construction of the recombination substrates

The plasmid pVβ14DβJβ carrying the 4.5 kb *Hin*dIII restriction fragment and the 3.2 kb *Hin*dIII–*Bam*HI restriction fragment containing respectively the germline Vβ 14 gene (Malissen *et al.*, 1986) and the germ-line Dβ 1.1, Jβ 1.1; Jβ 1.2 gene segments (Siu *et al.*, 1984; Gascoigne *et al.*, 1984)

has previously been described (Ferrier *et al.*, 1989). This plasmid was cleaved with *Eco*RI and partially cleaved with *Hin*dIII in the polylinker to isolate the 7.7 kb insert which was subcloned in the *Hin*dIII−*Eco*RI sites of a 4.8 kb vector derived from the cosmid pMCS (Grosveld *et al.*, 1982), resulting in cosmid cVβ14Dβ1β. The VβDβJβCμ construct was derived by ligating the 10.5 kb *Eco*RI fragment from genomic clone pRI-Cμ (Alt *et al.*, 1981) containing the IgH Cμ gene into the single *Eco*RI site of cVβ14Dβ1β. The pRI-Cμ insert corresponds to the embryonic 12.5 kb *Eco*RI fragment which contains the Cμ exons but has undergone a 2 kb deletion several kb 5′ to the first Cμ exon (Alt *et al.*, 1982a). In construct VβDβJβECμ, a 1126 bp *Nae*I−*Eco*RI restriction fragment (Gough and Bernard, 1981; Gillies *et al.*, 1983) containing the IgH transcriptional enhancer was inserted downstream of the TCR variable gene segments (using the *Sma*I restriction site originally from the pUC8 polylinker) before the ligation of the pRI-Cμ *Eco*RI insert.

Probes

DNA fragments used as probes were isolated and prepared as previously described (Alt *et al.*, 1981). The Vβ 14, 3′Vβ, 5′Dβ, Jβ and 5′Cμ probes (all schematized in Figure 1A) were previously described (Ferrier *et al.*, 1989). The 3′Dβ probe was a *Pst*I−*Bgl*II restriction fragment containing 350 bp of Dβ 1.1 3′ flanking sequences. The pMCS probe was a 2.0 kb *Hin*dIII−*Nru*I restricton fragment isolated from cosmid pMCS (Grosveld *et al.*, 1982). The pM.2 N-*myc* probe was previously described (Zimmerman *et al.*, 1986). The Vβ 14-specific probe used for S1 nuclease protection analyses contained a 340 bp *Hae*III−*Acc*I fragment from the 3′ portion of the Vβ 14 gene and 70 bp of polylinker plasmid sequences.

Production and analysis of transgenic mice

The constructs were linearized with restriction enzymes *Nru*I and *Pvu*I, which cut into the cosmid vector sequences. The 20.2 and 21.4 kb inserts containing, respectively, the VβDβJβCμ and VβDβJβECμ sequences were purified on agarose gels and by banding the CsCl, then resuspended at a concentration of 2 μg/ml. DNA micro-injection in fertilized (C57BL/6 × CBA/J)F_2 eggs and isolation of tail DNA were as previously described (Costantini and Lacy, 1982; Hogan *et al.*, 1986). Analysis of tail DNA for the presence of intact transgenes was performed by Southern Analysis (see below), using the pMCS probe and either *Kpn*I or *Eco*RI restriction enzymes which both cut twice at different regions in the two micro-injected constructs (see Figure 1A) generating predictable hybridizing fragments of, respectively, 9.4 and 10.3 kb (VβDβJβCμ) or 11.5 kb (VβDβJβECμ) when several intact copies of the construct are integrated in a head-to-tail configuration.

Cell separation

Peripheral B and T cells were purified from a mixed cell suspension prepared from spleen and lymph nodes of transgenic mice. Immunofluorescence analyses showed that such cell suspension were primarily constituted by B and T lineage cells in roughly equal proportion. B cells were purified by depletion of T cells using anti-Thy 1.2 monoclonal antibody (mAb) (New England Nuclear, Boston, MA) + rabbit complement (Pel-freez, Rogers, AR), whereas T cells were purified by panning of B cells on anti-surface Ig (Zymed, San Francisco, CA)-coated plates using previously described procedures (Malynn and Wortis, 1984; Wisocki and Sato, 1978). Cell purification was monitored by immunofluorescence (FACS) analysis using anti-Thy 1.2 (Becton Dickinson, Mountain View, CA) and Fab′2 anti-surface Ig (Zymed) fluorescent antibodies.

Production of A-MuLV-transformed cells

A-MuLV-transformation of newborn fetal liver (with Moloney helper) or adult thymus (with Rad LV helper) from transgenic mice was performed as previously described (Rosenberg and Baltimore, 1976; Cook, 1985).

Analysis of integrated transgenes and transgene rearrangements

DNA preparation, restriction enzyme digests, agarose gel electrophoresis, DNA blotting procedures, ^{32}P-labeling of DNA fragments by nick translation and hybridization procedures were performed as previously described (Alt *et al.*, 1981). Densitometric analyses were performed with a Joyce Loebl Chromoscan 3. For DNA cloning of the transgene rearrangments, genomic DNA from thymus of a transgenic 4 week old female mouse (line 392) was digested with enzymes *Hin*dIII and *Bgl*II and cloned in λ bacteriophages charon 21 and λDash (Stratagene, La Jolla, CA) respectively, using previously described procedures (Alt *et al.*, 1984). Recombinant bacteriophages were selected for hybridization to both Vβ 14- and 5′Cμ-specific probes. The inserts of the selected recombinants were mapped and restriction fragments shown to contain transgene rearranged regions were subcloned in M13 (mp19) bacteriophage or pUC19 plasmid. DNA rearrangements were sequenced by the Sanger dideoxy procedure.

Analysis of Vβ 14 and gene expression

Preparation of total RNA, Northern blotting and S1 nuclease protection methods were as described previously (Yancopoulos and Alt, 1985).

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