

# Junctional Sequences of Fetal T Cell Receptor $\beta$ Chains Have Few N Regions

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## Summary

T cell receptors (TCRs) and immunoglobulins (Igs) derive a large fraction of their repertoire from diversity generated at the junctions of the V, D, and J coding segments. This diversity is derived both from the random deletion of nucleotides from the ends of coding regions and from the subsequent addition of nontemplated N region nucleotides. While the vast majority of TCRs and Igs from adult mice have N regions, <5% of both TCR- $\gamma/\delta$  and Ig from fetal and neonatal mice have N regions. This study analyzed the ontogeny of junctional diversity of TCR- $\alpha/\beta$ . Genomic DNA or C $\beta$ -primed cDNA was prepared from thymocytes of mice at varying stages in ontogeny, and the rearranged V $\beta$ 8 or V $\beta$ 5 sequences were amplified by polymerase chain reactions. Sequencing of the V $\beta$ -D $\beta$ -J $\beta$  junctions showed few N regions early in ontogeny, although the fraction of sequences with N regions exceeded that previously reported for Ig and for TCR- $\gamma/\delta$ . N regions were found in 13% of V $\beta$  junctional sequences from day 18–19 fetal thymocytes, 33% of sequences from newborn thymocytes, 76% of sequences from day 4 postnatal thymocytes, and 88% of sequences from 5-wk-old thymocytes. In addition, nonrandom usage of the D $\beta$  and J $\beta$  segments was observed in both fetal and adult TCR sequences. While the usage of each of the six J $\beta$  segments was different, the same pattern of usage was seen regardless of whether D $\beta$ 1 or D $\beta$ 2 was used, suggesting that a factor controlling the rate of usage of each J segment is intrinsic to the J gene itself. Since TCRs derive so much of their diversity from N regions, the relative paucity of N regions in fetal  $\alpha/\beta$  T cells would create a fetal TCR- $\alpha/\beta$  repertoire that would be quite different from, and smaller than, the adult repertoire. The lack of N regions might be predicted to limit the range of affinities of TCR-MHC + peptide interactions, which may have important consequences for positive and negative selection of fetal and newborn T cells.

The three types of lymphocyte antigen receptors (Ig, TCR- $\alpha/\beta$ , and TCR- $\gamma/\delta$ ) are each heterodimers whose variable regions are encoded by somatically recombined gene segments. To obtain their vast repertoire, T cells and B cells use the same mechanisms of combinatorial and junctional diversity (1–3). Combinatorial diversity is derived from the large number of V, D, and J gene elements used to create the variable regions, and from the random association of the two chains to make a complete receptor. Additional diversity is generated at the junctions of these gene segments during somatic recombination by two mechanisms. First, there is deletion of a variable number of nucleotides from the ends of the coding segments. Second, there is addition of a variable number of N region nucleotides to the junctions of the IgH chain and all junctions of the TCR before ligation of the DNA. The N region nucleotides are nontemplated and are thought to be added by the enzyme terminal deoxynucleotidyl transferase (TdT)<sup>1</sup> (4–7). Junctional diversity is

even more important in generating the total diversity in the TCR repertoire than in the Ig repertoire, since combinatorial association alone leads to a much smaller repertoire for T cells than B cells, and since all TCR junctions can have N regions, while light chains of Ig do not have N regions (3).

T cells are subdivided into two lineages on the basis of which type of TCR they use. T cells with TCR- $\gamma/\delta$  arise first in ontogeny, but are soon outnumbered by  $\alpha/\beta$  T cells, the predominant T cell type in the adult (8). Fetal  $\gamma/\delta$  T cells, and some of their adult progeny, do not have N regions in their receptors (9–11), and IgH chains from fetal and newborn B cells also are devoid of N regions (12, 13). Since adult levels of TdT are not reached in the thymus until  $\sim$ 1 wk of age (14, 15), and since  $\alpha/\beta$  T cells are the most predominant T cell type in the mouse thymus by day 18 of gestation (8), this study addressed the question of whether  $\alpha/\beta$  T cells, like  $\gamma/\delta$  T cells and B cells, would express few N regions in their receptors early in ontogeny. The results show that this is the case, although the frequency of junctions with N regions is higher than is observed in TCR- $\gamma/\delta$  and Ig for age-matched mice. It has been proposed that the complemen-

<sup>1</sup> Abbreviations used in this paper: RSS, recombination signal sequence; TdT, terminal deoxynucleotidyl transferase.

tarity determining region 3 (CDR 3)-equivalent regions of the TCR- $\alpha/\beta$  (which are composed of all the junctions, all of the D region in the  $\beta$  chain, and part of the J region) are mainly responsible for interacting with antigenic peptides (3, 16). The paucity of N regions in fetal/newborn  $\alpha/\beta$  T cells will restrict the diversity in CDR3, and will therefore limit the fetal T cell repertoire.

## Materials and Methods

**Mice.** BALB/c mice were bred at Medical Biology Institute. Fetal thymuses were obtained from timed pregnancies, with day 0 being the day that a vaginal plug was observed. The newborn mice were used within 24 h of birth. At least five mice from a litter were pooled for the fetal and newborn samples. Each litter was prepared as a separate sample. Young adult thymus RNA was obtained from a pool of two 5-wk-old mice, and young adult spleen RNA was prepared from a pool of two different 5-wk-old mice.

**RNA and cDNA Preparation.** Total cellular RNA was extracted from thymuses by the guanidinium thiocyanate/cesium chloride method (17), and cDNA was prepared as previously described (12, 18) with a C $\beta$  primer. DNA was prepared by the freeze-thaw method previously described (12).

**PCR Primers.** All oligonucleotide primers were made by Genosys (The Woodlands, TX). Initially, a C $\beta$  primer (5'-CAT-AGAATTCCTACTTGGCAGCGGAAGTGGT, kindly provided by Dr. Daniel Gold, La Jolla Institute of Experimental Medicine, La Jolla, CA) that was complementary to a sequence in the third exon of C $\beta$  was used. Later experiments used a C $\beta$  primer (5'-GGG-TGAATTCACATTTCTCAGATC) that ended three nucleotides from the J/C junction. These C $\beta$  primers were used both for cDNA synthesis and for subsequent PCR, and both contained an EcoR1 site. The 5' V $\beta$ 8 PCR primer (5'-CGACAAGCTTTGGTATCG-GCAGGAC) has a HindIII site and is homologous to the three V $\beta$ 8 genes (19). The 5' V $\beta$ 5 PCR primer (5'-TCAGAAGCTT-CTGGGGTTGTCCAG) also contains a HindIII site, and is ho-

mologous to the two V $\beta$ 5 genes (19). Genomic DNA was amplified with a nested V $\beta$  primer set and a primer complementary to J $\beta$ 2.6. The external V $\beta$ 8 primer was (5'-GAGGAAAGGTGACATTG-AGC), and the internal primer was the V $\beta$ 8 primer described above. For V $\beta$ 5, the primer listed above was the external primer, and the internal primer was (5'-GAAGAAGCTTCCCATCTCTGGACA). The J $\beta$ 2.6 primer was (5'-GCCTGAATTCGGGACCGAAGTA).

**PCR Amplification and Sequencing.** PCR amplification, cloning, and sequencing was performed as previously described (12, 18) with the following exceptions. Annealing temperature for the PCR was 60–64°C. For genomic DNA, 35 cycles of amplification were performed using the external V $\beta$ 8 primer and the J $\beta$ 2.6 primer. 15  $\mu$ l of that primary PCR was directly added to a secondary PCR with the internal V $\beta$ 8 primer and the J $\beta$ 2.6 primer, and was amplified for 30 cycles. The amplified DNA was then processed as previously described (12).

## Results

**Analysis of Junctional Sequences and Choice of PCR Primers.** Analysis of junctional sequences requires knowledge of the complete germline sequence of the V, D, or J gene segment up to the heptamer of the recombination signal sequence (RSS). The germline sequences of the D $\beta$  and J $\beta$  segments are known (20–22), but not all V $\beta$  genes are sequenced. The three-member V $\beta$ 8 gene family has been sequenced (19), and is used in ~25% of TCR<sup>+</sup> thymocytes and peripheral T cells (23). Additionally, to assess the generality of the data, a small number of sequences were generated using V $\beta$ 5-specific primers. The two functional V $\beta$ 5 genes are also sequenced on the genomic level, and are used in 8% of thymocyte  $\beta$  chain transcripts (19, 24). The use of both V $\beta$ 8 and V $\beta$ 5 primers should sample a significant proportion of the TCR repertoire.

N region nucleotides were defined as any nucleotides that

ADULT THYMUS RNA										
	V $\beta$	Pv	N	Pd	D $\beta$ 1.1	Pd	N	Pj	J $\beta$ #deleted	
<b>V<math>\beta</math>8.1</b>	GCC AGC AGT GAT G	CA		CC	GGGACAGGGGGC	GC				
100-4N	-----		<b>T</b>		GGGGC		<b>GTTG</b>	1.4	4	
100-4AH	-----		<b>AG</b>		AGG	G		2.2	2	
<b>V<math>\beta</math>8.2</b>	GCC AGC GGT GAT G	CA								
100-4A	-----		<b>A</b>		AGGGG		<b>A</b>	1.3	2	
100-4D	----- (-)				GGACAG		<b>AAA</b>	2.6	2	
100-4AL	-----			CC	GGG			1.2	0	
100-4AC	-----				GGGACAGGG		<b>TG</b>	2.5	4	
100-4AE	-----			CC	GGGACA		<b>AGCGGG</b>	1.2	4	
100-4AF	-----		<b>GAA</b>		GGGA		<b>A</b>	1.1	0	
100-4AG	-----		<b>GC</b>		ACAGGG (G)			1.1	0	
<b>V<math>\beta</math>8.3</b>	GCC AGC AGT GAT G	CA								
100-4B	-----		<b>GCC</b>		GACAGG		<b>TC</b>	1.1	0	
100-4C	-----		<b>CTCT</b>		GGGA (C) **			2.6	3-4	
100-4F	-----		<b>AAGGC</b>		ACA		<b>CCC</b>	2.1	3	
100-4H*	-----		<b>TT</b>		GGGGC**			2.1	8	
100-4L	-----				GGGACAGGGGGC		<b>TCTT</b>	2.1	6	
100-4M	-----		<b>CGG</b>		GGGGC		<b>AGG</b>	1.2	4	
100-4AD	-----		<b>C</b>		GGACA		<b>A</b>	1.4	2	
<b>V<math>\beta</math> 6</b>	GCC AGC AGT ??									
100-4J	-----		<b>A</b>		GGGACA		<b>AT</b>	2.6	3	
<b>V<math>\beta</math>8.2</b>	GCC AGC GGT GAT G	CA		Pd	D $\beta$ 2.1	Pd				
100-4E	-----			CC	GGGACTGGGGGGC	CC				
100-4I	----- (-)				GGACTGG		<b>C</b>	CT	2.3	0
100-4AB	----- (-)				TGGGGGGC	G(C)			2.5	3-4
<b>V<math>\beta</math>8.3</b>	GCC AGC AGT GAT G	CA			GGACTGGGG				2.4	0
100-4K	-----				GGGACTGGGG		<b>CG</b>		2.3	5
100-4AK	-----		<b>CCCC</b>		GACTGGGGGGC				2.1	3
100-4AI	-----		<b>CG</b>	CC	GGGACTG		<b>C</b>		2.1	6
100-4AJ	-----		<b>GGA</b>		ACTGG (G)				2.2	0

**Figure 1.** Sequences of V $\beta$ -D $\beta$ -J $\beta$  junctional regions from thymocytes of 5-wk-old mice. Junctional antibodies that could be encoded by P regions (11) are listed under the Pv, Pd, or Pj columns. Nucleotides that could be encoded by either of two adjacent germline genes (including P dinucleotides as extensions of the germline gene sequence) are listed in parentheses in the more 5' location. The 3' end of the V $\beta$ 7 sequence is the consensus from the sequences that we obtained in this study, and therefore is only an estimate of the germline sequence. N region nucleotides are written in bold type. Sequences that result in the V region being out-of-frame with the J region are indicated by an asterisk after the sequence number. D region sequences that cannot be definitively assigned to D $\beta$ 1 or D $\beta$ 2 are indicated by a double asterisk. The number of nucleotides deleted from the J $\beta$  sequence proper (i.e., not including P region dinucleotides) are listed in the far right column. 21 of 24 sequences (88%) had N region nucleotides in at least one of the two junctions.

DAY 18 FETAL THYMUS RNA

Vβ	Pv	N	Pd	Dβ1.1	Pd	N	Pj	Jβ #deleted
Vβ8.1	GCC AGC AGT GAT G	CA	CC	GGGACAGGGGGC	GC			
126-1AA	--- -- (-)			ACAGG (G)			1.2	0
126-1AC	---	A		GACA			1.2	1
126-1AE	---			GGAC (A)			1.1	2-3
126-1CC	--- -- (-)			G (A) **			2.3	0-1
126-1CH	---			GGGACAGGGG			2.6	4
Vβ8.2	GCC AGC GGT GAT G	CA						
126-1F	---			CA			1.2	0
126-1H	---			C (AG)			2.3	0-2
126-1L	---			GGAC (AG)			2.6	0
126-1AB	--- -- (-)			GGG		TT	2.5	2
126-1BA	---			GGGACAGGGGGC	(G)		2.4	1-2
126-1BE	--- -- (-)			GACAGGGGG (C)			1.1	0-1
126-1BF	---		C	GGGACA			2.4	3
Vβ8.3	GCC AGC AGT GAT G	CA						
126-1AD	--- -- (-)			CA			1.2	0
126-1AH	---			GG (GA) **			2.3	6-8
126-1AI	---			GGACAGGGG (GC)			2.1	7-9
126-1BB	---			GGGG (C) **			2.5	2-3
126-1BI	---			GGG**			2.6	4
126-1CF	---			GGG (G)			2.3	6-7
126-1CG	---			C (A)			1.1	2-3
126-1CI	---			(AC)			2.6	14-16
Vβ8.2	GCC AGC GGT GAT G	CA	Pd	Dβ2.1	Pd			
126-1C	--- -- (-)		CC	GGGACTGGGGGGC	GC		2.6	4
126-1G	---			GGACT			2.5	5
126-1AL	---			GGGACTGGGGGG			2.6	4
126-1AF	---	A		GGGGGGC		C	2.6	7
126-1BD	---			TGGGGGG (C)		T	2.3	0
126-1BG	---			G		T	2.3	0
126-1BH	---		CCT	GGGACTGGGGG (G)			2.4	1-2
126-1CE	---			ACTGGGGGG (C)			2.5	3-4
Vβ8.3	GCC AGC AGT GAT G	CA						
126-1I	---			ACTGG			2.3	0
126-1AK	---	ACA		ACTGGGGGGG (C)			2.1	3-4
136-3A	---			ACTGGGG			2.4	5

Figure 2. Sequences of Vβ-Dβ-Jβ junctional regions from RNA from day 18 fetal thymocytes. Sequences are displayed as described in the legend to Fig. 1. 5 of 31 sequences (16%) had N region nucleotides in at least one of the two junctions.

could not have been accounted for by any of the germline gene segments. This is a minimal estimate of N region nucleotides, since it is possible that a nucleotide could be added by TdT that would coincidentally be identical to a germline encoded nucleotide. Any junctional nucleotides that could be encoded by P regions (11) were counted as such.

**Reduction in N Region Use in cDNA-derived Junctional Sequences from Fetal and Newborn Thymocytes.** RNA from thymocytes derived from mice at varying stages of ontogeny was converted into cDNA with a Cβ primer. The cDNA was then amplified by PCR with a Vβ and a Cβ primer, and the amplified DNA was cloned and sequenced. Fig. 1 shows the sequences derived from thymocytes of 5-wk-old mice, where 88% of the sequences contained N regions (N region-containing sequences are hereafter referred to as N+). Likewise, 82% of the sequences from splenocytes from 5-wk-old mice were N+ (data not shown). In Fig. 2, the sequences derived from day 18 fetal thymocytes are displayed, and 16% of those have N regions. 10% of the sequences from day 19 fetal thymocytes are N+ (Fig. 3). Fig. 4 shows representative cDNA junctional sequences from four separate litters of mice <24 h old. 33% of the 83 total sequences are N+. There was some litter-to-litter variation: the four samples contained an average of 23, 33, 36, and 44% N+ sequences. 76% of the sequences derived from thymocytes of 4-d-old mice contained N regions (Fig. 5). All of the results are summarized in Fig. 6. Analysis of only the sequences that had N regions showed a trend towards increasing the number of N nucleotides per N+ sequence with time.

**Reduction in N Region Use in Genomic DNA-derived Junctional Sequences from Newborn Thymocytes.** Amplification of

genomic DNA ensures that each cell is equally represented in the analysis, whereas analysis of RNA is subject to the possibility of cell-to-cell variation in the amount of mRNA (25). To address this issue of whether the analysis of RNA was representative of the cellular population in the thymus, we also analyzed sequences derived from genomic DNA. These sequences were amplified with a 3'PCR primer complementary to the frequently used Jβ2.6 segment (see Fig. 9). The decision to sample only that fraction of rearranged genomic sequences using Jβ2.6 was necessitated by the lack of a consensus Jβ sequence, and by the long intron between Jβ and Cβ, which precludes use of a Cβ 3' primer. 12% of the sequences derived from fetal day 18 DNA had N regions (Fig. 7). Two preparations of newborn genomic thymus DNA were made, and, as with the newborn RNA samples, variation between samples was observed. One sample (PCR 161-2, 163-2, and 165-3) contained 13% N regions, and the other (PCR 135-1 and 140-2) contained 29% N regions. Since this time point is the stage at which N regions begin to be seen, some sample-to-sample variation might be expected at this time. Overall, 22% of the newborn sequences contained N regions (Fig. 8).

Genomic DNA contains both productive and nonproductive alleles, and both are amplified. 28% of the sequences derived from genomic DNA were out-of-frame, whereas only 4% of the sequences from RNA were out-of-frame. The percentage of N regions was lower in the out-of-frame than in the in-frame rearrangements. Comparison of only in-frame rearrangements reveals that the percentage of N regions is very similar in both RNA and DNA. Thus, on the level of the expressed allele, there does not appear to be any skewing

DAY 19 FETAL THYMUS RNA

Vβ	Pv	N	Pd	Dβ1.1	Pd	N	Pj	Jβ #deleted
<b>Vβ8.1</b>	GCC AGC AGT GAT G	CA	CC	GGGACAGGGGGC	GC			
141-1CE	---			GGGACAGGG			T	2.3 0
141-1CF	---			GGA (CA)				1.2 0-2
141-1CH	---			GGACA				2.1 1
<b>Vβ8.2</b>	GCC AGC GGT GAT G	CA						
136-1A	---			GGACAGGG (G)				1.1 0
141-1C	---			CAG		AG		2.6 0
141-1F	---		<b>CTGG</b>	CAGG				2.6 6
141-1I	---							2.6 6
141-1CM	---							1.2 1
141-1CO	---			CAGG				1.2 2
141-1CC	---			A (G)				1.2 0
<b>Vβ8.3</b>	GCC AGC AGT GAT G	CA						
141-1Q	---			GACAGGG				1.3 3
141-1P	---							1.5 0-1
141-1G	---			GACAGG		<b>ACT +</b>		2.4 10
141-1BC	---			GACAGG (G)				2.3 1-2
141-1BD	---			GG (G)				2.3 1-2
<b>Vβ7</b>	GCT AGC AGT TTA	TA						
141-1N	---		<b>GGGG +</b>	CAGGGG (GC)				2.3 3-5
<b>Vβ5.1</b>	GCC AGC TCT CTC	GA						
159-2B	---			GGGACA				2.4 4
<b>Vβ5.2</b>	GCC AGC TCT CTC	GA						
159-1A	---			GACAGGG (GC)				2.1 1-3
159-2A	---			AC				1.1 0
159-2C	---			GGGGG**				2.4 1
<b>Vβ8.1</b>	GCC AGC AGT GAT G	CA		<b>Pd</b>	<b>Dβ2.1</b>	<b>Pd</b>		
141-1M	---			CC	GGGACTGGGGGGC	GC		
141-1BA	---				ACTGGGGGGC (G)			2.3 1-2
141-1CN	---				CTGGGGGG (GC)			2.3 3-5
					CTGGGGGG (GC)			2.1 7-9
<b>Vβ8.2</b>	GCC AGC GGT GAT G	CA						
141-1BF	---				GAC (TG)			2.1 6-8
141-1CB	---				GACTGGGGGG (GC)			2.5 3-4
<b>Vβ8.3</b>	GCC AGC AGT GAT G	CA						
141-1A	---				GGACTGGG			2.1 3
141-1B	---				GACTGGGGG			2.1 3
141-1BE	---				GACT			2.5 2
141-1CD	---				CTGGGGGGC (G)			2.5 6-7
<b>Vβ5.1</b>	GCC AGC TCT CTC	GA						
159-1F	---				GGGGG		CT	2.3 0
159-2F	---				TGGGGG		CT	2.4 0

**Figure 3.** Sequences of Vβ-Dβ-Jβ junctional regions from RNA from day 19 fetal thymocytes. Sequences are displayed as described in the legend to Fig. 1. In two cases, indicated at the N region (+), if the putative N region nucleotide that is not in bold type is a PCR error, then the whole N region could be germline. However, all such nucleotides are considered as true N region nucleotides in all calculations. 3 (including the two just mentioned) of 31 sequences (10%) had N region nucleotides in at least one of the two junctions.

of the analysis by amplifying RNA vs. DNA. Both data sets lead to the conclusion that the percentage of N<sup>+</sup> sequences in fetal and newborn TCR-α/β is greatly reduced as compared with adult TCR-α/β.

**Nonrandom D and J Usage.** The number of sequences that use each of the Jβ segments in association with either of the D segments is shown in Fig. 9. Only sequences derived from Cβ-primed cDNA were used, since all of the sequences derived from DNA were generated using a Jβ2.6 primer. Since the genomic organization of the D/J region is Dβ1, Jβ1.1-6, Dβ2, Jβ2.1-6, the Jβ1 segments can only use Dβ1, whereas the Jβ2 segments can join to either D segment (26). There are approximately equal frequencies of Dβ1-Jβ1, Dβ1-Jβ2, and Dβ2-Jβ2 rearrangements, resulting in the use of Dβ1 twice as often as Dβ2. Also, all three D region reading frames are used to a similar extent. These data, obtained from >200 unselected junctional sequences, confirm and expand previous analyses of T cell clones and hybridomas (20, 27, 28). There is significant nonrandom usage of Jβ segments. It is striking that the pattern of usage of the six Jβ2 segments is the same regardless of the Dβ segment used, as discussed below.

**Discussion**

The results presented here show that TCR β chains have a low frequency of N regions early in ontogeny. It has previ-

ously been shown that <5% of TCR-γ/δ from fetal and newborn mice have N regions, and we have recently shown that IgH junctions from newborn liver and spleen DNA are essentially devoid of N regions (10-13). Thus, the paucity of N regions early in ontogeny is a general phenomenon in all lymphocytes. The level of TdT, the enzyme thought to add N region nucleotides, is very low in fetal and newborn thymus, and does not reach adult levels until 1 wk after birth (14, 15). TdT is also not observed in fetal liver, the site of fetal B cell lymphopoiesis (15). Thus, the late appearance of TdT is the most likely explanation for the low frequency of N regions in fetal/neonatal TCR and Ig.

The percentage of N regions observed in TCR β chains from newborn mice is much higher than in TCR-γ/δ and IgH from newborn mice. By postnatal day 4, the percentage of N regions in TCR β chains is almost at the adult level. In contrast, only 29% of IgH junctional sequences from day 9 postnatal mice contain N regions (A.J. Feeney, unpublished results). There are two alternative explanations for the earlier appearance of N region nucleotides in α/β T cells as compared with γ/δ T cells and Ig. The first is that TdT could be differentially regulated in γ/δ T cells, α/β T cells, and B cells. The second explanation is that the intrinsic rate of N region addition could be equally low in all lymphocytes. However, α/β T cells undergo both positive selection for reactivity with self-MHC, as well as negative selection (toler-

NEWBORN THYMUS RNA

Vβ	Pv	N	Pd	Dβ1.1	Pd	N	Pj	Jβ	#deleted
<b>Vβ8.1</b>	GCC AGC AGT GAT G	CA		GGGACAGGGGGC	GC				
100-2AA	---			GGGACAGGGGG		<b>A</b>	1.3	4	
100-2AE	---			AC (A)			1.3	0	
111-1B	---			CAGG (G)			1.2	0	
111-1Q	---			ACAGGGG			1.6	9	
111-1AE	---	<b>A</b>		GGAC (AG)			2.6	0	
120-1C	---			AG		<b>C</b>	2.1	1	
120-2K	---			AC (A)			1.2	2-3	
<b>Vβ8.2</b>	GCC AGC GGT GAT G	CA							
100-2F	---			GGGACAGGG			1.6	5	
100-2AC	---			(G)			1.2	0	
111-1A	---			GACAGGG			1.3	9	
120-1L	---			CAGGGGGC	(G)		2.6	7-8	
120-1S	---			CAGG			2.6	4	
120-2J	---			GGAC (A)			1.2	2-3	
120-2CF	---			CAGGGGGC			2.6	5	
120-2CM	---			GGGAC (A)			2.1	2-3	
<b>Vβ8.3</b>	GCC AGC AGT GAT G	CA							
100-2E	---		<b>AT</b>	CA			1.6	4	
100-2AG	---			GGACAGGGG			2.4	7	
111-1J	---						1.1	4	
111-1L	---			GGGACAGGG		<b>CA</b>	2.1	10	
120-1H*	---			G (A) **			1.5	1-2	
120-1R	---			ACAGGG			2.6	4	
120-1G	---			GGC**		<b>TCT</b>	2.1	0	
120-2CA	---			AC (A)			2.5	0-1	
120-2CK	---		<b>CCCTC</b>	GACAGG (G)			2.4		
120-2CN	---			ACAGGG			2.3	0	
120-2CO	---		<b>CGT</b>	CAGGGG		<b>AGG</b>	1.4	7	
<b>Vβ7</b>	GCT AGC AGT TTA TA								
111-1M	---			GGGACAGG			2.4	4	
111-1P	---			CA			1.2	0	
111-1AB	---		<b>G</b>	GGGACAGGGG	CC		2.6	5	
120-1K	---			GGACA		<b>A</b>	2.6	3	
120-2S	---			ACAGG (G)			2.4	1-2	
120-2CJ	---						1.2	0	
<b>Vβ8.1</b>	GCC AGC AGT GAT G	CA		<b>Pd</b> Dβ2.1	<b>Pd</b>				
100-2H	---			CC GGGACTGGGGGGC	GC				
111-1AG	---			GGACTGGGGG		<b>T</b>	2.1	8	
				ACTGGGGG			2.1	0	
<b>Vβ8.2</b>	GCC AGC GGT GAT G	CA							
100-2AH	---			CTGGGGG		<b>C</b>	2.4	2	
111-1C	---			ACTGGG			2.4	7	
111-1I	---			GGGACTGGG (G)	CC		2.5	6-7	
120-1A	---			GGGACTGGGGGG	CC	<b>A</b>	2.1	8	
120-2C	---			(CT)			2.6	3-5	
121-2CL	---			CTGGGGGGC		<b>AGG</b>	2.4	6	
<b>Vβ8.3</b>	GCC AGC AGT GAT G	CA							
100-2N	---		<b>C</b>	GA**			2.3	1	
100-2AT	---			CTGGGG (G)			2.4	1-2	
111-1K	---			CTGGGG			2.6	4	
111-1N	---			GGACTGGGGG (GC)			2.1	7-9	
111-1AA*	---			ACTGGGGGGC	G	<b>AGA</b>	2.3	2	
120-1J	---			CTGG			2.6	4	
120-2R	---			CTGGG		<b>TT</b>	2.5	0	
<b>Vβ6</b>	GCC AGC AGT ??								
111-1H	---			GACTGGGGGG		<b>T</b>	2.3	0	
<b>Vβ7</b>	GCT AGC AGT TTA TA								
111-1E	---		<b>C</b>	GGGACTGGGG			2.2	2	
111-1G	---			ACTG (G)			2.3	1-2	
111-1AI	---			CTGGG			2.6	4	

**Figure 4.** Representative sequences of Vβ-Dβ-Jβ junctional regions from RNA from four separate litters of mice (PCR nos. 100-2, 111-1, 120-1, and 120-2) that were <24 h old. All of the sequences are used for the calculations in the summary figure, Fig. 6. Sequences are displayed as described in the legend to Fig. 1. 27 of 83 sequences (33%) had N region nucleotides in at least one of the two junctions.

ance) in the thymus (29, 30). The diversity afforded to TCR by N regions is likely to be important in generating the range of receptor affinities able to successfully undergo positive and negative selection, and emerge as mature T cells. Thus, if the lack of N regions restricts the ability of many TCRs to have sufficient affinity to interact with self-MHC (plus peptide) for positive selection, then the selection process could skew the percentage of TCR-α/β with N regions from the rate with which they were initially generated. In support of this latter hypothesis is the fact that there are fewer N regions in the nonproductive (8% N<sup>+</sup>) than the productive (28% N<sup>+</sup>) rearrangements in the sequences derived from newborn DNA.

TCRs have significantly less diversity generated by com-

binatorial usage of germline gene elements than do Igs, since there are fewer germline gene segments. However, this is compensated for by extensive diversity in CDR3-equivalent regions. This difference in the generation of diversity of T and B cell repertoires may have been selected for on the basis of the different ways in which T and B cells interact with antigen. Igs bind to antigens directly, and all IgH CDRs can be involved in antigen binding (31, 32). In contrast, α/β T cells are known to respond to antigenic peptides that are thought to be presented in the groove of class I or class II MHC (33, 34). On the basis of sequence comparisons between TCRs and Igs, it has been proposed that the CDR3 regions of the α and β chains of the TCR bind to the peptide, while the other CDRs bind to MHC (3, 16). This model

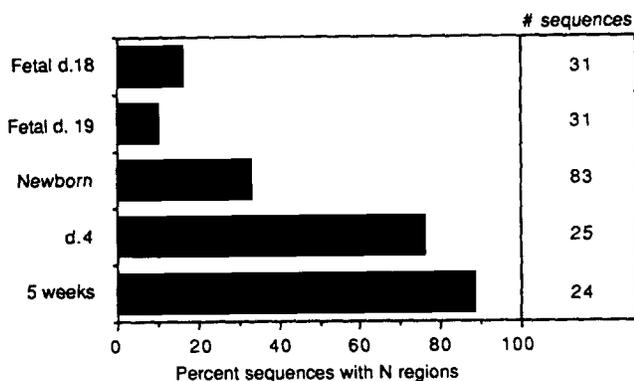
DAY 4 POSTNATAL THYMUS RNA

Vβ	Vβ	Pv	H	Pd	Dβ1.1	Pd	H	Pj	Jβ #deleted
Vβ8.1	GCC AGC AGT GAT G	CA		CC	GGGACAGGGGGC	GC			
121-1B	--- --- --- (-)				CAGGG				1.2 3
121-3J	--- --- ---				AG				1.6 5
121-3BK	--- --- ---		<b>A</b>		GGGACA		<b>T</b>		1.1 0
121-3BC	--- --- ---		<b>C</b>		AC (A)				1.2 2-3
121-3BF	--- --- ---				ACAGG				2.1 6
Vβ8.2	GCC AGC GGT GAT G	CA							
121-3C	--- --- ---		<b>CCTC</b>		GGACA		<b>A</b>		1.1 1
121-1E*	--- --- ---		<b>TGC</b>		CA		<b>A</b>		1.5 1
121-3H	--- --- ---		<b>A</b>		AGGGGG		<b>AAAGG</b>		2.4 5
121-1BK	--- --- ---		<b>CC</b>	CC	GGGACA		<b>A</b>	AG	2.6 0
121-3BE	--- --- ---		<b>GG</b>		CAGGGG				1.2 4
Vβ8.3	GCC AGC AGT GAT G	CA							
121-1J	--- --- ---		<b>A</b>		GGA				1.4 1
121-1K	--- --- --- (-)				GACAGG (G)				2.6 0
121-3E	--- --- ---		<b>T</b>		GGGAC (A)				2.5 0-1
121-3K	--- --- ---		<b>CG</b>		CAGG (G)				1.1 8-9
121-1BL	--- --- ---		<b>A</b>		AGGG				2.1 0
121-1BV	--- --- ---		<b>T</b>		GGACA (G)				1.1 0
121-3BA	--- --- --- (-)				GACAGGGG		<b>AG</b>		2.3 3
Vβ8.1	GCC AGC AGT GAT G	CA							
121-3L*	--- --- --- (-)				GGGACTGGGGGGC	GC	<b>A</b>	G	2.4 8
Vβ8.2	GCC AGC GGT GAT G	CA			GACTGGGGGG	GC			
121-2L	--- --- ---		<b>ACA +</b>	CC	GGGACTGGGGGGC	GC	<b>A</b>		2.1 8
121-3BM	--- --- ---		<b>T</b>		GACT		<b>TT</b>	AG	2.6 0
121-3BD	--- --- --- (-) (C)				TGGGGGG		<b>AC</b>		2.3 2
Vβ8.3	GCC AGC AGT GAT G	CA							
121-2I	--- --- ---				GGGGGGGC	GC			2.6 7
121-3BB	--- --- --- (-)				GGACTGGG (G)				2.3 3-4
121-3BH	--- --- ---				GGGGGGC		<b>CGGG</b>		2.4 8
Vβ7	GCT AGC AGT TTA TA								
121-1BN	--- --- ---		<b>CT</b>		CTGGGGGGC	GC	<b>ATG</b>		2.1 4

**Figure 5.** Sequences of Vβ-Dβ-Jβ junctional regions from RNA from thymocytes of 4-d-old mice. Sequences are displayed as described in the legend to Fig. 1. In one case, indicated at the N region (+), if the putative N region nucleotide that is not in bold type is a PCR error, then the whole N region could be germline. 19 of 25 sequences (76%) had N region nucleotides in at least one of the two junctions.

is supported by extensive sequence analysis of many anti-cytochrome *c* TCRs (35-37). Therefore, the paucity of N regions that we have observed in fetal/newborn α/β T cells as well as in fetal/newborn Ig (12) would limit the effective diversity of the fetal T cell repertoire even more drastically than the fetal B cell repertoire. However, the fetal TCR-α/β repertoire will still be sizeable, due to combinatorial diversity from the use of many V, D, and J segments, use of all three D region reading frames, and deletion of varying numbers of nucleotides from the coding regions.

The reduction in N region in fetal and newborn TCR was true not only for Vβ8 sequences, but for the limited number of Vβ5 and Vβ7 sequences that we generated. Since together these six Vβ genes account for >50% of the Vβ transcripts



**Figure 6.** Percent N region usage in sequences derived from thymic RNA from mice of varying ages. The total number of sequences analyzed for each time point is shown on the right.

in the thymus (24), and since TdT levels are low at this time in ontogeny, it is likely that this phenomenon will hold true for other TCR-α/β as well. It is unlikely that the minor thymocyte subpopulation of CD4<sup>-</sup>CD8<sup>-</sup> α/β<sup>+</sup> cells (which are greatly enriched in Vβ8.2 expression) has skewed our analysis since these cells arise late in ontogeny, and therefore would be absent from our fetal and newborn thymocyte samples (38-40).

In addition to the absence of N regions in fetal γ/δ T cells, it has been suggested that there was less "exonucleolytic nibbling" of coding regions in junctional sequences from fetal γ/δ T cells than in adult γ/δ T cells (11). The sequences shown here show a slightly increasing number of nucleotides deleted from Vβ8 with age; however, this difference was not significant (*p* = 0.135). Further data should clarify the significance of this trend.

Other parameters that could affect the size and composition of the neonatal and adult TCR-α/β repertoire were also analyzed. There was wide variation in the frequency of use of the 12 Jβ segments, and this pattern was consistent throughout ontogeny. Strikingly, although the six Jβ2 segments were used with very different frequencies, the pattern of usage was the same regardless of whether Dβ1 or Dβ2 was used, as shown in Fig. 9. These observations strongly suggest that each Jβ segment itself controls the frequency with which it is used, and rejects any hypothesis based on relative proximity of Dβ and Jβ segments. It has been shown that the frequency with which recombination occurs in a transfected recombination substrate is related to the exact sequence of the heptamer or nonamer of the RSS (41). The pattern of Jβ usage observed here is most easily explained by proposing that the RSS (or other closely flanking sequences) 5' of each

DAY 18 FETAL THYMUS DNA

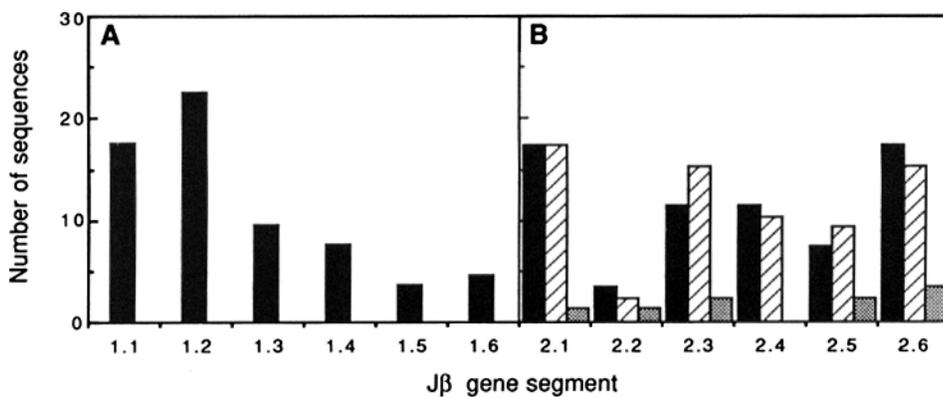
Vβ	Pv	N	Pd	Dβ1.1	Pd	N	Pj	Jβ #deleted
<b>Vβ8.1</b>	GCC AGC AGT GAT G	CA	CC	GGGACAGGGGGC	GC			
140-1K	---			GGGACAGGGGGC	G		2.6	4
140-1BG*	---			CAGGG			2.6	4
154-1F	---			CA			2.6	5
<b>Vβ8.2</b>	GCC AGC GGT GAT G	CA						
140-1G	---			CA			2.6	2
140-1AC	---	C	CC	GG**			2.6	5
140-1AL*	---			GGACAGGGGG			2.6	6
140-1BH	---			GG**			2.6	2
154-1L	---						2.6	4
154-2J	---			AC(AG)			2.6	0
<b>Vβ8.3</b>	GCC AGC AGT GAT G	CA						
154-1J	---						2.6	3
154-2C	---			GGGGC** (G)			2.6	7
<b>Vβ8.1</b>	GCC AGC AGT GAT G	CA	CC	GGGACTGGGGGGC	GC			
140-1B	---			ACT			2.6	3
140-1N	---			TGGGGGG			2.6	4
140-1BK	---			GGGGGG			2.6	6
154-1C	---			CTGG			2.6	1
154-2D	---			ACTGGG			2.6	4
<b>Vβ8.2</b>	GCC AGC GGT GAT G	CA						
140-1A*	---			GGA(CT)			2.6	3-5
140-1D*	---			GGACTGGG			2.6	3
140-1AF	---			GGACTGGGGG			2.6	6
140-1BD	---			ACTGG			2.6	6
154-2K	---	ACCGA		GGACTGGG		AG	2.6	0
<b>Vβ8.3</b>	GCC AGC AGT GAT G	CA						
140-1M*	---			C(TG)			2.6	6-8
154-1A	---		C	ACTG			2.6	6
154-1H	---			A(CT)			2.6	0-2

Figure 7. Sequences of Vβ-Dβ-Jβ junctional regions from DNA from day 18 fetal thymocytes. Sequences are displayed as described in the legend to Fig. 1. 3 of 24 sequences (12%) had N region nucleotides in at least one of the two junctions.

NEWBORN THYMUS DNA

Vβ	Pv	N	Pd	Dβ1.1	Pd	N	Pj	Jβ #deleted
<b>Vβ8.1</b>	GCC AGC AGT GAT G	CA	CC	GGGACAGGGGGC	GC			
135-1D	---			ACAGGGGGC		C	2.6	5
135-1AA	---						2.6	5-8
135-1BF	---		TT	ACA			2.6	4
135-1BP	---			GAC			2.6	2
140-2L	---			GA**			2.6	7
161-2A*	---			AGGG(GC)			2.6	0-1
161-2E	---			GGACA			2.6	4
161-2AA	---			GACAGGG(GC)			2.6	0-1
<b>Vβ8.2</b>	GCC AGC GGT GAT G	CA						
135-1F	---			GG**			2.6	4
135-1DJ	---		AGAA	GACA			2.6	4
135-1DT	---			GACAG			2.6	4
135-1DU	---			GGGAC**			2.6	0
140-2H	---			GACAG(G)			2.6	7-8
161-2B	---			AC(AG)			2.6	0
161-2K	---		CCA	GACA			2.6	2
161-2AE*	---						2.6	6
161-2AF	---						2.6	2-3
<b>Vβ5.1</b>	GCC AGC TCT CTC	GA						
165-3B	---			ACA		C	2.6	0
165-3C*	---			GG**			2.6	6
165-3I	---			GGGACA			2.6	0
<b>Vβ5.2</b>	GCC AGC TCT CTC	GA						
165-3A	---			GGGACAGGG			2.6	6
<b>Vβ8.1</b>	GCC AGC AGT GAT G	CA	CC	GGGACTGGGGGGC	GC			
135-1AD*	---			ACTGGGGGGC	(G)		2.6	7-8
140-2B*	---			GGGGG			2.6	6
140-2D	---			GACTGGGG		TT	2.6	0
140-2F	---		T	GGGACTGGGGG			2.6	5
140-2J	---			GGGGGG(C)			2.6	2-3
140-2K*	---			ACTGGGGG		A	2.6	2
<b>Vβ8.2</b>	GCC AGC GGT GAT G	CA						
135-1G*	---			ACTGGGGG			2.6	6
135-1AF*	---			GACTGGGGGGC			2.6	9
140-2C*	---			GGACTGGGGG			2.6	4
140-2I*	---			GACTGGGGGG			2.6	6
161-2D	---			GGACTGGGG			2.6	3
161-2F*	---			GACTGGGG			2.6	4
161-2H	---			TGGGGGGC			2.6	4
<b>Vβ8.3</b>	GCC AGC AGT GAT G	CA						
135-1AG	---			CTGGGG			2.6	6
<b>Vβ5.1</b>	GCC AGC TCT CTC	GA						
163-2C*	---			GGGGGGG			2.6	6
<b>Vβ5.2</b>	GCC AGC TCT CTC	GA						
165-3E	---			GGGGGGC	(G)		2.6	0

Figure 8. Sequences of Vβ-Dβ-Jβ junctional regions from DNA from thymocytes of newborn mice <24 h old. Sequences are displayed as described in the legend to Fig. 1. 8 of 37 sequences (22%) had N region nucleotides in at least one of the two junctions.



**Figure 9.** (A) Distribution of  $J\beta 1$  use among all the sequences derived from  $C\beta$ -primed cDNA in this study. (B) Distribution of  $J\beta 2$  use among all the sequences derived from  $C\beta$ -primed cDNA in this study. Sequences using  $D\beta 1$  are indicated by the solid bars, and sequences using  $D\beta 2$  are indicated with the striped bars. There are eight  $J\beta 2$  sequences that either lacked D segments or whose D regions could not be definitively assigned to  $D\beta 1$  or  $D\beta 2$ . Those sequences are marked with asterisks in Figs. 1–4 and are indicated here with the stippled bars.

$J\beta$  segment controls the rate with which each  $J\beta$  is used. Significant variation in usage for the four Ig-Jh segments and for the Ig-Dh segments has also been observed (12, 13, 42). Interestingly, the sequence of the heptamer of the RSS of both  $J\beta 2.2$  and Ig-Jh1, GACTGTG, is not used by any other IgJh or  $J\beta$  segment (22, 43), and these two Js are the least frequently used J segments of IgH and TCR- $\beta$ . These data are consistent with the hypothesis, proposed for IgVh genes (41), that the frequency with which all V, D, and J segments of all lymphocyte receptors are used may be controlled by their flanking RSS.

T cells and B cells use the same recombinational enzymes to somatically recombine their receptors (44). In addition, both lineages presumably use TdT to add N region nucleotides, and probably use the same exonuclease to delete nucleotides from the coding region. This study shows that N region addition is a developmentally regulated step in the generation of diversity of all lymphocytes. The result of this is that the immunological repertoire of T and B cells early in ontogeny is more restricted than, and differs from, the adult repertoire. The impact of this paucity of N region nucleotides will be more pronounced for  $\alpha/\beta$  T cells than for B cells because T cells depend upon junctional diversity much more than combinatorial diversity to generate a large repertoire, and because N regions are in the CDR3-equivalent region of the TCR that is proposed to recognize antigenic peptides (3, 16).

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