

# Quantitative and Qualitative Changes in V-J $\alpha$ Rearrangements During Mouse Thymocytes Differentiation: Implication For a Limited T Cell Receptor $\alpha$ Chain Repertoire

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## Abstract

Knowledge of the complete nucleotide sequence of the mouse TCRAD locus allows an accurate determination V-J rearrangement status. Using multiplex genomic PCR assays and real time PCR analysis, we report a comprehensive and systematic analysis of the V-J recombination of TCR  $\alpha$  chain in normal mouse thymocytes during development. These respective qualitative and quantitative approaches give rise to four major points describing the control of gene rearrangements. (a) The V-J recombination pattern is not random during ontogeny and generates a limited TCR  $\alpha$  repertoire; (b) V-J rearrangement control is intrinsic to the thymus; (c) each V gene rearranges to a set of contiguous J segments with a gaussian-like frequency; (d) there are more rearrangements involving V genes at the 3' side than 5' end of V region. Taken together, this reflects a preferential association of V and J gene segments according to their respective positions in the locus, indicating that accessibility of both V and J regions is coordinately regulated, but in different ways. These results provide a new insight into TCR  $\alpha$  repertoire size and suggest a scenario for V usage during differentiation.

Key words: TCR-diversity • TCRAD locus • rearrangement • development • quantitative PCR

## Introduction

Mature T lymphocytes express a clonotypic TCR on their surface. The TCR is activated through recognition of an antigenic peptide presented by molecules of the major histocompatibility complex. The TCR is composed of  $\alpha\beta$  or  $\gamma\delta$  heterodimers, in which each chain consists of a variable and constant region, in association with the CD3 complex composed of  $\epsilon$ ,  $\gamma$ ,  $\delta$ , and  $\zeta$  chains (1). During T cell development, TCR  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  chains are assembled following the rearrangement of independent gene segments contained on the corresponding TCRAD (coding TCR $\alpha$  and TCR $\delta$ ), TCRB, and TCRG loci. This rearrangement pro-

cess uses an enzymatic complex (the V(D)J recombinase) that selectively targets recombination signal sequences (RSS)\* flanking the coding sequences of dispersed V, D (only for TCRB and TCRD), and J gene segments (2, 3). In addition to the combinatorial process, TCR repertoire diversity is enhanced by various mechanisms which include imprecise joining at V(D)J junctions, addition or removal of junctional nucleotides, and pairing constraints of different  $\alpha$  and  $\beta$  (or  $\gamma$  and  $\delta$ ) chain molecules (4, 5). V(D)J recombination is a highly regulated process in terms of both cell lineage and stage of T cell development (6). TCRB rearrangement is initiated at the CD4<sup>-</sup>CD8<sup>-</sup> double-negative stage (7). The expression of a productively rearranged TCR $\beta$  protein associated with the invariant pT $\alpha$  chain and

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\*Abbreviations used in this paper: DP, double positive; FTOC, fetal thymic organ culture; IMGT, ImMunoGeneTics database; RSS, recombination signal sequence.

the CD3 complex leads to progression to the CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) stage and the initiation of rearrangement at the TCRAD locus (for a review, see reference 8).

Recently, our laboratory has established a detailed map of the BALB/c mouse TCRAD locus (9). This map tallies closely with a map of the TCRAD locus derived from the 129/SvJ mouse strain which has recently been constructed (NCBI accession nos.: AE008683–AE008686). Based on restriction length polymorphism it has been found that BALB/c and 129/SvJ mice strains share the same TCRA haplotype (10). Sequence alignments of the BALB/c and 129/SvJ TCRAD loci reveal that the level of sequence polymorphism is very low and that the distribution of V segments is identical (unpublished data). For instance, the majority of sequences from numerous TCRA haplotypes have been found to be closely related with few nucleotide substitutions (11, 12). Sequencing analysis of alleles of V genes belonging to the same V family reveals that the polymorphisms between TCRA haplotypes arose by duplication or deletion events which result in a gain or loss of V segments. Thus, as for human immunoglobulin VH gene polymorphism, allelic differences are likely to have little influence on the shape of the repertoire (13).

BALB/c and 129/SvJ TCRAD loci are 1,200 kb in length and contain 104 V and 60 J segments. The D, J, and C genes encoding  $\delta$  chains are found between the V and J  $\alpha$  segments. The V elements can be organized into 23  $\alpha$  and 6 delta families in which different members of the same family are at least 75% identical at the nucleotide level. Members of the same family are interspersed along the locus. Interestingly, single-membered families (e.g. V12, V19, V6) tend to localize at the extremities of the V region. With the exception of one member of the V1 family, the orientation of all segments is consistent with V-J joining occurring by a deletional mechanism. 18 V segments have been described as pseudogenes due to either the absence of an open reading frame (ORF) or because they encode a polypeptide defective on structural grounds (i.e., unable to fold correctly). However, all the nonfunctional V segments possess a canonic heptamer and nonamer RSS and thus can potentially rearrange normally with J segments (14). The 60 J segments span a region of 60 kb (15), 17 are considered as pseudogenes, but their status in terms of rearrangement are not clearly elucidated, except J60, 59, 51, 29, and 25 which do not rearrange (16).

Although gene rearrangement is only the first step involved in the establishment of the TCR repertoire it is frequently the less considered step in the estimation of total potential TCR diversity. In theory, any given V segment can rearrange with any D and/or J segment, giving a simple combinatorial formula: "rearrangement number =  $x(V) \times y(D) \times z(J)$ ," where x, y, and z correspond to the numbers of each segment present on the locus. Several studies have launched a debate as to whether combination between V, (D), and J gene segments is random or not. Some data, point to a biased expressed TCR $\alpha$  chain repertoire with a preferential rearrangement of the most J-proximal V segments to the closest J segments and of the most J-distal V

segments to the furthest J segments (17–20). In addition, use of both V and J gene segments appears developmentally regulated (17, 21). Thus, the progressive and coordinated utilization of the TCR-V and -J genes points to a rule resembling a modified version of the bidirectional, coordinated nibbling model proposed for Ig genes (22). However, a locus-wide analysis of TCRA rearrangements in human and mouse  $\alpha\beta$  T cell clones reported a loose correlation between the 5' or 3' position of the V and J segments used in a given rearrangement, leading to the conclusion that the erratic use of V genes appears inconsistent with the bidirectional and coordinated nibbling model (23). Finally to date, available informations encompass essentially either analysis at the transcriptional level or gene analysis for only a few V families, thus precluding a general synthetic overview of gene rearrangements.

In this report, to eliminate the biases due to transcriptional regulation and the effect of positive selection on the emergence of T cell clones, we have used a sensitive multiplex PCR assay at the genomic DNA level. This allows a systematic, qualitative screening of the rearrangements effected by 22 out of the 29 V families in a whole thymus. We have studied samples from BALB/c mice at a number of time points throughout ontogeny. In addition, for certain V-J rearrangements, these rearrangement events were analyzed quantitatively, again at the DNA level. Taken together, our findings indicate that the number of V-J combinations is lower than that predicted by a random rearrangement model and that the combinatorial diversity of the TCR $\alpha$  chain is significantly skewed during mouse T cell development.

## Materials and Methods

**Nomenclature.** Nomenclatures for V genes and for J segments are according to ImMunoGeneTics (IMGT) database (<http://imgt.cnusc.fr>). NCBI accession nos.: for V region, AE008683–AE008686, for J region, M64239.

**Mouse.** BALB/c mice were purchased from IFFA CREDO. RAG-2<sup>-/-</sup> (24) were raised in our SPF animal facility. Fetal thymi were obtained from timed pregnancies, where fetal day 1 corresponds to the day of detection of a vaginal plug. Thymic lobes from embryonic or neonatal mice were pooled and mechanically dissociated in PBS before DNA extraction.

**Fetal Thymic Organ Culture.** BALB/c fetal thymi were isolated on day 16 of gestation and placed on 0.8- $\mu$ m pore size membranes (Pall Corporation) floating on complete IMDM medium in 24-well plates, as described (25). After 6 d in culture, DNA was extracted from thymic lobes.

**Multiplex PCR Assay.** Genomic DNA was extracted and amplified as described (9). Multiplex PCR were performed as described (16). Briefly, using an upstream primer specific for a given V<sub>x</sub> family and a downstream primer specific for a given J<sub>y</sub> segment, the multiplex PCR assay allows the detection of a V<sub>x</sub> to J<sub>y</sub> rearrangement as well as that of V<sub>x</sub> genes to a limited set of 5' J segments spanning from J<sub>y</sub> to J<sub>y</sub>-4 position. The multiplex PCR strategy could potentially amplify DNA fragments corresponding to rearrangement of a V<sub>x</sub>+1 gene 3' to that targeted by the V<sub>x</sub> specific oligonucleotide. Actually, we did not detect amplicon corresponding to a fragment containing such rearrangement, ex-

cept for one band at 3594pb obtained with V3x primer which is compatible with a rearrangement of V4 gene located downstream to the targeted V3x gene. Amplifications were performed with 1.3 U/reaction of Expend High Fidelity PCR system (Roche Diagnostics). The cycling conditions were 5 min at 94°C, 26 cycles of 1 min at 94°C, 1 min at 58°C, 6 min at 72°C, and one cycle of 10 min at 72°C. In these assay conditions, maximum amplicon size was ~5 kb. Normalization of the quantity of DNA in each reaction was determined by amplification of either a C $\alpha$  gene exon or the p53 gene in the same PCR run. Negative PCR controls included DNA isolated from RAG-2<sup>-/-</sup> thymi, in which no rearrangements take place. Oligonucleotides specific for V, J segments, and C $\alpha$  segment were as described (9, 16, 17) with additional primers (Table I).

PCR products were separated on 1.5% agarose gels and Southern blotted. Higher bands were separated on 0.6% agarose gel during 24 h. To confirm the specificity of the hybridization signal, the same membrane was successively hybridized with V- and J-specific probes. Amplified fragments were considered as specific products of rearranged gene segments when they migrated at the expected position and were hybridized with both appropriate V- and J-probes. Autoradiograms were exposed and quantitative analysis performed on a Personal Molecular Imager FX (Bio-Rad Laboratories) using Quantity One 4.2.1 software (Bio-Rad Laboratories).

**Quantitative PCR.** PCR were performed on a Light Cycler™ (Roche Diagnostics) using the FastStart kit, 1 U/reaction. 25 ng matrix DNA was diluted in 25 ng of salmon sperm DNA. The cycling conditions were as follows: 94°C 10 min, 40 cycles (94°C 15 s, 60°C 15 s, 72°C 10 s). Melting curves of PCR products were determined according to the manufacturer's instructions. With a PCR elongation time of 10 s, only the rearrangement to the J segment closest to the downstream J primer is amplified. The specificity of the unique amplification product was determined by melting curve analysis and by migration on agarose gels followed by Southern blotting.

**Table I.**

Primer name	Sequence (5'–3')	S/A
AJ56do	TCAAAACGTACCTGGTATAAACTCAGAAC	A
AJ40	TCTTCTGCTTAACCTGTCCCTCATG	A
AJ33do	TTAGCTTGGTCCCAGAGCCCC	A
AJ33	CATGCATTATTACAGCCAGTGCCTTCT	A
AJ16	CTTGCTTCCCGTGATGTCTGGATGA	A
AJ9do	ACCGAAAGCCACAGGTAACCTCCTTATCTCC	A
AJ2	TACCGGGTTGCAAATGGTGCCACTT	A
X6.5 (P53)	ACAGCGTGGTGGTACCTTAT	S
X7 (P53)	CACATGTAAGTGTAGTGGATGG	A
Probe name		
AJ40p	TCTTGCTTGTACTACTTACGT	
AJ16p	ACCCCACTAACATGTCTAAAAG	
AJ9p2	GTAATTTAAATCAAGTTTCTCATTGCACTC	
AJ2p2	GGGGTAACTCACAGATATTACCGTCACT	

S, primer in transcription orientation; A, primer in reverse of transcription orientation.

## Results

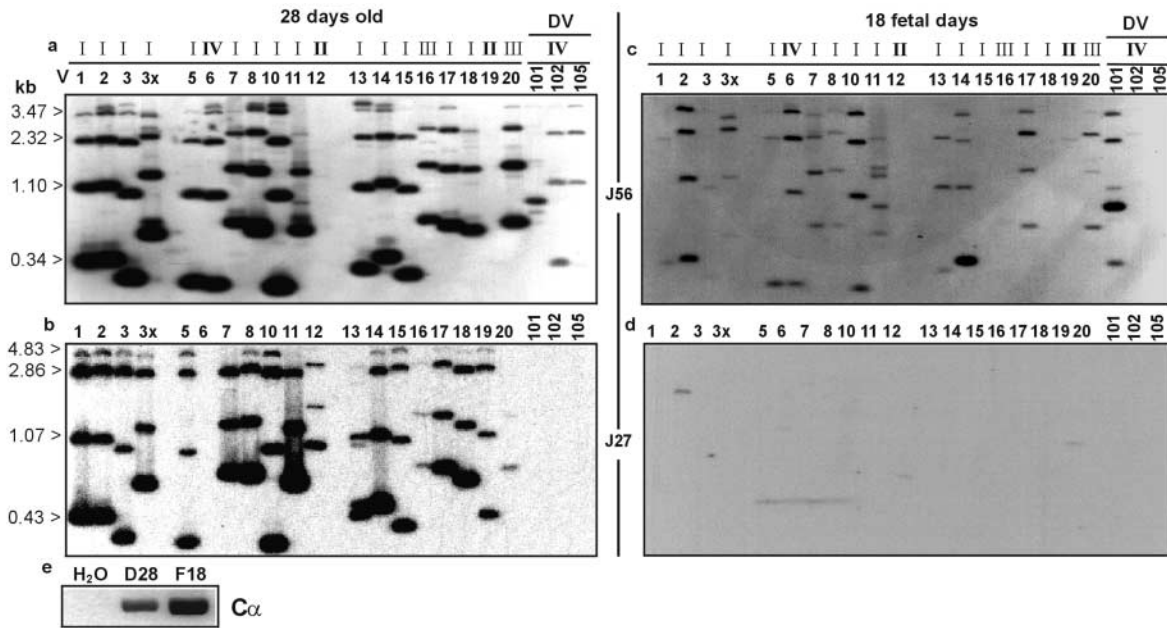
**Global Analysis of V-J Rearrangements in the Adult Thymus.** To determine the number of V-J recombinations, we performed a systematic analysis of the genomic rearrangements of 22 V families with a defined set of J segments. We used a multiplex PCR technique allowing one-tube amplification of DNA rearrangements of a given V family with four to five juxtaposed J segments contained within a targeted region. We first focused on two J segments, J56 located 5' of the J region and close to the V segments and J27 in the center of the J region.

Detailed analysis of the rearrangement patterns of adult thymocytes reveals that V families can be divided into four groups (Fig. 1, a and b). The group I which is the major group includes most of the V families located in the middle part of the V locus. These families comprise 2 to 10 members each, rearranging to approximately the same extent with both 5' and central J segments. The group II encompasses V12 and V19, located on the 5' distal end of the V locus. These segments rearrange with the central J27 to J30 segments only. The last two groups contain the V families most proximal to the J region. Group III is composed of V16 and V20, which rearrange mainly with J56 to J61 segments but also rearrange weakly with J segments from the central region. Group IV is restricted to V6 and DV101, 102, and 105, families which are only found rearranged with the most proximal J56 to J61 segments. The same pattern of rearrangement was detected in five independent preparations of thymocytes from individual adult mice, indicating that this global rearrangement pattern is identical from one individual thymus to another.

**Comparison of V-J Rearrangements Between Fetal and Adult Mice.** To determine if this global rearrangement pattern is a property of the adult thymus or whether it is already established during fetal life, the same experiment was performed with DNA extracted from fetal thymi at 18 d of gestation (F18; Fig. 1, c and d). A comparison of adult and F18 rearrangement patterns reveals that: (a) rearrangement signals from fetal samples are much weaker than those in adult samples; (b) while rearrangements amplified with the J56 primer are readily detectable in the F18 sample (Fig. 1 c), no rearrangements are detected with the J27 primer (Fig. 1 d); (c) the V families found not to rearrange with J56 to 61 segments in the adult thymus (V12, 19) are also found not to do so in fetal samples.

According to C $\alpha$  gene detection F18 sample was more concentrated than postnatal day 28 (D28) sample (Fig. 1 e). Prolonged exposure of hybridized filters did not reveal additional rearrangement bands in Fig. 1, c and d (unpublished data). The difference in intensity of signal between F18 and young adult D28 samples therefore reflects differences in rearrangement levels.

**The J Region Becomes Gradually Accessible.** The preceding results indicate differential use of the J segments in fetal and adult mice. To extend our observations we analyzed V2-J rearrangements during the early stages of thymic development in more detail. The six V2 family members,

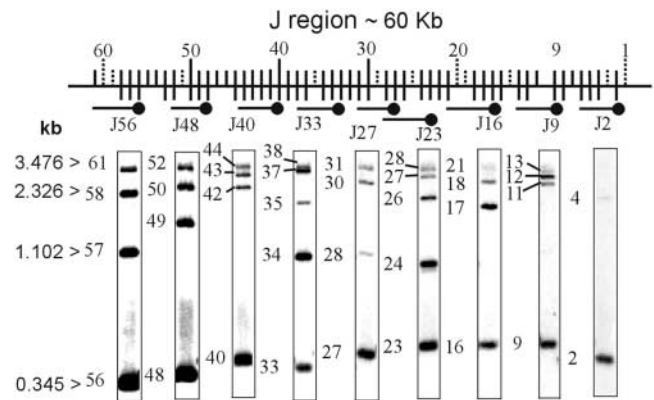


**Figure 1.** Analysis at F18 and D28 of rearrangements of 22 V families with J56 and J27 primers. Multiplex PCR reactions were performed on genomic DNA using individual V primers in conjunction with primers downstream of J56 (a and c) and J27 (b and d). Products were separated by gel electrophoresis, Southern blotted, and probed with radioactively labeled oligonucleotides specific for either J56 or J27. Each band corresponds to a rearrangement event as determined by distance migration. (e) For each PCR reaction, amplification of the C $\alpha$  exons served as a positive control. The quantity of DNA in F18 samples was increased compared with D28 in order to maximize the possibility of amplifying rearranged fragments.

which are dispersed over the V region, were amplified with a consensus V2 oligonucleotide in a multiplex assay using nine different J segment primers spread over the J region, as shown in Fig. 2. Our assay allows us to refine the analysis of the number of potential J segments used in V-J rearrangements. Until now, the status of J segments in terms of pseudogenes, which can be defined by an inability to rearrange and/or to be translated as a functional protein, has not been completely elucidated. As seen in Fig. 2, 34 different J segments were found rearranged with V2. A longer migration of the gel allowed us to detect a total of 49 J segments successfully rearranged (unpublished data). Among the 11 remaining nonrearranging J segments, seven have previously been described as incapable of rearrangement: J36 and J1 (IMGT <http://imgt.cnusc.fr>), and J60, J59, J51, J29, and J25 (16). Our study confirms these results and identifies four additional J segments unable to rearrange with members of the V2 family, namely J20, J19, J14, and J3. On the other hand, J61 which has been denoted as a nonrearranging pseudogene is detected in rearrangements in BALB/c mice.

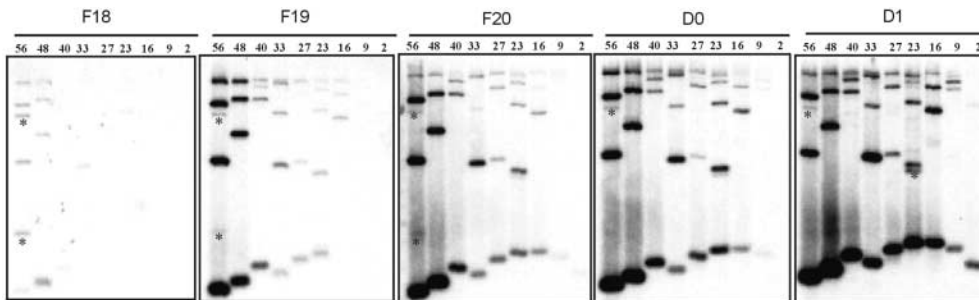
In our experimental conditions, rearrangements are detected from the 18th day of gestation with J segments in the 5' quarter of the J gene cluster (Fig. 3). Eight out of a possible eight V2-J rearrangements are readily detected in this region, a further two rearrangements are detectable with J40 and J34 while no rearrangements are detected beyond J34. From fetal day 19, the J locus opens up toward the 3' end of the V region and rearrangements involving J17 become detectable. From day 20 of gestation, rearrangements

are detected up to J2 indicating that the whole J region is accessible for recombination. From this time point, band intensities for the 3' J segment rearrangements increases



**Figure 2.** Multiplex analysis of J rearrangements for V2 genes. Multiplex PCR products were Southern blotted and probed with a pool of radioactively labeled primers specific for the nine J segments. Multiplex PCR analysis was performed as described in Fig. 1. Molecular weight indicators are given to the left of the figure. Individual lanes correspond to individual PCR reactions. Each band corresponds to a rearrangement with the J segment indicated to the left of the lane. The 9 J segments chosen are spread along the J region as indicated. Representation of J region (not to scale): dots indicate the position of J primers and probes, the corresponding lines indicate the range of J segments detectable in each reaction lane. The J region is composed of 43 functional J segments on a total of 60 segments. Dashed lines indicate 6 untranscribed J pseudogenes, J55, 54, 47, 46, 11, 7 according to IMGT database (<http://imgt.cnusc.fr>); cross lines indicate 11 unrearranged J pseudogenes according to IMGT and correlated with our data, J60, 59, 51, 36, 29, 25, 20, 19, 14, 3, (J1 according to IMGT). J10 has never been described in the mouse.





**Figure 3.** Analysis of rearrangements of the V2 family with J segments during ontogeny. The 9 J segments studied are indicated for each lane. Rearrangements were analyzed by multiplex PCR on DNA extracted from F18, F19, F20, D0 and D1 BALB/c thymi. Products were revealed using an internal V2 probe. Asterisks indicate nonspecific products, as determined by distance migration.

while those of the 5' J segments remain similar up to 4 wk after birth. However, it is notable that band intensities for rearrangements involving V2 and 3' distal J segments are lower than those of rearrangements with V-proximal J segments at all time points analyzed. Taken together, our results show a programmed opening of the TCRAD locus in the J region, moving from 5' J segments, close to the V region, toward 3' J segments closer to the C $\alpha$  gene, as has been demonstrated previously in studies at the transcript level (16). This demonstrates that the progressive J usage, as observed by analysis of mRNA is regulated at the level of gene rearrangements.

*The V Region Becomes Gradually Accessible.* We have previously demonstrated at the transcript level that, in addition to the opening of the J region of the TCRAD locus, there is a similar opening of the V region (17). We further investigated whether V localization is a factor that dictates the rearrangement to particular J segments. We availed of the multiplex technique to study the families identified in Fig. 1 that showed differential rearrangement to J56 or J27 genes. Thus, we compared in adult and newborn thymus two V families situated proximal to the J cluster, V6 (340 kb from C $\alpha$  coding region) and V20 (380 kb) with two V families distal to the J cluster, V12 (1,420 kb) and V19 (1,580 kb). As expected from the results in Fig. 1, V6 and V20 rearrangements are mainly detected with proximal, whereas V12 and V19 rearrange with a more distal group of J segments (Fig. 4 a). Nevertheless, there is a notable difference in the range of utilization of proximal J segments between V6 and V20. In five different experiments, we detect V6 rearranged to J segments located between J61-J33 and V20 rearranged to J segments located between J6-J23. Taking into account that between the J61 gene and either J33 or J23, 24 or 32 J segments contain a classical RSS, we calculate that V6 and V20 can be recombined with 49 and 65% of the functional J segments, respectively.

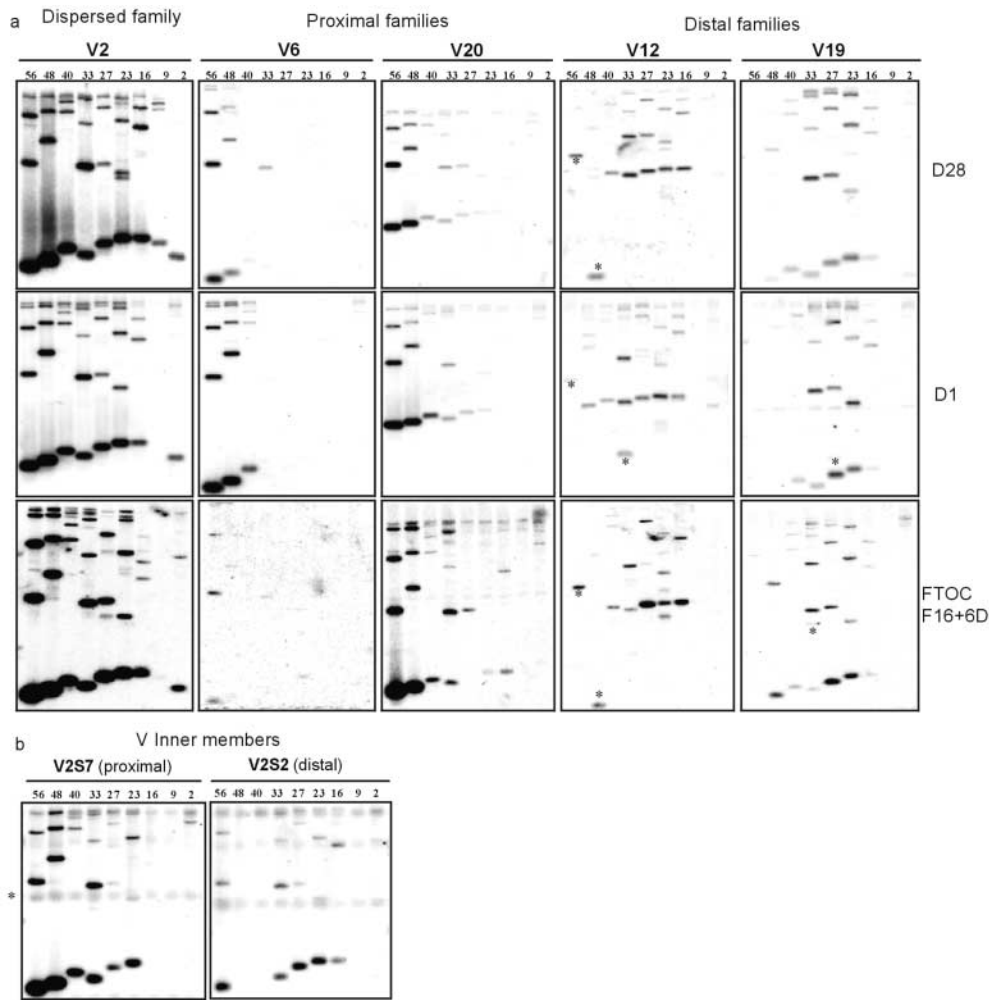
Conversely, V12 and V19 show similar patterns of rearrangement: considering that the rearrangements of both these variable genes are observed with a set of J segments from J49 to J16, this suggests that these variable genes can rearrange with  $\sim$ 67% of the J locus.

Furthermore, to test whether the V segments located in the center of the region also use a coordinated set of J segments, we performed the same analysis with V2S2 (1,140 kb from C $\alpha$ ) and V2S7 (490 kb) using specific primers for

each gene (Fig. 4 b). As expected, V2S2 rearrangements are centered on J27 and give a very similar pattern to that of V12 and V19. Conversely, V2S7 preferentially uses J48 (Fig. 4 b). To confirm these results, similar analyses using the four V7 family members, whose genes are located between V2S2 and 2S7 segments, show that the V7 genes preferentially use J elements in the middle of J locus (unpublished data).

In addition, the V families closest to the J region (V20 and V6) are detected in their rearranged form as early as F18 whereas those furthest from the J cluster (V19 and V12) only become detectable from day 1 after birth (Fig. 5, and unpublished data). These data are in agreement with the idea that particular V families show preferential rearrangement to certain J segments, both in adult and in newborn thymi, indicating that there is a stable mechanism throughout ontogeny.

*V-J Rearrangement Patterns Are Determined in the Thymus Before Birth.* Due to the spatial and temporal nature of the V-J rearrangements described above, later-appearing V12- and V19-J rearrangements could be the result of a new wave of T cell progenitors entering the thymus around the time of birth. To test this hypothesis, we investigated the rearrangement of various V families to the J region in DNA from day 16 fetal BALB/c thymic lobes maintained in fetal thymic organ culture (FTOC) for 6 d. The timing of isolation of fetal thymic lobes allowed us to study cells derived from the first wave of precursors entering the thymus. Thymic lobes were cultivated for 6 d in vitro, corresponding in age to those of 1-d-old mice. Very similar V-J rearrangement patterns were observed with DNA originating either from FTOC or from thymi taken from 1-d-old mice (Fig. 4 a). Thus, in DNA extracted from FTOC, V2 genes rearranged to the whole J region, V20 and V6 mainly rearranged to proximal J segments, whereas V12 and V19 were found rearranged to more distal J segments, results similar to those obtained with DNA from newborn and adult mice. These results show that the rules of V-J rearrangement are conserved in FTOC, suggesting that the relatively late V12- and V19-J rearrangements are not induced solely in the progeny of a postnatal wave of thymic precursors. In addition, these data underline the presence of an intrathymic program controlling the opening of the TCRAD locus. This program is independent of signals coming from other organs and of precursors entering the thymus after fetal day 16 (F16).

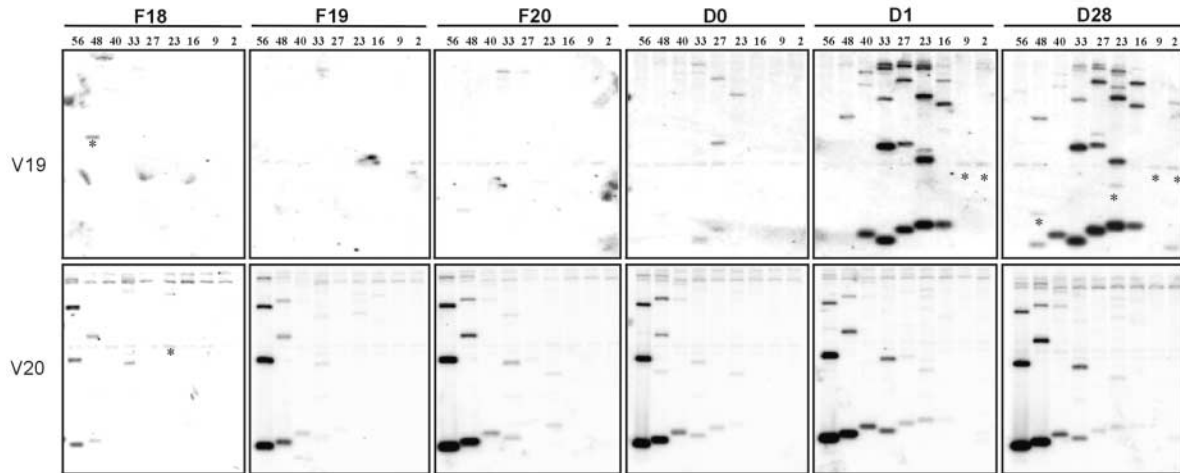


**Figure 4.** Kinetics of V2, V6, V20, V12, and V19 rearrangements. (a) Rearrangements were analyzed by multiplex PCR on DNA extracted from thymus of 28-d-old, day 1 BALB/c mice, and FTOC from F16 after 6 d of culture. Specific V family primers are indicated on panels, these were used in conjunction with 9 J segments primers, as indicated on lanes. (b) Comparison of V2S2 versus V2S7 members at D28. PCR products were revealed with a pool of appropriate J-segments probes. Asterisks indicate nonspecific products, as determined by distance migration.

*Quantitative Differences in V-J Rearrangement.* Taken together, our results show that each V gene only rearranges with a restricted set of J segments with differences in the relative use of particular J genes. To analyze these differences more precisely, we have developed a real-time quantitative PCR assay for genomic DNA matrices. This assay was adapted from our previous study where we quantified either V-C $\alpha$  or V-C $\delta$  transcripts (9).

Rearrangements were analyzed on DNA from the adult (D28) thymus. Nine different PCR reactions were run in parallel using a single oligonucleotide in the V segment coupled with each one of the nine oligos for the J segments. To check the amplification efficiency of the nine J primers, we used the V2 family which, as shown in Fig. 2, rearranges with J segments located across the whole J $\alpha$  region. The relative order of detection of the nine PCR products using this technique correlates with the relative abundance of rearrangements in the sample as detected by amplification of rearrangements to individual J gene clusters (Fig. 2). Thus, the first product to be detected corresponds to the most abundant rearrangement. Using the Light Cycler<sup>TM</sup> technology where the quantity of amplicon and fluorescent signal doubles every cycle if efficiency is 100%,

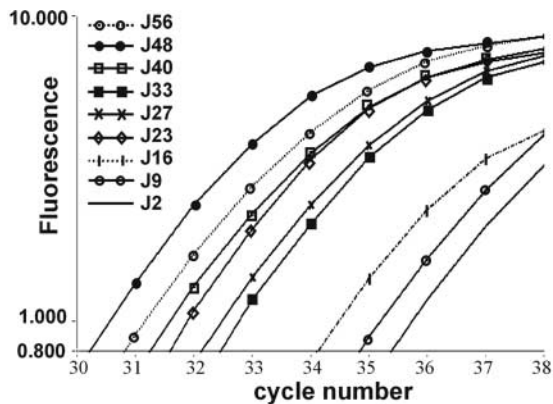
the maximal attainable slope for a PCR curve is  $-3.3$ . According to the manufacturer's instructions, this slope value is used as follows:  $10^{(-1/-3.3)} = 2$ , where 2 corresponds to a 100% increase (or doubling) of amplicon yield per cycle. For the nine PCR reactions of V rearrangements, the slopes of the amplification curves were similar, ranging from  $-3.7$  to  $-3.75$ , indicating equivalent reaction efficiencies and thus allowing comparison of the levels of V-J rearrangements in a given set of reactions (Fig. 6). The average yield per reaction was 85–86% (calculated as follows:  $10^{(-1/-3.7)} = 1.86$  or 86%), equivalent to a 1.7-fold increase of PCR product per cycle. For the V6 gene, only rearrangements to J56, J48, and J40 were detectable (Fig. 7 a). The quantitative comparison between rearrangements involving any two genes is calculated by multiplying their amplification factor (1.7) by the difference in cycle number at which their PCR products were initially detectable. For each V6-J40 rearrangement event, 6 V6-J48 and 6.7 V6-J56 rearrangements were detected. This demonstrates a significant skewing of the frequency of utilization of J segments by a given V gene and suggests a preferential distribution around J56. Similar experiments were performed with the V19 gene, which uses a different set of J segments.



**Figure 5.** Multiplex analysis of rearrangements for V19 and V20 families during ontogeny. Specific V family primers are indicated on panels, these were used in conjunction with 9 J segment primers, as indicated on lanes. Asterisks indicate nonspecific products, as determined by distance migration.

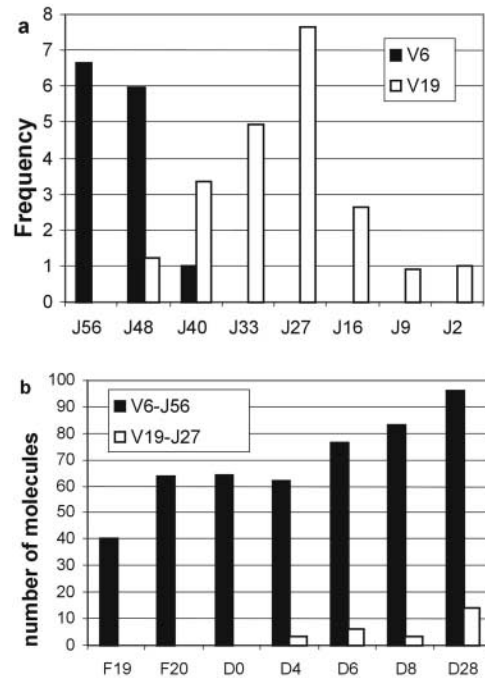
In this case, we find a symmetric distribution of rearrangements around the J27 segment, suggesting a gaussian pattern of J usage around the central part of the J region. V19-J27 rearrangements were 7.7 times more frequent than rearrangements involving J2. Thus, each V gene rearranges with a given cluster of J segments, but within this cluster, frequencies of rearrangements differ greatly. Taken together, this has a strong influence on the total number of V-J rearrangements contributing to the TCR $\alpha$  repertoire as a whole.

*Different V Segments Show Different Frequencies of Rearrangement.* The absolute number of DNA molecules corresponding to V6-J56 and V19-J27 rearrangements in a particular sample was calculated. We chose these two rearrangement events as they are the most abundant for the two variable segments studied in the previous section. PCR products corresponding to V6-J56 and V19-J27 were purified and dilutions used as PCR substrate to generate a standard curve alongside test samples. This allows the determi-



**Figure 6.** Quantification of V2 rearrangement on 9 J segments, in D28 thymus. V2 family rearrangements were amplified using cDNAU 5' primer in conjunction with 9 different 3' primers. The slope of the amplification curves was calculated to determine comparability of data.

nation of the number of each rearrangement in the starting population. The DNA contents of test samples were normalized by amplification of p53, as a nonrearranging gene, present at two copies per cell (unpublished data). At F19, 40 V6-J56 rearrangements are detectable in 25 ng DNA, which corresponds to a cell equivalent of 4167 (calculated as follows:  $25 \cdot 10^{-9}$  g of DNA divided by  $6 \cdot 10^{-12}$  g of DNA per cell; Fig. 7 b). From F20 to D4, V6-J56 is present at



**Figure 7.** Quantitative genomic rearrangement of V6 and V19 segment. (a) Frequency of utilization of 8 J segments by V6 and V19 in D28 thymus, determined by real time PCR. (b) Quantification of V6-J56 and V19-J27 rearrangement during BALB/c ontogeny. For each rearrangement the exact number of molecules is calculated from the corresponding DNA external standard curve.

~60 molecules before rising from 76 to 96 molecules between D6 and D28. For V19-J27, 4 rearrangements are detectable at D4, rising to 14 molecules at D28. These results show that in adult thymus, 1.15% of the chromosomes in the 4167 cell equivalents have rearranged V6-J56 (calculated as follows: 96 molecules divided by  $2 \times 4167$  equivalent cells, assuming that both chromosomes are fully rearranged), compared with 0.16% which have rearranged V19-J27. This corresponds to ~7-fold difference between the two variable gene segments, indicating that V6-J56 rearrangement is seven times more frequent than V19-J27 recombination. In addition, the RSS of V19 unlike that of V6, is very similar to the RSS consensus sequence of mouse V  $\alpha$  elements (26), indicating that the low level of V19 rearrangement is not due to a defect in its RSS. Taken together, these data point out a significant influence of the distance from the J region on the utilization of V segments.

## Discussion

In this report, using multiplex genomic PCR analysis, quantitative and qualitative analysis of representative V-J combinations, we conducted a systematic analysis of the V-J rearrangement profile of normal mouse thymocytes during development. There are four major conclusions from this study. First, the V-J rearrangement pattern is not random either in the fetal or adult thymus thereby imposing severe restrictions on the potential TCR $\alpha$  repertoire. Second, from analysis of FTOC, establishment of V-J rearrangement pattern is intrinsic to the thymus. Third, for individual V genes, rearrangement targets particular sets of J genes and within these particular sets, there is differential usage of individual J genes. Finally, there is quantitatively more rearrangement involving V genes at the 3' than at the 5' end of the V locus. Taken together, these results lead us to conclude that accessibility of the TCRA locus is coordinately regulated but that this regulation affects the V and J regions differently.

Previous analyses of V-J rearrangements in mouse thymocytes have used Southern blot analysis of genomic DNA (27). These studies provided initial indications that there was progressive 3' to 5' usage of J genes during development. However, due to the difficulty of quantifying a decrease in the hybridization signal from the nonrearranged genomic fragments and weak hybridization signal from rearranged bands, the Southern blot approach is relatively insensitive. Limited PCR analysis of the transcribed repertoire using a restricted set of V and J primers has also been performed. These studies have proposed an ordered and coordinated expression of the TCRA locus (17–20). To eliminate the biases due to transcriptional regulation, we have used a recently developed multiplex PCR analysis of genomic DNA (16). The added sensitivity of this approach allows us to draw important conclusions regarding the development and diversity of the murine TCR $\alpha$  repertoire. The screening of all V families located closest to the C $\alpha$  coding region (between -400 and -345.7 kb) indicates

that these V segments rearrange predominantly with the most proximal J segments. Reciprocally, the V families situated in the most distal part of V region (between -1584.2 and -1422.2 kb) are preferentially rearranged with the J segments found in the midsection and the most distal parts of the J region. This indicates a programmed opening of the V region from 3' to 5', incompatible with the idea of a random repartition of recombination during thymocyte development. Consequently, it is not surprising that during fetal life only the rearrangements between the 3' V and the 5' J segments are detected. However, it has been proposed for the regulation of the human TCRA locus, that the coordinated model concerns solely the V segments located at the extremities of the V region (23). The following grounds could explain the apparent disagreement. The human V rearrangements analyzed were derived from a panel of T cell clones. The rearrangement events were studied by cloning and sequencing of RT-PCR products or from published sequences. In our case, the whole study was done *ex vivo* at the genomic DNA level and at different stages of thymus development. This approach gives a direct view of rearrangement status and decreases the bias due to thymic selection. The human locus is less complex in term of V elements than its mouse counterpart and therefore the possibility of analyzing coordinated and polarized V segment use is restricted. In spite of the high level of homology between the two loci, some structural constraints may be divergent, leading to the emergence of different rules or factors governing the accessibility of the V and J regions between the human and the mouse loci.

Recently we proposed, using RNA level quantification, that TCR $\alpha$  chains are produced in regulated waves during thymic development (9). These regulated waves could corroborate with either the set of new thymic immigrants during ontogeny or an internal regulation in the TCRA locus. Our analyses of TCR $\alpha$  rearrangement in FTOC are more in accordance with the second hypothesis, as a similar profile of TCR $\alpha$  repertoire was observed between isolated and *in vivo* thymocytes. This assumes that the rearrangements of TCRA locus are controlled within the locus and depend mostly on the strict regulation of the opening and accessibility of chromatin.

As reported in this work, during rearrangement each individual V gene targets specific sets of J genes, leading to the restriction of the potential V repertoire. Thus, among the J set used by a given V segment, the J segments are used with a gaussian distribution centered on about 15 J segments. This differential frequency leads to the emergence of preferential recombinations and consequently to a reduction of TCR $\alpha$  diversity. Thus, before pairing with TCR $\beta$  chain and before the selection process during T cell development, the genomic repertoire of TCR $\alpha$  chain is already restricted. As we show here that all the rearrangements between V and J libraries are not equivalent and that their frequencies are diverse, one can assume that the diversity of TCR $\alpha$  chain expression is probably lower than the  $1.18 \times 10^4$  proposed by Cabaniols et al. (28). Taking into account that a given V gene can rearrange with a maxi-



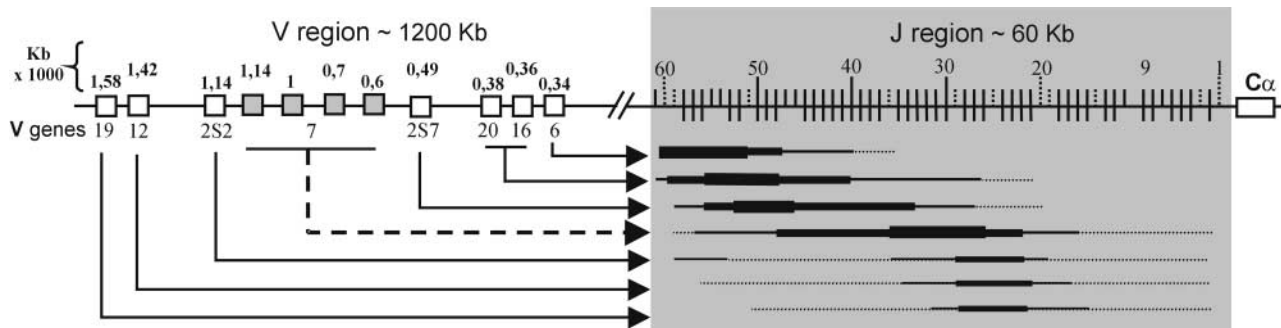
imum of 67% of J segments, we propose that the V diversity is in the order of  $0.67 \times 1.18 \times 10^4$ . Thus, assuming a TCR $\beta$  diversity in the order of  $6 \times 10^5$  (29), the number of potential  $\alpha\beta$  combinations should be inferior to  $4.7 \times 10^9$ . It will be interesting now to determine whether the predominant V-J rearrangements have an influence on thymocyte maturation and thereby limit the repertoire of TCR $\alpha\beta$  actually expressed.

We attempted for the first time to make an accurate comparison of the relative rearrangement and transcriptional status of a single V gene (V19) located at the 5' extremity of the locus. This analysis revealed that in adult mice, whereas the V19 gene is rearranged sevenfold less than V6, nevertheless, as we have recently reported (9), V19 transcripts are twice as abundant as those for V6. Likewise, we detected DV101, 102, and 105 rearrangements to proximal J genes, but again at the transcription level they were barely detected. During fetal life, initial TCRA rearrangements utilize a spatially restricted set of V genes located at the 5' end of the J region. Taken together, these results indicate that during development, 3' V region genes become accessible to rearrangement before those in the more distal 5' end of the locus. This would give an initial 3' V bias to the expressed V repertoire. As proposed previously (30, 31) use of more distal V segments more than likely occurs as a result of secondary V to J rearrangements. This process would normally occur at the DP stage of thymocyte development and its extent would be limited by cell survival time as well as the duration of RAG gene expression and protein function. Studies recently published (32, 33) would indicate that secondary V-J rearrangements favor utilization of J genes close to those used in the initial rearrangement. In this scenario the combined activity of the E $\alpha$  enhancer, TEA element and promoter of the initially rearranged V gene, result in a remodeling of V/J chromatin thereby allowing a new set of 3' J segments to become accessible to secondary recombination.

For the V region, recent data as well as those reported in this study favor a model of progressive tracking down the V locus. The primary rearrangement would mainly involve 3'

V genes either because a substantial portion of the V and J region becomes accessible to RAG activity or because the first V genes are rearranged behind an initial V-D rearrangement (34). For subsequent rearrangements, it is unclear whether the entire remaining V locus now becomes accessible or whether small windows are sequentially opened. We favor the former hypothesis given that: (a) contrary to the J region (35, 36), there is no interallelic coincidence in the chromosomal position of the two rearranged V genes (23); (b) the number of secondary rearrangements is limited by DP thymocyte lifespan; and (c) individual V genes show a gaussian-like utilization of J genes. To account for these observations, it would seem that the V region would be scanned faster than the J region in order for DP thymocytes to rearrange their most distal V genes. This would also explain why 3' V genes rearrange primarily to 5' J genes whereas distal 5' V genes use both central and 3' J elements (Fig. 8).

For the J locus, Guo et al. (32) have recently proposed a "local service" model to account for the pattern of J gene utilization, suggestive of sequential V-J rearrangements proceeding in small steps to J segments adjacent to the V-J joint to be replaced. This "local service" was in comparison to the term "express service" which they dismissed as a way of describing J gene utilization. However, in order to take into consideration kinetic and topographical aspects of V gene rearrangement, we would favor the "express service" model to describe V gene utilization. For an "express service" model to function there needs to be a large window of gene accessibility in the V locus, a situation more compatible with control by enhancer or enhancer-like elements rather than by V gene promoter activity. Overall, our results are in agreement with a bidirectional and coordinated model of TCRA recombination thereby generating TCRA diversity, yet maximizing the possibility of secondary rearrangements. When applied to situations of altered thymocyte development, the comprehensive quantitative and qualitative approach described herein will be useful for dissecting the complex molecular control involved in TCRA locus rearrangement.



**Figure 8.** Representation of V-J segment utilization in adult thymus. The V region (not to scale): V member position is indicated by open boxes. V7 family is in gray boxes. Distances (in kb) indicated above V segments are measured from the last base of V gene and first base of C $\alpha$  exon 1. The thickness of the line under the J region of the locus is an indication of the frequency of J utilization by the different V segments studied. The J region (not to scale) is presented as described in Fig. 2. V20 and V16 show the same distribution of rearrangement. The four V7 members located between V2S2 and 2S7 show an intermediate rearrangement pattern when compared with these two genes.

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