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# Regulated expression and structure of T cell receptor γ/δ transcripts in human thymic ontogeny

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**Gamma delta (γδ) T cells have been found in all vertebrates examined, yet their function *in vivo* remains unknown. Because γδ T cell receptors are related to immunoglobulin, and because they are encoded by rearranging, multi-gene families, the receptors are thought to be antigen recognition molecules. However, a capacity to recognize naturally diverse antigens has not yet been shown. In this work, the expression and structure of human γδ transcripts have been examined in the fetal and early post-natal thymus. The data indicate that many γ and δ genes are rearranged and expressed throughout ontogeny, but that as ontogeny proceeds, quite dramatic changes occur in the patterns of gene expression and rearrangement. In particular, receptors encoded by early to mid-gestation fetal thymic transcripts would be of quite retricted diversity. Only later in ontogeny can receptors of substantial diversity be generated. These properties are very similar to the patterns of γδ gene activation in the mouse, and they serve to reiterate similarities both in gene rearrangement and in γδ across vertebrate species.**

*Key words:* gene rearrangement/receptor diversity/thymus

## Introduction

In all vertebrates in which it has been examined, there are three sets of lymphocytes—B cells, αβ T cells, and γδ T cells—that encode their major cell surface receptors from genes assembled by somatic recombination. This recombination can join any one of a number of variable, V, gene segments with any one of a number of joining, J, gene segments, in some cases via the incorporation of diversity, D, gene segments (Tonegawa, 1983). This mechanism can potentially confer great diversity on the encoded receptors, exemplified by the diverse ligands recognized by B lymphocytes, and αβ T cells. However, it remains unclear the degree to which γδ T cell recognize diverse ligands (Asarnow *et al.*, 1988; Janeway *et al.*, 1988; O'Brien *et al.*, 1989; Parker *et al.*, 1990).

The extent of diversity shown by receptors encoded by rearranging genes is clearly regulated by a number of factors. The underlying regulatory factors are the size and the activation of the receptor gene families involved. Both in humans and in mice, the T cell receptor (TCR) γ and δ gene families are relatively small, theoretically limiting the combinatorial diversity (Tonegawa, 1983) that can be achieved through random assortment of multiple gene segments. However, rearranged TCR δ genes can be very diverse at the V−D−J junction region, since they can include D−D recombinations which potentially increase the number of possible sequence combinations, and introduce additional sites for variable, template-independent N nucleotide insertion (Davis and Bjorkman, 1988).

The extent to which these theoretical considerations of diversity are borne out in γδ cell populations *in vivo* will depend on the regulation of the expression and the recombination of the different γδ gene segments. The rearrangement and the expression of TCR genes are thought to commence in large part in the thymus, in T cell progenitors that have entered from the fetal liver, but presently, little is known about how the activation and recombination of any of the known vertebrate rearranging gene loci are regulated. In this paper, the expression and the structure of rearranged γδ transcripts during fetal and early post-natal human thymic development have been studied. Distinct patterns of expression and recombination are described. Provocatively, these patterns are strikingly similar in many respects to patterns of γδ gene expression and recombination seen in the murine fetal thymus (Elliott *et al.*, 1988; Carding *et al.*, 1990; Itohara *et al.*, 1990). In particular, in the fetal thymus the expression of a single Vδ gene is predominant, and junctional diversity is limited both by restricted gene segment usage, and by limited N nucleotide insertion. The implications of these findings are discussed.

## Results

### *Human TCR γ and TCR δ genes*

The human TCR γ locus (Figure 1A) contains fourteen variable (V) gene segments, that can be somatically recombined to any one of five joining (J) gene segments (Lefranc *et al.*, 1986a,b; Forster *et al.*, 1987; Huck and Lefranc, 1987; Huck *et al.*, 1988; Lefranc *et al.*, 1989). By sequence relatedness, the V genes fall into four families ($V_{I-IV}$) (Lefranc and Rabbitts, 1989). DNA probes and oligonucleotide primers can be designed that specifically detect the expression of genes from each of these four families (Table I). Families $V_{II}$, $V_{III}$, and $V_{IV}$ are single copy; family $V_I$ contains nine genes, of which four are pseudogenes.

The human TCR δ locus (Figure 1B) contains at least six V gene segments, that lie 5′ of Cδ within the TCR Vα gene segment cluster, with the exception of Vδ3, that lies 3′ to Cδ (Tighe *et al.*, 1987; Baer *et al.*, 1988; Boehm *et al.*, 1988; Brenner *et al.*, 1988; Hata *et al.*, 1988; Loh *et al.*, 1988; Satyanarayana *et al.*, 1988; Takihara *et al.*, 1988). Vδ4 and Vδ6, which were identified by sequencing cDNA clones (Takihara *et al.*, 1988) have not been accurately mapped; however an unidentified Vδ gene segment lies between Vα17.1 and Vδ2 (Hata *et al.*, 1989). Primers can

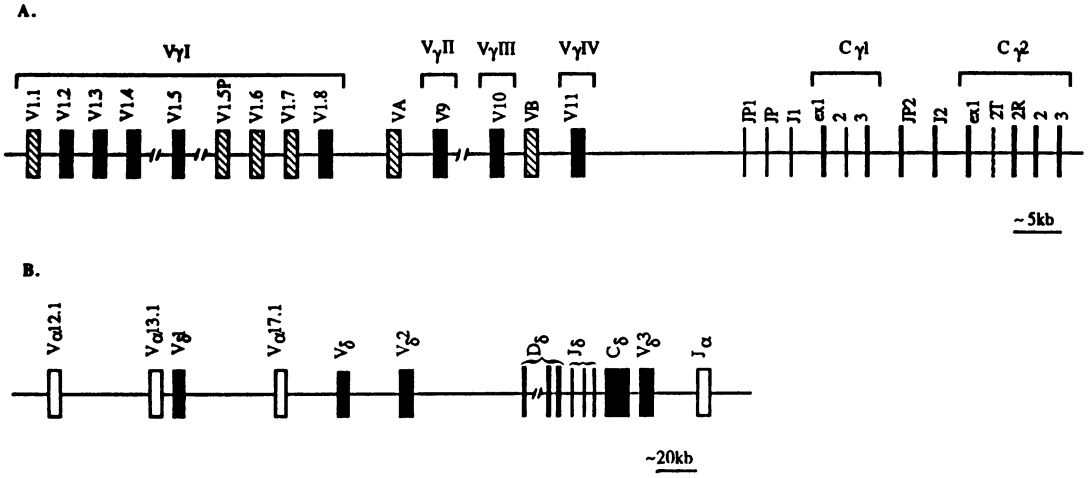


**Fig. 1. A**. Organization of the human T cell receptor γ locus. Vertical bars are gene segments. The intron−exon organization of the Cγ genes are shown. Pseudogenes are hatched (Lefranc and Rabbitts, 1989) 5′−3′, let to right. **B**. Organization of the human T cell receptor delta locus (Tighe *et al.*, 1987; Baer *et al.*, 1988; Boehm *et al.*, 1988; Hata *et al.*, 1988, 1989). Vδ gene segments are dark, vertical bars, within the locus of TCR α gene segments (open verical bars). Vδ4, Vδ5 and Vδ6 were identified by sequencing cDNA clones (Takihara *et al.*, 1988). Linkage between Vδ4 and Vδ6, and other segments has not been established. Dδ1 lies upstream of Dδ2, but has not been precisely mapped. 5′−3′, left to right.

**Table I.**

a. Oligonucleotides used for PCR Amplification of TCR γ and δ cDNAs:

| | |
|---|---|
| G V I | 5'-TACATCCACTGGTACCTACAC - 3' |
| G V II | 5' - GCAACCTCgAATTcCCAGTACT - 3' |
| G V III | 5' - ACTGGatCCGGCAGAAACCAAA - 3' |
| G V IV | 5' - CTGGTACTGGCAGAAACCAAAC - 3' |
| Cγ exon 1 | 5' - ATTCTGACGTCTGACGATACA - 3' |
| D V1 | 5' - GTGGTCGCTATTCTGTCAACTTCAAGAAAG - 3' |
| D V2 | 5' - GCTCCATGAAAGGAGAAGCGATCGGTAACT - 3' |
| D V3 | 5' - CCAGATTTATTCTGGTACCGGATAAGGCCAG - 3' |
| D V4 | 5' - GCAGCCCAGCAGTGGGGAAATCGATTTTTCTT - 3' |
| D V5 | 5' - CTGAAGGTCCTACATTCCTGATATCTATAAG - 3' |
| D V6 | 5' - TATCATGGATTCCCAGCCTGGAGACTCAGC - 3' |
| D C1 | 5' - TTACCAAGCTTGACAGCATTGTACTTCCCA - 3' |

b. J segment-specific oligonucleotides used for Southern blot hybridization to dectect PCR amplified TCR products:

| | |
|---|---|
| G J1J2 | 5' - AATTATTATAAGAAACTCTTTGGCAGTGGA - 3' |
| G JP | 5' - GGGCAAGAGTTGGGCAAAAAAATCAAGGTA - 3' |
| G JP1 | 5' - ACCACTGGTTGGTTCAAGATATTTGCTGAA - 3' |
| G JP2 | 5' - AGTAGTGATTGGATCAAGACGTTTGCAAAA - 3' |
| D J1 | 5' - GGAACCCGTGTGACTGTGGAACCAAGT - 3' |
| D J2 | 5' - GGAACACAACTCATCGTGGAACCAAGT - 3' |
| D J3 | 5' - GGAACTGGCATCAAACTCTTCGTGGAG - 3' |

[Letters in lower case indicate nucleotides introduced to create restrition sites for cloning.]

c. Expected size (nt) of PCR amplified products using TCR γ and δ gene-specific oligonucleotide primers.

| Vγ - Cγ | | Vδ - Cδ | |
|---|---|---|---|
| G V I - Cexon1 | 540 | D V 1 - Cd | 330 |
| G V II-Cexon1 | 625 | D V 2 - Cd | 465 |
| G V III-Cexon1 | 530 | D V 3 - Cd | 425 |
| G V IV-Cexon1 | 520 | D V 4 - Cd | 398 |
| | | D V 5 - Cd | 392 |
| | | D V 6 - Cd | 277 |

be designed that specifically detect expression of each of these 6 V segments (Table I). The Vδ segments can be somatically recombined to any one or more of three known Dδ segments that lie 5′ to three Jδ gene segments.

### *Expression of human TCR γ and TCR δ genes*

Expression of TCR γ and δ genes in human fetal thymus samples was detected directly *ex vivo*, using a sensitive polymerase chain reaction (PCR) technique, that was previously modified for analysis of murine fetal TCR γ and δ gene expression (Carding *et al.*, 1990). This level of sensitivity was required because γδ cells and their progenitors are a very small percentage of the cells in the thymus, yet we wished to avoid any techniques for enriching them that might have biased the analyses of gene expression. The method utilizes primers specific for Vγ and Vδ gene families to amplify random-primed cDNA in concert either with Cγ gene primers that anneal to both Cγ1 and Cγ2 genes, or with a primer for the single Cδ gene (Table I). The products of amplification are electrophoresed and blotted, and the specificity of amplification determined by the capacity of an appropriately sized fragment to hybridize to Jγ and Jδ oligonucleotides that lie internal to the V and C primers. In this way, the expression of only conventionally (V−J−C, and V−D−J−C) rearranged genes is detected.

The authenticity of PCR products detected was confirmed by direct DNA sequencing (see below: Figures 4−6). The specificity of the primers was further assessed by the use of cDNA from γδ+ and αβ+ cloned T cell clones, from leukemia cell lines and from other primary tissues. All primer combinations failed to amplify a product when cDNA was omitted from the reaction, or when irrelevant (fibroblast) cDNA was used as substrate (see below: Figure 3). Over a substrate concentration range of $\sim 10^3$-fold, the PCR signal strengths detected by hybridization are approximately proportional to the amount of substrate cDNA. This was determined by the amplification of a range of concentrations of T cell cDNA diluted into fibroblast cDNA, keeping the total amount of cDNA constant (Carding *et al.*, 1990). However, in this communication, the conclusions reached are largely limited to qualitative gene expression patterns.

A total of 21 independent fetal thymic samples, and two post-natal thymic samples were examined (Table II). The capacity of random-primed cDNA generated from tissue RNA to be amplified by PCR was in every case first tested by use of primers for the ubiquitously expressed tubulin gene (Figure 2).

### *Human δ gene expression*

The pattern of TCR gene expression in fetal thymus tissue directly *ex vivo* showed some variability among age-matched samples, possibly reflecting the genetic heterogeneity of human material (see Discussion). However, there was clearly a consensus pattern of TCR δ gene expression shown by over 50% of samples, and depicted in Figure 2. Expression of all TCR δ genes (except δ4) was evident in the earliest sample examined (11 weeks). The predominantly expressed gene throughout fetal thymic development, up until the latest fetal time point examined (22 weeks) was Vδ2. The most significant change during this time appeared to be a decrease in the use of Vδ6. By contrast, analysis of post-natal thymus [e.g. 3 years old (Figure 2)] examined directly *ex vivo* showed that expression of Vδ2 was no longer detectable. Hence, expression of rearranged Vδ2 genes appears to be strongly developmentally regulated. It is not clear at what point the decrease in Vδ2 expression occurred, since late fetal samples were not available. The data are consistent with the analysis of cell surface TCR expression on human

**Table II.** Human fetal thymus samples used to analyse γ/δ Tc receptor gene expression:

| Age | No. of Samples |
|---|---|
| 11 weeks | 1 |
| 13 weeks | 1 |
| 14 weeks | 1 |
| 15 weeks | 5 |
| 15.5 weeks | 2 |
| 16 weeks | 5 |
| 17 weeks | 3 |
| 18 weeks | 1 |
| 19 weeks | 1 |
| 22 weeks | 1 |
| 6 months | 1 |
| 3 years | 1 |
| Total | 23 |

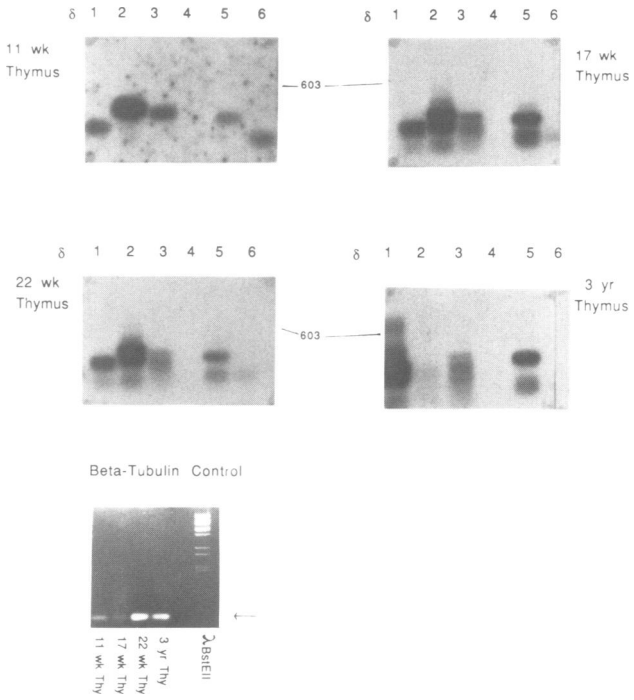


**Fig. 2.** Southern blot hybridizations of 11 week, 17 week, 22 week and 3 year thymus samples PCR amplified using Vδ and Cδ gene-specific oligonucleotides, denoted by the numbers above each panel. Nomenclature corresponds to that used in Figure 1. Probe was a pool of Jδ segment-specific oligonucleotides. Exposure to film was for 4 days. The migration of a molecular weight marker (603 nt) is indicated. Use of this and other markers indicated that the main products detected were of the expected size. [Occasional doublets that are apparent here have previously been seen in PCR analyses of other TCR genes (Kyes *et al.*, 1989; Takagaki *et al.*, 1989).] 1 μg of each cDNA was PCR amplified with human β-tubulin primers. A product of the expected size (arrowed) verified the integrity of random hexamer-primed cDNAs (see text; also Carding *et al.*, 1990).

**Table III.** TCR expression on human fetal thymocytes

| Fetal age | % cells positive | | | | |
|---|---|---|---|---|---|
| | CD3+ | αβ+ | γδ+ | Vδ1+ | Vδ2+ |
| 13 weeks | 34 | 15 | 2.0 | N.D. | 1.6 |
| 16 weeks | 37 | 23 | 1.5 | 0.3 | 1.2 |
| 17 weeks | 30 | 21 | 1.6 | 0.1 | 1.3 |
| 19 weeks | 47 | 34 | 1.4 | N.D. | 1.0 |
| 6 months | 64 | 55 | 1.1 | 1.0 | N.D. |

Fetal thymocytes were stained with saturating amounts of antibodies specific for CD3 (Leu3a), αβ TCR (WT-31), γδ TCR (TCRδ-1), Vδ1 (δTCS-1), and Vδ2 (Y-15D), followed by secondary antibodies conjugated to FITC, and analysed on a FACScan or FACS 440.

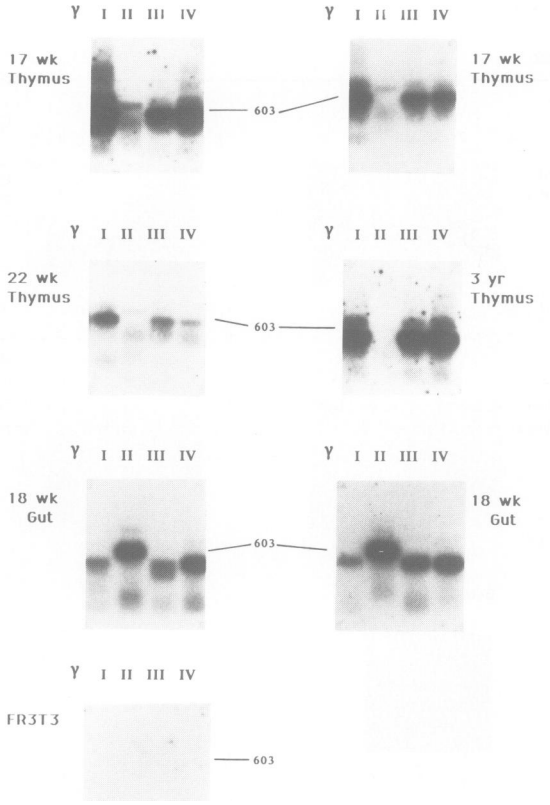


**Fig. 3.** Southern blot hybridization of products amplified from 17 and 22 week fetal and 3 year post-natal thymus samples, two independent 18 week fetal gut samples, and a fibroblast sample, using Vγ and Cγ gene-specific oligos. Exposure time of 4 days. Size markers, and nomenclature, see legend to Figure 2.

fetal thymocytes (Table III). Up until 19 weeks, most γδ+ cells express Vδ2, as indicated by staining with a Vδ2-specific antibody. However, post-natal thymocytes were predominantly Vδ1+.

### *Human γ gene expression*

Expression of all human Vγ genes was detected at all fetal time points examined. No amplification was detected using any primer combination on fibroblast cDNA (Figure 3). As for TCR δ genes, there was a distinct consensus pattern of expression, depicted in Figure 3. The expression of VγII was relatively low but detectable during fetal thymic ontogeny. By contrast, in post-natal thymus samples, no expression of VγII could be readily detected. The low level of detection of VγII does not reflect poor priming efficiency by the VγII primer: in other samples (e.g. fetal gut tissue) VγII expression is the predominant γ gene expression (Figure 3, and McVay,L., Hayday,A., Bottomly,K. and Carding,S., manuscript in preparation).

### *Sequences of human δ transcripts*

Since Vδ1 and Vδ5 genes are clearly expressed throughout fetal ontogeny and in post-natal thymus, the DNA sequences of these transcripts were examined at different time points to determine whether there were qualitative changes in the structure of the transcripts that occurred during development (Figures 4 and 5). The sequences are aligned according to their most probable derivation from either germline gene segments, palindromic ('P') syntheses (Lafaille *et al.*, 1989), or template-independent ('N') nucleotide insertions. Such alignments are sometimes equivocal. In this paper, wherever nucleotides could plausibly be P rather than N insertions, they were assigned as such. Usually, sequences were assumed to be derived from gene segments if they matched over three or more contiguous nucleotides. In one set of joins (post-natal Vδ1), a dinucleotide was assigned to a D1 element because of its recurrence in several transcripts.

The V−D−J junctional sequences of rearranged Vδ1 genes expressed at 11, 17 and 22 weeks of fetal life are shown in Figure 4. At 11 and 17 weeks, 12 out of 14 expressed genes represent V1−D3−J rearrangements. There is a very low level of N nucleotide insertion between D3 and J. Assigning, wherever possible, nucleotides as 'P insertions' (Lafaille *et al.*, 1989), 4 out of 7 sequences at each time point entirely lack 'N4' nucleotides (Figure 4). Similarly, there is negligible N nucleotide insertion between V and D ('N1'). By contrast, post-natal transcripts invariably include both D2 and D3 sequences, with widespread 'N3' nucleotide insertion between them. Between V and D2, there is also an increased frequency of nucleotides that may be assigned to N1, D1, or N2.

The junctional sequences of rearranged Vδ5 genes expressed in the same tissue samples as the sequenced Vδ1 genes, show both striking similarities to and differences from the Vδ1 gene sequences (Figure 5). The 11 week Vδ5 sequences, like the 11 week Vδ1 sequences, are simple. Again, D3 is the first D element utilized. Similarly, a high fraction (5/7) of the 17 week sequences entirely lack 'N4' nucleotides, and there are cases of direct fusions of germline sequences. However, compared with Vδ1 transcripts, there is clearly more frequent N1 insertion, plus some recurrent use of D1, and D2 elements, occasionally with 'N2' insertion (Figure 5). There were, however, no 'N3' insertions (see Discussion). By contrast, post-natal Vδ5 structure, like post-natal Vδ1 sequences, showed more extensive N nucleotides. Relative to 17 week samples, there was widespread use of N4 and, by now, use of N1 was invariable. There was greater use of D2−N3, but clearly to a lesser extent than in post-natal Vδ1 transcripts (see Discussion). The structures both of Vδ1 and of Vδ5 transcripts showed an increased representation of rearrangements to Jδ1 in post-natal thymus.

### *Sequences of fetal human γ transcripts*

Genes of the VγI family are expressed throughout ontogeny. Examples of these transcripts were examined at 17 weeks. Expression was attributable to a number of members of the VI family, that were rearranged to an assortment of J segments. However, as with the Vδ genes expressed at 17


**Fig. 4.** Nucleotide sequences of Vδ1−Dδ1−Dδ2−Dδ3−Jδ 1/2/3 junctions from 11 and 17 week fetal and 3 year post-natal human thymus. At the top are shown the germline sequences of Vδ1, Dδ1, Dδ2, Dδ3, Jδ1, Jδ2, and Jδ3 gene segments. Below are shown the joins derived from larger cDNA sequences cloned from fetal thymus, aged 11 weeks, and 17 weeks, and from 3 year old post-natal thymus. Sequence assignments are made as described in the text. Strong candidates for template-independent, N nucleotide insertions are underlined. In-frame joins are labeled (+), out of frame (−). In each case the identity of the J segment used is indicated by a number, 1, 2 or 3, and was determined by downstream sequences not shown here. In some cases, the D−J fusion involved extensive 5′ deletion of the J segment (denoted by . . . ). In all but one of these cases, the join was abortive. In one abortive join, N4 was so extensive that the sequence of 15 nucleotides within it are not shown here.

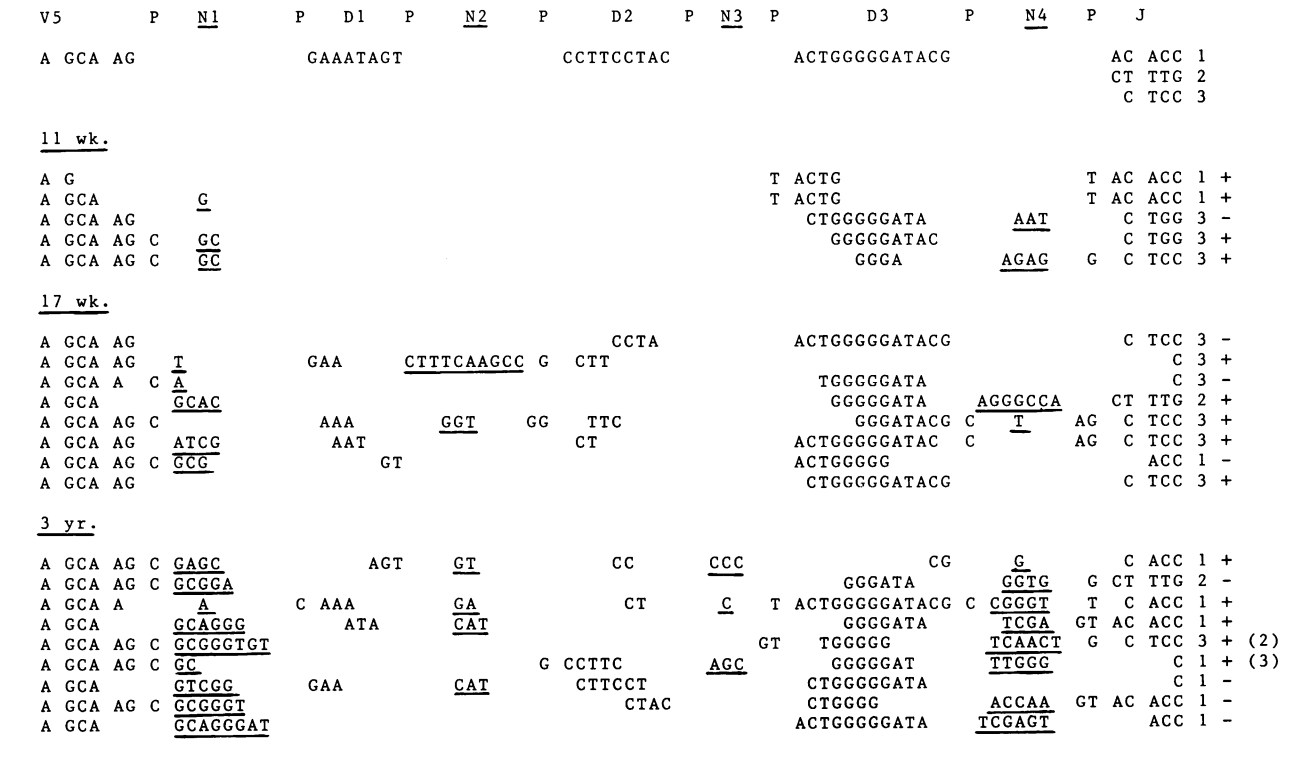


**Fig. 5.** Nucleotide sequences of Vδ5−Dδ1−Dδ2−Dδ3−Jδ 1/2/3 junctions from 11 and 17 week fetal, and 3 year post-natal human thymus. At the top are shown the germline sequence or Vδ5, Dδ1, Dδ2, Dδ3, Jδ1, and Jδ2, and Jδ3 gene segments. Below are the joins derived from cDNA sequences cloned form fetal thymus aged 11 weeks and 17 weeks, and 3 year post-natal thymus. Sequence assignments are made as assigned in the text. Strong candidates for template-independent, N nucleotide insertions are underlined. In frame joins are labelled (+), out of frame (−). The identity of the J segment used is indicated by a number 1, 2, or 3 and was determined by downstream coding sequences not shown here. Sequences represent single frequency clones, except in 3 year post-natal thymus where (2) and (3) indicate the number of identical sequences cloned.

17 Week Thymus:

| Germline Sequence: | Vγ8 | P | N | P | Jγ | | Frequency | In Frame |
|---|---|---|---|---|---|---|---|---|
| ACC TGG GAT AGG | | | | | | | | |
| ACC TGG GAT | | | | | ATTATTATAAG | J1 | 1/5 | (-) |
| ACC TGG GAT | | | | | ATTATTATAAG | J2 | 2/5 | (-) |
| ACC TGG GAT | | | | | GTTGGTT | JP1 | 1/5 | (-) |
| ACC TGG GAT | | | | | ACCACTG | JP1 | 1/5 | (+) |

| Germline Sequence: | Vγ5 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| TGT GCC ACC TGG GGC AGG | | | | | | | | |
| TGT | | | | | ACACTT | J1 | 2/2 | (+) |

| Germline Sequence: | Vγ4 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ACC TGG GAT GGG | | | | | | | | |
| ACC TGG GAT G | | | | | ATACCAC | JP1 | 1/6 | (+) |
| ACC TGG GAT GG | | | AAGG | | TATAAGAAA | J1 | 2/6 | (+) |
| ACC TGG GAT | | | | | ACCACTGG | JP1 | 1/6 | (+) |
| ACC TGG GAT G | | | AT | | ACCACTGG | JP1 | 1/6 | (+) |
| ACC TGG G | | | | | ATTATTAT | J2 | 1/6 | (+) |

| Germline Sequence: | Vγ3 | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ACC TGG GAC AGG | | | | | | | | |
| ACC TGG GAC AGG | | | CC | | ACCACTGG | JP1 | 1/8 | (-) |
| ACC TGG GAC AGG | | C | GGGGC | | AAGAAACTC | J1 | 1/8 | (+) |
| ACC TGG GAC AGG | | C | | | TATTATAAG | J2 | 1/8 | (-) |
| ACC TGG GAC A | | | | | AATTATTAT | J1 | 1/8 | (-) |
| ACC TGG GAC A | | | AA | | AATTATTAT | J1 | 1/8 | (+) |
| ACC TGG | | | ACA | | AATTATTAT | J1 | 1/8 | (+) |
| ACC TGG | | | ACAGAGG | | TTATTATAA | J2 | 1/8 | (-) |
| ACC TGG | | | ACAGGCC | | ATTATTATA | J2 | 1/8 | (+) |

**Fig. 6.** Nucleotide sequences of Vγ−Jγ junctions from members of the VI family of T cell receptor γ genes in 17 week human fetal thymus. At the top left of each section of Figure 6 are shown the germline sequence of 4 members of the VI gamma gene family, Vγ8, Vγ5, Vγ4, and Vγ3, respectively. Strong candidates for template-independent, N nucleotide insertions are underlined. The identity of the J segment used is indicated by a number 1, 2, or 3 and was determined by downstream coding sequences not shown here. The frequency of each sequence is indicated. In frame joins are labelled (+), out of frame (−).

weeks, there was little incorporation of N nucleotides (Figure 6). The junctions were not only simple, but in some cases recurrent (Figure 6). The independence of recurrent junctions was indicated by, among other things, sequence 3′ of the joins that revealed use of different J gene segments.

## Discussion

These experiments have examined the expression of γδ TCR genes directly in human fetal and post-natal thymus samples. No cell culture was applied, nor any other techniques that might have selected particular populations. The data reveal a number of points.

At time points between 11 and 22 weeks of human fetal ontogeny, all Vγ and Vδ families (with the possible exception of Vδ4) are expressed as rearranged genes in the thymus. The expression pattern of Vδ genes in post-natal thymus is distinguished from that in fetal thymus, most clearly by the developmental change in Vδ2 expression. Vδ2 is the most conspicuously expressed Vδ gene in the fetal thymus. Hence, a high abundance of Vδ2-encoded γδ TCRs in the fetal thymus (Table III; also S.Carding, unpublished) may at least in part be determined at the level of differentially regulated Vδ gene expression. (Vδ2 may be the earliest Vδ gene to be expressed, but we could not test this, because we were unable to obtain 7−8 week fetal thymus samples.) The loss of Vδ2 expression soon after birth is consistent with the rarity of Vδ2-encoded TCRs in the post-natal thymus (Bottino *et al.*, 1988; Triebel *et al.*, 1988; Casorati *et al.*, 1989). Therefore, the nature of the γδ TCRs expressed in the human thymus seems in part to be determined by the differential expression of a single Vδ gene. This is strikingly similar to the situation in mice, where Vδ1 is expressed more strongly early in fetal ontogeny than other γδ genes (Carding *et al.*, 1990), at a time when most γδ TCR protein includes the Vδ1 chain (Havran and Allison, 1988).

It was not possible to identify a predominantly expressed Vγ gene, throughout fetal thymic ontogeny. In particular VγII is not the predominantly expressed Vγ gene in the fetal thymus, despite the propensity of its product to pair with Vδ2 (Triebel *et al.*, 1988; Casorati *et al.*, 1989), and the ready capacity to identify VγII+ cells from fetal thymus, using an anti-VγII specific antibody (Kabelitz and Conradt, 1988). Therefore, selective activation of human Vγ gene is not a reliable predictor of TCRγδ protein composition on fetal thymocytes. This too is similar to the pattern of γ gene activation in the mouse, where all Vγ genes are expressed throughout fetal thymic ontogeny while the γδ TCRs expressed on fetal thymocytes are of restricted types (Carding *et al.*, 1990). In post-natal thymus, the expression of VγII,

like that for Vδ2, has declined dramatically. This is consistent with the reduction in VγII-containing TCRs in the post-natal thymus (Triebel *et al.*, 1988; Casorati *et al.*, 1989).

The expression of a broader range of γδ genes than γδ cell surface protein in the murine fetal thymus, implied that the predominance of particular cell surface γδ receptors at different times was at least in part due to a capacity of those receptors to interact with thymic stroma (Carding *et al.*, 1990). Such interactions could also explain the recurrence of particular junctional sequences within the restricted receptors of the early murine fetal thymus (Lafaille *et al.*, 1990). Developmental changes in interactions between murine γδ progenitors and thymic stroma have also been implied by recent chimera experiments (Havran and Allison, 1990). The striking parallels between the mouse gene expression data and the gene expression data presented here for humans suggest that such a mechanism of γδ cell development may be conserved in both species. In summary, a restricted set of γδ receptors on fetal thymocytes may, in both species, be determined in part by similar patterns of selective gene activation, and in part by thymocyte-stroma interactions. 'Successful' interactions might be between γδ chains and cellular equivalents of *Staphylococcus aureus* antigens. Such antigens can strongly interact with VγII, at least (Rust *et al.*, 1990).

The expression of the majority of γ and δ gene families throughout human and murine fetal thymic ontogeny does not imply that any cell can indiscriminantly express any gene segment. On an individual basis, cells that pass through the thymus on different schedules may be limited to the expression of particular genes, regulated by genetic and transient environmental factors. This could explain the frequent (but not invariable) rearrangement of the same V gene family both productively and abortively within the same cell (Ito *et al.*, 1989), and possibly the rarity of human γδ cells that have rearrangements to both Cγ1 and Cγ2 (Triebel and Hercend, 1989). The regulated utilization of different gene segments in different cells is also supported by the sequence data that shows for instance more widespread use of Dδ1 in Vδ5 joins than in Vδ1 joins, largely irrespective of the time in ontogeny at which rearrangements were sampled (see below).

A comparison of Vδ1 and Vδ5 sequences expressed in post-natal thymus with the same sequences in fetal thymus reveals increased junctional diversity, through increased N nucleotide insertion. In post-natal transcripts, N4 insertions are widespread both in Vδ1 and in Vδ5 sets, and N1 inserts occur in all Vδ5 transcripts. By comparison, incorporation of N nucleotides is much less common in fetal Vδ transcripts. Like fetal Vδ joins, fetal Vγ junctions at 17 weeks show very restricted N nucleotide insertion. In many cases, junctions are simple fusions of gene segments. The pattern of increasing N nucleotide insertion as human thymic ontogeny proceeds, also parallels the pattern seen in the mouse, where joins formed early in thymic ontogeny are simple gene segment fusions, and are recurrent. Some fetal human γ junctions sequenced here were recurrent, but this was not as common as in the mouse.

As ontogeny proceeds, there is also some increased nucleolytic activity, especially evident in post-natal D3 and Jδ sequences. This seems to reflect true changes in enzymatic activity, since most transcripts in which the Jδ segment is truncated at the 5′ end represent abortive rearrangements that could not be reflective of cell surface selection on the γδ protein product. For some reason, the increased nucleolytic activity does not seem to act on the Vδ gene segments: the 3′ breakpoints of both Vδ1 and Vδ5 segments are strikingly limited, and similar at all fetal and post-natal time points examined.

In addition to the increase in N nucleotide diversification and nucleolytic activity, there are changes in gene segment utilization as ontogeny proceeds. The earliest Vδ−Dδ recombinations involve only Dδ3. This parallels the predominant use of a single Dδ element (Dδ2) in murine fetal thymus. At 17 weeks, Vδ5 joins show use of Dδ1 and Dδ2 elements only rarely apparent in Vδ1 joins at the same time. It could be argued that the Vδ1 joins expressed at 17 weeks were formed before the expressed Vδ5 joins (they are very similar to Vδ1 11 weeks joins), and that by the time the 17 week Vδ5 joins were formed, there was increased Dδ1 and Dδ2 incorporation, reflecting increased use of D elements as ontogeny proceeds. However, this would be an over-simplification, since post-natal Vδ1 joins while having hallmarks of 'late-formed' junctions (high N4, N3, and invariable Dδ2 usage), still have little Dδ1 contribution (less frequent than in 17 week Vδ5 transcripts). By contrast, the use of Dδ2 in all post-natal Vδ1 joins, whether abortive or functional, is clearly in excess of Dδ2 usage by post-natal Vδ5 transcripts (~50% of cases). These data further support a model for regulated utilization of different gene segments in different sets of cells, with several different populations of cells being present in the thymus at any one time. Regulated utilization of different gene segments is also suggested by the distinct shift to Jδ1 usage in post-natal thymus.

The changes that occur in γδ rearrangements as ontogeny proceeds do not occur exactly in parallel. For example, in 17 week Vδ5 joins, Dδ2 sequences are incorporated in some transcripts, but they are never flanked by N3 sequences. In post-natal joins, Dδ2 elements are almost always flanked by N3 nucleotides. Also, the increased utilization of Dδ1 and Dδ2 elements in 17 week Vδ5 joins relative to 17 week Vδ1 joins is not paralleled by increased use of N4 nucleotides, relative to 17 week Vδ1 joins. By analysing total thymocyte RNA *ex vivo*, the data almost certainly include transcripts in immature cells. This approach, we believe, minimizes the influence of selection for cell surface TCR molecules on the rearrangement patterns analysed. The similarity of patterns shown by productively and non-productively rearranged genes supports this. One unresolved issue is whether different rearrangement events that occur on one allele occur according to different schedules in different cells. For example, do 17 week Vδ1 and Vδ5 cells both use Dδ3-J joins formed at the same time, but use V−D joins made at different times?

The data clearly show that neither Vδ1 nor Vδ5 genes are rearranged at only one time point in thymic development, but that progenitors attempt arrangement of these (and other) different families throughout ontogeny. Data from the mouse are similar (Carding *et al.*, 1990). As ontogeny proceeds, junctional diversity increases, through at least three mechanisms—regulated utilization of more gene segments, increased N nucleotide insertions, and increased variability in gene segment breakpoints. Many of the patterns of human thymic γδ gene expression described here are very similar to what has been described for the mouse γδ genes (Elliott *et al.*, 1988; Carding *et al.*, 1990; Itohara *et al.*, 1990). Futhermore, the patterns are likely to be relevant to the activation of immunoglobulin and TCR αβ genes, that are

more difficult to analyse because of the larger size of their gene families. The hypothesis that different sets of progenitor cells at any one time may be activating different sets of gene segments for rearrangement implies the likely complexity of the regulation of such processes *in vivo*.

The similarities of human and murine γδ gene activation in the fetal thymus—in particular the prodominance of one Vδ−Dδ rearrangement, and the limited junctional diversity—suggest that in humans, as in mice, any γδ T cells exported from the early to mid-gestation fetal thymus will be relatively restricted in diversity. Hence, the issue of how broad the specificity of such cells can be remains an issue in the human, as it does in the mouse (Asarnow *et al.*, 1988; Janeway *et al.*, 1988; Havran and Allison, 1990). Is a population of such cells localized in some peripheral human tissue, in a manner similar to the homing of murine early fetal thymocytes to epithelia?

While there were clear consensus patterns of Vγ and Vδ gene expression representative of the majority of samples analysed, other samples departed from this picture. Such variability may not be apparent in mice because of the restricted set of inbred genetic strains examined. The nature of the genetic determinants that combine with environmental influences to shape the thymic γδ repetoire is unresolved and understandably a major focus of study. Furthermore, various results suggest that for murine γδ cells, the thymus may not be the sole site of development (Takagaki *et al.*, 1989; Carding *et al.*, 1990; Parker *et al.*, 1990). In response to this, the patterns of γδ gene expression in other fetal human tissues needs to be studied in some detail.

## Materials and methods

### *Tissue samples*

Human fetal tissue was obtained after elective abortions from the International Institute for the Advancement of Medicine (Essington, PA) with the approval of the Human Investigations Committee at Yale. Gestational age was determined by fetal morphology and menstrual records (Moore, 1973).

### *cDNA preparation*

Total cellular RNA was isolated from human fetal thymus (Chirgwin *et al.*, 1979) in the presence of carrier tRNA. The integrity of mRNA was verified by optical density and ethidium bromide staining after electrophoresis on formaldehyde−agarose gels. Approximately 10 μg of total mRNA was used to synthesize random hexamer primed-cDNA, the integrity of which was confirmed by amplification with human β-tubulin primers (Carding *et al.*, 1990).

### *Polymerase chain reaction*

cDNA was used in 25-cycle PCR amplifications (Roth *et al.*, 1988; Kyes *et al.*, 1989). PCR primers were gene-specific and are described in Table I. PCR products were electrophoresed in 1.7−2% agarose gels, transferred to nitrocellulose and hybridized with a pool of kinased J segment-specific probes capable of detecting all known Jγ or Jδ gene segments (Table I, Figure 1A and 1B).

### *DNA sequencing*

Amplified PCR products were digested with restriction enzymes, purified and cloned. DNA was sequenced (Toneguzzo *et al.*, 1988) and analysed on 6−9% acrylamide−7 M urea gels.

### *Antibody staining*

The murine monoclonal antibodies reactive with human CD3 and TCR-α/β (WT-31) were purchased from Becton-Dickinson (Sunnyvale, CA). The anti-human TCR δ chain-specific antibody δ TCS1, was purchased from T Cell Sciences (Boston, MA) and TCR δ-1 was kindly provided by Dr Michael Brenner (Boston, MA). The antibody Y-TCR(Vδ2)-1 (15D) has been shown to recognize the Vδ2 chain of human TCR δ (S.R.Carding and K.Bottomley, manuscript in preparation). Phenotypic analysis of human fetal thymocytes was performed by indirect immunofluorescence as described previously (Carding *et al.*, 1990). Stained cells were analysed on either a FACSCAN or FACS 440.

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## References

Asarnow,D.M., Kuziel,W.A., Bonyhadi,M., Tigelaar,R.E., Tucker,P.W. and Allison,J.P. (1988) *Cell*, **55**, 837−847.

Baer,R., Boehm,T., Yssel,H., Spits,H. and Rabbits,T.H. (1988) *EMBO J.*, **7**, 1661−1668.

Boehm,T., Baer,R., Lavenir,I., Forester,A., Waters,J.J., Natcheva,E. and Rabbitts,T.H. (1988) *EMBO J.*, **7**, 385−394.

Bottino,C., Tambussi,S., Ferrini,S., Ciccone,E. Varese,P., Mingari,M.C., Moretta,L. and Moretta,A. (1988) *J. Exp. Med.*, **168**, 491−498.

Brenner,M.B., Stromigner,J.L. and Krangel,M.S. (1988) *Adv. Immunol.*, **43**, 133.

Carding,S.R., Kyes,S., Jenkinson,E.J., Kingston,R., Bottomly,K., Owen,J.T.T. and Hayday,A.C. (1990) *Genes Dev.*, **4**, 1304−1315.

Casorati,G., de Libero,G., Lanzevecchia,A. and Migone,N. (1989) *J. Exp. Med.*, **170**, 1521−1535.

Chirgwin,J.M., Przbyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) Biochemistry, **18**, 5294.

Davis,M.M. and Bjorkman,P.J. (1988) *Nature*, **334**, 395−402.

Elliott,J.F., Rock,E.P., Patten,P.A., Davis,M.M. and Chien,Y.-H. (1988) *Nature*, **331**, 627−631.

Forster,A. Huck,S., Ghanem,N., Lefranc,M.-P. and Rabbitts,T.H. (1987) *EMBO J.*, **6**, 1945−1950.

Hata,S., Satyanarayana,K., Devlin,P., Band,H., McLean,J., Strominger, J.L., Brenner,M.B. and Krangel,M.S. (1988) *Science*, **240**, 1540−1544.

Hata,S., Clabby,M., Devlin,P., Spits,H., De Vries,J.E. and Krangel,M.S. (1989) *J. Exp. med.*, **169**, 41−57.

Havran,W.L. and Allison,J.P. (1988) *Nature*, **335**, 443−445.

Havran,W.L. and Allison,J.P. (1990) *Nature*, **344**, 68−70.

Huck,S. and Lefranc,M.-P. (1987) *FEBS Lett.*, **224**, 291−296.

Huck,S., Dariavich,P. and Lefranc,M.-P. (1988) *EMBO J.*, **7**, 719−726.

Ito,K., Bonneville,M., Takagaki,Y., Nakanishi,N., Kanagawa,O., Krecko,E.G. and Tonegawa,S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 631−635.

Itohara,S., Farr,A.G., Lefaille,J.J., Bonneville,M., Takagaki,Y., Haas,W. and Tonegawa,S. (1990) *Nature*, **343**, 754−757.

Janeway,C.A., Jones,B. and Hayday,A.C. (1988) *Immunol. Today*, **9**, 73−76.

Kabelitz,D. and Conradt,P. (1988) *J Exp. Med.*, **168**, 1941−1946.

Kyes,S., Carew,E., Carding,S.R., Janeway,Jr.,C.A. and Hayday,A.C. (1989) *Proc. Natl. Acad. Sci. USA*, **84**, 5527−5531.

Lafaille,J.J., DeCloux,A., Bonneville,M., Takagaki,Y. and Tonegawa,S. (1989) *Cell*, **59**, 859−870.

Lefranc,M.-P., Forster,A. and Rabbitts,T.H. (1986a) *Nature*, **319**, 420−422.

Lefranc,M.-P., Forster,A., Baer,R., Stinson,M.A. and Rabbitts,T.H. (1986a) *Cell*, **45**, 237−246.

Lefranc,M.-P., Chuchana,P., Dariavich,P., Nguyen,C., Huck,S., Jordan,F.B. and Lefranc,G. (1989) *Eur. J. Immunol.*, **19**, 989.

Lefranc,M.-P. and Rabbitts,T.H. (1989) *Trends Biochem. Sci.*, **14**, 214−218.

Loh,E.Y., Cwirla,S., Serafini,A.T., Phillips,J.H. and Lanier,L.L. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 9714−9718.

Moore,K.L. (1973) In: *The Developing Human: Clinically Oriented Embryology*. W.B.Saunders Publishing Co., Philadelphia, PA.

O'Brien,R.L., Happ,M.P., Dallas,A., Palmer,E., Kobo,R. and Born,W. (1989) *Cell*, **57**, 667−674.

Parker,C.M., Groh,V., Band,H., Porcelli,S., Morita,C., Fabbi,M., Glass,D., Strominger,J.L. and Brenner,M.B. (1990) *J. Exp. Med.*, **171**, 1597−1612.

Roth,M.E., Lacy,M.J., McNeil,L.K. and Kranz,D.M. (1988) *Science*, **241**, 1354−1358.

Rust,C.J.J., Verreck,F., Vietor,H. and Koning,F. (1990) *Nature*, **346**, 572–574.
Satyanarayana,K., Hata,S., Devlin,P., Roncarlo,M.G., de Vries,J.E., Spits,H., Strominger,J.L. and Krangel,M.S. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8166–8170.
Takagaki,Y., DeCloux,A., Bonneville,M. and Tonegawa,S. (1989) *Nature*, **339**, 712–714.
Takihara,Y., Tkachuk,D., Michalopoulos,E., Champagne,E., Reiman,J., Minden,M. and Mak,T.W. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6097–6101.
Tighe,L., Forster,A., Clark,D., Boylston,A., Lavenir,I., Rabbitts,T.H. (1987) *Eur. J. Immunol.*, **17**, 1729–1736.
Tonegawa,S. (1983) *Nature*, **302**, 575–581.
Toneguzzo,F., Glynn,S., Levi,E., Mjolsness,S. and Hayday,A. (1988) *BioTechniques*, **6**, 460–469.
Triebel,F. and Hercend,T. (1989) *Immunol. Today*, **10**, 186–188.
Triebel,F., Faure,F., Mami-Chouaib,F., Jitsukawa,S., Griscelli,A., Genevee,C., Roman-Roman,S. and Hercend,T. (1988) *Eur. J. Immunol.*, **18**, 2021–2027.

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