DIVERSITY AND ORGANIZATION OF HUMAN T CELL RECEPTOR $\delta$ VARIABLE GENE SEGMENTS

By SHINGO HATA,* MARTHA CLABBY,* PETER DEVLIN,* HERGEN SPITS;† JAN E. DE VRIES;† AND MICHAEL S. KRANGEU

From the *Division of Tumor Virology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115; and the †Unicet Laboratory for Immunological Research, Dardilly, France

Two CD3-associated TCR complexes have now been identified. The first, TCR-$\alpha/\beta$, is expressed on the majority of T lymphocytes. TCR-$\alpha$ and -$\beta$ polypeptides mediate the specific recognition of antigen in association with MHC-encoded class I and class II molecules (1). TCR-$\alpha/\beta$ diversity is high, since in man there are 50-100 $V_{\alpha}$ and 50-100 $J_{\alpha}$ gene segments, as well as 70 $V_{\beta}$, 13 $J_{\beta}$, and two $D_{\beta}$ gene segments (2, 3). Combinatorial and imprecise joining, polymerization of template independent N region nucleotides, and combinatorial association of TCR-$\alpha$ and -$\beta$ polypeptides help to generate an extensive TCR-$\alpha/\beta$ repertoire.

The second, TCR-$\gamma/\delta$, is expressed on a small fraction of peripheral blood and thymic T lymphocytes (4-7), although it may be expressed on the majority of T lymphocytes in certain epithelial locations (8, 9). The function of TCR-$\gamma/\delta$ lymphocytes is unclear at present, although one example of murine TCR-$\gamma/\delta$ lymphocytes that recognize an MHC-linked gene product has been reported (10). The repertoire of germline TCR-$\gamma$ and -$\delta$ gene segments is apparently more limited than that of TCR-$\alpha$ and -$\beta$. In man, for example, there are only seven functional $V_{\gamma}$ segments and five $J_{\gamma}$ segments, with additional diversity added at the V-J junction due to N region nucleotide incorporation (7, 11-15). Similarly, initial studies have identified only a single human $V_{\delta}$ segment ($V_{\delta}1$) and three $J_{\delta}$ segments ($J_{\delta}1$, $J_{\delta}2$, and $J_{\delta}3$), with all productive rearrangements involving the $J_{\delta}1$ segment (16-18). The TCR-$\gamma/\delta$ repertoire is nevertheless likely to be large, since the use of two $D_{\delta}$ elements ($D_{\delta}1$ and $D_{\delta}2$) in tandem, coupled with imprecise joining and extensive N region nucleotide incorporation, generates marked variability in the TCR-$\delta$ V-J junctional region (18-23).

The location of the human TCR-$\delta$ gene is unusual in that it is nested within the TCR-$\alpha$ locus at chromosome 14q11 (19, 20, 23-26). The $D_{\delta}$, $J_{\delta}$, and $C_{\delta}$ gene segments are located just upstream of the $J_{\alpha}$ region, between $V_{\alpha}$ and $J_{\alpha}$ segments. It is striking that although the $V_{\delta}1$ segment is located among $V_{\alpha}$ segments and at a large distance from $C_{\delta}$ (20), the $V_{\delta}1$ segment appears to rearrange selectively to $D_{\delta}1$. This suggests that the $V_{\alpha}$ and $V_{\delta}$ repertoires may be distinct, and that rearrangement at the TCR-$\alpha/\delta$ locus may be highly controlled. By contrast, as many

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as nine murine $V_{\alpha}$ gene segments have now been identified (22). Two of these are nearly identical to previously characterized $V_{\alpha}$ gene segments, indicating the possibility that some $V_{\delta}$ segments may function as $V_{\alpha}$ segments as well.

To better understand the diversity of human TCR-$\delta$ gene segments and the relationship between the TCR-$\gamma/\delta$ and TCR-$\alpha/\beta$ repertoires, we have examined TCR-$\delta$ rearrangements in a new panel of cloned TCR-$\gamma/\delta$ lymphocytes. Through this analysis we have identified and determined the structures of two new $V_{\delta}$ segments, designated $V_{\delta}2$ and $V_{\delta}3$. These segments are distinct from known $V_{\alpha}$ segments, and are widely separated within the TCR-$\alpha/\beta$ locus both from $V_{\delta}1$ and from each other.

**Materials and Methods**

**TCR-$\gamma/\delta$ Cell Lines.** The derivation and characteristics of peripheral blood-derived cell line IDP2 (4) and leukemic cell line Molt-13 (17, 27) have been described previously. The cloned T cell line WM-14 was derived from umbilical cord blood cells (28). The T cell clones LB117, LB207, LB210, LB213, and LB220 were obtained by stimulating the PBL from a healthy donor with Burkitt's lymphoma cells, followed by cloning using limiting dilution and feeder cell mixtures as described previously (29). The derivation and properties of T cell clone F7 will be described in a subsequent publication. The T cell clones were maintained in Yssel's medium and were expanded in IL-2 by weekly stimulation with feeder cells (29).

**DNA Probes.** The TCR-$\delta$ DNA probes used in this study were as follows: $C_{\delta}$, cDNA O-240 (16); $J_{\delta}$, a 1.7-kb genomic Xba I fragment (18, 20); $V_{\delta}1$, a 300-bp Eco RI-Sca I fragment of cDNA O-240/47 (16); $V_{\delta}2$, a 430-bp Eco RI-Nco I fragment of cDNA LB11781.7; and $V_{\delta}3$, a 435-bp Eco RI-Taq I fragment of cDNA WM142.1. The TCR-$\gamma$ DNA probes used in this study were as follows: $J_{\gamma}$, 1.3/2.3, an 800-bp Jy1.3 genomic Hind III-Eco RI fragment (14); $V_{\gamma}1$, a 350-bp Eco RI-Kpn I fragment of $V_{\gamma}1.3$ cDNA M13k (27); $V_{\gamma}2$, a 445-bp Eco RI-Acc I fragment of cDNA PBLCl15 (30); $V_{\gamma}3$, a 300-bp Eco RI fragment of cDNA pT,15 (W. Strauss, personal communication); $V_{\gamma}4$, a 400-bp Sep I-Eco RI fragment of rearranged genomic clone pT,R4 containing both V and J sequences (W. Strauss, personal communication). Fragments were purified through low gelling temperature agarose and labeled to high specific activity by the random priming method (31).

**Preparation and Blot Hybridization Analysis of RNA and DNA Samples.** Total cellular RNA and high molecular weight genomic DNA were prepared from a single frozen pellet of $\sim 5 \times 10^7$ cells in each instance. Cells were lysed for RNA preparation by the urea-lithium chloride method (32). 90% of the lysate was sheared and used for RNA preparation, whereas 10% of the lysate was diluted into 15 vol of 150 mM NaCl; 10 mM NaEDTA; 10 mM Tris HCl (pH 8.0); 0.4% SDS; 100 $\mu$g/ml proteinase K, and was used for the preparation of genomic DNA (33). Yields averaged 85 $\mu$g RNA and 50 $\mu$g DNA.

RNA was subjected to electrophoresis through 1.5% agarose gels containing 2.2 M formamide, and was then blotted onto and UV crosslinked to Hybond-N membranes (Amer sham Corp., Arlington Heights, IL), according to the manufacturer's instructions. Genomic DNA was digested with the appropriate restriction enzyme, subjected to electrophoresis through 0.7% agarose gels, and was similarly blotted onto and UV crosslinked to Hybond-N membranes. Filters were prehybridized at 50°C and hybridized at 42°C in 50% formamide; 4.8 x SSC; 5 x Denhardt's; 0.5% SDS; 200 $\mu$g/ml boiled salmon sperm DNA; 50 mM Hepes (pH 7.0). High stringency washes were in 0.1 x SSC; 0.1% SDS at 50°C for Northern blots and at 65°C for Southern blots. Blots were stripped before reprobing by incubation for 1-2 h at 68°C in the prehybridization solution.

**cDNA Library Construction and Analysis.** cDNA libraries containing 2-5 $\times$ 10^5 recombinants were constructed from LB117 RNA, WM14 RNA, and a mixture of LB207, LB210, and F7 RNA. Poly(A)⁺ RNA prepared from $\sim 80$ $\mu$g total RNA was converted to ds cDNA using AMV reverse transcriptase, klenow, mung bean nuclease, and Eco RI methylase. After ligation of Eco RI linkers, size-fractionated cDNA was cloned into the Eco RI site of the vector
λgt10 (35). After screening with the appropriate probes, the 5′ Eco RI fragments (VJCs) of positive clones and subfragments thereof were subcloned into either Bluescript (Stratagene) or M13mp18, and nucleotide sequences were determined on both strands by the dideoxy chain termination method (36, 37) using modified T7 polymerase (38) (Sequenase; United States Biochemical Corp., Cleveland, OH).

**Analysis of Genomic Clones.** An 11-kb Bam HI-Kpn I fragment carrying the 3′ portion of Vg3, and a 3-kb Kpn I-Bam HI fragment carrying the 5′ portion of Vg3 were isolated from cosmid K7A (20) and subcloned into Bluescript. Sequences were determined by the dideoxy chain termination method using double-stranded templates and exonuclease III-generated deletions (39, 40) (Erase-a-base System; Promega Biotec, Madison, WI) according to the manufacturer.

**Results**

**Two Novel Human Vg Segments.** In previous studies it was shown that the TCR-γδ peripheral blood T cell lines IDP2, PBL C1, and PBL L1, and the leukemic T cell lines PEER and Molt-13, all displayed functional rearrangements using the Vg1 and Js1 gene segments (16-18). To identify TCR-γδ cell lines using distinct Vg segments, RNA samples from a panel of seven TCR-γδ T lymphocyte peripheral blood clones were examined by blot hybridization using Vg3 and Cs probes. As in RNA from the previously studied IDP2 cell line (16), a Cs probe revealed TCR S mRNA species indicative of differentially polyadenylated transcripts arising from fully rearranged genes (2.2 and 1.3 kb) in all cell lines (Fig. 1A). In addition, this probe detected differentially polyadenylated transcripts arising from partially or unrearranged genes (1.7 and 0.8 kb) in some cell lines. However, aside from IDP2 RNA, only a single cell line (F7) revealed transcripts (2.2 and 1.3 kb) detected by a Vg1 probe (Fig. 1A). The remaining cell lines (LB117, LB207, LB210, LB213, LB220, and WM14), which expressed full-length Vg1-β transcripts of 2.2 and 1.3 kb, were presumed to be using distinct Vg gene segments.

To determine whether the Vg1− cell lines all used the same novel Vg segment, Xba I digests of genomic DNA samples were analyzed by blot hybridization using Js1 and Js3 probes (Fig. 2, A and B). The Js1 probe detects a single germline fragment of 1.7 kb in SB (B cell) and HL60 (myeloid cell), and two rearrangements in the TCR-γδ cell lines IDP2 and Molt-13. Of these rearrangements, the 6.4-kb fragment shared by IDP2 and Molt-13 represents rearrangement to Vg1 (18). The 2.9-kb rearrangement in IDP2 is quite common, and is thought to represent either D-J or D-D-J, whereas the 7.2-kb rearrangement in Molt-13 has not been detected elsewhere, and is unidentified. As expected, the Vg1− cell line F7 shares the 6.4-kb Vg1-Js1 rearrangement with IDP2 and Molt-13. On the other hand, the Vg1− cell lines display three distinct Js1 rearrangements, two of which (4.2 and 1.9 kb) had not been observed previously. These were presumed to reflect rearrangement to two novel Vg segments, designated Vg2 (4.2 kb) and Vg3 (1.9 kb).

Based upon the above assignments, neither LB207 nor LB213 carries a functional Js1 rearrangement. We have previously described a distinct Js segment (Jg2) that is homologous to the murine Js2 element and is located ~10 kb 3′ of Js1. Since it is now clear that an additional Js segment lies between these two J segments (19, 23), it seems appropriate to rename the most 3′ Js segment as Js3. Our Js3 probe detects a 5.0-kb germline Xba I fragment that carries both Js2 and Js3, and therefore, should detect rearrangements to either of these Js segments. Notably, the Js3 probe detects a 3.4-kb rearrangement specific to LB207 and LB213, indicating the
Figure 1. Blot hybridization analysis of TCR-δ and TCR-γ transcripts. 5-μg samples of total RNA was subjected to electrophoresis through 1.5% agarose containing 2.2 M formaldehyde, transferred to Hybond-N membranes, and serially probed with the indicated 32P-labeled DNA fragments.

The likelihood of functional rearrangement to either Jδ2 or Jδ3 in these cell lines. Based upon the disposition of Xba I sites flanking Jδ1, Jδ2, and Jδ3, we predicted that in these instances Jδ3 had rearranged to the same V segment (Vδ2) identified by the 4.2-kb Jδ1 rearrangement.

To confirm these interpretations and characterize the novel V segments, we generated cDNA libraries from LBI17 and from WM14 poly(A)+ RNA. Using a Cδ probe we identified cDNA clones representing the transcripts of the functionally rearranged TCR-δ gene in each cell line. The nucleotide and deduced amino acid sequences of these cDNA clones are presented in Fig. 3, A and B, and the deduced amino acid sequences for the V segments are compared both with each other and with that of Vδ1 in Fig. 3 C. LBI17 (Vδ2) and WM14 (Vδ3) clearly use Vδ segments distinct from the Vδ1 segment and from each other. Both cDNAs display potential contributions from the Dδ1 and Dδ2 elements as well as from N region nucleotides within the junctional region, both utilize the Jδ1 segment, and both maintain an open reading frame across the V-D-D-J junction.

The Vδ1, Vδ2, and Vδ3 gene segments are all distinct from previously reported Vα segments, further supporting the notion that the human Vα and Vδ repertoires
are distinct. Furthermore, they display only low levels of amino acid sequence identity with each other, matching at 23–33% of the residues in various pairwise comparisons (Fig. 3 C). Nevertheless there are striking homologies with particular murine V\_\delta segments (Fig. 3 C). For example, human V\_\delta 1 matches a member of the murine V\_\delta 6 family at 58% of the residues, and human V\_\delta 3 matches murine V\_\delta 5 at 66% of the residues (22). Such high levels of interspecies sequence conservation despite low levels of intraspecies sequence conservation most probably indicate that these pairs of V segments have evolved from distinct ancestral V segments whose existence predates the divergence of mouse and man. Similar observations have been made for particular human and murine V\_\beta (41) and V\_\gamma (42) gene segments.

**Rearrangement and Expression of TCR-\delta and TCR-\gamma Gene Segments.** To further characterize the TCR-\delta rearrangements in the remaining TCR-\gamma/\delta cell lines in the panel, V\_\delta 2- and V\_\delta 3-specific probes were generated and were used to probe both Northern
Figure 3. Nucleotide and deduced amino acid sequences of Vg2 and Vg3 cDNA clones. (A) Sequences of Vg2 cDNAs from cell lines LB117, LB210, and LB207 are presented from near the Eco RI linker at the 5' end of each clone to the Eco RI site situated 77 bp into the Cg segment. Putative contributions of N and D segments within the junctional region are noted. (B) Sequence of a Vg3 cDNA from WM14. (C) Homologies among the deduced amino acid sequences of human and murine Vg segments. Mouse Vg1 and Vg6 sequences are from reference 22. Dashes denote identities and blanks denote gaps introduced to maximize the alignment. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00792.
blots (Fig. 1, C and D) and Southern blots (Fig. 4, A and B). All Val- cell lines expressed either Vs2 or Vs3 transcripts, although one cell line, LB210, expressed both. The Vs2 gene segment was found on a 3.7-kb germline Xba I fragment that was rearranged either to a 4.2-kb fragment (corresponding to rearrangement to J61) or to a 3.4-kb fragment (corresponding to rearrangement to J62 or J63). The Vs3 gene segment was found on a 9.4-kb germline Xba I fragment that was rearranged to a 1.9-kb fragment (corresponding to rearrangement to J61) in two cell lines. As expected from the analysis of Northern blots, LB210 displayed rearrangements of both Vs2 and Vs3. An additional Vs3 rearrangement of ~1.0 kb, not detected by J61 and J63 probes (not shown), may reflect rearrangement of Vs3 to D6. Such V-D rearrangements have been documented for murine TCR-β (21).
To determine which of the two rearrangements (Vs2 or Vs3 to J81) in LB210 was productive, and which of the J8 segments (J82 or J83) was rearranged to Vs2 in LB207, a cDNA library generated from RNA of both cell lines was screened with a Vs2 cDNA probe, as well as with J81- and J83-specific oligonucleotide probes. Two cDNA clones that used the Vs2 segment along with either J81 (LB210) or J83 (LB207) were characterized. Their nucleotide and deduced amino acid sequences are presented in Fig. 3A. Both cDNA clones maintain an open reading frame across the V-D-D-J junction, indicating that they represent the functional transcripts in the respective cell lines. Thus, in LB210, which displays both Vs2 and Vs3 rearrangements and transcripts, the Vs2 rearrangement is productive. Further, in LB207, and likely in LB213, productive rearrangements of Vs2 to J83 have occurred. This is the first evidence for functional utilization of the human J8 element.

We assessed Vγ and Jγ segment usage within this panel by analyzing Northern blots with Vγ1 family, Vγ2, Vγ3, and Vγ4 probes (Fig. 1, E, F, G, and H, respectively) and Southern blots of Kpn I-digested genomic DNA with Jγ1.3/2.3 and Vγ2 probes (Fig. 5, A and B, respectively; nomenclature according to references 12 and 14). As shown previously by Huck and Lefranc (15), the linkage of human Jγ segments predicts that a Jγ1.3/2.3 probe can detect all TCR-γ gene rearrangements in Kpn I digests of human genomic DNA. Additional analysis using a Vγ2 probe served to specifically identify Vγ2 rearrangements, and further, to distinguish between rearrangements to Vγ1 and Vγ3, since the germline Vγ2 gene segment would be deleted upon rearrangement of Vγ1 gene segments, but retained upon rearrangement of Vγ3.

All cell lines within the panel displayed two Jγ rearrangements (Fig. 5A). In some cases Northern blots allowed an assessment of which rearrangement was productive, since only one transcript was detected (Fig. 1, E, F, G, and H). Further, cells that had productively rearranged the Vγ2 gene segment could be identified using the mAb anti-TiyA, which specifically stains the surface of lymphocytes using this V segment (43). All cells that displayed a Vγ2 to Jγ rearrangement were found to be TiyA+ by surface fluorescence (data not shown). As a result, unambiguous assignment of the productive rearrangement could be made in most instances.

A summary of the Vγ and Vs transcripts and Jγ and J8 rearrangements detected in all of the clonal cell lines that we have examined so far is presented in Table I. All of the cell lines studied appear to productively rearrange one of the three characterized human V8 segments, and either a Vγ1 family member or Vγ2. A striking finding of this analysis was that all cell lines within the panel that display productive rearrangement of the Vs2 segment also display productive rearrangement of the Vγ2 gene segment. This relationship is not reciprocal, in that the Vγ2 gene segment is used in conjunction with either Vs1 or Vs2. The Vγ2-Vs2 cell lines within the panel use either Jγ1.2 (JγP; 12.0-kb rearrangement) or Jγ1.3/2.3 (7.5-kb rearrangement) (Fig. 5 and Table I), and either Js1 or Jγ3, indicating no specific restrictions on J segment utilization. Since the panel of cell lines examined is small, any conclusions must be tentative. However, these data may suggest that TCR-δ chains using the Vs2 gene segment may only be correctly assembled and expressed on the cell surface in association with TCR-γ chains using the Vγ2 gene segment. Alternatively, the data may reflect either positive selection for this particular receptor, or negative selection for receptors expressing Vs2 in conjunction with other Vγ segments. Fur-
FIGURE 5. Blot hybridization analysis of TCR-γ gene rearrangements. Samples of genomic DNA (5 μg) were digested with Kpn I, subjected to electrophoresis through 0.7% agarose, transferred to Hybond-N membranes, and serially probed with the indicated 32P-labeled DNA fragments. (A) The Jy1.3/2.3 probe detects both the Jy1 and Jy2 clusters (germline fragments [GL] of 9 and 16 kb, respectively). The migration of Hind III fragments of phage λ DNA are noted on the left border of the figure, whereas the predicted sizes (in kb) and assignments of rearrangements according to Huck and Lefranc (15) are shown on the right border. (B) The Vy2 probe detects two apparent germline fragments in Kpn I digests (GL). The explanation for this is unclear, although the analysis of Xba I digests with this probe reveals that Molt-13, LB17, LB207, and WM14 retain no germline copies of Vy2, a result that correlates with the presence or absence of the larger germline Kpn I fragment (data not shown). It is on this basis that distinction is made between Vy1 and Vy3 rearrangements on the nonproductive chromosome (Table I). The 11-kb fragment in F7 DNA corresponds to neither of the Jy rearrangements in these cells. Whether this represents an unusual rearrangement or a polymorphism is unclear.

Further studies will be necessary to determine the significance of the relationships observed in this panel, and to determine the mechanisms by which these relationships are maintained.

Additional Js1 Rearrangements in Newborn Thymus DNA. Since there was no evidence for the utilization of additional Vs segments within this panel of cell lines, we sought to determine whether additional Js rearrangements could be detected in Xba I digests of newborn thymus DNA (Fig. 6). A total of seven Js1 rearrangements
Table 1

| Cell line | Vγ and Vδ Transcripts and Jγ and Jδ Rearrangements |
|-----------|--------------------------------------------------|
|           | TCR-δ Rearrangements | Transcripts | TCR-γ Rearrangements | Transcripts |
|           | p   | n | p | n | p | n | p | n |
| IDP2      | Vδ1-Jδ1 Deletions | Vδ1 | - | Vγ2-Jγ2.3 | Vγ3-Jγ1.1 | Vγ2 | (Vγ3) |
| PBL C1    | Vδ1-Jδ1 Deletions | Vδ1 | - | Vγ2-Jγ2.3 | ND | Vγ2 | ND |
| Molt-13   | Vδ1-Jδ1 Deletions | Vδ1 | - | Vγ1.3-Jγ2.3 | Vγ1-Jγ1.1 | Vγ1 | ND |
| PEER      | Vδ1-Jδ1 Deletions | Vδ1 | - | Vγ1.8-Jγ2.3 | Vγ2-Jγ2.3 | Vγ2 | Vγ2 |
| LB117     | Vδ2-Jδ1 Deletions | Vδ2 | - | Vγ2-Jγ1.2 | Vγ1-Jγ2.1 | Vγ2 | - |
| LB207     | Vδ2-Jδ1 Deletions | Vδ2 | - | Vγ2-Jγ1.2 | Vγ3-Jγ1.3 | Vγ2 | - |
| LB210     | Vδ2-Jδ1 Vδ3-Jδ3 | Vδ2 | (Vδ3) | Vγ2-Jγ1.2 | Vγ3-Jγ1.3 | Vγ2 | Vγ3 |
| LB213     | Vδ2-Jδ3 Deletions | Vδ2 | - | Vγ2-Jγ1.2 | Vγ3-Jγ1.1 | Vγ2 | (Vγ3) |
| LB220     | Vδ2-Jδ1 Deletions | Vδ2 | - | ND | ND | Vγ2 | Vγ2 |
| WM14      | Vδ3-Jδ1 Deletions | Vδ3 | - | Vγ1-Jγ2.1 | Vγ1-Jγ1.1 | Vγ1 | - |
| F7        | Vδ1-Jδ1 Deletions | Vδ1 | (Vδ1) | Vγ1-Jγ1.1 | Vγ4-Jγ1.3 | Vγ4 | - |

Nomenclature used to denote TCR-γ gene segments is that of references 12 and 14. The assignments reflect the data obtained in this study as well as in references 17 and 18. The assignment of rearrangements and transcripts as either (p) productive or (n) nonproductive results from a combination of blot hybridization data, cDNA sequences, and staining with mAb anti-TiγA. Unambiguous assignment of the productive rearrangement could be made in each case, except for the TCR-γ rearrangements in WM14, which are bracketed. Vγ family segments are denoted explicitly when the information is available from sequence analysis (e.g., Vγ1.8), or simply as Vγ1 when the assignment relies solely on hybridization data that does not distinguish Vγ1 family segments from one another. Transcripts detected at very low levels are in parentheses.

Figure 6. Additional Jδ1 rearrangements in newborn thymus DNA. Samples of genomic DNA (5 μg) were digested with Xba I, subjected to electrophoresis through 0.7% agarose, transfected to Hybond-N, and probed with the 32P-labeled Jδ1 DNA fragment. SB displays two germline Jδ1 alleles, IDP2 displays Jδ1 rearrangements to Vδ1 and to Dδ, and LB210 displays Jδ1 rearrangements to Vδ2 and Vδ3. F LIV represents 20-wk fetal liver DNA, and N THY represents two samples of newborn thymus DNA (4 d and 12 wk). Rearrangements in the thymus samples that are represented within the clonal cell lines are marked by filled arrowheads, whereas those not represented within the clonal cell lines are marked with open arrowheads.
were detected in each of two DNA samples. Those of 6.4, 4.2, and 1.9 kb correspond to rearrangement to \( \text{V} \text{s}1 \), \( \text{V} \text{s}2 \), and \( \text{V} \text{s}3 \), respectively, whereas that of 2.9 kb corresponds to the common rearrangement (thought to be partial D-J or D-DJ) shared by many cell lines. Of the three remaining \( \text{J} \text{s}1 \) rearrangements (8.2, 3.4, and 2.4 kb; Fig. 6, open triangles), one might reflect the second partial rearrangement (D-J or D-D-J). Thus, the \( \text{J} \text{s}1 \) probe may detect rearrangements to two or three as yet unidentified V segments. The low frequency of rearrangements detected by the \( \text{J} \text{s}3 \) probe precluded an analysis of rearrangements to the \( \text{J} \text{s}2 \) or \( \text{J} \text{s}3 \) gene segments in total thymocyte populations. However, these data suggest strongly that the number of frequently rearranged human \( \text{V} \text{s} \) segments will not be large.

**Dispersed Organization of Human \( \text{V} \text{s} \) Segments within the TCR-\( \alpha/\delta \) Locus.** Our initial studies of the genomic organization of the TCR-\( \alpha/\delta \) locus revealed that the \( \text{V} \text{s}1 \) genomic segment was a large distance from the \( \text{C} \text{s} \) locus, with one \( \text{V} \alpha \) segment (\( \text{V} \alpha13.1 \)) immediately 5' of \( \text{V} \text{s}1 \), and at least one \( \text{V} \alpha \) segment (\( \text{V} \alpha17.1 \)) between \( \text{V} \text{s}1 \) and \( \text{D} \alpha \text{J} \alpha \text{s} \text{C} \alpha \) (20). These data indicated that \( \text{V} \alpha \) and \( \text{V} \text{s} \) segments were interspersed within the locus. To determine whether \( \text{V} \alpha \) segments were clustered or dispersed, we determined the locations of the newly identified \( \text{V} \text{s}2 \) and \( \text{V} \text{s}3 \) segments. As seen in Fig. 2 C, the F7 cell line, which has rearranged \( \text{V} \text{s}1 \) to \( \text{J} \text{s}1 \) on both chromosomes (Fig. 2, A and B and reference 20), has deleted both copies of the \( \text{V} \text{s}2 \) gene segment. This places the \( \text{V} \text{s}2 \) gene segment in the region between \( \text{V} \text{s}1 \) and the \( \text{C} \alpha \) locus. However, whereas previous studies have indicated that the \( \text{V} \alpha17.1 \) gene segment maps by field inversion gel electrophoresis to the \text{Sal} \text{I} and \text{Sfi} \text{I} fragments carrying \( \text{V} \text{s}1 \) (20), \( \text{V} \text{s}2 \) maps to the \text{Sfi} \text{I} and \text{Sal} \text{I} fragments carrying \( \text{D} \alpha \text{J} \alpha \text{s} \text{C} \alpha \) (data not shown). Thus, \( \text{V} \text{s}2 \) lies between \( \text{V} \alpha17.1 \) and \( \text{D} \alpha \text{J} \alpha \text{s} \text{C} \alpha \), and must be within 100 kb 5' of the latter (Fig. 7).

It is intriguing that like F7, Molt-13 also has deleted both copies of the \( \text{V} \text{s}2 \) gene segment. Molt-13 displays a productive \( \text{V} \text{s}1 \) to \( \text{J} \text{s}1 \) rearrangement on one chromosome, and an unidentified \( \text{J} \alpha1 \) rearrangement on the other chromosome, with one

![Figure 7: Dispersed genomic organization of \( \text{V} \alpha \) gene segments. \text{Sfi} \text{I} fragments of 190 and 180 kb carrying the \( \text{V} \text{s}1 \) and \( \text{C} \alpha \) segments are shown. The linkage between these fragments has not been established. The order of the gene segments mapping onto these fragments has been established in genomic Southern blotting experiments and by the mapping of cosmid clones (in the regions covered by the bold horizontal lines). However, the precise distances between the segments mapping outside the regions covered by cosmid clones are uncertain. The nomenclature for \( \text{V} \alpha \) segments is according to Klein et al. (46). Since we have only attempted to map a limited number of \( \text{V} \alpha \) segments, additional unidentified \( \text{V} \alpha \) segments may also map to these fragments. A more detailed restriction map of two adjacent subcloned \text{Bam} \text{HI}–\text{Kpn} \text{I} fragments that carry the \( \text{J} \alpha3 \), \( \text{C} \alpha \), and \( \text{V} \alpha3 \) segments is also presented. Transcriptional orientations are denoted by arrows. The orientation of the \( \text{J} \alpha3 \) and \( \text{V} \alpha3 \) segments with respect to mapped restriction sites has been directly established by nucleotide sequence analysis. The orientation of the \( \text{C} \alpha \) segment has been established elsewhere.](https://example.com/figure7.png)
copy of V61 in the germline configuration (18). This, together with field inversion gel electrophoresis mapping (data not shown), suggests that the nonproductive J61 rearrangement in Molt-13 involves an unidentified region mapping between Va17.1 and Va2.

Unlike Vs2, the Vs3 gene segment is not deleted upon rearrangement of V61 to J61 (Fig. 4 B), indicating that it does not lie between these gene segments. Further, V63 rearrangements to J61 were detected by a Cs probe (Fig. 4, B and C), indicating that Vs3 lies extremely close to Cs. The analysis of cosmide clones spanning the Cs region demonstrated that Vs3 lies in an inverted orientation, roughly 2-3 kb 3' of the Cs segment (Fig. 7). After subcloning and fine mapping of the Kpn I-Bam HI fragments spanning this region, the nucleotide sequence of the germline Vs3 segment was determined. This analysis revealed a structure typical of other TCR V segments, including separate exons encoding the leader peptide and the main body of the V segment, and heptamer and nonamer recombination signals flanking the 3' end of the second exon, thereby establishing its boundary (Fig. 8). The location and orientation of the Vs3 segment relative to Cs is analogous to that described for murine Vp14 relative to Cp2 (44), and implies that rearrangement of Vs3 occurs by inversion of Ds-Js-Cs. These data provide further support for the notion that a limited number of human Vs segments are dispersed within the human TCR-α/δ locus.

Discussion

The present study represents an extension of previous work carried out by ourselves and others detailing the diversity and organization of human TCR-δ gene segments. Previous studies have identified two Ds segments, three Js segments, and one Cs segment, nested within the TCR-α locus, and located just upstream of the Ja region (20, 23-26). An estimated 50-100 Va segments are situated in the region 5' of the Ds, Js, Cs, Ja, and Ca segments (2, 26). Despite the large number of V segments available for recombination, all productive TCR-δ gene rearrangements in previously examined TCR-γ/δ cell lines involve a single V segment, denoted V61 (16-18). This V segment is distinct from previously characterized Va segments, yet is situated among Va segments, at least 180 kb 5' of J61 (20). These observations

![Figure 8. Nucleotide and deduced amino acid sequence of the V63 genomic segment. Spliced donor and acceptor sequences, as well as heptamer and nonamer recombination signals, are underlined. These sequence data have been submitted to the EMBL/GenBank Data Libraries under the accession number Y00792.](image-url)
suggested a limited repertoire of germline TCR-δ gene segments, and a highly controlled rearrangement process that is able to segregate TCR-δ and TCR-α recombinational events and repertoires, despite the interspersion of TCR-δ and TCR-α gene segments.

Our analysis of a larger panel of TCR-γ/δ cell lines has now allowed the identification of two additional Vδ segments, termed Vδ2 and Vδ3. Like Vδ1, these Vδ segments are distinct from previously characterized Vα segments. Blot hybridization experiments indicate that Vδ2 lies within 100 kb 5' of the Cδ region, and analysis of cosmid clones reveals that Vδ3 lies ~2–3 kb 3' of Cδ. These results clearly demonstrate that Vδ segments are not present as a discrete cluster, but rather are dispersed within the locus. It follows that the segregation of Vα and Vδ rearrangements may be mediated by local signals marking particular V segments, rather than long range signals marking large segments of the locus. Such signals might include unique promoter or other elements that could help to modulate the accessibility of particular V segments to recombinases at a specific time in development (45), but probably do not include the heptamer and nonamer recombination signals flanking the V segments, since they are indistinguishable.

Of the three human Vδ segments already characterized, two display quite high amino acid sequence identity with particular murine Vδ segments. Identity is 58% in a comparison of human Vδ1 with a murine Vδ6 family member, and 66% in a comparison of human Vδ3 with murine Vδ5. Conservation extends to genomic organization as well, since murine Vδ5, like human Vδ3, lies 3' to the Cδ segment, in an inverted orientation (A. Korman, personal communication), whereas murine Vδ6, like human Vδ1, lies at some distance 5' to Cδ. This conservation strengthens the emerging close relationship between the global organizations of the human and murine TCR-α/δ loci.

The analysis of Jδ1 rearrangements in newborn thymus DNA supports the likelihood that there may be two or three additional Vδ segments utilized within this polyclonal population that are not represented within the clonal cell lines that we have examined to date. Since the rearrangements in question occur at low frequencies within the thymus, their characterization probably awaits the identification of clonal cell lines displaying these rearrangements. It is also possible that distinct and novel Vδ segments may be rearranged at a selective time in thymic development and/or in TCR-γ/δ cells populating distinct sites in the periphery and hence might not be represented in the polyclonal samples we have analyzed.

It is important to note that the vast majority of Jδ1 rearrangements detected in newborn thymocyte DNA must occur in cells that do not express functional TCR-γ/δ on their surfaces, since only 0.5–1.0% of the thymocytes in these samples stained with the mAb anti-TCR-δ1 (V. Groh, personal communication). Based upon the intensity of the rearrangements relative to control single copy examples in clonal cell lines, we assume that the fraction of thymocytes displaying Jδ1 rearrangements is much higher. This would argue that the detection of a limited number of Jδ1 rearrangements could not reflect selection for the expression of functional receptors, but rather must reflect specificity in the rearrangement process per se.

Current information indicates that the TCR-γ/δ V segment repertoires are both limited and of similar size in man and mouse. In man there are at most seven functional Vγ segments (in four families), and at least three functional Vδ segments. In
mouse there are six functional V\(_\gamma\) segments (in five families) and at least nine functional V\(\delta\) segments (in six families) (7, 22). These numbers are significantly lower than those estimated for TCR-\(\alpha/\beta\), which range from 30 to 100 for each V segment (2, 3). Based upon our limited panel analysis, it may be inappropriate to assume combinatorial association of V\(\gamma\) and V\(\delta\) segments, since five of five examples of TCR-\(\gamma/\delta\) cell lines using the V\(\delta\)2 segment also used the V\(\gamma\)2 segment. Whether this might reflect constraints imposed on chain pairing or on receptor specificity is unclear, but it nevertheless suggests that the total number of V\(\gamma\)-V\(\delta\) pairs may be limited. Such data could be interpreted to indicate that TCR-\(\gamma/\delta\) might interact with a discrete set of putative restricting elements or antigen-presenting molecules. The resolution of this issue clearly awaits the derivation and study of antigen-specific TCR-\(\gamma/\delta\) T cell lines and clones.

Summary

Previous studies of the human TCR-\(\delta\) gene identified a single commonly used V\(\delta\) segment, denoted V\(\delta\)1. To better understand the extent of the human TCR-\(\delta\) V gene repertoire, TCR-\(\delta\) transcripts and gene rearrangements were examined in a new panel of cloned human TCR-\(\gamma/\delta\) lymphocytes. Through this analysis we identified and determined the structures of two new V\(\delta\) segments, denoted V\(\delta\)2 and V\(\delta\)3. These V\(\delta\) segments are different from previously characterized V\(\alpha\) segments, supporting the notion that the human V\(\alpha\) and V\(\alpha\) repertoires are distinct. Examination of V\(\gamma\) gene segment usage in these cells reveals that the V\(\delta\)2 gene segment is used in conjunction with the V\(\gamma\)2 gene segment. Blot hybridization indicates that the V\(\delta\)2 gene segment lies between V\(\delta\)1 and D\(\delta\)-J\(\delta\)-C\(\delta\), and within 100 kb of the latter. Analysis of genomic clones indicates that the V\(\delta\)3 gene segment lies in an inverted orientation, \(\sim\)2 kb \(^{3}\prime\) of C\(\delta\). This implies that rearrangement of V\(\delta\)3 to D\(\delta\)-J\(\delta\)-C\(\delta\) occurs by inversion. Together with previous mapping studies, these results indicate that human V\(\delta\) segments are dispersed, rather than clustered, within the TCR-\(\alpha/\delta\) locus. The analysis of rearrangements in polyclonal thymocyte DNA suggests that there may be a limited number of additional V\(\delta\) gene segments yet to be characterized.

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Note added in proof: We have identified two additional T cell clones that apparently express a V\(\gamma\)2-V\(\delta\)2 receptor.

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References

1. Marrack, P., and J. Kappler. 1987. The T cell receptor. Science (Wash. DC). 238:1073.
2. Toyonaga, B., and T. W. Mak. 1987. Genes of the T-cell antigen receptor in normal and malignant T cells. Annu. Rev. Immunol. 5:585.
3. Kronenberg, M., G. Siu, L. E. Hood, and N. Shastri. 1986. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu. Rev. Immunol. 4:529.
4. Brenner, M. B., J. McLean, D. P. Dialynas, J. L. Strominger, J. S. Smith, F. L. Owen, J. G. Seidman, S. Ip, F. Rosen, and M. S. Krangel. 1986. Identification of a putative second T-cell receptor. Nature (Lond.). 322:145.

5. Bank, I., R. A. DePinho, M. B. Brenner, J. Cassimeris, F. W. Alt, and L. Chess. 1986. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. Nature (Lond.). 322:179.

6. Lew, A. M., D. M. Pardoll, W. L. Maloy, B. J. Fowlkes, A. Kruisbeek, S.-F. Cheng, R. N. Germain, J. A. Bluestone, R. H. Schwartz, and J. E. Coligan. 1986. Characterization of T-cell receptor gamma chain expression in a subset of murine thymocytes. Science (Wash. DC). 234:1401.

7. Brenner, M. B., J. L. Strominger, and M. S. Krangel. 1988. The γδ T-cell receptor. Adv. Immunol. 43:133.

8. Koning, F., G. Stingl, W. M. Yokoyama, H. Yamada, W. L. Maloy, E. Tschachler, E. M. Shevach, and J. E. Coligan. 1987. Identification of a T3-associated γδ T-cell receptor on Thy-1+ dendritic epidermal cell lines. Science (Wash. DC). 236:834.

9. Goodman, T., and L. Lefrançois. 1988. Expression of the γδ T-cell receptor on intestinal CD8+ intraepithelial lymphocytes. Nature (Lond.). 333:855.

10. Matis, L., R. Cron, and J. A. Bluestone. 1987. Major histocompatibility complex-linked specificity of γδ receptor-bearing T lymphocytes. Nature (Lond.). 330:262.

11. Forster, A., S. Huck, M. Ghanem, M.-P. Lefranc, and T. H. Rabbitts. 1987. New subgroups in the human T-cell rearranging Vγ gene locus. EMBO (Eur. Mol. Biol. Organ.) J. 6:1945.

12. Strauss, W. M., T. Quertermous, and J. G. Seidman. 1987. Measuring the human T-cell receptor γ-chain locus. Science (Wash. DC). 237:1237.

13. Huck, S., P. Dariavich, and M.-P. Lefranc. 1988. Variable region genes in the human T-cell rearranging (TRG) locus: Vγ J junction and homology with the mouse genes. EMBO (Eur. Mol. Biol. Organ.) J. 7:719.

14. Quertermous, T., W. M. Strauss, J. J. M. van Dongen, and J. G. Seidman. 1987. Human T-cell γ chain joining regions and T-cell development. J. Immunol. 138:2687.

15. Huck, S., and M.-P. Lefranc. 1987. Rearrangements to the JPl, JP and JP2 segments in the human T-cell rearranging gamma gene (TRγ) locus. FEBS (Fed. Eur. Biochem. Soc.) Lett. 224:291.

16. Hata, S., M. B. Brenner, and M. S. Krangel. 1987. Identification of putative human T-cell receptor δ complementary DNA clones. Science (Wash. DC). 238:687.

17. Loh, E. Y., L. L. Lanier, C. W. Turck, D. R. Littman, M. M. Davis, Y.-H. Chien, and A. Weiss. 1987. Identification and sequence of a fourth human T-cell antigen receptor chain. Nature (Lond.). 330:569.

18. Hata, S., K. Satyanarayana, P. Devlin, H. Band, J. McLean, J. L. Strominger, M. B. Brenner, and M. S. Krangel. 1988. Extensive junctional diversity of rearranged human T-cell receptor δ genes. Science (Wash. DC). 240:1541.

19. Boehm, T., L. Buluwela, D. Williams, L. White, and T. H. Rabbitts. 1988. A cluster of chromosome 11p13 translocations found via distinct D-D and D-D'J rearrangements of the human T-cell receptor δ chain gene. EMBO (Eur. Mol. Biol. Organ.) J. 7:2011.

20. Satyanarayana, K., S. Hata, P. Devlin, M. G. Roncarolo, J. E. De Vries, H. Spits, J. L. Strominger, and M. S. Krangel. 1988. Genomic organization of the human T-cell receptor α/δ locus. Proc. Natl. Acad. Sci. USA. In press.

21. Chien, Y.-H., M. Iwashima, D. A. Wettstein, K. B. Kaplan, J. F. Elliott, W. Born, and M. M. Davis. 1987. T-cell receptor δ gene rearrangements in early thymocytes. Nature (Lond.). 330:722.

22. Elliott, J. F., E. P. Rock, P. A. Patten, M. M. Davis, and Y.-H. Chien. 1988. The adult T-cell receptor δ-chain is diverse and distinct from that of fetal thymocytes. Nature (Lond.). 331:627.
23. Takihara, Y., D. Tkachuk, E. Michalopoulos, E. Champagne, J. Reimann, M. Minden, and T. W. Mak. 1988. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell β-chain locus. *Proc. Natl. Acad. Sci. USA.* 85:6097.

24. Boehm, T., R. Baer, I. Lavenir, A. Förster, J. J. Waters, E. Nacheva, and T. H. Rabbitts. 1988. The mechanism of chromosomal translocation t(11;14) involving the T-cell receptor Cδ locus on human chromosome 14q11 and a transcribed region of chromosome 11p15. *EMBO (Eur. Mol. Biol. Organ.)* J. 7:385.

25. Isobe, M., G. Russo, F. G. Haluska, and C. M. Croce. 1988. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. *Proc. Natl. Acad. Sci. USA.* 85:3933.

26. Griesser, H., E. Champagne, D. Tkachuk, Y. Takihara, M. Lalande, E. Baillie, M. Minden, and T. W. Mak. 1988. The human T cell receptor α-δ locus: a physical map of the variable, joining and constant region genes. *Eur. J. Immunol.* 18:641.

27. Hochstenbach, F., C. Parker, J. McLean, V. Gieselmann, H. Band, I. Bank, L. Chess, H. Spits, J. L. Strominger, J. G. Seidman, and M. B. Brenner. 1988. Characterization of a third form of the human T cell receptor γδ. *J. Exp. Med.* 168:761.

28. Alarcon, B., J. De Vries, C. Pettey, A. Boylston, H. Yssel, C. Terhorst, and H. Spits. 1987. The T-cell receptor γ chain-CD3 complex: Implication in the cytotoxic activity of a CD3+CD4+CD8- human natural killer clone. *Proc. Natl. Acad. Sci. USA.* 84:3861.

29. Yssel, H., J. E. De Vries, M. Koken, W. V. Van Blitterswijk, and H. Spits. 1984. A serum free medium for the generation and propagation of functional human cytotoxic and helper T cell clones. *J. Immunol. Methods.* 72:219.

30. Krangel, M. S., H. Band, S. Hata, J. McLean, and M. B. Brenner. 1987. Structurally divergent T cell receptor γ proteins encoded by distinct Cγ genes. *Science (Wash. DC).* 237:64.

31. Feinberg, A., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6.

32. Auffray, C., and F. Rougeon. 1980. Purification of mouse immunoglobulin heavy chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem.* 107:303.

33. Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacey, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from procaryotes and eukaryotes. *Cell.* 16:777.

34. Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker. 1977. RNA molecular weight determination by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry.* 16:4743.

35. Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Construction and screening cDNA libraries in λgt10 and λgt11. In DNA Cloning, A Practical Approach, Vol I. D. M. Glover, editor. IRL Press Limited, Oxford. 49-78.

36. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463.

37. Biggin, M. D., T. J. Gibson, and G. F. Hong. 1983. Buffer gradient gels and [35S] label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA.* 80:3963.

38. Tabor, S., and C. C. Richardson. 1987. DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. *Proc. Natl. Acad. Sci. USA.* 84:4767.

39. Chen, E. Y., and P. H. Seeberg. 1985. Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA (NY).* 4:165.

40. Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene.* 28:351.

41. Lai, E., P. Concannon, and L. Hood. 1988. Conserved organization of the human and murine T-cell receptor δ-gene families. *Nature (Lond.)* 331:543.
42. Pelkonen, J., A. Traunecker, and K. Karjalainen. 1987. A new mouse TCR Vγ gene that shows remarkable evolutionary conservation. *EMBO (Eur. Mol. Biol. Organ.)* J. 6:1941.

43. Treibel, F., F. Faure, M. Graziana, S. Jitsukawa, M.-P. Lefranc, and T. Hercend. 1988. A unique VJ-C rearranged gene encodes a γ protein expressed on the majority of CD3⁺ T cell receptor-α/β⁺ circulating lymphocytes. *J. Exp. Med.* 167:694.

44. Malissen, M., C. McCoy, D. Blanc, J. Trucy, C. Devaux, A.-M. Schmitt-Verhulst, F. Fitch, L. Hood, and B. Malissen. 1986. Direct evidence for chromosomal inversion during T-cell receptor β gene rearrangements. *Nature (Lond.)* 319:28.

45. Alt, F., T. K. Blackwell, and G. D. Yancopoulos. 1987. Development of the primary antibody repertoire. *Science (Wash. DC)*. 238:1079.

46. Klein, M. H., P. Concannon, M. Everett, L. D. H. Kim, T. Hunkapiller, and L. Hood. 1987. Diversity and structure of human T-cell receptor α-chain variable region genes. *Proc. Natl. Acad. Sci. USA.* 84:6884.