proteins. Mitochondrial transformation, however, should provide a generally applicable technique for mutational studies of mitochondria-encoded proteins as well as permit many studies of the function and evolution of mtDNA sequences that could not be done with the techniques previously available.

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Extensive Junctional Diversity of Rearranged Human T Cell Receptor δ Genes

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The human T cell receptor δ (TCR δ) gene encodes one component of the TCR γδ-CD3 complex found on subsets of peripheral blood and thymic T cells. Human TCR δ diversity was estimated by characterizing rearrangements in TCR γδ cell lines and determining the structures of complementary DNA clones representing functional and nonfunctional transcripts in these cell lines. One Vδ segment and one Jδ segment were identified in all functional transcripts, although a distinct Jδ segment was identified in a truncated transcript. Further, one Dδ element was identified, and evidence for the use of an additional Dδ element was obtained. Thus human TCR δ genes appear to use a limited number of germline elements. However, the apparent use of two Dδ elements in tandem coupled with imprecise joining and extensive incorporation of N nucleotides generates unprecedented variability in the junctional region.

TWO DISTINCT CD3-ASSOCIATED T cell receptor (TCR) structures have been identified on the surface of mature, peripheral blood T lymphocytes. The first receptor structure, TCR αβ (*1*), is expressed on most peripheral blood T lymphocytes. These cells, which are typically either $CD4^+CD8^-$ or $CD4^-CD8^+$, are involved in antigen-specific, major histocompatibility complex–restricted recognition and display either helper or cytotoxic activity (*2*). The second receptor structure, TCR γδ (*3, 4*), is expressed on a much smaller subset of peripheral T lymphocytes (0.5% to 12%), as well as on thymocytes and other cells (*5–8*). These cells are either $CD4^-CD8^-$ or $CD4^-CD8^+$ and have cytotoxic activity, but the nature of the antigens and restricting elements recognized by these lymphocytes is unknown.

All four receptor chains are encoded by immunoglobulin-like gene segments that rearrange to form a functional gene during T cell differentiation (*9–14*). The human TCR α locus is composed of approximately 50 variable (V) segments, 50 joining (J) segments, and a single constant (C) segment, whereas the human TCR β locus is composed of approximately 70 V segments, two diversity (D) segments, 13 J segments, and two C segments (*15*). Germline diversity, combinatorial and imprecise V-(D)-J joining, the insertion of template-independent N-region junctional nucleotides, and combinatorial association of α and β polypeptides generate an extensive TCR αβ repertoire (*15, 16*).

Characterization of the human TCR γ locus (*17, 18*) and the isolation of cDNA clones encoding human TCR δ (*11–13*) allow a similar assessment of the human TCR γδ repertoire. In contrast to the TCR α and β loci, the human TCR γ locus is composed of only six or seven functional V segments, five J segments, and two C segments (*17, 18*). Furthermore, the two most commonly used J segments are identical in amino acid sequence. Thus germline diversity is limited, and imprecise joining and N nucleotides play important roles in generating variability in TCR γ (*17*).

In order to investigate the variability of TCR δ, we obtained and analyzed cDNA clones representing seven distinct TCR δ transcripts from four TCR γδ cell lines. These clones display use of a single TCR δ V segment and a predominant TCR δ J segment, suggesting limited germline diversity. However they show unprecedented variability in the V-J junctional region.

We used a V-specific probe derived from the IDP2 TCR δ cDNA clone 0-240/47 (*11*) to examine TCR δ rearrangements in Xba I digests of genomic DNA from five different TCR γδ cell lines (Fig. 1A). This probe detects a single germline fragment of 7.5 kb that is rearranged to a 6.2-kb fragment on one chromosome in Molt-13 (*12, 19*), IDP2 (*3*), PEER (*5–7*), PBL L1 (*6*) (Fig. 1A) and PBL C1 (*6*). We also subcloned a genomic fragment encoding the Jδ segment present in the IDP2 TCR δ cDNA clone (Jδ1) (*20*). This probe detects a single 1.7-kb germline fragment that is rearranged on both chromosomes in all TCR γδ cell lines examined (Fig. 1B). One rearranged band, of 6.2 kb, corresponds exactly to the rearranged fragment detected by the V-specific probe, indicating the rearrangement

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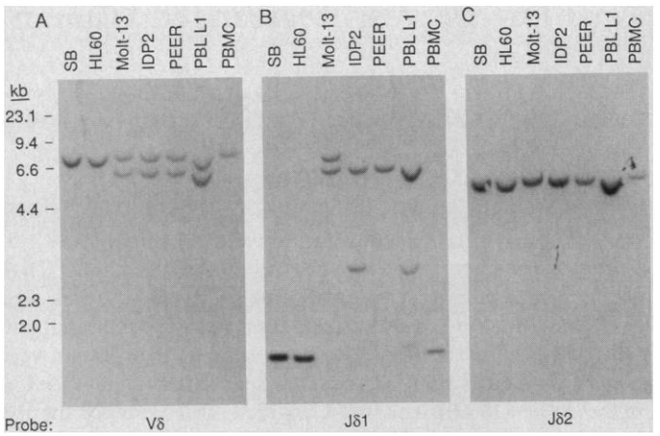

Fig. 1. TCR δ gene rearrangements in TCR γδ cell lines. High molecular weight genomic DNA was digested with Xba I, electrophoresed through 0.7% agarose, and transferred to Hybond membranes (Amersham). Filters were probed with appropriate DNA fragments labeled by hexamer priming (*26*), and were washed with 1× SSC and 0.5% SDS at 23°C and then with 0.1× SSC and 0.1% SDS at 60°C. SB (B cell line), HL60 (myeloid cell line), and PBMC (fresh peripheral blood mononuclear cells) serve as germline controls. Molt-13 and PEER are TCR γδ leukemic cell lines, IDP2 is a long-term IL-2–dependent TCR γδ cell line, and PBL L1 is an oligoclonal IL-2–dependent TCR γδ cell line. Ethidium bromide staining revealed that PBL L1 DNA was overloaded, and PBMC DNA underloaded, relative to the other samples. A phage λ Hind III digest provided molecular weight markers. (**A**) Vδ probe, a 300-bp Eco RI–Sca I fragment of IDP2 TCR δ clone 0-240/47 (*11*). (**B**) Jδ1 probe, a genomic 1.7-kb Xba I fragment carrying the Jδ1 segment (*20*). (**C**) Jδ2 probe, a genomic 1.1-kb Xba I–Bam HI fragment carrying the Jδ2 segment (*20*). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

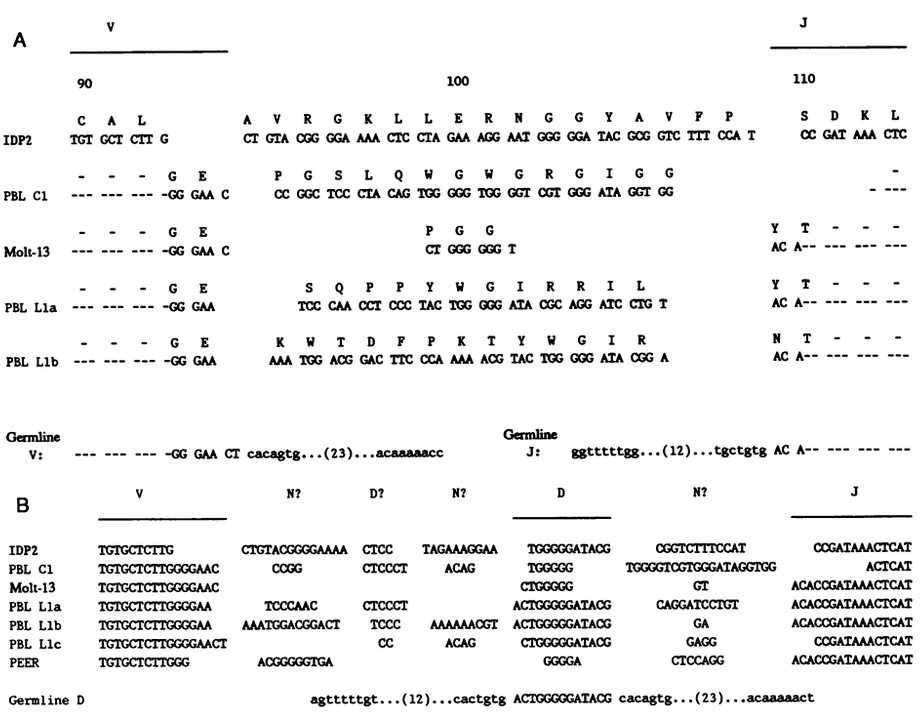

Fig. 2. Structure of human TCR δ V-J junctional regions. (**A**) Nucleotide and deduced amino acid sequences of the junctional regions of cDNA clones representing functional TCR δ transcripts in PBL C1, Molt-13, and PBL L1 (PBL L1a and PBL L1b) are presented and compared with those of IDP2. Lambda gt10 cDNA libraries (*19, 27*) were screened with probe IDP2 0-240 (*11*) labeled by nick-translation, the 5′ Eco RI fragments (extending from the Eco RI linker at the 5′ end to the natural Eco RI site 75 bp into the C segment) were subcloned, and their sequences were determined on both strands by the dideoxy chain termination method (*28*). The sequences proved identical to the V, J, and C sequences of IDP2 TCR δ, except in the junctional region. Only the junctional region is shown. Sequences are aligned in the V and J segments with (–) denoting identities. The boundaries of the germline V and J segments (*20*), including heptamer and nonamer recombination signals, are presented. Junctional sequences are not aligned. Codon numbering is for IDP2 TCR δ (*11*). (**B**) Nucleotide sequences presented in (A) are compared with those of a TCR δ cDNA clone representing an out-of-frame transcript in PBL L1 (PBL L1c) (PBL L1a, b, and c were found in ratios of 8:4:1) and that reported for PEER TCR δ (*12*). Sequences are aligned to emphasize segments shared by the majority of the clones. The sequence of one germline D element (*20*), including heptamer and nonamer recombination signals, is presented. Assignments of other potential N and D nucleotides are based on homologies and should be considered tentative.

of a common V segment to a common J segment in Molt-13, IDP2, PEER, PBL L1 (Fig. 1B), and PBL C1. The Jδ1 probe also detects a second rearranged band of 2.7 kb that is shared by IDP2, PBL L1, and PBL C1. By contrast, Molt-13 displays a distinct rearranged fragment of 7.2 kb detected by the Jδ1 probe, whereas PEER displays no detectable second rearrangement and has presumably deleted Jδ1 on this chromosome. The polyclonal TCR γδ cell line PBL L1 displays an additional minor Jδ1 rearrangement of 1.8 kb. It is likely that some of the rearrangements detected by the Jδ1 probe reflect partial (D-J and D-D-J) joins on one chromosome.

In order to investigate in more detail the functionally rearranged TCR δ genes in these cells, we isolated and characterized cDNA clones encoding the expressed TCR δ polypeptides. Nucleotide sequence analysis identified one functional transcript in TCR γδ peripheral blood clone PBL C1, one functional transcript in leukemic TCR γδ cell line Molt-13, and two functional transcripts in the oligoclonal TCR γδ peripheral blood cell line PBL L1 (PBL L1a and PBL L1b) (Fig. 2A). The V, J, and C segments of these clones are identical to those of the previously characterized IDP2 (*11*) and PEER (*12*) TCR δ cDNA clones. However, by comparison to the structures of the germline V and J segments (*20*) (Fig. 2A), the 3′ boundaries of the V segment and the 5′ boundaries of the J segment vary. Strikingly, the regions between the 3′ end of the V segment and 5′ end of the J segment range from as few as 9 to as many as 51 nucleotides. The junctional segments are therefore longer, and the variability among sequences more extensive, than is typically observed for other rearranged TCR genes (*15, 16*).

An alignment of the junctional sequences of these cDNA clones as well as of a cDNA clone representing a nonfunctional TCR δ transcript in PBL L1 (PBL L1c) and a cDNA clone representing a functional TCR δ transcript in PEER (*12*) cells is presented in Fig. 2B. Although there is wide variation in the junctional sequences, two discrete blocks of nucleotide homology are apparent. Segments of the sequences CTCCCT and ACTGGGGGATACG appear in most cDNA clones. Because these sequences are shared among the clones, they are likely to be germline encoded and represent at least portions of two distinct Dδ elements. The germline structure of the 3′ Dδ element, flanked by conserved heptamer and nonamer recombination signals (*21*), has been determined (*20*) (Fig. 2B). This D element conforms to the known properties of TCRβ D elements in that it is G-rich, is joined in an

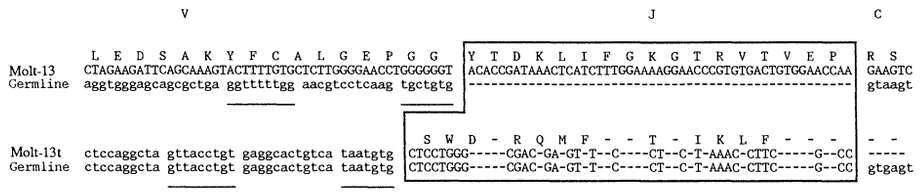

Fig. 3. Structure of the Molt-13t TCR δ cDNA clone. The nucleotide and deduced amino acid sequences of the Molt-13t (truncated) cDNA are aligned with the Molt-13 (functional) cDNA. These sequences are compared with the sequences of the germline J segments (*20*), and the J coding segments are boxed. Identities in the J and C segments are noted (–). Heptamer- and nonamer-like elements are underlined. The underlined nonamer-like element flanking the Molt-13t J segment (Jδ2) diverges significantly from the consensus nonamer.

imprecise fashion, and is translatable in all three reading frames (*22*). Two reading frames are actually used among the five functionally rearranged TCR δ genes analyzed here (Fig. 2A). The use of a 5′ Dδ element is inferred from the sequence conservation noted. However, this remains to be confirmed by genomic analysis.

The sequences interspersed between the boundaries of the V, D, and J elements display little identity with each other. Some of these nucleotides may derive from the putative 5′ D element or perhaps from additional D elements. However, most of them almost certainly do not, and therefore likely represent the incorporation of template-independent N region nucleotides, a process thought to be mediated by the activity of terminal transferase (*23*). The tentative assignments in Fig. 2B suggest extensive N nucleotide incorporation at multiple sites in the junctional region.

We also identified a cDNA clone representing a second transcript from the Molt-13 cell line (Molt-13t). This cDNA has C sequences identical to those of other cDNA clones but lacks V sequences and thus appears to represent a truncated nonfunctional transcript. Notably, the region immediately 5′ of the C segment is related to, although distinct from, the J segment (Jδ1) found in the functional cDNA clones (Fig. 3). The two J sequences display only 53% identity (26/49) at the nucleotide level and 44% identity (7/16) at the amino acid level. Further, the Molt-13t J segment (Jδ2) is 8 bp longer. Despite these differences, Jδ2 differs from the core of most highly conserved TCR J residues (*15*) (FG-G[T/S]-L-V; FG-GT-L-V-P for Jα) in only a single position, although this substitution (I for T) is unusual. At its 5′ boundary the Molt-13t Jδ2 sequence is bounded by the consensus heptamer-like sequence TAATGTG. This is separated by 12 bp from the sequence GTTACCTGT, which displays only limited similarity to the consensus nonamer recombination signal GGTTTTTGT (*21*). A genomic fragment encoding the Jδ2 segment detects a single germline fragment of 5.1 kb that is unrearranged in all TCR γδ cell lines examined (Fig. 1C). Since Molt-13 has undergone two different Jδ1 rearrangements, and since the region upstream of the germline Jδ2 segment displays a sequence identical to that of Molt-13t (Fig. 3) (*20*), the Molt-13t cDNA clone must represent a transcript initiating upstream from Jδ2 on a chromosome that has undergone rearrangement at Jδ1. The lack of rearrangement to Jδ2 in this panel of TCR γδ cells may reflect the divergent nonamer-like recombination signal flanking this element.

Heptamer- and nonamer-like elements separated by 23 bp flank the 3′ ends of the Vδ segment and the identified Dδ segment, whereas similar elements separated by 12 bp flank the 5′ ends of the Dδ and Jδ segments. On the basis of the so-called 12/23 rule (*21*) and the apparent incorporation of two Dδ elements in tandem into the junctional region, the putative 5′ Dδ segment would be predicted to be flanked by recombination signals spaced like those of the 3′ Dδ element. This arrangement of recombination signals would allow for the incorporation of 0, 1, 2, or perhaps more D elements between the V and J segments, further increasing the potential for diversity at the junction. Indeed, the alignments in Fig. 2B suggest that rearranged TCR δ genes in peripheral blood cell lines (IDP2, PBL C1, and PBL L1) incorporate both Dδ elements, whereas those in the leukemic cell lines (PEER and Molt-13) incorporate only the 3′ Dδ element.

These results are consistent with data in the murine system. Chien *et al.* (*24*) identified two Jδ segments highly homologous to those identified here (44/51 and 53/59 matches at the nucleotide level to Jδ1 and Jδ2, respectively). Further, they have identified two Dδ elements that may be incorporated in tandem into the junctional region. Murine Dδ2 matches the identified human Dδ element at 10 of 13 nucleotides that can be compared, although the murine segment is 3 bp longer. Perhaps in analogy with the apparent difference in human Dδ element use between the leukemic and peripheral blood cell lines characterized in this study, the murine Dδ2 element is selectively incorporated into rearranged murine fetal thymocyte TCR δ genes (*24*). Multiple murine Vδ segments have already been defined (*10, 24*).

Our initial studies indicate that germline human TCR δ diversity is quite limited. That only a single Vδ and Jδ segment are found in all functionally rearranged genes characterized here is unlikely to be due to the selective expansion of certain clones of TCR γδ lymphocytes as a result of a common cell source or particular culture conditions, since IDP2, PBL C1, and PBL L1 are peripheral blood cell lines whereas Molt-13 and PEER are leukemic cell lines and all are derived from different individuals. Because our sampling of TCR δ transcripts is admittedly small, any conclusions must be tentative. For example, preliminary characterization of a distinct panel of TCR γδ cell lines indicates the use of two additional Vδ segments (*25*). However, the repertoire of germline TCR δ V and J segments appears to be much closer to that of TCR γ than to that of TCR α or TCR β and in fact might be smaller than that of TCR γ.

In contrast to the limited germline diversity indicated by these sequences, the level of TCR δ junctional diversity is unprecedented. The apparent incorporation of multiple D elements in tandem distinguishes TCR δ from both the TCR β and immunoglobulin heavy chain genes. This feature, in conjunction with imprecise joining, multiple sites of insertion and extensive incorporation of N nucleotides, and translation in multiple reading frames, generates tremendous diversity in a small segment of the polypeptide. This suggests that the TCR δ variable domain may form a relatively conserved framework, with a single hypervariable region at the V-D-J junction. Junctional variability appears to be an important factor in TCR γ diversity as well. The contrasts between these patterns of diversity and those of TCR α and TCR β should reflect differences in the types of antigens and restricting elements recognized by the αβ and γδ TCRs. High junctional diversity might imply that TCR γδ could indeed recognize a diverse universe of antigens. However, the limited germline repertoire might indicate that such antigens would be presented on a restricting element displaying limited polymorphism. These studies may therefore serve as important clues to functional differences between TCR αβ and TCR γδ lymphocytes.

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Inhibition of Cellular Proliferation by Antisense Oligodeoxynucleotides to PCNA Cyclin

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The proliferating cell nuclear antigen (PCNA or cyclin) is a nuclear protein recently identified as a cofactor of DNA polymerase δ. When exponentially growing Balb/c3T3 cells are exposed to antisense oligodeoxynucleotides to PCNA, both DNA synthesis and mitosis are completely suppressed. A corresponding sense oligodeoxynucleotide has no inhibitory effects. These experiments indicate that PCNA (cyclin) is important in cellular DNA synthesis and in cell cycle progression.

THE PCNA WAS ORIGINALLY described as a nuclear antigen detectable by immunofluorescence, and its appearance has been correlated to the proliferative state of the cell (*1*). A similar nuclear protein described by Bravo and co-workers was called cyclin (*2*). PCNA and cyclin were shown to be identical (*3*) and were identified as an auxiliary protein for DNA polymerase δ (*4*), necessary for the replication of simian virus 40 DNA (*5*). A partial-length cDNA clone of human PCNA was isolated and sequenced by Almendral *et al.* (*6*), and a full-length human cDNA clone was isolated (*7*) from an Okayama-Berg library (*8*).

We have constructed oligodeoxynucleotides complementary to either the sense or the antisense strand of the first 36 nucleotides of the PCNA sequence, beginning from the AUG codon.

The relevant PCNA sequence for these experiments includes the last 48 nucleotides that precede the AUG codon, as well as the first 72 nucleotides of the coding sequence (Fig. 1). Four 18-mer oligodeoxynucleotides were synthesized (Fig. 1). The first 18-mer (cyclin 1) extends from nucleotide 4 to nucleotide 21, inclusive. Cyclin 3 extends from nucleotide 22 to nucleotide 39 inclusive. Cyclin 2 and cyclin 4 are the antisense complementary strands of cyclin 1 and cyclin 3, respectively. The oligonucleotides were made on an Applied Biosystems 380B DNA synthesizer by means of β-cyanoethyl phosphoramidite chemistry. The overall yield of 18-mer was 80 to 85%, and the lyophilized product was redissolved in culture medium.

In these experiments we used Balb/c3T3 cells, exponentially growing on cover slips in the presence of Dulbecco's medium supplemented by 10% fetal bovine serum. The oligodeoxynucleotides [a mixture of cyclins 1 and 3 (sense) or cyclins 2 and 4 (antisense)] were added at time 0 to cultures without serum, as follows. The growth medium was removed, and the cultures were incubated in Dulbecco's medium (without serum) to which oligodeoxynucleotides (30 μ*M*) were added. After incubation for 30 min with oligodeoxynucleotides, serum was added to a final concentration of 10% without changing the medium. The cells were incubated for up to 24 hours, labeled with [^{3}H]thymidine (6.7 μCi/ml) for 30 min, and then fixed in methanol. Addition of antisense oligodeoxynucleotides markedly inhibited the proliferation of Balb/c3T3 cells. Cultures incubated with cyclins 2 and 4 remained sparse, whereas the corresponding controls and the cells exposed to sense oligodeoxynucleotides grew to 70% confluence, as evaluated by visual observation.

The labeling indices of these cultures, as measured after autoradiography, are shown in Fig. 2. We show two experiments in which the labeling index of the controls (no treatment) is slightly different, but these experiments have been repeated seven times and the results are reproducible. With this protocol, the labeling index of exponentially growing control cells should remain roughly constant, with time, as was observed (Fig. 2). Cells exposed to antisense oligodeoxynucleotides had a markedly decreased labeling index. The decrease began at 6 hours, and by 16 hours no labeled cells could be detected. In cells exposed to sense oligodeoxynucleotides the labeling index remained constant, at about the same level of their controls. Cells exposed to the sense oligodeoxynucleotides had a decrease in the number of autoradiographic grains per nucleus (20 to 30; the grains in the control could not be counted), although the percentage of labeled cells remained the same as in controls. The decrease is due, we believe, to the high amounts of sense and antisense oligodeoxynucleotides added to the cultures. It is conceivable that the oligodeoxynucleotides are

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