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Distinct and Opposite Activities of Human Terminal Deoxynucleotidyltransferase Splice Variants

To-Ha Thai2*† and John F. Kearney3*†

Evidence for potential human TdT (hTdT) isoforms derived from hTdT genomic sequences led us to identify the short isoform (hTdTS), as well as mature long transcripts containing exon XII (hTdTL1) and another including exon VII (hTdTL2) in lymphoid cells. Normal B and T lymphocytes express exclusively hTdTS and hTdTL2, whereas hTdTL1 expression appears to be restricted to transformed lymphoid cell lines. In in vitro recombination and primer assays, both long isoforms were shown to have 3′→5′ exonuclease activity. Overexpression of hTdTS or hTdTL2 greatly reduced the efficiency of recombination, which was reverted to normal levels by the simultaneous expression of both enzymes. Therefore, alternative splicing may prevent the adverse effects of unchecked elongation or diminution of coding ends during V(D)J recombination, thus affecting the survival of a B or T cell precursor during receptor gene rearrangements. Finally, the newly discovered hTdTS isoforms should be considered in future screening of human leukemias. The Journal of Immunology, 2004, 173: 4009–4019.

To date, Ag receptors on B (Igs) and T cells (TCRs) of all higher vertebrate taxa examined, including mammals (1), birds (2), reptiles (3), amphibians (4), teleosts (5), and cartilaginous fish (6) are generated through V(D)J recombination (7). V(D)J recombination, which occurs at specific stages of B and T cell development (8), is a mechanism by which the Ag-binding region or the CDR3 of Ig H and L chains and TCRs is generated through combinatorial rearrangements of the V, D, and J gene segments. The V(D)J recombination reaction is initiated by lymphoid-specific MLR RAG-1 and RAG-2 proteins and is completed by ubiquitously expressed nonhomologous end-joining and double-stranded break repair proteins, including Ku70, Ku86, the catalytic subunit of DNA-dependent protein kinase, XRCC4 and DNA ligase IV, and Artemis (9–15). Although these proteins are required for the production of Igs and TCRs, most of the junctional diversity was shown to be contributed by nontemplated (N)5 addition and nucleotide deletion at V(D)J joins (16, 17).

TdT is a nuclear enzyme belonging to the X family of polymerases. The sequence of all members of this family contains the conserved X signature domain that mediates nucleotide interaction. The short isoform of mouse TdT (mTdTS; 509 aa long) is responsible for N addition at coding joins (CJs) (18–22). We showed that the long isoform of mTdT (mTdTL; 529 aa long) has 3′→5′ exonuclease (exo) activity capable of catalyzing the deletion of nucleotides from coding ends with either 3′ or 5′ extensions, but not from blunt signal ends (23). Recently, Artemis has been shown to have both endonuclease and exo activities, and the suggestion that there may be redundant exons has been confirmed by the residual exo activity observed in an Artemis-deficient ES cell line (12–15).

In vertebrates where V(D)J recombination occurs, the TdT gene (Dmtt) is conserved (24–27). Moreover, N addition and nucleotide deletion from coding ends contribute to the majority of junctional diversity in Igs and TCRs of these respective species; however, long isoforms of TdT (TdTLs) have only been identified in mice and cattle (28). The failure to identify TdT isoforms in other species may be due to their lack of sequence homology to one another.

Studies thus far, show that the human Dmtt is expressed in fetal life, and is generally restricted to T and B cell progenitors in the thymus and the bone marrow, although there may be exceptions (29–31). In addition to its role in diversifying Ag receptors, human TdT (hTdT) has been used as a marker in the diagnosis of certain human leukemias (32–34). The consequences of hTdT overexpression in malignancies have yet to be determined.

To determine whether the long isoform of hTdT (hTdTL) exists, and to gain insight into its functions during V(D)J recombination and leukemogenesis, the genomic sequence of the hTdT gene, located on chromosome 10, was examined. This search revealed that, in human, as in cattle, three potential TdT isoforms exist: the short isoform (hTdTS), and two long isoforms 1 (hTdTL1) and 2 (hTdTL2). The deduced amino acid sequences of hTdTL1 and hTdTL2 are highly homologous to those of the long isoform of bovine TdTL1 (bTdTL1) and bTdTL2. The generation of expression cDNA clones of hTdTL1 and hTdTL2 in vitro permitted us to show that hTdTL1 and hTdTL2 can localize to the nucleus, and like hTdTS, hTdTL2 transcripts are detected during B and T cell development. hTdTL1 transcripts were not detectable, yet both hTdTL1 and hTdTL2 have 3′→5′ exo activity in recombination assays. However, hTdTL1 could be detected in transformed lymphoid cells. Moreover, in a standard recombination assay, the overexpression of hTdTS or hTdTL2 adversely affects the recombination efficiency, which is rescued by the concomitant expression of the two enzymes in the same cell.

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4Abbreviations used in this paper: N, nontemplated; P, palindromic; m, mouse; h, human; b, bovine; S, short isoform; L, long isoform; exo, exonuclease; BRCT, BRCA-1 C-terminal; s, surface; DN, double-negative; DP, double-positive; CJ, coding join, CAM, chloramphenicol acetyltransferase.
Materials and Methods

Human samples and Abs

The Birth Defects Research Laboratory at University of Washington (Seattle, WA) provided human thymi, and University of Alabama at Birmingham Cancer Center Tissue Procurement program provided human bone marrows. All Abs used for FACS were purchased from BD Pharmingen (San Diego, CA).

RT-PCR

Total RNA from sorted B cells was prepared with Tri-Reagent (Molecular Research Center, Cincinnati, OH), treated with DNase, and reverse transcribed with oligo(dT) primers. TdT, tdTL1, or tdtL2 sequences were amplified by cDNA with the following primers: forward primers, 5'-CCGAAGACTCCACCAATTGCTG-3' and 5'-GCTGGTTAAAGAGCCTGTCG-3' (within the L1 insert); and hTdTL2 reverse primer (R3) 5'-GACCGCCAGCCGATGACG-3' (within the L2 insert). Conditions were as described (23). PCR conditions were 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The human cell line EU12 was a gift from H. Findley (Emory University School of Medicine, Atlanta, GA). The cell lines 697 and nalm 16 were purchased from American Type Culture Collection (Manassas, VA).

Generation of hTdTS, hTdTL1, and hTdTL2 cDNAs

cDNAs were generated by PCR insertion mutagenesis with the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) with modifications. The L1 or the L2 insert sequence was inserted into an hTdTS cDNA obtained from M. Ehrenstein (Division of Medicine, Royal Free and University College, London, U.K.) by PCR with the following primers: L1 forward, 5'-TGCCATATTTCTGGTTGTTCAGAG ATATTCTCAAGCCAGAAAAG-3'; L1 reverse, 5'-CTGGAAACATACACTAAGAAATATGCTGACCTGTGCTGATA AAGG-3'; L2 forward, 5'-CTTAAAAGTCATTGTTGCTATGGGCTGGTCGGGTCGTGGATTTC TGTTATATGAAGACCTTG-3'; and L2 reverse, 5'-CATAGCGCCAAATGACTTITAGGAACCAATTACCTATGTCCGGTCTGGTAATTTC TC-3'. PCR conditions were identical with those described for RT-PCR above. Resulting cDNAs were analyzed to verify the integrity of their sequence.

Immunoblots

Protein immunoblots were performed by standard methods. hTdTS isoforms were probed with a commercial rabbit polyclonal Ab against bTdT (Sigma-Aldrich, St. Louis, MO) and then detected with an HRP-conjugated goat anti-rabbit IgG Ab (Molecular Probes, Eugene, OR). F-actin (mouse mAb; Piscataway, NJ). DMRB microscope.

Immunohistochemistry

Transfected cells recovered from recombinant assays were stained as follows. Briefly, cyt centrifuge smears of transfected cells were dried, fixed in absolute methanol, stained for TdT expression with the same Ab used for immunoblots, and then developed with an Alexa 488-coupled goat anti-rabbit IgG Ab (Molecular Probes, Eugene, OR). F-actin filaments were stained with phallolidin conjugated to Alexa Fluor 546 at 5–10 U (Molecular Probes). Fluorescent images were captured with a Leica/Leitz (Deerfield, IL) DMRB microscope.

Primer modification assay

Purified primers (Invitrogen Life Technologies, Carlsbad, CA) were labeled with [γ-32P]ATP at the 5' end with T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Labeled primers were then purified with Bio-Spin 6 chromatography columns (Bio-Rad, Hercules, CA). To generate dsDNA, labeled primers were annealed with complementary strands. Primers (6 pmol) were incubated with purified TdT or TdTL (250 nM each, dialyzed against Tris-acetate buffer at pH 7.2 and 10% glycerol) in reaction buffer (0.2 M potassium cacodylate at pH 7.2, 4 mM MgSO4, 0.1 mM DTT, 100 μM b-TdTp, 10 μM ZnSO4, and 50 μM dNTPs) for 30–90 min at 37°C. The reaction was terminated by the addition of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). Samples were resolved on denaturing acrylamide gels and autoradiograms were obtained with Kodak (Rochester, NY) x-ray films.

Protein expression

To express N-terminal GST-tagged fusion proteins, hTdTS, hTdTL1, or hTdTL2 cDNA was cloned into the pGEX-4T-1 vector (Amerham Pharmacia Biotech). Expression was induced with 0.1 mM isopropyl β-D-thiogalactoside (Roche, Indianapolis, IN) for 3 h at 25–30°C. Fusion proteins were purified with glutathione Sepharose 4B (Amerham Pharmacia Biotech). For mammalian expression, hTdT, hTdTL1, or TdTl2 cDNA was subcloned into pcDNA 1.1/Amp vector (Invitrogen Life Technologies).

Recombination assays

Assays were done as described (23). Briefly, cells were transfected with Fugene 6 Transfection Reagent, according to the manufacturer's suggestions (Roche). Each subconfluent T-25 flask (~106 cells) was cotransfected with 1.5 μg of recombination substrate, 1.5 μg of truncated (core) RAG-1, 2.1 μg of truncated (core) RAG-2, and 1–2 μg of TdTS- or TdTTL-pcDNA 1.1. After 48 h, DNA was recovered with the Hirt method (23). PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA) instead of treating with Esol and alkaline phosphatase, and purified products were sequenced with primer DR09, which primed the coding ends.

GenBank numbers

GenBank accession numbers are as follows: mTdTL, accession no. AF316014; hTdTL1, accession no. A23595; and human chromosome 10, accession no. 10834424.

Results

Identification of three TdT isoforms in human

We have recently shown that mTdTL has 3→5' exo activity. However, hTdTL isoforms have only been identified in mice and cattle, although nucleotide deletion during receptor gene rearrangements is observed in the many vertebrates in which V(D)J recombination has to be studied. To resolve this issue, we examined the hTdTL genomic sequence to identify hTdTL isoforms. An search of the National Center for Biotechnology Information (NCBI) database revealed that, like that of bovine, the human Dntt has two additional exons. The first extra exon is 27 nt long, whereas the second is 51 nt long (Fig. 1A). The first and second extra exons could potentially encode 9-aa (L1) and 17-aa (L2) peptide fragments, respectively (Fig. 1B). An examination of the mtDnt genomic sequence, obtained through the Celera database, also revealed the presence of an additional exon, which could potentially encode a second mtDnt (mtTdTL2), the transcript of which was detected in the mouse cell line HTX-1 (T.-H. Thai and J. F. Kearney, manuscript in preparation). The deduced amino acid sequences of hL1 and hL2 inserts were highly homologous to those of bovine: hL1/hL1 had 67% aa identity, and hL2/hL2 had 77% aa identity (Fig. 1B). In addition, human and bovine L1 inserts were half the size of mL1. Moreover, all three extra core motifs were found in the hTdTL sequence, and Asp residues shown to confer the majority of exo activity to mTdTL (23) were also conserved (Fig. 1C; amino acids important for activity are in green). Based on these results and those of others (35), the genomic organization of hTdTL isoforms. The Dntt organization of these two species was considered cryptic. Like other DNA-modifying proteins such as BRCA-1, all three hTdTL isoforms contain one BRCA-1 C-terminal (BRCT) domain in the N terminus, which is thought to mediate protein-protein and
protein-DNA interactions (36, 37). Moreover, all domains important for TdT activity, such as nucleotide, DNA, and cation binding are conserved in all three hTdT isoforms (38–41). In addition, three cAMP-dependent phosphorylation sites are also present, as well as the putative tyrosine phosphorylation motif (Fig. 1E).

Regulated expression of hTdTL1 and hTdTL2

Because hTdTL1 and hTdTL2 were identified from hTdT genomic sequence, it was necessary to determine whether they were transcribed in lymphoid cells. During human B cell development, IgH chain genes rearrange at the pro-B stage where N addition and nucleotide deletion occur. Successful production of the H chain is followed by L chain gene rearrangement at the pre-B stage (42). hTdTL1, hTdTL2, and hTdTS expression during these stages was assessed by RT-PCR. Total RNA was prepared from pro-B (CD34+CD19+ surface δlglM−), pre-B (CD34−CD19+δlglM−), and mature B cells (CD34−CD19+δlglM−) sorted from human adult bone marrow. Transcripts of hTdT isoforms were detected in the pro-B population; hTdTS expression was most abundant, hTdTL2 expression was intermediate, and hTdTL1 was lowest (Fig. 2A). This population may contain small numbers of class-switched B cells, but TdT is not expressed in this mature population (J. F. Kearney, unpublished observations). In contrast, only hTdTS and hTdTL2 mRNAs were present in the pre-B cell stage (Fig. 2A). As expected, none of the isoforms were seen in the mature B cell population (Fig. 2A). These results show that the expression of hTdT isoforms is regulated during B cell development. However, this ordered pattern of expression was altered in transformed cell lines, because along with the other isoforms, hTdTL1 was detected in the human cell line 697 representative of the pre-B cell stage (Fig. 2A), where it is normally not detected (4).
To date, the expression of hTdT isoforms during normal human thymocyte development has not been formally assessed. Similar to adult bone marrow pre-B cells, hTdTTL1 was not detected in fetal thymocytes at any age (Fig. 2B). However, hTdTTL1 was detected in transformed T cell lines (Fig. 2E). In a semiquantitative RT-PCR assay, both hTdTTS and hTdTTL2 were expressed in all stages of thymocyte development (double-negative (DN); CD4+CD8+), double-positive (DP; CD4+CD8+), CD4+, and CD8+ thymocytes), as well as in transformed cell lines (Fig. 2, B and E, respectively). The level of hTdTTL2 transcripts was consistently higher than that of hTdTTS. At fetal day 91, all four populations studied (DN, DP, CD4+, and CD8+ thymocytes) expressed less hTdTTL2 than hTdTTS, and this level of differential expression persisted until fetal day 111, albeit slightly less pronounced. Moreover, TdT proteins are readily detected in the respective thymocyte subpopulations by FACs analyses using pan-TdT-specific mAbs (data not shown). To verify the developmental stages of sorted fetal thymocytes, the expression of RAG genes was analyzed. DP cells expressed the highest levels of RAG-2 mRNA, whereas RAG-1 expression appeared to be stage and age independent. Moreover, there appeared to be a higher level of RAG-2 transcripts in the single CD4+ and CD8+ populations in 91-day-old fetal thymocytes compared with those from 111 days (Fig. 2C). In contrast, both hTdTTS and hTdTTL2 were expressed at similar levels in all stages of adult thymocyte development (Fig. 2D). hRAG1 expression was not detectable in adult thymocytes, whereas hRAG2 transcripts were present, especially in the single-positive populations (Fig. 2D). The absence of hRAG1 in adult thymocytes may be due to transcript instability and/or age-dependent expression.

Therefore, in normal human B and T cells, exon XII of hTdT gene is always excluded, whereas exon VII can be included (generating hTdTTL2) or excluded (generating hTdTTS). In contrast, bovine exons XII and VII can be included (producing hTdTTL1 and hTdTTL2, respectively) or excluded (producing hTdTTS) (28) (Fig. 2F). Thus, alternative splicing, which appears to be species specific, seems to regulate human and bovine TdT isoforms expression.

Nuclear localization of hTdTTL1 and hTdTTL2

We have demonstrated that mature transcripts of both hTdTTL isoforms are made in normal and transformed lymphoid cells; however, their activities and cellular localization are not known. To carry out these studies, respective cDNAs were generated by PCR insertion mutagenesis. Sequence analyses of hTdTTS, hTdTTL1, and hTdTTL2 cDNAs revealed no changes when compared with both the NCBI and Celera genomic sequences (data not shown).

To establish whether the conserved nuclear localization motifs in hTdTTL1 and hTdTTL2 sequences do indeed mediate their localization into the nucleus, all three hTdT isoforms, subcloned into the pcDNA1.1+ mammalian expression vector, were expressed in the human embryonic kidney cell line 293T. After transfections, protein expression and cellular localization were verified by immunofluorescence using a rabbit polyclonal Ab raised against TdT. hTdTTL1 and hTdTTL2, as well as hTdTTS, localized in nuclei of 293T cells transfected with the respective plasmid (Fig. 3, green), whereas actin filaments (stained with phallolidin) localized mainly in the cytoplasm (Fig. 3, red). All three isoforms exhibited punctuated nuclear staining similar to that observed in normal lymphocyte precursors.

In vitro activities of hTdTTL isoforms

The presence of hTdTTL isoforms in the nucleus of normal lymphocytes suggests that they may function as DNA-modifying enzymes. To test this hypothesis, primer modification assays were done with purified TdTTS- and TdTTL-GST fusion proteins. Samples were resolved on 7 or 15% denaturing acrylamide gels. As expected, hTdTTS efficiently catalyzed the addition of dNTPs to the 21-bp ssDNA (Fig. 4, lane 1). In contrast, hTdTTL1 and -2 showed 3'→5' exo activity, producing species progressively smaller than the original 21-bp primer (Fig. 4, lanes 2 and 3); the primer was not modified by buffer alone (lane 4).

During V(D)J recombination, coding and signal ends are generated as a result of RAG-1- and RAG-2-mediated DNA cleavage. Coding ends are thought to possess 3' or 5' extensions, whereas signal ends are blunt. Thus, if hTdTTL1 and -2 were involved in the deletion of nucleotides from coding ends, dsDNA with 3' or 5' extensions would be expected to undergo nucleotide loss. We found that hTdTTL1 and -2 efficiently degraded dsDNA with 3' extensions (Fig. 4, lanes 6 and 7, respectively), and TdTTS added nucleotides to the same substrate (lane 5). dsDNA with recess 3' ends or 5' extensions were also modified by all three hTdT isoforms (Fig. 4, lanes 9–11). Moreover, dsDNA with blunt ends were similarly modified by all three isoforms (Fig. 4, lanes 13–15). The substrates were not modified in the absence of hTdT isoforms (Fig. 4, lanes 4, 8, 12, and 16). The low amount of smaller species is due to spontaneous degradation of the primers. Because the extent of transferase activity catalyzed by hTdTTS was extensive, all

<table>
<thead>
<tr>
<th>Exon</th>
<th>Position in Open Reading Frame</th>
<th>Size of Exon (bp)</th>
<th>5′-splice donor</th>
<th>intron</th>
<th>3′-splice acceptor</th>
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a Upper case letters denote exon sequence, and lowercase letters denote intron sequence. Newly identified exons are in bold. Putative cryptic donor and acceptor sites are in italics. Nearly invariant dinucleotides and flanking either end of the intron sequence are underlined.
FIGURE 2. Regulated expression of hTdT splice variants during B and T cell development. A, Adult human bone marrow pro-B and pre-B cells were isolated by FACS with forward-light scattering and the markers CD19⁺ IgM⁺ CD34⁻ and CD19⁺ IgM⁺ CD34⁻, respectively. Mature B cells were identified as CD19⁺ IgM⁺ CD34⁻. Left panel, RT-PCR products were generated from sorted bone marrow subpopulations with hTdT isoform-specific primers. Right panel, RT-PCR products from human cell lines: Nalm 16, pro-B stage; EU12, pro-pre-B stage; 697, pre-B stage. B, Fetal DN (CD4⁻ CD8⁻), DP (CD4⁺ CD8⁻), CD8⁺, and CD4⁺ thymocytes obtained from days 91 and 111 thymi were purified by FACS with forward-light scattering and the markers CD3, CD8, and CD4. RT-PCR products were generated with hTdT isoform-specific primers. C, RT-PCR analyses were performed to detect human RAG-1 and RAG-2 genes in the same fetal thymocyte subpopulations as defined in B. D, In adult thymocytes, both hTdTS and hTdTTL2 were expressed at similar levels, in all stages of T cell development. hRAG2 transcripts were readily seen in the single-positive populations, whereas hRAG1 was not present, perhaps due to transcript instability or aging. E, All three hTdT isoform transcripts were detected in Molt-4, Cem-6, and Jurkat human T cell lines by RT-PCR.
reactions shown for hTdTS were resolved on 7% denaturing acrylamide gels. The low-percentage gels allowed better resolution of the DNA ladder, which would otherwise stay at the top of a 15% gel (data not shown). The exo activity of hTdTL1 and -2 was similarly compressed when resolved on a 7% gel (data not shown); thus, all reactions shown for hTdTL1 and -2 were resolved on 15% gels.

These data show that, in vitro, hTdTL isoforms exhibited 3′→5′ exo activity with no detectable 5′→3′ exo activity. Their substrate specificity included ssDNA and dsDNA with 3′ or 5′ extensions. Transferase activity was not observed in any reactions conducted with either hTdTL1 or hTdTL2.

In vivo activities of hTdTL isoforms

To determine whether hTdTL isoforms also exhibited exo activity in vivo, recombination assays were done (23). In these assays, expression vectors encoding the truncated active core RAG-1 and RAG-2 recombinase proteins were transiently transfected into Chinese hamster ovary (CHO) cells along with a CJ recombination substrate plasmid, with or without expression vectors encoding hTdTS, hTdTL1, or hTdTL2. CHO cells were chosen for these experiments because hRAG1 protein, and hTdTS and hTdTL isoform messages were readily detected in the human 293T cell line; therefore, in our hands, 293T cells give unacceptable background recombination (T.-H. Thai and J. F. Kearney, unpublished observations); the status of hRAG2 protein expression in 293T cells cannot be determined, because quality Abs useful for immunohistochemistry and/or immunoblots are not available. The integrity of CJs from CHO transfecants was evaluated by sequence analyses (Fig. 5, A and B). We hypothesized that, if hTdTL1 and hTdTL2 were indeed exos in vivo, CJs formed in the presence of hTdTL1 and hTdTL2 would exhibit more nucleotide loss than joins formed with hTdTS or without hTdT. Analyses of CJs from CHO transfecants supported this hypothesis. On average, 4.7 ± 0.6 nt (p = 0.0028) were deleted from coding ends in the presence of hTdTL1 and 5.7 ± 0.6 nt (p < 0.0001) in the presence of hTdTL2, whereas without hTdT or in the presence of hTdTS, only 1.9 ± 0.7 and 2.3 ± 0.3 nt were lost from coding ends, respectively (Table II and Fig. 5C). N addition was not observed for hTdTL2 in this assay; 1 sequence of 16 with 1 nt added was recovered from hTdTL1 transfecants. In addition, few sequences with palindromic (P) additions were recovered from either hTdTL1 (12%) or hTdTL2 (5%) transfecants. In contrast, considerably more sequences contained P additions in the presence of hTdTS (44%) or without hTdT (29%), suggesting that hTdTL isoforms are responsible for the removal of P nucleotides from coding ends.

It is likely that, during V(D)J recombination, hTdTS and hTdTL2 are present simultaneously during the formation of CJs. To recapitulate this situation, recombination assays were done in the presence of both enzymes. When hTdTS was included in the assay, the mean ± SEM nucleotide loss exerted by hTdTL2 decreased from 5.7 ± 0.6 to 3.1 ± 1.0 (Table II and Fig. 5C), indicating that deletion by hTdTL2 was attenuated by hTdTS in vivo. Likewise, hTdTL1 exo activity was reduced by hTdTS, although hTdTL1 does not appear to be expressed during normal B and T cell development. The inclusion of all three isoforms did not additively lessen nucleotide loss from coding ends. The number of sequences with P additions was similar to that with (44%) or without hTdTS (29%) when isoforms were coexpressed (hTdTS plus hTdTL1, 33%; hTdTS plus hTdTL2, 30%; all three isoforms, 44%). It is also noteworthy to point out that the average length of N nucleotides was not decreased by the simultaneous expression of hTdTS with either hTdTL1 or hTdTL2 (average nucleotides added with hTdTS alone, 2.1; with hTdTS plus hTdTL1, 1.2; with hTdTS plus hTdTL2, 2.2). These observations suggest that, in this situation, deletion may occur before addition.

In vitro as well as in vivo, hTdTL1 and hTdTL2 behave as exons capable of catalyzing nucleotide loss from coding ends during...
Discussion

We have shown that humans, like cattle and mice, have three TdT splice variants, hTdTS, hTdTL1, and hTdTL2. hTdTL1 and hTdTL2 are derived from mature transcripts that include exons XII and VII, respectively. The amino acid sequences of human L1 and L2 inserts are highly homologous to those of bovine, having 67 and 77% identity, respectively. In contrast, the mL1 insert shows a low degree of amino acid identity to either human or bovine. Moreover, human and bovine L1 inserts are half the size of that of mouse, whereas the L2 insert of human and bovine has approximately the same size as that of mL1 insert. It is puzzling that the amino acid sequence of mL1 insert is so divergent from those of human and bovine inserts, whereas the genomic organization of their TdT genes is conserved. Taken together, these data suggest that the L1 and L2 inserts may serve as structural rather than protein interaction domains, which allow TdTTL1 and TdTTL2 to assume conformations different from those of TdTS. The change of conformation, in turn, may confer different DNA-modifying activities, i.e., 3′→5′ exo activity, to TdTTL1 and TdTTL2. The lack of nucleotide sequence homology between mouse and human TdTTL isoforms may explain why past attempts to clone hTdTL, based on mouse sequences, were unsuccessful. It is conceivable that TdT splice variants may also exist in other vertebrate species in which modification of Ig and TCR genes rearrangements is necessary to generate diverse repertoires. Indeed, identification of three TdT splice variants from the rat genomic database, and analysis of the nucleotide sequences shows that the deduced amino acid sequence of rat L1 and L2 inserts are quite divergent from those of mouse (T.-H. Thai and J. F. Kearney, manuscript in preparation). Because of the lack of homology between sequences, the search for TdT isoforms in other species may be difficult in the absence of a genomic database for the respective species. The nucleotide sequences of our hTdTS, hTdTL1, and hTdTL2 cDNAs are identical with genomic DNA sequences retrieved from both the NCBI and Celera human genome databases, suggesting that there is no polymorphism in the coding sequences.

Like mL1TdT, mL2TdT also possesses 3′→5′ exo activity. Furthermore, mL2TdT, consistently expressed during normal B and T lymphocyte development, is devoid of transferase activity, as evidenced by the complete absence of N addition in CJs derived from the recombinants. The inability of mL2TdT to add nucleotides is corroborated by the in vitro primer modification assay. Thus, in human, mL2TdT has the same enzymatic qualities as mL1TdT (23). Although mL1TdT, containing an insert half the size of mL2TdT, is not expressed during normal B and T cell development, it does exhibit 3′→5′ exo activity, albeit at a lower level. Unlike mL2TdT, 1 of 16 CJs obtained from mL1TdT transfectants

recombination. However, in normal B and T cell progenitors, mL1TdT expression is suppressed by constitutive exclusion of exon XII via alternative splicing mechanisms. Therefore, the propensity to exclusively express one exo, mL2TdT, suggests that perhaps the presence of both during V(D)J recombination might be detrimental to the survival of the rearranging B or T cell.

**Inefficient CJ formation caused by an excess of nucleotide deletion or addition**

To determine whether the concomitant expression of two exos adversely affects CJ formation, the standard recombination assay (described above) was performed. In this assay, recombination of the CJ substrate plasmid (the same substrate used in the previous section) activates expression of the chloramphenicol acetyltransferase (CAM) gene by removing the transcriptional terminator that lies between two coding/RS sequences. Plasmids recovered from transfected CHO cells were introduced into bacteria by transformation to determine the relative recombination efficiency by assaying for the ratio of total plasmids recovered (ampicillin-resistant colonies) over recombined plasmids (ampicillin/CAM-resistant colonies). In this assay, the presence of mL2TdT clearly impaired CJ formation compared with no TdT control (Fig. 6A). In contrast, the overexpression of mL1TdT, which consistently causes a lower level of deletion, did not have a great effect on CJ formation. In addition, mL2TdT overexpression effected a similar degree of reduction in CJ formation as mL1TdT. However, the impairment of CJ formation was corrected by the concomitant expression of mL2TdT with mL1TdT, but not by the coexpression of all three isoforms, which were expressed at equivalent levels as shown by immunoblotting (Fig. 6B). Together, these data and those shown for human TCRβ chain (43) suggest that the uncontrolled elongation (through N addition by mL2TdT) or reduction (through nucleotide deletion by mL1TdT) of V(D)J joins or CDR3 length, has the potential to adversely affect V(D)J recombination, thus generating nonproductive BCR or TCR, and reduces the survival of a rearranging B or T cell.
contains 1 nt (C) that could be derived from N addition. As expected, hTdTS efficiently catalyzes N addition without causing deletion above background. The concomitant expression of hTdTS with either long isoform reduces the average number of nucleotides deleted to background levels, and the coexpression of all three isoforms does not enhance nucleotide removal, suggesting that CJs with extensive deletion were not recovered, because they could not recombine. Therefore, it is likely that, because B and T cells normally express both isoforms during recombination, their Ig and TCR V(D)J junctions would not display a high degree of

FIGURE 5. Deletion of nucleotides from coding ends but not from signal ends by hTdTL1 and hTdTL2 in the standard recombination assay. A and B, Coding-end sequences were obtained from PCR products with primer DR99 (23). Underlined sequences are unmodified coding ends. Numbers in parentheses indicate repeated sequences recovered from multiple independent transfections, not within one transfection. No TdT. Three transfections; hTdTS, three transfections; hTdTL1, four transfections; hTdTL2, four transfections; hTdTS plus hTdTL1, three transfections; hTdTS plus hTdTL2, four transfections; hTdTS plus hTdTL1 plus hTdTL2, three transfections. C, Mean ± SEM nucleotides deleted from coding ends recovered from different transfectants. Values of \( p \) were calculated with unpaired two-tailed Student’s \( t \) tests.

FUNCTIONAL CHARACTERIZATION OF hTdTL ISOFORMS
expression is higher at fetal day 91, and by fetal day 111, the level of hTdTS has increased, but not to that of hTdTL2. Our data support previous studies demonstrating that the degree of N addition in TCR-β DJ junctions of human fetal thymocytes increases with age, but the extent of nucleotide nibbling remains constant or slightly decreased (50). This decrease of nibbling is probably affected by N addition through the increased expression of hTdTS in adult, as discussed above. The persistent expression of hTdTS and hTdTL2 in all stages of thymocyte development also explains the presence of N addition and nucleotide deletion in human TCR-β, -α, -γ, and -δ chain genes (51–55).

The absence of hTdTL1 in normal lymphocytes could be due to transcript instability, low abundance, or constitutive splicing of exon XII. In addition, there is no difference in expression levels of hTdTS and hTdTL2 in transformed cells. Thus, hTdT RNA processing and/or steady-state maintenance may be tightly controlled during normal human B and T cell development, and this control is lost upon cellular transformation.

Clinically, hTdT has been used as a marker for the diagnosis of leukemias due to its abundant expression. Until now, the presence of long isoforms has not been described, and a full understanding of the consequences of TdT isoform overexpression has not been explored. One possible outcome of hTdT isoform overexpression is that, in leukemias as in many other tumors, cells undergo a high rate of DNA synthesis and cellular proliferation, thus supplying a large pool of DNA breaks as substrates for hTdT isoform modification. This unwarranted modification by hTdT isoforms may create damages in the genome, which, in turn, activates cell cycle checkpoints to arrest DNA replication and mitosis. The recruitment of

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**Table II. In vivo analyses of nucleotide deletion, N addition, and P addition**

<table>
<thead>
<tr>
<th>Transfectants</th>
<th>Deleted Nucleotides</th>
<th>Sequences with &gt;4 nt Deleted (%)</th>
<th>Sequences with N Addition (%)</th>
<th>Sequences with Presumed P Addition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No TdT</td>
<td>1.9 ± 0.7</td>
<td>0/7 (0)</td>
<td>0/7 (0)</td>
<td>2/7 (29)</td>
</tr>
<tr>
<td>hTdTS</td>
<td>2.3 ± 0.3</td>
<td>0/18 (0)</td>
<td>13/18 (72)</td>
<td>1/16 (6)</td>
</tr>
<tr>
<td>hTdTL1</td>
<td>4.7 ± 0.6</td>
<td>7/16 (44)</td>
<td>1/16 (6)</td>
<td>2/16 (12)</td>
</tr>
<tr>
<td>hTdTL2</td>
<td>5.7 ± 0.6</td>
<td>12/19 (63)</td>
<td>0/19 (0)</td>
<td>1/19 (5)</td>
</tr>
<tr>
<td>hTdTS + hTdTL1</td>
<td>3.6 ± 0.9</td>
<td>4/9 (44)</td>
<td>6/9 (67)</td>
<td>3/9 (33)</td>
</tr>
<tr>
<td>hTdTS + hTdTL2</td>
<td>3.1 ± 1.0</td>
<td>4/10 (40)</td>
<td>8/10 (80)</td>
<td>3/10 (30)</td>
</tr>
<tr>
<td>All three isoforms</td>
<td>3.0 ± 0.6</td>
<td>1/9 (11)</td>
<td>7/9 (78)</td>
<td>4/9 (44)</td>
</tr>
</tbody>
</table>

*Data are from multiple independent transfections.*

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nucleotide loss. It is noteworthy to point out that the presence of hTdTL2 or hTdTL1 together with hTdTS in CJs does not shorten the length of N regions but diminishes the average nucleotides deleted, suggesting that nucleotide deletion may take place before addition.

The overexpression of either hTdTS or hTdTL2 effects a dramatic reduction in the recombination frequency, which can be rectified by the concomitant expression of these two isoforms in the same cell. However, the presence of the third isoform during CJ formation does not rescue the decrease in recombination frequency, but rather diminishes it. These results demonstrate that there may be a strong evolutionary selection for the coexpression of one transerase (hTdTS) and one exo (hTdTL2), but against the coexpression of two exos during V(D)J joining. This differential expression of the two long isoforms is regulated at the level of transcriptional termination; a minuscule amount of hTdTL1 transcript is detected in transformed cells representative of pre-B cells. The expression pattern of hTdTS (transerase) and hTdTL2 (exo) in pre-B cells is consistent with data showing that human L chains sustain N addition as well as nucleotide deletion (43–49).

Both hTdTS and hTdTL2 are coexpressed in all thymocyte subpopulations (DN, DP, CD4+, and CD8+) during normal human fetal thymocyte development. However, the level of hTdTL2 expression is higher at fetal day 91, and by fetal day 111, the level of hTdTS has increased, but not to that of hTdTL2. Our data support previous studies demonstrating that the degree of N addition in TCR-β DJ junctions of human fetal thymocytes increases with age, but the extent of nucleotide nibbling remains constant or slightly decreased (50). This decrease of nibbling is probably affected by N addition through the increased expression of hTdTS in adult, as discussed above. The persistent expression of hTdTS and hTdTL2 in all stages of thymocyte development also explains the presence of N addition and nucleotide deletion in human TCR-β, -α, -γ, and -δ chain genes (51–55).

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**FIGURE 6.** Inefficient CJ formation caused by an excess of nucleotide deletion or addition. The standard recombination assay was performed as described in Materials and Methods. A. In this assay, recombination of the CJ substrate plasmid activates expression of the CAM gene by removing the transcriptional terminator that lies between two coding/RS sequences. Plasmids recovered from transfected cells were introduced into bacteria via transformation to determine the relative recombination efficiency by assaying for the ratio of total plasmids recovered (ampicillin-resistant colonies) over recombined plasmids (ampicillin/ CAM-resistant colonies). B. Immunoblots were done to confirm protein expression. hTdT isoforms were detected with the same rabbit Ab as in Fig. 3.
Acknowledgments

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References

into biased usage of TCRβD and TCRβJ genes and diversity of CDR3 region length. *Hum. Immunol.* 60:1090.


