

# The Long Isoform of Terminal Deoxynucleotidyl Transferase Enters the Nucleus and, Rather than Catalyzing Nontemplated Nucleotide Addition, Modulates the Catalytic Activity of the Short Isoform

By Cindy L. Benedict,\* Susan Gilfillan,‡ and John F. Kearney\*

From the \*Division of Developmental and Clinical Immunology, Department of Microbiology, University of Alabama at Birmingham, Alabama 35294; and ‡The Basel Institute for Immunology, CH-4005 Basel, Switzerland

## Abstract

During variable/diversity/joining (V[D]J) recombination, the enzyme terminal deoxynucleotidyl transferase (Tdt) adds random nucleotides at the junctions of the rearranging gene segments, increasing diversity of the antibody (Ab) and T cell receptor repertoires. Two splice variants of Tdt have been described, but only one (short isoform of Tdt [TdtS]) has been convincingly demonstrated to catalyze nontemplated (N) addition in vitro. We have expressed each splice variant of Tdt in transgenic (Tg) mice and found that the TdtS transgene catalyzes N addition on the endogenous Tdt<sup>-/-</sup> background and in fetal liver, but that the long isoform of Tdt (TdtL) transgene does neither. In contrast to previous in vitro results, both TdtS and TdtL are translocated to the nucleus in our model. Furthermore, TdtL/TdtS double Tg mice exhibit less N addition in fetal liver than do TdtS Tg mice. Whereas the TdtS transgene was shown to have functional consequences on the antiphosphorylcholine (PC) B cell repertoire, TdtL Tg mice exhibit a normal PC response, and Tdt<sup>-/-</sup> mice actually exhibit an increase in the PC response and in TEPC 15 idiotype<sup>+</sup> Ab production. We conclude that TdtL localizes to the nucleus in vivo where it serves to modulate TdtS function.

Key words: V(D)J rearrangements • terminal deoxynucleotidyl transferase • nontemplated regions • terminal deoxynucleotidyl transferase isoforms • phosphorylcholine

## Introduction

Terminal deoxynucleotidyl transferase (Tdt)<sup>1</sup> is a nuclear enzyme that catalyzes addition of nontemplated (N) nucleotides to the free 3'-OH ends of fragmented or nicked DNA (1). Thus far, the only known physiological function of Tdt is the random addition of nucleotides to the V(D)J junctions of Ig H chain and TCR gene rearrangements (2–6) and, rarely, of Ig L chain rearrangements (7, 8). N addition effectively increases diversity in the repertoire of Ig and TCR receptors on lymphoid cells. In vitro, the enzymatic activity of Tdt can be measured in crude cell extracts (1, 9–11)

as well as in cellular recombination systems using transfected Tdt and recombination substrates (12–14). Additionally, Tdt activity has been shown to be associated with the Ku protein (15), and to be altered by the DNA-dependent protein kinase, of which Ku is a subunit (15, 16).

Two mRNA splice variants of Tdt have been described in mice (17, 18). Koiwai et al. initially isolated and characterized the first two murine Tdt cDNA clones (M11-3 and M16-1b; reference 19). The complete sequence of clone M11-3 was highly homologous to human and bovine Tdt cDNAs except for a 60-bp insertion encoding a unique 20 amino acid (aa) region near the carboxy terminus. Later, Doyen et al. (18) cloned a second shorter isoform (TdtS) from mice lacking this insertion, and for which the mRNA is expressed at much higher levels than the long form (TdtL). TdtL was also formally shown to contain a 20-aa insertion encoded by an additional exon “Xbis” located between exons X and XI of the murine Tdt gene (18). Although Tdt genes have since been identified and cloned from many vertebrate species, TdtL has been de-

C.L. Benedict's present address is Princeton University, 401 Schultz Laboratory, Washington Rd., Princeton, NJ 08544.

Address correspondence to J.F. Kearney, 378 WTI, 1824 6th Ave. South, University of Alabama at Birmingham, Birmingham, AL 35294-3300. Phone: 205-934-3370; Fax: 205-934-1875; E-mail: john.kearney@ccc.uab.edu

<sup>1</sup>Abbreviations used in this paper: aa, amino acid(s); LM, littermate; N, nontemplated; nt, nucleotide; PC, phosphorylcholine; RT, reverse transcription; T15, TEPC 15; Tdt, terminal deoxynucleotidyl transferase; TdtL, long isoform of Tdt; TdtS, short isoform of Tdt; Tg, transgenic.

scribed only in mice (19–23). Two alternative insertions have also been described for bovine Tdt, one of which bears some similarity to the insertion found in murine TdtL (24). Initially, Koiwai et al. (19) reported that a TdtL clone, M16-1b, produced Tdt protein with enzymatic activity in COS cells. The other clone, M11-3, was presumed to be neither expressed nor functional, because it contained an additional 5' out-of-frame ATG. Later, functional N addition by TdtS was shown using an episomic rearrangement construct in NIH 3T3 fibroblasts by Doyen and colleagues (18). The 5' out-of-frame ATG did not cause aberrant translation in the latter experiments, perhaps because the ATG lacks a favorable Kozak context (18). These data together suggested that both Tdt isoforms are functional. However, direct comparison of TdtS and TdtL clones (one of the original Koiwai TdtL clones as well as an independent TdtL clone) showed that TdtS added N nucleotides much more efficiently than the two different TdtL clones. The observation that in NIH 3T3 and COS transfectants, TdtS translocated to the nucleus whereas TdtL remained in the cytoplasm and did not enter the nucleus, was used to explain the difference in TdtS versus TdtL activity in this assay (25). However, lysates from the two TdtL COS cell transfectants contained 10–15 times less terminal transferase activity than TdtS. TdtL protein was also shown to be less stable than TdtS protein. Although no direct comparisons have been made, this result appears to contradict the original COS cell transfection data with the Koiwai TdtL M16-1b clone. Functional analysis of the two mouse Tdt isoforms has therefore produced contradictory and seemingly irreconcilable results. At this point, it is difficult to draw any conclusions about the function of TdtL, in contrast to TdtS, which is clearly functional as a transgene in vivo (26, 27).

We chose to investigate the function of the TdtL isoform in vivo. This is quite difficult in normal mice because they express both isoforms. Although it is relatively easy to distinguish TdtL from TdtS mRNA, identifying the proteins is more difficult. Anti-Tdt mAbs that discriminate between the two splice variants are not available and TdtS and TdtL proteins, differing only slightly in size, are not easily resolved on a standard protein analysis gel. Therefore, to determine whether TdtL can catalyze N addition or serve another function in vivo, we generated Tdt transgenic (Tg) mice in which a TdtL transgene was placed under the control of the same IgH promoter and enhancer used in our TdtS Tg mice that catalyzed N addition in both fetal and adult B lineage cells (27). As expected, TdtL Tg mRNA was detected in bone marrow, thymus, and spleen. Although TdtL protein was detected in the nuclei of mouse B cells, TdtL expression neither complemented the N addition defect in adult bone marrow of Tdt-deficient mice nor introduced N nucleotides into fetal/neonatal liver Ig rearrangements. In contrast, Tg TdtS catalyzed N addition in adult bone marrow of endogenous Tdt-deficient mice to the extent that H chain Ig rearrangements resembled those of normal Tdt-sufficient mice. Notably, TdtL/TdtS double Tg mice display a phenotype intermediate be-

tween TdtS or TdtL single Tgs, with respect to N addition. 7183 V<sub>H</sub>-D-J<sub>H</sub> rearrangements display this phenotype, but it is more strikingly revealed by D-J<sub>H</sub> rearrangements amplified from day 16 fetal liver of TdtL/TdtS double Tg mice. The observation of this intermediate phenotype in double Tg mice derived from three independent TdtL lines crossed with TdtS line V, the most effective adder of N nucleotides, suggests that TdtL can downregulate TdtS-mediated N addition. Biochemical studies addressing a possible mechanism for this downregulation are currently in progress. As a functional analysis to confirm the role of TdtL in Ab repertoire formation, the antiphosphorylcholine (PC) response was induced by immunization with *Streptococcus pneumoniae*. In contrast to the loss of the dominant canonical TEPC 15 (T15) Id normally encoded by N-less CDR3 regions that occurs in TdtS Tg mice (27), the T15 anti-PC immune response remains intact in TdtL Tg and is elevated in Tdt knockout mice. Thus, TdtL must serve a different function than its counterpart, TdtS. We propose that TdtL may play a regulatory role in N addition or V(D)J recombination.

## Materials and Methods

**TdtL Cloning.** All primers used for amplifying and sequencing the TdtS and TdtL forms were based on the sequence of a mouse Tdt clone isolated from RL-12 lymphoma cells (C57Bl/Ka) reported by Koiwai (19). All numbering is based on this sequence (sequence data are available from EMBL/GenBank/DBJ under accession no. X04123). Initially, a 1.9–2-kb Tdt fragment was amplified from cDNA generated from C57Bl/6 (B6) thymus RNA using the following primers: 5'-GCTGATACATTCTGGAGACACCACCTGATGG-3' (includes 1–23) and 5'-GGTGATCAGACACACAGGAGTCAGGTGGGC-3' (1947–1972). This fragment was blunt cloned into EcoRV-digested pBluescript. One clone was sequenced (both strands) from the 5' end to 1,749 (>100 bp past the stop codon) using the dideoxy chain termination method. All differences from the Koiwai sequence were confirmed in independent clones, by direct sequencing of independently amplified PCR fragments and, where possible, a genomic clone. This clone, the short form (TdtS), was then truncated at the 5' end to eliminate the first (out-of-frame) ATG, leaving 17 bp 5' to the appropriate ATG in the final short form clone.

To generate the long form (TdtL), primers flanking the region 1351–1590, 5'-ACAGCGAGAAGAGCGGCCAGCAG-3' (1,298–1,320) and 5'-CCATGGTTCAATGTAGTCCAGTC-3' (1,604–1,626) were used to amplify B6 thymus cDNA. The longer, much less abundant, of two fragments was isolated, cloned, and sequenced. It contained a 60-bp insertion identical to that published by Koiwai. To generate a long form, full-length Tdt clone, a BglII fragment (1,351–1,590) containing the long form insertion was used to replace this region in the fully sequenced Tdt short form clone. All cloning junctions and the insertion were confirmed by sequencing. For the full sequence of our TdtL and TdtS clones, refer to EMBL/GenBank/DBJ accession nos. AF316014 and AF316015, respectively.

**Mice.** TdtL Tg mice were generated as described previously using a plasmid construct containing the cDNA for TdtL, the V<sub>H</sub>81X promoter, E<sub>μ</sub>, and the EcoRI fragment of C<sub>μ</sub> (27). 10 founder lines were generated and bred onto the BALB/c back-

ground. Tg mice were identified by PCR and Southern blot as described previously (27). To discriminate between the TdtL and TdtS transgenes, PCR primer 1450Tdt (5'-GCTATGCCACACACGAGAGGAAGAT-3') was designed in the 10th exon of Tdt, just upstream of the 60-bp insert in TdtL. When paired with primer exJh3 (5'-CTGCAGAGACAGTGACCAGAGT-3'), located in the J<sub>H</sub>3 exon downstream of the Tdt cDNA in the transgene construct, 1450 Tdt gives a 520-bp product from the TdtS transgene and a 580-bp product from the TdtL transgene which can be easily resolved on a 3% MetaPhor agarose gel (FMC BioProducts). Amplification conditions were 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min.

After several generations of breeding onto the BALB/c background, mice of lines I, B, H (TdtL), U, and V (TdtS) were crossed with Tdt-deficient C57Bl/6 × SJL mice (28). Lines I, B, and H had estimated TdtL transgene copy numbers of 2 or 3, 10–12, and 5 or 6, respectively. Tdt-deficient mice were identified using primers Tdta, Tdtb, and Tdtc as described by Gavin and Bevan (29). To detect the presence of our Tdt transgene on the homozygous Tdt-deficient background, it was necessary to design a primer pair to detect endogenous Tdt that anneals to neither the Tdt knockout construct nor the TdtL/TdtS transgene. Such a primer pair was obtained by sequencing into the intron between exons 4 and 5 of mouse genomic Tdt and designing a primer complementary to the fifth exon of Tdt, primer Ex5-NC-Tdt: 5'-GCTTCAGAACTTCTCCATCTTC-3' to generate a PCR product in combination with primer Tdta in the fourth exon of Tdt. This PCR product was cloned and sequenced. A partial sequence of this intron is available from EMBL/GenBank/DBJ under accession no. AF318947. From this intronic sequence we designed the primer Tdt4-5Intron: 5'-GTAGCAAATTCACCTTCTCC-3'. Primer Tdt4-5Intron anneals from positions 6 through 25 bp immediately downstream of exon 4 of wild-type, endogenous Tdt and produces a product of 200-bp in length when used in combination with primer Tdta. Three PCR reactions are performed on TdtS or TdtL Tg mice bred to the Tdt<sup>-/-</sup> background: the presence of the transgene is detected by a transgene-specific PCR as described previously (primers IgP-1 and TL-3); the presence of the Neo-inserted Tdt allele is detected by primers Tdtc and Tdtb; and the presence of the endogenous Tdt allele is detected by primers Tdta and Tdt4-5Intron. TdtL and TdtS mice were bred for two generations to Tdt<sup>-/-</sup> mice and the second generation was screened by PCR for the presence of the transgene, the presence of the Neo-Tdt construct, and the absence of endogenous Tdt. All mice were maintained in a specific pathogen-free barrier facility in Microisolation (Lab Products) caging. All experiments and animal procedures conformed to protocols approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

**Reverse Transcription PCR.** TdtS and TdtL Tg mRNA were detected using 5' primer 1450Tdt and a 3' primer within either the J<sub>H</sub>3 gene segment exon (exJ<sub>H</sub>3: 5'-CTGCAGAGACAGTGACCAGAGT-3') that lies just downstream of the Tdt cDNA or a primer within the first exon of C<sub>μ</sub> (5' plus 1 C<sub>μ</sub> FM-1: 5'-GACATTTGGGAAGGACTGACT-3'; for C<sub>μ</sub> primer, reference 30). The product generated from TdtL cDNA when using 1450Tdt and exJ<sub>H</sub>3 is 580 bp. 1450Tdt and the C<sub>μ</sub> primer yield a 600-bp product indicating, in 17 out of 18 Tdt Tg lines of either splice variant, that the mRNA was spliced such that the C<sub>μ</sub> exon is juxtaposed with the J<sub>H</sub> segments just downstream of the Tdt cDNA.

Messenger RNA was extracted from whole bone marrow, spleen, thymus, or fetal liver using the TRI reagent (Molecular

Research Center, Inc.). All samples were treated with DNase (GIBCO BRL) before reverse transcription (RT) to eliminate genomic DNA contamination. cDNA was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase (GIBCO BRL) and PCR was performed as described previously (27) using primers described above.

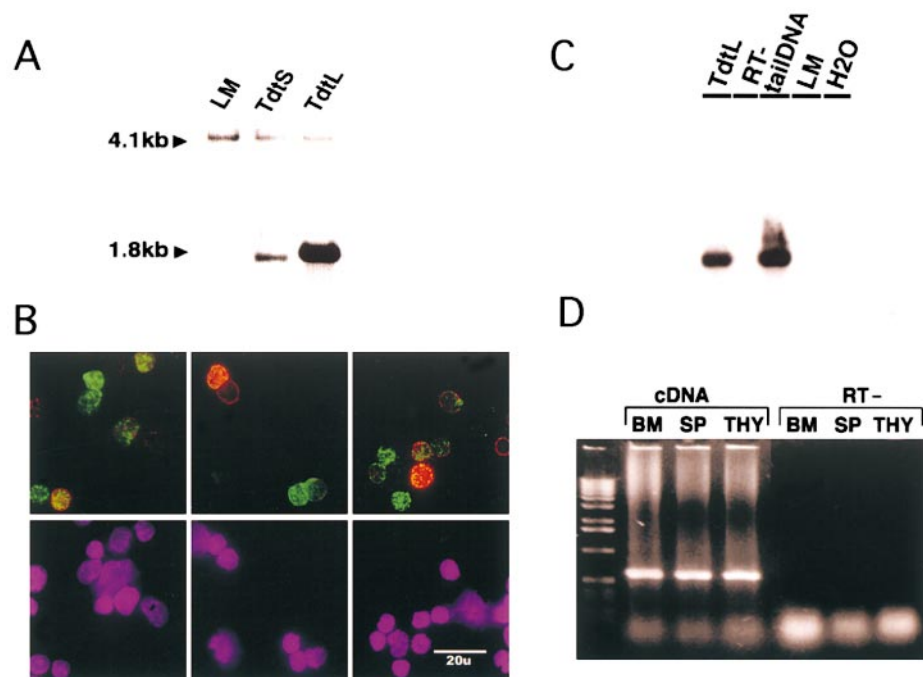
**Imaging of LPS-stimulated Splenocytes and Fetal Liver Cells.** Cyto-centrifuge smears were made from LPS-activated spleen cells from adult Tg and non-Tg littermate (LM) mice of each of the 10 TdtL lines at intervals up to 7 d. Fetal liver cells were prepared and smears made as described previously (27). Smears were dried, fixed in absolute methanol, and stained for Tdt expression with rabbit anti-Tdt (Supertechs) developed with an Alexa 488-coupled goat anti-rabbit IgG Ab (Molecular Probes) and rhodamine isothiocyanate (RITC) goat anti-IgM as described previously (27). Images were acquired on a Leica DMRB fluorescence microscope equipped with appropriate filter cubes (Chromatechnology) and a C5810 series digital color camera (Hamamatsu Photonic System) as described previously (30). The fluorescence intensity of Tdt staining of fetal liver cells from the three TdtL Tg mice was measured from digital images using the Density Slicing and Advanced Measurement Modules of OpenLab Imaging Software (Improvision Inc.). Fluorescence intensity of Tdt staining was calculated as the product of mean fluorescence and area and is expressed as arbitrary units. Confocal microscopy was performed on cyto-centrifuge smears of LPS-activated spleen cells using a confocal laser scanning microscope (Leica).

**Sequencing of Ig Gene Rearrangements.** Genomic DNA was prepared from day 16 fetal liver, adult bone marrow, and spleen of Tdt Tg and LM mice. V<sub>H</sub>7183 family to J<sub>H</sub>1–4 gene rearrangements were amplified, cloned, and sequenced as described (27). DFL16.1-JH1 gene rearrangements were amplified using primers and conditions as described (31) and sequences with ambiguous bases in the CDR3 region were discarded. N nucleotide status was assigned as described previously (27).

**PC Immunization and T15 Quantitative ELISA.** Mice were immunized with heat-killed *S. pneumoniae*, strain R36A. Serum levels of anti-PC Ab and Ids were quantified by ELISA as described previously (27).

## Results

**Cloning of Mouse cDNA for TdtL and TdtS.** Mouse Tdt was amplified, cloned, and sequenced from C57Bl/6 thymus cDNA. Initially, a 1.9–2.0-kb Tdt fragment was amplified and was found to differ from the Koiwai sequence by a deletion of 60 bp in the downstream region of the clone. Primers flanking the deleted region were used to amplify the “missing piece” needed to generate the long form of Tdt from C57Bl/6 thymus cDNA. The longer, much less abundant, of the two fragments amplified was shown to contain a 60-bp insertion identical to the murine Tdt sequence published by Koiwai (19). Comparison of our mouse TdtL sequence to that of Koiwai (sequence data are available from EMBL/GenBank/DBJ under accession no. X04123) and, more recently, Doyen et al. (18) revealed only one region between nucleotides 1,373 and 1,382 within exon IX in which our sequence differed from both of these. This region is present in both TdtL and TdtS and our sequence contains an arginine codon inserted between 1,376 and 1,379. Aa translation of this region from mouse,



**Figure 1.** Southern blot, RT-PCR, and protein expression from TdtL transgene. (A) Genomic DNA was analyzed by Southern blot from non-Tg LM, TdtS, and TdtL Tg mice. The 1.8- and 1.86-kb bands correspond to the TdtS and TdtL transgenes, respectively. The 4.1-kb band corresponds to a fragment of endogenous Tdt as seen in the non-Tg LM. (B) Embryonic day 19 fetal liver cells from TdtL Tg mouse lines I, B, and H (from left to right) were stained for  $\mu$  chains (red) and Tdt (green) in the top panels with corresponding nuclei revealed with Hoechst 33258 (blue) below. Original magnification indicated by bar inset. (C) TdtL expression was demonstrated by RT-PCR analysis of TdtL mRNA from newborn liver. Lanes 1 and 4 are RT-PCR products from TdtL and non-Tg LM newborn livers. Lane 2 is an RT-negative control product from liver mRNA. Lane 3 is a PCR product from a genomic tail DNA template used as a size control for the RT-PCR product. Lane 5 is a template-negative control. The blot was probed with the 1.8-kb BamHI fragment. TdtS PCR primers were 1450Tdt and 5' plus 1 C $\mu$  FM-1. (D) Ethidium

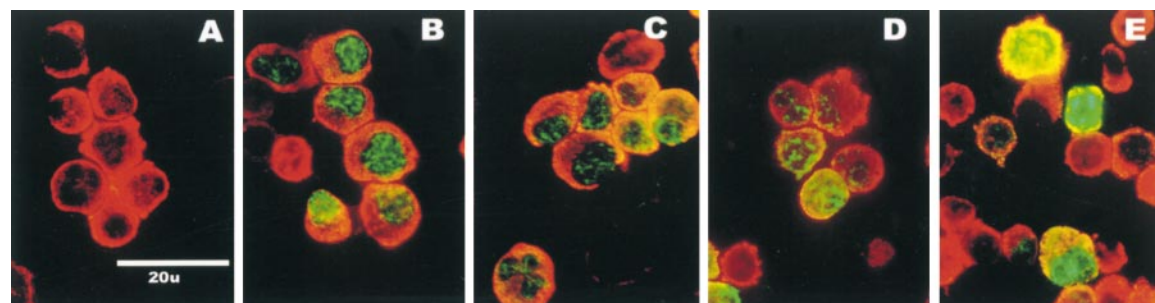
bromide (EtBr)-stained agarose gel of RT-PCR products from bone marrow (BM), spleen (SP), and thymus (THY) of an adult TdtL Tg mouse (lanes 2-4). Lanes 5-7 are corresponding RT-negative controls for each tissue. Lane 1 is a 1-kb ladder size standard (GIBCO BRL). PCR primers were 1450Tdt and 5' plus 1 C $\mu$  FM-1.

human, and bovine sequences reveals that the independent mouse clones (TdtL and TdtS) contain other minor differences which may be strain dependent (Koiwai, accession no. X04123 [19], and Doyen et al. [18]).

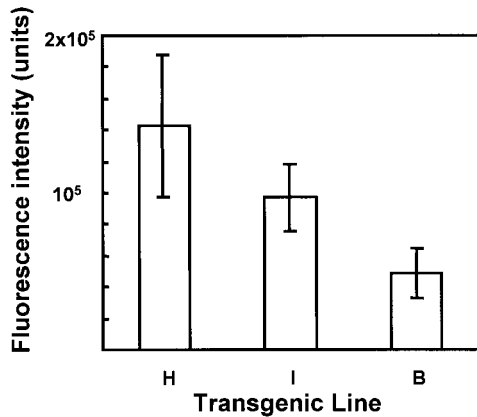
**Generation of TdtL Tg Mice.** A plasmid construct containing our TdtL cDNA was designed identical to that described for TdtS Tg mice (27). 10 lines of TdtL Tg mice were generated containing the TdtL cDNA under control of the V<sub>H</sub>81X promoter and the  $\mu$  enhancer, with the C $\mu$  exons placed downstream to enable mRNA processing. TdtL Tg mice were identified by PCR and by Southern blot of genomic tail DNA (Fig. 1 A) and, because of the 60-bp difference in size, could be clearly discriminated from the TdtS transgene in PCR, Southern blot, and RT-PCR analyses. TdtL mRNA was detected by transgene-

specific RT-PCR in newborn liver and in adult bone marrow, spleen, and thymus (Fig. 1, C and D).

**TdtL Protein Is Detectable in the Nucleus of Lymphoid Cells.** Because there was no prior evidence for TdtL protein expression in vivo, we screened our TdtL Tg mouse lines by immunofluorescent staining of LPS-stimulated splenocytes, the method that previously yielded the greatest abundance and most easily detected Tg protein in our TdtS Tg lines. Using this approach, TdtL protein was detected in 7 of our 10 independent TdtL Tg lines, three of which (lines I, B, and H) were chosen for detailed study. By immunofluorescence microscopy, TdtL Tg B cells appeared to express TdtL in the nucleus similarly to the nuclear staining seen in TdtS Tg B cells (line V; Fig. 2). These data are in direct contrast to in vitro expression data of Bentolila



**Figure 2.** Immunofluorescence of Tdt Tg splenocytes by fluorescence microscopy. Immunofluorescence of LPS-activated adult spleen B cells from (A) non-Tg LM, (B) line V (TdtS), (C) line I, (D) line B, and (E) line H (TdtL) Tg mice, stained for cytoplasmic  $\mu$  chains (red) and Tdt (green). All Tg lines exhibit nuclear staining for Tdt. Original magnification indicated by bar inset.



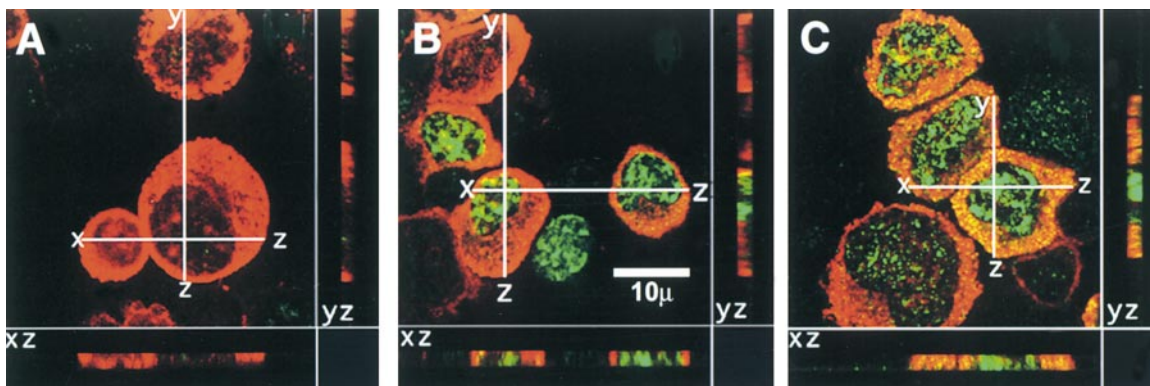
**Figure 3.** Three independent lines of TdtL mice exhibit different levels of Tdt expression as determined by measuring the intensity of nuclear immunofluorescence. The level of fluorescence of individual fetal liver cells was calculated as explained in Materials and Methods and is expressed as arbitrary units on the y axis. The standard deviation expressed as error bars on each column represents actual differences in the intensity and size of cell nuclei. Overall, line B stained two- to threefold less than did lines H and I.

et al. (25) who demonstrated by confocal microscopy that TdtL was excluded from the nucleus. In our experiments, the TdtL Tg splenocytes appear to stain more brightly for cytoplasmic Tdt expression than do TdtS cells, but TdtL is not excluded from the nucleus in any of our TdtL Tg lines. Non-Tg LM control splenocytes were negative for Tdt expression in both the nucleus and the cytoplasm after LPS stimulation (Fig. 2). Additionally, we screened for TdtL expression in newborn liver (Fig. 1 B), a tissue in which our TdtS transgene was expressed whereas endogenous Tdt is not (27). We found that TdtL was also expressed in the nucleus of fetal liver cells of TdtL lines B, I, and H, although there were apparent differences in the levels of expression. By measuring the mean fluorescence intensity of TdtL staining by density slicing, we found that H and I expressed

two- to threefold higher levels of Tdt than did line B (Fig. 3). These findings opened the possibility that, if functional, TdtL might affect the development of the adult B cell repertoire as did expression of our TdtS transgene.

To confirm that Tg TdtL enters the nucleus of TdtL Tg B cells, we compared Tdt expression in LPS-stimulated splenocytes from line V (TdtS) Tg mice to those from line I (TdtL) Tg mice by confocal microscopy (Fig. 4, B and C, respectively). Confocal images clearly demonstrate that TdtL can enter the nucleus of lymphoid cells. Again, Tdt can be detected in the cytoplasm of LPS-stimulated TdtL Tg B cells, but Tdt is not excluded from the nucleus. This is also the first demonstration of TdtL protein expression in nontransformed mouse B cells. Non-Tg LM splenocytes do not express detectable levels of nuclear or cytoplasmic Tdt with or without LPS stimulation (Figs. 2A and 4A), and also provide a control for the specificity of the rabbit anti-Tdt Ab. The absence of peripheral expression of Tdt has been described and is shown previously as a footnote in the work of Han et al. in agreement with our data (32–34).

*TdtL Does Not Catalyze N Addition in Fetal Liver but May Reduce TdtS Activity.* Intracellular endogenous Tdt cannot be detected by immunofluorescence in fetal liver; however, trace amounts can be detected by Western blot (Thai, T.-H., and J.F. Kearney, manuscript in preparation). As a consequence, fetal Ig gene rearrangements are normally N-less. TdtS Tg mice generated from a construct similar to the TdtL transgene exhibited N addition in fetal Ig gene rearrangements (27), so we assayed fetal and newborn liver of TdtL Tg mice for N addition to Ig gene rearrangements (Fig. 5). Of three TdtL Tg mouse lines that expressed TdtL protein, as demonstrated by immunofluorescence, none displayed significant N addition in fetal liver. V<sub>H</sub>7183 Ig rearrangements from TdtL Tg fetal and newborn livers were identical to those of non-Tg LM mice with respect to N addition, palindromic (P) nucleotide addition, and nibbling. Previously published sequences from the TdtS Tg line V contained adult-like N addition (more



**Figure 4.** Immunofluorescence of non-Tg TdtS and TdtL splenocytes by fluorescence confocal microscopy. Confocal microscopy of LPS-activated spleen B cells stained for cytoplasmic  $\mu$  chains (red) and Tdt (green) from (A) non-Tg LM, (B) line V (TdtS), and (C) line I (TdtL) Tg mice. In each panel the x-slice is shown in the large box, with the corresponding yz and xz sections displayed in the rectangles on the right and bottom, respectively, of each panel. Nuclear localization of Tg TdtS and TdtL is discernible in B and C, although in C the yellow granular staining indicates that TdtL is also found in the cytoplasm. Original magnification indicated by bar inset.

than 1 nucleotide at this junction) in 90% of  $V_H$ -D junctions and 45% of D- $J_H$  junctions of  $V_H$ 7183 family H chain rearrangements as early as day 16 of gestation (27). Strikingly, when we crossed TdtS Tg line V mice with TdtL Tg line I mice, only 50% of  $V_H$ -D junctions and 13% of D- $J_H$  junctions of  $V_H$ 7183 H chain rearrangements from day 16 fetal liver contained N addition of more than 1 nucleotide, indicating that expression of TdtL protein may affect TdtS activity or expression. The average number of N nucleotides added per junction was similar between line V and line V × I.

*TdtL/TdtS Double Tgs Display More Homology-directed Joining and Less N Addition in Fetal Liver than do TdtS Tg Mice.* To investigate the possible downregulation of TdtS activity by TdtL, we examined DFL16.1- $J_H$ 1 rearrangements from day 16 fetal liver (Table I). D- $J_H$  rearrangements allow for unambiguous assignment of N nucleotide

status because we know the definitive boundaries of the DFL16.1 and  $J_H$ 1 gene segments. N addition occurs in 36% of the D- $J_H$  sequences amplified from TdtS line V fetal liver, but in two of the TdtL/TdtS double Tg lines (V × I and V × H) N addition is only found in 7–10% of D- $J_H$  sequences (Table I). V × B does not exhibit such a striking reduction most likely because TdtL expression appears to be two- to threefold less in line B (and also has the highest copy number, which may interfere with expression) than in lines I and H. At day 16 of gestation, even in line V, the N additions were not very long. 10 of 12 D- $J_H$  sequences from line V that contained N addition contained only one or two N nucleotides. However, if one assesses the number of sequences that utilize short sequence homologies (based on the number of overlapping nucleotides that could have originated from either the D or the  $J_H$  germline segment), TdtS line V is strikingly different from the TdtL/TdtS dou-

Gene	Clone	93	VH	V/N?	N	DH	N	JH	102	DH	JH	IF	RF
TG+ B	9A	BB12.01	GCAAGA	C		ATTACTACGGTAGTAG		TCGTATGGACTAC		FL16.1	4	Y	1
	7A	BB12.03	GCAAGA	C		ATGGTA		ACTA TCGTATGGACTAC		SP2.5,7,8	4	Y	1
	21B	BB12.05	GCAAG					TCGTATGGACTAC			4	Y	
	23B	BB13.03	ACAA			TTATTACTACGGTAGTAGCTAC		GCTATGGACTAC		FL16.1	4	N	
	7A	BB13.05	GCAAGA	C		ACAGCTCGGGC		TAC TATGCTATGGACTAC		ST4	4	Y	1
TG+ H	6A	BB13.06	GCAAGA			GTATGGTAAC		TAC TATGCTATGGACTAC		SP2.8	4	N	
	1A	485.01	GCAAGAC			ACTATGATTACG		AC TATGCTATGGACTAC		SP2.2	4	Y	1
	21B	485.02	GCAAGA	G		ATTACTACGGTAGTAG		CTA TCGTATGGACTAC		FL16.1	4	Y	1
	22B	486.05	GCAAG	GC		TTACTACGGTAGTAGCTACG		TACTATGCTATGGACTAC		FL16.1	4	Y	2
	1A	486.06	GCAAGA			CATATTA	C	TTTGCTTAC		FL16.1E	3	Y	1
TG+ I	1A	4842.37	GC		G	TTACTACGGTAG		TCGTATGGACTAC		FL16.1	4	N	
	1A	4842.38	GCAAGAC			ATGGTAAC		TAC TATGCTATGGACTAC		SP2.5	4	Y	1
	16A	4842.39	GCAAG	GGATC		ACTACGGTAGTAG		CTAC		FL16.1	4	Y	1
	7A	4842.42	GCAAGA			TTACTACGGTAGTA		TTACTATGCTATGGACTAC		FL16.1	4	N	
	9A	4842.43	GCAAGA	C		ACTACGGTAGTAGC		TAC TATGCTATGGACTAC		FL16.1	4	Y	1
	2A	4842.44	GCAAGA			(GATG) (CTATAGG) CACAA (CGTA)		TTACTATGCTATGGACTAC	SP2.9,10/FL16.1	4	Y	2,3	
	2A	4842.45	GCAAGA	G		AGGTACG		AC TATGCTATGGACTAC		SP2.10	4	N	
	2A	4842.47	GCAAGA	G		ATTGCTACGGCT		A TTACTATGCTATGGACTAC		FL16.2	4	Y	1
	6A	4842.48	GCAAGA			TCTACAATGATTACG		AC TATGCTATGGACTAC		SP2.2	4	N	
	TG- LM	BB9.03	GCAAG									4	N
BB9.06		GCAAGA	CAT			AACTGGG		AC TATGCTATGGACTAC		Q52	4	Y	3
6A		BB11.01	GCAAGA			TATTACTACGGTAGTAGC		TAC TATGCTATGGACTAC		FL16.1	4	Y	1
21B		BB11.02	GCAAG			TGATGGTACTA		TATGCTATGGACTAC		SP2.9	4	N	
5A		BB11.06	GCAAGA	C		ATGGTAAC		TAC TATGCTATGGACTAC		SP2.5,7,8	4	Y	1
1A		490.01	GCAAGAC			ACTATAGGTAC		TACTATGCTATAGACTAC		SP2.10	4	Y	1
1A		490.02	GCAAG			GTA		ACTATGCTATGGACTAC			4	N	
7A		490.03	GCAAGA			CAACTGGGACG		TCGTATGGACTAC		Q52	4	Y	2
22B		490.04	GCAAGA			TTACTACGGTAGTAG		CTATGCTATGGACTAC		FL16.1	4	N	
7A		490.07	GCAAGA	C		ATGGTAAC		TAC TATGCTATAGACTAC		SP2.5	4	Y	1
DBL TG VxI	7A	490.08	GCAAGA			(CATTACTACGG) (TACTAT)		CTATGCTATGGACTAC		FL16.2/SP2.4	4	Y	1,2
	10A	490.09	GCAA	CA		TTTATTACTACGGTAGTAGC		GCTATGGACTAC		FL16.1	4	Y	1
	9A	490.11	GCAAGA			TCTATGATGGTTAC		TAC TATGCTATGGACTAC		SP2.9	4	N	
	2A	490.12	GCAAGA	G		ATGATGGTTACT		A TTACTATGCTATGGACTAC		SP2.9	4	Y	1
	4847.05	GCAAGA	AAAA			GGTAACTACG		ACTGGTACTTCGATGTC		SP2.5	1	N	
	4843.01	GCAAG	GCAA			ACTATGATTACGAC		TAGCTACTGGTACTTCGATGTC		SP2.2	1	Y	2
	2A	4843.49	GCAAGA	G		(ATAG) (TTACTACGGTAG)	C	TACTATGCTATGGACTAC	16.1E/FL16.1	4	Y	1,1	
	2A	4843.50	GCAAGA	GATCANT		TCTACTGTAGGTNC		TACTATGCTATGGACTAC		SP2.10	4	Y	1
	9A	4843.51	GCAAG	GCT		TAGTAGC		TACTATGCTATGGACTAC		FL16.1	4	Y	1
	2A	4843.52	GCAAGA	G		(ATAG) (TTACTACGGTAG)		TTACTATGCTATGGACTAC	16.1E/FL16.1	4	Y	1,1	
TG+ V	2A	4843.55	GCAAGA			(GATCACG) (TNTACTATAGGTACG)		ACTATGCTATGGACTAC		SP2.2/2.10	4	Y	1,1
	5A	4843.57	GCAAGA	G		CGGTAGTAGC	GG	ATTACTATGCTATGGACTAC		FL16.1	4	Y	2
	1A	V1.01	GCAAGACA		GAG	(AGGG) (TACTATGATTACGA)	TAC	TGGTTT	Q52/SP2.2	3	N		
	1A	V1.02	GCAAG		G	ATAGGTACGA	G	GCTATG	SP2.10	4	N		
	1A	V1.13	GCAAGAC		GGG	ATGGTA		ACTATGCTATG	SP2.8	4	Y	1	
	1A	V4.14	GCAAGA		AGGA	ATGGTAACTACG	T	GTTT	SP2.8	3	Y	1	
	2A	V1.07	GCAAGA	gatAGAG		TACTACGGTAGTAGCTACG	TTT	TACTATGCTATG	FL16.1	4	N		
	2A	V1.09	GCAAGA	gatTA		GATTACGACG		ATGCTATG	SP2.2	4	N		
	2A	V1.11	GCAAGA	ga		TATGG	GTC	TACTATGCTATG	SP2.4	4	N		
	2A	V4.15	GCAAGA	ga		AGTA		TGGTTT	FL16.1	3	Y	2	
6A	V1.05	GCAAGA	tGG		CTATGGTAA	TTT	TG	SP2.5	4	N			

**Figure 5.** Ig H chain rearrangements from fetal or neonatal liver genomic DNA.  $V_H$ 7183- $J_H$  rearrangements were amplified, cloned, and sequenced from whole liver of TdtL Tg line B, line H, line I, and corresponding non-Tg LM mice. The ages of the mice at the time of sacrifice were as follows: line B, newborn (<24 h after birth); line H, newborn; line I, day 18 of gestation; line V × I, day 16 of gestation. LM sequences originated from newborn non-Tg LMs of line B and line H and have been combined in the table. For  $V_H$ 81X (7183.1A) the entire coding sequence is known, but for the remaining  $V_H$ 7183 genes, each  $V_H$  was truncated after Arg codon 94 (AGA) and any non-V, non-D nucleotides placed in the V/N column. Nucleotides in the V/N column that likely originated from the V segment (based on repeated occurrence in multiple clones) are in lowercase letters. Parentheses delineate the boundaries of D-D fusions. P nucleotides are underlined. Nucleotides that may originate from either the germline D or germline  $J_H$  segments appear in italic. IF, in frame; RF, reading frame; DBL, double. Amplifications were performed with a  $J_H$ 4 primer. Sequence data are available from EMBL/GenBank/DDJB under accession nos. AF318407–447.



**Table I.** D-J<sub>H</sub> Junctions in TdtS, TdtL, and TdtS/TdtL Tg Mice

Mouse line	Total seq	Unique seq	Seq with N	D-J overlap				
				Seq without N	4nt	2nt	1nt	0nt
	<i>n</i>	<i>n</i>	<i>n</i> (% total)	<i>n</i> (% total)	<i>n</i> seq (% seq without N)			
V-TdtS ( <i>n</i> = 1)	33	28	12(36)	21(64)	5(24)	1(5)	4(19)	11(52)
V × B ( <i>n</i> = 2)	28	22	9(32)	19(68)	6(32)	1(5)	6(32)	6(32)
V × I ( <i>n</i> = 2)	28	14	2(7)	26(93)	14(54)	3(11)	1(4)	8(31)
V × H ( <i>n</i> = 1)	31	13	3(10)	28(90)	17(61)	1(4)	3(11)	7(25)
H-TdtL ( <i>n</i> = 1)	10	5	0(0)	10(100)	6(60)	1(10)	1(10)	2(20)
non-Tg LM ( <i>n</i> = 1)	4	2	0(0)	4(100)	3(75)	1(25)	0(0)	0(0)

Summary of DFL16.1-Jh1 joins from day 16 fetal liver genomic DNA. Sequence (seq) with number (and percentage) that were found to contain N nucleotide addition ( $\geq 1$ nt). In parentheses in the mouse line column is the number of mice from which liver DNA was prepared. 4nt of overlap indicates possible homology-directed recombination. 0nt indicates no overlap and that homology-directed recombination was not likely used. No sequences were found that contained an overlap of 3nt. Primary data from which this table was derived are available from EMBL/GenBank/DDJB under accession nos. AF318759–768, and AF318771–894.

ble Tgs. As has been shown previously (35, 36), homology-directed recombination is a feature of fetal Ig gene rearrangement, occurring normally in the absence of Tdt. In TdtL/TdtS double Tgs, the amount of homology-directed recombination is consistently intermediate between TdtS line V alone, which has very little homologously derived D-J<sub>H</sub> joining, and the high frequency of homologously derived D-J<sub>H</sub> joins that occurs in normal fetal liver (35, 36), or in our TdtL Tg and non-Tg LM mice. This observation further supports the idea that TdtL is downregulating TdtS activity. One caveat of these data is that when one amplifies D-J<sub>H</sub> rearrangements from fetal liver, recurrent sequences are found because there is less diversification by which to verify independent sequences. The numbers in Table I are based on the total number of sequences we amplified because it is very likely that many sequences do in fact recur in independent cells at the level of D-J<sub>H</sub> joining. However, we also calculated the frequency of N addition and N-less short sequence homologies when only distinctly independent sequences are counted. In this case, 57% of line VD-J<sub>H</sub>S lack N addition and only 29% of those contain short sequence homologies (4 nucleotide [nt], 2nt, and 1nt combined). In contrast, 59, 86, and 69% of D-J<sub>H</sub>S from V × B, V × I, and V × H lacked N addition, respectively. Of those lacking N addition, 54, 50, and 50% of V × B, V × I, and V × H D-J<sub>H</sub>S contain short sequence homologies. Thus, even when repetitive sequences are omitted, the trend holds true that TdtL and TdtS transgenes together result in less N addition and more homology-directed recombination than does TdtS alone. Furthermore, the average length of D-J<sub>H</sub> joins from TdtL/TdtS Tg mice was three nucleotides shorter than the average length of D-J<sub>H</sub> joins from TdtS line V alone. Because we observed previously that CDR3 lengths from fetal liver of TdtS Tg mice were not significantly different from those of non-Tg LMs, it is likely that these differences will be either compensated by nibbling during V<sub>H</sub>-D rearrangement or that these

longer junctions will be selected against after V<sub>H</sub>-D rearrangement is complete.

*TdtS, but Not TdtL, Restores N Addition in Tdt Knockout Mice.* To exclude the possibility that TdtL is simply not active in fetal liver because of a microenvironmental requirement, we tested its ability to add N nucleotides in bone marrow, the usual site of N nucleotide addition in B cells of adult mice. TdtS or TdtL Tg mice were crossed to endogenous Tdt-deficient (Tdt<sup>-/-</sup>) mice (28) for two generations. First, we confirmed that in the TdtS transgene lines (U and V), TdtS is sufficient to catalyze N addition to Ig H chain rearrangements (27) in the absence of endogenous Tdt (Fig. 6). Although fetal CDR3 lengths were maintained in TdtS fetal liver Ig H chain rearrangements, CDR3 lengths were increased to adult proportions in TdtS/Tdt<sup>-/-</sup> bone marrow. N addition was also observed in  $\kappa$  and  $\gamma$  L chain rearrangements of TdtS/Tdt<sup>-/-</sup> bone marrow (data not shown). V<sub>H</sub>7183 family Ig H chain gene rearrangements were amplified from bone marrow of TdtL Tg mice of lines B, H, and I on the Tdt<sup>-/-</sup> background. The H chain rearrangements isolated from the TdtL/Tdt<sup>-/-</sup> mice did not contain N addition (Fig. 6) nor did L chain rearrangements from TdtL/Tdt<sup>-/-</sup> mice contain N addition (data not shown). Thus, an adult bone marrow context for TdtL expression does not facilitate the addition of N nucleotides by TdtL.

*Tdt Deficiency Increases the T15 Id<sup>+</sup> Protective Anti-PC Response.* The T cell-independent immune response to PC is dominated by the protective T15 Id Ab, encoded by an N-less Ig H chain rearrangement (37, 38). The T15 anti-PC response proved to be an effective functional readout for TdtS activity in our TdtS Tg mice (27). Because the premature N addition results in a loss of T15 Abs expressing canonical CDR3 joins in TdtS Tg mice, we hypothesized that decreased, or absent, N addition might result in an increase in T15 Abs in the PC response of Tdt<sup>-/-</sup> or TdtL Tg mice. We immunized Tdt<sup>-/-</sup> mice, TdtL/Tdt<sup>-/-</sup>,

Gene	Clone	93	VH	V/N?	N	DH	N	JH	102	DH	JH	IF	RF
TG+	11B	2745.29	GCAAGA	c				ATGCTATGGACTAC				4	Y
	1A	2743.03	GCAAGACA		CTCCT	TATGATGGTTACT		GCTTAC		SP2.9	3	N	
	1A	2745.06	GCAAGA		GGG	GATGGTTACT	GGACCCCT	TTACTATGCTATGGACTAC		SP2.9	4	Y	1
	1A	2745.27	GCAAGAC		TCG	(CTATGAT)CG(CGGG)		TACTTTGACTAC		SP2.2/ST4	2	N	
	23B	2745.09	ACAAGA			(GTGG)(ACTATGGTAAAC)	C	TTGACTAC	FL16.1E/SP2.5	2	Y	1	
	3B	2745.17	GCAAGA	CT		(CTGGG)GTA(TATTACTA)		TGCTATGGACTAC	Q52/FL16.1	4	N		
	5A	2745.03	GCAAGA	ca		(CCTACTATAGTAAAC(T)TACGTCTAC)GT		ACTACTTTGACTAC	SP2.7/FL16.2	2	Y	3	
TG+	5A	2745.21	GCAAGA	ca		TGGGACG	AGG	GCTATGGACTAC		Q52	4	Y	2
	7A	2745.05	GCAAGA	catgC		GATCTATGATGGTTAC	GAGC	GGTACTTCGATGTC		SP2.9	1	Y	1
	11B	2603.03	GCAAGA	GGG		TATTACTACGGTAGTAGCT	T	CTACTGGTACTTCGATGTC		FL16.1	1/4	Y	1
	1A	2603.04	GCAAGACA		A	(GGGA)(ATGGTTACTAC)	CC	CTATGCTATGGACTAC		Q52/SP2.9	4	N	
TG+	6A	2603.3b			CACAGC	(CTATAGTAACTAC)GTGGAC(GTGG)		GGTTTGCTTAC	SP2.5/FL16.1E	3	N		
	7A	2603.16	GCAAGA	caGGCAC		TCTATGATGGTTACTACGT	GAGA	GCTATGGACTAC		SP2.9	4	Y	1
	7A	2603.18	GCAAGA	caTC		GTAGTAGCTAC	GAT	TACTATGCTATGGACTAC		FL16.1	4	Y	1
	1A	2553.14	GCAAGA			CTACTATAGTA		ACTGGTACTTCGATGTC		SP2.5	1	N	
LM	21B	2553.21	GCAAGA	C		ATTACTACGGTAGTA		GCTAC TGSTACTTCGATGTC		FL16.1	1	Y	1
	21B	2553.20	GCAAGA			TATGATTACG	C	GCTATGGACTAC		SP2.2	4	N	
	6A	2553.07			A	TATGATGGT		TACTAC TTTGACTAC		SP2.9	2	N	
	7A	2553.02	GCAAGA			CTATAGT		ACTAC		SP2.10	2	Y	3
	7A	2553.06	GCAAGA	cat		TTACTACGGTAGTAGC		TGGACTAC		FL16.1	4	Y	3
	7A	2553.10	GCAAGA	ca		CTATA		TTGCTTAC		SP2.10	2	Y	2
	TG+	5B	6266.06	GCAAGA	C		ATGATTAC		TACTATGCTATGGACTAC		SP2.2	4	Y
6A		6266.08	GCAAGA			TATAGCTACG		AT TACTATGCTATGGACTAC		SP2.10	4	Y	1
6A		6266.14	GCAAG	G		TACTACGG		TATGCTATGGACTAC		FL16.1.2	4	N	
9A		6266.13	GCAAGA	catg		TCTGTATGGTA		ACTA TGCTATGGACTAC		SP2.8	4	Y	1
18A		6266.12	ACAAGA	G		ATTACTACGGTAGTAG		CTA TGCTATGGACTAC		FL16.1	4	Y	1
TG+	8B	6266.05	TTGAGA			CTATGATTACGA		CTACTGGTACTTCGATGTC		SP2.2	1	N	
	7B	6052.04	GCAAGA	GA		TACTATAGTAA		CTACTGGTACTTCGATGTC		SP2.5	1	N	
	22B	6052.01	GCAAG	gc		TTTATTACTACGGTAGTA		GGTACTTCGATGTC		FL16.1	1	Y	1
	23B	6052.09	ACAAGA	gCC		ATTACTACGGTAGTAG		ACTGGTACTTCGATGTC		FL16.1	1	Y	2
	24B	6052.14	GCAAGA	g		ATGGTA		ACTGGTACTTCGATGTC		SP2.X	1	Y	1
	24B	6052.07	GCAAGA	g		ATGATTACGA		CTACTGGTACTTCGATGTC		SP2.2	1	Y	1
	TG+	VH11	2486.40	TCAAGA			TACTACGGTAGTAG		CTAC TGSTACTTCGATGTC		FL16.1	1	Y
1A		2486.13	GCAAGACA			TG GTTACGAC		GCTTAC		SP2.4	3	Y	1
1A		2486.42	GCAAGAC			AT TACTACGGTAGTAGC		TTTGCTTAC		FL16.1	3	Y	1
1A		2486.44	GCAAGAC			AT GGTA		CTAC TATGCTATGGACTAC		SP2.X	4	Y	1
23B		2486.34	ACAAGA	gAC		(AGCT)(CAG)	G	CTATGCTATGGACTAC		FL16.1E/Q52	4	Y	1
5A		2486.36	GCAAGA	c		ATTACTACGGTAGTAGC		TTTGCTTAC		FL16.1	3	Y	1
7A		2486.27	GCAAGA	catg		GTTACTACG		ACTAC		SP2.9	2	Y	1
7A		2486.41	GCAAGA			GATCTACTATGATTACGAC		GCTTAC		SP2.2	3	N	
11B	2486.09	GCAAGA			CTACTATGATTACGAC		CTGGTTGCTTAC		SP2.2	3	N		

**Figure 6.** Ig H chain rearrangements from bone marrow genomic DNA of TdtS/Tdt<sup>-/-</sup> or TdtL/Tdt<sup>-/-</sup> mice. V<sub>H</sub>7183-J<sub>H</sub> rearrangements were amplified, cloned, and sequenced from whole bone marrow of TdtS Tg lines U, V (26) for TdtL Tg lines B, H, or I adult mice on an endogenous Tdt-deficient background. Thus, in this figure the non-Tg LM is actually a Tdt<sup>-/-</sup> mouse. All mice were at least 8 wk of age at time of killing. Some amplifications were performed with a mixture of J<sub>H</sub> primers, others with only a single J<sub>H</sub> primer. IF, in frame; RF, reading frame. Sequence data are available from EMBL/GenBank/DDBJ under accession nos. AF31448–488.

TdtS/Tdt<sup>+/+</sup>, and normal LM (Tdt<sup>+/+</sup>) mice with a heat-killed R36A pneumococcal vaccine, as described previously (27). Table II shows that Tdt<sup>-/-</sup> and TdtL/Tdt<sup>-/-</sup> mice exhibit higher overall levels of total anti-PC Abs as well as the T15 and other Id<sup>+</sup> Abs in serum ELISA assays of their anti-PC response. TdtL mice on the BALB/c background exhibit similar T15 responses to normal BALB/c mice. These results indicate that the absence of Tdt results in the generation of more T15 B cells, possibly because the window of time for their generation is extended. Additionally, the T15 Id requires an aspartic acid residue at position 95 involving the V<sub>H</sub>-D junction of V<sub>H</sub>S107.1 Ig H chain that is encoded by the V<sub>H</sub>S107.1 segment. TdtL has little effect on the PC response, regardless of whether endogenous Tdt is present or absent (Table II), in contrast to TdtS that causes a drastic decrease in the T15 response (27).

## Discussion

In this work, we address the role of the two splice variants of Tdt in vivo. We independently amplified, cloned, and sequenced TdtS and TdtL cDNA to confirm the expression of these two variants and used them to help resolve the contradictions in the literature regarding whether the

long splice variant catalyzes N addition or not (19, 25). The confirmatory test of a gene's function is to complement a defect with that gene, and we have taken advantage of the Tdt<sup>-/-</sup> mouse model to test the function of our TdtS and TdtL transgenes (28). Our results indicate that TdtL does not catalyze N addition in vivo, but that TdtS alone is sufficient to complement the loss of the endogenous Tdt gene. That the failure of TdtL to catalyze N addition may be partially due to its inability to translocate to the nucleus (25) is called into question by our data, which clearly show TdtL expression in the nucleus. This is an important point because any physiological role for TdtL in V(D)J recombination obviously requires its presence in the nucleus.

The literature contains many examples of genes for which mRNA transcripts can be variably spliced, resulting in structurally and functionally distinct protein products. Several immunologically relevant genes have been described for which multiple mRNA splice variants have been isolated. In the case of Tdt, the inclusion or exclusion of a single exon results in two splice variants. Among the nuclear factor (NF)-κB/IκB (39), Oct (40), and Ikaros (41) transcription factors, and the Bcl-2 survival gene family (42), ranges of 2–10 alternatively spliced mRNA products have been described, which carry out distinct roles in regu-



**Table II.** Anti-PC Responses of TdtL, TdtS, and Tdt<sup>-/-</sup>

Genotype	Mice	IgM	PC	Serum Abs			
				TC68*	TC139*	AB1.2*	GB4.10*
	<i>n</i>	$\mu\text{g/ml}$ (log 10 SD)	$\mu\text{g/ml}$ (log 10 SD)	$\mu\text{g/ml}$ (log 10 SD)	$\mu\text{g/ml}$ (log 10 SD)	$\mu\text{g/ml}$ (log 10 SD)	$\mu\text{g/ml}$ (log 10 SD)
Tdt <sup>-/-</sup>	20	477.8 (2.5)	149.3 (2.0)	159.3 (1.9)	120.8 (2.0)	121.8 (2.0)	162.8 (2.1)
TdtL I <sup>+</sup> /Tdt <sup>-/-</sup>	5	475.3 (2.3)	127.5 (1.6)	140.4 (1.7)	109.1 (1.5)	119.7 (1.4)	148.9 (1.7)
Tdt <sup>+/+</sup>	13	414.7 (2.2)	83.6 (1.6)	92.9 (1.8)	74.3 (1.7)	79.7 (1.7)	86.4 (1.7)
TdtL I <sup>+</sup> /Tdt <sup>+/+</sup>	11	437.5 (2.1)	79.9 (1.7)	81.0 (1.8)	60.6 (1.6)	60.5 (1.7)	77.0 (1.9)
TdtS T <sup>+</sup> /Tdt <sup>+/+</sup> ‡	5	416.8 (2.2)	40.0 (1.3)	38.4 (1.9)	20.4 (1.2)	20.0 (1.3)	22.5 (1.4)
TdtS V <sup>+</sup> /Tdt <sup>+/+</sup> §	5	426 (1.3)	178 (1.7)	160 (1.8)	ND	19 (1.1)	15 (0.95)

Adult mice of each genotype were immunized with heat-killed R36A. Serum anti-PC and Id (\*) titers were measured by quantitative ELISA as described previously (reference 27). Each column represents the amount of total serum IgM, total anti-PC antibody ( $\mu\text{g/ml}$ ), and in the columns marked (\*) serum antibody, in  $\mu\text{g/ml}$  detected by the following Id markers. TC68 is specific for all V<sub>H</sub>S107.1 heavy chains; TC139 is specific for V $\kappa$ 22, the L chain expressed by T15 Id<sup>+</sup> Ab. AB1.2 is specific for the T15 CDR3-associated Id; and GB4.10 is specific for the T15 D-J<sub>H</sub> junction Id. Values are given as means of each group tested.

‡Values for the line T(TdtS) on the same background as line I mice in this experiment.

§The values for line V(TdtS) on a BALB/c background from reference 27.

lation of cellular pathways and functions. This regulation is usually achieved by altering, temporally or proportionately, levels of various splice products generated. For some differentially spliced genes described, distinct functions have been identified for the protein products of the correspondent mRNA splice variants. For Tdt, the specific function of the TdtL splice variant has not been revealed.

Tdt is a unique protein initially described decades ago; its enzymatic function was first studied biochemically (43), and a biological function was later discovered in lymphoid cells (3). More recently, two splice variants for Tdt were described (18). The reported full-length sequence of one (of two) original mouse Tdt cDNA clones isolated from a mouse lymphoma RL-12 library turned out to be the TdtL splice variant (19). Terminal transferase activity was clearly demonstrated in lysates from COS cells that had been transfected with the original TdtL clone. However, recent testing of one of the original TdtL clones, in parallel with an independently isolated TdtL clone, indicated that TdtL is excluded from the nucleus (25). Although this discrepancy between the two studies might be explained by the putative failure of TdtL to translocate to the nucleus, results with lysates from COS cell transfectants cannot. In these assays, Bentolila et al. (25) reported that the two TdtL clones conferred negligible activity and were at least 10–15 times less efficient than the TdtS clone. This seemingly contradicts the original Koiwai et al. (19) COS cell transfection data, although a direct comparison of all cDNAs used would be required to draw any useful conclusions about the function of TdtL. Thus, to date, results regarding the function of TdtL are contradictory, inconclusive, and limited to in vitro assays of transfected cells.

Therefore, to elucidate a possible function for TdtL and ascertain unequivocally in vivo whether TdtL can add N

nucleotides to rearranging Ig or TCR genes, we generated TdtL Tg mice. Using a similar construct bearing TdtS cDNA, we demonstrated previously nuclear expression of TdtS protein and N addition to Ig rearrangements (27). Consequently, this construct is well suited for testing the expression and capacity for N addition of TdtL. Nuclear TdtL expression in mouse B cells was demonstrated in LPS-stimulated splenocytes of seven independent TdtL Tg lines, similar to the pattern of expression in spleen cells from our TdtS Tg mice. This is the first demonstration of TdtL protein expression in mouse B cells and in a nuclear location. TdtL appears to be retained in the cytoplasm at a higher level than TdtS protein in our LPS-cultured cells, but TdtL is clearly translocated to the nucleus of B cells, where it presumably has access to rearranging Ig genes.

To determine whether nuclear-expressed TdtL can add N regions to rearranging Ig genes in vivo, we sequenced Ig gene rearrangements from adult bone marrow of TdtL Tg mice crossed with endogenous Tdt<sup>-/-</sup> mice. Ig H and L chain rearrangements from both Tdt<sup>-/-</sup> and TdtL/Tdt<sup>-/-</sup> mice contained no N addition; however, TdtS/Tdt<sup>-/-</sup> Ig H and L chain rearrangements contained convincing N addition. Thus, TdtL was incapable of adding N regions when it was the only splice variant of Tdt available. These results have also been corroborated by primer extension assays in vitro as a measure of Tdt catalytic activity (Thai, T.-H., and J.F. Kearney, manuscript in preparation). However, in Tdt Tg mice, expression of TdtL in conjunction with TdtS resulted in a decrease in N addition in comparison to N addition catalyzed by TdtS alone. This effect of TdtL/TdtS Tg coexpression was observed in complete H chain rearrangements and was further verified in D-J<sub>H</sub> rearrangements from three independent crosses of TdtL Tg with TdtS Tg mice. Several possible mechanisms for a

downregulation of N addition can be postulated. For example, there may be competition between the two proteins for binding to DNA and nucleotides. The formation of a TdtS–TdtL complex could inactivate TdtS or inhibit TdtS translocation to the nucleus. Alternatively, the reduced N addition observed may be the result of exonuclease activity which is associated with the TdtL isoform (Thai, T.-H., and J.F. Kearney, manuscript in preparation).

We have shown previously that premature expression of TdtS has functional consequences that alter the adult anti-PC Ab repertoire in BALB/c mice (27). Expression of TdtS in fetal life increased diversity in the fetal repertoire and concomitantly decreased the window of opportunity for generation of important germline-encoded specificities such as T15, the protective Id in the immune response against virulent pathogens like *S. pneumoniae*. It follows that the T15 response provides a readout for repertoire alterations associated with TdtL activity or a Tdt<sup>-/-</sup> phenotype. Immune responses against complex Ags that stimulate large polyclonal B cell responses appear to be intact in Tdt<sup>-/-</sup> mice (44), but T cell-independent, canonical responses in B cells have not been examined previously in Tdt-deficient mice. Here, we demonstrate that there is a phenotypic alteration in the immune response of Tdt<sup>-/-</sup> mice when immunized with heat-killed *S. pneumoniae*. In contrast to TdtS mice that exhibit normal or reduced levels of anti-PC Abs (Table II) comprised mostly of nonprotective, non-T15 clones (27), Tdt<sup>-/-</sup> mice exhibit higher than normal levels of both anti-PC Abs and T15 Id<sup>+</sup> Abs. This is the first demonstration of a convincing phenotypic alteration in the B cell repertoire of Tdt<sup>-/-</sup> mice. Several explanations for this phenotype are possible: (a) the adult B cell repertoire may be less diverse in general (more fetal-like) because of decreased junctional diversity caused by a lack of Tdt; (b) the window of opportunity for generation of germline-encoded Ids may be extended into adult life, perhaps indefinitely, by the lack of Tdt; and (c) the restriction of CDR3 lengths required to produce functional Abs may lead to the generation or selection of more clones in which nibbling is reduced in the absence of Tdt. In each case, there is an increase in the likelihood of preserving aa at the V<sub>H</sub>-D and D-J<sub>H</sub> critical to forming the T15 Id.

The TdtL mice produce anti-PC and anti-T15 Abs at levels greater than those found in non-Tg LM mice. CDR3 lengths are restricted in Ig rearrangements, indicating that junctional diversity must be regulated. This regulation may be accomplished during V(D)J recombination, by selection of optimal H/L chain pairing, by selection after the Ig goes to the cell surface, or by a combination of these. We propose that regulation of CDR3 length is accomplished in part during V(D)J recombination by the activities of both TdtS and TdtL.

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