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*J Immunol* 2004; 172:5478-5488; doi: 10.4049/jimmunol.172.9.5478

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Mutational Analysis of Terminal Deoxynucleotidyltransferase-Mediated N-Nucleotide Addition in V(D)J Recombination

Jamie A. E. Repasky, Elizabeth Corbett, Cristian Boboila, and David G. Schatz

The addition of nontemplated (N) nucleotides to coding ends in V(D)J recombination is the result of the action of a unique DNA polymerase, TdT. Although N-nucleotide addition by TdT plays a critical role in the generation of a diverse repertoire of Ag receptor genes, the mechanism by which TdT acts remains unclear. We conducted a structure-function analysis of the murine TdT protein to determine the roles of individual structural motifs that have been implicated in protein-protein and protein-DNA interactions important for TdT function in vivo. This analysis demonstrates that the N-terminal portion of TdT, including the BRCA-1 C-terminal (BRCT) domain, is not required for TdT activity, although the BRCT domain clearly contributes quantitatively to N-nucleotide addition activity. The second helix-hairpin-helix domain of TdT, but not the first, is required for activity. Deletional analysis also suggested that the entire C-terminal region of TdT is necessary for N-nucleotide addition in vivo. The long isoform of TdT was found to reduce N-nucleotide addition by the short form of TdT, but did not increase nucleotide deletion from coding ends in either human or rodent nonlymphoid cells. We consider these results in light of the recently reported structure of the catalytic region of TdT. The Journal of Immunology, 2004, 172: 5478–5488.

The enormous diversity in Ig and TCR Ag binding sites is created during assembly of their genes from individual V, D, J, and K gene segments in a process called V(D)J recombination (1). This diversity is generated at multiple levels. Combinatorial diversity arises from the use of different combinations of V, D, J and K segments. Superimposed on this is junctional diversity, created by the imprecise joining of the gene segments during V(D)J recombination. Finally, Igs and TCRs are heterodimers of rearranged receptor genes, with both subunits contributing to Ag recognition, thus creating an additional layer of combinatorial diversity.

The first phase of V(D)J recombination begins with binding by the recombination-activating gene 1 (RAG1)3/RAG2 proteins to a pair of recombination signal sequences, followed by double-strand cleavage of the DNA to generate two hairpin sealed coding ends and two blunt signal ends. The second phase (end processing and joining) is performed primarily by the ubiquitously expressed nonhomologous ending joining machinery (DNA ligase IV, XRC4, Ku70, Ku80, DNPK, and Artemis) and results in the formation of a coding joint and a signal joint. Coding joints are typically imprecise, exhibiting both nucleotide deletion and the addition of templated and nontemplated nucleotides. Signal joints are often precise fusions of the two recombination signal sequence, although frequent addition of nontemplated nucleotides to signal joints has been observed for several Ag receptor loci (2–4) and with extrachromosomal recombination substrates (5).

Nontemplated nucleotide (N-nucleotide) addition to coding and signal joints in V(D)J recombination is due almost entirely to the action of the enzyme TdT. TdT is a template-independent DNA polymerase that adds random nucleotides (with a GC bias) to the 3’-hydroxyl of single- or dsDNA (reviewed in Ref. 6). TdT-deficient mice are viable and perform V(D)J recombination normally, although virtually no N-nucleotides are found in the assembled Ig and TCR genes (7, 8). In addition, in the absence of TdT, coding joints show a significant increase in the use of short stretches of microhomology near the ends of the coding segments to mediate joining, suggesting that TdT also influences the joining process (9). How TdT interferes with microhomology-mediated joining is uncertain (reviewed in Ref. 10). TdT is not expressed before birth in the mouse; in the adult it is expressed at most stages of thymocyte development and in pro-B, but not pre-B, cells (reviewed in Ref. 11). Hence, adult TCR and IgH genes, but not Ig L chain genes, routinely contain N-nucleotides.

TdT belongs to the pol X family of polymerases (12), a subgroup of an ancient nucleotidyltransferase superfamily defined by homologies within nucleotide binding domains and active site motifs (13). Although DNA polymerases β (pol β), μ (pol μ), and λ (pol λ), preferentially add nucleotides complementary to those on a template strand, TdT is unique in that it adds nucleotides in an untemplated fashion. Homology among members of the pol X family is not limited to their catalytic center. Additional domains shared by several of these polymerases include BRCA-1 C-terminal (BRCT) domains as well as helix-hairpin-helix (HhH) domains (see Fig. 1A for schematic of the TdT protein). The BRCT domain is a phosphopeptide binding motif (14, 15) that mediates protein-protein interactions and is commonly found in proteins involved in DNA recombination and repair, such as BRCA-1, XRCC4, and DNA-PKcs, catalytic subunit of the DNA-dependent protein kinase; H2, second HhH domain; HhH, helix-hairpin-helix; Kan, kanamycin; N, nontemplated; NLS, nuclear localization signal; pol, polymerase; TdT-FL, full-length TdT, TdTL, long isoform of TdT.

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2 Received for publication June 26, 2003. Accepted for publication February 10, 2004.

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3 Abbreviations used in this paper: RAG, recombination-activating gene; BRCT, BRCA-1 C-terminal; Cam, chloramphenicol; CHO, Chinese hamster ovary; DNAPK, catalytic subunit of the DNA-dependent protein kinase; H2, second HhH domain; HhH, helix-hairpin-helix; Kan, kanamycin; N, nontemplated; NLS, nuclear localization signal; pol, polymerase; TdT-FL, full-length TdT, TdTL, long isoform of TdT.

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DNA ligase IV (16–18). One such BRCT domain was also identified in the N-terminal regions of TdT, pol β, and pol λ (19).

Support is growing for the possibility that TdT is recruited to coding ends during V(D)J recombination by components of the nonhomologous end-joining machinery. The lack of N-nucleotides in the rare CJs found in Ku80−/− and scid mice led to the suggestion that the Ku heterodimer and/or DNAPKcs plays this role (5, 20, 21). Furthermore, an in vitro study demonstrated that the Ku heterodimer interacts with the N-terminal portion of TdT in coimmunoprecipitation experiments (22). In contrast, Mickelsen et al. (23) reported that in gel shift assays, TdT was able to associate with DNAPKcs on DNA ends, but not with the Ku heterodimer. DNAPKcs could modulate the activity of TdT in vitro with respect to the length and composition of nucleotide addition (23), suggesting that DNAPKcs directs and controls TdT activity during V(D)J recombination. Perhaps most intriguing is a recent biochemical study demonstrating that TdT and pol β can be stably recruited to DNA by virtue of interactions with Ku-XRCC4-DNA ligase IV, but that Ku alone or XRCC4-ligase IV alone is not sufficient (24). If active recruitment of TdT to coding ends does occur during V(D)J recombination, the domain(s) of TdT involved has not been defined, although the BRCT domain remains an attractive candidate.

HhH domains are nonsequence-specific DNA binding motifs that contact DNA by interactions of peptide backbone nitrogen atoms with phosphate groups of the DNA (25). The two putative HhH domains identified in TdT, pol β, pol μ, and pol λ, are thus expected to bind to the substrate DNA and to position it properly for nucleotide addition. Although both HhH domains in pol β match the consensus motif (GxG, with Φ being a hydrophobic residue, typically isoleucine or valine), in TdT, HhH differs from the consensus sequence (GxG instead of GxG). Because the two glycine are critical for the sharp hairpin characteristic of HhH domains, it is not clear whether this region of TdT can adopt this structure. Interestingly, a similar variation of the HhH motif is also present in DNA pol μ and λ (19).

TdT from a number of species has been reported or predicted to undergo alternative splicing (26). In mice, alternative splicing results in the production of a long form of TdT (TdTL) containing a portion of TdT containing no TdT or containing the short form of TdT (which we will refer to in this report as full-length TdT (TdT-FL)) (26). It also explained the observation that TdTL was able to down-modulate the activity of the short isoform of TdT in transgenic mice (29) and thus refers to in this report as full-length TdT (TdT-FL)) (26). It also explains the observation that TdTL was able to down-modulate the activity of the short isoform of TdT in transgenic mice (29) and the entire C terminus of TdT. We were unable to obtain evidence for an interaction between TdT and Ku by coimmunoprecipitation from cell extracts or for a nuclease activity associated with TdTL in human or rodent nonlymphoid cell lines.

Materials and Methods

Cloning of TdT mutants

The untagged TdT mutants were PCR-amplified using Pfu polymerase (Stratagene, La Jolla, CA) from the pDTS TdT cDNA (27) and cloned into the pEBoB-RAG1 vector after digestion with EcoRI to remove the RAG1 cDNA. Constructs generated included TdT-FL (primers MUT11 and MUT3prim), TdT-ΔN (MUT10 and MUT3prim), TdT-ΔB (MUT11 and MUT3), NLS alone, MUTS and MUT3prim (digested with XhoI and ligated before EcoRI cloning), TdT-ΔN+B (MUT19 and MUT3prim), TdT-322 (MUT22 and MUT3prim), TdT-280 (MUT23 and MUT3prim), TdT-421 (MUT11 and MUT18), TdT-453 (MUT11 and MUT21), and TdT-481 (MUT11 and MUT20). TdT sequences were PCR-amplified from the mTdTL cDNA (provided by T.-H. Thai and J. Kearney, University of Alabama, Birmingham, AL). Mutations were confirmed by DNA sequencing.

Site-directed mutagenesis

The TdT cDNA was subcloned into the pBluescript KS+ vector and mutagenized using the QuikChange kit and Pfu polymerase (Stratagene) according to the manufacturer’s instructions. The mutants included TdT-ASM (primers D343/SE-F and D343/SE-B, template TdT-FL), TdT-ΔH1 (HHH1TOP and HHH1BOT, template TdT-FL), TdT-ΔH2 (HHH2TOP and HHH2BOT, template TdT-FL), and TdT-ΔH1/2 (HHH2TOP and HHH2BOT, template TdT-ΔH1). Mutations were confirmed by DNA sequencing. The mutants were PCR-amplified and cloned into pEBoB.

Transient transfection V(D)J recombination assay in 293T and CHO-K1 cells

293T cells were transfected by a calcium phosphate method as described previously (35), using 5 μg of each plasmid: recombination substrate pS290 or pS200 (35), pEBoB-RAG1, and pEBoB-RAG2, with or without pEBoB-TdT mutants. After 48 h at 37°C, extrachromosomal DNA was harvested by rapid alkaline lysis as described previously (35). DNA was then transformed into MC1061 bacteria and plated on agar containing kanamycin (Kan) and chloramphenicol (Cam). When transformed into bacteria, pS290 and pS200 confer resistance to Kan in both the unrearranged and the rearranged form, and after successful V(D)J recombination they confer resistance to Cam. To analyze the structure of the joints, individual colonies were picked, and the portion of the plasmid containing the relevant junction was sequenced.

CHO-K1 cells were transfected using FuGene 6 (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Briefly, FuGene 6 (3 μg/μg DNA) was mixed with serum-free medium to a final volume of 100 μl, and DNA was then added (2.5 μg of the recombination substrate pH290 (36) and 2 μg each of pEBoB-RAG1, pEBoB-RAG2, and, as appropriate, pEBoB-TdT-FL and/or pEBoB-TdT) and plated on agar containing kanamycin (Kan) and chloramphenicol (Cam). Transfections were cultured at 37°C until cells adhered to the plate. DNA was then extracted and sequenced.
and transformed into bacteria as described above. Recombined substrates were isolated from Amp\(^{\beta}\)+Cam\(^{\beta}\) bacterial colonies, and coding joints were analyzed by sequencing.

**Immunoprecipitation**

Cells were washed with PBS, and the cell pellet was resuspended with 0.5–1 ml of lysis buffer (1 mM PMSF, 20 \(\mu\)g leupeptin, 8 \(\mu\)g pepstatin, 1 mM benzamidine, and 1% Nonidet P-40 in TBS, pH 7.5). The cells were sonicated three times for 10 s each time on ice. The solution was rotated for 30 min at 4°C and spun at 14,000 rpm for 15 min at 4°C. Five micrograms of the Abs (TdT\(\beta\), Ku70 (clone N3H10; Kamiya Biomedical, Thousand Oaks, CA), Ku80 (clone 111; Kamiya Biomedical), DNA-PK\(\alpha\) (clone 18-2; Neomarker), or IgG (Sigma-Aldrich, St. Louis, MO)) were added to each sample and rotated overnight at 4°C. The samples were spun again at 14,000 rpm for 10 min, and 30 \(\mu\)l of a washed protein G slurry was added to the supernatant and rotated for 2 h at 4°C. Each sample was washed five times with 1 ml of lysis buffer. The pellet was resuspended with 30 \(\mu\)l of 2× loading buffer, boiled, and loaded on an SDS-polyacrylamide gel.

**Immunofluorescence**

Forty-eight hours after transfection, cells were split onto coverslips in a 24-well plate (1 \(\times\) 10\(^5\) cells/200 \(\mu\)l) and incubated overnight at 37°C. Cells were washed with PBS, fixed in methanol for 5 min at 4°C, washed with PBS four times, and permeabilized with PS+ buffer (RPMI 1640, 0.1% newborn calf serum, 0.05% saponin, 10 mM HEPES, and 10 mM glycine) for 5 min at room temperature. The anti-TdT Ab (Supertech) was diluted 1/10 in 30 \(\mu\)l of PS+ buffer, and the cells were incubated upside down for 30 min at room temperature in a moisture chamber. The coverslip was returned to the 24-well plate, and the cells were washed four times in PS+. The secondary Ab (1/300 goat anti-rabbit, 1/5000 SYTOX) was diluted in 30 \(\mu\)l of PS+ and incubated on the coverslips for 30 min at room temperature in the moisture chamber. Coverslips were washed twice with PS+ PBS, dipped briefly in water, vacuum-dried, and mounted in 5 \(\mu\)l of mounting solution.

**Chromosomal V(DJ) recombination in 3TGR cells**

3TGR cells were seeded in a 24-well plate (1 \(\times\) 10\(^5\) cells/200 \(\mu\)l) and incubated overnight at 37°C. Lipofectamine 2000 (3 \(\mu\)l; Invitrogen, Carlsbad, CA) was diluted in 50 \(\mu\)l of Opti-MEM medium (Life Technologies, Gaithersburg, MD) and incubated for 5 min at room temperature. A solution of pEBB-RAG1 and pEBB-RAG2, with or without pEBB-TdT mutants (3 \(\mu\)g of DNA total), was added to the Lipofectamine/Opti-MEM solution, incubated at room temperature for 20 min, added to the cells, and incubated for 48 h at 37°C. Cells were harvested, washed with PBS, resuspended in 500 \(\mu\)l of lysis buffer (50 mM Tris (pH 8), 20 mM EDTA, 1% sodium lauryl sulfate, and proteinase K), and incubated at 55°C overnight. The genomic DNA was phenol/chloroform-extracted twice, precipitated in ethanol, and resuspended in 50 \(\mu\)l of TE (10 mM Tris, pH 8.0, 1 mM EDTA). CJs were amplified using nested PCR and Taq polymerase (Life Technologies). First-round amplification (25\(\mu\)l reaction) was performed for 10 cycles using primers V2 (5'-CTCTCCATCTATCTGGGATCCC-3') and BR-1 (5'-GGAGAAGCAGAAGAATCTGGGATCCC-3'). One microliter of the first-round reaction was added to a fresh mix containing primers BR-3 clone (5'-GCCGGTTCACCACTAGATCGTCCG-3') and V5 clone (5'-GCCGGAATTCCAACCTAGAATCTGGGATCCC-3') and amplified for an additional 25 cycles.

**Statistical analysis**

Statistical analysis of N-nucleotide addition, coding end deletion, and microhomology use was conducted using a two-sample \(t\) test with DataDesk version 5.0 (Data Description, Ithaca, NY).

**Results**

**TdT mutants**

The first set of mutants was designed to inactivate individual predicted functional domains (all mutants are depicted schematically in Fig. 1B). The respective regions were either deleted entirely, or critical amino acids within these domains were mutated to abolish their function: TdT-FL (TdT-FL cDNA, short form; Fig. 1A), TdT-ASM (pol X active site motif mutant, D343E, D345E, identical with the catalytic mutant used by Yang et al. (33)), TdT-ΔN (deletion of putative NLS, aa 1–27 (37)), TdT-ΔB (deletion of the BRCT domain, aa 26–143), TdT-ΔH1 (mutated first HhH domain, GIP\(_{213-215}\) AAA), TdT-ΔH2 (mutated second HhH domain, GVGV\(_{357-259}\) AAA), and TdT-ΔH1/2 (both HhH domains mutated).

The second panel of mutants comprised a series of C- and N-terminal truncations: TdT-ΔN+B (aa 1–143 deleted), TdT-232 (N-terminal truncation after the first HhH, lacking aa 1–232), TdT-280 (N-terminal truncation after the second HhH, lacking aa 1–280), TdT-421 (C-terminal truncation, deletion of aa 421–501), TdT-453 (C-terminal truncation, deletion of aa 453–501), and TdT-481 (C-terminal truncation, deletion of aa 481–501). All TdT mutants were expressed without an epitope tag, because in preliminary experiments the presence of even a short C-terminal Myc-epitope tag was found to dramatically reduce activity (data not shown). Finally, we also analyzed the long isoform of murine TdT, TdTL, which contains 20 additional aa near its C terminus as a result of alternative splicing (TdTL cDNA provided by T.-H. Thai and J. Kearney).

All TdT mutants were tested for their ability to add N-nucleotides to coding and signal joints. For selected TdT mutants, the assays were performed with either full-length or truncated core RAG proteins to determine whether there exists a functional interaction between TdT and the noncore regions of the RAG proteins (which are dispensable for V(D)J recombination in such assays). For a subset of TdT mutants, including TdTL, activities were measured in both extrachromosomal and chromosomal V(DJ) recombination assays. In all cases duplicate sequences obtained from a single transfection were counted only once. Histograms showing the distribution of coding end nucleotides deleted or N-nucleotides added for each mutant are available upon request.

**N-nucleotide addition to coding joints using episomal recombination substrates**

The activity of all TdT mutants was first assessed using a V(DJ) recombination assay involving episomal substrates transfected into human 293T cells. Cells were transfected with expression vectors for RAG1, RAG2, and TdT and with artificial recombination substrate pSF290 or pSF200 to test for CJ or SJ formation, respectively. After 48 h the plasmids were harvested from the cells and transformed into bacteria, individual Kan\(^{\beta}\)+Cam\(^{\beta}\) colonies were grown, and the portion of the plasmid containing the relevant junction was sequenced. Coding junctions were analyzed for the frequency of addition of N-nucleotides, the number of N-nucleotides added, the number of nucleotides deleted from the coding ends, and whether microhomologies were present. Signal joints were analyzed for the frequency of addition of N-nucleotides. The results obtained in assays using the full-length RAG proteins are shown in Fig. 1B, whereas those obtained with the core RAG proteins are shown in Fig. 2. Western blotting demonstrated that all mutants of TdT were expressed at levels comparable to that of TdT-FL, with the exception of TdT-ΔN+B, which accumulated to lower levels (data not shown).

With full-length RAG1/RAG2, wild-type TdT (TdT-FL) added N-nucleotides to 59% of CJs and 44% of SJs (Fig. 1B, line 2), whereas in the absence of TdT, only a single, 1-bp addition was observed in the 36 junctions examined (line 1). For TdT-FL, the average number of N-nucleotides found in all CJs sequences was 2.0, whereas this value rose to 3.3 when only those CJs with N-nucleotides added were considered. The nucleotides added by TdT in the in vivo assay showed a preference for the addition of G and C nucleotides (60%), as observed in endogenous Ag receptor genes (6).

To confirm that the pol X catalytic core motif is required for enzymatic activity, an active site mutant, TdT-ASM, was examined. This TdT variant (DD343,345EE) is identical with the catalytic mutant analyzed in a previous biochemical study (33). These
mutations maintain the charge and chemical nature of the side chains and thus are not expected to have a significant effect on the global structure of the protein. As expected, this TdT mutant was found to be inactive in vivo; no N-nucleotides were detected in the 22 joints obtained from four independent transfections (Fig. 1B, line 3). Thus, the active site identified within the pol X family catalytic core of TdT is essential for polymerase activity in vivo and in vitro.

Of the other 12 TdT mutants examined, four were found to be active in vivo: TdT-ΔN, TdT-ΔB, TdT-ΔN+B, and TdT-ΔH1 (Table 1B, lines 4–7). Although three of these mutants, TdT-ΔN (80%), TdT-ΔB (55%), and TdT-ΔH1 (63%), demonstrated frequencies of N-nucleotide addition to CJ comparable to TdT-FL (59%), TdT-ΔN+B added N-nucleotide to only 34% of joints. As noted above, TdT-ΔN+B was not expressed as well as the other TdT proteins, and this could explain its reduced activity. The average length of the N regions added to CJ was, however, reduced in all four cases. Although the average number of nucleotides added by TdT-ΔN was 80% of the wild-type level (2.7 nt compared with 3.3 nt), for TdT-ΔB, TdT-ΔN+B, and TdT-ΔH1, N regions were, on the average, at least 1 bp shorter, containing only 1.9–2.2 N-nucleotides (Fig. 1B). This difference is statistically significant for TdT-ΔN+B (p < 0.05) and borderline significant for TdT-ΔB (p = 0.057). Whether this reduction is due to partial misfolding of the mutant proteins, reduced expression (in the case of TdT-ΔN+B), or a direct contribution of these deleted domains to the catalytic activity remains to be elucidated. In summary, neither the NLS, the

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**FIGURE 1.** Activity of TdT mutants in 293T cells with full-length RAG proteins. A, Structure of TdT-FL. The domains of TdT are depicted as rectangles and labeled as follows: NLS, BRCA-1 C-terminal domain (BRCT), helix-hairpin-helix (HhH1 and HhH2), and pol X active site. Numbers refer to the murine TdT-FL sequence. The 20-aa insertion found in TdTL occurs after aa 482. B, 293T cells were transfected with vectors expressing the full-length RAG proteins, a recombination substrate (pSF290 or pSF200), and full-length (FL) or mutant TdT, as indicated. CJs and SJs were amplified and sequenced. Mutation, deleted or mutated amino acids; RX, number of independent recombination reactions; CJ N-nt addition, percentage with N-nt added (number of sequences with N-nucleotide added/total number of sequences); Avg Length N-nt addition, average number of N-nucleotides added to CJs based only those sequences with N-nucleotides (based on all sequences); statistical analysis of the N-nucleotide addition in comparison with N-nucleotide addition by TdT-FL is indicated as follows: *, p < 0.05; **, p < 0.001); microhomology use, number of sequences with at least one nucleotide of homology at the CJ (percentage); statistical analysis in comparison with microhomology use in presence of TdT-FL is indicated as above); SJ N-nt addition, percentage of SJs with at least one N-nucleotide added (number of sequences); Avg Nt-deletion, average total number of nucleotides deleted from the two coding ends (std dev); ND, not determined.
BRCT domain, nor the first HhH motif is essential for the addition of N-nucleotide to CJs by TdT. In particular, the first 27 aa, spanning the putative NLS, are largely dispensable for activity in this assay. In contrast, all seven remaining mutants, TdT-/H9004/H2, TdT-/H9004/H1/2, TdT-232, TdT-280, and TdT-421, TdT-453, and TdT-481, show no appreciable activity in vivo. This indicates that the second HhH domain is critical for TdT activity, and that TdT cannot undergo even a 20-aa deletion at its C terminus without a complete loss of activity.

N-nt addition to signal joints

A somewhat different picture was seen when nucleotide addition to SJs was analyzed. The wild type protein, TdT-FL, was the only form that added N-nt to a significant number of SJs (44%). Three mutants with partial activity at CJs exhibited a much greater deficit in N-nt addition to SJs (Fig. 1B, lines 4, 5, and 7). Deletion of the putative NLS, which has almost no effect on activity at CJs, reduces activity at SJs by >60%, and deletion of the BRCT domain or mutation of HhH1 essentially eliminates N-nt addition to SJs. The other mutant forms of TdT analyzed for N-nt addition to SJs were also defective in this activity (lines 8, 9, and 12). As observed for CJs, the N-nucleotides added to SJs were G/C rich (57% with full length RAG proteins; 67% with core RAG proteins) (data not shown). Altogether, TdT is less active at signal ends as compared with coding ends, and the activity at signal ends is particularly sensitive to mutations in TdT. This is consistent with the previous observation that higher levels of TdT are required for addition of N-nucleotides to SJs than to CJs (38), and is in keeping with the relatively poor activity of TdT on blunt ends (39), such as signal ends.

N-nucleotide addition in the context of the core RAG proteins

The core RAG proteins appear to perform DNA cleavage in vivo efficiently, as measured by steady state levels of signal ends and coding ends, but they are significantly compromised for SJ and CJ formation relative to the full-length proteins (40–42). To determine whether the noncore regions of the RAG proteins are required for efficient recruitment of TdT to coding ends, TdT-FL, TdT-ASM, TdT-ΔB, and TdT-421 were analyzed using the core RAG proteins by transient transfection of 293T cells. As expected, TdT-ASM and TdT-421 were inactive (Fig. 2, lines 3 and 5). TdT-FL and TdT-ΔB both mediated N-nucleotide addition at CJs, but deletion of the BRCT domain resulted in a reduction in the frequency and average length of N-nucleotide addition (39%, 2.6 nt for TdT-ΔB; 68%, 3.9 nt for TdT-FL; p = 0.046; Fig. 2, lines 4 and 2). These results further support the conclusion that the BRCT domain of TdT is not essential for function, but contributes to the overall level of activity.

Interestingly, N-nt addition to SJs was reduced using the core RAG proteins compared with their full-length counterparts (compare Fig. 2 with Fig. 1). Previous results led to the suggestion that the nonessential portions of the RAG proteins, missing from the core proteins, facilitate remodeling of postcleavage complexes and thereby enhance signal joint formation (42). It may be that postcleavage complexes containing the core RAG proteins only inefficiently assume a conformation conducive to TdT activity.

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**TABLE 1.** Activity of TdT mutants in 293T cells with core RAG proteins. 293T cells were transfected with vectors expressing the core RAG proteins, a recombination substrate (pSF290 or pSF200), and full-length (FL) or mutant TdT, as indicated. CJs and SJs were amplified and sequenced. Other symbols and abbreviations are explained in Fig. 1.

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<th>Mutation</th>
<th># Rx</th>
<th>CJ N-nt addition</th>
<th>Avg Length N-nt addition</th>
<th>micro-homology use</th>
<th>SJ N-nt addition</th>
<th>Avg Nt deletion</th>
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<td>3</td>
<td>68% (23/34)</td>
<td>3.9 [2.6]</td>
<td>5/34 (15%)</td>
<td>19% (5/27)</td>
<td>5.4 (2.9)</td>
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<tr>
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<td>4</td>
<td>6% (2/36)</td>
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<td>22/36 (61%)</td>
<td>9% (1/11)</td>
<td>6.4 (3.4)</td>
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<tr>
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<td>del 26-145</td>
<td>39% (17/44)</td>
<td>2.6 [1.0]</td>
<td>10/44 (23%)</td>
<td>13% (3/21)</td>
<td>5.3 (2.9)</td>
</tr>
<tr>
<td>5 TdT-421</td>
<td>del 421-501</td>
<td>0% (0/29)</td>
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<td>14/29 (48%)</td>
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</tr>
<tr>
<td>6 TdT-L</td>
<td>insert at 482 20aa</td>
<td>23% (7/30)</td>
<td>1.7 [0.4]</td>
<td>12/30 (40%)</td>
<td>0% (0/12)</td>
<td>5.7 (2.7)</td>
</tr>
<tr>
<td>7 TdT-FL + TdT-FL</td>
<td>4</td>
<td>48% (14/29)</td>
<td>2.4 [1.2]</td>
<td>6/29 (21%)</td>
<td>0% (0/16)</td>
<td>6.2 (2.3)</td>
</tr>
<tr>
<td>8 TdT-ASM + TdT-FL</td>
<td>2</td>
<td>67% (10/15)</td>
<td>3.9 [2.9]</td>
<td>1/15 (11%)</td>
<td>13% (2/16)</td>
<td>5.5 (4.2)</td>
</tr>
</tbody>
</table>

---

**FIGURE 2.** Activity of TdT mutants in 293T cells with core RAG proteins. 293T cells were transfected with vectors expressing the core RAG proteins, a recombination substrate (pSF290 or pSF200), and full-length (FL) or mutant TdT, as indicated. CJs and SJs were amplified and sequenced. Other symbols and abbreviations are explained in Fig. 1.
at signal ends, perhaps because the 3’-hydroxyl groups are not made accessible.

**TdT- and microhomology-mediated coding joint formation**

Previous results indicated that TdT reduces the use of microhomologies to mediate CJ formation (see introduction). Our results confirm this. The presence of TdT-FL significantly reduces the use of microhomologies in CJs relative to the situation where only the RAG proteins were expressed (16 vs 63%; Fig. 1B, lines 2 and 1). To separate possible effects of TdT on microhomology joining due to its enzymatic activity as opposed to the mere presence of TdT in the end-joining machinery, the CJs formed in the presence of the catalytic mutant, TdT-ASM, were analyzed. As noted above, the subtle mutations in this protein maintain the charge and chemical nature of the side chains and are not expected to have an effect on the global structure of the protein. Thus, putative interactions with the DNA substrate and other proteins should remain normal. Interestingly, TdT-ASM does not reduce microhomology-based joining compared with TdT-FL. Sixty-four percent of the CJs in the presence of TdT-ASM were created by the microhomology pathway, nearly identical with the 63% observed in the absence of TdT (Fig. 1B, lines 1 and 3). This suggests that the enzymatic activity of TdT, i.e., the addition of N-nucleotides, and not recruitment of the enzyme to the DNA ends, plays an important role in the choice of the joining pathway. Two additional findings provide further support for this model. First, all TdT mutants that lack polymerase activity show frequencies of microhomology-mediated CJ formation comparable to those found in the absence of TdT (Fig. 1B, compare line 1 to lines 1 and 3). Second, all active mutants, TdT-ΔN, TdT-ΔB, TdT-ΔN+B, and TdT-ΔH1, just like the wild-type form TdTL-FL, reduce microhomology use in CJs to less than half the value observed when expressing the RAG proteins alone (compare line 1 to lines 2 and 4–7).

**TdT and nucleotide deletion from coding ends**

TdT is only one factor in a group of enzymes involved in processing of coding ends before joining. We were interested in determining whether TdT or mutants thereof have any effect on other end-processing activities. We thus analyzed all of our sequences, mining whether TdT or mutants thereof have any effect on other end-processing activities. We were interested in determining whether TdT or mutants thereof have any effect on other end-processing activities.

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Our data in 293T cells are consistent with a subset of these observations. Coexpression of TdT with TdT-FL reduced the average length of N-nucleotide additions compared with TdT-FL alone: from 3.3 to 1.6 with full-length RAG proteins (Fig. 1B, line 16) and from 3.9 to 2.4 with core RAG proteins (Fig. 2, line 7). The frequency of CJs containing N-nucleotides was not reduced (full-length RAG proteins; Fig. 1) or was modestly reduced (core RAG proteins; Fig. 2). These results are consistent with those previously reported (26, 29) and support the model that TdT acts to down-modulate the activity of TdT-FL without substantially affecting its recruitment to coding ends. Interpretation of these data is complicated by our observation that coexpression of TdT-ASM with TdT-FL resulted in a comparable reduction in N-nucleotide length, but only in reactions using the full-length RAG proteins (Fig. 1B, line 17), not with the core RAG proteins (Fig. 2, line 8). We conclude that TdT-FL is indeed capable of reducing the average length of N regions added in the presence of TdT-FL (discussed below).

In contrast with previously published results in CHO cells (26), we did not observe any increase in nucleotide deletion from coding ends when TdT-FL was expressed in 293T cells. This was true when TdT was expressed by itself or when it was coexpressed with TdT-FL, and was true in reactions using either the full-length or core RAG protein (Fig. 1B, lines 15 and 16, and Fig. 2, lines 6 and 7). Hence, TdT does not enhance nucleotide deletion from coding ends using an extrachromosomal substrate in 293T cells.

The difference between our results and those reported by Thai et al. (26) might be due to the use of different cell lines derived from different species. We therefore repeated a subset of these experiments in the CHO-K1 cell line using the coding joint recombination substrate pH290 (36). The results were, for the most part, very similar to those we obtained in 293T cells. The expression of TdT in CHO-K1 cells (confirmed in all experiments by Western blot) resulted in detectable N-nucleotide addition and did not cause an increase in deletion of nucleotides from coding ends (Table 1). In contrast to results in 293T cells, we did not detect a decrease in N-nucleotide addition in transfections containing TdT-FL and TdT compared with those containing only TdT-FL (Table 1). Hence, using a hamster cell line similar to that used by Thai et al. (26), we did not detect evidence for an exonuclease activity associated with TdT-FL capable of removing nucleotides from coding ends. As noted in the next section, TdT also did not enhance nucleotide deletion from coding ends using a chromosomal recombination substrate in mouse 3T3 fibroblasts. Hence, in both human and rodent nonlymphoid cells, with both extrachromosomal and chromosomal substrates, TdT-FL was not able to increase nucleotide deletion from coding ends. Possible explanations for the difference between our results and those obtained by Thai et al. (26) are discussed below.


**Table I. Activity of TdT-FL and TdT-L in CHO cells with full-length RAG proteins**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No. Rx</th>
<th>CI N-nt addition</th>
<th>Avg. Length N-nt addition</th>
<th>Avg. Nt deletion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Full-length RAGs alone</td>
<td>7</td>
<td>0% (0/25)</td>
<td>0/0</td>
<td>5.1 (3.1)</td>
</tr>
<tr>
<td>TdT-FL</td>
<td>4</td>
<td>57% (16/28)</td>
<td>2.3/1.3</td>
<td>5.3 (4.1)</td>
</tr>
<tr>
<td>TdT-L</td>
<td>7</td>
<td>19% (4/21)</td>
<td>1.5/0.3</td>
<td>5.2 (5.1)</td>
</tr>
<tr>
<td>TdT + TdT FL</td>
<td>6</td>
<td>61% (14/23)</td>
<td>3.2/2.0</td>
<td>5.3 (4.4)</td>
</tr>
</tbody>
</table>

*a* Number of independent recombination reactions.  
*b* Percentage of coding joints with N-nucleotides added (number of sequences with N-nucleotides added/total number of sequences).  
*c* Average number of N-nucleotides added to CI based only on those sequences with N-nucleotides/based on all sequences.  
*d* Average total number of nucleotides deleted from the two coding ends (SD).

**TdT activities on a chromosomal substrate**

Assays using extrachromosomal substrates in 293T or CHO cells have some limitations. First, the proteins, such as RAG1/2 and TdT, are typically expressed at supraphysiological levels. This could obscure an important role for nonessential domains, such as the BRCT domain of TdT. Second, the chromatin configuration of extrachromosomal substrates is probably not identical with that of a chromosomal substrate. Third, it is formally possible that some recombination of extrachromosomal substrates occurs outside the nucleus, and this would obscure the importance of certain motifs (e.g., NLS). To address some of these issues and further examine the activity of TdT, we tested several of the TdT mutants in the murine 3T3 fibroblast cell line, 3TGR. 3TGR contains two copies of a retroviral V(D)J recombination substrate, each integrated at a different chromosomal location, and has been used previously for the study of V(D)J recombination (43, 44). Expression vectors encoding TdT-FL or TdT mutants were transiently transfected into 3TGR cells along with full-length RAG1/2 expression plasmids. After 48 h, genomic DNA was prepared from the cells, and DNA fragments containing coding joints were amplified by PCR, subcloned, and sequenced.

TdT-FL added N-nucleotide to 56% of the CJs, comparable to the frequency of 59% observed using an extrachromosomal substrate (Fig. 3, line 2), indicating that the polymerase is efficiently recruited to coding ends on both types of substrates. The average length of the additions, however, was smaller with the chromosomal substrate (1.6 vs 3.3 nt with the episomal substrate; Fig. 3, line 2). This could reflect differences in TdT expression levels in the two assays, or differences in the repair process and/or TdT activity with chromosomal vs episomal substrates. Both TdT-ΔN and TdT-ΔB were also able to add N-nucleotide to CJs with the integrated substrate, albeit at lower frequencies than in the transient assay (23 and 31%, compared with 80 and 55%, respectively; Fig. 3, lines 3 and 4). Despite this reduced frequency of N-nucleotide addition, the average number of nucleotides added to CJs by TdT-ΔN and TdT-ΔB was nearly identical with that for TdT-FL in this system (1.4 and 1.6 nt, respectively, vs 1.6 nt for TdT-FL). Thus, in the case of a chromosomal V(D)J break, both the putative NLS as well as the BRCT domain are more important for recruitment than for polymerase activity. As in the episomal assay, TdT had detectable terminal transferase activity in the chromosomal assay (Fig. 3, line 6).

We also analyzed the chromosomal CJs for nucleotide deletion from the coding ends. None of the TdT mutants analyzed, including TdT-L, exhibited a substantial change in the extent of nucleotide deletion compared with reactions lacking TdT (Fig. 3). Therefore, under none of the conditions tested was any mutant of TdT, including TdT-L, able to affect nucleotide deletion from coding ends.

**Immunofluorescence localization of TdT**

To address directly the question of whether TdT variants are properly transported to the nucleus, TdT proteins were expressed by transient transfection in 3TGR cells and localized by immunofluorescence with anti-TdT Abs. A nuclear stain (SYTOX) was used to delineate the nucleus (Fig. 4). As expected, no TdT was detectable in untransfected cells. TdT-FL, TdT-ΔN, TdT-ΔB, and TdT-L all yielded a strong fluorescence signal with the anti-TdT Ab, and in all cases this signal colocalized with nuclear staining. This indicates that these TdT variants are transported efficiently into the nucleus, and that differences in N-nucleotide addition between different variants is probably not due to differences in subcellular localization. In particular, the putative NLS is not required for nuclear localization of TdT.

**Protein-protein interactions**

Mahajan et al. (22) reported an interaction between TdT and Ku70/80 and proposed that this association was important for the recruitment of TdT to the V(D)J recombination complex. The first 131 aa of TdT were found to be essential for this interaction. We were interested in determining more precisely which region(s) of TdT was responsible, using coimmunoprecipitation to assess interactions. This effort was greatly facilitated by a novel panel of anti-TdT mAbs (a gift from J. Kearney, University of Alabama, Birmingham, AL). 293T cells were transfected with an Myc-tagged TdT-FL expression vector and lysed 48 h post-transfection, and proteins were immunoprecipitated using the anti-Myc, anti-

![FIGURE 3. Activity of TdT mutants in 3TGR cells with full-length RAG proteins. 3TGR cells were transfected with vectors expressing the full-length RAG proteins and full-length (FL) or mutant TdT, as indicated. CJs were amplified from the recombination substrate stably integrated into the genome of 3TGR cells and sequenced. Other symbols and abbreviations are explained in Fig. 1.](image-url)
Ku70, anti-Ku80, and a control IgG Ab. The proteins were then analyzed by Western blot analysis using anti-TdT and anti-Ku70/80 Abs. Under mild conditions in which precipitates were not washed, TdT was clearly coprecipitated by the anti-Ku70 and anti-Ku80 Abs (Fig. 5A, lanes 4 and 5). This was not open to straightforward interpretation, however, because nonspecific IgG also efficiently precipitated TdT (Fig. 5A, lane 2).

We then repeated the immunoprecipitation experiment, expressing untagged TdT-FL in 293T cells and including several wash steps so as to reduce the nonspecific background. Under these conditions, TdT did not coprecipitate with Ku70 or Ku80 (Fig. 5B, lanes 4 and 5), nor did Ku70 or Ku80 coprecipitate with an anti-TdT Ab (lane 3). These results suggest that the interaction between the Ku complex and TdT in cell extracts does not occur to a significant extent or is of low affinity. It seems unlikely that both the anti-Ku70 and the anti-Ku80 Abs interfere with binding of Ku to TdT. To determine whether the catalytic subunit of the DNA-dependent protein kinase, DNAPKcs, interacts with TdT, the immunoprecipitation experiment was repeated using anti-DNAPKcs Abs. Again, no significant interaction between TdT and DNAPKcs was detectable (Fig. 5B, lane 6; the very faint band visible in this lane was not reproducible). To analyze whether the interaction of TdT with components of DNAPK occurs only in the context of V(D)J recombination, similar experiments were performed using cells that were cotransfected with an extrachromosomal recombination substrate. Again, no association of TdT with either the Ku proteins or DNAPKcs was observed (data not shown). Even though protein-protein interactions between TdT and Ku or between TdT and DNAPKcs were not detected, an interaction between these proteins may still be relevant to V(D)J recombination.

FIGURE 5. Immunoprecipitation (IP) of TdT, Ku, and DNA-PKcs. Cell extracts from 293T cells transfected with TdT were immunoprecipitated with the Abs indicated above the lanes. Bead-bound proteins were analyzed by Western blots using anti-TdT or anti-Ku70/80 Abs, as indicated to the right. A. Extracts were prepared from 293T cells transfected with Myc-tagged TdT, immunoprecipitated with the indicated Abs and protein G beads, and then loaded on the gel after washes of the beads (22). Lane 1, 1/10 dilution of the input for the immunoprecipitation; lane 2, immunoprecipitation with control IgG; lane 3, immunoprecipitation with anti-Myc (TdT); lane 4, immunoprecipitation with anti-Ku70; lane 5, immunoprecipitation with Ku80. B. Extracts were prepared from 293T cells transfected with untagged TdT-FL, immunoprecipitated with the indicated Abs and protein G beads, and then loaded on the gel after four washes of the beads, Lane 1, 1/20 dilution of the input; lane 2, immunoprecipitation with control IgG; lane 3, immunoprecipitation with 29-1 (anti-TdT); lane 4, immunoprecipitation with anti-Ku70; lane 5, immunoprecipitation with Ku80; lane 6, immunoprecipitation with anti-DNA-PKcs.

Discussion
Role of the HhH, putative NLS, and BRCT domains

This study represents the first extensive analysis of predicted functional domains within TdT. With the V(D)J recombination system used in this study, the putative NLS domain, the BRCT domain, and the first HhH domain were found to be nonessential for TdT activity in V(D)J recombination. Variants of TdT harboring deletions of or point mutations within these motifs (TdT-ΔH1, TdT-ΔAB, and TdT-ΔH1) show a reduction in the frequency of N-nucleotide additions compared with the wild type protein, suggesting that these mutants are recruited less efficiently to the coding ends within the recombination complex and/or that they associate less tightly with DNA ends. As both of our assays involved overexpression of TdT protein, the effects of inactivating these domains could be more dramatic in V(D)J recombination of the endogenous Ag receptor loci.

It is unclear whether the putative NLS (defined as a basic region at the N terminus) of TdT has any functional significance. Although overexpression in 293T cells might have masked the relevance of this sequence for nuclear transport in our transient transfection assay, TdT-ΔN was able to localize to the nucleus in 3TGR cells and was able to mediate N-nucleotide addition with a chromosomal recombination substrate. Our results indicate a nonessential or redundant role for this motif.

Nearly as enigmatic is the role of the BRCT domain of TdT. N-nucleotide addition by TdT-ΔB was easily detectable, but was reduced compared with TdT-FL in all of our assays. In the transient transfection assay, both the frequency and the length of N-nucleotide addition were reduced by deletion of the BRCT domain, whereas in the chromosomal assay only the frequency was decreased. BRCT domains are found in many DNA repair proteins
and are thought to mediate interactions between them. Our data are consistent with the hypothesis that the TdT BRCT domain is involved in nonessential interactions that help localize and/or retain TdT at the site of coding end repair. Previous findings indicated that the BRCT domain within TdT is required for the interaction between TdT and Ku70 (22). Using coimmunoprecipitation assay, however, we could not find evidence for an interaction between Ku70 and TdT. Recent biochemical studies from the Ramsden laboratory (24) provide a means of reconciling our findings with those demonstrating a requirement for Ku in N-nucleotide addition (5, 20). Mahajan et al. (24) demonstrated that the combination of DNA ligase IV, XRCC4, and Ku, but not Ku by itself, is able to recruit pol μ or TdT to DNA ends. It is plausible, therefore, that the interaction between TdT and Ku is quite weak (consistent with our data) and that Ku plays a significant role in the recruitment of TdT to coding ends through interactions involving DNA ligase IV and XRCC4. Our results are also consistent with the idea that the BRCT domain of TdT is involved in this interaction and hence in the recruitment of TdT to coding ends, but they clearly demonstrate that this domain is not essential for N-nucleotide addition in V(D)J recombination, at least under the assay conditions used in this study.

The second HhH domain (H2) was found to be essential for TdT activity. In contrast to the dispensable first HhH motif, the second one matches the consensus sequence (GHG) and was thus thought likely to represent the domain responsible for protein-DNA interactions near the active site. The recent crystal structure of TdT supports this model (30). In this study three carbonyl atoms in the second HhH domain were found to coordinate a sodium ion, and molecular modeling suggested that this sodium ion is directly involved in contacting and positioning of the second nucleotide of the primer strand. Our results support a critical role for the second HhH domain. Based on the crystal structure of TdT, no clear functional role can be assigned to the first HhH domain, and consistent with this, mutating critical residues of the motif did not have a clear deleterious effect on TdT activity. This region of TdT may not have a critical function, or alternatively, this region might perform a function that our assays do not measure. As a similar non-consensus HhH domain is present in DNA pol μ and λ, it is tempting to speculate that this domain will also prove to be nonessential for their activity.

**TdT and end joining involving microhomologies**

Two general mechanisms can be envisioned to explain the inhibition of microhomology-mediated joining by TdT. The mere presence of TdT in the repair complex could inhibit the process, perhaps by interfering with some aspect of end alignment. Alternatively, the catalytic activity of TdT might be essential to direct the reaction into a pathway less able to make use of microhomologies. In addition, N-nucleotide addition could prevent the detection of homology-mediated joining, because microhomology-mediated joining involving N-nucleotides at one or both coding ends could not be identified as such. Our data reveal a tight correlation between N-nucleotide addition and suppression of microhomology use. Of particular note is the finding that mutation of the active site of TdT results in a protein that lacks catalytic activity and the ability to suppress microhomology-mediated joining, but presumably retains the ability to be recruited to sites of coding end repair. We conclude that it is likely to be the catalysis of N-nucleotide addition, and not the presence of TdT at coding ends, that suppresses microhomology-mediated joining.

**Role of the C-terminal domain of TdT**

Any truncation of the C-terminal α domain of TdT abolishes polymerase activity. This could be a result of improper protein folding or a conformational change that renders the mutant proteins catalytically inactive. The crystal structure of TdT reveals a strong similarity to the closed conformation of DNA polymerase β. This closed conformation of TdT is stabilized by interactions between N-terminal as 148–163 and the C-terminal residues 498–506 and 508–510 (Fig. 6) (30). The inactivity of truncation mutants TdT-232, TdT-280, TdT-453, and TdT-481 could therefore easily be explained by a loss of exactly those interactions required for maintenance of the closed conformation (although in the case of TdT-232, residues in addition to those involved in this interaction were removed). In the case of pol β, the closed conformation is formed by an induced fit mechanism upon interaction of the incoming free nucleotide with the template strand (45) and thus represents the active state of the polymerase. TdT is not required to distinguish between different nucleotides and hence has been proposed to remain permanently in the active, i.e., closed, state. Our mutagenesis study provides the first experimental support for the model that the closed configuration is the one and only active state of TdT.

**TdT vs TdT-FL**

Several lines of evidence support the idea that TdT modulates the structure of V(D)J coding joints by virtue of an associated exonuclease activity. First, biochemical studies demonstrated a robust 3' to 5' exonuclease activity for TdT, with no evidence of terminal transferase activity (26). Second, TdT was able to reduce the length of N-nucleotide additions by TdT-FL in transfected CHO cells and in transgenic mice and to enhance nucleotide deletion from coding ends in the CHO cell assay (26, 29). There is, however, evidence for a thermolabile terminal transferase activity associated with TdT-FL (28), although in no case has N-nucleotide addition in vivo by TdT been reported previously.

Consistent with previous reports, we found that coexpression of TdT with TdT-FL reduced the average length of N-nucleotide additions in both 293T and 3T3 cell lines. Curiously, the catalytic mutant TdT-ASM had almost as large an effect on N-nucleotide addition by TdT-FL as did TdT when the full-length (but not the core) RAG proteins were used. This raises the possibility that the


