Evidence That the Long Murine Terminal Deoxynucleotidyltransferase Isoform Plays No Role in the Control of V(D)J Junctional Diversity

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J Immunol 2004; 172:6764-6767; doi: 10.4049/jimmunol.172.11.6764
http://www.jimmunol.org/content/172/11/6764
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Two TdT isoforms have been found in the mouse. The short isoform is known to add N regions to gene segment junctions during V(D)J recombination, but the role of the long (TdTL) isoform is controversial. We have shown that TdTL, although endowed with terminal transferase activity, is thermally unstable and unable to add N regions in vivo. In this study, we demonstrate that TdTL is devoid of 3′-5′ exonuclease activity, and provide an analysis of nucleotide deletion and addition patterns in large series of V(D)J coding joins, arguing against a role of TdTL in the control of junctional diversity in Ig and TCRs. The Journal of Immunology, 2004, 172: 6764–6767.
mix, and electrophoresed on a 16% acrylamide denaturating gel. Products were visualized after exposure of the wet gel under a Kodak film (Biomax MR) at \(-70^\circ C\).

Results

TdTL does not modify V(D)J recombination coding ends

We had previously compared the functional properties of the two murine TdT isoforms by analyzing the nucleotide additions at the V(D)J recombination junctions of an episomal substrate transfected into NIH 3T3 fibroblasts. This work established the role of TdT in N region addition (1) and the lack of N region addition by TdTL (5). Following the report by Thai et al. (8) that murine TdT and TdTL have “distinct and opposite diversifying activities,” we conducted a retrospective analysis of various sets of junction sequences obtained in transfection experiments from NIH 3T3 and COS cell transfectants (1, 5). The junction sequences from NIH 3T3 fibroblasts and COS cells are displayed in Fig. 1, a and b, respectively, with their characteristics summarized in Table I. In the presence of TdT, N regions are present in \(~75\%\) of the rearranged plasmids (79 and 67%, respectively, in 3T3 and COS cells), whereas in the absence of TdT or in the presence of TdTL, only 4–14% of the junctions contain nucleotide additions (see Table I and Fig. 1, a and b). As previously reported (5), the nucleotide insertion frequencies observed with TdTL are in the same range as those observed in the absence of TdT or in the junctions retrieved from TdT knockout mice (2, 3). Presumed palindromic (P) nucleotides, which are believed to result from asymmetric opening of coding end recombination intermediate hairpins, are present in no-TdT, TdT, and TdTL sequences, and in both 3T3 (4, 44, and 19%, respectively) and COS cells (4, 21, and 18%, respectively). They are more frequent at recombined junctions in TdTS- or TdTL-transfected 3T3 or COS cells than at junctions retrieved from cells not expressing TdT, as if the presence of TdT influenced hairpin or P nucleotide processing, but there is no significant difference in the percentage of P nucleotides added when sequences from TdT-transfected cells and TdTL-transfected cells

![Figure 1](https://example.com/fig1.png)

**FIGURE 1.** Sequences of the junctions formed in pBlueRec or pH2Rec recombination substrates after cotransfection with plasmids coding for RAG1, RAG2, and either no TdT, TdT, or TdTL in NIH 3T3 fibroblasts (a) and in COS cells (b). Recombined sequences are aligned with the underlined unmodified coding ends of pBlueRec (a) and pH2Rec (b). Numbers in parentheses indicate repeated sequences recovered from independent transfections: 9 and 5 transfections in the absence of TdT, 13 and 5 transfections in the presence of TdT, and 6 and 5 transfections in the presence of TdTL, in 3T3 and COS cells, respectively. Sequences already published (1) are marked with an asterisk. Putative P nucleotides are underlined. nt deleted, Represent the number of nucleotides deleted from the coding ends. ¶, Runs of nucleotides are indicated as follows: nN/4 N n.
are compared. The analyses of deletion patterns at recombination junctions in 3T3 and COS cells reveal no significant differences among no-TdT, TdTS, and TdTL sequences (Fig. 1 and Table I). The nucleotide loss, calculated on average per sequence, is the same (4 nt) whether recombination occurred in the absence of TdT or in the presence of TdTS or TdTL. About one-half of the TdTS and TdTL sequences have deletions >4 nt (40–52%). Deletions >4 nt are also present in about one-half of the no-TdT sequences in COS cells (46%) and in 22% of the sequences in 3T3 cells.

**Murine TdTL exhibits terminal transferase activity in vitro and is devoid of 3′-5′ exonuclease activity**

Comparison of the two murine TdT isoform activities was performed on a 32P-labeled (dA)10 primer substrate in enzyme excess (enzyme/primer > 5), with proteins highly purified from bacterial extracts (see Materials and Methods for details). The chain length distributions of the products obtained in kinetics studies over a 60-min time course, in the absence or presence of nucleotides, are shown in Fig. 2. In the presence of nucleotides, both TdTS and TdTL exhibit terminal transferase activity, with TdTS synthesizing more of the longer polymers. Synthesis by TdTL is less efficient, but within 15 min almost all of the (dA)10 primer has been elongated. In the absence of nucleotides, there is no degradation of the primer by TdTS or TdTL. Preincubation of TdTS or TdTL at 35°C for 1 h leads to the reduction of terminal transferase activity, as previously reported (7), but does not reveal any cryptic exonuclease activity (data not shown).

**Discussion**

It has been shown that in transfected cells, only TdTS adds N nucleotides to V(D)J recombination junctions (5, 8). The absence of N region addition by TdTL is not due to a lack of terminal transferase activity (7). As we showed in this study, TdTL is, under appropriate in vitro conditions, also able, like TdTS, to synthesize very long DNA chains. However, TdTL is much more unstable than TdTS in transfected cells (5) and is thermosensitive in vitro (7). This fragility could explain why TdTL, which has the same nuclear localization sequence as TdTS, does not accumulate in transfected cells and does not add N regions (5). The lack of terminal transferase activity recently reported for TdTL in vitro cannot be explained by sequence differences in the clones used, and is most likely due to the experimental conditions that...
are, considering the thermosensitivity of the enzyme, highly unfavorable (8).

Our exonuclease assay was free of nucleotides because the competing polymerase activity of TdT and TdTL would in the presence of nucleotides mask any associate 3′-5′ exonuclease activity, whether intrinsic or contaminant. In these conditions, no 3′-5′ exonuclease activity is detected in vitro, even at high enzyme/primer ratio, with either TdTS or TdTL. In the work of Thai et al. (8), the presence of nucleotides in the assay precludes to assert that only TdTL has exonuclease activity because the temperature-sensitive polymerase activity of TdT is, as explained above, suppressed under the experimental conditions used.

Using a recombinant assay, we found no significant difference among no-TdT, TdTS, and TdTL deletion patterns of V(D)J recombination junctions in two different transfected cell lines (3T3 or COS cells), indicating that TdTL does not have any effect on coding end trimming in these cells. Interestingly, a lack of effect of TdTL on coding end processing was also found in sequences retrieved from transgenic mice only expressing TdT (6). Thai et al. (8) had deduced from results obtained in similar transfection experiments, but using Chinese hamster ovary cells, that murine TdTS and TdTL have “distinct and opposite diversifying activities.” It is conceivable that TdTL recruits an exonuclease specifically expressed in Chinese hamster ovary cells. However, the observation that the effect of TdTL on V(D)J junctions is limited to specific experimental conditions makes it unlikely that such properties contribute to shaping the immune repertoire in the mouse.

TdT is conserved across the vertebrate phylum, from cartilaginous fish to humans (12), yet the mouse is the only species in which expression of TdTS and TdTL isoforms has been demonstrated. Three alternatively spliced mRNAs have been isolated by RT-PCR from calf thymus (13), and potential splicing sites corresponding to the bovine splicing sites have been identified in human genomic sequences (8). However, the two bovine TdT isoforms, with none identical with murine TdT, have not been shown to be expressed during lymphocyte differentiation. Although it is now believed that a high percentage of human genes undergoes alternative splicing (see Sorek et al. (14) and references therein), identification of potential alternative splice sites is not sufficient to ascertain a functional and regulated expression of spliced variants. As discussed previously (7), TdT may result from an evolutionary happenstance or represent an ancestral, maybe vestigial, form of the enzyme. It is interesting to note that in a few members of the family X of DNA polymerases, a subclass of an ancient nucleotidyltransferase superfamily to which TdT belongs, the position of the TdT 20-aa insertion coincides with an insertion zone of sequences of variable length (15). Structural analyses localize the position of the insertion within the thumb subdomain, in a region, between two conserved domains, which forms a short loop pointing outward the structured catalytic core (16–18).

In the case of TdT, the extension of the loop does not modify the catalytic activity, but has functional consequences. The underlying mechanisms of the functional inhibition caused by the presence of the loop in TdT are unknown, but may involve specific protein-protein interactions (14).

References