Terminal deoxynucleotidyl transferase (TdT) catalyzes the condensation of deoxyribonucleotides on 3'-hydroxyl ends of DNA strands in a template-independent manner and adds N-regions to gene segment junctions during V(D)J recombination. Although TdT is able to incorporate a few ribonucleotides in vitro, TdT discrimination between ribo- and deoxyribonucleotides has never been studied. We found that TdT shows only a minor preference for incorporation of deoxyribonucleotides over ribonucleotides on DNA strands. However, incorporation of ribonucleotides alone or in the presence of deoxyribonucleotides generally leads to premature chain termination, reflecting an impediment accommodation of ribo- or mixed ribo/deoxyribonucleic acid substrates by TdT. An essential catalytic aspartate in TdT was identified, which is a first step toward understanding the apparent lack of sugar discrimination by TdT.

A traditional classification of polymerases, as DNA or RNA polymerases, relies on their ability to use dNTPs or rNTPs as nucleotide substrates (1, 2). Sugar selectivity is extremely high, several orders of magnitude (3–7), allowing nucleic acid polymerases to accomplish specialized tasks in the presence of competing nucleotide substrates. Terminal deoxynucleotidyl transferase (TdT, EC 2.7.7.31) is a DNA polymerase, which contributes to the diversification of antigen receptors by adding nucleotides, called N-regions, to gene segment junctions during V(D)J recombination (8, 9). In vitro, TdT most efficiently polymerizes deoxynucleotides on 3'-hydroxyl ends of DNA strands in a template-independent manner (10, 11). However, shortly after the isolation of TdT, it was shown that TdT could add a few ribonucleotides to oligodeoxynucleotide initiators and incorporate ribonucleotides into growing DNA chains (12, 13). The methodology at that time allowed neither accurate determination of the reaction kinetic parameters nor calculation of the TdT selectivity factor for deoxyribonucleotides over ribonucleotides. Having recently developed a new method for producing large quantities of pure and unproteolyzed recombinant TdT in bacteria (14), we have been able to compare the activity of the two TdT isoforms found in the mouse (15), to undertake three-dimensional structural analyses (16), and to further improve the enzymatic characterization of the protein.

Here, we present our analysis of the sugar selectivity of TdT, and our investigation of its enzymatic behavior in vitro in the presence of both ribo- and deoxyribonucleotides. We report that the short murine TdT, which is the isozyme conserved across the vertebrate phylum (17), has only minor preference for the incorporation of deoxyribo- over ribonucleotides on DNA strands in vitro and that under conditions that reproduce the in vivo ribo/deoxyribonucleotide pool imbalance (18), incorporation of ribonucleotides by TdT leads to premature termination of chain elongation. As a first step toward analyzing the mechanisms underlying the apparent lack of sugar discrimination by TdT, unique among nucleic acid polymerases, we identified by directed mutagenesis the position of the third carboxylate residue of the conserved catalytic triad.

EXPERIMENTAL PROCEDURES

Purification of Wild Type and Mutant Forms of Murine TdT—The detailed protocol used for expression and purification of recombinant TdT has been described (14). The same protocol was used to obtain Asp170Ala, Asp170Glu, and Asp434Ala histidine-tagged protein variants. Purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis using an 8% acrylamide gel. Densitometric analysis of the Coomassie Blue-stained gels led to an estimation of the purity of the proteins as greater than 80%.

TdT Enzymatic Assays and Determination of Kinetic Parameters—(dA)10 and (dA)rA oligonucleotides were purchased from Genset (Paris, France). Ultrapure ribo-, deoxy-, and dideoxyribonucleotide solutions were purchased from Amerham Pharmacia Biotech. Standard kinetic assays were carried out at 35 °C by incubating recombinant proteins in 200 mM potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.25 mg/ml bovine serum albumin, 4 mM MgCl2, 4 μM ZnSO4, and various concentrations of the reaction substrates (oligonucleotide initiator and nucleotides). Measurements of incorporation of α-32P-labeled nucleotides into acid insoluble material, and chain extension assays used to visualize elongation products were done as described previously for the polymerization of dATP (14). Specific conditions are given in the legends of the figures. Michaelis-Menten kinetic parameters (Km, kcat, Kcat/Km, kcat/Km, and kcat/km) were obtained by titrating each substrate (nucleotide or initiator) in the presence of saturating concentrations of a second substrate, as previously described (14).

Determination of TdT Sugar Selectivity—40 nM TdT were incubated with 10 μM 5'-32P-labeled (dA)10 and 0.5 mM of each pair of nucleotides (dATP+rATP, dTTP+rTTP, dGTP+rGTP, or dCTP+rCTP). Aliquots were taken after 15, 30, 45, and 60 s of incubation, and the reaction was stopped with the addition of a formamide dye mix (10 mM NaOH, 95% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol). The samples were loaded onto a 16% polyacrylamide gel in order to separate the products of the reaction. Under these conditions, the (dA)r0 initiator was only extended by one nucleotide, either dNTP or rNTP. (dA)r4N migrating faster than (dA)r2N, the two products were easily separated by electrophoresis. The gel was scanned on a 445 SI PhosphorImager (Molecular Dynamics), and band intensities within each lane of migration were quantified with Bio-Rad Quantity One software.
The quantification of each band, in arbitrary units, was plotted against the incubation time, allowing the determination of initial velocity ($v_i$) for single incorporation of either ribo- or deoxyribonucleotide. To minimize the effect of local background heterogeneity caused by contaminating bands in the (dA)$_{10}$ preparation, the selectivity factor was obtained by dividing the initial velocities of deoxyribonucleotide incorporation ($v_{dNTP}$) by the initial velocity of ribonucleotide incorporation ($v_{rNTP}$).

SITE-DIRECTED MUTAGENESIS OF MARINE TdT cDNA—Site-directed mutagenesis was carried out according to the method described by Kunkel (19) using the Muta-Gene kit from Bio-Rad. Oligonucleotides were purchased from Genent (Paris, France). Mutagenesis of amino acid Asp$^{27}$(19) was performed in plasmid pET22(b)-TdT (14). The presence of the mutations was verified by double-stranded DNA sequencing using the primer 5’-ATATCGTCTTGGCGAAGAAC located 30 base pairs upstream from the mutagenesis site. Mutated cDNA was subcloned into plasmid pET28(b) at the NdeI/EcoRI sites and sequenced again before transformation into our expression strain (14). Mutation of amino acid Asp$^{434}$ was performed directly in plasmid pET28(b)-TdT (14). Verification of the change of the codon was done by double-stranded DNA sequencing using the primer 5’-CCAGAATAATGGCTTCCTGATTC located 79 base pairs downstream from the mutagenesis site. Sequences of mutagenic primers were as follows: 5’-ATATCAAGGGCGGCGTGGATTTAGTT (Asp$^{434}$Ala), 5’-patATACAGGGCTCTCCGAATGTT (Asp$^{434}$Glu) and 5’-gCCAGAGCTACACG (Asp$^{434}$Ala).

RESULTS

The Lack of Discrimination Between Ribo- and Deoxyribonucleotides by TdT—The kinetic parameters of the reaction of addition of ribonucleotides to single-stranded DNA have as yet not been determined. We first measured TdT catalytic activity using a standard kinetic assay with a (dA)$_{10}$ oligonucleotide substrate at saturating concentration (150 μM) and dATP or rATP nucleotide substrates at varying concentrations (between 8 and 1000 μM). Initial velocities were measured using linear regression on appropriate time courses (every 30 s for 3.5 min) and were normalized to the concentration of TdT. Michaelis-Menten kinetic parameters are presented in Table I. TdT exhibits the same apparent affinity ($K_m$) for both ribo- and deoxyriboadenosine triphosphate and shows only an $R$ that equals a 3-fold lower polymerization efficiency for rATP than for dATP ($R = k_{cat}/K_m^{\text{dATP}}/k_{cat}/K_m^{\text{rATP}} = 3$) indicating that TdT does not discriminate strongly against rATP. The ratio $R$ of the $k_{cat}/K_m$ values determined independently for two substrates is commonly taken as a good indicator of the selectivity S of the enzyme when the two substrates are simultaneously present (20, 21). A strict $R = S$ equality holds when the enzyme follows Michaelis-Menten kinetics and, more generally for kinetic mechanisms in which the substrate binds to a single conformational state of the enzyme (Fig. 1A in Ref. 22). The value of the TdT selectivity factor $S$ for each ribo/deoxyribonucleotide pair (rATP/dATP, rUTP/dTTP, rGTP/dGTP, and rCTP/dCTP) was experimentally determined in a chain elongation assay, under conditions where both types of nucleotides are present and where there is a single nucleotide addition to the nucleic acid initiator. Because the initiator terminated by a deoxyribonucleotide migrates faster than the initiator terminated by a ribonucleotide, the products are easily separated by gel electrophoresis and quantified. The selectivity factor is given by the ratio of the efficiencies for dNTP and rNTP addition ($v_{dNTP}/v_{rNTP}$). A representative experiment and the selectivity factors calculated from several quantifications of single incorporation chain extension assays (see “Experimental Procedures” for details) are presented in Fig. 1. The S values range between 2.0 ± 0.3 for dCTP over rCTP and 4.9 ± 1.0 for dTTP over rUTP. They are several orders of magnitude smaller than the sugar selectivity factor values reported for other nucleic acid polymerases (3–7). The $S$ value for dATP over rATP differs from the $R$ value by a factor of only 1.5 indicating that under our experimental conditions the enzyme follows Michaelis-Menten kinetics.

Analysis of Incorporation of Ribonucleotides by TdT—The analysis of the behavior of TdT (250 nM) over a 60-min time course, at 5 μM (dA)$_{10}$ with a large excess of rATP (1 mM), shows that the incorporation of rATP reaches a plateau after about 20 min (Fig. 2A). Only 8% (2 nmols) of the total rTTP present in the reaction are polymerized. The chain length distribution pattern is presented in Fig. 2B. TdT elongates all of the (dA)$_{10}$ primer within 5 min but stops after the incorporation of only a limited number of ribonucleotides. After an hour, the major products are elongated by one and two ribonucleotides. Under the same reaction conditions, with dATP as the nucleotide substrate, TdT synthesizes very long polymers (15), and polymerization of 1 mM dATP is completed within an hour (data not shown). The ribonucleotide-terminated products are only weakly elongated with the addition of 1 mM dATP after a

<table>
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<tr>
<th>Nucleotide</th>
<th>$K_m$</th>
<th>$v_{cat}$</th>
<th>$v_{cat}/K_m$</th>
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<tbody>
<tr>
<td>dATP</td>
<td>300 ± 6</td>
<td>180 ± 5</td>
<td>0.6 ± 0.02</td>
</tr>
<tr>
<td>rATP</td>
<td>368 ± 64</td>
<td>76 ± 6</td>
<td>0.2 ± 0.08</td>
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$a$ $K_m$ and $v_{cat}$ mean values ± S.D. were obtained from a nonlinear least-squares fit of the kinetic data to the Michaelis-Menten equation.
15-min incubation (Fig. 2C, lanes 1–2). Addition of 1 mM (dA)10 and 1 mM dATP after 15 min leads to the extension of the newly added nucleic acid substrate (Fig. 2C, lanes 3–4), indicating that the enzyme is still active. When the enzyme/substrate ratio is raised from 0.05 to 0.8, with rATP as a unique nucleotide substrate, TdT is able to synthesize long polymers of rATP, with more than 20 rATP added (Fig. 2D). We compared TdT utilization of (dA)10 and (dA)10rA initiators and found that TdT extends within 5 min 95% of the (dA)10 in the presence of dATP and 80% of the (dA)10 in the presence of rATP. In the presence of dATP or rATP, only 55 and 30% of the (dA)10rA initiator, respectively, are extended (Fig. 3). Michaelis-Menten parameters for (dA)10rA were determined by measuring the rate of dATP polymerization at varying concentrations of (dA)10rA initiator. In Table II, the Km(dA)10rA and kcat(dA)10rA values can be compared with the Km(dA)10 and kcat(dA)10 values previously determined (14). The apparent affinity for the (dA)10rA is slightly higher (2.5-fold) than for the (dA)10, and the rate of dATP polymerization to (dA)10rA is decreased (10-fold). Our results demonstrate that the enzyme cannot efficiently elongate nucleic acids terminated by riboadenosine residues.

Characteristics of Deoxyribonucleotide, Ribonucleotide, and Mixed Ribo/Dexoxyribonucleotide Polymerization by TdT—The indiscriminate incorporation of ribo- and deoxyribonucleotides and the effect of ribonucleotide incorporation on oligomer extension rate by TdT led us to analyze polymer synthesis and growth in the presence of the two types of nucleotides. We compared the extension by TdT of a (dA)10 initiator with dATP (at 10 and 100 μM) or dATP and rATP at equimolar ratio or with rATP in excess (10- and 100-fold) (Fig. 4A). Under all conditions, addition of rATP modifies the pattern of chain length distribution. When rATP is added at equimolar ratios, all the (dA)10 initiators are elongated, but there is a slight reduction in the size of the products. The population of polymers becomes heterogeneous for each length, reflecting the mixed incorporation of dATP and rATP. As the rATP/dATP ratio is raised, the polymers are enriched in rATP leading to a dramatic reduction in the size of the polymers and to an increase in the heterogeneity of the population. The lengths of the products obtained with 100 μM dATP and 1 mM rATP are similar to those obtained with dATP and ddATP at equimolar concentration (100 μM). However, in the presence of dideoxynucleotides, which act as chain terminators, the polymer population is more homogeneous because there is only one ddATP incorporated per chain.

We verified the generality of the profile of mixed polymerization described for dATP/rATP. As shown in Fig. 4B, TdT is unable to add more than 3 rUTP, 3 rCTP, and 4 rGTP residues to the (dA)10 initiator. When we compared the polymerization of individual deoxyribonucleotide to the corresponding ribo/deoxyribonucleotide mixed polymerization (dTTP/rUTP, dGTP/rGTP, and dCTP/rCTP), in the presence of a 10-fold excess of ribonucleotides over deoxyribonucleotides mimicking the cellular pool imbalance (18), we observed again that all the (dA)10...
initiators are elongated, and the polymerization products are shorter than at equimolar concentrations (Fig. 4C). Interestingly, the elongation of all the (dA)_{10} is also observed when TdT polymerizes dCTP alone (Fig. 4C), reflecting the preference of the enzyme for (dA) oligomers over dCMP-terminated oligomers (12). The rapid elongation of all the (dA)_{10} in the presence of both ribo- and deoxyribonucleotides is a consequence of the poor ability of TdT to elongate initiators containing ribonucleotides at the 3’-hydroxyl end. This suggests that the enzyme dissociates from the DNA initiator after the incorporation of one nucleotide, whether a ribo- or a deoxyribonucleotide, and uses deoxynucleotide-terminated initiators preferentially. This hypothesis was tested with the following experiments.

**Analysis of TdT Mode of Polymerization of Ribo- and Deoxyribonucleotides**—Early investigations of polymerization of deoxyribonucleotides showing that the length of TdT products could be predicted by the nucleotide/initiator concentration ratio suggested a non-processive mode of synthesis (23). The mode of polymerization of ribonucleotides was not addressed at the time. We compared the degree of processivity of TdT when polymerizing dATP or rATP by analysis of chain length distributions at various enzyme/initiator ratios. As shown in Fig. 5, the sizes of the polymers synthesized by TdT at high (dA)_{10} initiator concentrations (100 μM), in the presence of an excess of nucleotides (1 mM dATP or rATP), decrease with TdT concentration. At the lowest enzyme concentration (4 nM), most of the initiators have not been elongated. The major product is an 11-mer. Only traces of a 12-mer product are visible. This pattern of chain length distribution, which was also observed for all other ribo- and deoxyribonucleotides (data not shown), is only expected if the probability is very high that the enzyme leaves the nucleic acid substrate after the incorporation of just one nucleotide (24). Thus, the mode of polymerization of both ribo- and deoxyribonucleotides by TdT is distributive.

**Indiscriminate Incorporation of rNTPs and dNTPs by TdT**

Indiscriminate incorporation of rNTPs and dNTPs by TdT—TdT belongs to the family X polymerases (25), a subclass of an ancient nucleotidyltransferase superfamilly (26). Replicative polymerases and nucleotidyltransferases share a catalytic carboxylic residue triad. The single aspartate motif A in nucleotidyl transferases is located downstream from the double aspartate motif C and not upstream as in replicative polymerases (27–32). The double aspartate motif C in human TdT, identified by mutagenesis, is around Asp^{256} and Asp^{434} (33). The position of the third catalytic aspartate was not experimentally determined. Based on amino acid sequence comparison to replicative polymerases from other classes, the single aspartate motif A of DNA polymerase β (pol β), another family X polymerase, had been initially mislocated around Asp^{17} (27). Elucidation of the pol β structure suggested instead that Asp^{17} was the third catalytic aspartate (34), and this was later confirmed by mutagenesis experiments (35). Sequence comparisons of pol β and TdT would suggest that Asp^{256} is functionally equivalent to pol β Asp^{176}. Because TdT Asp^{170} is homologous to pol β Asp^{17}, we compared the activity of TdT variants, mutated at position 170 and position 256, to the activity of wild-type TdT. As shown in Fig. 6, the Asp^{170}Ala and Asp^{170}Glu mutants both retain significant TdT activity, but the Asp^{256}Ala mutant is totally inactivated. This confirms the involvement of the TdT Asp^{256} amino acid in catalysis.

**DISCUSSION**

TdT is defined as a DNA-dependent, non-replicative DNA polymerase despite its known ability to add ribonucleotides to single-stranded deoxyribonucleic acids under standard experimental conditions (12, 13). This natural nucleotide substrate ambivalence had not been characterized. We have analyzed TdT discrimination between ribo- and deoxyribonucleotides by determining the influence of the sugar moiety of the nucleotide on the kinetic parameters of the reaction and by measuring TdT selectivity in single nucleotide addition assays.
in the presence of both ribo- and deoxyribonucleotides. The selectivity factors obtained in single nucleotide addition assays with the four ribo/deoxyribonucleotide pairs are in the 2–5 range indicating that TdT incorporates almost indifferently the ribose or the deoxyribose version of a nucleotide, regardless of the base. This property of TdT, unique among all known polymerases (3–7), led us to test the effect of ribonucleotide incorporation on polymer growth. We demonstrate that TdT extends a ribonucleotide-terminated initiator with a lower catalytic rate than a deoxyribonucleotide-terminated initiator. TdT polymerization efficiency under standard conditions decreased dramatically after the incorporation of only a limited number of ribonucleotides. However, at high enzyme/initiator ratio, TdT was able to synthesize long polymers of ribonucleotides. In the presence of the two types of nucleotides, TdT makes mixed polymers of ribo- and deoxyribonucleotides. The products are shorter than the all-DNA products, reflecting the lack of sugar discrimination of TdT, its preference for deoxyribonucleotide initiators and its distributive mode of synthesis.

The sugar specificity of nucleic acid polymerases can be

FIG. 4. Effects of indiscriminate incorporation of ribo- and deoxyribonucleotides by TdT on polymer growth. 250 nM TdT were incubated with 5 μM 5-32P-labeled (dA)10 and nucleotide substrates as indicated. Aliquots of the reaction were withdrawn at 5 and 15 min (A and B) or 5, 15, and 30 min (C). Products were analyzed on a 16% polyacrylamide denaturing gel. TdT polymerization of dATP alone or in the presence of ddATP or rATP at various concentrations is shown in A. The relative migration of polymers of identical length depends on the nucleotide sugar composition: (dA)ₙ-ddATP migrates faster than (dA)ₙ₋₁ and (dA)ₙ₋₁ faster than (dA)ₙ-rATP. Depending on the relative number of dATP and rATP incorporated, (dA)ₙ₋₁-dATP/rATP mixed polymers migrate to intermediate positions. Polymerization products obtained with the three other conventional ribonucleotides, individually or in the presence of the homologous deoxyribonucleotide, are displayed in B and C respectively.

FIG. 5. Determination of TdT mode of polymerization of ribo- and deoxyribonucleotides. TdT was incubated at various concentrations (500, 100, 20, and 4 nM) with 100 μM 5-32P-labeled (dA)₁₀ and 1 mM dATP or rATP as indicated. Aliquots of the reaction taken at 5 and 15 min of incubation were analyzed on a 16% polyacrylamide denaturing gel.

FIG. 6. Identification of the third catalytic aspartate of TdT. Wild type TdT (filled circles), Asp170Ala (filled diamonds), Asp170Glu (filled squares), and Asp434Ala (filled triangles) mutants were assayed for terminal transferase activity by incorporation of dATP on a (dA)₁₀ primer. 250 nM of purified proteins were incubated at 35 °C for 60 min with 1 mM nucleotide and 100 μM initiator substrates, in the presence of 4 mM Mg²⁺ and 4 μM Zn²⁺. Aliquots of the reactions were taken at 0, 5, 10, 15, 30, 45, and 60 min, and the amount of dATP incorporated was quantified. Each experiment was repeated three times, and results were averaged. Error bars represent S.D.
relaxed under particular conditions, by using Mn$^{2+}$ in place of Mg$^{2+}$ as a catalytic divalent cation (7, 21, 36, 37) or by mutating specific positions within the active site, but it never leads to a complete switch of specificity (6, 38). Incorporation of the “wrong” sugar into the growing chain promptly induces a change of conformation of the nucleic acid substrate, and the reaction stops (21). This scenario has been documented for several RNA and DNA polymerases (3–5, 7). We have shown that the inability of TdT to use RNA substrate efficiently is due to its poor accommodation of single-stranded RNA. Crystallographic studies and construction of mutants should allow an understanding of the structural basis of nucleic acid specificity in TdT and enable the design of a terminal ribonucleotidyl transferase.

For several replicative DNA polymerases sugar discrimination has been shown to depend on a single residue in motif A acting as a steric barrier blocking the 2'-hydroxyl of an incoming ribonucleotide (6, 38). Crystalllographic analyses of pol β suggest a different mechanism in which exclusion of the ribose moiety is caused by a three residue peptide backbone in the flexible hinge region following the single aspartate motif A (39, 40). The topology of the catalytic domain of pol β differs from that of all other replicative polymerases in several aspects (34, 41), and pol β belongs with TdT to an ancient nucleotidyltransferase superfamily (26) in which the single carboxylate motif A is located downstream from the double aspartate motif C (29–32). As a first step toward understanding the mechanisms underlying TdT sugar ambivalence, we confirmed that the third catalytic aspartate is located downstream of motif C.

TdT catalyzes the addition of anti-templated nucleotides to junctions of rearranging V, D, and J gene segments. Despite high concentrations of TdT in lymphocytes, N-regions made by TdT are only 3–4 nucleotides long on average (42), much shorter than those made in a V(D)J recombination cell-free assay (43). A recent study on adenosine deaminase (ADA) deficiency, an inherited lymphoid-specific metabolic disease associated with abnormal purine metabolism (44), showed that alterations in intracellular deoxynucleotide pools can modify the composition of N-regions (45). If nucleotide availability at the sites of V(D)J recombination is not regulated (e.g. by channeling, Ref. 46), the lack of sugar discrimination of TdT, in light of the ribonucleotide excess in cells (18), could affect N-region synthesis. Functional properties of TdT sugar discrimination mutants will be explored in a V(D)J recombination transfection assay (47).

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