Volume 16 Number 7 1988 Nucleic Acids Research

Initiator role of double stranded DNA in terminal transferase catalyzed polymerization reactions

David J.Robbins and Mary Sue Coleman

Department of Biochemistry and the Lucille P.Markey Cancer Center, University of Kentucky Medical Center, Lexington, KY 40536-0084, USA

Received August 31, 1987; Revised and Accepted February 5, 1988

ABSTRACT

Binding of the 58 kDa monomer and 44 kDa $\alpha\beta$ dimer forms of terminal deoxynucleotidyl transferase to double stranded DNA was demonstrated by gel retardation and tryptophan fluorescence quenching. The dissociation constants and cooperativity parameters were similar to those that have been determined for binding of these two forms of terminal transferase to single stranded DNA. However, the double stranded DNA binding site size of 10 nucleotides was half the size expected. The efficacy of blunt ended DNA as an initiator in the polymerization reaction catalyzed by terminal transferase was demonstrated by radiometric assays and product analyses on agarose gels. The initial reaction kinetics indicated that dGTP but not dATP was added efficiently to a blunt double stranded DNA 3' end. These results are correlated with current models for in vivo terminal transferase function.

INTRODUCTION

Terminal deoxyribonucleotidyltransferase (EC 2.7.7.31) is a DNA polymerase which in vitro catalyzes a condensation reaction. The enzyme requires dNTP substrates and a DNA initiator, but no template. It has been postulated that, in vivo, terminal transferase catalyzes the incorporation of N regions (small numbers of nucleotides not coded for by the chromosome) into gene recombination junctions such as the junctions of variable, diversity, and joining regions of the immunoglobin heavy chain (1) and T cell receptor (2) genes. The structural details of a putative DNA initiator for terminal transferase have not been addressed in these models.

The kinetic parameters of reactions initiated with single stranded DNA have been determined under a variety of divalent cation and buffer conditions (3, 4). A random sequential mechanism has been proposed (4). We have recently examined the parameters of terminal transferase binding to single stranded DNA with the aid of fluorescence quenching experiments. Two forms of terminal transferase, a 58 kDa monomer (the principal enzyme form detected in vivo) and a 44 kDa $\alpha\beta$ dimer (a proteolyzed form of the enzyme purified from thymus and also present in vivo), were found to bind cooperatively to poly(dA) and single stranded M13 DNA. A binding site size of approximately 11 nucleotides was calculated (5).

Terminal transferase is commonly employed in recombinant DNA technology to polymerize short homopolymers at the 3' end of DNA vectors and cDNAs (6, 7). Because blunt ended double stranded DNA has been observed to be a poor initiator for terminal transferase (8), most studies reported in the literature advise the use of DNA fragments with 3' overhanging ends. In the studies reported herein, the interaction of terminal transferase with double stranded DNA was probed. We took advantage of the observation that tryptophan fluorescence in the protein is quenched upon DNA binding to study terminal transferase and DNA interactions. Gel retardation experiments allowed us to monitor the formation of protein-DNA complexes. Kinetics experiments revealed differential initial rates of purine nucleotide catalysis with DNA substrates. The implications of these results for current models of terminal transferase function are discussed.

MATERIALS AND METHODS

Buffer compositions. The following buffers were used: 10 mM sodium phosphate, pH 7.4, 0.14 M NaCl (PBS), 90 mM Tris-borate, pH 8.0, 2 mM EDTA (TBE), and 10 mM Tris-HCl, pH 7.4, 0.154 M NaCl (TBS). Cacodylic acid was passed through a Chelex column to remove heavy metal contaminants.

Initiators. ϕX174 double stranded DNA was purchased from BRL. Single stranded DNA-cellulose was purchased from Sigma (St. Louis, MO). Double stranded DNA was isolated from M13mp19 by the method of Maniatis et al. (9). Restriction of ϕX174 and M13 double stranded DNA to form blunt or overhanging 3' ends was performed in a 20 μL reaction mix containing 0.1 mg/mL DNA and the buffers suggested by the manufacturers for each enzyme used. Most reactions were complete after one hour of digestion. The cleaved DNA was used directly without further purification. The two strands of the trp A transcription terminator sequence

5'-d[AGCCCGCCTAATGAGCGGGCTTTTTTTT]-3'

3'-d[TCGGGCGGATTACTCGCCCGAAAAAAAA]-5'

were synthesized using an ABI oligonucleotide synthesizer and reannealed by incubation at 57^{0}C for 2 hours followed by slow cooling to room temperature. Contaminating single stranded DNA was removed by digestion with S1 nuclease (9). The final product was examined for traces of contaminating single stranded DNA on a 25% acrylamide gel in TBE stained with Stains-All (Eastman Kodak). $(dA)_{50}$ was synthesized by the terminal transferase reaction using $(dA)_6$ as the initiator and purified as described (10).

Enzyme purification. The 44 kDa $\alpha\beta$ form of terminal transferase was isolated from calf thymus by the method of Chang and Bollum (11) as modified by Deibel and Coleman (12). The extinction coefficients of 35,000 $M^{-1}cm^{-1}$ at 275 nm and 11,000 $M^{-1}cm^{-1}$ at 295 nm were assumed (5, 13). The 58 kDa monomer form of terminal transferase was isolated

as detailed (5). The specific activities of enzyme preparations were approximately 100,000 units/mg at 35°C for the 44 kDa $\alpha\beta$ form (5,12) and 76,000 units/mg for the 58 kDa form (5). All preparations were $\geq$95% pure as estimated from SDS-polyacrylamide gels (5). Extinction coefficients of 84,000 $M^{-1}cm^{-1}$ at 275 nm and 23,000 $M^{-1}cm^{-1}$ at 295 nm (5) were assumed.

Determination of binding parameters

The binding of the two forms of terminal transferase to double stranded DNA was monitored by quenching of the intrinsic protein fluorescence. The experimental design was identical to that employed for binding of terminal transferase to single stranded DNA (5). All binding data were analyzed only by the graphical method of Schwarz and Watanabe (14, 15) for an infinite lattice (5). An example of the plots of the protein fluorescence vs. the total concentration of terminal transferase used to determine the site size, the observed dissociation constant, the cooperativity parameter, and the intrinsic dissociation constant is shown in Figure 1.

Gel Retardation Assays

Reaction volumes of 20 μL containing 5 μg of DNA and 25 mM potassium cacodylate, pH 7.4 were incubated in the absence or presence of terminal transferase for one hour on ice. All binding reactions were stopped by addition of 5 μL of a solution containing 0.1 M EDTA, pH 8.0, 0.1% bromophenol blue, and 50% glycerol. Samples were loaded onto 10 cm long 0.8% (ϕX174 DNA) or 0.7% (M13 DNA) agarose gels in TBE and run for 3 hours at 80 V. Gels were stained with ethidium bromide and photographed under ultraviolet illumination.

Enzyme Kinetics

Standard radiometric assays for terminal transferase activity employing single stranded DNA, $p(dA)_{50}$, as the initiator and [^{3}H]-dATP or [^{3}H]-dGTP as the monomer (16) were compared to assays employing the 28 base pair double stranded DNA trp A terminator sequence as a 3' blunt ended initiator. Kinetic experiments were performed at constant enzyme concentration; for each series of measurements one of the two substrates was varied while the other was at or near saturating concentration. Initial reaction velocities at 35°C were measured in triplicate and the average values were used to calculate K_m for each substrate (17). One unit of terminal transferase catalyzes the incorporation of 1 nmol of dNTP/hour under these conditions (16).

Product analysis

Hour-long polymerizations of dATP onto restriction digested double stranded DNA by terminal transferase were carried out in a 30 μL reaction volume at 37°C in 200 mM potassium cacodylate, pH 7.4, 8 mM $MgCl_2$. Aliquots of 4 μL were removed at various time intervals and mixed with 2 μL of a solution containing 0.1 M EDTA, pH 8.0, 0.1%

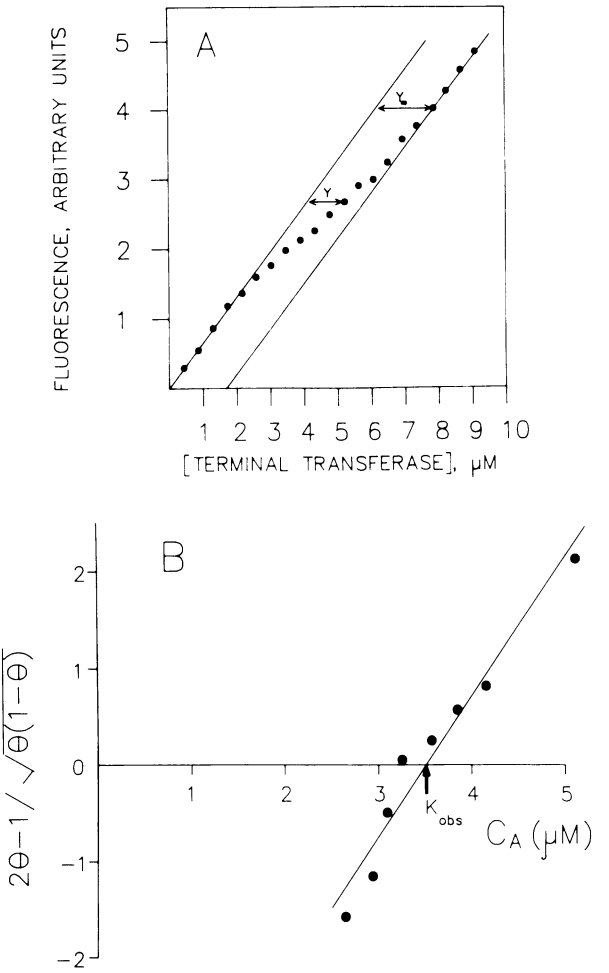


Figure 1. Fluorescence quenching of the 44 kDa αβ form of terminal transferase by M13 double stranded DNA. [A] The protein fluorescence plotted vs. total protein concentration C_A. Data are from an experiment with 0-9.6 μM 44 kDa αβ terminal transferase and 10 μM base pairs (20 μM nucleotides) M13 double stranded DNA in 25 mM potassium cacodylate, pH 7.2. The parallel lines indicate the expected linear increase in unbound protein fluorescence prior to any binding of DNA and after all available binding sites are saturated. The degree of binding, θ, = Y/Y_∞, where Y is the horizontal distance from the initial line to the data point and Y_∞ is the distance between the two lines. The binding site size in nucleotide residues n = ZC_p/C_A where Z is the maximum amount of protein fluorescence quenching possible, C_p is the nucleotide concentration, and C_A is determined by extrapolation of the final line to the C_A axis. In this experiment, n = 11.6 residues. [B] Graphical analysis of the data from A) as described by Schwarz and Watanabe (14). $(2\theta-1)/\sqrt{\theta(1-\theta)}$ vs. the unbound terminal transferase concentration $C_A = C_A - (\theta C_p/n)$. Linear regression using the points corresponding to $0.20 \leq \theta \leq 0.80$ (15) yields a line crossing the abscissa at $C_A = K_{obs}$ and the ordinate at $(2\theta-1)/\sqrt{\theta(1-\theta)} = -\sqrt{q}/n$ where q is the cooperativity parameter. For this experiment, K_{obs} = 3.5 μM, q = 305, and K_{int} ($=qK_{obs}$) = 1.07 mM.

bromophenol blue, and 50% glycerol after 0, 5, 10, 20, 40, and 60 min. Samples were then applied to an agarose gel as described above.

RESULTS

Intrinsic protein fluorescence quenching

The binding of terminal transferase to double stranded DNA was initially demonstrated by titrating a solution of DNA with increasing amounts of 58 kDa or 44 kDa terminal transferase and measuring the intrinsic protein fluorescence quenching. The maximum fluorescence quenching (Z) in the protein-DNA complex was determined to be 0.8, the same value obtained for the protein-single stranded DNA complexes (5). The binding site size n, dissociation constant K_{obs}, cooperativity of binding factor q, and intrinsic dissociation constant K_{int} for the protein-DNA complexes were calculated from the titration data as described previously (5) and in the legend to Figure 1. The data, presented in Table I, indicated that both forms of terminal transferase bind to double stranded DNA in a cooperative manner (q ranged from 115 to 260). The 44 kDa form of terminal transferase exhibited greater cooperativity than the 58 kDa form. The observed dissociation constant K_{obs} was about 3.5 μM for both enzyme forms; the intrinsic dissociation constant K_{int} was 0.5 mM for the 58 kDa enzyme and 0.9 mM for the 44 kDa enzyme. The double stranded DNA binding site size was approximately 11 nucleotides regardless of the form of terminal transferase used in the titration experiment.

Gel retardation analysis

The binding of terminal transferase to double stranded DNA was confirmed by gel retardation analyses. Figure 2A shows the effects of 58 kDa terminal transferase concentration on ϕX174 double stranded DNA band position. At low concentrations of 58

Table I. Parameters derived from titrations of double stranded DNA by terminal transferase as monitored by intrinsic protein fluorescence

Enzyme form	DNA	binding site size (nucleotides)	K_{obs} (μM)	K_{int} (mM)	cooperativity factor (q)
58 kDa					
	calf thymus dsDNA	10.5	3.3	0.56	170
	M13 dsDNA	11.0	3.9	0.45	115
	poly(dA)[a]	11	4.5	0.4	75
44 kDa					
	M13 dsDNA	11.1 ± 0.6	3.5	0.92 ± 0.2	260 ± 60
	M13 ssDNA[a]	10	3.9	0.6	150
	poly(dA)[a]	8	4.5	1.7	400

[a]Values taken from ref. 5 for comparison.

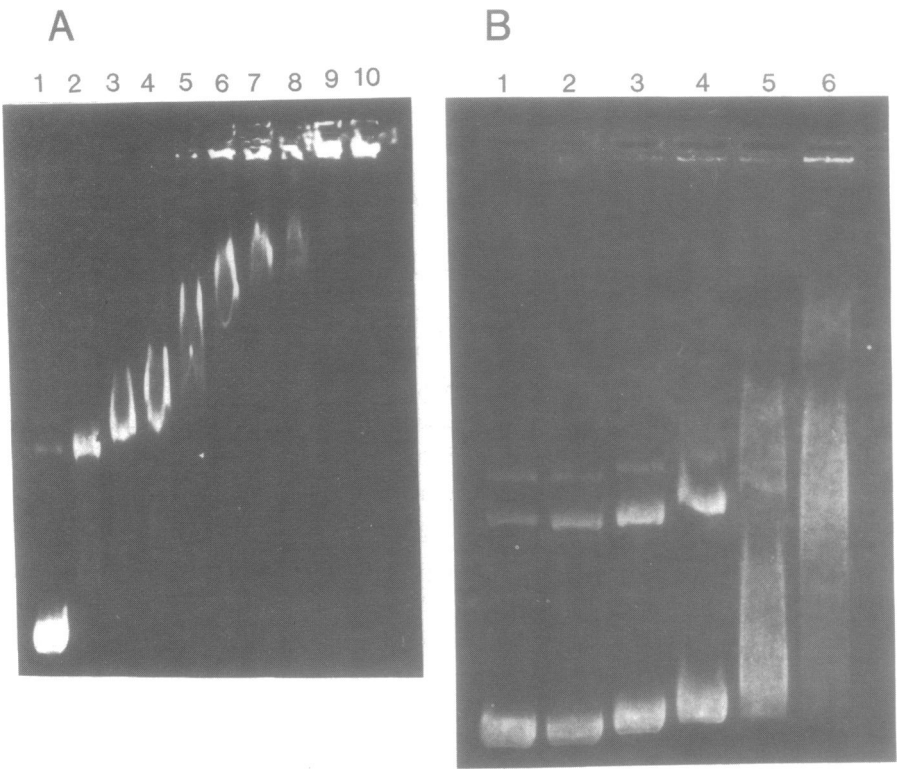


Figure 2. Agarose gel analysis of protein-DNA complexes. [A] Complexes of 58 kDa terminal transferase and supercoiled ϕX174 double stranded DNA. The concentrations of terminal transferase in each gel lane were 1) 0 μM, 2) 1.1 μM, 3) 2.2 μM, 4) 3.2 μM, 5) 4.3 μM, 6) 6.5 μM, 7) 8.6 μM, 8) 10.8 μM, 9) 16.2 μM, and 10) 21.6 μM terminal transferase. [B] Complexes of 44 kDa terminal transferase and supercoiled ϕX174 double stranded DNA. The concentrations of enzyme in each lane were 1) 0 μM, 2) 1 μM, 3) 2 μM, 4) 4 μM, 5) 6 μM, and 6) 12 μM terminal transferase.

kDa terminal transferase, the band corresponding to intact circular DNA was retarded in the gel. The retardation of the complex increased with increasing concentrations of 58 kDa terminal transferase until all of the DNA remained in the wells. Binding of double stranded DNA from M13 phage by the 58 kDa form of terminal transferase was demonstrated in analogous experiments (data not shown).

Endonuclease activity has been detected in preparations of terminal transferase (18). We therefore investigated the possibility that the slower migrating species of ϕX174 DNA observed in the presence of terminal transferase constituted nicked DNA. Electrophoretic anaylsis of DNA purified from binding reactions by phenol extraction (Figure 3A) revealed a DNA ladder spanning intact and nicked DNA. This ladder

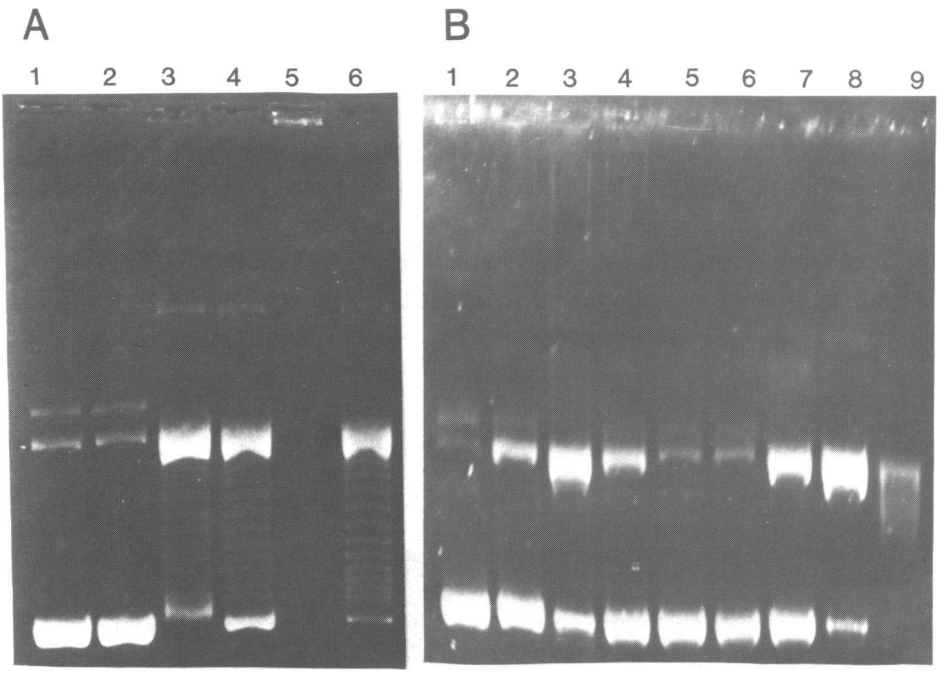
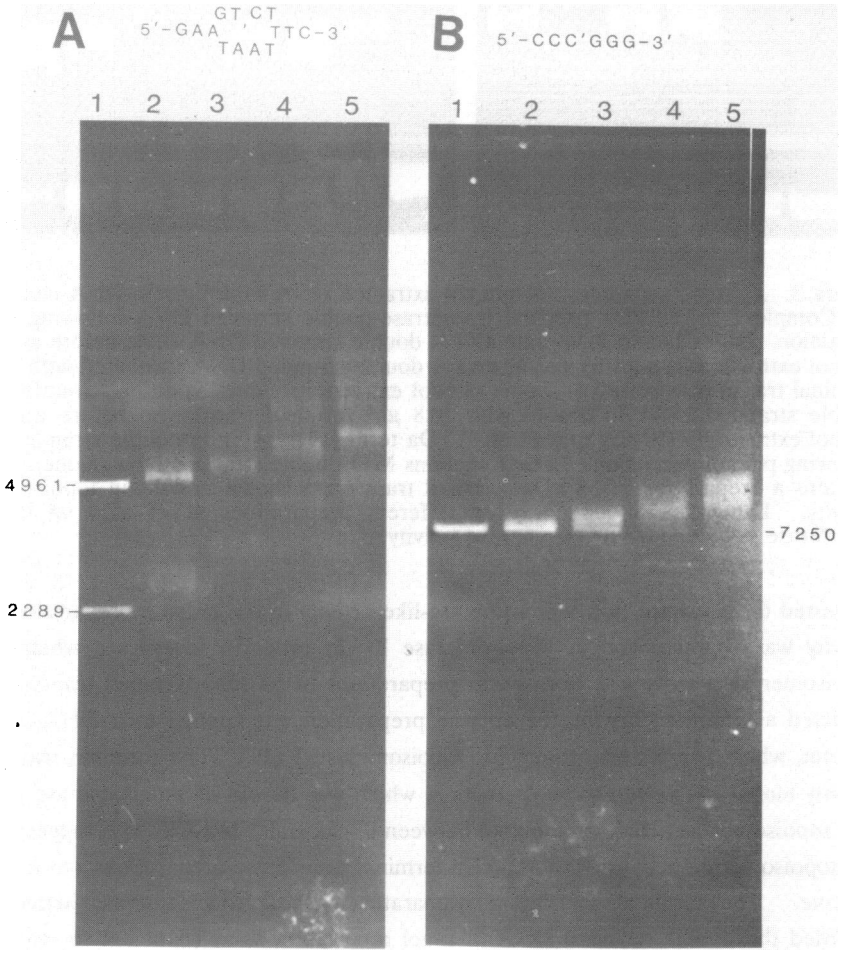


Figure 3. Agarose gel analysis of phenol extracted DNA from protein-DNA complexes. [A] Complexes of 58 kDa terminal transferase-double stranded DNA following phenol extraction. Lanes 1) and 2) contain ϕX174 double stranded DNA alone before and after phenol extraction. Lanes 3) and 4) ϕX174 double stranded DNA incubated with 1.1 μM terminal transferase before and after phenol extraction. Lanes 5) and 6) contain ϕX174 double stranded DNA incubated with 10.8 μM terminal transferase before and after phenol extraction. [B] Complexes of 44 kDa terminal transferase-double stranded DNA following phenol extraction. Lane 1 contains M13 double stranded DNA alone. Lane 9 contains a preparation of 58 kDa terminal transferase shown to contain topoisomerase activity. Lanes 2 - 8 contain seven different preparations of 44 kDa $\alpha\beta$ terminal transferase examined for topoisomerase activity.

suggested the presence of a topoisomerase-like activity in the enzyme preparation. The activity was characterized as topoisomerase I. In order to investigate whether this topoisomerase activity was intrinsic to preparations of 58 kDa terminal transferase or reflected a separate enzyme, the enzyme preparation was applied to a DNA-cellulose column, which has a high affinity for topoisomerase I (19). The terminal transferase activity eluted in the run-through fraction, which was devoid of topoisomerase activity. The topoisomerase activity was eluted between 0.4 M and 2 M KCl. This suggested that the topoisomerase activity in the 58 kDa terminal transferase preparations was a distinct enzyme. The terminal transferase preparation lacking topoisomerase activity also retarded the double stranded DNA in a gel retardation assay (data not shown). This

result indicated that the 58 kDa terminal transferase bound to double stranded DNA.

In agreement with the fluorescence data, the gel retardation experiments also indicated that the 44 kDa $\alpha\beta$ dimer form of terminal transferase bound double stranded DNA (Figure 2B). The higher concentration of protein required to retard DNA suggested that the 44 kDa $\alpha\beta$ dimer bound less tightly than the 58 kDa monomer form (compare Figures 2A and 2B). No topoisomerase activity was detected in any of the 44 kDa $\alpha\beta$ terminal transferase preparations examined (Figure 3B). Because the two enzyme forms were isolated by completely different procedures (a single monoclonal antibody column, versus a multistep conventional scheme), we tested whether topoisomerase activity was present at any stage in the 44 kDa preparation. Crude extracts displayed the 58 kDa form of



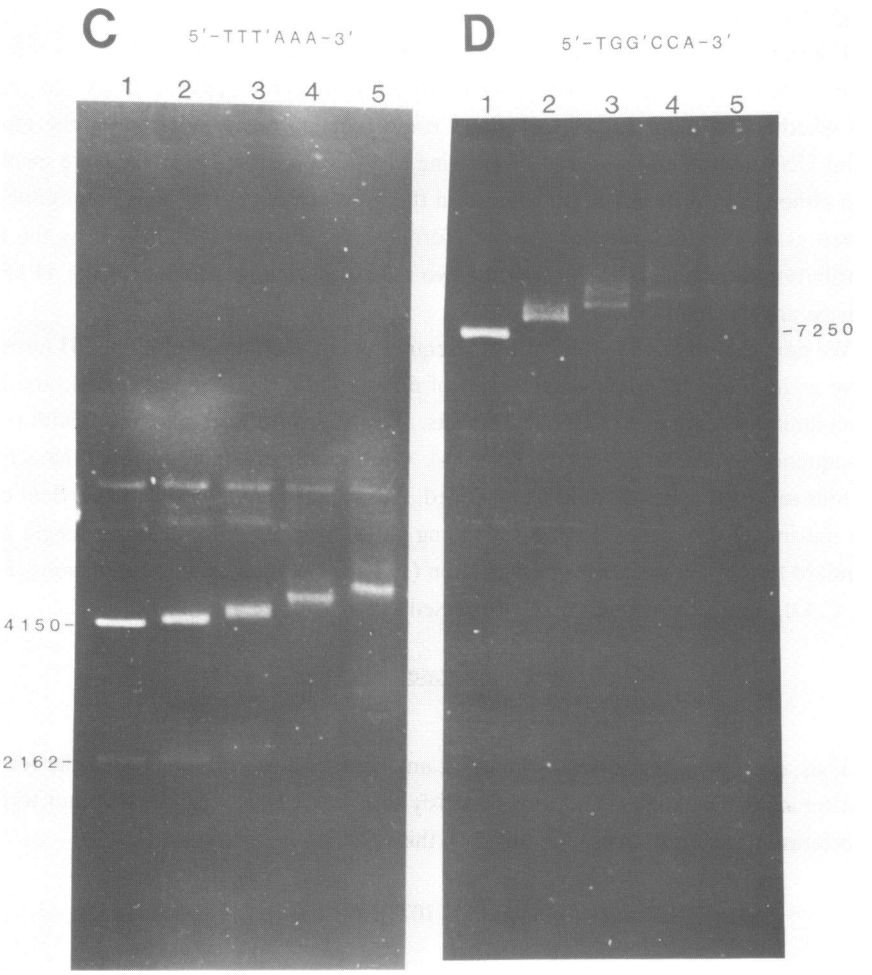


Figure 4. Agarose gel analysis of products produced with blunt-ended double stranded M13 DNA initiators labeled with dATP by 44 kDa $\alpha\beta$ terminal transferase. One strand of the restriction site(s) is indicated for each enzyme. Lanes 1 - 5 are blunt-ended DNA labeled for 0, 15, 30, 45, and 60 min. [A] 0.3 μg of XmnI-cut M13 DNA (two restriction sites per M13 molecule). [B] 0.15 μg of SmaI-cut M13 DNA (one restriction site per M13 molecule). [C] 0.6 μg of DraI-cut M13 DNA (five restriction sites per M13 molecule; the smaller fragments of 400 bp or less do not remain on our gels). [D] 0.15 μg of BalI-cut M13 DNA (one restriction site per M13 molecule).

terminal transferase and topoisomerase activity. The latter persisted through the early stages of purification, but was not present in the final preparation which consisted primarily of 44 kDa $\alpha\beta$ terminal transferase, proteolytically derived from the 58 kDa form (data not shown).

Product analyses

The implication of the fluorescence quenching and gel retardation data was that both forms of terminal transferase could bind efficiently to double stranded DNA. In order to test whether the blunt end 3' OH group could function as an initiator for the enzyme, XmnI DNA fragments, prepared by cleaving M13 double stranded DNA, were incubated with either form of terminal transferase in the presence of dATP. This blunt ended site served as an efficient initiator for both forms of the enzyme as indicated by the equal distribution of extended products in the two reactions (data generated for the 44 kDa $\alpha\beta$ enzyme are shown in Figure 4A).

We next examined the effect of DNA sequence on the ability of blunt 3' OH termini to serve as initiators by employing a series of differentially cleaved (SmaI, BalI, and DraI) blunt double stranded M13 DNA fragments. The fragments generated differ with respect to sequence and chain length (see Figure 4). Our preliminary data suggested that whereas all four sets of fragments could be extended, thus ruling out a sequence-dependent effect, the maximal DNA concentration for tailing varied as a function of chain length at the standard terminal transferase concentration (135 units) employed in our reactions (Figure 4B, C, D). This relationship can be expressed as:

$$\frac{[\text{terminal transferase}]}{\text{nucleotides}} \propto \frac{\text{no. of 3' ends}}{\text{total nucleotides}}$$

Thus, efficient labeling was achieved at any blunt end by adjusting either the terminal transferase or DNA concentrations to satisfy this relationship. At the constant terminal transferase concentration used in Figure 4, this equation becomes:

$$\mu\text{g DNA in 30 } \mu\text{L reaction} = 1078[\text{No. of 3' ends/No. of bases}]$$

Thus, for example, M13 DNA contains 14,500 bases and a single SmaI site, generating two 3' ends. According to the above formula, we found that 0.15 μg of SmaI-cut M13 DNA could be used as an efficient substrate (Figure 4B) but 0.3 μg of DNA inhibited the terminal transferase polymerization reaction (data not shown). If the DNA concentration is to be kept constant, the terminal transferase concentration can be increased or decreased to obtain efficient extension of the 3' OH at a blunt end.

A kinetic analysis over the course of an hour compared dATP incorporation onto M13 double stranded DNA cut with SmaI with incorporation onto M13 double stranded DNA cut with KpnI, which leaves a four-base 3' overhang as an analog to single stranded DNA at the site of catalysis. The total molar addition of dATP was comparable for both blunt and 3' overhang substrates (Table II).

A synthetic 28-mer, the trp A terminator sequence, was used in order to increase the

Table II. Comparison of the efficiencies of blunt-ended and 3' overhang or single-stranded DNA as initiators for terminal transferase-mediated dNTP polymerization[a]

			Enzyme Form			
			44 kDa αβ dimer		58 kDa monomer	
DNA	dNTP	time interval	pmols incorporated	ratio of pmols	pmols incorporated	ratio of pmols
M13-blunt[b]	dATP	15 min	1291		874	
M13-overhang[b]	dATP	15 min	1567		1237	
				0.82		0.71
M13-blunt[b]	dGTP	15 min	425		100	
M13-overhang[b]	dGTP	15 min	460		175	
				0.92		0.56
trp A term.[c]	dATP	10 min	11786		6700	
dA_{50} [c]	dATP	10 min	14738		10811	
				0.80		0.62
trp A term.[c]	dGTP	10 min	507		599	
dA_{50} [c]	dGTP	10 min	1052		739	
				0.48		0.81
trp A term.[c]	dATP	20 sec	74		106	
dA_{50} [c]	dATP	20 sec	786		819	
				0.09		0.13
trp A term.[c]	dGTP	20 sec	518		555	
dA_{50} [c]	dGTP	20 sec	903		895	
				0.57		0.62

[a]Assays were performed in 0.2 M potassium cacodylate, pH 7.4, containing 8 mM $MgCl_2$, saturating dNTP concentrations, and 76 units of 58 kDa or 135 units of 44 kDa αβ terminal transferase. Data were calculated by linear regression analysis of four consecutive time points. [b]M13mp19 double stranded DNA was digested with SmaI (blunt) or KpnI (3' overhang). 1.23×10^{-13} moles of 3' ends were incubated with saturating [^{32}P]-dNTP (specific activity 200-300 cpm/pmol). [c]Assays contained saturating [^{3}H]-dNTP (specific activity varied from 35 to 170 cpm/pmol) and 1 μM (3' ends) $p(dA)_{50}$ or annealed trp A terminator blunt double stranded DNA.

concentration of the blunt 3' ends to saturation (10 μM) in the assay. The trp A terminator sequence was chosen to ensure that all DNA was blunt (as opposed to annealing undefined homopolymers such as poly(dA) and poly(dT)), was long enough to ensure double strandedness, and was short enough so that the concentration of 3' hydroxyl ends could be maintained at saturation in the assay. Under the conditions of the assay, product analysis early in the reaction gave information about the rate of the first nucleotide polymerized at the 3' OH initiator. With this short double stranded DNA we could directly compare polymerization onto blunt 3' ends to polymerization onto the single stranded $p(dA)_{50}$ (16). After 10 minutes of reaction the radioactive dATP incorporated was comparable for the blunt double stranded DNA and the $p(dA)_{50}$ initiators (80% as efficient for the 44 kDa terminal transferase and 60% for 58 kDa terminal transferase). In contrast, the initial rate (during an 80 sec reaction) of dATP

Table III. Kinetic data of 44 kDa αβ terminal transferase with trp A terminator sequence as initiator.

variable substrate	saturating substrate[a]	K_m (μM)[b]	comparable K_m values for single stranded initiator (μM)[c]
trp A	dGTP	2.5 ± 0.8	4.4 (ref. 28)
	dATP	14 ± 3	7 (ref. 28), 4 (ref. 5)
dGTP	trp A	50 ± 10	120 ± 20 (ref. 28)

[a]Concentrations were 50 μM (3' ends) trp A terminator double stranded DNA or 1.25 mM dNTP. [b]Errors are propagated from the least squares analysis. [c]Values from the literature employing $p(dA)_{50}$ as the initiator.

polymerization (Table II) is very inefficient with a blunt 3' initiator as compared with a single stranded initiator (9% and 13% for the 44 kDa and 58 kDa forms of terminal transferase, respectively). When dGTP was used as the monomer substrate, entirely different reaction rates were observed. The initial rates (during an 80 sec reaction) of dGTP polymerization to blunt double stranded DNA and to single stranded $p(dA)_{50}$ were comparable (about 60% for both enzyme forms).

The K_m values for nucleotide and double stranded initiator substrates were determined and compared to those previously obtained with a single-stranded initiator (Table III). The K_m values reflected the observations of the initial rate experiments. The K_m for the trp A terminator was lower with dGTP as the saturating substrate than with dATP. The K_m for dGTP was two-fold lower with a double stranded blunt end initiator than with a single stranded DNA initiator.

DISCUSSION

We report here that two forms of terminal transferase bound double stranded DNA with an efficiency equal to single stranded DNA. The binding of enzyme to double stranded DNA was cooperative and the binding parameters K_{obs} and K_{int} were comparable for each enzyme form to those found to describe single stranded DNA interactions under low salt conditions. The differences in binding between the two terminal transferase forms were also consistent with our earlier work (5). The 58 kDa monomer form had an intrinsic dissociation constant twice that of the 44 kDa αβ form, indicating that it bound more tightly to the DNA. This observed tighter binding to both single and double stranded DNA is in agreement with kinetic experiments which reveal that the 58 kDa enzyme has a lower K_m than the 44 kDa αβ form for high molecular weight initiators (20).

The double stranded DNA binding experiments also revealed a topoisomerase activity in all preparations of the 58 kDa terminal transferase, but in none of the 44 kDa enzyme

samples. The existence of the topoisomerase activity in the enzyme preparations is intriguing, but does not appear to be associated with the capacity of terminal transferase to bind double stranded DNA. The topoisomerase is not intrinsic to terminal transferase protein. While it is a closely associated enzyme that co-purifies on a monoclonal antibody column, it can be separated on a DNA cellulose column. The terminal transferase devoid of topoisomerase (as well as the 44 kDa enzyme) still bound the double stranded DNA.

Previous calculations using single stranded DNA initiators indicated that the binding site size on terminal transferase is 11 nucleotides (5). In the case of double stranded DNA initiators there are several ways to envision protein-DNA interactions. On the basis of the single stranded DNA studies, it could be postulated that the enzyme covered both strands with a binding site size of 11 base pairs or 22 total nucleotides. In fact, the data we report here revealed that only 11 nucleotides comprised the binding site. This observation suggests the possibility that the terminal transferase is interacting with only one strand, leaving the complementary strand free to bind a second enzyme molecule. Alternatively, the geometry of binding double stranded DNA to the enzyme could be entirely different. For example, the terminal transferase might be binding the DNA in a tight loop of 5 base pairs. The method of analysis used in these studies does not differentiate between these two possible binding modes.

Blunt-ended double stranded DNA was shown to be a competent initiator for terminal transferase. The important determinants in efficient extension of the DNA were enzyme concentration and DNA chain length. There was no indication of terminal sequence specificity. These observations are consistent with our earlier finding that terminal transferase binds equally well to interior as well as terminal portions of the DNA (5). Only interactions of the enzyme with DNA termini allow catalysis, and binding of enzyme to internal regions effectively lowers the concentration of enzyme available for polymerization. Because binding at internal sites increases with chain length, the concentration of terminal transferase relative to both DNA chain length and DNA concentration must be optimized. Consideration of these critical ratios in the experiments reported herein most likely accounts for the differences in polymerization rates observed in these as opposed to previous studies by others (21).

In blunt ended DNA initiated reactions terminal transferase exhibited substrate specificity that is not observed with single stranded initiators. We found that dATP was initially polymerized to the 3' OH of the blunt ended trp A terminator sequence at only 10% of the rate for the single stranded $p(dA)_{50}$, while dGTP was initially added at 60% of the rate for $p(dA)_{50}$. The K_m value for initial nucleotide addition confirmed the favored rate for dGTP polymerization at a blunt end 3' OH initiator. This efficient initial addition of dGTP occurred without recourse to methods designed to disrupt the double stranded ends such as inclusion of Co^{2+} ion (cf. 8, 21, 22).

The significance of the preference for dGTP at initial addition to blunt 3' OH ends is not clear given information about the nucleotide composition of non-genomic segments of rearranged immunoglobulin and T-cell receptor genes. When the N regions were first discovered, the sequences described were highly enriched for dG nucleotides. However, as more of these N regions are elucidated (1, 2, 23-27), an overwhelming preference for G residues is not apparent. Based on our calculations, of the 22 separate sequences that have been published from murine N regions, assuming that terminal transferase mediated DNA synthesis occurred at the J terminus, 31 of the residues are G, C = 26, A = 20, and T = 19.

Many questions remain concerning details of the in vivo function of terminal transferase. While considerable circumstantial evidence supports hypotheses linking terminal transferase to gene rearrangement events, no definitive demonstration of the involvement of this enzyme has yet been made. It is not known if terminal transferase is capable of polymerizing nucleotides on one strand of the D region gene or the other strand of the J region gene of the immunoglobin heavy chain gene. The work presented here indicates that functional models of terminal transferase need not invoke a single stranded DNA terminus. We did detect a distinct topoisomerase I activity that co-purifies with 58 kDa terminal transferase. Whether this activity or others function along with terminal transferase to introduce variability and for joining segments of immunoglobin genes as well as T cell receptor genes (23) will be of interest in future investigations.

ACKNOWLEDGEMENTS

This work was supported by National Cancer Institute Grants CA19492 and CA26391. We are grateful to Ms. Karen Gathy for technical assistance in enzyme purification.

REFERENCES

1. Alt, F.W., and Baltimore, D. (1982) Proc. Natl. Acad. Sci. USA 79, 4118-4122
2. Goverman, J., Minard, K., Shastri, N., Hunkapiller, T., Hansburg, D.,Sercarz, E., and Hood, L. (1985) Cell 40, 859-867
3. Kato, K.-I., Goncalves, J.M., Houts, G.E., and Bollum, F.J. (1967) J. Biol. Chem. 242, 2780-2789
4. Deibel, M.R., Jr., and Coleman, M.S. (1980a) J. Biol. Chem. 255, 4206-4212
5. Robbins, D.J., Barkley, M.D., and Coleman, M.S. (1987) J. Biol. Chem. 262, 9494-9502
6. Okayama, H., and Berg, P. (1982) Mol. Cell. Biol. 2, 161-170
7. Heideker, G., and Messing, J. (1983) Nucl. Acids Res. 11, 4891-4906
8. Michelson, A.M., and Orkin, S.H. (1982) J. Biol. Chem. 257, 14773-14782
9. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory, NY
10. Coleman, M.S., and Hutton, J.J. (1981) in Methods in Hematology: The Leukemic Cell (Catovsky, D., ed) Vol. 2, pp. 203-219, Churchill Livingstone, Edinburgh
11. Chang, L.M.S., and Bollum, F.J. (1971) J. Biol. Chem. 246, 909-916
12. Deibel, M.R., Jr., and Coleman, M.S. (1980b) Arch. Biochem. Biophys. 202, 414-419
13. Robbins, D.J., Deibel, M.R., Jr., and Barkley (1985) Biochemistry 24, 7250-7257
14. Schwarz, G., and Watanabe, F. (1983) J. Mol. Biol. 163, 467-484

15.Watanabe, F., and Schwarz, G. (1983) J. Mol. Biol. 163, 485-498
16.Coleman, M.S. (1977) Arch. Biochem. Biophys. 182, 525-532
17.Cleland, W.W. (1967) Adv. Enzymol. 29, 1-32
18.Deibel, M.R., Jr. (1983) Biochem. Biophys. Res. Commun. 115, 909-916
19.Liu, L.F., and Miller, K.G. (1981) Proc. Natl. Acad. Sci. USA 78, 3487-3491
20.Augl,C., Lee, S., Breviario, D., Chang, L.M.S., and Bollum, F.J. (1983) Fed. Proc. 42, 2278
21.Roychoudhury, R., Jay, E., and Wu, R. (1976) Nucl. Acids Res. 3, 863-877
22.Deng, G., and Wu, R. (1981) Nucl. Acids Res. 9, 4173-4188
23.Yancopoulos, G.D., Blackwell, T.K., Suh, H., Hood, L., and Alt, F.W. (1986) Cell 44, 251-259
24.Desiderio, S.V., Yancopoulos, G.D., Paskind, M., Thomas, E., Boss, M.A., Landau, N., Alt, F.W., and Baltimore, D. (1984) Nature 311, 752-755
25.Siu, G., Kronenberg, M., Strauss, E., Haars, R., Mak, T.W., and Hood, L. (1984) Nature 311, 344-350
26.Alessandrini, A., Pierce, J.H., Baltimore, D., and Desiderio, S.V. (1987) Proc. Natl. Acad. Sci. USA 84, 1799-1803
27.Hagiya, M., Davis, D.D., Shultz, L.D., and Sakano, H. (1986) J. Immunol. 136, 2697-2700
28.Deibel, M.R., Jr., Liu, C.-J., and Barkley, M.D. (1985) Anal. Biochem. 144, 336-346