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Interactions of Photoactive DNAs with Terminal Deoxynucleotidyl Transferase: Identification of Peptides in the DNA Binding Domain†

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ABSTRACT: Terminal deoxynucleotidyl transferase (terminal transferase) was specifically modified in the DNA binding site by a photoactive DNA substrate (hetero-40-mer duplex containing eight 5-azido-dUMP residues at one 3′ end). Under optimal photolabeling conditions, 27–40% of the DNA was covalently cross-linked to terminal transferase. The specificity of the DNA and protein interaction was demonstrated by protection of photolabeling at the DNA binding domain with natural DNA substrates. In order to recover high yields of modified peptides from limited amounts of starting material, protein modified with ^{32}P-labeled photoactive DNA and digested with trypsin was extracted 4 times with phenol followed by gel filtration chromatography. All peptides not cross-linked to DNA were extracted into the phenol phase while the photolyzed DNA and the covalently cross-linked peptides remained in the aqueous phase. The ^{32}P-containing peptide–DNA fraction was subjected to amino acid sequence analysis. Two sequences, Asp^{221}–Lys^{231} (peptide B8) and Cys^{234}–Lys^{249} (peptide B10), present in similar yield, were identified. Structure predictions placed the two peptides in an α-helical array of 39 Å which would accommodate a DNA helix span of 11 nucleotides. These peptides share sequence similarity with a region in DNA polymerase β that has been implicated in the binding of DNA template.

The events involved in the rearrangements of immunoglobulin and T-cell receptor genes appear to be mediated in part by terminal deoxynucleotidyl transferase (terminal transferase)[1] (Alt & Baltimore, 1982; Alt et al., 1986). This enzyme is non-template-directed and catalyzes the addition of deoxynucleoside triphosphates onto a 3′-OH group of a DNA initiator in a distributive manner. A 58-kDa polypeptide chain represents the primary translated sequence of this protein, and polymerase activity is the sole catalytic function identified with the protein. Terminal transferase can be cleaved by limited proteolysis to two small polypeptides, α (11 kDa) and β (33 kDa), both of which are required for catalytic activity (Deibel & Coleman, 1980a; Bollum & Chang, 1981; Chang et al., 1982).

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[1] Abbreviations: TdT, terminal deoxynucleotidyl transferase; azido-dU-DNA, random-sequence 40 base pair DNA that contained 5-azidouracil in place of thymidine; DS DNA, nonradioactive DNA(a) shown in Figure 1 in which 5-azidouracil is replaced by thymidine; TS DNA, template strand of DS DNA; CS DNA, complementary strand to TS DNA; 8-azido-dATP, 8-azido-2′-deoxyadenosine 5′-triphosphate; 5-azido-dUTP, 5-azido-2′-deoxyuridine 5′-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FPLC, fast-performance liquid chromatography; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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The location of the polymerase catalytic site on terminal transferase has been approached by using chemical modification studies since X-ray crystallography of this protein has not yet been feasible. The deoxynucleotide binding site on terminal transferase has been probed by using UV light mediated cross-linking of substrate and substrate analogues. Direct cross-linking of terminal transferase using dTTP (Pandey & Modak, 1988) resulted in isolation of two modified peptides[2] identified as Asp^{221}–Lys^{231} and Cys^{234}–Lys^{249}. In contrast, when a photoaffinity analogue, 8-azido-dATP, was used to modify the enzyme active site, two peptides corresponding to a different region, which encompassed the primary amino acid sequence of Ile^{351}–Lys^{381}, were identified (Evans & Coleman, 1989; Evans et al., 1989). These latter peptides bear striking similarity to active-site sequences found in other nucleotide binding proteins (adenylate kinase, DNA polymerase β, and three viral DNA polymerases).

A similar strategy for probing the DNA binding site of terminal transferase has been devised. Preliminary experiments have shown that DNA polymerase or terminal transferase mediated incorporation of 5-azido-dUTP or 8-azido-dATP (Evans & Haley, 1987) into double- or single-stranded DNA initiators produced DNAs that functioned as efficient substrates for terminal transferase (Farrar et al., 1990; unpublished data of Evans and Coleman). Moreover, in the presence of UV light, these DNAs labeled the protein, and photolabeling was light-dependent. In this paper, we present a detailed characterization of the photolabeling of calf thymus terminal transferase with photoactive DNA probes, and the identification of two photolabeled peptides. These DNA binding peptides reside within the region of the protein that shares sequence similarity with the template-dependent DNA polymerase β (Matsukage et al., 1987) and bind nucleotides within 10 base pairs of the 3′ end of the DNA substrate. Taken together, the properties of this terminal transferase domain suggest that it is in close proximity to the catalytic site of the polymerase.

Experimental Procedures

Materials. [^{3}H]dATP and [α-^{32}P]dATP were purchased from New England Nuclear. dA_{50} was prepared as reported by Coleman and Hutton (1981). dA_{16} and dT_{25-30} were purchased from Pharmacia. $(dAdT)_{20}$ was chemically synthesized on an Applied Biosystems 380B DNA synthesizer (Macromolecular Structure Facility, University of Kentucky). The photoprobes 5-azido-dUTP and 8-azido-dATP were synthesized according to Evans and Haley (1987) and Czarnecki et al. (1979). All other chemicals were of reagent grade or higher from various commercial sources.

Enzymes. Klenow fragment of *Escherichia coli* DNA polymerase I was purchased from Promega Corp. Terminal transferase was purified from calf thymus according to Diebel and Coleman (1980a). The purified enzyme preparations had a specific activity of greater than 100 000 units/mg. Enzyme units are defined as 1 nmol of mononucleotide polymerized to DNA initiator per hour.

Preparation of Photoactive DNAs. The template strands and the 24-base primers (24 bases complementary to the 3′-OH end) of DNA(a), DNA(b), and DNA(c) shown in Figure 1 were chemically synthesized on an Applied Biosystems 380B DNA synthesizer. The template strand containing a 5′-dimethoxytrityl group was separated from short failure sequences

[2] The numbering of amino acid residues begins with the amino terminus of the 58-kDa form of TdT. This form of the enzyme contains a total of 520 amino acid residues.

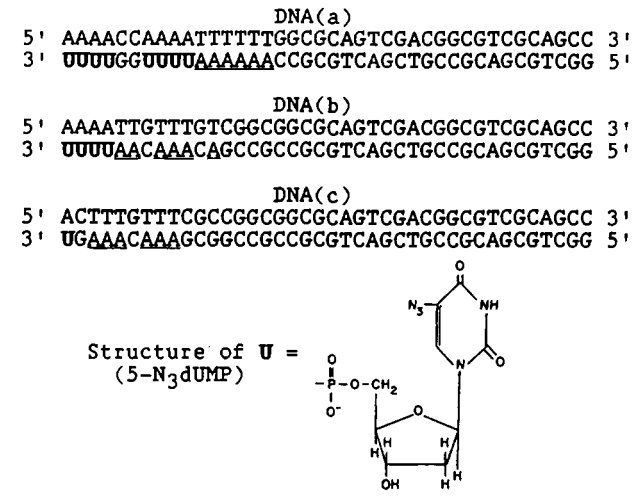

FIGURE 1: Structure of photoactive DNAs. U and A represent 5-azido-dUMP and [^{32}P]dAMP, respectively.

by C_{18} reverse-phase HPLC using a 30-min linear gradient of 100 mM triethylamine and methanol (Applied Biosystems User Bulletin 14, 1984). Newly synthesized (1 μmol) 24-base primers for the three DNAs (described in Figure 1) were dissolved in 100 μL of deionized water and ethanol-precipitated. The template and primer were annealed in 10 mM Tris-HCl (pH 7.8), 100 mM NaCl, and 0.1 mM EDTA as described in Maniatis et al. (1982), with minor modifications. The photosensitive 5-azido-dUMP-containing DNA samples shown in Figure 1 were prepared by filling in the remaining 16 bases under the template direction of Klenow fragment. The fill-in reactions, containing 20 mM Tris–acetate (pH 7.4), 10 mM magnesium acetate, 0.1 mM EDTA, 1 mM dAMP, and either 5 μM (50 μCi)[α-^{32}P]dATP or 40 μM nonradioactive dATP, either 40 μM 5-azido-dUTP or dTTP, 40 μM dCTP, 40 μM dGTP, 0.6 μM template/primer, and Klenow fragment (2–10 units), were incubated at 25 °C for 15–30 min, and then terminated by the addition of diethyl ether with vortexing. Following the evaporation of ether, the reaction mixture was applied to a small Sephadex G-25 column to separate the DNA from mononucleotides. DNA was eluted with a buffer containing 10 mM Tris–acetate (pH 8.2), 50 mM NaCl, 5% glycerol, and 0.1 mM EDTA.

Photoactive and radiolabeled ^{32}P single-stranded DNA [dA_{16}(azido-dA/dA)$_5$] was synthesized by terminal transferase mediated copolymerization of azido-dATP and [α-^{32}P]dATP onto a dA_{16} initiator. The reaction mixture contained 200 pmol of dA_{16}, 1 nmol of 8-azido-dATP, 1 nmol of dATP, 100 μCi of [α-^{32}P]dATP, 200 mM potassium cacodylate (pH 7.5), 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 3000 units of terminal transferase in 214 μL. Following incubation at 37 °C for 4.5 h, the reaction was terminated by the addition of diethyl ether, and the DNA was separated from mononucleotides as described above.

Gel Electrophoresis. Nondenaturing polyacrylamide gel (12%) electrophoresis was carried out according to a protocol described by Maniatis et al. (1982) and SDS–polyacrylamide gel (12%) electrophoresis according to Laemmli (1970). Following electrophoresis, nondenaturing gels were dried and autoradiographed. SDS–polyacrylamide gels were stained with Coomassie Blue, destained, dried, and autoradiographed.

Terminal Transferase Assays. Reactions containing 1 mM [^{3}H]dATP (20 cpm/pmol), 0.01 mM dA_{50}, 0.2 M potassium cacodylate (pH 7.5), 1 mM 2-mercaptoethanol, and 8 mM $MgCl_2$ were preincubated for 2 min at 25 °C prior to the addition of terminal transferase. The reactions were incubated

at 25 °C, and aliquots were removed at designated time points. The incorporation of radioactive mononucleotides into dA_{50} was monitored by using the GF/C filter paper assay as described by Diebel and Coleman (1980a). Any deviation from this standard assay scheme is described in figure legends.

Photoaffinity Labeling. Reaction mixtures (50 μL) containing 16 mM Tris–acetate (pH 8.2), 10 mM NaCl, 1% glycerol, 0.02 mM EDTA, and appropriate amounts of photoactive DNA and terminal transferase were placed on a parafilm surface and irradiated for 1 min with a 302-nm UV lamp (Spectronics Corp., Model EB-28) from a distance of 0.5 cm (5500 μW/cm²). Parallel control reactions were irradiated in the absence of terminal transferase. Following photolysis, heparin (final concentration of 4 mM) was added to all reactions to reduce the interaction between DNA and the glass fiber filter during quantitation.

Quantitation of Photolabeled Terminal Transferase. Aliquots of photolysis reactions were spotted on glass fiber filters (34, Schleicher & Schuell) and precipitated with 2 M ammonium acetate. The filters were then washed with deionized water, dehydrated by three washes in cold 95% ethanol and one ether wash, air-dried, and monitored for radioactivity by scintillation spectroscopy.

Preparative Cross-Linking of DNA to Terminal Transferase and Isolation of Photolabeled Peptides. The preparative reaction mixtures contained 2.5 nmol of DNA(a) (Figure 1) (primer/template DNA was filled in and ^{32}P labeled as described above substituting 5-azido-dUTP for dTTP). The DNA was purified by ethanol precipitation and resuspended in 50 μL of 60 mM Tris–acetate, pH 8.2. Purified terminal transferase (5 nmol) was diluted in 2 mL of 60 mM Tris–acetate, pH 8.2, and concentrated on a 30k Centricon (Amicon Corp.) to 200 μL in order to remove glycerol from the preparation. The protein concentrate was added to the photoactive DNA, and the mixture was diluted to 1 mL with water and divided into four equal aliquots. Photolysis was carried out by using a 302-nm UV lamp (5500 μW/cm²) for 1 min at a distance of 0.5 cm. The entire sample (1 mL) was concentrated to 150 μL with repeated anhydrous butanol extractions. Three ether extractions removed all traces of butanol. Following ether evaporation, 50 μL of 8 M urea was added, and the volume was adjusted to 200 μL with water to yield a final concentration of 2 M urea. Trypsin was added at a final concentration of 10% (w/w) and digestion continued 4 h at 37 °C. Peptides not cross-linked to DNA were removed from the aqueous phase with four consecutive phenol extractions using 200 μL of Tris-saturated phenol at each step (Beach et al., 1990). Residual phenol was removed with three diethyl ether extractions. The remaining ether was evaporated and the volume adjusted to 100 μL with 50 mM NH_4HCO_3. The sample was applied to a NAP-10 column (Pharmacia) equilibrated in 50 mM NH_4HCO_3, washed into the matrix with 900 μL of 50 mM NH_4HCO_3, and eluted with 500 μL of 50 mM NH_4HCO_3. The photolabeled peptides were dried under vacuum, resuspended in 100 μL of 25% acetonitrile, and dried again. The residue was resuspended in water. The amino acid sequence of the peptides was determined on an Applied Biosystems 477A peptide sequencer with on-line phenylthiohydantoin amino acid identification.

Results

The photoactive double-stranded DNAs synthesized for this work contained blunt-end 3′-OH groups (Figure 1) which, as we previously have demonstrated, serve as satisfactory initiators for DNA polymerization catalyzed by terminal transferase (Robbins & Coleman, 1988). The effect of the azide group

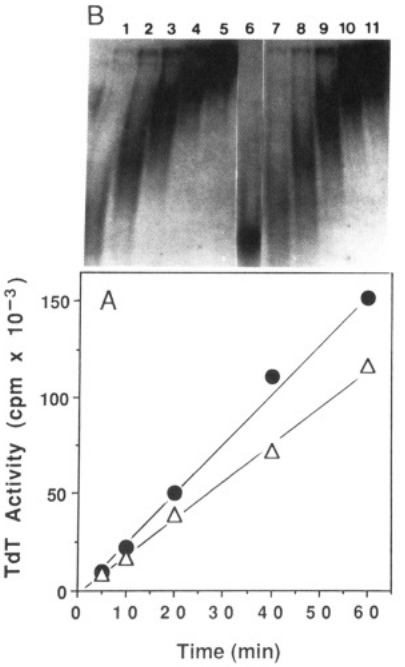

FIGURE 2: (A) Polymerization of [α-^{32}P]dATP onto nonradioactive azido-dU-DNA [DNA(a) from Figure 1] (●) and nonradioactive dTMP-containing control DNA (△). Reactions contained 0.064 μM DNA, 1 mM [α-^{32}P]dATP (570 cpm/pmol), 14.4 μg/mL terminal transferase, and other components as described under Experimental Procedures. (B) Electrophoretic analysis (12% nondenaturing gel) of products synthesized in the reactions (described above) incubated for 5, 10, 20, 40, and 60 min and initiated with control dTMP-containing DNA (lanes 1–5) or azido-dU-DNA (lanes 7–11). Nonpolymerized ^{32}P-labeled azido-dU-DNA [DNA(a) in Figure 1] was electrophoresed in lane 6. Shown is an autoradiogram of the gel.

on the capacity of the DNA to serve as an initiator was assessed by comparing the reaction rates obtained with photoactive DNA [DNA(a) in Figure 1] and control DNA containing dTMP in place of azido-dUMP under conditions optimal for terminal transferase activity (Diebel & Coleman, 1980a). Virtually identical reaction rates were obtained (Figure 2A). Moreover, analysis of products synthesized during reaction revealed that the average number of residues added to each DNA initiator was comparable (Figure 2B). These results indicated that azido-dUMP containing DNA bound as a substrate DNA within the terminal transferase catalytic site.

Optimization of the conditions for maximal photoincorporation of DNA into terminal transferase was accomplished by comparing Tris-HCl, Tris–acetate, and Hepes–acetate at concentrations ranging from 12.5 to 100 mM, as well as various salt and Mg^{2+} concentrations. Maximum levels of photoincorporation were obtained with 16 mM Tris–acetate, pH 8.2, and 10 mM NaCl. Photolabeling was independent of Mg^{2+} (data not shown). The standard exposure to UV light (302 nM) was 1 min at a distance of 0.5 cm. Terminal transferase did not lose enzymatic activity after 3 min of irradiation at this wavelength (Evans & Coleman, 1989).

The specificity of binding and photoincorporation of 5-azido-dUMP containing double-stranded DNA to terminal transferase were initially assessed in control reactions containing prephotolyzed DNA(a). Following rephotolysis, no cross-linking of DNA(a) to terminal transferase was observed (data not shown). Photoincorporation of DNAs(a), -(b), and -(c) (Figure 1) into varying amounts of terminal transferase was then quantitated. The sigmoidal saturation curves obtained (Figure 3) demonstrated that the binding of terminal transferase to DNA was cooperative with a Hill coefficient

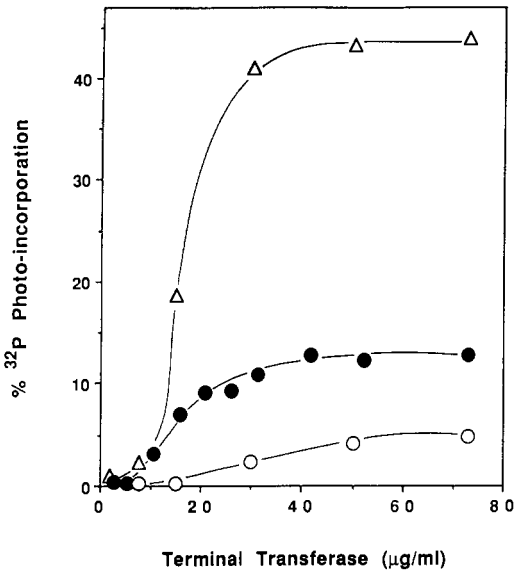

FIGURE 3: Saturation of photoactive DNA by terminal transferase. Photolabeling reactions contained 4.6 nM DNA and increasing amounts of terminal transferase. After photolysis, additional terminal transferase was added to each reaction mixture to make the final concentration of the enzyme equal to the highest concentration of terminal transferase used [68 μg/mL for DNA(a), 83 μg/mL for DNA(b), and 125 μg/mL for DNA(c)] since the total amount of protein affected the degree of the interaction between DNA and the filter paper. All other reaction components and conditions were as described under Experimental Procedures. (△) DNA(a); (●) DNA(b); (○) DNA(c).

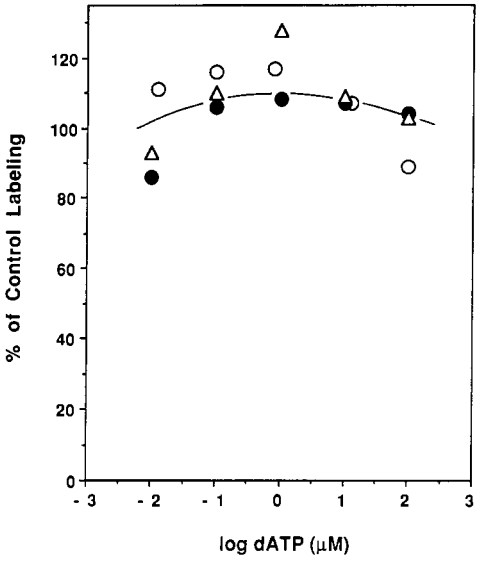

FIGURE 4: Effect of Mg·dATP on the labeling of terminal transferase with photosensitive DNA. Reaction components were 9.6 nM DNA, 2.6 mM $MgCl_2$, and 29, 48, and 70 μg/mL terminal transferase for photolabeling with DNA(a), DNA(b), and DNA(c), respectively. All other conditions were as described under Experimental Procedures. The control labeling was based on a reaction lacking dATP. (○) DNA(a); (●) DNA(b); (△) DNA(c).

(Segal, 1975) of 3.8 for DNA(a). While the calculations of Hill coefficients from the data obtained with DNA(b) and DNA(c) were less accurate, similar values (approximately 3.5) were consistently obtained. These results indicated that DNA(a), which contains eight azido-dUMP residues, was saturated effectively with terminal transferase and that sufficient cross-linking to DNA occurred to make subsequent isolation of photolabeled peptides feasible.

The interaction of photoactive DNA with terminal transferase was further investigated in competition experiments

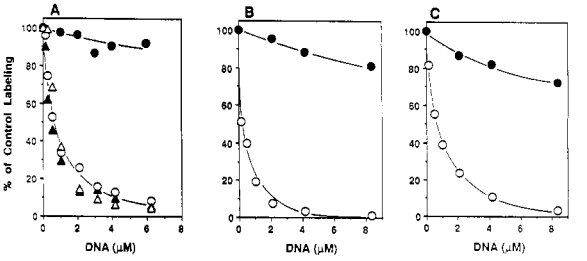

FIGURE 5: Effect of single- or double-stranded DNAs on the labeling of terminal transferase with photosensitive DNA. Reaction components were 9.4 nM radioactive photosensitive DNA and 29, 48, and 70 μg/mL terminal transferase for photolabeling with DNA(a) (panel A), DNA(b) (panel B), and DNA(c) (panel c), respectively. (▲) is template strand DNA (TS); (○) is complementary strand DNA (CS); (△) is double-stranded DNA (DS); (●) is dA_{50}. All other conditions were as described under Experimental Procedures.

Table I: Competitive Reduction in Binding of Photoactive DNAs to Terminal Transferase

	% DNA cross-linked to terminal transferase	
competitor DNA	DNA(a)[a]	dA_{16}(8-azido-dA/dA)$_5$[b]
none	100	100
CS DNA	7	3
dA_{16}	93	22
dA_{50}	71	18
dT_{25-30}	30	33
dA_{16} + dT_{25-30}	0	6
$(dA\text{-}dT)_{20}$	0	1

[a] DNA(a) (4 nM) was photoincorporated into 30 μg/mL terminal transferase in the presence of 7.5 μM competing DNA. In the reaction containing both dA_{16} and dT_{25-30}, the concentration of each competitor DNA was 3.75 μM. [b] Photo-cross-linking terminal transferase with dA_{16}(8-azido-dA/dA)$_5$ was accomplished in the presence of 16 nM photoactive DNA, 20 μM competing DNA, and 40 μg/mL terminal transferase. In the reaction containing both dA_{16} and dT_{25-30}, the concentration of each DNA was 10 μM. All other reaction components and conditions were as described under Experimental Procedures.

employing Mg·dATP as a competitor in the photoincorporation reactions. No decrease in the cross-linking of DNA to protein was observed over a concentration range of 10 nM to 100 μM Mg·dATP (Figure 4). These data were consistent with the random sequential mode of polymerization of the reaction catalyzed by terminal transferase (Deibel & Coleman, 1980b) and indicated that photoactive DNA probes did not label the nucleotide binding site.

In order to determine the relative binding affinities of photosensitive DNA and natural single- or double-stranded DNAs, a competition experiment was performed in which a constant concentration of radiolabeled DNA(a) was photolyzed in the presence of terminal transferase and increasing concentrations of competitor DNAs. When DNA(a) was the DNA probe in the reaction, efficient competition was obtained with the template strand hetero-40-mer DNA (TS DNA), with the complementary strand hetero-40-mer DNA (CS DNA), and with the hybrid double-stranded 40-mers (DS DNA). Virtually identical competition by CS DNA was observed when DNA(b) and DNA(c) were the photoactive components in the reaction (Figure 5).

The inefficient competition of protein–DNA cross-linking by dA_{50} (Figure 5) was investigated in greater detail by cross-linking terminal transferase with DNA(a) or with the single-stranded homooligomer dA_{16}(8-azido-dA/dA)$_5$ in the presence of various competitor DNAs (Table I). As expected, the heterooligomers and double-stranded DNAs competed effectively with both photoactive species for protein binding. The oligomers containing only dA or dT residues were less

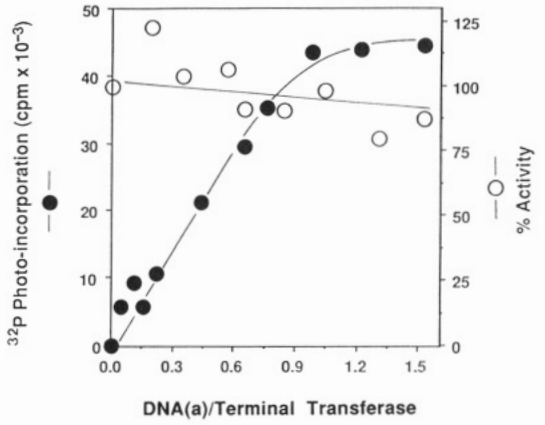

FIGURE 6: Saturation of terminal transferase with photosensitive DNA(a). For ^{32}P photoincorporation (●), the reaction mixture contained 0.46 μM terminal transferase and various concentrations of radioactive DNA(a). For measurement of enzyme activity remaining after photolysis (○), nonradioactive DNA(a) was used to photolabel terminal transferase. Terminal transferase activity employing the standard assay system was measured for the first 4 min subsequent to photolysis during which time the enzyme activity was linear. All other conditions were as described under Experimental Procedures.

effective competitors apparently because they bound the enzyme with lower affinities than heterooligomeric DNAs. However, both types of photoactive DNA could be completely inhibited from cross-linking to terminal transferase, indicating that these DNA analogues occupied the DNA binding domain.

The final test of binding specificity by the photoactive DNAs was a determination of the stoichiometry of binding. Saturation of photoincorporation was achieved when the molar ratio of DNA(a)/terminal transferase approached 1 (Figure 6). However, in order to achieve saturation, an excess of protein had to be employed. At this ratio, repetitive cross-linking experiments revealed that 3–7% of the protein in the reaction was cross-linked to DNA (data not shown). The un-cross-linked terminal transferase (93% of the enzyme in the reaction) would be expected to remain active. Because of the low percentage of cross-linked enzyme, the kinetics of enzyme inactivation could not be assessed (Figure 6). The activity of the enzyme after photoincorporation did indicate that no damage resulted from exposure to UV light.

The DNA-photolabeled terminal transferase was visualized by subjecting photolyzed reaction products to electrophoresis in SDS–polyacrylamide gels. Figure 7 shows the electrophoretic patterns of DNA(a), terminal transferase, and cross-linked products under various conditions. In the absence of UV light, the DNA was not cross-linked to terminal transferase (lane 3). However, when DNA(a) was irradiated in the presence of terminal transferase (lane 4), the two polypeptides (33 and 11 kDa) of the enzyme were cross-linked. Addition of competing double-stranded DNA to the reaction significantly reduced the cross-linking (lane 5). Lane 6 contains a photograph of the Coomassie Blue stained gel illustrating the position of the unmodified β (33 kDa) and α (11 kDa) peptides. A comparison of lanes 4 and 6 indicates that the peptides cross-linked to DNA migrate more slowly in the gel. The patterns on SDS–PAGE using photoactive DNA(b) and DNA(c) were identical (data not shown), but labeling efficiencies were lower (see Figure 3).

In order to identify peptides in the DNA binding domain of terminal transferase, the photolabeling reactions were scaled-up, and a new technique for isolating peptides covalently bound to DNA was developed. The preparative photolabeling reactions were carried out with 2.5 nmol of ^{32}P-labeled double-stranded DNA(a) containing eight azido-dUMP residues in the primer strand and 5 nmol of terminal transferase. A photolysis time of 1 min was sufficient for optimal labeling of the protein (data not shown). Under these conditions, the incorporation of radioactivity into terminal transferase peptides was highly specific using criteria of saturation and protection of photolabeling. Approximately 27% of the DNA was cross-linked to protein (data not shown). Tryptic digestion of the DNA-labeled terminal transferase followed by four separate phenol extractions (in the presence or absence of 10 mM β-mercaptoethanol) and gel filtration chromatography of the peptide mixture resulted in the elimination of all non-cross-linked peptides. This extraction procedure provided removal of these species because the peptides cross-linked to DNA remained in the aqueous phase and the unmodified peptides were extracted into phenol. No radioactivity was detected in the phenol phase. The DNA–peptide mixture was then subjected to N-terminal amino acid sequence analysis.

Two amino acid sequences corresponding to Asp^{221}–Lys^{231} (peptide B8) and Cys^{234}–Lys^{249} (peptide B10), obtained in similar yields, were unambiguously identified by alignment along the published amino acid sequence of terminal transferase (Evans et al., 1989) (Table II). These two peptides were obtained whether or not β-mercaptoethanol was present during isolation.

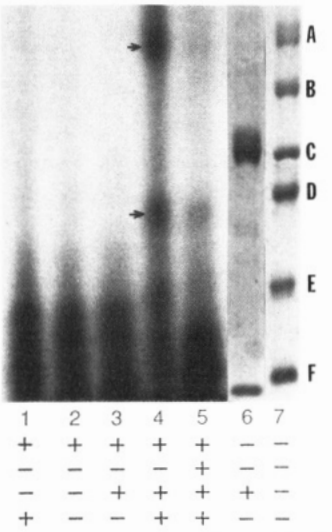

FIGURE 7: SDS–PAGE of terminal transferase photolabeled by DNA(a). The samples were boiled for 1 min before being loaded onto the gel. Molecular weight markers are A = egg albumin (45000), B = glyceraldehyde-3-phosphate dehydrogenase subunit (36000), C = carbonic anhydrase (29000), D = trypsinogen (24000), E = trypsinogen inhibitor (20100), and F = α-lactalbumin (14200) (lane 7). Following electrophoresis, the gel was stained with Coomassie Blue, dried, and autoradiographed. Autoradiograms of lanes 1–5 are shown. The terminal transferase preparation shown in lane 6 contained doublets of β polypeptide (33.5 and 32 kDa) and α polypeptide (11 kDa), both of which were photolabeled by radioactive DNA(a) as seen in lane 4 (indicated by arrows). All other conditions were as described under Experimental Procedures.

Table II: Primary Sequence of DNA-Cross-Linked Tryptic Peptides Purified by Repetitive Phenol Extraction

peptide[a]	amino acid sequence[b]	associated radioactivity
B8	DTEGIP(C)LGDK	yes
B10	(C)II(E)EIIEDGESSEV(K)	yes

[a] The numbering of the tryptic peptides is from Figure 1 in Evans et al. (1989) in which the first tryptic peptide at the amino-terminal end of the terminal transferase β subunit was designated peptide B1. [b] Amino acids in parentheses were not identified.

Additional evidence that these peptides were isolated during the procedure only as a consequence of the DNA cross-linking was obtained from control experiments. The photoactive DNA was preexposed to UV light for 1 min, terminal transferase was added, and the photolysis was repeated. The mixture was then subjected to tryptic digestion and phenol extractions as before. When the resulting aqueous phase was subjected to protein sequence analysis, no identifiable sequence was obtained (data not shown).

DISCUSSION

This study was designed to test the suitability of 5-azido-dUMP-containing DNAs as probes for active-site labeling of the $\alpha\beta$ form of calf terminal transferase, and to establish conditions for the identification of peptides involved in forming the DNA binding site. Several photolabeling conditions were evaluated to optimize photoinsertion efficiency and specificity so that labeled peptides could be isolated and identified from limited amounts of the enzyme. By several criteria, 5-azido-dUMP-containing DNAs were shown to photolabel the active site of terminal transferase and to interact specifically and reversibly with the protein when activated by UV light. Photosensitive double-stranded DNAs containing 5-azido-dUMP and single-stranded DNAs containing 8-azido-dAMP were as effective DNA initiators as their respective natural DNAs. Similar kinetic characteristics have been reported earlier for terminal transferase with a double-stranded blunt-end DNA substrate (Robbins & Coleman, 1988). Since incorporation of the precise number and location of 5-azido-dUMPs into DNA can be controlled for the synthesis of double-stranded DNA, these latter probes were employed for the photolabeling experiments.

Variation of the number and position of photoactive nucleotides in the DNA duplex revealed that the photolabeling reaction was not linear. Four azido-dUMP residues at the 3′ terminus [DNA(b)] gave 8% photolabeling while the addition of four more azido residues (for a total of eight) at the 3′terminus [DNA(a)] resulted in photolabeling of 40% of the DNA molecules in the reaction. This observation suggests that closer or more accessible contacts between DNA and protein occur within the interior of the DNA strands. The photoinsertion reaction exhibited cooperativity over the concentration range employed, and this is consistent with the binding parameters we have generated for terminal transferase using fluorescent measurements to monitor protein–DNA interactions (Robbins et al., 1987; Robbins & Coleman, 1988). Virtually all of the photolabeling was competitively inhibited by natural substrate DNAs, and both the β and α peptides of calf terminal transferase were specifically modified by the photoactive DNAs.

Using the optimal conditions for photolabeling, we isolated and identified two specifically modified peptides in the β subunit designated as B8 and B10. The use of a simple phenol extraction procedure to separate labeled from unlabeled peptides was a significant improvement over more conventional methods of peptide isolation involving HPLC. This technique, in which the peptides covalently bound to DNA remained in the aqueous phase, permitted peptide sequence analysis starting with 5 nmol of terminal transferase. Because only two peptides were recovered, the sequences could be determined directly from the mixture. The specific and light-dependent cross-linking of these peptides to the DNA was indicated by their absence in control experiments in which the DNA was photolyzed prior to the addition of terminal transferase. In addition, the recovery of both B8 and B10 from control experiments in which 10 mM β-mercaptoethanol was present during

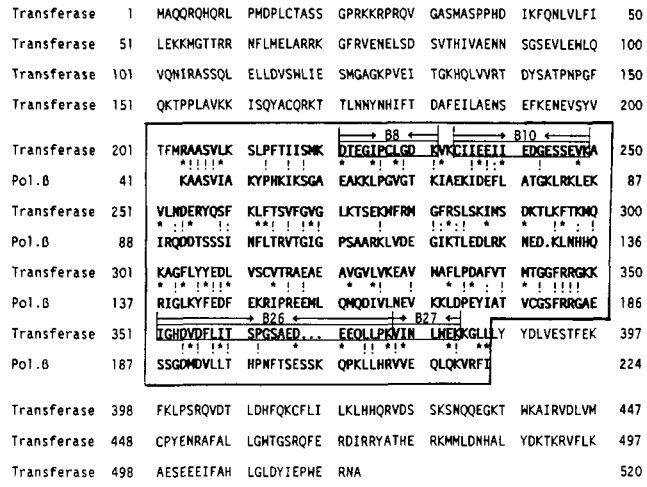

FIGURE 8: Comparison of the amino acid sequence between the putative active site of calf terminal transferase (transferase) and conserved regions in rat DNA polymerase β. The amino acid sequence of calf terminal transferase is from Evans et al. (1989) and Koiwai et al. (1986). Sequence similarity with rat DNA polymerase β peptides is shown in the boxed area and is from Matsukage et al. (1987). Peptides B8 and B10 were photolabeled with azido-dUMP containing DNA [(DNA(a)], and peptides B26 and B27 were photolabeled with 8-azido-dATP. The symbols between the two sequences are defined as follows: (!) identical amino acid; (*) conservative substitution; (:) similar substitution.

the phenol extraction indicated that peptides B8 and B10 were both cross-linked to the DNA rather than to each other through a disulfide bridge. Additional peptides may participate in the catalytic site of terminal transferase. The α subunit is consistently photolabeled with nucleotide and DNA probes, but photolabeled α peptides have not yet been identified. Access to recombinant polymerases and terminal transferase (Kumar et al., 1990; Medin et al., 1990) will facilitate extended structural studies.

One other DNA polymerase (*E. coli* Pol 1) has been photolabeled. In this study, an 11 azido/20-mer containing a photoactive derivative of 5-(3-phthalimidopropyl)-2′-deoxyuridine served as the DNA analogue. Fifty nanomoles of the protein was used in photolabeling reactions (Catalano et al., 1990). While 40% of the protein molecules were covalently labeled, only 20–70 pmol of the DNA-cross-linked peptides was isolated after FPLC and HPLC procedures. By contrast, the optimization of buffer conditions coupled with the use of the substrate analogue 5-azido-dUMP containing double-stranded DNA(a) and phenol extraction of modified peptides employed in the terminal transferase studies reported herein resulted in cross-linking of 27–40% of the DNA (using only 5 nmol of protein) and unequivocal identification of two photolabeled peptides (in amino acid yields of 20–65 pmol).

The two peptides (B8 and B10) identified on the β subunit of terminal transferase (see Figure 8) are identical with those previously isolated and implicated in forming the nucleotide binding domain of terminal transferase by Pandy and Modak (1988). Using direct cross-linking of the protein with dTTP, they isolated peptides B8 and B10 and proposed that the two Cys residues at positions 227 and 234 were involved in nucleotide binding. Since Cys^{234} is not conserved in the human enzyme (Evans et al., 1989), it does not appear likely that this residue is important for catalysis. Using 8-azido-dATP as a photoaffinity probe, we (Evans et al., 1989) identified two different peptides as forming the nucleotide binding domain (peptides B26 and B27; see Figure 8). This region (204–385) of the terminal transferase molecule shares significant sequence similarity with other DNA polymerases and nucleotide binding

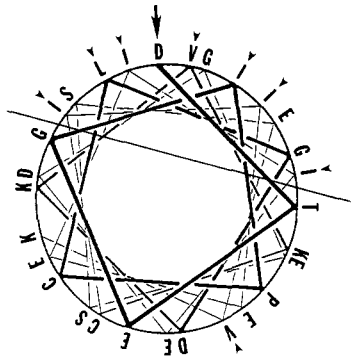

FIGURE 9: Radial projection (predicted α helix) of the terminal transferase sequence for residues 221–249. The starting residue, Asp^{221}, is identified by a large arrow. Hydrophobic residues along one face of the helix are identified by small arrows. Note that a single charged residue, Glu^{247}, interrupts the uncharged span.

proteins (Matsukage et al., 1987). The results of the current study, in which specific modification of B8 and B10 was demonstrated with a DNA photoprobe, suggest an explanation for the earlier correlation of this site with nucleotide binding. It is possible that the conditions used for direct cross-linking generate species that react with peptides that normally make contacts with DNA. Pandy and Modak (1988) did not indicate whether the covalent modification of terminal transferase by dTTP was protectable by DNA. We have demonstrated that azido-dATP photolabeling of terminal transferase is not altered by DNA (Evans, unpublished results) and that DNA photolabeling is not altered by dATP (this paper).

The region of terminal transferase that we have correlated with DNA binding is similar to a domain within rat DNA polymerase β that has been correlated with the DNA template binding site. This DNA polymerase is organized as two protease-resistant segments linked by a short protease-sensitive region (Kumar et al., 1990). The segment that comprises the amino terminus of the protein (about 75 amino acids) binds single-stranded DNA with a strong affinity. This amino-terminal region of DNA polymerase β shares similarity with sequences of peptides B8 and B10 (Figure 8). In addition, a region proximate to peptide B8 (residues 41–51 on DNA polymerase β) is similar to the ribonucleoprotein consensus sequence found in many single-stranded DNA and RNA binding proteins (Adam et al., 1986; Merrill et al., 1988). Another site on DNA polymerase β has been linked to the binding of single-stranded DNA. By site-directed mutagenesis and characterization of altered proteins, Arg-183 was associated with binding to the primer/template DNA (Date et al., 1990). In terminal transferase, this residue falls within a segment that forms part of the nucleotide binding domain (Evans et al., 1989), and in adenylate kinase, it is believed to control access to the nucleotide binding domain (Fry et al., 1986).

The interaction between DNA and terminal transferase has been previously investigated in our laboratory by measurements that monitored the quenching of protein fluorescence (Robbins et al., 1987). Those studies revealed that terminal transferase binding is not accompanied by the release of cations from the DNA phosphate backbone and that the DNA binding site size spans about 11 nucleotides. We proposed that the terminal transferase interaction with DNA is essentially nonelectrostatic and likely involves the nucleotide bases.

In order to test this hypothesis, we examined the computer-generated secondary structure predictions for terminal transferase in the region encompassing peptides B8 and B10. These two peptides are predicted to be in an α-helical conformation, with an intervening dipeptide (Matsukage, 1987). This region is illustrated in Figure 9 with a projection of residues around the proposed α helices (assuming they are in phase). Aromatic, hydrophobic, and charged residues are localized such that uncharged interactions could occur along one face of the helix. This stretch along the face of the helix would extend for 39 Å interrupted by only one charged residue within three amino acids of the end of B10 if the helices predicted for B8 and B10 were continuous. Correlation of the 39-Å length along the α helix of the protein with the DNA duplex helix rise (3.4 Å/base) yielded a predicted span of 11.4 nucleotides. This value is virtually identical with the size of the DNA binding site within terminal transferase predicted by a totally different experimental approach (Robbins et al., 1987).

Hydrophobic interactions between aromatic amino acids and bases of nucleic acids have been observed for several DNA polymerases and eukaryotic RNA binding proteins (Chase & Williams, 1986), and it appears that terminal transferase may share this mechanism of DNA binding. These models, and the significance of the similarities among various polymerases, will require extensive experimental verification including X-ray analysis of crystal structures and site-directed mutagenesis. Identification of peptides in terminal transferase involved in DNA and nucleotide binding provides targets for these studies.

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Wild-Type and Mutant Bacterioopsins D85N, D96N, and R82Q: High-Level Expression in *Escherichia coli*†

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ABSTRACT: The integral membrane protein bacterioopsin, found in the extremely halophilic archaebacterium *Halobacterium halobium*, was expressed in *Escherichia coli* as a fusion protein containing 13 heterologous amino acids at the amino terminus. The expressed protein was localized primarily to the *E. coli* cytoplasmic membrane (>80%) and had an in vivo half-life of 26 min. The amount of bacterioopsin in *E. coli* crude lysates was quantitated immunologically from Western blots and was expressed at 10–20-fold higher levels than seen previously (i.e., 17 mg/L; 5.6% of the total protein). Three distinct forms of the protein were detected immunologically: two of the forms were generated by the removal of either one or four amino acid residues at the amino terminus; the third form remained unaltered.

Bacterioopsin (BO)[1] complexed with the chromophore retinal constitutes bacteriorhodopsin (BR) in the purple membrane (PM) of *Halobacterium halobium* (Stoeckenius & Bogomolni, 1982). Under conditions of low oxygen tension and high light intensity, BR pumps protons across the cell membrane as it cycles through a number of short-lived (femtosecond to millisecond) photointermediates (Oesterhelt & Stoeckenius, 1973). The resultant electrochemical gradient is used to drive energy-requiring metabolic processes (e.g., ATP synthesis) and provides sufficient energy to sustain phototrophic growth (Oesterhelt & Krippahl, 1983). The formation of highly ordered two-dimensional crystalline patches of BR in the purple membrane has facilitated structural analyses which have been performed by electron imaging to about 3.5 Å in-plane by ~10 Å perpendicular to the membrane plane (Henderson et al., 1990).

In vivo experimental approaches involving alteration of specific amino acids of BO to determine which residues are responsible for proton pumping have been hampered by the high spontaneous mutation frequencies found in *H. halobium* (Pfeifer et al., 1981). Spontaneous purple membrane deficient mutants[2] occur at a frequency of 10^{-4} and with few exceptions are due to the integration of insertion elements in or near the bacterioopsin (*bop*) gene (Betlach et al., 1984). However, mutagenic agents have been used successfully in vivo to produce point mutations in the purple membrane producing halobacterial strain GRB (Soppa & Oesterhelt, 1989; Soppa et al., 1989). Strain GRB has a much lower frequency of spontaneous *bop* mutations than *H. halobium* and lacks all

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[1] Abbreviations: BO, bacterioopsin; BR, bacteriorhodopsin; PM, purple membrane; e-BO, bacterioopsin expressed from pβgbop in *E. coli*; e-BR, e-BO complexed with retinal; IPTG, isopropyl β-D-thiogalactopyranoside; SMG, supplemented M9 glycerol; bp, base pair(s); kbp, kilobase pair(s); PVDF, poly(vinylidene difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

[2] Mutant: A "mutant" is a strain carrying one or more mutations. For clarity and consistency with overwhelming usage within the literature, "mutant bacterioopsin", as used in the title, is defined as a bacterioopsin protein containing a substituted amino acid residue.

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