Photoaffinity Labeling of Terminal Deoxynucleotidyl Transferase. 2. Identification of Peptides in the Nucleotide Binding Domain†

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ABSTRACT: Terminal deoxynucleotidyl transferase (terminal transferase) was specifically modified in the nucleotide binding site by the substrate photoaffinity analogue [γ-^{32}P]-8-azido-dATP. The α and β polypeptides of photolabeled terminal transferase were resolved by high-performance liquid chromatography. The β polypeptide was digested with trypsin and fractionated by reverse-phase chromatography. Two ^{32}P-containing fractions were isolated and subjected to amino acid sequence analysis. Peptides were identified as Ile^{209}–Lys^{232} (B26) and Val^{233}–Lys^{239} (B27). Peptide B26 was further resolved into two overlapping species; one contained an additional lysine residue at the N-terminus which resulted from tryptic cleavage between Lys^{207} and Lys^{208}. In order to ensure that the sequenced peptides corresponded to the photolabeled species, we devised an anion-exchange procedure to isolate photolabeled peptides from the mixture. Analysis of photolabeled peptides from terminal transferase $\alpha\beta$ using DEAE-cellulose chromatography followed by reverse-phase HPLC confirmed that the photolabeled species were peptides B26 and B27. Peptide B26, the major photolabeled species, contained a conserved octapeptide region found in several eucaryotic DNA polymerases. In addition, peptide B27 was flanked by a sequence that has been implicated in triphosphate binding in other proteins. Structure predictions, based on sequence data, place the two peptides identified by photolabeling in spatial proximity consistent with the participation of both in the nucleotide binding domain.

RNA and DNA polymerases efficiently bind and distinguish between ribonucleotides and deoxyribonucleotides and carry out polymerization reactions. The contributions of amino acid sequence or topology of polymerase nucleotide binding domains to substrate selection are not understood. A combination of genetic and chemical techniques has made active site domains of both RNA and DNA polymerases accessible. One of the most powerful of these, for localization of substrate domains, is photoaffinity labeling. The substrate binding domains of one DNA polymerase (Pol I) (Joyce et al., 1986) and one RNA polymerase (Woody et al., 1984), both procaryotic, have been characterized with the aid of 8-azido(deoxy)adenine nucleotides. In contrast, the greater complexity of eucaryotic polymerases has impeded similarly detailed characterizations. Terminal deoxynucleotidyl transferase (terminal transferase)[1] offers a unique opportunity to focus on the delineation of polymerase substrate binding sites.

Terminal transferase catalyzes a linear condensation polymerization reaction in the absence of DNA templates. The polymerization reaction is distributive and follows a rapid equilibrium random mechanism (Deibel & Coleman, 1980). Moreover, the enzyme is synthesized as a single polypeptide (60 kDa), which is specifically proteolyzed to produce a small two-polypeptide catalytically active form consisting of an 11-kDa (α) and a 33-kDa (β) polypeptide (Chang et al., 1982).

In the accompanying paper (Evans & Coleman, 1989), we demonstrated that 8-azido-dATP was an efficient substrate for the $\alpha\beta$ form of calf terminal transferase and that 21% of the enzyme active sites were radiolabeled in a UV light dependent reaction. In the present paper we describe the isolation and identification of two photolabeled substrate binding peptides from calf terminal transferase. Both photolabeled peptides (B26 and B27) are similar to the corresponding sequence in human terminal transferase. Within peptide B26 is a conserved region also found in rat DNA polymerase β (Matsukage et al., 1987), vaccinia, Epstein–Barr virus, and adenovirus 2 DNA polymerases (Earl et al., 1986).

Materials and Methods

Materials

Nonradioactive 8-azido-dATP was synthesized according to Czarnecki et al. (1979) and was a generous gift from Dr. Boyd Haley (University of Kentucky). [γ-^{32}P]-8-Azido-dATP was synthesized according to a standard procedure (Glynn & Chappell, 1964). Other chemicals were of reagent grade or higher.

Methods

Sequencing of the Terminal Transferase α and β Polypeptides. The methods and materials described in Beach et al. (1985) are pertinent to this study. Aliquots of purified terminal transferase were acidified with trifluoroacetic acid (Pierce, Rockville, IL) and centrifuged to pellet-suspended material. The supernatant was injected onto a 4.6 mm × 5 cm Vydac (Hesperia, CA) C_4 reverse-phase HPLC column (TP silica) and eluted with a linear gradient of CH_3CN in 0.1% trifluoroacetic acid (TFA), pH 2.1 (mobile phase 1). Fractions containing the UV-absorbing α and β polypeptides were collected and dried for further manipulations. The method described by Tar: (1986) was used to modify the

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[1] Abbreviations: terminal transferase, terminal deoxynucleotidyl transferase; 8-azido-dATP, 8-azido-2′-deoxyadenosine 5′-triphosphate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; SDS, sodium dodecyl sulfate; Endo Glu-C, endoproteinase Glu-C or *Staphylococcus* V-8 protease; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography.

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cysteine residues of the polypeptides with 4-vinylpyridine before enzymatic digestion. The modified polypeptide was precipitated with 2 parts water and 9 parts cold acetone. The precipitate was dried in a vacuum centrifuge. For digestion by trypsin, 2–3 nmol of protein was dissolved in 200 μL of solution containing 2 M urea and 20 mM NH_4HCO_3. One aliquot of 4–6% (w/w) L-1-(tosylamino)-2-phenylethyl chloromethyl ketone treated trypsin (Cooper Biomedical) was added, and the mixture was incubated at 37 °C for 24 h.

To obtain larger peptides from a tryptic digest, the lysine (amino) residues of the polypeptides were modified with citraconic anhydride according to Tarr (1986). This modification is easily reversible by incubation at low pH. Digestion of the modified polypeptide was accomplished in 2 M urea with 4% trypsin (w/w) at 37 °C over 20 h. Citraconyl groups were removed prior to application to the reverse-phase column.

Insoluble chymotrypsin bound to agarose (Sigma) was also used to cleave the TdT αβ polypeptides. Under these conditions, α polypeptide was cleaved, but not β polypeptide. Soluble chymotrypsin (Worthington) was used to digest β polypeptide. Fractionation of peptides by HPLC indicated that digestion did occur, but amino acid sequence analysis of peptides revealed that digestion at Phe residues was not complete.

To create peptides by specific cleavage after glutamic acid residues, the pyridylethylated polypeptides were digested by endoproteinase Glu-C (Boehringer-Mannheim) according to Drapeau, (1977). Fractionation by reverse-phase HPLC revealed peptides from α polypeptide but none from the β polypeptide, probably because of insolubility of the β polypeptide. When pyridylethylated β polypeptide was citraconylated (to improve solubility) and then digested by the procedure above, many peptides were fractionated by reverse-phase HPLC.

The enzyme pyroglutamate aminopeptidase (Boehringer-Mannheim) was successfully used to cleave cyclized glutamine residues from the N-termini of two peptides. The procedure used was essentially that described by Podell and Abraham (1978). The treated peptide was desalted by reverse-phase HPLC.

To cleave the α and β polypeptides by CNBr, roughly 2 nmol of separated α or β protein was dried, and about 70 mg of CNBr in 75% TFA was added. After being allowed to stand at room temperature for 1 h, the solution was repeatedly dried and treated with 40 μL of aminoethanol. The fragments were separated by reverse-phase HPLC on a 4.6 mm × 5 cm Vydac C_4 column using mobile phase 1. All other peptide fractionation employed the same mobile phase and either 4.6 mm × 5 cm or 4.6 mm × 25 cm Vydac C_{18} reverse-phase HPLC columns.

The amino acid sequence of the peptides was determined on an Applied Biosystems 470A gas-phase sequencer with isocratic phenylthiohydantoin amino acid identification (Beach et al., 1985).

Direct application of unmodified α and β polypeptides to the sequencer did not yield any sequence information. However, CNBr fragmentation of terminal transferase α polypeptide produced three fragments, one of which was blocked to sequencing. When that fragment was treated with pyroglutamate aminopeptidase and subsequently sequenced, two amino acid sequences were produced, one beginning with Gln^{291} and the other beginning with Glu^{292}. It is possible that the N-terminus of α was heterogeneous, but we feel that it is more likely that N-terminal cyclized Gln^{290} was cleaved by pyroglutamate aminopeptidase, exposing Gln^{291} which cyclized and was also cleaved.

We suspected that the N-terminus of the β polypeptide was Ser^1-Ala^2-Thr^3-... because that peptide was generated as a result of a variety of different specific cleavage conditions. As it was not chemically blocked, it seemed likely that the N-terminus of the β polypeptide was buried and not physically accessible to Edman degradation. We directly sequenced the N-terminus of terminal transferase β polypeptide by denaturing the protein by pyridylethylation and then citraconylation of amino groups. The modified polypeptide was loaded onto the sequencer and then treated with gas-phase TFA at 45 °C for 10 min to remove the citraconyl groups from the Lys residues and the amino terminus of the protein. Subsequent sequencing yielded information consistent with our suspected β polypeptide N-terminus.

Photoaffinity Labeling of Terminal Transferase. The irradiation mixture (final volume of 1.0 mL) was divided into four equal aliquots and placed in a polystyrene 96-well microtest assay plate (Becton Dickinson Labware) or on white porcelain spot plates (Coors) at 4 °C. Each photolabeling reaction contained 50 mM Bis-Tris-HCl, pH 7.1, 25 mM NaCl, 8 mM $MgCl_2$, various concentrations of [γ-^{32}P]-8-azido-dATP, and 3.5–5.0 nmol of homogeneous terminal transferase. Each reaction was photolyzed for 3 min with a 302-nm UV lamp (Spectronics Corp., Model EB-28, 1500 μW/cm^2 at 15 cm) from a distance of 0.5 cm. Specificity of photolabeling was shown by reactions in which 2 mM dATP was included to compete with [γ-^{32}P]-8-azido-dATP photoincorporation.

In preparation for tryptic digestion of photolabeled enzyme, the labeled terminal transferase was precipitated by the addition of 300 μL of cold 15% trichloroacetic acid. After a 1-min incubation on ice and a 2-min centrifugation at 12000*g*, the pellet was resuspended in 50 μL of 8 M urea. Ammonium bicarbonate (1 M) was added (30 μL) followed by the addition of 120 μL of water and 20 μg of trypsin. The trypsinization was allowed to proceed for 4 h at 35 °C with agitation of sample to ensure adequate mixing.

DEAE-Sephadex Separation of Tryptic Peptides from Photolabeled Terminal Transferase. DEAE-Sephadex was packed into a 2 mm × 2 cm guard column housing, attached to the HPLC, and equilibrated at 0.5 mL/min with 10 mM Tris-HCl, pH 7.6. The entire photolabeled peptide sample was loaded onto the column at 0.1 mL/min, and 5 mL was collected at 0.5 mL/min (stage I). The mobile phase was changed to 0.1% acetic acid–TFA, pH 3.0 (stage II), 0.1% TFA, pH 2.1 (stage III), and 0.1% TFA plus 0.2 M NaCl (stage IV), successively. Ten 0.5-mL fractions were collected from each stage of elution and were assayed for ^{32}P radioactivity by Cerenkov counting. Fractions of interest were rechromatographed on a 4.6 mm × 25 cm Vydac C_{18} reverse-phase column by a gradient of mobile phase 1 at 1 mL/min.

Results

Amino Acid Sequence of Terminal Transferase α and β Polypeptides. A full-length cDNA coding for calf terminal transferase has been isolated and sequenced, thus allowing prediction of the entire amino acid sequence for the enzyme (Koiwai et al., 1986). Our initial protein sequencing permitted the alignment of β and α polypeptides within the predicted sequence (Beach et al., 1985).

We have now determined the sequence and boundaries of terminal transferase α and β polypeptides. Overlapping peptides generated by trypsin, chymotrypsin, Endo Glu-C, and CNBr digestion of the HPLC-purified α or β polypeptide were sequenced. Treatment of the N-terminal CNBr fragment of α with pyroglutamate aminopeptidase was successfully used

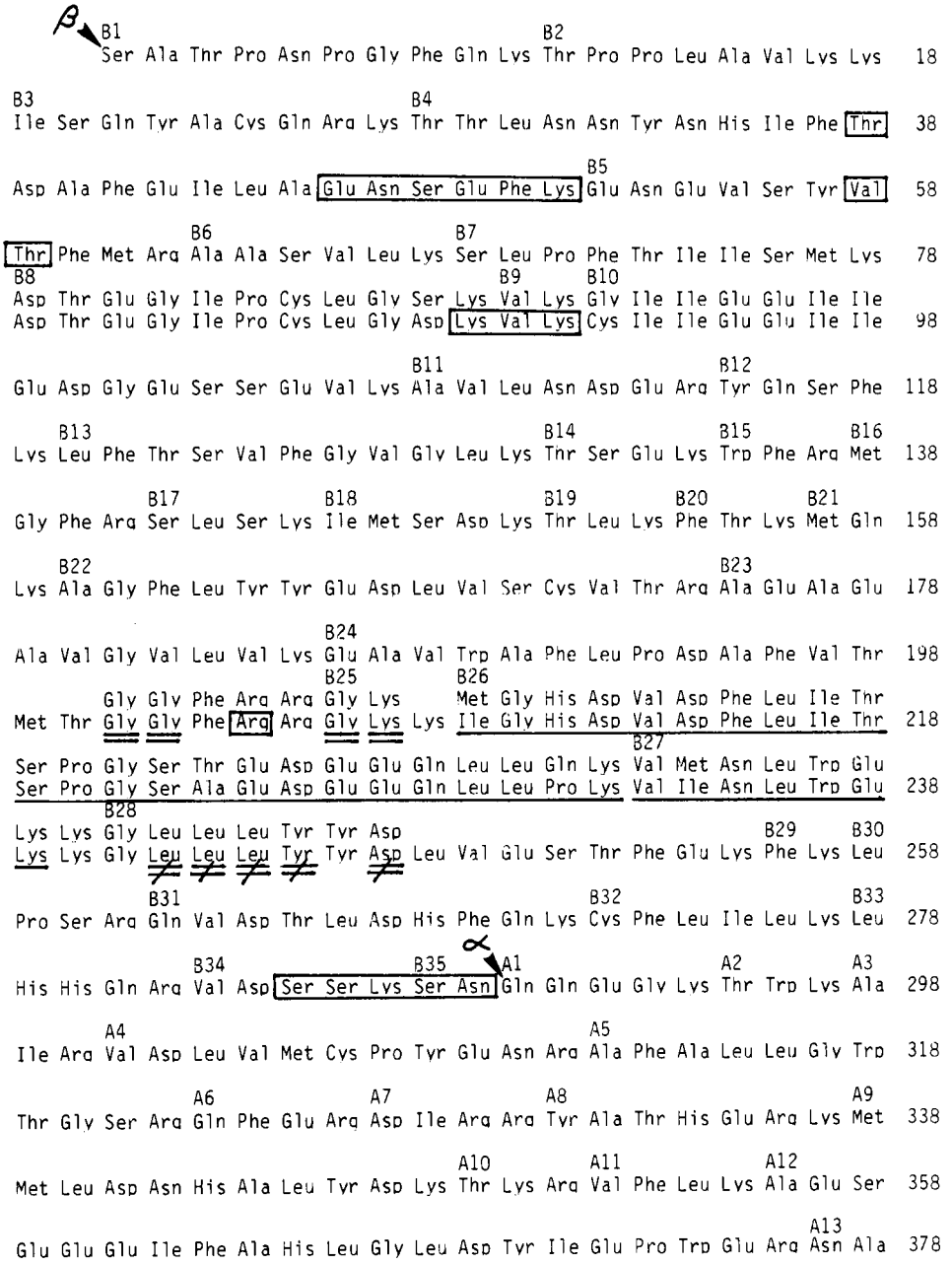

FIGURE 1: Amino acid sequence of calf terminal transferase $\alpha\beta$. Sequence was determined from overlapping peptides generated as described under Methods. The amino acids are numbered consecutively beginning with the N-terminal Ser of the β polypeptide. Amino acids contained within boxed regions have not yet been identified by sequence analysis, and the amino acids predicted from the bovine cDNA sequence are shown. The amino acid sequence of human terminal transferase (as predicted from cDNA sequence) is shown in some regions above the calf sequence. Tryptic peptides are numbered consecutively beginning with the N-terminal peptide of each polypeptide and are identified above the first residue of each tryptic peptide. The peptides which were photolabeled with 8-azido-dATP are identified by a single underline. Also shown are the glycine-rich segment (double underline) and hydrophobic region ($\neq$) shown in the legend to Figure 7.

to remove the cyclized N-terminal glutamine. Completion of the α polypeptide sequence revealed that the N-terminal glutamine corresponded to Gln^{290} in the cDNA predicted sequence (Figure 1).[2] The N-terminus of the β polypeptide was identified as Ser^{1}. Sequencing of the C-terminal region of the β polypeptide has progressed through Asp^{284}. The intervening pentapeptide (Ser^{285} through Asn^{289}) has not yet been identified. With the boundaries of β as Ser^{1} to Asn^{289} the molecular weight of the β polypeptide is 32857 and consists of 289 amino acids. The α polypeptide contains 89 amino acids with an M_r of 10721. Figure 1 shows the amino acid sequence of calf terminal transferase $\alpha\beta$ as determined by direct analysis. All of the predicted amino acid sequence with the exception of 18 amino acids in the β polypeptide has been verified by direct sequence analysis.

Purification of Photolabeled Terminal Transferase α and β Polypeptides. The preparative photolabeling reactions were carried out with 20 μM [γ-^{32}P]-8-azido-dATP. At this concentration of photoprobe, incorporation of radioactivity into terminal transferase peptides is highly specific, and approximately 6% of the terminal transferase molecules were photolabeled (Evans & Coleman, 1989). Terminal transferase α and β polypeptides were resolved by reverse-phase HPLC in order to generate unique sets of tryptic peptides (Figure 2). Recovery of protein-bound radioactivity in α and β was greater than 90%, and the β polypeptide contained 83% of the in-

[2] The amino acid numbering system in this paper designates the amino terminus of β as 1. To convert residue members to the 60-kDa form of terminal transferase, it is necessary to add 142 to each residue number.

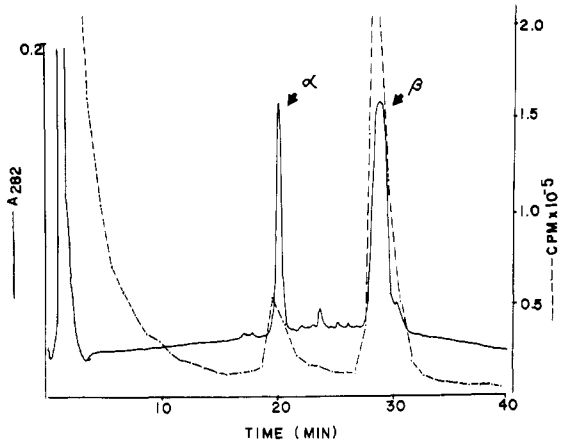

FIGURE 2: Terminal transferase $\alpha\beta$ was photolabeled with 20 μM [γ-^{32}P]-8-azido-dATP, as described under Methods. The entire reaction mixture was injected onto a reverse-phase column. Either a Beckman Ultrapore C_3 (4.6 mm × 7.5 cm) or a Vydac C_4 (TP silica, 4.6 mm × 5 cm) column was eluted with a linear gradient of mobile phase 1. Fractions were collected and subjected to analysis for radioactivity. The broken line indicates ^{32}P cpm (Cerenkov counting).

Table I: Primary Sequence of Tryptic Peptides Purified by Reverse-Phase HPLC

peptide	amino acid sequence	associated radioactivity
A4	VDLVMCPYEN R	yes
A12	AESEEEIFAH LGLDYIEPWE R	no
B10	CIIEEIIEDG ESSEVK	no
B13	LFTSVFGVGL K	no
B26a	KIGHDVDFLI TSPGSAEDEE QLLPK	yes
B26b	IGHDVDFLIT SPGSAEDEEQ LLPK	yes
B27	VINLWEK	yes

corporated label. In contrast, when terminal transferase was covalently modified with a saturating concentration of [γ-^{32}P]-8-azido-dATP (120 μM), a similar resolution profile was obtained, but the percentage of protein-bound radioactivity in β decreased from 83% to ~73%. This difference in labeling pattern reflected an increase in nonspecific nucleotide binding which was primarily localized on the α polypeptide (Evans & Coleman, 1989).

HPLC Separation of Tryptic Peptides from Photolabeled Terminal Transferase β Polypeptide. The purified photolabeled β polypeptide was digested with trypsin and subjected to reverse-phase HPLC on a 4.6 mm × 7.5 cm C_3 column. The UV and radioactivity profiles of resolved β tryptic peptides are shown in Figure 3A. Major peaks in the UV absorbance profile at ~13- and 32-min retention time appeared to have associated radioactivity. Close inspection of the UV peak eluting near 32 min revealed a doublet. Amino acid sequence analysis of fractions collected at the beginning of the doublet elution indicated the presence of two major peptides corresponding to β tryptic peptides B13 (Leu^{120}–Lys^{130}) and B26a (Lys^{208}–Lys^{232}) (Table I, Figure 1). When the eluting gradient was made shallower in this region, the doublet was resolved into three peaks (Figure 3B). Sequence analysis revealed fractions comprising peak 1 to contain peptide B13 with no associated radioactivity, while material in peaks 2 and 3 was identified as peptides B26a and B26b (Table I). The two overlapping forms of peptide B26 resulted from tryptic cleavage carboxyl terminal to Lys^{207} or Lys^{208}, and both peptides had equal, but low, amounts of associated radioactivity.

In an effort to resolve the radioactive peak at ~13-min retention time (Figure 3A), several modifications of the separation conditions were evaluated. The most successful approach involved the use of a C_{18} reverse-phase HPLC column for tryptic peptide resolution and reduction of the period of time between the isolation of β polypeptide and chromatography of β tryptic peptides (Figure 4). Fractions comprising two radioactive peaks contained the majority of the protein-bound label and appeared to correspond to two UV absorbance peaks at 37- and 44-min retention time. Amino acid sequence analysis of the fractions corresponding to peak 2 revealed the presence of peptides B26a and B26b (Table I). Amino acid sequence analysis of the fractions associated with peak 1 in-

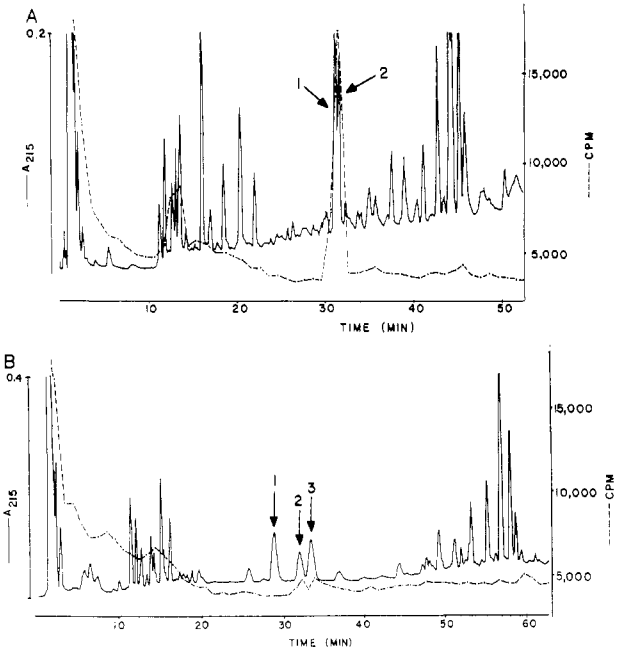

FIGURE 3: Photolabeled terminal transferase β polypeptide (isolated as in Figure 2) was dried, dissolved in 2 M urea–0.05 M NH_4HCO_3, and digested with 6% (w/w) trypsin for 4 h. The peptides were eluted from a 4.6 mm × 7.5 cm Beckman Ultrapore C_3 column with a gradient of mobile phase 1. (A) Elution with a linear gradient; (B) elution with a linear gradient as in (A), but made more shallow in the region of the numbered UV peaks. Fractions were collected and subjected to analysis for radioactivity (Cerenkov counting).

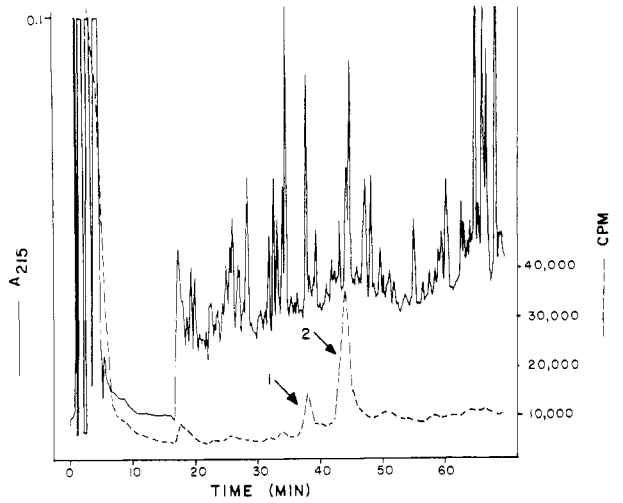

FIGURE 4: Photolabeled terminal transferase β polypeptide (isolated as in Figure 2) was dried, dissolved in 2 M urea–0.025 M NH_4HCO_3, and digested with 4% (w/w) trypsin for 4 h. the peptides were eluted from a 4.6 mm × 25 cm Vydac C_{18} (TP silica) reverse-phase column with a gradient of mobile phase 1. The gradient consisted of 0.7% CH_3CN/min except for a 0.35% CH_3CN/min segment from 37 to 53 min. Fractions were collected and analyzed for radioactivity (Cerenkov counting).

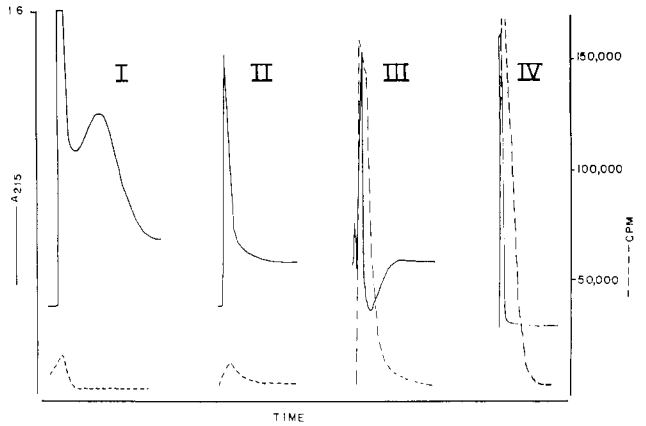

FIGURE 5: Elution of tryptic peptides from photolabeled terminal transferase $\alpha\beta$ from a 2 mm × 2 cm DEAE-Sephadex column with a step pH gradient. The tryptic peptides were generated as described under Methods. (I) Peptides were bound to the resin in 10 mM Tris-HCl, pH 7.6. (II) Unlabeled peptides were eluted with 0.1% acetic acid–TFA, pH 3.0. (III) Peptides cross-linked to [γ-^{32}P]-8-azido-dATP and a small number of acidic peptides were eluted with 0.1% TFA, pH 2.1. Fractions were collected and analyzed for radioactivity (Cerenkov counting).

dicated the presence of a single peptide, B27 (Table I).

Use of DEAE-Sephadex To Separate Labeled from Unlabeled Tryptic Peptides. In order to ensure that the sequenced peptides corresponded to photolabeled species and did not represent unlabeled coeluting peptides, we devised a technique to separate labeled from unlabeled tryptic peptides. DEAE-Sephadex chromatography was employed. Tryptic peptides containing specifically modified amino acids should become acidic due to the triphosphate group present in the photoprobe. By exploiting this property, we separated labeled from unlabeled peptides by decreasing the pH of the eluting buffer in a stepwise fashion. Terminal transferase was photolabeled with 120 μM [γ-^{32}P]-8-azido-dATP as described under Methods. The protein was precipitated by the addition of cold trichloroacetic acid (final concentration 3.5%) followed by resuspension of the pellet in 8 M urea. After dilution of the urea to 2 M and a 4-h digestion with trypsin, the entire sample was applied to a 2 mm × 2 cm DEAE-Sephadex column in 10 mM Tris-HCl, pH 7.6. Figure 5 shows the procedure used to elute the photolabeled peptides specifically. Stage I elution illustrated that most of the radioactivity remained bound to the resin at 10 mM Tris-HCl, pH 7.6. At stage II peptides with little associated radioactivity were eluted with 0.1% acetic acid–TFA, pH 3.0. When the column was eluted with 0.1% TFA (pH 2.1), a large release of radioactivity was observed with a corresponding release of UV-absorbing material (stage III). Stage IV shows additional release of both radioactivity and UV-absorbing material when 0.1% TFA was combined with 0.2 M NaCl as a column eluent.

To determine the nature of the radioactivity released during the last two stages of DEAE-Sephadex elution, reverse-phase HPLC was used to analyze the fractions. Figure 6A shows the reverse-phase separation of the contents of stage III of DEAE-Sephadex elution. Six UV-absorbing peaks were observed, and the fractions comprising three of these peaks had associated radioactivity. These results suggested that the photolabeled peptides (containing radioactivity) were eluted at pH 2.1, but not at pH ≥3.0. Quantitation of ^{32}P eluted from the DEAE column in stage III showed that 3.5 nmol of peptide-bound [γ-^{32}P]-8-azido-dATP was eluted when 5.4 nmol of terminal transferase was photolabeled, trypsinized, and fractionated. This corresponds to 65% photolabeling efficiency

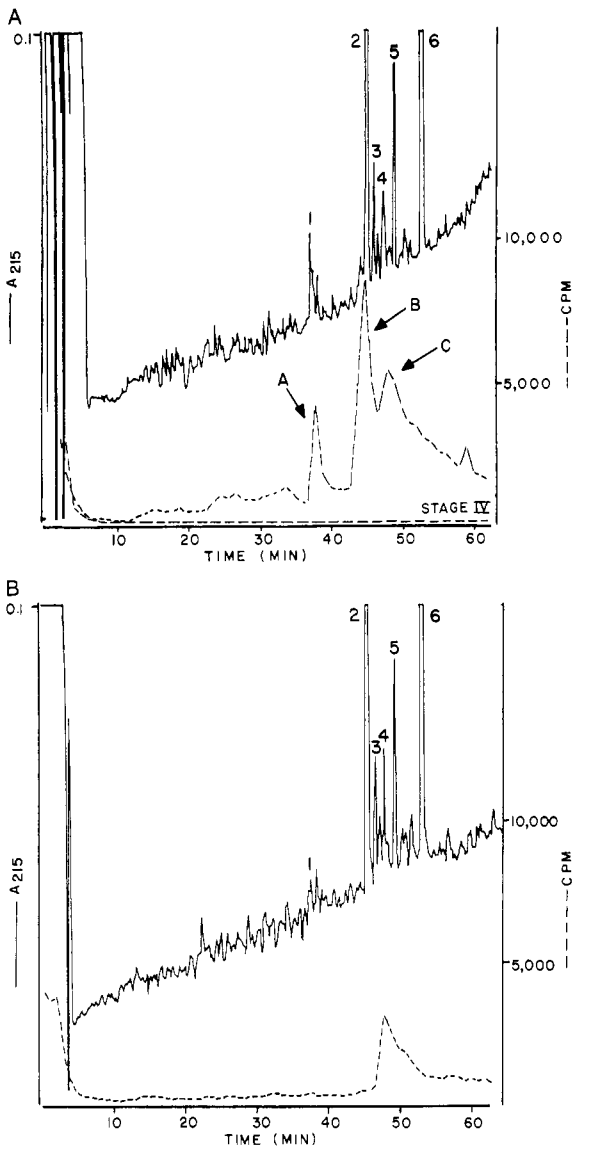

FIGURE 6: Photolabeled tryptic peptides eluted by stage III from the DEAE-Sephadex column were resolved by reverse-phase HPLC. A 4.6 mm × 25 cm Vydac C_{18} (TP silica) column was eluted by a linear gradient of mobile phase 1. Fractions which were subjected to amino acid sequence analysis were dried to about 20-μL volume and applied to an Applied Biosystems gas-phase sequencer (see Methods). (A) The upper curve denoting ^{32}P cpm (Cerenkov counting) with peaks labeled A–C represents the radioactivity detected in the fractions corresponding to the UV absorbance trace. The lower broken line denotes radioactivity detected in a similar reverse-phase fractionation of stage IV from the DEAE-Sephadex column. There were no corresponding UV-absorbing peaks above background except immediately after injection. (B) Same as (A) except the photolabeling reaction included 2 mM dATP. The remaining radioactive fractions denote nonspecifically labeled peptide(s).

and quantitative recovery of protein-bound radioactivity (Evans & Coleman, 1989). When stage IV of DEAE elution was subjected to reverse-phase HPLC, the only UV-absorbing or radioactive peaks observed were within 1 min of the injection (Figure 6A), indicating that the material in this fraction was unicorporated photoprobe, which will not bind to the reverse-phase resin under these conditions. To confirm that the radioactive peaks observed at stage III elution were due to protein-bound radioactivity and not free photoprobe, a sample of photolyzed [γ-^{32}P]-8-azido-dATP was subjected to the same elution conditions. The results (not shown) indicated that photolyzed [γ-^{32}P]-8-azido-dATP eluted only during stage IV of elution and not during stage III.

To determine the identity of each photolabeled peptide (in stage III elution), the contents of five UV-absorbing peaks were subjected to amino acid sequence analysis. Fractions from peak 1 (Figure 6A) did not yield any sequence information; however, the material did elute with a radioactive peak at the same retention time seen earlier for peptide B27. Several repetitions of this experiment revealed that while the radioactivity corresponding to peaks 1 and 2 was always present, the UV-absorbing peak at position 1 was not consistently observed. Radioactive material in peak 2 was shown by sequence analysis to correspond to peptide B26b. Peptide B26a was not detected, probably because the trypsin cleavage at Lys^{208} was complete. The radioactivity in fractions comprising the third peak (c) eluted near 49-min retention time and appeared to correspond to UV peak 5. However, the radioactive profile was broad. Amino acid sequence analysis showed that the material in peak 4 was peptides B10 and A4 (Table I), while the fractions comprising peak 5 contained peptide B10 (Table I). The fractions containing the large UV-absorbing peak eluting near 53 min (peak 6) contained only peptide A12.

To determine which of the radioactive peptides isolated by DEAE chromatography were photolabeled active site peptides, a competition experiment was performed. The photolabeling reaction was carried out in the presence of 2 mM dATP. As seen in Figure 6B, under these conditions only one broad radioactive peak was recovered after DEAE-Sephadex and reverse-phase HPLC. This radioactive peak appeared to correspond to UV absorbance from peptides B10 and A4 (peak 4, Figure 6B). The persistence of label in peptides B10 and A4 in the presence of excess substrate indicates that these peptides are not contained in the enzyme active site. This protection experiment confirms that photolabeling of peptides B26 and B27 reflected labeling at the active site.

Discussion

In the accompanying paper, we demonstrated that 8-azido-dATP effectively photolabeled the nucleotide binding site of calf terminal transferase. When terminal transferase was photolabeled at low photoprobe concentrations, greater than 85% of the photolabeling with [γ-^{32}P]-8-azido-dATP was eliminated by competition with the substrate dATP. Using a low concentration of photoprobe, we isolated and tentatively identified two specifically modified β peptides designated as B26 and B27.

In order to confirm that B26 and B27 were part of the nucleotide binding domain, an anion-exchange fractionation procedure was devised to purify peptides modified by the azido analogue. Since the triphosphate group from the incorporated photoprobe is more acidic than the carboxylic acid containing amino acids (Scheit, 1980), this separation exploited the differential binding of negatively charged peptides to DEAE-Sephadex as the pH of the eluting buffer was decreased. The effectiveness of the procedure was optimized by generating peptides with trypsin since each resulting peptide contained only two positive charges (except internal His residues). Therefore, at sufficiently low pH (to protonate internal Asp and Glu residues), all tryptic peptides were positively charged except those containing the acidic triphosphate group or large numbers of Asp or Glu residues. This procedure allowed quantitation of incorporated [γ-^{32}P]-8-azido-dATP since all the labeled peptides eluted with 0.1% TFA but the free label eluted only with 0.2 M NaCl. Because of the small size of the column and the high capacity of the resin, recovery of peptides was sufficient to allow sequence analysis after an additional HPLC separation starting with 3.5–5 nmol of terminal transferase.

When a tryptic digest of [γ-^{32}P]-8-azido-dATP-photolabeled terminal transferase was applied to a 2 mm × 2 cm DEAE-Sephadex column and eluted as described, all unlabeled tryptic peptides were eluted at pH 3.0 except for peptides B10, B26, B27, A4, and A12. Each of these contained either six or seven internal Asp or Glu residues or the triphosphate photoprobe. Of these, only peptides B26 and B27 were protected from photolabeling by excess substrate.

Direct UV cross-linking using [α-^{32}P]dTTP has also been employed to label terminal transferase nucleotide binding peptides (Virendranath & Modak, 1988). It was reported that dTTP appeared to cross-link to two cysteine-containing peptides identified as Asp^{79}–Lys^{89} (peptide B8 in Figure 1) and Cys^{92}–Lys^{107} (peptide B10 in Figure 1). On the basis of these results a model was proposed describing the involvement of the two cysteine residues in forming hydrogen bonds to the substrate nucleotide bases. Specifically, Cys^{85} and Cys^{92} (numbered according to Figure 1) were proposed to act as hydrogen-bond donors to the 4(O) and 2(O) carbonyl oxygens in dTTP, respectively. In addition, it was stated that Cys^{85} and Cys^{92} may also act as hydrogen-bond donors to the N(1) and N(3) positions of the adenine ring and possibly account for the enzyme's ability to bind both purine and pyrimidine nucleotides. While it is theoretically possible that these two cysteine residues may act as hydrogen-bond donors to thymine and adenine bases, this model does not allow binding of cytosine or guanine bases. The participation of thiol groups as hydrogen-bond donors has been shown to be chemically possible (Paul, 1974), but these bonds are substantially weaker than hydrogen bonds between more electronegative atoms (Pimentel & McClellan, 1960). The importance of Cys^{85} and Cys^{92} in terminal transferase nucleotide binding is further diminished by the fact that Cys^{92} is not conserved in the human enzyme (Figure 1). The glycine which replaces Cys^{92} in the human enzyme cannot form hydrogen bonds; thus, hydrogen-bond donation by Cys^{92} in the calf enzyme is probably not involved in nucleotide binding. Peptides B8 and B10 have not been shown to exhibit sequence similarities with other DNA polymerases. In contrast, B26 and B27, the peptides identified herein by photoaffinity labeling, occur in a region of protein sequence that has been shown to be conserved in other polymerases.

Recently Earl et al. (1986) identified a highly conserved region of 14 amino acids in vaccinia virus, adenovirus 2, and Epstein–Barr virus DNA polymerases. The predicted structure for this region was a turn flanked by β-pleated sheets. Within this 14 amino acid region a conserved octapeptide occurred in all three polymerases. These authors suggested that the conserved structure might relate to a conserved function, i.e., nucleotide binding.

Similar secondary structures in rat DNA polymerase β and terminal transferase were predicted by Matsukage et al. (1987). This region contains an octapeptide that is similar to the consensus sequence in the viral polymerases. The conserved sequences and the flanking regions are shown in Figure 7. In the terminal transferase octapeptide there are three positions of identity, while in rat DNA polymerase β there is identity at four of the eight residues. Secondary structure predictions for both polymerases indicated β-structure within the octapeptide with a turn or coil beginning at approximately Pro^{220} (Figure 7) of terminal transferase or Pro^{198} of DNA polymerase β. These structures are reminiscent of those predicted for the viral polymerases.

As shown in Figure 7, the octapeptide consensus sequence is contained within peptide B26, which was identified as the

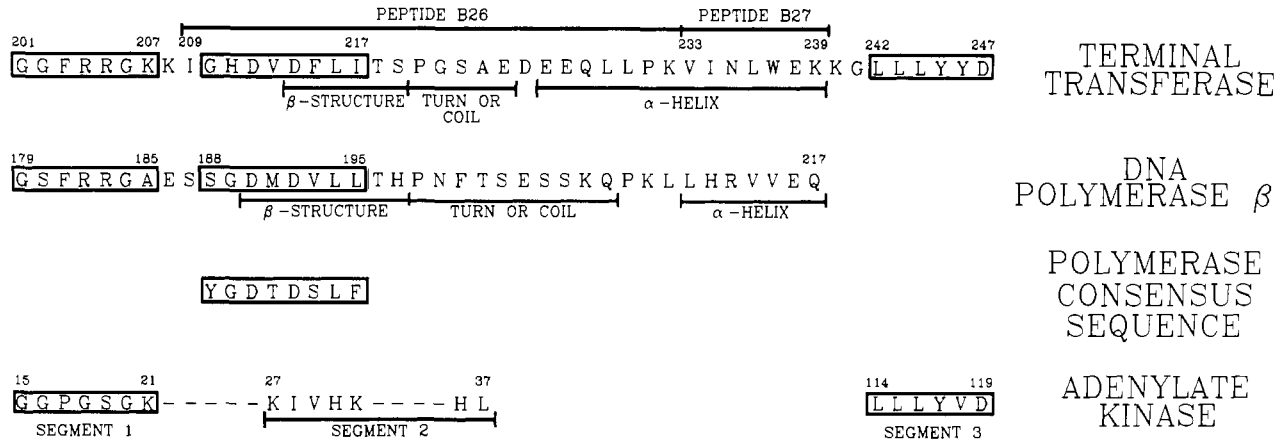

FIGURE 7: Comparison of the amino acid sequence between the putative nucleotide binding region of terminal transferase and conserved regions in rat DNA polymerase β, viral polymerases, and adenylate kinase. The two terminal transferase tryptic peptides labeled with 8-azido-dATP (B26 and B27) are indicated above the amino acid sequence. Three segments in the nucleotide binding domain of adenylate kinase and other nucleotide binding proteins are designated as segments 1–3. The predicted structure within these regions for terminal transferase and rat DNA polymerase β is shown below the corresponding amino acid sequence.

major specifically photolabeled peptide from terminal transferase. The second photolabeled peptide (B27) is adjacent to peptide B26 (at the C-terminal end). Juxtaposition of these two peptides would require an intervening turn. Indeed, the secondary structure predictions of Matsukage et al. (1987) suggest that a turn or coil occurs at approximately Pro^{220} and continues for about five residues.

Similarities to other nucleotide binding proteins in flanking regions (bordering peptides B26 and B27) in the terminal transferase sequence also suggest that this segment of the enzyme may be responsible for nucleotide binding and catalytic activity. Two segments in terminal transferase are similar to those found in adenylate kinase and in several nucleotide binding proteins (Fry et al., 1986). The first segment (identified as segment 1 in Figure 7) is a glycine-rich area spanning approximately seven residues which controls access to the nucleotide binding domain in adenylate kinase. The four most conserved positions of the seven-residue segment occur in terminal transferase (Figure 7). This segment is also found in rat DNA polymerase β (Figure 7). A hydrophobic segment (segment 3, Figure 7), believed to form part of the triphosphate binding pocket in adenylate kinase, is also found in terminal transferase (five of the six residues of the segment are identical, and a conservative substitution is at the remaining position). It may also be significant that the adenine-ribose binding region from adenylate kinase (segment 2) is not highly conserved in terminal transferase. Instead, it appears that the octapeptide region found in several other polymerases replaces this adenine-ribose binding region in terminal transferase and DNA polymerase β. This might be explained by the fact that the polymerases require a nucleotide binding domain capable of binding all four deoxynucleoside triphosphates but adenylate kinase requires binding only of ATP. In terminal transferase the nucleotide binding domain appears to span 47 residues (Gly^{201}–Asp^{247}) with the glycine-rich segment at the extreme N-terminal end, the hydrophobic segment at the C-terminal end, and peptides B26 and B27 in the middle.

In summary, three lines of evidence now identify a region in the terminal transferase sequence as the nucleotide binding domain: (1) photoaffinity labeling has identified peptides B26 and B27 (this paper); (2) conservation of sequences within and flanking B26 and B27 has been reported for several eucaryotic DNA polymerases and other nucleotide binding proteins; (3) structural similarities in this region among several DNA polymerases have been predicted.

The strategies described in this paper for photolabeled peptide isolation should allow more detailed mapping of terminal transferase active site peptides. Future studies will involve the identification of specifically labeled peptides in the α polypeptide of terminal transferase as well as the localization of the amino acids within tryptic peptides B26 and B27 that contain cross-linked photoprobe. These studies will provide more detail about the topology of the active site from which testable hypotheses of polymerization mechanisms can be generated.

Registry No. 8-Azido-dATP, 117679-32-0; terminal transferase, 9027-67-2.

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Temperature-Dependent CD and NMR Studies on a Synthetic Oligonucleotide Containing a B-Z Junction at High Salt†

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ABSTRACT: It is now accepted that two or more conformations may exist within the same DNA molecule, thereby generating conformational junctions. The presence of B-Z junctions between right- and left-handed DNA conformations has been detected in plasmids by a number of techniques. Preliminary characterization of the first example of a B-Z junction is a short DNA oligonucleotide has recently been reported [Sheardy, R. D. (1988) *Nucleic Acids Res. 16*, 1153-1167]. We report additional CD and NMR data that support the existence of the junction in this model oligomer. These studies indicate that only three base pairs are involved in the junction and only one of these is dramatically distorted. Furthermore, the NMR saturation-transfer experiments suggest the junction's internal motion is temperature dependent.

It is now recognized that a segment of DNA may possess contiguous left- and right-handed conformations, mandating the existence of a conformational junction within that segment. Most of the studies to date on these B-Z junctions are concerned with junctions contained within plasmids [for example, Singleton et al. (1982) and Johnston and Rich (1985)]. In these systems, (dG-dC) oligomers are cloned into plasmids that are then subjected to conditions conducive to Z formation of the inserts, thereby generating B-Z junctions at either end of the insert. Although these types of studies can lead to broad generalizations about the nature of B-Z junctions, they give little detailed structural information at the molecular level.

We have recently reported the preparation and initial characterization of the synthetic DNA hexadecanucleotide (Sheardy, 1988):

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5'-C*--G--C*--G--C*--G--C*--G--A--C--T--G--A--C--T--G-3'

3'-G--C*--G--C*--G--C*--G--C*--T--G--A--C--T--G--A--C-5'

   1  2  3  4  5  6  7  8  9  10 11 12 13 14 15 16
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(C* is 5-methyldeoxycytidine.[1] The numbering is for exchangeable proton chemical shift assignments.) The sequence of this duplex was designed such that the first eight base pairs (as written) could assume a left-handed conformation under dehydrating conditions, while the last eight base pairs should remain right-handed under all conditions. The existence of both conformations within the molecule would mandate a conformational junction between the two (i.e., a B-Z junction). Such a model could provide insight into the biological significance of conformational junctions in terms of their roles in gene expression and regulation.

The initial CD, UV, and NMR studies reported indicate that this molecule assumes a right-handed conformation at low salt. However, under conditions of high salt (5.0 M NaCl), the duplex contains regions of both left- and right-handedness and thus must contain a B-Z junction. The preliminary ^{31}P NMR studies also indicated that the span of the junction is on the order of four to six base pairs.

We report additional CD and NMR data that support the existence of the B-Z junction in the model oligomer. These studies indicate that only three base pairs are involved in the junction, with one of these dramatically distorted. Furthermore, data from both ^{1}H NMR and saturation-transfer experiments suggest that the internal motion of the junction is temperature dependent.

Experimental Procedures

Materials. The oligonucleotide was synthesized and purified as previously described (Sheardy, 1988). Phosphate buffer (0.01 M sodium phosphate, 1 mM EDTA, pH adjusted to 7.0 with NaOH) was used in all experiments. The low-salt buffer had no added NaCl; the high-salt buffer had NaCl added to a final concentration of 5.0 M. Stock oligonucleotide duplex solutions were prepared by combining solutions of the individual strands to give 1:1 mixtures, followed by heating to 90

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[1] Abbreviations: C*, 5-methyl-2'-deoxycytidine; C, 2'-deoxycytidine; G, 2'-deoxyguanosine; T, 2'-deoxythymidine; A, 2'-deoxyadenosine; CD, circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement.

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