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Cis-Syn Thymine Dimers Are Not Absolute Blocks to Replication by DNA Polymerase I of *Escherichia coli* in Vitro†

John-Stephen Taylor*,‡ and Christine L. O'Day
Department of Chemistry, Washington University, St. Louis, Missouri 63130
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ABSTRACT: Both *Escherichia coli* DNA polymerase I (pol I) and the large fragment of pol I (Klenow) were found to bypass a site-specific cis-syn thymine dimer, in vitro, under standard conditions. A template was constructed by ligating d(pCGTAT[*c,s*]TATGC), synthesized via a cis-syn thymine dimer phosphoramidite building block, to a 12-mer and 19-mer. The site and integrity of the dimer were verified by use of T4 denV endonuclease V. Extension of a 15-mer on the dimer-containing template by either pol I or Klenow led to dNTP and polymerase concentration dependent formation of termination and bypass products. At ~0.15 unit/μL and 1-10 μM in each dNTP, termination one prior to the 3'-T of the dimer predominated. At 100 μM in each dNTP termination opposite the 3'-T of the dimer predominated and bypass occurred. Bypass at 100 μM in each dNTP depended on polymerase concentration, reaching a maximum of 20% in 1 h at approximately 0.2 unit/μL, underscoring the importance of polymerase binding affinity for damaged primer-templates on bypass. Seven percent bypass in 1 h occurred under conditions of 100:10 μM dATP:dNTP bias, 1% under dTTP bias, and an undetectable amount under either dGTP or dCTP bias. At 100 μM in each dNTP, the ratio of pdA:pdG:pdC:pdT terminating opposite the 3'-T of the dimer was estimated to be 37:25:10:28. Sequencing of the bypass product produced under these conditions demonstrated that >95% pdA was incorporated opposite both Ts of the dimer and that little or no frame shifting took place. A mechanism whereby products terminating in pdA opposite the 3'-T of the dimer are preferentially elongated by pol I was proposed to account for the higher sequence specificity of the bypass product than the termination product. On the basis of the results of this study, a mechanism was proposed that could account for the origin of the major mutation induced by ultraviolet light in bacteria, the C → T transition mutation at TpdC sites.

The precise mechanisms by which mutagens lead to mutations is not known. One general mechanism is thought to involve error-prone DNA synthesis past DNA damage (lesions) produced by reaction of the mutagen with DNA. The extent and mechanisms by which DNA polymerase are able to synthesize past lesions and the bases that are introduced as a consequence are not well-known or understood [for a review see Strauss (1985)]. One of the best characterized DNA polymerases is pol I[1] of *Escherichia coli* and its large

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* To whom correspondence should be addressed.
‡ Alfred P. Sloan Foundation Fellow.

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fragment (Klenow) (Kornberg, 1980; Kuchta et al., 1988). The primary role of pol I is thought to involve filling in of gaps produced during excision repair and semidiscontinuous replication. Gaps containing lesions could arise as a consequence of excision repair of DNA containing closely opposed lesions or during discontinuous replication of damaged DNA (Friedberg, 1985). Pol I might therefore play a role in mutagenesis caused by a particular agent if it were able to bypass the lesions produced by that agent in an error-prone fashion.

One of the most intensively studied class of environmentally produced lesions are pyrimidine photodimers, of which the cis-syn cyclobutane and (6–4) products are the major members (Wang, 1976). Both of these photoproducts have been implicated in ultraviolet light induced mutagenesis and cancer [for recent discussions see Hutchinson (1987) and Brash (1988)]. It has been the conclusion of recent in vitro studies that the pyrimidine photodimers are *absolute* (Chan et al., 1985) or *permanent* blocks (Larson & Strauss, 1987) to pol I under standard conditions. If true, this would imply that mutagenesis induced by pyrimidine dimers either does not involve pol I or, if it does, requires additional factors to enable pol I to bypass such lesions in an error-prone manner.

The conclusion that pyrimidine dimers are absolute blocks to pol I under standard conditions was based on studies conducted with templates produced by direct photolysis of DNA. Templates produced in this manner are inhomogeneous and contain cis-syn, (6–4), and possibly Dewar products (Taylor et al., 1988) among others. The relative amounts of cis-syn and (6–4) products have been shown to depend on the DNA sequence and photolysis conditions (Chan et al., 1985). It has been recognized that analysis of data derived from such studies cannot reliably establish whether or not bypass of a particular lesion occurs to the extent of a few percent (Strauss, 1985). Since mutagenesis is an infrequent event, any mechanism by which it can take place must be important. In order to rigorously establish whether or not a particular lesion can be bypassed by a polymerase and to understand the mechanism by which such an event can take place, pure well-characterized site-specific lesion-containing templates are required. Such an approach has been successfully used to study polymerase bypass of aminofluorene (AF) adducts (O'Connor & Stohrer, 1985; Michaels et al., 1987) and models of abasic sites (Takeshita et al., 1987).

DNA containing site-specific cis-syn thymine dimers have been used in high-field NMR (Kemmink et al., 1987a,b), photolyase binding (Husain & Sancar, 1987), DNA bending (Husain et al., 1988), in vivo mutagenesis (Banerjee et al., 1988), and T4 denV endonuclease V (Inaoka et al., 1989) studies. The DNAs utilized in these studies were all constructed from oligonucleotides containing a site-specific thymine dimer prepared by photolysis of an oligonucleotide. In order to reduce the number of products formed in photolysis and thereby facilitate the isolation of the dimer-containing product in a reasonable amount and purity, the sequence of the oligonucleotide is restricted to having one dipyrimidine site. As a result, this method limits the study of sequence effects on termination and bypass of dimers.

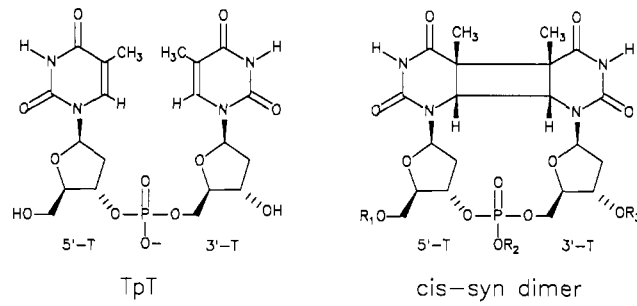

FIGURE 1: Structure of TpT, its cis-syn dimer ($R_1 = R_3 = H$, $R_2 = {}^-$), and the building block used to incorporate the cis-syn dimer into oligonucleotides by solid-phase DNA synthesis [R_1 = DMT, $R_2 = CH_3$, $R_3 = P(OCH_3)(NC_4H_8O)$].

As part of a program aimed at unraveling the structure–activity relationships in sunlight-induced mutagenesis we have been developing methods for the general synthesis of DNA containing site-specific photoproducts. In order to avoid the sequence limitations of the direct photolysis route, we developed a building block (Figure 1) for the incorporation of cis-syn thymine dimers into oligonucleotides by solid-phase DNA synthesis technology (Taylor et al., 1987). Our method is general and can be used to incorporate cis-syn thymine dimers into any site, of any sequence, any number of times, and in high purity. Herein, we describe the combined chemical and enzymatic synthesis of a 41-mer containing a site-specific cis-syn thymine dimer and demonstrate that both Klenow and pol I can bypass this dimer in vitro under standard buffer conditions. We also demonstrate that Klenow bypasses the dimer in a nonmutagenic fashion and propose a mechanism by which this might take place. The latter result also leads us to propose a new mechanism for the origin of C → T mutations at TpdC sites.

Materials and Methods

Enzymes and Reagents. T4 polynucleotide kinase, T4 DNA ligase, pol I (5–10 units/μL, 9000–10 000 units/mg), and Klenow for the time study (3–9 units/μL, 12 000–15 000 units/mg) were from Bethesda Research Labs. Klenow for all other studies was from Promega (9 units/μL, 11 000 units/mg). One unit is that required to polymerize 10 nmol of dNTPs in 30 min at 37 °C on a poly(dA-dT) template-primer. Sequenase was from United States Biochemical. Phage T4 denV UV endonuclease V was from Applied Genetics Inc., Freeport, NY. [γ-^{32}P]ATP was from Amersham. β-Cyanoethyl phosphoramidites were from American Bionetics. dNTPs were from Boehringer Mannheim.

General Procedures. Concentrations of oligonucleotides were estimated from their absorbance at 260 nm, using calculated extinction coefficients (Fasman, 1975). Solutions of duplexes and template-primers were annealed by heating to 70 °C in a 300-mL water bath which was then allowed to cool to room temperature. Primer-extension reactions were conducted in polymerase buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, and 5 mM BME) and quenched by addition to an equal volume of 15 mM EDTA. Samples were prepared for electrophoresis by lyophilization and dissolution in 99% formamide and 5 mM EDTA. Analytical gel electrophoresis was carried out at 1400 V on 0.4 mm thick, 15%, 20:1 cross-linked, acrylamide gels prepared in TBE and 8 M urea. Preparative gel electrophoresis was carried out at 400 V on 1.5 mm thick gels. Analytical gels were autoradiographed at −8 °C with Kodak SB-5 film and band densities quantified by densitometry on a Joyce-Loebel, Chromscan 3, scanning densitometer. Percent primer-extended products were

[1] Abbreviations: BME, β-mercaptoethanol; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid, dd, doubly distilled; dNTP, deoxynucleotide triphosphate; Klenow, the large fragment of pol I; PEI, poly(ethylenimine); Pol I, *Escherichia coli* polymerase I; PP_i, inorganic pyrophosphate; Py, pyrimidine; TAE, 100 mM Tris–acetate, 0.2 mM EDTA, pH 7.8; TBE, 130 mM Tris, 44 mM boric acid, 2.5 mM EDTA; TE, 10 mM Tris-HCl, pH 8, 1 mM EDTA; TEAB, triethylammonium bicarbonate.

computed from autoradiograms in the linear response range as (100 × integrated optical density of the product band)/(total integrated optical density of all primer-extended products).

Oligonucleotide Synthesis. Oligonucleotides were synthesized and purified according to general procedures (Taylor et al., 1987; Taylor & Brockie, 1988). The 10-mers and 19-mer were 5′-phosphorylated by incubating 100 pmol of the oligonucleotides with 20 units of polynucleotide kinase in 20 μL of 1 mM ATP, 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM DDT, and 0.1 mM spermine at 37 °C for 30 min. 5′-^{32}P-End-labeled oligomers were similarly prepared with [γ-^{32}P]ATP.

Template Construction. The 12-mer, 34-mer, and 5′-phosphorylated 10-mer and 19-mer (100 pmol of each) were annealed in 60 μL of 100 mM NaCl and 20 mM Tris-HCl, pH 8. The solution was then made up to 100 μL in 40 μM ATP, 42 mM Tris-HCl, pH 8.0, 4 mM $MgCl_2$, 60 mM NaCl, 10 mM DTT, and 1 mM EDTA and incubated overnight at 4 °C with 6 units of DNA ligase. The reactions were quenched by heating to 65 °C for 15 min and electrophoresed for 5 h on a preparative gel. The sections of the gel containing the 41-mers were located by reference to the position of 5′-^{32}P-end-labeled 41-mers, cut out, macerated, and eluted in TE buffer at 4 °C for 2 days. The eluants were passed through C-18 Prep Sep columns (Fisher) which had been preequilibrated with 25 mM TEAB. After washing the column with dd H_2O, the 41-mers were eluted with 30% acetonitrile in 100 mM TEAB, lyophilized, and precipitated from 0.4 M sodium acetate, pH 5.2, with 3 volumes of ethanol.

T4 denV Endonuclease V Assay. 5′-^{32}P-End-labeled templates were annealed to 0.06 pmol of the complementary 41-mers in 10-μL of 50 mM Tris-HCl, pH 8.0, 75 mM NaCl, and 1 mM EDTA. The solutions were then made up to 1 mg/mL in BSA and treated with 8000 units of phage T4 denV endonuclease V for 2 h at 37 °C.

Primer-Extension Studies. Unlabeled template (33 nM) was annealed to 33 nM 5′-^{32}P-end-labeled 15-mer in 2× polymerase buffer and then made up to twice the volume by addition of dNTPs and polymerase. After the appropriate time at 22 °C, 10-μL aliquots were quenched and electrophoresed.

Strand Exchange Study. CS-41-mer (0.15 pmol) was separately annealed to 0.5 pmol of labeled 15-mer and 1 pmol of unlabeled 15-mer in 10 μL of polymerase buffer, and both were incubated with 1.5 units of Klenow at 22 °C. After 0.5 h, 0.5 pmol of labeled primer was added to the unlabeled reaction and 1 pmol of unlabeled primer to the labeled reaction, along with an additional 1.5 units of Klenow each. After another 0.5 h, the reactions were quenched and electrophoresed.

Nucleotides Terminating Opposite Dimer. Primer extension was carried out at 22 °C with 1.5 units of Klenow on 0.3 pmol of CS-41-mer annealed to 0.3 pmol of unlabeled 15-mer in 10 μL of polymerase buffer containing 1 μM of each dNTP. After 0.5 h, the volume was made up to 20 μL with an additional 3 units of Klenow and a final concentration of 100 μM in each [α-^{32}P]dNTP of equal specific activity. After 0.5 h, the reaction was quenched and electrophoresed on an analytical gel. The 24-mer band was excised, eluted with TE, ethanol precipitated, and digested with 0.2 unit of snake venom phosphodiesterase at 37 °C for 0.5 h in 50 mM Tris-HCl, pH 8.9, 100 mM NaCl, and 15 mM $MgCl_2$. The crude reaction mixture was subjected to two-dimensional chromatography on PEI paper as previously described (Randerath & Randerath, 1967) except that the second dimension was run in 0.8 M ammonium sulfate buffer. The chromatogram was autoradiographed, and the percentages of each dNMP were quantified by spot densitometry.

TEMPLATE STRAND

```
10-mer                  5'-CGTAxyATGC-3'
12-mer  5'-TGCATGCCTGCA-3'
19-mer                                  5'-AATTCGTAATCATGGTCAT-3'
41-mer  5'-TGCATGCCTGCACGTAxyATGCAATTCGTAATCATGGTCAT-3'
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COMPLEMENTARY STRAND

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14-mer                                  3'-TAAGCATTAGTACC-5'
15-mer                                   3'-GCATTAGTACCAGTA-5'
34-mer     3'-GTACGGACGTGCATAATACGTTAAGCATTAGTAC-5'
41-mer  3'-ACGTACGGACGTGCATAATACGTTAAGCATTAGTACCAGTA-5'
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FIGURE 2: Synthetic oligonucleotides used in this study. The prefix ND (nondimer) is used when xy is TpT, and CS is used when xy is the cis-syn dimer.

Sequence of the Bypass Product. CS-41-mer (12 pmol) annealed to 10 pmol of 14-mer in 750 μL of polymerase buffer was made up to 100 μM in each dNTP and incubated with 72 units of Klenow for 4 h at 22 °C. The reaction mixture was quenched, phenol extracted, made up to 1 M LiCl, precipitated with 6 volumes of 3:1 acetone/EtOH, and electrophoresed on an analytical gel. The section corresponding to a 37-mer was excised and electroeluted into a 200-μL volume of TAE in an ISCO Model 1750 electrophoretic concentrator. The solution was lyophilized and the residue precipitated from 1 M LiCl. The 37-mer was sequenced with 1.2 units of Sequenase, 0.1 pmol of 5′-^{32}P-end-labeled 12-mer, 12 μM ddNTP, and 80 μM in each dNTP in 40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$ 50 mM NaCl, and 7 mM DTT. Reactions were run for 15 min at 37 °C.

RESULTS

Design of the Template. The template was chosen to be 41 nucleotides long with the cis-syn thymine dimer at positions 17 and 18 in order to minimize possible end effects on both initiation of primer extension and bypass. In this way a 15-mer complementary to the 3′-end of the template could be used as a primer and still leave an eight-nucleotide gap between its 3′-terminus and the dimer site. In addition, the length was such that would enable the use of a 12-mer as a primer for dideoxy sequencing a bypass product. The sequence of the 41-mer was chosen for a number of reasons. First, the complementary duplex d(CGTAT[*c*,*s*]TATGC)·d(GCATAATACG) is currently the subject of high-field ^{1}H NMR studies in our group aimed at determining its structure and properties. Second, the same dimer-containing decamer has been site-specifically incorporated into a bacteriophage DNA for parallel mutagenesis studies in *E. coli* (Taylor & O'Day, 1989). The sequence of the 41-mer (Figure 2) was therefore chosen to be the same as that in the bacteriophage DNA in order to facilitate comparisons between the in vitro and in vivo studies.

Synthesis and Characterization of the Template. The template containing the cis-syn dimer, CS-41-mer, was prepared by ligating together CS-10-mer, 12-mer, and 19-mer in the presence of the complementary 34-mer. The template containing no dimer, ND-41-mer, was similarly prepared as a control. It was found that the CS-41-mer had to be properly purified in order to observe polymerase bypass of the cis-syn dimer. Whereas CS-41-mer eluted from a gel by diffusion followed by either ethanol precipitation or passage through a C-18 column was suitable, ethanol-precipitated electroeluted

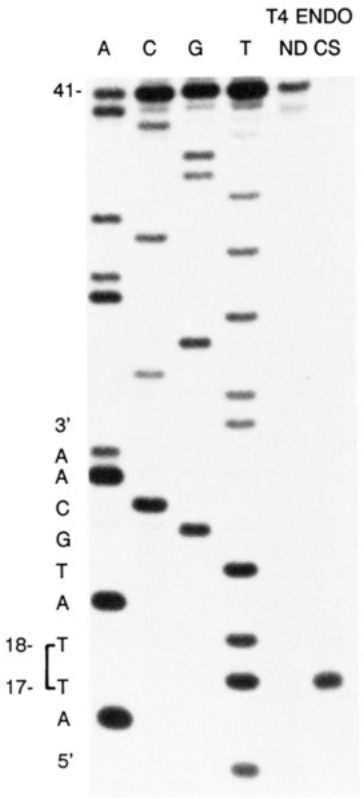

FIGURE 3: Verification of the site and integrity of the cis-syn dimer. Autoradiogram of an analytical denaturing acrylamide electrophoresis gel. Left lanes: Dideoxy sequencing reactions conducted with Klenow on 5′-^{32}P-end-labeled 12-mer annealed to the complementary 41-mer. Lane headings refer to the dideoxy nucleotide used. Right lanes: Reaction of T4 denV endonuclease V with 5′-^{32}P-end-labeled ND-41-mer (ND) or CS-41-mer (CS) annealed to the complementary 41-mer. The sequence of the ND- and CS-41-mers is on the left, with the dimer indicated by a bracket.

material was not, unless subsequently passed through a C-18 column.

The site and integrity of the cis-syn thymine dimer were established by incubating T4 denV endonuclease V with 5′-^{32}P-end-labeled CS-41-mer annealed to its complementary 41-mer. This endonuclease hydrolyzes the 5′-glycosyl bond of a cis-syn cyclobutane dimer after which it cleaves the internucleotide phosphate bond (Friedberg, 1985). The CS-41-mer was cleaved in essentially quantitative yield (>98%) to give the expected 17-mer whereas the duplex formed from ND-41-mer remained intact (Figure 3).

Primer-Extension Reactions. Primer extension of 5′-^{32}P-end-labeled 15-mer on both the CS-41-mer and ND-41-mer was first carried out for 0.5 h as a function of dNTP concentration at a polymerase concentration of approximately 0.15 units/μL (Figure 4). The total amount of primer extended by Klenow at all dNTP concentrations was approximately 60% on the CS-41-mer and 35% on the ND-41-mer. The lack of complete primer utilization is not fully understood but may be due in part to overestimation of the template concentrations.

Of the Klenow-catalyzed primer-extended products on the CS-41-mer, approximately 80% were 23- and 24-mers, corresponding to termination prior to and opposite the 3′-T of the dimer, and 10% were 18- and 19-mers. The 23-mer:24-mer ratio depended on dNTP concentration and was 19:1 at 1 μM, 3.4:1 at 10 μM, and 1:1.3 at 100 μM in each dNTP for the gel shown. The precise ratio, however, was found to depend on the lot of polymerase used. On the ND-41-mer, approximately 65% of the primer-extended products corresonded to a 41-mer and 25% to 18- and 19-mers. Pol I led to similar

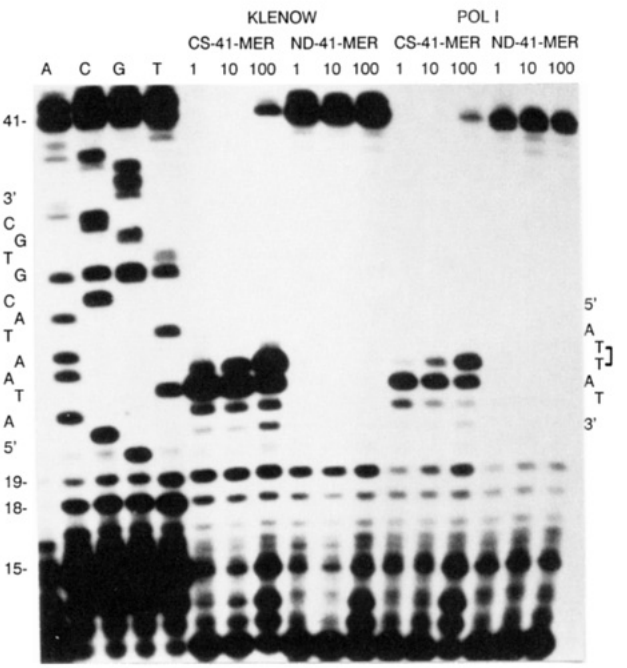

FIGURE 4: Effect of dNTP concentration on termination and bypass. Autoradiogram of an analytical denaturing acrylamide electrophoresis gel. Left lanes: Dideoxy sequencing reactions conducted with Klenow on 5′-^{32}P-end-labeled 15-mer annealed to the ND-41-mer. Lane headings refer to the dideoxy nucleotide used. Middle and right lanes: 5′-^{32}P-End-labeled 15-mer (17 nM) annealed to either CS-41-mer or ND-41-mer was incubated with 0.15 units/μL ($\sim$180 nM) Klenow and 0.12 $\pm$ 0.04 unit/μL ($\sim$120 $\pm$ 45 nM) pol I at 22 °C for 0.5 h as a function of dNTP concentration. Lane headings refer to the concentration of each dNTP in micromolar. The sequence of the primer-extended strand is on the left and that of the template on the right, with the dimer indicated by a bracket.

product distributions and extents of primer extension.

The presence of significant amounts of 18- and 19-mer in both the CS-41-mer and ND-41-mer reactions suggests that termination of polymerization may have been due to base damage in the 19-mer used to construct the template or to some sort of sequence-related effect. A number of minor products corresponding to termination two or more prior to the dimer were also observed. The product terminating two prior to the dimer was not very dNTP concentration dependent and averaged 3% in the Klenow-catalyzed reactions and 7% in the pol I catalyzed reactions.

In addition to termination products, a reproducible amount of a 41-mer corresponding to bypass of the dimer was produced in both the Klenow and pol I reactions conducted at 100 μM in each dNTP. The rate of 41-mer production and the 24-mer:23-mer ratio were also found to depend on polymerase concentration at this dNTP concentration (Figure 5). In contrast, no 41-mer was observed when primer extension was conducted on a 41-mer template containing a trans-syn thymine dimer in place of the cis-syn dimer (Taylor and O'Day, unpublished results).

In order to unambiguously establish whether or not the 41-mer was the result of polymerization past the dimer and not to some other process, a number of experiments were undertaken. The first was to determine the kinetics of its production. If the 41-mer resulted from primer extension of a small amount of nondamaged template, it would also appear at short incubation times and not change in amount with time. Consistent with trans-dimer synthesis, primer extension by pol I led to an almost linear increase in the production of the 41-mer, ultimately reaching 14% after approximately 1 h (Figure 6A). The decreasing intensity of the bands on the

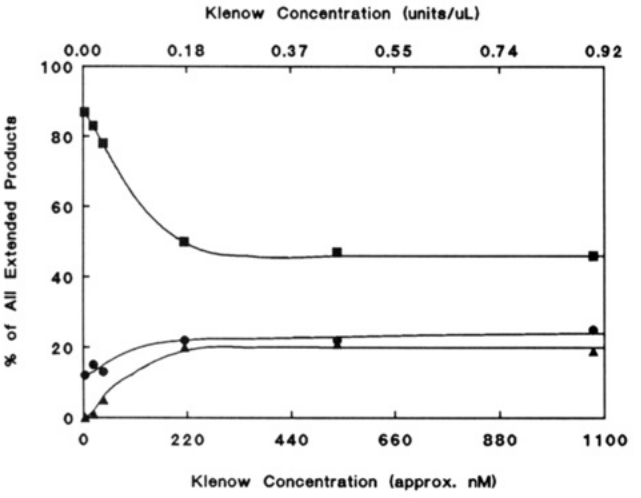

FIGURE 5: Effect of polymerase concentration on termination and bypass. 5′-^{32}P-End-labeled 15-mer (15 nM) annealed to CS-41-mer was incubated with serial dilutions of Klenow for 1 h at 22 °C in 100 μM of each dNTP. Percent bypass (▲), termination opposite the 3′-T of the dimer (■), and termination prior to the 3′-T of the dimer (●) are plotted as a function of polymerase concentration (0.1 unit/μL $\approx$ 120 nM).

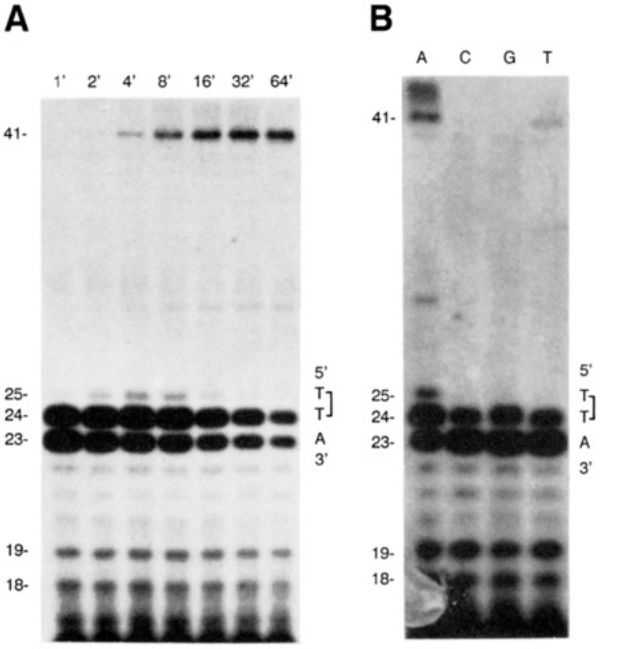

FIGURE 6: Effect of time and dNTP bias on termination and bypass. Autoradiograms of analytical denaturing acrylamide electrophoresis gels. Gel A: 5′-^{32}P-End-labeled 15-mer (17 nM) annealed to CS-41-mer was incubated with 0.06 ± 0.02 unit/μL (~60 ± 20 nM) pol I at 22 °C in 100 μM of each dNTP for the indicated times in minutes. Gel B: 5′-^{32}P-End-labeled 15-mer (17 nM) annealed to CS-41-mer was incubated with 0.15 unit/μL (~180 nM) of Klenow at 22 °C for 0.5 h in 100 μM of the indicated nucleotide and 10 μM of each of the other nucleotides. The length of the primer-extended fragment in nucleotides is on the left of each gel, and the sequence of the template is on the right, with the dimer indicated by a bracket. The origin of the band appearing between the 24-mer and 41-mer as well as that appearing above the 41-mer in the A lane of Gel B is not known.

gel with increasing time can be attributed to the loss of the 5′-^{32}P-label resulting from the 5′→3′ exonuclease activity of pol I. Klenow led to a linear increase in the production of the 41-mer, reaching 11% in 1 h (at twice the polymerase concentration, Figure 5, it reached 20%). Most importantly, no 41-mer was observed in either reaction within the limits of detection at 1 min, though the total amount of 23- and 24-mer was already at a maximum. This puts an upper limit of about 0.5% on the amount of undamaged template that might have been present. Because the T4 denV endonuclease V assay established that the template contained >98% cis-syn thymine dimer, it was concluded that if bypass were occurring, it was the dimer that was being bypassed and not some minor product.

We next considered the possibility that the time-dependent production of the 41-mer was due to repetitive replication of a small amount of contaminating undamaged template via a strand exchange mechanism. In such a process, a primer would invade the double-stranded form of the undamaged template and be extended by the polymerase with concomitant displacement of the complementary strand. In order to test for this process, unlabeled CS-41-mer was incubated with a 5-fold excess of unlabeled 15-mer for 0.5 h at which time it was challenged with a 5-fold excess of labeled 15-mer. After an additional 0.5-h incubation no labeled primer-extended products were observed, consistent with the absence of a strand-exchange mechanism. Parallel experiments in which the original primer was labeled demonstrated that the expected amount of 41-mer was being produced under these conditions.

Additional evidence for bypass by trans-lesion synthesis comes from the results of primer-extension reactions conducted under conditions of 100:10 μM dNTP concentration bias (Figure 6B). In 0.5 h, 7% 41-mer was detected under dATP bias and 1% under dTTP bias. Neither dGTP nor dCTP bias led to detectable amounts of the 41-mer. Bypass by strand exchange would not be expected to be affected by dNTP bias, though bypass by trans-lesion synthesis would.

Sequence Specificity of the Termination and Bypass Products. The specificity of termination opposite the 3′-T of the dimer was determined by analysis of the [α-^{32}P]dNMPs resulting from degradation of the 24-mer produced by Klenow-catalyzed extension of unlabeled 23-mer in the presence of [α-^{32}P]dNTPs. The ratio of radiolabeled dAMP:dCMP:dGMP:dTMP was approximately 37:10:25:28. The relative amount of pdT incorporated opposite the 3′-T of the dimer may be less than indicated by this ratio due to the possible additional incorporation of [^{32}P]pdT into position 23 of the 24-mer by an excision–polymerization mechanism.[2]

The identity and frequency of the nucleotides incorporated opposite the dimer in the bypass product were determined by isolating and dideoxy sequencing the 37-mer produced by Klenow extension of the 14-mer (Figure 7). The 14-mer was used to enable separation of the bypass product from the template on a denaturing gel and thereby facilitate its sequencing. Due to the presence of a small amount of nonspecific termination in all of the sequencing reactions, it was not possible to identify nucleotides present in the bypass product with a frequency of less than about 5%. Within these limits, however, pdA was the only nucleotide incorporated opposite both Ts of the dimer and the sequences flanking the dimer were replicated with complete fidelity.

Discussion

We have demonstrated that a cis-syn thymine dimer is not

[2] Added in proof: Recent primer-extension experiments conducted under infinite dNTP bias with a 16-mer primer terminating one prior to the dimer suggest that the amount of pdT (and possibly pdC) terminating opposite the 3′-T of the dimer under unbiased conditions may have been indeed overestimated. It was found that the amount of the 16-mer extended to a 17-mer in the presence of excess template and 100 μM of a single dNTP under conditions otherwise similar to those described for Figure 6B was 76% for dATP, 0% for dCTP, 36% for dGTP, and 7% for dTTP.

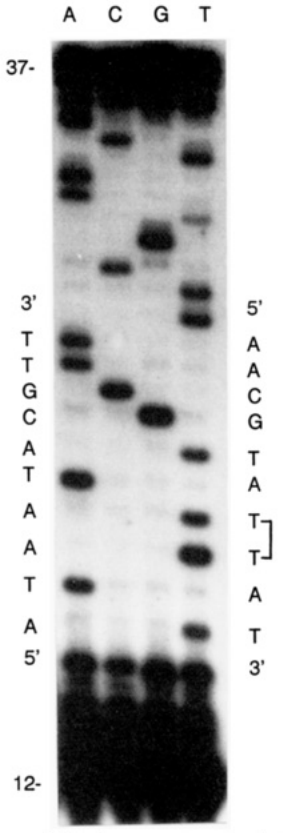

FIGURE 7: Sequence of the bypass strand. Autoradiogram of an analytical denaturing acrylamide gel. The bypass product resulting from Klenow-catalyzed extension of the 14-mer on CS-41-mer was isolated and subjected to dideoxy sequencing with Sequenase and 5′-^{32}P-end-labeled 12-mer. Lane headings refer to the dideoxy nucleotide used. The sequence of the bypass product is on the left and that of the template strand on the right, with the dimer indicated by a bracket.

an absolute block to replication by either pol I or Klenow in vitro under standard conditions. We were able to demonstrate bypass by conducting primer-extension reactions on a homogeneous template containing a site-specific cis-syn thymine dimer constructed by a combination of synthetic and enzymatic steps. Four criteria were used to establish that bypass occurred by trans-dimer synthesis rather than by a template-switching process. First, bypass increased with increasing concentration of dNTPs. Second, bypass increased with increasing time. Third, bypass was affected by dNTP bias. Fourth, challenging a reaction containing unlabeled primer with labeled primer did not lead to labeled products.

In all of the previous studies of pol I action of photodimers, nonhomogeneous templates were used. With such templates, the ability to detect bypass depends on the ability to detect small changes in the relative amounts of termination products upon changing dNTP concentration or reaction time. On the basis of our results, the combination of polymerase, dNTP, and template concentrations and reaction times used in the previous studies were in the range that might have lead to undetectable amounts of bypass. We also found that the template had to be properly purified in order to obtain bypass. The only set of conditions under which pol I had been previously demonstrated to bypass dimers involved a three-stage process, the second stage of which utilized the divalent cation Mn^{2+} in place of Mg^{2+}, the one present in vivo (Rabkin et al., 1983; Strauss, 1985).

The ability of pol I to bypass bulky and distorting damage is not unique to cis-syn thymine dimers as pol I has been shown to bypass site-specific aminofluorene adducts (O'Connor & Stohrer, 1985; Michaels et al., 1987). Studies on multiply damaged templates suggest that pol I can also bypass (diethylenetriamine)platinum(II) adducts (Pinto & Lippard, 1985), 4′-(hydroxymethyl)-4,5′,8′-trimethylpsoralen (HMT) monoadducts (Piette & Hearst, 1983), thymine glycols (Hayes & LeClerc, 1986), and apurinic sites (Schaaper et al., 1983). It is interesting to note that the structure of an HMT adduct of thymine resembles that of a cis-syn thymine dimer, in that both result from a photo [2 + 2] reaction involving the 5,6 double bond of thymine.

Origin of the Sequence Specificity of the Bypass Product. We have found that, within the limits of detection, the bypass product had the sequence that would have been expected had there been a TpT in place of the dimer during bypass. There was no evidence of bypass products resulting from insertion or deletion of nucleotides. In accord with these results, bypass of the dimer was most efficient under conditions of dATP bias. Under unbiased conditions, the preference for nucleotide termination opposite the 3′-T of the dimer was pdA > pdT $\approx$ pdG > pdC, though the amount of pdT may be an overestimation (see Results). With the exception of pdT, this preference is similar to that of dATP > dGTP $\gg$ dCTP $\approx$ dTTP for effecting elongation opposite a thymine dimer under conditions of infinite dNTP concentration bias (Rabkin et al., 1983). As had been pointed out in that study, pdAs incorporated opposite a thymine dimer during bypass would not result in a mutation.

One possible explanation for the highly specific incorporation of pdA opposite the cis-syn thymine dimer during bypass has been suggested in previous studies on dimers and relates to the A rule in mutation [Larson and Strauss (1987) and references therein]. This rule states that pdA is preferentially inserted opposite noninstructional lesions by polymerases. The rule was proposed to account for the high percentage of N $\rightarrow$ A mutations and the fact that elongation opposite pyrimidine dimers takes place to the greatest extent under conditions of infinite dATP concentration bias. If only this mechanism were operating, the selectivity for incorporation of pdA opposite the 3′-T of the dimer would be the same in the termination products as it would in the bypass product. In contrast, we observe that the 1.5-fold preference for terminating in pdA over pdG opposite for 3′-T of the dimer increases to >20-fold in the bypass product. This requires a mechanism for increasing selectivity over that provided by preferential incorporation.

One possible mechanism for increasing selectivity would involve preferential elongation of DNA terminating in pdA opposite the dimer. Preferential elongation of a correctly matched primer terminus has been shown to contribute a 6- to 340-fold increase to fidelity of replication by pol I (Kuchta et al., 1988). A preference for elongation of DNA terminating in pdA opposite the cis-syn thymine dimer, as well as for insertion of pdA opposite the dimer, can be readily understood on the basis of its structure. Because the cis-syn dimer of TpT retains the same pattern of hydrogen-bonding donor and acceptors as that of its precursor TpT unit (Figure 1), it is still expected to prefer to base pair with As. The orientation and bonding properties of the hydrogen bond donors and acceptors as well as the electronic structure and conformation of the dimer are different, however, than those of a TpT unit. Such differences might lead to diminished base-pairing and π-stacking interactions. Recent NMR studies of the complementary duplex octamer d(GCGTTGCG)·d(CGCAACGC) indicate that dimerization of a TpT unit causes only a small amount of helix distortion and that hydrogen bonding to the

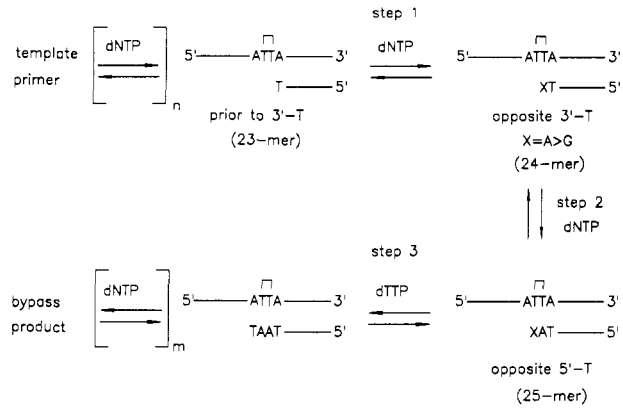

FIGURE 8: Steps and intermediates involved in bypass of the cis-syn thymine dimer by pol I. The dimer is indicated by a bracket.

opposed pdAs, though substantially diminished, still takes place (Kemmink et al., 1987a,b). Similar conclusions concerning the effect of dimerization on helix structure and hydrogen-bonding interactions were obtained by an earlier modeling study (Rao et al., 1984). In contrast, a later study concluded that a cis-syn thymine dimer would bend DNA (Pearlman et al., 1985), a prediction that was latter verified experimentally (Husain et al., 1988).

Given the likelihood that each T of the cis-syn thymine dimer base pairs best with A, the overall high selectivity for incorporation of pdA opposite the dimer in the bypass product can be explained by the coupling of a number of modestly selective sequential events (Figure 8). In the first step, incorporation of pdA opposite the 3'-T of the dimer takes place in preference to other nucleotides due to weak, but complementary, base-pairing interactions and pol I's preference for binding and catalyzing phosphodiester bond formation with dATP. Additionally, conformational changes are expected to contribute to the overall nucleotide preference at this step, as they do in the high-fidelity replication of DNA (Kuchta et al., 1988). In the second step, DNA terminating in pdA opposite the 3'-T of the dimer is preferentially elongated because it forms the best matched primer terminus. Elongation is expected to take place preferentially with pdA for the same reasons as in the first step. In the third and final step, DNA containing pdAs opposite both Ts of the dimer is again preferentially elongated, this time by pdT. The final step is then followed by a rapid series of elongation steps leading to a bypass product containing almost exclusively pdAs opposite the dimer. Detailed kinetic studies will be required to assess the relative contribution of each step to the overall selectivity of the bypass reaction.

Role of dTTP in Bypass. Bypass of the dimer takes place under dTTP concentration bias with approximately 15% the efficiency observed under dATP bias. That the 41-mer band is due to bypass of the dimer, and not to primer extension of some small amount of non-dimer-containing impurity, was supported by the fact that no such product was observed under conditions of dGTP and dCTP bias. One explanation for the bypass is that there is a preference for insertion of pdT opposite the dimer. This is consistent with our finding that pdT was the next most abundant nucleotide, after pdA, terminating opposite the 3'-T of the dimer, though this may be an overestimation (see Results). No evidence, however, was found for incorporation of pdT opposite the dimer in the bypass product under unbiased conditions.

Another explanation for bypass under conditions of dTTP bias takes into account the step involving elongation past the damage site (Strauss, 1985). Since the dimer in our template is flanked by pdA, the rate of this elongation step is expected to be greater at higher concentrations of dTTP. After this step, which serves to lock in the bypass event, further elongation proceeds rapidly to the end of the template. Consistent with this explanation, the greatest amount of 25-mer (4%, Figure 6B) was observed under dATP bias, conditions in which the concentration of dATP is high but that of dTTP is low. Under these connditions, the 25-mer builds up to a higher steady-state level than under unbiased conditions because its rate of elongation has been attenuated by the low concentration of dTTP. The low rate of bypass under dTTP bias and the absence of the 25-mer can be explained by a low rate of production of 25-mer due to the low dATP concentration.

Factors Influencing Termination and Bypass. The relative amounts of termination and bypass were found to depend on both dNTP and polymerase concentrations. At ~150 nM polymerase, increasing the dNTP concentration led to an increase in the amount of bypass and an increase in the 24-mer:23-mer ratio. A similar change in sites of termination on photolyzed templates upon changing dNTP concentration had been previously observed (Larson & Strauss, 1987). The 24-mer:23-mer ratio at a given dNTP concentration can be attributed (Strauss, 1985) to a competition between a dNTP-dependent elongation step and a dNTP-independent 3' → 5' exonucleolytic degradation step resulting in a steady-state mixture of products. Very little 25-mer is observed at 100 μM in each dNTP and high polymerase concentrations, indicating that its elongation and/or degradation is much faster than elongation of the 24-mer. Under these conditions, elongation of the 25-mer must be faster than degradation in order to account for the increase in the steady-state amount of the 25-mer under dATP bias. All this suggests that the rate-limiting step in bypass at high dNTP and polymerase concentrations is elongation of the product terminating opposite the 3'-T of the dimer.

At 100 μM in each dNTP, increasing the polymerase concentration led to a decrease in the 24-mer:23-mer ratio and an increase in the amount of bypass. A similar increase in bypass with an increase in polymerase concentration has been observed with AF sites (Michaels et al., 1986) and model abasic sites (Takeshita et al., 1987). All these results can be attributed to reversible dissociation of the polymerase from the damaged template-primer. The binding affinity of a polymerase for a damaged template-primer has been suggested to to be an important factor in bypass [see Shwartz et al. (1988) and references therein]. The high 24-mer:23-mer ratio at low polymerase concentrations suggests that the dissociation constant of the polymerase is greater for the template-primer terminating opposite the 3'-T of the dimer than it is for that terminating one prior. Preliminary analysis of the data in Figure 5 assuming steady-state conditions for the 24-mer and 23-mer (the time study indicated that this was reached in 16 min) indicates that the former dissociation constant is on the order of 100 nM, whereas the latter is on the order of 20 nM. These values can be compared to 5 nM for a normal template-primer (Kuchta et al., 1987). At polymerase concentrations >200 nM, the 24-mer:23-mer ratio reaches a minimum and the amount of bypass reaches a maximum. At these concentrations, the 24- and 23-mer template-primers are expected to be completely bound by the polymerase, and in such a case their steady-state ratio would approximately equal the ratio of the polymerase-catalyzed elongation and degradation rates.

The sites at which pol I terminates relative to the 3'-T of a pyrimidine dimer have been previously reported as one prior

(Moore & Strauss, 1979); two prior, one prior, and opposite (Chan et al., 1985); and one prior and opposite (Larson & Strauss, 1987). These reported differences may now be understood to be due in part to differences in dNTP and polymerase concentrations, as well as differences in the relative proportions of cis-syn and (6–4) products. Flanking sequences are also known to lead to differences, as termination opposite the 3′-T of putative dimers has only been found to occur significantly at d(GTTA), d(GTTG) (Larson & Strauss, 1987), and d(ATTA) (Chan et al., 1985; this study). These results suggest that flanking purines may play an important role in bypass of cis-syn thymine dimers by pol I, as primer extension into the dimer site is a necessary prerequisite for bypass.

Implications for Mutagenesis by Ultraviolet Light. The principal mutation induced by ultraviolet light in *E. coli* is the C → T transition, which is found to occur almost exclusively at PyPy sites [see Hutchinson (1988) for a discussion]. There has been a lot of controversy surrounding the photoproduct class responsible for this mutation. Arguments in support of (6–4) products were based on an observed correlation between the frequency of (6–4) product formation at dTpdC sites with that of C → T transitions at the same sites. However, cis-syn dimers are also produced at these sites which slowly deaminate to the cis-syn dimers of TpU [Liu & Yang, 1978; Ruiz-Rubio and Bockrath (1989) and references therein]. This led to the proposal that C → T mutations could result from photoreactivation of a cis-syn dimer of TpdU followed by trans dU synthesis. The cis-syn dimer of TpdU is identical in structure with that of TpT except that the C5 methyl of the 3′-T has been replaced by a hydrogen. On the basis of the results of our work, pol I bypass of the cis-syn dimer of TpdU would be expected to lead to a bypass product in which pdA is incorporated opposite the pdU of the dimer. Overall, such a series of steps would result in a C → T mutation and might represent a general mechanism for the formation of this class of mutations.

Conclusion

A cis-syn thymine dimer can be bypassed by pol I of *E. coli* under standard conditions at a rate that depends on both dNTP and polymerase concentration. The dependence of bypass on polymerase concentration supports the notion that the binding affinity of a polymerase for damaged DNA is an important factor in bypass and that the role of some SOS-induced proteins may be to enhance such binding [Shwartz et al. (1988) and references therein]. The sequence specificity of the bypass product was found to be higher than that of the termination product, leading to a explanation involving a preferential elongation mechanism. Such a mechanism may play an important role in the bypass of other lesions, in addition to that involving preferential incorporation. Clearly, the sequence specificity of a termination product is not a reliable indicator of the sequence specificity of a bypass product, as it has sometimes been used. On the basis of the sequence of the bypass product, pol I bypass of cis-syn thymine dimers in vivo would be expected to result in a low frequency of mutation. On the other hand, pol I bypass of cis-syn dimers of TpdU, resulting from deamination of cis-syn dimers of TpdC, might result in a high frequency of C → T transition mutations, the major UV-induced mutation observed in bacteria. The effect of flanking sequences on the ability of pol I to bypass a cis-syn dimer and on the identity and frequency of the nucleotides inserted opposite the dimer remains to be determined. By studying the pol I/cis-syn pyrimidine dimer system in more detail, we hope to uncover the fundamental structure-activity principles involved in bypass of DNA damage.

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O^6-Methylguanine in Place of Guanine Causes Asymmetric Single-Strand Cleavage of DNA by Some Restriction Enzymes†

Jeffrey M. Voigt‡ and Michael D. Topal*

Lineberger Cancer Research Center and Departments of Pathology and Biochemistry, University of North Carolina Medical School, Chapel Hill, North Carolina 27599-7295

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ABSTRACT: The interactions of restriction enzymes with their cognate DNA recognition sequences present a model for protein-DNA interactions. We have investigated the effect of O^6-methylguanine on restriction enzyme cleavage of DNA; O^6-methylguanine is a carcinogenic lesion and a structural analogue of the biological restriction inhibitor N^6-methyladenine. O^6-Methylguanine was synthesized into oligonucleotides at unique positions. The oligonucleotides were purified and analyzed by high-pressure liquid chromatography to assure that, within the limits of our detection, O^6-methylguanine was the only modified base present. These oligonucleotides were annealed with their complement so that cytosine, and in one case thymine, opposed O^6-methylguanine. DNA cleavage by restriction enzymes that recognize a unique DNA sequence, *Hpa*II, *Hha*I, *Hin*PI, *Nae*I, *Nar*I, *Pvu*II, and *Xho*I, was inhibited by a single O^6-methylguanine in place of guanine (adenine for *Pvu*II) within the appropriate recognition sequences. However, only the modified strand was nicked by *Hpa*II, *Nae*I, and *Xho*I with O^6-methylguanine at certain positions, indicating asymmetric strand cleavage. For all the restriction enzymes studied but *Aha*II, *Ban*I, and *Nar*I, lack of double- or single-strand cleavage correlated with inability of the O^6-methylguanine-containing recognition sequence to measurably bind enzyme. None of the restriction enzymes studied were inhibited by O^6-methylguanine outside their cognate recognition sequences.

The interaction of restriction enzymes with their cognate DNA recognition sequences presents a model system to study protein-DNA interactions; the proteins are relatively small yet have high specificity for simple DNA sequences as short as 4 bases [reviewed in Modrich (1979) and Yuan (1981)]. Such protein-DNA interactions lie at the heart of most biological processes, so the ability to probe such interactions is important.

The simplest type of restriction enzyme, type II, is a single-function enzyme that only requires Mg^{2+} as a cofactor. Type II enzymes recognize sequences with twofold symmetry, and they generally cleave within their cognate recognition sequences. Several approaches have been used to study DNA sequence recognition by type II restriction enzymes including (i) determination of the crystal structure of *Eco*RI complexed with the cognate oligonucleotide duplex TCGC*GAATTC*GCG (McClarin et al., 1986), (ii) chemical substitution of base analogues for bases in the recognition sequence to probe the requirements for specific functional groups (Berkner & Folk, 1979; Dwyer-Hallquist et al., 1982; Bodnar et al., 1983; Brennan et al., 1986; Jiricny & Martin, 1986; Jiricny et al., 1986; McLaughlin et al., 1987), and (iii) chemical protection experiments to indicate where *Eco*RI endonuclease contacts DNA (Lu et al., 1981).

Some points to emerge from the large amount of work represented by these papers, as well as others, include (i) *Eco*RI "hugs" the DNA making contact with phosphates and the DNA major groove. The latter provides the major means of sequence discrimination by specific hydrogen bonding, as proposed by Seeman et al. (1976), and by sensing the presence or absence of a methyl at C-5 of pyrimidines; interactions with the minor groove may be important for recognizing some sequences. *Eco*RI interaction with its cognate recognition sequence is facilitated by kinking at the center of the recognition site, concomitant with enzyme binding, to allow better contact with the major groove (type I neokink) and two more moderate kinks that span the scissile bond (type II neokink). (ii) *Eco*RI contacts at least 10 base pairs, indicating that interactions beyond the recognition sequence are important for stability. (iii) Different restriction enzymes react to the same base modifications differently in terms of binding and cleavage. Thus, the specific modes of enzyme-DNA recognition may be as varied as the sequences to be recognized.

We present studies that place a methyl group at the O^6 position of unique guanines in the recognition sequence of several restriction enzymes. Methylation of guanine at O^6 is the natural product of some chemical carcinogens. This

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* Address correspondence to this author.
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