

The Ability of a Variety of Polymerases to Synthesize Past Site-specific *cis-syn*, *trans-syn-II*, (6–4), and Dewar Photoproducts of Thymidylyl-(3' → 5')-thymidine*

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The role of photoproduct structure, 3' → 5' exonuclease activity, and processivity on polynucleotide synthesis past photoproducts of thymidylyl-(3' → 5')-thymidine was investigated. Both Moloney murine leukemia virus reverse transcriptase and 3' → 5' exonuclease-deficient (*exo*⁻) Vent polymerase were blocked by all photoproducts, whereas *Taq* polymerase could slowly bypass the *cis-syn* dimer. T7 RNA polymerase was able to bypass all the photoproducts in the order *cis-syn* > Dewar > (6–4) > *trans-syn-II*. Klenow fragment could not bypass any of the photoproducts, but an *exo*⁻ mutant could bypass the *cis-syn* dimer to a greater extent than the others. Likewise T7 DNA polymerase, composed of the T7 gene 5 protein and *Escherichia coli* thioredoxin, was blocked by all the photoproducts, but the *exo*⁻ mutant Sequenase 2.0 was able to bypass them all in the order *cis-syn* > Dewar > *trans-syn-II* > (6–4). No bypass occurred with an *exo*⁻ gene 5 protein in the absence of the thioredoxin processivity factor. Bypass of the *cis-syn* and *trans-syn-II* products by Sequenase 2.0 was essentially non-mutagenic, whereas about 20% dTMP was inserted opposite the 5'-T of the Dewar photoproduct. A mechanism involving a transient abasic site is proposed to account for the preferential incorporation of dAMP opposite the 3'-T of the photoproducts.

Dipyrimidine sites are the major sites of UV-induced photoproducts and mutations (1–7). The four main classes of photoproducts formed by ultraviolet light at dipyrimidine sites (shown in Fig. 1 for a TpT¹ site) are the *cis-syn* and *trans-syn* (*trans-syn-I* (8) and *trans-syn-II* (9)) cyclobutane dimers and the (6–4) pyrimidine-pyrimidone photoproducts and their Dewar valence isomers (10–13). All of these photoproducts have been found to lead to mutations in *Escherichia coli* under

SOS conditions by use of site-specific photoproduct-containing bacteriophage vectors, but the (6–4) and Dewar photoproducts are far more mutagenic than either the *cis-syn* and *trans-syn* isomers (14–18). The extent to which a particular photoproduct contributes to UV-induced mutations at a particular site not only depends on its mutagenicity, but also depends on its rate of induction, repair, and DNA synthesis bypass (7, 12). At the moment, the relative contribution of individual DNA photoproducts to UV-induced mutations is not known, nor are the detailed mechanisms by which DNA photoproducts are repaired or bypassed. Recently, we have prepared homogeneous 49-mer oligonucleotides containing the four major photoproduct classes of TpT (19)² for use as substrates for the necessary *in vitro* and *in vivo* mechanistic studies. Herein, we report the use of these 49-mers and 72-mers containing a T7 promoter to study the role of photoproduct structure and the 3' → 5' exonuclease activity and processivity of polymerases on DNA and RNA synthesis past these photoproducts.

EXPERIMENTAL PROCEDURES

Enzymes, Reagents, and Equipment—The preparation of the photoproduct-containing 49-mers has been reported elsewhere (19).² Other oligonucleotides were purchased at a local facility and purified by ion exchange high performance liquid chromatography. Oligonucleotide concentrations were measured by absorbance at 260 nm using estimated extinction coefficients (20). T4 polynucleotide kinase and *exo*⁻ Vent (21) were purchased from New England Biolabs. *Taq* DNA polymerase, wild-type T7 DNA polymerase, Sequenase Version 1.0 (22), Sequenase 2.0 (δ28 K118-R145; Ref. 23), Klenow fragment (KF), *exo*⁻ KF (D355A/E357A; Ref. 24), T7 RNA polymerase, and deoxynucleotide triphosphates were purchased from U. S. Biochemical Corp. Concentrations of commercial enzymes were calculated from data obtained from the supplier. The D5A/E7A T7 gene 5 protein (25) and *E. coli* thioredoxin components of T7 DNA polymerase were a generous gift of Isaac Wong and Kenneth Johnson (University of Pennsylvania). Moloney murine leukemia virus reverse transcriptase (MMLV RT) was purchased from Life Technologies, Inc. dNTPs were from Fisher, and NTPs were from Sigma. [γ -³²P]ATP (2 μM, 10 μCi/μl) was purchased from Amersham Pharmacia Biotech. Dideoxy sequencing mixes were prepared in Sequenase buffer (40 mM Tris-HCl, 10 MgCl₂, and 5 mM DTT) with each nucleotide triphosphate at 300 μM, with the eponymous nucleotide triphosphate in a ratio of 1:3 ddNTP to dNTP. Unless otherwise stated, all electrophoresis was conducted on 0.4-mm-thick, 37.5-cm-long, 7 M urea, 1:19 cross-linked, 15% acrylamide gel at 1800 V. DNA fragments were visualized by autoradiography with Kodak XAR-5 film. Densitometry was performed on a Joyce-Loel Chromoscan 3 or a Molecular Dynamics computing densitometer model 300A. The percentage of a primer-elongated product is computed as the percentage of the total amount of extended products.

Comparative Primer Extension Reactions at 1, 10, and 100 μM dNTPs—Reactions were carried out by incubating 12.5 nM 15-mer primer annealed to 125 nM 49-mer template with 1, 10, or 100 μM

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¹ The abbreviations used are: Trp, thymidyl-(3' → 5')-thymidine (6–4), pyrimidine-(6–4)-pyrimidone; Dewar, pyrimidine-(6–4)-Dewar pyrimidone; KF, Klenow fragment of *E. coli* DNA polymerase I; *exo*⁻, 3' → 5' exonuclease-deficient; DTT, dithiothreitol; pol, polymerase; MMLV, Moloney murine leukemia virus; RT, reverse transcriptase; BSA, bovine serum albumin; PCNA, proliferating cell nuclear antigen; AF, 2-aminofluorene; AAF, acetylaminofluorene; BPDE, benzo[*a*]pyrenedioloxide; SOS, a cellular response to DNA damage; ddNTP, dideoxy NTP.

² The *trans-syn* isomer reported in this earlier paper was erroneously assigned the *trans-syn-I* structure. It has now been established to be the *trans-syn-II* structure (M. Wang, Y. Ren and, J.-S. Taylor, unpublished results).

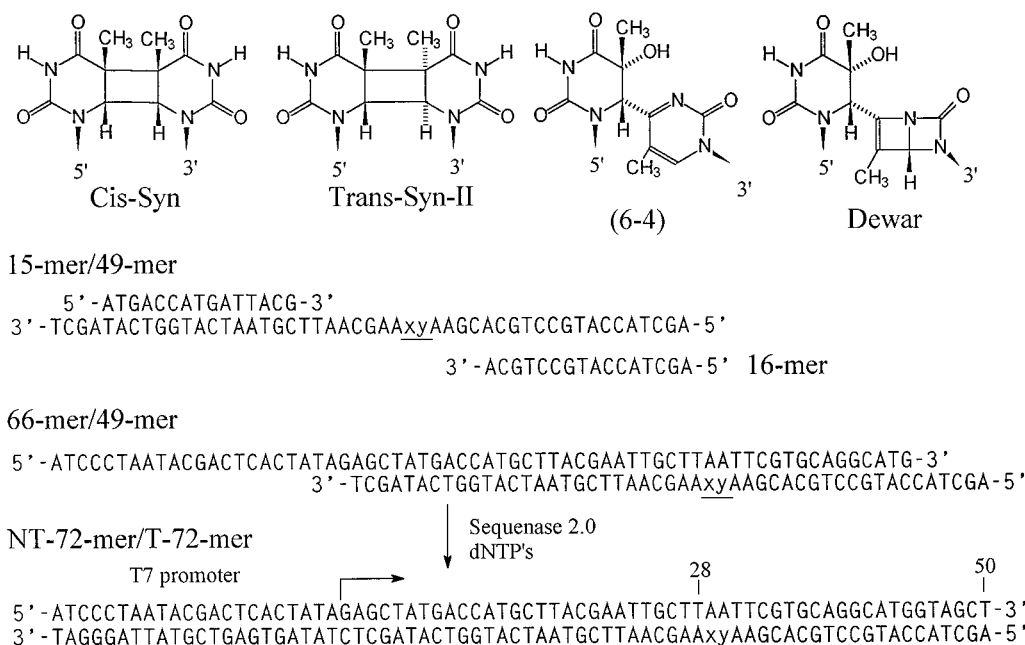


Fig. 1. Oligonucleotides used in this study. The 15-mer/49-mer was used to study DNA synthesis opposite the photoproduct-containing templates, and the NT-72-mer/T-72-mer was used to study RNA synthesis. *XY* refers to either a *cis-syn*, *trans-syn-II*, (6-4), or Dewar photoproduct of TpT, or to the undamaged TpT site. *NT* refers to the non-transcribed strand and *T* to the transcribed strand. The 16-mer was used in sequencing the products of DNA synthesis past the photoproducts.

dNTPs and polymerase in a total volume of 4 μ l (160 units of MMLV, 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 6 mM MgCl₂, and 1 mM DTT; 0.8 units of *exo*⁻ Vent, 20 mM Tris-HCl, pH 8.8 at 25 °C, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, and 10 mM DTT; 2 units of *exo*⁻ KF, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 10 mM DTT; 5.2 units of Sequenase 2.0, 40 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, and 5 mM DTT), for 15 min at 37 °C, and quenched with 11 μ l of 95% formamide, with the exception of *exo*⁻ Vent, which was incubated for 5 min at 37 °C followed by 15 min at 50 °C.

Primer Extension by *Taq* Polymerase—Primer extensions were conducted by incubating 7 nM 15-mer primer annealed to 70 nM 49-mer template with 100 or 200 μ M dNTPs and 0.5 units of enzyme in a total volume of 10 μ l (67 mM Tris-HCl, pH 8.8 at 25 °C, 17 mM (NH₄)₂SO₄, 6.7 mM MgCl₂, 1 mM DTT, and 20 μ g/ml BSA) for 30 min at 60 °C. The polymerase was added last to the prewarmed solution. The reactions were quenched by the addition of 15 μ l of 95% formamide.

Primer Extension by *KF* and *exo*⁻ *KF*—Primer extensions were conducted by incubating 7 nM 15-mer primer annealed to 70 nM 49-mer template with 100 nM enzyme (0.8 units of *KF*, 1.9 units of *exo*⁻ *KF*) and 100 μ M dNTPs in a total volume of 10 μ l (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, and 100 μ g/ml BSA) for 30 min at 37 °C. The reactions were quenched by addition of 15 μ l of 95% formamide.

Primer Extension by Wild Type *T7*, Sequenase 2.0, and *D5A/E7A* Gene 5 Protein with and without Thioredoxin—Primer extension were conducted by incubating 7 nM 15-mer primer annealed to 70 nM 49-mer template with 100 μ M dNTPs and 100 nM enzyme (0.1 units of wild-type *T7*, 1.2 units of Sequenase 2.0), preincubated with or without 2 μ M thioredoxin in a total volume of 10 μ l (40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 20 mM MgCl₂, and 10 mM DTT) for 30 min at 37 °C. The reactions were quenched by the addition of 15 μ l of 95% formamide.

Sequencing the Sequenase 2.0 Bypass Products—Thirty pmol of 5'-labeled primer was annealed to 20 pmol of template. To each primed template was added 13 units of Sequenase 2.0 with buffer and dNTPs for a total volume of 5 μ l (200 μ M dNTPs, 40 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, and 5 mM DTT). The reaction was incubated at 37 °C for 90 min, quenched with 20 μ l of 95% formamide, and electrophoresed. The full-length product was excised, eluted, and dialyzed. An estimated 50 fmol or less of each bypass product was annealed to 25 fmol of 5'-end labeled 16-mer primer, d(AGCTACCATGCCTGCA). Sequenase version 1.0 (2.6 units) was added, for a final volume of 8 μ l (40 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, and 5 mM DTT). To each of four tubes containing 5 μ l of dideoxy mix was added 2 μ l of the annealed primed template. The reactions were incubated for 5 min at 37 °C, and quenched by the addition of 9 μ l of 95% formamide. The reaction mixtures were heat-denatured, electrophoresed, and visualized by autoradiography.

Transcription Reactions—One pmol of the 49-mer was annealed to 2 pmol of complementary 66-mer in 40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 50 mM NaCl in a total volume of 8.5 μ l by heating to 90 °C and cooling slowly to room temperature. The DNA was then extended by adding 0.5 μ l of Sequenase 2.0 and 100 μ M dNTPs. The DNA polymerase was inactivated by brief heating at 90 °C and slow cooling to allow reannealing of the DNA duplex. Transcription was carried out in 56 mM Tris-HCl, pH 8, 23 mM MgCl₂, 20 mM NaCl, 5 mM DTT, 0.5 mg/ml BSA, and 800 μ M NTPs and 10 μ Ci of [α -³²P]GTP and 50 units of *T7* RNA polymerase in a total volume of 25 μ l. The mixture was incubated at 37 °C and 2- μ l aliquots were taken at 0, 5, 15, 30, and 60 min and quenched into 5 μ l of loading buffer. The products were electrophoresed at 1000 V for 4 h on a 0.6-mm 7 M urea, 1:19 cross-linked 19% polyacrylamide gel.

RESULTS

Substrates—The photoproduct-containing 49-mers (Fig. 1) were designed to be suitable for a variety of repair and replication studies, and their preparation and characterization have been reported previously (19).² The substrate for transcription was constructed by primer extension of a hybrid between a 66-mer containing a *T7* RNA promoter (26) and the photoproduct-containing 49-mers. DNA and RNA synthesis reactions opposite the photoproducts with well characterized polymerases were principally undertaken to determine the enzymatic properties and conditions that facilitate bypass of DNA photoproducts. For the experiments described herein, the important design feature of the 49-mers is that the photoproducts are centrally located in a deoxyoligonucleotide long enough to serve as a template for primer extension by polymerases. With the exception of the *trans-syn-II* containing 49-mer, these photoproduct-containing 49-mers have also been incorporated into M13 vectors and used to obtain photoproduct mutation spectra in *E. coli* under SOS conditions (18).

Effect of Polymerase and dNTP Concentration on Photoproduct Bypass—The results of the primer extension reactions as a function of polymerase and dNTP concentration are displayed in Figs. 2 and 3. All polymerases were able to fully extend the primers on the undamaged templates even at the low dNTP concentration, except for MMLV RT, which did not fully extend at 1 μ M dNTPs. Not surprisingly, then, MMLV RT was almost

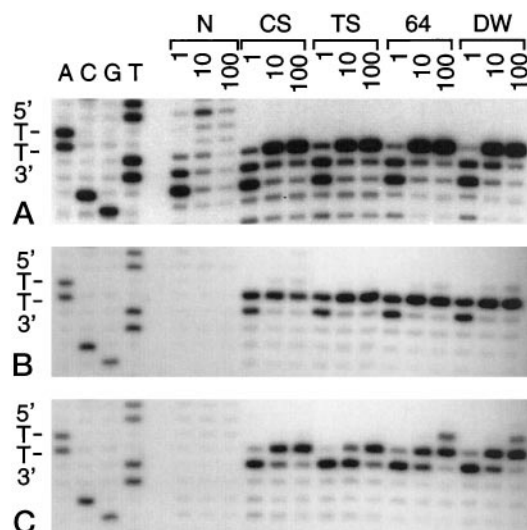


FIG. 2. **Extension by MMLV RT, *exo*⁻ KF and *exo*⁻ Vent.** Autoradiogram of a denaturing electrophoresis gel of the extension of the 15-mer primer opposite excess 49-mer templates by MMLV reverse transcriptase (A), *exo*⁻ KF (B), and *exo*⁻ Vent (C) at 37 °C for 15 min. The dideoxy sequencing reactions of the undamaged template with the indicated ddNTP, and the positions of the 3'-T and 5'-T of the photoproducts in the template are shown to the left. The other lane headings refer to the undamaged (N), *cis-syn* (CS), *trans-syn-II* (TS), (6-4) (64), and Dewar (DW) templates and the micromolar concentration of dNTPs.

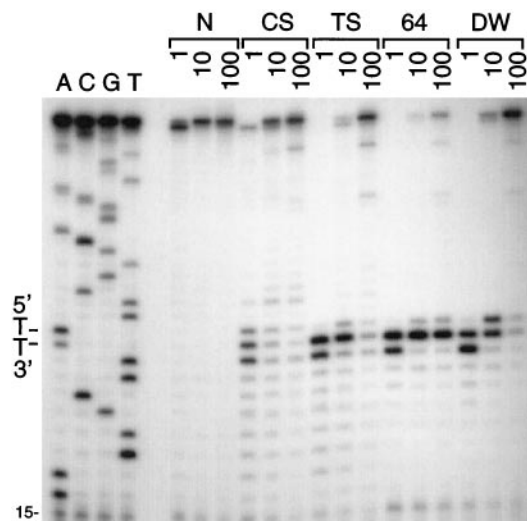


FIG. 3. **Extension by Sequenase 2.0.** Autoradiogram of a denaturing electrophoresis gel of the extension of the 15-mer primer opposite excess 49-mer templates by Sequenase 2.0 at 37 °C for 15 min. Annotations are as described in Fig. 2.

completely blocked by all the photoproducts even at 100 μM dNTPs, stopping primarily one nucleotide prior to the 3'-T of the photoproducts, and terminating primarily three nucleotides prior to the photoproducts at low dNTPs (Fig. 2A). The *exo*⁻ KF primarily stopped one nucleotide prior to and opposite the 3'-T of all the photoproducts at low dNTPs, and primarily opposite the 3'-T at high dNTPs (Fig. 2B). In this experiment, *exo*⁻ Klenow was also able to bypass the *cis-syn* dimer in 2, 8, and 19% yields at 1, 10, and 100 μM dNTPs, respectively. The *exo*⁻ Vent could not bypass any of the lesions, and stopped primarily one prior to all the photoproducts at low dNTPs, but stopped primarily opposite the 3'-T at high dNTPs (Fig. 2C). Interestingly, Vent was able to advance the primer opposite the 5'-T for the (6-4) and Dewar products, but not the *cis-syn* and *trans-syn-II* products. Previous unpublished work in this laboratory found that *Taq* DNA polymerase was able to bypass the

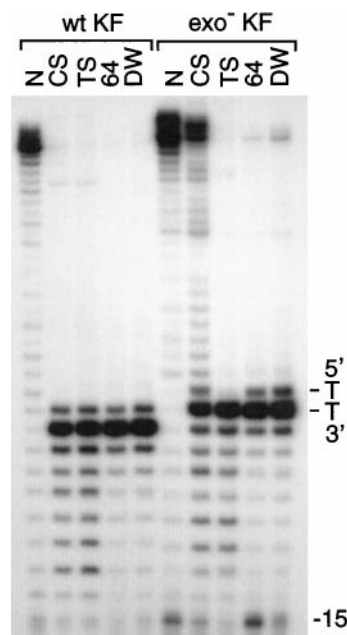


FIG. 4. **Effect of exonuclease activity on extension by Klenow fragment.** Autoradiogram of a denaturing electrophoresis gel of the extension of the 15-mer primer opposite excess 49-mer templates by wild-type (*wt* KF) and exonuclease-deficient Klenow fragment (*exo*⁻ KF) at 100 μM dNTPs at 37 °C for 30 min. Annotations are as described in Fig. 2.

cis-syn dimer, but not the *trans-syn-I* dimer at 60 °C (27). In this study, *Taq* polymerase was also found to bypass the *cis-syn* dimer in about 10% yield at 60 °C in the presence of either 100 or 200 μM dNTPs, but could not bypass the *trans-syn-II*, (6-4), or Dewar products, even at 200 μM dNTPs (data not shown). In all cases, synthesis stopped primarily opposite the 3'-T of all of the photoproducts (>53%) with significant amounts of termination one prior and opposite the 5'-T of the photoproducts (7-15%). Sequenase 2.0 could bypass all the lesions at high dNTP concentrations, and stopped primarily opposite to the 3'-T of the photoproducts at 10 μM dNTPs (Fig. 3). Extension opposite the undamaged template was too fast to measure, but the pseudo first-order rate constant was at least 15 min^{-1} , and should be greater than 500 min^{-1} based on published kinetic data (25). Of all the photoproducts, the *cis-syn* dimer was bypassed the fastest by Sequenase 2.0, with a pseudo first order rate constant of 0.49 min^{-1} . The Dewar product was bypassed the second fastest at 0.071 min^{-1} and the *trans-syn-II* dimer slightly more slowly at 0.040 min^{-1} . The (6-4) product was bypassed the slowest of all with a rate constant of 0.0063 min^{-1} .

Effect of Exonuclease Activity and Processivity on Bypass—The effect of the 3' \rightarrow 5' exonuclease activity was determined by comparing the action of wild-type KF and T7 polymerase with corresponding exonuclease-deficient mutants on the photoproduct containing templates (Figs. 4 and 5). There was a dramatic increase in the ability of both polymerases to extend opposite and past the lesions in the absence of exonucleolytic proofreading ability. Although wild-type KF led to <3% bypass of any of the photoproducts at 100 μM dNTPs, *exo*⁻ KF led to 47% bypass of the *cis-syn* dimer, and smaller, but significant amount (2-4%) of bypass of the other photoproducts under identical conditions (Fig. 4). Likewise, wild-type T7 DNA polymerase was unable to bypass any of the photoproducts at 100 μM dNTPs, but Sequenase 2.0 led to >23% bypass of all the photoproducts (Fig. 5). The effect of processivity on bypass was determined by comparing the action of the exonuclease-deficient T7 polymerase with and without its processivity cofactor (Fig. 6). T7 polymerase is composed of a 1:1 complex of the T7

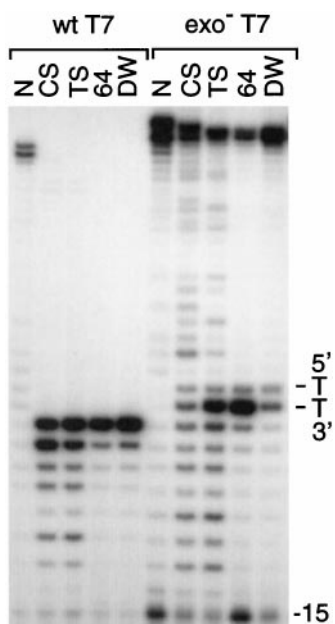


FIG. 5. Effect of exonuclease activity on extension by T7 DNA polymerase. Autoradiogram of a denaturing electrophoresis gel of the extension of the 15-mer opposite excess 49-mer templates by 100 nM wild-type T7 DNA polymerase (*wt T7*) and Sequenase 2.0 (*exo⁻ T7*) at 100 μ M dNTPs at 37 °C for 30 min. Annotations are as described in Fig. 2.

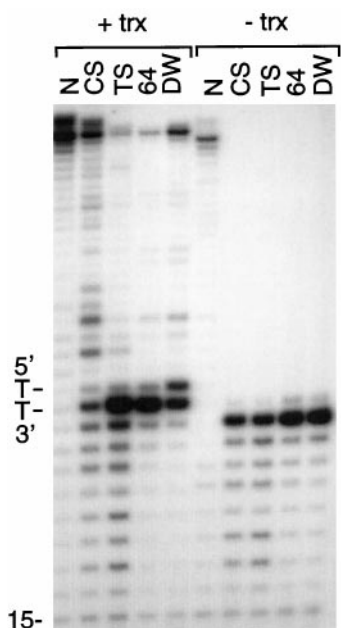


FIG. 6. Effect of the thioredoxin processivity factor on extension by an *exo⁻ T7* gene 5 protein. Autoradiogram of a denaturing electrophoresis gel of the extension of the 15-mer opposite the 49-mer templates by the *exo⁻ D5A/E7A* T7 gene 5 protein (100 nM) with (+*trx*) and without (*-trx*) thioredoxin (2 μ M) at 100 μ M dNTPs at 37 °C for 30 min. Annotations are as described in Fig. 2.

gene 5 protein and *E. coli* thioredoxin (28). Without thioredoxin, a D5A/E7A exonuclease-deficient T7 gene 5 protein was unable to bypass any of the photoproducts and terminated primarily prior to the 3'-T of the photoproducts. Upon the addition of thioredoxin, however, a significant fraction of the *cis-syn* and Dewar products were bypassed (>23%) and termination occurred primarily opposite the 3'-T of the photoproducts (Fig. 6). Unlike what was observed for the Δ 28 exonuclease-deficient Sequenase 2.0, only about 3% of the *trans-syn*-II and (6-4) products were bypassed by the D5A/E7A exonucle-

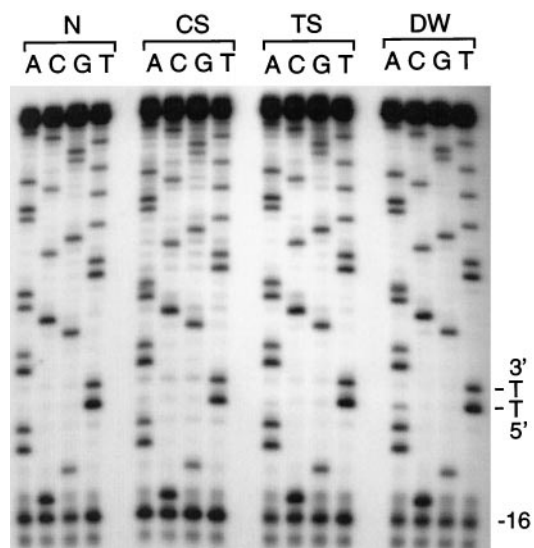


FIG. 7. Sequence of the Sequenase 2.0 photoproduct bypass products. Autoradiogram of a dideoxy sequencing gel of the full-length products resulting from Sequenase 2.0 extension of the 15-mer on the 49-mer templates. Lane headings are as described in Fig. 2. The positions corresponding to the 3'-T and 5'-T of the photoproducts and the 16-mer sequencing primer are indicated on the right.

ase-deficient mutant in the presence of excess thioredoxin.

Sequence Determination of the Sequenase 2.0 Bypass Products—With the exception of the (6-4) product, the bypass products of the photoproduct-containing 49-mers were obtained in sufficient quantity to be sequenced several times by the dideoxy method (Fig. 7). The bypass products of the undamaged, *cis-syn* dimer, and *trans-syn*-II dimer templates did not appear to have any mutations above a background level of about 5% for all sites that are produced during sequencing. Densitometric analysis of the dideoxy sequencing bands of the bypass product of the Dewar photoproduct in comparison to that of the undamaged template indicated that about 20% of thymidine had been introduced opposite the 5'-T of the photoproduct in place of deoxyadenosine.

To confirm that T could indeed be incorporated by the polymerase opposite the 5'-T of the Dewar product, and to investigate the selectivity of nucleotide incorporation opposite the (6-4) product, the rates of dTMP and dAMP incorporation opposite both products were determined (data not shown). Extension of a 15-nucleotide primer terminating in A opposite the 3'-T of the (6-4) product with 100 μ M dATP occurred 25 faster than with 100 μ M dTTP, compared with 7.4 times faster for the Dewar product. Extension of a 16-nucleotide primer terminating in T opposite the 5'-T of the (6-4) product was 26 times faster than that terminating in A, compared with 40 times faster for the Dewar product, demonstrating that the mutagenic products could be further elongated. Because the polymerase is exonuclease-deficient, and both mutagenic and non-mutagenic products can be readily elongated, the selectivity of nucleotide incorporation opposite the 5'-T in the bypass product is solely governed by the selectivity of nucleotide incorporation in the elongation step opposite the 5'-T. Thus, one might expect that dTMP is also incorporated opposite the 5'-T of the (6-4) product in the bypass product, but possibly at a lower frequency than for the Dewar product.

Transcription Past the Photoproducts—RNA synthesis opposite the dimers was also briefly investigated with T7 RNA polymerase and the 72-mer duplex containing the T7 RNA promoter. At 800 μ M NTPs, all the photoproducts could be bypassed with almost the same relative order as observed for the *exo⁻ T7* DNA polymerase, Sequenase 2.0, except that the

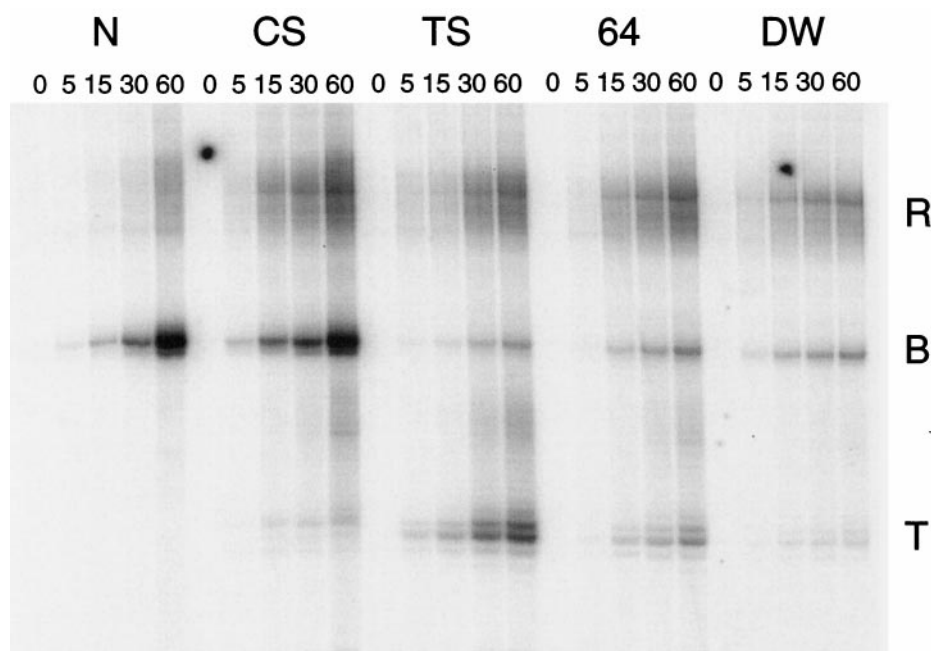


FIG. 8. **Transcription past DNA photoproducts by T7 RNA polymerase.** Autoradiogram of a denaturing electrophoresis gel of the products of transcription by T7 RNA polymerase on the 72-mer duplexes in the presence of 800 μM NTPs and [α - ^{32}P]GTP for the indicated times at 37 $^{\circ}\text{C}$. Lane headings are as described in Fig. 2. The positions corresponding to termination (*T*), bypass (*B*), and supposed runoff (*R*) products are shown on the right.

trans-syn-II isomer was bypassed the slowest (Fig. 8). In 1 h, the ratio of bypass products to termination products was 4.9, 0.4, 0.72, and 2 for the *cis-syn*, *trans-syn*-II, (6-4), and Dewar photoproducts, respectively. Arrest was found to occur at multiple sites surrounding the photoproduct site, but could not be accurately assigned, though it does appear that the T7 RNA polymerase could advance one nucleotide further opposite the *cis-syn* photoproduct than any of the other photoproducts. In addition to the predominant full-length products, small amounts of +1 and -1 full-length product were observed, which have also been observed by others (29), as well as some nonspecific longer products that may be self-encoded run-offs (30).

DISCUSSION

One of our primary goals was to determine how differences in polymerase and lesion structure and properties would affect extension opposite and past DNA photoproducts. A second was to find a DNA polymerase that could bypass all the lesions with high enough efficiency to allow the effects of 3' \rightarrow 5' exonuclease activity and processivity on the bypass reactions, and to allow isolation and sequencing of the bypass products and eventually study the bypass mechanisms in detail. Polymerases deficient in 3' \rightarrow 5' exonuclease activity were selected for study first, because 3' \rightarrow 5' exonucleolytic cleavage is known to compete with elongation opposite and past DNA damage and slow down or completely inhibit DNA damage bypass (31-33). Primer extension opposite the photoproducts was examined as a function of dNTP concentration, as the rate of bypass was expected to increase with increasing dNTP concentration based on early studies on heterogeneous templates (31) and later studies with site-specific *cis-syn* dimers (34). Of the six 3' \rightarrow 5' exonuclease-deficient polymerases studied, MMLV RT, *Taq*, *exo*⁻ Vent, *exo*⁻ KF, Sequenase 2.0, and T7 RNA polymerase, only four of them were able to bypass at least one of the photoproducts, and only two were able to bypass all four photoproducts. Both T7 RNA polymerase and the *exo*⁻ T7 DNA polymerase Sequenase 2.0 were able to bypass all the lesions (Figs. 3 and 8), whereas *exo*⁻ KF and *Taq* were only able to significantly bypass the *cis-syn* dimer. When slightly different conditions were used, including a longer reaction time and the addition of 100 $\mu\text{g}/\text{ml}$ BSA, *exo*⁻ KF was able to synthesize past a small fraction of the *trans-syn*-II, (6-4), and

Dewar products (Fig. 4).

The ability of Sequenase 2.0 to bypass all four products is not unexpected, as T7 DNA polymerases have been reported to bypass a wide variety of bulky adducts and intrastrand cross-linked species. The *exo*⁻ T7 polymerases have been reported to bypass the bulky 2-aminofluorene (AF) adduct of guanine (35, 36), and the bulky 7-bromomethylbenzo[*a*]anthracene (37) and styrene oxide (38) adducts of adduct of dA. The *exo*⁻-deficient T7 polymerase also bypasses photochemically cross-linked TA sites (39), and *cis*-diamminedichloroplatinum(II) cross-linked purines in GG, AG, and GCG (40) though no bypass was observed in different sequence context for the GG and AG sites (41). On the other hand, *exo*⁻ T7 polymerase has not been found to bypass acetylaminofluorene (AAF) (32, 36) or benzo[*a*]pyrenediol-epoxide (BPDE) (42) adducts. KF has also been found to bypass a variety of bulky lesions, including the AF adduct of guanine (35, 43), and the bulkier AAF adduct of guanine (43) and C4'-modified bases (44). It can also bypass model estrogen DNA adducts (45), styrene oxide adducts (38) and certain stereoisomers of BPDE adducts of G (46). In contrast to what we observe for the dipyrimidine photoproducts, the TA* photoproduct is more easily bypassed by *exo*⁻ KF than by Sequenase 2.0 (39). A similar trend was observed for a *cis*-platinum adduct of a GG site in one sequence context (41) but not in another (40).

The ability of the thermostable *Taq* polymerase to bypass some forms of DNA damage is not unprecedented, as it has been recently reported to bypass the *cis-syn* thymine dimer and a (6-4) product to a small extent (47), as well as 7, 8-dihydro-8-oxoadenine, a lesion that causes little distortion to the DNA duplex (48). Because *exo*⁻ Vent did not bypass any of the photoproducts, it may be a better choice for quantifying these products in genes by methods based on quantifying full-length polymerase chain reaction products (49, 50) or polymerase chain reaction termination products (47, 51, 52).

Our finding that all the photoproducts studied, which have often been classified as bulky adducts, can be bypassed by T7 RNA polymerase, contrasts with results observed for prokaryotic and eukaryotic RNA polymerases. Cyclobutane dimers have been shown to halt *Escherichia coli* RNA polymerase both *in vitro* (53) as well as *in vivo* (54). In transcription-coupled

repair, RNA polymerase arrest initiates a series of events that involve excision of a small section of DNA containing the damage followed by new gap-filling DNA synthesis (Ref. 55; reviewed in Ref. 56). Similarly, eukaryotic RNA polymerase II is also fully inhibited by UV-induced adducts (57), and proceeds to initiate what is thought to be a similar series of events as its prokaryotic counterpart. On the other hand, T7 RNA polymerase is often found to be able to transcribe past many types of DNA damage. For example, modified bases such as 8-oxoguanine and an abasic site analog do not block transcription, whereas AF and AAF adducts show an increasing ability to block transcription (58). Cytosine arabinoside (29), as well as single nucleotide gaps (59–61) also prove unable to arrest T7 RNA polymerase, though they may result in miscoding by the polymerase. The bulky BPDE DNA adducts inhibit transcription by T7 RNA polymerase to varying degrees which depend on the stereochemistry of the adduct (62).

Polymerases That Were Incapable of Bypassing the Photoproducts—MMLV RT led to very little if any bypass of any of the photoproducts, stopping almost exclusively one nucleotide prior to all four photoproducts at 100 μ M dNTP concentrations, which may make it useful for mapping the location of photoproducts in irradiated DNA. Although we are not aware of any reports of MMLV RT used in studies of damage bypass, the reverse transcriptase from avian myeloblastosis virus has been found to terminate at DNA photoproducts, but unlike MMLV RT, termination appeared to occur opposite the 3'-T of the dimer (63). Avian myeloblastosis virus RT has also been found to bypass *cis*-thymine glycol lesions (64) and abasic sites (65). Human immunodeficiency virus RT has been shown to be blocked by all but one of the six stereoisomeric BPDE adducts of G (66). The *exo*⁻ Vent was also unable to bypass any of the lesions, but stopped at different positions depending on the lesion and dNTP concentration (Fig. 2C). At 100 μ M dNTPs, *exo*⁻ Vent stalled opposite the 3'-base (first base) of the *cis-syn* and *trans-syn-II* dimers, but partly extended opposite the 5'-base (second base) of the (6–4) and Dewar products. It is difficult to understand why incorporation opposite the 5'-base of the (6–4) and Dewar products is easier than for the *cis-syn* and *trans-syn-II* dimers, and we are unaware of any other reports using *exo*⁻ Vent on damaged templates with which to compare our results.

Effect of Photoproduct Structure on Bypass—Of all the photoproducts, the *cis-syn* dimer was bypassed most easily by any given polymerase. Sequenase 2.0 bypassed the *cis-syn* dimer about 7 times faster than the Dewar product, about 11 times faster than the *trans-syn-II* dimer, and about 77 times faster than the (6–4) product. We interpret this as a consequence of relatively close resemblance of the *cis-syn* dimer structure to a undamaged dithymine site (Fig. 1), and to the relatively little distortion that it causes to normal DNA duplex (67–69). The correspondingly slow bypass of the (6–4) product of TT can likewise be attributed to the fact that it has been found to greatly distort DNA structure and not to base pair to an opposed A based on an NMR structure (69) or only weakly so based on an unrestrained molecular dynamics calculation (70). Though no structure for the *trans-syn-II* dimer in a duplex exists, its lower rate of bypass can be attributed to its 3'-T, which is locked into a *syn* orientation and places the methyl group in the base pairing region, thereby sterically blocking the addition of nucleotides to the primer terminus (Fig. 1). The finding that the Dewar product is more rapidly bypassed than the (6–4) product was predicted previously on the basis of molecular modeling studies of the dinucleotide products indicating that the Dewar product could be fit to a B DNA structure better than could the (6–4) product (71). Photoisomerization of the pyrimidone ring of the (6–4) product converts it from an

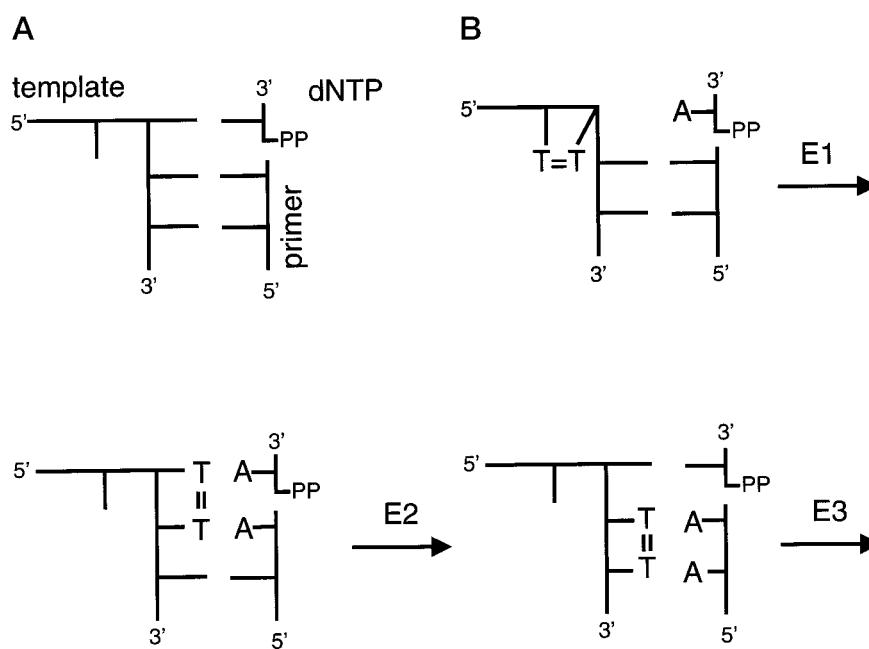
extended flat planar ring to a more compact tentlike structure.

Effect of Exonuclease Activity and Processivity on Bypass—Two properties that have been suggested as important in the ability of a polymerase to bypass a lesion are the presence and activity of a proofreading (3' \rightarrow 5') exonuclease (31, 64) and the processivity of the polymerase (72). KF and T7 DNA polymerases were chosen to study the effects of exonuclease activity on bypass, because both enzymes could be obtained in exonuclease-proficient and -deficient versions, and have been the subject of a number of detailed kinetic studies (25, 73, 74). The *exo*⁻ Klenow was able to extend one nucleotide further than wild-type Klenow on all the lesions before stalling, and was able to bypass 47% the *cis-syn* dimer compared with 3% for the wild-type Klenow under otherwise identical conditions (Fig. 4). Likewise, no bypass was seen with wild-type T7 polymerase, but Sequenase 2.0 gave substantial amounts of bypass of all lesions (Fig. 5). These results are in accord with the observation that synthesis opposite irradiated templates was increased when *exo*⁻ KF and T7 DNA polymerase were used in place of the wild-type enzymes (75). Recently, elimination of the 3' \rightarrow 5' exonuclease activity of KF, has been shown to greatly accelerate bypass of an abasic site analog (33).

To examine the effects of processivity on bypass, we again made use of the T7 DNA polymerase system, by taking advantage of the 1000-fold increase in processivity conferred on T7 gene 5 polymerase subunit by thioredoxin, (28). Thioredoxin enhances the processivity of the gene 5 protein by increasing the lifetime of the polymerase-DNA complex (76). The difference in bypass ability of an *exo*⁻ T7 gene 5 protein (25) in the presence or absence of thioredoxin (Fig. 6), was similar to the difference seen in the absence or presence of the exonuclease (Fig. 5). In the presence of thioredoxin, the *exo*⁻ gene 5 protein was able to bypass all the photoproducts, stalling opposite the first and second bases of the photoproducts. In the absence of thioredoxin, however, the gene 5 protein was not able to bypass any of the photoproducts, and stalled one base prior to every photoproduct site. These results are similar to those that we observed to occur in the bypass of *cis-syn* and *trans-syn-I* dimers by calf thymus polymerase δ (pol δ) in the presence and absence of its processivity factor, proliferating cell nuclear antigen (PCNA) (77). Addition of PCNA has also been shown to greatly increase the bypass of abasic sites by pol δ (78).

The A-rule Revisited: Origin for the Incorporation of A Opposite the 3'-T of Photoproducts by Sequenase 2.0—One general hypothesis for the preferential incorporation of A opposite DNA damage is the A-rule (79, 80), which proposes that preferential dATP binding by the polymerase governs nucleotide incorporation when it encounters a non-instructional lesion, typified by an abasic site. Because the mutation spectra of UV-irradiated DNA could be explained by incorporation of A, dipyrimidine photoproducts were originally classified as non-instructional. Recently this classification has been called into question, and hence the use of the A-rule to explain mutations caused by DNA photoproducts. Lawrence and co-workers have argued that the *cis-syn* dimer must be an instructive lesion by virtue of its high coding specificity in *E. coli* relative to abasic sites (81), and its ability to engage in near normal hydrogen bonding to adenine (67–69). Furthermore, G is incorporated opposite the C in a *cis-syn* dimer of TC in *E. coli* (82) and opposite the 3'-base in the (6–4) products of both TT and TC (83). In contrast, Sequenase 2.0 puts A opposite the 3'-T of all the photoproducts, irrespective of structure. Why then would one polymerase add G opposite the 3'-T of the (6–4) product and another polymerase add A? The argument that DNA photoproducts are instructional and not subject to the A-rule was based on observations in *E. coli* under SOS conditions and may in fact not apply to all polymerases. A possible expla-

FIG. 9. Proposed model for elongation opposite dipyrimidine photoproducts by T7 DNA polymerase. *A*, model for elongation opposite undamaged DNA, which is based on the crystal structure of a primer-template, and ddNTP complex with T7 DNA polymerase, which reveals that the template is forced to make a right angle turn after the catalytic site for primer extension. *B*, a model for elongation opposite a photoproduct between the two Ts (denoted by an equal sign). Each successive step in elongation opposite a photoproduct of TT is indicated by *E_n*. Because the bases in dipyrimidine photoproducts are covalently linked together, the 3'-nucleotide of these photoproducts cannot be accommodated in the active site during primer elongation opposite this site.



nation for the incorporation of A opposite the 3'-T of all the photoproducts by Sequenase 2.0 comes from examination of the recent crystal structure of a complex between an exo^- T7 polymerase and a template primer in the presence of a ddNTP. In this structure, the template is forced to take a sharp 90° turn at the polymerase active site following the nucleotide opposite which the dNTP is incorporated (84). Because all dipyrimidine photoproducts covalently link two nucleotides together, the 3'-pyrimidine of a photoproduct cannot be made to occupy the site opposite which the dNTP resides, and is instead forced out of the active site with the rest of the template (Fig. 9). This would create an empty site, much like an abasic site, which would therefore be non-instructional, and lead to the preferential incorporation of A. Bending the template at the active site may be an important and general mechanism for preventing or greatly attenuating translesion synthesis at the sites of intrastrand cross-linked nucleotides such as dipyrimidine photoproducts, *cis*-platinum adducts, and other intrastrand cross-linked nucleotides, and has been observed in human $\text{pol } \beta$ (85) and *Bacillus* polymerase I (86). Once the A is incorporated, the template can move by one nucleotide, and the entire photoproduct can now be bound in the active site. Incorporation of a nucleotide opposite the 5'-pyrimidine will then be mediated by the instructional properties of the 5'-pyrimidine and the fit of the photoproduct in the active site.

Origin of the Preference for Incorporation of A Opposite the 5'-T of the Photoproducts—In considering the rate and selectivity of nucleotide incorporation opposite the 5'-pyrimidine, it is useful to examine the results of the primer extension reactions by Sequenase 2.0 opposite the 5'-T of the photoproducts in light of the mechanism by which T7 DNA polymerase maintains high fidelity on undamaged templates (74). The rate-determining step during processive synthesis is a conformational change after dNTP binding, and before bond formation. In this induced-fit model, it is speculated that the conformational change selects the correct dNTP by recognizing its correct Watson-Crick geometry. Likewise, 3'-end mismatches drastically slow the conformational change necessary to incorporate the next nucleotide. By analogy, dNTP incorporation opposite and past lesions will be governed by the how closely the nascent base pair resembles the Watson-Crick geometry of normal B DNA. This model argues against the importance of hydrogen bonding, and argues for the importance of resemblance to Watson-Crick geometry as providing the instruction to the polymerase. Indeed, the selection of correct ge-

ometry has been suggested by several groups as the primary means by which polymerases maintain high fidelity (74, 87–91). This is also borne out by recent crystal structures of a *Bacillus* DNA polymerase I complexed to template-primers and T7 DNA polymerase and $\text{pol } \beta$ complexed to template-primers and ddNTPs (84,85). For the case of the dinucleotide photoproducts studied, all have a 5'-T that retains the H-bonding properties of T, though the conformation of the 5'-T depends on the particular photoproduct. Thus, the preferential incorporation of A opposite the 5'-T of all these products is likely to be the result of the ability to form a Watson Crick-like base pair. The differing rates of bypass of the photoproducts is probably the result of deviations of the geometry from an ideal Watson-Crick base pair caused by distortions induced by the photoproducts that slow phosphodiester bond formation during the rate-determining step in bypass. For all the photoproducts, the major termination band corresponds to termination opposite the 3'-T of the dimer suggesting that the slowest or rate-determining step in bypass involves the extension step opposite the 5'-T (step *E2* of Fig. 9).

Biological Implications—Of the four major dipyrimidine photoproducts of TT, the *cis-syn* dimer was found to be the most easily bypassed by the polymerases studied, which correlates with the fact that it is the least disruptive of DNA structure (69). The *cis-syn* thymine dimers have also been found to be more easily bypassed than *trans-syn*-I dimers by $\text{pol } \delta/\text{PCNA}$ (77), and a vector containing a *cis-syn* thymine dimer was found to be more efficiently replicated by lagging strand synthesis than a (6–4) product in cell free HeLa extracts (92). The greater efficiency of *cis-syn* dimer bypass would support the notion that *cis-syn* dimers have the highest mutagenic potential of the four major photoproducts (12), as they are also the most slowly repaired by excision repair systems (93–95). Although there is no direct evidence at this point, it may be that *cis-syn* dimers may also be the most easily bypassed by transcription systems, and therefore also the least readily repaired by transcription-coupled repair. It is known, however, that *cis-syn* dimers of TT sites are not very mutagenic when bypassed by KF *in vitro* (34, 96) or in *E. coli* under SOS conditions or in yeast (14, 97). Likewise, bypass of *cis-syn* thymine dimer in an SV40 vector by HeLa cell free extracts appears to be non-mutagenic (92). On the other hand, it has been shown that the deamination products of C-containing *cis-syn* dimers are highly mutagenic, almost exclusively causing C \rightarrow T mutations,

the major mutation induced by UV light (15, 98–100). Thus, *cis-syn* dimers have four features (7) that make them prime candidates as the principal products involved in mutagenesis at dipyrimidine sites. 1) They are the major photoproducts induced by UV light, 2) they are the least rapidly repaired of the dipyrimidine photoproducts, 3) they are the most easily bypassed, and 4) they can be highly mutagenic. The observation that the Dewar isomer of the (6–4) product is more easily bypassed than the (6–4) product by Sequenase 2.0, as previously predicted based on its structure (71), would also confer a higher mutagenic potential on this product than its (6–4) isomer.

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