In Vivo Evidence That UV-Induced C → T Mutations at Dipyrimidine Sites Could Result from the Replicative Bypass of Cis-Syn Cyclobutane Dimers or Their Deamination Products†

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ABSTRACT: The major mutations induced by UV light are C → T transitions at dipyrimidines and arise from the incorporation of A opposite the C of dipyrimidine photoproducts. The incorporation of A has most often been explained by the known preference of a polymerase to do so opposite noninstructional DNA damage such as an abasic site (A rule). There are also mechanisms that suppose, however, that cis-syn dipyrimidine photodimers are instructional. In one such mechanism (tautomer bypass), the incorporation of A is directed by the tautomer of a C of a dimer that is equivalent in base-pairing properties to U [Person et al. (1974) *Genetics 78*, 1035–1049]. In another mechanism (deamination bypass), the incorporation of A is directed by a U of a dimer that results from the deamination of the C of a dimer [Taylor & O'Day (1990) *Biochemistry 29*, 1624–1632]. The viability of these mechanisms was tested by obtaining the mutation spectrum of a TU dimer in *Escherichia coli* by application of a standard method for site-directed mutagenesis. To this end, a 41-mer containing a site-specific TU dimer was constructed via ligation of a dimer-containing decamer that was produced by triplet-sensitized irradiation and used to prime DNA synthesis on a uracil-containing (+) strand of an M13 clone containing a double mismatch opposite the dimer. The reaction mixture was used to transfect a uracil glycosylase proficient, photoproduct repair deficient *E. coli* host, and all progeny phage weakly hybridizing to the parental (+) or (−) strands were sequenced. Under non-SOS conditions the TU dimer almost completely blocked replication, while under SOS conditions it directed the incorporation of two As with much higher specificity (96%) than would an abasic site. The implications of these results to the mechanism of the UV-induced TC → TT mutation, and by extension to the CT → TT, CC → TC, CC → CT, and the tandem CC → TT mutations, are discussed.

Ultraviolet light (UV) causes mutations, many of which are targeted by the DNA photoproducts it produces. Targeted mutations arise when nucleotides are inserted, deleted, or substituted opposite the damaged DNA during replication. The predominance of UV-induced G·C → A·T mutations in *Escherichia coli* under SOS conditions was first discovered by Drake (1963) and later established to be targeted by the C of the G·C base pair (Howard & Tessman, 1964). Subsequent sequence analysis showed that these mutations occur at dipyrimidines, the principal site for UV-induced damage, and most frequently at TC sites (Coulondre & Miller, 1977; Brash & Haseltine, 1982; LeClerc et al., 1984; Wood et al., 1984; Miller, 1985; Schaaper et al., 1987). Similar mutation spectra have also been found in human cells (Bredberg et al., 1986; Brash et al., 1987; Seetharam et al., 1991). Most recently, C → T mutations, and in particular, the CC → TT tandem mutation, have been found in the p53 tumor suppressor gene of human squamous cell carcinomas (Brash et al., 1991). Because the CC → TT mutation appears to be uniquely induced by UV light (Coulondre & Miller, 1977), a strong case was made that the UV light in sunlight was both the mutagenic and carcinogenic agent. How these mutations arise is therefore of great interest.

Establishing the origin of the UV-induced C → T mutations is made difficult by the complex photochemistry of dipyrimidines (Cadet & Vigny, 1990) as illustrated in Figure 1 for a TC site (Taylor et al., 1990b). The cis-syn cyclobutane dimer and the (6–4) product are the major products of UV-B and -C[1] irradiation, and both have been correlated with C → T mutations [for a review see Hutchinson (1987)]. The C of a cis-syn dimer is not chemically stable, however, and can tautomerize, or deaminate to U. The (6–4) product, while being chemically stable, is converted to its Dewar valence isomer by UV-A and -B light. The formation of UV-induced C → T mutations at dipyrimidines and the low UV mutability of TT sites can be readily seen to result from the incorporation of As opposite both the Ts and Cs of dipyrimidine photoproducts by a polymerase. Which of the photoproducts is primarily responsible for a C → T mutation at a given site and the precise mechanisms by which the As are incorporated remain unknown.

One general mechanism that has often been invoked to explain the incorporation of As opposite dipyrimidine photoproducts during replicative bypass was originally proposed by Tessman in 1976 [see Tessman (1985)] and has come to be called the "A rule" [see Larson and Strauss (1987) and references therein]. According to this rule, polymerases preferentially incorporate As opposite noninstructional DNA damage, typified by abasic sites. Recent in vitro and in vivo evidence suggests, however, that cis-syn dimers and (6–4)

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[1] Abbreviations: ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; dNTP, 3′-deoxynucleotide triphosphate; DTT, dithiothreitol; exo⁻, 3′→5′ exonuclease deficient; IPTG, isopropyl thio-β-D-galactoside; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; SSC, standard sodium citrate; SDS, sodium dodecyl sulfate; UV-A, 320–400 nm; UV-B, 280–320 nm; UV-C, 240–280 nm; XP, *Xeroderma pigmentosum*.

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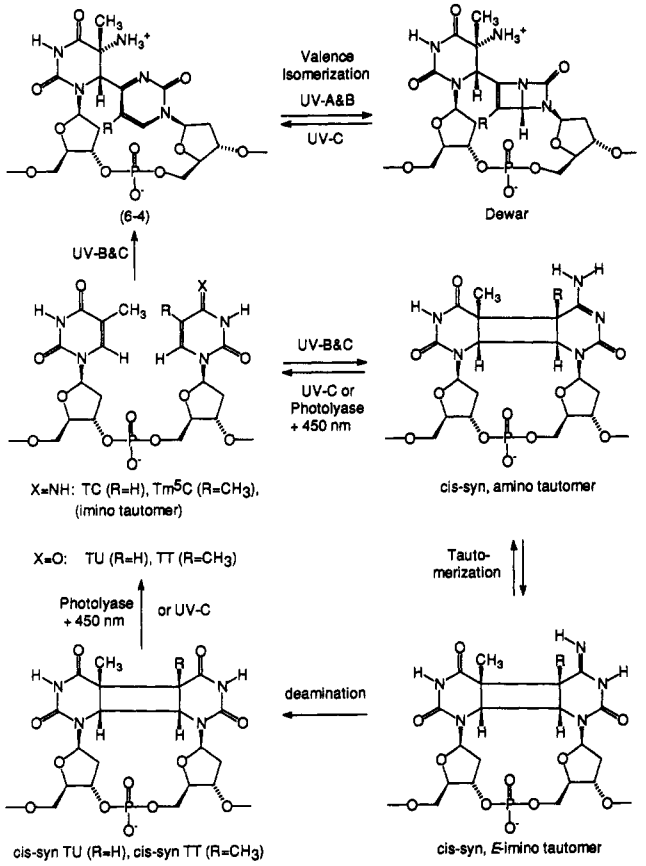

FIGURE 1: Photochemistry of TC and Tm^{5}C sites. The chemistry shown also applies to CT and CC sites and their m^{5}C derivatives. In *E. coli*, methylation occurs at the 5′-C of CC sites (Adams & Burdon, 1985), whereas in mammalian cells, methylation occurs primarily at the 3′-C of TC and CC sites (Pfeifer et al., 1990, 1991).

products are instructional (or misinstructional) rather than noninstructional [for recent discussions see Lawrence et al. (1990a), Taylor and O'Day (1990), and LeClerc et al. (1991)]. In *E. coli* under SOS conditions, As were found to be incorporated opposite both Ts of the cis-syn dimer with a frequency of 94%, much higher than the prototypical noninstructional abasic site, opposite which A was incorporated with an average frequency of 65% (Lawrence et al., 1990b). Likewise, A was incorporated opposite the 5′-T of the (6–4) product 95% of the time, but was incorporated opposite the 3′-T only 11% of the time, while G was incorporated 85% of the time. Because the (6–4) and Dewar products of TC are isostructural with their TT counterparts (Taylor et al., 1990b), the (6–4) product of TC is expected to be relatively nonmutagenic and lead to only a small amount of C → T mutation, contrary to an earlier proposal based on the A rule (Wood et al. 1984). Unlike the other two photoproducts, A was incorporated opposite the 5′- and 3′-Ts of the Dewar product of TT with frequencies of 79% and 60%, respectively, much like that for an abasic site (LeClerc et al., 1991), suggesting that the Dewar product of TC would lead to a substantial amount of a C → T mutation. Because the Dewar product is a long-wavelength UV product of the (6–4) product, it is expected to be formed in low relative yield in sunlight and not at all by UV-C (Figure 1) and therefore is unlikely to contribute significantly to UV-induced C → T mutations.

In contrast to mechanisms based on the A rule, three distinct mechanisms have been proposed for the C → T mutation that are based on the inherent base-pairing or instructional properties of cis-syn dimers (Figure 2) or their photoreversal products. In the tautomer-bypass mechanism (Figure 3), both

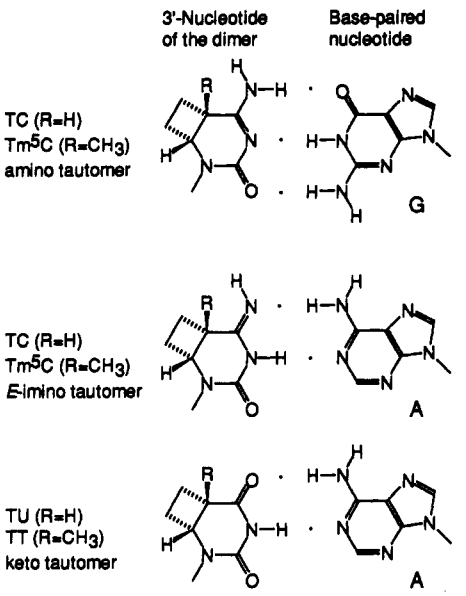

FIGURE 2: Proposed base-pairing properties of the cis-syn cyclobutane dimers of TC, Tm^{5}C, TU, and TT. Similar base pairing would apply to the dimers of CT and CC and their m^{5}C derivatives and deamination products, as well as to the 5′-pyrimidine of (6–4) and Dewar products.

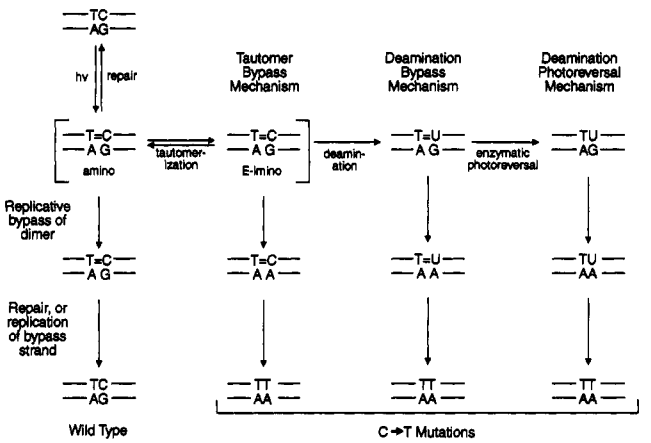

FIGURE 3: Proposed mechanisms for the origin of UV-induced C → T mutation at TC sites. This scheme can also be applied to CT and CC sites and their m^{5}C derivatives.

the T and the (*E*)-imino tautomer of the C of a dimer, which have the same base-pairing properties as T and U, direct the incorporation of A, while the amino tautomer of C directs the incorporation of G (Bockrath & Cheung, 1973; Person et al., 1974). When the C5–C6 bond of cytosine becomes saturated, aromatic stabilization of the amino tautomeric form is no longer possible, resulting in an increase in the proportion of otherwise minor imino tautomers (Brown & Hewlins, 1968). Evidence for such an increase comes from molecular orbital calculations (Dupuy-Mamelle & Pullman, 1967) and spectrophotometric studies (Brown & Hewlins, 1968) on 5,6-dihydrocytosine. In the latter study, it was found that whereas the imino tautomer was only present at about 4% in water, it was the exclusive tautomer in chloroform. Most significantly, in transcription systems 5,6-dihydrocytidine triphosphate can substitute for either CTP or UTP (Grossman et al., 1966), and A is incorporated exclusively opposite a C6 methoxylamine adduct of C (Phillips & Brown, 1966). Likewise, 5,6-dihydrothymidine triphosphate is only incorporated opposite As by polymerase I of *E. coli* (Ide & Wallace, 1988), indicating that saturation of the 5,6-double bond of thymidine does not affect its base-pairing properties and hence its tautomeric state.

In the deamination–photoreversal mechanism (Figure 3), it is the U that results from enzymatic photoreversal of a photodimer in which the C has undergone deamination that directs the incorporation of A during replication (Setlow & Carrier, 1966; Fix & Bockrath, 1981). In support of this mechanism, UV-induced glnU and glnV tRNA suppressor mutations (C → T transitions) in *E. coli* become resistant to photoreactivation following thermal treatment in *ung*⁻ cells with a half-life of about 10 h (Fix & Bockrath, 1981). The deamination of C to U has been shown to occur readily in irradiated DNA (Setlow & Carrier, 1966), and in the cyclobutane dimer of TpdC (Liu & Yang, 1978). Photodimerization causes the loss of the 5,6-double bond and hence aromatic stabilization, thereby lowering the activation energy, and consequently raising the rate of deamination. The deamination rate was found not to be sufficient, however, to account for the observed rate of mutation in *E. coli* (Ruiz-Rubio & Bockrath, 1989), implying that the mutagenic event in this case involves the C-containing dimer itself. Furthermore, the U generated by enzymatic photoreversal would be expected to be rapidly repaired by uracil glycosylase (Duncan & Miller, 1980), thereby diminishing the overall efficiency of the deamination–photoreversal mechanism for producing C → T mutations.

In the deamination-bypass mechanism (Figure 3) (Taylor & O'Day, 1990; Taylor & Nadji, 1991), the U of a dimer that results from deamination of a C of a dimer directs the incorporation of A during replicative bypass. This mechanism was based on the observation that the cis-syn dimer of TU is isostructural to that of TT, which is bypassed as though it were TT >95% of the time in vitro by *E. coli* polymerase I (Taylor & O'Day, 1990) and 94% of the time in *E. coli* under SOS conditions (Banerjee et al., 1988). On the basis of these observations alone, the deamination-bypass mechanism could explain the origin of UV-induced C → T mutations at Tm^5C sites in mammalian cells (Pfeifer et al., 1990, 1991), because the deamination products of the dimers of these sites are in fact TT dimers (Figure 1). The deamination-bypass mechanism could be an important contributor to mutagenesis in those cases where the rates of replication and repair are slower than the rate of deamination, and evidence for this mechanism has been obtained in *E. coli* (Tessman & Kennedy, 1991). Furthermore, because this mechanism does not depend on photoreversion, it is not subject to attenuation by uracil glycosylase and may therefore be fairly efficient.

Of the three mechanisms involving cis-syn cyclobutane dimers, the deamination–photoreversal mechanism is the only one for which all the chemical and enzymatic steps are well precedented and understood. In contrast, nothing is directly known about replication past C- or U-containing dimers, the critical step in the tautomer-bypass and deamination-bypass mechanisms. We decided to first study the TU dimer because it is the stable deamination product of the unstable dimer of a TC, the principal site of UV-induced C → T mutations in both bacterial and mammalian cells. Furthermore, the TU dimer was chosen because it is isostructural with, and hence serves as a good model for, the unstable imino tautomer of the dimer of TC which is the key intermediate in the tautomer-bypass mechanism. Herein, we report that replicative bypass of the TU dimer in *E. coli* under SOS conditions results in the essentially exclusive incorporation of adenines opposite the dimer as postulated by both the tautomer-bypass and deamination-bypass mechanisms. We also discuss the implications of this result to C → T mutations at CT and CC sites.

(+) STRANDS (5' → 3')

12-mer TGCATGCCTGCA

19-mer AATTCGTAATCATGGTCAT

10xy-mer CGTAxyATGC

xy = TT, TC, GG, T(c,s)T, T(c,s)U

41xy-mer TGCATGCCTGCACGTAxyATGCAATTCGTAATCATGGTCAT

xy = TT, T(c,s)T, T(c,s)U

17xy-mer GCACGTAxyATGCAATT

xy ≡ TT, GG

(-) STRANDS (3' → 5')

18xy-mer ACGTGCATxyTACGTTAA xy = AA, AG, CC

34-mer GTACGGACGTGCATAATACGTTAAGCATTAGTAC

41-mer ACGTACGGACGTGCATAATACGTTAAGCATTAGTACCAGTA

FIGURE 4: Oligonucleotides used in this study.

MATERIALS AND METHODS

Materials. T4 polynucleotide kinase (10 units/μL) was from Bethesda Research Labs, T4 DNA ligase (40 000 units/μL) from New England Biolabs, the T4 DNA polymerase (10 units/μL) from Promega. Sequenase Version 1.0 (10 units/μL) (Tabor & Richardson, 1987) was purchased from United States Biochemical. Uracil glycosylase (1 unit/μL) was obtained from Perkin Elmer Cetus. Phage T4 *denV* endonuclease V was from Applied Genetics Inc., Freeport, NY, or as a generous gift from S. Lloyd of Vanderbilt University. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham. *E. coli* bacterial strains used were as follows: DH5αF′ was from Gibco BRL, CJ236 (*dut*1, *ung*1) was from Bio-Rad, CSR06 (*phr*-1, *uvr*A6) (Sancar & Rupert, 1978), strain CGSC 5831, was kindly provided by B. Bachmann, curator of the *E. coli* Genetic Stock Center, Department of Biology, Yale University, and CSR06F′ [CSR06/F⁺::Tn3(Amp^r)] was kindly prepared by R. Landick, Department of Biology, Washington University, by conjugation of CSR06 with WB110F′:Tn3(Amp^r,Str^r). Nucleotides were purchased from Sigma and Boehringer Mannheim GmbH. IPTG was purchased from Fisher Biotech (Biotech Grade). Ampicillin (molecular biology grade) and bovine albumin (fraction V) were obtained from Sigma. Ficoll (type 400) was purchased from Pharmacia. Poly(vinylpyrrolidone) was purchased from Kodak. SDS (ultrapure) was obtained from USB. LB and YT media, LB plates, top agar, and Denhardt's solution were all prepared according to standard protocols (Sambrook et al., 1989). All other chemicals were obtained from standard suppliers of molecular biology reagents. Oligonucleotides (Figure 4) were synthesized by standard automated solid-phase phosphoramidite chemistry and purified by anion-exchange HPLC as previously described (Taylor et al., 1987). Oligonucleotide concentrations were determined spectroscopically (Fasman, 1975). Preparation of the templates by ligation, acrylamide gel electrophoresis, autoradiography, and densitometry were performed as previously described (Taylor & O'Day, 1990; Wang & Taylor, 1992) unless otherwise noted.

Preparation of the cis-syn-TU Dimer-Containing Decamer. A 20% solution of CH_3CN in water containing 30 μM decamer and 3 mM acetophenone was degassed by bubbling a stream of nitrogen through the solution for 15 min and then irradiated with a medium-pressure mercury lamp filtered through Pyrex (wavelengths >280 nm) in an ice bath for 1 h. Reaction products were separated on an analytical C-18 column [LichroCART 125-4, Lichrospher 100 RP-18 (5-μm particle size)] with a 0.5-h 10–40% linear gradient of methanol in 75 mM KH_2PO_4/K_2HPO_4, pH 6.6. The collected fractions were

loaded on a C-18 cartridge (100 mg of C-18 resin packed in a 1-mL disposable syringe) and washed with 10 mL of water to remove salts. The oligonucleotide was then eluted with 2 mL of 15% aqueous CH_3CN, lyophilized, and stored in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA at −20 °C.

Preparation of U-(+)-NJ1-CC and U-RF-NJ1-GA. Replicative-form NJ1-CC was prepared according to a previously reported scheme (Taylor & O'Day, 1989) by cloning 5′-phosphorylated 18CC-mer/10GG-mer into *ECo*RI- and *Pst*I-cleaved M13mp18 utilizing a standard procedure (Hanahan, 1985). NJ1-GA was similarly prepared from 5′-phosphorylated 18GA-mer/10CT-mer, and both clones were characterized by Sanger sequencing. The construction of NJ1-AA by cloning of 18-AA-mer/10TT-mer has been previously reported (Taylor & O'Day, 1989). Uracil-substituted NJ1-CC (+) strand [U-(+)-NJ1] was prepared in a large scale as described by Kunkel (1987) by isolating the single-strand form of NJ1-CC from CJ236, an *E. coli dut⁻ ung⁻* strain, grown in 2 × YT medium containing 300 μg/L uridine. Uracil-substituted replicative-form NJ-GA (U-RF-NJ1-GA) was prepared similarly except that the RF form was isolated instead.

Preparation of Site-Specific Photodimer-Containing Bacteriophage DNA. Either 1.6 or 3.2 pmol of 5′-phosphorylated dimer-containing 41-mer was annealed to 2 μg (0.8 pmol) of U-(+)-NJ1 in 20 μL of SSC buffer (150 mM NaCl and 15 mM sodium citrate). Primer extension and ligation were initiated by adding 10 units of T4 DNA polymerase and 6 Weiss units of T4 DNA ligase in 50 μL of reaction buffer (20 mM HEPES, pH 7.8, 2 mM DTT, 10 mM $MgCl_2$, 500 μM dNTPs, and 1 mM ATP). The reaction mixture was first incubated for 5 min at 0 °C and then 5 min at 22 °C followed by incubation at 37 °C for 2 h. After incubation, 25 μL of the reaction mixture was treated with 2 units of uracil glycosylase in 30 μL of buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl) at 37 °C for 1 h.

Transformation of CSR06F′ by Dimer-Containing Bacteriophage DNA. The preparation and transformation of competent CRS06F′ cells was accomplished according to a standard procedure (Hanahan, 1985) utilizing freshly thawed competent cells and 5 μL of the in vitro DNA synthesis mixture described above. Competent SOS-induced CRS06F′ cells were prepared as follows. Log-phase CRS06F′ cells (OD_{600nm} 0.7) grown in 30 mL of LB medium containing 25 μg/mL ampicillin were pelleted at 4 °C. After resuspension in 60 mL of ice-cold 0.15 M NaCl solution, the cells were poured into a 150- × 15-mm Petri dish, irradiated with 254-nm light in an intensity of about 50 μJ/cm² for 8 s, and pelleted again at 4 °C. The cells were then resuspended in 15 mL of LB medium and incubated in a shaker at 37 °C for another 30 min. The cells were then made immediately competent in the same way as the uninduced cells. As a control, competent cells were transformed with U-(+)-NJ1-CC and U-RF-NJ1-GA. Transformed cells were then added to top agar prepared with log-phase CSR06F′ cells and plated on LB plates containing 25 μg/mL ampicillin (LB/amp plates).

Screening of Progeny Virus by Hybridization and Dideoxy Sequencing. Clear plaques developed on the LB/amp plates after 8–10 h at 37 °C and about 500 separated plaques were picked and transferred to master LB/amp plates (50/plate) and to two other sets of replica plates covered by nitrocellulose filters. After overnight incubation at 37 °C, the master plate was stored at 4 °C and the filters from the other two plates were treated to lyse the cells (colony side up) in 1–2 mL of the following solutions: 10% SDS for 3 min, 1.5 M NaCl and 0.5 M NaOH for 5 min, 1.5 M NaCl and 1 M Tris-HCl, pH 8.0, for 5 min, and 2 × SSC for 5 min, and then they were baked at 80 °C for 2 h. The dried filters were then prehybridized by immersing in a solution containing 4 × SSC, 50 mM Na_2HPO_4/NaH_2PO_4, pH 7.0, 5 × Denhardt's, 0.1% $Na_4P_2O_7$, 0.1% SDS, and 50 μg/mL salmon sperm DNA for 3–4 h at 65 °C. Then 10^5 cpm of 5′-^{32}P-labeled 17TT-mer or 17CC-mer was added to above solution followed by overnight incubation at 37 °C. Autoradiograms were taken after filters were washed twice with 4 × SSC and 1% *N*-lauroylsarcosine, sodium salt, for 15 min per wash. The filters were further washed once at 50–55 °C for another 30 min, and the autoradiograms were taken again. Plaques that did not hybridize to either probe at 37 °C or failed to hybridize to either probe at 55 °C were picked from the master plate and grown up in LB medium for the small-scale DNA preparation. Single-stranded DNA was then separated and sequenced using the M13 (−40) sequencing primer by the Sanger method utilizing Sequenase Version 1.0. Plaques able to hybridize with either probe were also randomly selected and sequenced.

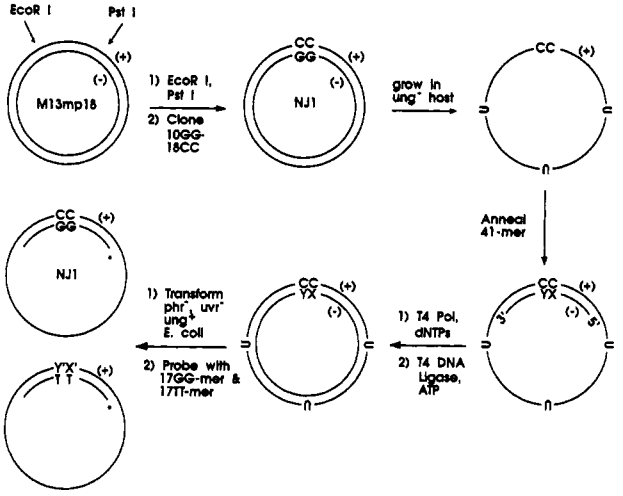

FIGURE 5: Scheme used for obtaining the mutation spectra.

RESULTS

The in vivo mutation spectra of the cis-syn photodimers of TU and TT were obtained (Figure 5) by application of a general method for efficient site-directed mutagenesis that had been envisioned to be useful for such a purpose (Kunkel, 1985; Kunkel et al. 1987). This method relies on the rapid degradation of the uracil-containing strand of a heteroduplex replicative form (RF) bacteriophage by a uracil glycosylase active (*ung*⁺) *E. coli* host, thereby insuring replication of complementary strand. The required hybrid RF DNA is easily and rapidly prepared by extension of a site-specifically damaged primer on a (+) strand grown in a *dut⁻*, *ung⁻* host. Progeny arising from the replication of the (+) and (−) strands are then distinguished by hybridization probes. Mutants are identified by probing the (−) strand under more stringent conditions and sequencing any weakly hybridizing colonies.

Construction of TU Cis-Syn Dimer-Containing Primers. Our standard approach to constructing site-specific photoproduct-containing oligonucleotides has been to design and synthesize an appropriate photoproduct building block for use in standard automated DNA syntheses [for a review see Taylor (1990)]. Early attempts to synthesize a TU dimer building block were thwarted by a number of problems [for a detailed discussion see Taylor and Nadji (1991)]. We

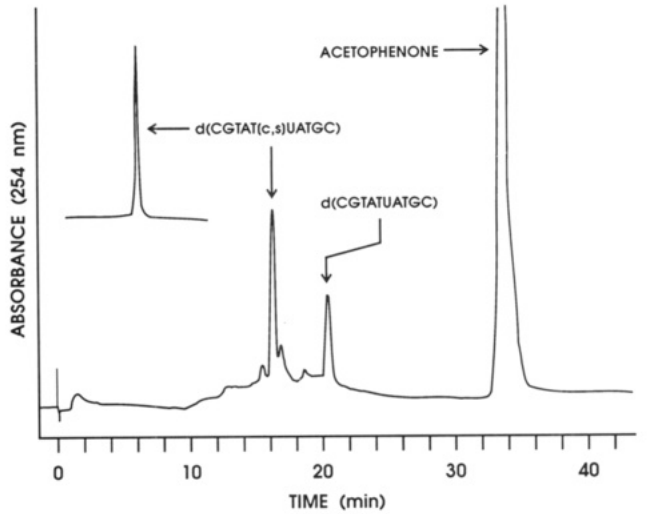

FIGURE 6: Analytical gradient HPLC chromatogram of the reaction mixture following acetophenone-sensitized photolysis of d(CGTATUATGC) for 1 h. The inset is a small section of the chromatogram of d(CGTAT[c,s]UATGC) following purification on an analytical C-18 column.

therefore pursued a more direct, but less general, approach to preparing site-specific TU dimer-containing oligonucleotides that entailed the photolysis of an oligonucleotide containing a unique TU site as the only dipyrimidine site. This approach has been successfully used to prepare site-specific TT cis-syn thymine dimer-containing oligonucleotides for NMR (Kemmink et al., 1987a) and in vitro enzymological (Inaoka et al., 1989) and in vivo mutagenesis (Banerjee et al., 1988) studies.

The sequence into which we incorporated the TU cis-syn dimer was the same as that into which we had previously incorporated the TT dimer for use in replication (Taylor & O'Day, 1990) and NMR studies (Taylor et al., 1990a), namely, d(CGTAxyATGC), where x and y correspond to any two pyrimidines, or a pyrimidine dimer. This sequence had been intentionally designed to have a unique dipyrimidine sequence to facilitate the preparation of authentic samples by sensitized photolysis. Thus, the cis-syn dimer-containing decamer, d(CGTAT[c,s]UATGC), was prepared by irradiating d(CGTATUATGC) with Pyrex-filtered light (wavelengths >280 nm) in the presence of acetophenone as a sensitizer. After 1-h irradiation approximately 73% of the starting material had been converted to photoproducts, of which 60% was the cis-syn dimer-containing decamer and 18% was thought to be the trans-syn dimer-containing decamer (Figure 6). The cis-syn dimer-containing decamer could be cleanly separated from the other products by reverse-phase HPLC.

To construct longer dimer-containing oligonucleotides, the TU dimer 10-mer was ligated to the appropriate oligonucleotides with T4 DNA ligase. For comparison purposes, the sequence of the TU dimer 41-mer was chosen to be identical to the template used in the previous in vitro study of the replication of the TT cis-syn dimer (Taylor and O'Day, 1990). The total yield of the templates was typically 20% on the basis of the 10-mer used. The site and integrity of the cis-syn dimers of TT and TU were established by the essentially quantitative cleavage of the dimer-containing templates by T4 *denV* endonuclease V at the expected site (shown for the TU dimer in Figure 7).

In Vivo Mutation Spectra. RF DNA containing uracil in the (+) strand and a site-specific photodimer in the (–) strand was prepared by priming replication of a uracil-containing (+) single-strand bacteriophage DNA by T4 DNA polymerase

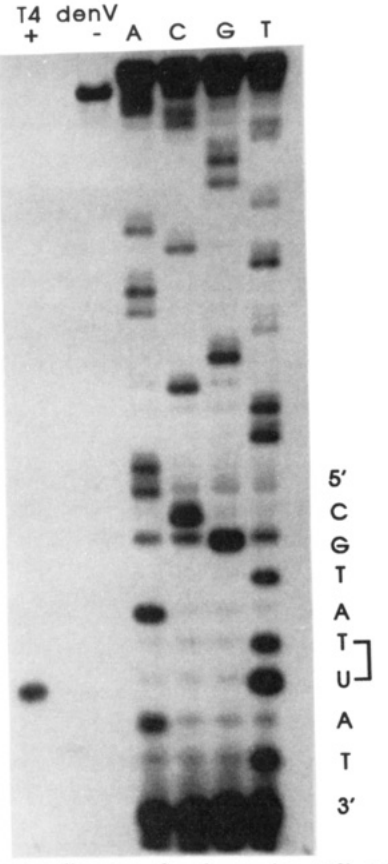

FIGURE 7: Autoradiogram of a denaturing 15% polyacrylamide electrophoresis gel of the products of T4 *denV* endonuclease V cleavage of the TU dimer-containing 41-mer template: (–) without treatment, (+) with treatment. 50 nM ^{32}P-end-labeled dimer-containing 41-mer annealed to its complementary strand was incubated for 2 h at 37 °C with 800 units/μL T4 *denV* endonuclease V in 10 mM Tris HCl, pH 7.6, 10 mM EDTA, and 100 mM KCl. Dideoxy sequencing reactions were conducted with the 5′-end-labeled 12-mer as a primer on the 41-mer complementary to the dimer-containing template and are labeled according to the dideoxy nucleotide used. The sequence on the right is that of the TU dimer-containing 41-mer.

and dNTPs with site-specifically damaged 41-mers followed by ligation with T4 DNA ligase and ATP. The required (+) strand was prepared by constructing an M13mp18 clone (NJ1-CC) that is otherwise fully complementary to the 41-mer with the exception of the two bases directly opposite the dimer. These two bases were made to be Cs to aid in distinguishing the progeny of the (+) and (–) strands by hybridization probes as was done in a study of the cis-syn TT photoproduct (Banerjee et al., 1988). The uracil-containing (+) single-strand form of NJ1 was then isolated from CJ236 (*ung*$^-$, *dut*$^-$) that was grown in the presence of uridine.

Reaction mixtures resulting from primer extension and ligation were used to transform CSR06F′ cells according to a standard technique (Hanahan, 1985). The *E. coli* strain CSR06 was chosen because it is deficient in both photorepair (*phr*-1) and excision repair (*uvr*A6) of cyclobutane dimers and was rendered infectable by M13 phage (F′) by mating with WB110F′:Tn3(Ap^r,Str^r). The SOS response of CSR06F′ was induced by exposing cells to 3.5–4 J m^{-2} of 254-nm UV light and incubating at 37 °C for 0.5 h immediately before the cells were made competent. A 0.5-h incubation time was used to ensure sufficient time for the production of SOS proteins. Plaques started appearing on the plates after the transformed competent cells were plated and incubated for 4–5 hours and were fully developed after 8–10 h of incubation. When the same amount of in vitro synthesis products (5 μL)

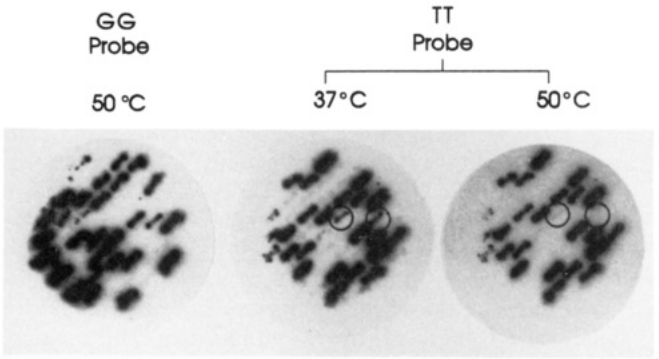

FIGURE 8: Autoradiograms of radioactively probed nitrocellulose paper replica of an agarose gel plate containing phages resulting from the transformation of SOS-induced CSR06F′ cells with the TU dimer-containing bacteriophage. The replica were probed with 5′-^{32}P-labeled 17TT-mer and 17GG-mer at the indicated temperatures. The colonies corresponding to the circled sites are those that failed to hybridize as well as 50 °C with the 17TT-mer probe and were plaque-purified and sequenced.

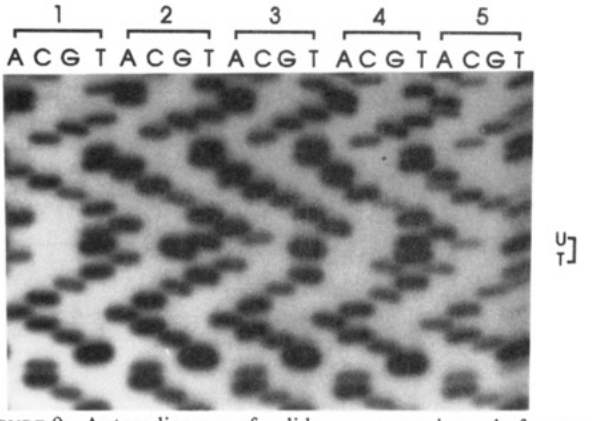

FIGURE 9: Autoradiogram of a dideoxy sequencing gel of mutants resulting from the in vivo replication of the TU dimer in the SOS-induced CSR06F′ cells. The sequencing reactions were conducted with Sequenase Version 1.0 and primed with the M13 (–40) universal primer. Lane headings refer to the dideoxy nucleotide used, and the label to the right indicates the original site of the cis-syn dimer in the (–) strand. Mutant 1 has a –1 frameshift, resulting from the loss of an A 5 nucleotides to the 3′-side of the dimer, mutant 2 has a T → G transversion at the 5′-T of the dimer, mutants 3 and 5 have U → C transitions (which would be nonmutagenic if the U arose from a C, see Figure 10), and mutant 4 has an A → G transition at the nucleotide at the 5′-side of the dimer.

was used for transformation, more plaques were generated from infection of the uninduced cells (10^6 transformants/μg of RF M13mp18) than the SOS-induced cells (10^5–10^6 transformants/μg of RF M13mp18). The actual difference depended on the batch of competent cells used. The transformation efficiency of the nondimer and dimer-containing vectors could not be directly compared because the efficiency of in vitro DNA synthesis for each primer could not be quantified readily. No plaques were observed in the transformations with uracil-containing U-(+)-NJ1-CC or U-RF-NJ1-GA.

Progeny of the (+) strand could be readily identified by the 17GG-mer hybridization probe at either 37 or 50 °C, while progeny of the dimer-containing (–) strand could be identified by the 17TT-mer probe at 37 °C. Phages containing one noncomplementary base would hybridize well at 37 °C, but poorly at 50 °C (Figure 8), while those with more than one noncomplementary base failed to hybridize well to either probe, even at 37 °C. Phages that hybridized to the 17TT-mer at 37 °C but not at 55 °C, and those that did not hybridize to either probe at 37 °C, were sequenced (Figure 9). The results of three separate transformation experiments are given in Table I and Figure 10. The sequence specificity of the hybridization conditions was verified by use of authentic (+)-NJ1-AA and (+)-NJ1-GA.

Table I: Analysis of Progeny Virus from Transfection of *ung*$^+$ *phr*$^-$ *uvr*$^-$ *E. coli* CRS06 with *cis-syn*-TT and -TU Dimer-Containing Uracil-Containing Heteroduplex Bacteriophage under SOS and Non-SOS Conditions[a]

		xy in (–) strand[b]		
conditions	progeny	TT	T[c,s]U	T[c,s]T
–SOS	(+) strand	29/51/19 (50)	199/240/74 (>99)	25/295/187 (98)
	(–) strand	24/60/16 (50)	1/0/0 (<1)	2/5/3 (2)
	XY → TT	24/60/16 (100)	1/0/0 (100)	2/5/3 (100)
+SOS	(+) strand	24/70/38 (36)	96/18/63 (57)	66/78/66 (61)
	(–) strand	38/137/62 (64)	83/16/37 (43)	35/47/52 (39)
	XY → TT	36/137/62 (99)	78/16/37 (96)	34/47/50 (98)

[a] The results of three transfections are separated by slashes. [b] Values in parentheses are in percent (%).

DISCUSSION

The in vivo mutation spectrum of the TU dimer in *E. coli* was obtained to test the deamination-bypass and tautomer-bypass mechanisms for the origin of UV-induced C → T mutations at dipyrimidine sites. Whereas replication past the dimers in uninduced cells was virtually completely blocked, a significant amount of bypass occurred in the SOS-induced cells. Under these latter conditions all the dimers were replicated primarily as though they were a TT site (>96%), with a low frequency of base substitutions at or adjacent to the dimer site. The highly specific (96%) introduction of two As opposite the *cis-syn*-TU dimer (Table I, Figure 10) demonstrates the viability of both the tautomer-bypass and deamination-bypass mechanisms for the origin of the C → T mutations.

Methodology for Obtaining in Vivo Mutation Spectra. A number of approaches have been reported for obtaining mutation spectra of site-specific DNA damage (Basu & Essigmann, 1988; Singer & Essigmann, 1991), the most successful of which have utilized the gapped heteroduplex (Green et al., 1984; Wood et al., 1990) and ligation-scaffold (Banerjee et al., 1988) methods for introducing the damage into single-stranded vectors. The requirement for using a single-stranded vector, at least when attempting to obtain mutation spectra of bulky or otherwise distorting lesions, was indicated by the low mutation frequency obtained with duplex vectors which was attributed to the preferential replication of the undamaged strand (Koffel-Schwartz et al., 1987). We experienced similar problems when trying to obtain mutation spectra from a site-specific cis-syn thymine dimer-containing duplex bacteriophage that we had constructed by the gapped duplex method (Taylor & O'Day, 1989). The method that we have described for obtaining in vivo mutation spectra has many advantages over the methods currently employed. First, because it relies on a primer extension to generate the damage-containing strand, it does not require the presence of any restriction sites as do the gapped heteroduplex and ligation-scaffold methods and can thus be targeted to any portion of a genome. Indeed, primer extension has been used effectively by others to site-specifically incorporate DNA damage into duplex genomes (Preston et al., 1986; Kodadek & Gamper, 1988). Second, because the cell rapidly degrades the undamaged, uracil-containing strand, the damage-containing

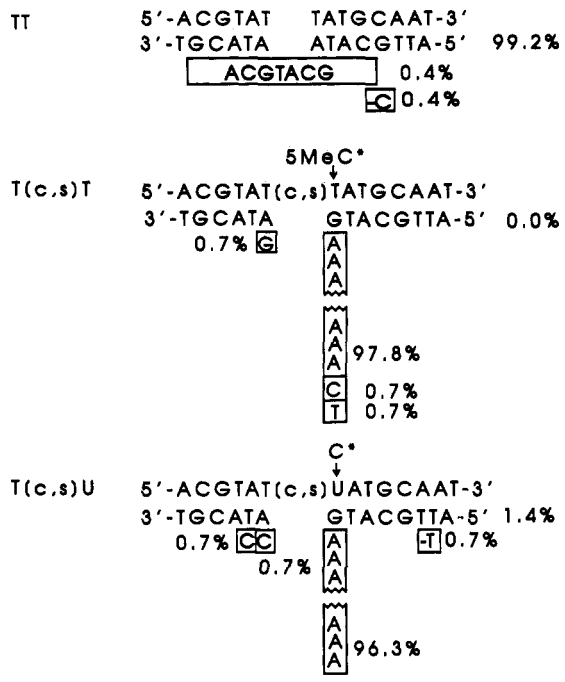

FIGURE 10: Mutation spectra of the cis-syn dimers of TU and TT along with the TT nondimer sequence as a control, obtained in SOS-induced CSR06F′ cells. Each box corresponds to an individual mutant and encloses the nucleotide that was incorporated opposite the dimer-containing strand during replicative bypass. Numbers next to the boxes refer to the frequency of the mutation. The sequence of the strand complementary to the dimer-containing strand corresponds to the sequence that would have originally been present prior to formation of the photoproduct. Hence, there is a G opposite the 3′-U or -T of the dimer because the dimer arose from the photolysis and subsequent deamination of either a TC or a Tm^{5}C site. The asterisk on the C or m^{5}C denotes the (*E*)-imino tautomer which would be expected to lead to the same mutation spectrum.

duplex vector can be directly used in the transformation without prior denaturation and strand separation. Uracil-containing damaged DNA templates have been effectively used to determine the mutagenic consequences of in vitro replicative bypass of DNA damage (Sahm et al., 1989) and site-specific damage introduced by a gapped heteroduplex method (Reid et al., 1990). Third, because primers of 40 or more nucleotides in length are used, hybridization is virtually quantitative and one can use one or less equivalents of primer to vector. In this way, one can be assured that the chemical integrity of the damaged vector used to transform the host is the same as that established for the primer. This is in contrast to the general use of 100 or more equivalents of short damage–containing oligonucleotides in the gapped heteroduplex and ligation-scaffold approaches that may result in the incorporating a minor, but better hybridizing impurity. Fourth, because damage-containing primers of 40 or more nucleotides are used, they can also be used for parallel in vitro replicative bypass studies.

Replicative Bypass in Uninduced Cells. In control transformations with RF DNA constructed from the undamaged TT 41-mer, 50% of the progeny were derived from the (–) strand in uninduced cells, and 64% under SOS conditions. In contrast, 2% or less of the progeny were derived from the dimer-containing (–) strands in uninduced cells, whereas the fraction was comparable to that of the undamaged control under SOS conditions. Given that the fraction of the progeny derived from a dimer-containing (–) strand reflects the relative ease by which the dimer is bypassed, the cis-syn dimer of TT was found to be slightly more easily bypassed than that of TU under non-SOS conditions (Table I). The low relative bypass efficiency of the cis-syn dimers under non-SOS conditions is in accord with the low transformation efficiency (average of 0.4%) observed by Banerjee et al. (1990) for a site-specific cis-syn thymine dimer in the (+) single strand of an M13mp7-based vector. That dimers can be bypassed under non-SOS conditions, albeit poorly, is also consistent with earlier studies with inhomogeneous photolyzed DNA substrates in vivo (Bridges & Woodgate, 1985; Tessman, 1985) and in vitro with Pol III holoenzyme (Livneh, 1986). Unfortunately, we have been unsuccessful to date in demonstrating that the cis-syn-TT dimer in the 41-mer template can be bypassed with Pol III holoenzyme under any of a number of conditions tried.

Replicative Bypass under SOS Conditions. Under SOS conditions, both dimers were found to be bypassed with frequencies approaching that of the nondimer (Table I), consistent with what was observed by Banerjee et al. (1988, 1990) for the cis-syn dimer of TT. The increased replication efficiency of damaged DNA under SOS conditions has been suggested to be a consequence of the suppression of the 3′ → 5′ exonuclease activity of Pol III by *rec*A (Villani et al., 1978) and the association of the UmuC and D′ proteins [for a recent review see Echols and Goodman (1991)]. Replicative bypass of the dimers under SOS conditions resulted in the highly specific introduction of a two As opposite the dimers, 96% for the *cis-syn*-TU dimer and 98% for the *cis-syn*-TT dimer, though Lawrence et al. (1990) observed only 94% for the in vivo bypass of a *cis-syn*-TT dimer.

In the SOS-induced cells, nucleotides other than A were also found to be incorporated opposite the cis-syn dimers in a low frequency (Figure 10) and can be understood to arise from a weakening of the originally encoded Watson–Crick hydrogen-bonding interactions (Kemmink et al., 1987b; Taylor et al., 1990a). Our results are quite similar to those found by Banerjee et al. for a site-specific cis-syn thymine dimer, only differing in the relative frequencies of the various types of substitutions. The average frequency of incorporation of T opposite the 3′-pyrimidine of the cis-syn dimers of TT and TU that we found (0.4%) was not nearly as high as they found for TT (5%), although the average frequency of incorporation of G opposite the 5′-pyrimidine (0.4%) and 3′-pyrimidines (0.7%) was comparable to theirs (0.2% and 1.1%). In addition, we found that C was incorporated opposite the 3′-pyrimidine (0.7% for TT) and 5′-pyrimidine (0.7% for TU) of the cis-syn dimers. A base substitution was also detected at the base flanking the 5′-T of the *cis-syn*-TU dimer which could also have resulted from the effect of the dimer on the neighboring base-pairing interactions (Hayes et al., 1971). The observed deletions of a C and a larger section of DNA in the nondimer study, and a T in the TU dimer study, may have been introduced during the construction of the RF DNA, or due to nontargeted mutagenesis. The significance of the minor differences in the mutation spectra obtained by us and by Banerjee et al. is hard to assess given their low frequency and the limited number of progeny that we were able to screen. Differences are nonetheless expected to exist between our study and their study because of the differences in the sequence surrounding the dimers (GTAxyATG vs AAGxyGGA) and the hosts (CRS06 vs AB1186).

Cis-Syn Dimers as Instructive Rather Than Noninstructive Lesions. The idea that DNA photoproducts are noninstructive lesions as required if the A rule were to apply was seriously questioned in 1981 by Lawrence following analysis of mutagenesis data (Lawrence, 1981). Comparison of the mutation spectra of site-specific cis-syn, trans-syn, and (6–4) products of TT in *E. coli* to those of abasic sites in place of

either T confirmed the idea that these photoproducts are instructive or misinstructive, rather than noninstructive (Lawrence et al., 199a; LeClerc et al., 1991). The cis-syn dimer was found to code primarily as TT (94%) and the (6–4) product as TC (85%), whereas the corresponding two abasic (ab) sites, the prototypical noninstructional lesions, coded for T with much less specificity (50% for abT and 77% for Tab). In vitro bypass studies of cis-syn dimers by Klenow (Taylor & O'Day, 1990) and exo⁻ Klenow and Sequenase Version 2.0 (Wang & Taylor, 1992) have shown a similar high degree of specificity for incorporating A (>95%). Model building (Rao et al., 1984; Pearlman et al., 1985; Raghunathan et al., 1990), NMR, and melting temperature studies (Kemmink et al., 1987a,b; Taylor et al., 199a) support the idea that the cis-syn dimer can be accommodated in a B-DNA helix and maintain standard Watson–Crick hydrogen-bonding interactions with As in the complementary strand. The A rule, therefore, appears to have been fortuitously successful in explaining UV-induced mutations simply because the majority of the UV-induced DNA photoproducts are cis-syn pyrimidine dimers, all of which can adopt the same base-pairing properties as TT, as described in the following section.

Implications for the Origin of TC → TT, CT → TT, and CC → TT Mutations. We have established that the TU cis-syn dimer and, by implication, the (*E*)-imino tautomer of the precursor TC dimer, would lead to the C → T mutation with high efficiency, but the extent to which they contribute to the observed mutation frequency depends on what fraction of the TC dimer that they represent. A case was made in the introduction that the imino tautomer should be a prevalent, if not the most prevalent, tautomer, while the fraction of U-containing dimer will depend on time (see next section). Because of their structural similarity to the TT and TU dimers, it is highly probable that the UT and UU dimers and the corresponding (*E*)-imino tautomers of the TC, CT, and CC dimers will also code as though they were TT. Thus, both the tautomer-bypass or deamination-bypass mechanism could explain the origin of the C → T mutation at TC, CT sites and the tandem CC → TT mutation.

Of particular relevance to the mechanism of skin cancer induction is the fact that C → T mutations at dipyrimidine sites, and in particular the CC → TT mutation, have been found in the p53 gene of squamous cell tumors (Brash et al., 1991). Examination of existing in vivo mutation data shows that the tandem CC → TT mutation is indeed a prevalent class of mutations uniquely induced by UV in *E. coli* (Coulondre & Miller, 1977; Wood et al., 1984; Schaaper et al., 1987) and mammalian cells (Protic-Sabljic et al., 1986; Brash et al., 1987; Drobetsky et al., 1987; Keyse et al., 1988; Hsia et al., 1989; Vrieling et al., 1989; Seetharam et al., 1991). That a large percentage of this tandem mutation must result from the replicative bypass of the CC dimer or its deamination products comes from comparison of mutation spectra of photodamaged DNA that had, or had not, been photoreversed prior to transfection. It was found that 100% of 254 nm-induced tandem CC → TT mutations in the shuttle vector pZ189 were removed by enzymatic photoreversal when propagated in an monkey cell line (Protic-Sabljic et al., 1986) whereas only 50% were removed when propagated in an XP cell line (Brash et al., 1987).

The deamination–photoreversal mechanism can also explain the origin of C → T mutations, but only in those organisms capable of enzymatic photoreversal of dimers. Even in these systems, it is not expected to contribute significantly to the observed mutation frequency because of competitive repair of the intermediate U by uracil glycosylase. The (6–4) photoproducts are likewise unlikely to contribute as greatly to the C → T mutation frequency as the cis-syn dimers because (1) they are formed with lower frequency than dimers (Mitchell, 1988; Mitchell et al., 1990), (2) they are often repaired faster than dimers (Mitchell & Nairn, 1989), and (3) they are not expected to be efficient at causing the observed C → T mutations, at least in *E. coli*. For example, the (6–4) product of TC is expected to be weakly mutagenic because of its structural similarity to the (6–4) product of TT (Taylor et al., 1990b) which was found to code as though it were a TC site 85% of the time in *E. coli* under SOS (LeClerc et al., 1991). At the moment, the coding properties of the (6–4) products of CT and CC are unknown, though the 5′-Cs may well undergo the same type of tautomerization and deamination reactions. The latter reaction has been observed for the (6–4) and Dewar product of dCpT, though at a much lower rate than for a dimer (Douki et al., 1991). These reactions would render the (6–4) products of CT and CC isostructural with those of TT and would be predicted to result primarily in CT → TC and CC → TC mutations, only the latter of which falls into the mutation category of interest. Despite the possibility that the Dewar products could cause the C → T mutation, they are not considered significant contributors because of their much lower yield relative to other photoproducts (see the introduction).

Mechanistic Time Domains and the Origin of CC → CT, TC, and TT Mutations. Whether the tautomer-bypass or deamination-bypass mechanism or both play a significant role in a living system would depend on the delay time between the UV-damaging event and replication. In systems undergoing rapid cell division, deamination would be expected to be slow relative to replication and the tautomer-bypass model would apply. In systems undergoing slow cell division, deamination would be fast relative to replication and the deamination-bypass mechanism would apply. This analysis of course would require that repair of the damage is likewise slow relative to deamination. In repair-competent human cell lines it has been observed that a large fraction of cis-syn dimers remain unrepaired after 10 h (Mitchell, 1988; Nairn et al., 1989), the approximate half-life for their spontaneous deamination (Fix, 1986). Evidence that there are two mechanistic time domains comes from a direct comparison study in which tandem CC → TT mutations were found to be more prevalent in human cells than in faster dividing *E. coli* cells (Hsia et al., 1989). This could be readily explained if the fraction of the (*E*)-imino tautomer of C in a dimer is less than 1, while the fraction of keto tautomer of the U in the deaminated dimer is close to 1, as the results of our bypass study indicate. For example, if the fraction of imino tautomer was 50%, then a CC dimer would have an equal probability of coding as though it were TT, TC, CT, and CC. When fully deaminated, however, it would have close to a 100% probability of coding as TT.

A mechanism that is a hybrid of the A rule and deamination-bypass mechanisms has recently been proposed to explain a short delay in the production of C → T mutations in *E. coli* under SOS conditions (Tessman et al., 1992). In this hybrid mechanism, elongation opposite a C-containing cyclobutane dimer is postulated to occur according to the A rule, but bypass is postulated to only occur following deamination of the C to U. While not refuted by the present study, this mechanism is unlikely in view of the observation of both CC → TC and CC → CT mutations and the comparative absence of CC → TT mutations in *E. coli* under conditions in which enzymatic

photoreversal of dimers is suppressed (LeClerc et al., 1984; Schaaper et al., 1987). The hybrid mechanism would predict that one should only observe CC → TT mutations as it postulates that only As are inserted opposite dimers. The tautomer-bypass mechanism, on the other hand, can nicely account for the observed CC → TC and CC → CT mutations if the tautomeric equilibrium between the (*E*)-imino tautomer, which would lead to a C → T mutation, and the amino tautomer, which is not mutagenic, is close to 1. These mutations could also be explained if the C existed primarily as the amino tautomer and there was only partial deamination of the CC dimer. The relative contributions of these two mechanisms would require knowledge of the deamination rate and the equilibrium constant between the tautomers.

CONCLUSION

We have presented evidence that the major mutation induced by UV light, the TC → TT mutation, and by extension, the CT → TT, CC → CT, CC → TC and the tandem CC → TT mutations, could result from the replicative bypass of the cis-syn dimers of these sites or their deamination products. Because all of these mutations have also been found in the p53 gene in squamous cell carcinomas (Brash et al., 1991), we also conclude that these mechanisms could also explain, at least in part, how sunlight leads to the mutations implicated in skin cancer induction.

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