Accelerated Deamination of Cytosine Residues in UV-Induced Cyclobutane Pyrimidine Dimers Leads to CC→TT Transitions†

Wu Peng and Barbara Ramsay Shaw*

Department of Chemistry, P. M. Gross Chemical Laboratory, Duke University, Durham, North Carolina 27708

Received January 2, 1996; Revised Manuscript Received April 16, 1996⊗

ABSTRACT: The rate of UV-induced deamination of cytosine to uracil at a specific site in double-stranded (ds) DNA was monitored using a genetic reversion assay. M13mp2C141 ds DNA was exposed to 160 J/m^2 UV (254 nm), incubated at 37 °C, pH 7.4, for various time intervals to allow for deamination, and treated with *Escherichia coli* photolyase in the presence of 365 nm light to reverse cyclobutane-type pyrimidine dimers. Upon transfection into uracil-glycosylase deficient (*ung*$^-$) *E. coli* cells, the mutation (i.e., reversion) frequencies in the CCCC target sequence increased greatly with post-UV time of incubation at 37 °C, nearly doubling every day that the DNA had been held at 37 °C. After 8 days, the reversion frequencies had increased by two orders of magnitude upon transfection into *ung*$^-$ cells, relative to isogenic *ung*$^+$ cells, indicating that most of the mutations arising in UV/photolyase-treated ds DNA were C→T mutations mediated by a uracil intermediate. Sequencing of the revertants revealed that all mutations were single C→T or tandem double CC→TT mutations. An increasing percentage of tandem double CC→TT mutations was found with longer post-UV incubation times, yet none occurred if the post-UV delay time step was omitted before photoreversal. After a 4-day delay between UV and photoreversal at 37 °C, greater than 84% of the total revertants had tandem double CC→TT mutations. Thus, the generation of a tandem double mutation is a time-dependent process that arises in DNA after the initial UV exposure. The rate of appearance (with a pseudo-first-order rate constant ca. 10^{-6} s^{-1}) of tandem double mutations during incubation of UV-irradiated DNA is inconsistent with two random, independently occurring mutational events and suggests a concerted deamination of both residues in a tandem cytosine pyrimidine (C<>C) dimer. Considering that deamination in a C<>C dimer occurred here with a half-life of ca. 5 days, in contrast to the measured half-life of ca. 20 000 years for spontaneous (non-UV-treated) cytosine deamination for the same target, these studies show that the formation of pyrimidine dimers in DNA increases the rate of deamination by six orders of magnitude, leading to the accelerated formation of single C→T and tandem double CC→TT mutations.

Hydrolytic deamination of cytosine leads to the formation of uracil due to replacement of the C4 exocyclic amino group by an oxygen atom (Figure 1). Deamination is among the most common spontaneous mutational processes in DNA, second only to depurination (Shapiro, 1981). The uracil formed by cytosine deamination is potentially mutagenic, changing the coding information during DNA replication and RNA transcription, and resulting in altered base pairs in the genome (Lindahl, 1977, 1979). Uracil formed in DNA is removed by a specific enzyme, DNA uracil-glycosylase (Lindahl, 1979). Organisms which are defective in the removal of uracil from DNA have an increased spontaneous mutation rate (Duncan & Weiss, 1978) and more G:C→A:T base-pair transitions (Duncan & Miller, 1980). Hydrolytic deamination is a spontaneous process dependent on pH and temperature (Lindahl, 1979). Frederico et al. (1990) were able to directly determine the rates of cytosine deamination at specific cytosine residues in both ss and ds DNA[1] at physiologically relevant conditions by a sensitive genetic assay. Their measured site-specific rate constants for cytosine deamination were 7×10^{-13} s^{-1} for ds DNA and 1×10^{-10} s^{-1} for ss DNA at 37 °C, pH 7.4, thus directly demonstrating that the formation of a secondary structure protects DNA from deamination. Treating DNA with bisulfite (Shapiro, 1983; Chen & Shaw, 1993, 1994), acid, or base (Notari et al., 1970; Garrett & Tsau, 1972; Lindahl & Nyberg, 1974) can noticeably accelerate the rate of cytosine deamination. The deamination in cytosine-containing pyrimidine dimers in poly(dC)·poly(dI) was also greatly increased by UV (Setlow et al., 1965), yet the rates for site-specific deamination of photodimers in natural DNA is unknown.

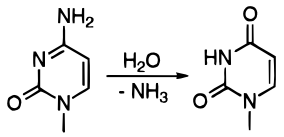

FIGURE 1: Hydrolytic deamination of cytosine to uracil.

UV irradiation of DNA produces a variety of photoproducts, consisting principally of (i) unstable monomeric cytosine photohydrates resulting from addition of water to the 5,6-double bond (Fisher & Johns, 1976) that can revert back to cytosine or produce uracil photohydrate which undergoes conversion to uracil (Boorstein et al., 1990) as well as (ii)

† This work was supported by NIH Grant CA-62176 to B.R.S.

* Address correspondence to this author.

⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1996.

[1] Abbreviations: ds, double-stranded; ss, single-stranded; C<>C dimer, tandem cytosine cyclobutane pyrimidine dimer; β-Gal, β-galactosidase; PL buffer, *E. coli* DNA photolyase reaction buffer; RF, reversion frequency; TDM, tandem double mutation; SM, single mutation.

S0006-2960(96)00001-3 CCC: $12.00

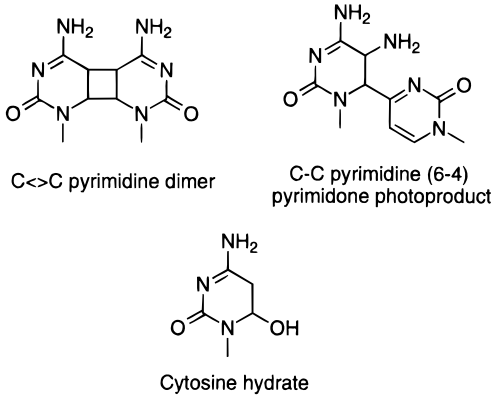

FIGURE 2: Main UV products: cyclobutane pyrimidine dimer (reversable by *E. coli* photolyase), pyrimidine (6−4) pyrimidone photoproduct, and cytosine hydrate.

dimeric cyclobutane pyrimidine and pyrimidine(6−4)-pyrimidone adducts (Patrick & Rahn, 1976; Brash & Haseltine, 1982) (Figure 2). Since UV exposure is strongly correlated with human skin cancer, UV-induced mutagenesis has been the focus of many studies both *in vivo* and *in vitro*. Mutations caused by UV radiation consist predominantly of C→T transitions, including CC→TT tandem double mutations, within dipyrimidine sites (Coulondre et al., 1977; Miller, 1985; Brash et al., 1987, 1991; Drobetsky et al., 1987; Brash, 1988; Armstrong & Kunz, 1990; McGregor et al., 1991; Hutchinson, 1994).

Because most C→T and CC→TT mutations caused by UV are at dipyrimidine sites, a number of hypothesis involving pyrimidine dimers have been presented to account for the mechanism of UV mutagenesis. (1) *Misincorporation/bypass*. Bridges and Woodgate (1985) proposed the "misincorporation" model to explain UV mutagenesis in excision-defective *Escherichia coli*. In this model, an incorrect nucleotide (usually A) is first inserted opposite the pyrimidine dimer under the direct influence of the *rec*A gene product. Replication bypass (in the presence of the *umu*D and C genes products at induced levels) fixes the mutation, producing C→T transitions. (2) *Deamination*. In studies where bacteria were first exposed to UV and then allowed time for metabolism after irradiation prior to exposure to visible light for photoreversal of pyrimidine dimers (*delayed photoreversal mutagenesis)*, the deamination of cytosine in pyrimidine dimers was proposed to explain the G:C→A:T transitions found in *ung*$^-$ but not in *ung*$^+$ cells (Fix & Bockrath, 1981; Fix, 1986; Ruiz-Rubio & Bockrath, 1989). This hypothesis was consistent with studies of model compounds by Green and Cohn (1958), who showed that cytosine nucleoside monomers with a saturated 5,6-bond as in a photohydrate or pyrimidine dimer could deaminate to uracil, as well as studies by Setlow et al. (1965) demonstrating that 280-nm UV light produced pyrimidine dimers in poly(dC)·poly(dI) with an estimated 2-h half-life for deamination. Tessman and co-workers (1991, 1992) invoked both the "deamination" and bypass models to explain the delayed appearance of C→T transitions after exposure to UV light and delayed photoreversal in a S13 system. They suggested that the replication enzyme is blocked at a cytosine-containing dimer but, after deamination, is able to replicate correctly past uracil-containing dimers. Ruiz-Rubio and Bockrath (1989) showed a delayed photoreversal mutagenesis at thymine-cytosine dimers in *E. coli* and indicated that both models can exist in *E. coli*. Jiang and Taylor (1993) presented evidence that UV-induced C→T mutations *in vivo* at pyrimidine dimer sites could be caused by the replicative bypass of cyclobutane dimers or their deamination products.

Beginning with Setlow et al. (1965), who first reported the existence of uracil in cytosine-containing pyrimidine dimers in DNA homopolymers, there have been a number of reports on the deamination of cytosine in pyrimidine dimer nucleotides (Liu & Yang, 1978; Skalski et al., 1988; Douki & Cadet, 1992; Lemaire & Ruzsicska, 1993). Estimates of rates and activation energy for *in vivo* cytosine deamination deduced from photoreversal studies were also published (Fix & Bockrath, 1981; Fix, 1986). Barak et al. (1995) used a forward assay to deduce the *in vitro* rate constant of deamination in cytosine-containing pyrimidine dimers in plasmid DNA. Yet the direct rate of *in vitro* cytosine deamination in a tandem cytosine cyclobutane (C<>C) photodimer in ds DNA is so far unknown.

We set out to measure directly, at a specific site in DNA, the deamination rate of cytosine in a C<>C photodimer, and to test the underlying mechanism of delayed deamination, whereby deamination in a C<>C dimer formed by UV irradiation could lead to CC→TT tandem mutations. Cytosine deamination to uracil at specific sites (C141, C142) in DNA of phage M13 can be measured directly using a sensitive genetic assay (Frederico et al., 1990). The genetic assay enables us to score mutations that occur at a specific site (codon) in one out of 10^6 molecules and to deduce a deamination rate constant. Previous work has explored the deamination of cytosine induced spontaneously (Frederico et al., 1990; Shaw, 1994) and by agents like bisulfite (Chen & Shaw, 1993, 1994) and cupric ion (Fang and Shaw, manuscript submitted), which induce surprisingly high levels of tandem double CC→TT mutations in addition to single C→T transitions. Although tandem double CC→TT mutations have been thought to be a signature of UV damage (Brash et al., 1991), the finding that tandem dimer mutations might be caused by chemical (Reid & Loeb, 1992, 1993; Chen & Shaw, 1994) as well as by UV damage led us to ask whether it is possible that a C→T transition at one site could accelerate a second C→T transition at a neighboring cytosine. In the present study, we show that (1) cytosine in C<>C cyclobutane dimers rapidly deaminates to uracil, relative to undimerized cytosine, and (2) the proportion of tandem double CC→TT mutations in a ds DNA target increases with the elapsed time allowed after initial UV exposure. The rate of deamination following UV treatment is six orders of magnitude greater than that for spontaneous deamination at the same site.

MATERIALS AND METHODS

Materials. Wild-type (*ung*$^+$) *E. coli* strains MC1061 [F$^-$*araD139* Δ(*ara-leu*)7696 *galE15 galK16* Δ(*lac*)*X74 rpsL*(Str^r) *hsdR2*($r_k^-m_k^+$) *mcrA mcrB1*] and NR9099 [Δ(*pro-lac*), *recA56*, *thi*, *ara*/F′(*pro*AB, *lac* I^{q}ΔM15)] were from Drs. Thomas Kunkel and Roel Schaaper (NIEHS), respectively. The isogenic *E. coli* (*ung*$^-$) strain NR9404 [F$^-$*araD139* Δ(*ara-leu*)7696 *galE15 galK16* Δ(*lac*)*X74 rpsL*(Str^r) *hsdR2*($r_k^-m_k^+$) *mcrA mcrB1 ung*] was constructed by Dr. Roel Schaaper (NIEHS). M13mp2C141 DNA, a mutant M13mp2 derivative, was the same as that used in Frederico et al. (1990). *E. coli* DNA photolyase was a generous gift

from Dr. Aziz Sancar (UNC, Chapel Hill). Sephadex G-50F was purchased from Pharmacia, Inc. All other chemicals were from previous sources (Frederico et al., 1990).

Genetic Assay. We used a sensitive genetic assay developed by Frederico et al. (1990) to measure the rate of cytosine deamination in DNA at a single cytosine residue. The reversion assay monitors the activity of the *lac*Z gene product, β-galactosidase (β-Gal), produced by α-complementation in a mutant phage M13mp2C141 DNA target, which has a CCC proline codon instead of wild-type GCC alanine codon for the 34th codon (at sites 141−143) of the α-peptide portion of the *lac*Z α gene in M13mp2. The sequence around the target site is ^{132}CCCCCTTT^{140}**C<u>CCC</u>**^{143}-AGCTGGC^{150} (the sequence in bold is the target site and the underlined is the 34th codon in *lac*Z α gene). The starting M13mp2C141 mutant DNA cannot produce an active β-Gal through α-complementation and results in a colorless or white plaque phenotype when it transfects an NR9404 *ung*$^-$ cell and is plated in the presence of *X*-Gal. M13mp2C141 DNA that has deaminated to uracil at either of the first two cytosines in the 34th (CCC) codon permits expression of an active β-Gal and leads to a wild-type phenotype, giving a dark blue plaque phenotype (revertant). Transfection of M13mp2C141 revertants DNA into *E. coli ung*$^-$ cells, which are unable to remove uracil from DNA, produces a C→T transition (because U codes like T in *ung*$^-$ cells), and thus the deamination event can be detected.

Preparation of ds DNA. Double-stranded M13mp2C141 DNA was prepared as described (Chen & Shaw, 1994) and resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA) to a final concentration of 8.2 μg/μL.

Irradiation of ds DNA with UV at 254 nm. Twenty-microliter aliquots of ds DNA (100 ng/μL) in TE buffer were irradiated with UV light at 254 nm for 160 s on parafilm which was placed on ice. The parafilm had been soaked in 95% ethanol and dried just before the experiment. The UV source was a UVGL15 lamp (UVP Product) suspended 15 cm above the parafilm. The UV dose rate, as read by a UVX radiometer (UVP Product), was 1 W/m^2. After irradiation, DNA samples were sealed in sterile capillary tubes, and those tubes were wrapped in aluminum foil and placed in an incubator at 37 °C for various time intervals.

Treatment with E. coli Photolyase. Irradiated DNA (20 μL), held at 37 °C from 0 to 16 days, was removed from the incubator and added to 200 μL of *E. coli* photolyase reaction buffer (PL buffer) (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 50 μg/mL BSA, 8% glycerol) and 0.5 μg of *E. coli* photolyase kindly provided by Dr. Aziz Sancar. (For some control samples, PL buffer was not added to the samples, and these were transfected directly.) To reverse cyclobutane pyrimidine dimers, the whole solution was irradiated with 365 nm light (from a UVGL 15 lamp) in a 1.5-mL Eppendorf tube on ice for 1 h, placing a band-pass filter (SBG-38 from CVI) over the Eppendorf tube to cut off the light below 300 nm (Sancar, 1994). The flux of light was 5.25 W/m^2 as read by UVX radiometer. Unless specifically indicated, the DNA sample was thereafter wrapped with aluminum foil to avoid extra light irradiation.

Transfection and Plating. After treatment with *E. coli* photolyase, the DNA solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) to remove photolyase, and the DNA sample was passed through a Sephadex G-50F column [for details, see Chen and Shaw (1993), except that the column buffer here was TE buffer] for desalting and then frozen at −20 °C until transfection. Various amounts (1−15 ng) of ds DNA were transfected into NR9404 or MC1061 *E. coli* cells by electroporation and then plated onto minimal media plates. The detailed procedure was described by Chen and Shaw (1993). Then, the plates were maintained in a 37 °C incubator for about 16−20 h to allow for plaque formation.

Revertant Scoring. Revertants appeared as dark blue plaques against the colorless plaques background. Reversion frequency (defined as the ratio of number of blue plaques to that of total plaques) could thus be obtained. Every blue plaque was picked from the plate, put into 1 mL of 50 mM sodium borate, and replated to verify the phenotypic color change. Only dark blue phenotype (and no light blue) revertants were seen in these experiments, which is expected if reversion occurs in the target CCC codon.

DNA Sequencing. In order to determine the specific DNA base change corresponding to the reversion, DNA from up to 25 randomly selected blue plaques at each time point was sequenced by the chain terminator method (Sanger et al., 1977).

RESULTS

Target Sites and Design of the Experiment. Our target DNA comes from M13mp2C141 phage (M13mp2 mutant) which has a G→C base change at position 141 of the *lac*Z gene that creates a CCC (proline) codon for the 34th amino acid of the α-peptide and yields a defective polypeptide unable to complement β-Gal activity in transfected *E. coli* host cells. Thus, cells transfected with the target M13mp2C141 DNA yield colorless plaques, while cells transfected with wild-type M13mp2 generate a blue plaque phenotype when plated on *X*-Gal indicator plates.

In the experiment here, we scored for reversions in the 34th (CCC) proline that revert to the wild-type (blue) β-Gal activity. Specifically, the first two cytosine residues (at positions 141 and 142 of the *lac*Z α gene) of the 34th codon are the targets for scoring cytosine deamination. C→T base changes at either C141 or C142 will produce Ser and Leu, respectively, giving wild-type blue plaque phenotypes. C→A transversion mutations also can be scored at sites 141 and 142 and C→G transversions can be scored at site 141. It should be noted that although single base revertants cannot be scored at sites 140 and 143, tandem double CC→TT mutations at sites (140−141), (141−142), and (142−143) can be scored because they give the same blue plaque phenotypes as revertants with C→T base change at site 141 or 142 [see Figure 1 in Chen and Shaw (1994)]. Since the target DNA is double-stranded, the assay can also score for complementary mutations in the (−) strand ^{140}GGGG^{143} sequence.

In order to directly observe the process of cytosine deamination in C<>C pyrimidine dimers at the target sites and measure the *in vitro* rate of deamination (Figure 3), we first induced the formation of pyrimidine dimers by irradiating the DNA and then waited various intervals, designated "post-UV incubation time", for deamination to occur. Following incubation, DNA was treated with the enzyme *E. coli* DNA photolyase which reverses cyclobutane pyrimidine dimers to monomers in the presence of 365 nm light (Sancar,

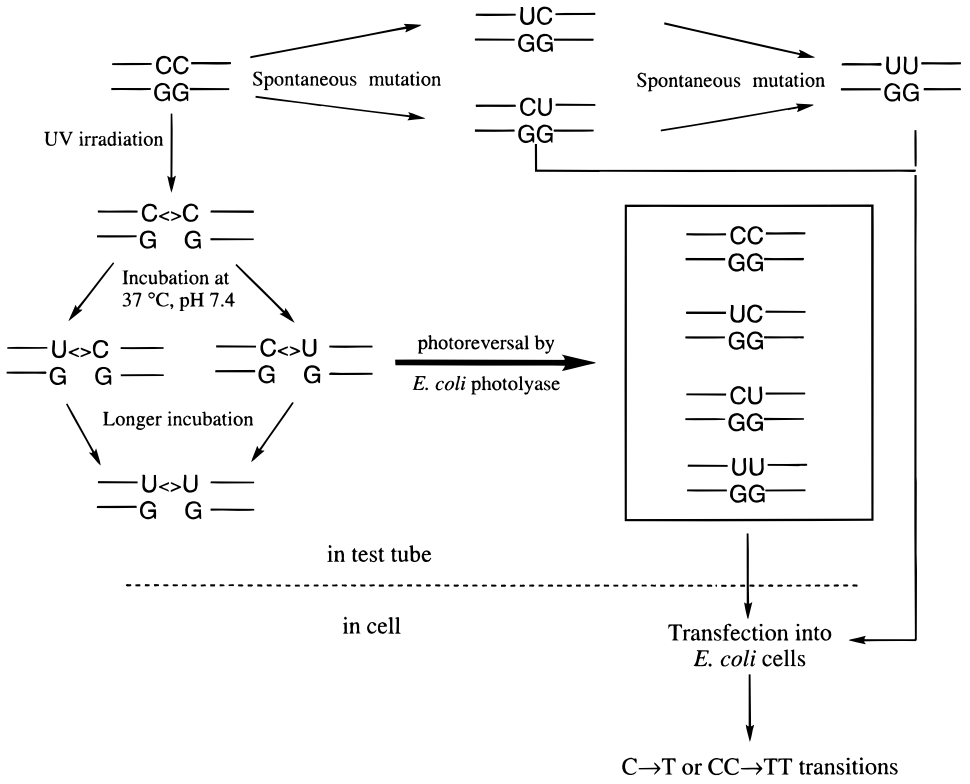

FIGURE 3: Experimental design.

1994). *E. coli* photolyase only reverses cyclobutane pyrimidine dimers among those UV-induced photoproducts. The quantum yields of splitting for T<>T is 0.9, T<>U 0.8, U<>U 0.6, and C<>C 0.05 (Kim & Sancar, 1991). The reversal of photodimers to monomers thereby permits normal replication (which was blocked by a photodimer). Any uracils that were formed by deamination during the incubation period will now code as thymines unless they are repaired. Transfection of this DNA into *ung*$^-$ cells and plating on X-Gal reveal mutations at the target site, and the ratio of reversion frequencies in isogenic *ung*$^-$ and *ung*$^+$ cells gives the proportion of mutations arising from deamination of cytosine to uracil compared to mutations that arise by other processes.

Time Dependence of the UV-Induced Mutations in ds DNA. After UV (254 nm) irradiation, the DNA was incubated at 37 °C, pH 7.4, for varying time periods, treated with *E. coli* DNA photolyase, and transfected into *ung*$^-$ cells (NR9404) after removal of the protein. The revertants were scored as blue plaques against a background of colorless plaques (see Table 1). The RF increased with the extent of post-UV incubation time, i.e., the holding time between the initial UV (254 nm) irradiation event and the photolyase treatment. Over the course of 16-days post-UV incubation time, the RF increased by nearly 100-fold to 1.27×10^{-3}. As seen in Figure 4, the curve of RF (in *ung*$^-$ cells) vs incubation time first increased and then leveled off at longer incubation times, as expected for a first-order reaction that approaches completion.

Ung Dependence. In order to determine whether UV-induced mutations in the target site were caused by cytosine deamination, aliquots of the same UV/photolyase treated-DNA samples were transfected into an isogenic *E. coli ung*$^+$ cell strain (MC1061) which is proficient in uracil glycosylase. Data in Table 1 demonstrate that the *ung*$^+$ reversion frequencies were reduced 100-fold compared to those in *ung*$^-$ cells at the same time point and increased less than 2-fold during the 16-day incubation period. This increase is negligible compared to the increase in the *ung*$^-$ (NR9404) cells. *E. coli* MC1061 cells are isogenic with *E. coli* NR9404 cells with the exception that the former are capable of removing uracil from DNA, while the latter are not. In the absence of uracil glycosylase, the C→U conversion would yield G:C→A:T transitions. If the C→T transitions are mediated by uracil, then uracils in the DNA samples treated with UV/photolyase will be removed by endogenous uracil-glycosylase in *E. coli ung*$^+$ strains, resulting in DNA with abasic sites that can undergo excision repair (Lindahl, 1979) and leading to proportionately fewer revertants. Therefore,

Table 1: Reversion Frequencies of M13mp2 C141 ds DNA Irradiated with 254 nm UV and Then Incubated at 37 °C, pH 7.4, for Varying Time Periods, Followed by Treatment with *E. coli* Photolyase, Purification, and Transfection into *ung*$^-$ or *ung*$^+$ Cells

	post-UV incubation time (days)	revertants	total plaques (10^4)	*RF* (10^{-5})	$1 - (RF/\eta)^a$
ung$^-$	0[b]	11	85.0	1.3	0.99
	0.17	12	27.1	4.4	0.97
	1	37	26.7	14	0.90
	4	122	15.7	78	0.44
	8	104	9.42	110	0.19
	16	111	8.81	130	0.09
ung$^+$	0[b]	15	188	0.80	
	0.17	10	160	0.63	
	1	11	149	0.74	
	4	10	90.7	1.1	
	8	13	86.6	1.5	
	16	14	113	1.2	

[a] $\eta = 1.4 \times 10^{-3}$. It is the fraction of pyrimidine dimers, containing cytosine, formed at our target site. [b] The zero time meant that after UV irradiation, ds DNA was treated immediately with photolyase and 365 nm light (a delay of not more than 5 min).

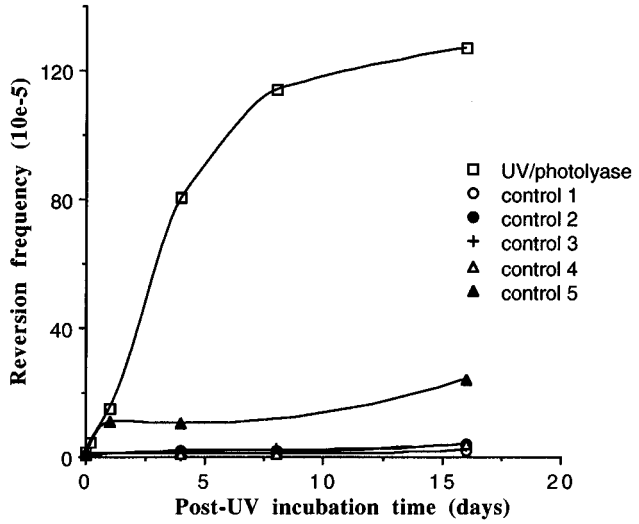

FIGURE 4: Reversion frequencies of treated ds DNA (data from Table 1) and controls transfected into *ung*$^-$ cells. An average of seven transfections were done for controls 1−5, to obtain a minimum of eight revertants per data point; 72 000 to 2.2 million total plaques were scored at each time point. The different treatments of DNA, listed in sequential order of execution, were as follows:

Sample	irradiation at 254 nm	post-UV incubation at 37 °C in TE buffer	PL buffer	addition of photolyase	irradiation at 365 nm	total revertants scored per time point
UV/photo-lyase	+	+	+	+	+	11-111
control 1	-	+	+	-	+	8-18
control 2	-	+	+	+	+	11-37
control 3	-	+	-	-	-	14-18
control 4	-	+	+	-	-	8-15
control 5	+	+	-	-	-	8-62

the RF in *ung*$^+$ cells will be lower than in *ung*$^-$ cells, as observed. The fact that the reversion frequencies were up to 100-fold higher in *ung*$^-$ cells than in *ung*$^+$ cells indicates that most of the mutations in UV/photolyase-treated ds DNA arose via a uracil intermediate.

UV Irradiation Increases the Probability of Cytosine Deamination. Spontaneous deamination of a cytosine residue in ds DNA is an extremely slow reaction with a rate constant of 7×10^{-13} s^{-1} and a half-life of ca. 20 000 years (Frederico et al., 1990), and thus it would be negligible in comparison with the huge reversion frequencies found in Table 1. To determine whether factors other than UV irradiation could have brought about cytosine deamination in ds DNA at rates above the very low spontaneous level, various control experiments were carried out (see Figure 4). DNA controls number 1−4 had various combinations of treatment of UV (254 nm) irradiation, photolyase, and 365-nm irradiation (for details, see the legend of Figure 4). The much lower RF for controls 1−4 compared with UV/photolyase treatment showed that photolyase reaction buffer and/or photoreversal light (365 nm) by themselves had little effect on cytosine deamination, giving low mutation levels. Only UV irradiation, which is known to produce pyrimidine dimers, had a dramatic influence on cytosine deamination. Control 5, which was irradiated with UV (254 nm) and transfected into *E. coli* cells without any photoreversal, showed a somewhat higher RF (particularly at the long incubation times) than controls 1−4, indicating some translesional bypass replication of the lesions; yet the RF of control 5 was much lower than UV-treated samples that underwent photoreversal.

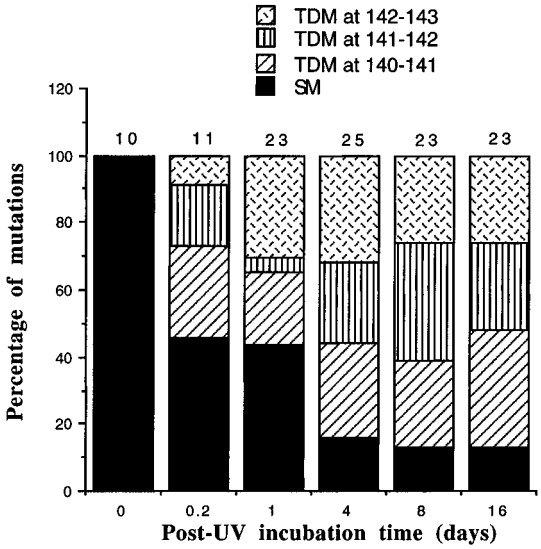

FIGURE 5: Distribution of single C→T mutation (SM) and tandem double CC→TT mutations (TDM) (from *ung*$^-$ cell transfections) at target sites out of the total mutations. Mutants were sequenced from DNA samples with UV/photolyase treatments and different time periods for incubation at 37 °C, pH 7.4. Every revertant sequenced was a C→T or CC→TT transition. The number over each bar was the number of revertants sequenced.

Tandem Double CC→TT Mutations. Revertants able to be scored with this assay include C→T transitions and C→A transversions at sites 141 or 142, C→G transversions at site 141, and CC→TT transitions. Since the target site contains four adjacent cytosines, there are three possible combinations of C<>C dimers: C_{140}<>C_{141}, C_{141}<>C_{142}, and C_{142}<>C_{143}; we sequenced up to 25 revertants from each time point (Figure 5) in order to determine the base changes in the target sites. Only single C→T or tandem double CC→TT mutations were found. Neither transversion mutations nor nonconsecutive CXC→TXT double mutations about sites 140−142 or 141−143 were found. At the zero incubation time point, no tandem double mutations were obtained; C→T single mutations constituted 100% of the mutants. But within 4 h following UV irradiation, tandem double CC→TT mutations constituted 54% of the total mutants, and these numbers increased to 87% with longer incubation times (Figure 5). The tandem double mutations occurred with almost equal probability at sites (140−141), (141−142), and (142−143). By comparison, the non-UV irradiated DNA sample treated with photolyase (control 2 in Figure 4) gave mostly single C→T mutations, and a ca. 100-fold lower RF for tandem double CC→TT mutations relative to UV treatment (as depicted in Figure 6). From the results in Figures 4 and 5, the time dependence of RF for total tandem double mutants (at target sites 140−141, 141−142, and 142−143) could be obtained as in Figure 6.

Calculation of the Deamination Rate Constant of Cytosine Residues in C<>C Pyrimidine Dimers in ds DNA. In order to calculate the rate of deamination of cytosine in a pyrimidine dimer, we made the following assumptions: (1) The background mutation contributed nothing to the cytosine deamination in this experiment as verified by the negligible increase in RF of control 2 (no UV) relative to the sample with UV/photolyase treatment (Figure 4). (2) Every revertant scored was a C→T mutation involving deamination of at least one cytosine (the conversion of cytosine to uracil being substantiated by the *ung* dependence and sequencing data

in Table 1 and Figure 5). (3) Deamination of 5,6-dihydrocytosine (as in a cyclobutane dimer) is a pseudo-first-order reaction where the rate-determining step is nucleophilic attack of water on C4 of the cytosine ring [by analogy with the rate-determining step in the substitution of an amino by a carboxyl group in spontaneous deamination (Shapiro & Klein, 1966; Frederico et al., 1990)]. The *ung*$^-$ plot (Figure 4) of reversion frequency vs post-UV incubation time is characteristic of a first-order reaction that has gone almost to completion at the longest incubation time (see eq 5 below).

In this model, the rate constant for cytosine deamination in a pyrimidine dimer can be calculated as follows. If R represents the number of M13mp2C141 molecules which at a given time have a C<>C pyrimidine dimer at the target sites, i.e., (140−141), (141−142), (142−143), and P represents the number of M13mp2C141 molecules which have a uracil produced by cytosine deamination at the target sites, then the reaction rate equation for the reaction $R \rightarrow P$ is

$$dR/dt = -kR \quad (1)$$

where k is the rate constant. Equation 1 can be integrated to give

$$R/R_0 = e^{-kt} \quad (2)$$

where R_0 is the initial number of M13mp2C141 ds DNA molecules which have a C<>C photodimer caused by the initial UV (254 nm) exposure. Since one plaque originates from one DNA molecule and, in the absence of DNA uracil-glycosylase the C→U conversion would yield a C→T transition upon replication, we can define RF′ as the ratio of the number of revertant plaques (with thymine at the target sites) to the total number of plaques with pyrimidine dimers at the target sites (prior to photoreversal), expressed here as $RF' = P/R_0 = (R_0 - R)/R_0$. Equation 2 can be written as

$$1 - RF' = e^{-kt} \quad (3)$$

Since not every M13mp2C141 molecule has a pyrimidine dimer at one of the above three target sites (140−141), (141−142), and (142−143), the reversion frequency, which is the number of revertants divided by the number of total plaques, obtained from the experiment will not equal the RF′ in eq 3, and thus we could not use RF directly in eq 3. Only a fraction η of M13mp2C141 molecules will have a pyrimidine dimer in one of the target sites, and

$$RF = \eta RF' \quad (4)$$

and this fraction will depend on the dose of the initial UV irradiation.

Combining eqs 3 and 4, then

$$RF = \eta(1 - e^{-kt}) \quad (5)$$

or

$$1 - (RF/\eta) = e^{-kt} \quad (6)$$

Given sufficient time, every C<>C photodimer at the target sites will eventually convert to a C<>U, U<>C, or U<>U, such that as $t \rightarrow \infty$, $\eta = RF_\infty$. Equation 5 is a reasonable expression for the UV/photolyase sample in Figure 4 and allows us to approximate a value of $\eta = 1.4 \times 10^{-3}$ at the given dose of UV irradiation (i.e., one in 700 M13mp2 DNA molecules will have a pyrimidine dimer at the CC target site). We can also approximate η independently from the literature. Shavitt and Livneh (1989) reported an average of 10 cyclobutane pyrimidine dimers per molecule formed in M13 mp18 ds DNA exposed to the UV dose of 100 J/m^2. Assuming the same proportionality of dose to dimer ratio, this translates for our experiment (160 J/m^2) to the formation of 16 pyrimidime dimers per DNA molecule. Given the efficiency ratio for T<>T, C<>T, T<>C, and C<>C dimer formation of 68:13:16:3 (Mitchell et al., 1992), and knowing that there are 1465 T-T dimer sites, 872 C-T sites, 774 T-C sites and 707 C-C sites in M13mp2 ds DNA, then the probability of forming C<>C dimers at the target site will be $16 \times 3 \times 3/[(1465 \times 68) + (872 \times 13) + (774 \times 16) + (707 \times 3)] = 1.14 \times 10^{-3}$. This calculation is based on the assumption that every type of pyrimidine dimer forms randomly among its potential sites. We found that the value of 1.14×10^{-3} for η as calculated from the literature is approximately equal to the 1.4×10^{-3} value obtained from our experimental data using eq 6.

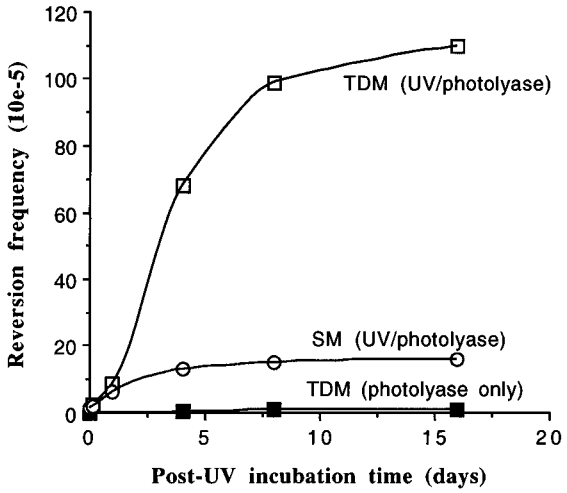

FIGURE 6: Reversion frequencies for tandem double CC→TT mutations and single C→T mutations at the target site in *ung*$^-$ cells. Reversion frequencies were obtained for DNA samples treated with only photolyase in the presence of 365-nm light or both UV (254 nm) and photolyase (at 365 nm). These RF were calculated based on the sequencing results (Figure 5). (Open square) Tandem double mutations with UV/photolyase treatments; (open circle) single mutations with UV/photolyase treatments; (solid square) tandem double mutations with photolyase treatment only.

By sequencing revertants (blue plaques), we obtained the ratio of single C→T transitions to tandem double CC→TT mutations and calculated a RF for each type of mutation (Figure 6). The RF of single mutations increased slightly and leveled off after 3-days post-UV incubation, while the RF of tandem double mutations increased with incubation time from 0 to 1.27×10^{-3} (Figure 6). We are not aware of any known mechanism whereby two cytosines in a pyrimidine dimer could simultaneously deaminate to two uracils; we therefore assume that tandem double CC→TT mutations are caused by a stepwise reaction process, expressed as $R \rightarrow P_1 \rightarrow P_2$, where P_1 is the total number of single mutants in pyrimidine dimers (unknown), P_2 is the total number of tandem double mutants, k_1 is the rate constant of step $R \rightarrow P_1$, and k_2 the rate constant of step $P_1 \rightarrow P_2$. Assuming these two steps are both first-order reactions, we obtain from the appropriate rate equations

$$R = R_0 e^{-k_1 t} \quad (7)$$

and

$$P_1 = k_1 R_0 (e^{-k_1 t} - e^{-k_2 t})/(k_2 - k_1) \quad (8)$$

$$P_2 = R_0 [1 - k_2 e^{-k_1 t}/(k_2 - k_1) + k_1 e^{-k_2 t}/(k_2 - k_1)] \quad (9)$$

We know that the decrease of R over time behaved as a simple first-order reaction, so we can use eq 6 to get the rate constant for the rate-determining step, i.e., the first deamination event in a dimer (as seen in Figure 7), which gives k_1 equal to 1.8×10^{-6} s^{-1}.

If the step from single mutants to tandem double mutants involves some kind of concerted deamination, and $k_2 \gg k_1$, then eq 9 can be simplified to give

$$P_2 = R_0 (1 - e^{-k_1 t}) \quad (10)$$

The RF for tandem double CC→TT mutations alone (from Figure 6) together with eq 6 can be used to obtain a rate constant of 1.2×10^{-6} s^{-1}, which is close to the value of 1.8×10^{-6} s^{-1} obtained from the total mutations. The near equivalence of these two k_1 values supports the assumption that k_2 is greater than k_1.

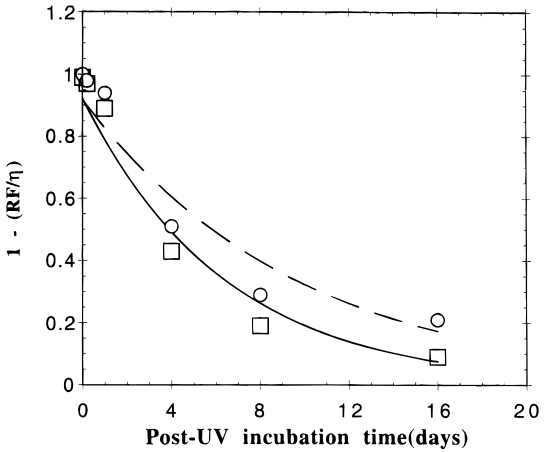

FIGURE 7: Curve fit of $(1 - RF/\eta)$ vs time to obtain rate constants of cytosine deamination from total mutations and tandem double mutations at the target site. The meaning of RF/η is explained in the text. (Square) total mutations, k_1 obtained here was 1.8×10^{-6} s^{-1}; (circle) tandem double mutations, k_1 obtained here was 1.2×10^{-6} s^{-1}.

DISCUSSION

The mutational specificity and rate of cytosine deamination in C<>C pyrimidine dimers at specific sites in ds DNA have been studied here by a sensitive genetic reversion assay. A double-stranded DNA plasmid was first treated with UV irradiation and then incubated for varying times at 37 °C, pH 7.4, and finally treated with *E. coli* DNA photolyase in the presence of 365-nm light to reverse the photodimers. Such *in vitro* treated DNA was then transfected into isogenic *E. coli* strains which differ only in their proficiency to repair uracil. The number of C→T mutations in *ung*$^-$ cells increased dramatically with the time intervals between the initial UV exposure and the photoreversal treatment. Further, the reversion frequency was 100-fold higher in *ung*$^-$ cells than in *ung*$^+$ cells, suggesting that most of the base changes appearing in DNA were caused by cytosine deamination to uracil. DNA sequencing showed that all mutants were single C→T or tandem CC→TT transitions, and that the percentage of tandem double mutations increased remarkably during the 16-day incubation period. Notably, immediately following UV and photoreversal treatment, no tandem double mutations were observed. However, after 4 h of incubation post-UV, the majority of transitions were tandem double mutations. These results indicate that much of the damage that fixes the tandem CC→TT mutations arises not at the time of UV treatment, but in the hours and days following the initial exposure.

UV irradiation causes a variety of modified bases in DNA besides cyclobutane pyrimidine dimers, including pyrimidine (6−4) pyrimidone photoproducts and cytosine photohydrates (Figure 2) (Wang, 1976; Patrick & Rahn, 1976; Brash & Haseltine, 1982). Therefore, it was expected that a number of different products besides cyclobutane pyrimidine dimers formed at our target site (^{140}CCCC^{143}). However, our data argue against a role for these other products in mutagenesis here. We found that, after photoreversal treatment, the RF of UV-irradiated samples transfected into *E. coli ung*$^+$ cells (Table 1) approached the background level (control 2 in Figure 4) in *ung*$^-$ cells. Since it is known that *E. coli* DNA photolyase will specifically reverse only cyclobutane pyrimidine dimers, but not (6−4) photoproducts (Sancar, 1994), we can thus safely assume that the large *ung*$^-$ increase in RF upon photolyase treatment was primarily due to reversal of cyclobutane dimers in which cytosine had deaminated to uracil. If pyrimidine(6−4)pyrimidone photoproducts or photohydrates had been significant contributors to mutations in this system [for example, via replication errors made by bypassing such photoproducts (Lecomte et al., 1981; Glickman et al., 1986)], then significant amounts of mutations caused by polymerase bypass of (6−4) products should have been observable also in *ung*$^+$ cells, and we would have expected the reversion frequencies in *ung*$^+$ cells to be higher than background. The near equality of reversion frequencies of the UV/photolyase samples in *ung*$^+$ and the unirradiated DNA in *ung*$^-$ cells (compare the *ung*$^+$ RF in Table 1 with control 2 in Figure 2) indicates that (6−4) products were not mutagenic in our system. This lack of significant mutagenesis by (6−4) photoproducts at our CCCC target site is consistent with a report by Douki and Cadet (1995) that far UV will generate dTpdT and dTpdC (6−4) dimers but not dCpdC (6−4) photoproducts. Furthermore, no treatment except 254 nm UV caused the remarkable increase in mutations observed with post-UV incubation time (Figure 4), indicating that mutations were not simply a result of photolyase treatment or other factors. We therefore conclude that cytosine or cytosine derivatives, which deaminate to uracil or uracil derivatives in the cyclobutane pyrimidine dimers, were responsible for most of the C→T and CC→TT transitions.

Whereas our experiments strongly demonstrate that all mutations arose from deamination, they do not allow us to distinguish the reaction pathway. A single C→T mutation could arise by deamination of cytosine in a pyrimidine dimer, as discussed above, or by deamination of a cytosine photohydrate. Boorstein et al. (1990) showed that, with a 1000-fold higher dose (100 kJ/m^2) of 254-nm light than we used here, 2.2% of cytosine residues in poly(dG-dC) were

Table 2: Deamination Rate Constants of Cytosine Residues in Cyclobutane Pyrimidine Dimers

substrate	rate constant of deamination (s^{-1})	half-life (h)	temperature	pH	reference
dimers					
mixture of *cis*−*syn* and *trans*−*syn* dT[p]dC		1	50 °C		Liu and Yang (1978)
Cyt-$(CH_2)_3$-mo^{5}Ura	9.0×10^{-5}	2	room temperature	6.7	Skalski et al. (1988)
cis−*syn* dC[p]dT[a]	1.2×10^{-4}	1.6	room temperature		Douki and Cadet (1992)
cis−*syn* dC[p]dT[b]	2.8×10^{-5}	6.8	room temperature	7	
cis−*syn* dT[p]dC	2.5×10^{-5}	7.7	25 °C	7	Lemaire and Ruzsicska (1993)
trans−*syn* dC[p]dT	6.5×10^{-6}	30	25 °C	7	
DNA					
poly(dI)·poly(dC)		∼2[c]	37 °C	7	Setlow et al. (1965)
mixed sites[d] in plasmid pOC2	3.9×10^{-5}	5	37 °C	7.5	Barak et al. (1995)
E. coli[e]	5×10^{-6}	38	48 °C		Fix and Bockrath (1981)
E. coli[e]	1.1×10^{-5}	17	48 °C		
CCCC target in M13mp2C141 DNA[f]	$1.2-1.8 \times 10^{-6}$	107−160	37 °C	7.4	this study

[a] In aqueous solution. [b] In 0.01 M phosphate buffer. [c] Estimated half-life. [d] Different sites of cytosine-containing pyrimidine dimers in UV-treated DNA, incubated in 10 mM Tris-HCl/1 mM EDTA (pH 7.5). [e] Different sites, *in vivo*. [f] In 10 mM Tris-HCl/0.1 mM EDTA (pH 7.4).

converted to cytosine hydrate (6-hydroxy-5,6-dihydrocytosine) at pH 8.0. Cytosine hydrate can lose water and revert to cytosine with a half-life of 25 h at 37 °C, or it can go on to deaminate to uracil hydrate (6-hydroxy-5,6-dihydrouracil), which dehydrates and converts to uracil. These hydrates can persist in DNA for days at a time. Conversion of uracil hydrate to uracil has been measured for only poly(dA-dU) and occurs with a half-life of 6 h at 37 °C (Boorstein et al., 1990). Thus, some of the single C→T mutations which we found after several days of incubation may have arisen from photohydrates. We can, however, rule out photolyase treatment or reversal with long wavelength light (controls 1 and 2 in Figure 4) as causes of single C→T mutations because their mutation frequencies were about equal to background.

UV light also produces 8-hydroxydeoxyguanine (8-OHdGua) (Fischer-Nielsen et al., 1993; Wei et al., 1996), which during translesional synthesis can mispair with A, giving rise to G→T (i.e., C→A) transversions in the ds DNA (Wood et al., 1990; Moriya & Grollman, 1993). Since mutations here were scored at C:G base pairs, it was possible that the C→T mutations scored here could have arisen from UV damage to the guanine strand. However, we think 8-OHdGua is a highly unlikely cause of mutations here, for several reasons. First, up to 99% of the mutations were eliminated in the *ung*$^+$ cells. Uracil-DNA glycosylase is known to excise from DNA only uracil and two uracil derivatives, 5,6-dihydroxyuracil (Zastawny et al., 1995) and 5-hydroxyuracil (Hatahet et al., 1994). If incorporation of dATP opposite damaged G_{141} or G_{142} had occurred, the uracil-glycosylase should have had no effect, and we should have seen little difference in *ung*$^+$/*ung*$^-$ reversion frequencies, in contrast to our observations. Furthermore, mutations in our system increased with the longer post-UV incubation times, but misincorporation opposite 8-OHdGua should not increase with time of incubation. We thus exclude the possibility that the mutations in *ung*$^-$ were caused by simple nucleotide misreplication of a UV-damaged G_{141} or G_{142} in the (-) strand. The apparent lack of G→T transversions provides further support for the absence of a significant amount of 8-OHdGua. We conclude that UV damage of guanine in the (−) strand is not responsible for the enhanced mutagenesis in *ung*$^-$ cells.

One can use the measured reversion frequencies to gain insight into mechanisms of tandem double CC→TT mutation formation. If we assume that cytosine deamination occurs randomly and independently, and that a single C→T mutation occurs with reversion frequency of 10^{-5} to 10^{-3} (as in Table 1), then the probability of finding two independently arising CC→TT tandem double mutations at our target sites should be the product of the individual events, i.e., 10^{-10} to 10^{-6}. Such frequency is below the background levels and, as such, would be too small to be detected with our assay. Our observation that the reversion frequency of tandem double mutations was about 10^{-4} after 1 day indicated that this mutation could not be the result of two independent cytosine deamination (or other rare) events happening at neighboring sites but suggested the occurrence of two cooperative or interrelated events whereby one event enhances the probability of occurrence of the second event. It is likely, we believe, that the second event giving rise to a double mutation arose during the post-UV incubation period, since CC→TT mutations increased with time of incubation. The fact that only C→T, and not CC→TT, mutations appeared at the very beginning of the incubation at 37 °C suggested to us that deamination of one cytosine residue in the C<>C dimer could promote deamination of the neighboring cytosine residue by a concerted process. We suggest that formation of CC→TT tandem double mutations involves a rate enhancement of the second deamination event at a neighbor site by the first cytosine deamination event.

Since Setlow et al. (1965) first reported cytosine deamination in the UV-irradiated dI·dC homopolymer and estimated the half-life to be 2 h at 37 °C and pH 7 by chromatographic analysis of the acid hydrolyzed radioactive polymer, there has been growing evidence that such deamination may also occur in natural DNA. In several types of *dinucleotide cyclobutane pyrimidine dimers* exposed to UV, rate constants of cytosine deamination have been reported to occur with half-lives of 2−30 h at 25 °C (see Table 2). In natural DNA duplexes, the half-lives are not all that different, varying from 5 to 10^2 h. The deamination rate of cytosine in C<>C dimers *in ds DNA* might have been predicted to be slower than in model dimers, based on measurements showing that normal duplex formation protects cytosine from deamination [its rate being 100-fold slower than in ss DNA (Frederico et al., 1990, 1993)]. *Cis*−*syn* cyclobutane pyrimidine dimers in ds DNA unwind DNA by about 15° (Ciarrocchi & Pedrini, 1982) and distort the helix, possibly reducing hydrogen-bonding and base stacking within

the dimer region (Kemmink et al., 1987; Taylor et al., 1990; Kim et al., 1995). The local structural and conformational changes could alter solvent exposure about the dimer and lead to increased levels of deamination compared to normal ds DNA. Notably, the deamination rate for C<>C photodimers in ds DNA found here is a million-fold faster than that for normal ds DNA and 10 000-fold faster than ss DNA (Frederico et al., 1990). Given the large rate constants measured for deamination here, we conclude that the distortions to the pyrimidine ring upon cyclobutane formation, and accompanying changes in protonation and solvent exposure of cytosine in the dimer, apparently override the usual protecting effect that the duplex has in preventing cytosine deamination in B-form DNA. It will be instructive to compare the deamination rates of C<>C in single-stranded and double-stranded DNA.

Our data is the first direct measurement of the rate of cytosine deamination in C<>C cyclobutane dimers in natural ds DNA. From data in Figure 7 obtained at physiological temperatures and pH values, we calculate the site-specific rate constant of cytosine deamination in UV dimers to average ca. 1.5×10^{-6} s^{-1}, corresponding to a half-life of 5 days at 37 °C. This value falls within the same order of magnitude as *in vivo* deamination rate constants (k) of cytosine in C<>T pyrimidine dimers (upon photoreversal in *E. coli* DNA at 48 °C) deduced by Fix and Bockrath (1981) where k equaled 5×10^{-6} and 11×10^{-6} s^{-1} for different sites (corresponding to half-lives of 38 and 17 h, respectively). They assumed that all dimers were monomerized by photoreactivation, that an adenine was inserted with 100% efficiency opposite a uracil in place of cytosine in DNA, and that each C<>T dimer site had equal probability of forming a dimer. In a similar photoreactivation experiment where *E. coli* cells were held at different temperatures to allow for "deamination" prior to photoreversal, Fix (1986) found the *in vivo* activation energy for deamination in C<>T pyrimidine dimers to be 17 kcal/mol, which was 10−12 kcal/mol less than values found for deamination of cytosine in ss DNA (Lindahl & Nyberg, 1974; Frederico et al., 1990), indicative of a more readily accessible transition state for cytosine in the dimer compared to cytosine in normal ss DNA. Recently, Barak et al. (1995) used a forward assay to measure an *in vitro* k of 3.9×10^{-5} s^{-1} for cytosine deamination at multiple cytosine-containing pyrimidine dimer sites in UV-irradiated plasmid DNA at 37 °C, corresponding to a half-life of 5 h. Their value (presumably for deamination in both C<>T and T<>C as well as C<>C dimers) is 26-fold greater than our rate constant for deamination in a C<>C dimer.

Although we have not yet measured deamination in C<>T and T<>C dimers with our reversion assay, it appears from comparison of our data with forward assay data of Barak et al. (1995) that deamination of cytosine in C<>T and T<>C dimers may be faster than in a C<>C dimer. Lemaire and Ruzsicska (1993) have suggested that the deamination rate constant of the first cytosine in a *cis*−*syn* C<>C dimer would be lower than that obtained for a C<>T dimer because the neighboring amino group may not stabilize the iminium group by dipole reaction as well as a carbonyl group, causing the pK_a of that cytosine to be lower than the pK_a in a C<>T dimer. For comparison, it should be noted that the rate constant for spontaneous cytosine deamination at 37 °C, pH 7.4 in ds DNA is 7×10^{-13} s^{-1} (Frederico et al., 1990), which is six orders of magnitude smaller than the value for cytosine deamination in C<>C pyrimidine dimers, as measured here.

When UV light activates DNA, causing the two plus two addition reaction of 5,6-double bonds of the neighboring cytosines to form a C<>C cyclobutane dimer, the deamination of the saturated cytosine is greatly accelerated and results in single C→T and tandem CC→TT double mutations. It should be noted that tandem CC→TT double mutations are caused not only by UV but also by chemical damage to DNA. Chen and Shaw (1994) found high percentages of tandem double CC→TT mutations attributed to deamination after incubating ds DNA in the presence of bisulfite at pH 7.4, in which the active species was proposed to be either an oxygen centered $S\dot{O}_3^-$ radical or sulfur centered $S\dot{O}_3^-$ radical, saturating the 5,6-double bond and causing deamination at a rate that is much faster than normal, yet much slower than with UV. Tandem double CC→TT mutations are also caused by metal ions (Reid & Loeb, 1993; Fang and Shaw, manuscript submitted) and the bis-intercalating agent echinomycin (Moyer et al., 1993).

From results of the UV studies, it should be born in mind that deamination in pyrimidine dimers can readily take place with half-lives of hours and days, rather than years as with spontaneous deamination (Frederico *et al*, 1990), and therefore precautions should be taken in experiments dealing with UV-treated DNA to avoid conditions that will induce delayed deamination initiated by UV irradiation.

ACKNOWLEDGMENT

We are very grateful to Dr. Aziz Sancar for the gift of *E. coli* DNA photolyase and to Dr. Sang-Tae Kim in Dr. Sancar's lab for advice on working with photolyase. We also want to thank Dr. Hong Chen, Mr. Gang Fang, Dr. Faqing Huang and Dr. Kunal Merchant for discussions on the mathematical approach of the kinetics and Drs. Ted Gonzalez and Shereen Barry for the help in the experiments. Special thanks to Dr. E. I. Budowsky for his critique of the manuscript.

REFERENCES

Armstrong, J. D., & Kunz, B. A. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9005−9009.

Barak, Y., Cohen-Fix, O., & Livneh, Z. (1995) *J. Biol. Chem. 270*, 24174−24179.

Boorstein, R. J., Hilbert, T. P., Cunningham, R. P., & Teebor, G. W. (1990) *Biochemistry 29*, 10455−10460.

Brash, D. E. (1988) *Photochem. Photobiol. 48*, 59−66.

Brash, D. E., & Haseltine, W. A. (1982) *Nature 298*, 189−192.

Brash, D. E., Seetharam, S., Kraemer, K. H., Seidman, M. M., & Bredberg, A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3782−3786.

Brash, D. E., Rudolph, J. A., Simon, J. A., Lin, A., McKenna, G. J., Baden, H. P., Halperin, A. J., & Ponten, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10124−10128.

Bridges, B. A., & Woodgate, R. (1985) *Mutat. Res. 150*, 133−139.

Chen, H., & Shaw, B. R. (1993) *Biochemistry 32*, 3535−3539.

Chen, H., & Shaw, B. R. (1994) *Biochemistry 33*, 4121−4129.

Ciarrocchi, G., & Pedrini, A. M. (1982) *J. Mol. Biol. 155*, 177−183.

Coulondre, C., & Miller, J. H. (1977) *J. Mol. Biol. 117*, 577−606.

Douki, T., & Cadet, J. (1992) *J. Photochem. Photobiol. B: Biol. 15*, 199−213.

Douki, T., & Cadet, J. (1995) in *Interface between Chemistry and Biochemistry* (Jolles, P., & Jörnvall, H., Eds.) pp 173−197, Birkhaüser Verlag Base, Switzerland.
Drobetsky, E. A., Grosovsky, A. J., & Glickman, B. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 9103−9107.
Duncan, B. K., & Weiss, B. (1978) in *DNA Repair Mechanism (*Hanawalt, P. C., Friedberg, E. C., & Fox, C. F., Eds.) pp 183−186, Academic Press, New York.
Duncan, B. K., & Miller, J. (1980) *Nature 287*, 560−561.
Fischer-Nielsen, A., Loft, S., & Jensen, K. G. (1993) *Carcinogenesis 14*, 2431−2433.
Fisher, G. J., & Johns, H. E. (1976) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., Ed.) Vol. 1, pp 169−225, Academic Press, New York.
Fix, D. F. (1986) *Mol. Gen. Genet. 204*, 452−456.
Fix, D. F., & Bockrath, R. (1981) *Mol. Gen. Genet. 182*, 7−11.
Frederico, L. A., Kunkel, T. A., & Shaw, B. R. (1990) *Biochemistry 29*, 2532−2537.
Frederico, L. A., Kunkel, T. A., & Shaw, B. R. (1993) *Biochemistry 32*, 6523−6530.
Garrett, E. R., & Tsau, J. (1972) *J. Pharm. Sci. 61*, 1052−1061.
Glickman, B. W., Schaaper, R. M., Haseltine, W. A., Dunn, R. L., & Brash, D. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6945−6949.
Green, M., & Cohn, S. S. (1958) *J. Biol. Chem. 228*, 601−605.
Hatahet, Z., Kow, Y. W., Purmal, A. A., Cunningham, R. P., & Wallace, S. S. (1994) *J. Biol. Chem. 269*, 18814−18820.
Hutchinson, F. (1994) *Mutat. Res. 309*, 11−15.
Jiang, N., & Taylor, J.-S. (1993) *Biochemistry 32*, 472−481.
Kemmink, J., Boelens, R., Koning, T., van der Marel, G. A., van Boom, J. H., & Kaptein, R. (1987) *Nucleic Acids Res. 15*, 4645−4653.
Kim, J.-K., Patel, D., & Choi, B.-S. (1995) *Photochem. Photobiol. 62*, 44−50.
Kim, S. T., & Sancar, A. (1991) *Biochemistry 30*, 8623−8630.
Lecomte, P., Boiteux, S., & Doubleday, O. (1981) *Nucleic Acids Res. 9*, 3491−3451.
Lemaire, D. G. E., & Ruzsicska, B. P. (1993) *Biochemistry* 32, 2525−2533.
Lindahl, T. (1977) in *DNA Repair Processes* (Nichols, W. W., & Murphy, D. G., Eds.) p 225, Symposium Specialists Inc, Miami.
Lindahl, T. (1979) *Prog. Nucleic Acid. Res. Mol. Biol. 22* , 135−192.
Lindahl, T., & Nyberg, B. (1974) *Biochemistry 13*, 3405−3410.
Liu, F.-T., & Yang, N. (1978) *Biochemistry 17*, 4865−4876.
McGregor, W. G., Chen, R.-H., Lukash, L., Maher, V. M., & McCormick, J. J. (1991) *Mol. Cell. Biol. 11*, 1927−1934.
Miller, J. H. (1985) *J. Mol. Biol. 182*, 45−68.
Mitchell, D. L., Jen, J., & Cleaver, J. E. (1992) *Nucleic Acids Res. 20*, 225−229.
Moriya, M., & Grollman, A. P. (1993) *Mol. Gen. Genet. 239*, 72−76.
Moyer, R., Stewart, U., Briley, D., Johnson, A., & Shaw, B. R. (1993) *Mutat. Res. 288*, 291−300.
Notari, R. E., Chin, M. L., & Cardoni, A. (1970) *J. Pharm. Sci. 59*, 28−38.
Patrick, M. H., & Rahn, R. O. (1976) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., Ed.) Vol. 2, pp 35−95, Academic, New York.
Reid, T. M., & Loeb, L. A. (1992) *Cancer Res. 52*, 1082−1086.
Reid, T. M., & Loeb, L. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3904−3907.
Ruiz-Rubio, M., & Bockrath, R. (1989) *Mutat. Res. 210*, 93−102.
Sancar, A. (1994) *Biochemistry 33*, 2−9.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Setlow, R. B., Carrier, W. L., & Bollum, F. J. (1965) *Proc. Natl. Acad. Sci. U.S.A. 53*, 1111−1118.
Shapiro, R. (1981) in *Chromosome Damage and Repair* (Seeberg, E., & Kleppe, K., Eds.) pp 3−18, Plenum Press, New York.
Shapiro, R. (1983) in *Induced Mutagenesis* (Lawrence, C. W., Ed.) pp 35−60, Plenum Press, New York.
Shapiro, R., & Klein, R. S. (1966) *Biochemistry 5*, 2358−2362.
Shavitt, O., & Livneh, Z. (1989) *J. Bacteriol. 171*, 3530−3538.
Shaw, B. R. (1994) in *Structural Biology: The State of the Art* (Sarma, R. H., & Sarma, M. H., Eds.) Vol. 2, pp 367−378, Adenine Press, New York.
Skalski, B., Wenska, G., Paszyc, S., & Stefaniak, Z. (1988) *Can. J. Chem. 66*, 1027−1031.
Taylor, J.-S., Garrett, D. S., Brockie, I. R., Svoboda, D. L., & Telser, J. (1990) *Biochemistry 29*, 8858−8866.
Tessman, I., & Kennedy, M. A. (1991) *Mol. Gen. Genet. 227*, 144−148.
Tessman, I., Liu, S-K., & Kennedy, M. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1159−1163.
Wang, S. Y., Ed. (1976) *Photochemistry and Photobiology of Nucleic Acids*, Vols. 1 and 2, Academic, New York.
Wei, H., Cai, Q., & Rahn, R. O. (1996) *Carcinogenesis 17*, 73−77.
Wood, M. L., Dizdaroglu, M., Gajewski, E., & Essigmann, J. M. (1990) *Biochemistry 29*, 7024−7032.
Zastawny, T. H., Doetsch, P. W., & Dizdaroglu, M. (1995) *FEBS Lett. 364*, 255−258.

BI960001X