Deamination of Cytosine-containing Pyrimidine Photodimers in UV-irradiated DNA

SIGNIFICANCE FOR UV LIGHT MUTAGENESIS*

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The realization that cytosine in cyclobutyl pyrimidine dimers rapidly deaminates to uracil raised the possibility that this chemical transformation, rather than an enzymatic polymerase error, is the major mutagenic step in UV mutagenesis. We have established a sensitive bioassay system that enabled us to determine the rate of deamination of cytosine in cyclobutyl pyrimidine dimers in plasmid DNA. This was done by in vitro UV irradiation and deamination of a plasmid carrying the cro gene, followed by photoreactivation, and assaying uracils in DNA by their ability to cause Cro+ mutations in an indicator strain that was deficient in uracil DNA N-glycosylase. DNA sequence analysis revealed that 27 out of 29 Cro+ mutants carried GC → AT transitions, as expected from deamination of cytosine. Deamination of cytosines in the cro gene in UV-irradiated plasmid pOC2 proceeded at 37°C with first-order kinetics, at a rate of $(3.9 \pm 0.6) \times 10^{-3} \text{s}^{-1}$, corresponding to a half-life of 5 h. Physiological salt conditions increased the half-life to 12 h, whereas decreasing the pH increased deamination. The temperature dependence of the rate constant yielded an activation energy of $13.6 \pm 3.3 \text{kcal/mol}$. These kinetics data suggest that deamination of cytosine-containing dimers is too slow to play an important role in UV mutagenesis in Escherichia coli. However, it is likely to play an important role in mammalian cells, where the mutagenic process is slower.

UV radiation produces in DNA a multiplicity of photoproducts, of which two are believed to be responsible for most of the killing and mutagenic effects of UV radiation (1). These are the cyclobutyl pyrimidine-pyrimidine dimers, and the pyrimidine-pyrimidone 6-4 adducts (2, 3). Since GC → AT transitions constitute a major class of UV mutations both in prokaryotes and eukaryotes (4–6), cytosine-containing dimers are likely to constitute a major class of UV mutations both in prokaryotes and eukaryotes (4–6), cytosine-containing dimers are likely to constitute a major mutagenic class of UV lesions.

It is believed that the key step in UV mutagenesis involves a bypass synthesis reaction, whereby a DNA polymerase replicates through a UV lesion in DNA (reviewed in Refs. 6 and 7). According to this model, the polymerase incorporates with high frequency an incorrect nucleotide opposite the lesion, a potentially mutagenic event. The chemical nature of the DNA lesion is a major parameter in determining the efficiency and specificity of misincorporation opposite the lesion (1). Thus, if a UV lesion undergoes a secondary chemical transformation to yield a different product, this may have a significant effect on its mutagenicity. This is the case with cytosine-containing photodimers which undergo spontaneous deamination to yield uracil-containing photodimers (8). The special interest in this reaction stems from the fact that a C → U change is potentially mutagenic, since uracil has the coding properties of thymine (9). Thus, if the spontaneous deamination proceeds fast enough, it adds a component of a non-enzymatic mutagenic reaction to the process of UV mutagenesis (10). Clearly, the rate of deamination is a major factor needed in order to evaluate its biological importance.

The deamination of normalcytosines in DNA into uracils is a slow process under physiological conditions. At 37°C and pH 7.4 it proceeds with a half-life of approximately 200 and 30,000 years in single-stranded and double-stranded DNA, respectively (11). This means a deamination of approximately 100 cytosines/human genome/day. However, saturation of the 5,6-bond of cytosine greatly facilitates the rate of deamination. For example, 5,6-dihydrocytosine deaminates at pH 7.0 and 37°C with a half-life of only about 2 h (12). Since in both types of photodimers the 5,6-double bond is saturated, the rate of deamination of cytosine-containing pyrimidine dimers is likely to be much higher than that of cytosine. Previous attempts to determine the rate of deamination of cytosine-containing cyclobutyl pyrimidine dimers (CPDs) led to conflicting results. One set of results suggested that CPDs deaminate in Escherichia coli with a half-life of 5–6 h (13, 14), whereas a second set of results suggested that deamination of CPDs in DNA proceeds with a sharp step kinetics, and is completed within 55 min in phage λ (10) or in purified phage S13 dsDNA (15), and within 30 min in ssDNA purified from phage S13, or in the virion state (10, 15). This time difference is critical for evaluating the significance of deamination, since in E. coli, for example, mutations are believed to be formed within 30 min after UV irradiation (6, 16–18).

EXPERIMENTAL PROCEDURES

Materials—Plasmid pOC2 is a 5-kilobase pair pBR322 derivative constructed in our laboratory (19). It carries the cro gene of phage λ, and the bla and kan genes, which confer ampicillin and kanamycin resistance, respectively. DNA photolyase was a gift from A. Sanar (University of North Carolina, Chapel Hill). Radiolabeled (α-32P)dATP at 3000 Ci/mmol and Hybond-N nylon membranes were obtained from the Radiochemical Center, Amersham. Dithiothreitol was purchased from Boehringer Mannheim, MOPS was purchased from Sigma, and eosin yellow and methylene blue were obtained from Riedel-de Haen.

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1 The abbreviations used are: CPD, cyclobutyl pyrimidine dimer; ds, double-stranded; ss, single-stranded; MOPS, 4-morpholinepropanesulfonic acid; MF, maximal mutation frequency; MF,t, mutation frequency at time t.
Bacterial Strains—The bacterial strains used in this study are listed in Table I. All the strains constructed in this study originated from E. coli (recA::Tn10) (19). The desired mutations were transferred by P1 transduction (20) from strains RW82 (Δlac-pro, thi, met) (21), BD2314 (ung::Tn10) (22), and WBM535 (ΔrecA::Tn10) (19). In order to construct a ΔrecA::Tn10 derivative of an ung::Tn10 strain, non-revertible Tet” mutants of the ung::Tn10 strain were selected on fusaric acid plates as described (23). This enabled using Tet selection for the subsequent 3-day transduction. The lacI gene was then introduced into the constructed strains by mating (20) with DP90C/R (22) which contains an F’ episome carrying Lac“-Y”-Pro”. The cells were then lysogenized (20) with x200ind in which lacZ is fused to the OpaP operator-promoter of phage λ (24). The reCA genotype was checked by its extreme sensitivity to UV irradiation at 254 nm. The Ung phenotype was verified by a comparison of the transformation efficiency of a normal plasmid to that of a uracil-containing plasmid. An Ung“ strain does not distinguish between these types of plasmids, but Ung“ strains attempt to repair the uracil-containing plasmid by releasing it from free uracils, thus producing AP sites (25). When those are present in small amounts they are rapidly repaired by excision repair initiated by AP endonucleases (26). However, if the plasmid is heavily substituted with uracil, it will be degraded and lost. The result is that the transformation efficiency of both plasmids is similar in Ung“ strains, but in Ung“ strains the transformation efficiency of the normal plasmid is at least 20-fold higher than that of a uracil-containing plasmid. The uracil-substituted plasmid was prepared from a culture of C. Bimung dut cells. The Umu“ phenotype was checked by assay for loss of UV-mutability to rifampicin resistance as described (27).

UV Irradiation of Plasmid DNA—DNA was UV irradiated essentially as described (28). DNA (0.1 μg/μl in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) was spread on parafilm as droplets (3 μl each) and UV irradiated at 254 nm on ice, using a low pressure mercury germicidal lamp. The dose rate was 2.2 J/m2 s-1 as determined by a UV Products radiometer using a UV-25 sensor. The average number of pyrimidine photodimers/DNA molecule, determined in our laboratory, was 5 × 10-6 photodimers/nucleotide2-2 DNA. DNA preparations of pOC2 plasmids with an average of 2.5 (50 μm), 5 (100 μm), 7.5 (150 μm), 10 (200 μm), and 20 (400 μm) photodimers per molecule were used throughout this study.

Plasmid Mutagenesis—The photoreactivation mixture (30 μl) contained 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 μg/ml bovine serum albumin, 10 mM diethothreitol, UV-irradiated plasmid DNA (0.3 μg), and DNA photolyase (10 ng). The mixture was spread on parafilm as droplets (3 μl each) and UV irradiated at 365 nm using a long-wavelength UV lamp (Black-ray model UVI-56) which was at a distance of 13 cm from the droplets. Irradiation was for 15 min at a dose rate of 5 J/m2 s-1, as determined by a UV Products radiometer using a UV-36 sensor. The enzyme was heat inactivated for 10 min at 65 °C and the mixture stored at -20 °C. This procedure eliminates cyclobutyl pyrimidine dimers by direct reversal to the original pyrimidine-pyrimidine sequence.

TABLE I

| Name | Genotype | Source |
|------|----------|--------|
| BD2314 | fha-2, lacY1 Tss-70, glv44A5, gal-6, trp-45, his-68, purC50, ung-152::Tn10, tyrA2, rpsL125, malT1, xylA7, nthA2, thi-1, λ+ | E. coli Genetic Stock Center Laboratory stock |
| Cj 236 | duf-1, ung-1, thi-1, rdaA lacZ105 (camR F’+) | E. coli Genetic Stock Center Muller-Hill |
| CSH26 | ara, Δ(lac-pro), thi, met | Laboratory stock |
| DP90C | Δ(lac-pro), thi, nalA, x200ind/F’ Lac“-Y”-Pro” | Muller-Hill |
| RW82 | F-1, thi-6, lacZ14, ay-A, rha1, galK2, ara-14, yhi-S, thy-1, proA2, his-4, rpsL31, rax-L, supE44, vil-225, trp-uvA6E, (ΔumuDC)1595“cat”, λ | R. Woodgate |
| WBM535 | ara, thi, met, Δ(lac-pro), lacZ105::Tn10, (Δ(lac-pro), x200ind/F’ Lac“-Y”-Pro” | Our Laboratory |
| WBY07 | as CSH26, x200ind/F’ Lac“-Y”-Pro” | This work |
| WBY11T | as WBY07, Δ(lac-pro), lacZ105::Tn10, (ΔumuDC)1595“cat” | This work |
| WBY13T | as WBY11T, ung-152 | This work |
| WBY20T | as WBY07, ung-152::Tn10 | This work |

RESULTS

An in Vivo Assay for Deamination of Cytosine-containing Cyclobutyl Pyrimidine Dimers—Our approach was to UV irradiate and deaminate plasmid DNA under defined in vitro conditions, and then to measure the deamination of the dimers by assaying the formation of uracils in the DNA using a bioassay. In preparation for the bioassay the DNA was treated with purified DNA photolyase. This enzyme uses light as a cofactor for a direct reversal reaction that converts all types of cyclobutyl pyrimidine-pyrimidine dimers (including uracil-containing dimers) into the original pyrimidine-pyrimidine sequence (31). The net result of deamination and photoreactivation of UV-irradiated DNA was the conversion of cytosine residues, that were originally part of cyclobutyl dimers, into uracils (Fig. 1). Since the latter have the coding properties of thymine, this transformation of a GC base pair into a GU base pair is mutagenic if the uracil is not removed from DNA (32). When such a plasmid is introduced into a Ung“ strain the uracil will be eliminated and the DNA will be repaired to the original GC base pair, thus preventing mutations. In an Ung“ strain, the GU mismatch will persist, and after replication, one of the daughter chromosomes will carry an AU base pair instead of the original GC base pair, resulting in an elevated frequency of GC → AT transitions (Fig. 1).

To assay deamination-induced mutations we used the cro mutagenesis system developed in our laboratory (24). It is based on the repressor properties of the cro gene product of bacteriophage λ (33), carried on plasmid pOC2. The tester strains have a Lac“ Z”+ LacB“ + genetic background and contain a λ200ind prophage, in which lacZ is fused to the OpaP operator-promoter of phage λ (200 itself has the immunity region of phage 21). Upon transformation, the plasmid which overproduces the Cro repressor is introduced into the tester strain cells. Under normal conditions, the overproduced repressor binds to the OpaP operator and represses expression of lacZ, thus blocking the production of β-galactosidase. In this case, cells
are unable to ferment lactose and thus give rise to white colonies on lactose-EMB indicator plates. A mutation that sufficiently reduces the binding of the repressor to the Orec region will cause full or partial derepression of lacZ, enabling lactose fermentation. This will lead to a local reduction of pH on the indicator plates and production of dark red colonies. To avoid loss of the plasmid from the cells during growth, kanamycin is included in the plates (19, 24, 30).

The indicator strains used were an isogenic pair of Ung (and Ung) strains. In order to eliminate any contribution from the SOS system, we engineered the strain to carry ΔrecA and ΔumuDC mutations. This inactivates the SOS response in general (34), and the SOS-mutagenic pathway in particular (35, 36), thus not allowing the processing of UV lesions into mutations. The conversion of unrepaired GU mismatches to mutations is unaffected by the lack of the UmuDC and RecA proteins.

As can be seen in Table II, when an indicator Ung strain was transformed with unirradiated plasmid pOCC2, a background mutation frequency of (0.2 ± 0.1) × 10⁻⁴ was observed. A plasmid irradiated with 400 J m⁻² gave only a marginal increase in mutation frequency (0.6 ± 0.3) × 10⁻⁴, as expected from the non-mutability phenotype of the ΔrecA ΔumuDC indicator strain. When the UV-irradiated plasmid was heated at 37°C for 5 hours, then photoreactivated, and assayed in an Ung strain, a dramatic increase of up to 200-fold was observed (mutation frequency 40.3 ± 8.4) × 10⁻⁴. These mutations were almost entirely dependent on the combined treatments of UV irradiation, deamination (heating), and photoreactivation of the plasmid, and on the use of an Ung strain.

Mutations arising from deamination of cytosines should give rise to GC → AT transitions. Indeed, DNA sequence analysis of Cro mutants revealed that 27 out of 29 mutants (93%) contained GC → AT transitions, as expected. The remaining two mutations were GC → CG transitions. We analyzed also 12 mutants obtained from unirradiated plasmid, and found 8 GC → AT transitions, 2 GC → CG transitions, one deletion, and one insertion. All GC → AT mutations (in both irradiated and unirradiated DNA) occurred at cytosines that have one or more adjacent pyrimidines. For UV-irradiated DNA this is expected for mutations initiated by a cyclobutyl photodimer. Taken together, these results strongly suggest that our system assays the deamination of cytosine-containing cyclobutyl pyrimidine dimers, and that this reaction occurs at 37°C on a time scale of hours.

Rate of Deamination of Photodimers in cro—The rate of deamination of CPDs is expected to be dependent on their concentration, and thus on the UV dose for a given DNA concentration. Indeed, as can be seen in Fig. 2, Cro™ mutation frequency was dependent on UV dose, increasing nearly linearly up to 400 J m⁻², a dose at which the plasmid contained an average of 20 pyrimidine dimers/molecule. In order to determine the rate of deamination of CPDs we used the plasmid irradiated at 400 J m⁻², and incubated it for various periods of time at 37°C. The time-dependent accumulation of Cro™ mutations is represented in Fig. 3. The mutation frequency in the Ung™ strain was very low, in the range of 0.4 × 10⁻⁴, indicating rapid repair of uracils prior to plasmid replication. In contrast, when assayed in the Ung™ strain, mutation frequencies were dramatically higher, and increased with deamination time (Fig. 3).

The deamination reaction is expected to follow first-order kinetics, described by the rate equation (C*+ = (C+)₀.exp(-kt) where (C+)₀ and (C+) are the concentrations of cytosines in pyrimidine dimers before incubation (t = 0) and after t hours of incubation. (U*)₀, the concentration of uracils arising from the deamination of the cytosines is (C+)₀ - (C+) (Fig. 3). After rearrangement the equation becomes, ln(1 - (U*)₀/(C+)₀) = -kt. How can U* be calculated from the observed mutation frequencies? Since the assay detects essentially only mutations at CPD sites, (U*) is proportional to the mutation frequency at time t (MFₜ), and the initial concentration of CPDs, (C+)₀ is proportional to the maximal mutation frequency (MFₘ) observed in our system. Thus, one can assume that (U*)₀/(C+)₀ = MFₜ/MFₘ. In order to determine MFₘ we have conducted several experiments for prolonged incubation times at 42°C or at pH 6.0, conditions that enhance deamination (see below). From these experiments, for a plasmid irradiated at 400 J m⁻², MFₘ is (70 ± 14) × 10⁻⁴ (see “Experimental Procedures”). Thus, the first-order rate constant, k, can be obtained by plotting ln(1 - MFₜ/MFₘ) as a function of time. Such a plot is shown in Fig. 3B. The rate constant is (3.9 ± 0.6) × 10⁻⁵ s⁻¹, implying a half-life of 5 h for the deamination of CPD in the cro gene.

Temperature Dependence of Deamination of CPD—Deamination of cytosines is temperature dependent. In order to examine the effect of temperature on deamination of UV lesions plasmid pOCC2 was irradiated at 400 J m⁻² and incubated for an hour at different temperatures. The temperature range examined was 25–47°C, a range which includes physiological growth temperatures. The plasmids were then photoreactivated, and assayed in strains WBY11T Ung™ and WBY13T Ung™ (Fig. 4). Consistent with previous experiments, the mutation frequency of
pOC2 in the Ung⁻ strain was constant, about $0.4 \times 10^{-4}$, at all temperatures, indicating rapid repair of uracils prior to replication. In contrast, a large increase in the mutation frequency of pOC2 in the Ung⁻ strain was observed, from $7 \times 10^{-6}$ at 25°C to $42 \times 10^{-6}$ at 47°C. The increase was exponential, typical of an Arrhenius temperature dependence of reaction rate, and represents most likely the temperature dependence of deamination of cytosine-containing pyrimidine dimers. The first-order reaction rate constants are shown in Table III. They indicate that within the range of physiological temperatures (25–42 °C), the half-life of deamination of CPDs varies 9-fold: from 7.4 to 0.8 h. The Arrhenius plot of the rate constants (Fig. 4B) gave an activation energy of 13.6 ± 3.3 kcal/mol.

Effects of Salt and pH Conditions on the Deamination Rate of CPDs—The intracellular environment contains salts at an estimated concentration of 0.1–0.2 M, and in both prokaryotes and eukaryotes K⁺ is the major intracellular cation (37, 38). In E. coli the major anion is believed to be glutamate (37), whereas in eukaryotes a major free anion is chloride (38). In order to evaluate the effect of the intracellular salt environment on deamination, we measured reaction rates of deamination of CPDs in plasmid pOC2 in the presence of 0.15 M KCl, or 0.15 M potassium glutamate. The presence of 0.15 M KCl reduced the reaction rate 2.4-fold ($k = (1.6 \pm 0.2) \times 10^{-5}$), representing a half-life of 12 h (Fig. 5). The rate was the same in 0.15 M potassium glutamate (not shown).

The effect of pH on the deamination of CPDs in UV-irradiated plasmid pOC2 was determined at 37 °C. As can be seen (Fig. 6) deamination increased as the pH decreased, with the
Deamination of UV Lesions in DNA

First-order rate constants varying nearly 50-fold in the tested pH range of 5–9. This implies half-lives of deamination varying from 0.7 h at pH 5 to 34.4 h at pH 9.

**DISCUSSION**

The degree of fidelity by which DNA polymerases copy damaged nucleotides is critical to the mutagenic effect of DNA lesions. Thus, any secondary chemical reaction that affects the primary DNA lesion might be a major factor in its final mutagenic outcome. This was suggested to be the case for UV light that produces two major types of DNA lesions: the cyclobutyl pyrimidine dimers, and the 6-4 pyrimidine-pyrimidone adducts. The TT cyclobutyl dimers are stable in DNA, and are not known to undergo any further chemical transformation. However, it was noticed in the early days of nucleic acid photochemistry that cytosines in cyclobutyl dimers were rapidly deaminated to uracils (8). This occurs generally in cytosine derivatives in which the 5‘-6’ bond is saturated. Since uracil has the coding properties of thymine, this secondary chemical reaction is potentially mutagenic.

Tessman and co-workers (10, 15) have proposed that the major mutagenic step in UV mutagenesis is not due to a polymerase error, but rather due to deamination of CPDs, followed by accurate replication of the uracil-containing dimers. This suggestion is consistent with the observation that site-specific TT and UT cyclobutyl photodimers in M13 ssDNA were copied with relatively high fidelity in E. coli in vivo (39, 40), however, it is not clear whether it provides a general explanation for chromosomal UV mutagenesis in E. coli (41). Clearly, a critical parameter in assessing the importance of deamination in UV mutagenesis is the rate of deamination of pyrimidine dimers in DNA.

The assay system that we have developed measures in vitro deamination of CPDs in UV-irradiated plasmid DNA utilizing a mutagenesis bioassay. The presence of uracils produced by the deamination reaction was assayed by their ability to cause GC → AT transitions in the cro reporter gene. This is a sensitive bioassay that enabled us to determine rates of deamination in a biologically active DNA molecule, under physiological temperature and salt conditions.

In our assay system deamination of CPDs follows first-order kinetics, as expected, with a rate constant of \( 3.9 \pm 0.6 \times 10^{-5} \) s\(^{-1} \) at 37°C in 10 mM Tris HCl, 1 mM EDTA (pH 7.5), corresponding to a half-life of 5 h. This result is in agreement with the deamination rate in E. coli in vivo, evaluated to be 2.2 \times 10^{-3} \) min\(^{-1} \) (half-life 5.3 h (14)). Our results differ from the results of Tessman and co-workers, who reported a sharp step kinetics for the deamination of both ssDNA and dsDNA, with deamination completed within 55 min in dsDNAs from phages \( \lambda \) (10) or S13 (15), and within 29 min in ssDNA from phage S13 (10, 15). We do not know the reason for this difference, especially since some of the experiments were performed under similar buffer and temperature conditions.

Extensive chemical studies were performed on the deamination of defined synthetic dinucleotide cyclobutyl dimers. As can be seen in Table IV, in all cases studied, a first-order deamination kinetics was observed, and the rate constants were close to the one calculated based on our bioassay. For example, the half-life of the cis-syn cyclobutyl dimer of pdCpdT in 10 mM phosphate buffer at pH 7.0 was 6.8 h at room temperature (42) (Table IV). Since the reaction conditions were not identical in the different studies, the results are not fully comparable. However, the emerging consensus from the data presented in
Table IV is that deamination of CPDs in DNA proceeds by first-order kinetics, and with a half-life of several hours, depending on conditions. It is noteworthy that one set of conditions (10 mM Tris-HCl, 1 mM EDTA, pH 7.5, 37 °C) gave a half-life of 5 h, similar to that evaluated for E. coli in vivo (14). The activation energy for the deamination of CPDs in DNA calculated based on the bioassay gave a value of 13.6 ± 3.3 kcal/mol, remarkably similar to the activation energy of the cis-syn isomer of the cyclobutyl dimer of the dinucleotide dTdcP, which was determined to be 13.7 kcal/mol (43). These two values are within the error margin of the activation energy of UV mutations in glutamine tRNA in E. coli in vivo (44).

What is the significance of our data to in vivo UV mutagenesis? The rates of deamination are affected primarily by salt conditions and pH. Under physiological salt conditions (0.15 M KCl or 0.15 M potassium glutamate), deamination was slowed pending on conditions. It is noteworthy that one set of conditions and pH. Under physiological salt conditions (0.15 M KCl, 1 mM EDTA, pH 7.5, 37 °C) gave a half-life of 5 h, similar to that evaluated for E. coli in vivo (14).

The activation energy for the deamination of CPDs in DNA calculated based on the bioassay gave a value of 13.6 ± 3.3 kcal/mol, remarkably similar to the activation energy of the cis-syn isomer of the cyclobutyl dimer of the dinucleotide dTdcP, which was determined to be 13.7 kcal/mol (43). These two values are within the error margin of the activation energy of UV mutations in glutamine tRNA in E. coli in vivo (44).

The 6-4 adducts could not be assayed in our system since they do not interfere with our assay since they are most likely deaminated by the repair of 6-4 adducts is not likely to be significant since the average interlesion distance in the UV-irradiated plasmid is longer than 500 nucleotides (20 or less lesions in a 5-kilobase pair plasmid), whereas the repair patches are of the order of 10–30 nucleotides (1). Any remaining 6-4 adducts will not give rise to mutations due to the ΔεαAumuDC genetic background of the indicator strain. Studies on the deamination of the 6-4 adduct in the dinucleotide dCpT have shown that it deaminates with a half-life of 152–413 h (depending on conditions), nearly two orders of magnitude slower than CPDs (42). Extrapolating to DNA, this suggests that the deamination of the 6-4 adducts is unlikely to be a significant process under physiological conditions.

In E. coli UV mutations are believed to be fixed within 30 min (16–18). Thus, our results lead us to conclude that deamination of pyrimidine dimers does not play a significant role in UV mutagenesis in E. coli. On the other hand, it is likely to be a significant factor in mammalian cells, where mutagenesis, repair, and replication are much slower (1).