Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8141–8145, November 1988
Genetics

Frequency and spectrum of mutations produced by a single cis–syn thymine-thymine cyclobutane dimer in a single-stranded vector

(UV mutagenesis/*Escherichia coli*/translesion synthesis)

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Communicated by Richard B. Setlow, July 29, 1988 (received for review, May 16, 1988)

ABSTRACT **We have constructed a single-stranded vector that contains a uniquely located cis-syn T-T cyclobutane dimer by ligating a synthetic oligomer containing this UV photoproduct into M13mp7 viral DNA linearized with *Eco*RI. In the absence of SOS induction, transfection of a *uvrA6* mutant of *Escherichia coli* with this vector gave very few progeny plaques, and the data imply that a single dimer blocks replication in at least 99.5% of the molecules. *In vitro* photoreactivation completely abolished this inhibition. Transfection of cells irradiated with UV at 4 $J \cdot m^{-2}$ to induce the SOS response gave 27% of the number of plaques found with a dimer-free control. Nucleotide sequence analysis of 529 progeny phage showed that translesion synthesis was usually accurate: the normal sequence was found in 93% of them. Where mutations occurred, all were targeted single-nucleotide substitutions, with ≈90% being targeted at the 3′ nucleotide of the lesion: of a total of 26 mutations, 15 were 3′ T → A, 8 were 3′ T → C, and 3 were 5′ T → C. No T → G mutations were found. In addition to these results with the normal construct, data were also obtained from vectors in which the M13mp7 cloning site, which forms a hairpin in single-stranded DNA, was present 4 nucleotides on the 3′ side of the T-T dimer. These hairpin-containing vectors gave a very similar mutation frequency (8% versus 7%) but altered mutation spectrum: all 12 mutations detected were 3′ T → A transversions, a difference from the previous set of data that is significant ($P = 0.03$).**

Several investigators have analyzed the spectrum of nucleotide sequence changes induced by UV in phage or plasmid genomes (1–5), chiefly with the aim of understanding the molecular processes responsible for induced mutagenesis. From this perspective, however, the interesting patterns evident in such data raise more questions than they settle: their interpretation is uncertain because of the heterogeneous and ill-defined nature of UV-irradiated genomes. UV induces a variety of photoproducts, and controversy exists over their relative mutagenic potential (1–10). These photoproducts are distributed quasi-randomly within and between UV-irradiated genomes, and their low frequency at any one genetic site is a major reason for the rarity of mutations. As a consequence, mutation detection or selection methods based on change in gene function must be used, a procedure that fails to identify all nucleotide sequence changes and biases the spectrum of events recovered. It also precludes estimation of the error rate of translesion synthesis. In short, UV-irradiated genomes are far from ideal substrates with which to investigate mutagenic mechanisms.

One means of avoiding the problems inherent in experiments with UV-irradiated genomes is the use of artificially constructed vectors that contain a single defined photolesion located at a uniquely specified genetic site. In this report, we describe a rapid, flexible, and general method for making such vectors that was designed to meet two criteria, (*i*) detection of a full range of sequence changes without the use of a reporter gene or sequence and (*ii*) the insertion of the photoproduct specifically and directly into a single-stranded vector. In this latter respect, our method differs from those of others who, for similar reasons, have used this approach to examine the mutagenic properties of a variety of non-UV lesions (ref. 11 and references therein).

We report here results from transfection of a *uvrA6* mutant of *Escherichia coli* with a single-stranded M13 hybrid phage vector containing a unique cis-syn T-T cyclobutane dimer. Our eventual aim is to determine the error rate and mutation spectrum of each of the major UV photoproducts, to provide a basis for analysis of data from irradiated genomes, and in general to use these molecules as tools for the investigation of mutagenic mechanisms.

MATERIALS AND METHODS

Vector Construction. The principal elements of the method are given in Fig. 1. As shown by Been and Champoux (12), M13mp7 viral DNA can be uniquely linearized by restriction enzymes because the symmetrical cloning site of this hybrid phage forms a duplex hairpin in the otherwise single-stranded genome. Purified viral DNA cut with *Eco*RI was recircularized noncovalently by annealing with a synthetic 51-mer scaffold, the two ends of which were complementary to the terminal 20 nucleotides at the ends of the linearized vector. The central segment of the 51-mer was largely complementary to a synthetic purine-rich 11-mer, modified to contain a single T-T cyclobutane dimer, which could be ligated efficiently into the viral DNA by using a 100-fold molar excess. The scaffold was removed immediately before transfection by heating the ligation mix at 90°C for 2 min, followed by rapid cooling. The scaffold molecule contained a tandem C-C to form mismatched base pairs opposite the site of the T-T dimer, to allow the detection of the rare progeny phage that had resulted from the use of scaffold, rather than viral-strand, information in this region.

In addition to the construct diagrammed in Fig. 1, the procedure also produced molecules containing the M13mp7 hairpin immediately on the 3′ side of the inserted 11-mer. These were formed when *Eco*RI digestion occurred at the 5′, but not the 3′, site within the recognition sequence. With only a minor disturbance of normal base pairing, singly cut molecules of this kind can anneal with the 51-mer scaffold, leaving a normal gap of 11 nucleotides (Fig. 2). Restriction endonuclease digestion at 37°C gave ≈70% singly cut molecules, but this could be reduced to ≈10% by cutting at 23°C. Such molecules presumably arise when the 5′ site is cut first, followed immediately by rupture of the G·C base pair at the bottom of the hairpin, which prevents the occurrence of the 3′ cut by destroying the recognition sequence. When the 3′ site

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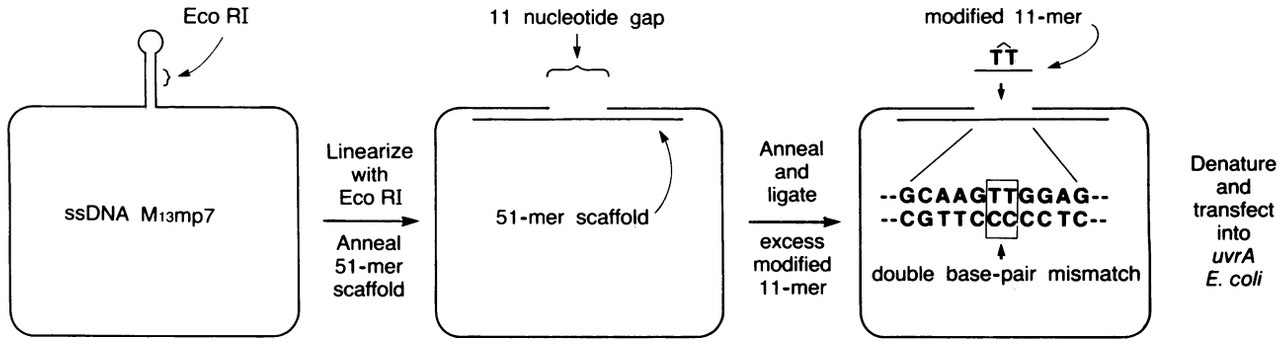

FIG. 1. Method for inserting photoproduct-containing oligonucleotide into the single-stranded M13mp7 vector. ssDNA, single-stranded DNA.

is cut first, this train of events is less likely, and more easily reversed. Single cutting was much reduced in M13mp7L1, a derivative of the normal phage in which four extra base pairs had been added to stabilize the bottom of the hairpin. Results reported here came exclusively from normal M13mp7, however.

Purification and Photochemical Modification of Oligomers. Oligonucleotides synthesized on an Applied Biosystems (Foster City, CA) model 380A oligonucleotide synthesizer were deprotected and prepared for purification according to manufacturer's instructions. The 51-mer scaffold oligomer was partially purified by electrophoresis through a 20% polyacrylamide gel; chain lengths between 48 and 51 bases were recovered from the gel. The 11-mer was purified by HPLC with a Macherey–Nagel model 60-7 DEAE-silica column and a linear gradient from 300 to 400 mM LiCl in 20 mM sodium acetate, pH 5.8/20% (vol/vol) acetonitrile over a period of 18 min, followed by a linear gradient to 1 M LiCl over a period of 12 min at a flow rate of 1 ml/min. Fractions containing the 11-mer were evaporated to a volume containing 4 M LiCl and the oligomer was precipitated at −20°C with 5 vol of 95% ethanol/acetone, 1:1 (vol/vol), and then reprecipitated with ethanol.

The 11-mer was photochemically modified by exposing 100 μl of an anoxic aqueous solution containing 50–100 μg of purified oligomer and 2×10^{-2} M acetophenone to ≈1.8 MJ·m^{-2} of radiation from fluorescent sunlamps (FS40, Westinghouse) filtered through a 0.005-inch Mylar film (1 inch = 2.540×10^{-2} m). T-T cyclobutane dimer-containing oligomer was separated from other photoproducts and the parental species by HPLC with a Waters radial compression Nova-Pak C_{18} reversed-phase column and a linear gradient of 7%–9% (vol/vol) acetonitrile in 0.1 M triethylammonium acetate (pH 7.0) over 20 min, followed by a linear gradient to 70% acetonitrile over 10 min at 1 ml/min (Fig. 3). In some cases the initial gradient was 6%–9% acetonitrile, and in general it was necessary to fine tune the gradient for replacement columns. Modified oligomer was repurified by the same procedure, treated with T4 polynucleotide kinase, and again purified by HPLC. Quantification of the final chromatogram indicated that the purified material contained no more than 0.4% of the unmodified oligomer. Fractions containing the unmodified parental species were also purified from the photochemical reaction mix and handled similarly to provide control material for the experiments.

Other Procedures. M13mp7 was grown in JM101 and the constructs were transfected at a multiplicity of 0.2–0.5 into SMH10, an F$^+$, Δ(*pro-lac*) *leu*$^+$ *uvrA6* derivative of AB1157, made competent by calcium chloride treatment. For SOS induction, SMH10 cells were irradiated with 254-nm UV at 4 J·m^{-2}—or for one experiment, 2 J·m^{-2}—immediately before they were made competent. Nucleotide sequence of progeny phage was determined by the dideoxy method (13) by using a synthetic primer complementary to nucleotides 37–57 in the *lacZ* gene, counting from the first base on the 3′ side of the *Eco*RI site. Photoreactivation was carried out with purified *E. coli* photolyase (kindly supplied by Gwendolyn and Aziz Sancar, University of North Carolina, Chapel Hill) by using buffer (14) and blacklight (model F20T12 BLB, General Electric) illumination.

RESULTS

Analysis of Modified 11-Mer. The nature of the photochemically induced alteration in the modified 11-mer was exam-

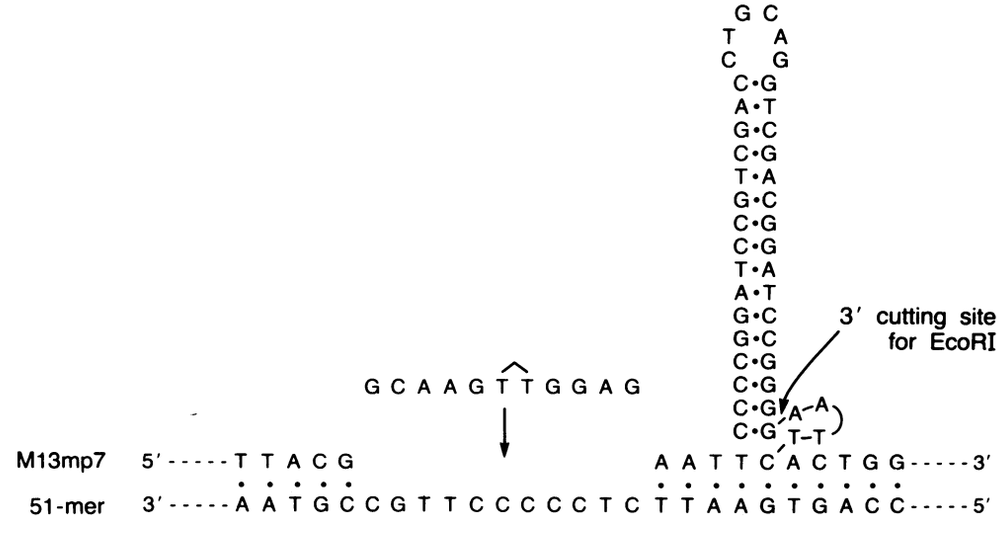

FIG. 2. Origin of the hairpin-containing construct.

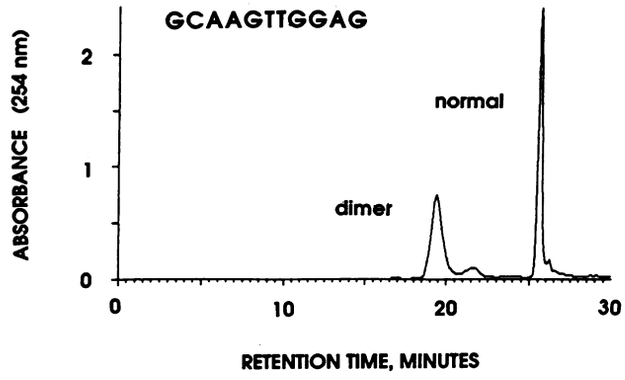

FIG. 3. Chromatogram of 11-mer treated with 320-nm UV light, in anoxia, and 2×10^{-2} M acetophenone. The DNA eluting at 19.5 min contains cis-syn T-T dimer, the material eluting at 21.5 min probably contains trans-syn dimer, and the material eluting at 25.5 min is the unmodified parental species.

ined by treatment with purified *E. coli* photolyase and then by analysis with HPLC (Fig. 4). As shown in Fig. 4 *D–H*, photolyase quantitatively converted nearly all of the modified oligomer into a species that was chromatographically identical with the parent molecule, in a reaction that depended on the presence of blacklight (*D*) as well as enzyme (*H*). In view of the known selectivity of the acetophenone-sensitized reaction (15) and the substrate specificity of photolyase (16, 17), there can be little doubt that the modified molecules contain cis-syn T-T cyclobutane dimer. Further evidence supporting this conclusion was obtained (J.E.L., unpublished data) from analysis of ^{32}P-labeled oligomer by electrophoresis through 20% polyacrylamide gels, in which the modified oligomer migrates slightly more slowly than the parent material. In addition to confirming that virtually all of the dimer-containing oligomer could be converted into the parent species by photolyase, such analysis showed that a high proportion could be converted nonenzymatically by exposure to 254-nm UV, a treatment that is known to selectively monomerize cyclobutane dimers (16). The interaction of photolyase with this material has also been studied by others (18, 19). In addition, no abasic sites could be detected in the modified 11-mer, by the method of Lindahl and Anderson (20), and the oligomer was not susceptible to hot alkali, a treatment that cleaves DNA containing the pyrimidine-pyrimidone (6-4) photoproduct (21). Finally, it is unlikely that the purified material is significantly contaminated with oligomer containing the trans-syn isomer of the

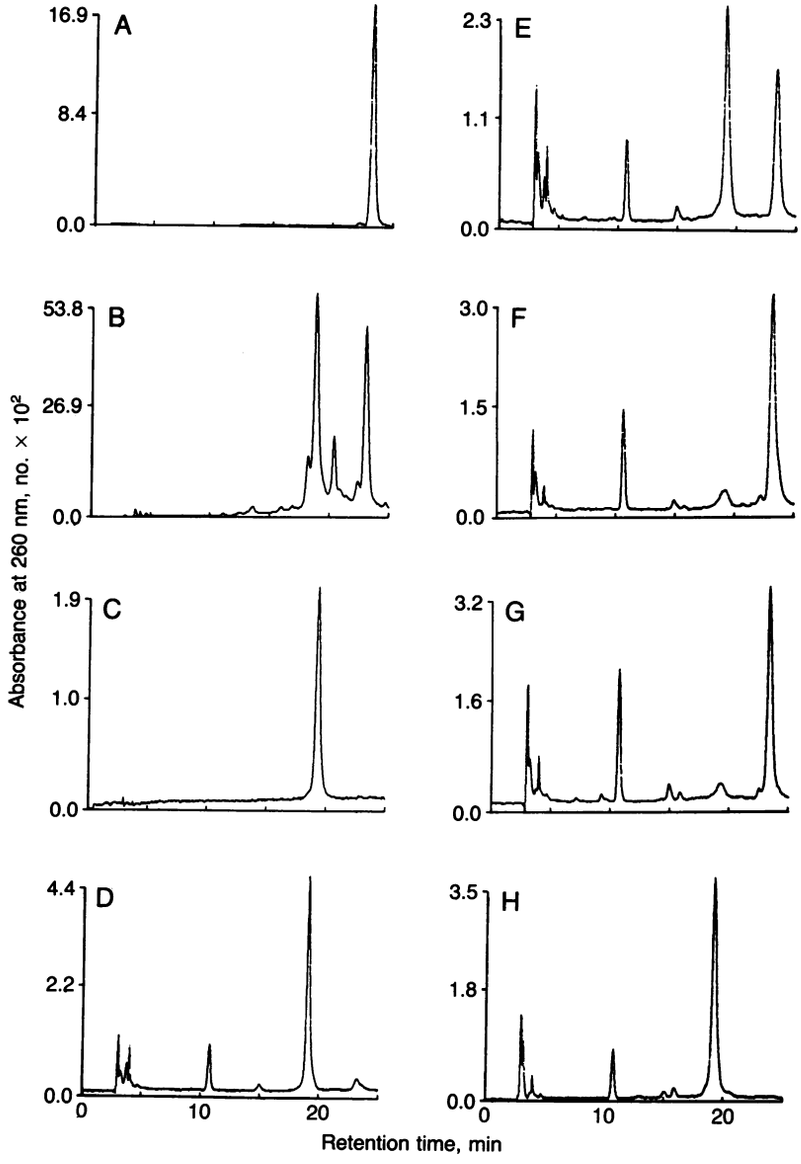

FIG. 4. Analysis of modified 11-mer. (*A*) Unmodified 11-mer. (*B*) An 11-mer treated with 320-nm UV, in anoxia, and 2×10^{-2} M acetophenone. (*C*) Purified modified 11-mer. (*D*) Modified 11-mer plus *E. coli* photolyase but no blacklight. Material eluting before 18 min comes from the buffer and enzyme preparation. (*E*) Modified 11-mer plus *E. coli* photolyase and exposed to 30 min of blacklight. (*F*) Modified 11-mer plus *E. coli* photolyase and exposed to 60 min of blacklight. (*G*) Modified 11-mer with 2.5-fold more photolyase and exposed to 120 min of blacklight. (*H*) Modified 11-mer but with no photolyase and exposed to 8 hr of blacklight.

T-T photoproduct, since this species appears to elute ≈2 min later (Fig. 3). Electrophoretic analysis (J.E.L., unpublished data) of this minor product, eluting at 21.5 min, showed that while it was susceptible to photoreversal by 254-nm UV, it was insusceptible to enzymatic photoreactivation, properties expected of DNA containing the trans-syn isomer (22). Further, this isomer is expected to comprise 10%–15% of the total photoproduct when single-stranded DNA is used (22). Thus we conclude that virtually all of the modified molecules contained cis-syn T-T cyclobutane dimer and that they are unlikely to contain other alterations.

Dependence of Transfection Efficiency on SOS Induction. The capacity of control and SOS-induced cells to replicate dimer-containing vectors was examined by transfecting them with matched samples of control and photoproduct-containing construct, at multiplicities of infection of between 0.2 and 0.5. Matched samples were made by ligating modified and normal oligomer, both purified from the photochemical reaction mixture (Fig. 3), into equal amounts of the same preparation of annealed material.

As shown in Table 1, only 0.4%–1.2% of the normal frequency of plaques were found when uninduced cells were transfected with dimer-containing M13. In SOS-induced cells, however, transfection efficiencies were increased 50- to 100-fold, resulting in phage survival levels that were ≈30% of control values. The inhibition of replication of photoproduct-containing DNA in uninduced cells could be completely reversed by treatment of the construct with purified *E. coli* photolyase and blacklight before transfection (Table 1), showing that the toxic photoproduct was indeed the T-T cyclobutane dimer, rather than any other unidentified lesion. Further, it cannot be ruled out that a single T-T dimer represents an absolute block to replication in uninduced cells. Sequence analysis of 28 progeny phage recovered from uninduced cells showed that 12 of them, presumably the progeny of uncut M13mp7, did not contain 11-mer sequence; 9 contained 11-mer sequence with G-G replacing T-T and arose, therefore, by use of 51-mer scaffold information; and only 7 had a sequence of the kind expected from a normal ligation product. Combining these data with those from Table 1, the frequency of normal phage relative to the control is 0.1%–0.3%. Given that the lesion-containing oligomer may contain up to 0.4% of the parental species, we cannot be sure that these phage result from translesion synthesis. Further, the presence of the single dimer may induce SOS functions in a small proportion of the unirradiated cells. In view of these uncertainties, we cannot distinguish between a very low frequency of translesion synthesis in uninduced cells and a complete block to such replication.

Mutation Frequency and Spectrum. The mutagenic consequences of replicating phage containing the T-T cyclobutane dimer were examined by sequencing a total of 529 progeny phage, the result of transfecting SOS-induced cells of SMH10 with the construct (Table 2). In addition, 59 phage obtained by transfecting SOS-induced cells with dimer-free M13 were analyzed and found to be completely normal over a region starting ≈30 nucleotides on the 3′ side of the T-T site and extending 50 or more nucleotides on the 5′ side of it (data not shown).

Table 1. Plaque-forming efficiency, relative to the control, of M13 DNA carrying a single T-T cyclobutane dimer

Construct	Exp.	Survival, %		Photoreactivation, %	
		− SOS	+ SOS	− PR	+ PR
Control	1	100	132	100	91
	2	100	76	100	72
	3	100	91	—	—
Average		>100	100	100	82
Dimer	1	0.5	41	1.2	95
	2	0.0	17	1.1	74
	3	0.7	22	—	—
Average		0.4	27	1.2	85

In the SOS induction experiments, 100% = 192, 53, and 261 plaques per 2 ng of vector in the three experiments. In the photoreactivation experiments, 100% = 66 and 176 plaques per 2 ng of vector. Vectors were photoreactivated *in vitro* and transfected into uninduced cells. PR, photoreactivation.

Among the 373 phage analyzed that lacked the M13mp7 hairpin sequence, 347 (93%) contained a normal T-T sequence at the site of the cyclobutane dimer (Table 2), indicating that *E. coli* cells possess a significant capacity to replicate past the lesion accurately. Among the remaining 26, all contained single-nucleotide substitutions. Twenty-three of these (88%) were targeted at the 3′ thymine. Slightly more than half of the mutations were T → A transversions and the remainder were T → C transitions; no T → G transversions were found. With the exception of one questionable case, none of the 373 phage sequenced contained untargeted mutations in a region comprising ≈30 nucleotides on the 3′, and 50 or more nucleotides on the 5′, side of the T-T target site. The possible exception was a single nucleotide deletion, in which the 5′ guanine of the 11-mer sequence was missing. The presence of such a mutation at a ligation junction point raises the possibility that it arose from exonucleolytic attack or from a contaminating 10-mer, rather than from a replication error.

In addition to the above results, sequence data were also obtained, in the same set of experiments, from 156 progeny phage that contained the M13mp7 cloning site hairpin located 4 nucleotides on the 3′ side of the T-T target sequence (see Fig. 2). Results from these phage resemble those from the hairpin-free construct with regard to the high proportion that possess normal T-T sequence (92%) and the absence of untargeted mutations. They differ, however, with respect to mutation spectrum; only 3′ T → A transversions were found. The difference between the two sets of data is significant (P = 3.2% for data from the 3′ site only, and P = 1.7% for data from both sites).

Finally, all mutations were likely to be the consequence of the T-T cyclobutane dimer itself, rather than any other undetected photoproduct. Treatment of dimer-containing DNA with photolyase and blacklight prior to transfection completely abolished all mutability (0 mutants out of 193 phage sequenced). Matched samples exposed to photolyase but not to blacklight gave normal levels of mutation (12 mutants out of 181 phage sequenced).

Table 2. Targeted mutation frequency and spectrum from T-T cyclobutane dimer

Type of construct	5′ thymine substitution, no.			3′ thymine substitution, no.			Total no. of phage sequenced	Mutation frequency, %
	C	A	G	C	A	G		
Normal	3	0	0	8	15	0	373	7
+ hairpin	0	0	0	0	12	0	156	8

Data tabulated are the total from experiments with three independently constructed samples of vector by using two samples of modified 11-mer.

DISCUSSION

Experiments with single-stranded M13 vectors that carry a uniquely located cis-syn T-T cyclobutane dimer show that this UV photolesion is mutagenic as well as toxic. The frequency of targeted mutations resulting from translesion synthesis on this DNA, determined by direct sequencing and without the aid of a reporter gene, was ≈7%, showing that *E. coli* cells possess appreciable capacity to carry out such replication accurately. In part, this accuracy may result from polymerase bias in favor of the noninstructive insertion of adenine (23). Experiments with depurinated M13 viral DNA (24) show that adenine is selected in 60% of such cases. Acting alone, bias of this magnitude would result in a mutation frequency of ≈65%, rather than 7%, as in the present experiment. It therefore appears likely, as argued (25), that T-T cyclobutane dimers retain significant capacity to form correct base pairs, a conclusion that is supported by an NMR study of this lesion (26).

With one possible exception, all mutations were targeted at the site of the photoproduct, suggesting that near-targeted or hitchhiking mutations, whose occurrence is tied specifically to the presence of a lesion, are unlikely to be common. Untargeted mutations resulting from a general reduction in replicational fidelity in irradiated cells would not be detected in these experiments; their frequency, unlike that of targeted events, is unlikely to be increased appreciably by the presence of the T-T dimer in all phage genomes.

All targeted mutations analyzed were single-nucleotide substitutions. About 90% of these were targeted at the 3′ thymine of the lesion, the first encountered by polymerase. The results also demonstrate that a single lesion can give rise to more than one type of mutation, though the spectrum was not random: T → A transversions were more common than T → C transitions, and no T → G transversions were observed with the construct used. The extent to which this spectrum represents a general property of T-T cyclobutane dimer is not yet clear, however, since it would appear to depend on genetic context; when the M13mp7 hairpin sequence was present 4 nucleotides on the 3′ side of the dimer, only 3′ T → A transversions were found. The mechanism by which this sequence, which will have already been replicated at the time of translesion synthesis, exerts its influence on polymerase error remains an intriguing question.

Finally, our results show that a single T-T dimer blocks replication in >99.5% of the phage transfecting *uvrA6* cells uninduced for SOS functions. After exposure of these cells to irradiation with UV at 4 $J \cdot m^{-2}$, plaque-forming efficiencies that were ≈30% of the control values were observed. Irradiation with UV at 2 $J \cdot m^{-2}$ resulted in much lower phage survival (though the same mutation frequency), while UV irradiation with 5 $J \cdot m^{-2}$ was about equally as effective as with 4 $J \cdot m^{-2}$. Such results suggest that these cells have only a limited capacity for coping with the T-T dimer. It is possible, however, that the need to make the UV-irradiated cells competent prevents the full development of the SOS response, and our estimate of this capacity, therefore, may be a minimal one. The extreme inhibition of replication caused by the T-T dimer in uninduced cells contrasts curiously with the accuracy of this process in SOS-induced cells, perhaps implying that elongation and misincorporation are unrelated activities or that accuracy depends on SOS induction.

We thank Angela Borden for help with some of the sequence analysis and Gwendolyn and Aziz Sancar for the purified photolyase. This work was supported by Public Health Service Grants GM21858 and GM32885 from the National Institutes of Health and by Grant DE-FG02-85ER60281 from the U.S. Department of Energy.

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